

ANALYSIS OF THE MODE OF ACTION OF SOME NEUROTOXIC  
INSECTICIDES ON LOCUSTA MIGRATORIA

by

P.M. Cook.

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## II. INTRODUCTION.

There are in use today, in the battle against insect pests, a considerable number of organic chemicals, collectively called "insecticides". While some of these substances are the outcome of systematic search for suitable insecticides, many of them are produced as by-products in other industries, and have been examined for insectidal properties, together with other routine tests, before being classified as waste products. Some of these substances, such as DDT and  $\gamma$ BHC, appear to be of these substances, such as DDT and  $\gamma$ BHC, appear to be to owe their virtual specificity to differences in penetrability between insect cuticle and mammalian skin (Dresden and Krijgsman, 1948). Others, such as parathion and DNOC are equally toxic to mammals, as evidenced by effects on operators where insufficient precautions have been taken. Many show differential toxicity with different species of insects. Many of these so called insecticides produce symptoms which would indicate some interference with neural mechanisms, for example, violent tremors, ataxia and paralysis.

The purpose of this work is to take insecticides in current or recent use in the field against locusts, and

using Locusta migratoria migratorioides as the test animal, to study their effect on this insect by an electrophysiological technique; further, to select those which have a direct effect on the nervous system and are therefore truly neurotoxic from those which affect the nervous system only secondarily and are consequently pseudo-neurotoxic, and attempt to understand the mode of action of the former. This is, perhaps, a somewhat ambitious aim since little is known of the functioning of the insect central nervous system, particularly of the chemistry inherent in these processes. It is possible, however, that such a comparative study may throw some light on the more fundamental problems.



### III. BRIEF SURVEY OF PRESENT STATE OF KNOWLEDGE.

The problem of keeping down the vast numbers of insect pests has been a major concern for a very long time. The use of chemical substances has been, and is at present the most common way of dealing with this problem. The literature dealing with these substances, their properties, uses and mode of action is immense, and has recently been reviewed by Shepard, 1939, Haller, 1947, De Ong, 1948, Metcalf, 1948, Dresden, 1949, West and Campbell, 1950. This work is not concerned with insecticides in general but with the mode of action of a limited number on Locusta migratoria. There are several suggestions regarding the mode of action of the more common insecticides. Discounting those substances which act by purely mechanical means, for instance the sticky trap materials, or substances which block stigmata, it is clear that a substance which is to be used as an insecticide must first be introduced into the body of the insect. This can be done via the digestive system or by penetration through the cuticle. The insecticides can thus be divided into two groups, viz., stomach and contact poisons. The aim of the producers of insecticides is to obtain substances which are specifically

toxic to insects. Some insecticides are toxic to mammals also, but even those which seem to be specifically toxic to insects only derive their specificity solely from the fact that they penetrate the insect cuticle easily but not the mammalian skin, which acts as an effective barrier against these substances. Such substances injected beneath the skin were found to be as toxic to mammals as to insects (Dresden and Krijgsman, 1948).

The most obvious requisite of an insecticide is that it shall be so toxic to the insect as to result in death. An insecticide may itself fulfil the requirement, or it may be converted into a toxic substance in plants which are sprayed with the insecticide or by the animal, either during penetration or inside the body; such a compound is octamethylpyrophosphoramidate (OMPA).

In discussing the toxicity of an insecticide we may approach the problem from the chemical side by finding out which configuration in the chemical structure determines toxicity; or we may approach the problem by analysing the mode of action of a drug in the animal body. Usually only the chemical approach has been considered when theories of toxicity have been advanced. For the present paper I shall deal only with the biological approach.

Dismissing the aspects of penetration and in vivo changes in the chemical constitution and considering only the mechanism of action of the ultimate form of the insecticide at the site of action, there seem three broad ways in which a substance can act. (1) By surface action on nervous tissue, (2) by competition with an essential metabolite of a vital biological reaction, and (3) by destruction of such an essential metabolite.

(1) Welsh and Gordon (1947) suggested that some substances might have a surface effect on the nerve axon and that this action is characteristic of substances having a high lipoid/water solubility ratio. They were considering a variety of substances including nicotine, pyrethrum and DDT. While it is unlikely that their theory holds for all these compounds, it is, nevertheless, one which should not be forgotten. Inorganic substances which upset the ionic relationships would presumably produce similar effects.

(2) The possibility that an insecticide may act as a competitive metabolite has been suggested by Slade (1945) for the action of  $\gamma$  BHC. It has a similar spatial configuration to meta-inositol and may thus interfere with inositol in some vital process (Buston, Jacobs and Goldstein, 1946).

(3) Destruction by the introduced substance of some essential metabolite has been suggested for phenothiazine, and such destruction may be brought about by a reversible chemical reaction resulting in products which interfere further with metabolic processes. Alternatively, the destruction may be the result of an irreversible combination, such as the formation of carboxyhaemoglobin. This example is not, however, strictly valid since the carbon monoxide could also be described as competing with oxygen. Zukel (1944) suggests that phenothiazine interferes with cytochrome oxidase while De Eds and Thomas (1941) consider that it causes permanent oxidation of the respiratory enzymes by the leucothionolthionol system. A number of insecticides have been shown to destroy the enzyme cholinesterase. DFP (Masur and Bodansky, 1946), HETP (Dübois and Mangun, 1947), (Chadwick and Hill, 1947), Fluorophosphonates (Adrian, Feldberg and Kilby, 1947), etc., etc. DDT was suspected to be an anticholinesterase, but Richards and Gutkomp (1945) showed that it did not inhibit cockroach cholinesterase in vitro. Tobias et al. (1946) were also unable to demonstrate any cholinesterase inhibiting action of DDT.

For some insecticides these theories on mechanisms of action are still very much in the hypothetical stage, despite the fact that a great deal is known of their in vitro reactions, where the experimental difficulties are obviously considerably reduced, in comparison with the in vivo reactions.

#### IV. ANATOMY OF THE LOCUST CENTRAL NERVOUS SYSTEM.

The present experimental study of the toxic action of insecticidal compounds is based on an electrophysiological technique. Such techniques have been widely applied in the study of nerve and muscle physiology in both vertebrate and invertebrate animals. The reviews of Prosser (1946), Welsh and Schallek (1946) and Bullock (1947) deal specifically with electrophysiological study of invertebrate nervous systems. Amongst the four important fields of application of the method which Bullock mentions are "the comparative study of synaptic mechanisms" and "physiological neuronography - which has been applied especially to giant nerve fibre systems".

The method developed by Pumphrey and Rawden-Smith (1937) to record the electrical activity from the ventral nerve cord of a cockroach offers a simple preparation for the study of a central synapse in an invertebrate. It is the synapse in the last abdominal ganglion between the primary vibration receptor afferents from the cercal nerve and the ascending axons of the giant neurons in the abdominal cord. This preparation involves a single synapse in each of a relatively few ascending pathways, thereby simplifying analysis of the records and approximating to a single fibre preparation. Lowenstein (1942)

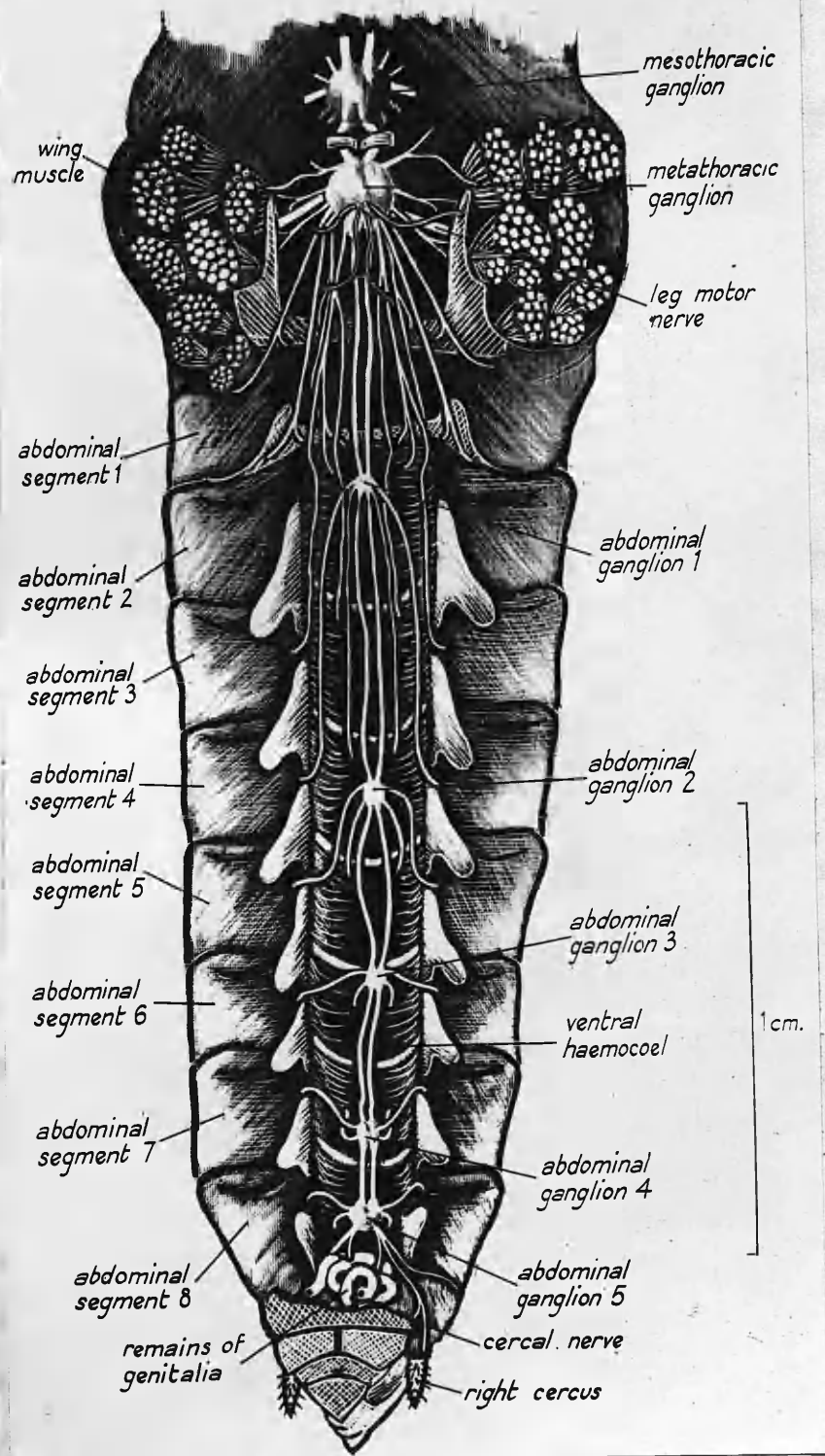


Figure 1. Dissection of adult male *Locusta migratoria*, dorsal aspect, to show posterior region of CNS. (Fig. 1 from Cook, 1951).

used the time of abolition of the response to "air-puff" stimulation in this preparation as a method of bio-assay of pyrethrum extracts. Roeder et al. (1947) used the preparation for the study of the action of various drugs on the cockroach synapse and Roeder (1948) extended the study of the organisation of the giant fibre system both electrically and histologically.

A similar preparation was used for the study of the mode of action of the neurotoxic insecticides. Since the literature contains little or no detailed description of this region of the nervous system in the locust, a histological study was undertaken, partly as a necessary background for the physiological work, and partly to provide anatomical information as yet not available in the case of the locust. The information gained from this study is important for comparison of the functions of the giant fibre systems in Periplaneta and Locusta.

Only the more posterior region of the locust central nervous system has been studied in detail, since, for the purposes of this work it is desirable to restrict neurophysiological preparations to the simplest and most easily obtainable. Figure 1 shows the region studied. In Locusta the nervous system lies in a part of the haemocoel which is divided from the main haemocoel by a thin sheet



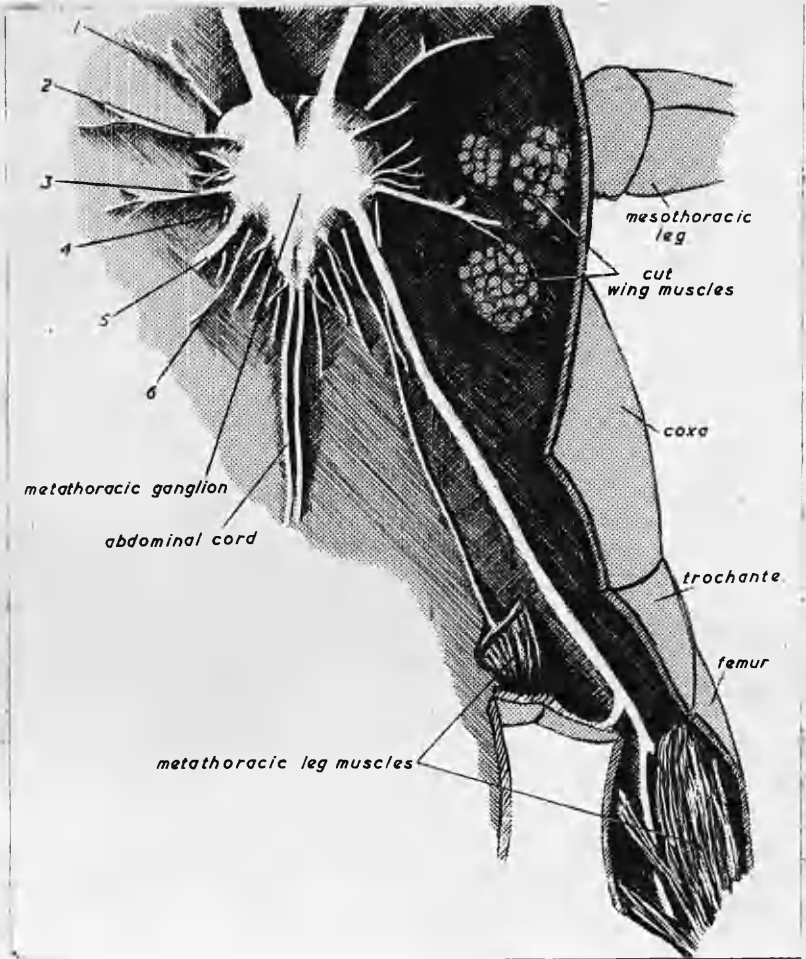
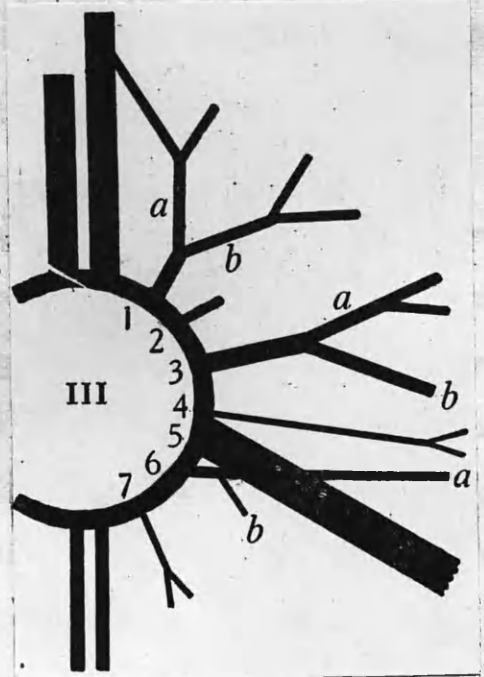


Figure 2. Dissection of adult locust, ventral aspect, showing metathoracic ganglion and its nerves. The nerves are numbered, as far as possible, according to Pringle.

Figure 3. Diagram of the nerves leaving the metathoracic ganglion of *Periplaneta*. Nerve 1 does not supply the leg; 2 and 7 are tracheal trunks accompanied by a few fibres not part of the leg motor systems. (After Pringle).



of muscular tissue stretched across a trough, the sides of which are formed by endophragmal skeleton. In female locusts the nervous system is longer than the normal body length and therefore folded, to allow for extension during oviposition. The abdominal nerve chain contains only five ganglia. From the distribution of the paired nerves and from the relative sizes of ganglia it is likely that the first four ganglia are associated each with a single segment, namely abdominal segments 4, 5, 6 and 7. Ganglia associated with segments anterior to segment 4 have become incorporated in the metathoracic ganglion, whilst the fifth abdominal ganglion represents an agglomeration of that of segment 8 and those more posterior.

Figure 2 shows a locust dissected from the ventral aspect to show the metathoracic ganglion and its nerves. Nerve 5 corresponds to that similarly named by Pringle (1939) (see Figure 3) and has been exposed along its course in the metathoracic leg.

It is, perhaps, useful at this stage to compare the giant fibre system of the locust with that of the cockroach. Periplaneta americana has been most commonly used in insect neurophysiological investigations, due mainly to its large size and availability. Thus it is the only species amongst the Orthoptera in which the giant fibre

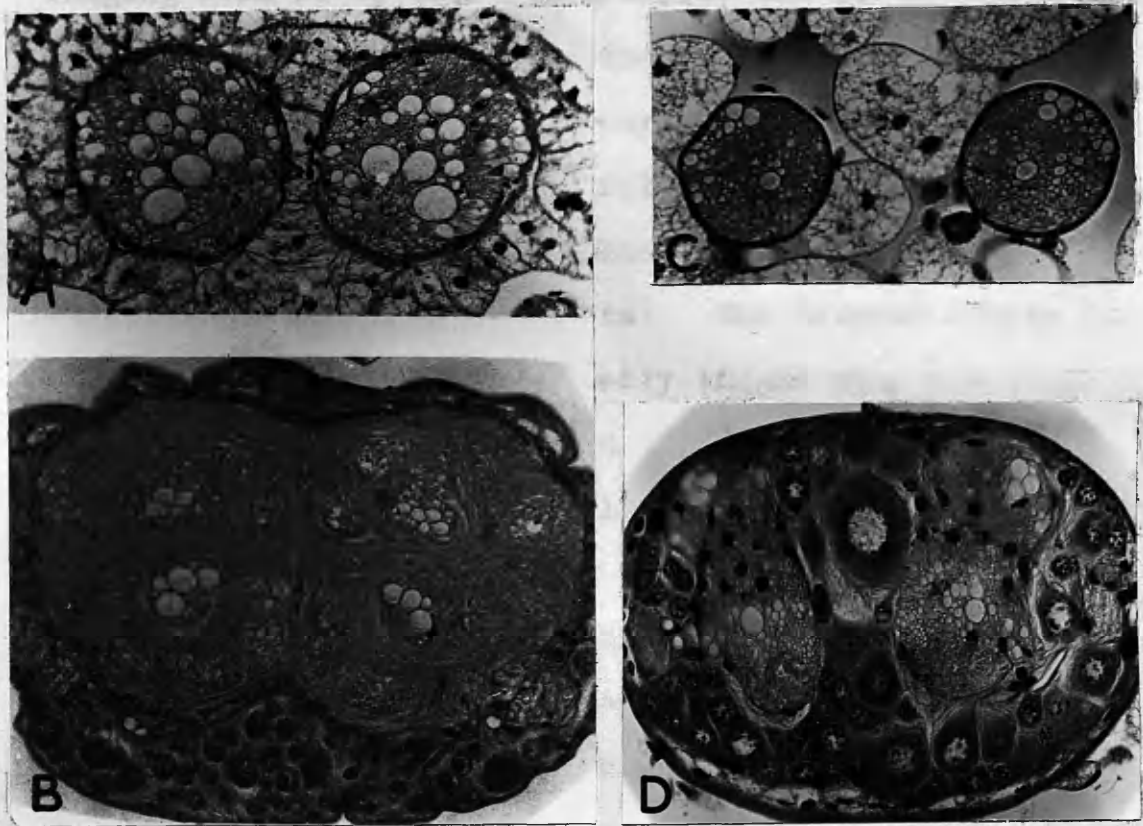


Figure 4. Photomicrographs showing relative sizes of cords and ganglia and arrangement of giant fibres in A and B, Periplaneta, and C and D, Locusta. A: abdominal cord x 150. B: 5th abdominal ganglion x 150. C: abdominal cord x 200. D: 1st abdominal ganglion x 200.

system has hitherto been studied histologically. Roeder (1948) describes six giant fibres in each abdominal cord ranging from 20 to 45  $\mu$  in diameter and 10 to 12 fibres of 5 to 20  $\mu$  in diameter. The fibres are arranged in two main groups, a ventral group formed by the three largest fibres and a smaller fibre and a dorsal, similarly arranged, quartet of fibres which are all slightly smaller than their ventral counterparts. The largest fibres in Periplaneta are thus considerably bigger than the four large fibres in Locusta, which only reach a diameter of 12 to 15  $\mu$  (Figure 4). Both in Periplaneta and in Locusta the giant fibres pass uninterruptedly through the abdominal ganglia and are considerably reduced in diameter while doing so. Roeder was not able to trace connexions of these fibres with individual cell bodies, but thought that they are multicellular, and arise from groups of cell bodies situated in the posterior peripheral region of the last abdominal ganglion. This region is close to the point of entrance of the cercal nerve. While it was not possible in the cockroach to pin-point the synaptic region between giants and afferents, there was no morphological evidence for the presence of interneurons.

Not only are the giant fibres of Locusta thinner than those of Periplaneta: there are also fewer of them,

viz. only five fibres of a diameter of  $5 \mu$  or over. The most medianly situated of these fibres could be traced to its connexion with a cell body and is, in all probability, unicellular. The other three large fibres were not traced to individual cell bodies, although they disappear in the region of large cell bodies in the peripheral part of the posterior region of the fifth abdominal ganglion. The possibility cannot be excluded that the cell bodies of these fibres may lie in the metathoracic ganglion. This does not seem likely since these fibres, like the median fibre, taper towards their anterior ends.

Roeder (1945) considered it probable that one of the quartets of fibres in Periplaneta served as a pathway for ascending impulses, the other quartet probably being concerned with descending impulses. In Locusta, both natural and electrical cercal stimulation evoke spikes in the abdominal cord which are both larger and faster than those recorded in the cercal nerve. These large spikes are not uniform in size, so that it would seem probable that more than one large fibre is involved in the response.

This account represents a summary of the anatomical and histological details relevant to the main theme of the present thesis and is taken from a publication "Observations on giant fibres of the nervous system of Locusta migratoria" (Cook, 1951) which is included in Appendix A.

## V. METHODS.

### a). The Preparation.

The legs and wings are removed from a living locust and a median dorsal strip of body wall removed, usually by inserting the scissors at the joint between two posterior abdominal segments, a little to one side of the mid line, and cutting forwards into the thorax and back on the other side. The alimentary canal is then freed and removed by two cuts, the dissection pinned out with four pins in the abdominal wall. As much of the fat tissue and reproductive organs are removed as is necessary to expose the nervous system. Unless otherwise stated, male locusts have been used throughout this work, since the cercal nerve is more readily exposed in this sex. In order to simplify the oscillographic picture, by eliminating descending impulses from the head region, the nerve cord is then usually transected between ganglia 1 and 2 and the whole locust anterior to this region is removed. Electrodes can then be placed in contact with any part of the nervous system thus remaining. This whole operation can be performed rapidly, in one or two minutes and with considerable reliability.

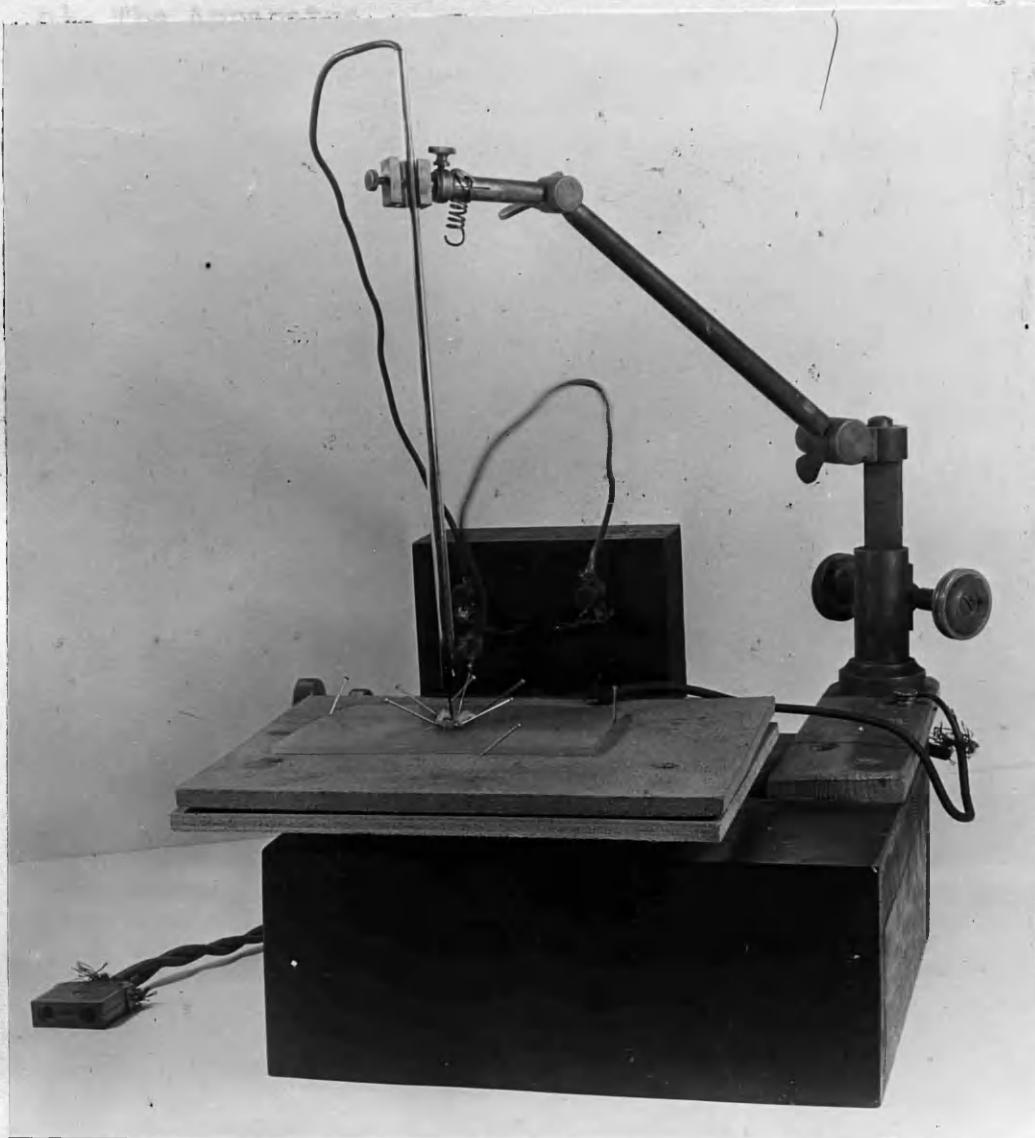


Figure 5. Electrode holder, in which grid electrode can be placed in position by semi-micro manipulation. The cork platform is movable in an horizontal plane by means of the fine adjustment controls just visible behind the platform. The grid electrode is movable in a vertical plane.

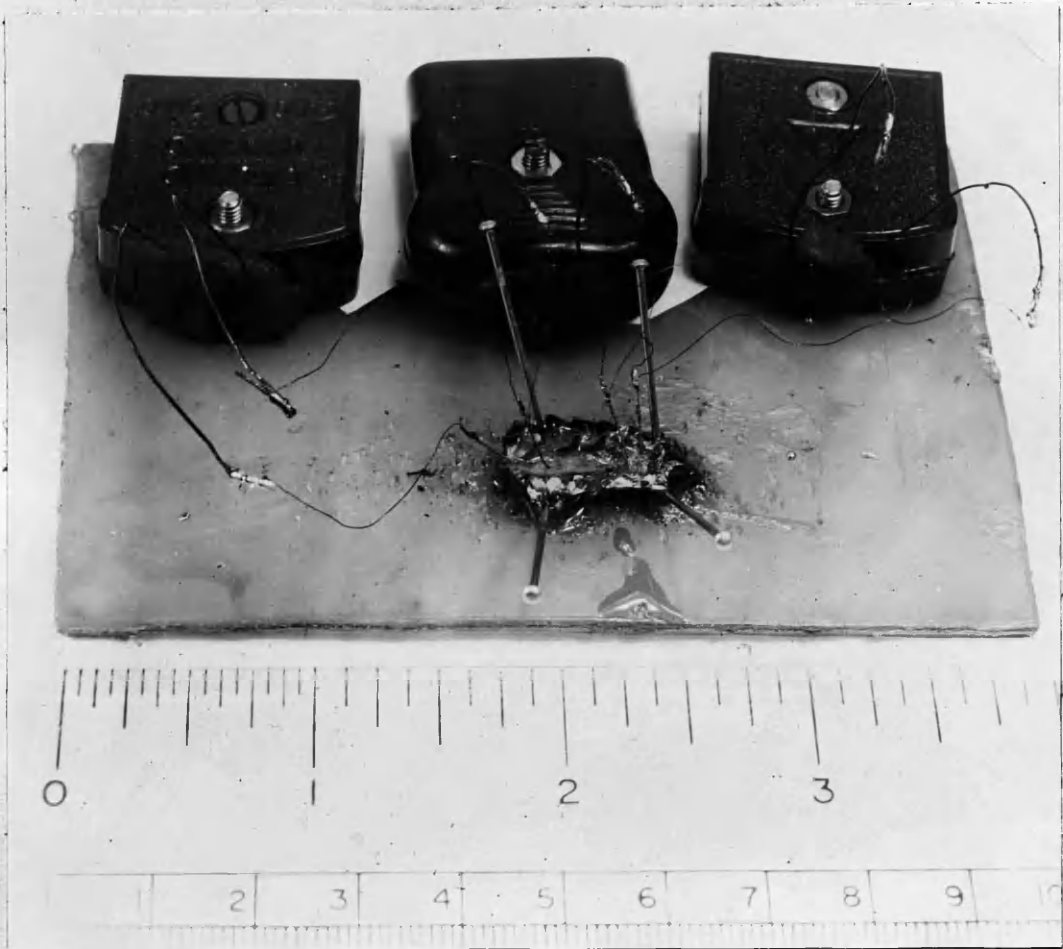
b). The Apparatus.

During the course of the work various electrodes and electrode holders were used. Two electrode holders in particular were in common use and are figured here. One, illustrated in fig.5, consists of a platform, movable by means of a microscope mechanical stage, and a dissecting lens holder which carries a glass tube. Insulated copper wire was passed through the glass tube and soldered at the lower end to fine gauge silver wire. The silver wire was insulated either with Dhekotinsky cement or with shellac, and the lower end snipped off with scissors. This electrode could then be lowered on to a ganglion, usually the fourth abdominal. The second electrode is merely a silver pin soldered on to wire and placed anywhere in the preparation, preferably as far away from the first as possible, but median to the endophragmal skeleton in order to avoid being in a region liable to dry up.

Another simple electrode holder, fig.6, which allows recording from several positions, consists of a cork or polythene base to which are attached three biscuit flex connectors with insulated copper wire. Soldered on to this wire at the free ends is very fine gauge insulated copper wire bearing extra fine silver entomological pins. It is easy to stick these pins into



the preparation by means of fine tweezers - that they are adjacent to and in contact with the electrode or



**Figure 6.** The simple 3 position electrode holder used for most of the insecticide studies.

the preparation by means of fine forceps, so that they are adjacent to and in contact with the nerve cord or cercal nerve. If they are stuck into the cord, contact is so good that too many fibres come into the picture and analysis is difficult. It was not necessary to insulate these electrodes, since the preparation was usually covered with a film of medicinal liquid paraffin; when saline was used, however, insulation was essential. This was accomplished by coating the electrodes with insulating varnish and stoving them in an oven at 180 - 200°C. A small patch of insulation to one side of the tip was removed before use by means of a fine file. The electrode was then placed with the bare region in contact with the nerve.

In order to record from three positions the dissection procedure was modified. The wings and first two pairs of legs and head were all removed. A small portion only of the dorsal body wall was removed and the alimentary canal and genital organs withdrawn through this window and cut out. Another window was cut in the chitin ventrally over the region of the metathoracic ganglion and the proximal portion of nerve 5. Great care was needed in this operation since the ganglion is very close to the ventral body wall. The preparation was then pinned down

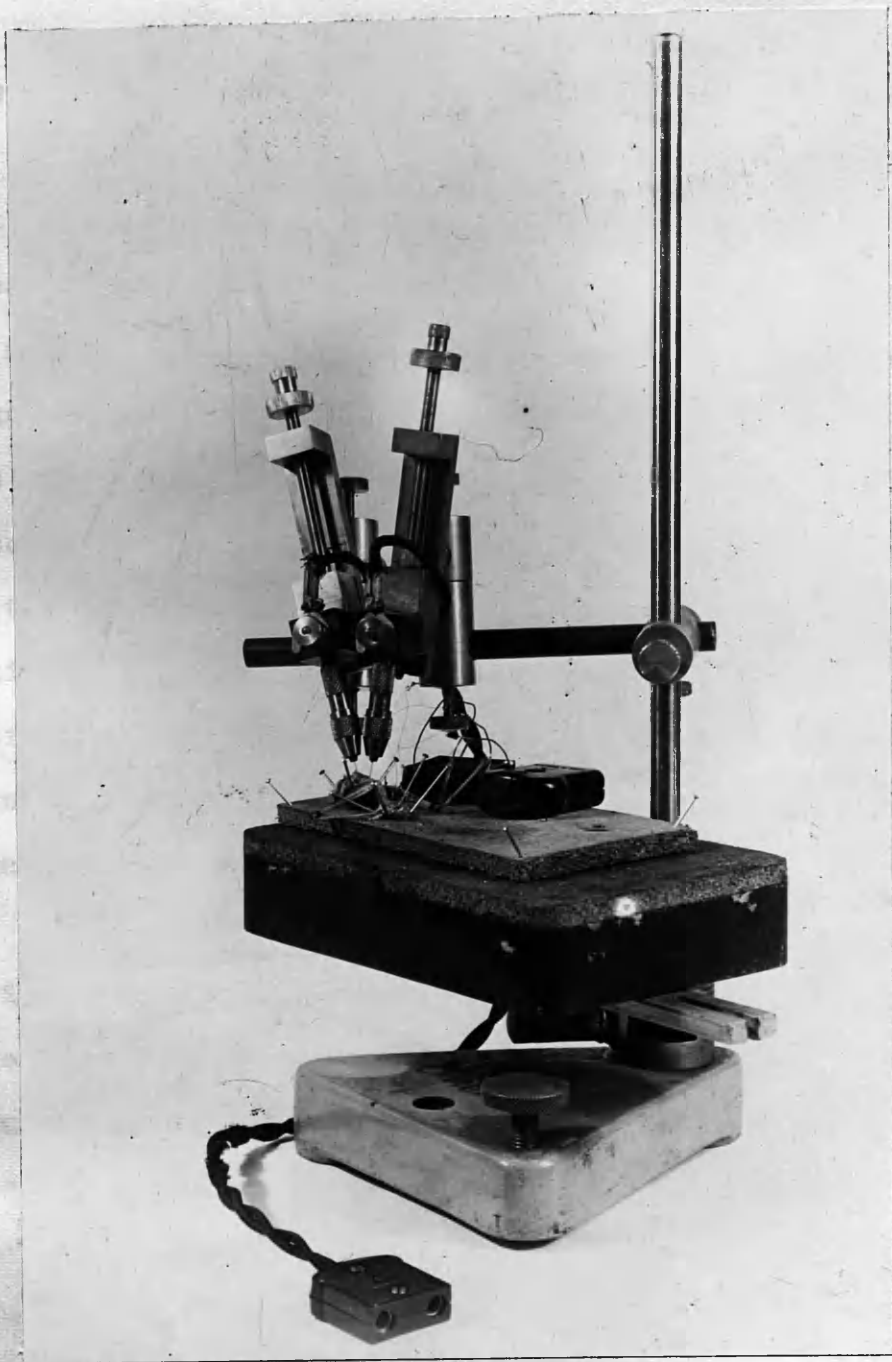


Figure 7. Electrode holder used when recordings were made from Nerve 5 and from the abdominal nerve cord and cercal nerve in the same preparation.

in such a way that the thorax was ventral side uppermost and the posterior region of the abdomen dorsal side uppermost. Silver pins were then placed in contact with the cercal nerve and abdominal cord as previously described, but nerve 5 was lifted on silver pins bent into small hooks and held in screw chuck electrode holders (fig.7). This preparation dries up very quickly and must therefore be covered with paraffin at the start of the experiment. All the preparations are subject to drying so that precautions to prevent this must be taken. They can be covered with paraffin or saline or walled in with saline soaked cotton wool and so preclude evaporation of the animal's own body fluid.

The leads from the preparation were connected to the input of a standard resistance-capacity coupled four stage amplifier with a push-pull input (fig.8). The output from the amplifier was connected both to the Y plates of a cathode ray oscillograph and to a monitor stage and speaker. Permanent records were made either by recording from the C.R.O. directly on to photographic paper, or by acoustic recording on to a Ferrograph tape recorder. This acoustic record could be played back on to the C.R.O. and recorded photographically at a later date.

A time marker was projected through the camera lens on to the film. This consisted of rectified A/C mains fed to a small neon tube causing it to flash at the rate of 50/second.

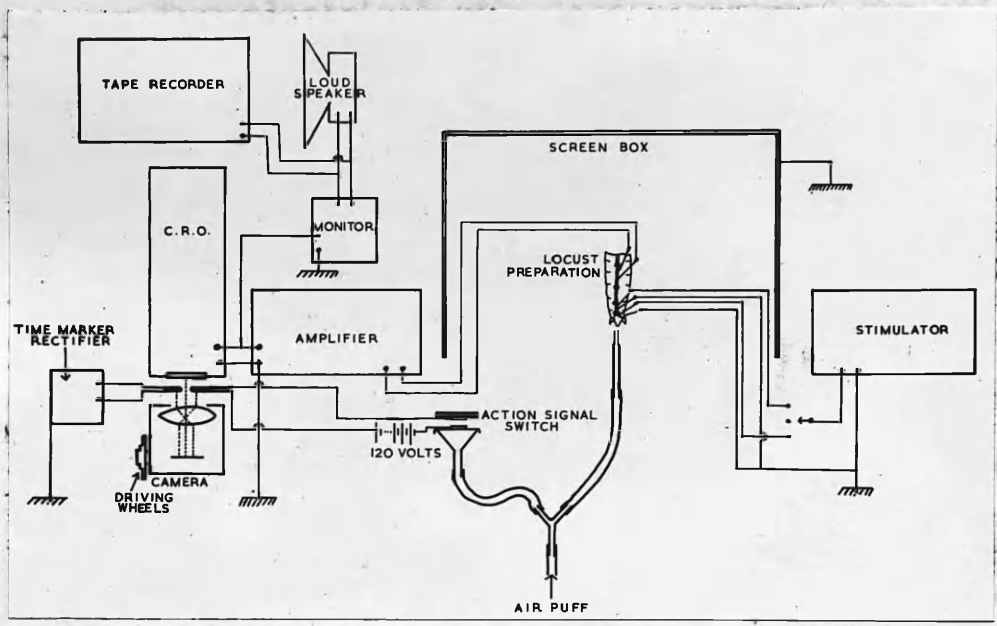


Figure 8. Diagrammatic representation of apparatus used for recording action potentials from the CNS of locusts. This diagram is composite, showing the means of both electrical and natural stimulation and the arrangements for both direct photographic recording or indirectly via the tape recorder.

A time marker was projected through the camera lens on to the film. This consisted of rectified A/C mains fed to a small neon tube causing it to flash at the rate of 50/second.

When electrical stimulation was used the stimulus artifact served as sufficient indication of the moment of stimulation, but for natural stimulation an action signal was needed. When photographic records were made directly this action signal was provided by means of another neon tube, the beam from which was also directed through the camera lens on to the paper. The jet of air, providing the natural stimulus, was divided into two and half was led to a filter funnel across which was stretched a rubber membrane. A small piece of metal was glued on to this rubber sheet, each puff of air caused the membrane to balloon out and the metal to make contact with a second piece of metal, and so complete the circuit of an appropriate voltage supply to the neon signal. By careful adjustment of the lengths of rubber tubing involved, the duration of time the neon was alight could be made to coincide exactly with the duration of stimulation.

This type of action signal, however, was useless for acoustic recording on to a tape recorder. Here a similar set-up was used but the switch completed a circuit through

a small resistor across the terminals of a charged condenser. This resistor was hanging loose in the screen box, the condenser charge adjusted until the discharge was picked up by the amplifier at a suitable size on the C.R.O. screen. A signal was picked up both at the make and break of contact, so that the duration of stimulation was admirably marked. The signal was both audible and visible when played back from the tape on to the C.R.O. for photographic recording.

Preparations were stimulated electrically either by a square pulse generator which was capable of delivering either single stimuli or repetitive stimuli over a wide range of frequencies, from 45/minute to 1300/second with variable width and intensity, all these variables were independent of one another; or from a simple thyatron stimulator which has a frequency range from 2/second to 400/second or more.

VI. MEASUREMENT OF RATES OF CONDUCTION.

Before going on to the description of the insecticide studies, mention should be made here of attempts to measure relevant rates of conduction.

By applying a stimulus to the central nerve and recording from two points in the abdominal cord channel amplification model 1049 photographs were made either by photographing and an ordinary photostatic from a distance of 25 cm. The pulse of this kind of apparatus could be delayed by a variable length of time after the trigger, so that it appeared in a convenient position on the screen. This method of recording was more satisfactory for such measurements than the usual method

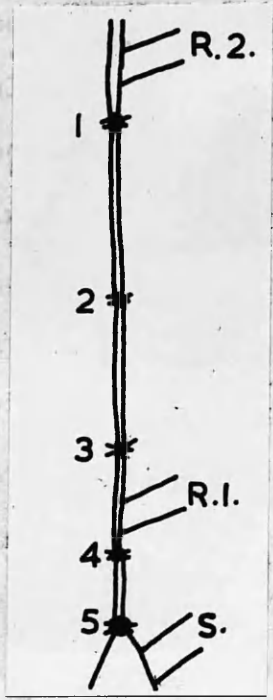


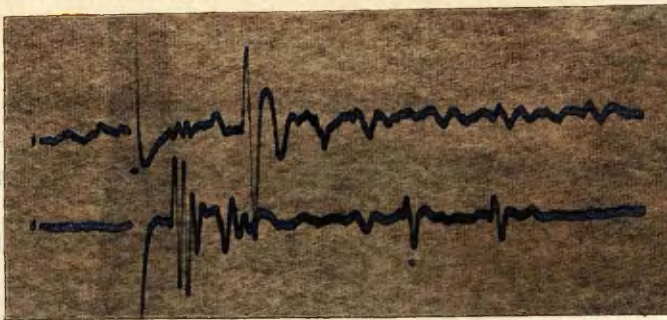
Figure 9. Diagram showing arrangement of stimulating and recording electrodes in experiments to determine the rate of conduction in giant fibres in locust abdominal cord.

Some measurements could then be studied. Some measurements with the other method of film recording, the method of varying the electrical stimulation the

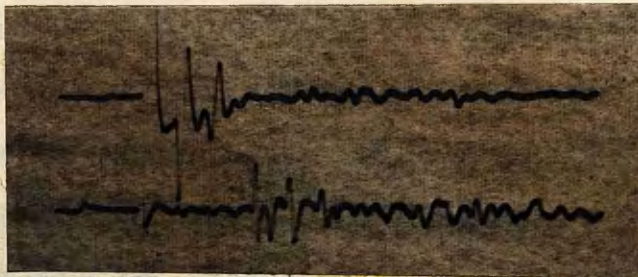


VI. MEASUREMENT OF RATE OF CONDUCTION.

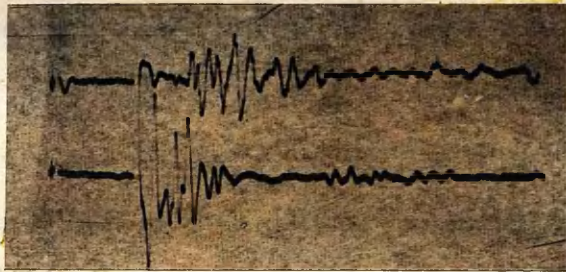
Before going on to the description of the insecticides studies, mention should be made here of attempts to measure relevant rates of conduction in the locust preparation. By applying a stimulus just above threshold value to the cercal nerve and recording simultaneously from two positions in the abdominal cord (fig.9) by means of double channel amplification and a double beam Cossor G.R.O. model 1049 photographic records were made. These records were made either by the usual moving film method or by photographing one sweep of the oscillograph beam on an ordinary photographic plate. In the latter case a thyatron stimulator was used to trigger the sweep and also the oscillator of a square pulse generator. The pulse of this piece of apparatus could be delayed by a variable length of time after the trigger, so that it appeared in a convenient position on the sweep. This method of recording was more satisfactory for such measurements than the usual method of recording since the beam could be spread out more than the fastest rate of film travel allowed, the spike complex evoked by stimulation could then be studied. Some measurements were made with the other method of film recording, however, instead of response to electrical stimulation the



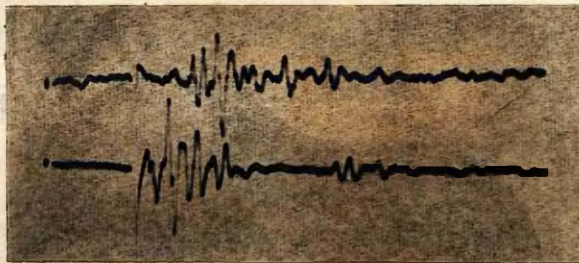
Prep. No. 342 (1)



Prep. No. 341 (2)



Prep. No. 343 (4)



Prep. No. 343 (6)



Calibration - 50 Cyc./Sec.

Figure 10. Selection of records used in calculation of conduction rate of action potentials in giant fibres in abdominal cord of locust. Amplification of two channels is approximately the same. Explanation in text.

rate of travel of occasional spontaneous action potentials was measured.

In the first attempts to measure conduction rates the preparation was set up as usual, the distance between recording electrodes along the nerve cord was measured by first making a camera lucida drawing of the preparation. The length of nerve cord could then be measured on a large scale and the necessary reduction calculated. This method was subject to certain inaccuracies arising from the difficulty of getting the whole preparation in the field of view at one time. Later measurements were made in preparations which had previously been stretched so that the nerve cords were pulled out straight. Turner (1951) has shown that the rate of conduction in nerves is unaltered by stretching.

Figure 10 shows a selection of records which were made in this way. An ordinary plate camera was used and was set up in front of the cathode ray oscillograph screen on a tripod. In order to avoid blurring of the plates the spot and first part of the sweep was dimmed. All the records were not made with the camera in a fixed position; the exposures therefore show the total sweep at varying lengths. The sweep speed and actual sweep length, however, remained unaltered throughout these experiments, so that calculations were based on fractions of the sweep length

and not the actual measured distance.

The preliminary measurements showed conduction to be of the order of 4 metres per second. The sweep speed, calculated by means of the 50 cycle calibration shown in figure 10 was 44.6 milliseconds. The distance between electrodes was measured directly with dividers and the time taken for the response to travel this distance measured from the records, the rate of conduction accordingly calculated. Table I shows the results of these calculations. Considering the variations in pattern of the spike complex obtained at the two recording positions the results show surprisingly good agreement, again giving an average of 4 metres per second.

TABLE I.     Measurements of rate of conduction in abdominal cord giant fibres.

| Prep. No. | Distance between electrodes | Time delay | Rate of Cond. |
|-----------|-----------------------------|------------|---------------|
| 342 (1)   | 2 cms.                      | 4.95 msec. | 4.2 m./sec.   |
| 341 (2)   | 2 cms.                      | 5.58 msec. | 3.6 m./sec.   |
| 341 (3)   | 2 cms.                      | 5.09 msec. | 3.9 m./sec.   |
| 343 (4)   | 1.6 cms.                    | 3.33 msec. | 4.8 m./sec.   |
| 343 (5)   | 1.6 cms.                    | 3.99 msec. | 4.0 m./sec.   |
| 343 (6)   | 1.6 cms.                    | 3.99 msec. | 4.0 m./sec.   |
| Average   |                             |            | 4.1 m./sec.   |

Roeder (1948) gives the rate of conduction in the giant fibres in the abdominal cord of the cockroach as 6 metres per second. Since these fibres are three times the diameter of those found in the locust, this difference in conduction rate is to be expected. Attempts to measure synaptic delay were unsuccessful due to difficulties caused by the stimulus artifact when stimulating and recording electrodes had to be placed in close proximity.

## VII. INSECTICIDE STUDIES.

Where possible insecticide solutions were made up in medicinal liquid paraffin or white oil. Both are inert substances which have no effect on the locust preparation over a period of several hours.

Check tests on intact locusts were carried out with the insecticide solutions before using them on the locust preparation. These check tests were those in standard use, namely the observation of the effect of small quantities of insecticide solution injected intra-abdominally into locusts. The quantity used was, as nearly as possible, similar to that used on the neurophysiological preparation. No.17 hypodermic needles were used, since finer needles would not allow the viscous liquid paraffin to pass through. The needle was inserted ventrally, to one side of the median line, in the soft joint between adjacent segments and directed forwards. Trials with coloured liquid, e.g., eosine, showed that, provided adequate care was taken, only rarely did the needle enter the alimentary canal.

Locusts into which liquid paraffin was injected showed no odd behaviour and lived quite normally after the operation: some marked specimens lived for weeks.

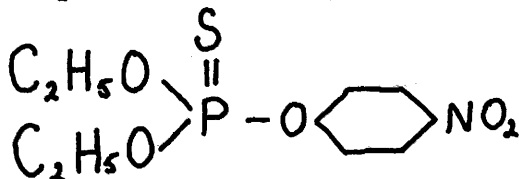
Intact locusts were also allowed to come into contact

with filter paper painted with the appropriate insecticide solutions.

These preliminary tests were the only tests in the course of the whole work where the substance could be subjected to physical or chemical action either during penetration through the cuticle, or due to the metabolic activity of an intact animal. In all other tests the substance was applied direct to the organ concerned, in this case the C.N.S., and no allowances were made for possible alteration in the chemical or physical constitution which could take place in an intact animal.

VIII. PARATHION.a). Review of literature.

Parathion belongs to the class of substances which are collectively and somewhat loosely termed organic phosphates. It is para-nitrophenyl diethyl thiophosphate



and also masquerades under the names E.605, DNT., and thiophos. This substance together with others in the same class, such as di-isopropyl fluorophosphonate (DFP), hexa-ethyl tetraphosphate (HETP) and tetra-ethyl pyrophosphate, was first studied by Michaelis in Germany (1890-1915). Just prior to and during the last war these substances were studied intensively and secretly in Germany by Schrader and to a lesser extent in Britain (Adrian, Feldberg and Kilby, 1947) with a view to their use in chemical warfare. A considerable amount of effort in this country was devoted to methods of defence against chemical warfare. Since it was very likely that the enemy would use new "secret" gases, one of the jobs was to try to anticipate the gases and find suitable antidotes. A publication in a German chemical journal in 1932 on alkyl fluorophosphonates had led a team of workers in Cambridge to examine



these substances further with the end of chemical warfare defence in view. Consequently a considerable amount of work on their toxicity to and toxicology in mammals has been carried out. Before the war Schrader had considered E.605, as he called it, a suitable substance for use as an insecticide. Since the war "converting swords into ploughshares" (Sir Henry Dale, 1947), many of these substances have been developed as insecticides. Most of the symptoms of poisoning in man and other mammals suggest that poisoning is due to an excess of acetylcholine. In vitro and in vivo experiments confirm this further by demonstrating that these substances are powerful cholinesterase inhibitors (Adrian, Feldberg and Kilby, 1947; Du Bois and Mangun, 1947; and Masur and Bodansky, 1946). It is thought that these compounds owe their insecticidal properties to this cholinesterase inhibition. It is, however, by no means certain that the transmission of nerve impulses in insects is, in fact, cholinergic. Roeder et al. (1940) have studied the action of DFP on the cockroach preparation originally used by Pumphrey and Rawdon-Smith, and find that the effects it produces fit in with this idea. DFP produces alternative periods of facilitation and block. According to Roeder these effects can be explained by accumulation of acetylcholine at the synapse due to inhibition of

the enzyme. Roeder suggests that at first the synapse appears "leaky" due to the persistence of Ach. This results in repetitive firing, i.e., several postganglionic impulses follow each preganglionic one. This "leaky" period is the period of "facilitation". The concentration of Ach. at the synapse continues to rise until a certain critical concentration is reached; at this point synaptic "block" ensues. Meanwhile the acetylcholine gradually diffuses through the tissues away from the site of action so that the concentration at the synapse falls again below the critical level and facilitation recurs. Ach. concentration builds up again and another block follows, and so on.

Roeder put forward this hypothesis in a tentative fashion and it is not supported entirely by his observations on the action of Ach. itself. This substance itself has no effect on trans-synaptic conduction in the cockroach, probably because it is broken down by cholinesterase present before it is able to produce any recordable reaction. But it was surprising to find that none of the other known anti-cholinesterases such as physostigmine, prostigmine or strychnine produce alternate periods of block and facilitation, as observed after application of DFP. In the isolated nerve cord of *Periplaneta*, however, Ach. in high

concentration ( $10^{-4}$ M. and above) produces facilitation (Roeder and Roeder, 1939). Further, when applied to the cockroach preparation after previous treatment with  $10^{-5}$ M. DFP, Ach. ( $5 \times 10^{-6}$ M.) produces immediate synaptic block not preceded by facilitation.

These discrepancies could be explained on the assumption that transmission across insect synapses is effected by Ach. like substances rather than Ach. itself, or that the inhibitors of mammalian cholinesterase do not affect the insect tissue in the same way. In this connexion it is interesting to note that specific cholinesterase occurring in frog brain, also that found in the invertebrate Planaria is relatively insensitive to eserine (Hawkins and Mendel, 1946). Inhibition by eserine cannot therefore be regarded as a fundamental property of all "true cholinesterases". Certainly biochemists are discovering that certain enzymes do not possess anything like the specificity that they were formerly thought to have (Kilby, personal comm.) and it may be that some inhibitors are less specific than others, i.e., affect a wider range of substances than do others.

b). Experimental results with Parathion.

Since the time this work was begun (October, 1948) a great deal of information has been amassed regarding this

class of compounds, but despite Roeder's work with DFP on the cockroach, considerable doubt still existed regarding their mode of action in insects.

When I began work I had available only a crude sample of parathion, X.Y.191, which was supplied by the Plant Protection Co. and had the following composition:-

|   |        |
|---|--------|
| p-nitrophenyl diethyl phosphate                           | 70.3%  |
| triethyl phosphate  | 16.7%  |
| di-p-nitrophenyl monoethyl thiophosphate                  | 6.4%   |
| nitrophenol   | 2.6%   |
| Unaccounted for - incl. Na salts of ethyl phosphoric acid | 4.0%   |
|   | <hr/>  |
|   | 100.0% |

This crude substance was dark brown in colour with a strong odour. It was not soluble in medicinal liquid paraffin, but a relatively fine emulsion was made by subjecting the mixture to mechanical stirring for twenty minutes before use, using a glass stirrer.

1). Effect of crude parathion on the locust cercal nerve - giant fibre preparation.

The following series of dilutions were made up:-  
%age parathion in liquid paraffin

1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125,  
and applied to the locust cercal nerve - giant fibre

preparation by means of a No.1 squirrel hair paint brush. Each experiment with a different test animal was allowed to run for a period of two hours. For the purposes of comparison with Lowenstein's results with pyrethrum (1942) the length of time in seconds for the giant fibre action potentials to disappear was noted. In these early experiments both male and female locusts were used. The results are given in the following table:

TABLE II. Reaction time in seconds for various percentage concentrations of crude parathion.

| Conc.              | 1.0 | 0.8 | 0.6 | 0.4 | 0.2 | 0.1 | 0.05 | 0.025 | 0.0125    |
|--------------------|-----|-----|-----|-----|-----|-----|------|-------|-----------|
|                    | 82  | 97  | 90  | 130 | 308 | 165 | 274  | 708   | > 2 hours |
|                    | 23  | 95  | 129 | 227 | 205 | 260 | 404  | 447   | 2295      |
|                    | 130 | 125 | 109 | 87  | 119 | 275 | 170  | 350   | 3000      |
|                    | 104 | 87  | 123 | 110 | 120 | 220 |      |       | 630       |
|                    |     | 94  | 127 | 127 |     | 140 |      |       | 1620      |
|                    |     | 91  |     |     |     | 210 |      |       |           |
|                    |     |     |     |     |     | 200 |      |       |           |
| Total<br>No.Expts. | 4   | 6   | 5   | 5   | 4   | 7   | 3    | 3     | 5         |

Of these 42 experiments one, with the lowest concentration of parathion, failed to have an effect within the two hour limit. Analysis of variance on the time in seconds for the remaining 41 gives a value for F of 13.25. The value exceeded in only 1% of the cases is 3.12. Similarly analysis of variance calculated for adjacent groups shows that each concentration has significantly

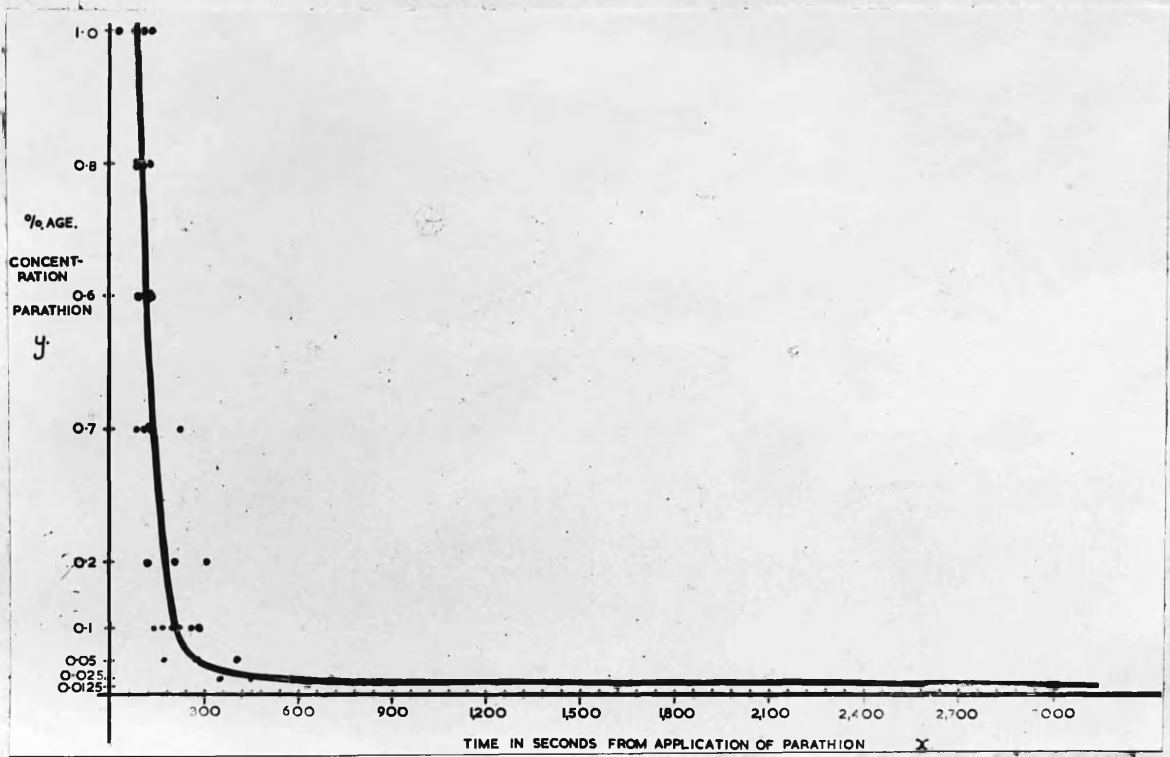


Figure 11. Time concentration curve for parathion. All the readings are plotted; where two or more points occur so closely as to overlap, they are displaced vertically. The concentrations marked on the y axis are actual concentrations used.

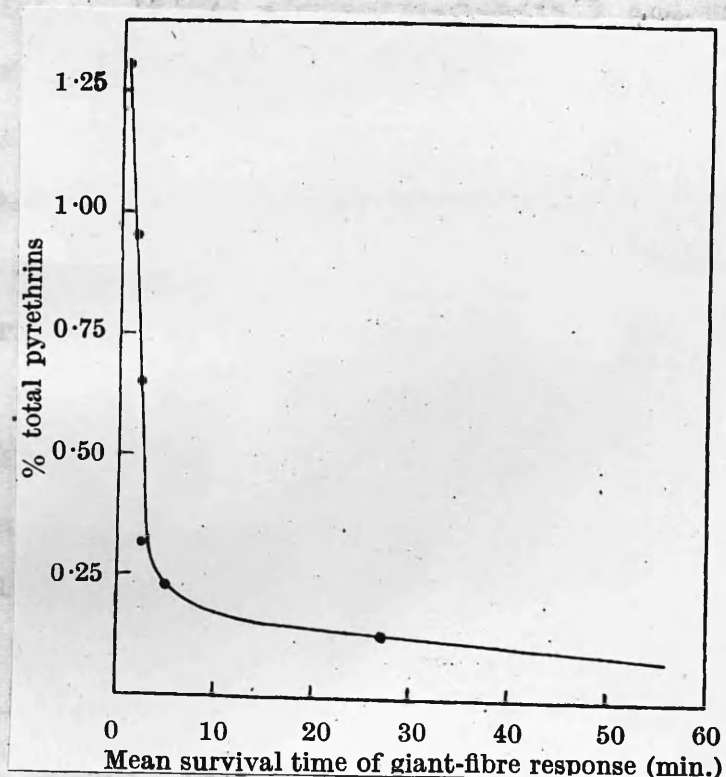


Figure 12. Time concentration curve for pyrethrum. The test animal was Periplaneta. (after Lowenstein, 1942).

different reaction times from that on either side of it. These results when plotted give a time-concentration curve similar to that for pyrethrum (Lowenstein, 1942), Figs. 11 and 12.

This curve is not a perfect rectangular hyperbola and will not yield a straight line on direct logarithmic conversion. It is clear that the curve will not cut either of the ordinates but that two thresholds are present. The concentration will have a lower limit, or threshold value, below which no effect will be produced. At the other end of the scale the time of reaction cannot be reduced below a certain limiting value, no matter how high the concentration applied. If the concentration is  $y$  and the time of reaction  $x$ , these threshold values  $k$  and  $b$  respectively, then the  $\log.(y-k)$  plotted against  $\log.(x-b)$  should yield a straight line. The values  $k$  and  $b$  could only be obtained by visual inspection of the curve. Since in one of the five experiments with the lowest concentration, 0.0125%, no effect was produced, it is fairly certain that this concentration is near the threshold value.  $k$  was therefore taken as 0.01%.  $b$  must obviously be less than the lowest value obtained with the highest concentration. Two values of  $b$  were chosen and the regression co-efficient and standard error calculated for each of these. When  $b$  is

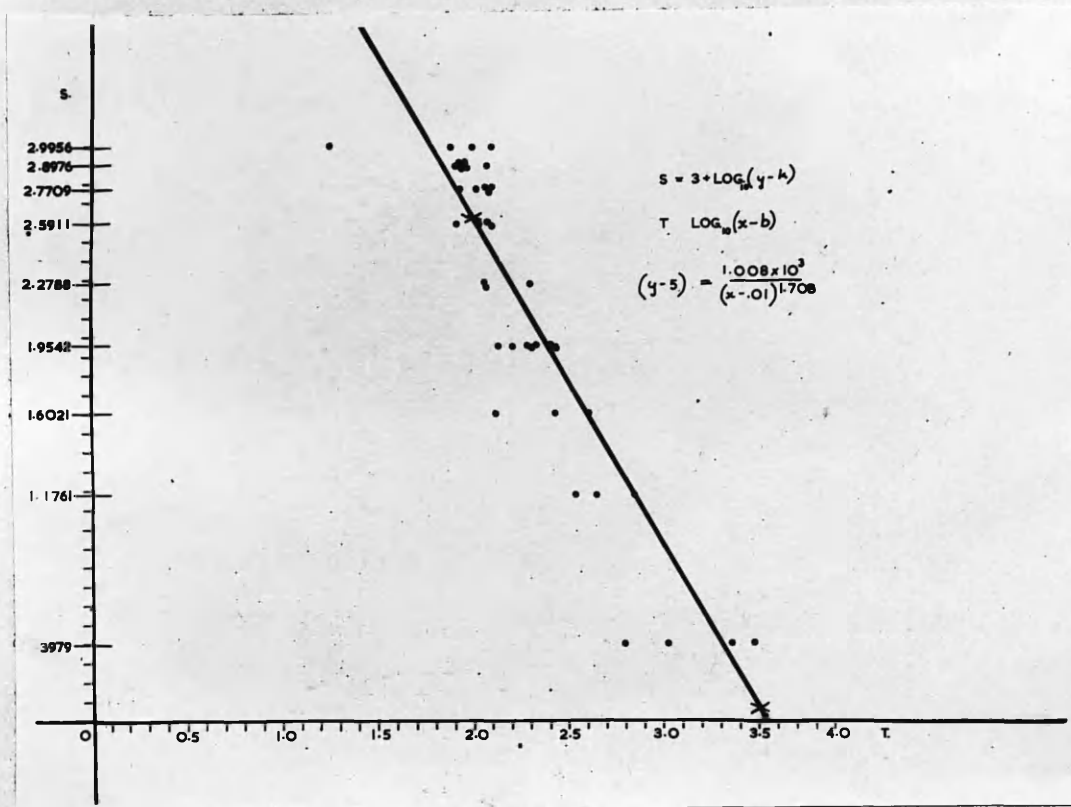


Figure 13. Time concentration curve for parathion subjected to modified logarithmic conversion. Adjacent points are displaced on vertical axis.

experiments the preparation was subjected to repetitive  
 external stimulation, at the rate of one puff of air  
 approximately every 90 seconds. During the block period  
 notice is made of the initial disappearance of action



15 seconds the standard error is 4.9015 and when b is 5 seconds the standard error is 4.7767. The latter value was therefore considered to be nearer the true value. Knowledge of the true value of b was not considered sufficiently valuable to warrant further calculation. The regression co-efficient for this latter value of b is -1.7082. The linear regression when tested for significance gives a value for F of 155.748: the value allowed for 1% of the cases is 7.33.

The graph is shown in Fig.13, where  $S = 3 \log.(y-0.01)$  and  $T = \log.(x-5)$ . From this it can be calculated that the concentration varies with the time of reaction in the following manner:-

$$(y-5) = \frac{1.008 \times 10^3}{(x-0.01)^{1.708}}$$

The reaction time referred to in these calculations was not, however, equivalent to the length of time taken to kill (the usual quantity denoted in insecticide literature). In all these experiments the giant fibre potentials returned in varying lengths of time. In almost all these experiments the preparation was subjected to repetitive natural stimulation, at the rate of one puff of air approximately every 20 seconds. During the block period which followed the initial disappearance of action

potentials there would be no detectable response to stimulation. Then usually an odd fibre would begin to fire spontaneously. The term "spontaneously" is used here to denote return of "basic activity" which occurs not in response to deliberate major stimulation. This "basic activity" must not be regarded as entirely "spontaneous". Some of it probably arises as the result of unavoidable spurious stimulation, since it is considerably reduced in preparations where the cerci are removed. Cutting the cercal nerve, however, does not result in a completely "silent" preparation. Since it is not possible at present to eliminate this spurious stimulation these stray action potentials will be termed "basic activity". Their re-appearance after a temporary disappearance or an increase in existing level, if not produced by obvious or deliberate stimulation, will be called "spontaneous" even though it does not necessarily mean spontaneous in the usual sense.

Almost immediately after the spontaneous appearance of action potentials following a block period, response to stimulation returned. Often this response was considerably more marked than normal. In other words, spike density, measured as the number of impulses/second, was higher than usual. This period was followed by another

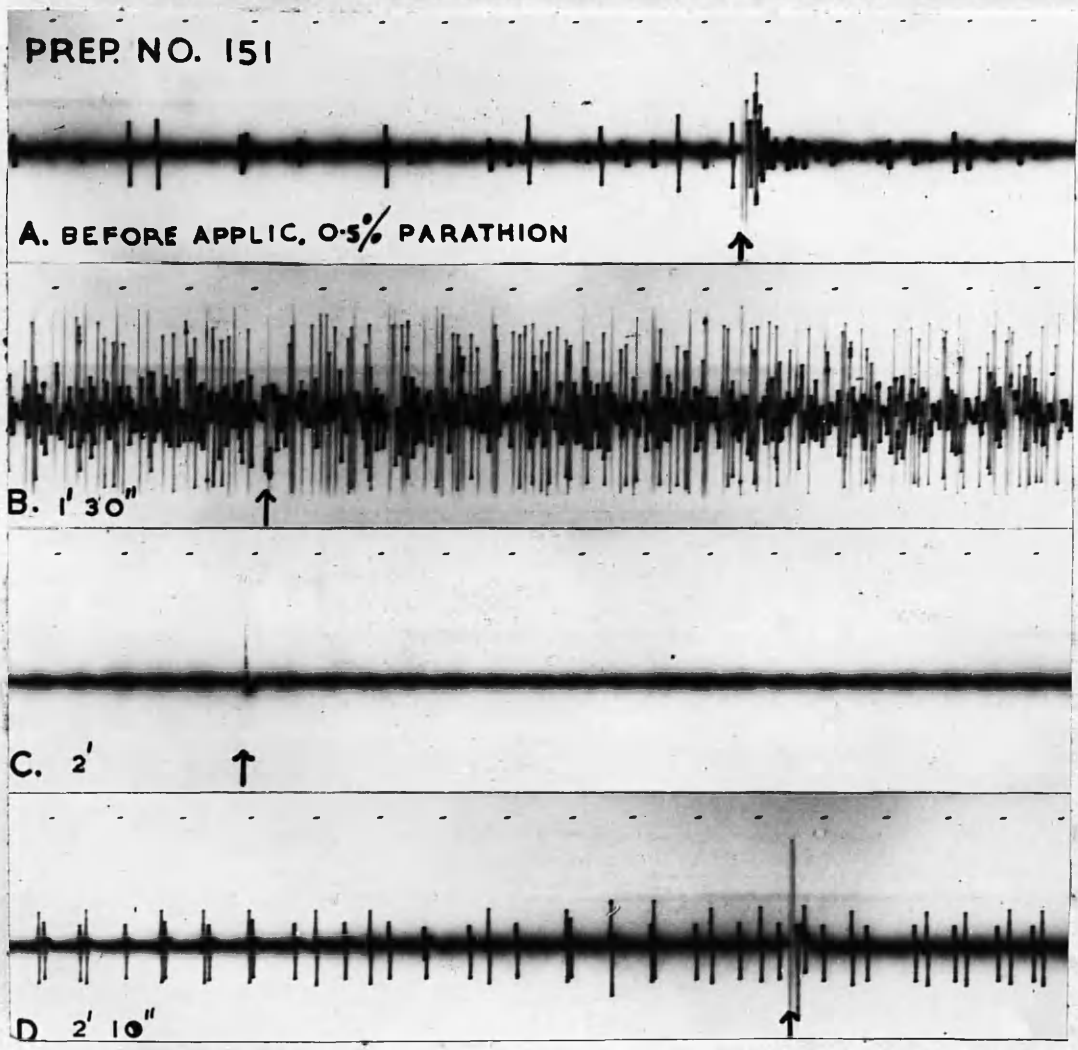


Figure 14. Oscillographic records showing various stages in parathion poisoning. A, normal preparation; B, C and D, various stages after application of insecticide. Upper time marker approximately 20/second. Arrows indicate electrical stimulation which was being applied at the rate of 48 pulses/minute. Record C shows complete block following facilitation period B. During the block period there is no response to stimulation, only the stimulus artifact is discernable. D shows recovery from block.

block, and so on. The alternation of periods of facilitation and block described by Roeder caused by the action of DFP on Periplaneta was produced almost identically by parathion on Locusta. After the initial block, time relationships appeared to have no significance, but varied between successive blocks in one preparation and both with concentration applied and with individual animals.

Fig. 14 shows the sequence of events in a typical preparation. These records were made at a later date with a different sample of parathion and when a square pulse electrical stimulator was available. The effects, however, are indistinguishable from those produced by the crude material.

When using "natural" stimulation, it was not always convenient to subject the preparation to regularly repeated stimuli since no mechanical device was used. Consequently some preparations received stimuli at random intervals. An interesting difference was noted between preparations subjected to these two sets of conditions.

As has already been mentioned, the cercal nerve - giant fibre preparation when set up as described has a certain level of "basic activity" and a certain response density to natural stimulation, provided the latter is

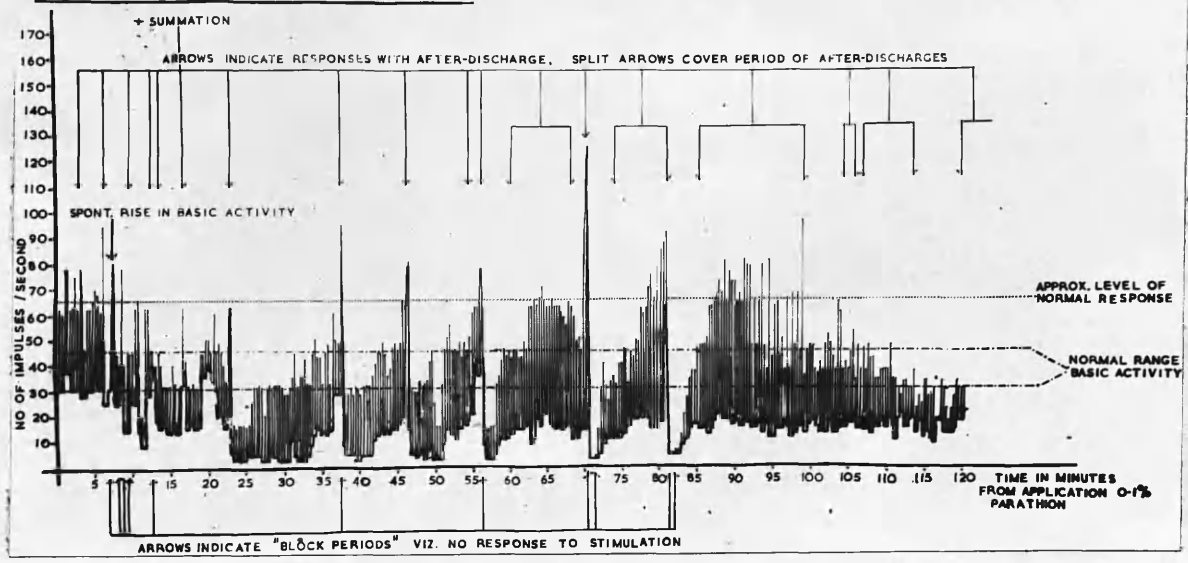


Figure 15. Graph showing number of impulses per second plotted against time, in a parathion poisoned preparation subjected to repetitive natural stimulation. Each vertical line represents increase from basic level to maximum response density. During block periods the counter reading does not return to zero due to small artifacts caused by stimulation.

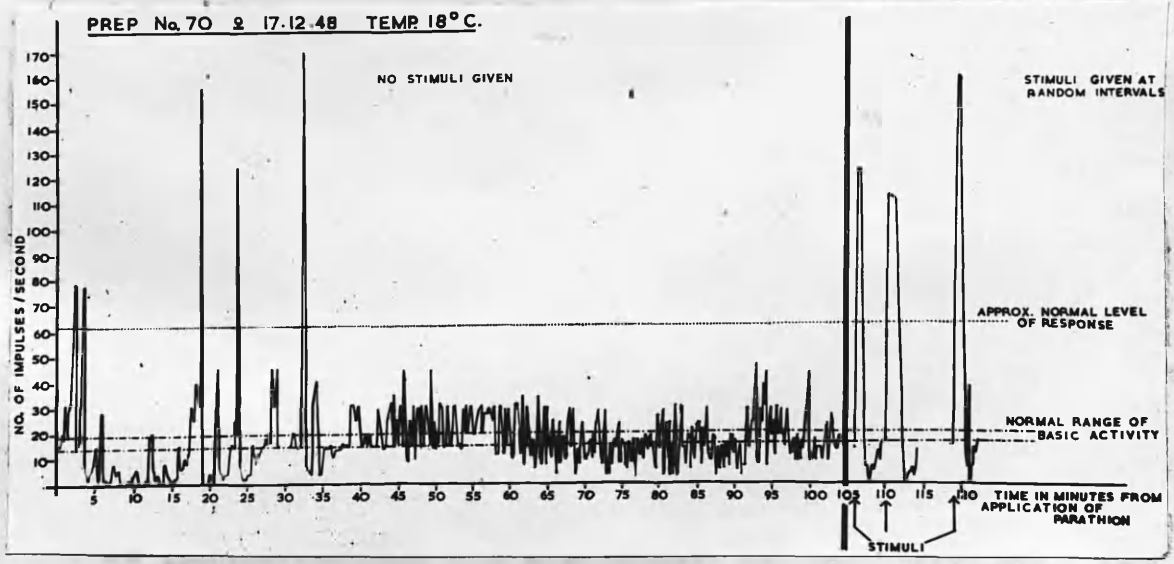


Figure 16. Graph showing number of impulses per second plotted against time in a parathion poisoned preparation without stimulation. This graph then, to the left of the double line, at 105 minutes, represents only changes in basic activity. After 105 minutes 3 stimuli were given at random intervals.

uniform. These levels remain fairly constant for a given preparation while conditions are maintained as near normal as possible. By putting an electronic counting-rate-meter across the output terminals of the amplifier they can be measured quantitatively. The measurements given in Figs.15 and 16 are, however, not exact measurements but relative ones, since it was necessary to parasitise the input to the counter with a resistor in order to count only the giant potentials: the instrument was not calibrated for these conditions.

Figs.15 and 16 show counter readings plotted against time taken every 10 seconds throughout the length of two experiments each lasting for two hours. In that illustrated by Fig.13 a puff of air was directed at the cerci every 20 seconds throughout the duration of the experiment. The counter readings represent alternately response density and basic level between successive stimuli. Each vertical line in the graph is the increase from this artificial basic activity to response level. In the other experiment no stimuli were given except for 3 at random towards the end of the two hour period. The curve in Fig.16 then is comparable with the fluctuations in basic activity under the influence of parathion. It must not be forgotten however that much of this basic activity is due to spurious stimulation which, under these

conditions, may initiate a whole sequence of events which cannot therefore be justly termed spontaneous.

Where there is repetitive stimulation there is a gradual stepwise increase in both response density and basic level until block occurs. This stepwise building up process recurs in each facilitation period. After-discharge where it occurs (marked by arrows in the diagram) is slight. Maximum response density is above normal but not spectacularly so.

When the preparation is left undisturbed the basic activity builds up, prior to a block, to a total firing density, of all fibres, of spectacular dimensions much higher than in the previous type of experiment. In some preparations this is repeated many times. In the experiment figured, however, an equilibrium seemed to be reached after a few times. Stimulation at this stage evoked an enormous response with considerable after-discharge.

It seems, then, that periods of facilitation occur much more suddenly and are much more marked, although often shorter, under the latter conditions than the former.

In preparations where both conditions were introduced alternately, it appeared that recovery from block could be induced by stimulation slightly before it would have occurred of its own accord. Stimulation at this

crucial moment produced an enormous response but probably also shortened the facilitation period thus begun. A facilitation period without a high density of action potentials would in all probability last longer.

2). Effect of a purified sample of parathion on the cercal nerve - giant fibre locust preparation.

In January 1949 a purified sample of parathion was obtained from Albright and Wilson and later in the year a square pulse stimulator was completed. A similar series of experiments was conducted with this as with the crude preparation. In each experiment an electrical stimulus was applied to the cercal nerve; the intensity of the pulse was adjusted to a value just above threshold for that individual. The frequency in all cases was 48 per minute. The response evoked in the ventral nerve cord was complex. It was made up of several spikes of varying sizes and conduction rates, the larger spikes being closer to the stimulus artifact than the smaller, as would be expected. Direct stimulation of the abdominal cord produced a more simple response, presumably since the possibility of spatial summation at the synapse was eliminated, and perhaps only those giant fibres nearest the electrodes were involved in the response. The threshold for each individual was found by gradually increasing the intensity



of the pulse from nil until a response was elicited. The threshold was determined for cercal nerve stimulation and then the same intensity applied directly to the cord.

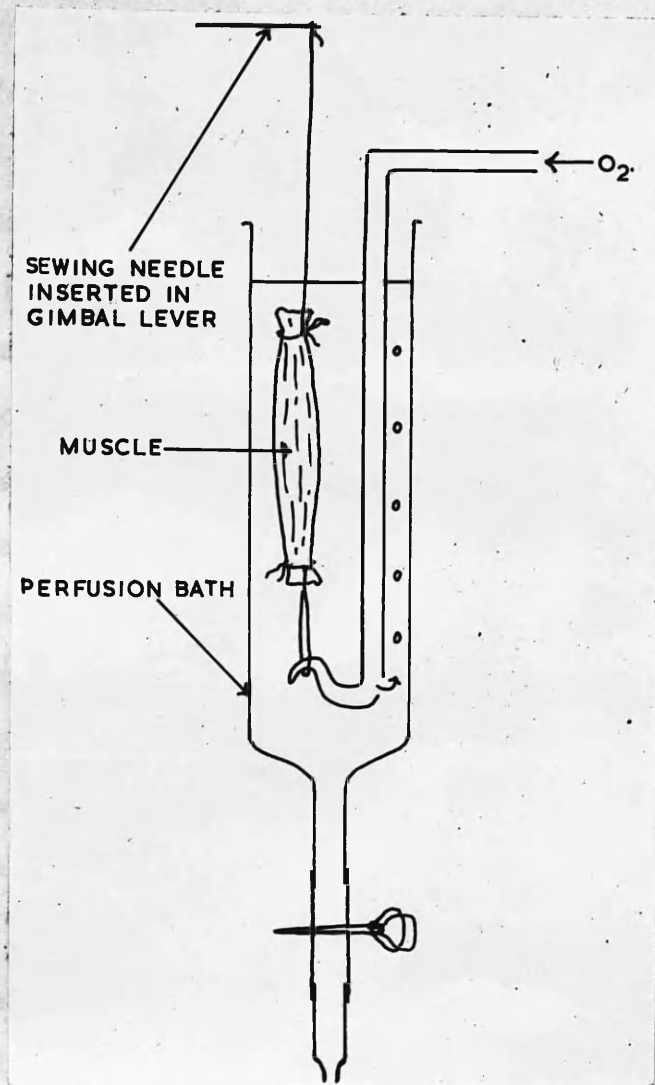
The general picture produced with this sample of parathion was identical with that produced by the crude sample, both with natural and artificial stimulation. The frequency of electrical stimulation (48/min.) was much higher than that for natural stimulation (3/min.); neither, however, was sufficient to fatigue the preparation. Since the response from natural stimulation was not strictly comparable with that from electrical, this difference in frequency was thought to be immaterial. The above described anomaly in behaviour between preparations subjected to constant repetitive stimulation and those with no stimulation or only few occasional stimuli was again observed.

Can all these observed phenomena, i.e., alternation of periods of facilitation and block and the different behaviour between preparations subjected on the one hand to repetitive stimulation and on the other occasional or no stimulation, be explained on the basis of cholinesterase inhibition by parathion? Obviously an anti-cholinesterase ought only to be effective in a cholinergic system where cholinesterase is present at the synapse.

c). The presence of cholinesterase in the fifth abdominal ganglion of the locust.

It is impracticable at this stage to determine

whether or not the cercal nerve is cholinergic owing to the smallness of the nerve concerned and its close association with other nerves whose functions are not certain. The technique involved in ascertaining the presence or absence of cholinesterase, however, is not a difficult one and reliable results can be obtained with little previous experience, as evidenced by students' results in practical classes. Frog rectus abdominis muscle can be made to contract by acetylcholine provided that its own cholinesterase has been previously destroyed. This rectus abdominis muscle preparation can be used as a sensitive test for the presence or absence of cholinesterase. The tissue to be tested is ground up and made into a homogenate with frog Ringer solution. The tissue is usually divided into two portions. One is made up with eserinated Ringer and the other with ordinary Ringer. Acetylcholine is then added to both portions and the solutions incubated at 37°C for at least 30 minutes. 1 cc. of each of these suspensions is added in turn, the perfusion fluid bathing the rectus abdominis muscle. If cholinesterase is present then the Ach. will be destroyed in the homogenate without eserine and the muscle will not be affected. In the eserinated sample the Ach. will remain and the muscle will then contract. For class



**Figure 17.** Muscle preparation used to show the presence of cholinesterase in the 5th abdominal ganglion of locust. Perfusion fluid was added to bath by means of a 50 cc. pipette and run out by opening tap at the bottom.

experiments rabbit brain is normally used and in the present experiments the technique was checked using rabbit brain. From stock frog Ringer 3 solutions were made up, eserinated Ringer, Ringer containing Ach., and eserinated Ringer containing Ach. The concentration of eserine was  $1/154,000$  parts and Ach.  $1/(5 \times 10^3)$ . The frog rectus abdominis muscle was dissected out and placed in eserinated Ringer 2 hours before being required. The fifth abdominal ganglion was dissected out from about thirty locusts, ground in an agate mortar and made into a homogenate with Ringer containing Ach. Ganglia from another thirty locusts were treated similarly but this time the homogenate was made up with eserinated Ringer containing Ach. Both solutions were incubated for thirty minutes in a water bath maintained at  $37^{\circ}\text{C}$ . The frog rectus abdominis muscle was set up as shown in Figure 17. Oxygen was bubbled through the bath sufficiently slowly not to interfere with the recording of the gimbalever. From the results shown in Figure 18 it is evident that a cholinesterase is present in the fifth abdominal ganglion. Similar experiments were also carried out in which leech muscle was used instead of the frog rectus abdominis muscle. These results are shown in Figure 19.

It thus seems not unreasonable to suppose that

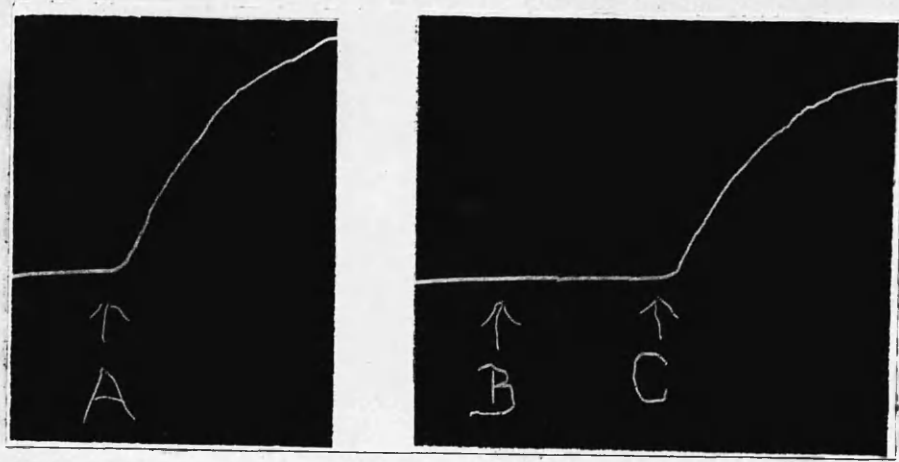


Figure 18. Kymographs showing the effects of A, acetylcholine, B, homogenate of 5th abdominal ganglia with Ach., and C, homogenate of 5th abdominal ganglia with eserine and Ach., on frog rectus abdominis muscle.

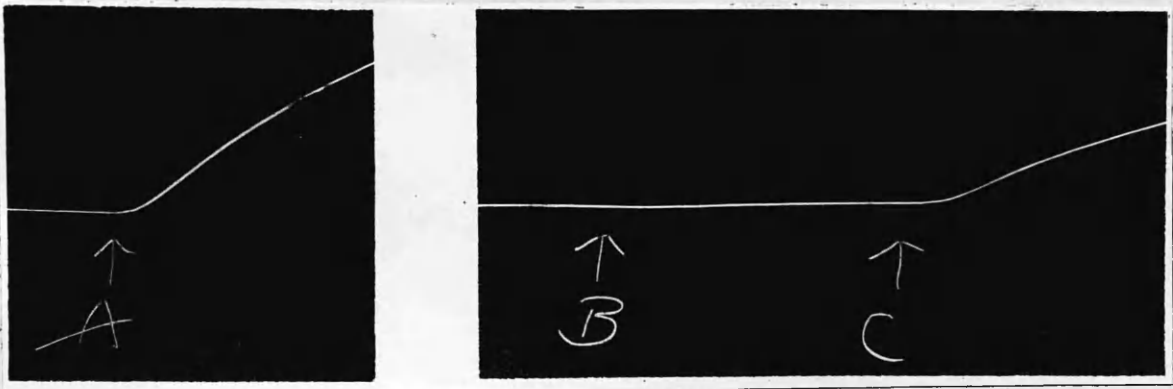


Figure 19. Kymographs showing effects of solution A, B and C, as in figure 15, on leech muscle.

cholinesterase is present at the synapse between the afferent cercal nerve fibres and the post-ganglionic giants and that transmission across this synapse is effected by the liberation of Ach. Presumably Ach. is liberated only when an impulse arrives at the synapse on the preganglionic side. It is not possible to shield the preparation entirely from all stimulation so that impulses will be arriving at the synapse more or less continuously. Some of these impulses will be responsible for part of the basic activity of the locust preparation, although a great many will in all probability be subthreshold and will evoke no post-ganglionic action potentials.

d). Hypothesis to explain the behaviour differences between preparations with repetitive stimulation and those without.

Roeder et al. (1947) have suggested that the facilitation period of D.F.P. poisoning is due to an accumulation of Ach. which persists and hence the one to one relationship of presynaptic to postsynaptic impulses no longer exists. Each successive stimulus will increase this accumulation of Ach. until a critical concentration is reached above which the synapse is impassable or blocked. This stepwise accumulation might well cause the stepwise increase in response to stimulation and in basic activity

leading up to block observed in the locust preparations poisoned with parathion and subjected to repetitive stimulation (Fig.19). The recovery from block is more difficult to account for. Ach. will still be liberated at the presynaptic nerve endings by the arrival of impulses even during a block period. It is difficult to suppose that diffusion through the tissues away from the synaptic region should be faster during a block period than during a period of facilitation, and it seems, therefore difficult to accept Roeder's hypothesis. There is however another possibility of explaining the recovery from block in terms of acetylcholine. This will be discussed later.

Before this, let us consider a preparation without repetitive "natural" stimulation. Under these circumstances the accumulation of Ach. will be much slower and more gradual, since it is dependent solely on chance stimulation. It may be that all presynaptic impulses whether sub-threshold or not cause Ach. to be liberated, or it may be that only those above threshold produce Ach. Whether or not these impulses evoke post-synaptic impulses and whether or not all liberate Ach., the concentration at the synapse will gradually increase if the cholinesterase has been destroyed. This increase will be relatively smooth since the successive steps will be of negligible

size. Possibly due to the gradualness of this increase, facilitation will occur at a higher concentration of Ach. at the synapse than in the previous case. A phenomenon comparable with super-cooling in freezing point estimations occurs. Once facilitation begins, however, it is more marked and sudden, due perhaps to the higher concentration (cf., the jump back to normal freezing point level in the cooling curve analogy). A sudden stimulus applied at a crucial moment would evoke a facilitation period (as stirring will cause solidification in the super-cooled liquid).

This explanation would require the normal transmission to be a function not of the presence or absence of Ach. but the amount of Ach. released relative to the concentration already present. In normal preparations the two theories are equivalent since the initial concentration is zero. When the concentration of Ach. is allowed to build up the difference between these two theories becomes an important one. When there is no Ach. present only a very small quantity is needed to produce post-synaptic potentials. As Ach. accumulates the quantity liberated at the presynaptic endings is only effective when it is considerably larger, and probably only when a number of presynaptic impulses arrive simultaneously would sufficient Ach. be liberated.



e). A new hypothesis to explain alternating periods of facilitation and block.

The block period and the alternation of periods of facilitation and block have been explained by Roeder by accumulation and diffusion of acetylcholine. In discussing this matter with Dr. Feldberg another possibility was envisaged which is based on some recent findings about the acetylcholine output from perfused eserinated cervical sympathetic ganglia of cats during prolonged periods of preganglionic stimulation as shown by Dr. Perry (personal communication).

In Perry's experiments the output of Ach. starts at a high level but quickly falls down to a low steady level. If stimulation is then interrupted for a few minutes, a new period of stimulation does not start with high Ach. output but continues at the low level finally reached in the first period. If the first period of stimulation was in the absence of eserine the second period starts with a high level. Nevertheless the reduction in the output of Ach. is not due to an action of eserine itself because perfusion of the ganglion with eserinated solution for a period as long as the stimulation period, does not reduce the Ach. output of a subsequent stimulation period, which starts at the high level. These results clearly

show that when Ach. is not destroyed but bathes the nerve ending, the ability of the latter to produce more Ach. is reduced.

A simple explanation of this fact would be to assume that the persistence of Ach. in the neighbourhood of the nerve ending has a toxic effect on it. It is difficult to explain Perry's results in any other way and in any case, it is known that anticholinesterases in large concentrations have a paralysing effect on nerve conduction. If in the presence of Parathion the accumulation of undestroyed Ach. causes overdepolarisation so leading to a blocking effect and if the accumulated Ach. poisons the nerve ending, the alternation between facilitation and block can be easily explained. The block would be due not only to overdepolarisation, but also to cessation of Ach. release. If, however, the cessation of Ach. release took no part in the sequence of events leading up to and causing block, but merely occurred as a side reaction, the block would still come to an end, since Ach. escaping into the haemocoel would reduce the concentration at the synapse. When the concentration reaches a sufficiently low level, facilitation will recur; recovery of the nerve endings with consequent liberation of Ach. will allow the concentration to build up again until block once more occurs.

This theory would also explain why a sudden stimulus near the end of a block period, just as the preganglionic fibres are recovering, may cause a premature liberation of Ach. and so cause the enormous response and after-discharge characteristic of a preparation which has not previously been stimulated for some time.

These explanations are at present hypothetical and must await experimental confirmation. With the present available methods, the possibility of measuring Ach. liberation in the locust, such as was done with cat cervical ganglion (for complete list of references see Feldberg, 1950), seems rather remote. Measurements of depolarisation in the giant neurons both in normal preparations when an impulse is conducted across the synapse and in preparations treated with various substances might give valuable information in favour or against one or the other of the theories suggested. Since at least one of the giant neurons in each cord arises from a cell body of diameter of about  $50\ \mu$  and is situated in the posterior peripheral region of the ganglion, the technique involved should not be too difficult. Further attempts should also be made to obtain a preparation with an isolated nervous system.

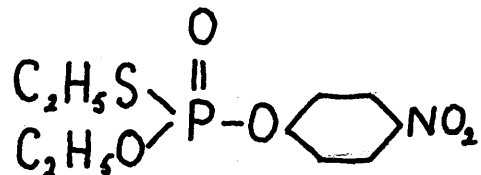
f). Effect of repetitive electrical stimulation.

Electrical stimulation made it possible to confirm the supposition that parathion has a ganglionic effect and therefore, presumably, a synaptic effect. Pre-ganglionic stimulation during block evoked no response, whereas post ganglionic stimulation produced a normal response. In other words, axonic conduction was not affected. In some preparations it was noticed that if the stimulus was switched from the preganglionic position to the post ganglionic immediately on the onset of block, there did appear to be a fleeting axonic block. The possibility that this might be due to an artifact caused either by switching or by an occasional missed stimulus was not completely eliminated however. Also it was easy to miss the moment when the stimulus should be switched. Some preparations were left with stimulation of the cord going on. These also showed fleeting moments of apparent axonic block which may have coincided with synaptic block onset, although it was difficult to be certain. If this effect is not an artifact it would fit in with the idea of extensive depolarisation of post-ganglionic neurons due to Ach. accumulation, and would support the hypothesis regarding the nature of the parathion effects as suggested on pages 46 - 48. The present evidence is very slight

and until further experimental evidence can be brought forward, for instance depolarisation measurements and simultaneous comparison of cord and axonic block, it must be treated with considerable wariness.

g). Comparison of the cholinesterase inhibition by parathion with that by the S-ethyl isomer.

Diggle and Gage (1950) pointed out that cholinesterase inhibition in vitro of parathion decreased with decreasing contamination with the S-ethyl isomer



Samples of parathion and its isomers obtained in October 1949 from Messrs. Albright and Wilson confirmed this observation, on the locust preparation. A 4% mixture of parathion with liquid paraffin produced only facilitation, i.e., after-discharge to stimulation and some spontaneous repetitive discharge, but no block. The S-ethyl isomer at this concentration produced enormous upheaval and death almost instantaneously.

More samples were obtained from Mr. Aldridge of the M.R.C. Unit of Toxicology, Carshalton in 1951. These were made up into 2% solutions in medicinal liquid paraffin.

When injected intra-abdominally into locusts both parathion and the S-ethyl isomer produced characteristic symptoms, but the latter much more rapidly than the former. When applied to the locust cercal nerve - giant fibre preparation both solutions produced the expected sequence of events leading to block. Facilitation, spontaneous repetitive discharge and finally silence. The parathion itself, however, did not show these symptoms until about 15 minutes after application of the poison, whereas symptoms occurred in the isomer within 40 seconds after application. The block period in the parathion preparations was very short and after the first block became less frequent, transitory and finally apparently disappearing altogether. Preparations with S-ethyl isomer did not recover at all from the initial block within a two hour period after application of the poison. This picture was not altered when the solutions were added to preparations already covered with paraffin, thereby reducing the concentration, the only difference being one in the "reaction time", which was extended to about 30 minutes for parathion and 3 - 5 minutes for the isomer.

These experiments show that the S-ethyl isomer has a higher toxicity than pure parathion. However, the symptoms produced by the latter if not identical with those

produced by the commercial substance are very like them, and removal of all the impurities, TEPP and the isomers, does not eradicate those symptoms which are thought to be due to accumulation of Ach. as a consequence of cholinesterase inhibition. The symptoms produced by the S-ethyl isomer, on the other hand, do not conform with the symptoms of either parathion or DFP. This is apparently not due to concentration, since even when the first block does not occur until 7 minutes after application, no recovery takes place within 2 hours.

Diggle and Gage (1951) showed that parathion is converted in vivo to a more powerful cholinesterase. Aldridge and Barnes (1952) demonstrated that parathion has greater cholinesterase inhibition when injected intraperitoneally or intravenously into rabbits than when an equivalent concentration is incubated with rabbit blood in vitro. The substance therefore must in vivo be converted into an inhibitor. The locust experiments lend support to this idea. Also it would seem that the symptoms observed after application of the less pure substance are a combination of those produced by the substance itself together with its impurities. Further in vivo studies with these substances should, therefore, be undertaken.

## IX. DNOC.

### a). The Preparation of a Solution and Experimental Results.

2,4-dinitro-ortho-cresol is supplied in the pure state as a yellow powder, with a melting point of  $86.25^{\circ}\text{C}$ . It is soluble in organic solvents such as alcohol, ether, acetone, etc., but only very slightly soluble in water, viz., 1 part in 7813 at  $15^{\circ}\text{C}$ . It soon became evident that it was only very slightly soluble in medicinal liquid paraffin. It was not possible to dissolve a known small quantity of DNOC in a known volume of liquid paraffin. A solution could however be made by stirring a large excess of DNOC with liquid paraffin for a considerable time and then filtering off the excess DNOC through a sintered glass filter. The problem then was to make a quantitative chemical estimation of the strength of the solution. Direct titration with potassium hydroxide of a known volume shaken with water, using phenol phthalein as an indicator, was not satisfactory; since with both oil and water phases present only a very indeterminate end point was reached. Extraction with alcohol and then titration created a pH value 8 which was unsuitable for this indicator. Extraction with water was more successful, but such a large quantity was required, about 1500 ccs., to extract 5 ccs. of solution that the blank titre formed 30 to 40% of the



total titre. Volumetric methods were accordingly abandoned and a quantitative recovery method tried. 5 ccs. of solution were extracted with 5% sodium hydroxide of which a relatively small quantity only is needed. This solution was evaporated to small bulk, acidified and extracted with ether. The ether was distilled off, the residue redissolved in ether, which was then distilled off again. The final residue was rather large and did not have the characteristic bright yellow appearance. It was, therefore, regarded with suspicion and the method abandoned. No further attempts were made at quantitative analysis; a solution of known concentration was made up as follows. A known weight of DNOC was dissolved in re-distilled, peroxide free ether, mixed with a known volume of paraffin, and the ether distilled off under reduced pressure, the flask being kept in a boiling water bath. The following series of dilutions were then made up from the resultant solution:

$1.2 \times 10^{-2} \text{M.}$ ,  $6 \times 10^{-3} \text{M.}$ ,  $3 \times 10^{-3} \text{M.}$ ,  $1.5 \times 10^{-3} \text{M.}$ ,  
 $1.2 \times 10^{-3} \text{M.}$ ,  $1.2 \times 10^{-4} \text{M.}$

These solutions were then applied to the locust cercal nerve - giant fibre preparation and the time of disappearance of spikes recorded. The results are given in the following tables. Those given in table III are from

preliminary experiments carried out to ascertain whether these concentrations were within a suitable range for this technique. There, the concentrations were not known accurately.

TABLE III. Time in minutes for giant fibre potentials to disappear.

| Conc. | $2 \times 10^{-2} M.$ | $2 \times 10^{-3} M.$      | $2 \times 10^{-4} M.$          |
|-------|-----------------------|----------------------------|--------------------------------|
|       | 1' 20"<br>32'         | 5' 30"<br>5' 25"<br>4' 50" | 27'<br>50'<br>no effect in 60' |

TABLE IV. Time in minutes for giant potentials to disappear.

| M. Conc. | $1.2 \times 10^{-2}$              | $6 \times 10^{-3}$ | $3 \times 10^{-3}$          | $1.5 \times 10^{-3}$ | $.12 \times 10^{-3}$       | $1.2 \times 10^{-4}$ |
|----------|-----------------------------------|--------------------|-----------------------------|----------------------|----------------------------|----------------------|
|          | 2' - 3'<br>1' 20"<br>1' 30"<br>1' | no effect          | 2' 30"<br>2' - 3'<br>7' 45" | 5' 30"               | 6' 47"<br>9' 30"<br>8' 50" | no effect            |

Although there is a general trend towards a normal time - concentration curve, there are certain gross anomalies in these few experiments. It had previously been noticed, during the chemical analysis, that there is a selective

accumulation of DNOC at the oil/water interface, which assumes a deep yellow colour. It is probable that there will be selective accumulation at the oil/water interface in the locust preparation which will give rise to anomalous quantitative results. Although it was not absolutely vital for the purposes of the present work to obtain a time - concentration curve, it was felt desirable to seek a medium for application of this insecticide, where some reliability could be placed on quantitative results. The obvious medium was saline.

Control experiments were carried out with Bělár (1929) saline which has the following constitution:

|                    | gms./litre |
|--------------------|------------|
| Sodium chloride    | 9.0        |
| Potassium chloride | 0.2        |
| Calcium chloride   | 0.2        |
| Sodium carbonate   | 0.2        |
| Dextrose           | 4.0        |

Na:K ratio 33.7 approximate osmotic pressure in mM 181.67  
salt:sugar ratio 2.4.

This saline maintained the locust cercal nerve - giant fibre preparation in a satisfactory condition over the arbitrary experimental period of one hour. Since the saline readily grows moulds, it was not stored ready for use but individual stock solutions of higher concentration of each of the constituents were kept and small quantities of saline made as required, sterilized if necessary before the addition of the buffer.

The main difficulty experienced in attempts to prepare aqueous solutions of DNOC was concerned with the wetability of the compound. Centrifuging, grinding in agate mortar, solution in vacuum all proved unsuccessful. Finally a stock solution was prepared by pouring distilled water at 70°C through a sintered glass filter containing an excess of DNOC and the filtrate collected. It was difficult to rid the residue in the filter of the remaining traces of water; the concentration was therefore estimated volumetrically by titration with carbonate free barium hydroxide or with potassium hydroxide using phenol phthalein as an indicator. This method repeatedly yielded stock solutions of  $10^{-3}$ M. The pH value for this solution is in the region of from 3 to 4. The value is only approximate since this solution has a very low conductivity.

It is clear then that a further difficulty is encountered here. DNOC, due to the presence of free phenolic groups, behaves as a pseudoacid. The pH value for Bělř saline is 7.8 and for DNOC in saline 6.5.

The dissociation constant (pK) 2:4-dinitrophenol is given as approximately 4.2. It is likely that the cresol will have a similar value.

Since the acid dissociates into its ions thus:



$$K = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]}$$

$$K = \frac{[\text{H}^+] \times [\text{A}^-]}{[\text{HA}]}$$

$$-\log K = -\log \text{H}^+ - \log \frac{[\text{A}^-]}{[\text{HA}]}$$

$$\text{or } \text{pK} = \text{pH} - \log \frac{[\text{A}^-]}{[\text{HA}]}$$

$$\text{H}^+ \rightleftharpoons \sqrt{K_a c}$$

$$\text{pH} = \frac{1}{2} \text{pK}_a - \frac{1}{2} \log c \quad \text{where } \text{pK}_a = \text{pK for the acid}$$

$c = \text{concentration}$

since pH measured = 3.4

concentration = .0011 M.

$$\begin{aligned} -\frac{1}{2} \text{pK}_a &= -\text{pH} - \frac{1}{2} \log c \\ &= -3.4 - \frac{1}{2} \log .0011 \\ &= -3.4 - \frac{1}{2} (3.0414) \\ &= -3.4 + 1.52 \\ &= -1.9 \end{aligned}$$

calculated  $\text{pK} = 3.8$  ( $\pm 0.3 = \text{estimated error}$ ).

Alternatively if  $\text{pK}$  is assumed to be 4.2, calculated pH is 3.3, agreeing closely with the observed value of 3.4.

Measurement of the depression of freezing point  $\Delta$  for DNOC confirmed that the estimated concentration was of

the right order of magnitude.

It is, therefore, not unreasonable to assume the pK value for DNOC to be 4.2.

When pH = pK ratio of free acid/salt is almost equal to 1  
 at pH 3.2 \_\_\_\_\_ " \_\_\_\_\_ 10/1  
 at pH 6.2 \_\_\_\_\_ " \_\_\_\_\_ 1/100

DNOC solution in Belar saline will contain a negligible quantity of DNOC relative to its sodium salt. The literature on the subject seems equally divided as to whether the sodium salt of DNOC is as effective an insecticide as the free acid (Decker and Drake, 1940, Dierrick, 1943, and Goble and Patton, 1946). Since the purpose of this work is an attempt to analyse the mode of action of DNOC as an insecticide, the object will not be attained by a study of the action of this solution. If the sodium carbonate buffer is omitted from the saline, the pH more nearly approximates to that of the insecticide alone.

Further control experiments were undertaken with both buffered and unbuffered saline. These experiments were so arranged that the identity of the solutions was concealed to the experimenter until after a series of twelve tests had been completed. It was not possible to distinguish between the two salines by a study of the behaviour of the locust preparation. It was clear that precautions

must be taken to prevent evaporation of the saline and possible concentration of the salts. This could be done by surrounding and roofing over the preparation with saline-soaked cotton wool. Here precautions must be taken to prevent flooding, or disturbance of recording by A/C pick up becomes considerable. Another method used in some preparations was to add a small drop of the solution, using a hyperdermic syringe and number 20 needle, at fairly frequent intervals during the course of the experiment. Roeder (1947) found that pH's of 2.5 and above did not affect a similar cockroach preparation, whereas pH 2.0 blocked both synaptic and axonic conduction. As the pH of all solutions of DNOC are above 3.4, we can discount any observed effects being due to reduced pH and consider them as true toxic effects of DNOC.

Since the stock solution was  $10^{-3}$  M., the most concentrated solution possible with saline was  $8 \times 10^{-4}$  M. This concentration is very near the lower end of the preliminary "paraffin range".

TABLE V.

DNOC Solutions.

| Label | Saline     | Concentration                   |
|-------|------------|---------------------------------|
| A     | Buffered   | $8 \times 10^{-4} \text{M}$ .   |
| B     | Buffered   | $4 \times 10^{-4} \text{M}$ .   |
| C     | Unbuffered | $8 \times 10^{-4} \text{M}$ .   |
| D     | Unbuffered | $4 \times 10^{-4} \text{M}$ .   |
| E     | Unbuffered | $2 \times 10^{-4} \text{M}$ .   |
| G     | Unbuffered | $1.5 \times 10^{-4} \text{M}$ . |

A few preliminary experiments were carried out with solutions of concentrations  $8 \times 10^{-4} \text{M}$ ,  $4 \times 10^{-4} \text{M}$ ,  $2 \times 10^{-4} \text{M}$  made up in unbuffered saline. The reaction times are given in table VI.

TABLE VI. Time in minutes for giant fibre potentials to disappear.

| C $8 \times 10^{-4} \text{M}$ | D $4 \times 10^{-4} \text{M}$ | E $2 \times 10^{-4} \text{M}$ |
|-------------------------------|-------------------------------|-------------------------------|
| 3'<br>6'                      | 10'<br>11' 10"                | no action<br>24'              |

These experiments, together with the earlier ones with paraffin solutions, showed that DNOC had no spectacular neurotoxic effects. The final disappearance of action potentials is preceded by an increase in the basic



rate of firing. In order to gain a clear picture of the sequence of events it was desirable to spread this sequence out in time. Solutions C and D were not, therefore, very suitable. A solution which approximated to the threshold concentration of these particular experimental conditions would obviously be most suitable. A further two solutions F and G were made up, with concentrations  $1 \times 10^{-4} \text{M}$  and  $1.5 \times 10^{-4} \text{M}$  respectively. Table VII gives the reactant times.

TABLE VII. Time in minutes for giant fibre potentials to disappear.

| E. $2 \times 10^{-4} \text{M}$ | G. $1.5 \times 10^{-4} \text{M}$ | F. $1 \times 10^{-4} \text{M}$ |
|--------------------------------|----------------------------------|--------------------------------|
| 20' 40"                        | 12'                              | 8 no effect                    |
| 12'                            | 15'                              |                                |
| 30'                            | 16'                              |                                |
| 6' 30"                         | 15'                              |                                |
| 36'                            | 59'                              |                                |
| 7' 10"                         | 15 no effect                     |                                |
| 8'                             |                                  |                                |
| 2' 45"                         |                                  |                                |
| 9'                             |                                  |                                |
| 13' 25"                        |                                  |                                |
| 26'                            |                                  |                                |
| 11'                            |                                  |                                |
| 33'                            |                                  |                                |
| 6 no effect                    |                                  |                                |
| 19                             | 20                               | 8 total number of expts.       |

Analysis of variance, using as data the reactant<sup>ion</sup> times, i.e., the length of time in minutes for the giant spikes to disappear, gave a value for F of .875. The permitted value of F was 4.6. There was, therefore, no significant difference between the two series in this respect.

In order to ascertain whether it was justifiable to suppose that the threshold, for these experimental conditions, lay between these two concentrations on the basis of these comparatively few experiments (39) a  $\chi^2$  test was applied: the numbers with effect and without effect being considered.

F value was 7.547  
giving a value for P of .01 - .001.

The two series are significantly different in this respect and it is likely that the threshold concentration lies between  $2 \times 10^{-4}M$  and  $10^{-4}M$  DNOC dissolved in unbuffered Belar saline.

At this stage attempts to obtain quantitative data with this particular insecticide were abandoned in favour of a purely qualitative observation of the effects of DNOC on the behaviour of the preparation. No attempt was made during the course of these experiments to maintain regular natural stimulation, but stimuli were given at random, at comparatively infrequent intervals throughout the course of

A BEFORE APPLIC.  $4 \times 10^{-4} M$  DNOC

PREP. NO. 299

B 2' 0"

B

C 3' 0"

D 3' 10"

E 3' 20"

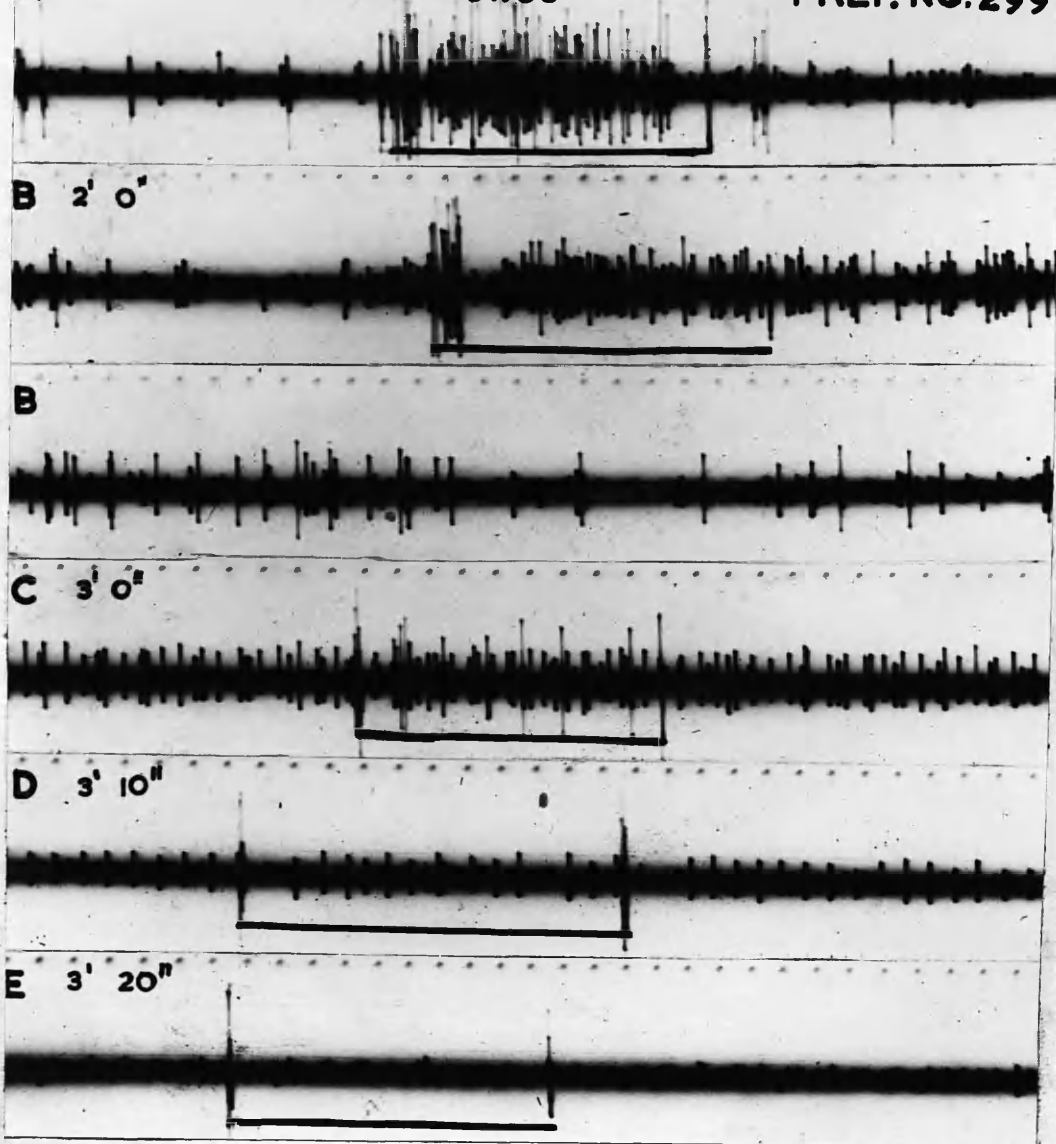


Figure 20. Oscillographic records showing various stages in the effect of DNOC on the locust cercal nerve - giant fibre preparation. Time marker 50/second. These records were made on play back from a tape recorder. Continuous line indicates duration of natural stimulation; when there is no response the condenser discharge which marks the beginning and end of each stimulus can be seen. B and C show increase in excitability and basic level of activity. D shows a decline of this phase and E final stage after all action potentials have disappeared.

In those experiments with concentrations

the following sequence of events occurred

the experiment.

The most usual effect was an increase in basic activity, the onset of which was often gradual and at the same time a decrease in action potential density in response to stimulation, until shortly before cessation of all activity there was no response to stimulation, Figure 20. The moment of cessation of firing was not marked by trains of spikes of high frequency such as usually occur in a dying fibre or preceding block in a parathion poisoned preparation. All fibres did not necessarily cease to fire at the same instant, although the individual moments of cessation were not widely spaced in time: usually all occurred within a single minute. In no case was there any recovery but occasionally a single fibre would begin firing spontaneously a short time after the bulk had reached their critical maximum density and stopped. The rate of firing of this fibre would increase and then it too would stop. This was considered to be simply a belated reaction.

This sequence of events happened in all DNOC experiments with the exception of some of those where near-threshold concentrations were used, although the reaction time could not be predicted with accuracy as with parathion or pyrethrum. In those experiments with concentrations near threshold value the above sequence of events occurred

in some, but in others it appeared to be masked by muscle potentials which behaved similarly. In others again, the preparation would behave normally for some time and then these symptoms would all be sandwiched together in a very short time interval. In a few, the action potentials disappeared without any preliminary increase in basic activity. Many showed no effect within the experimental time limit of one hour. This unreliability of preparations poisoned with concentrations of DNOC approximating to threshold values supports the hypothesis that DNOC is a metabolic poison affecting the C.N.S. only secondarily. A primarily neurotoxic substance would have an all or nothing effect: above threshold it would show its characteristic symptoms and below threshold it would be expected to have no effect. On the other hand, a drug affecting some other part of the metabolism might well be expected to have intermediate effects, if the observed effects in the C.N.S. were due to the metabolic disturbance and its products rather than the drug itself. This idea is in agreement with the conclusions of some other workers. Viado (1941) says that dinitrophenols are stomach poisons and seem to affect more than the nerves. Stellwaag and Standenmeyer (1940) suggest that the toxic effect of DNOC is due to coagulation of protein in living cells.

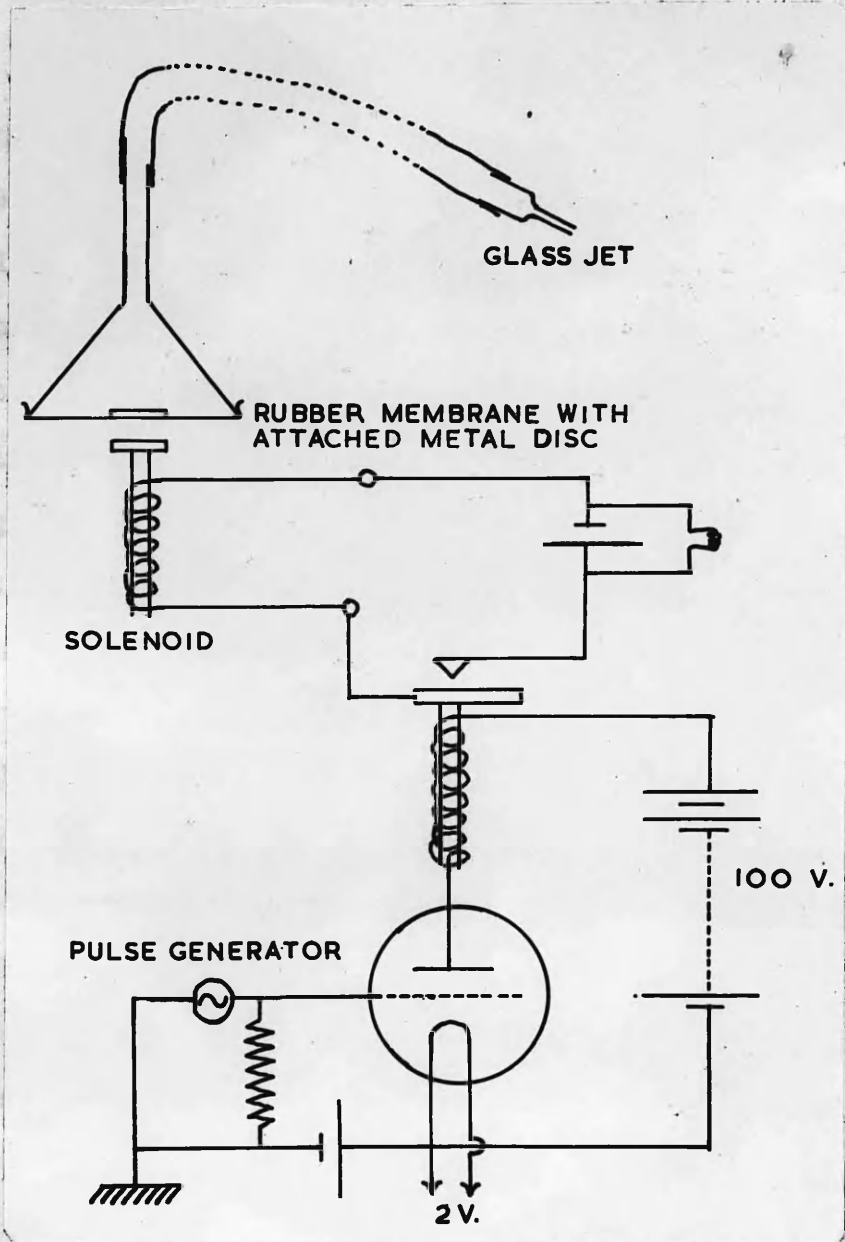


Figure 21. Diagram of apparatus used to deliver repetitive "air-puff" stimuli at the rate of 48/minute.

Parathion preparations which were subjected to prolonged repetitive stimulation showed a markedly different behaviour from those which received only sporadic stimulation. A few experiments were carried out with DNOC to discover whether this variation existed with this insecticide. Since it was not possible to use electrical stimulation when using saline solutions, the stimulator was arranged so that it delivered natural stimuli (Figure 21). The stimulator was made to work a relay which was connected to a small electromagnet: at each pulse the magnet attracted a piece of metal resting on a rubber membrane stretched across a glass funnel. By this means a small puff of air was directed at the cerci at the rate of 45 per minute. Control experiments with unbuffered saline showed that this rate of stimulation had no deleterious effect on the preparation within the experimental time limit. The response to stimulation at the end of the hour was in no way different from that at the beginning of the experiment. 12 preparations were observed, 6 with solution E and 6 with D. 4 of those with solution E had effect and 3 with solution D. In each of these 7 the sequence of events was identical with the previous experiments and the time of onset of symptoms comparable with the earlier preparations.

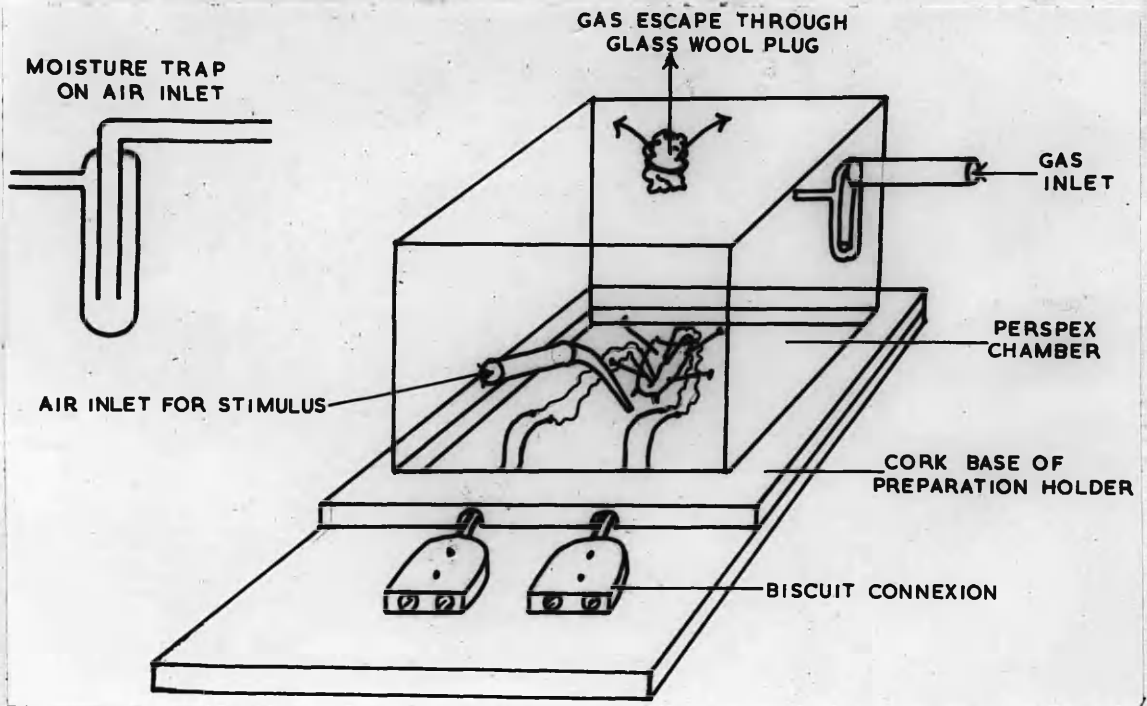


Figure 22. Anaesthetic chamber used in studying the action of carbon dioxide on the locust cercal nerve-giant fibre preparation.



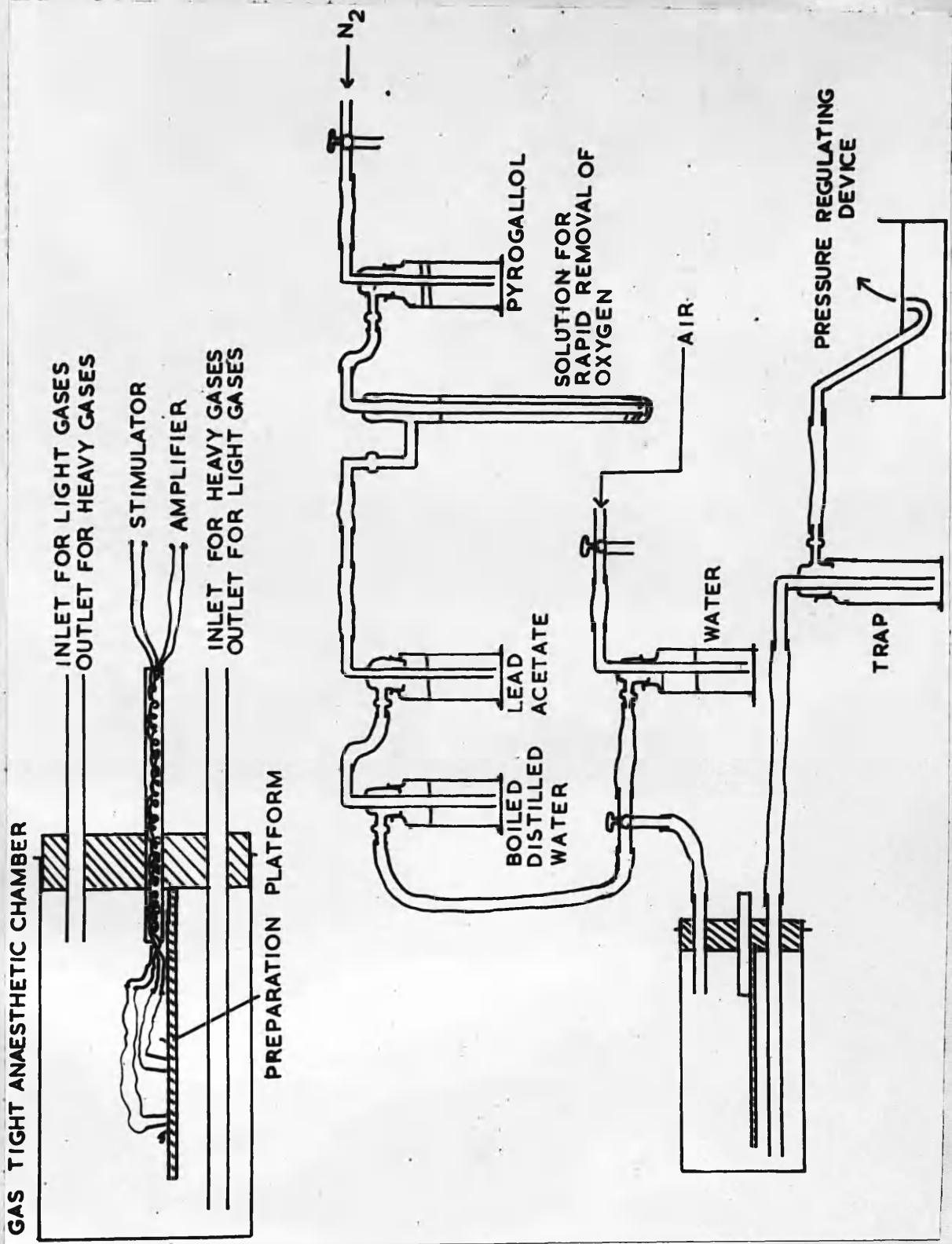


Figure 23. . Gas tight anaesthetic chamber. Below, apparatus used for studying the action of oxygen free nitrogen.

b). Comparison of DNOC effects with those of narcotics and various gases.

The general effects of DNOC on the locust cercal nerve - giant fibre preparation are reminiscent of the effects caused by anaesthesia, namely, an excitatory phase followed by a quiescent one, except that the quiescent phase in the case of DNOC is irreversible. The effects of various anaesthetics on the locust preparation were studied. The narcotics chosen were, carbon dioxide, chloroform, ether and ethyl alcohol. The effects of nitrogen and oxygen were also studied.

Early experiments with the narcotics were carried out using a perspex anaesthetic chamber as shown in Fig. 22. This box was by no means air tight, but allowed a concentration of gas to accumulate over the preparation without increasing the pressure. It also had the advantage that puffs of air could be directed at the cerci at intervals. This arrangement was useless where nitrogen effects were under consideration. In this case an air tight chamber became a necessity. Accordingly the apparatus figured in Fig. 23 was devised. An arrangement of Dreschel bottles and three-way taps allowed a change over from one gas to another without interference with the system. The pressure in the chamber was kept more or less

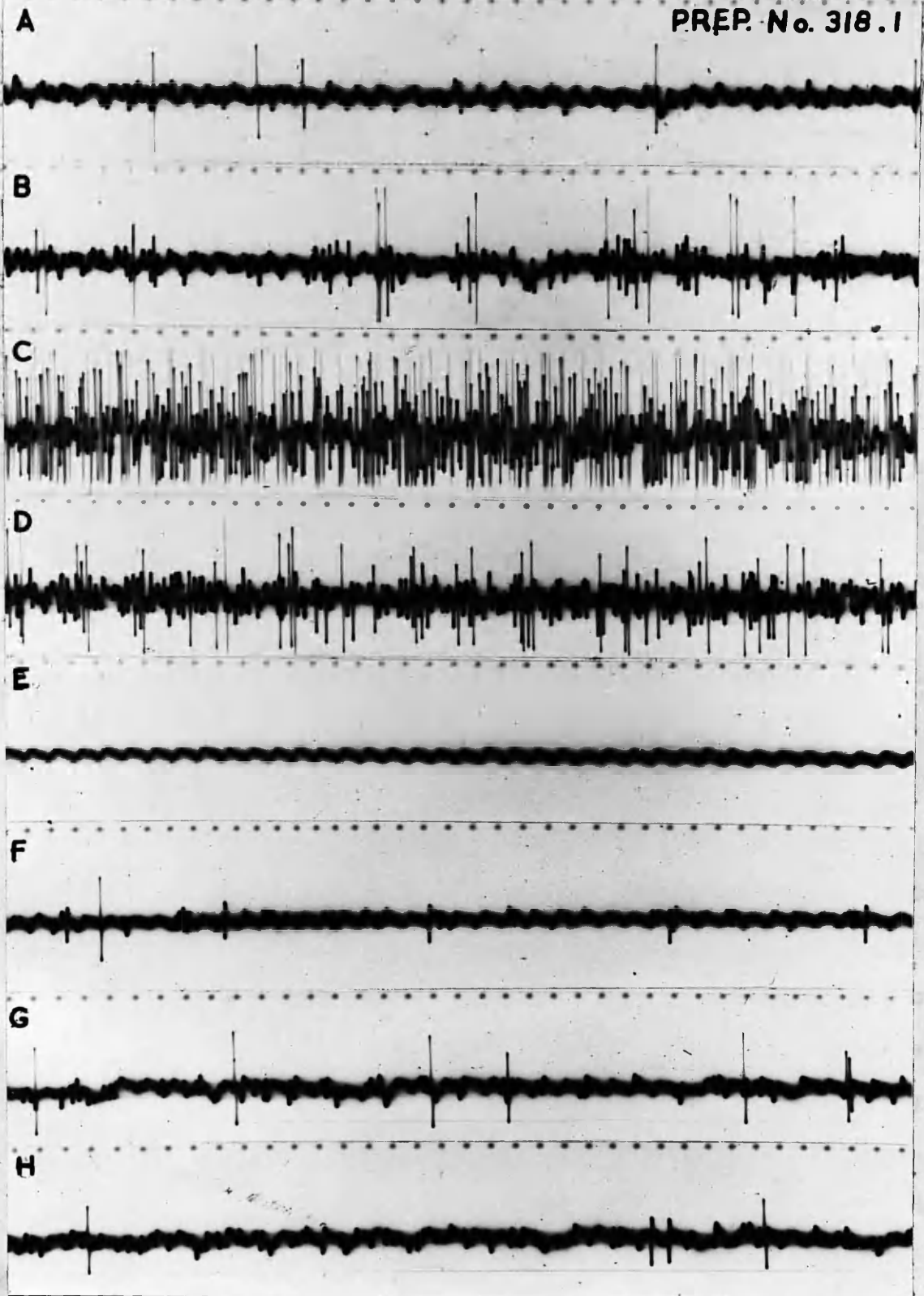


Figure 24. Stages in carbon dioxide anaesthesia in a preparation subjected to natural stimulation only. A, normal preparation; B, preparation in air stream; C,D,E, progressive stages in carbon dioxide anaesthesia; F, G, stages in recovery in air; H, air stream off.

equal with atmospheric pressure by means of a water outlet valve.

This apparatus allowed both natural and electrical stimulation, electrical stimulation usually via the cercal nerve, and natural stimulation by means of the stream of air or gas. Preliminary experiments with moist air demonstrated only that the preparation showed no fatigue to such continuous stimulation even over long periods, of an hour or more. If the rate of flow of gas stream was reduced so that air escaped from the chamber in bursts instead of continuously, the preparation responded to each release so that the effect of frequent bursts of stimulation was obtained.

#### 1. Carbon Dioxide.

Using the perspex chamber only natural stimulation was given. The effects varied depending on the rate of flow of carbon dioxide. When the flow was sufficient to produce complete anaesthesia inside 10 minutes, there was an increase in basic activity, often to very high density, followed by a decrease to nil. Response to stimulation usually disappeared shortly after the basic activity. Recovery in moist air reversed the sequence. Response to stimulation appeared first, although often with a low density, then basic activity (Figure 24).

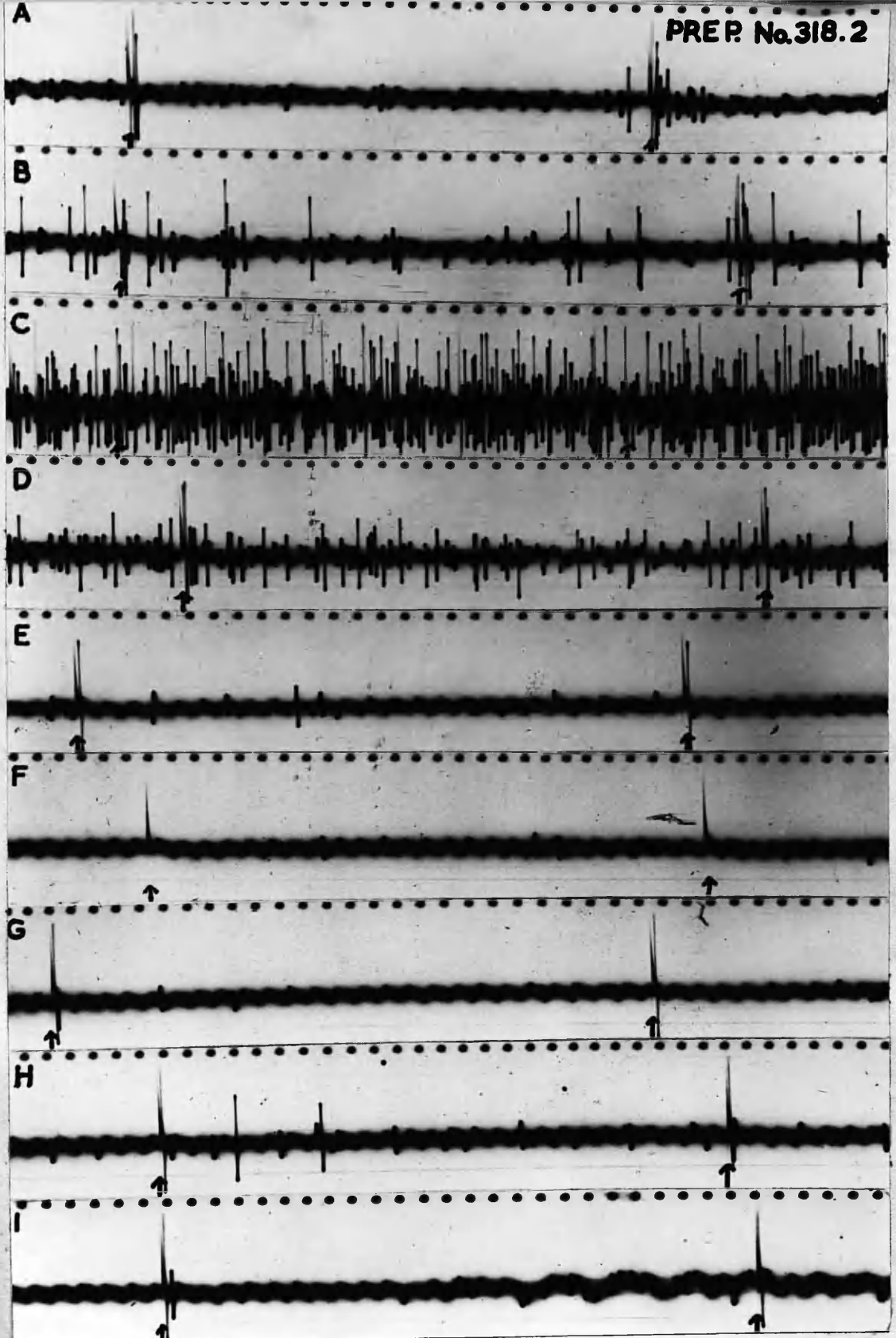


Figure 25. Stages in carbon dioxide anaesthesia in preparation subjected to repetitive electrical stimulation. A, electrical stimulation only; B, airstream and stimulation; C-F, stages in anaesthesia; G,H, recovery in air; I, air stream off.

Experiments with the air-tight chamber showed a similar result except that effects were naturally much more marked. The excitatory phase was considerable, during which response to electrical stimulation could not be distinguished from general activity. As this phase passed into the quiescent one, the firing density decreased and the response to electrical stimulation could again be discerned (Figure 25, D & E). Not until all the basic activity and/or response to natural stimulation had gone did the response to electrical activity finally disappear. In recovery this response reappeared first.

With the perspex chamber it was possible to adjust the flow of carbon dioxide so that incomplete anaesthesia could be maintained. Here there was first a marked response to natural stimulation and then a reduced one. Basic activity showed signs of reduction very shortly after this. All response to stimulation disappeared but spasmodic basic activity remained. This condition appeared to be an equilibrium one. If during this stage the rate of flow of carbon dioxide was increased, basic activity increased accordingly. Response to natural stimulation did not reappear. Suddenly basic activity disappeared, leaving the normal quiescent phase. Recovery in moist air was normal.

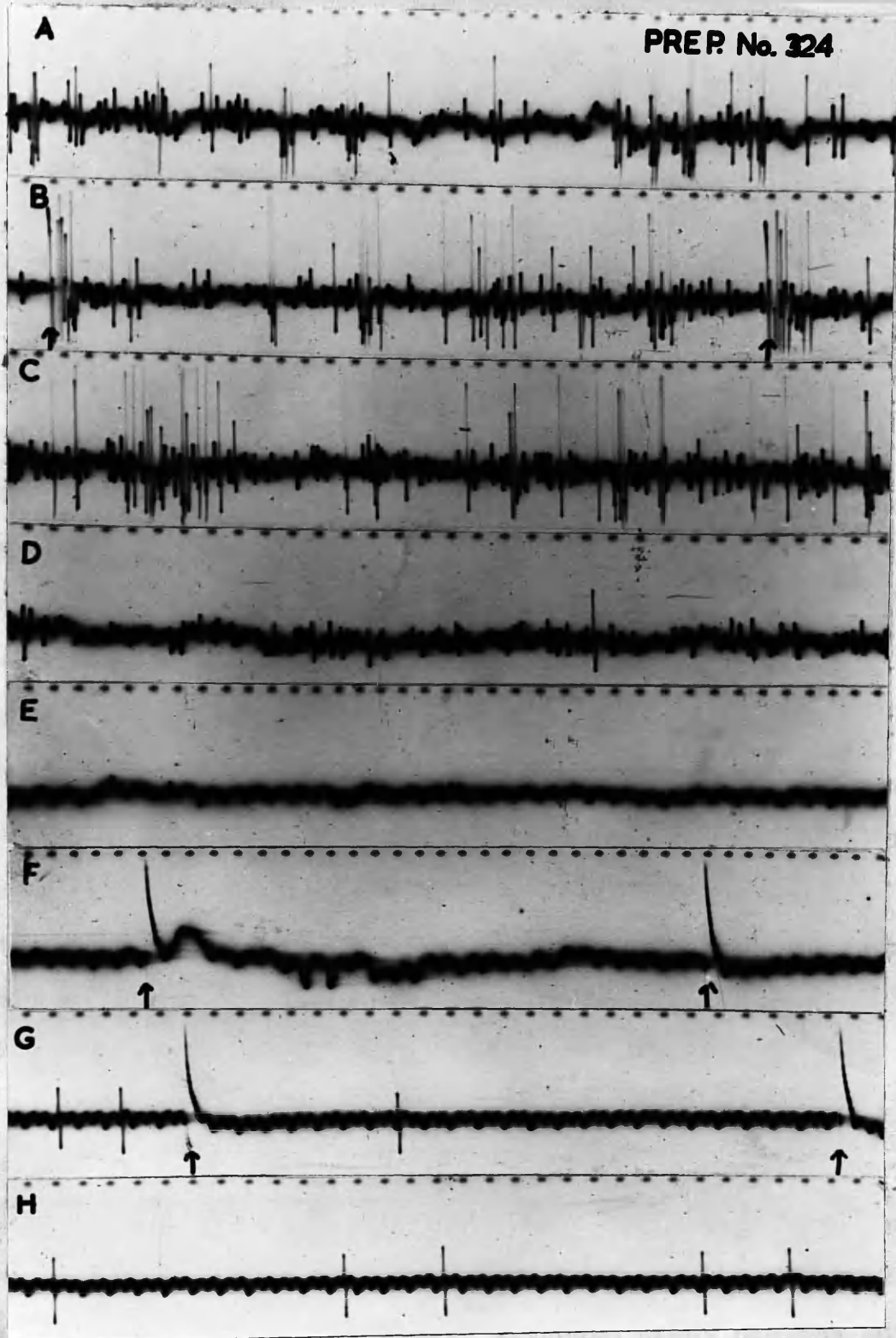


Figure 26. Chloroform anaesthesia. A, normal preparation in air stream; B, air and electrical stimulation; C,D,E, Chloroform, no stimulation; F, Chloroform and stimulation; G,H, recovery in air.

## 2. Chloroform.

In none of the experiments with this substance did the preparation return to normal within a period of two hours. The events preceding anaesthesia were similar to but not identical with those preceding carbon dioxide anaesthesia. The main difference was in the extent of excitability. The giant spikes did not reach anything like the firing density found with carbon dioxide (Figure 26). Small action potentials were more numerous. Response to electrical stimulation vanished almost co-incidentally with basic activity. During recovery in air, response to electrical stimulation did not return, even after  $1\frac{1}{2}$  hours had elapsed from the time the chloroform was switched off. Some giant spikes did appear, although firing seemed spontaneous, not associated with any stimulus either electrical or natural.

## 3. Ether.

The sequence of events which occurred with this narcotic was very similar to chloroform. The excitatory phase was associated mainly with smaller fibres. Response to electrical stimulation disappeared almost immediately after cessation of this phase, although giant neurons continued to fire for a short time. In recovery electrical stimulation initiated a burst of activity comparable with



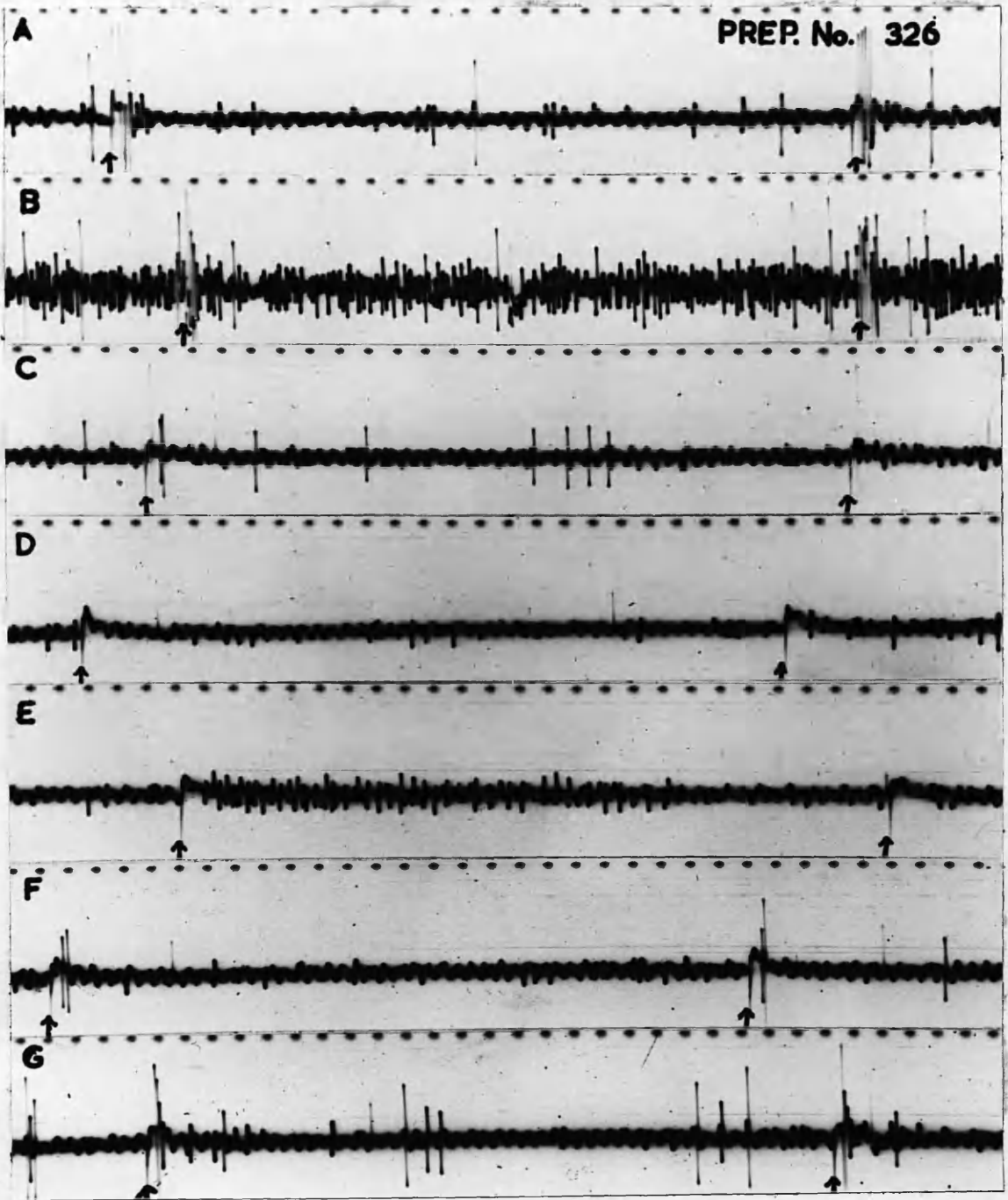


Figure 27. Ether anaesthesia. A, normal preparation; B,C,D, stages in anaesthesia; E,F,G, stages in recovery.

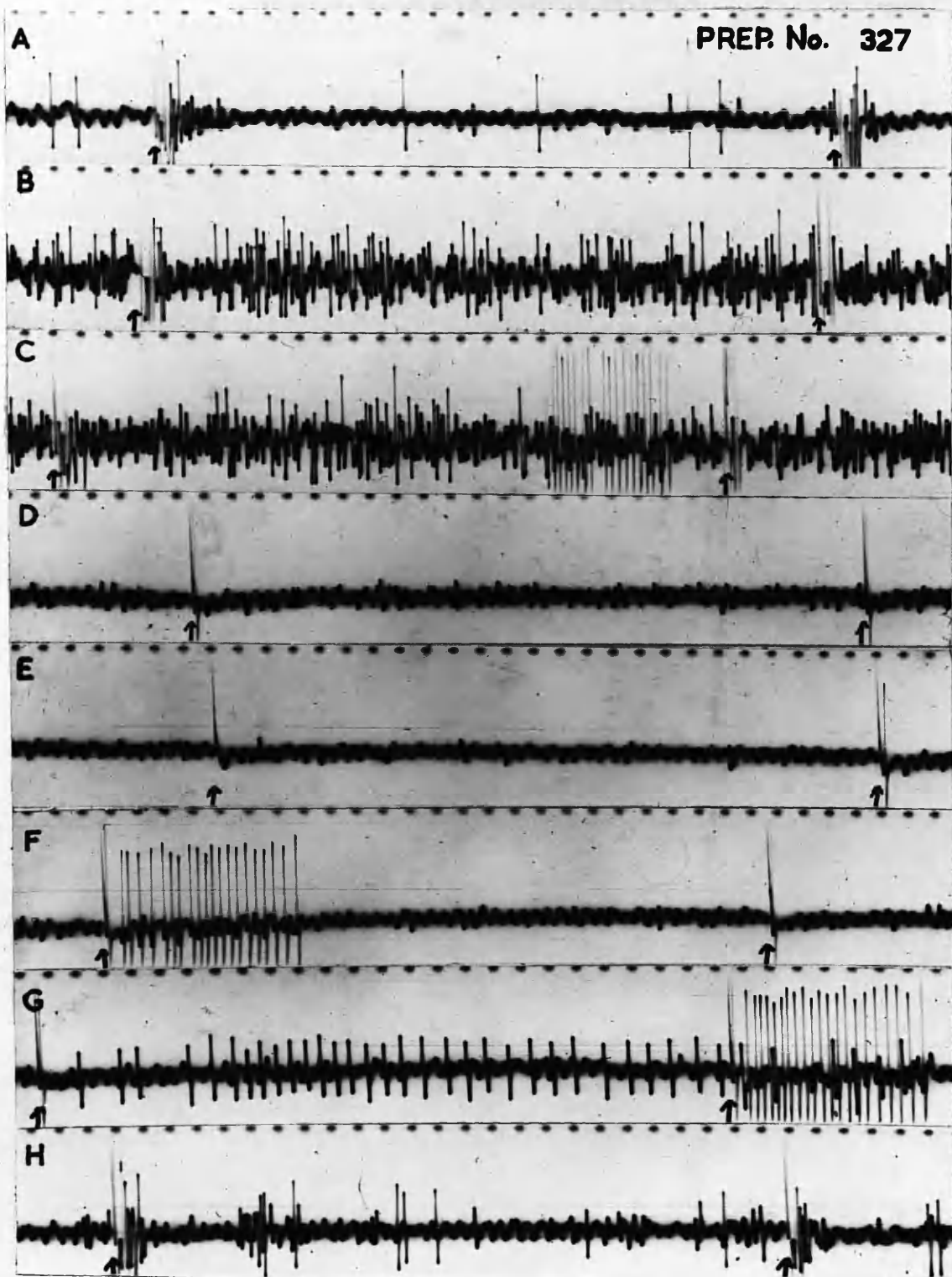


Figure 28. Alcohol anaesthesia. A, air stream and electrical stimulation; B-E, stages in anaesthesia; E-H, stages in recovery in air.

that of the excitatory phase before it evoked a more normal response (Figure 27, E & F). Full recovery did occur with this narcotic.

#### 4. Ethyl Alcohol.

In some respects the effects of this narcotic are much more like those of carbon dioxide than either of the others. In other ways the effects are characteristic of alcohol. The oscillograph records can readily be picked out from the others without any risk of confusion (Figure 28). The excitatory phase involves all sizes of spikes and electrical stimulation provokes a response throughout. The firing density was never so high as with carbon dioxide. For short periods, approximately 1/10 second trains of giant spikes occurred at a frequency of 150-200/second. These trains probably did not involve more than one fibre at a time. Response to electrical stimulation was last to disappear. In recovery the sequence of events was completely reversed. Response to electrical stimulation reappeared first, then trains of spikes, often triggered off by electrical stimulation, a phase of hyper-activity and then full recovery. It is in this respect that it differs from other narcotics.

#### 5. Nitrogen.

No effect at all was produced by this gas unless it

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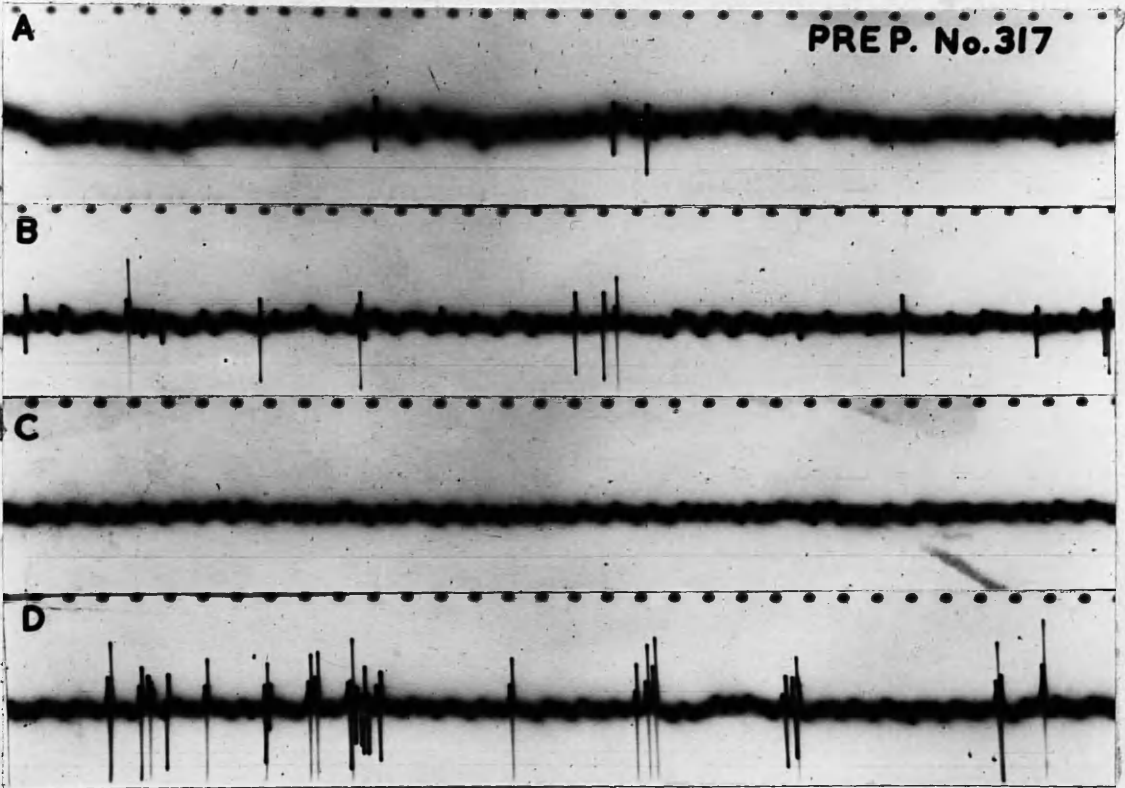


Figure 29. Effect of nitrogen on a preparation not subjected to electrical stimulation. A, normal preparation no gas flow; B, preparation in air stream; C, after 6 minutes in nitrogen; D, recovery in air.

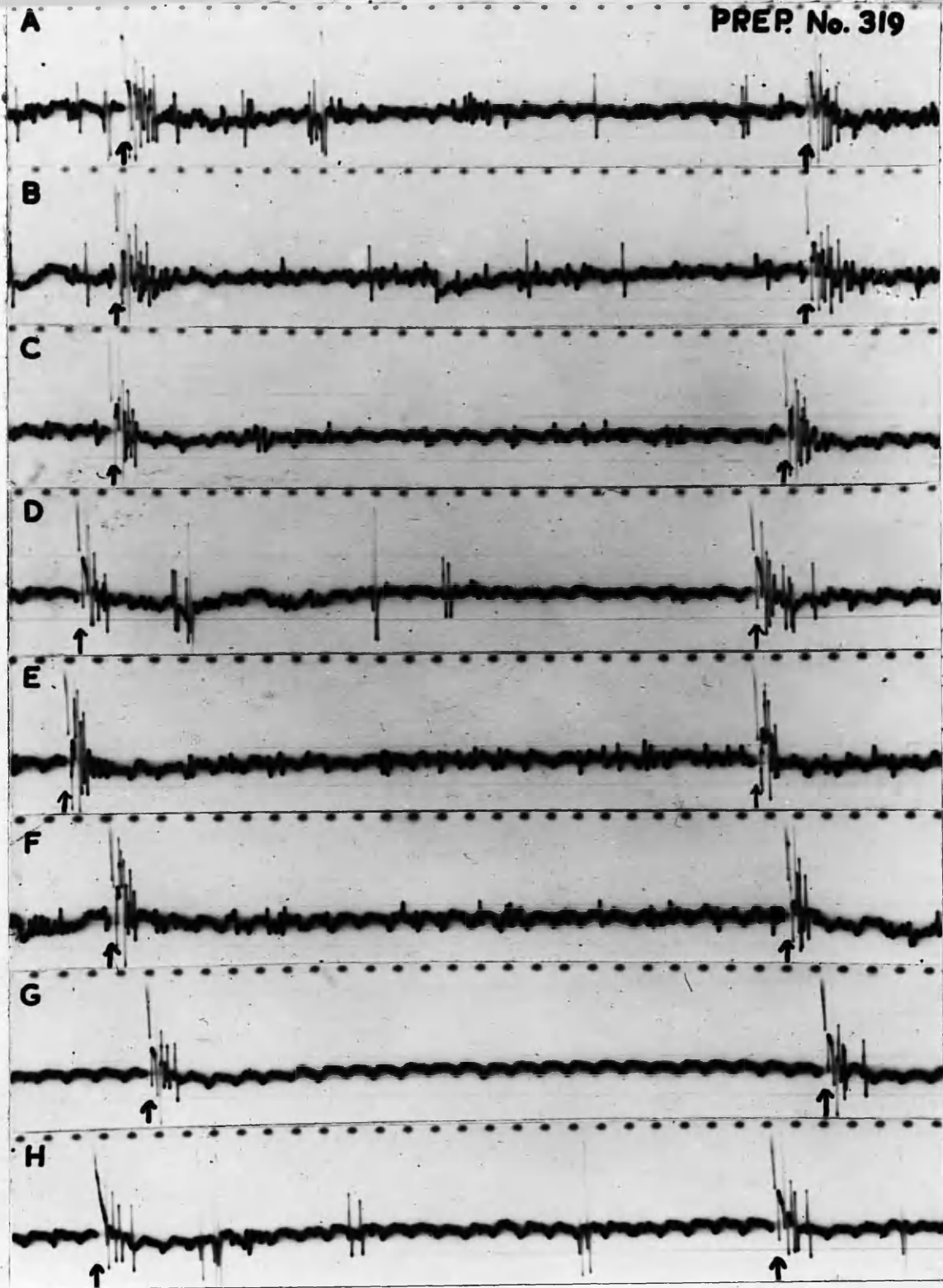


Figure 30. Oscillograms showing the effect of pure nitrogen. A, air stream and electrical stimulation; B, nitrogen stream after 1 minute; C, nitrogen after 8 minutes; D, recovery in air; E, nitrogen again; F, nitrogen stream turned off but not replaced by air; G, air stream on; H, full recovery.

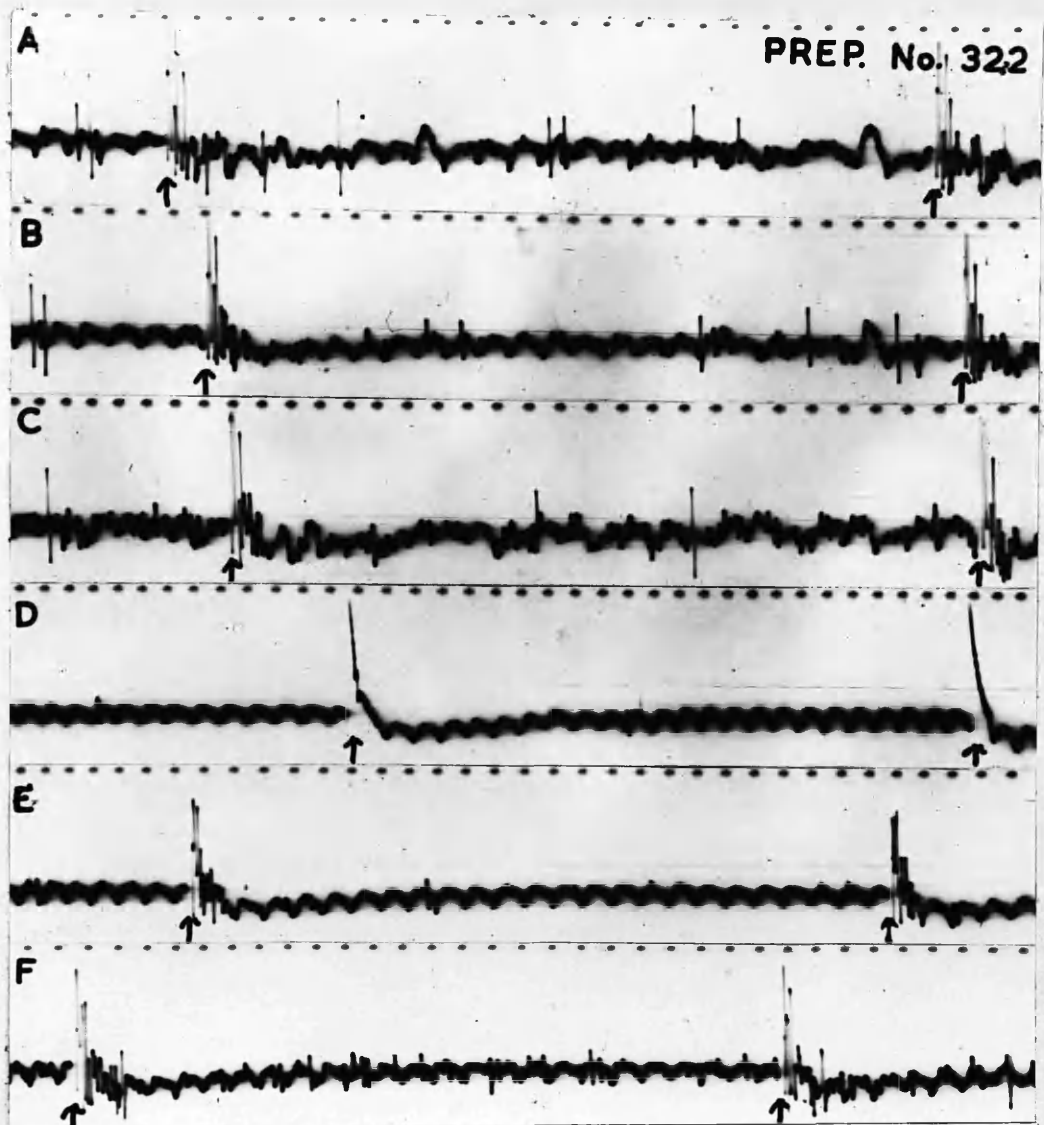


Figure 31. Effect of nitrogen on a paraffin covered preparation. A, air stream and electrical stimulation; B, C, D, effects of nitrogen stream; E, F, recovery in air.

paration was left in contact with moist air, but slightly reduced covered preparations (Flanagan)

was applied in an oxygen free condition in an air tight chamber. Then after some minutes all basic activity ceased suddenly. Except for one occasion, this cessation was not preceded by an excitatory phase. Recovery occurred immediately moist air was passed over the preparation (Figure 29). Response to electrical stimulation continued normally even though the preparation remained in nitrogen for as much as 30 minutes after cessation of "basic activity" (Figure 30, A-D). In many preparations after the giant action potentials had disappeared small action potentials remained and these showed an increased rate of firing. This appeared to be a direct effect of nitrogen, since it was not due to natural stimulation it remained after the gas stream was turned off, although the preparation was still in an atmosphere of nitrogen (Figure 30, E & F). Immediately moist air was passed over the preparation this activity disappeared (Figure 30 G).

It is interesting to note here, that if the preparation is covered with medicinal liquid paraffin, response to electrical stimulation can be removed by nitrogen. In all previous anaesthetic experiments the preparation was left uncovered. This response returns with moist air, but complete recovery is delayed in paraffin covered preparations (Figure 31).

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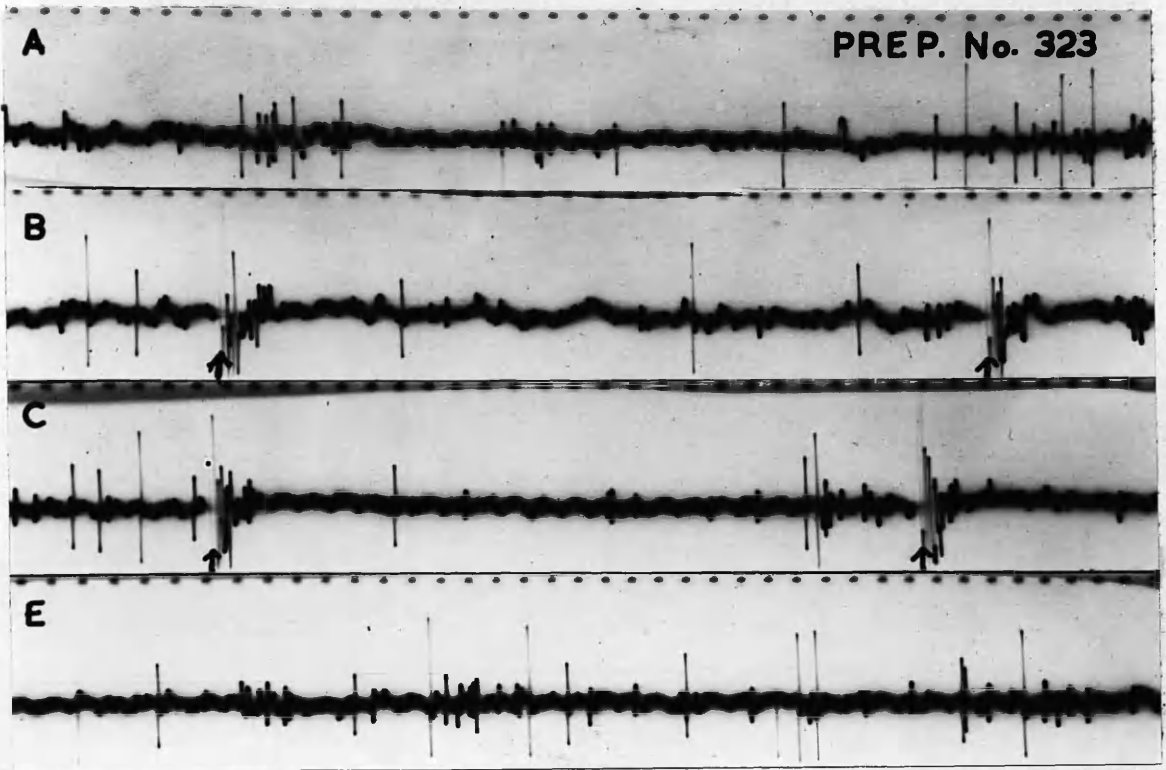


Figure 32. Effects of oxygen. A, preparation after 30 minutes in oxygen; B, as in A but with electrical stimulation; C and D, preparation after 10 minutes in air.



## 6. Oxygen.

No effects were observed, even after the preparation had been in moist pure oxygen for 60 minutes (Figure 32).

## 7. Conclusions.

From these observations it is clear that the excitatory phase, so characteristic of the preliminary stages of anaesthesia, is a product of anaesthesia and not due to asphixia. Both experiments with anaesthetics and with nitrogen seemed to indicate that the threshold for the sensory receptors is lower than that for nerve conduction because response to natural stimulation disappears before that of electrical stimulation. Superficially DNOC effects resemble carbon dioxide and alcohol effects if the bursts of firing of the latter are discounted. However, with DNOC the response to natural stimulation decreases quite markedly before the basic activity has increased to its maximum. It may well be that all the neurons which would be concerned in the response are already firing at a maximum rate and are therefore unable to respond. However, as this period passes, it becomes clear that the response is quite definitely gone. Since, however, the DNOC effects are irreversible, it may be that the nerve mechanism has been upset in a way similar to that which occurs under anaesthesia, but in a more

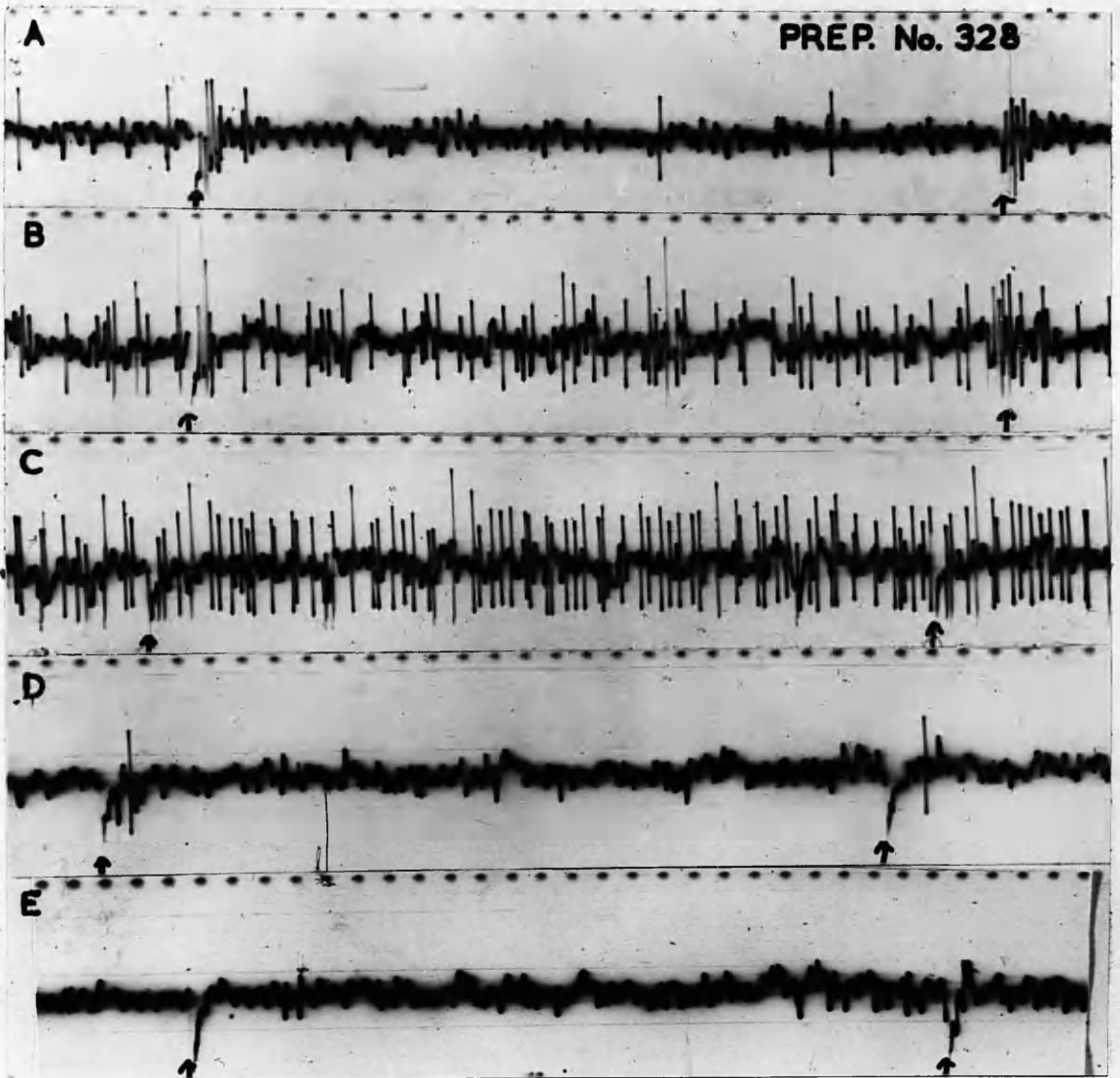


Figure 33. The effect of DNOC on a preparation subjected to repetitive electrical stimulation. A, normal preparation; B-E, DNOC effects, see text.

permanent way. All the DNOC observations had been made with natural stimulation only, since the preparation was covered with saline. It was obviously desirable at this stage to attempt some observations with electrical stimulation.

#### 8. Electrical stimulation with DNOC.

These anaesthetic experiments showed that it was possible to use electrical stimulation without first covering the preparation with paraffin or without using an elaborate mechanism for lifting the electrodes and nerve away from a preparation flooded with saline such as was described by Roeder and Weiant (1945). It was therefore decided to attempt the use of this method for the electrical stimulation of a preparation poisoned with DNOC saline solution and to add a very small quantity only of DNOC solution, this added, if possible, at a place some distance from the immediate vicinity of the electrodes. This was accomplished using a hyperdermic syringe fitted with a No.20 needle to apply the solution. No interference occurred provided great caution was used when adding the solution.

Figure 33 shows records from a preparation with stimulation via the cercal nerve. Response to stimulation did continue beyond the main excitatory phase, although

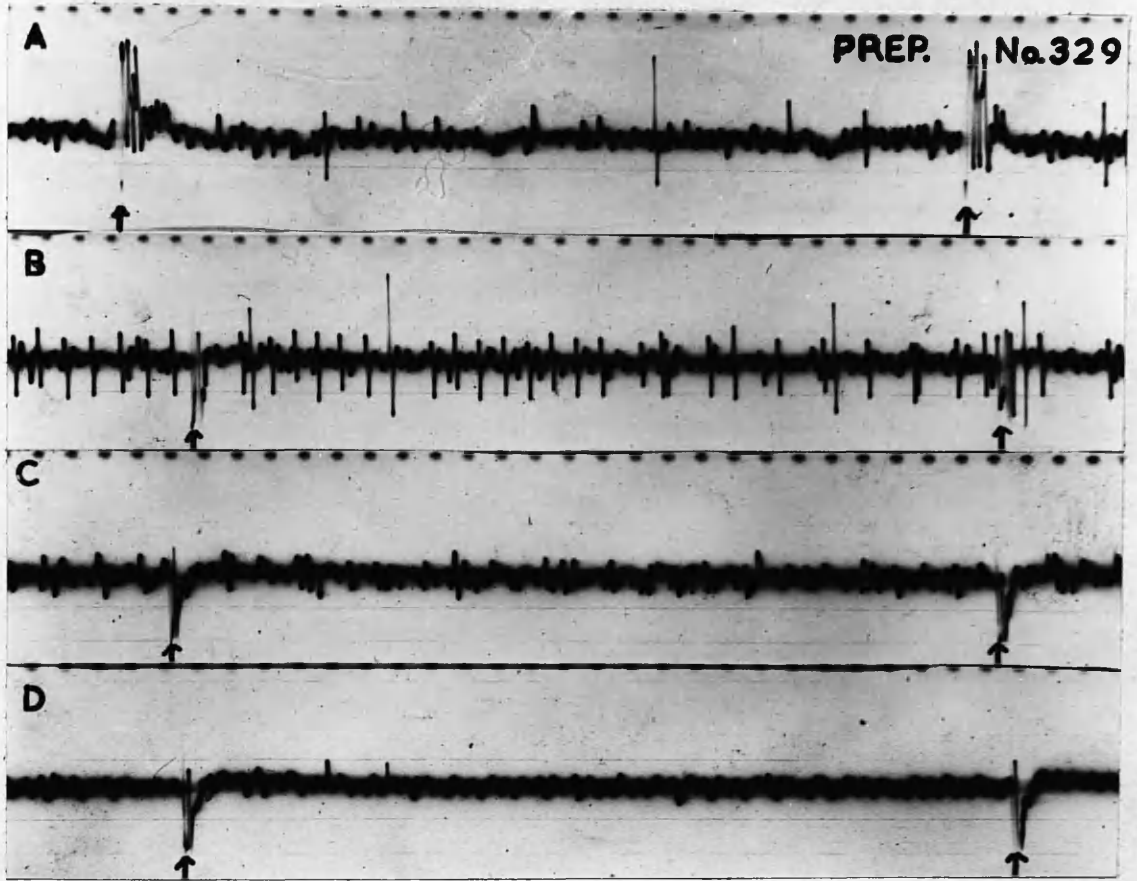


Figure 34. Effect of DNOC on a preparation subjected to electrical stimulation via abdominal cord. A, normal preparation with stimulation; B, C, D, DNOC effects.

DNOC might be responsible for the

was first observed by [unclear]

added, oxygen was passed over the

line. Figure 35 shows [unclear]

reduced as in anaesthesia, but there was an increased delay in appearance of the response. This could be due either to a decrease in conduction rate or to an increased delay in response time. Finally the response disappeared altogether. A similar preparation subjected to electrical stimulation directly on to the posterior region of the abdominal cord showed that axonic conduction was unaffected by DNOC (Figure 34) since response to axonic conduction continued after the disappearance of other activity. The response, however, was again reduced. Some change takes place which prevents the spread of the stimulus.

c). An attempt to counteract the effects of DNOC.

It has been repeatedly recorded that DNOC increases the rate of oxygen uptake in both insects and mammals (Ambrose, 1942, Bodine and Boell, 1936, Goble and Patton, 1946 and Roeder et al., 1948). It has already been shown here that it is unlikely that the effects of DNOC on the locust cercal nerve - giant fibre preparation are produced solely by a lack of oxygen. However, it was thought possible that some if not all of the effects of DNOC might be counteracted by oxygen. The preparation was first accustomed to oxygen and then the DNOC solution added, oxygen was passed over the preparation all the time. Figure 35 shows the results. The usual sequence

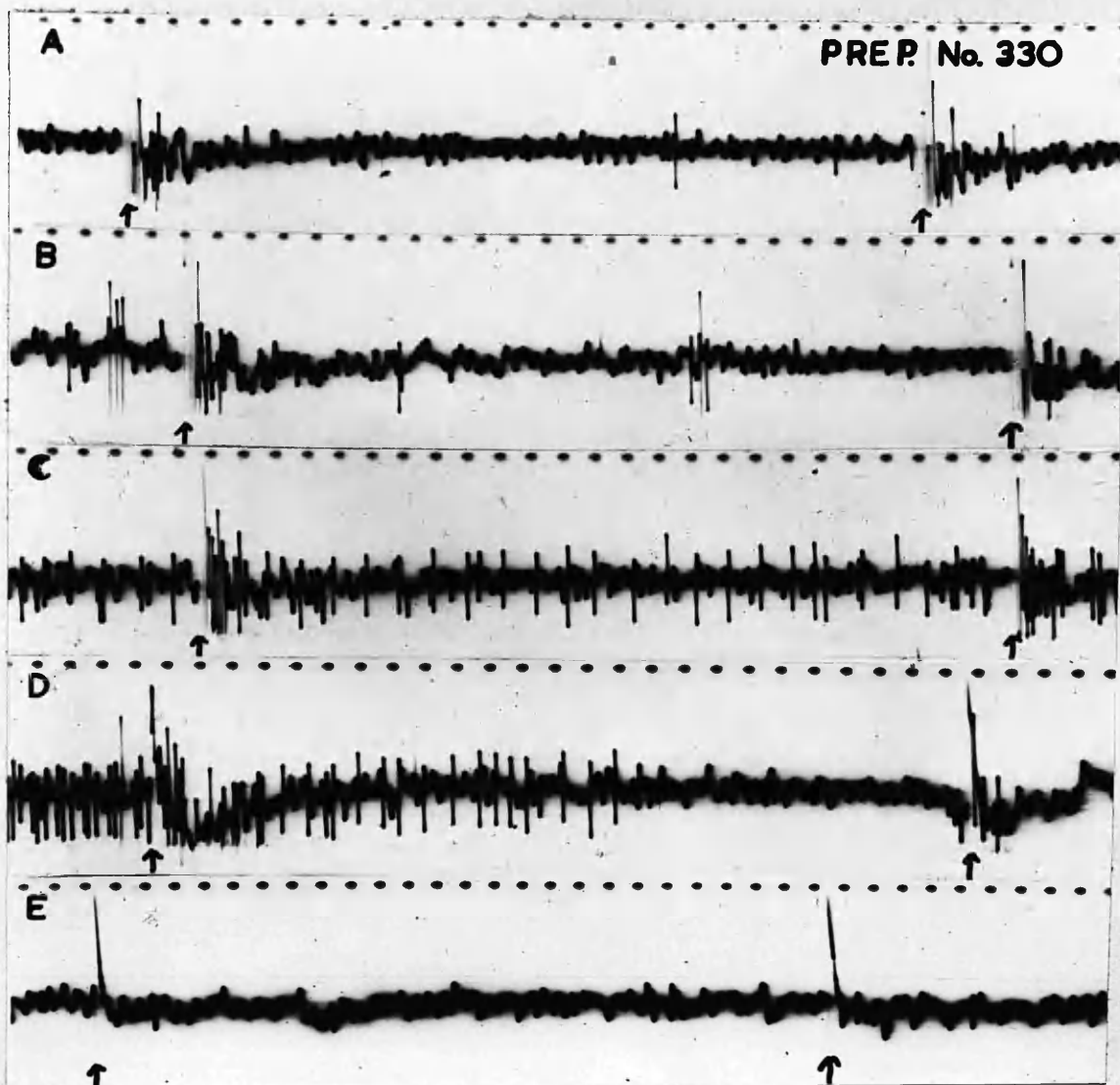


Figure 35. Effects of DNOC on a preparation kept in a stream of oxygen. A, normal preparation; B, preparation in oxygen stream; C, D, E, DNOC effects in oxygen.

of events occurred in comparable time with other preparations. Response to electrical stimulation disappeared shortly after cessation of other activity. No recovery was observed in two hours. It must thus be assumed that the effects of DNOC cannot be counteracted by oxygen.

d). Comparison of the toxicity of DNOC with its sodium salt.

The anion of DNOC is said to have only 1/40 of the toxicity of the undissociated molecule when used as a fungicide (Brian, P.W., 1945). Certainly it was noticed that in buffered saline solution containing DNOC fungal growth occurred as in both buffered and unbuffered salines alone, whereas in unbuffered saline DNOC solutions no such growth occurred. Opinions are divided, however, on the efficiency of the dissociated anion as an insecticide.

Solutions of  $8 \times 10^{-4}$ M. and  $4 \times 10^{-4}$ M. DNOC were made up in buffered saline (solutions A and B respectively) to correspond with solutions C and D in unbuffered saline. Preliminary experiments with both these solutions showed that both strengths produced toxic effects within experimental time limits. A series of 10 experiments were then carried out with solution B and the reactant times compared with those of solution E by analysis of variance.  $F = 0$ , so that no significant difference existed. It has already

been shown that no significant difference in reactant times existed between solutions D, E and G of strength  $4 \times 10^{-4}M.$ ,  $2 \times 10^{-4}M.$ , and  $1.5 \times 10^{-4}M.$  If, however, the dissociated anion had a toxicity of the order of  $1/40$  the undissociated molecule, the reactant times of solution B would be significantly different from any of these three. Under these conditions therefore the neurotoxicity is but little affected by increased pH and corresponding increase in the ratio of sodium salt to free acid.

Later it was possible to obtain a sample of the sodium salt of DNOC. This substance is considerably more soluble in water than DNOC itself, so that a  $10^{-3}M.$  aqueous solution was prepared directly by weight. Aqueous DNOC solution was prepared in the usual way. Carbonate free potassium hydroxide was standardised by titration with standard potassium hydrogen phthalate in an atmosphere of nitrogen, using phenol phthalein as an indicator. This potassium hydroxide was then titrated against the DNOC solution which was found to be  $9.4 \times 10^{-4}M.$  by this method.

Solutions of both the sodium salt and the free acid were made up in buffered and unbuffered saline respectively of concentration  $4 \times 10^{-4}M.$  Both solutions had similar effects within similar reactant times, on the locust cercal nerve - giant fibre preparation (Figure 36).



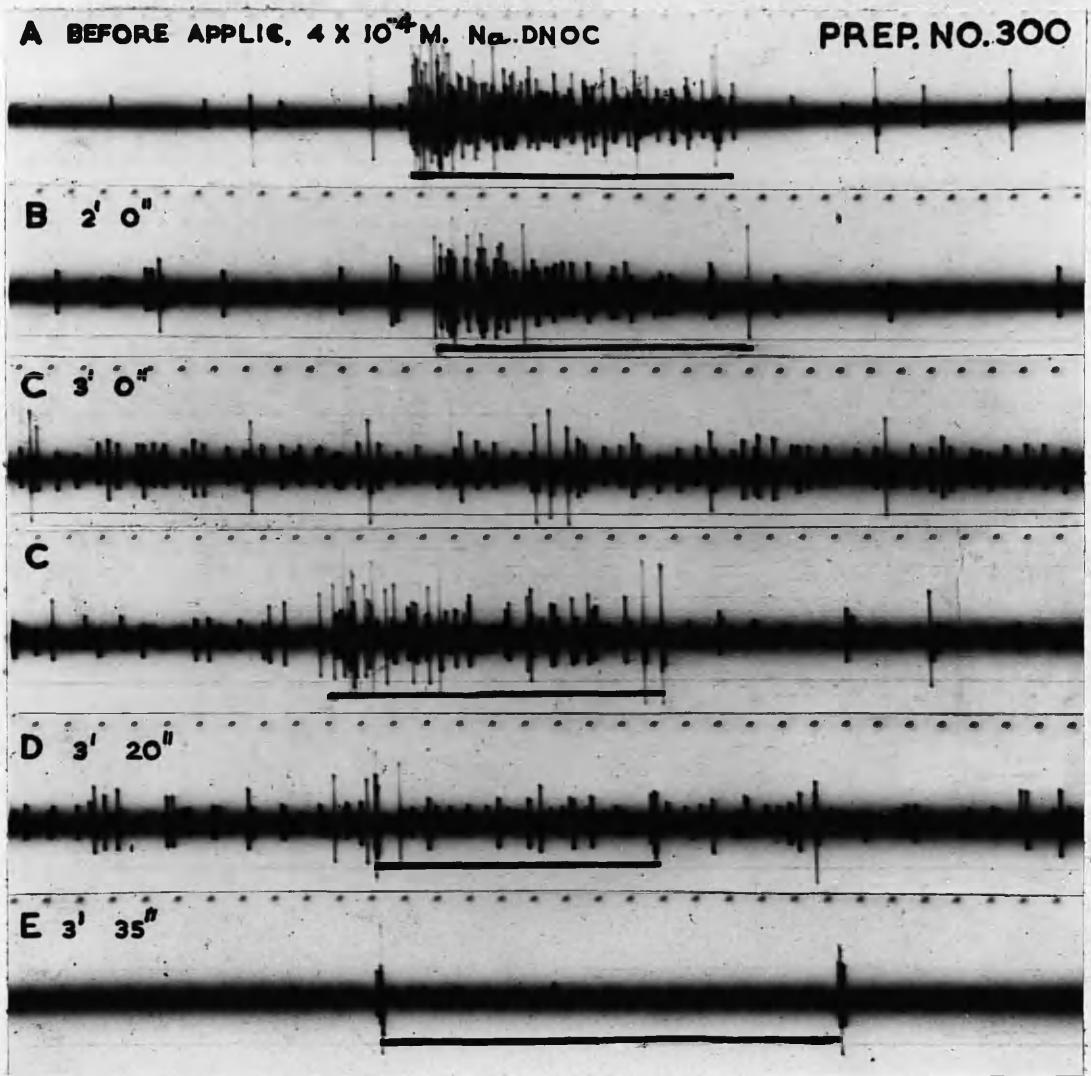


Figure 36. Oscillographic records showing the effect of a solution of the sodium salt of DNOC on the locust cercal nerve - giant fibre preparation. Records made on play back from a tape recorder. A, normal preparation; B, C and D, decline of response and increase of basic activity; E, final stage showing no activity. Compare with figure 20.

The effect of these substances was also compared on intact animals. In one experiment 12 male locusts were taken and were injected intra-abdominally with 0.1 cc. of solution, four with  $4 \times 10^{-4}$  M. DNOC, four with  $4 \times 10^{-4}$  M. NaDNOC and four with unbuffered saline. After six hours the saline controls were all normal and active. All the others showed symptoms to a certain degree; three with DNOC and two with the sodium salt were unable to stand. The rest were far from normal but moving about.

A similar experiment was conducted in which three sets of four male locusts were allowed to come into contact with filter paper painted with one or other of these three solutions. After twenty four hours the controls were normal and the other eight dead.

Since the locust body fluid is buffered it is unlikely that there will be much difference in degree of dissociation at the locus of action, whichever original solution is applied, unless the quantities be sufficiently great to swamp the buffer.

X. GAMMA-BENZENE HEXACHLORIDE.

a). Review of the literature.

Gamma-benzene hexachloride or gamma-hexachloro-cyclohexane is a colourless crystalline substance, m.p. 108-111°C., usually called either Gammexane or  $\gamma$  BHC. The insecticidal properties of the  $\gamma$  isomer are considerably more marked than those of any of the other isomers. Like D.D.T.,  $\gamma$  BHC has an apparently relatively low toxicity to mammals as compared with insects. Dresden and Krijgsman (1948), however, have shown that this effect is due to differential penetration and not to differential toxicity.

The effect of  $\gamma$  BHC and its isomers on the CNS of rats has been studied by Herken (1951). Certain drugs such as Cardiazol produce convulsions similar to those occurring in epileptic conditions. Herken found that rats previously treated with sub-lethal doses of  $\gamma$  BHC or its isomers were resistant to subsequent injections of Cardiazol. The  $\gamma$  BHC itself produced initial convulsions which afterwards subsided. He also studied the action of  $\gamma$  BHC and its isomers in conjunction with the anti-cholinesterases physostigmine (eserine) and prostigmine. The effects of the latter could be counteracted to some extent by previous treatment with BHC. From these

experiments Herken concluded that BHC exerted an effect on the mammalian CNS and that this effect was unlike that of any of the neurotoxic drugs previously studied. He was unable to draw any conclusions regarding its mode of action, but had great hopes of its usefulness in future attempts to understand and even treat epileptic conditions.

Savit et al. (1946) describe the symptoms of  $\gamma$  BHC poisoning in Periplaneta as tremors followed by ataxia, convulsions, falling and prostration. These symptoms are not unlike those described by Tobias and Kollross (1946) in DDT poisoned cockroaches, namely, postural instability, tremors, ataxia, falling with fast tremors and slower isolated movements of appendages and finally prostration. Tobias et al. (1946a) have studied the effect of poisoning by  $\gamma$  BHC on the Ach. content of P. americana abdominal nerve cord. In the prostration stage of poisoning they found a definite increase in Ach. content from the normal level 38  $\mu\text{g/g}$  to 57  $\mu\text{g/g}$ . A similar increase (up to 100  $\mu\text{g/g}$ .) was found in DDT poisoned cockroaches in the same stage of prostration. The cholinesterase activity however was not affected. Dallemagne, Phillipot and Gernay (1948) thought that the action of  $\gamma$  BHC did seem to involve the activity of cholinesterase.

Another theory of the mode of action of  $\gamma$  BHC is

based upon the isosteric resemblance of the molecular configuration of meso-inositol, one of the vitamin B complex compounds, and that of  $\gamma$  BHC. Slade (1945) believes that  $\gamma$  BHC may act as a competitive metabolite and thereby block some vital reaction of the organism. It has been shown that Saccharomyces cerevisiae must have meso-inositol for normal growth. Kirkwood and Philips (1946a) studied the effect of adding isomers of hexachlorocyclohexane to the culture medium of this yeast.

$\alpha$  and  $\beta$  isomers produced slight inhibition and  $\delta$  more marked inhibition. Addition of meso-inositol did not reverse this inhibition.  $\gamma$  BHC markedly inhibited the growth of this yeast and this inhibition could be progressively reversed by increasing addition of meso-inositol. However, this inhibition could not be completely reversed. So far attempts to counteract poisoning effects in insects by administration of meso-inositol have not proved successful. Liquid triglycerides inhibit toxicity of  $\gamma$  BHC to fourth instar larvae of mosquitoes Aedes aegypti and Culex fatigans (Thorp and de Meillon, 1947). The mechanism of poisoning therefore still remains very obscure.

b). Experimental results with  $\gamma$  BHC.

A solution of  $\gamma$  BHC in liquid paraffin was made by stirring a warmed mixture of  $\gamma$  BHC and liquid paraffin for some time. The saturated solution thus obtained when injected intra-abdominally into locusts produced the characteristic symptoms, tremors, ataxia and prostration. The insects were unable to stand within 10-15 minutes after injection. When locusts were allowed to come into contact with filter paper painted with this solution, symptoms began to appear within 10-15 minutes, falling, tremors, violent wing beating and finally prostration after an hour or so.

This solution was then applied to the locust cercal nerve - giant fibre preparation. Of twelve preparations observed, all were still conducting impulses at the end of the experimental period of one hour. In most preparations the response to stimulation seemed slightly enhanced and basic activity increased. In other words, the preparation was hypersensitive. This hypersensitivity occurred both in preparations in which the cerci were left intact and stimulation was natural; and in a-cercal preparations subjected to repetitive or sporadic electrical stimulation.

TABLE VIII

Preparation 277. Intervals in seconds between successive bursts of action potentials.

Columns read from top to bottom. Time in minutes after application indicated by figure to the left at top of each column.

| 24 | 32 | 40 | 48 |
|----|----|----|----|
| 15 | 13 | 14 | 12 |
| 14 | 10 | 11 | 11 |
| 16 | 11 | 13 | 15 |
| 12 | 16 | 13 | 7  |
| 18 | 7  | 12 | 10 |
| 14 | 5  | 9  | 12 |
| 10 | 10 | 12 | 17 |
| 11 | 14 | 13 | 10 |
| 10 | 8  | 11 | 4  |
| 7  | 12 | 12 | 11 |
| 12 | 10 | 10 | 27 |
| 11 | 9  | 10 | 12 |
| 10 | 11 | 10 | 8  |
| 10 | 12 | 10 | 9  |
| 11 | 11 | 10 | 14 |
| 9  | 15 | 9  | 6  |
| 11 | 11 | 15 | 11 |
| 12 | 12 | 16 | 9  |
| 10 | 13 | 14 | 12 |
| 10 | 11 | 16 | 9  |
| 14 | 11 | 10 | 11 |
| 20 | 12 | 9  | 19 |
| 12 | 15 | 10 | 11 |
| 10 | 12 | 12 | 9  |
| 9  | 12 | 10 | 13 |
| 11 | 12 | 13 | 13 |
| 8  | 11 | 11 | 7  |
| 11 | 10 | 13 | 8  |
| 11 | 13 | 14 | 10 |
| 13 | 10 | 4  | 12 |
| 13 | 11 | 7  | 8  |
| 12 | 13 | 19 | 7  |
| 17 | 12 | 15 | 5  |
| 8  | 13 | 11 | 16 |
| 10 | 12 | 11 | 8  |
| 12 | 11 | 11 | 6  |
| 13 | 11 | 9  | 5  |
| 11 | 13 | 12 | 15 |
| 11 | 12 | 7  |    |
| 13 | 13 | 10 |    |
| 9  | 13 | 9  |    |
| 8  | 11 | 11 |    |

In seven of these twelve experiments a marked rhythm developed in the basic activity. This rhythm took the form of aggregations of action potentials which fired at intervals of 8-15 seconds. Table VIII shows the intervals between successive bursts in a single preparation from 24 minutes to 55 minutes after application of  $\gamma$  BHC.

In the other five preparations basic activity was increased but the preparation appeared otherwise normal. It is perhaps significant that three of these five were the first three experiments, and the rhythm may possibly have been overlooked. The other two were carried out after the solution had been standing for some time: settling might thus have taken place resulting in the application of a rather more dilute solution than was intended. In all later experiments the solution was stirred well before use.

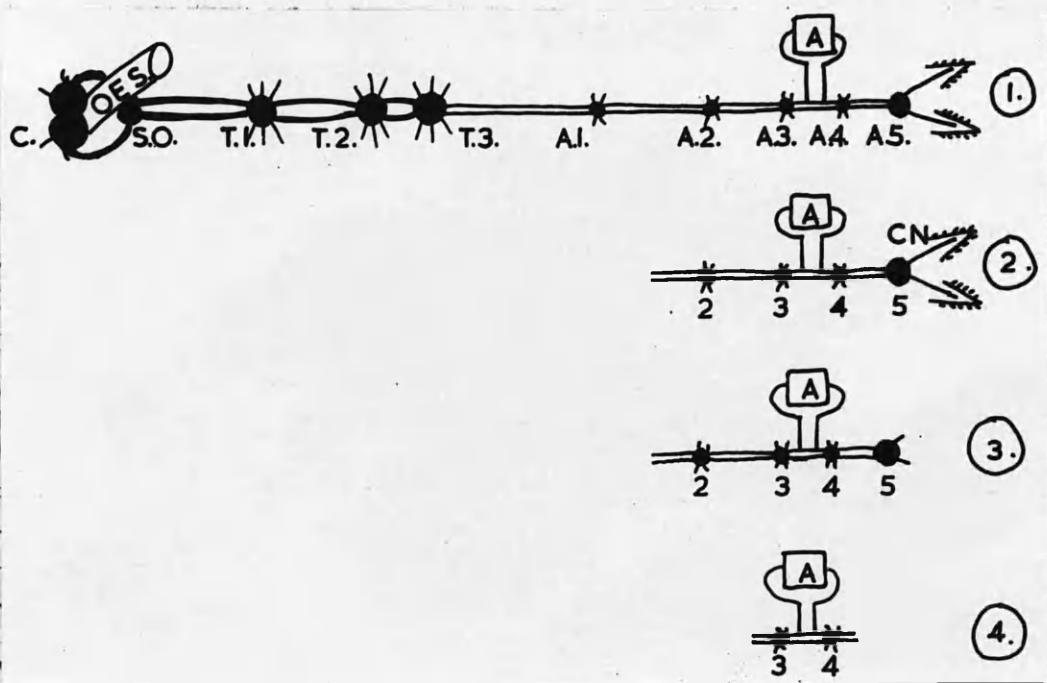
It was hoped to gain from these experiments some indication of the optimum range of concentrations and the best method of approach to the problem. It seemed clear that, despite the great efficacy of this saturated solution on whole locusts, dilution would not be desirable for the neurophysiological preparations. Since quantitative data <sup>er</sup> was not required, no attempt was made to estimate the concentration of the solution.



The disturbances produced in the nervous system did not seem sufficient to account for the violent symptoms produced in intact insects. Since conduction remained normal, the lack of co-ordination must be explained by other means. An obvious hypothesis would be to suppose direct interference with muscular innervation or function. As the present work is a comparative study, it was decided to leave this approach, which would necessitate an entirely new type of preparation, for the time being and to confine the investigation to purely neural disturbances.

The observations of previous workers and the results of these preliminary experiments suggested that  $\gamma$  BHC may act in a manner similar to DDT. The general symptoms are similar in both cases, and point to interference with nervous mechanisms. The origin of neurophysiological changes however is somewhat obscure. It was decided therefore to continue the investigation along similar lines to those used by Dresden (1949) in his physiological investigation into the action of DDT on the cockroach.

Dresden started from the idea that DDT acted somewhere on the nervous system. In order to discover whether DDT affected all parts of the nervous system equally or whether it acted selectively on any particular



**Figure 37.** Scheme of types of preparation used in study of effects of  $\gamma$  BHC on locust cercal nerve - giant fibre preparation. The nervous system was left attached to the locust but various cuts made as indicated. In (1) the nervous system was left intact. In the other preparations the nerves leaving abdominal ganglia 2, 3 and 4, were left intact. A, amplifier; A1-A5, abdominal ganglia 1-5; C, cerebral ganglia; CN, cercal nerve; Oes., cut oesophagus; S.O., subsophageal ganglia; T1-T3, thoracic ganglia 1-3.

part he carried out an analytical investigation of the entire nervous system from receptor to effector by electrophysiological methods. He confined his investigations in Periplaneta to a reflex arc concerned with the second leg and second thoracic ganglion.

The cercal nerve - giant fibre preparation in the locust does not offer such a complete reflex arc, since it makes no provision for recording from motor fibres or effector organs, unless the metathoracic leg is included in the preparation. Dresden's preparation included both sensory and motor fibres in the leg nerve, and receptors and effectors in the leg itself. The locust preparation does include receptors, on the cercus, a peripheral nerve, the cercal nerve which contains sensory fibres and the CNS in the abdominal cord. Spontaneous activity occurs in the abdominal ganglia and can be recorded in the abdominal cords simply by sticking electrodes into the cord. This preparation, therefore, provides an adequate starting point, and does allow the investigation of this insecticide to remain comparable with those of parathion and DNOC.

Figure 37 gives a diagrammatic representation of the types of preparation used. The ideal approach would be to study the effects of  $\gamma$  BHC on the isolated nervous system. Attempts to obtain reliable and reproduceable

preparations with isolated locust CNS have so far failed. For most purposes, however, the giant neurons can be considered as isolated when the anterior region of the abdominal cord is cut and the cercal nerve is cut close to the fifth abdominal ganglion. The smaller fibres probably have cell bodies situated in the intervening ganglia which still have nerves attached and therefore cannot be considered as isolated neurons.

The following experiments were all conducted using locusts dissected in the manner previously described. Since these experiments involved cutting the nervous system in various new ways, double channel recording was employed and a secondary preparation, treated identically except for the application of the insecticide, was made to act as a control. This control preparation was covered with liquid paraffin. At least five experiments of each type illustrated in figure 37 were carried out, each with a parallel control animal. The insecticide was not added until the requisite nerve transections had been made and these were not performed until after the electrode position had been checked by recording from them.

When the CNS is intact as in 1. Figure 37 and  $\gamma$  BHC applied, a rhythm developed in the basic activity while sensory reception and nerve conduction and

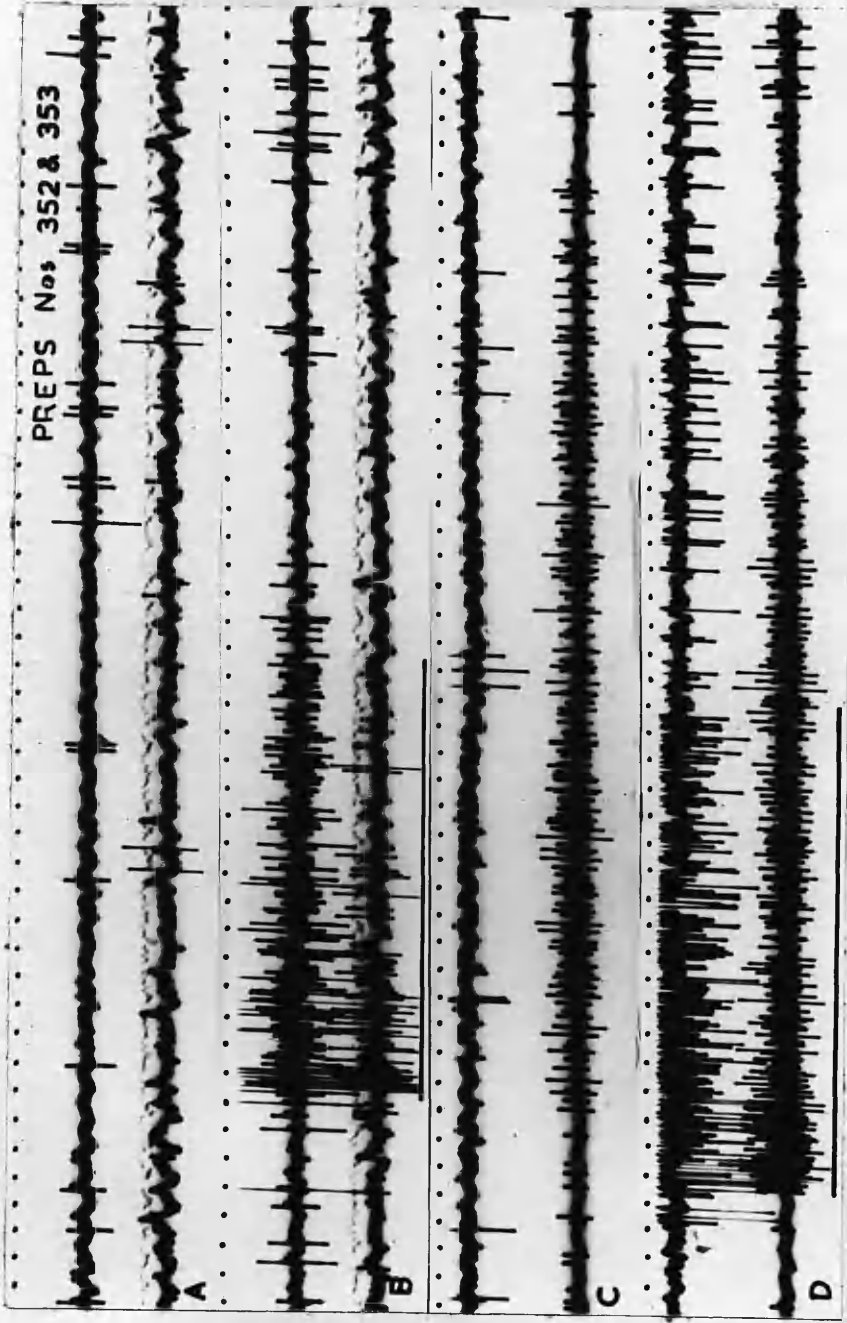


Figure 38. Oscillograph records showing the effect of  $\gamma$  BHC on the intact ventral CNS in the locust. Upper trace shows control preparation covered with medicinal liquid paraffin. Lower trace from preparation covered with  $\gamma$  BHC. A, B, 2 minutes after application; C, D, 40 minutes after application. A and C, basic activity; B and D, natural stimulus. C shows rhythmical burst in BHC preparation. Apologies must be made for displacement of upper trace in C and D.

transmission remained unaltered (Figure 38). The picture differed from the usual preparation (2. Figure 37) only in the amount of basic activity which was slightly higher when the CNS was intact. This basic activity, it must be remembered, is a combination of response to spurious stimulation and spontaneous activity. Preparations in which the cercal nerve was cut had therefore a basic activity composed entirely of spontaneous activity, provided that the cord was transected anteriorly (3. Fig.37). In these preparations rhythmical activity also developed. Preparations of type 4 (Figure 37) were unsuccessful, since all activity disappeared immediately the cord between ganglia 4 and 5 was cut. This cessation of activity took place whether this cut was made first with the CNS otherwise intact, or after an anterior cut had already been made. It was therefore concluded that action potentials could not occur spontaneously in the absence of the cell bodies of the neurons. It was surprising, though, that no other activity persisted, for there should be smaller neurons whose cell bodies are not situated in the fifth abdominal ganglion.

Further experiments were carried out in which the recording electrodes were placed on the abdominal cord and on the cercal nerve and  $\gamma$  BHC added. Natural stimulation evoked a response in both the cercal nerve and the

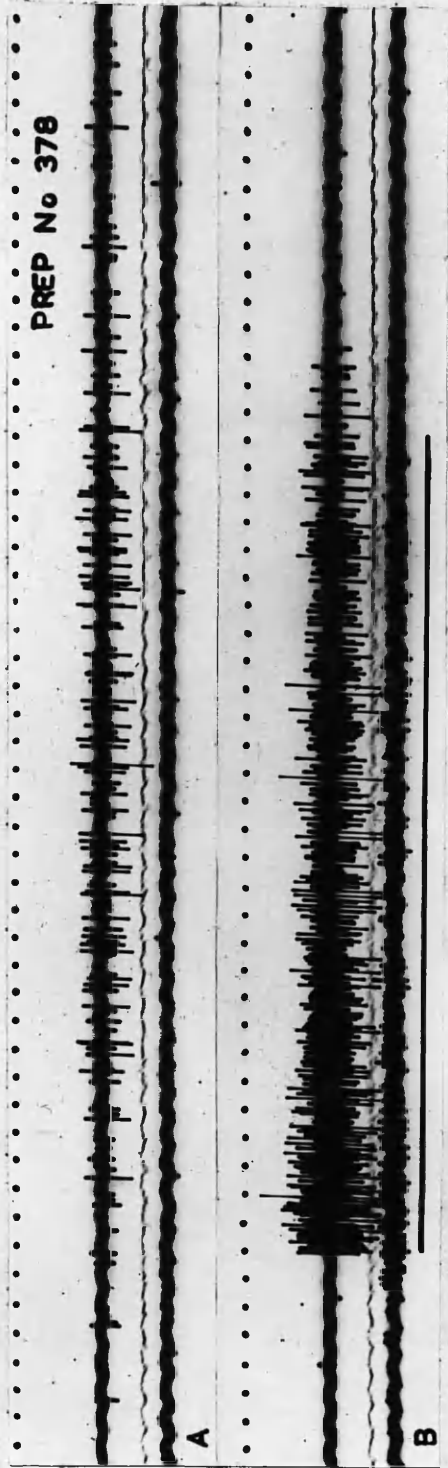


Figure 39. Records from a  $\gamma$  BHC poisoned preparation. Upper trace from abdominal cord, lower trace from cercal nerve. A shows a rhythmical burst in abdominal cord, cercal nerve shows no corresponding activity. B, response to natural stimulation.

cord although the action potentials were smaller and slower. Figure 39 is a record from this type of preparation. The amplification of the two channels is not the same, but has been adjusted so that action potentials in both positions are clearly visible.

In the cercal nerve no spontaneous activity occurred. Action potentials are apparently always correlated with stimulation of the cercal receptors. After application of  $\gamma$  BHC, characteristic rhythmical activity developed in the cord region. No corresponding activity occurred in the cercal nerve (Figure 39). Natural stimulation still evoked a burst of action potentials from both positions. Cutting the cercal nerve anterior to the recording electrodes did not produce any change in the picture, other than the elimination of response to natural stimulation in the cord region. The rhythmical bursts continued undisturbedly and the response remained unaltered in the cercal nerve. In some of these preparations the cord was cut between ganglia 4 and 5. Here, an interesting phenomenon was observed. Instead of cessation of all activity as in a normal preparation, some fibres continued to fire. Sometimes there were a few trains of action potentials immediately following the operation, then a temporary cessation of activity, after which a few sporadic



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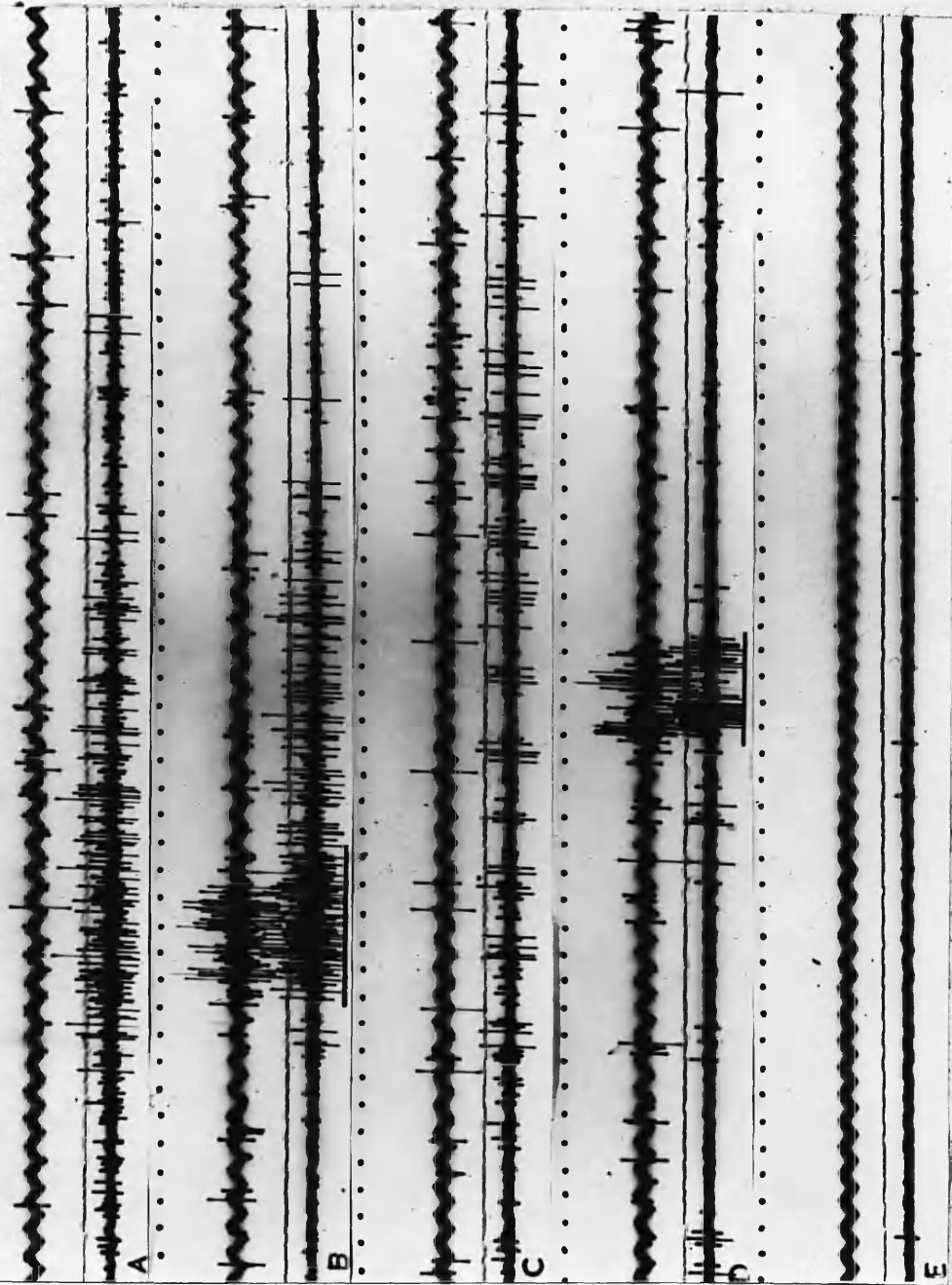


Figure 40. Records from preparations dissected 40 minutes after injection with either  $\gamma$  BHC (Prep. No. 383, lower trace) or paraffin (Prep. No. 384, upper trace). A, B, CNS intact; C, D, abdominal cord between ganglia 2 and 3 cut; E, 10 minutes after cord between ganglia 4 and 5 cut. B and D show response to natural stimulation. 50 cycle hum in upper trace is due to poor pick-up conditions.

TABLE IX.

Comparison of disturbances produced by  $\gamma$  BHC in the nervous system of the locust with stages in poisoning of intact insect.

| Approx. time after inject. | Symptoms in intact locust.  | Electrophysiological symptoms.  |
|----------------------------|---|---|
| 10-15 mins.                | Signs of inco-ordination in locomotary movements. Occasional spasm of violent wing movements. | Rhythmical activity in abdominal cord.<br>Normal response<br>Cercal nerve normal  |
| 20-30 mins.                | Decreased general activity. Marked tremors. Frequently falling over.                          | As above.   |
| 40 mins. -<br>2 hours      | Insects unable to stand. Tremors and often convulsions.                                       | As above.   |
| 2 - 4 hours                | Prostration with slight irregular tremors of limbs and mouth parts. No convulsions.           | Often considerable basic activity. Rhythms not present or present with very long intervals between bursts. Threshold for natural stimulation slightly raised, but response when evoked enormous with after-discharge. |

action potentials occurred. In some of the preparations, after a period of time, even a rhythmical activity reappeared, although many fewer fibres were involved.

Some further experiments were carried out using locusts which had previously received intra-abdominal injections of either  $\gamma$  BHC or liquid paraffin alone. Dissection of these poisoned locusts could then be carried out at various stages in the development of symptoms. By this method neural symptoms could be directly correlated with symptoms appearing in the intact animal. Locusts were dissected up to four hours after injection. The results are summarised in Table IX.

All control preparations were normal. The cercal nerve did not appear to be affected at any stage. Cutting the cercal nerve or the abdominal cord anterior to the third abdominal ganglion did not materially affect the oscillographic picture. Cutting the cord between the fourth and fifth ganglia did change the picture, but some activity always remained, although frequently giant neuron potentials were abolished. Rhythmical activity always disappeared, but in some preparations reappeared after a variable time, with never more than a few fibres involved (Figure 40).

c). Conclusions.

What conclusions then can be drawn from these experimental results? Firstly, it is clear that  $\gamma$  BHC does not affect all parts of the nervous system in the locust. Sensory reception, conduction and transmission seem unaffected, at any rate, in the initial stages of poisoning. In later stages some facilitation does occur. Peripheral nerves seem unaffected whereas spontaneous activity in the CNS is directly affected. This spontaneous activity is apparently enhanced by  $\gamma$  BHC. This much can be stated with some degree of certainty. These statements, however, do not go far towards offering any explanation of the mode of action of  $\gamma$  BHC. Neither are all the observations accounted for by these conclusions.

The continuation of activity in the abdominal cord of poisoned locusts after the cord has been transected between the fourth and fifth ganglia might be accounted for in either of two ways.

1).  $\gamma$  BHC might have a surface effect on the axon and thereby affect the resting potential so as to produce a series of action potentials. This is not very likely. If this were the case, then it is reasonable to suppose that all axons would be affected in the same way. Spontaneous activity should then appear in all neurons,

even in those where it does not normally occur, as well as in isolated pieces of axons. This is not the case: spontaneous activity does not appear in the cercal nerve up to four hours after poisoning, three and a half hours at least after the central neurons are affected.

2). Another explanation might be that  $\gamma$  BHC raises the potentiality of intact neurons to fire spontaneously. Hence small neurons which are unaffected by cutting the cord between ganglia 4 and 5 are induced to fire spontaneously, whereas in normal preparations they do not. This explanation is liable to the same objection as the previous one, namely, why are the neurons of the cercal nerve unaffected? This objection, however, offers less of a barrier than previously. All neurons do not normally fire spontaneously. There exists therefore some fundamental difference between those that do and those that do not. It might therefore be postulated that instead of two sharply defined categories there is a steady gradation from one sort to the other. Some central neurons, while not capable of producing spontaneous action potentials all the time, might under certain circumstances, some of which may occur naturally, be excited or otherwise altered in some way so as to fire spontaneously.

It must be emphasised that these suggestions are

nothing more than mere speculations. Before these results can be properly understood it is essential that further work be carried out both on the effects of  $\gamma$  BHC on various parts of the nervous system and on the normal functioning of the nervous system. It is not possible at the present stage, either, to offer any explanation as to why these spontaneous action potentials should be grouped into rhythmical bursts.

Clearly this work should be followed by similar studies on other parts of the insect nervous system, for instance on a preparation such as Dresden used involving effector organs in addition to sensory and CNS neurons. Further attempts to achieve satisfactory isolated nerve preparations should also be made. So far, however, it does seem that the mechanism of poisoning of  $\gamma$  BHC is not identical with that of DDT. Dresden concluded from his experiments that the increased activity produced by DDT originated neither in the periphery nor spontaneously in the centre, but probably depended on a lowering of the threshold of the synapses, in other words, on facilitation. Roeder and Weiant (1946) also considered that DDT had no central effect, but that both motor nerves and muscles were affected and that the increased activity was due to intense bombardment from the motor neurons. Whichever

of these two interpretations is correct, there seems to be a fundamental difference from  $\gamma$  BHC. While no statement can be made at present about the effect of the latter on muscles or motor neurons, the results so far show that  $\gamma$  BHC does exert a central effect.

## XI. GENERAL DISCUSSION.

The effects of any lethal poison on an animal are likely to be numerous. Some are major effects and as such responsible for the death of the animal, while others are mere side reactions. Analysis of the mode of action of such poisons involves the determination of the various effects produced by the poison and secondly the separation of side effects from the major ones. The ideal approach for such an analysis would be to study the biological effects of the poison on all the metabolic processes of the animal concerned and to correlate the findings with the symptoms preceding death. This is not a feasible proposition since it would involve a wide range of experimental techniques, outside the capacity of one person, and our knowledge of the physiological and biochemical processes involved is too incomplete to form the basis for such a thorough analysis. Another method is to confine the investigation to a particular process of an animal and, despite inadequate knowledge of normal functioning, make a comparative study of various poisons. This method might serve the dual purpose of increasing our knowledge of normal functioning and determining which poisons affect the system under study and how they do so. It was this approach which was attempted in the present



work. A particular region of the locust nervous system was chosen and the effects upon it of certain insecticides studied by electrophysiological methods. Three insecticides, Parathion, DNOC and  $\gamma$ BHC, have been studied in detail and the results of the investigations considered separately. This is a convenient point at which to consider the results of this method of attack on the problem and to see how far it has been successful in the achievement of its aims.

Most of our knowledge regarding the physiology of invertebrate nervous systems has been obtained by analogy with vertebrate nerve physiology rather than by direct analysis. This is necessarily the case since not all the experimental techniques used in vertebrate neurophysiological investigations can readily be applied to invertebrates. While this method is not the most desirable, it is useful, provided that its limitations are fully realised, particularly that such comparison rests on the assumption that there is a fundamental similarity of function between the nervous systems of the two groups, an assumption which is not necessarily valid or by any means proven.

The problem in this case was resolved into three questions:-

1) Does the insecticide interfere with the normal oscillographic picture of the locust cercal nerve - giant fibre preparation?

2) If so, can the effect be localised to a particular site of action?

3) Can the effect be explained in the light of current knowledge of the functioning of the nervous system?

Questions 1) and 2) can be answered directly by experimental investigation while the answer to question 3) is an interpretation of these experimental results. The answer to this latter question can be supported by further experimental investigation but it will remain a theoretical answer rather than a factual one.

All three insecticides affect the normal oscillographic picture recorded from the preparation concerned. The effect of parathion appears to be restricted to the synapse whereas that of  $\gamma$  BHC is restricted to the central neurons. The site of action of DNOC was not localised.

Parathion has been shown to be a truly neurotoxic substance, acting as a ganglion blocking agent. It is a known cholinesterase inhibitor and its effect on the locust cercal nerve - giant fibre preparation can be explained in terms of cholinesterase inhibition. Both Ach. and cholinesterase have been shown to be present in insect nerve

tissue (Stegwee, 1951) but that Ach. functions as a transmitter substance in insects has yet to be demonstrated conclusively. By comparison with the action of other known vertebrate cholinesterase inhibitors it seems likely that chemical transmission does take place in insects but differs in detail from that of vertebrates. Further work both with parathion and with these other substances (eserine, etc.) should throw considerably more light on the problem of nerve transmission in insects particularly if the studies can be limited to a single fibre preparation. The giant fibres of the locust abdominal cord should prove admirable for such experiments. Their cell bodies are comparatively large,  $50 \mu$ . in diameter, and are situated near the posterior surface of the last ganglion. They are, therefore, relatively accessible and both antidromic and preganglionic stimulation could be easily applied.

The investigations with DNOC and  $\gamma$  BHC have not been quite so successful. In both cases the starting points were not as advantageous as with parathion. There is available much less information regarding possible modes of action of these substances. From the results obtained it is probable that the effects on the nervous system produced by DNOC are secondary effects, resembling

in some degree an irreversible anaesthesia, brought about by the poisoning of some other metabolic process.  $\gamma$  BHC on the other hand affects the CNS directly, but this effect in itself may not be responsible for the death of the animal. Death may be brought about by neurological upset of some part of the nervous system not yet studied or by some other process altogether.

This work does not make any pretence to be an exhaustive study. The results, however, are hopeful, both concerning the question of mode of action of these substances and towards elucidating the normal physiology of the insect nervous system a little further. More work along similar lines should prove fruitful. There are several other preparations in the locust and in the cockroach which might be used; some have already been mentioned. Comparison with other substances both on this and on other preparations should also be undertaken.

While this method has considerable possibilities and has already yielded some useful information, oscillographic analysis, by itself, cannot be expected to produce a complete understanding of the normal functioning of the nervous system. Neither can the mode of action of these neurotoxic poisons be fully understood by means of this method alone. Living animals are not additive

sums of a number of simple component parts. Experimental limitations make it inevitable that in order to gain any understanding of these functions the animal shall be divided up into simple units. Interpretations of the results of these experiments must, however, be considered in relation to the integrated functional individual. Before this can be done, some simple hypotheses based on the experimental results must be formulated. It must be realised, though, that these hypotheses offer little more than the raw materials from which the final understanding will ultimately emerge. This latter may bear little or no relation to the theories on which it has been built.

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\*References marked thus have not been read by the present author.

XIII. APPENDIX A.

"Observations on giant fibres of the nervous system  
of Locusta migratoria" by P.M. Cook.

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