THE SENSITIVITY OF FROG HEARTS TO ACETYLCHOLINE AND OTHER NEUROHUMORAL TRANSMITTER AND ALLIED SUBSTANCES.

AND

THE COMPARATIVE TOXICITY OF MODERN PLASTIC AND SILICONE CONNECTING TUBING TESTED ON FROG HEARTS.

a thesis for the degree of Ph.D.

University of Glasgow.

1962.

by

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(Volume 1)

PART II

THE COMPARATIVE TOXICITY OF MODERN PLASTIC AND SILICONE CONNECTING TUBING, TESTED ON FROG HEARTS

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P R E F A C E.

The work incorporated in this thesis is out of a joint project between myself and Dr. I.A. Boyd, Senior Lecturer in Physiology, University of Glasgow, on the 'Biological Threshold for Drug Action'. A sensitive equipment specially suitable for continuously recording four cardiac parameters - venous pressure, heart rate, arterial pressure, and cardiac output, has been developed by Dr. Boyd and Mr. Eadie over a period of several years. The details of this equipment and technique of recording have been published, as mentioned in the section on Methods. All the work incorporated in the thesis was conducted by myself. Dr. Boyd has, however, very kindly supplied the results of a few experiments conducted by him in 1956 and 1957. These results of Dr. Boyd have been used only for evaluating the general trend of sensitivity of frog hearts to acetylcholine over a longer period and they are essentially in agreement with my own results of three years. These experiments of Dr. Boyd are clearly identified in this thesis, and I hereby thankfully acknowledge permission to refer to them.

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A C K N O W L E G E M E N T S.

I shall ever remain grateful to Professor R.C. Garry for the keen interest he has taken in the guidance of this work from beginning to end.

I am also thankful to Dr. I.A. Boyd for guidance, supervision and helpful suggestions during the course of this work, and also for permission to refer to some of his own experiments.

My thanks are also due to the Boyd Research Institute for providing the equipment, apparatus and technical staff to look after the working and maintenance of the equipment. My thanks are due to each of the members of the technical staff headed by Mr. Eadie and Miss Campbell, who individually took extraordinary interest in keeping the apparatus and equipment ready for use at all times. The success of the work is largely due to this promptness.

I am also thankful to all the members of the teaching staff of the Institute of Physiology for their helpful attitude in this work, and my thanks are particularly due to Dr. J.B. deV. Weir who helped in the statistical analysis of the results. Among the technical staff of the Institute of Physiology I am especially grateful to Mr. R. Callander, Miss J. Wilson and Mr. D. MacAllister for their help in obtaining the photographs of the Figures.

I N T R O D U C T I O N.

The present work was not intended to investigate the pharmacological actions of a drug or of a group of drugs. The aim was to investigate some physiologically important and well-documented substances to determine the minimum effective concentration (threshold sensitivity) which can influence the activity of a biological preparation. The neurohumoral transmitter substances, acetylcholine, adrenaline, noradrenaline, 5-hydroxytryptamine and nicotine, have been extensively investigated by many workers on a large variety of biological preparations. One would also expect most of these substances to be effective in small amounts. These substances were, therefore, selected for the present study.

The spontaneously beating perfused frog heart was selected as the preparation to test the threshold sensitivity to these substances for several reasons:

1. The frog heart is responsive to all substances under investigation.
2. The frog heart has been extensively used in the study of the actions of acetylcholine ever since Dale's classical experiments in the year 1914.
3. Some of the variables which fundamentally influence and determine cardiac performance can be readily controlled in the hearts of frogs.

4. Frog hearts can be made to survive for days under a suitable perfusion technique and, therefore, several series of doses can be tried on the same preparation with a sufficient interval of time.
5. A sensitive equipment could be developed to record several parameters of the activity of frog hearts simultaneously and continuously.

Acetylcholine was selected as the first substance for investig--ating the threshold sensitivity. Because it was intended to test a very wide range of concentrations of acetylcholine starting with very small concentrations, it was essential to eliminate all possible chances of an effect due to an artefact. Several types of control at different stages of each experiment were, therefore, introduced. To obtain satisfactory controls with this sensitive equipment was not easy. Several factors which are relatively unimportant while studying effects of large doses of drugs in high concentration with insensitive recording techniques are now of considerable importance. All factors liable to variation demand analysis and control when sensitive equip--ment is used to study the effect of very small quantities of substances. Such precautions are necessary simply because variables or contaminants can so easily simulate the effect of small quantities of potent physiological agents. My principal task in this type of work, therefore, was to obtain such experimental perfection as would provide satisfactory controls at all stages of each experiment.

The distilled water used for preparing solutions, the ionic

composition of fluids, the type of glass containers, the connecting tubing, the minor variations in temperature from place to place in the laboratory (and consequently of solutions standing at different places), minor variations in the pH of solutions and bacterial contamination of glassware and tubing, all were liable to influence the behaviour of the preparation. With conventional methods the influence of such factors would not be easily detected. Most of these variable factors were easily controlled once their importance was realised, though considerable time was devoted to analysing and controlling them. The choice of a suitable type of connecting tubing of really non-toxic material, however, was found to be a difficult problem and a special study had to be undertaken to find out the best available material. The results of this side study are described separately.

Since the sensitivity of frog hearts to acetylcholine seemed to vary from time to time, experiments were carried out over a period of several years to determine the exact periods of incidence of high sensitivity. The results of these experiments indicated that the incidence of high sensitivity was greater in certain periods of the year. The possibility of some relation between the incidence of high sensitivity (considered possibly greater in the female at one stage) to acetylcholine and the sex cycle in the frog was entertained. A scheme of injecting female sex hormones into intact frogs of either sex before using them for testing sensitivity to acetylcholine, was formulated with a view to increasing or stabilising the sensitivity. The

influence of the composition of the Ringer's solution on sensitivity to acetylcholine was also investigated.

A considerable time was devoted to conducting these different types of experiment on sensitivity to acetylcholine. The other substances have not been investigated in similar detail. However, experiments were designed to test the other substances on at least some hearts to find if the high sensitivity of a particular heart to acetylcholine was associated with a high sensitivity to other substances and if administration of female sex hormones was associated with a change in the sensitivity to any of these substances. The sensitivity of the hearts to adrenaline, noradrenaline and 5-hydroxytryptamine was, therefore, tested in a few uninjected frogs and in most of the injected frogs. Nicotine was investigated only in a few uninjected frogs.

For convenience of description the thesis is divided into two parts.

Part I - contains the experiments on the 'Sensitivity of Frog Hearts to Acetylcholine and Other Neurohumoral Transmitter and Allied Substances'.

Part II - is devoted to the 'Comparative Toxicity of Modern Plastic and Silicone Connecting Tubing, Tested on Frog Hearts'.

PART I

THE SENSITIVITY OF FROG HEARTS TO ACETYLCHOLINE AND OTHER NEUROHUMORAL TRANSMITTER AND ALLIED SUBSTANCES.

REVIEW OF LITERATURE.

A large volume of literature is available on the different aspects of the actions of acetylcholine, adrenaline, noradrenaline, and 5-hydroxytryptamine. A review of the literature relevant to the present work is presented here.

ACETYLCHOLINE

Sensitivity of Various Biological Preparations.

A number of biological tissues have been investigated for sensitivity to acetylcholine. The dorsal longitudinal muscle of the leech was found to be quite sensitive in a concentration of 10^{-6} g/ml. (Chang and Gaddum, 1933; Feldberg and Gaddum, 1934). Eserinised leech muscle may respond to 10^{-10} g/ml (Minz, 1932). Chang and Gaddum (1933) also found that the sensitivity of the rectus muscle of the frog varied from 10^{-6} to 10^{-8} g/ml depending upon the season. Goffart (1939) observed that the sensitivity could be improved by storing the frogs at 2 to 3°C for a few days prior to use. The frog heart has been extensively used for the study of actions of acetylcholine. (Dale, 1914; Kolm and Pick, 1920; Clark, 1926; Loewi, 1949; Bentley and Shaw, 1952). Dale (1914) and Clark (1926) observed that the minimum effective concentration in frog hearts was 10^{-9} g/ml. Guinea-pigs ileum (Bentley and Shaw, 1952; Bernheim and Gorfain, 1934) and rabbits' duodenum (Dale, 1914; Dale and Dudley, 1929; Feldberg and Gaddum, 1934; Fletcher, Best and Solandt, 1935) are sensitive to acetylcholine between 10^{-6} and 10^{-9}/

10^{-9} g/ml and have been used for assay of acetylcholine. The blood pressure response of anaesthetised cat is also sensitive to acetyl--choline in a dose of 0.01 x 10^{-6} to .000002 x 10^{-6} g/kg (Dale, 1914; Reid Hunt, 1928; Feldberg and Gaddum, 1934, Brown and Feldberg, 1936). Isolated rabbit auricle is sensitive to acetylcholine in concentrations of 10^{-6} g/ml to 10^{-9} g/ml (Clark, 1921; Feldberg and Gaddum, 1934; Webb, 1950; Burn, 1956). Recently the invertebrate tissues have been investigated for their sensitivity to several neurohumoral substances. The hearts of several molluscs have been found to be very sensitive (Welsh, 1943). The heart of the clam Venus mercenaria is sensitive to acetylcholine in a concentrat--ion of 10^{-11} g/ml (Prosser, 1940; Wait, 1943; Welsh and Taub, 1948). A seasonal variation in sensitivity (Prosser, 1940; Welsh and Taub, 1948) was also observed. The sensitivity was found to be maximum in late winter and spring up to June. An irregularity in the heart beat was observed in late summer. Isolated hearts of Cyprina islandica suspended in a bath responded to acetylcholine in concentrations of 10^{-9} to 10^{-11} g/ml. A graded effect on rate but not on amplitude was observed (Welsh, 1954). Similar findings were confirmed on the heart of the whelk Buccinium undatum (Welsh, 1954). Hughes (1955) found the isolated ventricle of Mya arenaria to be responsive to acetyl--choline around a concentration of 10^{-11} g/ml. An inhibitory response (both reduction in amplitude and slowing) was observed. He also found that this preparation was occasionally (April and May) sensitive to as low a concentration as 10^{-16} g/ml. Stimulating

action, both on amplitude and rate, of low concentrations of acetyl--choline was also observed. Stopping doses of acetylcholine occasionally improved the performance after washing out acetylchol--ine and made the heart more sensitive. Old hearts which beat sluggishly could be improved with ergometrine maleate without influencing sensitivity to acetylcholine.

Muscle strips of holothurians (echinodermata) have also been found to be sensitive to acetylcholine (Bacq, 1939). The longitud--inal body-wall muscle of Holothuria nigra responded to 10^{-9} g/ml of acetylcholine with little spontaneous activity. Eserine at a concent--ration of 10^{-8} g/ml produced 100 times increase in sensitivity but also increased the spontaneous activity (Welsh, 1954). Krnjevic and Mitchell (1961) reported that the rat duodenum was sensitive to acetylcholine 10^{-11} g/ml. Corsten (1941) found that acetylcholine was effective on frog lung preparation in a concentration of 10^{-16} g/ml. MacIntosh and Perry (1950) have discussed the sensitivity of different biological preparations used for assay of acetylcholine.

Influence of some Factors on Sensitivity to Acetylcholine.

(i) Between species and in the same species:-

The sensitivity of various biological preparations from different species has been considered above. A significant variation in sensitivity between members of same species was observed by many workers quoted above.

(ii) Spontaneous changes in sensitivity in the same preparation:-

Spontaneous changes in sensitivity to acetylcholine in isolated frog and rabbit hearts has been reported (Cori, 1921; Pines, 1934; Rothberger and Sach, 1938; Webb, 1950).

(iii) Sex:-

The difference in sensitivity to acetylcholine between two sexes has not been reported by previous workers. However, Cori (1921) observed a more marked and consistent cardiac inhibition from vagal stimulation in female frogs. Applerot (1930) reported similar findings in frogs and toads.

(iv) Season:-

A seasonal variation in the sensitivity in molluscs' hearts has been reported. The hearts of Venus mercenaria (Prosser, 1940; Welsh and Taub, 1948) showed maximum sensi--tivity in late winter and spring up to June. The heart of Mya arenaria was, however, found to be most sensitive in April and May (Hughes, 1955). A marked seasonal variation in the action of the vagus on the frog hearts was recorded by Clark (1927 b). He found vagal stimulation to be much less effective in the summer than in the winter.

(v) Temperature:-

No remarkable effect of the temperature of perfusion fluid on acetylcholine sensitivity was observed in frog heart (Clark, 1926) and in isolated rabbit auricles (Webb, 1950). However, minor variations in the intensity of action of

acetylcholine on the chronotropic response was reported (Webb, 1950).

(vi) Anoxia:-

No influence of anoxia on sensitivity to acetylcholine was observed in isolated rabbit auricles (Webb, 1950)

(vii) Adrenaline:-

Adrenaline potentiated the actions of vagus on frog hearts (Kolm and Pick, 1920) and acetylcholine antagonised the action of adrenaline on frog heart (Loewi, 1949). Burn (1945) has discussed the potentiating effect of adrenaline on the action of acetylcholine. Middleton and Talesnik (1949) observed an increase in sensitivity to acetylcholine in guinea-pig and cat hearts. The potentiating effect of adrenaline on acetyl--choline action was, however, not found in isolated rabbit auricles (Webb, 1950).

(viii) Composition of Ringer's solution:-

(a) Potassium

Bouckaert (1921) reported a decrease in the action of pilocarpine on frog hearts when the concentration of potassium was reduced. A reduced concentration of potassium and excess of hydroxyl ions was found to decrease the action of acetylcholine in frog hearts (Davis, 1931). Clark (1927, a,b), however, found that a low concentration of potassium increased while a high concent-

-ration of potassium decreased the action of acetylcholine in frog hearts. The findings of Clark have been confirmed in isolated rabbit auricles (Graham, 1949).

(b) Calcium:-

No change in the sensitivity of frog hearts to pilocarpine and muscarine was observed on changing the calcium concentration (Loewi, 1912). Kolm and Pick (1920) however reported a decrease in the action of acetylcholine and muscarine in frog hearts when the calcium concentration was increased. Zondek (1920) also observed a decrease in the action of muscarine in frog hearts on increasing the calcium concentration. Clark (1927,a) found that an increase in the concentration of calcium decreased the action of acetylcholine while a reduction of calcium concentration had no effect in frog hearts. Webb (1950) has reported a qualitative change in the action of acetylcholine on increas--ing the calcium concentration. He reported that acetyl--choline stopped the isolated rabbit auricles, suspended in normal Ringer's solution, without influencing the pacemaker; but in presence of a high concentration of calcium in the Ringer's solution, the stoppage by acetylcholine was associated with the failure of pacemaker to discharge the impulse.

(ix) Metabolism:-

Torda and Wolff (1946) observed that iodoacetate had no

effect on acetylcholine sensitivity while fluoride increased the sensitivity. Webb (1950) has studied the influence of several metabolic substrate and inhibitors on sensitivity of isolated rabbit auricles to acetylcholine. It was observed that succinate, fumarate, citrate, oxalacetate, glutamate, butyrate, β-hydroxybutyrate, caprylate, pyruvate, malate, acetate, malonate, pyrophosphate, and phloridzin, had no effect on acetyl--choline sensitivity. Cyanide, azide and iodoacetate potentiated acetylcholine action. Fluoride had a biphasic effect causing initial transient potentiation but subsequent decrease in the action of acetylcholine. Glucose depletion caused only minor changes in the action of acetylcholine.

Stimulating Action of Acetylcholine:

Sach (1937) and Rothberger and Sach (1938) showed that acetyl--choline and other choline derivatives gave an excitatory effect (more marked on inotropic than on chronotropic response) in lower concentrations (around 10^{-10} g/ml) and an inhibitory effect at higher concentrations (between 10^{-6} to 10^{-8}) in isolated rabbit auricles. They also observed that both negative and positive inotropic effects could be neutralised by atropine. Pines (1934) observed an excitatory effect at 10^{-7} g/ml. Spadolini and Domini (1940) made similar observat--ions on isolated guinea-pigs' heart. They noted an excitatory effect between the concentration of 10^{-9} and 10^{-6} g/ml and an inhibitory effect from higher concentrations. Prosser (1942) demonstrated

that low concentrations of acetylcholine increased the heart rate in Talorchestia, Bactrurus and Asellus. Hughes (1955) observed an increase in amplitude and rate of the ventricles of Mya arenaria at lower concentrations of acetylcholine. McDowall (1946) showed that small quantities of acetylcholine stimulated the unatropinised and larger quantities stimulated the atropinised cat hearts. Hoffman, Hoffman, Middleton and Talesnik (1945) also demonstrated the stimulant action of large quantities of acetylcholine on atropinised mammalian hearts. They showed that the effect of larger quantities of acetyl--choline was due to a nicotine-like effect on the ganglia and chrom--affin tissue present in the heart leading to a release of adrenaline. Burn (1956) has discussed the excitatory effect of acetylcholine in rabbit auricles and on ciliary movements.

Marshall and Vaughan Williams (1955) have demonstrated the stimulating action of acetylcholine in a different way. These workers observed that cooling of the isolated rabbit auricles to 20°C stopped the mechanical and electrical activity except small localised potent--ial swings presumably at the pacemaker. Addition of small quantities of acetylcholine to the bath restored the conducted action potential and the associated mechanical activity. Sometimes a concentration as low as 10^{-9} g/ml was found effective for this purpose. Webb (1950) observed a different type of stimulation (called 'post-wash stimulat--ion') on washing out acetylcholine from the organ bath in isolated rabbit auricles using acetylcholine in a concentration of 10^{-7} g/ml.

On the basis of the stimulating effects of small quantities of acetylcholine and other evidences Burn (1956) has proposed the possibility that an acetylcholine synthesis-destruction cycle may be responsible for the origin and maintenance of heart beat.

Mechanism of Action of Acetylcholine on Heart:

Acetylcholine and vagal stimulation increased the resting potential and decreased the action potential (Gilson, 1932). Acetylcholine slows the conduction rate (Dale and Mines, 1913; Wedd and Blair, 1945). Acetylcholine shortens action potential and excit--ation time (Bogue and Mendez, 1930) and decreases the refractory period (Gilson, 1932; Wedd and Blair, 1945). It has been considered highly probable that acetylcholine produces some change in the elect--rical properties of the membrane of the cells (Gaskell, 1887; Einthoven, 1908; Bogue and Mendez, 1930; Gilson, 1932, and many others). Acetylcholine is known to be a depolarising agent. Webb (1950) has discussed the possibilities whether acetylcholine exerts a direct depolarising action or it acts on the process of repolaris--ation. He also discussed the possibility of dependence of acetyl--choline action on metabolic process involved in the cardiac activity. A reduction in oxygen consumption during vagus stimulation and during acetylcholine action has been observed (Garrey and Boykin, 1934).

Relationship between Dose and Response to Acetylcholine:

Clark (1926) calculated the amount of acetylcholine entering the cells and concluded that there was no relationship between the

amount of drug entering the cells and the intensity of inhibitory response. Straub (1907) maintained that the action of a drug depend--ed on the difference of concentration outside and inside the cells. He stated that as muscarine gradually entered the cells and accumulated inside the cells its action on the heart diminished. The intensity of action of choline compounds was found to be inversely proportional to the rate at which they diffused into living tissue. (Wertheimer and Paffrath, 1925). These authors noted that acetylcholine had 1000 times as intense action as choline on isolated gut of guinea-pig but choline diffused through frog skin 1000 times more rapidly than acetyl--choline. A development of tolerance to the same dose of acetylcholine has been reported in isolated rabbit auricles (Webb, 1950). Vagal action was also found to be decreased during depression due to previous stimulation of vagus (Gilson, 1932).

Maintenance of Strength of Acetylcholine Solutions:

It is a general belief that acetylcholine solutions deteriorate rapidly in alkaline medium. Dale (1914) observed a slow hydrolysis of acetylcholine in Ringer's solution at room temperature after some hours. Clark (1926) stated that there was a 'fair decomposition' of dilute solutions of acetylcholine. Since these observations a majority of workers have used freshly prepared acetylcholine solutions and failure to demonstrate the effect of acetylcholine is often attrib--uted to the high alkalinity of the solutions leading to a rapid deterioration of acetylcholine.

ADRENALINE AND NORADRENALINE.

The sensitivity to catechol amines has been investigated on several biological preparations to find out one suitable for assaying small amounts of these substances in tissue fluids.

The blood pressure of spinal cats can be affected by adrenaline and noradrenaline in doses of about 10^{-8} g. The blood pressure of anaesthetised rats is the most sensitive (Crawford and Outschoorn, 1951). The isolated uterus of the rat has been used for assay and is as sensitive as rats' blood pressure to adrenaline but relatively insensitive to noradrenaline. The colon of the rat is sensitive both to adrenaline and noradrenaline (Gaddum and Lembeck, 1949, Gaddum, 1950). The duodenum of the rabbit has also been used for the assay of catechol amines (Burn, Finney and Goodwin, 1950). The caecum of the chicken is quite sensitive to adrenaline and noradrenaline (Euler, 1956). West (1943) used isolated frog heart for assaying small quantities of adrenaline up to a dilution of 10^{-8} g/ml. He found that 30% of the activity of adrenaline solutions was lost 5 minutes after preparation! West (1947) also compared the sensitivity of frog hearts to adrenaline and noradrenaline and found that adrenaline was 8 times more effective. The hearts of 'winter frogs' were more sensitive both to adrenaline and to noradrenaline than the hearts of 'summer frogs'. Cocaine and ephedrine slightly sensitised the frog hearts to the action of adrenaline.

5-HYDROXYTRYPTAMINE.

The vascular response of isolated ear of rabbit was found to be sensitive to 5-hydroxytryptamine (Page, 1942). Sheep carotid artery rings (Reid and Rand, 1952) and chicks' amnion (Ferguson, 1947) are other preparations sensitive to 5-hydroxytryptamine.

Isolated colon of rat (Dalgleish, Toh and Work, 1953; Feldberg and Toh, 1953) and isolated uterus of rat (Amin, Crawford and Gaddum, 1954) are sensitive to 5-hydroxytryptamine in a concentration of 10^{-9} g/ml. Recently the fundal strips of rats' stomach have been reported to be sensitive to 5-hydroxytryptamine around similar concen--trations (Vane, 1957).

Hearts of several marine invertebrates have been found to be sensitive to 5-hydroxytryptamine which stimulates them (Erspamer and Boretti, 1951). The heart of Venus mercenaria responds by an increase in the rate and amplitude (Welsh, 1953) and has been used for assay of small quantities of 5-hydroxytryptamine (Twarog and Page, 1953) in concentrations around 10^{-10} g/ml. Welsh (1953) tested other marine invertebrates and showed that the isolated hearts of Cyprina islandica was sensitive to 5-hydroxytryptamine to the same degree, giving a graded excitatory response with increasing concen--trations. Lysergic acid diethylamide antagonised this action. Isolated hearts of Spisula solida (Gaddum and Paasonen, 1955) Anodonta, Cygnea, and many other molluscs, snails and mussels are sensitive to 5-hydroxytryptamine in concentrations around 10^{-10} g/ml. The actions of 5-hydroxytryptamine (serotonin) have been reviewed recently (Page, 1958).

SUPERSENSITIVITY OF DENERVATED STRUCTURES.

The problem of supersensitivity of denervated structures has been of great interest since the publication of the monograph by Cannon and Rosenblueth (1949) on this subject. Rosenbleuth (1932) found that the denervated nictitating membrane of cat was supersensi--tive to substances as different as acetylcholine, pilocarpine, eserine, and histamine. Bacq and Rosenblueth (1934) found that it was also supersensitive to calcium and potassium salts. Later Bacq and Fredericq (1935) showed that the nictitating membrane had both cholinergic and adrenergic innervation.

The iris of cat's eye becomes more sensitive to acetylcholine after the removal of ciliary ganglion. Schofield (1952) found that the supersensitivity was associated with a fall in cholinesterase in the iris.

Ricker and Wescoe (1949) observed that injections of DFP (difluorophosphate) made the sub-maxillary gland more sensitive to acetylcholine and the amount of cholinesterase also fell. Similar observations have been made by Stromblad (1955) in the case of parotid gland after section of pre-and post-ganglionic fibres.

Spinks (1952) has shown that thyroid feeding decreases the amount of amine oxidase in the blood vessels and thus increased the pressure response to epinephrine. Burn and Robinson (1953) found a decrease in amine oxidase associated with supersensitivity of cats' nictitating membrane to epinephrine. They observed a correlation

between decrease in amine oxidase and degree of supersensitivity. However, the degree of amine oxidase inhibition by different drugs and potentiation of epinephrine response do not correlate. Burn and Philpot (1953) did not find a fall in amine oxidase in the dilator pupillae after denervation of superior cervical ganglion in cat.

Emmelin (1952) showed that removal of superior cervical ganglion sensitised the gland to the action of epinephrine. Division of chordatympani also leads to increased sensitivity to epinephrine. Emmelin and Muren (1952) also found that if the gland is maintained in activity after the division of chorda-tympani by injections of pilocarpine, epinephrine or carbachol, supersensitivity to epinephrine did not develop. Stromblad (1955) showed a fall in cholinesterase in sub-maxillary gland after divisions of chorda-tympani. He also noticed that increased sensitivity of the gland to epinephrine and fall in cholinesterase both could be prevented by injections of pilocarpine. Burn and Robinson (1953) have observed a correlation between the fall in the amine oxidase concentration in the nictitating membrane and increased sensitivity to norepinephrine.

The above work suggests that supersensitivity to neurohormones is related to the amount of inactivating enzymes present in the tissues. However, there are many exceptions where such a correlation does not exist. It is possible that the amount of neurohormones present in the tissue rather than the concentration of enzymes which

are responsible for their destruction, may be of more importance. Ruegg (1955) has observed that the effect of epinephrine and norepinephrine on the radial muscle of iris of the rabbit is potentiated by very small amount of acetylcholine, and also by eserine and neostigmine. A potentiation of response to acetylcholine by adrenaline on different tissues has been reported as reviewed earlier.

M E T H O D S.

EQUIPMENT.

The block diagram of the equipment is given in Fig.1. Photographs showing front and side views of the main operational parts of the equipment, presenting details of the perfusion system are given in Fig. 2 and 3 respectively. The various parts of the equipment are described below.

Perfusion System.

Six Perfusion Reservoirs:

1. A large glass reservoir (Fig.2,R) or
   A Mariotte bottle (Fig.3,b) of about 1500 ml. capacity from which the heart could be perfused for long periods especially over night.
2. Five horizontally-placed specially designed 'burettes' each of 100 ml capacity (Fig.3,B)

Above each of these reservoirs and burettes was a separate press button (Fig.2,P) which electrically moved a marker pen (Fig.4) to an appropriate position on the recording paper, showing the commencement and end of perfusion from any reservoir or burette. A green light (Fig. 2,G) above each button also kept the operator constantly informed as to which burette was in use and whether the marker had been moved to the appropriate position. The Mariotte bottle and burettes were mounted on a common carriage which could be moved up and down by an

electric motor (Fig.2,m) to change simultaneously the level of all the burettes. The levels of different burettes and reservoirs could be adjusted in relation to each other by separate mechanical arrangements (Fig.3,Sc). The perfusion pressure (venous pressure), therefore, could be adjusted in any of these reservoirs to any desired value or could be kept constant at a particular value. The large capacity of the reservoir and the horizontal position of the burettes avoided significant changes in the venous pressure over a period of several minutes and frequent adjustments were not needed. It was desirable not to adjust the venous pressure during a critical perfusion and these arrangements obviated the need for such readjustments.

Six Standpipes:

Each of these reservoirs and burettes was connected to one of the 6 standpipes (Fig. 2,S) by a flexible connecting tube (standpipe-connecting-tube). The reservoir and Mariotte bottle were connected together to one standpipe with the help of a T tube. Each standpipe was in turn connected by a short flexible tube (transducer-connecting-tube, Fig. 1) to the common perfusion chamber (Fig. 1) incorporated in the venous pressure transducer (Fig.3,t). Each standpipe had a separate exit (Fig. 1) for draining out the perfusing fluid. An artery

clip was placed on each of the six transducer-connecting-tubes. To perfuse the heart from a particular burette the clip was removed from the transducer-connecting-tube in front of the corresponding standpipe.

Connecting Tubing:

'Portex Crystal Vinyl' P.V.C. (Portland Plastics Ltd.) was initially used as standpipe-connecting-tubing. Manufacture of this material was later discontinued and 'Waterclear' P.V.C. (Esco Rubber Ltd.) was substituted. A sample of silicone tubing supplied by Esco Rubber Ltd. in 1956 was used as transducer-connecting-tubing. This was often responsible for unsatisfactory controls and was replaced by silicone 'DSR-551' (Dunlop Rubber Co. Ltd.). The reasons for selecting 'Waterclear' P.V.C. and 'DSR-551' silicone are discussed in Part II.

Venous Pressure Recorder

This comprised a transducer (pressure sensing head), associated electronic equipment and a pen recorder which gave a continuous record of the venous pressure on the paper. A monitoring meter, calib--rated to read the venous pressure in mm of water, was also provided (Fig.2,M). The movements of the needle of this meter with changes in venous pressure with each heart beat indicated a free flow of fluid into the heart. Changes in the venous pressure of a fraction of a mm of water could be appreciated and corrected, thus enabling the

venous pressure to be kept constant throughout the experiment. It should be noted here that both the amplitude and rate of heart beat are profoundly influenced by changes in intra-luminal pressure in isolated hearts (Pathak, 1957, 1958, a,b). Therefore, the three other recorded parameters of cardiac activity, i.e. the heart rate, the arterial pressure, and the cardiac output were all influen--ced by changes in venous pressure and special care had to be exercis--ed in designing and developing the recording of venous pressure and to minimise the variations in the venous pressure. During the change over of perfusion from one reservoir to the other insignificant variat--ions in the venous pressure were liable to occur but they could be promptly adjusted so that there was no alteration in the activity of the heart and the stability of record during the change over.

Perfusion Cannulae and Fluid Transit:

The perfusion fluid from the transducer chamber entered the heart through a glass cannula introduced into the inferior vena cava and came out through two glass cannulae, one in each aorta (Fig.9). The aortic cannulae were connected by flexible silicone tubing to a mercury manometer. The escaping fluid passed through one of the several artificial resistances made of drawn glass capillary tubes, into a common outlet chamber, finally passing out drop by drop through a narrow glass cannula (Fig. 1 and Fig.3,r).

Cardiac Output Recorder:

The drops escaping from the exit cannula of the common outlet chamber at the bottom of the resistance unit fell between two metal plates (Fig. 1 and Fig. 3,d) short-circuiting them and producing an electrical impulse each time a drop fell. These impulses were counted, integrated and fed into the output pen recorder which was calibrated to record the output in ml/min as a continuous line.

Arterial Pressure and Heart Rate Recorder:

Changes in the height of the column of mercury in the manometer (Fig. 2,Hg) with each heart beat altered the capacitance between the mercury and a brass plate (Fig. 1). The change in the capacitance was measured by the Fielden Proximity meter (Fig. 5) which fed the arterial pressure (blood pressure) recorder as well as a heart rate integrator, which in turn fed the heart rate pen recorder. The blood pressure recorder gave a continuous record of the pulse pressure as well as the systolic and diastolic pressures. The heart rate was counted and recorded as a continuous line. To provide an audible indication of each heart beat a loud speaker was connected in the circuit of the heart rate recorder.

Fig.4 is a photograph of the pen recorders and of the actual record on the chart papers. Fig.5 is a photograph of the electronic equipment associated with the pen recorders.

Parts of this equipment were described in the Journal of Physiology (Boyd and Eadie, 1957,a,b). Further improvements have been added to the equipment. The mains supply has been stabilised so that fluctuations in the mains voltage do not interfere with the working of the equipment and the pen recorders have also been modified to minimise pen to paper friction. Complete electronic details of the equipment have been published in J. Brit. Institution of Radio Engineers (Boyd and Eadie, 1961).

CLEANING AND STERILIZATION OF GLASSWARE AND TUBING.

All glassware and connecting tubing were thoroughly cleaned and sterilised each time before use. The sterilised apparatus was stored in sealed nylon bags which were opened just before use. The 'Calgon Method' was usually employed for the cleaning of glassware and connecting tubing. In a few experiments described in Part II of the thesis a 'Bicarbonate Method' of cleaning the tubing, as recommended by one of the manufacturers (Esco Rubber Ltd.) was also employed to compare the two methods of cleaning. Both these methods are described below.

Calgon Method. (Hanks, 1952; modified in the present work)

Reagents:

1. Calgon mixture

   Calcium metaphosphate 40 g. (Calgon, supplied by Albright and Wilson Ltd., Birmingham.)

   Sodium meta silicate 360 g.

   Distilled Water 4.5 litres

   Filter, dilute 1 : 100 before use.

2. Concentrated hydrochloric acid

   Dilute 1 : 100 before use

3. Soft potassium soap solution 1 to 2%

Procedure:

1. Rinse all glassware and connecting tubing thoroughly with tap water

2. Wash with soap solution and later with tap water.

3. Soak in a glass tank containing the diluted calgon mixture for at least 12 hours

4. Wash with distilled water.

5. Fill with and soak in 1 : 100 hydrochloric acid in a glass tank for 2 hours.

6. Wash thoroughly with distilled water. Fill with distilled water and leave in a glass tank containing distilled water for 1 hour.

7. Check pH of the distilled water of the tank with an indicator paper. If acid is present repeat steps 6 and 7.

Bicarbonate Method.

1. Rinse the tubing thoroughly with distilled water.

2. Boil for 30 minutes in 1% sodium bicarbonate solution and cool.

3. Wash with distilled water.

4. Soak in 1% hydrochloric acid for 2 hours.

5. Rinse with tap water.

6. Rinse in three changes of distilled water and dry.

Sterilization.

1. Put the cleaned apparatus in drying oven for 2 hours at 120°C.

2. Put into bags of autoclave nylon layflat tubing and seal with adhesive tape (both supplied by Portland Plastics Ltd.) and then put into oven for 2 hours at 150°C

A small beaker filled with distilled water is placed inside the oven to preserve tubing from becoming cracked or brittle.

The cleaning and sterilization of equipment was conducted in a separate room. Fig. 6 is a photograph of a set of cleaning tanks. The cleaning methods described above were developed by Dr. I.A. Boyd, and carried out by Miss J. Campbell and Miss A. Kirkland.

PREPARATION OF SOLUTIONS.

The preparation of Ringer's solution and the test solutions of acetylcholine and other substances was carried out in a separate room.

Ringer's Solution:

Stock solutions:

Sodium chloride	7.0%	(1.2M)	(isotonic x 10)
Potassium chloride	0.89%	(0.12M)	(isotonic)
Sodium bicarbonate	1.00%	(0.12M)	(isotonic)
Calcium chloride ($6.H_2O$)	1.88%	(0.086M)	(isotonic)
Sodium di-hydrogen phosphate	1.0%	(0.08M)	(not isotonic)

These stock solutions were prepared from 'Analar' reagents supplied by British Drug Houses Ltd. The Ringer's solution was prepared from the stock solutions in the following way:

Take NaCl	solution	400 ml	- make up to 4000 ml with double distilled water.
Add KCl	solution	140 ml	- shake
Add $NaHCO_3$	solution	160 ml	
Add $CaCl_2.6H_2O$	solution	80 ml	
Add NaH_2PO_4	solution	14 ml	
Add Glucose	solid	4 g	- shake

The final concentration of different ingredients was:

NaCl	109.0 mM
KCl	3.8 mM
$CaCl_2.6H_2O$	1.5 mM
$NaHCO_3$	4.3 mM
NaH_2PO_4	0.26 mM
Glucose	5.0 mM

Ringer's solution of this composition was used unless otherwise stated.

The double distilled water used in the preparation of Ringer's solution and test solutions was obtained from a special distilling unit in which the water was initially passed through a resin deioniser and then double-distilled in quartz glass. The use of a deioniser alone did not give satisfactory results. A photograph of the distilling unit is shown in Fig. 7.

Test Solutions:

The solutions of acetylcholine and other test substances were prepared in the Ringer's solution. Part of the Ringer's solution was used for preparing the test solutions and the rest was used for continuous perfusion and for controls. The ionic composition of the perfusing solution and control solution was, therefore, identical with the ionic composition of the test solutions. The test solutions, of course, contained minute quantities of acetylcholine or other test substances in addition.

Glassware of identical material and make (Pyrex) was used in order to avoid the possibility of any influence of the material of glass on the solutions during preparation and storage. All glassware (i.e. flasks, pipettes, beakers, cylinders, syringes) was clean and sterile (see p. 31), the seals of the nylon packages being broken just before use. The flasks had interchangeable Quickfit glass stoppers.

The wooden racks for holding the flasks had several rows of circular grooves, one behind the other. The front row was permanently labelled showing increasing concentrations from right to left, the extreme right position being allotted for the flask containing the 'control solution'. For labelling the flasks, printed labels bearing the concentration and name of a series of solutions (e.g. series a, b or c, etc.) were obtained. Each label was provided with a loop of wire to hang it round the neck of the flask.

When preparing a series of solutions, the flasks (each of 100 ml capacity) bearing labels were arranged in the front row in appropriate positions so that the concentration marked on a flask corresponded with the concentration marked on its groove on the rack. Flasks for the preparation of additional series, when needed, were similarly arranged in the back rows. For the transfer of solutions the fluid was sucked into the pipette up to the required mark and allowed to fall by gravity, the pipette tip being kept clear of the surface of the solution and the sides of the flask. A pipette was used only for one transfer and was then rejected for recleaning and sterilization. Each experiment

involved the use of a large number of clean sterile flasks and pipettes. When several series of acetylcholine were tested or when several substances were tested in the same heart, more than 50 flasks and pipettes were required.

Acetylcholine series

Acetylcholine chloride powder was obtained in sealed ampoules of 100 mg or 200 mg from Roche Products Ltd. An ampoule of sterile distilled water accompanied each ampoule of solid. A small error in the weight of the solid was possible. Reweighing of the solid was not feasible due to the extreme hygroscopic nature of the powder. The manufacturer stated that routine analytical check of samples from a batch showed a tolerance limit of $\pm$ 5%. This was rechecked by weighing the ampoules, filing them into two parts, dissolving and rejecting the contents, drying and reweighing the glass pieces. The weight of solid in two 100 mg ampoules was, in fact, 103 mg and 105 mg, while that in three 200 mg ampoules was 189 mg, 204 mg, 205 mg. On this basis a difference of up to 10% between corresponding concentrations of different series obtained from separate ampoules is to be expected.

The ampoule of acetylcholine chloride containing 100 mg and its accompanying ampoule of distilled water were opened and using an insulin syringe 2 ml of distilled water was withdrawn and injected into the ampoule containing the powder. When the powder had dissolved, the solution was sucked back into the syringe and was transferred to the flask marked 10^{-3} g/ml and the volume was made up to 100 ml with

Ringer's solution. The other concentrations were obtained from this concentration by appropriate dilution. For reasons to be considered later two different types of series covering various concentrations of acetylcholine were used.

1. Series of solutions covering a wide range of concentrations i.e. from 10^{-3} to 10^{-23} g/ml in steps of 1 in 100 dilution to determine the minimum effective concentration of acetylcholine.

The arrangement of flasks and the procedure of dilution is illustrated in Fig. 8. From flask 1 containing 10^{-3} g/ml one millilitre was pipetted into flask 2 and the volume was made up to 100 ml with Ringer's solution giving 10^{-5} g/ml. Using this procedure of diluting in steps of 1 in 100, the other concentrations were similarly prepared. A routine series covered the range from 10^{-3} to 10^{-15} g/ml (fig. 8A, left).

If a particular heart showed a pronounced effect with 10^{-15}, still lower concentrations were obtained by similar steps of 1 in 100 dilutions from the 10^{-15} g/ml flask (fig. 8A, right). Further, two or more additional series were prepared from different ampoules of solid acetylcholine to compare the effect of corresponding concentrations and to confirm that the effect of a given concentration was genuine.

2. Series of solutions covering a narrow range of concentrations i.e. from 10^{-3} to 10^{-10} g/ml in small steps of dilution for studying concentration-response relationship and rate of hydrolysis of acetylcholine.

The dilution procedure is illustrated in Fig. 8B. The

concentration of 10^{-3} g/ml was prepared in flask 1 by dissolving 100 mg or 200 mg of acetylcholine chloride in 100 ml or 200 ml of Ringer's solution, respectively, as described above. From this 10^{-5} was prepared in flask 3 by the step of 1 in 100 dilution. From 10^{-5}, the lower concentrations i.e. 10^{-6}, 10^{-7}, 10^{-8} etc. were prepared by steps of 1 in 10 dilution in flasks with odd numbers in the diagram. The strength of 5×10^{-5} g/ml was prepared in flask 2 by pipetting 5 ml of 10^{-3} g/ml and making up the volume to 100 ml with Ringer's solution. From this strength the other lower concentrations 5×10^{-6}, 5×10^{-7} etc. were prepared by steps of 1 in 10 dilution in flasks with even numbers. Further intermediate concentrations were prepared when required, as indicated in the diagram for 1.5×10^{-9}, 2.5×10^{-9}, 3.5×10^{-9} and 7.5×10^{-9} g/ml.

All solutions in dilution procedure 2 were prepared by myself. Assistance in the preparation of dilutions in procedure 1 was given by Miss J. Campbell, who had many years' experience of preparing solutions of substances at high dilution under sterile conditions. Where several series of acetylcholine were tested at great dilution on the same heart, separate series were often prepared by different operators on purpose.

Other Test Solutions:

Adrenaline: was supplied by B.D.H. in sealed ampoules of 1 ml of 1 in 1000 concentration.

Noradrenaline bitartrate: was obtained from Bayer Products Ltd. in sealed ampoules of 2 ml of 1 in 1000 concentration.

5-Hydroxytryptamine: was obtained from Roche Products Ltd. in a powder form as Serotonin-creatinine--sulphate.

Nicotine: 40% solution was supplied by T.and H. Smith Ltd. Glasgow.

Serial dilutions of these substances in Ringer's solution were prepared in the same way as described above for acetyl--choline solutions.

FROGS.

The Rana temporaria species of frog was used. They were stored in a pond. A record was kept of the source and batch number. Weighing of frogs was not routine but weights were recorded before and after injections of female sex hormones. These injected frogs were kept in tap water in a sink covered with wire gauze. Each injected frog had an identity number.

EXPERIMENTAL PROCEDURE.

Dissection and Setting Up of Experiments

The frog was stunned and decapitated and the spinal cord was destroyed. The animal was then pinned on a frog board. The abdominal wall was incised and the thorax was opened avoiding the pericardium. A flap of the abdominal and chest wall was freely excised so as to expose the heart. The portal veins were tied. The venous cannula attached to the venous pressure transducer, was introduced into the inferior vena cava and a ligature was tied round the cannula. The Ringer's solution was allowed to run into the heart from the reservoir at a pressure of about 10 to 12 mm of water. The aortae were cannul--ated and one of the aortic cannulae was connected by a flexible tube to the mercury manometer. The perfusion system was cleared of air bubbles. Fluid from the manometer and from the other aortic cannula ultimately passed through a common resistance suitable for a particular heart. The blood pressure was kept between 10 to 30 mm of Hg (Boyd and Mackay, 1957; Boyd and Eadie, 1958) by suitably adjusting the venous pressure and selecting an appropriate resistance. The optimum venous pressure and resistance required varied from frog to frog. Fig. 9 is a photograph of the heart in position.

Calibration of Equipment.

The equipment was switched on 4 hours before the start of experi--ment to allow for electronic stabilisation. The venous pressure was calibrated in mm of water using a vertical scale on one of the

standpipes. The heart rate was calibrated by counting the rate over a period of one minute. Two ranges were provided 0-50 and 0-100 beats/min. The chart is printed 0-50. Since in most of the experiments 0-100/min range was used the actual rate was twice that shown on the chart. The scale on the photographs to be presented in this thesis has been doubled so as to indicate the original rate. The blood pressure monitoring meter and pen recorder were calibrated directly in mm of Hg in accordance with the movement of the mercury in the manometer. The cardiac output over a period of 2 minutes was collected in a 10 ml graduated cylinder and the pen recorder was calibrated to write the actual output in ml per min.

Controls.

The perfusion was started from the main reservoir which was indicated on the chart by the baseline position of the marker. Record--ing was switched on and the heart was allowed to settle until a stable record was obtained for 10 to 15 minutes. One of the burettes was then filled with control Ringer's solution which was the same Ringer's solution as in the reservoir except that it had been standing beside the test solutions of acetylcholine for some time at a different place than the reservoir. A small amount of fluid was drained out through the standpipe exit so as to remove any possible contamination in the connecting tubing and the stagnant fluid in the standpipe. The clip from the transducer-connecting-tube of the corresponding standpipe was taken off and placed on the transducer-connecting-tube of the

reservoir so that the fluid from the burette now perfused the heart. At the same time the appropriate button for the marker was pressed so that the marker moved to the position of this burette on the chart. The heart was perfused from this burette for 2 minutes at the end of which the perfusion was changed back to the reservoir by reversing the position of the clip and at the same time pressing the button for the reservoir to move the marker back to the baseline. The change over of perfusion from the reservoir to the burette and back to the reservoir involved a minimum disturbance of the venous pressure and any unavoid--able minor change could at once be readjusted in a matter of a few seconds by suitably moving the burette or the reservoir carriage. This, however, required considerable practice. If no appreciable change took place in the venous pressure and if the cleaning and ster--ilisation of the burette and connecting tubing was satisfactory no change was observed in any of the recorded parameters during the period of perfusion with control Ringer's solution from the burette and also after changing back to the reservoir. This was accepted as a satisfac--tory initial control. If the control was not satisfactory it was repeated. Continued failure to obtain a satisfactory control would imply air bubbles in the perfusion system, especially in the common perfusion chamber of the venous pressure transducer which could be opened and cleared. This was easily recognisable by the type of change it produced in the record (see introduction to record). Unsatisfact--ory controls could also indicate contamination of the burette or connecting tube in which case a fresh burette and tube were substituted.

When several satisfactory controls had been obtained, the heart was alternately perfused with increasing concentrations of test solutions from the burette and Ringer's solution from the reservoir, each for a period of 2 minutes. This was the usual procedure.

In the case of acetylcholine, when a heart was found to be sensitive to a low concentration two or more series of solutions of acetylcholine were prepared and the effects of the corresponding strengths of each series were compared by perfusing with increasing concentrations of each series of solutions of acetylcholine from a common burette or a separate burette was used for each series of solutions. An initial control was done from each of these burettes to show that they were free from contamination.

The following additional methods of control were also used.

1. Conducting perfusion with the control Ringer's solution from a particular burette before using it for perfusion with an effective solution containing a low concentration of acetylcholine and repeating the perfusion with control Ringer's solution from the same burette after having thoroughly washed out the acetylcholine solution. The effect of a low concentration of acetylcholine was thus 'bracketed' in between two control perfusions with control Ringer's solution from the same burette.

2. Using two burettes one for perfusing the heart with acetylcholine solution and another for control perfusions with control Ringer's solution each time before and after the test perfusion with acetylcholine solution. The effect of the acetylcholine solution from one burette was thus 'bracketed' in between two controls from a second burette to show that the perfusion system and the process of change-over of perfusion from the reservoir to the burette and back was not responsible for any change in the record. Both burettes had, of course, been tested initially by conducting control perfusions from each.
3. Perfusing the heart with a certain effective strength of acetylcholine solution through several burettes after obtaining satisfactory controls from each of them.

These additional controls were only necessary when an effect was observed with acetylcholine solutions in a concentration of 10^{-11} g/ml or a still lower concentration.

The temperature of the laboratory was stabilized with thermostatically regulated fans so as to minimise the difference in the temperature of the solutions standing at different places and also to provide a nearly constant temperature for the heart.

ADMINISTRATION OF TEST SOLUTIONS.

The heart was continuously perfused from the reservoir except during control and test perfusions which were conducted from a different source. All test solutions were prepared in Ringer's solution and were administered by perfusion. The heart was constantly nourished in identical ionic and nutritional environment throughout the period of perfusion. The heart was not subjected to any mechanical disturbances like that involved in the washing of the isolated organ bath or renewal of fluid of the bath. No unnoticed or unrecorded disturbances in the venous pressure were possible. Such disturbances always occur during addition of the test solution to the perfusion cannulae when Syme's type of perfusion cannula is used or when test solutions are directly injected into flexible tubing.

Since the capacity of the common perfusion chamber of the venous pressure transducer (0.5 ml approx.) was less than the usual cardiac output (1.5 to 2.0 ml/min.) the chamber and the heart could be flushed completely at least twice in a two minute perfusion with normal Ringer's solution from the reservoir. Hence the activity of the heart and the levels of recorded parameters were quickly restored within about two minutes after the end of perfusion with the test solution, unless the test solution contained some substance in a high concentration, the effect of which took longer to pass off. The dilute test solutions, therefore, could be administered in rapid succession after every

4 to 6 minutes and a two minute perfusion period for the test solutions was enough to show any effect. Longer periods of interval between two doses did not change the response to an effective concentration significantly. Initial trials also indicated that perfusions with ineffective solutions for a longer period than two minutes did not make them effective. It was also essential to have a standard time period of exposure of the heart to different concentrations so as to keep uniformity of dose. The test solutions, therefore, were administered for a period of two minutes as a routine. If a weak solution gave a doubtful effect then the perfusion period with that particular solution was prolonged for 4 to 6 minutes to see if it gave a definite effect after a longer time. This in fact meant the administration of a larger total dose in the same concentration. The relevant control perfusions were also prolonged to the same extent. The two minute period of perfusion with test solution was also reduced in some cases in order to observe the relative effect of several concentrations in rapid succession if the heart was powerfully inhibited at a very low concentration. This also made it possible to record the effect of several concentrations in a short length of the recording paper to enable photographic reproduction of a continuous extract. In these cases it was already ascertained from previous trials which concentrations were likely to be effective in a particular heart and whether recovery was likely to be prolonged. Reference will be made in connection with the individual records where the routine procedure was altered in any way for any reason.

It may be mentioned here that the same burette was used only for testing increasing concentrations of the same substance. No burette was ever used for testing a concentration lower than the one which had already been tested from it. Different test substances were normally perfused from separate burettes. Occasionally, however, direct comparisons of similar concentrations of different substances were made on purpose from the same burette after initial tests from separate burettes.

SPECIAL PROCEDURES.

Alterations in the Composition of Ringer's Solution.

The stock solutions of the salts forming the Ringer's solution were all in isotonic or 10 x isotonic strength (except NaH_2PO_4). The concentration of any ion, therefore, could be easily altered simply by changing the volume of the stock solution added to prepare the Ringer's solution. Thus modified Ringer's solution containing high Ca^{2+}, low Ca^{2+}, low Na^+, low K^+, high Ca^{2+} plus low K^+ and low Na^+ plus low K^+ concentrations, were prepared. Na^+ was replaced by osmotically equivalent amounts of sucrose to obtain low Na^+ concentrations. The actual degree of alteration in the concentration of different ingredients is given along with the results.

Administration of Female Sex Hormones.

Oestradiol and progesterone were obtained from Organon Ltd. Suspensions of these steroids in Ringer's solution were injected in

the dorsal lymph sac or intraperitonially over a period of 3 to 4 consecutive days in daily divided doses. The total dose of oestradiol ranged from 0.005 mg to 15 mg and of progesterone from 1.5 mg to 5.0 mg. Oestradiol and progesterone were also injected simultaneously in a few frogs. Some of the details of these injections are given in the results. The injected frogs (of either sex) were weighed before and after treatment with female sex hormones and were subsequently used for perfusion experiments to determine the effect of female sex hormones on the sensitivity of their hearts to acetylcholine and other substances. Control experiments were also conducted simultaneously on the hearts of uninjected frogs.

GENERAL COMMENTS ON THE PROTOCOL OF EXPERIMENTS.

The technique of setting up of an experiment, the calibration of equipment, the special precautions and the methods of control have been described in the preceding pages. General comments on the protocol, programme of experiments and difficulties encountered in obtaining stable records are being mentioned here.

The first half of each week was occupied by the process of cleaning and sterilisation of all glassware, connecting tubing and other apparatus which came in contact with the perfusion fluid or the test solutions. The second half of each week was occupied by the actual experiment. Work was continued through the night and weekends if the circumstances of the experiment in progress so warranted. In some experiments more than one test substance was administered. Tests with different substances, however, were carried out at separate times in the experiment.

The complexity of electronic equipment imposed some restrictions and dependence on technical assistance. Major breakdowns in the equipment, however, were rare. Technical assistance was ready at hand. The equipment was calibrated on each occasion it was used. Once suitably calibrated, the equipment usually worked flawlessly.

The introduction of venous and aortic cannulae required considerable practice. The aortic cannulation was much easier than the cannulation of the inferior vena cava. The venous cannula required

to be introduced into the inferior vena cava was attached to the common perfusion chamber of the venous pressure transducer. The cannula, therefore, could not be moved. On opening the ventral body wall of the frog, the inferior vena cava was seen quite prominently but it soon collapsed to the thickness of a pin on further handling. The venous cannula had to be sufficiently thick so as to allow a free flow of fluid into the heart. The venous cannula, therefore, could be suitably introduced only by catching the cut margins of the vein and stretching them and at the same time pushing the venous pressure transducer by the chin in an appropriate direction and at a proper level. This procedure required considerable practice and occasionally a magnifying lens or a watchmaker's binocular was quite useful for this purpose. The resistance unit needed cleaning each time before use. The setting up of each experiment and calibration of equipment on an average required 3 to 4 hours.

The individual experiments lasted from 8 to 72 hours, most of the experiments covering 12 to 36 hours. The main events of each experiment were recorded at the time of occurence in the protocol books. The following extract from a protocol book illustrates the routine of working. This heart showed a high degree of sensitivity to acetylcholine (minimum effective concentration 10^{-11}, see results for different degrees of sensitivity).

Date: 26-1-61	Approximate time
Male frog. Uninjected	
Equipment switched on	9.0 a.m.

Material for perfusion assembled	10.0 a.m.
Solutions prepared	11.30 a.m.
Dissection completed and inferior vena cava cannulated and perfusion started	12.30 p.m.
	Lunch
Aortic cannulation completed. Resistance unit cleaned and all connections made.	2.30 p.m.
Calibration of equipment completed	3.0 p.m.
Nine control perfusions conducted and transducer opened and cleaned between control 5 and 6 to clear small air bubbles which were responsible for slightly unstable record.	4.15 p.m.
Leak detected and corrected in one of the connecting tubes. Suspected because of low diastolic pressure	4.30 p.m.
Two control perfusions repeated	4.45 p.m.
Spontaneous changes in the heart rate. Heart allowed to settle.	5.15 p.m.
Two control perfusions conducted and acetylcholine series tested	6.15 p.m.
Control perfusions conducted and test solutions from tubing tested.	7.15 p.m.
Perfusion changed to Mariotte bottle, pen switched off and heart left over night	7.30 p.m.

Date: 27-1-61

Same heart left over night. Equipment switched on	8.0 a.m.

All burettes and connecting tubing changed. Perfusion system checked. Venous pressure and resistance re-adjusted to suit the heart	10.0 a.m.
Fresh test solutions of acetylcholine in yesterday's sample of Ringer's solution prepared.	10.30 a.m.
Several control perfusions conducted and acetylcholine series tested.	12.15 p.m.
Controls conducted and the same test solution from tubing after a longer duration of soaking tested.	1.30 p.m.
	Lunch
Same series of acetylcholine tested	3.45 p.m.

Date: 27-1-61

Second heart. Female frog. Uninjected. Continued — Same solutions.

Spontaneous irregularity in the rhythm of frog hearts was rather common and such hearts had to be left to settle with the perfusion 'on' at a constant venous pressure. The impulse formation is profoundly influenced by perfusion pressure (Pathak, 1958 a). Test solutions were only administered when the heart had settled nicely on perfusion and a stable record had been obtained for 10 to 15 minutes. If the heart did not settle in a few hours it was rejected and another heart was set up. Records of erratic and unstable hearts have been rejected. Sometimes the heart became erratic in the course of the experiment after having initially settled to a regular

rhythm. In such a case if the heart was found to be relatively sensitive (by some pilot trials of test solutions) and if the record was otherwise stable (except for a slight constant irregularity of beat) the experiment was continued to the end. Otherwise the heart was rejected.

Considerable time in each experiment was occupied in conducting control perfusions. As a matter of fact the ratio between the number of controls and the number of trials with effective test solutions, on an average was 2 : 1. Sometimes the ratio was 4 : 1 especially in those hearts which were highly sensitive and where the effect of each concentration of acetylcholine was 'bracketed' in between several controls on either side to increase the accuracy and reliability of results.

The record of each heart is several feet long (average 20 feet). Photographs of extracts from these records are presented in the results. The actual size of original Figures is on an average 5 times the size of the photograph. Recording was continuous and it was, therefore, possible to observe short term as well as long term changes in the activity of the heart.

From the above comments it is clear that one successful experiment was the outcome of a considerable degree of perseverence and patience as there were several conditions which had to be satisfied before obtaining a good, stable and well controlled record. It took a considerable time to develop the special precautions and methods

of control and to standardise the procedure. It has been the aim throughout this work to obtain a high degree of accuracy and reliab--ility of results rather than to try to test a large number of hearts under uncontrolled or semi-controlled experimental conditions as done by many previous workers. The equipment was sensitive. The development of the experimental procedure eliminated most of the sources of contamination. Each step in an experiment was well controlled. It is, therefore, no wonder that this type of work was time consuming, but the results have been sufficiently productive.

DYNAMICS OF THE PREPARATION

Some details of the dynamics of frog heart under the experimental conditions of the present perfusion system have been described by Boyd and Eadie (1957, 1958, 1961). In the present work an optimum venous pressure and a suitable resistance were selected for each heart so as to obtain a systolic pressure of 20 - 40 mm of Hg (average for intact frog hearts being 30 mm Hg., Boyd and Mackay, 1957) and an output of 1.5 to 2 ml/min (Boyd and Eadie, 1958).

Usually the mercury manometer is considered to give an accurate value of mean pressure only, the pulse pressure given by the mercury manometer being much less than the true pulse pressure due to the high inertia of the system. Resonance is also likely at heart rates which are simple multiples of the natural frequency of oscillation of the mercury column, with resultant exaggeration of the pulse pressure. The diameter of the glass tubing was carefully selected (about 1mm) so that the resonance frequency of the mercury column was approximately 100/min. Since the usual heart rate of frogs is 20 to 40 beats / min, there could be no interference due to resonance. Moreover the volume displacement of the mercury and the distensibility of the rubber connecting tubing allowed accommodation of about half the stroke volume in the artificial arterial system. Under these conditions this mercury manometer was shown by direct comparison with an inductance transducer (National Electronic Products) to be giving about 90% of the true pulse pressure. Further, since the present work was concerned with changes

in the pressure rather than its absolute value, the mercury manometer was adequate for the purpose. It also provided a continual visual check on the accuracy of the electronic recording system.

The influence of changes in one parameter on the other parameters can be considered under the following headings.

1. Venous Pressure. The effect of graded changes in venous pressure (with a constant resistance) on the dynamics of the preparation has been described (Boyd and Eadie, 1957). Using isolated perfused frog hearts and its individual chambers (Pathak, 1957, 1958 a) and isolated mammalian hearts (Pathak, 1958 b) with the conventional method of perfusion, it was shown that the amplitude and the heart rate are both altered significantly on changing the intraluminal perfusion pressure. Both these parameters were shown to increase as the venous pressure increased, up to a certain value (called critical pressure) in frog hearts; a further increase in the venous pressure either produced no change or decreased the rate and amplitude. The value of the 'critical pressure' varied from heart to heart and could be different for amplitude and heart rate. Reducing the venous pressure in steps produced opposite effects. The majority of frog hearts showed optimum performance at a perfusion pressure of 20 to 25 mm H_2O.

Similar conclusions were reached independently by Boyd and Eadie (1957) and have been confirmed during the present work. Boyd and Eadie considered that the influence of venous pressure was more marked on the arterial pressure and output than on the heart rate and that the heart rate often decreased at high pressures.

2. Resistance. Boyd and Eadie (1958, 1961) studied the influence of changes in artificial arterial resistance and observed that an increase in the resistance produced an increase in the mean arterial pressure and a decrease in the output and usually no change in the heart rate. In the present work it was found that if the resistance was increased in very small steps from zero the output and heart rate might actually show a slight increase initially. At intermediate resistances the results were as given by Boyd and Eadie, while a change to a very high resistance sometimes produced a decrease in the heart rate.

The venous pressure and artificial resistance being constant, the cardiac activity remains fairly stable as shown by the stability of other recorded parameters, there being only a very slow progressive decline over a period of 24 hours.

3. Arterial pressure and output. The venous pressure and resistance being constant, two basic processes influence these parameters:

(a) change in the pacemaker activity (heart rate)

(b) change in the force of contraction of the cardiac muscle

The arterial pressure is determined by both these processes directly, while the outflow (from a constant resistance) depends on the mean arterial pressure and is equal to cardiac output in a state of equilibrium. Therefore, the outflow has been called output. For the sake of description the influence of a primary change in the heart rate or force of contraction can be considered separately.

1. Change in heart rate. A decrease in the heart rate results in

(i) a longer diastolic pause which permits greater decay of

arterial pressure due to outflow of fluid from the resistance and thus tends to lower the diastolic pressure.

(ii) a longer diastole also allows increased ventricular filling with a resultant increase in the force of contraction by Starling's effect. This tends to increase the pulse pressure.

The usual result of the combination of these factors is that when the heart slows the diastolic pressure decreases while the systolic pressure may rise or stay constant depending on the magnitude of the increase in the force of contraction. If the mean pressure remains constant the outflow also remains constant. If the mean pressure decreases, the outflow decreases, though the change may be too small to be seen on the reproduced output trace.

Conversely, increase in the heart rate if sufficient to raise the mean arterial pressure will increase the outflow.

2. Change in the force of contraction.

The individual excursions on the arterial pressure trace show the pulse pressure in each cardiac cycle. When the force of contraction of the ventricle increases, the stroke volume is greater, and the systolic pressure increases. The diastolic pressure also rises but to a lesser extent, so that there is an increase both in pulse pressure and mean arterial pressure. The increase in mean pressure is reflected in an increased outflow from the fixed resistance. When a state of equilibrium is reached this outflow, of course, is equal to the cardiac output. Conversely,

a decrease in the force of contraction leads to a fall in pulse pressure, mean pressure and cardiac output.

During the action of a test substance both heart rate and force of contraction are normally affected and the final result is a combination of the effects described above. The degree to which rate and force of contraction are each affected by a given concentration of test substance may differ considerably.

INTRODUCTION TO RECORD

Fig. 10 illustrates the stability of record and the technique of controls. For the sake of description the record of Fig. 10 can be considered under the following headings:

1. Chart paper: The two chart papers (Fig.4) have been joined together lengthwise in the middle. The numbers in the middle of each paper in relation to every fourth vertical line indicate 'points' on the chart and have been used for spatial and temporal reference to events occurring during the course of the experiments. The distance between two adjacent vertical lines was covered in 30 seconds. Hence a distance between two whole numbers means a duration of 2 minutes. The occurrence of events near the vertical lines bearing whole numbers is referred to as occurring at that point, e.g. the first downward displacement of the marker under the word 'control' occurred at point 11.0. Events near first, second and third vertical lines after a whole number are referred to by adding 0.15, 0.30 and 0.45 respectively to the whole number, e.g. there is a big fall in the output and blood pressure due to a fall in the venous pressure between points 9.30 and 9.45. The horizon--tal lines on the chart paper represent various scales which are described below in connection with different traces.

2. Traces: The upper chart paper shows three traces and the lower chart shows two traces. The five traces from above downwards are considered below:

(i) movements of the marker: this shows the commencement, duration and end of perfusion from different sources. The zero position of the marker means perfusion from the reservoir. The downward movement of the marker indicates the start of perfusion from a burette, the end of perfusion from the burette being shown by the return of the marker to the zero position after an overshoot.

(ii) output: this is recorded as a continuous line and gives output in ml/min. Ten original horizontal divisions on the paper represent 1 ml/min. A change in the output by 0.1 ml/min is reflected by a shift of the output trace by a distance equal to that between two adjacent horizontal lines and is clearly visible. Note the prominence of a small change in output by 0.15 ml/min at point 9.45.

(iii) blood pressure: the systolic, diastolic and pulse pressures during each cardiac cycle are recorded. The width of the trace indicates the pulse pressure which is proportional to the amplitude (force) of contract-ion. The blood pressure scale is in mm of Hg. The distance between two adjacent horizontal lines

represents a pressure of 1 mm Hg.

(iv) heart rate: the heart rate/min is recorded as a continuous line. The actual recorded rate was half of the original rate. The original scale on the paper has been doubled to indicate the original rate. Note the prominent display of occasional change in rate of 1 beat/min (half of the distance between two adjacent horizontal lines).

(v) venous pressure: scale reads directly in mm of water. Note the stability of the venous pressure and the ability to record changes of the order of less than 0.5 mm of water indicated by half of the distance between two adjacent horizontal lines.

3. Description of the events recorded: At the beginning of the record the zero position of the marker indicates perfusion from the reservoir. The heart had settled well on perfusion and the record is absolutely stable. The values of the different parameters at this time were as follows:

Cardiac output		1.6 ml/min
blood pressure -	systolic	25 mm Hg
	diastolic	15.5 mm Hg
	Pulse pressure	9.5 mm Hg
heart rate		36/min
venous pressure		26.5 mm H_2O

The first downward movement of the marker to the position of burette 1 (right top corner scale) indicates that the perfusion from burette 1 was started at this time while the perfusion from the reservoir was stopped. This was done simply by taking off the artery clip from the transduc--er-connecting-tube communicating with burette 1 (through standpipe 1) and putting it on the transducer-connecting--tube communicating with the reservoir (through the respect--ive standpipe) and at the same time the button for burette 1 was pressed to move the marker. The perfusion from burette 1 was continued for 2 minutes during which time the marker remained at this position. At the end of 2 minutes, the position of the artery clip was reversed to change back to the reservoir and at the same time the button for the reservoir was pressed and the marker moved up to the zero position after an overshoot. Note that there is no change in any of the recorded parameters in the process of change--over of perfusion from the reservoir to burette 1 and back to the reservoir. The reservoir and burette 1 contained the same sample of Ringer's solution as was used for prepar--ing the test solution. However, the test solution and the sample of Ringer's solution used in burette 1 had been standing together at a different place. This sample of Ringer's solution which stood with test solution was called 'control' Ringer's solution. The perfusion from burette 1

was called 'control perfusion' because of the following considerations:-

1. it showed whether the process of changing over the perfusion from the reservoir to burette 1 and back involved any significant change in the venous pressure and hence in the other parameters of cardiac activity.
2. it could detect minor differences in the pH and temperature of the Ringer's solution in the reservoir and the control Ringer's solution itself by showing a significant change in record without any change in the venous pressure. If some slight difference existed between the two solutions a similar degree of difference could be allowed for between the Ringer's solution and the test solution.
3. it could show whether there was any contamination in burette or its connecting tubes.
4. it could detect a possible obstruction in the perfus--ion system due to small air bubbles. The obstruction was indicated by a fall in the venous pressure, a decrease or stoppage of the excursions of needle of the venous pressure monitoring meter (in front of the operator) and also by the fall in the level of other parameters.

Returning to the description of Fig. 10, during the first control there was no change in any parameter. Hence this was a very satisfactory control. The second deflection of the marker to the position of burette 2 indicates a similar control perfusion from this latter burette. The third deflection of the marker indicates perfusion of a test solution from burette 1 with a very slight effect. The fourth movement of the marker shows a repetition of control perfusion from burette 2. Later on between point 9.30 and 9.45 the common perfusion chamber of the transducer was flushed back by opening the exit of standpipe 1 and removing the artery clip from the transducer- connecting tube in front of the standpipe. This procedure flushed out the test solution completely from the common perfusion chamber and the transducer-connecting-tube and is referred to by 'F' on the chart. Since burette 1 was used for the test perfusion, it was rinsed thoroughly with Ringer's solution and a final control perfusion was conducted from burette 1 to show that it was still satisfactory. Thus the slight effect of the test solution was not due to any defect in the perfusion system or any contamination in burette 1. The momentary small change in the record (well marked in the venous pressure and the blood pressure) at the start of the final control perfusion is due to a small change in venous pressure at the time of

change over of perfusion from the reservoir to burette 1. The venous pressure was adjusted at once and the blood pressure returned to its original level. It is obvious from the record that this degree of change in the venous pressure during change over of perfusion from one source to the other was of no significance.

Appreciation of the following points is useful in interpretation of the records.

1. The time constant of the recording system -

(a) Heart rate - The ratemeter is triggered by the impulses generated as a result of changes in pressure due to individual beats. When the recording system is connected at the beginning of the experiment, the pen moves upwards from the zero position to the appropriate level reaching a stable value, the rate of movement of the pen being determined by the time constant of the integrating circuit which is about 1 minute. Once a stable value has been reached any gradual smooth change in the heart rate moves the pen to a new position showing the prevailing rate per minute.

During the action of a test substance the change in rate is usually smooth and gradual and the new stable position gives accurate readings except under two circumstances -

(i) When the heart is powerfully inhibited and the individual excursions of the mercury fail to produce an impulse sufficient to trigger the rate meter.

(ii) When a further sudden change in the heart rate occurs before a new stable level has been reached.

In both these cases the true heart rate is derived by counting individual beats in the blood pressure trace. During failure of triggering or during stoppage of the heart, the pen moves towards the zero position with the same time constant.

(b) Cardiac output- The recording system for the output behaves in the same way as that of the heart rate, with a time constant of about 0.5 minute. If during a change a new stable level has not been reached before the next change occurs, the correct output is derived by counting the small fluctuations on the output trace, each small fluctuation representing one drop of fluid. Counting of drops is also a useful check when the output is very small.

2. Spontaneous changes in heart rate or a change in rate during control perfusions of up to 5% and occasionally up to 10% may occur without any significant change in the other parameters. Hence any rate effects within these limits of control have been considered insignificant.

3. The action of a test solution usually produced a change in the heart rate as well as in the force of contraction. As mentioned

in the section on dynamics, these parameters influenced both arterial pressure and output records. Usually the action of a test substance registered a change in all the other recorded parameters (except venous pressure which was maintained constant).

4. The record of heart rate reflects pacemaker activity and a smooth progressive change in the trace reflects a change in the pacemaker activity. Arrhythmias (commonest being coupling of beats and extrasystoles) and interference with conduction of impulse give rise to irregularities in the record of heart rate, blood pressure and output.

RESULTS.

DETAILS OF SUCCESSFUL EXPERIMENTS

The results of successful experiments on 151 frog hearts in connection with sensitivity to different substances are considered in this part of the thesis. Other experiments in which the rhythm of the heart was very erratic and the record was consequently unstable have been rejected. Still others in which only a few concentrations of different substances were tried without any effect and the whole series of solutions could not be tried, have also been excluded. The details of the frogs used and the number of frogs used for different purposes are given in table 1.

The duration of individual experiments varied from a few hours to 3 days. The records are continuous in time except for short breaks. Recording was discontinued during the night if the experiment was not in progress. Momentary stoppage of recording for some technical reason during the middle of the experiment is indicated by a prominent 'S' on the record and is usually self-evident. Photographs of extracts from the record showing important observations are presented here. The strength of all test solutions is in g/ml.

The solutions of all test substances were administered in increasing concentrations to exclude the possibility of alterations in the response of the heart due to a high initial dose. Also preliminary experiments conducted by Dr. Boyd on acetylcholine suggested that there might be two peaks of action of acetylcholine - one at lower concentrations between 10^{-11} and 10^{-13} g/ml and the other at higher concentrations. It was considered possible that the

same might be true for other substances. Hence it was necessary to start with very low concentrations.

For the purpose of investigating the incidence of hearts sensitive to low concentrations it was considered sufficient to obtain an approximate estimate of minimum effective concentrations by testing a standard series of solutions covering a wide range. The standard series (prepared as illustrated in Fig. 8A) consisted of the following strengths: 10^{-5}, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13} and 10^{-15} g/ml.

The term minimum effective concentration (or threshold of sensitivity or simply threshold sensitivity) has been used in a relative sense for discriminating the degree of sensitivity of different hearts e.g. when it is stated that the minimum effective concentration in a particular heart was 10^{-11} g/ml, it implies that the actual threshold lay between this and the next lower concentrations tested i.e. between 10^{-11} and 10^{-13} g/ml.

pH OF SOLUTIONS

An electronic pH meter model 700 with a readability to 0.02 of a pH unit (Analytical Measurements Ltd., Surrey) was used. This meter is mains operated, gives quick and reproduceable readings and only small samples of solutions are required.

The degree of variation in the pH of solutions, the measures adopted to control variations in pH, and the responsiveness of the frog heart to changes in pH of Ringer's solution are considered below.

Variations in the pH of Ringer's solution

(a) Samples of Ringer's solution prepared with water from different sources may show a difference in the pH as illustrated by the following measurements in one experiment -

Sample of Ringer's solution	pH
1. in tap water	8.19
2. in glass distilled water	8.10
3. in deionised water	8.16
4. in water deionised and then glass distilled	8.10
5. in water deionised and then double distilled in quartz glass	8.01

The maximum variation in pH was less than 0.2 units

(b) A difference in the pH of two samples of Ringer's solution prepared from the same stock solutions at different times in one experiment was also possible. The difference was usually within 0.1 unit but occasionally it was up to 0.2 units.

(c) Alteration in the ionic composition of Ringer's solution produced only a small change in the pH except when the concentration of Ca^{2+} was changed. The following values were recorded in one experiment -

Sample of Ringer's solution	pH
1. usual composition (see page 34.)	8.30
2. containing a low concentration of K^+	8.26
3. containing a low concentration of both Na^+ and K^+	8.22
4. containing a high concentration of Ca^{2+}	7.76

The low pH of the 4th sample is possibly due to the acidic nature of the solution of $CaCl_2$ which is a salt of a strong acid and a weak base.

(d) Samples of the same Ringer's solution stored in different containers (e.g. 5-litre flask, Winchester bottle, 100 ml flask, perfusion burette and reservoir) sometimes exhibited a difference in the pH as illustrated below -

Date of experiment	Sample	pH
5. 8. 60	from control (100 ml) flask	8.25
	from perfusion burette	8.33
25. 8. 60	from reservoir	7.83
	from Winchester bottle	7.78
	from control flask	7.72

A maximum difference of 0.2 units was possible but the usual difference was within 0.1 units.

(e) When the solutions were allowed to stand for a long time, the pH usually rose slightly as illustrated by the following example -

Date of experiment	Sample of Ringer's solution	pH
24. 8. 60	just after preparation	7.72
	48 hours after preparation	7.82

Measures adopted to minimise changes in pH

Although the variation in pH under each condition considered above was small, their combined effect could be greater. Hence the following measures were adopted to control each source of variation and to reduce the overall variation in the pH during individual experiments -

1. Use of water from only one source, i.e. water which was deionised and then double distilled in quartz glass was used throughout.
2. Preparation of a large volume of Ringer's solution (5 litres) at one time in each experiment. All other solutions were prepared in this.
3. Use of glassware of one material and make (Pyrex) for the preparation and storage of all solutions.
4. If a test solution was to be tested after it had been standing for some time, the Ringer's solution and the control solution were also allowed to stand for the same period.
5. Use of analytical reagents from one manufacturer (British Drug Houses Ltd.).
6. Addition of NaH_2PO_4 to the Ringer's solution.

These measures were generally sufficient to reduce the overall variation in pH during individual experiments to 0.1 unit or less. Occasionally, the maximum difference in the pH of different solutions was up to 0.2 unit i.e. a change in pH by ± 0.1 unit.

Responsiveness of Frog Heart to Changes in pH

After having controlled the overall variation in the pH of solutions to ± 0.1 unit, the influence of changes in the pH of Ringer's solution on the activity of the heart was investigated. The pH was adjusted to different values in two ways

1. by adding small volumes of $\frac{N}{1}$ hydrochloric acid.
2. by changing the volume of isotonic solution of $NaHCO_3$ added to the Ringer's solution.

A change in the pH between 7.2 and 8.5 primarily influenced the chronotropic response, a decrease of pH slowing the heart while an increase in the pH produced slight acceleration. Changes in the mean arterial pressure and output were small and could be accounted for largely by changes in the heart rate. Reduction of pH below 7.2 produced a negative inotropic effect. The effect on the chronotropic and inotropic responses was completely reversible after exposure to different pH values for 4 to 8 minutes.

Fig. 11 illustrates the responsiveness of the frog heart to changes in pH. Fig. 11a shows that a sudden lowering of pH from 8.5 to 6.0 (i.e. by 2.5 units) for 4 minutes produced a small effect. A progressive decrease in the pH in smaller steps from 8.5 to 7.2

produced only a 3 to 6% decrease in the heart rate without any change in the mean arterial pressure or output even when the perfusion period was prolonged to 7 minutes. Lowering of pH below 7.2 produced a greater slowing, the mean arterial pressure and output now also decreased and clear evidence of a negative inotropic effect was visible. The heart, however, did not completely stop at 5.8.

Fig. 11b shows that lowering of pH from 8.5 to 8.1 (by 0.4 units) produced a small decrease in the heart rate, mean arterial pressure and output. The decrease in these parameters was almost of the same order when the pH was lowered in larger steps i.e. from 8.5 to 7.5 (a change of one full unit) and from 8.5 to 7.2 (by 1.3 units).

These records illustrate that the frog heart can withstand considerable changes in pH and can adapt to quite a low pH (6.0 in this case). From a practical point of view it was clear that if the pH is changed by $\pm$ 0.3 around 7.5 or 8.0 or around any value within this range, there is very little or no change in the activity of the heart.

In this work the action of various test substances was usually tested for 2 minutes. As seen in Fig. 11 the onset of effect due to lowering of pH was slow and progressed to a stable value over a period of several minutes. If the reduction in pH were to be restricted to 2 minutes only, the effect would obviously be still small, for example in Fig. 11a it is seen that lowering the pH from 8.5 to 8.0 (i.e. by 0.5 units) for 2 minutes produced a 3% decrease in the heart rate while lowering by the same degree for 4 minutes reduced the rate by 6%.

Changes in pH by ± 0.3 unit (or sometimes even more) for 2 minutes within the range of 7.5 to 8.5 was, therefore, likely to produce either no change at all or a small change in the heart rate by 3 to 6%. As mentioned elsewhere a change in the heart rate by 5 to 10% could occur during control perfusions and was considered to be of no significance, and a change in the pH of solutions by ± 0.3 units within the range of 7.5 to 8.5 could be regarded as a reasonable tolerance limit in this work. The possibility of an overall variation in the pH of solutions by ± 0.1 unit during individual experiments was of little practical importance and certainly could not influence the results.

Equilibration of solutions with gaseous CO_2 was considered unnecessary because

1. Simple measures adopted for the stabilization of pH were shown to be adequate.
2. To maintain a stable pH after initial equilibration requires continuous bubbling with CO_2. Without continuous bubbling a greater change in pH is liable to occur than if the solutions are not equilibrated in the first place. Equilibration with CO_2 was also not very practical in view of the very large number of solutions and containers required in a single experiment.
3. It was considered essential to keep all solutions completely free from contamination and a CO_2 cylinder and bubbling equipment were a potential source of contamination.

pH of Test Solutions

Most of the test substances were in the form of salts containing acid radicals. Ionisation was likely to provide additional H^+ ions when these substances were dissolved in Ringer's solution. Measurements of the pH of concentrated solutions indicated that the measures adopted for the stabilization of the pH of Ringer's solution were adequate to deal with minor changes in H^+ ion concentration. The following examples from different experiments illustrate that the difference in the pH of Ringer's solution and a concentrated solution of acetylcholine prepared in it was very small and insufficient to influence the heart

Date	pH of Ringer's solution	pH of acetylcholine 10^{-7} g/ml prepared in the same Ringer's solution
13. 8. 60	8.27	8.33
18. 8. 60	Four samples of Ringer's solution	
	1. 8.18	8.13
	2. 8.10	8.12
	3. 8.24	8.20
	4. 8.15	8.15
20. 7. 62	8.2	8.2 (series a)
		8.2 (series b)

The pH of all dilute solutions prepared from acetylcholine 10^{-7} g/ml was measured in some experiments and all values were found to be within the range of $\pm$ 0.1 unit as compared with the pH of Ringer's solutions. As already established this degree of

variation was definitely insufficient to influence the heart. Routine testing of all dilute solutions was considered unnecessary for the following reasons

1. There was only a small difference in pH between a concentrated solution (i.e. 10^{-7} g/ml) of acetylcholine and the Ringer's solution which was used for obtaining the dilute solutions from this concentration.
2. Any difference in the pH between the concentrated solution and the Ringer's solution would be reduced with each step of dilution.

However, the pH of solutions containing a very low concentration of acetylcholine was checked occasionally when acetylcholine was effective in surprisingly small quantities. For example, the pH of the solution of acetylcholine 10^{-15} g/ml which had a powerful inhibitory effect on the heart (Fig. 37) was measured immediately after the test. The difference between this solution (10^{-15} g/ml) and the corresponding control solution was 0.01 unit. There was, therefore, no doubt that the inhibitory effect was due to acetylcholine. Similarly the pH of acetylcholine 10^{-17} g/ml (A_{17}, Fig. 62) was 7.6 measured immediately after the test. The pH of the Ringer's solution in the reservoir was 7.4 and of the control solutions was 7.5. No significant difference (i.e. greater than the limit of $\pm$ 0.1 unit described above) between the pH of a solution of acetylcholine and the control solution or Ringer's solution in the reservoir was ever observed.

The above remarks made in connection with acetylcholine also apply to solutions of other test substances.

ACETYLCHOLINE

Stability of Acetylcholine in Alkaline Ringer's Solution

It is a commonly held view that solutions of acetylcholine in an alkaline medium hydrolyse fairly rapidly at room temperature, as described by authorities like Dale (1914) and Clark (1926) and many subsequent workers. During the present work it became obvious that the solutions of acetylcholine in Ringer's solution with a pH of about 8 maintained their activity for periods of many hours.

To obtain more precise information about the rate of hydrolysis of acetylcholine solutions a special series of experiments was carried out on 11 hearts. These experiments were conducted during the months of July and August. During this period frog hearts are relatively insensitive to acetylcholine (see later, Fig. 41). The minimum effective concentration was about 5×10^{-9} g/ml and that required to stop the hearts was between 2.5×10^{-7} and 5×10^{-7} g/ml in most cases (Fig. 39). A series of acetylcholine solutions (labelled series 'a'), ranging in concentration from 5×10^{-7} g/ml to 10^{-10} g/ml, was prepared from an ampoule of the solid chloride as illustrated in Fig. 8B. Additional series b, c, etc. were prepared from separate ampoules as required. A set of test perfusions were conducted with increasing concentrations starting with 10^{-10} g/ml which was ineffective in all 11 hearts. The following types of experiment were carried out:

1. A set of test perfusions as described above using one series of solutions as soon after preparation as possible (commencing within 0.5 hr), followed by another set of tests on the same heart using the same solutions after they had been standing

for periods varying from 3 to 30 hours.

2. A comparison of the action of a fresh series of solutions with the action of solutions of one or more series prepared from 3 to 94 hours previously, on the same heart. A complete set of tests with the full range of solutions of one series was followed by a set of tests with another series, or tests with corresponding concentrations from different series were interdigitated.

3. A comparison of a completely fresh series of solutions with a second series prepared by diluting a relatively concentrated solution of acetylcholine (about 10^{-5} g/ml in Ringer's solution) which had been standing for a long period, and testing the dilute solutions shortly thereafter.

In Fig. 12a a record is shown of the action of a freshly prepared series of solutions (0.5 hr old at the first test) in concentrations from 10^{-9} g/ml, which was ineffective, to 5×10^{-7} g/ml, which stopped the heart. The same series of solutions, after standing for 3 hours, produced changes in the activity of the same heart of almost exactly the same degree (Fig. 12b) as the corresponding fresh solutions. The maximum percentage inhibition of cardiac output, heart rate, and mean arterial pressure during each test with the fresh series of solutions is given by the filled circles, while the values for the tests with solutions 3 hours old are plotted as open circles, in Fig. 13a. The horizontal arrow on each ordinate scale indicates the maximum change in that parameter during any control perfusion in the experiment.

Vertical arrows near the curves indicate onset of conduction block. Arrows with continuous lines indicate complete heart block while arrows with dotted lines indicate partial heart block. The continuous line in this and subsequent figures connects the values obtained with the fresh series of solutions, any hydrolysis being indicated by a downward displacement of the open circles from this continuous line. Some degree of variation in the action of the same solution on the same heart at different times is to be expected as described later, which accounts for the fact that some old solutions which have not undergone any hydrolysis may have a slightly greater (or smaller) inhibitory effect than was produced by the same solutions when fresh. Fig. 13a and the filled circles in Fig. 13b show the action of a freshly prepared series of solutions (series c) on another heart. Further tests with the same solutions after standing for 5 hours gave almost identical results (Fig. 13b, open circles) showing that no detectable hydrolysis had taken place in this period.

The relationship between concentration of acetylcholine and the response of another heart is shown in Fig. 13c. The actions of solutions from one series tested when fresh (filled circles) and of the same solutions after standing for 3 hours (open circles) were very similar to each other and to the actions of corresponding solutions of a different series which had been standing for 5

hours (circles containing dots). Again there is no evidence of hydrolysis within a 5 hour period.

An experiment in which solutions of two different series which had been standing for 3 and 25 hours were tested is illustrated in Fig. 15a. The concentration-response relationship of this heart with the 3 hours old series (open circles), and with the 25 hours old series (circles containing dots), are very similar. On the other hand, in an experiment on another heart (Fig. 15b) there was evidence of some hydrolysis in a series of solutions which had been standing for 29 hours (open circles) when its action was compared with the action of a fresh series of solutions (filled circles). In general, there was evidence of hydrolysis in some solutions after they had been standing for long periods, especially if they had been handled e.g. part of their contents used for earlier tests.

In one experiment two series of solutions (a and b) were obtained from concentrations of 10^{-5} g/ml or 5×10^{-5} g/ml of acetylcholine (in Ringer's solution) which had been prepared from separate ampoules of solid and allowed to stand for 94 hours (series a) and 77 hours (series b). Tests from certain solutions of these series on one heart were interdigitated with tests with a series only a few hours old (series c), and the degree of inhibition produced by corresponding solutions of all three series was almost identical (Fig. 14b). Thus, no significant hydrolysis occurred in concentrations of the order of 10^{-5} g/ml within a period of nearly 100 hours.

Some evidence on the extent of hydrolysis in more dilute solutions of acetylcholine than 10^{-10} g/ml was obtained from experiments carried out on very sensitive hearts. In one experiment (Fig. 52a) concentrations ranging from 10^{-15} g/ml to 10^{-9} g/ml produced slightly greater inhibition of the heart when 8 hours old (open circles) than they did when freshly prepared, presumably due to a slight increase in the sensitivity of the heart. However, a separate series of solutions which had been standing for 24 hours (triangles) produced much less inhibition indicating that these latter solutions had hydrolysed considerably.

In another experiment (Fig. 54c) concentrations of 10^{-15} g/ml and 10^{-13} g/ml of a series which had been standing for 17.5 hours (open circles) produced a similar degree of inhibition (in repeated tests) to that produced by the same solutions, tested when fresh on the same heart on the previous day (filled circles). Thus, there was no hydrolysis of these solutions in 17.5 hours. Also Fig. 54a shows that in another experiment concentrations of 10^{-9} g/ml and 10^{-11} g/ml produced considerably greater inhibition on the second day than that produced by the same solutions on the same heart on the first day i.e. there was an increase in sensitivity of the heart and no obvious hydrolysis in the solutions in about 24 hours. Finally, Fig. 54b shows that a solution of acetylcholine in a concentration of 10^{-13} g/ml, which was prepared and kept in stoppered, sterile flasks without being handled for 24 hours before being tested, produced about 50% inhibition of the heart. Any

hydrolysis taking place must have been of small degree.

Table 9 summarises the experiments in which low concentrations of acetylcholine were effective after different durations of standing.

It was assumed throughout most of the present work that the solutions of acetylcholine maintained their strength because they were prepared using chemically clean and bacteriologically sterile glassware. One experiment was conducted in which two series of solutions were prepared from the same ampoule of solid acetylcholine chloride, the one series in clean and sterile stoppered flasks while the other series was contained in unstoppered flasks which had been standing in a laboratory cupboard and were not subjected to the usual cleaning process. The activity of the two sets of solutions was compared when they were freshly prepared (Fig. 16a) and, on the same heart, when they had been standing for about 18 hours (Fig. 16b). The results are plotted in Fig. 15c, and there is, surprisingly, no indication of any hydrolysis in any of the solutions at all. Sterile pipettes were used, however, in the preparation of both series of solutions in this experiment, and it may be that any hydrolysis which occurs is primarily due to contamination introduced in the pipetting process. It is clear from this experiment, however, that under some circumstances, solutions of acetylcholine will maintain their activity for long periods without the elaborate precautions in cleanliness and sterility used throughout the present work.

The general conclusion which emerges from this study is that

if acetylcholine solutions are prepared in Ringer's solution using clean and sterile glassware and the solutions are not handled in any way thereafter, all of them will maintain their full activity for periods of more than 8 hours, and often for more than 24 hours. Further, concentrated solutions of acetylcholine (e.g. 10^{-5} g/ml) may maintain their full strength for periods of several days. Opening the flasks and handling the solutions may, however, result in an unpredictable degree of hydrolysis of the remaining contents of the flasks, negligible within about 8 hours, but varying from nil to almost complete hydrolysis over longer periods.

Threshold Sensitivity to Acetylcholine and Stoppage Concentrations of Acetylcholine.

Fig. 22 illustrates the routine technique of conducting several initial control perfusions with 'control' Ringer's solution from two burettes. These initial controls demonstrated that the perfusing system was completely satisfactory and the process of change-over of perfusion from the reservoir to burettes and back did not produce any change in the record. Since burette 1 was to be used for testing the influence of acetylcholine solutions several control perfusions were conducted from it to show that it gave satisfactory controls repeatedly. Subsequently burette 1 was used for testing the effect of increasing concentrations of acetylcholine. The heart was found to be sensitive only to 10^{-7} which stopped it within 30 seconds. The common perfusion chamber was flushed back (F) at point 2.0 to wash out acetylcholine. Soon after the heart restarted but stopped again

due to the entrance of residual amount of acetylcholine from the venous cannula into the heart. However the heart recovered quite quickly afterwards. Near point 3.30 the common perfusion chamber was again flushed back to wash out remaining acetylcholine before the final control perfusion from burette 2. There was no necessity for adopting any special methods of control as this was a very definite effect and, as will be considered later on, this concentration influenced all hearts. No change in the venous pressure occurred during the test perfusion with 10^{-7} and the effect was completely reversible.

The lowest concentration of acetylcholine which influenced all the three parameters (i.e. cardiac output, arterial pressure and heart rate) represented the threshold of sensitivity (threshold sensitivity) for that heart. Usually the force of contraction (as evidenced by changes in the record of cardiac output and arterial pressure) and heart rate were almost simultaneously inhibited at an effective concentration. At times, however, small changes in the heart rate occurred without any significant effect on other recorded parameters. Changes in heart rate of less than 10% were within the limit of controls and have been ignored. Further details regarding the action of acetylcholine are considered along with the concentration-response relationship.

If the first noticeable effect with any concentration was complete stoppage of the heart without any effect of lower concentrations or in which the lower concentrations were not tested, that concentration has been taken as threshold

sensitivity as well as stoppage concentration. For example it has been seen in Fig. 22 that 10^{-7} was the lowest effective concentration which, however, also stopped the heart. Fig. 23 is an example of another heart in which the first significant effect was stoppage at 10^{-9}. In such cases the stoppage concentration has also been regarded as threshold sensitivity.

The sensitivity to acetylcholine varied considerably from heart to heart. For simplicity of description the hearts can be classified according to threshold sensitivity to acetylcholine into three categories.

1. Relatively insensitive hearts (hearts with low sensitivity). This includes those hearts which showed a threshold sensitivity at 10^{-7}. Fig. 24 is an example of a relatively insensitive heart showing threshold at 10^{-7} and stoppage at 10^{-5}. The heart shown in Fig. 22 also falls under this category.
2. Hearts with medium sensitivity. those hearts which had a threshold sensitivity at 10^{-9} are classified under this category. Fig. 25 is an example of a heart with medium sensitivity showing a threshold at 10^{-9} and stoppage at 10^{-7}. Fig. 21 shows the record of another heart in which the effectiveness of 10^{-9} g/ml was confirmed by 7 repeated tests. There was only a slight variation in the response of the heart to the same concentration.
3. Hearts with high sensitivity. This includes those hearts which showed a threshold effect at 10^{-11} or still lower

concentrations. Records of some of the highly sensitive hearts are being presented in Figs. 26 to 33. Fig. 26 illustrates the record of a heart which showed a threshold effect at 10^{-11}. The initial control perfusions from burette 1 showed no effect. Acetylcholine solution in concentrations of 10^{-15} and 10^{-13} from the same burette showed no effect while 10^{-11} produced a large effect. Two subsequent controls from the same burette after rinsing several times with Ringer's solution showed no effect. The changes in all the three variable parameters (i.e. output, arterial pressure and heart rate) during perfusion with 10^{-11} could not be due to an artefact because of the following reasons:

(i) The perfusion system was satisfactory before and after the test perfusion with 10^{-11} as shown by several controls on either side (indeed 10^{-15} and 10^{-13} also serve as controls in the beginning).

(ii) The latency of onset of effect from the time of start of perfusion with 10^{-11} is about 30 seconds which was the time taken by the fluid to reach the heart.

(iii) The effect was gradual in onset with a steep fall up to the end of perfusion with 10^{-11} and was completely reversible within 90 seconds which was also the approximate period for which the heart was exposed to the drug.

(iv) The record is stable. There was no change in the venous pressure during the test as evident from the trace.

(v) The genuine nature of effectiveness of 10^{-11} in this heart was confirmed by repeating the tests (Fig.27).

Fig. 28 is a second example of a highly sensitive heart in which acetylcholine in a concentration of 10^{-13} produced very marked effect and 10^{-11} stopped the heart. The effects are gross and 'bracketed' between controls. Fig. 29 shows the record of another heart in which the minimum effective concentration was 10^{-15} g/ml. Fig. 30 is an example of still more highly sensitive heart showing a big effect at 10^{-15} and stoppage at 10^{-13}. The effects are again well controlled. Subsequently still lower concentrations of acetylcholine were prepared and tested on this heart the next day and it showed a threshold effect at 10^{-17} as shown in Fig. 31. The effectiveness of 10^{-15} was confirmed by several tests on both days. (Fig. 54c). Fig. 32 and 33 are extracts from the record of the most highly sensitive heart. Fig. 32 shows that this heart showed a very large effect at 10^{-15}; 10^{-13} and 10^{-11} gave still larger effects within a much shorter duration. These solutions belonged to a series named 'a'. Subsequently two completely new series of solutions were prepared from separate ampoules of solid acetylcholine chloride in the usual way and in the same sample of Ringer's solution which was used in the reservoir and in the control perfusions from burette 1 and 2. Dilutions up to 10^{-23} were prepared and tested after several hours

as shown in Fig. 33. By this time there was a slight decline in the cardiac activity as indicated by the decrease in output and arterial pressure. The reduced appearance of pulse pressure is partly due to the fact that in comparison to Fig. 32, Fig. 33 has undergone a much greater photographic reduction. The duration of experiment covered by Fig. 33 is about twice that covered by Fig. 32. In fact the original size of the extract of Fig. 33 was still greater due to several repetitions of tests with 10^{-23}, 10^{-19} and 10^{-17} of both series (b) and (c) and associated controls. Part of the extract from the middle, therefore, was removed to accommodate the Figure in this size. The junction can be seen as a thin white line between point 1 and point 7 after the second control from burette 2. The initial controls from burette 1 and 2 were satisfactory. Acetylcholine 10^{-23} (b) series produced no effect, the effect on rate being of the same order as from the initial control from burette 1. The effect of 10^{-23} (c) and 10^{-21} (b) were also not significant. However the effect of 10^{-21} (c) was significant as all the three parameters were involved; the effect of 10^{-19} (c) was gross. The effect of 10^{-19} (b) though greater than 10^{-21} (b) was less than that from 10^{-21} (c). Hence though both series showed progressive increase in the effect, the corresponding strengths of solutions of the two series were not exactly comparable. The possible explanation for the difference in the action of corresponding strengths of different series will be considered later. More details regarding this experiment will be given in reference to Fig. 55c. For this heart 10^{-21} represented the threshold sensitivity.

Figs. 52 a, b and c, 54 a and b, and 55 a and b illustrate the examples of 7 other highly sensitive hearts in each of which the action of low concentrations of acetylcholine was confirmed by several tests with solutions of one or more series. The record of these and other highly sensitive hearts will be presented in due course.

The distribution of 86 frogs according to the threshold sensitivity of their hearts to acetylcholine is given in Fig. 34. Although the majority (51 frogs) was sensitive to acetylcholine 10^{-7}, others (35 frogs) were influenced by still lower concentrations. The percentage of hearts showing different degree of sensitivity is given below -

Hearts with low sensitivity	(10^{-7})	59%
Hearts with medium sensitivity	(10^{-9})	27%
Hearts with high sensitivity	(10^{-11} to 10^{-21})	14%

In 2 out of the 4 female frogs affected by acetylcholine 10^{-15} (Fig. 34) the heart was actually stopped at this concentration (Fig. 35).

Fig. 35 shows the number of frogs in which the heart was stopped by different concentrations of acetylcholine. Out of the 86 frogs the stoppage of the heart was tested in 73 frogs. In a few cases where the heart was inhibited by 80 to 90% with some concentration which was tested for only about 30 seconds, that concentration was taken as stoppage concentration. The concentration which produced complete conduction block for 30 seconds or more was also regarded as stoppage concentration. The pattern of distribution of frogs in Fig. 35 is quite like that of Fig. 34 which shows the sensitivity distribution. The

hearts of 8 frogs required acetylcholine 10^{-5} for stoppage. Concentrations between 10^{-7} and 10^{-5} were not tested. Later experiments in which several intermediate concentrations were tested indicated that all hearts are actually stopped at 5×10^{-7} (Fig. 39). Out of the remaining 65 hearts 58 were stopped at 10^{-7} while 7 hearts were stopped at still lower concentrations. The significant point here is that some hearts can be stopped with as low a concentration as 10^{-15} (Figs. 36 and 62). Fig. 37 shows the record of a third heart in which 10^{-15} produced a very powerful inhibition, amounting to almost complete stoppage, within 1 minute.

In some hearts which were stopped at low concentrations the effect of higher concentrations was not investigated. If it is presumed that higher concentrations would have been effective if tried in these hearts (which is a reasonable presumption) and also if the action of all effective concentrations (so far only threshold and stoppage concentrations have been considered) in each heart is taken into account the true incidence of influence of different concentrations of acetylcholine can be assessed. Fig. 38 has been derived on this basis. It is at once clear that in the range of concentrations from 10^{-21} to 10^{-7} there is an exponential type of relationship between the number of frogs in which the heart was affected and the concentrations of acetylcholine. Fig. 38 also shows that hearts of all the 86 frogs would have been affected by acetylcholine 10^{-7}. In fact all hearts which were not stopped by lower concentrations when tested with acetylcholine 10^{-7} were shown to be influenced by this concentration. Thus out of the 86 frogs acetylcholine 10^{-7} was actually tested on the hearts of 73 frogs, 15

of which were grossly influenced by it, whereas the remaining 58 were stopped, hence it was effective in all the 73 frogs (100%).

Figs. 34 and 35 show that the majority of hearts, which were in the low or medium sensitivity range, were soon stopped by increasing the concentration of acetylcholine 100 times. Both the threshold sensitivity and stoppage concentration, therefore, fell into the narrow range of concentrations between 10^{-9} and 10^{-7}. Subsequent experiments in 17 other hearts in which a large number of intermediate concentrations between 10^{-10} and 10^{-5} g/ml prepared in small steps of dilution (Fig. 8B) were tested, showed that the actual minimum effective concentration in most of these less sensitive hearts was between 2.5×10^{-9} and 5×10^{-9} g/ml and the stoppage concentration ranged from 10^{-7} to 5×10^{-7} g/ml (Fig. 39).

Among the highly sensitive hearts there was a tendency towards a greater incidence of stoppage at concentrations lower than 10^{-7} g/ml as the threshold sensitivity increased, but a number of highly sensitive hearts only stopped at 10^{-7}.

Seasonal Variation in Sensitivity to Acetylcholine

The sensitivity of frog hearts varied considerably during different periods of the year. The results of experiments conducted in different months of the years 1959, 1960 and 1961 on these 86 frogs indicated that there were two peaks of sensitivity, one in winter and another in autumn. However, the number of frogs in October and in November was rather small. For this reason it was considered desirable to use Dr. Boyd's experiments on 22 frogs. These experiments were conducted in

the years 1956 and 1957. The total number of frogs now became 108 spread over a 5 year period. The seasonal distribution of the experiments on these frogs during this period is given in Fig. 40. Dr. Boyd's experiments are specified by blank areas. This amalgamation of results also made the distribution of frogs in each month slightly more even, except in the month of April. Fig. 41 gives the percentage distribution of frogs in which the heart was sensitive to concentrations in the medium or high sensitivity range, out of the total number of frogs shown in Fig. 40 in each month. The two peaks of sensitivity are well marked. April, May, June, July and December were characterised by a very low incidence of high sensitivity. January, February, October and November were characterised by a high incidence of highly sensitive hearts. The most highly sensitive hearts, one sensitive to 10^{-21} and another to 10^{-17} were observed in January and February respectively. The variation in the incidence of highly sensitive hearts with season is statistically significant ($0.025 > p > 0.01$).

Relation of Sex to Sensitivity to Acetylcholine

The incidence of high sensitivity as well as the incidence of stoppage of hearts at concentrations lower than 10^{-7} g/ml in the two sexes is given in Table 3. The differences in the percentage incidence between the two sexes do not prove to be statistically significant on applying the students 'T' test. Fig. 42 gives the percentage of male and female frogs in which the heart was sensitive to concentrations in the medium or high sensitivity range during each month of the year. The actual number of frogs from which the percentage has been derived is

given at the top of each column for the two sexes. The two peaks of incidence of high sensitivity are noticeable in each sex and the two peaks in each sex are quite comparable to the two peaks obtained for both sexes jointly in Fig. 41. The slight variation of sensitivity between the two sexes from month to month is not significant statistically.

Spontaneous Changes in Sensitivity to Acetylcholine

Occasionally a small variation in the threshold sensitivity was observed in some hearts. Slight decrease in sensitivity was more common than an increase when acetylcholine was tested twice or several times within 12 hours of perfusion of the heart.

Some experiments in which the sensitivity to acetylcholine was re-tested after the heart had been perfused for more than 12 hours are summarised in Table 4. It is seen that the sensitivity changed in 6 out of 8 hearts. Of the 6 hearts which showed a change in the sensitivity, 3 showed an increase while the other 3 showed a decrease. Spontaneous changes in sensitivity to acetylcholine have also been reported by previous workers (Cori, 1921, Pines, 1934, Webb, 1950) in the hearts of frogs and other species.

Relation of Adrenaline to Sensitivity to Acetylcholine

In some experiments a few drops of adrenaline were administered externally in a concentration of 10^{-6} to 10^{-3} g/ml to the hearts to obtain a stable record if the heart was irregular or feeble and occasionally it was observed that there was an increase in the sensitivity to acetylcholine (Table 5). The sensitivity increased within 15 minutes to 3 hours after administration of adrenaline. One

heart showed a progressive increase in the sensitivity when tested 1.25, 2.5 and 16 hours after the administration of adrenaline.

Marked spontaneous changes occurred in a few hearts after a longer durations (more than 12 hours) of perfusion. However, the the sensitivity increased only in 3 out of 8 hearts (Table 4). In the case of the 8 hearts treated with adrenaline (Table 5) the sensitivity increased in 4 within 12 hours and in the other 4 after 12 hours of perfusion. The degree of increase in the sensitivity was also greater in the adrenaline treated hearts. Although these experiments supported the impression that adrenaline somehow or other increased the sensitivity at least in some hearts, these do not represent a pre-planned series of experiments - adrenaline was not used with a view to influencing the sensitivity.

A few direct experiments in which the heart was perfused with Ringer's solution containing adrenaline in a concentration of 10^{-6} g/ml showed no change in the threshold sensitivity to acetylcholine within a period of 2 hours. Occasionally due to the irregular record of the heart, small effects which could be due to the action of weak solutions had to be rejected. The action became more definite when the rhythm became regular after treatment with adrenaline. In the case of one heart (27. 4. 60, table 5) this partly accounts for the apparent change in sensitivity from 10^{-7} to 10^{-13} g/ml.

COMPOSITION OF RINGER'S SOLUTION AND SENSITIVITY TO ACETYLCHOLINE

A programme was formulated to screen the influence of several changes in the composition of Ringer's solution on sensitivity to acetylcholine to find out if any particular alteration in the composition of Ringer's solution brought about some remarkable change in the sensitivity of the hearts to acetylcholine.

As was expected, changes in the concentration of different ions greatly altered the activity of the hearts, and the hearts had to be allowed to settle on each modified Ringer's solution for several hours before a stable record could be obtained. The influence of acetylcholine solutions prepared in the same modified Ringer's solution was then investigated. Moreover for comparing the influence of different changes in the composition of Ringer's solution on sensitivity to acetylcholine, it was considered more useful to test the effect of several changes in the same heart under well controlled conditions after it had been allowed to settle fully in the new ionic environment.

Several hearts became completely erratic in rhythm as a result of drastic changes in the composition of Ringer's solution and had to be rejected. However, 20 series of acetylcholine solutions were successfully tested on 5 hearts stabilised on modified Ringer's solutions involving several changes in the composition. The results of these experiments are summarised in Table **6**. Since there was no remarkable change in the sensitivity to acetylcholine from any of the several changes in the composition of Ringer's solution further experiments were not conducted. These findings are in agreement with

the results of many previous workers. A brief mention of the influence of these changes in individual components on cardiac activity and on sensitivity to acetylcholine is made below. The concentration of individual ions is indicated in terms of mM concentration of the constituent solid.

High Calcium Concentration

Increase in the concentration of calcium by 50% (from 1.2 to 2.3 mM) did not produce any change in the action of acetylcholine 10^{-7} (heart 4). Doubling the calcium concentration (from 1.1 to 2.3 mM) with or without simultaneous addition of sucrose either made the heart less sensitive to acetylcholine (heart 1) or there was an increase in the effect of a threshold concentration without any change in threshold sensitivity or stoppage concentration (heart 2). The duration of stoppage was, however, longer when the calcium concentration was raised. On the other hand a high concentration of calcium frequently brought about a gross irregularity in rhythm and many hearts had to be discarded because of the gross instability of record. Fig. 43 and 44 illustrate the regularisation of rhythm on changing from the Ringer's solution containing a high concentration of calcium (2.3 mM) to normal Ringer's solution (calcium 1.5mM).

Low Calcium Concentration

Reduction in the concentration of calcium to 25% (0.37 mM, heart 3) and to 75% (1.1 mM, heart 5) of normal concentration (1.5 mM) did not change the sensitivity to acetylcholine in these hearts. On the other hand the cardiac activity declined considerably resulting in lowering of all the parameters. This was expected on the basis of the well known

stimulating effect of calcium on the heart beat.

Low sodium Concentration:

The effect of reduction in the concentration of sodium to 50% (54.5mM, heart 3) of normal (109 mM) by replacing it with osmotically equivalent amounts of sucrose resulted in a marked reduction in the blood pressure and frequency of heart beat. There was no change in the sensitivity to acetylcholine. The blood pressure and frequency of heart beat were quickly restored to normal levels when the perfusion was changed back to normal Ringer's solution.

Low Potassium Concentrations:

A reduction in the concentration of potassium to 50% (1.9 mM) of normal was associated with an increase in the threshold sensitivity to acetylcholine from 10^{-7} to 10^{-9} in heart 4 but had no effect on the threshold sensitivity of heart 5. As slight changes in sensitivity of some hearts are liable to occur spontaneously, the slight increase in the sensitivity of heart 4 was not considered significant.

High Calcium plus Low Potassium Concentrations and Low Sodium plus Low Potassium Concentrations:

The influence of increase in the calcium concentration to 150% of normal with simultaneous reduction of potassium concentration to 50% of normal did not produce any change in the sensitivity (heart 5). Similarly a reduction in the concentration

of sodium to 50% of normal with a simultaneous reduction in potassium concentration to 50% of normal in the same heart also did not produce any change in the sensitivity to acetylcholine.

Sucrose, Glucose and Oxygen:

Addition of sucrose or glucose to the Ringer's solution or oxygenation of Ringer's solution did not produce any change in the sensitivity to acetylcholine. Addition of sucrose slightly slowed the heart. There was an associated increase in the force of contraction (as evidenced by increased pulse pressure and rise in systolic pressure) possibly because of greater diastolic filling. The osmotic influence of sucrose may be responsible for these effects.

FROGS INJECTED WITH FEMALE SEX HORMONES : SENSITIVITY OF HEARTS TO ACETYLCHOLINE

Oestradiol

As mentioned in the section on Methods oestradiol was suspended in Ringer's solution and was injected intraperitonially or in the dorsal lymph sac in daily divided doses over a period of 3 to 4 days. The total dose of oestradiol ranged from 0.005 mg. to 15 mg. (Table 7). Experiments were conducted on the hearts of 22 injected frogs in 1960-1961 to test the sensitivity to acetylcholine. The results of these experiments are given in Fig. 45. Of the 22 hearts 6 were highly sensitive (sensitive to 10^{-11} or still lower concentrations), 4 showed medium sensitivity (sensitive to 10^{-9}) while the remaining 12 were relatively insensitive. The injected frogs received varying total dosage of oestradiol as a trial. The results given in Fig. 45

suggested that the incidence of high sensitivity and the degree of sensitivity to acetylcholine was higher in the hearts of those frogs which received large doses of oestradiol. Therefore, experiments on the hearts of 7 other frogs injected with large doses of oestradiol (Table 7) were conducted in June (1961) when the hearts were expected to be relatively insensitive to acetylcholine. Control experiments on the hearts of 7 uninjected frogs were also conducted alternating with the experiments on the hearts of injected frogs. Of the 7 hearts of uninjected frogs 3 were sensitive to acetylcholine 10^{-9} and 4 to 10^{-7}. Five of these 7 hearts were stopped by 10^{-7} while the 2 remaining hearts required still higher concentrations for stoppage. Thus the heart of none of the 7 uninjected frogs was highly sensitive. Among the 7 hearts of oestradiol injected frogs, 2 were in the high sensitivity range (Table 7) but the difference between the incidence in these two groups was not statistically significant.

When the results of experiments on the hearts of all the 29 oestradiol injected frogs (22 of 1960-1961 and 7 of June 1961) were considered together the incidences of medium, and combined medium plus high sensitivity was 17%, 24% and 41% respectively. The corresponding values estimated previously for the hearts of uninjected frogs were 27%, 14% and 41%. However, these differences in the incidence of different degrees of sensitivity between the hearts of injected and uninjected frogs also were not significant statistically. There was also no positive or negative correlation between the

total dose of oestradiol and the incidence of high sensitivity to acetylcholine. Oestradiol pretreatment also produced no change in the concentration needed to stop most of the hearts. Direct experiments in which the hearts were perfused with Ringer's solution containing oestradiol 25 mg/100 ml, showed no change in the threshold sensitivity to acetylcholine. The heart rate decreased slightly and occasionally there was a slight increase in the response to an effective concentration of acetylcholine during oestradiol perfusion (Fig. 46), but this could be due to some other factor like minor spontaneous change in sensitivity. It can, therefore, be concluded that oestradiol pretreatment on the whole does not influence the sensitivity to acetylcholine.

The record of all the 29 frogs injected with oestradiol is summarised in Table 7. Weights before and after treatment with oestradiol were recorded for 23 frogs of which 16 were male and 7 female. The weight increased after oestradiol treatment in 15 frogs (11 male and 4 female) and decreased in 8 (5 male and 3 female). No correlation between the dose of oestradiol and the absolute or percentage increase or decrease in the weight can be

obtained statistically either for individual sex or for both sexes together. As the influence of oestradiol on the weights of frogs was not being investigated specifically, the other factors which could influence the weight were not controlled. This seems to be mainly responsible for the absence of any correlation. However, the only possible explanation of the increase in the weight in some frogs can be water retention due to oestradiol injections as these frogs lived in tap water during the period of injection. There was no relationship between the increase or decrease in the weight and sensitivity to acetylcholine.

Progesterone:

Progesterone was injected in daily divided doses in the same way as oestradiol over a period of 3 to 4 days. The total dose varied from 1.5 to 5 mg. The experiments on the hearts of progesterone injected frogs are summarised in Table 8. Of the 7 hearts, 6 showed an increase and 1 showed a decrease in the weight after the injections of progesterone. The absolute or percentage increase in the weight, however, does not correlate statistically with the dose possibly because of the same reasons as have already been discussed in the case of increase in the weight due to injections of oestradiol. The increase in the weight of progesterone treated frogs like those of the oestradiol treated frogs could only be due to retention of water as the progesterone injected frogs also lived in tap water. The heart of the first frog (Table 8) was tested in February 1961.

February has been shown to be the month characterised by the occurrence of highest sensitivity to acetylcholine. This was a male frog. It may be recollected here that the two most highly sensitive hearts among the hearts of uninjected frogs were also from male frogs and were encountered in the month of February - one in 1960 and another in 1961. Hence the high sensitivity of the heart of this frog can be explained on this basis. The hearts of the remaining 6 frogs were not affected at all by progesterone treatment. Even if the heart of frog 1 is included in the calculation of the incidence of high sensitivity among the hearts of these 7 frogs, the incidence would be 14.2% which is the average incidence of high sensitivity in the hearts of uninjected frogs throughout the year. These considerations lead to the conclusion that progesterone pretreatment did not influence the sensitivity of frog hearts to acetylcholine.

Oestradiol + Progesterone:

Oestradiol in the morning and progesterone in the evening were injected in daily divided doses over a period of 4 days to test if a synergistic effect of the two could influence the sensitivity of the hearts to acetylcholine. Two frogs (1 male and 1 female) were injected and tested in September 1961. Each frog received a total dose of 5.0 mg. of oestradiol and 5.0 mg of progesterone. There was a decrease in the weight of both frogs after the course of injections. Since there was no dramatic change in the sensitivity to acetylcholine and since oestradiol

and progesterone individually also did not produce any remarkable change in the sensitivity of the hearts to acetylcholine, no further experiments appeared useful.

ACTION OF ACETYLCHOLINE

Nature of Action of Acetylcholine

A clear excitatory effect of acetylcholine at any concentration, within the very wide range of concentrations tested, was never obtained. Occasionally there was a transient rebound type of increase in the heart rate and/or amplitude during the recovery of the heart from the inhibitory action of an effective concentration of acetylcholine. The rebound increase in the heart rate was more common. Where the rebound increase in rate was considerable the amplitude of beat decreased. During this rebound phase acetylcholine was being washed out of the heart by the normal Ringer's solution from the reservoir. These rebound changes in the cardiac activity never occurred during the course of the perfusion with test solutions of acetylcholine at which time the heart was actually exposed to the drug. Such rebound changes are likely to occur after inhibition by any depressant drug. They were as liable to appear after stopping concentrations as at any other effective concentration. These rebound changes are illustrated in Figs. 26, 47, 48, 49, 50. In this work, therefore, the action of acetylcholine was always inhibitory.

CONCENTRATION-RESPONSE RELATIONSHIP OF FROG HEARTS TO ACETYLCHOLINE

Each concentration of acetylcholine was usually tested for a uniform duration of 2 minutes. The maximum effect of all concentrations was visible within this period. Prolongation of administration for more than 2 minutes did not show any further increase in the effect, indicating that the magnitude of response was related to the concentration rather than to the total dose. Therefore, the use of the term 'concentration-response relationship' was considered more appropriate than 'dose-response relationship'.

Concentration-response relationship in hearts with a low sensitivity.

In experiments with series of acetylcholine covering a wide range of concentrations, obtained by diluting in steps of 1 in 100 (Fig. 8A) it was seen that acetylcholine 10^{-7} g/ml influenced all hearts (Fig. 38), most of them being actually stopped by this concentration (Fig. 35). The next lower concentrations tested in these experiments was 10^{-9} g/ml which was ineffective in about 60% of the hearts. It was obvious that these hearts responded to a narrow range of concentrations and that the minimum effective concentration was somewhere between 10^{-9} and 10^{-7} g/ml, the latter concentration always producing either a powerful inhibition or actual stoppage. Because of this relatively narrow range between minimum effective concentration and stoppage concentration, it was necessary to test a large number of intermediate concentrations between 10^{-10} and 10^{-7} g/ml to elucidate the form of the concentration-response curve. A series of experiments of this type were conducted on 17 hearts

during July and August. As expected the hearts during these months were not highly sensitive, the minimum effective concentration being between 10^{-9} and 5×10^{-9} and the stoppage concentration being between 10^{-7} and 5×10^{-7} g/ml in most of these hearts (Fig. 39).

The mean arterial pressure, the cardiac output and the heart rate were all inhibited at the minimum effective concentration and at other higher concentrations, the effect being more pronounced on the mean pressure and output than on the heart rate. Changes in the mean pressure and output are due to a combination of effects on both the frequency and the force of contraction. It was not possible to separate the precise contribution of change in frequency and change in force. However, inhibition of pulse pressure gave a fair estimate of inhibition of force of contraction i.e. action on the cardiac muscle.

The change in the frequency of heart beat indicated alteration in the activity of the pacemaker (the sinus venosus being the normal pacemaker in the frog heart) or interference with the conduction of impulses from the pacemaker to the ventricle. Evidence of conduction block was provided mainly by the trace of arterial pressure in which dropped beats were clearly seen during partial block. A discontinuity in the smooth change in the traces of heart rate and output provided additional confirmatory evidence. Complete conduction block was commonly preceded by a partial block produced by lower concentrations.

A sudden drop in all traces to zero (taking into account the time constants of decay in the rate and output recording systems) without a preceding gradual reduction in pulse pressure was taken as evidence of complete block. If, when the heart restarted, the pulse pressure was

initially very small this indicated that almost complete inhibition of the cardiac muscle had occurred in addition to the complete conduction block. If the pulse pressure of the initial beats during recovery was considerable, however, this indicated that complete block had occurred at a concentration insufficient to produce complete inhibition of the muscle. Frequently, the degree of inhibition of the muscle produced by a concentration sufficient to cause complete block was apparent before the block occurred (Fig. 18). In these cases the values of mean arterial pressure and output at the moment of onset of complete block have been indicated on the graphs by small circles.

Typical forms of concentration-response curves for cardiac output, mean arterial pressure and heart rate

Fig. 14a illustrates the typical response of a heart to increasing concentrations of acetylcholine. The inhibitory effect on cardiac output and mean pressure increased gradually and progressively as the concentration of acetylcholine increased from 10^{-9} to 10^{-8} g/ml. At 5×10^{-8} g/ml there was a sudden increase in the effect on these parameters but further increase up to 10^{-7} g/ml produced no substantial increase in the effect, indicating approach to a maximum effect in the range of concentration between 5×10^{-8} and 10^{-7} g/ml (plateau effect) before the heart finally stopped at 5×10^{-7} g/ml (100% inhibition). Also note the marked initial reduction in the pulse pressure at 10^{-7} g/ml indicating considerable inhibition of cardiac muscle before the rate was much affected. The ratemeter was actually not triggered when the pulse pressure was very small. Hence the initial steep fall in the rate trace does not represent a genuine change in rate.

The effect on the heart rate increased smoothly and progressively from 10^{-9} to 5×10^{-7}. Stoppage of the heart at 5×10^{-7} g/ml was very sudden suggesting complete conduction block, but the possibility of coincident complete inhibition of cardiac muscle cannot be excluded in this case.

The actual values of percentage inhibition of different parameters are plotted in Fig. 13b. The continuous lines represent the concentration-response curves. Occurrence of complete conduction block is indicated by continuous vertical arrows while the dotted vertical arrows indicate the onset of partial conduction block. The curves for the output and mean pressure are 'S' shaped. The upper limb of the 'S' represents a tendency to reach a plateau near the stoppage concentration. The concentration-response relationship for the heart rate is curvilinear.

The 'S' shape of the concentration-response curves for the mean pressure and output, and the curvilinear shape for the heart rate represent typical forms. Figs. 15b and 19a (filled circles) show the same forms of curves in two other hearts. Part of the record of the heart whose concentration-response curves are presented in Fig. 19a (filled circles), is shown in Fig. 17c. Note the plateau effect on cardiac output and mean pressure coincident with marked inhibition of cardiac muscle and some interference with the conduction process.

However, these typical forms of curves were not seen in all hearts. In many hearts an underlying similar form was probably present but was modified as considered below. Since the curves for mean pressure and output were similar and since most of the evidence regarding inhibition of cardiac muscle was derived from the record of the arterial

pressure (the record of output giving confirmatory evidence), the modifications in the curve for mean pressure only are described, but in general what applies to the curve for mean pressure also applies to the curve for output.

Modifications in the concentration-response curves for mean arterial pressure

The typical 'S' shape of curves for the mean pressure resulted from the tendency to reach a plateau coincident with marked inhibition of cardiac muscle near the stoppage concentration i.e. near 100% inhibition. Two basic changes occurred either together or separately which gave rise to modifications in the typical curves.

1. Early onset of complete conduction block i.e. occurrence of block at a concentration at which the cardiac muscle was not very powerfully inhibited. Due to the sudden onset of complete block all parameters suddenly dropped to the level of 100% inhibition.
2. Tendency to reach a plateau at lower levels of inhibition than 100%.

The curve for mean pressure in Fig. 15a shows the onset of complete conduction block at 10^{-7} g/ml when the curve was tending to reach 100% inhibition implying full inhibition of cardiac muscle in the absence of block. Fig. 13c shows that the curve for mean pressure was tending to reach a plateau at a level of about 90% inhibition in this heart, but the onset of complete block resulted in an abrupt fall of arterial pressure to zero.

The tendency to reach a plateau at still lower levels of inhibition was seen in some hearts. Figs. 19b, 20b, and 20c show the formation

of a plateau on the curves for mean pressure (filled circles) at about 80% inhibition in three different hearts. The record of the heart whose concentration-response curves are shown in Figs. 19b and 20b (filled circles) are shown in Figs. 17a and 18a, respectively. The formation of a plateau on the curves for mean pressure at still lower levels of inhibition in other hearts is seen in Figs. 13a, 15c and 20a. In all these instances the development of a full 'S' shape of the curve was prevented by the occurrence of complete conduction block as if the block sectioned off the curve at different places in different hearts.

Modifications in the concentration-response curves of heart rate

The typical curvilinear concentration-response relationship for heart rate was seen in 11 out of 17 hearts. In the remaining hearts the typical form of the curve was modified by the occurrence of conduction block. Partial heart block at any concentration resulted in a sudden drop in the heart rate (e.g. to half its former value in 2:1 block) which produced humps on the curves at different levels of inhibition in different hearts. Fig. 18c shows part of the record of the heart whose concentration-response curves are shown in Fig. 20a (filled circles) and illustrates different degrees of partial block at 10^{-7} and transient complete block at 1.5×10^{-7} g/ml. The production of a hump on the curve for heart rate due to partial block is well illustrated in Fig. 20b (filled circles). Partial heart block appeared to explain the hump on the heart rate curve in all cases except one where definite evidence of partial block was not visible. Sudden

occurrence of complete block (Figs. 13a, 15a) or the occurrence of partial and complete block in succession (Fig. 20a) gave rise to a sharp angularity in the curve for heart rate. Complete conduction block occurred in all 17 hearts at concentrations between 5×10^{-8} and 5×10^{-7} g/ml.

Electrocardiographic studies would be necessary for determining the actual site(s) of conduction block at different concentrations and also for determining the exact nature of block.

Inter-relationship between concentration-response curves for heart rate and mean arterial pressure

At lower concentrations the reduction in the pulse pressure is not obvious and it is not possible to separate exactly the effect of change in the heart rate from that of change in the force of contraction on the mean arterial pressure. However, the degree of reduction in mean pressure and output was much more pronounced than can be accounted for by the small decrease in the heart rate, the conclusion being that the force of contraction must have been inhibited as well. Inhibition of force of contraction was clearly visible in the form of reduced pulse pressure at higher concentrations.

The heart rate and mean pressure were powerfully inhibited at concentrations between 5×10^{-8} and 5×10^{-7} g/ml and as already mentioned conduction block also occurred over the same range of concentration. However, in individual hearts the action on the cardiac muscle (as judged from the reduction in pulse pressure and also from the magnitude of reduction in mean pressure and output) and on the heart rate (indicating action on the sinus venosus, the normal

pacemaker, in the absence of interference with conduction) over this range of concentrations could occur in any sequence.

In some hearts there was a considerable inhibition of cardiac muscle long before (i.e. at lower concentrations) there was any marked effect on the heart rate (Figs. 13b and c and 15a). In other hearts there was a marked inhibition of heart rate before there was any evidence of a marked inhibition of cardiac muscle (Fig. 15c). Only occasionally the concentration-response curves in a heart showed almost a parallel effect on the sinus and the cardiac muscle (Fig. 20c filled circles).

Since the range of concentrations over which complete muscle inhibition and complete heart block occur is the same, both effects are likely to occur coincidently (Fig. 17a and b), but if the complete block comes suddenly the muscle inhibition may be masked (Fig. 18a). If aomplete block occurs late (i.e. at a higher concentration) and the inhibition of cardiac muscle has been progressive without any tendency to reach a plateau at lower levels of inhibition, the full 'S' shape of the curve for mean pressure is seen. If the complete block occurs early (i.e. at lower concentrations) the portion of the 'S' curve on the right side of the concentration at which the block occurs is not seen.

In those hearts in which the complete block occurred at a concentration at which the mean pressure and output were inhibited by about 90% (Figs. 13c and 15a) it can be assumed reasonably that the typical full form of the 'S' shape would have been displayed eventually but for the occurrence of block. In the case of hearts

in which the mean pressure appeared to attain a plateau at about 40 to 80% inhibition before the onset of block, such an assumption does not seem justifiable because the level of the plateau shows that the cardiac muscle in these hearts might not have been completely inhibited at any concentration. On the other hand it appears most unlikely that there could be any basic difference in the behaviour of the hearts and if the cardiac muscle in some hearts can be inhibited completely, it should be inhibited in all hearts at some concentration. An indication of the true state of affairs was provided by the concentration-response curves of a few hearts in which the tendency to reach a plateau was followed by a phase of sudden increase in the inhibitory action on the muscle leading to complete stoppage with (or without) coincident conduction block (Fig. 19b).

CONCENTRATION-RESPONSE CURVES IN THE PRESENCE OF ESERINE

Five litres of Ringer's solution was divided into two parts. To one eserine was added to obtain a final concentration of between 10^{-6} and 10^{-4} g/ml in different experiments. Two series of solutions of acetylcholine were prepared concurrently from the same ampoule of solid, one in eserinised Ringer's solution (eserinised series) and the other in uneserinised Ringer's solution (uneserinised series) using the dilution procedure illustrated in Fig. 8B. All glassware including pipettes, burettes, flasks and reservoir was quite separate for the eserinised and uneserinised solutions.

The heart was first perfused continuously with uneserinised Ringer's solution and the uneserinised series was tested. Later

the heart was perfused with eserinised Ringer's solution for about 30 minutes and then the eserinised series was tested. Both series were tested within an hour after preparation. In this way the action of acetylcholine in the absence of, and in the presence of, eserine was compared in 6 hearts.

Perfusion of the heart with eserinised Ringer's solution resulted in a slight decrease in the heart rate with an associated increase in the cardiac output, arterial pressure and pulse pressure suggesting slight increase in the force of contraction. This is in agreement with the previous findings where the amplitude of contraction was recorded directly (Pathak, 1958 d).

Figs. 17a and b illustrate the action of increasing concentrations of acetylcholine in the absence (Fig. 17a) and in the presence (Fig. 17b) of eserine in the same heart. The effect of concentrations lower than 5×10^{-8} g/ml was smaller with the eserinised series than with corresponding concentrations of the uneserinised series, while in greater concentrations the eserinised solutions were more effective.

At a given concentration, the onset of effect was more gradual, and the rate of recovery was almost two times slower, in the presence of eserine (Fig. 17b) than it was in the absence of eserine (Fig. 17a). The stoppage concentration was lowered in the presence of eserine from 2.5×10^{-7} to 1.5×10^{-7} g/ml.

Figs. 18 a and b show the record of another heart with similar features. In the absence of eserine (Fig. 18a) partial block occurred at 5×10^{-8}, 7.5×10^{-8} and 1.5×10^{-7} g/ml. The normal rate in this heart was unusually high and the individual excursions in the trace of

arterial pressure were very close to each other. Photographic reduction has brought them still closer. Hence the evidence of partial block which is very clear in the original record, has been almost completely masked. The curves for this heart are shown in Fig. 20b which illustrates the production of a hump on the curve for heart rate due to partial block. In this case the stoppage concentration was lowered by eserine from 2.5×10^{-7} (Fig. 18a) to 10^{-7} g/ml (Fig. 18b) simply by potentiating the occurrence of complete conduction block.

Figs. 19 and 20 show the concentration response curves of 5 hearts in the presence of eserine (crosses with discontinuous connecting lines) and in the absence of eserine (filled circles with continuous connecting lines). The concentration-response curves for the heart whose record is illustrated in Fig. 17 a and b are shown in Fig. 19b while the curves for the heart whose record is illustrated in Fig. 18 a and b are shown in Fig. 20b.

It is clear from Figs. 19 and 20 that the minimum effective concentration with the eserinised and uneserinised series of solutions of acetylcholine was the same, indicating that eserine produced no change in the sensitivity of hearts to acetylcholine.

Complete conduction block occurred at a lower concentration in the presence of eserine (thus lowering the stoppage concentration) in all hearts (as seen in Figs. 19 a and b and 20 a and b) except in one in which the complete block actually occurred at a higher concentration in the presence of eserine (Fig. 20c).

Besides potentiating the occurrence of complete block, eserine also greatly potentiated the inhibition of heart rate (i.e. the

effect of acetylcholine on the pacemaker) shifting the concentration-response curves for heart rate to the left in 4 (Figs. 19 a and b and 20a and b) out of 6 hearts. However, the form of the curve for heart rate remained unaltered in the presence of eserine.

Potentiation of the action of acetylcholine on the mean pressure and output was observed in only one heart (Fig. 20a). In another heart (Fig. 20c) there was no evidence of any potentiation at all, while in 3 out of 6 hearts there was actually less inhibition of these parameters with low concentrations of acetylcholine in the presence of eserine (Figs. 19a and b). Since the heart rate was potentiated in these latter three hearts, the decreased inhibition at lower concentrations seems to imply a decrease in the action of acetylcholine on the cardiac muscle in the presence of eserine. This difference in the action of acetylcholine has already been illustrated in Fig. 17a and b. The reduction in the action on muscle was not related to the concentration of eserine. Both series were prepared concurrently and tested within 0.5 hr of each other and it is most unlikely that the decrease in the action of acetylcholine is due to such factors as hydrolysis of solutions or change in the response of the heart.

The curves for the mean pressure and output were modified by eserine, the concentration response curves now adopting a curvilinear shape in 3 out of 6 hearts. These were, in fact, the same 3 hearts in which the action of low concentrations of acetylcholine was less in the presence of eserine. The conversion of the 'S' or related shape into a curvilinear shape, therefore, seems to be due to;

1. Decrease in the action of acetylcholine at lower concentrations

shifts the initial part of the curve to the right.

2. Decrease in the concentration required to stop the heart shifts the upper part of the curve to the left and accounts for the crossover of the two curves.

CONCENTRATION-RESPONSE RELATIONSHIP IN HIGHLY SENSITIVE HEARTS

When a heart was found to be highly sensitive to acetylcholine principal attention was paid to establishing beyond question that the effect of a given low concentration was genuine. For this purpose repetition of tests with a limited number of concentrations at frequent intervals, under carefully controlled experimental conditions, with solutions of several completely different series (prepared from separate ampoules of solid) was necessary.

In these hearts a wide range of concentrations (from 10^{-7} to 10^{-15} or still lower concentrations) was tested, the difference between two successive concentrations being 100 times (Fig. 8A). Straight lines drawn through points showing values of percentage inhibition simply illustrate the pattern of response to increasing concentrations. For the sake of description they have been called concentration-response curves. Since the injection of female sex hormones did not produce any change in the response of hearts to acetylcholine, all highly sensitive hearts are considered together.

The typical concentration-response curves seen in less sensitive hearts were observed only in a few highly sensitive hearts. Fig. 51 illustrates the record of a heart in which concentrations ranging from 10^{-13} to 10^{-7} g/ml produced increasingly greater inhibition. The actual

stoppage concentration was not ascertained. The curves in this heart for all parameters were curvilinear (Fig. 52b).

Concentration-response curves of another heart showed similar features (Fig. 52a). In this heart two different series were tested. Tests with one series conducted when it was 24 hours old showed considerable hydrolysis of acetylcholine (triangles). The other series was tested twice once when fresh (filled circles) and a second time when 8 hours old (open circles). In this heart the effect at 10^{-11} and 10^{-9} g/ml was almost equal with fresh and with old solutions. Fig. 53 is the record of some of the tests with 8 hours old solutions showing equal effect at 10^{-11} and 10^{-9} g/ml. In some duplicate tests (not shown in Fig. 53) with the 8 hours old solutions conducted at different times partial conduction block occurred at 10^{-11} g/ml which accounts for the higher position of some open circles (inverted vertical arrow). The equal effect at 10^{-11} and 10^{-9} g/ml suggested a tendency to attain the plateau as seen in hearts with a low sensitivity. The curve for the heart rate does not show a full curvilinear form presumably because concentrations higher than 10^{-7} g/ml were not tested. This again illustrates that the action on the cardiac muscle and on the pacemaker may not be parallel.

In the majority of highly sensitive hearts the curves had a different form. Comparison of curves of hearts with a low sensitivity (Figs. 13, 15, 19 and 20) and of highly sensitive hearts (Figs. 52, 54 and 55) leads to the following ~~in~~conclusions -

1. The steep part of the curvilinear form seen in less sensitive hearts is also present in the highly sensitive hearts but instead

of being limited between 5×10^{-8} and 5×10^{-7} g/ml it occurs over a wider range (i.e. between 10^{-17} and 10^{-7} g/ml) . However, in individual hearts it is still limited to a narrow range of concentrations. Fig. 54c shows the curves for a heart in which the steep part lay in the concentration range between 10^{-15} and 10^{-13} g/ml. This was confirmed by testing the series at different times. In other hearts the steep part was seen at other positions. In those hearts in which 10^{-15} was the stoppage concentration, it lay in the concentration range between 10^{-17} and 10^{-15} g/ml, but never at still lower concentrations.

Although the steep part in some highly sensitive hearts lay at very low concentrations, a number of highly sensitive hearts still had the steep part between 10^{-9} and 10^{-7} g/ml as seen in Figs. 52, 54 and 55 i.e. although the minimum effective concentration was very low that required to stop the heart was of the same order as in the less sensitive hearts.

2. The initial part of the concentration-response curves in highly sensitive hearts shows many variations.

   (a) A hump was usually present on the initial part of the curve for all parameters. Unlike the less sensitive hearts, in the highly sensitive hearts the hump on the curve for heart rate was not due to partial conduction block.

   (b) The concentration corresponding to the peak on this hump varied from heart to heart, but the position of the peak on the curves for all three parameters in any one heart was the same.

Fig. 52c shows the concentration-response curves of a heart in which acetylcholine 10^{-11} g/ml produced a marked inhibitory effect on the mean pressure and output. The next higher concentration (i.e. 10^{-9} g/ml) produced a smaller effect. The position of the peak at 10^{-11} was confirmed by duplicate tests. In this case there was no hump on the heart rate.

Fig. 54c is an example of a heart in which repeated tests with fresh solutions (filled circles) showed a marked effect at 10^{-15} g/ml and 10^{-13} g/ml stopped the heart. Further tests after 17.5 hours with the same series of solutions of acetylcholine starting with still lower concentrations (open circles) showed a peak of effect at 10^{-17} g/ml.

Fig. 54a shows a peak at 10^{-11} g/ml. This was checked repeatedly. The initial part of the record of this heart is presented in Fig. 56. Tests with the same solutions 24 hours later are shown with open circles. Marked inhibition was again produced by 10^{-11} and 10^{-9} g/ml. The record of the heart whose concentration-response curves are shown in Fig. 54b is presented in Fig. 57. Tests confirming the peak at 10^{-13} g/ml are shown in Figs. 58 and 47.

Fig. 55a represents the concentration-response curves of a heart in which the peak on the hump was at 10^{-17} g/ml. This was confirmed by several tests with solutions of two completely different series. Figs. 59 and 60 are extracts from the record of this heart. Note the definite inhibition produced by 10^{-19} g/ml and the marked inhibition by 10^{-17} g/ml of both series b and c. The possible explanation for the difference in the effectiveness of corresponding concentrations of different series is considered later.

In another heart whose concentration-response curves are shown in Fig. 55b, three different series of solutions freshly prepared from separate ampoules were tested. Tests with series a (filled circles) and series b (triangles) showed a hump with a peak at 10^{-15} g/ml. Fig. 61 shows the record of the test conducted with solutions of series b. Tests conducted with series c (crosses) after many hours showed greater effect at 10^{-19} g/ml and 10^{-17} g/ml and the heart was now stopped at 10^{-15} g/ml due to the intervention of complete conduction block when the muscle itself was not greatly affected (Fig. 62). Some irregularity in rhythm was also produced by other solutions i.e. 10^{-15} b and 10^{-17} b.

The concentration-response curves of the most highly sensitive heart are shown in Fig. 55c. Part of the record of this heart has already been presented in Figs. 32 and 33. Fig. 32 showed that acetylcholine 10^{-15}, 10^{-13} and 10^{-11} g/ml produced progressively increasing and very gross inhibitory effects. Repetition of the tests with the complete range of solutions of the same series (i.e. series a) several hours afterwards showed a considerable change in the effectiveness of acetylcholine and there was a hump with a peak at 10^{-13} g/ml (Fig. 63). When two other series (b and c) were tested several hours later still, the hump had shifted to lower concentrations and the peak was now at 10^{-15} g/ml with series b and at 10^{-19} with series c (confirmed by three tests) and 10^{-21} g/ml of series c produced a definite inhibitory effect (Fig. 33). Thus it appears that the position of the hump in this heart was changing rapidly with time. Also note that the action of acetylcholine was more marked on the mean pressure and output (indicating involvement of cardiac muscle) at lower concentrations and the steep part of all

curves remained between 10^{-9} and 10^{-7} g/ml.

The effectiveness of corresponding concentrations of two or more series of solutions in one heart was remarkably similar. However, in some cases especially in highly sensitive hearts where very low concentrations were involved, a difference in the effectiveness of corresponding concentrations of different series was observed. Some degree of hydrolysis could be a possible explanation but this is least likely because very low concentrations were effective and the peak of effect usually moved with time to lower concentrations rather than to the higher concentrations. In fact this difference cannot be attributed to any single factor. It is possible that a combination of some of the following factors may be responsible for it.

1. The ± 5% difference in the weight of the solid acetylcholine chloride contained in different ampoules was likely to give rise to a 10% difference between corresponding concentrations of two series prepared from separate ampoules.
2. The responsiveness of the heart to the same concentration usually varied within a limit of about ± 5% but could be greater in some cases.
3. Possibility of variation in the actual molecular concentration of acetylcholine in very high dilutions prepared by a similar process from different stocks.

VARIATION IN RESPONSE TO THE SAME CONCENTRATION OF THE SAME SERIES OF ACETYLCHOLINE SOLUTIONS

The variation in response of individual hearts to repeated tests with the same solution within a short time of one another is best seen in Figs. 52, 54 and 55 showing the concentration-response curves of highly sensitive hearts. The information is summarised in table 2. The maximum scatter in repeated tests was usually within ± 5% of the mean value and was very often less than ± 3% (see records in Figs. 21, 27, 28, 59 and 60). Greater scatter in some cases was due to occurrence of conduction block in some tests and not in others (Fig. 52a).

If the tests with the same solution were separated by a much longer period (e.g. 6 hours or more) there could be a greater scatter in the values presumably due to a change in the responsiveness of the heart (see records in Figs. 47, 57, 58 and curves in Figs. 54a and b and 55c). However a change in the responsiveness of the heart with time was frequently absent even when two tests were separated by 17 hours (Fig. 54c) or more.

In general a ± 5% scatter in the values in response to the same concentration was possible. A greater scatter was rare. This degree of scatter in the values is not sufficient to explain the decrease in the effect of acetylcholine at some intermediate concentration(s) between the minimum effective and stoppage concentrations as observed in many highly sensitive hearts (see concentration-response curves in Figs. 52c, 54a, b, and c and 55a, b and c and also records in Figs. 57, 59, 60, 61 and 63) especially if it is borne in mind that each successive concentration was 100 times greater than the preceding one. The decrease in the action of acetylcholine at intermediate concentrations, therefore, appears to have some different explanation not understood at present.

ADRENALINE.

Sensitivity to Adrenaline:

A systematic study of the influence of different concentrations of adrenaline was conducted on the hearts of 22 frogs. These include 3 uninjected frogs and 19 out of those 22 frogs which were injected with oestradiol in 1960-1961 to test sensitivity to acetylcholine. Since there was no appreciable difference in the threshold sensitivity to adrenaline between the hearts of uninjected (Table 10) and the injected (Table 11) frogs, no further experiments were conducted on uninjected or injected frogs. The intensity of effect of 10^{-7} and 10^{-5} appears to be greater in the hearts of injected frogs than in the hearts of uninjected frogs but the two series are not strictly comparable because of the disproportion in the number of hearts. The experiments were not designed to test changes in the intensity of action of effective concentrations but were planned to test the influence of oestradiol on the incidence of sensitivity at different concentrations of adrenaline. However direct perfusion with a suspension of oestradiol in Ringer's solution containing 25 mg. of oestradiol /100 ml produced no effect on the threshold sensitivity to adrenaline or on the intensity of action of an effective concentration of adrenaline. In the heart of only one of the injected frogs (frog 15) oestradiol perfusion actually reduced the intensity of response to adrenaline by 50%. Thus there was no convincing indication of any definite influence of

oestradiol on the threshold sensitivity to adrenaline or intensity of response to effective concentrations of adrenaline. The results of all the 22 hearts from both injected and uninjected frogs, therefore, can be considered together to evaluate the incidence of sensitivity at different concentrations of adrenaline. Since few hearts were affected at concentrations less than 10^{-7}, threshold sensitivity at these lower concentrations has been taken as high sensitivity. Of the total of 22 hearts (3 from uninjected and 19 from injected frogs) 1 from uninjected and 4 from injected frogs showed a high sensitivity to adrenaline. Thus the incidence of high sensitivity was about 22%. The heart of frog 2 (Table 11) showed sensitivity to 10^{-11} g/ml, the highest threshold sensitivity observed. Some hearts (4 out of 22) showed no effect with 10^{-7}. Adrenaline 10^{-5} was always effective in all hearts.

Action of Adrenaline:

Commonly the heart rate was primarily influenced by lower concentrations of adrenaline but in some hearts the rate and amplitude were simultaneously involved ~~and~~ at the ~~maximum~~ minimum effective concentration. Of the 7 highly sensitive hearts 5 (2 from uninjected and 3 from injected frogs) showed small inhibitory effects at concentrations lower than 10^{-7}. In 4 of these 5 hearts, the inhibitory effect was also observed at 10^{-7}. The inhibitory effect was observed in the hearts of both male and

female frogs of both uninjected and injected series. Besides the 4 highly sensitive hearts which showed the inhibitory effects, one heart (frog 19, Table II) showed a complete but transitory stoppage after initial stimulation at 10^{-7}. No inhibitory action was noticed at 10^{-5} in any of the 22 hearts including the heart of frog 10 which showed a marked inhibitory effect at 10^{-7} (Fig.64). Thus 10^{-5} always produced excitatory effect. External administration of adrenaline to the outside of the heart also occasionally produced inhibitory effect and sometimes the inhibitory and excitatory influences of adrenaline operated together producing marked oscillating effects on the heart rate, the amplitude of contraction and the associated parameters i.e. output and blood pressure (Fig. 65). These changes could not be due to phasic or irregular absorption of adrenaline from the surface of the heart because the action of adrenaline takes a long time to wear off. Moreover the magnitude of reduction in the amplitude (pulse pressure)during the inhibitory phases cannot be explained on the basis of wearing off of the action of adrenaline.

NORADRENALINE.

Sensitivity to Noradrenaline:

Noradrenaline was studied in 21 hearts (3 from uninjected frogs and 18 from frogs injected with oestradiol). The injected frogs belonged to the same group of 22 frogs in which the sensitivity of the hearts to acetylcholine was tested and in 19 of which the sensitivity of hearts to adrenaline was tested. The results on hearts of uninjected and injected frogs are given in Table 12 and 13 respectively. Since the threshold sensitivity to noradrenaline was similar in the hearts of injected and uninjected frogs more experiments on uninjected or injected frogs were not considered necessary. Also since oestradiol pretreatment did not influence the sensitivity to noradrenaline the results of all the 21 frogs can be utilized for evaluating the incidence of threshold sensitivity. Like adrenaline and for similar reasons, a threshold sensitivity to noradrenaline at concentrations less than 10^{-7} g/ml has been considered as high. Only 1 heart out of the total 21 hearts was sensitive to 10^{-11} and another to 10^{-9} the incidence of high sensitivity to noradrenaline being about 10%. It appears from a comparison of Tables 12 and 13 that oestradiol treatment might have increased the intensity of effect to noradrenaline 10^{-7}. Actually the apparent difference in the results is not real but is due to the difference in the number of hearts in the two series. The intensity of effect at this concentration in the

hearts of frogs, 14,15,16 and 17 in which high doses of oestradiol were injected is similar to that in the heart of uninjected frogs. Also direct perfusion with the suspension of oestradiol in Ringer's solution containing 25 mg of oestradiol /100 ml produced no effect on the response of hearts to noradrenaline. Hence it can be concluded that oestradiol pretreatment did not alter the intensity of action of noradrenaline,

Action of Noradrenaline:

The heart rate was first involved quite commonly when noradrenaline was effective at lower concentrations. However, in some hearts the amplitude of contraction was the first affected parameter at the minimum effective concentration of noradrenaline (Fig. 66). In the heart of frog 3 (Table 13) the rise in the rate of 10^{-9} (the lowest effective concentration) was moderate so that the blood pressure and cardiac output also increased and the overall effect, therefore, was stimulation while in the heart of frog 3 (Table 12) the rise in the rate at 10^{-7} was considerable, resulting in a decrease in the cardiac output and blood pressure. In other hearts the rate and amplitude were simultaneously affected. The heart of frog 18 (Table 13) stopped for a short time after showing initial stimulation at 10^{-7}. This is the same heart which gave a similar response with adrenaline 10^{-7} (Fig. 67). A pure stimulation response from noradrenaline was observed at all concentrations in other hearts.

COMPARISON OF ACTIONS OF ADRENALINE AND NORADRENALINE

A comparison of results of experiments on sensitivity of the frog hearts to adrenaline (Tables 10 and 11) and noradrenaline (Tables 12 and 13) indicates that the frog heart is almost equally sensitive to adrenaline and noradrenaline. The intensity of effect at different concentrations was greater from adrenaline than from noradrenaline (Figs. 66 and 67). The duration of effect, on cardiac output and systolic blood pressure, of noradrenaline was greater and noradrenaline frequently produced a much more sustained rise in the diastolic blood pressure in comparison to the brief effect of the same concentration of adrenaline in the same heart. The latency of onset of effect of adrenaline was shorter and the peak of effect of adrenaline was also attained more quickly. Like adrenaline, noradrenaline also produced excitatory effect both on the inotropic and chronotropic responses. Commonly the chronotropic response was involved first during the action of both adrenaline and noradrenaline and lower concentrations of both frequently involved the chronotropic response primarily. The positive chronotropic effect of adrenaline was about twice as great as that of noradrenaline. The greater increase in the heart rate during the action of adrenaline often resulted in a considerable associated decrease in the amplitude of contraction due to which the rise in the cardiac output and blood pressure was not maintained for a long time. Noradrenaline never produced

a pure inhibitory effect whereas low concentrations of adrenaline frequently produced pure inhibitory effects. On the whole the stimulation produced by adrenaline was greater than that produced by the same concentration of noradrenaline.

5-HYDROXYTRYPTAMINE

Sensitivity to 5-hydroxytryptamine

5-hydroxytryptamine was tested on the hearts of 17 frogs, 3 of which were uninjected and 14 belonged to the same series of frogs which were injected with oestradiol and tested for sensitivity of their hearts to acetylcholine, adrenaline and noradrenaline. The results of experiments on the hearts of uninjected and injected frogs are given in Tables 14 and 15 respectively. Since the sensitivity of the hearts in injected and uninjected frogs was of the same order, no further experiments were considered useful on the hearts of uninjected or injected frogs. As done in the case of adrenaline and noradrenaline, the incidence of sensitivity to 5-hydroxytryptamine can be evaluated in the hearts of all the 17 frogs considered together. Also like adrenaline and noradrenaline, the threshold sensitivity to 5-hydroxytryptamine has been considered high at concentrations lower than 10^{-7} g/ml. Of the 17 hearts 2 showed threshold sensitivity at 10^{-9} (the minimum effective concentration observed for 5-hydroxytryptamine). Thus about 11% of the hearts were highly sensitive. Oestradiol pretreatment had no effect on the sensitivity to 5-hydroxytryptamine.

Action of 5-hydroxytryptamine:

The chronotropic influence of 5-hydroxytryptamine was well marked in the hearts of both injected and uninjected frogs. The fall in the blood pressure and output as a consequence of marked acceleration was seen in the many experiments. However, a positive

inotropic response was also obtained and the total effect was a resultant of the chronotropic and inotropic influences. In the hearts of frog 7 and 12 (Table 15) a pure inhibitory effect was noticed and in these two hearts the threshold sensitivity was higher than in all other hearts of injected and uninjected frogs. Fig. 68 illustrates the diphasic action of 5-hydroxytryptamine on the heart of frog 2 (Table 15). The initial rise in the rate under the influence of 10^{-5} does not appear to be sufficient to explain the fall in the blood pressure and output because the second rise in the rate which is much greater was associated with no such fall. Also note the second fall in blood pressure when the second rise in the rate was actually declining, and the prolonged effect on rate. The rise in the diastolic pressure was proportionately always greater than the rise in the systolic pressure. At times only a rise in the diastolic pressure without any significant change in the systolic pressure was noted with higher dilutions. Fig. 69 is the extract of record of frog 4 (Table 15) and illustrates this point. During the test perfusion with 10^{-7} g/ml there was a small increase in the heart rate. The increase in the diastolic pressure was much greater than the increase in the systolic pressure. The output increased as a result of increase in the mean pressure. The overall evidence suggests that the changes in other parameters were mainly due to the positive chronotropic effect. Under the action of 10^{-5} g/ml complex changes occurred in the arterial pressure and output traces. The positive chronotropic effect was very marked. The fluctuations in

the pulse pressure probably indicate a dual action on the force of contraction. Fig. 70 illustrates the record of frog 7 (Table 15). There was a graded increase in the inhibitory effect from increasing concentrations of 5-hydroxytryptamine. The action of 10^{-9} is well marked. This was 1 of the 2 hearts of injected frogs which showed highest threshold sensitivity (minimum effective concentration 10^{-9}) to 5-hydroxytryptamine. There was no evidence of any change in the intensity of action of 5-hydroxytryptamine in the hearts of oestradiol injected frogs.

NICOTINE

Trial experiments with nicotine were conducted in the hearts of 2 uninjected frogs. Perfusion of each heart was continued for 2 days and tests were conducted with several series of solutions of nicotine on both days in each heart. The minimum effective concentration observed was 10^{-3} g/ml. As this work was not concerned with such high concentrations, no further experiments were undertaken. Nicotine in a concentration of 10^{-3} g/ml produced a marked negative chronotropic effect accompanied with an increase in the amplitude of contraction. The magnitude of increase in the amplitude was far greater than that which could be expected on the basis of increased filling associated with the decrease in the frequency of heart beat, suggesting an active augmentation of the amplitude. These findings are similar to those reported earlier by the author (Pathak, 1958d). There was no alteration in the threshold sensitivity to acetylcholine when acetylcholine was administered along with ineffective concentrations of nicotine (concentrations lower than 10^{-3} g/ml).

SENSITIVITY OF SAME HEART TO DIFFERENT SUBSTANCES.

The results of experiments on those hearts on which several substances were tested for threshold sensitivity are given in Table 16. It has been mentioned already that sensitivity to adrenaline, noradrenaline and 5-hydroxytryptamine was regarded as high at concentrations lower than 10^{-7} g/ml. The sensitivity to acetylcholine was regarded high at concentrations below 10^{-9} g/ml. These values of high sensitivities for different substances were adopted for the purpose of comparison. They were based on the fact that the majority of hearts tested for sensitivity to each of these substances were not influenced at these concentrations. Using these criteria of high sensitivity it is seen from Table 16 that a high sensitivity of a particular heart to one substance was not associated with a high sensitivity to other substances. Only the heart of frog 4 was highly sensitive to 3 of the 4 substances tested.

D I S U S S I O N

ACETYLCHOLINE

Biological Significance of Effectiveness of Acetylcholine in Low Molecular Concentrations

It is clear from the results that very small quantities of acetylcholine can significantly influence the activity of some of the spontaneously-beating perfused frog hearts. However several possibilities which could vitiate the results should be considered and excluded before accepting the effect to be genuine. It has already been discussed at several places that the possibility of contamination was reduced to the absolute minimum. Minor variations in pH of solutions were shown to be insufficient to influence the heart and a systematic record of the pH of solutions was kept. Other factors such as artificial changes in the record due to obstruction to inflow of fluid or due to toxicity of tubing were well recognised. The frequent control perfusions, and the technique of 'bracketing' the effect of a low concentration between several controls, further increased the accuracy of results. There was no possibility of unrecognised changes in venous pressure or unrecognised changes in the record due to electronic artefacts.

Very strict precautions were undertaken to obtain reliable dilutions. Each flask was labelled beforehand. The shelves for preparing and keeping different concentrations were permanently labelled. Fresh labelled flasks and fresh pipettes were used for each step of dilution. All glassware was clean and sterile. Hence the possibility of any unrecognised mistake in dilution procedure was remote. Besides the routine control perfusions, special methods of control were always employed when a heart was found to be responsive to low concentrations of acetylcholine. Moreover,

the technique of testing with several series of solutions prepared from different ampoules of acetylcholine chloride powder on the same heart was additional check on the possibility of unrecognised mistakes in obtaining correct dilutions or accidental introduction of some extraneous unrecognised contamination. Such mishaps certainly could not have occurred in all the series of solutions tested on a particular heart. Several confirmatory tests were conducted with the minimum effective concentration and the next lower concentration was usually shown to be ineffective. Thus the author is forced to accept that the effect was due, in fact, to the weak solutions of acetylcholine.

Using radioactive isotopes W.E. Boyd verified that the actual molecular concentration in solutions up to the dilution of 10^{-13} g/ml prepared according to the procedure followed in this work, was very close to the theoretically expected molecular concentration. Unfortunately this technique could not be employed for estimating molecular concentrations in still weaker solutions because of the limit of the counting technique. However, there is no reason to believe that the distribution of molecules would be absolutely random at greater dilutions. A gradation in molecular concentration is likely to be present even in more dilute solutions of acetylcholine than 10^{-13} g/ml but a deviation from the theoretical value is quite possible. The conventionally called 10^{-21} g/ml solution of acetylcholine theoretically contained only 3.3 molecules /ml. Since not more than 4 ml of this solution could come in contact with the heart during a 2 minute perfusion a total of only 13 molecules of acetylcholine was possibly involved

(theoretically) when this concentration was effective. This conclusion may not be absolutely correct but the results do show that only a few molecules can produce significant inhibition of the heart in stray instances. It is also significant to mention that, although tested on several highly sensitive hearts, 10^{-23}, which theoretically contains no molecules of acetylcholine whatever, never produced any effect. Moreover, 10^{-19} and 10^{-17} were more often effective than 10^{-21}.

The demonstration that acetylcholine in such low concentrations can produce significant inhibitory effects and that a concentration of 10^{-15} g/ml (containing 3.3×10^{6} molecule/ml) can completely stop the heart, has great physiological significance. Although the effect from very small quantities of acetylcholine was observed only in a few instances on the cardiac tissue of frogs, nevertheless the very fact that it can be demonstrated, even though in rare instances, is of fundamental importance regarding the status of acetylcholine in biology.

The effectiveness of small quantities of acetylcholine on the heart has a very important bearing on the mechanism of regulation of cardiac activity through the vagus in particular and on the mechanism of neurohumoral transmission in general. Although the actual quantity (or quanta) of acetylcholine released from the post-ganglionic parasympathetic terminals in the heart is not known precisely, this quantity in terms of molecules per impulse is probably very small and, further, the maintenance of vagal tone may require still smaller amounts of acetylcholine. Acetylcholine influences not only the rate

and amplitude of beat but also finer processes like conduction (Dale and Mines, 1913, Wedd and Blair, 1932, Bogue and Mendez, 1930) and the refractory period (Gilson, 1932, Wedd and Blair, 1945).

The possible physiological significance of acetylcholine in the process of neurohumoral transmission has been inferred from the fact that nerve and muscle are capable of forming and destroying acetylcholine and that the presence of acetylcholine can be demonstrated at critical sites. Although most of these actions of acetylcholine are based on observation of effects of rather higher concentrations, it is commonly believed that the same actions may result from smaller quantities of acetylcholine secreted naturally. Recently del Castillo and Katz (1957) using microelectrode technique estimated that 10^6 molecules of acetylcholine are released per impulse at a single nerve ending in frog muscle. The effectiveness of acetylcholine in this molecular dose has never been actually demonstrated. Like most other workers they maintained that externally administered acetylcholine is less effective in producing depolarization.

Thus, there has constantly been a gap between theory and practice in as much as acetylcholine in quantities believed to be naturally effective in intact animals has not been shown previously to be effective experimentally. The present work fulfils this gap and demonstrates that acetylcholine is effective in all molecular concentrations.

Sensitivity to Acetylcholine

Acetylcholine has been studied extensively by physiologists and pharmacologists using different biological tissues as mentioned

earlier in the Review of Literature. The minimum effective concentrations in frog hearts were found to be between 10^{-9} to 10^{-6} g/ml by different workers (Dale, 1914, Kolm and Pick, 1920, Clark, 1926, Loewi, 1949, Bentley and Shaw, 1952). Occasionally a concentration of 10^{-13} was found to be effective in frog hearts (Schmidt, 1958). Isolated mammalian hearts have also been found to be sensitive to similar concentrations as frog hearts, i.e. between 10^{-9} to 10^{-6} g/ml. Occasionally mammalian hearts responded to still lower concentrations under certain circumstances. Thus McDowall (1946) noted occasional stimulation with 10^{-11} in the atropinised cat, rabbit and rat hearts, and Spadolini (1948) noted occasional effects with 10^{-13} in the guinea pig heart, Thus, on the whole, the minimum effective concentrations on amphibian and on mammalian hearts were found to be similar.

In the present work a very wide range of concentrations was investigated. The minimum effective concentrations (threshold sensitivity) varied from heart to heart ranging from 10^{-21} g/ml (theoretically containing 3.3 molecules /ml) to 5×10^{-9} g/ml (theoretically containing 16.5×10^{12} molecules /ml). The stoppage concentrations ranged from 10^{-15} to 5×10^{-7} g/ml. However, only a few hearts responded to very low concentrations like 10^{-17}, 10^{-19} and only one heart responded to 10^{-21}.

The following factors enabled the author to detect effects of acetylcholine in very low concentrations in the present study.

1. Use of clean and sterile glassware for the preparation and storage of acetylcholine solutions.

2. Sensitive equipment.
3. Large number of hearts tested.
4. Distribution of experiments throughout the year over a period of several years.

Variation in Sensitivity to Acetylcholine from Heart to Heart

The decrease in the cholinesterase activity in the tissue made supersensitive by denervation (see Review of Literature) suggests that this enzyme may determine the effectiveness of a given concentration. However, carefully controlled experiments during the present work showed that eserine produced no change in the threshold sensitivity to acetylcholine, though it potentiated the action of concentrations which were already effective in the absence of eserine. It is possible that factors like diffusion, permeability of membrane or other structural peculiarities may determine the effectiveness of low concentrations. Absence of cholinesterase may be an additional factor.

Spontaneous and Seasonal Variations in Sensitivity to Acetylcholine

Two peaks of incidence of highly sensitive hearts were observed, one in the months of January and February and the other in the months of October and November. Since the overall results (supported by statistical analysis) indicate that oestradiol pretreatment did not influence the sensitivity to acetylcholine, the injected frogs in which the heart was highly sensitive in the month of December can be included in a general evaluation of the incidence of high sensitivity.

The sex cycle in most of the species of frogs including Rana

temporaria is fairly characteristic (Matthews and Marshall, 1956). The period from September to February is the period of rest or winter hibernation. The principal breeding month is March, after which there is a short period of involution lasting up to June. During the following months the ovaries and testes undergo hypertrophy and attain full activity by September. Thus the period of high sensitivity to acetylcholine coincides with winter hibernation, during which the sex organs are fully mature whereas the period during which the hearts were relatively insensitive coincides with the breeding season and the period of reactivation of the sex glands.

MacLean (1908)observed that vagal stimulation in frogs and in many other lower vertebrates is less effective in summer. Clark (1927b) also observed that vagal stimulation in frogs was less effective in summer. McDowall (1946) mentioned that the sensitivity to acetylcholine in cat, rabbit and rat heart was greater in May and June. Prosser (1940) and Welsh and Taub (1948) found seasonal variation in the sensitivity of some mollusc hearts to acetylcholine, the sensitivity being maximum in late winter and spring up to June in the heart of Ven-us mercenaria. Hughes (1955) observed that the heart of Mya arenaria was more sensitive in April and May. Thus seasonal variation in the sensitivity of hearts of various species to acetylcholine is a regular feature for which no satisfactory explanation has been advanced.

Since injections of female sex hormones produced no effect on sensitivity of hearts to acetylcholine, the sex activity does not

seem to be directly related to sensitivity. Efforts to alter the sensitivity to acetylcholine by administration of adrenaline or by changing the composition of Ringer's solution have also not shown any dramatic results. The results of previous workers on the relationship between sensitivity to acetylcholine and allied substances and the ionic composition of Ringer's solution are conflicting. Clark (1927a) observed that an increase in the concentration of calcium decreased the action of acetylcholine while a reduction in concentration of calcium had no effect. Other workers have reported similar findings in frog hearts with acetylcholine (Kolm and Pick, 1920) and muscarine (Kolm and Pick, 1920; Zondek, 1920). Loewi (1912) found no change in the sensitivity of frog hearts to pilocarpine and muscarine on changing the concentration of calcium. Clark (1927a) found an increase, while Davis (1931) found a decrease in the action of acetylcholine in frog hearts on lowering the concentration of potassium. A decrease in the action of pilocarpine in frog hearts on reducing the concentration of potassium was noted by Bouckaert (1921). Graham (1949) noted an increase in the action of acetylcholine in rabbit auricles on reducing the concentration of potassium.

Nature of Action of Acetylcholine

A number of workers have observed a biphasic effect of acetylcholine, i.e. stimulation at lower concentration and inhibition at high concentrations in mammalian hearts (Pines, 1934; Sach, 1937; Rothberger and Sach, 1938; Spadolini and Domini, 1940; Hoffman et al 1945; McDowall, 1946; Burn, 1956) and in invertebrate hearts (Prosser, 1942; Hughes, 1955).

The stimulating effect was observed at lower concentrations (around 10^{-9} g/ml) whereas inhibition was observed at higher concentrations (around 10^{-6} g/ml). The author observed previously (Pathak, 1958c) that both acetylcholine and nicotine restarted stopped frog hearts but the concentration required for restarting the hearts was usually greater than 10^{-7} g/ml. Hoffman et al (1945) suggested that the stimulating effect is due to release of adrenaline, through a nicotine-like action of acetylcholine.

No clear excitatory effect of acetylcholine was ever observed during the present work on frog hearts using a very wide range of concentrations. On a few occasions some rebound type of increase in the rate and rarely in amplitude was observed during the recovery period following a powerful inhibitory action of acetylcholine. This rebound phenomenon appears to be similar to the 'post-wash' stimulation observed by Webb (1950) in isolated rabbit auricles but washing out of the organ bath introduces a mechanical factor which was avoided in the present work.

Actual Forms of Concentration - Response Curves.

For ease in presentation of results the concentration-response curves were plotted using a logarithmic scale for the concentrations of acetylcholine. In the hearts with low sensitivity only a narrow range of concentrations was effective and, therefore, the greater part of the curves could be re-plotted using, as abscissa, a linear scale of actual weight of acetylcholine chloride per ml. present in different concentrations.

Fig. 71 shows the re-plotted curves for mean arterial pressure and heart rate in the three hearts (a,b,c) whose original curves were shown in Figs. 19b, 19a and 20a respectively. The curves for the mean pressure (and hence for the output also) show an initial phase of increasing inhibition at lower concentrations followed by a tendency to attain a plateau at higher concentrations. The curves for the heart rate retain the curvilinear form in most of the hearts but become more expanded in the high concentration range (Fig.71a and b). In a few hearts the initial part of the curve for heart rate is similar to the initial part of the curve for mean pressure but the later part is not seen due to complete conduction block (e.g. Fig. 71a). In the presence of eserine, curves tend to adopt a linear form.

The curves of highly sensitive hearts cannot be re-plotted in this manner on a convenient length of graph paper. However, it is obvious that these curves will become considerably expanded and would tend to assume the same forms as in less sensitive hearts, with a superimposed hump at lower concentrations.

Mechanism of Action of Acetylcholine

From the results it is clear that acetylcholine acts on at least three processes in the heart.

1. On the activity of the pacemaker.
2. On the conduction of impulses.
3. On the contractile responses of the cardiac muscle.

Since the response of the highly sensitive hearts to very low concentrations of acetylcholine presented some peculiar features, the mechanism of action in these hearts may be slightly different. The action in less sensitive hearts is considered first and that in the highly sensitive hearts will be discussed later.

Hearts with a Low Sensitivity to Acetylcholine

(a) Action on the Pacemaker - The curvilinear shape of the concent--ration-response curve for heart rate implies two phases in the action of acetylcholine - a slow initial phase and a rapid phase. A simple explanation for the two phases could be that during the slow phase (at lower concentrations) the cholinesterase system succeeds in inactivating a proportion of the molecules of acetyl--choline before they reach the receptors, but once a concentration sufficient to overwhelm the cholinesterase receptors is reached, the action progresses rapidly on further increasing the concent--ration. The uniformly potentiating effect of eserine on the action of acetylcholine on the pacemaker supports this view. The presence of other forms of curves, i.e. an almost linear curve or a curve with an upward convexity can also be accounted for on this basis - a linear curve might be due to absence of cholinesterase and a curve with an upward convexity due to low cholinesterase

activity. A high cholinesterase activity at the pacemaker would also account for the lower position of the curve for heart rate than those of mean pressure and output (indicating involvement of contractile response) in the same heart. Another explanation for the two phases could be that small quantities of acetylcholine only slow the rate of development of the diastolic slope of depolarisation (pacemaker potential) while large quantities may exert an additional hyperpolarising action on the membrane of the pacemaker tissue, producing a greater inhibition of heart rate. These actions of acetylcholine are well known.

The two alternative explanations mentioned above are not mutually exclusive and they may be jointly responsible for the two phases. Cholinesterase activity also may partly determine the position of two phases (i.e. range of concentrations over which each phase occurs)in individual hearts and the relative position of the curves in different hearts, the curve lying more towards the left in those hearts which have a low cholinesterase activity.

(b) Action on Conducting System - In the mammalian hearts acetylcholine acts on the specialised conducting tissue and slows the conduction of impulses. No definite specialised conducting system has ever been demonstrated in frog hearts. It is usually presumed that the impulse travels through the muscular tissue.

One of the most interesting observations in the present work has been that acetylcholine in relatively high concentration induces conduction block in almost every heart. Electrocardiographic studies are clearly indicated to locate the site (or sites) of

block. However, the present work suggests the following two points.

1. The considerable potentiating action of eserine on the occurrence of block in all hearts strongly suggests that cholinesterase act--ivity is high in the conducting system. This may also be the reason why conduction block usually occurs at high concentrations.
2. Marked involvement of the contractile response at the concentrat--ions, at which conduction block usually occurs, suggests that at these concentrations the cardiac muscle in general becomes greatly involved by some mechanism.

(c) Action on the Cardiac Muscle (Contractile Response)

The curves for the mean pressure and cardiac output suggest a rapid initial phase followed by a phase of reduced action (plateau effect). The tendency to attain a plateau indicates some kind of opposing process. The dual action of eserine (i.e. potentiation of action at higher concentrations, and from little potentiating effect to actual antagonistic effect at lower concentrations of acetylcholine) also supports the possibility of presence of two phases of action.

The antagonistic action of eserine at low concentrations of acet--ylcholine suggests that the commonly known anticholinesterase effect is not the only mode of its action. The antagonistic effect may be due to competitive attachment to cholinesterase receptors as well as to acetylcholine - sensitive receptors. Complete absence of potent--iation in some hearts may be due to a low cholinesterase activity.

Hearts with a High Sensitivity to Acetylcholine

Since eserine does not influence the threshold of sensitivity in the hearts, the presence or absence of cholinesterase cannot be respon-

-sible for determining the threshold of sensitivity. The effectiveness of acetylcholine in very small quantities in highly sensitive hearts must, therefore, be due to some other factors like rate of diffusion, permeability of membrane or other structural peculiarities. Absence of cholinesterase may be an additional factor.

More work is obviously needed to determine the details of the concentration-response relationship in highly sensitive hearts espec--ially with very low concentrations. The complex pattern of response seen at very low concentrations has no simple explanation.

Fig. 72 is a diagram in which an attempt has been made to correlate theoretically the behaviour of less sensitive and highly sensitive hearts. It has already been mentioned that the tendency for the concentration-response curves to form a plateau before 100% inhibit--ion is attained may be due to some opposing process. Fig. 72a shows that in the absence of cholinesterase (i.e. in presence of eserine) the curve is more steep and displaced to the left. Presence of cholinester--ase (as in normal hearts) shifts it to the right also making it less steep. The opposing process further modifies the curve to the approp--riate shape. The action of eserine was not investigated in the highly sensitive hearts. Hence a dotted hypothetical line has been drawn in Fig.72b which shows that the final curves in highly sensitive hearts may have the same basis as those of the less sensitive hearts with the additional feature that in the highly sensitive hearts the opposing process is activated earlier and assumes considerable proportions so that the curve is pushed down below the expected level, giving rise to a hump on the curve at lower concentrations.

ADRENALINE

Sensitivity to Adrenaline:

The sensitivity of frog hearts to adrenaline is not as high as that to acetylcholine. The frog heart is occasionally sensitive to low concentrations of adrenaline (10^{-9} and 10^{-11}) but the incidence of hearts showing such a high degree of sensitivity is not very high. However, a concentration of 10^{-7} was quite effective in producing a marked stimulation in the majority of hearts. The concentration of 10^{-8} was not included in the series of adrenaline solutions. However, from the trend of results it appears that 10^{-8} would be quite effective. West (1943) used frog hearts for assay of adrenaline and found that concentrations up to 10^{-8} could be assayed fairly accurately. West (1943) also found that the sensitivity to adrenaline could be slightly increased by sensitising the heart with cocaine.

Action of Adrenaline:

The general conclusion from the results of the present experiments on the sensitivity of frog hearts to adrenaline is that adrenaline is liable to produce inhibitory effects on some hearts at concentrations lower than 10^{-5}. The incidence of inhibitory effects became progressively less while the incidence of excitatory effects increased progressively at higher concentrations till only an excitatory effect was obtained in all the hearts at a concentration of 10^{-5}.

An inhibitory action at low concentrations of adrenaline has been observed by other workers on isolated frog hearts (Kolm &

Pick, 1921; Sollman & Barrow, 1926) on isolated rabbit heart (Claes, 1924) on isolated fish heart (MacDonald, 1925) and on isolated hearts of mollusc (Gaddam & Paasonen, 1955). The mechanism of the inhibitory action is not clearly understood. One possibility could be that the inhibitory action could be due to decomposition of adrenaline. The inhibitory action at lower concentrations may imply that possibly in the higher concentrations there was still sufficient undecomposed adrenaline not only to counteract the inhibitory action of the product of decomposition but also to produce a stimulation. Occasionally, it was seen that a fresh solution of adrenaline (in the same concentration in which an old solution produced inhibition), when tested soon after the old solution, produced a stimulation instead of inhibition. This indicated a difference in the two solutions. It is however, possible that the reversal of action could be due to repetition of administration of adrenaline in these cases. Thus whereas the decomposition of adrenaline solution seems to be a possible basis to explain the inhibitory action of lower concentrations, it may not be the correct explanation. In the heart of frog 10 (Table II) a 3 hours old adrenaline solution in concentrations of 10^{-9} and 10^{-7} produced inhibitory effects whereas a 5.5 hours old solution of adrenaline in a concentration of 10^{-9} produced excitatory effect in the heart of frog 3 (Table II) and a 4.5 hours old solution in a concentration of 10^{-7} produced an initial marked stimulation followed by stoppage (frog 19, Table II) followed by alternate stoppage and acceleration for about 15 minutes. Thus the duration of standing of solutions from the time of preparation up to the time of use was not definitely related to the nature of effect. Euler &

Hamberg (1949) studied the rate of oxidation of adrenaline and noradrenaline and mentioned that spontaneous oxidation of solutions takes months and the rate of oxidation is slow even in alkaline pH.

Further the author had observed previously (Pathak, 1958d) that as high concentrations of adrenaline as 10^{-4} also produced inhibitory effect in frog hearts. It would thus appear that adrenaline is liable to produce inhibitory as well as excitatory effects in frog hearts. The incidence of inhibitory effect is, however, low and decreases progressively further as the concentration of adrenaline increases. The significance of the inhibitory action is not clear. It might however be similar in nature to the reversal of pressure-response which has never been satisfactorily explained.

West (1949) reported vasodepressor action of noradrenaline. Serin (1952 a,b) observed that dibenamine and piperidinomethyl benzodioxane (933F) reversed the stimulating action of adrenaline and noradrenaline in isolated rat hearts. In the present work noradrenaline always produced excitatory effects. The transitory stoppage of heart following initial stimulation with noradrenaline 10^{-7} g/ml in one case was possibly due to the exhaustion of heart as a result of repeated administration of adrenaline and noradrenaline.

Maintenance of Strength of Adrenaline Solutions:

West (1943) observed that about 30% activity of adrenaline solutions was lost in 5 minutes. During the present work the

adrenaline solutions were prepared in alkaline Ringer's solution (pH 7.8 - 8.4) and the time schedule of the experiments was such that these solutions stood for some time before being used. The period of standing ranged from 1 hour to as long as 8 hours in some experiments. The solutions used in the heart of frog 2 (Table II) (one of the most highly sensitive hearts, sensitive to 10^{-11}) were 1.5 hours old. The concentration of 10^{-7} was effective up to 8 hours or more. These solutions were also prepared and stored under sterile conditions as adopted for the preparation and storage of acetylcholine solutions. There was no indication of substantial deterioration in the strength of those solutions up to a few hours in the case of concentrations lower than 10^{-7} and up to 8 hours or more in the case of more concentrated solutions.

NORADRENALINE

The frog hearts were almost as sensitive to noradrenaline as to adrenaline. However, only 10% of the hearts responded to noradrenaline in a concentration of less than 10^{-7} and about 10% not responding even to 10^{-7}. Noradrenaline 10^{-5} was effective in all the hearts. Noradrenaline always produced excitatory effects in frog hearts with the exception of the heart of one frog in which both adrenaline and noradrenaline in a concentration of 10^{-7} produced initial marked stimulation followed by transitory stoppage of the heart. Inhibitory effects from noradrenaline have not been reported by previous workers and are certainly not common. Occasional negative inotropic effect with noradrenaline in a concentration of 10^{-8} was observed by Serin (1952 a,b) in isolated rat hearts after administration of dibenamine and other adrenolytic compounds. Frog heart is suitable for assaying noradrenaline in concentrations up to 10^{-7} g/ml only. The observation that noradrenaline was less effective than adrenaline is in agreement with the results of previous workers. West (1947) found that adrenaline was about 8 times more effective whereas Gaddum, Pert & Vogt (1949) reported that it was 10 to 20 times more effective in frog hearts. Burn & Hutcheon (1949) also reported a greater effectiveness of adrenaline in cat hearts. Ahlquist (1948) reported that adrenaline was 2 times more effective in rabbit hearts. Other workers have observed no definite difference in the

effectiveness of adrenaline and noradrenaline in rabbit and cat hearts (Marsh, Pelletier & Ross, 1948) and in rat hearts (Serin, 1952a). In the present work it was found that the positive chronotropic effect of adrenaline was about 2 times greater than that of noradrenaline. The difference in the results of various workers could be due to such factors as species variations, variation in the criteria for effectiveness of the two substances and also possibly due to variations in the concentrated solutions obtained from the manufacturers. Adrenaline is the dominating neurohormone in frog hearts (Euler, 1946).

5-HYDROXYTRYPTAMINE

The sensitivity (minimum effective concentration) of frog hearts to 5-hydroxytryptamine was slightly less than that to noradrenaline. Obviously the sensitivity of frog hearts to 5-hydroxytryptamine is much less than sensitivity of other tissues discussed in the section on Review of Literature. Only 2 out of 17 hearts were sensitive to a concentration of 10^{-9} which produced a pure inhibitory effect in both. Inhibitory effects were also observed at high concentrations. However, in the majority of hearts the action of 5-hydroxytryptamine was excitatory. The inhibitory and excitatory effects involved both the chronotropic and inotropic responses. Like adrenaline and noradrenaline lower concentrations of 5-hydroxytryptamine frequently involved the chronotropic responses primarily. The inhibitory effect could not be related to the dose of 5-hydroxytryptamine nor to any other known variable.

Several workers have studied the actions of 5-hydroxytryptamine on cardiac tissues. Schneider & Yonkman (1954) observed both positive chronotropic and inotropic effects of 5-hydroxytryptamine in isolated hearts of dog, cat and rabbit. Sassine & Mauche (1955) observed that 5-hydroxytryptamine increased only the rate but not the strength of beat of isolated papillary muscles of dog, cat and rabbit. 5-hydroxytryptamine produces excitatory effects in molluscs and other vertebrate hearts which are very sensitive to it (see Review of Literature). Hence the observed pure inhibitory effect over a wide range of concentrations in some hearts seems

to be a unique effect of 5-hydroxytryptamine in frog hearts. There is nothing to suggest that the inhibitory effects observed with 5-hydroxytryptamine represented reversal of response due to oestradiol injections for minor inhibitory effects were observed in the hearts of uninjected frogs also. Moreover the inhibitory effect was observed in the hearts of only 2 of the 14 injected frogs and there was no relation of dose of oestradiol to the occurrence of inhibitory effect. Further a biphasic effect was often observed in the same heart with clear evidence of both inhibitory and excitatory components. The only conclusion is that in some hearts 5-hydroxytryptamine produces a pure inhibitory effect involving both the chronotropic and the inotropic responses. On the whole the excitatory responses to 5-hydroxytryptamine was much less in intensity than that to corresponding concentrations of adrenaline and noradrenaline.

1. The actions of a wide range of concentrations of acetylcholine, adrenaline, noradrenaline, 5-hydroxytryptamine and nicotine have been investigated to determine minimum effective concentrations (threshold sensitivity) in isolated perfused frog hearts. A specially designed sensitive electronic equipment consisting of a 4 channel direct ink writing recorder provided a continuous record of the venous pressure, heartrate, arterial pressures (systolic, diastolic and pulse pressures) and cardiac output. The venous pressure could be kept constant at any desired value throughout the period of perfusion lasting from 1 to 3 days.

2. Special methods of precaution and control were introduced at several stages of each experiment so that the effect of test solutions were well controlled and it was clearly shown on the record that there could be no possibility of unrecognised changes in the record due to artefacts - electronic or otherwise.

3. It was observed that the distilled water used for the preparation of solutions, the ionic composition of fluids, the types of glass container and connecting tubing, the minor variations in the temperature of the laboratory from place to place (responsible for slight differences in the temperature of solutions kept at different places), the minor variation in the pH of solutions and bacterial, chemical or organic contamination of glassware and connecting tubing, all

were liable to influence the behaviour of the preparation. Because of the sensitivity of the equipment, changes in the cardiac activity due to these factors were picked up and recorded as prominent changes which were often responsible for unsatisfactory controls. Most of these variables were easily controlled once their importance was realised, though a considerable time was devoted to analyse and control them.

4. The choice of a suitable type of connecting tubing of a really nontoxic material was a difficult problem and a special side study (described in Part II) was undertaken to compare the toxicity of different kinds of tubing. It was observed that all connecting tubing including natural rubber and plastic tubing were potentially toxic and must be cleaned thoroughly several times before being used.

5. It was also observed that a thorough cleaning and sterilisation of all apparatus coming in contact with the test solutions was essential for accurate work, and the solutions of such substances as acetylcholine and adrenaline maintained their strength for a much longer duration than commonly believed if prepared and stored using clean sterile glassware.

6. The dynamics of the heart under the present perfusion technique has been investigated and discussed. It has been concluded that the venous pressure and arterial resistance both influenced the inotropic and chronotropic responses leading to appropriate changes in associated parameters of cardiac

activity such as cardiac output and blood pressure.

7. It has been demonstrated that if the solutions of acetylcholine are prepared by using clean sterile pipettes and if stored in clean sterile containers, the solutions maintained their strengths in alkaline Ringer's solution at room temperature for a much longer period than that observed by previous workers. Solutions containing acetylcholine in a concentration of 10^{-7} g/ml maintain their strength up to 36 hours. More concentrated solutions (10^{-5} and 10^{-3}) keep for a much longer time- up to 72 hours or more. Dilute solutions containing acetylcholine in concentrations between 10^{-7} and 10^{-15} g/ml keep for 8 to 12 hours and undergo only a slow hydrolysis after this time. The duration of maintenance of strength varies with the molecular concentration.

8. The threshold sensitivity of frog hearts to acetylcholine varied from 10^{-21} to 5×10^{-9} g/ml. The concentration needed to stop the heart varied from 10^{-15} to 5×10^{-7} g/ml. The majority of hearts showed a threshold sensitivity at 5×10^{-9} g/ml and were stopped by 2.5×10^{-7} g/ml, only a few requiring 5×10^{-7} for stoppage.

9. When acetylcholine in a concentration of 10^{-21} g/ml was effective (even though only in a stray instance), theoretically only 13 molecules were involved. Greater concentrations were effective more frequently. The physiological significance of effectiveness of acetylcholine in all molecular concentrations has been discussed in relation to the nervous regulation of

cardiac activity in particular and in the process of neurohumoral transmission in general.

10. The demonstration of effectiveness of acetylcholine in all molecular concentration fulfil-s an important gap between the theoretically expected and experimentally demonstrated effectiveness of acetylcholine. The effectiveness of acetylcholine in all molecular concentrations has never been demonstrated previously. There is now no need to presume that endogenously produced acetylcholine is more effective than externally administered acetylcholine.

11. The incidence of hearts showing a high degree of sensitivity to acetylcholine (sensitive to concentrations lower than 10^{-9} g/ml) varies significantly with season, being maximum in winter (January and February) and minimum in spring and summer.

12. The threshold sensitivity to acetylcholine does not undergo any remarkable change on altering the composition of Ringer's solution, on administration of adrenaline and after injections of female sex hormones in intact animals.

13. Frequent spontaneous changes in the threshold sensitivity within 12 hours of perfusion were not common. In a few instances the sensitivity changed significantly after the heart had been on perfusion for more than 12 hours, the sensitivity decreasing in some and increasing in other cases.

14. A pure excitatory effect from acetylcholine was never observed at any concentration. A rebound increase in rate and/or amplitude during recovery from inhibitory action of acetylcholine observed in some cases cannot be regarded as an excitatory effect. The action of acetyl--choline was, therefore, always inhibitory.

15. The concentration-response relationship in hearts with a low sensitivity to acetylcholine leads to the following conclusions:

(i) acetylcholine acts on three sites - (1) pacemaker (2) conducting system (3) contractile response (cardiac muscle). The action on the pacemaker and on the contractile response are rarely parallel, the action being usually more pronounced on the latter at lower concentrations, though the chronotropic and inotropic responses are both simultaneously involved at the minimum effective concentration.

(ii) typical curve for heart rate is curvilinear. Eserine has a marked potentiating effect, shifting it to the left and making it more linear.

(iii) typical curves for contractile response (mean pressure and output) have a convexity upwards. It is 'S' shaped if the concentration of acetylcholine is expressed in a logarithmic scale. Eserine usually potentiates the action

of higher concentrations of acetylcholine but may antagonise the action of lower concentrations.

(iv) conduction block occurs in all hearts at higher concentrations and may change the form of different curves. Complete block stops the heart. Block occurs at a much lower concentration in presence of eserine.

16. Concentration-response curves in hearts with a high sensitivity to acetylcholine show the same features as in less sensitive hearts with the additional observation that the curves for all three parameters have a hump over a range of very low concentrations with a peak on the hump at a certain concentration.

17. The mechanism of action of acetylcholine which may possibly be responsible for these observations has been discussed.

18. The frog heart is not a suitable preparation for assaying acetylcholine in concentrations lower than 10^{-9} g/ml because of the very low incidence of highly sensitive hearts (only 14%)

19. The threshold sensitivity of frog hearts to adrenaline and noradrenaline varied from 10^{-11} to 10^{-5} g/ml. The incidence of highly sensitive hearts (hearts sensitive to concentrations lower than 10^{-7} g/ml) was 22% in the case of

adrenaline and about 10% in the case of noradrenaline. The majority of hearts were influenced by adrenaline and noradrenaline in a concentration of 10^{-7} g/ml, though some hearts were only influenced at 10^{-5} g/ml.

20. Frog heart is a suitable preparation for assaying adrenaline and noradrenaline in concentrations greater than 10^{-9} g/ml.

21. Lower concentrations of adrenaline (between 10^{-11} and 10^{-7}) often produced inhibitory effects. The incidence of inhibitory effects decreased at higher concentrations. It is possible that some decomposition products of adrenaline may be responsible for the inhibitory effects, the inhibitory effect being masked at higher concentrations which may still contain sufficient undecomposed adrenaline to produce a predominently excitatory action. However, this does not appear to be a complete explanation for the occurrence of inhibitory effects. The strength of adrenaline was maintained fairly well for several hours with the present technique of using clean sterile glassware for the preparation and storage of solutions and the duration of standing of solutions does not correlate with the occurrence of inhibitory effects. Noradrenaline always produced excitatory effects with the single exception of one heart in which both adrenaline and noradrenaline in a concentration of 10^{-7} g/ml produced a brief stoppage of the heart

preceded by a well marked stimulation.

22. The chronotropic response was first and primarily involved at lower concentrations of adrenaline and noradrenaline. The positive chronotropic effect of adrenaline was about 2.5 times greater than that of noradrenaline.

23. The latency of onset of excitatory action of adrenaline was shorter and the peak of effect was more quickly attained. The increase in the blood pressure (determined by the heart rate and amplitude of beat mainly, the resistance being constant) due to the action of adrenaline was greater but was of shorter duration than that due to the action of noradrenaline. The marked acceleration produced by adrenaline resulted in a greater reduction in the amplitude of contraction during the action of adrenaline than during the action of corresponding strength of noradrenaline. On the whole adrenaline produced a greater degree of stimulation than noradrenaline.

24. The frog heart is less sensitive to 5-hydroxytryptamine than to adrenaline and noradrenaline, the threshold sensitivity to 5-hydroxytryptamine ranging from 10^{-9} to 10^{-3} g/ml. Whereas adrenaline and noradrenaline were always effective in a concentration of 10^{-5} g/ml, 5-hydroxytryptamine was ineffective in some hearts in this concentration. Only about 11% of the hearts were sensitive to 5-hydroxytryptamine in a concentration of 10-9 g/ml, the majority being sensitive

to 10^{-7} or 10^{-5} g/ml. Thus frog heart is a suitable preparation for the assay of 5-hydroxytryptamine in concentrations greater than 10^{-9} g/ml.

25. 5-hydroxytryptamine produces both excitatory and inhibitory effects in frog hearts. Although the incidence of hearts showing a pure inhibitory effect was only about 11%, the pure inhibitory effects were observed at all concentrations, the inotropic and chronotropic responses both being inhibited. In the majority of hearts an excitatory effect influencing both the inotropic and chronotropic responses, was observed. The excitatory effect, however, influenced the chronotropic response more frequently and the increase in the heart rate was often associated with a decrease in the output and blood pressure. Frequently the excitatory and inhibitory components of action of 5-hydroxytryptamine were simultaneously operative at the same concentration in the same heart, the nett effect being a resultant of the two components.

26. The frog heart is relatively insensitive to nicotine, no effect being observed till such high concentrations as 10^{-3} are reached. In these high concentrations nicotine produces a powerful negative chronotropic effect.

27. The high sensitivity of a heart to one substance is not associated with a high sensitivity to other substances.

28. According to the effectiveness in lowest concentrations (highest degree of threshold sensitivity) in frog hearts, the various substances investigated can be arranged in the following descending order: acetylcholine, adrenaline, noradrenaline, 5-hydroxytryptamine, nicotine.

PART II

THE COMPARATIVE TOXICITY OF MODERN PLASTIC AND SILICONE CONNECTING TUBING, TESTED ON FROG HEARTS.

I N T R O D U C T I O N

The difficulties which were encountered in analysing and controlling the several relatively obvious variables which interfered with the tests designed to investigate the action of small quantities of acetylcholine and other substances and which were often responsible for unsatisfactory controls are discussed in Part I. After having controlled most of such possible variables, frequent unsatisfactory controls were still observed and ~~that~~ the inert nature of the so called non-toxic connecting tubing came under suspicion. It was considered necessary to undertake a detailed study of the relative toxicity of some commonly available modern connecting tubing made of plastic material (polyvinyl chloride) and silicone rubbers in order to select the best available type with a view to eliminating or minimising the occurrence of unsatisfactory controls and to enhance the accuracy of results.

REVIEW OF LITERATURE

The toxic properties of various kinds of natural rubber products have been recognised for a long time. Stokes & Busman (1920) were the first workers to notice that natural rubber tubing used in intravenous infusions could cause a reaction in human subjects. Rubbers were also found to be spermicidal (Ranson, 1937) and toxic to tissue culture cells (Gunz, 1948; Lajtha, 1952; Parker, Morgan & Morton 1951; MacDougall, 1953; Cruickshank, Caroline Hooper, Lewis & MacDougall, 1960). MacDougall (1953) also reported that the toxicity of rubber was due to sulphur and talc content.

Both rubber and plastic materials have been reported to inhibit growth of bacteria and this property of the tubing was found to correlate with the incidence of thrombophlebitis when such tubing was present in equipment used to give intravenous transfusions (Handfield - Jones & Lewis, 1952; Medical Research Council, 1957).

Several types of plastic material are carcinogenic in animals (Oppenhiemer, Oppenhiemer, Danishefsky, Stout & Eirich, 1955). The life span of red blood cells in the recipient after transfusion was reported to be considerably shortened when containers of plastic materials were used for storage of blood in the blood banks (Wall, Buckley & Doan, 1953; Strumia, Colwell,

Ellenberger & Maur, 1955). Strumia et al (1955) also observed that some plastic materials were better than others.

Silicone rubbers were found to be non-toxic to the chick embryo cultures and other tissue cultures (MacDougall, 1953; Cruickshank et al. 1960) but silicone implants were not well tolerated by the human brain and caused a delayed meningitic reaction (Dimant, 1955). Dimant (1955) suggested that the reaction might be due to a slow diffusion of a toxic substance, perhaps benzoic acid derived from the vulcanising agent.

Natural rubber and plastic materials have also been investigated for their toxicity by implantation of samples in intact animals (Ingraham, Alexander & Matson, 1947 a,b) and in certain instances severe inflammatory reactions were observed due to the liberation of irritant products.

Perspex (polymethylmethacrylate) (Beck, Russel, Small & Graham, 1945) and polyethylene (Polythene) (Ingraham et al. 1947 c) were found to be well tolerated by intact animals. Nylon, which can be sterilized by autoclaving, is well tolerated as a suture material.

It is clear from this review of short literature on the toxicity of various kinds of materials used for the manufacture of connecting tubing that the inert nature of various materials has been questioned from time to time and isolated studies have been conducted to substantiate such belief. It is rather surprising

that, although so widely used in biological laboratories, the dangers from the toxicity of the connecting tubing have not been fully realised nor systematic studies on isolated biological preparations have been conducted to investigate this problem.

METHODS

From amongst the commonly available modern tubing 4 kinds of P.V.C. (polyvinylchloride) and 3 kinds of silicone rubbers were selected for investigation. The names of these materials and the manufacturers from whom they were received are mentioned below.

P.V.C. tubing.

1. Portex Crystal Vinyl P.V.C. (Portland Plastics Ltd.,).
2. Portex Standard P.V.C. (Portland Plastics Ltd.).
3. X-lon P.V.C. (X-lon Products Ltd.,)
4. Waterclear P.V.C. (Esco Rubber Ltd.)

Silicone rubbers

1. Silicone-1956 (supplied by Esco Rubber Ltd. in 1956).
2. Silicone TC-156 (Esco Rubber Ltd.)
3. Silicone DSR-551 (Dunlop Rubber Co. Ltd.).

Having decided to undertake such a study, the author was faced with two immediate problems:

1. How to incorporate the presumed toxic product from the material of tubing into a 'test solution' which could be perfused in the frog hearts and its action observed under strictly controlled conditions so that any effect noticed could only be attributed to the toxicity of tubing and to nothing else.

2. Which kind of tubing could be presumed to be relatively non-toxic and could be used for connections to conduct the perfusion tests with the 'test solutions' obtained from other kinds of tubing to demonstrate their toxicity. As there was no known inert material to start with, and as the test solutions from other materials were to flow through the connecting tubing, this was a rather difficult decision. Analysis of the previous work indicated that among the several materials previously used as connecting tubing in the perfusion experiments 'Portex Crystal Vinyl' P.V.C. tubing was relatively satisfactory if it had been in use for a long time and had been subjected to a lengthy elaborate process of cleaning (and sterilization) by 'Calgon Method' a number of times repeatedly. It was decided to use this tubing as standpipe-connecting tubes while studying the effect of toxicity from other kinds of tubing. But a narrow bore and sufficiently flexible tubing, made of the same material needed for transducer-connecting-tube, was not available, and 'Silicone-1956' (also

cleaned repeatedly several times), had to be used for this purpose. This procedure, in fact, implied the provisional assumption that 'Portex Crystal Vinyl' P.V.C. and 'Silicone-1956' tubing cleaned repeatedly were relatively non-toxic. However, since all solutions (including the control and test solutions) were to pass through these two kinds of tubing, any toxicity still present in them could be regarded as a constant factor for all practical purposes.

The toxicity of 'Portex Crystal Vinyl' P.V.C. tubing itself and of 'Portex Standard' P.V.C. tubing was investigated initially using 'Portex Crystal Vinyl' and 'Silicone-1956' as standpipe-connecting-tubes and transducer-connecting-tubes respectively. The 'test solutions' for investigating the toxicity of these two kinds of tubing were prepared by the 'boiling of tubing' method described under the preparation of test solutions. By this method the presumed toxic product from the material of the tubing was expected to be extracted by boiling and the extract was subsequently incorporated in a sample of Ringer's solution for conducting the test perfusions to study the action of test solution, if any, on the activity of the isolated perfused heart. Although this procedure clearly demonstrated the toxicity of these two kinds of

tubing and the results were highly encouraging for conducting further tests with other materials, the method of preparing the 'test solution' by 'boiling' did not reproduce the actual circumstances under which interference in the experiments on the action of small quantities of acetylcholine and other substances, was encountered due to frequent occurrence of unsatisfactory controls. This interference resulted from the possible release of some toxic product into the test solutions when these solutions lay stagnant inside the connecting tubing for some time. It was abundantly clear from the previous work that if the control solution was allowed to remain in contact with the connecting tubing for about 10 minutes, the perfusion of the heart with such a solution often produced a significant effect on the activity of the heart. A better approach, therefore, was to test the toxicity of a solution in which pieces of tubing had actually been suspended for some time. Moreover, the 'boiling of tubing' method was also considered as a rather drastic procedure. To meet these requirements the method of 'soaking of tubing' for the preparation of test solutions (see below) was finally adopted.

When the method of preparing the test solutions from tubing by 'soaking of tubing' was being standardised another problem crept in. The manufacture of 'Portex Crystal Vinyl' P.V.C. tubing which was being used as standpipe-connecting-

tube, was discontinued. The subsequent work, therefore, was conducted by substituting 'Waterclear' P.V.C. as standpipe-connecting-tubes after subjecting them to repeated cleaning through the 'Calgon Method'. When these other changes were being made in the prodedure, it was also considered useful to try some other kind of tubing for transducer-connecting-tubes because the contact of solutions even with the short length (1.5") of 'Silicone -1956' which was being used as transducer-connecting-tubes often resulted in unsatisfactory controls and the transducer-connecting-tubes had to be flushed frequently to obtain satisfactory results. 'Silicone DSR-551', therefore, was substituted for transducer-connecting-tubes. After making all these changes, it was also felt that for uniformity of results all the 7 materials originally selected for investigation of toxicity should be investigated by the new technique, and consequently those two kinds of tubing (namely 'Portex Crystal Vinyl' and 'Portex Standard' P.V.C) which were initially investigated by obtaining 'test solutions' from them by the 'boiling of tubing' method, were re-investigated along with other materials using the 'soaking of tubing' method of preparing the test solutions and using 'Waterclear' P.V.C. and ' Silicone DSR-551' as connecting tubes.

Preparation of Solutions to Test for Toxicity (Test Solutions)

The two methods of preparing the test solutions presumably incorporating the toxic product from different kinds of tubing are detailed below:

1. 'Boiling of Tubing' Method: A one foot length of each kind of tubing was divided into small pieces each about 2 inches long. The pieces of each kind of tubing were boiled separately for half an hour in 250 ml of double distilled water in a large beaker. The pieces were then removed with the help of a glass rod and the fluid allowed to cool. The volumes of the different stock solutions needed to prepare 2 litres of the usual Ringer's solution were added together to obtain a concentrated mixture. Half of this mixture was made up to 1 litre by adding double distilled water and the Ringer's solution so obtained was used for the perfusion of the heart from the reservoir, the other half was divided into two equal parts and each part was transferred into a volumetric flask of 500 ml capacity. A label bearing the name of the tubing under investigation was affixed on one of the two flasks while the second flask was marked 'control'. The test solution was now prepared by

adding the fluid obtained by boiling the tubing into the appropriately labelled flask and the volume was made up to 500 ml with double distilled water. The control solution in the 'control' flask was prepared by adding the same volume of boiled, double distilled water (instead of the fluid obtained by boiling the tubing) and the volume was made up to 500 ml by adding more doubled distilled water. The control solution was, therefore, also a sample of Ringer's solution made with boiled double distilled water. It is obvious that the ionic composition of the different solutions, i.e. the Ringer's solution used for perfusion from the reservoir, the test solution presumably containing the toxic product extracted from the tubing by boiling and the control solution, was absolutely identical. The heart was perfused with the test solution and the effects due to toxicity were studied. Frequent perfusions with control solution showing no effect on the heart, served as routine controls.

2. 'Soaking of Tubing' Method: Stoppered cylindrical volumetric flasks, each of 100 ml capacity, were selected and a label bearing the name of one of the different kinds of tubing under investigation was affixed to each. A 3 foot length of each kind of

tubing was divided into small pieces each about 2 inches long. These pieces were then suspended in 100 ml of Ringer's solution in the appropriately labelled flask and were allowed to stand for 8 to 12 hours. Hundred ml of Ringer's solution in one of the same type of flasks marked 'control ' also stood near these test solutions for identical periods and served as a 'control solution' for conducting control perfusions. Samples were withdrawn from the test solutions at known intervals of time and were tested for toxicity by perfusing the hearts. Frequent perfusions with samples of control Ringer's solution, obtained after identical periods of standing, served as controls and indicated that the effect of test solution, if any, was not due to any other possible variable or contamination.

Tubing cleaned several times by the 'Calgon Method' was used for obtaining the test solutions by each of the two methods described above. Some experiments were also designed to substantiate the belief that the process of cleaning in some way reduced the toxicity of tubing. To demonstrate the

influence of cleaning on the toxicity of tubing, three types of test solutions were obtained from each kind of tubing by suspending in the Ringer's solution pieces (i) which had been cleaned several times (ii) which had been cleaned once, and (iii) which had never been cleaned at all. Samples from these test solutions were withdrawn after known intervals of soaking and were tested on the same heart. In adopting this procedure it was expected that if the process of cleaning altered the toxic property of tubing in any respect, the response of the heart ought to show a graded effect. Initially these experiments on the influence of cleaning on the toxicity of tubing, were conducted by obtaining test solutions from pieces which had been cleaned by the 'Calgon Method'. Since one of the manufacturers (Esco Rubber, Ltd.) recommended a 'Bicarbonate Method' for cleaning the tubing, it was considered useful to compare the influence of both Calgon and Bicarbonate methods of cleaning on the toxicity of the tubing. In some of the experiments, therefore, test solutions were prepared from some kinds of tubing by soaking pieces which had been cleaned once or several times by both methods, and these solutions were tested on the same hearts. The details of both Calgon

and Bicarbonate methods of cleaning are given in Part I.

The general technique adopted for investigating the effect of the test solutions obtained from different kinds of tubing was the same as that described in Part I for investigating the action of acetylcholine and other substances. The heart was continuously perfused from the reservoir except during control and test perfusions which were conducted from a different source. An initial control perfusion with control Ringer's solution was carried out for 2 minutes from one of the burettes, to show that the perfusion system and the burette were satisfactory. The burette was emptied and filled with one of the test solutions. The heart was perfused for 2 minutes with the test solution to record the effect due to toxicity of tubing, if any. The burette was then washed several times and the control perfusion repeated from it, thus 'bracketing' the effect of a test solution between two controls from the same burette. Each test solution was perfused from a separate burette and no burette or connecting tube was used twice, except when it was known from previous trials that a particular test solution produced a particular type and degree of toxic effect quite readily recognisable from the effect of another solution. Under these conditions two or more solutions were tested from the same burette, to obtain the record of effects of different solutions under absolutely identical conditions on a short

length of recording paper. This procedure simply increased the accuracy of comparison and also enabled photographic reproduction of short records. However, in all such cases a control perfusion from the same burette was conducted after washing out the first test solution and before filling with a second test solution. At times one burette was reserved for conducting only control perfusions which alternated with test perfusions from another burette (or burettes). An initial control perfusion was conducted from each burette before it was used for test perfusion.

The changes in the different recorded parameters due to alterations in the activity of the heart during test perfusions and the significance of controls should be interpreted exactly in the same ways as in Part I. The special methods of control mentioned in Part I were also occasionally used to increase the accuracy of results.

RESULTS

The 'boiling of tubing' method of preparing the test solution from the tubing was used only for 'Portex Crystal Vinyl' and 'Portex Standard' P.V.C. in a few experiments. Test solutions from these two kinds of tubing were also prepared by the 'soaking of tubing' method used for other kinds of tubing. As the analysis of the results involves frequent comparisons between different kinds of tubing, the results obtained by the 'boiling of tubing' method have been kept separate and will be referred to from time to time. Unless otherwise stated, the toxic action of a tubing, therefore, implies the effect of test solutions obtained by soaking pieces of different kinds of tubing which had been cleaned a number of times by the 'Calgon Method'.

The effects produced by the test solutions caused a change in the various parameters of cardiac activity to a different degree. For the purpose of comparison, therefore, it was decided to classify the effects into three grades, neglecting all doubtful effects (i.e. minor effects which were not significantly greater than those obtained from controls).

Grade I - indicates a change in at least two of the three variable parameters by 5 to 10%.

Grade II - means all variable parameters affected and at least two parameters affected by 10 to 15% or a smaller but prolonged effect.

Grade III - means all variable parameters affected and at least two parameters affected by more than 15%.

The different grades of effects from the test solutions of various kinds of P.V.C. tubing are shown in Figs. 73 to 80. Fig. 81 illustrates a grade III effect from one of the silicone materials.

From the examples of effects seen in Figs. 73 to 81 it is clear that the toxicity of tubing exerted an inhibitory effect on the heart, reducing the recorded heart rate, amplitude of contraction and associated parameters. In addition to the inhibitory effect, the test solutions from 'Portex Standard' P.V.C., 'Silicone-1956' and 'Silicone TC-156' sometimes also produced a delayed increase in the heart rate. The effect on the heart rate, therefore, was biphasic in these cases, the rate initially decreasing and then increasing above control value. During the phase of acceleration of the heart there was a further decrease in the systolic pressure and pulse pressure, indicating a further decrease in the amplitude of contraction. The effect on the cardiac output under these circumstances was variable, depending upon whether the increase in the heart rate was sufficient to balance the decrease in the amplitude (Fig. 86,87 and 88). The toxicity of all kinds of tubing was liable to upset the rhythm of the heart, but the incidence of gross arrhythmia was more common with P.V.C. tubing than with silicone tubing. The disturbances of rhythm produced by the

toxicity of various kinds of P.V.C. tubing are illustrated in Figs. 78,79,80,82,83 and 84, and by the toxicity of two kinds of silicone tubing in Figs. 87 and 88. The effects due to toxicity observed during or after the 2 minute-test perfusion were usually abolished by perfusing with normal Ringer's solution and permanent damage to the heart was not encountered. The latency of onset of irregularity in rhythm appeared to depend on the duration of soaking as well as on the material under investigation.

The summary of the results of experiments on the hearts of all the 35 frogs is given in graphic form in Fig. 85. The grades of effects shown represent the average effect evaluated from two or more test perfusions with the same test solution in each case. It is seen that in the hearts of 10 out of 35 frogs, no toxic effects were observed. This, however, does not directly imply that these 10 hearts were insensitive to the toxicity of tubing, because the occurrence of toxic effects in a particular heart depended on several factors:

1. The dose of toxic substance contained in the test solution. This was determined mainly by the duration of soaking because the volume of fluid in which the tubing was soaked and the total length of the pieces of tubing were identical. However, the total surface

area of tubing exposed to the fluid was not strictly controlled and small variations in the volume of fluid could also possibly occur when samples were withdrawn at intervals. Hence minor differences in the dose contained in apparently identically obtained solutions could occur.

2. The material of tubing tested. It will be considered later that the different kinds of tubing showed different degrees of toxicity. Naturally if more toxic materials were incidentally tested in some hearts and not in others, this will influence the incidence of effect. Similarly if several kinds of materials were tested in a heart, there would be greater statistical chances of occurrence of toxic effect. The rate of release of toxic product may also vary with the material of tubing and could influence the occurrence of effect.

3. The number of times the tubing had been cleaned. It will be seen later that the process of cleaning reduced the toxicity of the tubing. Although all tubing had been cleaned repeatedly several times before test solutions were obtained from them the actual number of times a particular material had been cleaned could influence the occurrence of effect. It should also be realised that the pieces of tubing from which test solutions were

obtained were cleaned before each experiment, and hence the toxicity present in these pieces was liable to be reduced progressively in succeeding experiments.

4. Factors related to individual hearts. In this category may be included such factors as threshold dose and dynamic condition of hearts. Some variation in threshold dose could be expected theoretically from heart to heart, as has been found in the case of many substances considered in Part I. The sensitivity of hearts, however, was not an important factor in determining the occurrence of toxic effects from tubing. The dynamic condition of the heart could possibly play a role in determining the occurrence of effect in some cases. A fresh preparation might tolerate short exposures to feeble doses of a toxic product. Similarly a given degree of inhibition could appear as a greater percentage change in a relatively hypodynamic heart than in a normal heart. Of course, it was not at all necessary to control these subtle factors in this work, as the toxicity of all varieties of tubing could be easily demonstrated and suitable methods of comparison were also evolved to find out the best available material.

The chief methods of comparing the toxicity of different materials adopted in this work were as follows:

1. Comparison of incidence of toxic effects among the

hearts in which different kinds of tubing were tested irrespective of the duration of soaking.

2. Comparison of incidence of toxic effects after definite intervals of soaking.
3. Comparison of actions of test solutions obtained after identical duration of soaking from different materials, in the same heart.

Method 1 is apparently inaccurate and has been used only as supportive evidence, whereas methods 2 and 3 provide direct comparison and have been used as confirmatory evidences in all cases. The results will now be considered in the light of these remarks.

The summary of results given in Fig. 85 shows that some kinds of tubing have been investigated more thoroughly than others. The manufacture of 'Portex Crystal Vinyl' P.V.C. was discontinued in the middle of the work, hence there was no point in investigating it on a larger number of hearts. Similarly 'Silicone-1956' has been replaced by newer materials e.g. 'Silicone TC-156'. Moreover, initial experiments suggested that 'Silicone DSR-551' was probably a better material, hence particular attention was paid to this silicone material. Among the P.V.C. materials particular attention was paid to 'X-lon' and 'Waterclear' tubing, as these materials were newer and transparent.

Although the duration of soaking varied from experiment to experiment, the results from the hearts of first 14 frogs suggest

that 'Portex Standard' P.V.C. was probably the worst of the four kinds of tubing tested in these hearts, because of the high frequency of occurrence of toxic effects, and also because of the frequent occurrence of large effects (grade II and grade III). Similarly the experiments on the hearts of frogs 15 to 35 suggest that 'Silicone DSR-551' was probably a better material among the three kinds of tubing investigated in these hearts. However, because the test solutions of different duration of soaking were tested in different hearts, a direct comparison of the effects of different tubing tested in all the experiments is not possible. In some hearts the test solutions from two or more kinds of tubing, obtained after an identical duration of soaking,were tested. These experiments (Table 17) could be used for direct comparison. The results from the heart of frog 1 indicate that 'Portex Standard' P.V.C. was worse than 'Portex Crystal Vinyl 'P.V.C., 'Silicone-1956' and 'Silicone TC-156'. The results from the heart of frog 14 show that, among the last three, 'Silicone-1956' was worst. Figs. 73,86,87 and 88 are extracts from the record of this heart. As mentioned in reference to Fig. 73, a solution of 'Portex Crystal Vinyl' P.V.C., after 24 hours of soaking, produced a grade I effect. Test solutions, obtained after the same duration of soaking from two kinds of silicone tubing received from the same manufacturer, were also tested in the heart, as shown in Fig. 86. The test solution from 'Silicone TC-156'

produced no effect, but a prolonged small effect (classified as grade II) was obtained from the test solution of 'Silicone-1956'. Samples from the same test solutions obtained after 36 hours of soaking were again tested in this heart. The test solution from 'Silicone TC-156' now exhibited a grade I toxicity (Fig. 87) but the test solution from 'Silicone-1956' showed no evidence of increase in the toxicity, still showing a grade II effect (Fig 88). There was also no increase in the toxicity of the test solution from 'Portex Crystal Vinyl' P.V.C. (Fig. 85). These results showed that 'Silicone TC-156' was slightly better than 'Portex Crystal Vinyl' P.V.C. and 'Silicone-1956'. 'Silicone TC-156' is a newer product than 'Silicone-1956' and was better than the old product in two respects: (i) the toxicity of 'Silicone TC-156' was evident after a longer duration of soaking, and (ii) 'Silicone TC-156' produced a lower grade effect. Table 17 also shows that 'X-lon' P.V.C., 'Waterclear' P.V.C. and 'Silicone DSR-551' all produced grade III effects in the heart of frog 15. The records of these tests are illustrated in Figs. 78,80 and89. Large toxic effects from 'X-lon' P.V.C. and 'Silicone DSR-551' were also observed in the heart of frog 16. The results from the hearts of subsequent frogs (frogs numbers 18,19,20,21 and 23), however, show that the high degree of toxicity exhibited by 'Silicone DSR-551', 'Waterclear' P.V.C. and 'X-lon' P.V.C. in the heart of frogs 15 and 16 was eliminated completely from 'Silicone DSR-551' and to a great extent from 'Waterclear' but very little

from the 'X-lon' P.V.C. tubing, as the tubing was subjected to further cleaning before each experiment. The high frequency with which 'X-lon' P.V.C. produced toxic effects, indicates that it was the worst material among these three kinds of tubings. 'Waterclear' and 'X-lon' P.V.C. were comparatively new products. Their toxic effect, therefore, was compared on some hearts. Figs 90 and 91 are illustrations from the record of the heart of frog 30. Fig. 90 shows that the test solution from 'Waterclear' P.V.C. cleaned several times, had no effect. Fig. 91 shows the effect of three test solutions from 'X-lon' P.V.C., obtained by soaking pieces (i) which had been cleaned several times, (ii) which had been cleaned once only, and (iii) which had not been cleaned at all. Each test solution (including that from 'Waterclear' P.V.C.) was obtained after 28 hours of soaking. A graded effect of the three test solutions from 'X-lon' P.V.C. was observed, indicating that the toxicity of 'X-lon' P.V.C. was reduced as a result of cleaning. Figs. 90 and 91 also illustrate that 'Waterclear' P.V.C. was better than 'X-lon' P.V.C.

The incidence of effect and the incidence of different grades of effect obtained with test solutions from different kinds of tubing soaked for 8 to 24 hours, are given in Table 18. The occurrence of toxic effect within this duration of soaking was not common. Tests for toxicity, therefore, were conducted mostly after 24 hours of soaking. Due to this reason, the number of hearts in which each kind of tubing was tested is small in

Table 18. Nevertheless, in comparative terms the occurrence of toxic effect within this duration from a material was an indication of the quick rate with which the toxic product was released into the surrounding fluid and could be regarded as an important point for comparing the toxicity of different materials. 'Portex Crystal Vinyl', 'Portex Standard', 'X-lon' P.V.C. and 'Silicone-1956' showed toxic effects within this duration and should, therefore, be regarded as unsuitable products. Among the P.V.C. materials 'Waterclear' was best and 'Portex Standard' P.V.C. was worst. Among the Silicones, 'Silicone-1956' was worst, showing a grade II effect within this period of soaking.

The incidence of effect after soaking the pieces of different kinds of tubing for 8 to 36 hours is given in Table 19. It is seen that the number of kinds of tubing showing toxic effect now increased, all materials showing toxic effect. Up to the duration of 36 hours of soaking 'Waterclear' was the best product among both P.V.C. and silicone materials, because of the low incidence of toxicity. 'Silicone DSR-551' was the next best material. The other kinds of tubing showed a very high incidence of toxic effects within 36 hours of soaking.

Table 20 gives the incidence of toxicity from different kinds of tubing after soaking for 8 to 72 hours. It was mentioned in the beginning that the results of experiments in which the test solutions obtained by 'boiling of tubing' method were used, have been kept separate. The 'boiling of tubing' method was considered

a rather severe test procedure. Since soaking for 72 hours was also a fairly severe procedure, the results of both the procedures have been considered together in Table 20. Only the results of two experiments in which test solutions were obtained by boiling pieces of 'Portex Crystal Vinyl'P.V.C. have been included. Other tests in which 'boiling method' was used have been ignored because the adjacent controls were not considered satisfactory or because the record was not stable. It is clear that the inclusion of these tests has not in any way influenced the general pattern of results. Table 20 shows that, although the percentage incidence of effect was high with 'Portex Crystal Vinyl'P.V.C. and 'Silicone TC-156', these materials never produced large toxic effects. Bothwere tested in the hearts of the same 4 frogs, in 2 of which neither gave any effect, while in the other 2 both gave a grade I effect. In these hearts the test solutions in which the tubing had been s-oaked for 24 to 72 hours were tested. Hence these materials exhibited only a low grade toxicity even after prolonged soaking. However, the high incidence of toxicity would suggest that the toxic substance from them is released quite quickly into the fluid and therefore was present in effective amounts in a large number of test solutions. The percentage incidence of effect with 'Waterclear'P.V.C. and 'Silicone DSR-551' was low, being lowest with 'Silicone DSR-551'. The percentage incidence of effect with other tubing i.e. 'Portex

Standard' P.V.C., 'X-lon' P.V.C. and 'Silicone -1956' was very high. 'Portex Standard ' P.V.C. proved to be the worst tubing, because of the high percentage incidence of effect and also because of the high incidence of grade II and III effects.

The total number of hearts in which 'Portex Crystal Vinyl', 'Silicone-1956' and 'Silicone TC-156' were tested is small. The number is further decreased when the results are sub-divided according to the duration of soaking as already seen in Table 18 and 19. The conversion of these small numbers of affected hearts into percentage may not be justified statistically. However, this seems to be a satisfactory way of comparing the toxicity of test solutions obtained after different durations of soaking. Such a comparison of percentage incidence of effects and the percentage incidence of various grades of effects after different durations of soaking, is given in Table 21. Whereas the percentage values may not indicate the absolute incidence of effect, there is a definite indication that the percentage incidence of effect increased with the duration of soaking, except in the case of 'Portex Crystal Vinyl' and 'Silicone-1956' tubing. However, the test solution from each of these two kinds of tubing, after 24 hours of soaking, was tested in only one heart. The combined incidence of grades II and III effects also roughly correlates with the duration of soaking. The data of Table 18,19,20 and 21 clearly show that as the duration of

soaking increased, the incidence of toxic effect, the degree of toxic effect and the number of kinds of materials showing toxic effect increased progressively, suggesting that the occurrence of these effects was directly related to the quantity of the toxic product released into the test solution.

The results so far considered indicate that 'Waterclear' was the best material among the P.V.C. tubing, and 'Silicone DSR-551' was the best material among silicone tubing. Further, between 'Waterclear' P.V.C. and 'Silicone DSR-551' the latter was slightly better, because of the lower incidence of toxic effect. However, it should be realised at this point that whenever an effect with 'Silicone DSR-551' was observed, it was always a grade III effect. The effect of this tubing in the hearts of frogs 15and 16 was related to the first recent supply of the material, and the effect in the heart of frog 33 was related to the second recent supply of the material. Although before being tested in each of these three hearts the tubing had been cleaned 3 to 4 times, these cleanings were not enough to remove the toxicity; but further cleanings completely removed the toxicity, so that no effect was observed in subsequent experiments. Thus it is essential that 'Silicone DSR-551' must be cleaned 6 to 8 times before the toxicity, present in the freshly supplied material, is completely eliminated. But the very fact that the toxicity from this material can be eliminated after 6 to 8 cleanings, places this tubing in a far better position in comparison to other kinds of tubing from which it is not possible to eliminate the toxicity completely,

even by several additional cleanings. Table 22 shows the effect of cleaning on the toxicity of three kinds of tubing, two of which have already been shown to be better, because of the low incidence of toxic effects. Three types of test solutions were obtained from each kind of tubing, by soaking the pieces (i) which had been cleaned several times, (ii) which had been cleaned once only and (iii) which had not been cleaned at all. Test solutions of 'X-lon' P.V.C. obtained by soaking pieces which had not been cleaned at all, or cleaned only once, affected all the 8 hearts, whereas'X-lon' cleaned several times affected 4 hearts. Thus repeated cleaning reduced the incidence of effect from 100% to 50%. The incidence of grade II and grade III effects was also reduced. 'Waterclear' was tested in 6 hearts of which 2 were affected, one by the test solution from pieces which had not been cleaned at all and the other by the test solution from pieces which had been cleaned only once. The effect was abolished in 1 of the 2 hearts by repeated cleaning. In those hearts where both were tested, 'X-lon' P.V.C. was worse than 'Waterclear' P.V.C. even after repeated cleanings. 'Silicone DSR-551' was tested on 2 hearts both of which showed no effect with any of the three types of test solutions. It has already been mentioned that the toxicity of 'Silicone DSR-551' was completely eliminated by cleaning the tubing 6 to 8 times. 'Waterclear' tubing, however, continued to

produce grade I effects intermittently, even after many cleanings.

In the case of 'X-lon' P.V.C. the tubing improved with cleaning in two respects (i) the incidence of toxic effects decreased, (ii) the incidence of large effects also decreased. Figs. 92,93 and 94 show graded effects from the three types of test solutions from 'X-lon' P.V.C., indicating a reduction in toxicity as a result of cleaning. However, 'X-lon' Material continued to produce both small and large effects even after many cleanings.

There was no difference between the incidence of effect obtained with the test solutions from a particular kind of tubing, when the two different methods of cleaning were used (Fig. 95). Since the duration of soaking of tubing cleaned by the two methods varied from experiment to experiment, the results from all the hearts were not strictly comparable. However, in a few cases the duration of soaking of tubing cleaned by the two methods was approximately equal. These experiments are summarised in Table 23. It is seen that there was no appreciable difference between the results obtained by the two methods of cleaning. Slight differences recorded in Table 23 can be accounted for by the slight difference in the duration of soaking. Also it should be realised that at times there was a very small difference between the grade I effect and a doubtful

effect. It was mentioned in the beginning of the section on Results that all doubtful effects were regarded as no effect and were neglected in the analysis of the results, but it seemed useful to retain them in this Table, so as to indicate the nature of difference involved.

DISCUSSION.

From the review of the literature it is clear that the inert nature of some kinds of rubber and plastic materials has been questioned from time to time. Previous work on the toxicity of these materials indicates a considerable concern both on the part of surgeons and of physicians. In clinical work these materials are used for several purposes e.g. as connecting tubing in transfusion-giving sets, in heart-lung machines, as containers for storage of blood, as catheters and as sutures in surgical operations. While the toxicity of natural rubber has been well recognised, at least in clinical work, the potential toxicity of silicone rubbers and plastic tubing, which have almost replaced natural rubber for many purposes, has not been appreciated.

It is rather surprising that the toxicity of these materials has been a concern more to clinicians than to laboratory workers who use them more frequently. Some form of detoxicating mechanism is likely to be operative in intact animals and in human beings and the effects, therefore, may not be manifested fully. The toxicity may not matter in acute pharmacological experiments for the demonstration of gross effects with high concentrations of drugs, but isolated biological preparations, which receive nutrition from a medium in contact with these materials, are likely to be dangerously influenced. This is of considerable importance to physiologists, pharmacologists, biochemists and cytologists as well

as to others. Where substances in low concentration are to be studied using sensitive methods of recording the toxicity of flexible connecting tubing, manufactured from these plastic materials, becomes very important. Besides producing a toxic effect on the isolated preparation and causing interference with the tests, the material of tubing may inactivate some delicate pharmacological drugs like penicillin (Cowan, 1945) and possibly other substances.

In the present work several kinds of plastic and silicone tubing have been investigated. The results clearly indicate that all types of tubing tested were potentially toxic unless they were subjected to repeated cleaning by a lengthy and elaborate process. The toxicity from different kinds of materials was detected after a variable interval of soaking, the incidence of toxicity increasing with the duration of soaking. Although the toxicity was commonly evident after 24 hours of soaking, it should not be inferred that the effects due to toxicity can be ignored in short-term experiments. The occurrence of toxic effects depends primarily on the concentration and dose of the toxic substance released into the fluid. The ratio of the total surface area of pieces of tubing to the volume of fluid in which the pieces were soaked to obtain test solutions, is of great importance. In actual experimentalwork a small volume of fluid is usually in contact with a large internal surface area of tubing

and the stagnant fluid is liable to contain effective quantities of toxic substance within a short time. The ability to record toxic effects no doubt depends on the sensitiveness of the recording equipment and to some extent on the sensitivity of the preparation. This is the explanation of the interference experienced in the initial stages of this work due to frequent occurrence of unsatisfactory controls. Even after repeated cleaning some kind of tubing may still exhibit a variable degree of toxicity depending on the material, duration of soaking and also possibly on the sensitivity of the frog heart.

Because of the occurrence of toxicity in practically all kinds of tubing the aim has been to select the best available. Four types of tubing manufactured from P.V.C. (polyvinyl chloride) were investigated. 'Waterclear' P.V.C. and 'Portex Crystal Vinyl' P.V.C. were found to be superior to 'Portex Standard' P.V.C. and 'X-lon' P.V.C. The manufacture of 'Portex Crystal Vinyl' P.V.C. has been discontinued and 'Portex Standard' P.V.C. tubing is being manufactured instead. The results indicate that the new material is much inferior to the old one. Extensive comparison of 'Waterclear' P.V.C. and 'X-lon' P.V.C. tubing indicated that the 'Waterclear' was better than 'X-lon', but 'Waterclear' continued to show intermittently a low grade toxicity even after it had been cleaned more than 10 times.

Among the three types of silicone rubber tubing investigated, 'Silicone DSR-551' was best. Although it gave a large effect on three

occasions when the tubing had been received from the manufacturer shortly before being tested, it never produced any effect after having been cleaned about 6 to 8 times. The other two silicone rubbers, i.e. 'Silicone-1956' and 'Silicone TC-156' were fairly toxic. 'Silicone TC-156' is a newer product and is better than 'Silicone-1956'. MacDougall (1953) and Cruickshank et al. (1960) investigated several silicone rubbers for their toxicity on the growth of bacteria and on cell growth in tissue culture and found them to be non-toxic. The latter workers also found that the toxicity of some kinds of P.V.C. was very great, being almost equal to that of natural rubber.

'Silicone DSR-551' tubing, after 6 to 8 cleanings, was superior to all other tubing tested in the present work. The P.V.C. materials are plasticized compounds obtained by adding several substances as 'stabilisers' and 'plasticizers'. It is quite possible that the greater toxicity of the P.V.C. tubing is due to these added compounds which may contain reactive chemical groupings which are not present in a pure plastic like nylon. These compounds seem to be washed out into the fluid in contact with the P.V.C. material. The silicones are synthetic organic compounds containing the element silicon. The release of the silicon compound or other active chemical grouping may be the source of toxicity.

Since the potential toxicity of many types of tubing has now been realised, a suitable method to compare the toxicity of

different materials on biological preparations would be very useful.

Implantation of samples of tubing in intact animals causes local trauma and the results are liable to be equivocal.

The inhibition of growth of bacteria has been used to detect toxicity of rubber and plastic materials. A test for rubber in transfusion equipment is based on the effect of the rubber upon the growth of Str. pyogenes. This is the test prescribed by British Standards 2463:1954.

Toxicity of materials has also been assessed by several workers on the growth of cells in tissue culture. The inhibition of the growth of bacteria and of the growth of cells in tissue culture along with determination of O_2 consumption by the cultured cells have all been used by Cruickshank et al. (1960) for this purpose. These workers observed no inhibition of bacterial growth by some materials yet their toxicity could be demonstrated by the other methods used. They noted that a definite inhibition of bacterial growth was a clear indication of toxicity, but that absence of inhibition of bacterial growth did not exclude toxicity in the material tested.

The toxicity of several types of silicone rubbers and P.V.C. tubing could be detected and compared by perfusing the frog hearts with test solutions obtained by soaking pieces of tubing in Ringer's solution. In fact the silicone rubbers said to be without effect on bacterial growth and on growth of cells in tissue culture, have been shown to be quite toxic to the frog heart.

Although the usual 'India rubber tubing' of the laboratory has not been systematically tested during the present work, a few trials on the perfused hearts of the frog showed it to be very toxic. Perfusion of the frog heart, therefore, can be applied as a test for toxicity in all kinds of materials and seems to be more delicate than other tests.

The ability to remove the toxicity partly or completely by repeated cleaning by any of the two elaborate methods (i.e. Calgon and Bicarbonate methods described in detail in the section on Methods in Part I), emphasizes the need to subject all kinds of tubing to repeated cleaning and sterilization at least 10 to 12 times before using the tubing in accurate work. Previous workers used various extraction procedures with hot and cold solvents (Parker, Morgan and Morton, 1951) and boiling (Cruickshank et al., 1960) to reduce the toxicity of rubber and plastic materials but without much success. The successful reduction or elimination of toxicity with the present technique of cleaning is probably due to the elaborate procedure followed and also due to the large number of times the tubing was cleaned, the cleaning and sterilization of tubing being a routine procedure before each experiment. It should be emphasized here that besides the cleaning solutions used, the technique of the cleaning procedure in reducing toxicity depends on a thorough internal flushing of the tubing. The solutions used in the different stages of cleaning must be passed through the tubing several

times and the tubing should be filled with the cleaning solutions and allowed to remain in the cleaning tanks for the specified length of time. Simple rinsing or soaking of tubing in the cleaning solutions is not enough to wash out toxic products. The present work indicates that repeated cleaning in this manner about 10 to 12 times, reduced the toxicity of all kinds of tubing. Whether the toxicity can be completely eliminated or can only be reduced by repeated cleaning, probably depends on the material. It seems clear that tubing made from plastic and silicone materials is, on the whole, superior to tubing made from natural rubber. Among the plastic and silicone materials tested 'Silicone DSR-551' is better than the others, provided that it is cleaned at least 6 to 8 times or preferably 10 to 12 times, before use. 'Waterclear' P.V.C. tubing is the next best choice. It must, however, be cleaned at least 12 times and it should be realised that 'Waterclear' P.V.C. can still occasionally produce low grade toxic effects. These require to be identified and allowed for in any accurate work.

The physical properties of the tubing may also influence selection. 'Silicone DSR-551' is not completely transparent and air bubbles cannot be seen clearly in this tubing. Its use, therefore, is limited to procedures where a visual control is not necessary. 'Waterclear' P.V.C. is completely transparent and air bubbles do not stick to the sides. On the other hand, silicone tubing is flexible and compressible and provides tight

connections whereas P.V.C. tubing, in general, does not possess these properties. In this work 'Waterclear' P.V.C. tubing was selected for standpipe-connecting-tubes because of the following considerations:

1. It was completely transparent and therefore useful for visual control. In the perfusion technique used in this work it was imperative to be able to see and to avoid air bubbles.
2. The type and degree of effect produced due to residual toxicity after repeated cleaning were well known and could be easily detected by the methods of control.
3. Since the incidence of effectwas considerably reduced after repeated cleaning, there was no interference at all in many experiments.

Although it was desirable to use the same material for all connections for the sake of uniformity, 'Waterclear' P.V.C. tubing could not be used for transducer-connecting-tubes because 'Waterclear' P.V.C, tubing did not provide tight connections and was not fully compressible. 'Silicone DSR-551' was ideally suitable for this purpose and was, therefore, selected for transducer-connecting-tubes.

It may be mentioned here that experience indicates that it is very useful to flush the solution which has been in contact

with any kind of tubing, each time before a critical test as an extra precaution. It has already been seen in the records of present work that this precaution was followed as a routine before each control and test perfusion.

SUMMARY.

1. Toxicity of modern connecting tubing made from plastic and silicone materials has been investigated. The presumed toxic product of tubing was incorporated in the Ringer's solution by two methods; (i) by boiling small pieces of the tubing in water and then using the water to prepare a sample of Ringer's solution (ii) by soaking small pieces of tubing directly in Ringer's solution for known intervals of time. Control solutions to match the test solutions exactly, were also prepared by both methods. The test solutions were used for perfusing the isolated frog hearts and the effects due to toxicity from the different kinds of tubing were investigated on 35 hearts. The effects due to toxicity of tubing were 'bracketed' between control perfusions with control solutions in each case.

2. Four types of P.V.C. (polyvinylchloride) and three types of silicones were investigated. No material was found to be inert. All tubing exhibited toxicity.

3. The incidence of toxicity was lowest from 'Silicone DSR-551'. This tubing also could be rendered completely non-toxic by repeated cleaning. It was considered the best material after 6 to 8 cleanings. 'Waterclear' P.V.C. tubing was the next best material. The incidence of toxicity from this tubing was slightly greater than that from 'Silicone DSR-551' but was considerably less than that of other materials. The

toxicity of 'Waterclear' P.V.C. tubing could be further reduced by repeated cleaning but in spite of all endeavours it continued to show occasional low grade toxic effects. This tubing must be cleaned 12 times before use and the occasional effects due to residual low grade toxicity must be well recognised.

4. Besides the consideration of incidence of toxicity and degree of toxicity, the physical properties of the tubing are also important in influencing selection. If it is not essential to have transparent tubing, 'Silicone DSR-551' is best after 6 to 8 cleanings. If transparency is essential, 'Waterclear' P.V.C. tubing can be used provided it is cleaned 12 times before use and its occasional low grade toxic effects recognised. Silicone tubing is compressible and provides a tight connection while P.V.C. tubing generally lacks these properties.

5. Details of Calgon and Bicarbonate methods for cleaning the tubing developed in the course of the present work have been described and practical suggestions have been made for cleaning the tubing to reduce toxicity. Both methods of cleaning were equally effective in this respect. Whether the toxicity can be partially reduced or can be completely eliminated depends on the material of tubing.

6. The modern tubing made from P.V.C. and Silicone materials are superior to the tubing made from natural rubber. On the

whole, tubing made from Silicone is better than tubing made from P.V.C.

7. The technique of detecting and comparing the toxicity of tubing, which has been developed in the course of the present work, seems to be more delicate than the methods used previously by other workers. The present approach is superior to the methods using inhibition of bacterial growth or of tissue culture as an index of toxicity.

8. The importance, in experimental work, of recognising and reducing the effects of toxicity in rubber, plastic and silicone materials, is discussed.

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SUMMARY

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SUMMARY OF THESIS

for

the degree of Ph.D.

UNIVERSITY OF GLASGOW

1962

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The thesis is divided into two parts dealing with separate topics:

Part I - The Sensitivity of Frog Hearts to Acetylcholine and Other Neurohumoral Transmitter and Allied Substances.

Part II - The Toxicity of Modern Plastic and Silicone Connecting Tubing, Tested on Frog Hearts.

The aim of the present work was to investigate the threshold sensitivity (minimum effective concentrations) of frog hearts to some well documented substances of physiological importance under critically controlled experimental conditions. Acetylcholine, adrenaline, noradrenaline, 5-hydroxytryptamine and nicotine were selected for this purpose.

Very sensitive electronic equipment specially designed and adapted to suit the frog heart was developed over a period of several years. The equipment permits a well controlled perfusion of frog heart and records the perfusion pressure (venous pressure) heart rate, arterial pressures (systolic, diastolic and pulse pressure) and cardiac output simultaneously and continuously. The electronic part of the equipment essentially consists of a specially designed 4 channel direct ink writing recorder. Photographs of different parts of equipment and operational details are given in the section on Methods.

The perfusion of frog heart was conducted by introducing a cannula into the inferior vena cava without opening the pericardium. The perfusate coming out of the two aortic cannulae passed through an 'arterial system' consisting of elastic connecting tubing and artificial resistance, and finally escaped out in the form of drops which were counted and integrated electronically. Since the constancy of venous pressure was a crucial point in providing a stable record and also in preventing the occurrence of undesirable changes in the different recorded parameters during a critical test perfusion, special care was exercised in designing and developing the venous pressure recorder. The venous pressure recorder and the carriages for the different sources of perfusion have been so developed that the venous pressure can be kept constant at any desired value throughout the period of experiment lasting from 1 to 3 days. At the same time unavoidable minor changes in the venous pressure (of course quite insufficient to influence the stability of record) of the order of 0.05 cm of even less, were recorded and displayed very prominently. A venous pressure monitoring meter constantly guided the operator.

Special methods of precaution and control were introduced at several stages of the experiment so that the effect of test solutions were well controlled and it was clearly shown on the record that there could be no possibility of unrecognised changes in the record due to artefacts - electronic or otherwise.

The distilled water used for preparing solutions, the ionic composition of fluids, the type of glass containers, the connecting tubing, the minor variations in temperature from place to place in the laboratory (and consequently of solutions standing at different places), minor variations in the pH of the solutions and bacterial, chemical or organic contamination of glassware and tubing, all were liable to influence the behaviour of the preparation. With conventional crude methods the influence of such factors would never be detected. Most of these variables were easily controlled once their importance was realised, though a considerable time was devoted to analysing and controlling them.

The choice of a suitable type of connecting tubing of a really nontoxic material was found to be a difficult problem, and a special study had to be undertaken to find out the best available material. The toxicity of modern flexible connecting tubing made of various synthetic products (polyvinylchloride and silicone) was investigated using the frog heart as the test object. The presumed toxic products of tubing were incorporated in the Ringer's solution by two different methods: (i) by boiling of small pieces of the tubing in distilled water and then using the water to prepare a sample of Ringer's solution, (ii) by soaking pieces of tubing directly in the Ringer's solution. The samples of Ringer's solution containing the presumed toxic products were called the 'test solutions'. The influence

of test solutions from different kinds of tubing was investigated by perfusing the test solutions in frog hearts. The results of this side study are considered in Part II.

Special care was exercised in the cleaning and sterilization of all glassware and other apparatus which came in contact with the solutions. Clean sterile glassware was used for the preparation, storage and use of the solutions. All test solutions were prepared in Ringer's solution and were administered by perfusion.

Since the sensitivity of frog hearts to acetylcholine seemed to vary from time to time, experiments were carried out over a period of several years to determine the exact period of incidence of high sensitivity. At one stage of the work it appeared that the sensitivity of hearts from female frogs was possibly greater than that of the hearts of male frogs. Some frogs (of either sex) were, therefore, injected with female sex hormones and the hearts of injected frogs were tested for sensitivity to acetylcholine. The influence of the changes in the Ringer's solution on the sensitivity of hearts to acetylcholine was also investigated in some pilot experiments. A considerable time was devoted to conducting these different types of experiments on acetylcholine. Moreover the analysis and control of several variables and the development of control procedures and standardisation of technique all required considerable time. The other substances, therefore, have not been investigated over

as long a period as that devoted to acetylcholine. However, experiments were designed to test the sensitivity of frog hearts to other substances both in the uninjected and injected frogs.

The difficulties encountered in the experimental procedure and in obtaining a stable record and satisfactory controls have been fully described in the section on Methods where comments on the protocol of experiments and an introduction of record is also included.

The hydrodynamics of the preparation was studied in detail to obtain information regarding the influence of experimental variables like the venous pressure and artificial resistance on the different parameters of cardiac activity. The venous pressure and arterial resistance both influenced the inotropic and chronotropic response leading to appropriate changes in the associated parameters of cardiac activity such as cardiac output and blood pressure.

Results and Conclusions.

Part I:

Acetylcholine:

1. The threshold sensitivity of frog hearts to

acetylcholine varied from 10^{-21} to 5×10^{-9} g/ml. The concentration needed to stop the hearts (stoppage concentration) ranged from 10^{-15} to 5×10^{-7} g/ml.

2. Acetylcholine in a concentration of 10^{-21} g/ml contains only 3.3 molecules/ml and since not more than 4 ml of the solution could reach the heart in a 2 minute test perfusion, only about 13 molecules of acetylcholine were involved when this concentration was effective. This implies that biologically acetylcholine is active in any molecular concentration. The effectiveness of acetylcholine in such a small molecular dose has never been demonstrated previously. The physiological significance of this observation in relation to the importance of acetylcholine in the nervous regulation of cardiac activity in particular and the process of neurohumoral transmission in general, has been discussed.

3. There has always been a gap between theory and practice regarding the effectiveness of small quantities of acetylcholine in biological systems. The knowledge about various actions of acetylcholine is largely based on experimental observations using rather high concentrations of

acetylcholine and it has been a common belief that endogenously produced acetylcholine is more effective than externally administered acetylcholine. This work clearly demonstrates the effectiveness of acetylcholine in all molecular concentrations and hence fills the gap which so far existed between theoretically expected and practically demonstrated effectiveness of acetylcholine. There is now no need to presume that endogenously produced acetylcholine is more effective than externally administered acetylcholine.

4. Contrary to the observations of previous workers and contrary to the common belief of physiologists and pharmacologists that acetylcholine hydrolyses fairly rapidly, it has been demonstrated that if the solutions of acetylcholine are prepared and stored using clean sterile glassware, the strength of the solutions containing concentrations up to 10^{-7} g/ml is maintained well up to 36 hours or more. Still more dilute solutions maintain their strength for 8 to 12 hours after which there is only a slow hydrolysis. The duration of maintenance of strength roughly depends on molecular

concentrations of acetylcholine.

5. The incidence of hearts showing high threshold sensitivity to acetylcholine varies significantly with season, being maximum in winter (January and February) and minimum in spring and summer.
6. The threshold sensitivity to acetylcholine does not undergo any remarkable change on altering the composition of Ringer's solution, on administration of adrenaline and after injections of female sex hormones in intact animals.
7. Frequent spontaneous changes in the threshold sensitivity to acetylcholine within 12 hours of perfusion were not common. When a change in sensitivity was observed within this period, the sensitivity usually decreased. Significant changes in the threshold sensitivity, however, occurred in a few instances after the heart had been on perfusion for more than 12 hours; the sensitivity decreased in some cases and increased in others.
8. A pure excitatory effect from acetylcholine was never observed at any concentration within a wider range of concentrations than has ever been systematically tested before. Occasionally a rebound increase in the heart

rate and/or amplitude of contraction was observed during the recovery from a powerful inhibitory action of acetylcholine. These changes never occurred during the actual exposure of the heart to acetylcholine but were always part of the recovery process. The action of acetylcholine was, therefore, always inhibitory and both the chronotropic and inotropic responses were involved at the minimum effective concentration, the action on the former usually being less pronounced.

9. Determination of the concentration-response relationship in hearts with a low sensitivity to acetylcholine led to the following conclusions;

(a) acetylcholine acts on three sites,

(i) pacemaker

(ii) conducting system

(iii) contractile response (cardiac muscle)

(b) Normally the action of acetylcholine at lower concentrations is more pronounced on the contractile response than on the pacemaker. The action at these two sites is rarely parallel but the chronotropic and inotropic responses are simultaneously involved at the minimum effective concentration.

(c) The typical form of concentration-response curve for the inhibition of mean arterial pressure and

cardiac output (indicating action on the contractile response) is 'S' shaped when the concentration of acetylcholine is plotted on a logarithmic scale. If a linear scale showing the actual weight of acetylcholine per ml. in each solution is used the curve has an upward convexity. Eserine potentiates the action of higher concentrations but has no effect or may actually antagonise the action of low concentrations of acetylcholine; the curves tend to adopt a more linear form.

(d) The typical form of concentration-response curve for heart rate (action on pacemaker) is curvilinear on the logarithmic as well as on the linear scale. Eserine has a marked potentiating effect on the action of all concentrations of acetylcholine.

(e) Acetylcholine induces conduction block in all hearts. The conduction block modifies the concentration-response curves. Eserine has a marked potentiating effect on the occurrence of conduction block i.e. it occurs at a much lower concentration in the presence of eserine.

(f) Stoppage of the heart by acetylcholine may be due to complete inhibition of muscle, or due to onset of complete conduction block, or both may occur coincidently.

10. The concentration-response relationship in highly sensitive hearts exhibits similar features with the addition that the curves for all parameters (i.e. output, mean pressure and heartrate) have a hump at lower concentrations, there being a peak on the hump at a certain concentration. When a heart was found to be sensitive to very low concentrations, principal attention was paid to establishing beyond question that the effect of the given concentration was genuine. More work is needed for investigating further details of the concentration-response relationship by testing concentrations prepared in small steps of dilution.

11. The curves representing the action of acetylcholine on the mean arterial pressure and cardiac output suggest involvement of the contractile response in two phases i.e. an initial rapid phase and a phase of reduced action. The curves for the action of acetylcholine on the pacemaker also suggest two phases which appear to be in the opposite sequence i.e. an initial slow phase and a rapid phase. The possible mechanism of action of acetylcholine has been discussed in the light of these interpretations.

12. The incidence of hearts sensitive to acetylcholine at concentrations less than 10^{-9} g/ml is only 14%. Because of this low incidence of highly sensitive

hearts and because of the occurrence of a rather complex pattern of response to progressively increasing concentrations of acetylcholine at very low concentrations, the frog heart is not a very suitable test object for the assay of acetylcholine in concentrations less than 10^{-9} g/ml.

Adrenaline and Noradrenaline:

1. The threshold sensitivity of frog hearts to both adrenaline and noradrenaline varied from 10^{-11} to 10^{-5} g/ml. The incidence of highly sensitive hearts (hearts sensitive to concentrations less than 10^{-7} g/ml) was 22% in the case of adrenaline and about 10% in the case of noradrenaline. The majority of hearts was influenced by adrenaline and noradrenaline in a concentration of 10^{-7} g/ml, though some hearts were only influenced at 10^{-5} g/ml.
2. Frog heart is a suitable preparation for assaying adrenaline and noradrenaline in concentrations greater than 10^{-9} g/ml.
3. Lower concentrations of adrenaline (between 10^{-11} and 10^{-7}) often produced inhibitory effects. The incidence of inhibitory effects decreased at high concentrations. It is possible that some decomposition product of adrenaline may be responsible for

attained. The increase in the blood pressure (determined by heart rate and amplitude of contraction only, the resistance being constant) due to the action of adrenaline was greater but was of a shorter duration than that due to the action of noradrenaline. The marked acceleration produced by adrenaline resulted in a greater reduction in the amplitude of contraction during the action of adrenaline than during the action of a corresponding strength of noradrenaline. On the whole, adrenaline produced a greater degree of stimulation.

5-Hydroxytryptamine:

1. The frog heart is less sensitive to 5-hydroxytryptamine than to adrenaline and noradrenaline, the threshold sensitivity to 5-hydroxytryptamine ranged from 10^{-9} to 10^{-3} g/ml. Whereas adrenaline and noradrenaline were always effective in a concentration of 10^{-5} g/ml, 5-hydroxytryptamine was ineffective in some hearts in this concentration. Only about 11% of the hearts were sensitive to 5-hydroxytryptamine in a concentration of 10^{-9} g/ml, the majority being sensitive to 10^{-7} or 10^{-5} g/ml. Thus frog heart is a suitable preparation for the assay of 5-hydroxytryptamine in concentrations

greater than 10^{-9} g/ml.

2. 5-hydroxytryptamine produces both excitatory and inhibitory effects in frog hearts. Although the incidence of hearts showing a pure inhibitory effect was only 11%, the pure inhibitory effects were observed at all concentrations. In the majority af hearts an excitatory effect influencing both the inotropic and chronotropic response was observed. The excitatory effect, however, influenced the chronotropic response more frequently and the increase in the rate was often associated with a decrease in the output and blood pressure. Frequently the excitatory and inhibitory components of action of 5-hydroxytryptamine were simultaneously operative at the same concentration in the same heart, the nett effect being a resultant of the two components.

Nicotine:

The frog heart is relatively insensitive to nicotine, no effect being observed till such high concentrations as 10^{-3} g/ml are reached. Nicotine produces an inhibitory effect on the heart rate, the action being quite reversible. Nicotine had no effect on the sensitivity of hearts to acetylcholine.

General Sensitivity of a Particular Heart:

The high sensitivity of a heart to one substance is

not associated with a high sensitivity to other substances.

PART II:

1. The toxic action of test solutions from 7 different kinds of modern flexible connecting tubing was investigated on 35 frog hearts, the test solutions being administered by perfusion. The general procedure of experiments and controls was the same as that described for other substances in Part I. Four types of P.V.C. (polyvinyl chloride) and three types of silicone materials were investigated.
2. No material was found to be inert. All tubing exhibited toxicity. The incidence of toxicity with 'Waterclear' P.V.C. and 'Silicone DSR-551' was lower than with other materials. The incidence of toxicity was lowest from 'Silicone DSR-551'. 'Silicone DSR-551' could be rendered completely non-toxic by repeated cleaning. The toxicity of 'Waterclear' P.V.C. was reduced considerably by repeated cleaning but in spite of all endeavours it continued to show occasional low grade toxic effects. If it is not essential to have transparent tubing, 'Silicone DSR-551' is best after 6 to 8 cleanings by 'Calgon' or 'Bicarbonate' method. If transparency is essential, 'Waterclear' P.V.C. can be used provided it is cleaned

10 to 12 times and its occasional low grade toxic effects recognised.

3. The Calgon and the Bicarbonate methods of cleaning are equally effective in reducing toxicity.

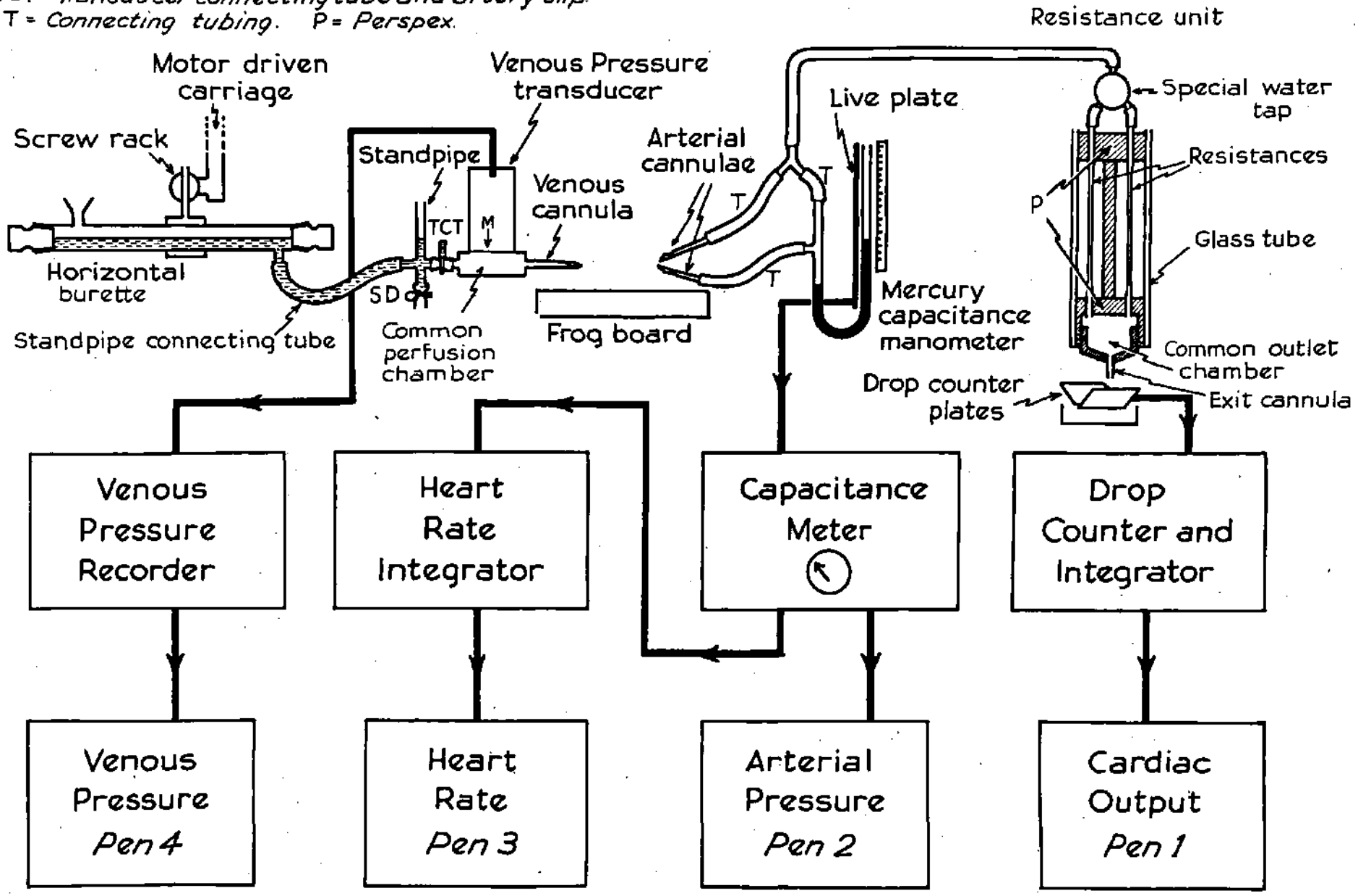
4. On the whole, tubing made from silicone is better than tubing made from P.V.C.

5. The technique of detecting and comparing the toxicity of tubing, which has been developed in the course of present work seems to be more delicate than the methods used previously by other workers. The present approach is superior to the methods using inhibition of bacterial growth or tissue culture as an index of toxicity.

6. The importance, in experimental work, of recognising and reducing the effects of toxicity in rubber and plastic materials is discussed.

Fig. 1 . Block diagram of the equipment.

SD = *Standpipe exit with a tube and clip.* M = *Rubber membrane*
TCT = *Transducer connecting tube and artery clip.*
T = *Connecting tubing.* P = *Perspex.*



Block Diagram of Equipment

Fig.

Fig. 2 Front view of the main operational parts of the equipment showing details of the perfusion system.

B = burettes held with clamps which are mounted on the electrically driven carriage.

G = green lights

Hg = mercury manometer.

M = monitoring meters. Only the meter on the extreme right monitoring the venous pressure, is in use at present.

m = electric motor which drives the common carriage on which the clamps for holding the burettes and Mariotte bottle are mounted. The motor is operated through a tapping key below the operating table. Movements of the carriage change the levels of all the burettes and the Mariotte bottle simultaneously.

P = press buttons for moving the markers.

R = reservoir.

r = resistance unit.

S = standpipes

T = operating table.

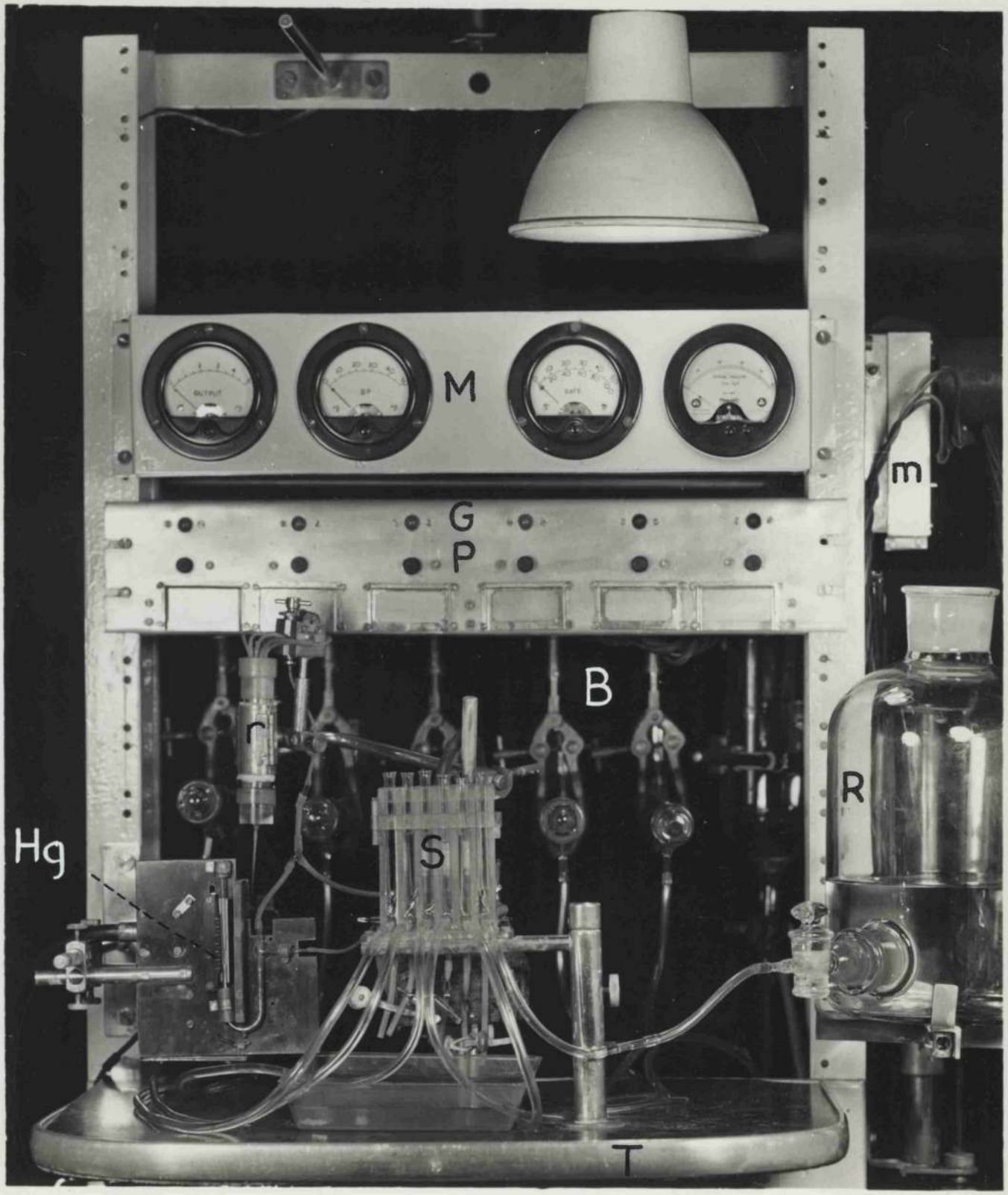


Fig. 2.

Fig. 3 Side view (seen from the left) of the main operational parts of the equipment showing the details of perfusion system.

Front portion (on the right side of the stand)

d = drop counting plates

F = frog board

r = resistance unit

t = transducer

Back portion (on the left side of the stand)

b = Mariotte bottle

B = burettes placed horizontally.

Sc = screws to adjust the relative position of the burettes.

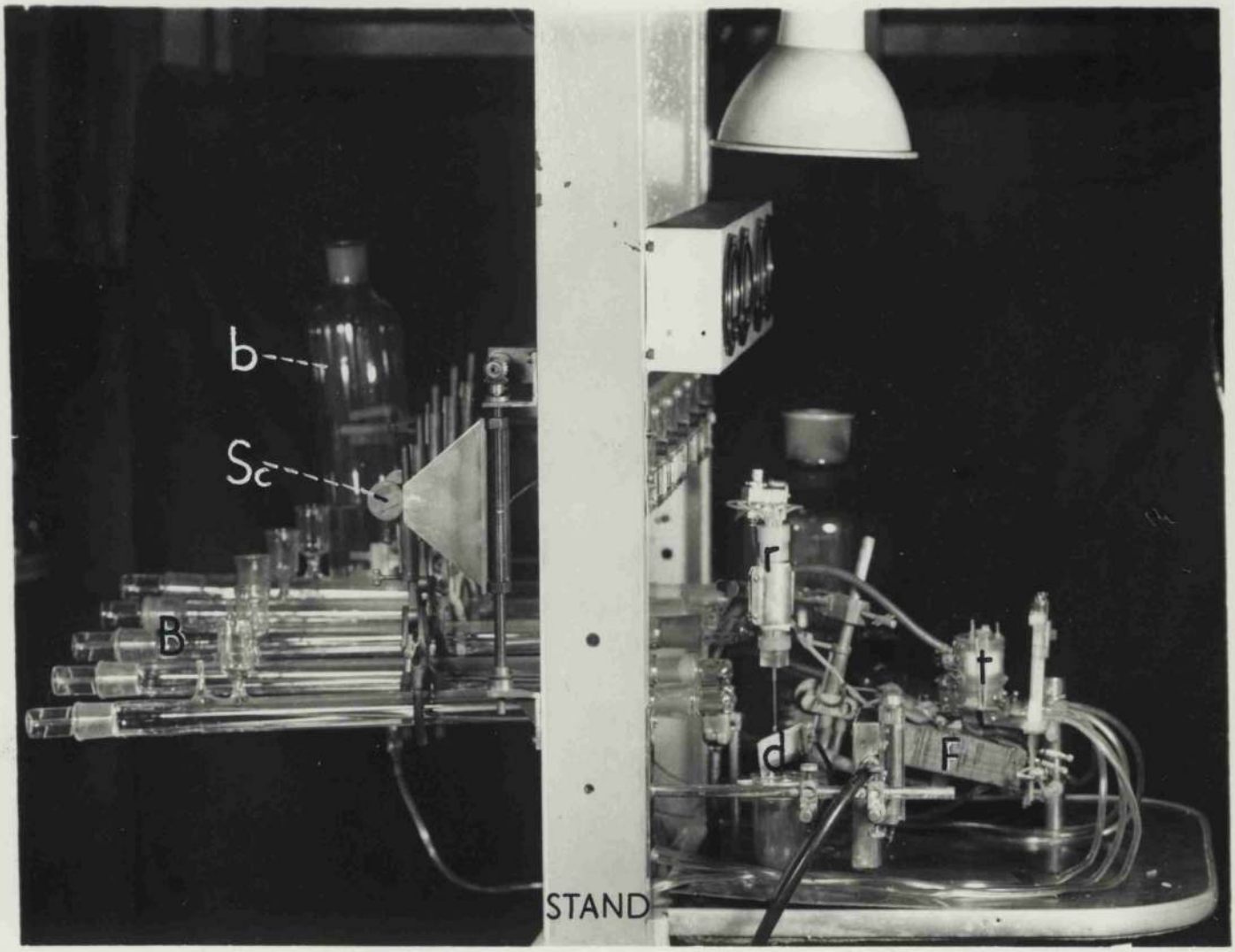


Fig. 3.

Fig. 4 Pen recorders and chart papers. The movement of the papers can be regulated at a slow or fast speed. The fast speed is used during the actual experiment. The slow speed is used to obtain general information about the activity of the heart during the night or during long breaks. The details of record are not clear when the slow speed is used. The marker moves a pen which records the commencement, duration and end of perfusion from the reservoir or from different burettes.

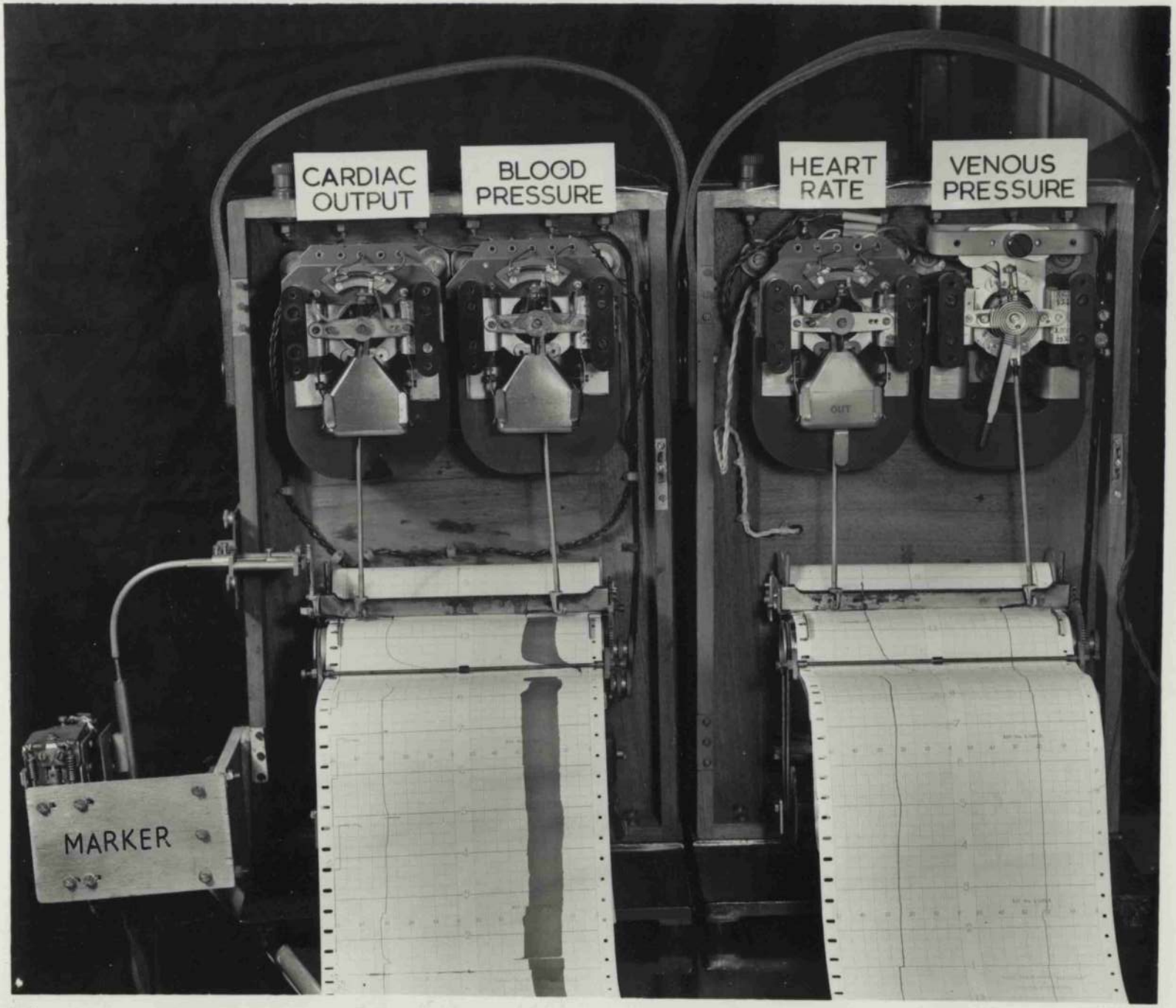

CARDIAC OUTPUT
BLOOD PRESSURE
HEART RATE
VENOUS PRESSURE
MARKER

Fig.4.

Fig. 5 Parts of the equipment associated with the pen recorders.

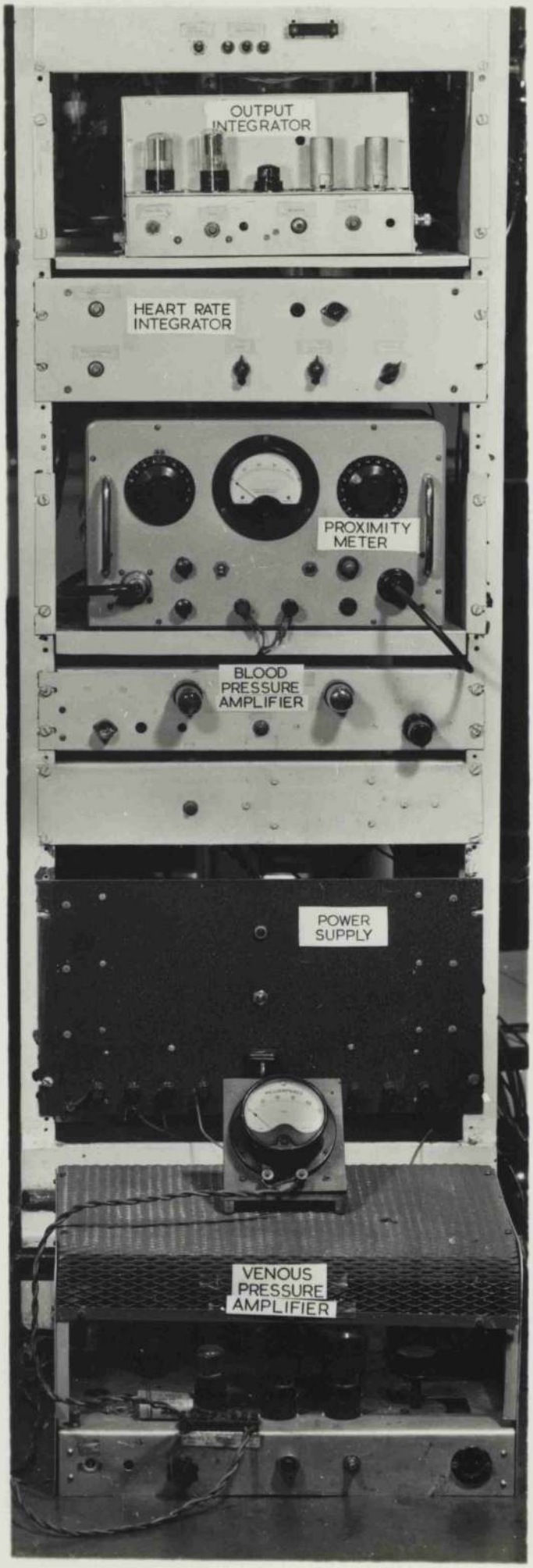

OUTPUT
INTEGRATOR
HEART RATE
INTEGRATOR
PROXIMITY
METER
BLOOD
PRESSURE
AMPLIFIER
POWER
SUPPLY
VENOUS
PRESSURE
AMPLIFIER

Fig. 5.

Fig. 6 A set of cleaning tanks. The glassware and tubing are seen in the different stages of cleaning procedure by 'Calgon Method'

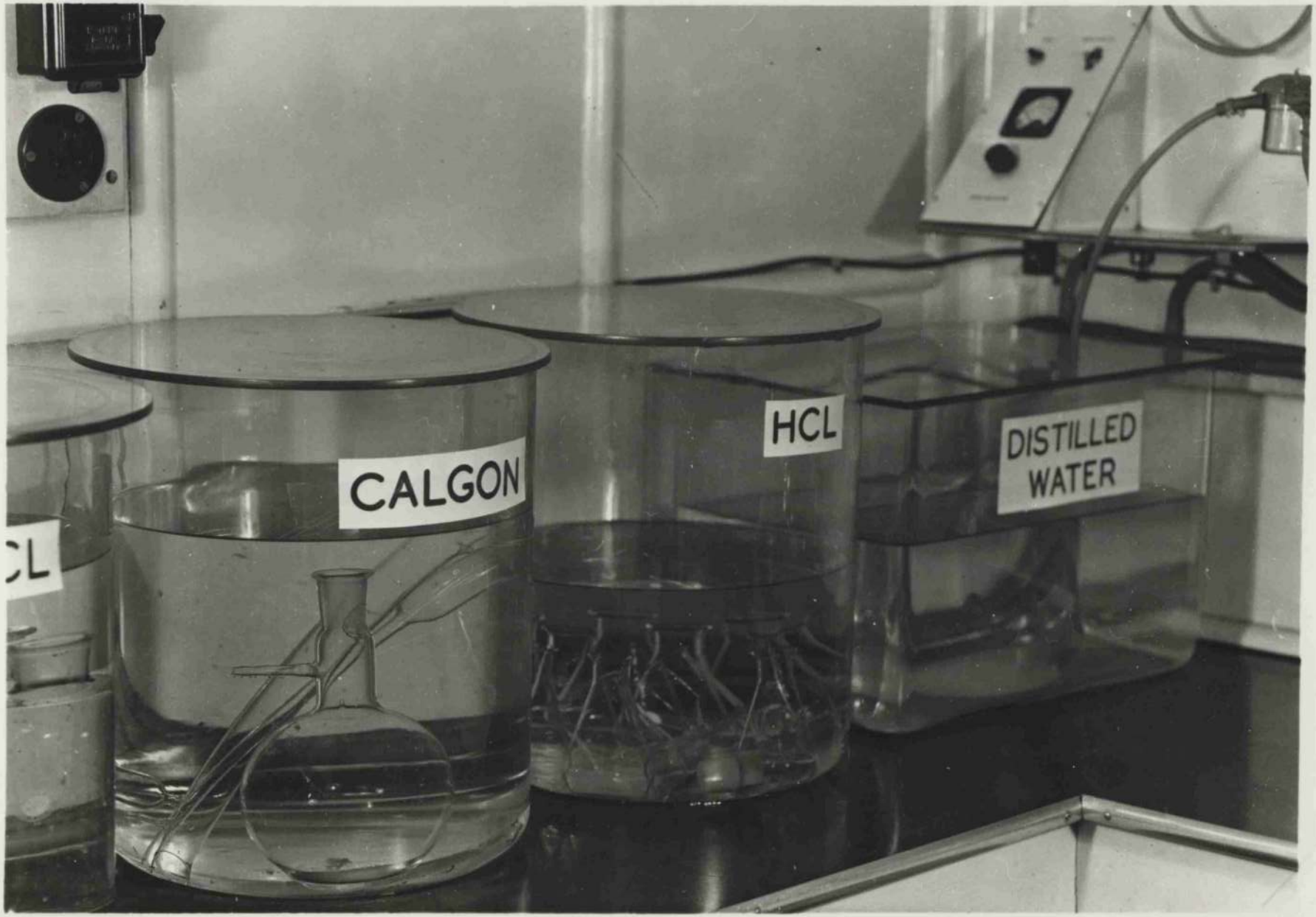


Fig. 6

Fig. 7 Distillation unit. The tank labelled 'Distilled Water' is a cleaning tank and not a storage tank. The distillation unit and cleaning tanks are in the same room, hence the cleaning tank has appeared in the photograph.

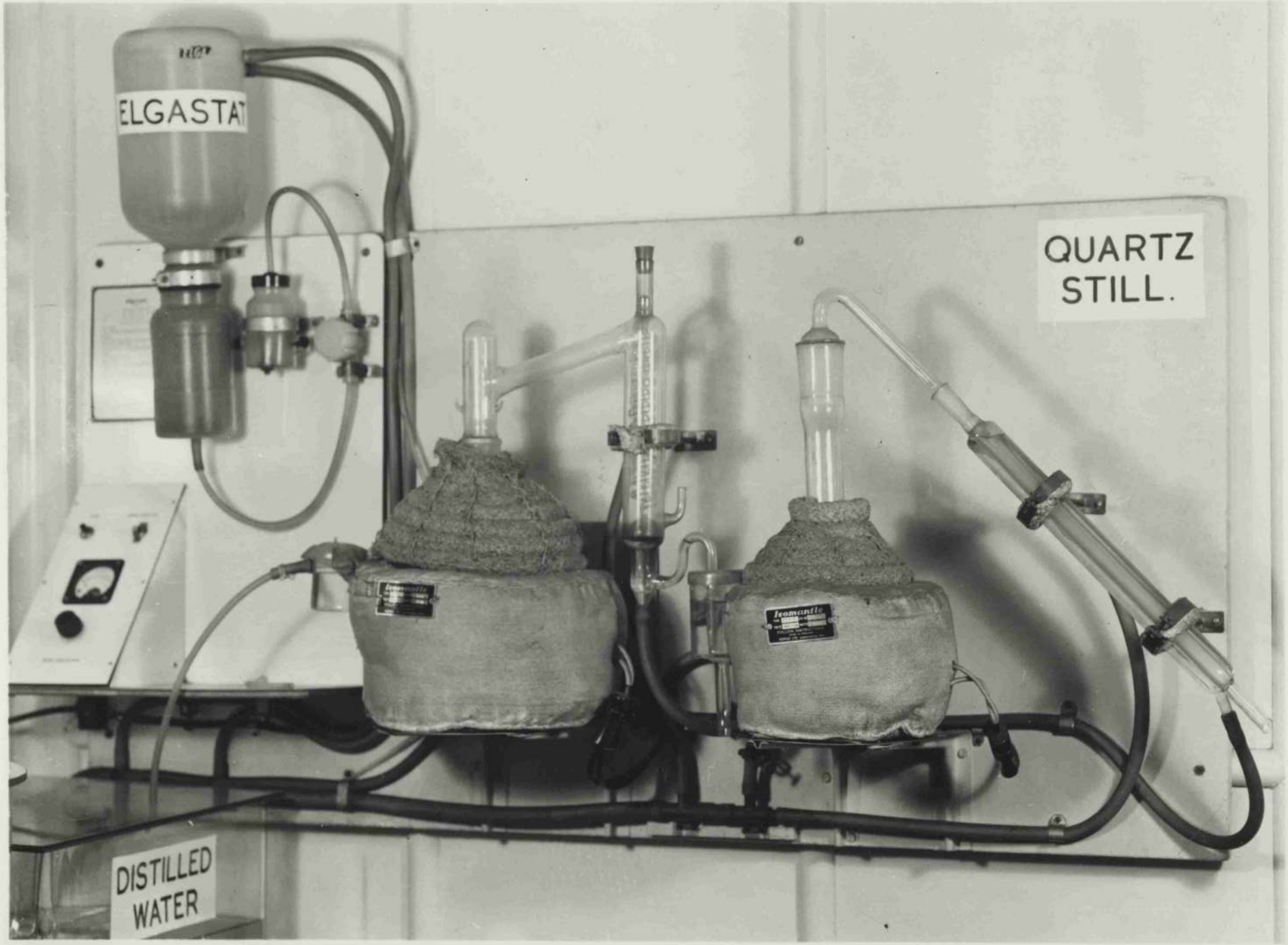
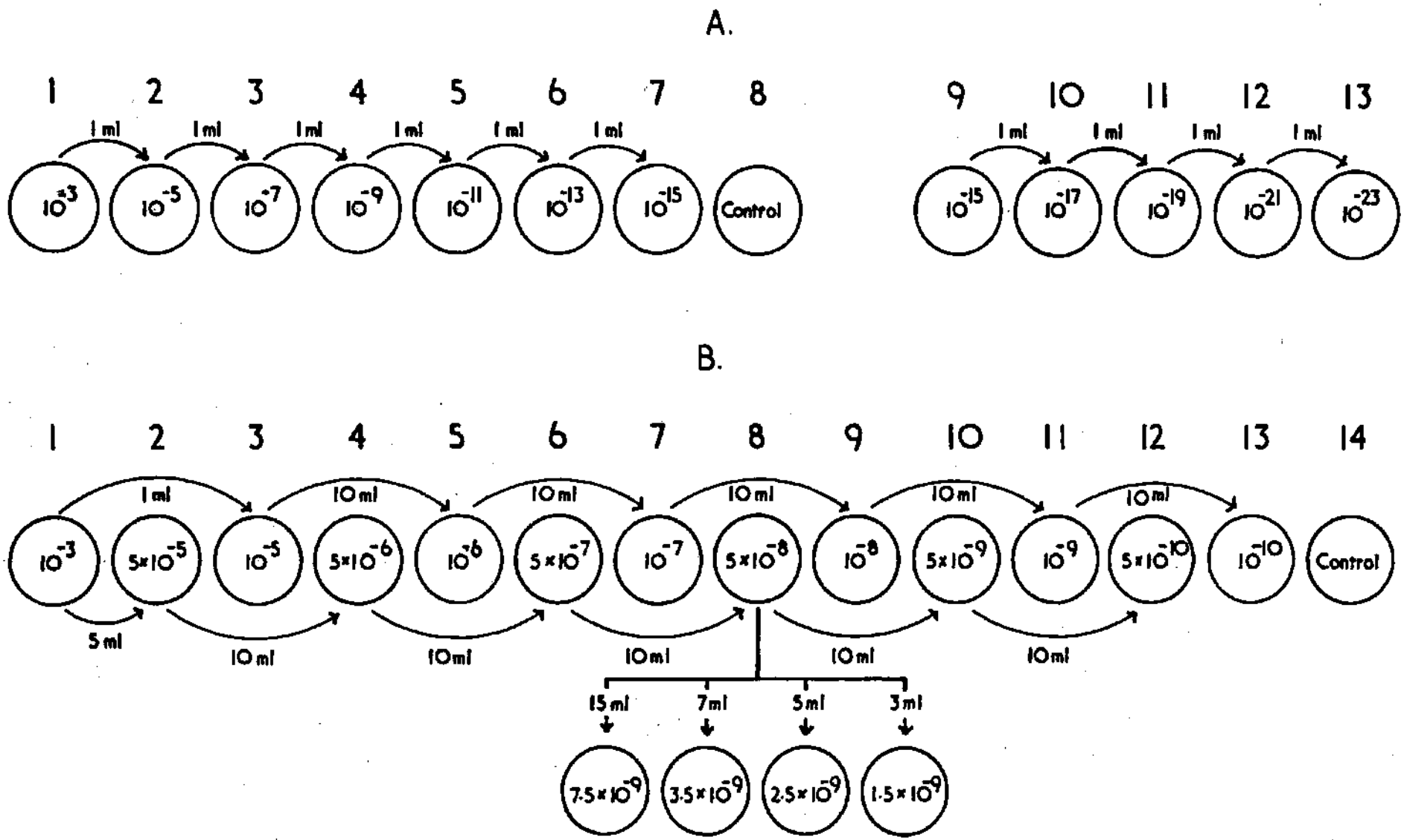


Fig. 7.

Fig.8. Dilution procedures employed for obtaining different concentrations of acetylcholine. Arrows indicate transfer of the specified volume of solution from one flask to the other. The volume in each flask was made up to 100 ml with Ringer's solution.



Each circle represents a 100 ml flask

Fig. 8

Fig. 9 The heart in position. The fluid enters the heart through the venous cannula and is pumped out through the aortic cannulae. Pericardium is intact.

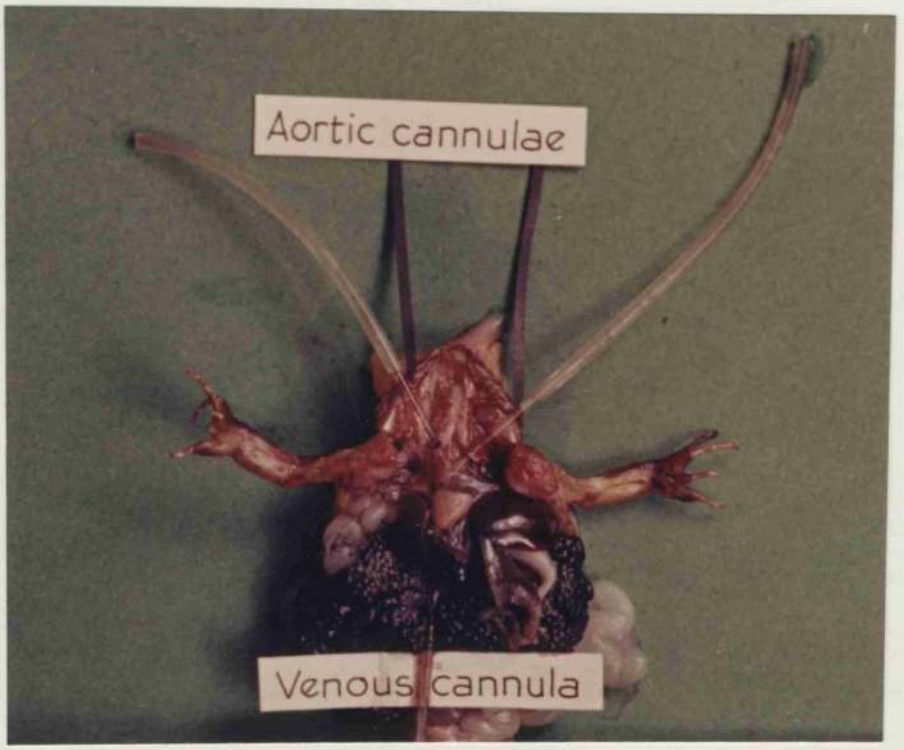


Fig. 9 Heart in situ.

Fig. 10 Stability of record and technique of controls. The downward displacement of marker from zero position to the position of a burette (right top corner scale) indicates commencement of perfusion from that burette, the duration of perfusion being given by the duration of downward displacement. The distance between 4 adjacent vertical lines represents 2 minutes. The end of perfusion from a burette and change back to the reservoir are indicated by the return of the marker to the zero position after an overshoot. The cardiac output (ml/min) the heart rate (original rate/min) and the venous pressure are recorded as continuous lines. The widetrace is the arterial pressure (blood pressure) trace. The upper level of the trace gives systolic pressure, the lower level gives the diastolic pressure and the width of the trace gives the pulse pressure. These pressure changes during each heart beat are recorded. Because of a comparatively higher frequency of this heart and also because of photographic reduction, the individual beats are very close together producing a dense trace. The pulse pressure during each cardiac cycle is proportional to the amplitude of contraction. Where the heart rate was slow individual beats could be distinctly seen in the blood pressure trace as in Fig. 50. All scales are prominently displayed with bold numerals on white vertical strips on the chart between point 3.0 and 3.30. For details of record and events recorded see text.

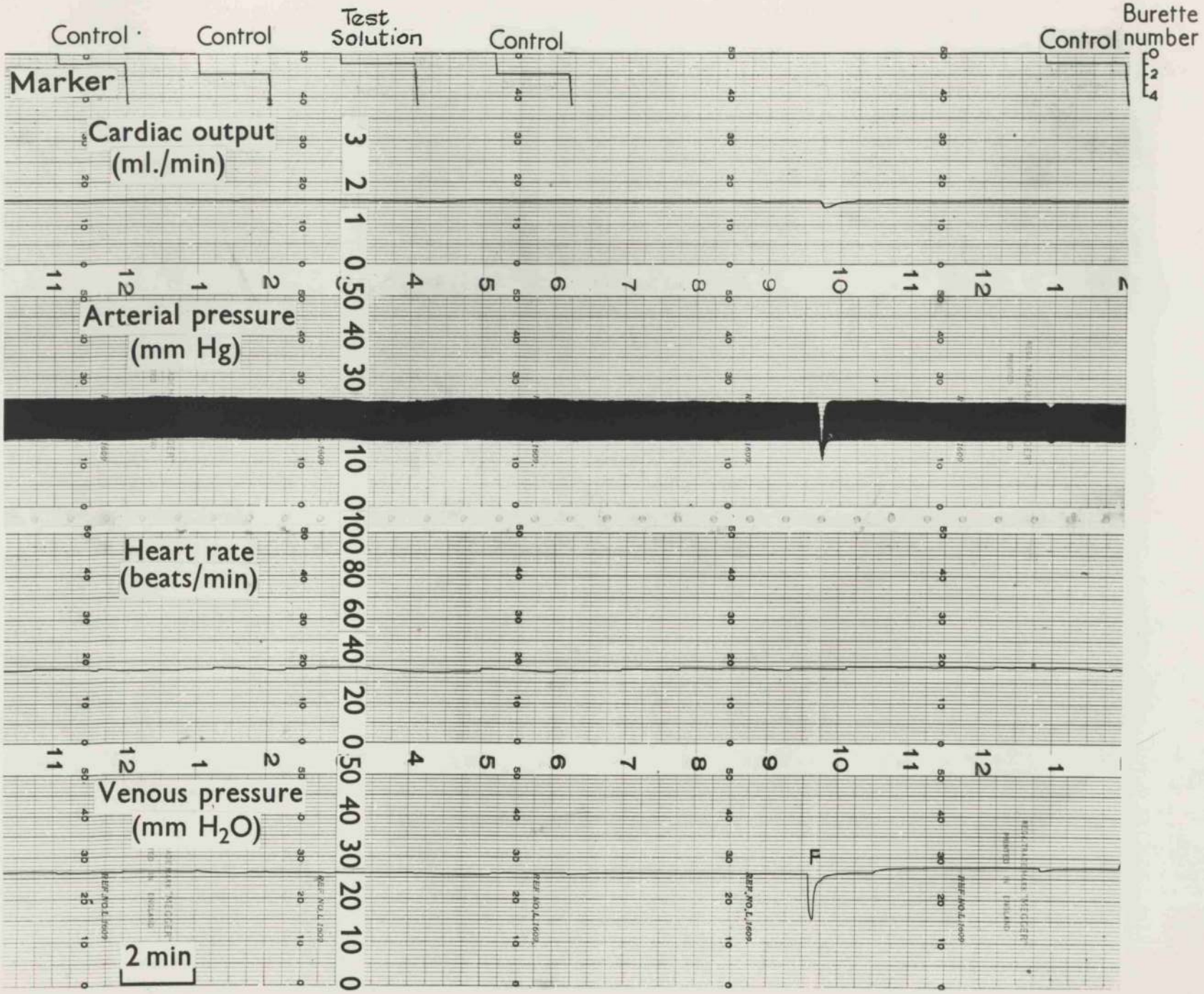

Control
Control
Test Solution
Control
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig. 10

Fig. 11. Record of a heart illustrating the degree of tolerance to wide fluctuations in the pH of Ringer's solution. The marker in this case shows steps of changes in the pH of Ringer's solution. The pH was adjusted to different values by adding $\frac{N}{1}$ hydrochloric acid (trace a) and by adjusting the volume of isotonic sodium bicarbonate solution in the Ringer's solution (trace b).

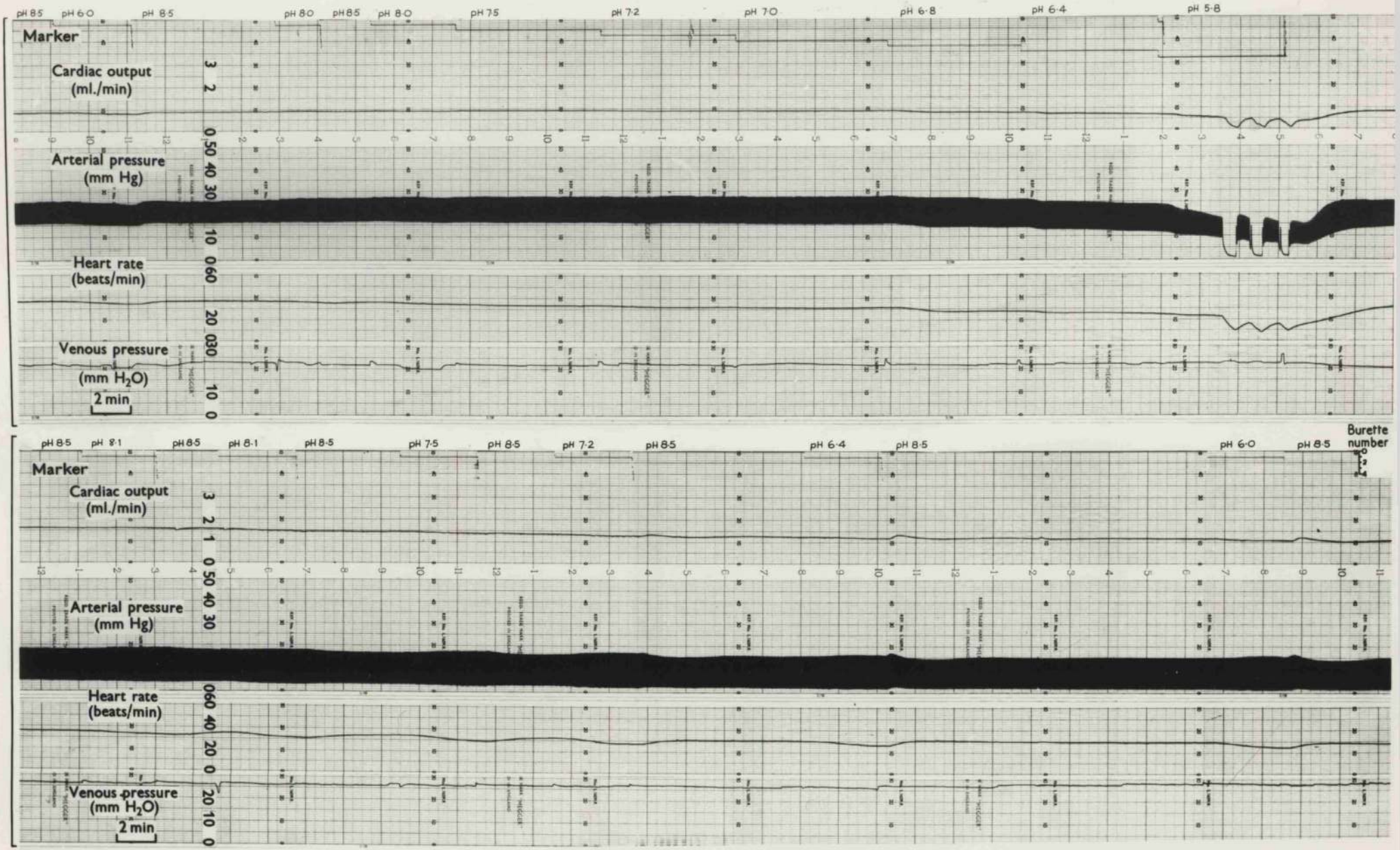

a
pH 8·5
pH 6·0
pH 8·5
pH 8·0
pH 8·5
pH 8·0
pH 7·5
pH 7·2
pH 7·0
pH 6·8
pH 6·4
pH 5·8
Marker
Cardiac output (ml./min)
3 2
Arterial pressure (mm Hg)
0 50 40 30
Heart rate (beats/min)
10 0 60
Venous pressure (mm H2O)
20 0 30 10 0
2 min
b
pH 8·5
pH 8·1
pH 8·5
pH 8·1
pH 8·5
pH 7·5
pH 8·5
pH 7·2
pH 8·5
pH 6·4
pH 8·5
pH 6·0
pH 8·5
Burette number
Marker
Cardiac output (ml./min)
3 2 1 0
Arterial pressure (mm Hg)
50 40 30
Heart rate (beats/min)
60 40 20 0
Venous pressure (mm H2O)
20 10 0
2 min

Fig. 11

Fig. 12. Almost equal effectiveness of the same solutions of acetylcholine When fresh (a) and when 3 hours old (b). The effect of 5×10^{-9} (trace b) is not very obvious due to photographic reduction. To minimise the reduction, the venous pressure trace was omitted. The movement of the pen for heart rate towards zero between point 4 and 5 during the test with 2.5×10^{-8} (trace b) was due to failure in triggering of the ratemeter (see introduction to record). The correct rate is indicated by the dotted line.

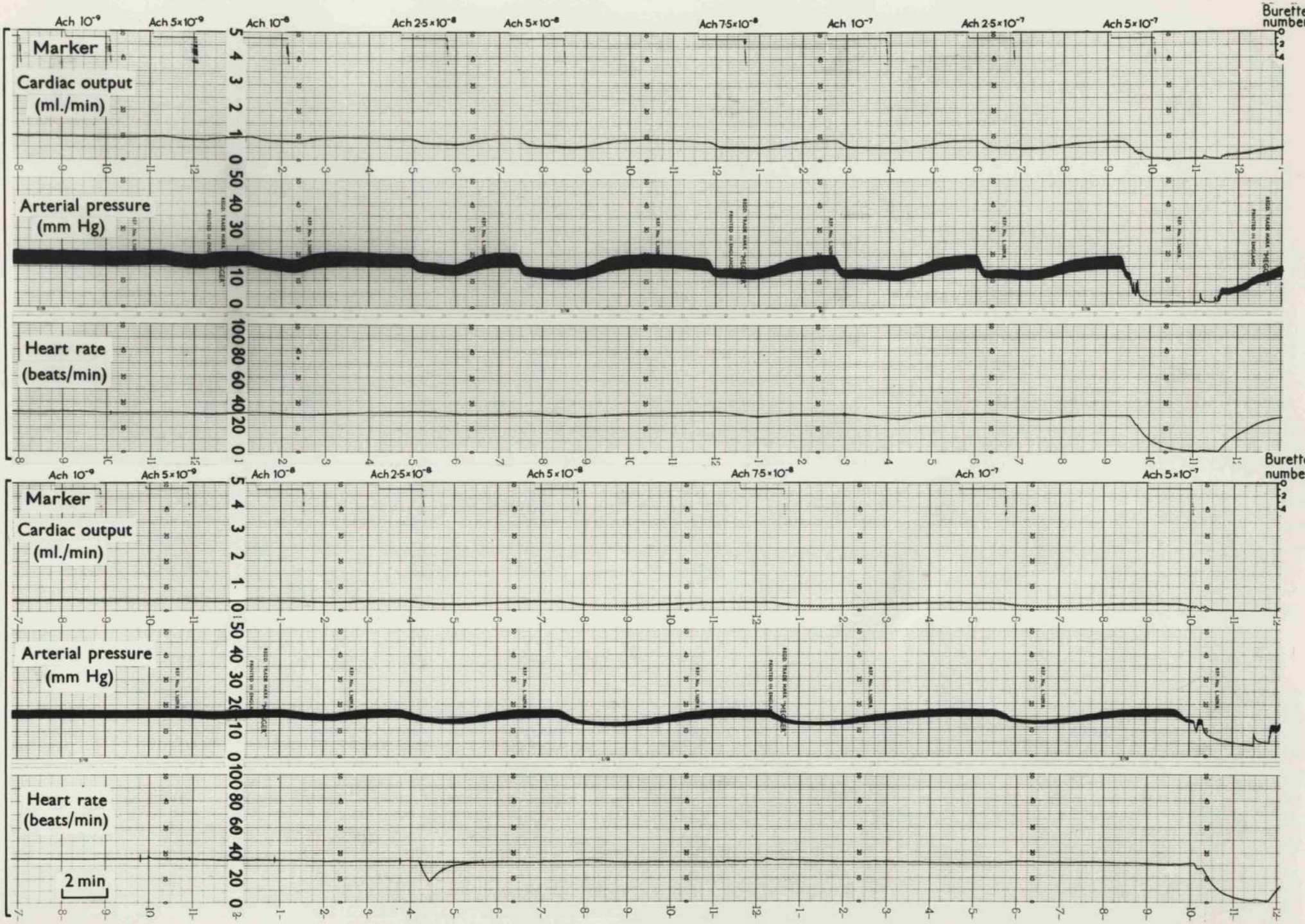

a
Ach 10⁻⁹
Ach 5×10⁻⁹
Ach 10⁻⁸
Ach 2·5×10⁻⁸
Ach 5×10⁻⁸
Ach 7·5×10⁻⁸
Ach 10⁻⁷
Ach 2·5×10⁻⁷
Ach 5×10⁻⁷
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
b
Ach 10⁻⁹
Ach 5×10⁻⁹
Ach 10⁻⁸
Ach 2·5×10⁻⁸
Ach 5×10⁻⁸
Ach 7·5×10⁻⁸
Ach 10⁻⁷
Ach 5×10⁻⁷
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
2 min

Fig 12

Fig. 13. Concentration-response curves of three different hearts (a, b and c) showing the action of increasing concentrations of acetylcholine. The small divisions on the abscissa between two whole logarithmic numbers (shown below the abscissa) indicate intermediate concentrat-ions. For example the numbers 2.5, 5 and 7.5 with a small cross on one side between 8 and 7 means 2.5×10^{-8}, 5×10^{-8} and 7.5×10^{-8} respectively.

Vertical dotted arrows	-	partial conduction block
Vertical continuous arrows	-	complete conduction block
Horizontal arrow on the ordinate	-	maximum limit of variation during controls in the whole experiment.
Small dot on the curve	-	value just before the onset of conduction block.

The details of solutions tested in different hearts are given below:

(a) filled circles	-	values obtained from tests with fresh solutions (tested within 1 hour of preparation)
open circles	-	values from tests with same solutions when 3 hours old
(b) filled circles	-	values obtained from tests with fresh solutions
open circles	-	values obtained from tests with same solutions when 5 hours old.
(c) filled circles	-	values from tests with fresh solutions.
open circles	-	values from tests with same solutions when 3 hours old
circle containing a dot	-	values from tests with solutions of a different series when 5 hours old.

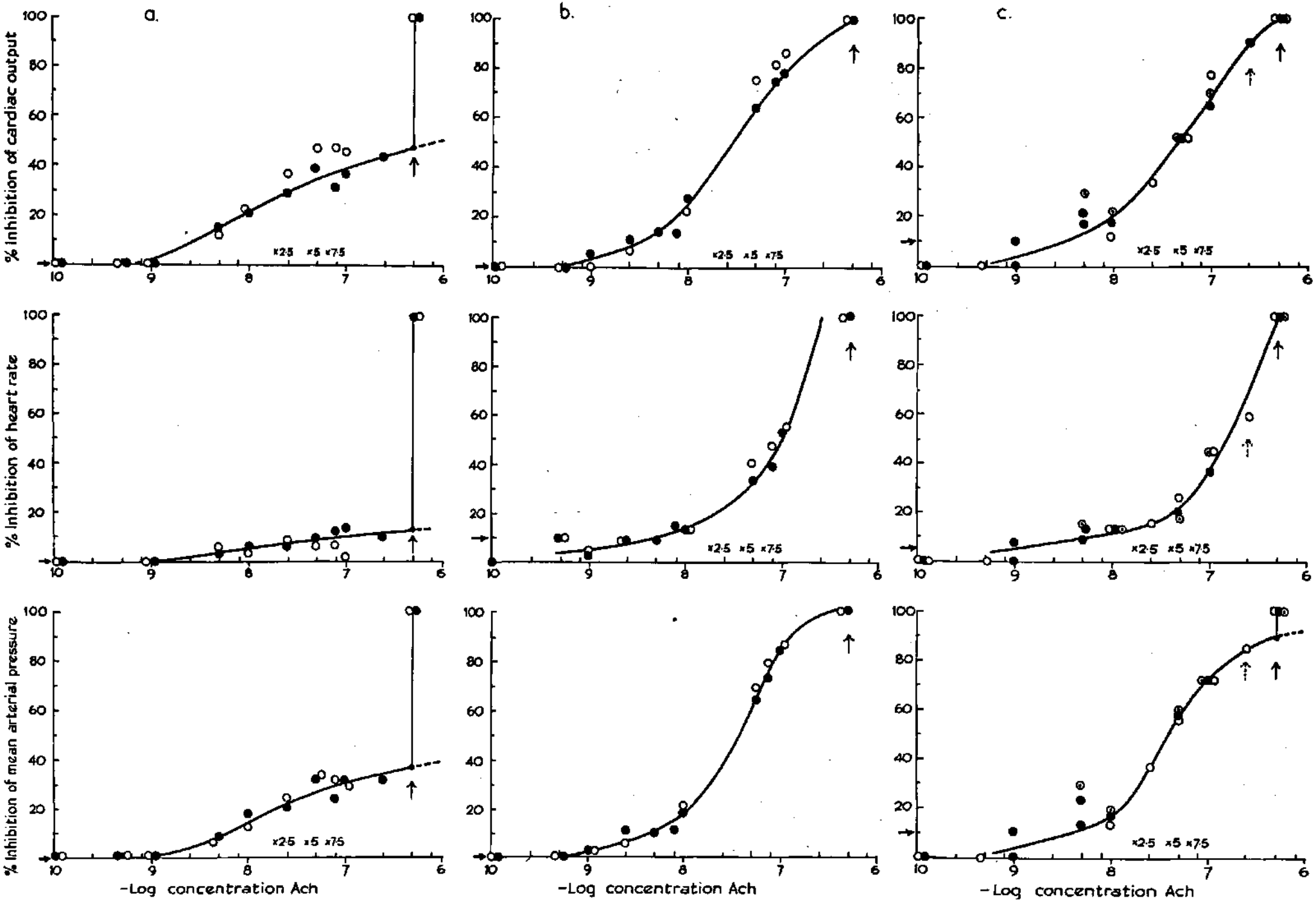

a.
b.
c.
% Inhibition of cardiac output
% Inhibition of heart rate
% Inhibition of mean arterial pressure
×2·5 ×5 ×7·5
-Log concentration Ach

Fig.1 3

Fig. 14. Almost equal effectiveness of solutions of three different series after different intervals of standing. Series c was tested when fresh (trace a) and when 5 hours old (trace b). Solutions of series a and b were prepared by diluting concentrated solutions (i.e. 5×10^{-5} and 10^{-5} prepared in Ringer's solution) which had been standing for 94 and 77 hours respectively and were tested soon afterwards. Tests with solutions of the three different series (trace b) were interdigitated for comparison, using a separate burette for each series. A portion of the record has been omitted (see dotted lines).

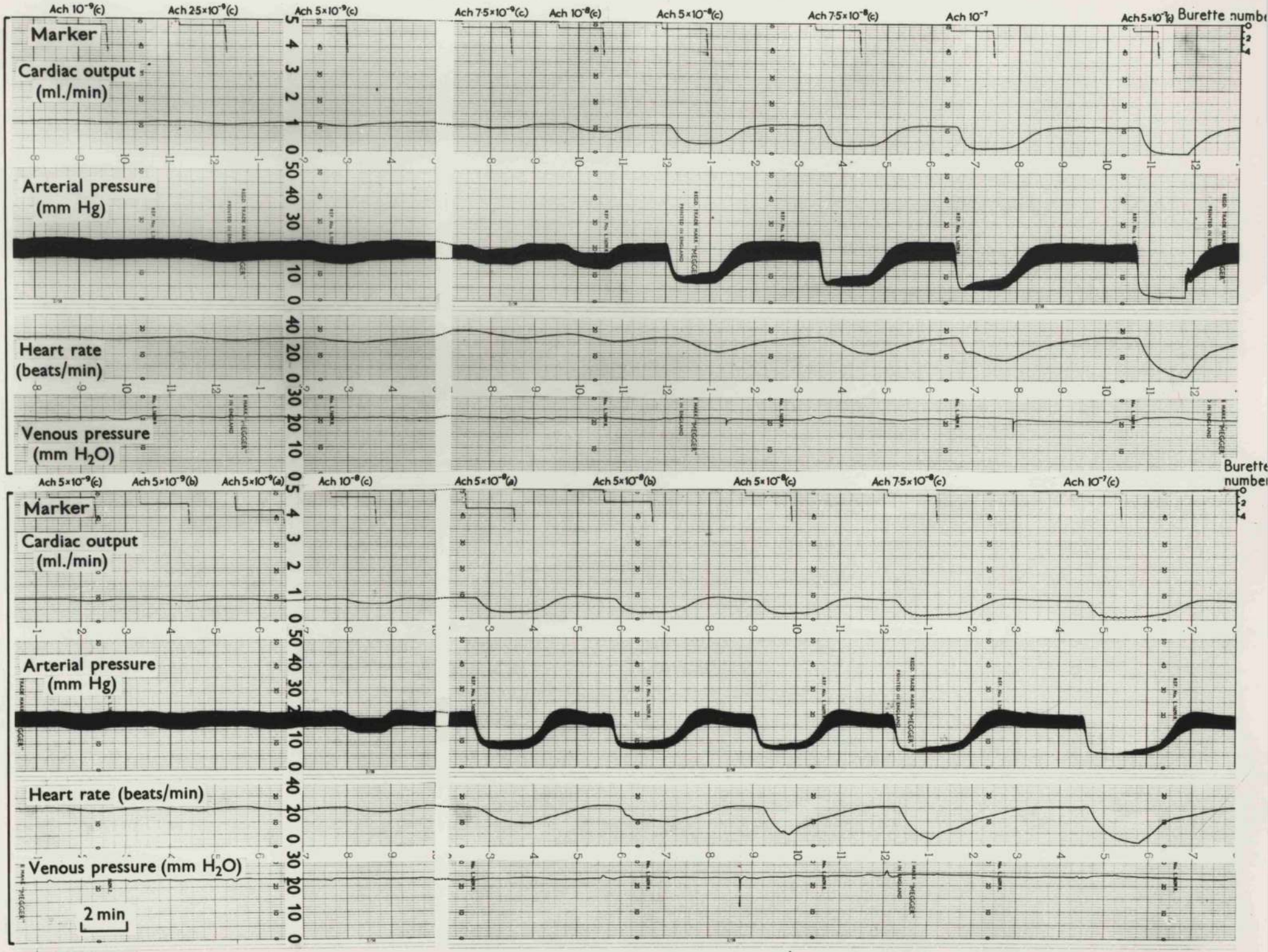

a
Ach 10⁻⁹(c)
Ach 2·5×10⁻⁹(c)
Ach 5×10⁻⁹(c)
Ach 7·5×10⁻⁹(c)
Ach 10⁻⁸(c)
Ach 5×10⁻⁸(c)
Ach 7·5×10⁻⁸(c)
Ach 10⁻⁷
Ach 5×10⁻⁷(c)
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
b
Ach 5×10⁻⁹(c)
Ach 5×10⁻⁹(b)
Ach 5×10⁻⁹(a)
Ach 10⁻⁸(c)
Ach 5×10⁻⁸(a)
Ach 5×10⁻⁸(b)
Ach 5×10⁻⁸(c)
Ach 7·5×10⁻⁸(c)
Ach 10⁻⁷(c)
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig 14

Fig. 15. Concentration-response curves of three hearts (a, b, c) showing the action of increasing concentrations of acetylcholine.

(a) open circles - values from tests with solutions of a series 3 hours old.

circles containing dots - values from tests with solutions of a different series which was 25 hours old

(b) filled circles - values from tests with a fresh series.

open circles - values from tests with another series which was 29 hours old

(c) filled circles - values from tests with fresh solutions of series a

open circles - values from tests with same solutions of series a when 18 hours old

circles containing plus signs - values from tests with fresh solutions of series b

circles containing dots - values from tests with same solutions of series b when 18 hours old.

series a prepared in clean and sterile flasks as usual

series b prepared in flasks not subjected to the usual process of cleaning and sterilisation.

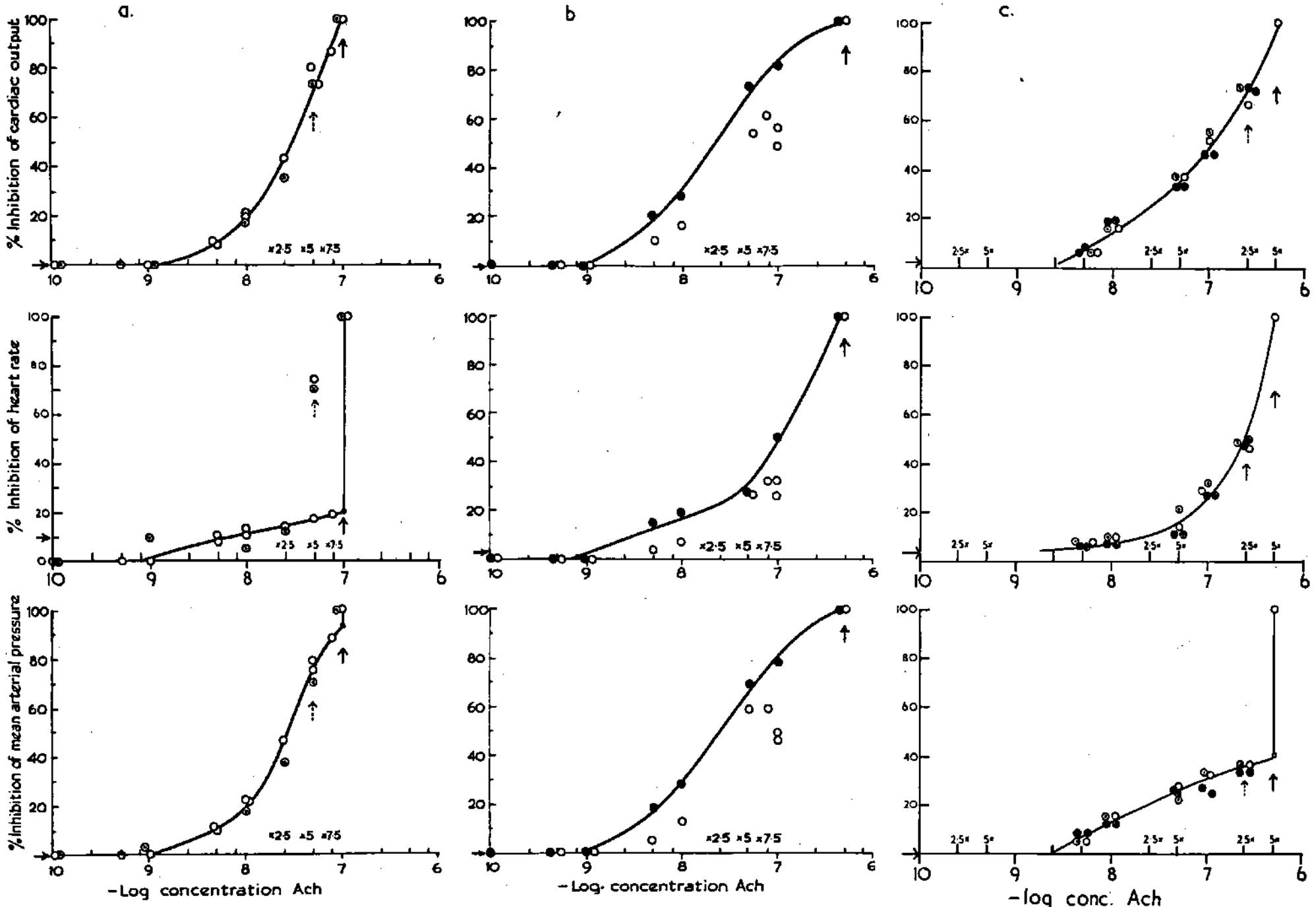

a.
b
c.
% Inhibition of cardiac output
% Inhibition of heart rate
%Inhibition of mean arterial pressure
×2·5 ×5 ×7·5
2·5× 5×
-Log concentration Ach
-Log· concentration Ach
-log conc. Ach

Fig.15

Fig. 16. Almost equal effectiveness of solutions of two series (a and b) when fresh (trace a) and when about 18 hours old (trace b). Solutions of series a were prepared in clean and sterile flasks as usual but solutions of series b were prepared in flasks which had been standing in a cupboard in the laboratory without being subjected to the usual process of cleaning and sterilisation. (see also Fig. 15c)

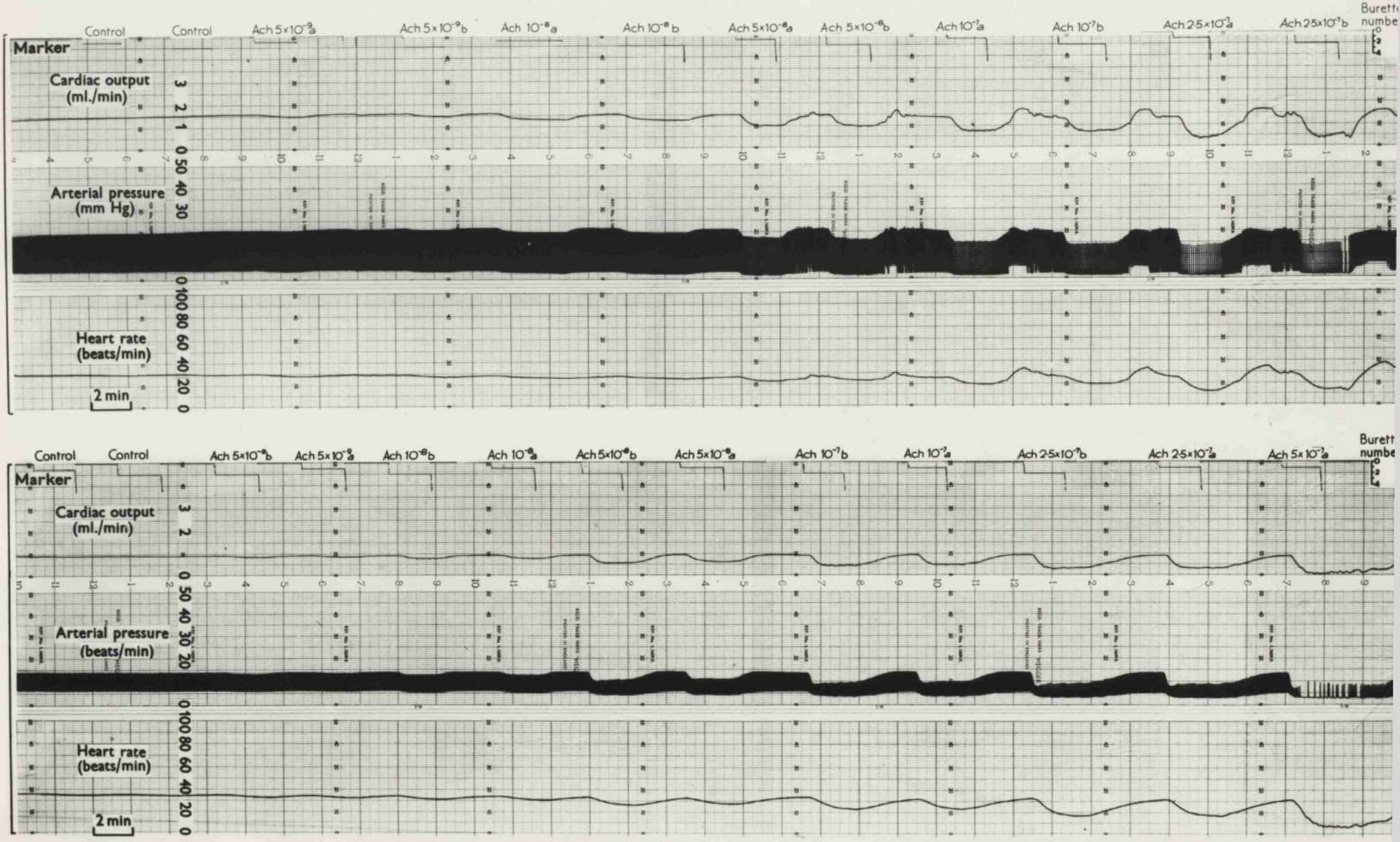

a
Control
Control
Ach 5×10⁻⁹a
Ach 5×10⁻⁹b
Ach 10⁻⁸a
Ach 10⁻⁸b
Ach 5×10⁻⁸a
Ach 5×10⁻⁸b
Ach 10⁻⁷a
Ach 10⁻⁷b
Ach 2·5×10⁻⁷a
Ach 2·5×10⁻⁷b
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
2 min
b
Control
Control
Ach 5×10⁻⁹b
Ach 5×10⁻⁹a
Ach 10⁻⁸b
Ach 10⁻⁸a
Ach 5×10⁻⁸b
Ach 5×10⁻⁸a
Ach 10⁻⁷b
Ach 10⁻⁷a
Ach 2·5×10⁻⁷b
Ach 2·5×10⁻⁷a
Ach 5×10⁻⁷a
Marker
Cardiac output (ml./min)
Arterial pressure (beats/min)
Heart rate (beats/min)
2 min

Fig. 16

Fig.17.

(a) Action of increasing concentrations of acetylcholine in the absence of eserine. There was only a small increase in the effect on mean pressure and output when the concentration was increased from 7.5×10^{-8} to 1.5×10^{-7} g/ml (plateau effect). The initial pulse pressure during the action of 2.5×10^{-7} also shows that the tendency for the attainment of a 'plateau' was persisting when partial block occurred. However, coincident with the block there was a progressive reduction in the individual excursions on the pressure trace (i.e. pulse pressure during each cardiac cycle) indicating sudden increase in the action of acetylcholine on the ventricular muscle. When the heart finally stopped there was full inhibition of muscle and probably a coincident complete conduction block also.

(b) Action of increasing concentrations of acetylcholine in the same heart as (a) in presence of eserine. Eserine in a concentration of 10^{-4} g/ml was incorporated in the Ringer's solution and in the solutions of acetylcholine. Traces a and b show that in the presence of eserine, the systolic pressure, pulse pressure and cardiac output increased while the heart rate decreased slightly; the onset of action of acetylcholine was more gradual and the recovery was also slower; acetylcholine in a concentration of 5×10^{-9} and 10^{-8} was less effective while the stoppage concentration was lowered from 2.5×10^{-7} to 1.5×10^{-7} g/ml.

(c) Part of the record of another heart showing very little increase in the effect on blood pressure and output on increasing the concentration from 7.5×10^{-8} to 1.5×10^{-7} g/ml (plateau effect). There was some interfer--ence with the conduction during tests with 10^{-7} and 1.5×10^{-7} g/ml. The trace for the heart rate during test with 1.5×10^{-7} is not showing the correct value because triggering of the ratemeter stopped due to very small pulse pressure (indicating powerful inhibition of cardiac muscle). The actual heart rate was higher as evidenced by the proximity of excursions on the pressure trace. This is an example of a heart in which acetylcholine involved the cardiac muscle long before there was a marked effect on the pacemaker.


a
Ach 10⁻⁹
Ach 5×10⁻⁹
Ach 7·5×10⁻⁹
Ach 10⁻⁸
Ach 1·5×10⁻⁸
Ach 2·5×10⁻⁸
Ach 3·5×10⁻⁸
Ach 5×10⁻⁸
Ach 7·5×10⁻⁸
Ach 10⁻⁷
Ach 1·5×10⁻⁷
Ach 2·5×10⁻⁷
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
b
Ach 10⁻⁹
Ach 5×10⁻⁹
Ach 10⁻⁸
Ach 5×10⁻⁸
Ach 10⁻⁷
Ach 1·5×10⁻⁷
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
C
Ach 7·5×10⁻⁸
Ach 10⁻⁷
Ach 1·5×10⁻⁷
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig.17

Fig. 18.

(a) Action of increasing concentrations of acetylcholine in another heart in the absence of eserine. The stoppage concentration in this case was 2.5×10^{-7} g/ml. Stoppage occurred due to sudden onset of complete conduction block. Recovery with very small excursions in the pressure trace indicates that the muscle was also fully inhibited. Partial conduction block occurred during tests with 5×10^{-8}, 7.5×10^{-8}, 10^{-7} and 1.5×10^{-7} g/ml. The normal rate of this heart was unusually high due to which the excursions on the pressure trace were very close together. Photographic reduction has brought them still closer, therefore the partial block is not visible in the photograph. Note the attainment of maximum effect on the cardiac output and arterial pressure between 5×10^{-8} and 1.5×10^{-7} (plateau effect)

(b) Action of increasing concentrations of acetylcholine in the same heart in the presence of eserine. Complete conduction block now occurred at 10^{-7} due to which all parameters fell towards zero. Tests with 10^{-8} and 10^{-7} of series a (uneserinised), after the control, on the eserinised heart gave comparable effects to those obtained with eserinised solutions.

(c) Part of the record of another heart illustrating different degrees of conduction block. During the test with 10^{-7} the heart rate suddenly decreased to half its initial value (near point 4) due to 2 : 1 block. The inhibition of the sinus was progressing when the block increased further resulting in clearly visible 'dropped beats' between points 4.30 and 4.45. Note the gradual recovery from the block, the heart rate returning to normal level suddenly near 5.15. During the short test with 1.5×10^{-7} transient complete conduction block occurred.

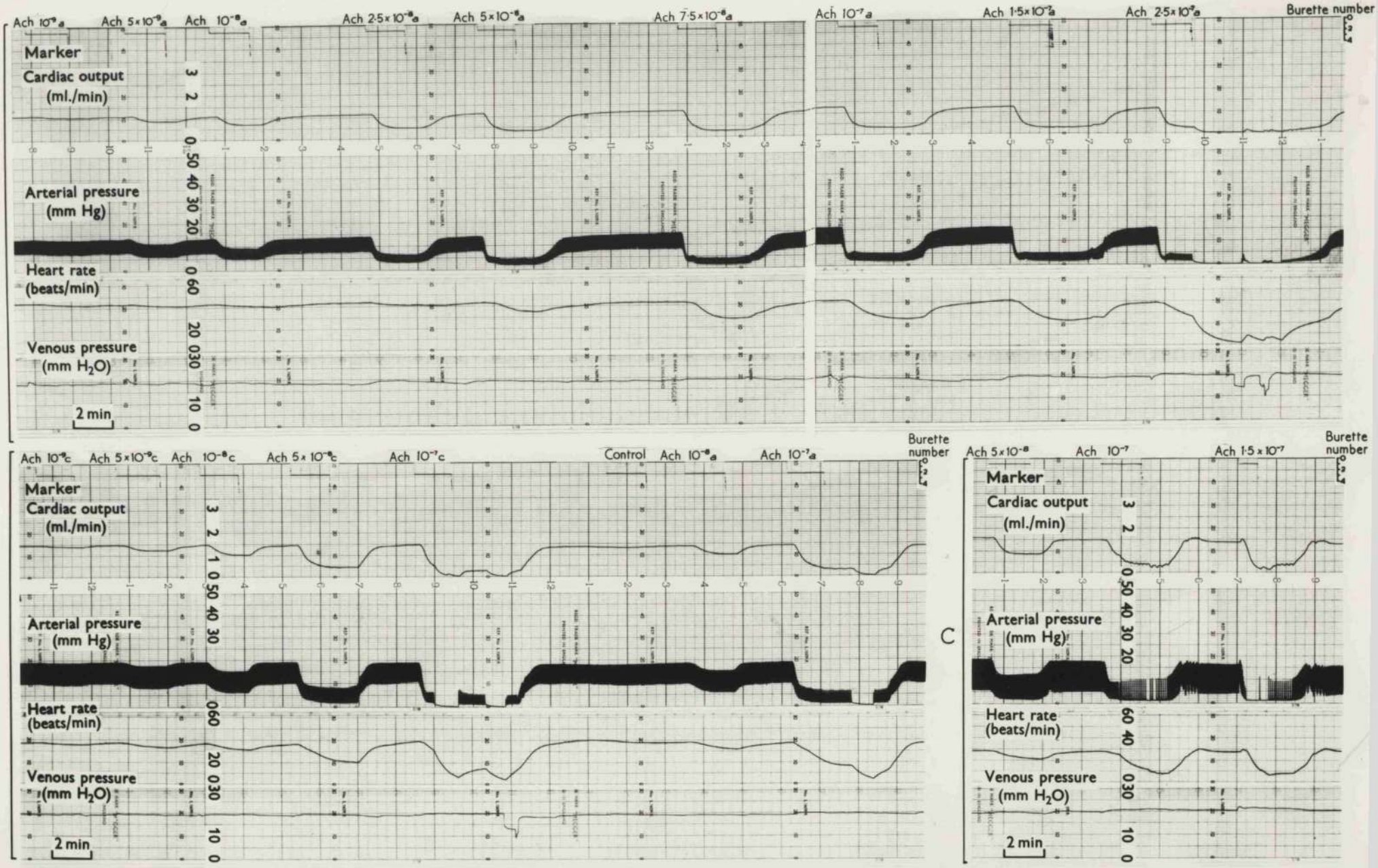

a
Ach 10⁻⁹a
Ach 5×10⁻⁹a
Ach 10⁻⁸a
Ach 2·5×10⁻⁸a
Ach 5×10⁻⁸a
Ach 7·5×10⁻⁸a
Ach 10⁻⁷a
Ach 1·5×10⁻⁷a
Ach 2·5×10⁻⁷a
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
b
Ach 10⁻⁹c
Ach 5×10⁻⁹c
Ach 10⁻⁸c
Ach 5×10⁻⁸c
Ach 10⁻⁷c
Control
Ach 10⁻⁸a
Ach 10⁻⁷a
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
c
Ach 5×10⁻⁸
Ach 10⁻⁷
Ach 1·5×10⁻⁷
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig. 19. Concentration-response curves of two hearts (a and b) in the presence (crosses with discontinuous lines) and in the absence (filled circles with continuous lines) of eserine. Symbols and notations as in Fig. 13. All solutions were tested within 1 hour of preparation
E - conduction block in presence of eserine.

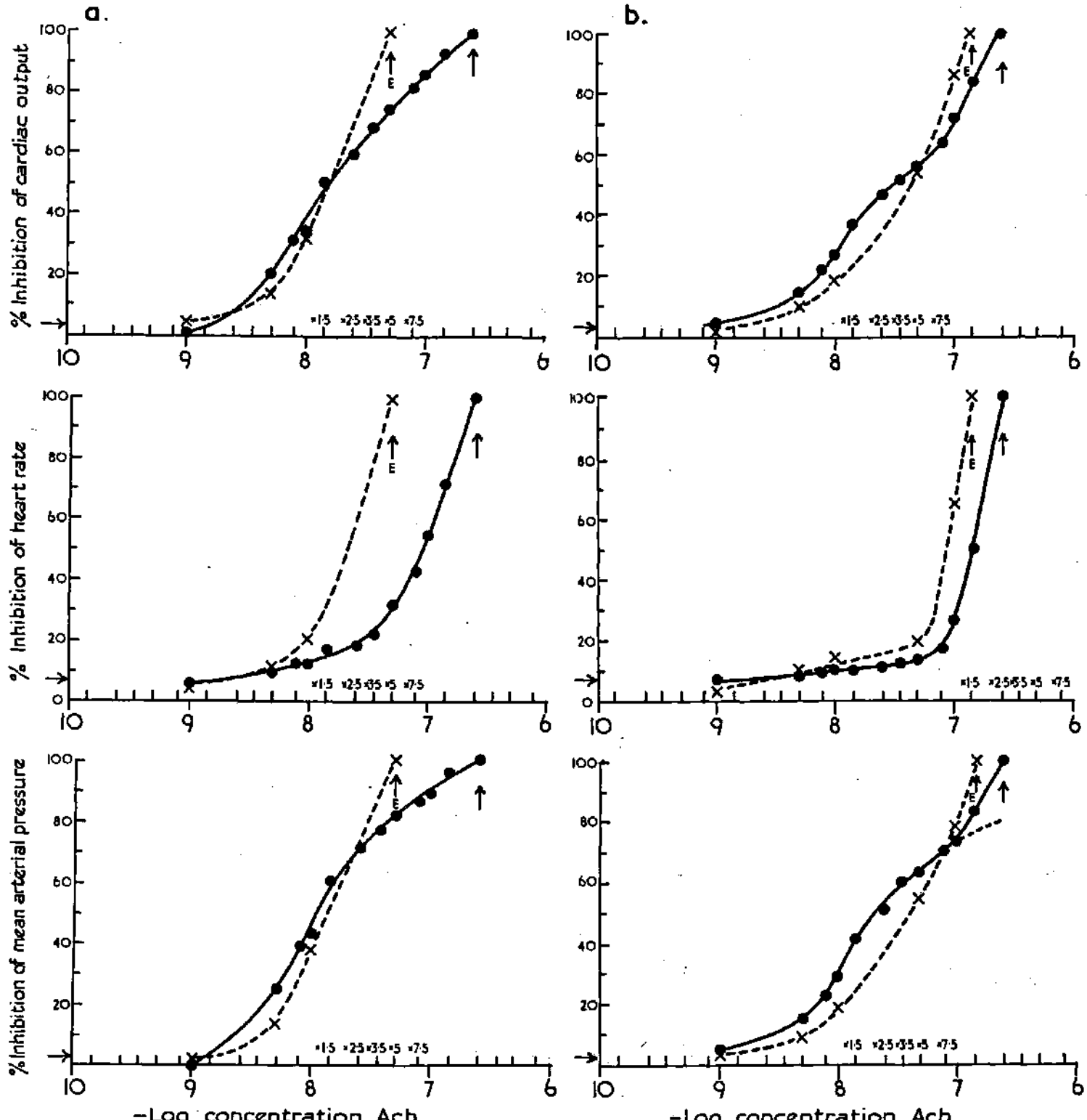

a.
b.
% Inhibition of cardiac output
% Inhibition of heart rate
% Inhibition of mean arterial pressure
E
×1·5 ×2·5 ×3·5 ×5 ×7·5
10 9 8 7 6
0 20 40 60 80 100
-Log concentration Ach

Fig 19

Fig. 20. Concentration - response curves of three other hearts in the presence (crosses with discontinuous lines) and in the absence (filled circles with continuous lines) of eserine. Other symbols and notations as in Fig. 13.

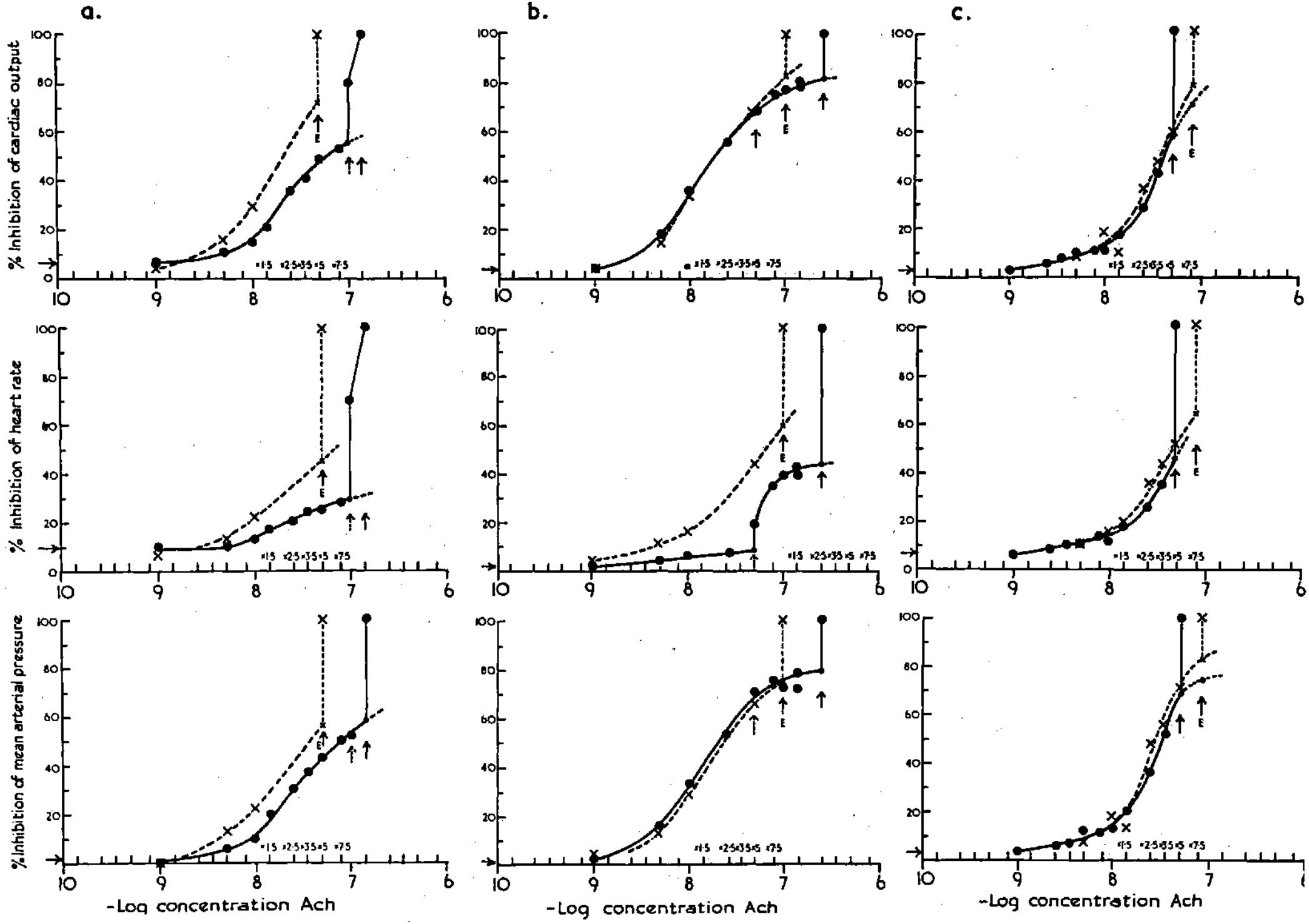

a.
b.
c.
% Inhibition of cardiac output
% Inhibition of heart rate
% Inhibition of mean arterial pressure
-Log concentration Ach
-Log concentration Ach
-Log concentration Ach

Fig. 2 O

Fig. 21. Only slight decrease of response to acetylcholine in a concentration of 10^{-9} g/ml. The inhibitory effect at this concentration persisted for quite a long time and decreased only slightly after the administration of 6 successive doses. The 6th and 7th tests with 10^{-9} were conducted for 3.5 minutes instead of 2 minutes as usual. At 'X' the control from burette 1 was started but it was soon realised that the burette had not been properly rinsed to wash out acetylcholine hence it was abandoned at this point and was conducted subsequently as seen at the end of the record. This figure has undergone considerable photographic reduction. It was considered useful to maintain the continuity of record.

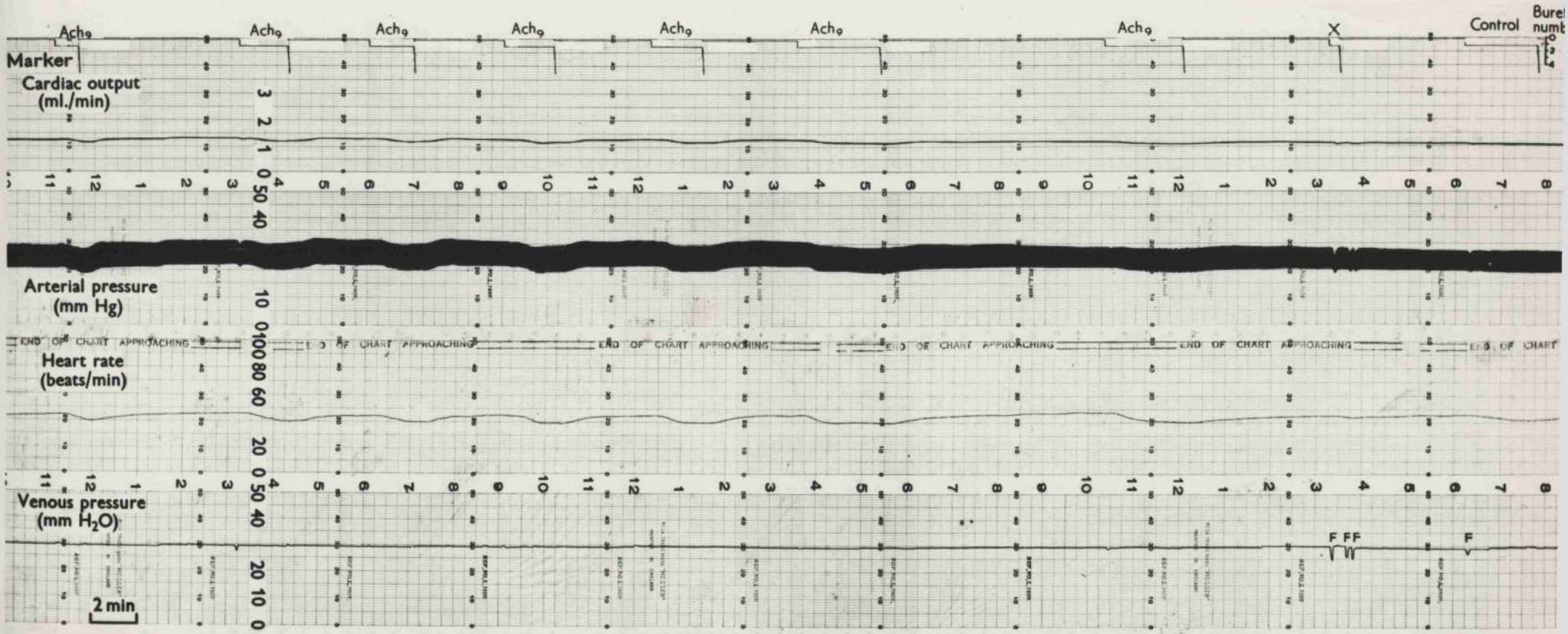

Ach
Ach
Ach
Ach
Ach
Ach
Ach
X
Control
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
END OF CHART APPROACHING
Heart rate
(beats/min)
Venous pressure
(mm H2O)
F F F
F
2 min

Fig.21

Fig. 22. The routine technique of testing a series of acetylcholine (Ach) solutions in increasing concentrations from 10^{-15} to 10^{-7} g/ml. Several initial control perfusions were conducted to show that the perfusion system was free from any contamination and that there was no possibility of any obstruction to the flow of fluid into the heart due to air bubbles. The final control perfusion showed that the perfusion system was still satisfactory. The concentrations of acetylcholine are indicated by suffixing the negative logarithm of the concentration in g/ml after the word Ach. Thus Ach 15 means acetylcholine in a connentration of 10^{-15} g/ml and so on. The minor changes in rate during test perfusions with acetylcholine solutions from 10^{-15} to 10^{-9} are similar to those during controls and are of no significance. The minimum effective concentration in this heart was 10^{-7} g/ml which stopped the heart within 30 seconds. The heart restarted between points 2.0 and 2.15 but stopped again for a brief period before the final recovery. The reason for the second stoppage of the heart is that as the heart emptied its contents during the first few beats, small amounts of residual

acetylcholine present in the venous cannula, entered the heart.

F = common perfusion chamber flushed back to wash out acetylcholine with normal Ringer's solution from the reservoir by removing the artery clip from the transducer-connecting-tube in front of standpipe 1 and allowing the fluid from the common perfusion chamber to escape through the standpipe exit. Because of the free outlet for the fluid of the common perfusion chamber, the venous pressure fell during this process of 'flushing back'. This was a routine procedure in each experiment.

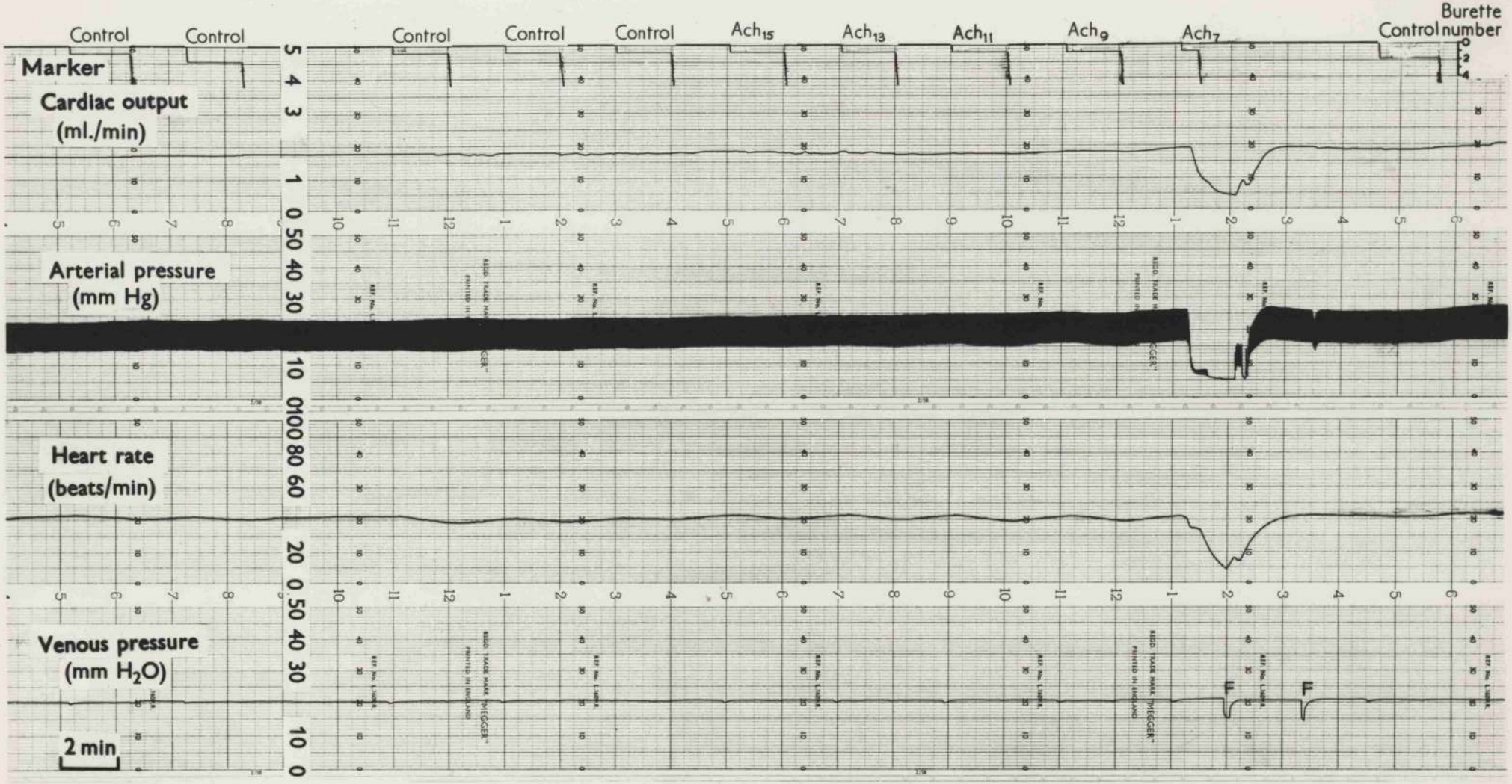

Control
Control
Control
Control
Control
Ach15
Ach13
Ach11
Ach9
Ach7
Control
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2 min

Fig. 22

Fig. 23 Example of a heart showing stoppage by acetylcholine in a concentration of 10^{-9} g/ml. The main source of perfusion in this case is burette 2 instead of the reservoir. There were also minor spontaneous changes in rate (not shown here) which were reflected in the output and blood pressure traces. Changes in the rate, output and blood pressure seen in the Figure during the test perfusions with 10^{-11} were of the same order of magnitude as the spontaneous changes and therefore fell within the range of experimental error. These changes therefore were ignored. However the effect of 10^{-9} was very gross resulting in typical stoppage of the heart.

Buriette number
0
2
4
Ach
Ach
Ach
Control
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2min
3 2 1 0 50 40 30
0 100 80 60 40 20 0 50 40 30 20 10 0
REF. No. L.1609/R.
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND

Fig.24. Example of a heart relatively insensitive to acetylcholine. The minimum effective concentration was 10^{-7} g/ml (Threshold sensitivity). The stoppage concentration was 10^{-5} g/ml. Burette 2 was in use throughout. Recording was discontinued at point 4.0 which represents the end of the experiment. There was no necessity for any final control as these concentrations were invariably effective in all hearts.

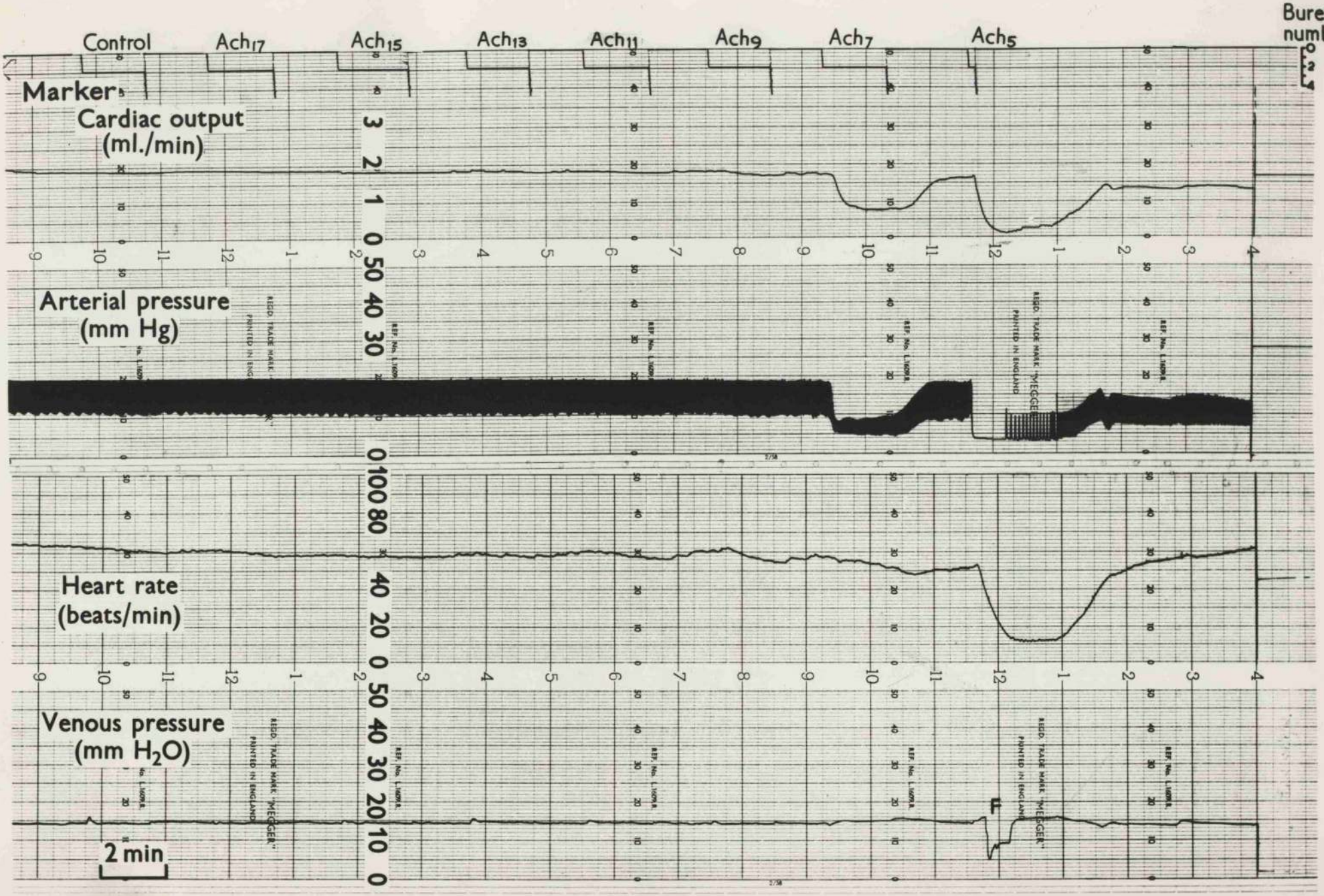

Control
Ach17
Ach15
Ach13
Ach11
Ach9
Ach7
Ach5
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig.24

Fig. 25. Heart with a medium degree of sensitivity to acetylcholine. Threshold sensitivity 10^{-9} and stoppage concentrations 10^{-7} g/ml. The minor effects at lower concentrations (from 10^{-15} to 10^{-11}) were of no significance. Several series of solutions of acetylcholine were tested in some hearts. The mention of symbol (b) after the concentrations of acetylcholine means solutions belonging to 'b' series. Burette 1 was used for initial and final controls. Test with ineffective concentrations of acetylcholine from burette 2 serve as controls from that burette.

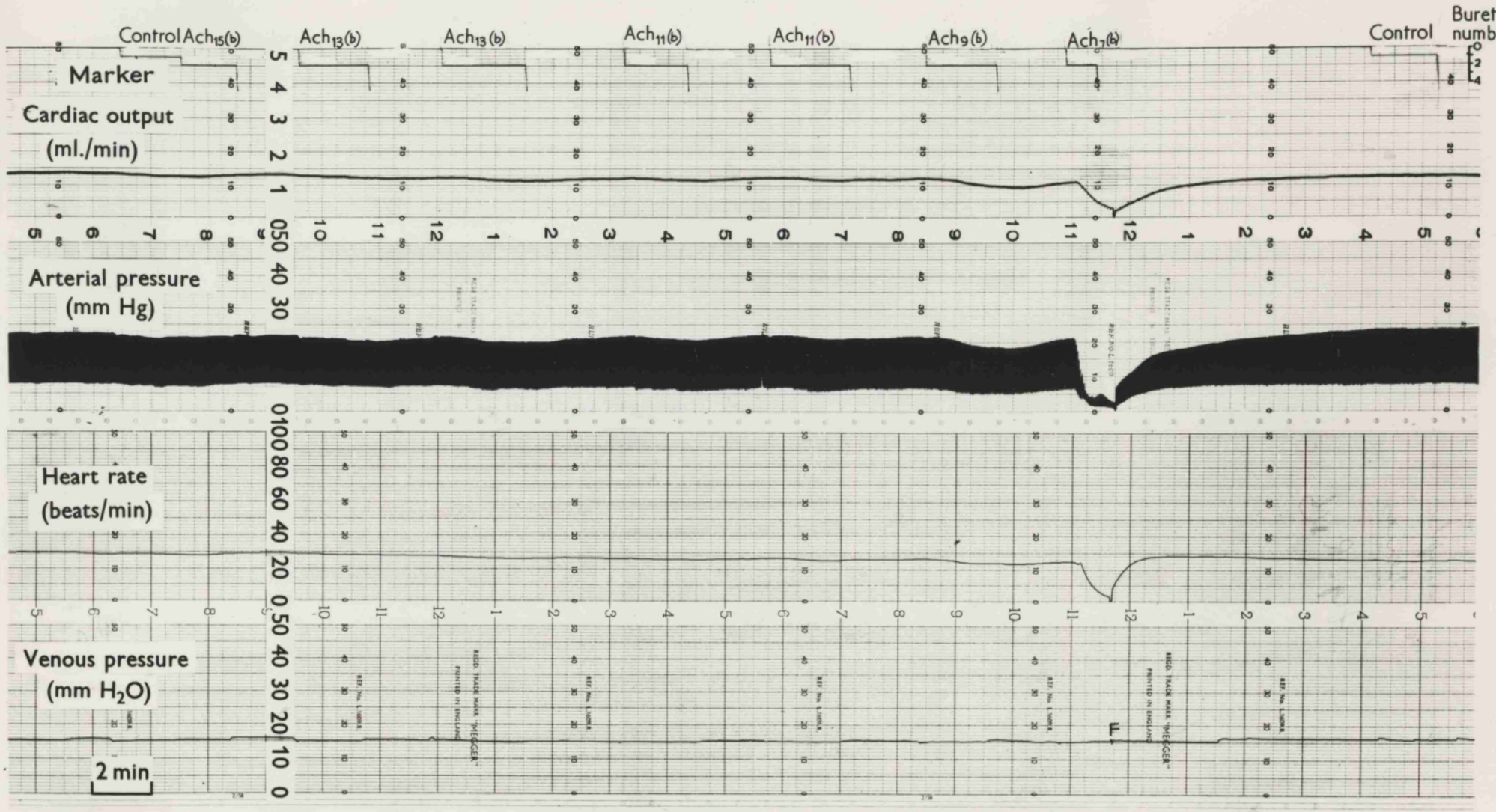

Control
Ach15(b)
Ach13(b)
Ach13(b)
Ach11(b)
Ach11(b)
Ach9(b)
Ach7(b)
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig. 25

Fig. 26. Highly sensitive heart: threshold sensitivity 10^{-11} g/ml. Burette 1 in use throughout. The venous pressure was constant throughout except between points 6.45 and 7.0 which represents 'flushing back' of the common perfusion chamber before the final controls. The effect of 10^{-11} is gross and is 'bracketed' between several controls from the same burette on either side; indeed tests with 10^{-15} and 10^{-13} in the beginning also serve as controls. The action of 10^{-11} influenced all the three variable parameters. The effect was completely reversible showing that there was no question of any obstruction to the flow of fluid into the heart. For further discussion regarding the genuine nature of the effect, see text. The slight rise in the output and blood pressure during the recovery from the action of 10^{-11} and afterwards, seems to be partly due to rebound changes in the amplitude of contraction (pulse pressure) and partly due to the decrease in the heart rate.

Burette number
0
2
4
Control
Control
Ach11
Ach13
Ach15
Control
Marker
Cardiac output (ml/min)
3 2 1 0
Arterial pressure (mm Hg)
50 40 30
Heart rate (beats/min)
10 0 100 80 60 40 20 0
Venous pressure (mm H2O)
50 40 30 20 10 0
2min
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND

Fig. 27 Record of the same heart as in Fig. 26 showing repetition of tests with the minimum effective concentrations. The second test perfusion with 10^{-11} (in this Figure) was conducted when the recorded parameters of cardiac activity had apparently recovered from the inhibitory action of 10^{-11} administered during the first test perfusion. The intensity of effect during the second test with 10^{-11} was slightly less probably because the first test perfusion was longer than the second.

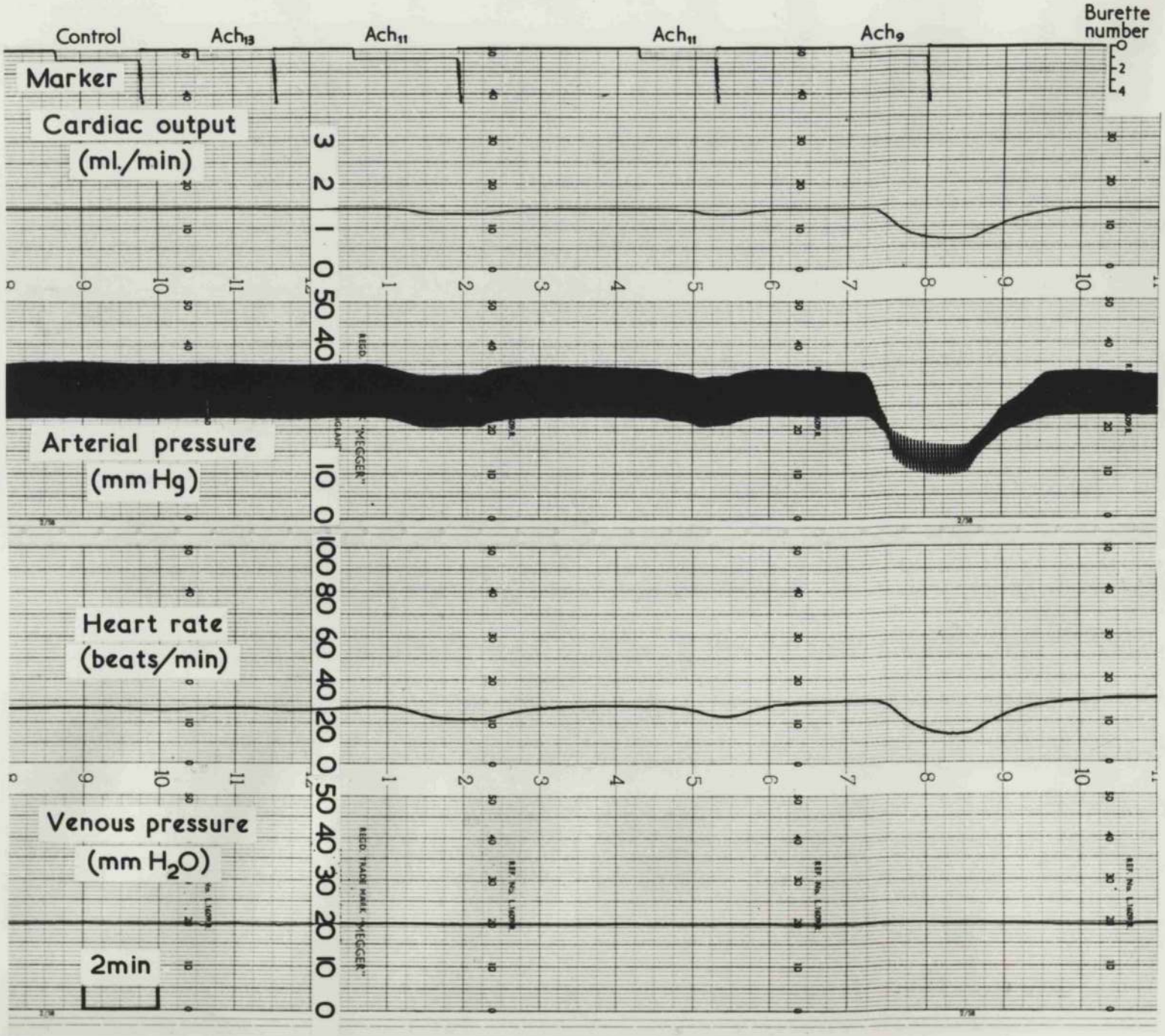

Control
Ach13
Ach11
Ach11
Ach9
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2min

Fig. 28. Highly sensitive heart: threshold sensitivity 10^{-13}, stoppage concentration 10^{-11} g/ml. The initial controls from burette 1 are satisfactory. The minor changes in rate during controls are not significant. Several other substances were tested on this heart previously. Towards the end of experiment sensitivity to acetylcholine was being tested casually starting with a concentration of 10^{-11} which however stopped the heart. Note the slight rebound stimulation during recovery. The subsequent control between points 10.30 and 11.30 is satisfactory except for minor disturbances in blood pressure due to adjustment in venous pressure. Subsequent two trials with 10^{-13} produced very large effects. The action of still lower concentrations could not be tested in this heart, hence 10^{-13} was taken as threshold sensitivity.

F = same significance as in previous Figures.

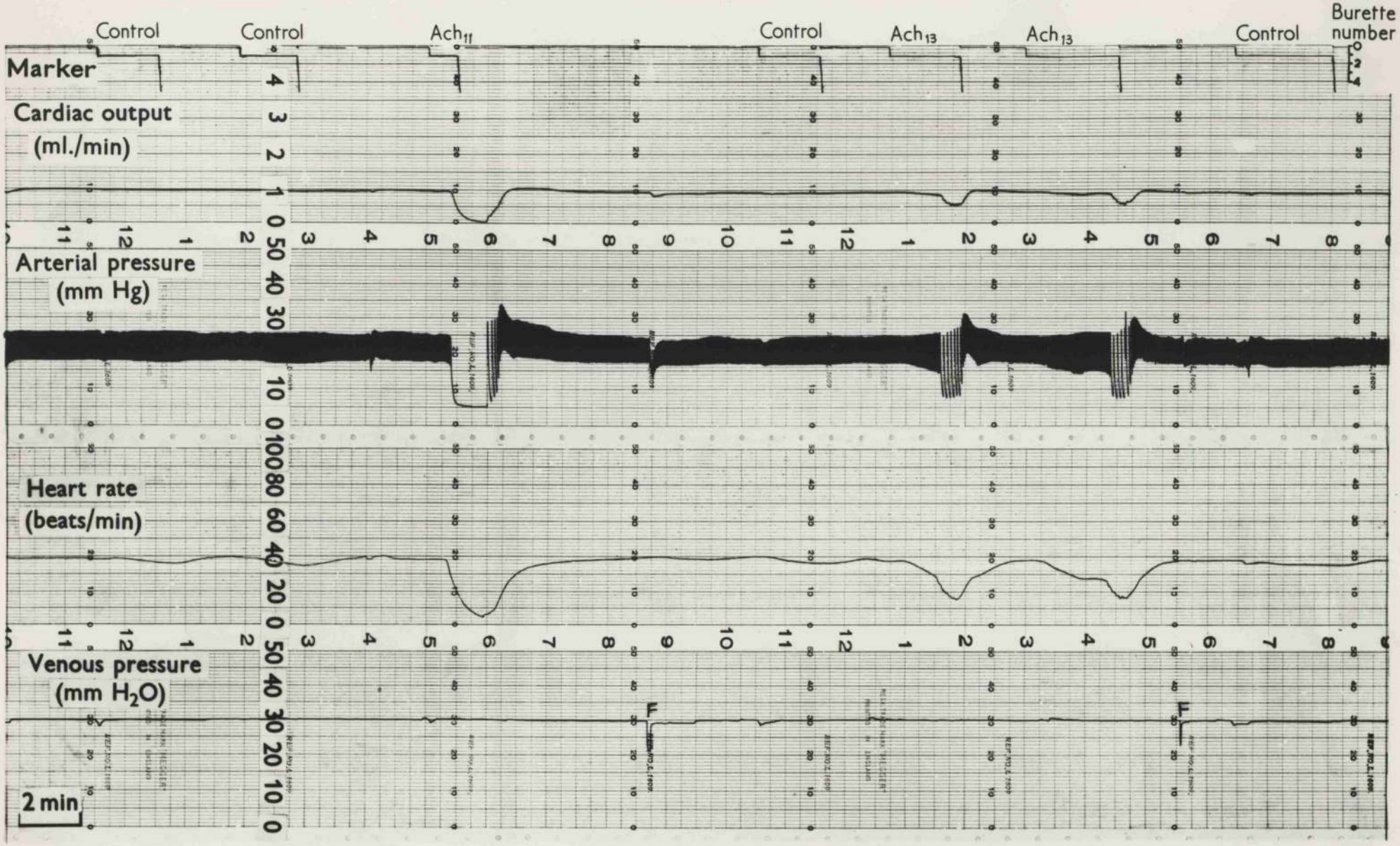

Control
Control
Ach11
Control
Ach13
Ach13
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig. 28

Fig. 29 Record of another highly sensitive heart in which the minimum effective concentration was 10^{-15}g/ml. The inhibitory action of acetylcholine first increased then decreased and finally again increased suddenly resulting in the stoppage of the heart, as the concentration was increased from 10^{-15} to 10^{-7}g/ml. Note the very small effect at 10^{-11} which is 100 times stronger than 10^{-13} and 10000 times stronger than 10^{-15}. Repetition of the test perfusion with 10^{-11} after an interval still showed small effect indicating that the decrease in the response at 10^{-11} was not due to administration of different concentrations in rapid succession. Also note that all the recorded parameters had completely recovered from the action of the previous concentration when the next concentration was tested. Slight initial increase in the heart rate before the control and the slow decrease in the rate after the control were due to spontaneous changes in the frequency of heart beat and are of no significance. The discontinuity in the record between the two test perfusions with 10^{-11} is due to the removal of part of the record during which no tests were conducted. This pattern of response to acetylcholine was seen in several hearts. The concentration - response curves of highly sensitive hearts are considered later in Fig. 52,54 and 55.

Control
Ach15
Ach13
Ach11
Ach9
Ach7
Ach11
Burette number
0
2
4
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
END OF CHART APPROACHING
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND
F

Fig. 30. Highly sensitive heart. Burette 5 in use. Controls satisfactory. Large effect from acetylcholine 10^{-15} and stoppage by 10^{-13} g/ml. The recording was temporarily switched off at point 9.15 (S) to allow for recovery and also to reduce the length of trace for photographic reproduction. Subsequently still lower concentrations were tested and acetylcholine 10^{-17} was found to be effective in this heart as shown in Fig. 31. The two chart papers are out of step by about two minutes.

Burette number
Control
Ach13
Ach15
Control
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
F
S
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND

Fig. 31 Same heart as in Fig.30. Fresh burette 5 and the same series of acetylcholine solutions in use. Controls satisfactory. Acetylcholine 10^{-17} g/ml produced gross effect. Hence for this heart the threshold sensitivity was 10^{-17} and stoppage concentration was 10^{-13} g/ml. Note that the effect of 10^{-15} is less than that from 10^{-17} g/ml.

Minor drifts in the venous pressure pen in Figs. 30 and 31 represent electronic artefacts and are of no significance.

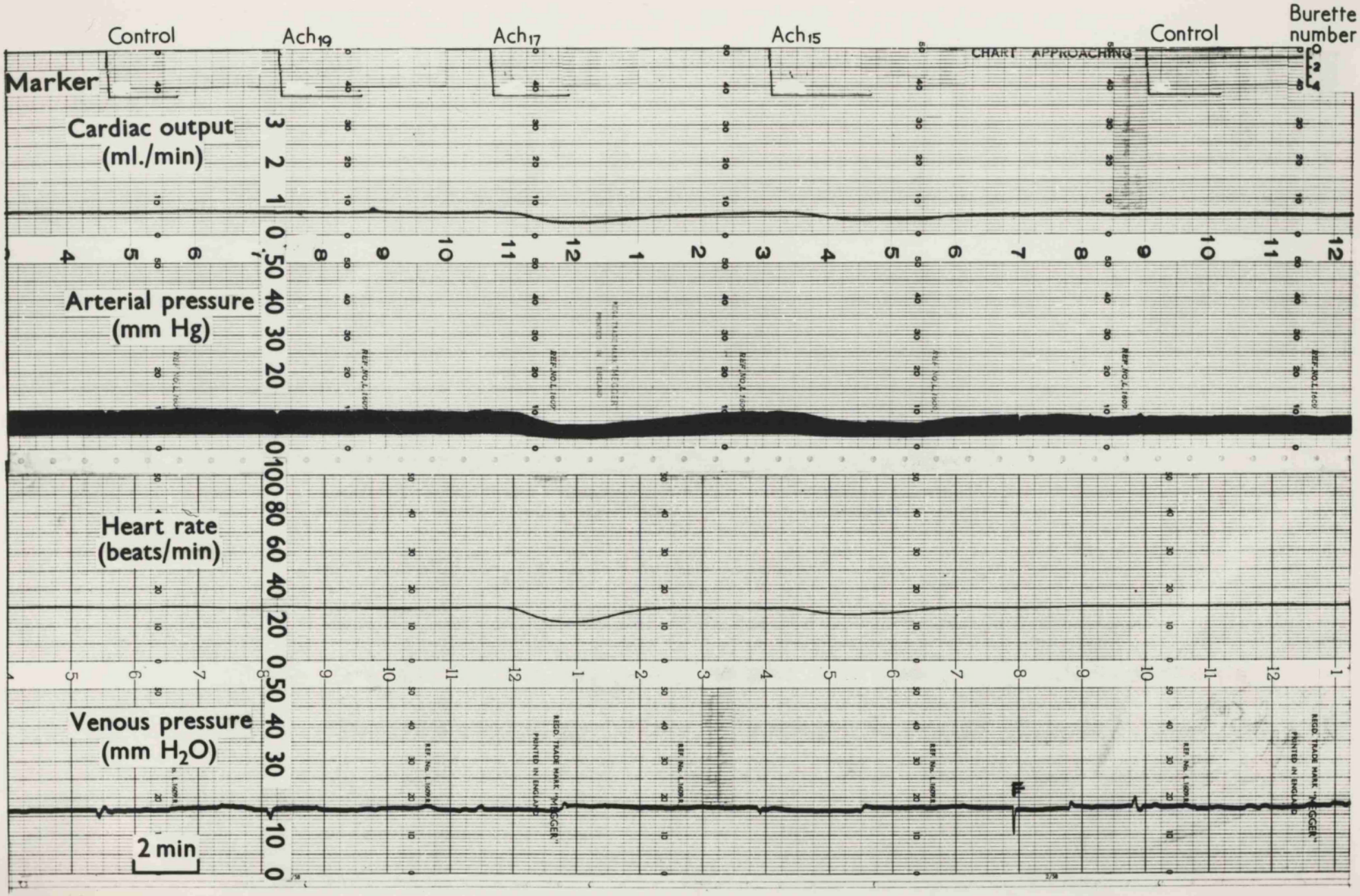

Control
Ach19
Ach17
Ach15
Control
Burette number
CHART APPROACHING
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig. 31

Fig. 32. Most highly sensitive heart. Initial control from burette 1 satisfactory. The word 'a' after the concentrations of acetylcholine mentioned at the top indicates a particular series of solutions called 'a' series. Acetylcholine 10^{-15} g/ml produced very large effect and still larger effects were produced by 10^{-13} and 10^{-11}. The concentrations 10^{-13} and 10^{-11} were tested for a lesser duration to allow a quick recovery. Subsequently two fresh series of solutions (called series b and c) were prepared and still lower concentrations of each series were tested and were found to be effective in this heart as shown in Fig. 33. Note the stability of venous pressure.

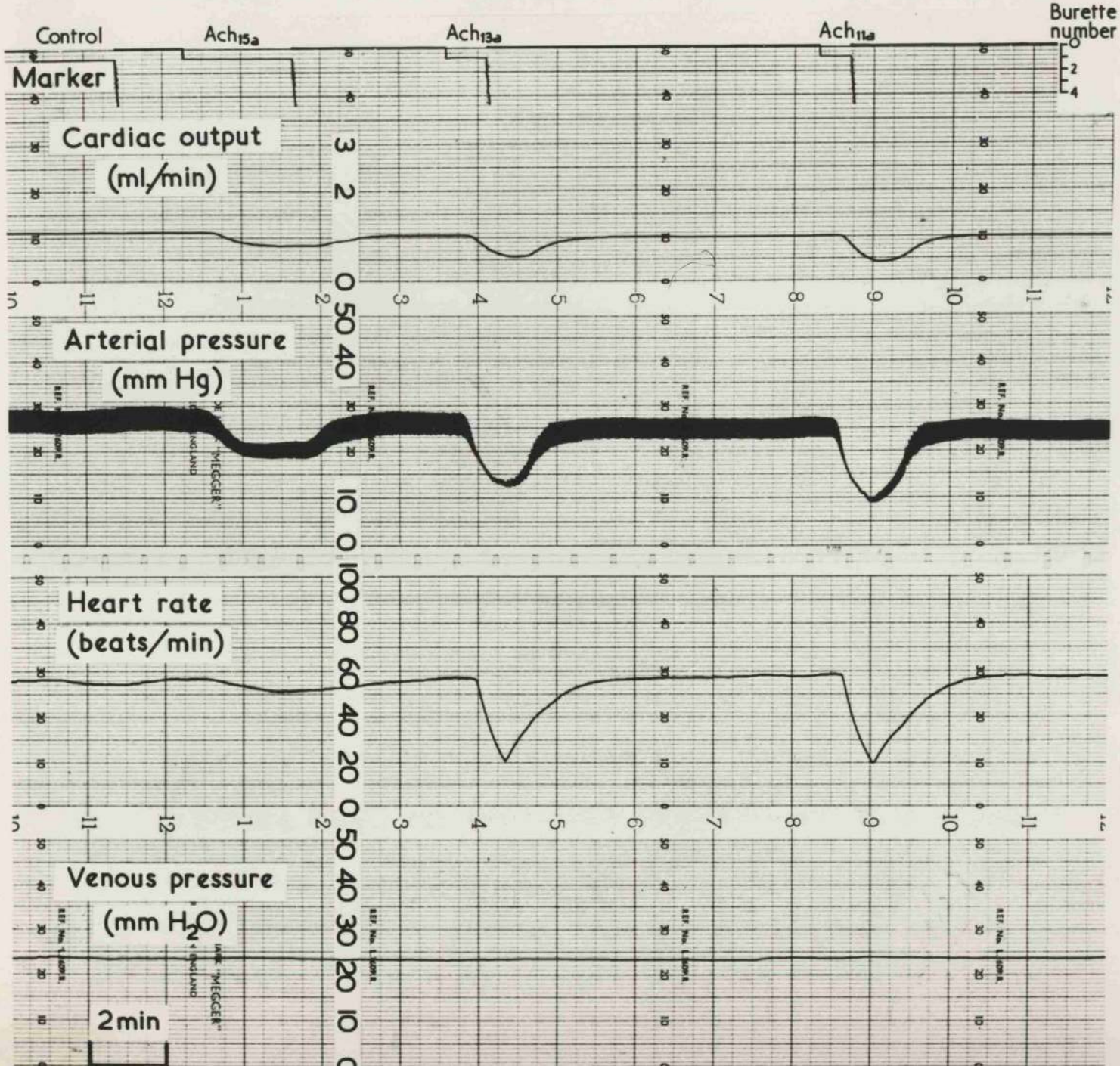

Control
Ach15a
Ach13a
Ach11a
Burette number
0
2
4
Marker
Cardiac output
(ml/min)
3
2
0
50
40
Arterial pressure
(mm Hg)
10
0
100
80
60
40
20
0
50
40
30
20
10
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2min

Fig. 33. Same heart as in Fig. 32. Both burette 1 and 2 fresh. Two fresh series (b and c) of solutions of acetylcholine in use. Controls satisfactory. Acetylcholine 10^{-23} g/ml of both (b) and (c) series produced only minor effects on the heart rate which are equal to those during controls and hence insignificant. Acetylcholine 10^{-21} (b) produced slightly greater effect than 10^{-23} (b) but it was also not very significant. The effect of 10^{-21} (c) was a still greater effect involving all the three variable parameters and was considered significant. The effect of 10^{-19} (b) was greater than that of 10^{-21}(b) but of the same order as that of 10^{-21}(c). The effect of 10^{-19} (c) was very large. The threshold sensitivity of this heart was considered to be 10^{-21} g/ml. The (b) and (c) series are obviously out of step by one dilution (i.e. there is 100 times difference in the corresponding strengths of solutions of two series) as shown by the tests though theoretically they contain the same amount of acetylcholine per ml. However both series of solutions individually produced progressively greater effect at higher concentrations. Note the absolute stability of venous pressure. It should also be appreciated that this Figure has undergone a much greater photographic reduction because of the length of the original record in

spite of the fact that part of the record showing repetition of the tests with 10^{-23}, 10^{-21} and 10^{-19} concentrations of both series, has been removed. The junction on the chart is visible as a faint white vertical line between points 1.0 and 7.0 after the second control from burette 2.

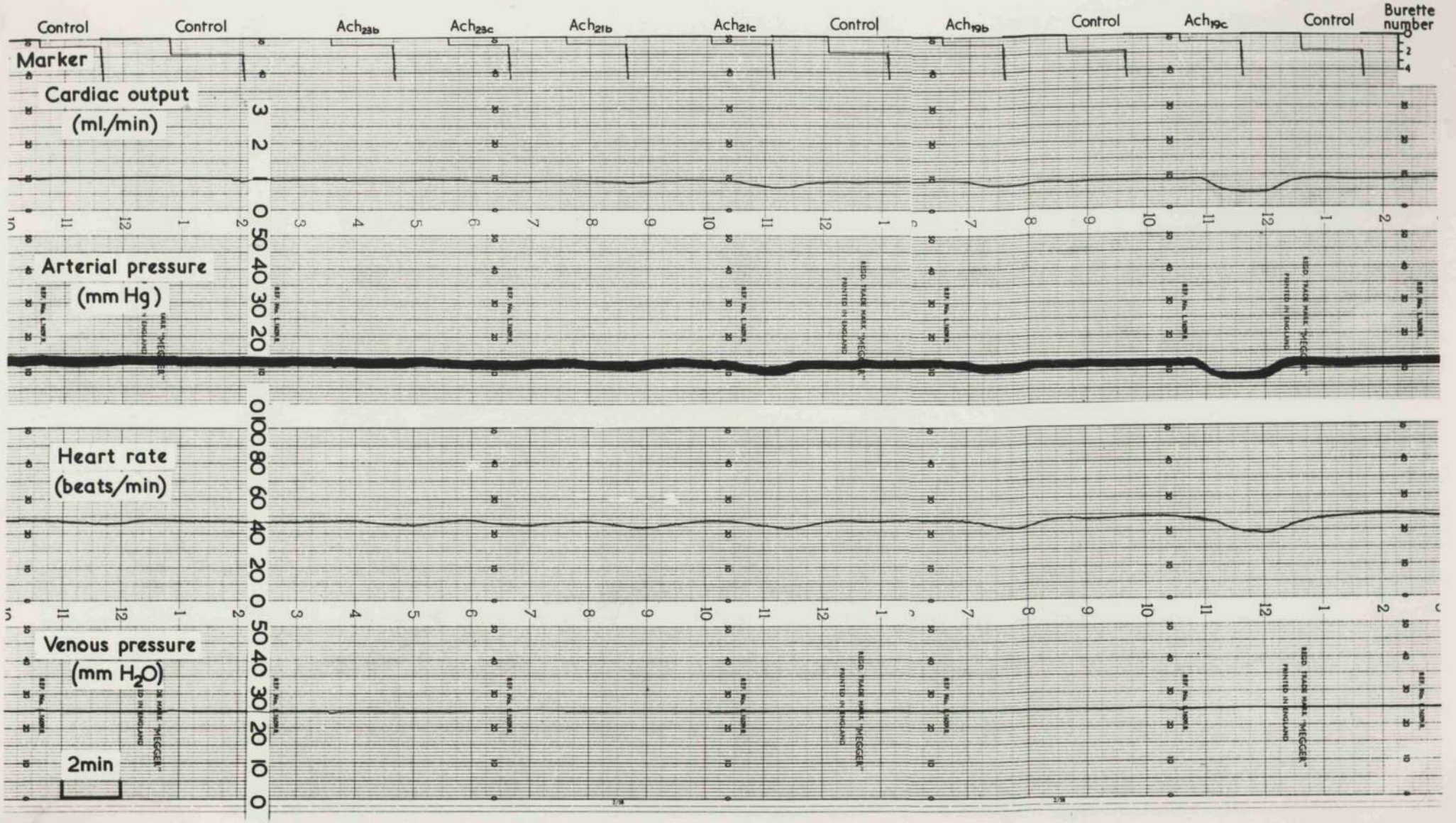

Control
Control
Ach23b
Ach23c
Ach21b
Ach21c
Control
Ach19b
Control
Ach19c
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2min

Fig. 33

Fig. 34. Distribution of 86 frogs according to the threshold sensitivity of their hearts to acetylcholine. Frogs in which the minimum effective concentration also stopped the heart are included in this Figure.

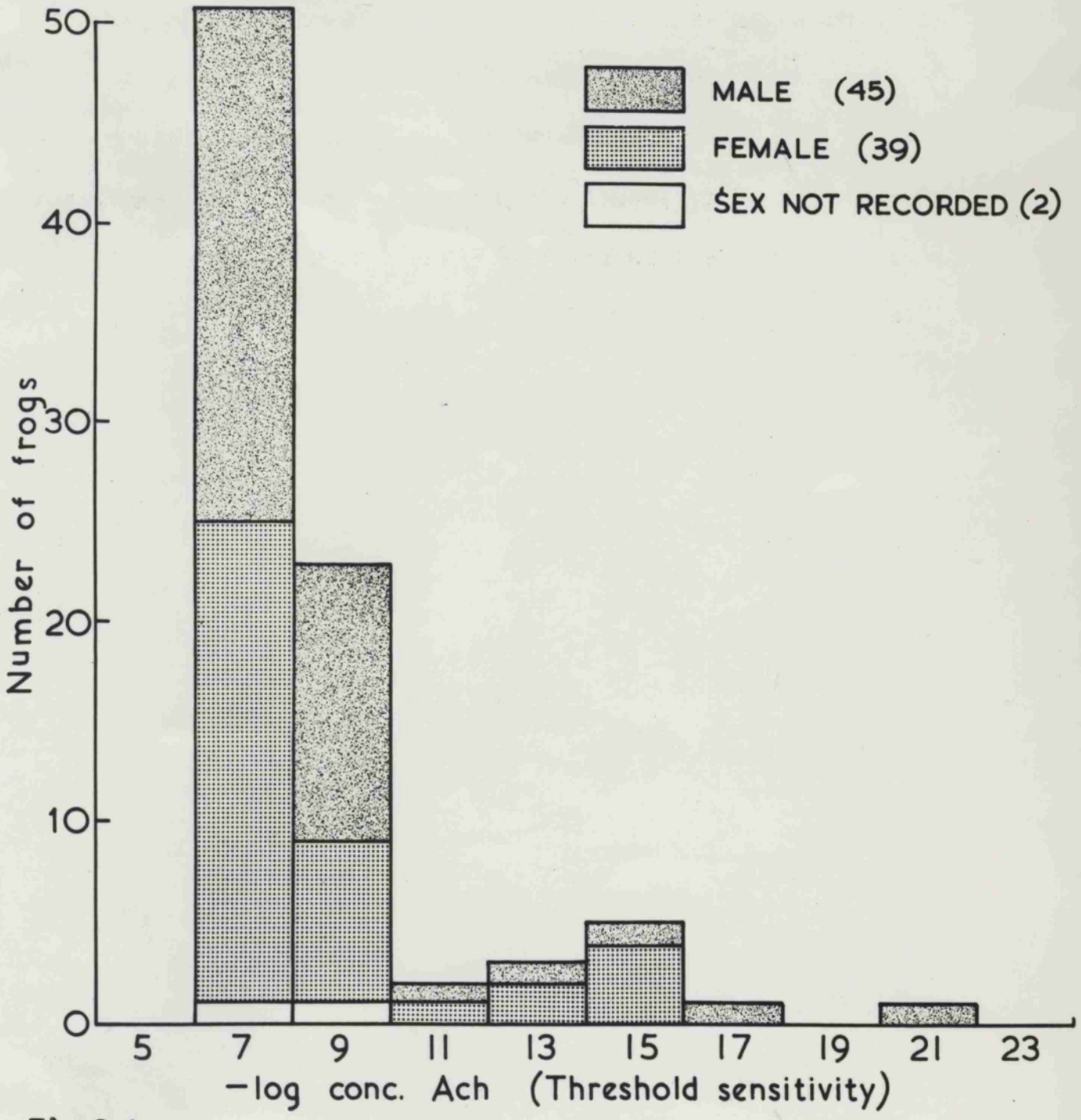

50
40
30
20
10
0
Number of frogs
MALE (45)
FEMALE (39)
SEX NOT RECORDED (2)
5
7
9
11
13
15
17
19
21
23
−log conc. Ach (Threshold sensitivity)

Fig.34

Fig. 35. Incidence of stoppage of heart in 73 frogs at different concentrations of acetylcholine. The actual number of frogs in which the heart was stopped at different concentrations is shown. The stoppage concentration was determined in the hearts of 73 out of the total of 86 frogs.

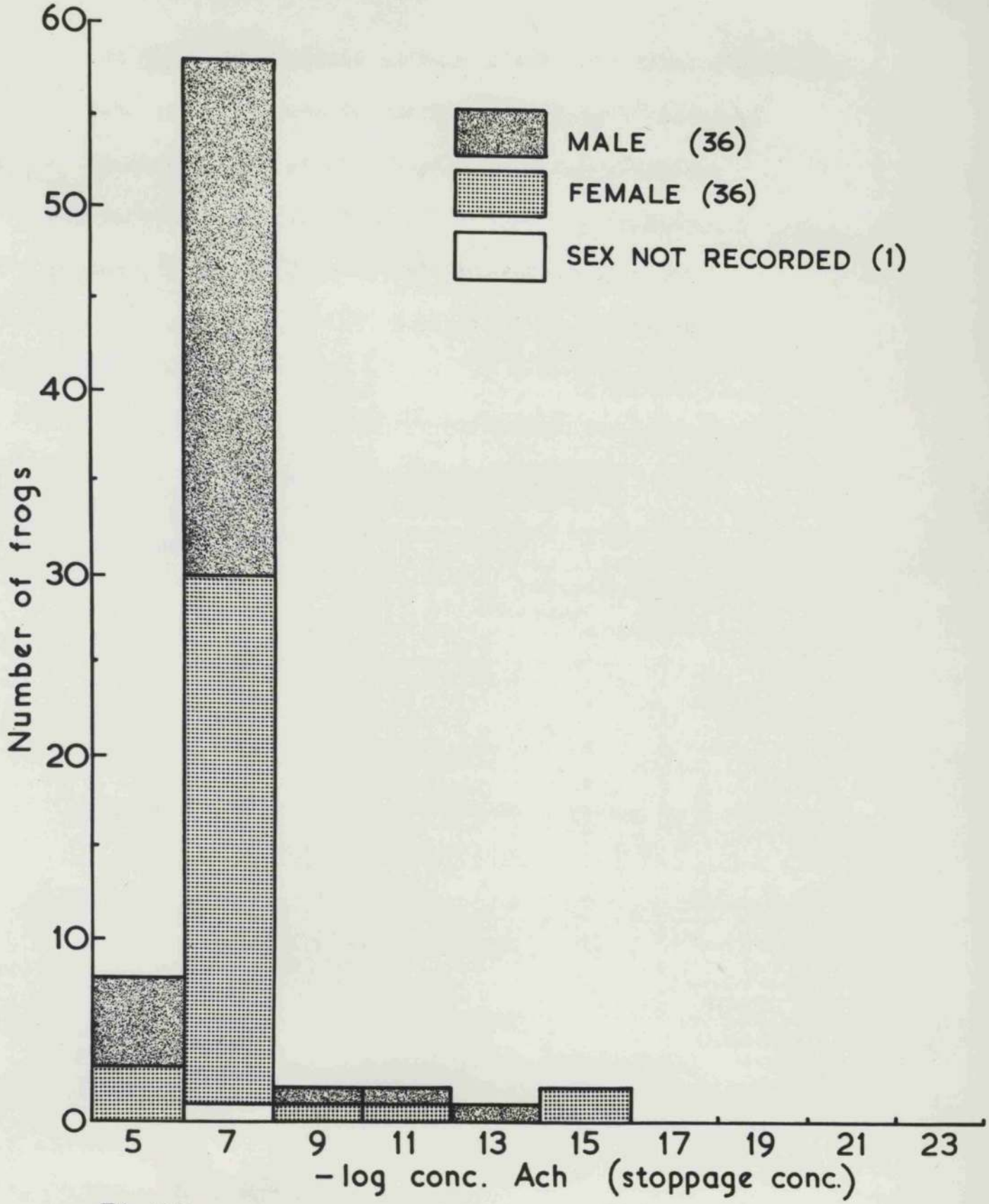

60
50
40
30
20
10
0
Number of frogs
MALE (36)
FEMALE (36)
SEX NOT RECORDED (1)
5
7
9
11
13
15
17
19
21
23
– log conc. Ach (stoppage conc.)

Fig.35

Fig. 36. Example of a heart showing stoppage at the concentration of 10^{-15} g/ml of acetylcholine. The initial controls from burette 1 and 2 are satisfactory. The rise in the output and blood pressure before the test perfusion with 10^{-15} is due to the rise in the rate, the venous pressure being quite constant throughout. Acetylcholine 10^{-15} stopped the heart within 30 seconds. Recording was stopped for a short time near point 6.45 to allow for recovery. The action is typical of acetylcholine. The heart recovered completely. Still lower concentrations were not tested in this heart.

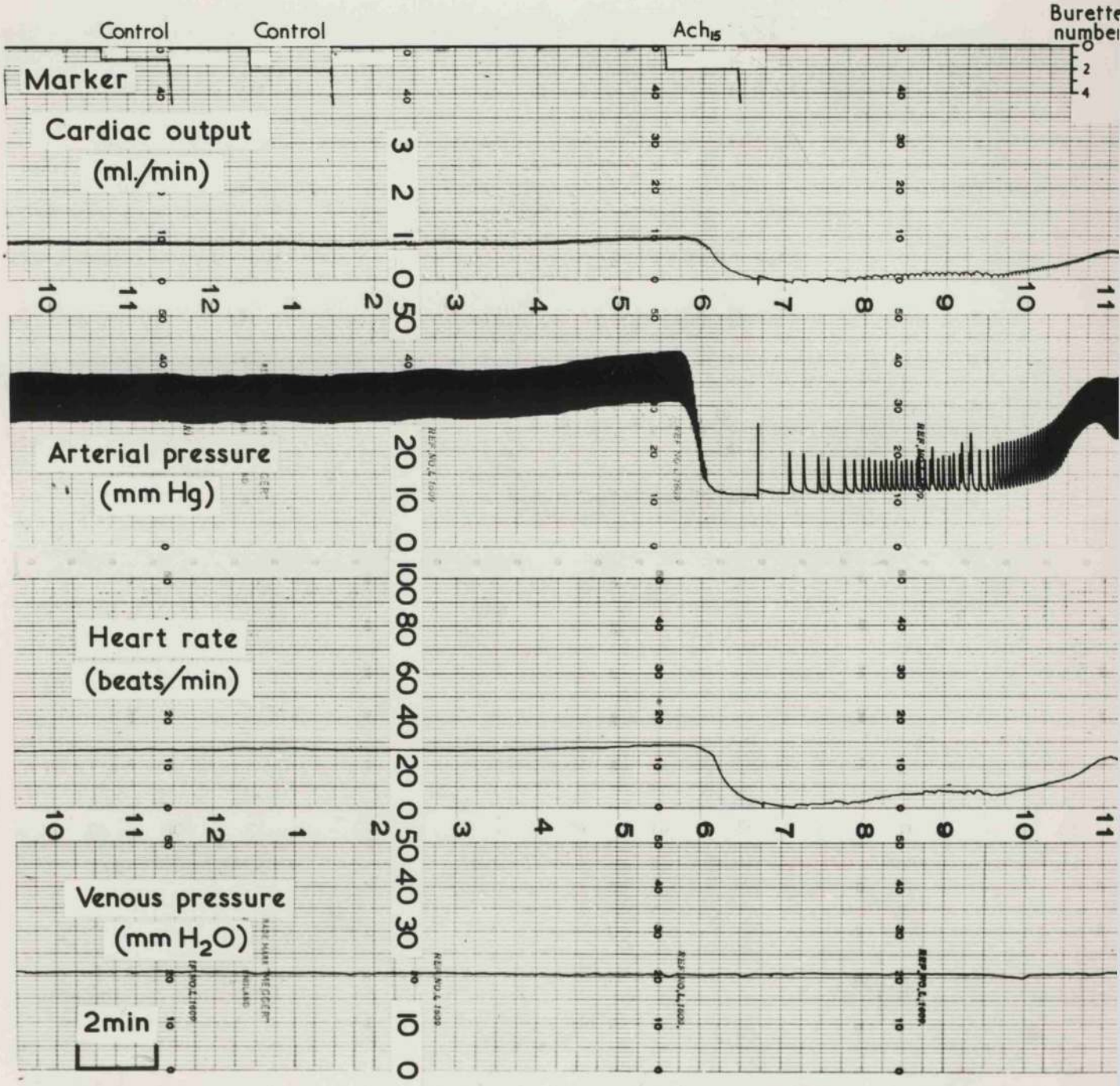

Control
Control
Ach15
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2min

Fig. 36

Fig. 37. Example of another highly sensitive heart showing stoppage for 30 seconds (due to complete conduction block) by acetylcholine 10^{-15} g/ml administered for only 1 minute (first test). Two subsequent tests with the same solution administered for still shorter periods produced powerful inhibitory effects due to transient complete conduction block. The large excursions in the pressure trace during the action of acetylcholine show that the muscle was not grossly inhibited. The pH of the control solution and of this solution of acetylcholine 10^{-15} g/ml was measured immediately after these tests and the difference in the pH was only 0.01 unit (known to be insufficient to influence the heart - see section on pH).

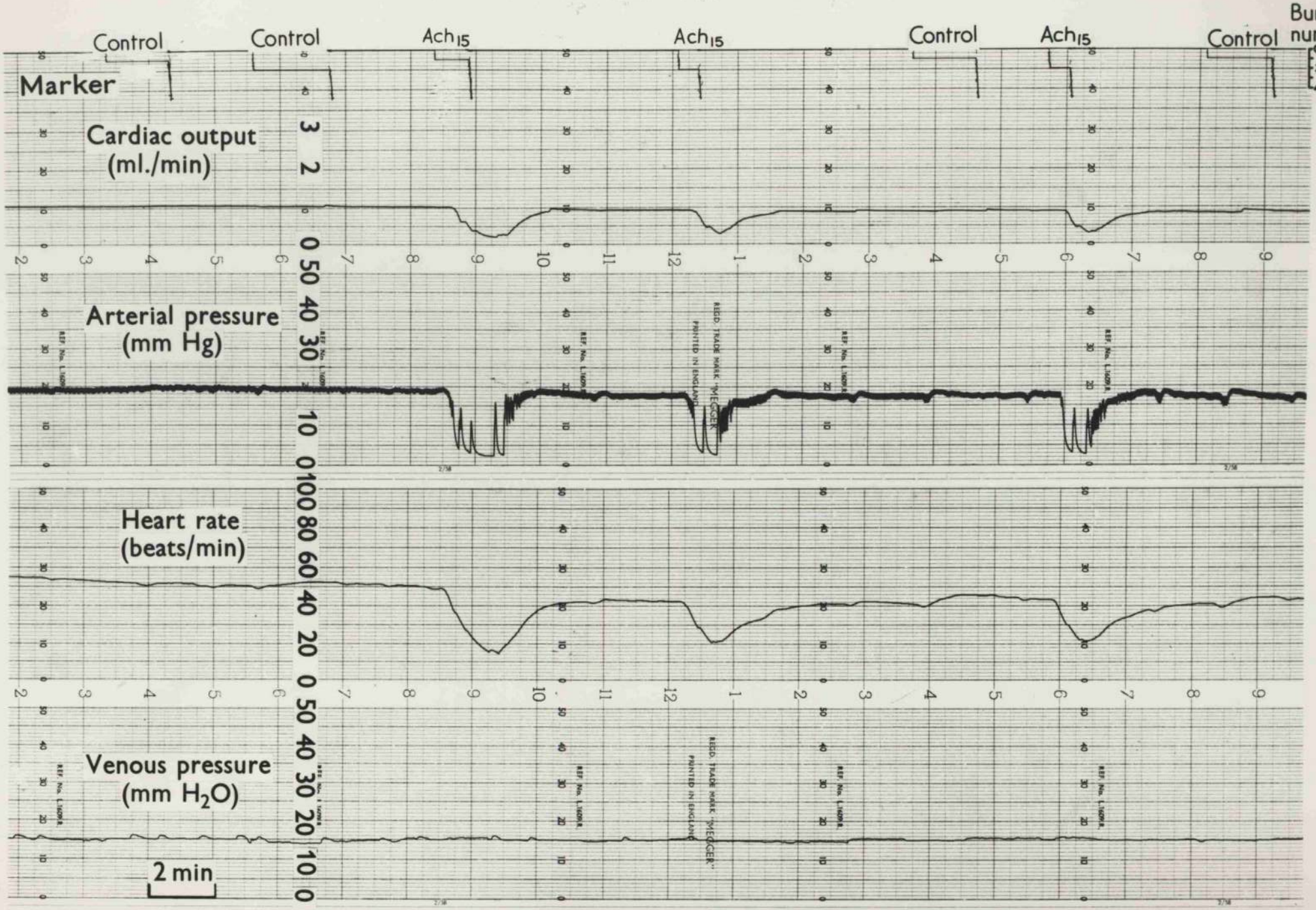

Control
Control
Ach15
Ach15
Control
Ach15
Control
Burett numbe
0
2
4
Marker
Cardiac output (ml./min)
3 2 1 0
Arterial pressure (mm Hg)
50 40 30 20 10 0
Heart rate (beats/min)
100 80 60 40 20 0
Venous pressure (mm H2O)
50 40 30 20 10 0
2 min

Fig. 37

Fig. 38 The probable incidence of effect from different concentrations of acetylcholine in the hearts of 86 frogs. The number of frogs in which the heart was or would have been affected by different concentrations of acetylcholine is shown (see text). The incidence of effect increased progressively as the concentration of acetylcholine increased, till the hearts of all the 86 frogs were involved at the concentration of 10^{-7} g/ml. The relationship between the concentration of acetylcholine and the number of frogs in which the heart would have been affected, is roughly exponential.

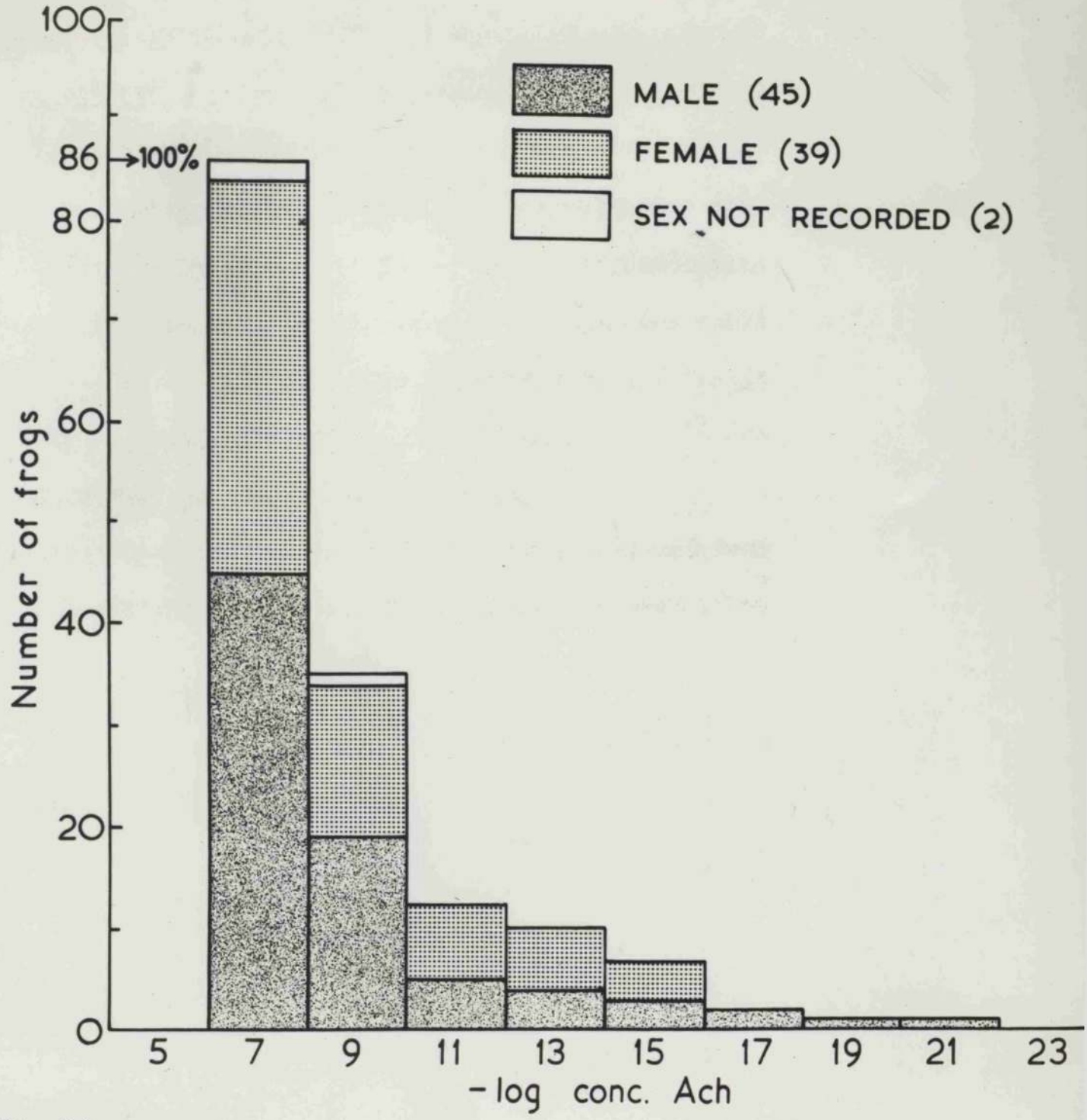

100
86
→100%
80
60
40
20
0
Number of frogs
MALE (45)
FEMALE (39)
SEX NOT RECORDED (2)
5
7
9
11
13
15
17
19
21
23
- log conc. Ach

Fig.38

Fig. 39. Histogram showing the minimum effective concentration (dark shading) and stoppage concentration (light shading) in 16 hearts showing a low sensitivity to acetylcholine.

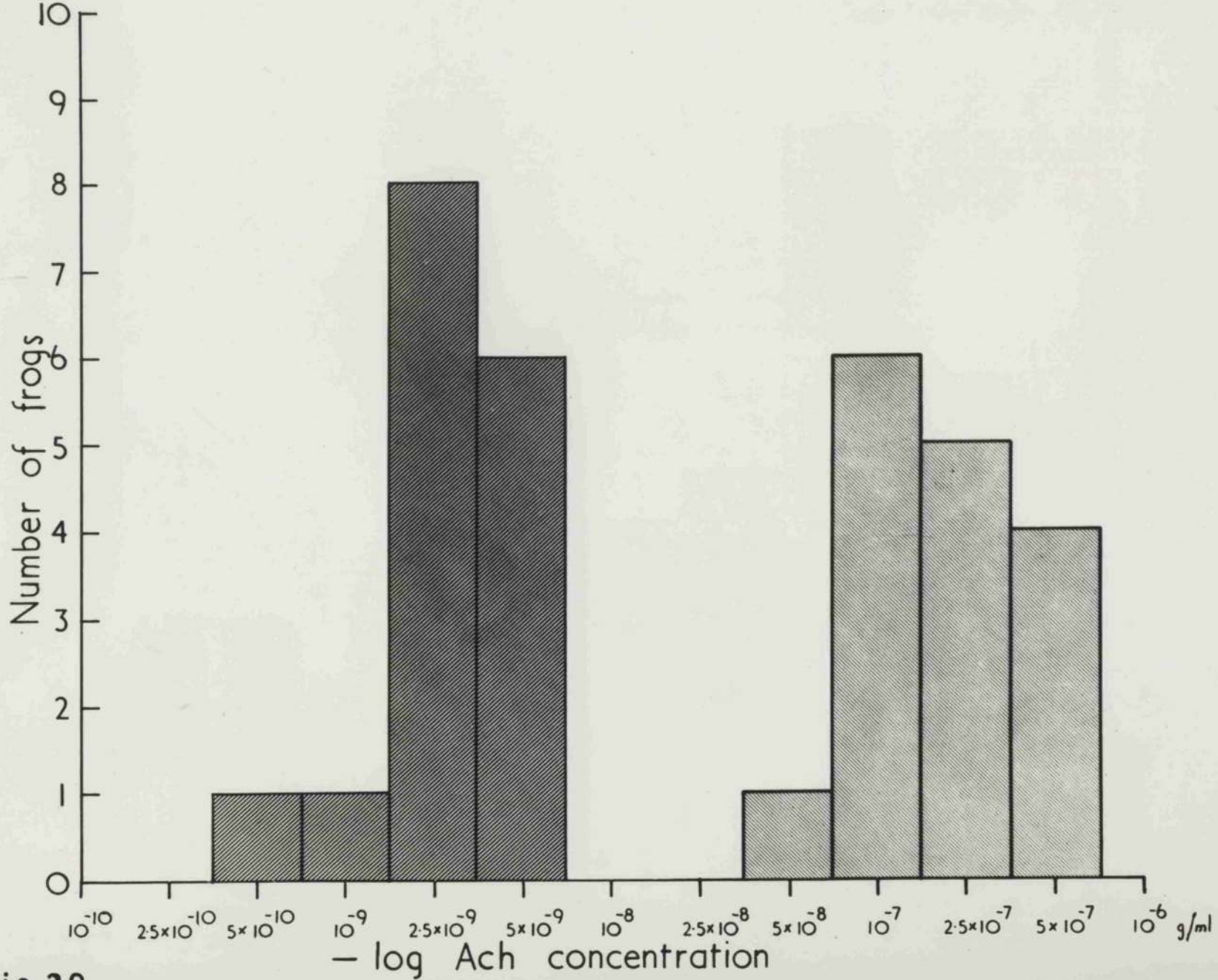

Number of frogs
0
1
2
3
4
5
6
7
8
9
10
10^-10
2·5×10^-10
5×10^-10
10^-9
2·5×10^-9
5×10^-9
10^-8
2·5×10^-8
5×10^-8
10^-7
2·5×10^-7
5×10^-7
10^-6 g/ml
— log Ach concentration

Fig. 39

Fig. 40. Seasonal distribution of experiments on 108 frog hearts. Dr. Boyd's experiments conducted in 1956 and 1957 on 22 hearts are shown by blank areas. The shaded area represents experiments conducted by me on 86 hearts as considered in Fig. 34.

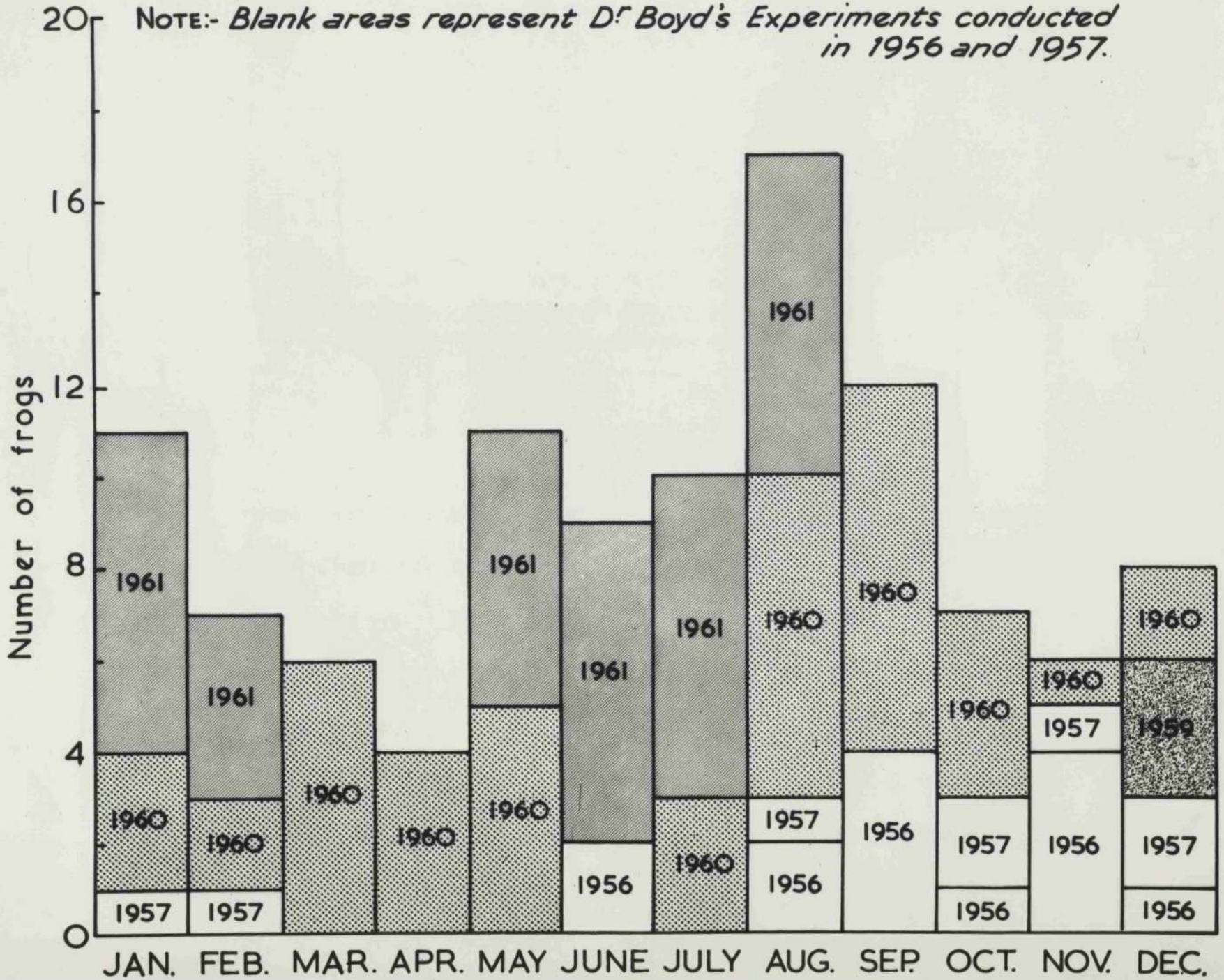

NOTE:- Blank areas represent Dr Boyd's Experiments conducted in 1956 and 1957.
Number of frogs
20
16
12
8
4
0
JAN.
FEB.
MAR.
APR.
MAY
JUNE
JULY
AUG.
SEP.
OCT.
NOV.
DEC.
1961
1960
1957
1956
1959

Fig. 41. Histogram showing seasonal variation in the percentage of incidence of sensitive hearts. The number at the top of each column indicates the total number of frogs in which the heart was tested in that month. The height of the unshaded column indicates the percentage of this number, in which the heart was sensitive to acetylcholine at concentrations lower than 10^{-7} i.e. from 10^{-21} to 10^{-9} g/ml. The height of the shaded column indicates percentage in which the heart was sensitive to concentrations lower than 10^{-9} i.e. from 10^{-21} to 10^{-11} g/ml.

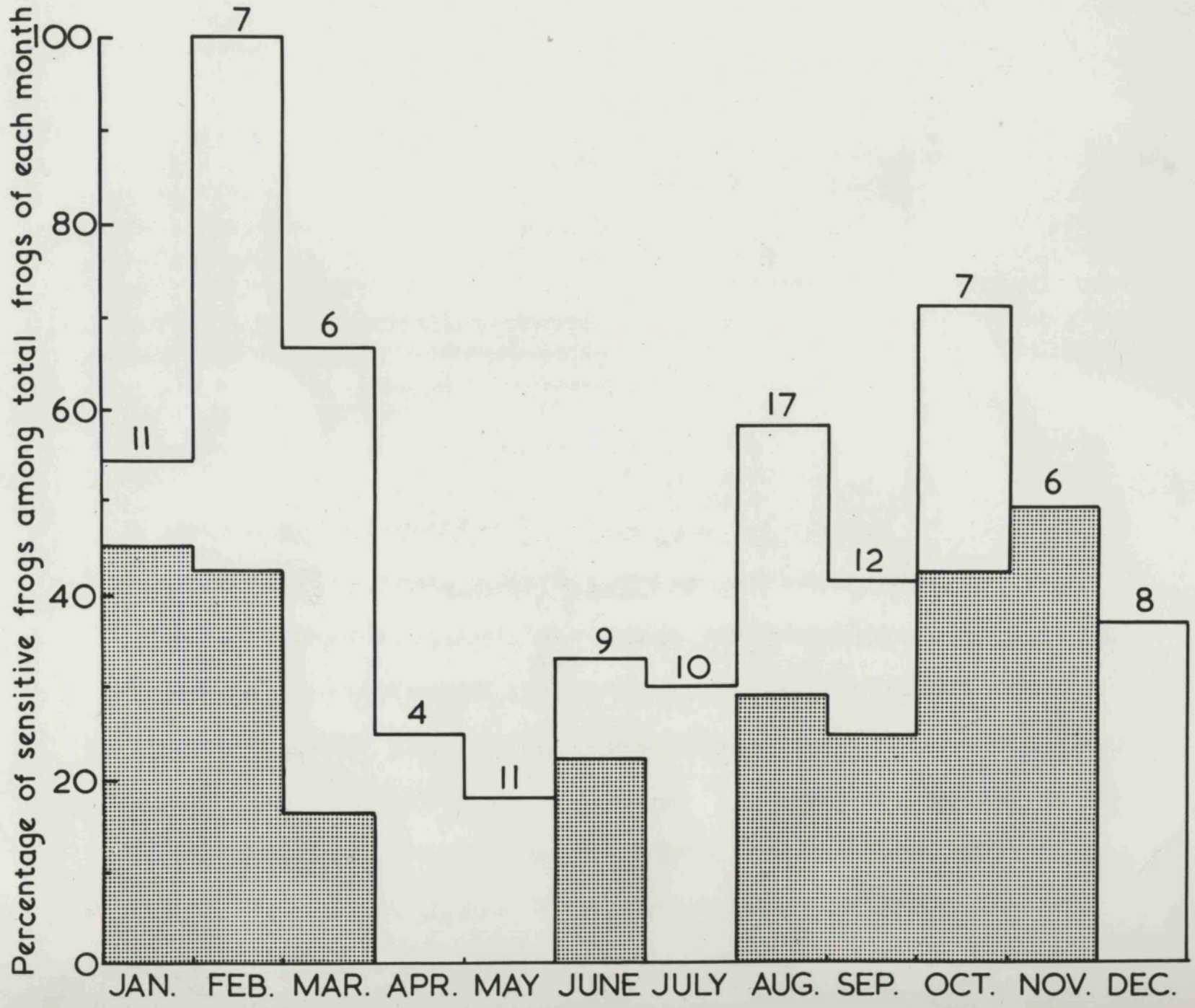

Percentage of sensitive frogs among total frogs of each month
100
80
60
40
20
0
7
6
11
4
11
9
10
17
12
7
6
8
JAN.
FEB.
MAR.
APR.
MAY
JUNE
JULY
AUG.
SEP.
OCT.
NOV.
DEC.

Fig. 42 Histogram showing the seasonal variation in the percentage of incidence of threshold sensitivity at concentrations lower than 10^{-7} g/ml (i.e. from 10^{-21} to 10^{-9}) in the heart of male and female frogs. The total number of frogs in which the heart was tested in each month is given at the top of the column for both sexes separately. The percentage incidence of sensitivity has been derived individually for the two sexes from the total number of each sex.

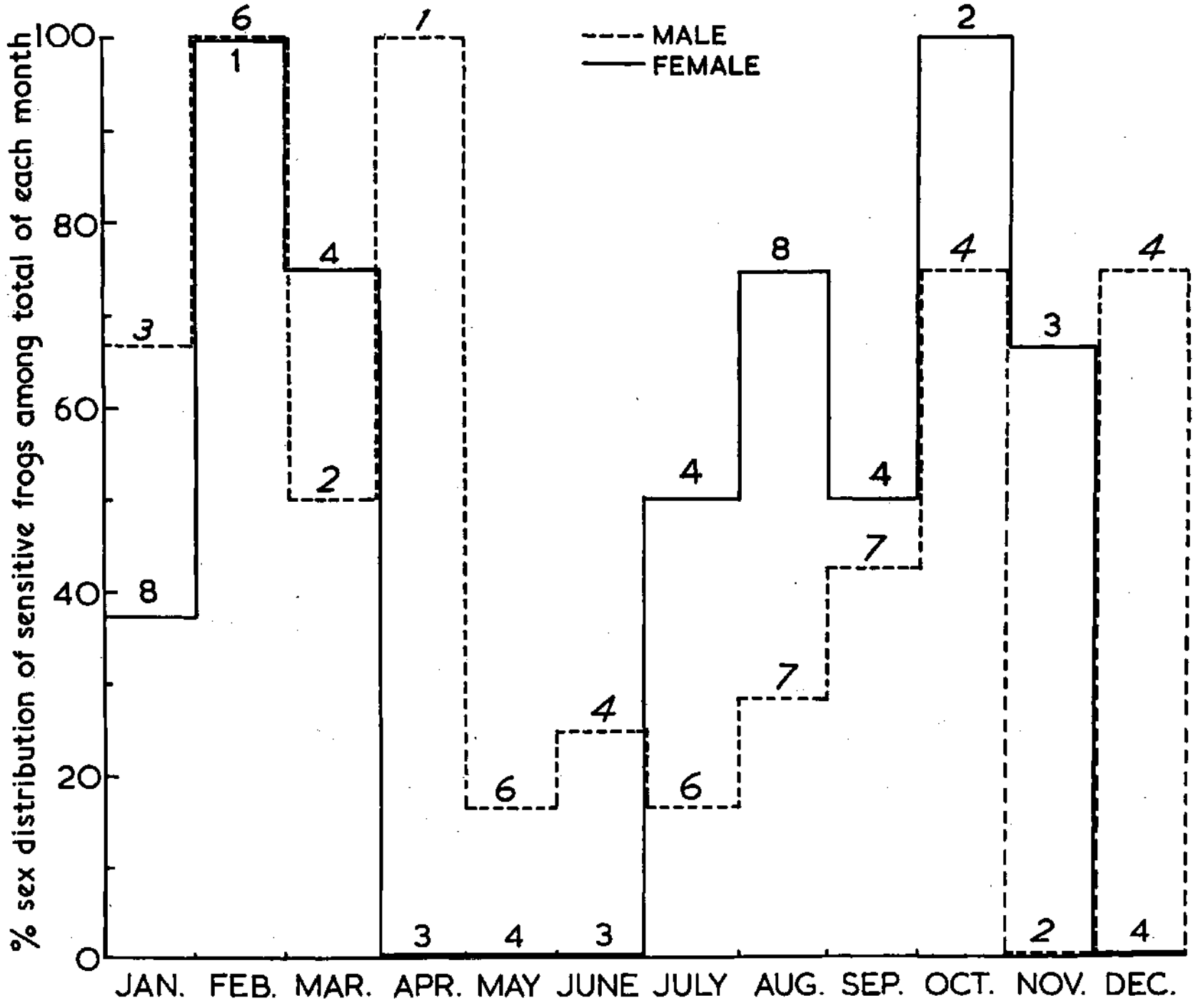

MALE
FEMALE
% sex distribution of sensitive frogs among total of each month
100
80
60
40
20
0
JAN.
FEB.
MAR.
APR.
MAY
JUNE
JULY
AUG.
SEP.
OCT.
NOV.
DEC.
8
1
6
3
4
2
3
6
4
4
6
4
7
7
8
4
2
3
4
4

Fig. 43. Gross irregularity of rhythm induced by the high concentration of calcium (150% of normal) in the Ringer's solution perfused from the reservoir and regularisation of rhythm on perfusing with normal Ringer's solution from burette 2. The square shaped elevation on the blood pressure trace is due to electronic artefact (A). The discontinuity in the record is due to the removal of a part of the original record for shortening the length. Changing to the Ringer's solution containing a high concentration of calcium between points 12.0 and 1.15 again caused irregularity of rhythm. The recurrence of irregularity is more clear in Figure 44 which represents nearly original size of the upper part of the record. The controls with normal Ringer's solution from burette 1 indicate that no other factor could be responsible for the irregularity of rhythm.

Burette number
0
2
4
Control
High Calcium
Control
Normal Calcium
High Calcium
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
A
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND

Burette number
0
2
4
7
6
5
4
3
2
1
12
11
50
40
30
20
10
0
Control
High Calcium
REF. No. L.
REF. No.
REGD. TRADE MAR
EGGER"
PRINTED IN
ND

Fig. 45. Threshold sensitivity to acetylcholine and stoppage concentration of acetylcholine in the hearts of frogs injected with oestradiol in 1960 - 1961.

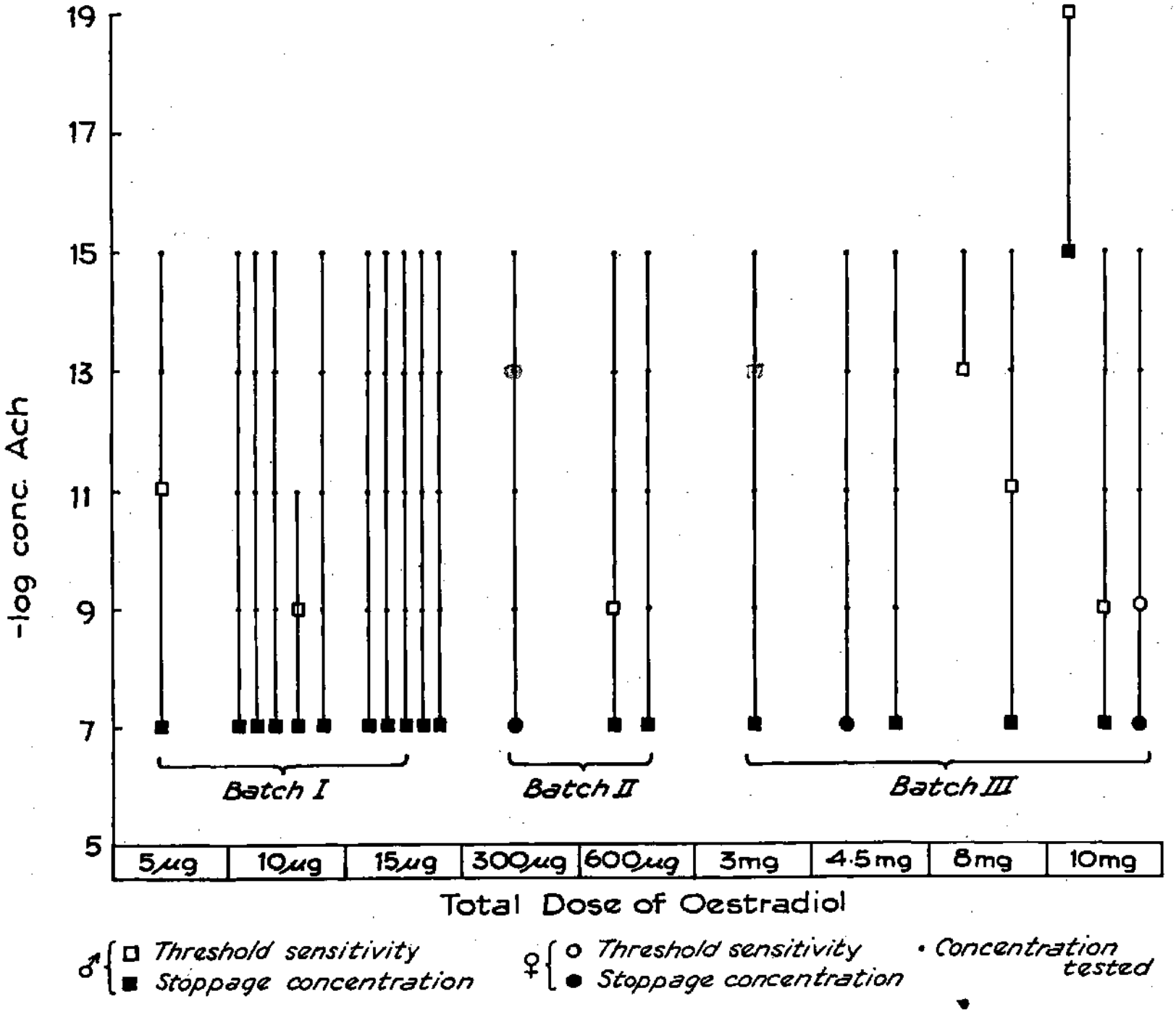

19
17
15
13
11
9
7
5
-log conc. Ach
Batch I
Batch II
Batch III
5μg
10μg
15μg
300μg
600μg
3mg
4·5mg
8mg
10mg
Total Dose of Oestradiol
♂ □ Threshold sensitivity
■ Stoppage concentration
♀ ○ Threshold sensitivity
● Stoppage concentration
• Concentration tested

Fig. 46. Slight increase in the effect of acetylcholine 10^{-9} g/ml as a result of perfusion with Ringer's solution containing oestradiol 25 mg/100 ml from burette 2 (2R + ostradiol). Initial tests with increasing concentrations of acetylcholine indicated that the heart showed threshold sensitivity at 10^{-9} g/ml and stoppage at 10^{-7} g/ml. The action of 10^{-9} was very definite although it is not very prominent in the photograph due to reduction in size. Part of the record at J between points 7.0 and 6.0 containing other tests has been removed. Subsequent test perfusion with 10^{-9} during the period when the heart had stabilised on Ringer's solution containing oestradiol showed a slightly greater effect. The threshold sensitivity, however, remained unaltered. The increase of response to an effective concentration was however observed only occassionally and cannot be directly attributed to perfusion with oestradiol.

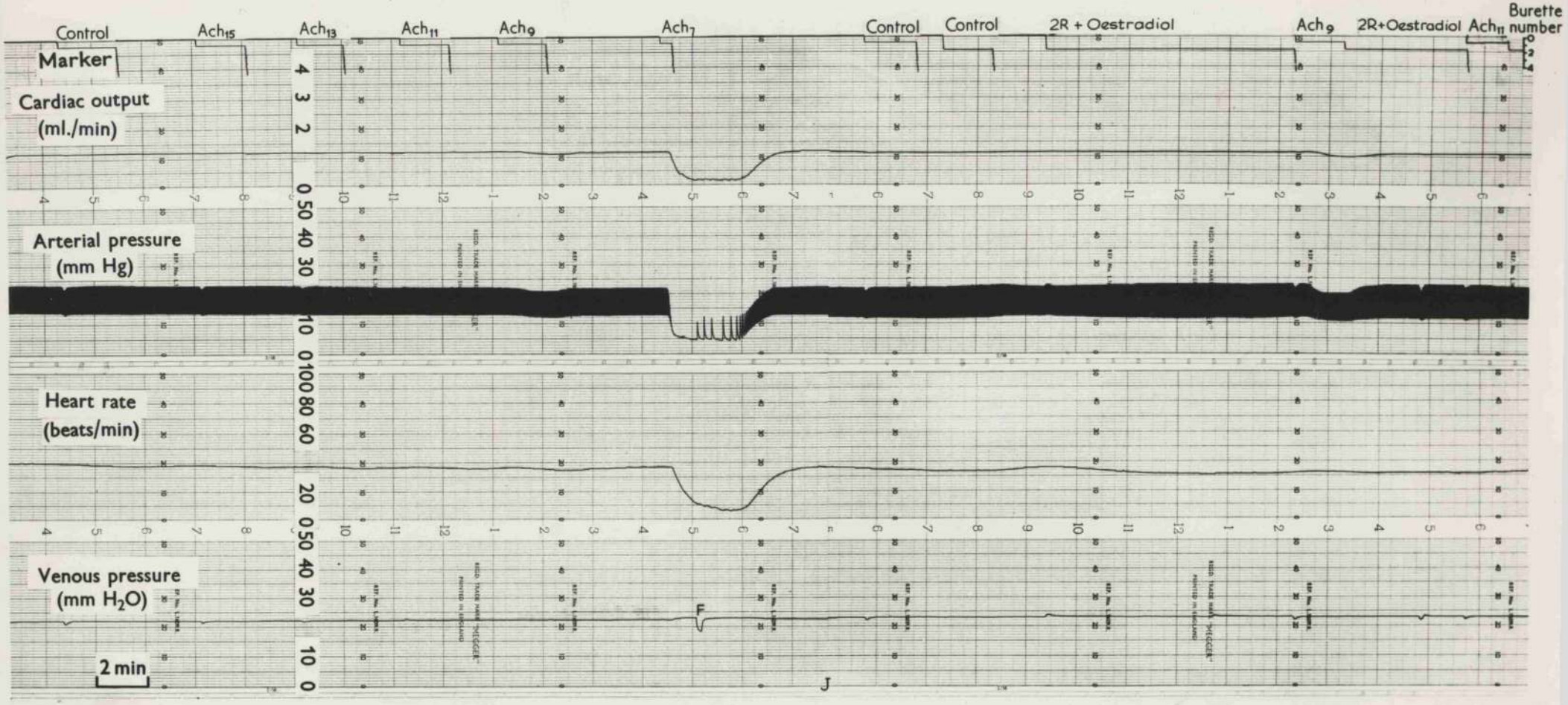

Control
Ach15
Ach13
Ach11
Ach9
Ach7
Control
Control
2R + Oestradiol
Ach 9
2R+Oestradiol
Ach11
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig. 4.6

Fig. 47 Rebound acceleration of heart with associated decrease in the amplitude (pulse pressure) after the action of acetylcholine in a concentration of 10^{-13} g/ml. The heart was being perfused continuously from burette 5 containing control Ringer's solution in this experiment. Test perfusions were conducted from burette 4. The maximum degree of acceleration was reached after the second test perfusion. The persistence of systolic pressure at a lower level after the first perfusion is mainly due to increase in the heart rate. Control perfusions from burette 4 were satisfactory (not shown here). Acetylcholine in a concentration of 10^{-15} had no effect. The rebound acceleration typically occurred during the recovery phase and never occurred during the test perfusion with acetylcholine when the heart was actually exposed to the drug.

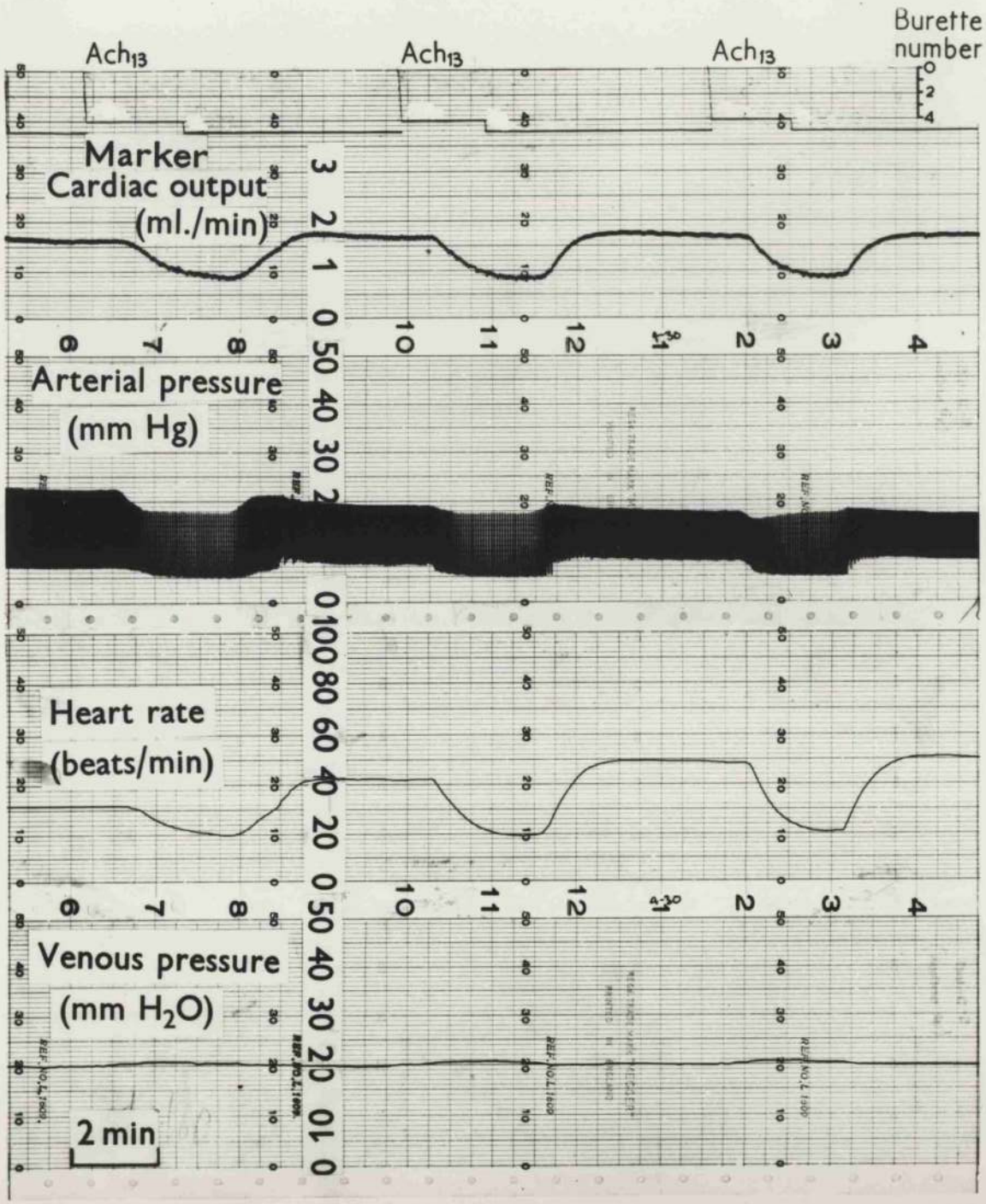

Ach13
Ach13
Ach13
Burette number
0
2
4
Marker
Cardiac output
(ml./min)
3 2 1 0
6 7 8 10 11 12 2 3 4
Arterial pressure
(mm Hg)
50 40 30 2
Heart rate
(beats/min)
0 100 80 60 40 20 0
Venous pressure
(mm H2O)
50 40 30 20 10 0
2 min

Fig 47

Fig. 48 Stoppage of the heart by acetylcholine in a concentration of 10^{-7} g/ml. Minor changes in heart rate during test perfusions with still lower concentrations are of no significance. Note the rebound increase in the amplitude and rate during the recovery from the action of acetylcholine 10^{-7} g/ml.

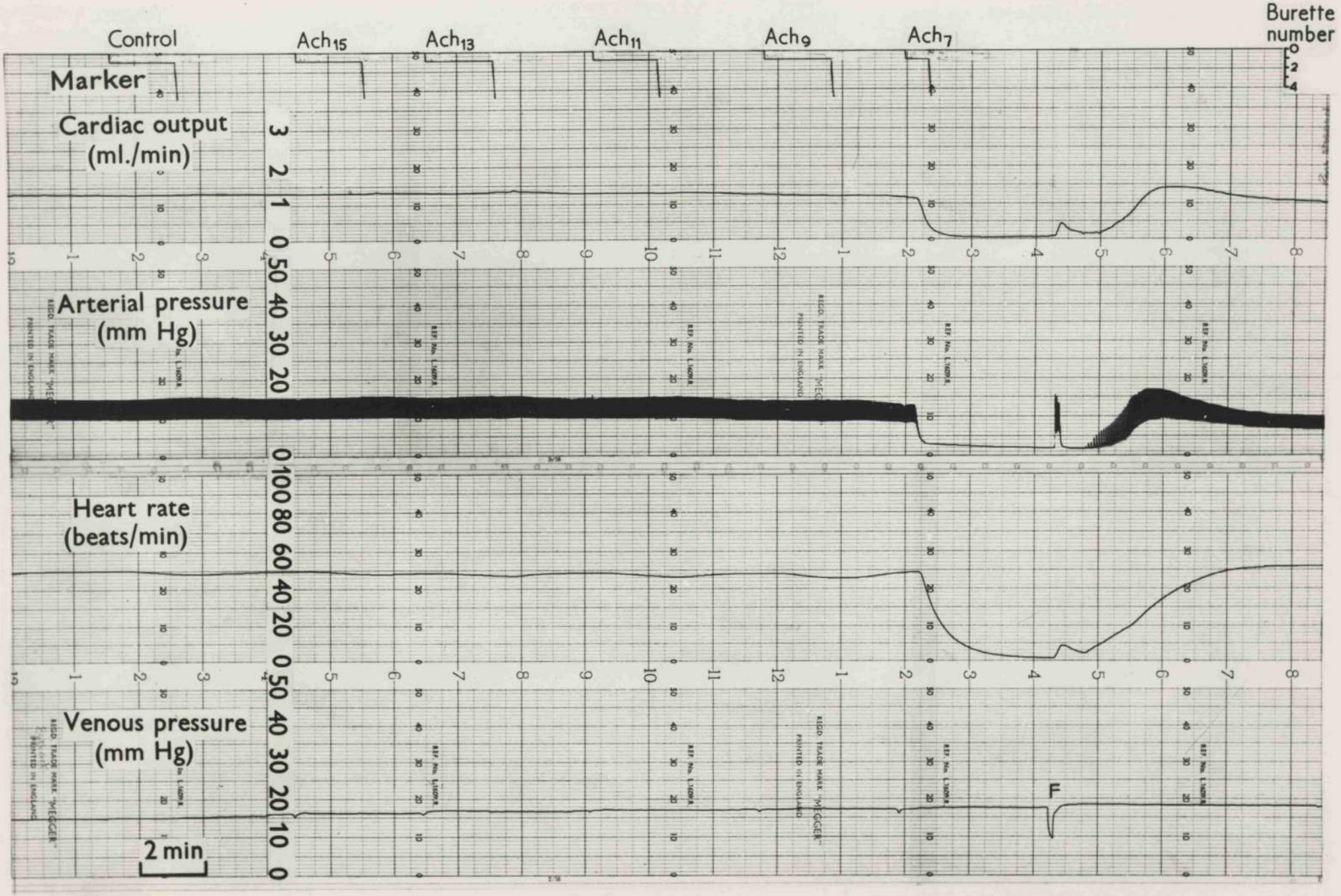

Control
Ach15
Ach13
Ach11
Ach9
Ach7
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm Hg)
2 min

Fig. 48

Fig. 49. Stoppage of the heart by acetylcholine in a concentration of 10^{-9} g/ml, the lowest effective concentration for this heart. The slight progressive decrease in the output and blood pressure are due to slow recovery of heart rate from the action of adrenaline tested previously in this heart. The amplitude had recovered completely as evident from the pulse pressure. Minor changes in the heart rate during the control and test perfusions with 10^{-12} and 10^{-11} are superimposed upon the progressive fall in the heart rate and are of no significance. The low value of output was due to the development of a leak in the common outlet chamber of the resistance unit and is also of no special significance. The action of 10^{-9} is typical of acetylcholine. The rise in the venous pressure during the stoppage of the heart is quite prominent in this case. The recording was stopped (S) near point 4.0 for a short time to allow for recovery and during this period the leak in the resistance unit was sealed. The subsequent increase in output, blood pressure and heart rate is due to several factors i.e. sealing of the resistance unit, possible rebound acceleration after the action of 10^{-9} and lowering of venous pressure. The action of 10^{-9} was confirmed later when the heart had become fully stable (not shown here).

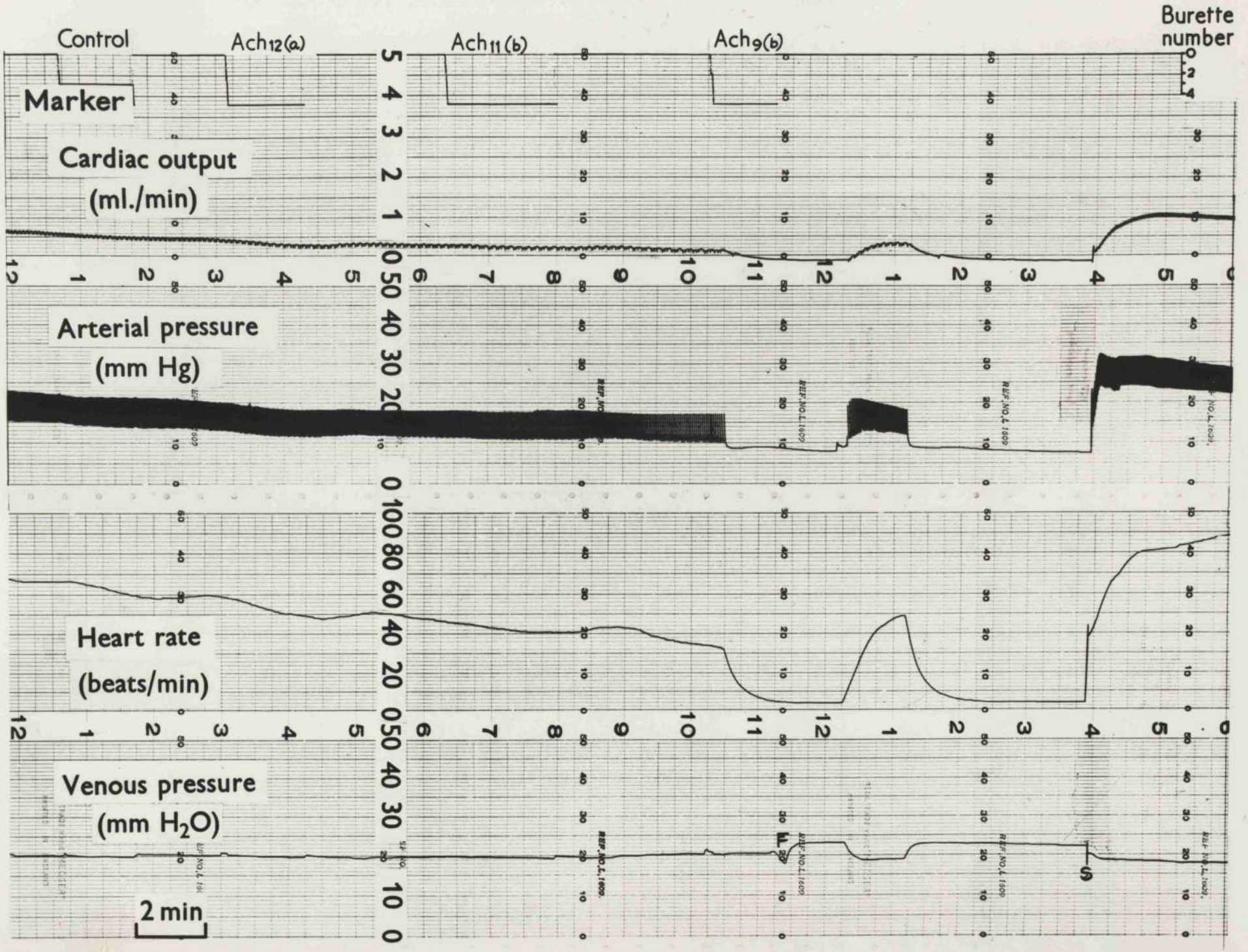

Control
Ach12(a)
Ach11(b)
Ach9(b)
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2 min

Fig. 50 Stoppage of the heart by acetylcholine in a concentration of 10^{-11} g/ml. The controls at the beginning and at the end (not labelled) from burette 5 were satisfactory. The test perfusion with 10^{-13}, without any effect, serves as a control from burette 4. Note the prolonged rebound acceleration during recovery, the acceleration being more marked after the second test perfusion with 10^{-11}. There is an associated decrease in the amplitude (pulse pressure). These changes are reflected in the output and blood pressure. The scale of 0 - 100/min on the heart rate trace is not applicable in this experiment, in which the full scale deflection was 0 - 50/min.

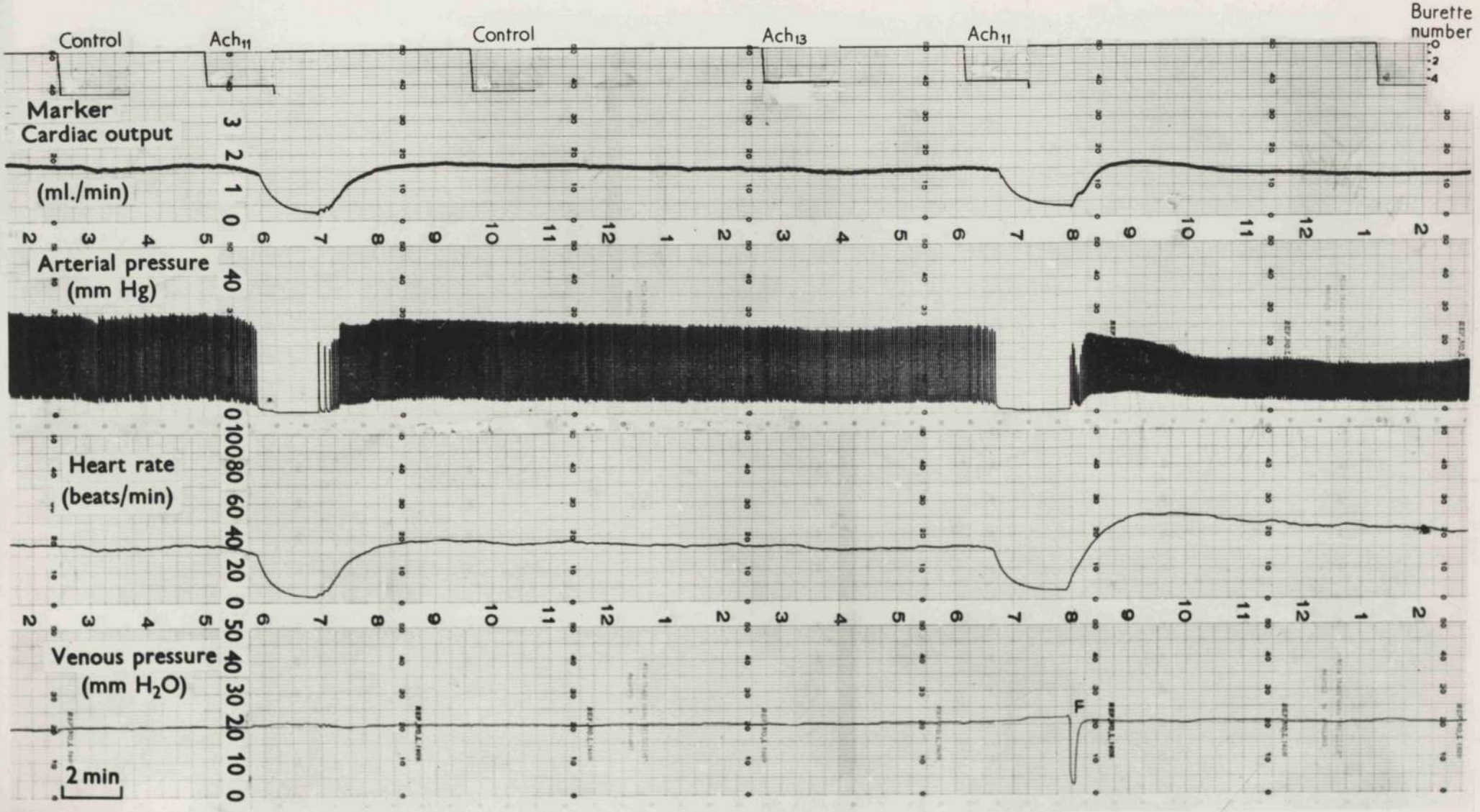
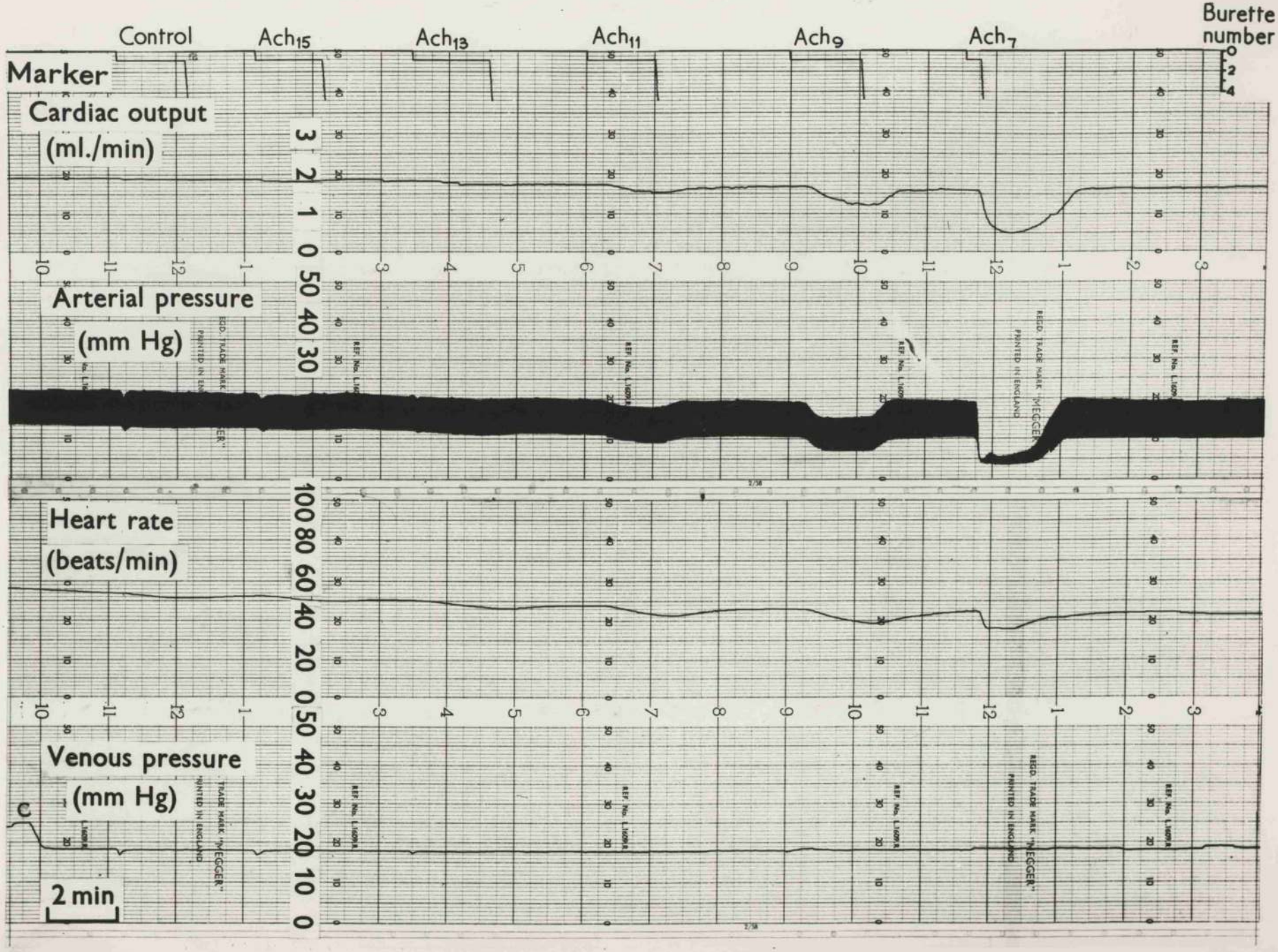

Control
Ach11
Control
Ach13
Ach11
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2 min

Fig. 50

Fig. 51 Progressively increasing response to increasing concentrations of acetylcholine. The inhibitory effect increased as the concentration of acetylcholine was increased from 10^{-11} to 10^{-7} g/ml. 10^{-7} g/ml administered only for 30 seconds produced powerful inhibition. If it had been perfused for two minutes as usual, probably it would have stopped the heart.



C = Calibration of V.P.

Fig 5.1

Fig. 52. Concentration-response curves of three highly sensitive hearts (a, b and c). Symbols and notations as in Fig. 13. Note wide range of concentration on abscissa. Several values at one concentration represent confirmatory tests.

(a) closed circles - values from tests with a fresh series (about 1 hour old).

open circles - values from tests with the same series when 8 hours old

triangles - values from tests with a second series which was 24 hours old.

(b) filled circles - values from tests with a fresh series

triangles - values from tests with another fresh series.

(c) closed circles - values from tests with fresh solutions.

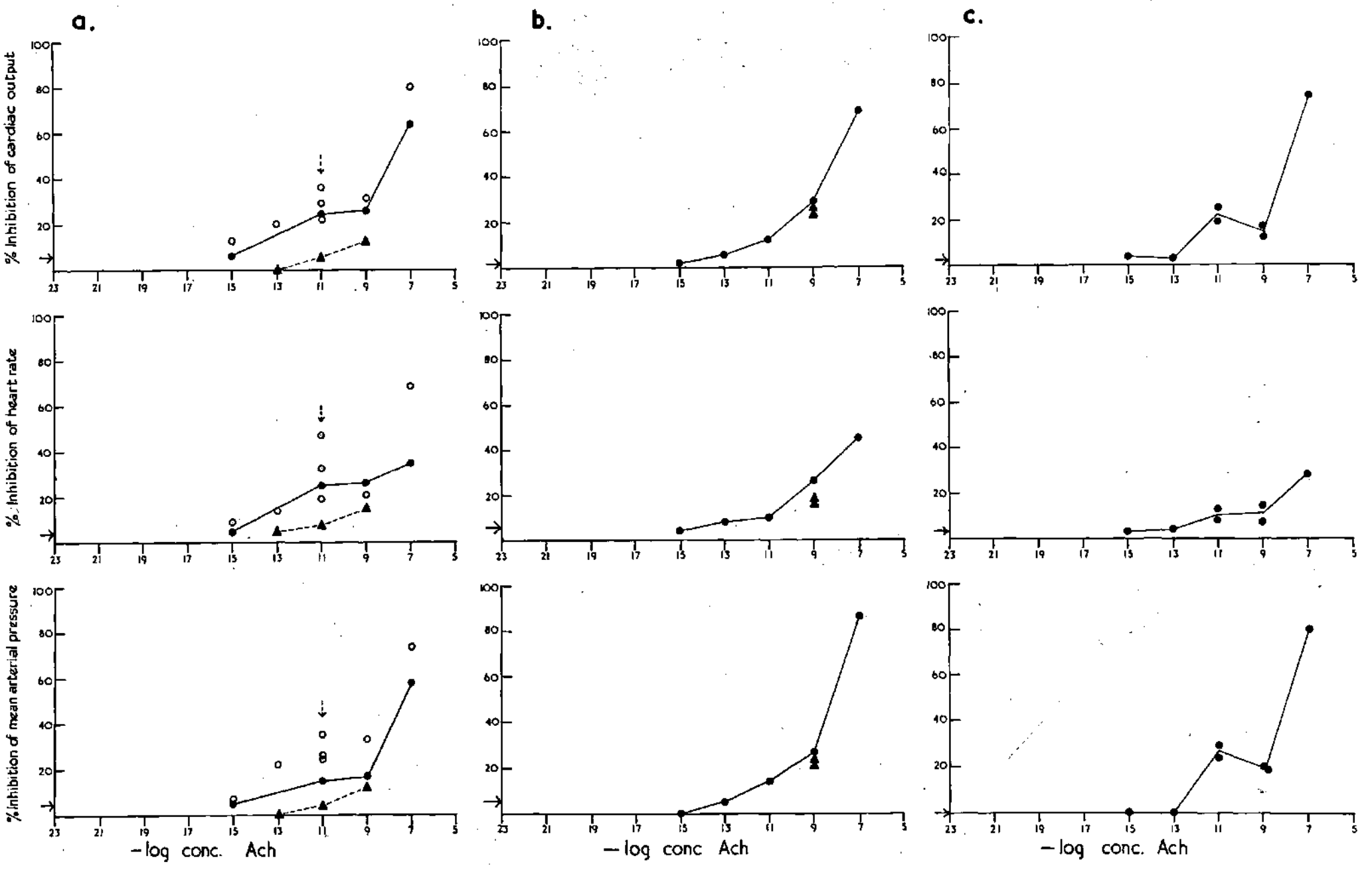

a.
b.
c.
% Inhibition of cardiac output
% Inhibition of heart rate
% Inhibition of mean arterial pressure
100
80
60
40
20
23
21
19
17
15
13
11
9
7
5
– log conc. Ach

Fig. 52

Fig. 53 Example of another heart in which the minimum effective concentration was 10^{-15} g/ml, while 10^{-11} and 10^{-9} produced very similar effects. 10^{-7} g/ml, administered for only 30 seconds produced marked inhibition, and if perfused for the usual 2 minutes would probably have stopped the heart.

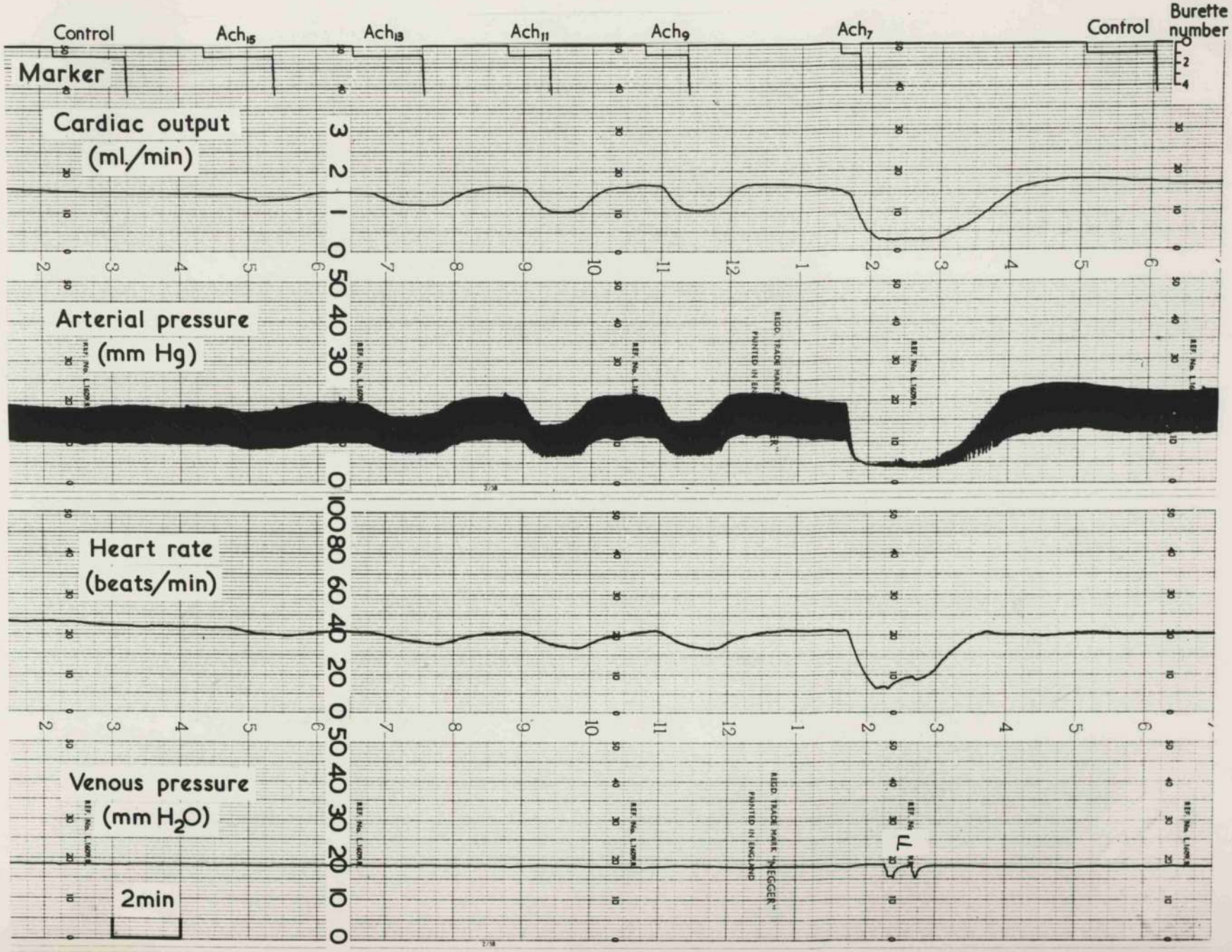

Control
Ach15
Ach13
Ach11
Ach9
Ach7
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2min

Fig. 54. Concentration-response curves of three other highly sensitive hearts (a, b and c). Symbols and notations as in Fig. 13

(a) filled circles - values from tests with a fresh series

open circles - values from tests with the same series almost 24 hours later.

(b) In this heart a series of solutions, prepared and allowed to stand untouched for 24 hours, were tested.

(c) filled circles - values from tests with fresh solutions of one series.

open circles - values from tests with solutions prepared from 10^{-13} solution of the same series after it had been allowed to stand for 175 hours.

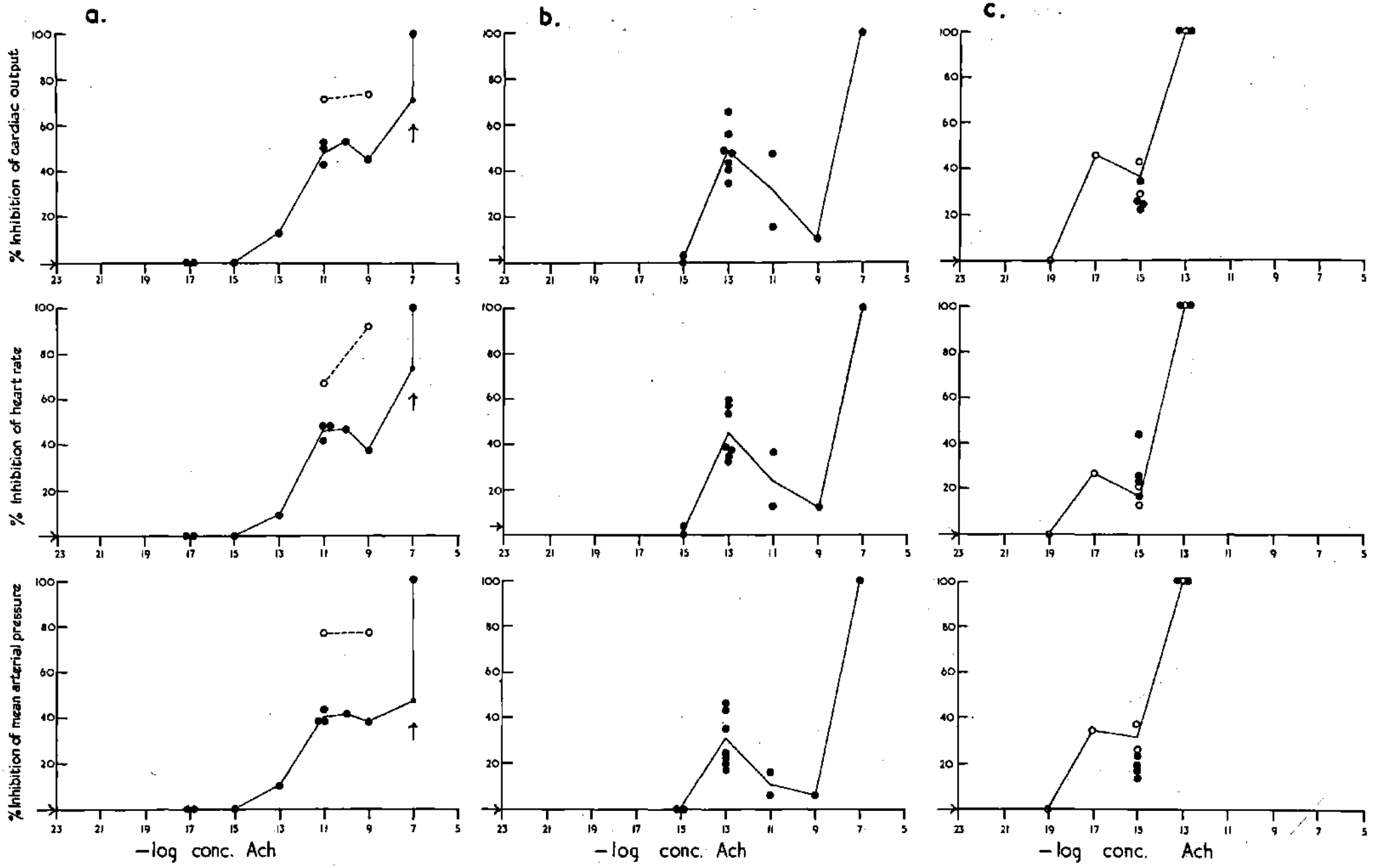

a.
b.
c.
% Inhibition of cardiac output
% Inhibition of heart rate
% Inhibition of mean arterial pressure
100
80
60
40
20
23
21
19
17
15
13
11
9
7
5
−log conc. Ach

Fig. 54

Fig.55. Concentration-response curves of three very highly sensitive hearts (a, b and c). Symbols and notations as in Fig. 13.

(a) filled circles - values from tests with fresh solution of series b.

triangles - values from tests with fresh solutions of series c

(b) filled circles - values from tests with fresh solutions of series a

triangles - values from tests with fresh solutions of series b

crosses - values from tests with fresh solutions of series c

(c) filled circles - values from tests with fresh solutions of series a

crosses - values from tests with fresh solutions of series b

triangles - values from tests with fresh solutions of series c

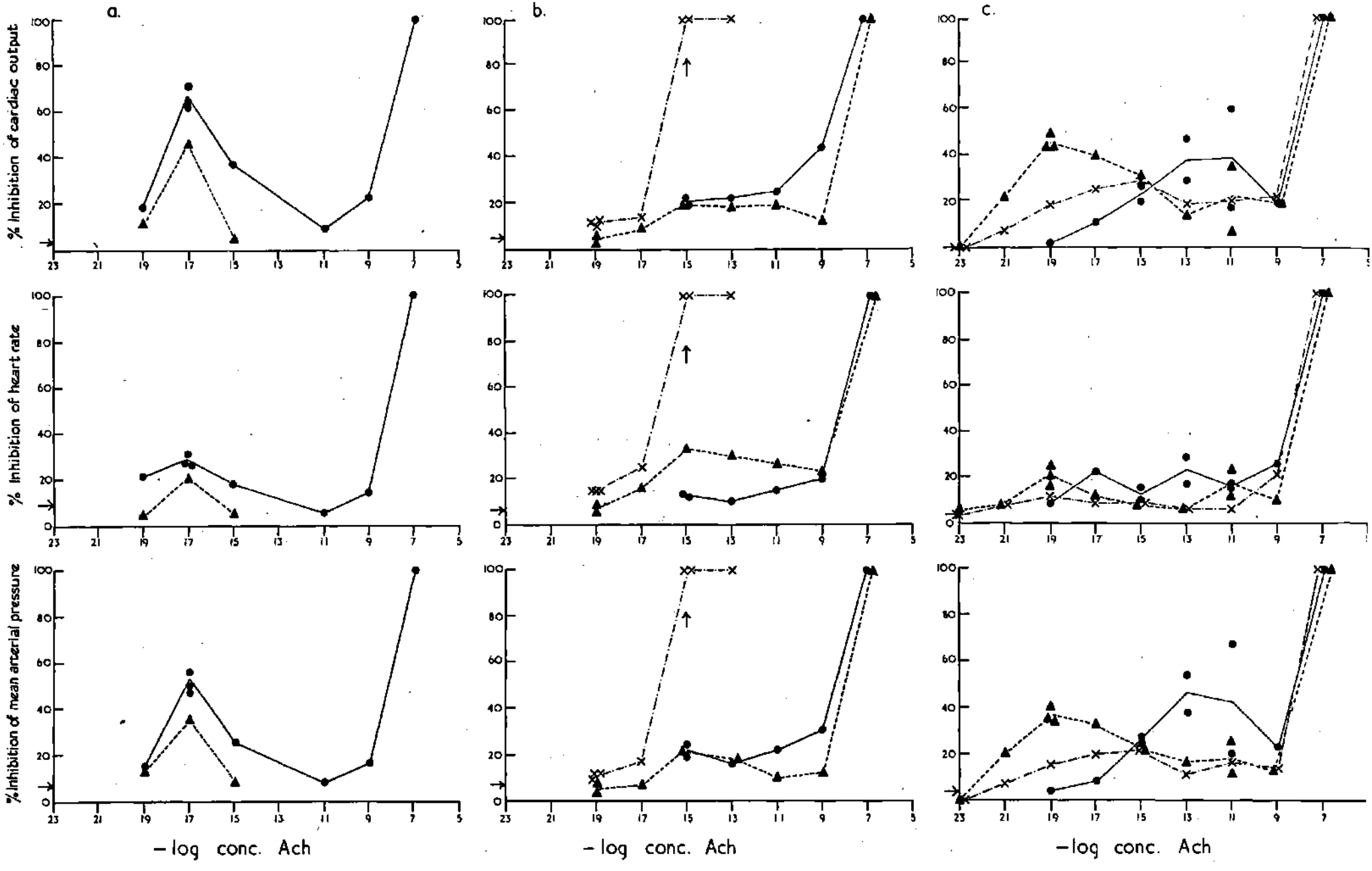

a.
b.
c.
% Inhibition of cardiac output
% Inhibition of heart rate
% Inhibition of mean arterial pressure
100
80
60
40
20
0
23
21
19
17
15
13
11
9
7
5
– log conc. Ach

Fig. 55

Fig. 56. Part of the record of a highly sensitive heart in which the minimum effective concentration of acetylcholine was 10^{-13} g/ml and 10^{-11} g/ml produced a large effect which was confirmed by several tests (see Fig. 54a)

Ach 10^{-11}
Ach 10^{-13}
Ach 10^{-15}
MARKER
CARDIAC OUTPUT (ml./min)
ARTERIAL PRESSURE (mm Hg)
HEART RATE (beats/min)
VENOUS PRESSURE (mm H_2O)
2MIN
REF. No. L1609.R
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND

Fig. 57 Record of tests with the complete series of solutions in the same heart as in Fig. 47 and Fig. 58. In this case there was a decrease in the response at intermediate concentrations (i.e. 10^{-11} and 10^{-9} gave less effect than 10^{-13}). The concentration response curves for this heart are shown in Fig. 54b.

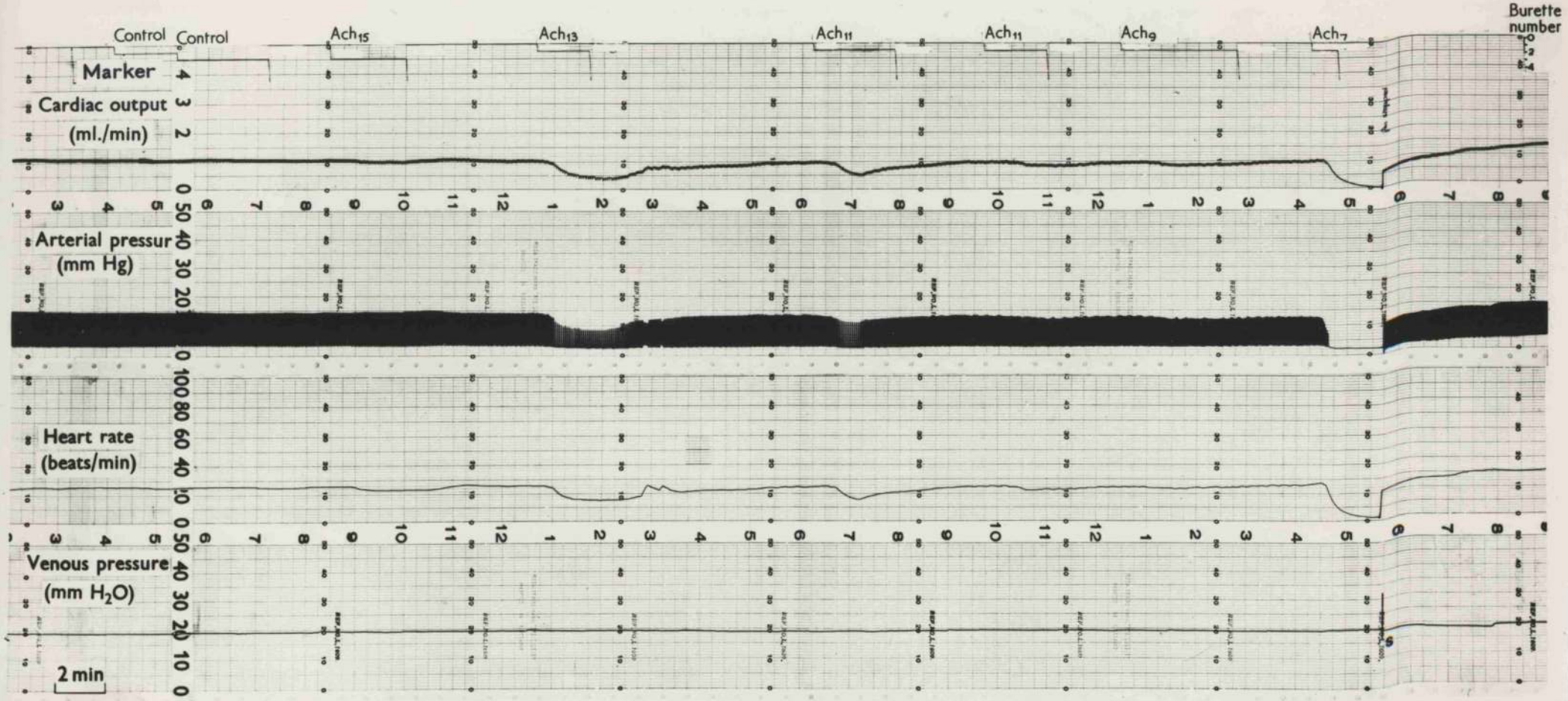

Control
Control
Ach15
Ach13
Ach11
Ach11
Ach9
Ach7
Burette number
Marker
Cardiac output (ml./min)
Arterial pressur (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig.57

Fig. 58. Part of the record of the same heart as in Fig. 57, showing a further test with 10^{-13} g/ml. Other tests with this concentration are shown in Fig. 47.

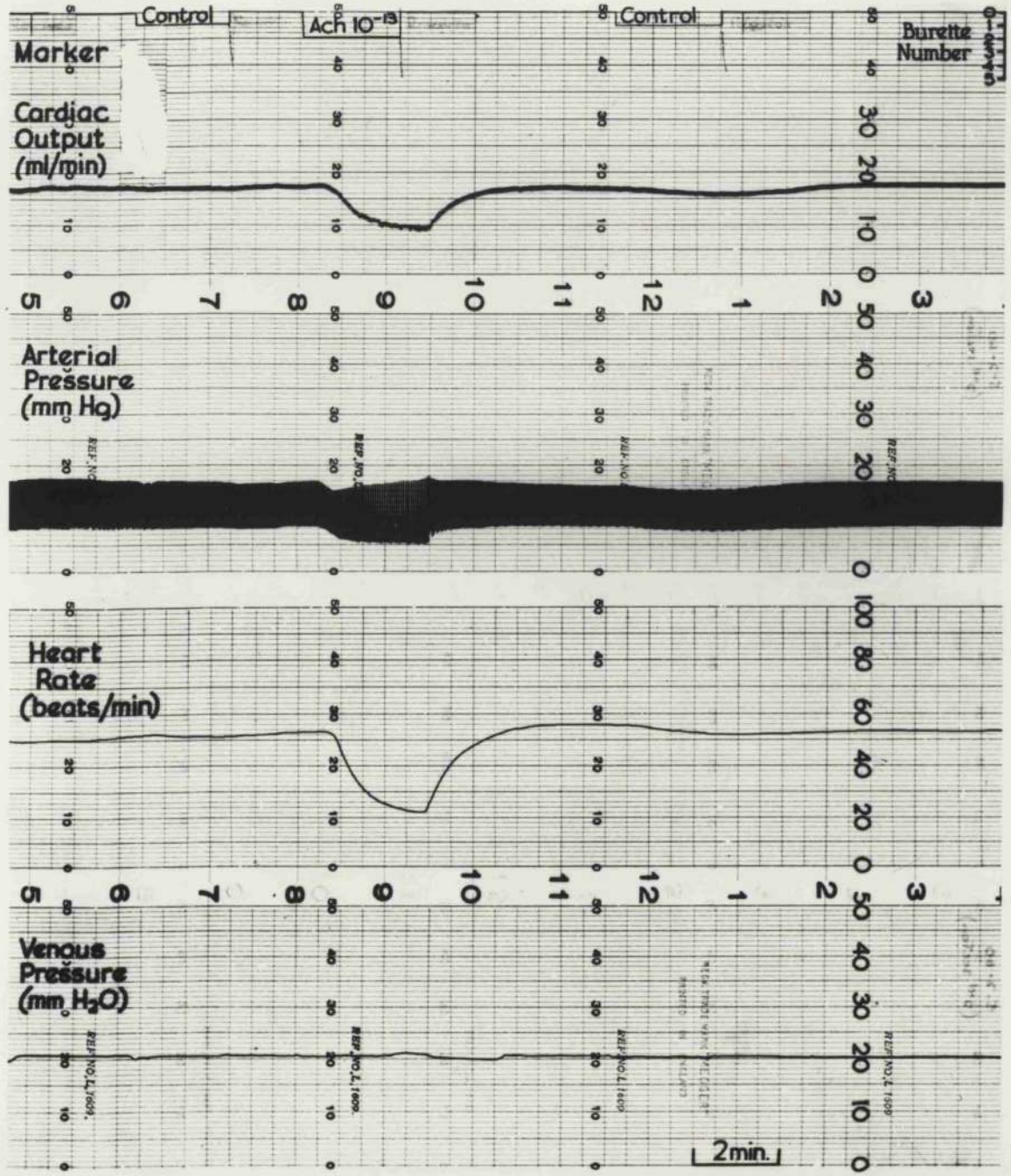

Control
Ach 10⁻¹³
Control
Burette Number
Marker
Cardiac Output (ml/min)
Arterial Pressure (mm Hg)
Heart Rate (beats/min)
Venous Pressure (mm H₂O)
2min.

Fig 58

Fig. 59 Record of a highly sensitive heart in which the minimum effective concentration was 10^{-19} while 10^{-17} produced a gross inhibitory effect. The actions of these concentrations are 'bracketed' between controls. Test with 10^{-17} was repeated to confirm the effect. Other tests conducted on this heart with these solutions and with solutions of a completely different series are shown in Fig. 60. The action of acetylcholine was more marked on cardiac muscle (arterial pressure and output) than on the pacemaker (heart rate).


Control
Ach19(b)
Ach17(b)
Control
Ach17(b)
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig 6.9

Fig. 60 Three parts (separated by dotted vertical lines) of the record of one of the very highly sensitive hearts. Interdigitated tests with corresponding concentrations of two different series of acetylcholine (b and c) show that minimum effective concentr-ation was 10^{-19} g/ml while a peak of effect was seen with 10^{-7} g/ml of both series. Initial tests with series b are shown in Fig. 59.

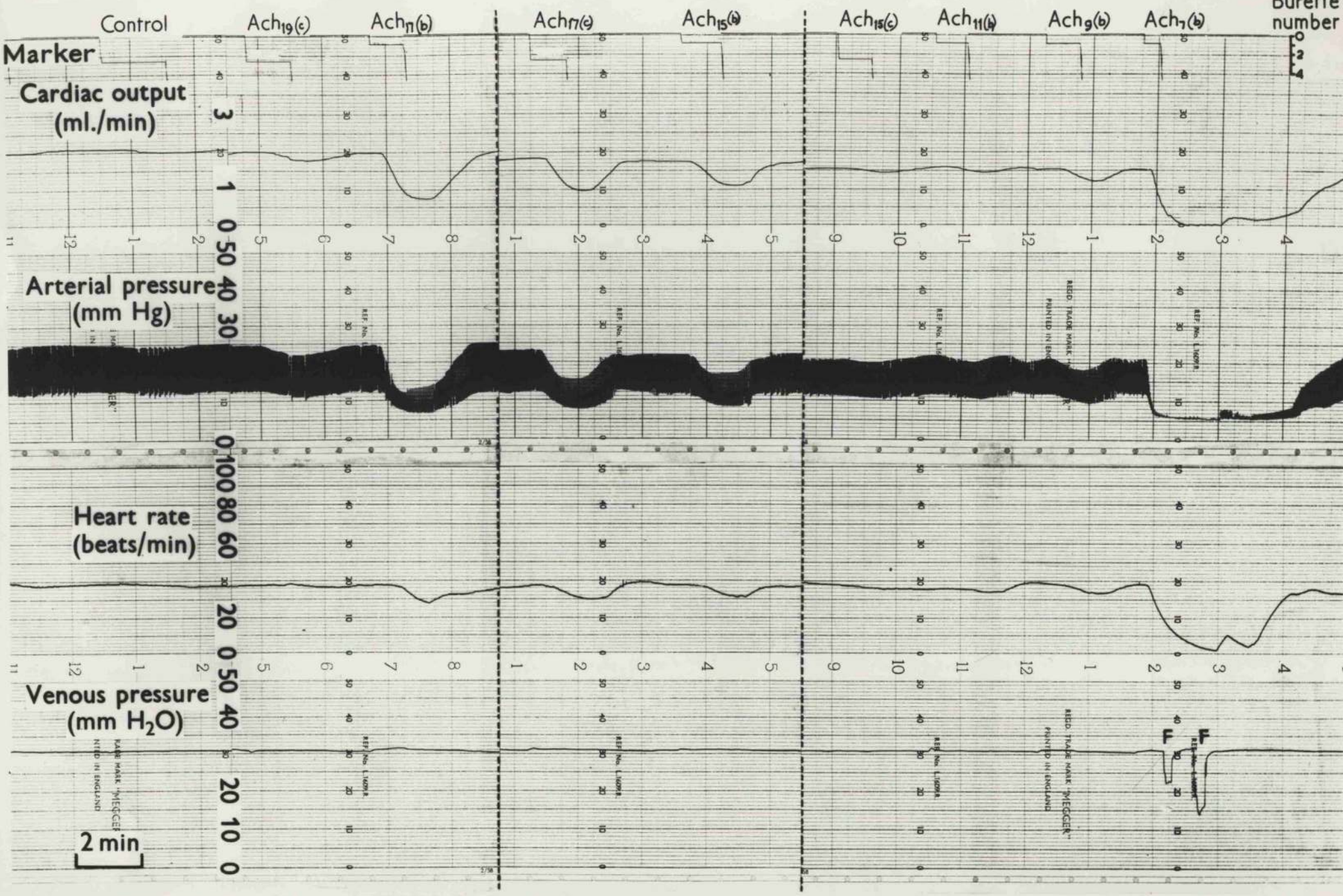

Control
Ach19(c)
Ach11(b)
Ach17(c)
Ach15(b)
Ach15(c)
Ach11(b)
Ach9(b)
Ach7(b)
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig. 60

Fig. 61 Record of tests with a complete range of solutions of acetylcholine (series *b*) in a highly sensitive heart. There was a peak of effect at 10^{-15} g/ml in this heart. Part of the record has been omitted (at vertical dotted line).

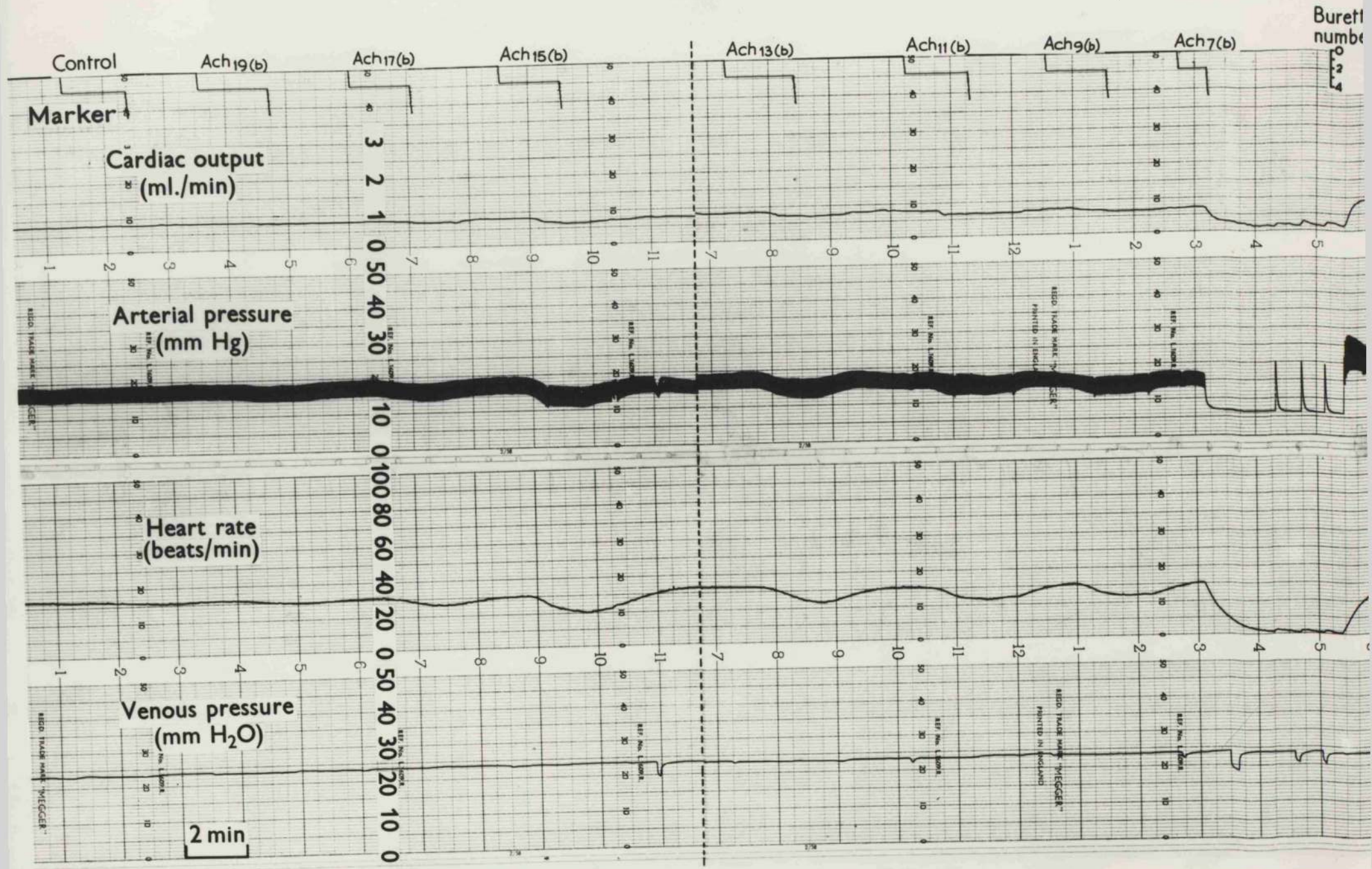

Control
Ach 19(b)
Ach 17(b)
Ach 15(b)
Ach 13(b)
Ach 11(b)
Ach 9(b)
Ach 7(b)
Buret
numbe
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2 min

Fig 6

Fig. 62. Record of another heart showing threshold sensitivity to acetylcholine at 10^{-19} g/ml and stoppage at 10^{-15} g/ml. Burette 1 in use throughout. The controls are very satisfactory. Note the rebound stimulation during the recovery from the action of 10^{-15}. Several series of solutions were tested. The action of test solutions of series (e) only are shown.

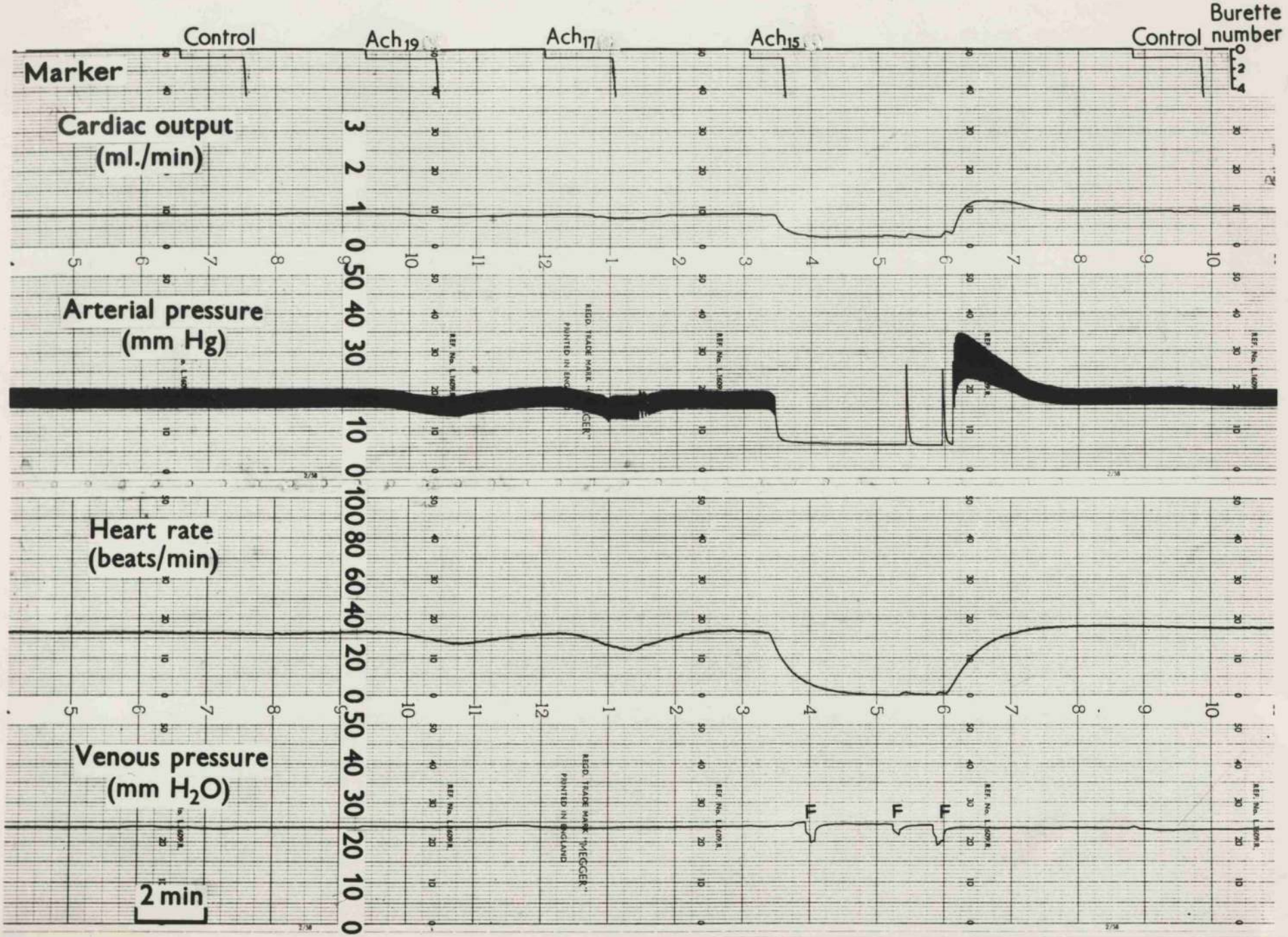

Control
Ach19
Ach17
Ach15
Control
Burette number
0
2
4
Marker
Cardiac output (ml./min)
3 2 1 0
Arterial pressure (mm Hg)
50 40 30
Heart rate (beats/min)
100 80 60 40 20 0
Venous pressure (mm H2O)
50 40 30 20 10 0
2 min

Fig. 63 Part of the record of the most highly sensitive heart showing a peak of effect at 10^{-13} g/ml of series 'a'. Initial tests with series a are shown in Fig. 32 while interdigitated tests with very low concentrations of series b and c are shown in Fig. 33.

Burette number
0
2
4
Control
Ach15a
Ach13a
Ach11a
Ach9a
Ach7a
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
TRADE MARK "MEGGER"
IN ENGLAND
REF. No. L.1609.R.

Fig.64 Biphasic action of adrenaline. Slight inhibition at 10^{-9}, marked inhibition at 10^{-7} and a marked and prolonged excitatory effect at 10^{-5} g/ml. These solutions of adrenaline were 3 hours old.

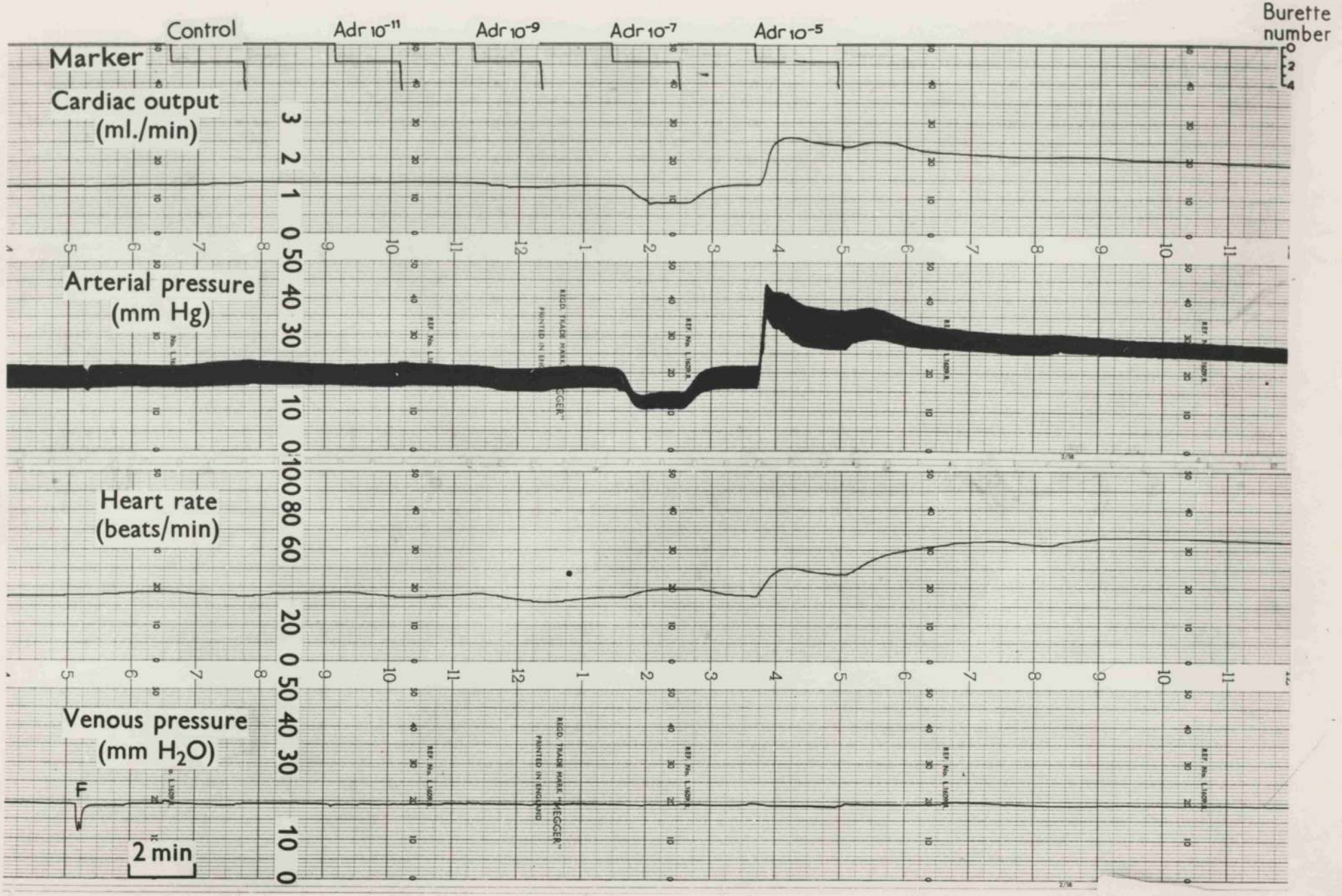

Control
Adr 10^{-11}
Adr 10^{-9}
Adr 10^{-7}
Adr 10^{-5}
Burette number
Marker
Cardiac output (ml./min)
3 2 1 0
Arterial pressure (mm Hg)
50 40 30 10 0
Heart rate (beats/min)
100 80 60 20 0
Venous pressure (mm H_2O)
50 40 30 10 0
F
2 min

Fig. 64

Fig. 65 Marked oscillating changes in the heart rate and amplitude of contraction as a result of external application of a drop of adrenaline in a concentration of 10^{-3} g/ml. The arrow at the top indicates the point of administration of adrenaline. The initial fluctuations in the record between point 11.0 and 1.0 are partly due to readjustments in the venous pressure and artificial resistance. These adjustments were made to provide optimum hydrodynamic conditions for the heart. After point 1.30 the resistance was constantly the same, and only the venous pressure was changed at points 2.45 and 8.45. The oscillating changes in the heart rate and amplitude (and consequently in the output and blood pressure) persisted for a long time.

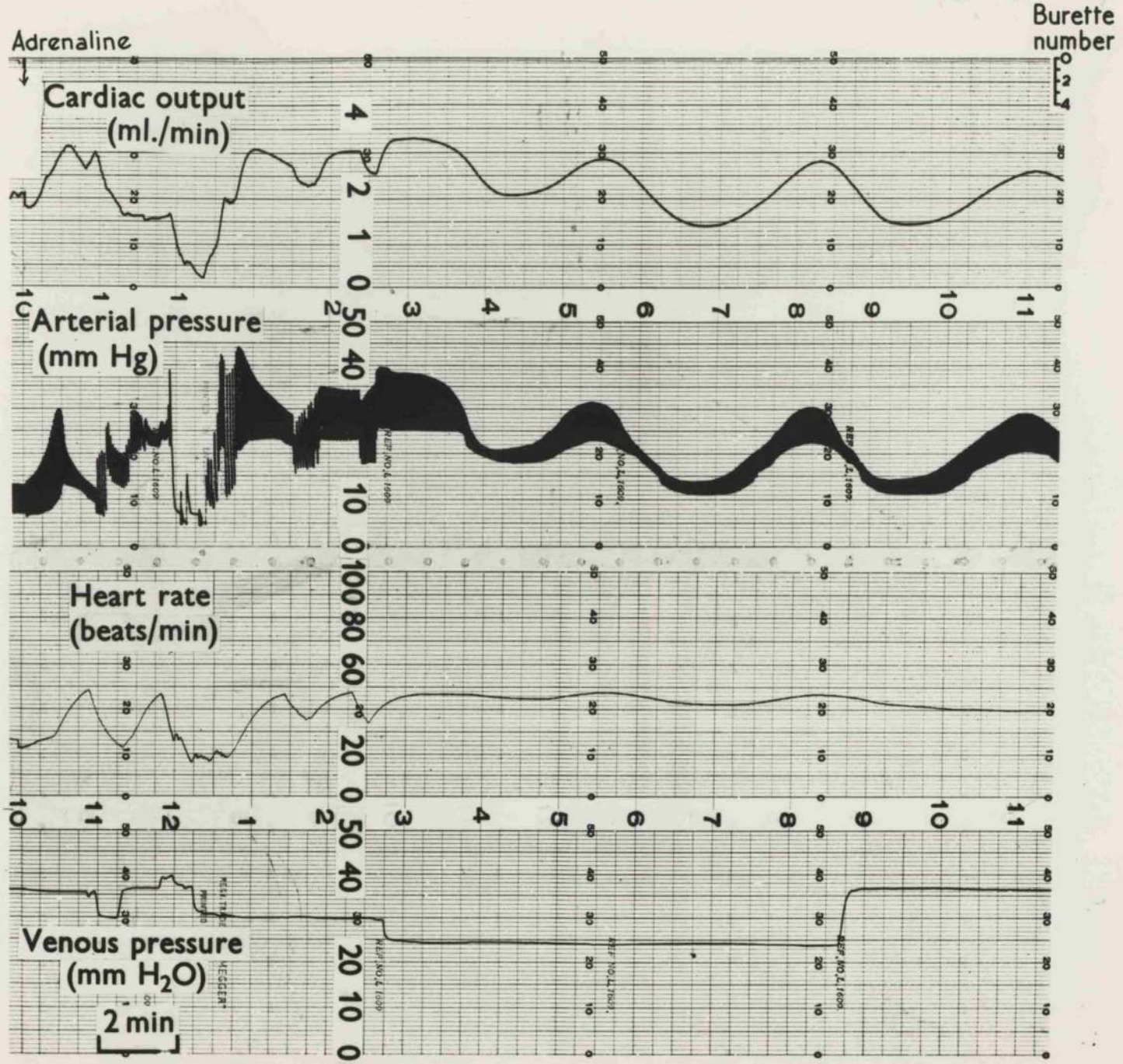

Adrenaline
Burette number
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig. 65

Fig. 66 Comparison of the stimulating effect of adrenaline and noradrenaline in the same concentration. Noradrenaline 10^{-11} produced a delayed effect. The delay in the onset of effect is not correctly shown in the Figure because the marker pen was actually put back by 30 seconds as it was striking against the output pen. The output was rather high and in spite of the change in the position of the marker pen it continued to strike against the output pen as evidenced from small momentary vertical deviations in the output trace below the 'overshoot' of the marker pen. The heart had not fully recovered from the action of noradrenaline 10^{-11} when noradrenaline 10^{-9} was tested. However it is clear that the action of noradrenaline 10^{-9} was greater than that of 10^{-11}. A comparison of the action of adrenaline and noradrenaline in a concentration of 10^{-7} g/ml indicates that both substances influenced the heart rate and amplitude of contraction and associated parameters e.g. output and blood pressure. The positive chronotropic effect of adrenalinewas more than two times greater than that of noradrenaline. Noradrenaline produced a greater increase in the output than adrenaline. Noradrenaline produced a proportionately greater rise in the diastolic blood pressure than in the systolic blood pressure. Adrenaline produced equal effect on systolic and diastolic pressures. The effect of adrenaline passed off more quickly than that of noradrenaline.

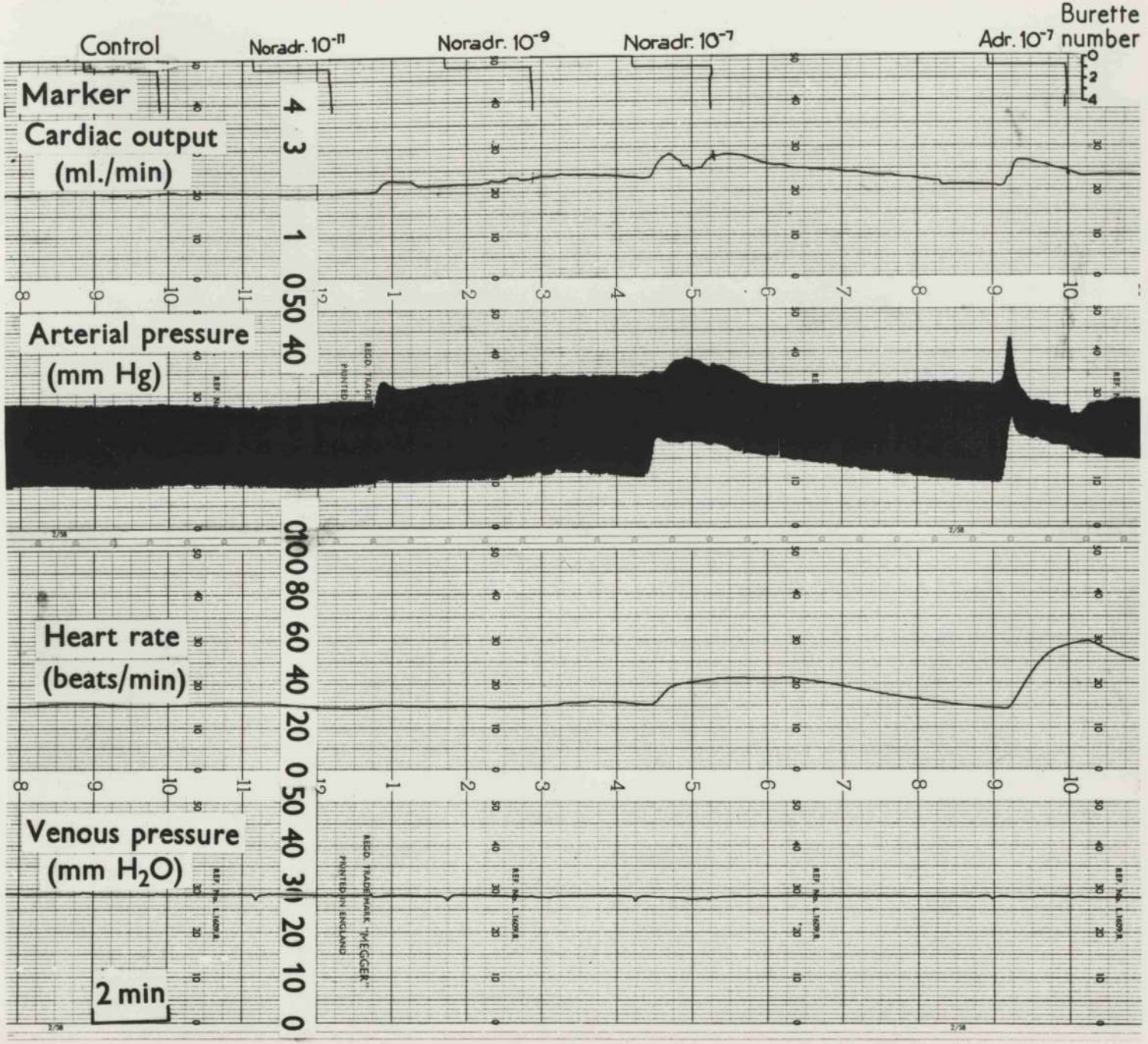

Control
Noradr. 10^{-11}
Noradr. 10^{-9}
Noradr. 10^{-7}
Adr. 10^{-7}
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig 66

Fig. 67 Stimulation followed by brief stoppage of the heart due to the action of noradrenaline and adrenaline in a concentration of 10^{-7} g/ml. The two sharp spikes on the venous pressure trace, one between points 11.0 and 11.15 and the other between points 3.15 and 3.30 are due to blowing of fluid in the standpipe to confirm that there was no obstruction to the flow of fluid into the heart which could account for the low levels of recorded parameters. The venous pressure was otherwise quite stable throughout. There was, of course, no question of any obstruction as spontaneous recovery occurred each time after the brief stoppage by noradrenaline and adrenaline. The irregularity in the rhythm of the heart persisted for a long time after the test with adrenaline and periods of stimulation and stoppage recurred alternately. The movement of the marker at R near point 3.0 indicates that more Ringer's solution was poured into the reservoir and the recording was temporarily stopped for this purpose.


Control
Noradr. 10^{-7}
Adr. 10^{-7}
R
Buret
numbe
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H_2O)
2 min

Fig. 67

Fig. 68 Diphasic action of 5-hydroxytryptamine. The threshold sensitivity of this heart to 5-hydroxytryptamine was at the concentration of 10^{-7}, the minor changes at 10^{-9} being insignificant. The fall in the output and blood pressure during the action of 10^{-7} is partly due to the decrease in the amplitude (as shown by decreased pulse pressure) associated with the increase in frequency. The actual biphasic action of 5-hydroxytryptamine is well seen at 10^{-5}. The initial fall in the output and blood pressure is not wholly due to the rise in the rate because the subsequent greater rise in the rate is actually accompanied with an increase in the output and blood pressure. The second fall in the output and blood pressure when the rate was actually decreasing also represents the inhibitory component of the action of 5-hydroxytryptamine. Note the prolonged effect of 10^{-5} on all the three variable parameters, the total effect being a resultant of the inhibitory and excitatory actions of 5-hydroxytryptamine on the chronotropic and inotropic responses. Near the end of perfusion with 10^{-11} the fluid ran out due to which the venous pressure, output and blood pressure registered a transient fall. The perfusion was quickly changed back to the reservoir and all the affected parameters returned to original levels. The control

at the end is satisfactory in view of the fact that it was carried out from the same burette which previously contained 5-hydroxytryptamine in a concentration of 10^{-5} g/ml which was not expected to be washed out entirely.

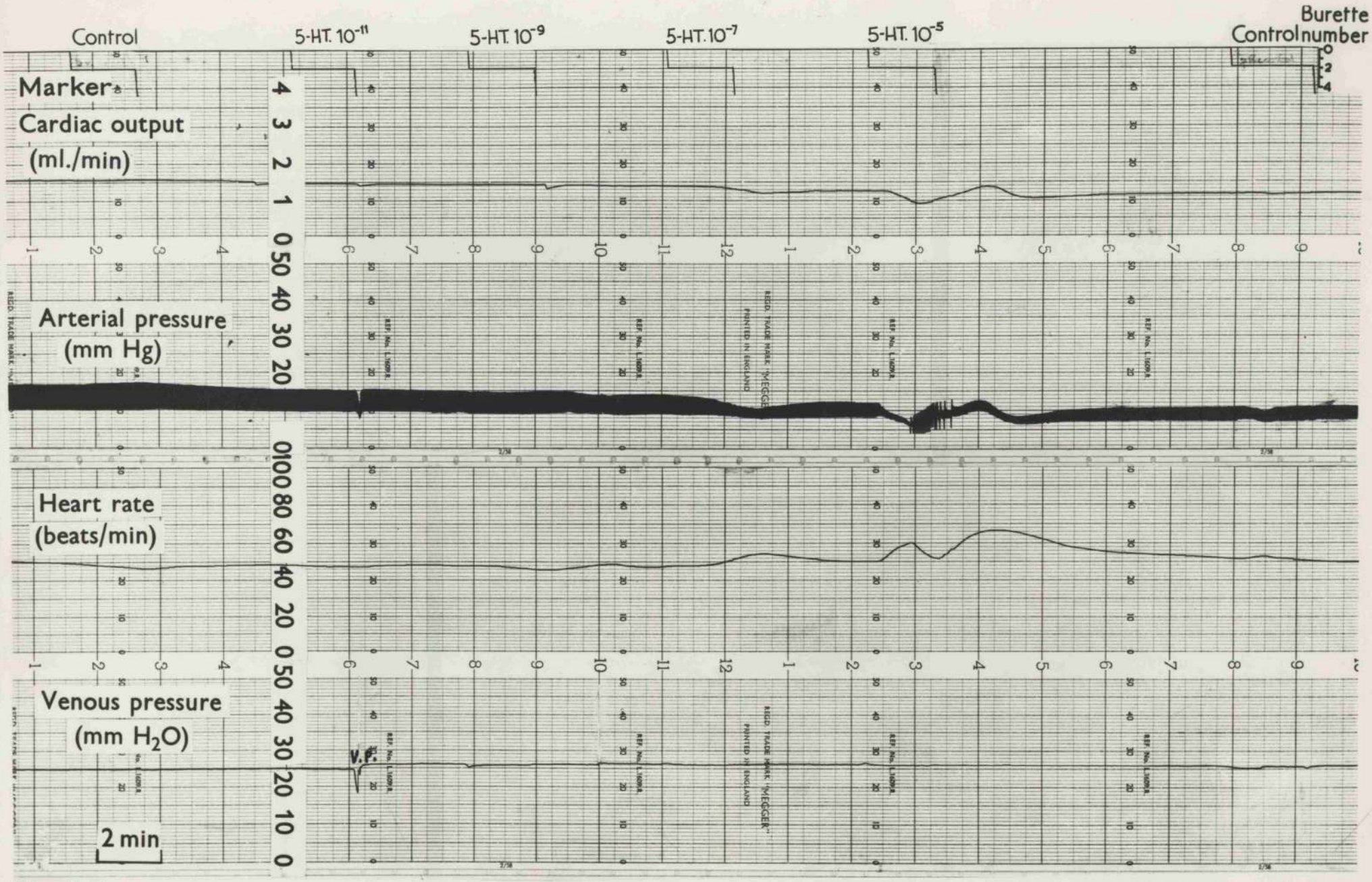

Control
5-HT. 10⁻¹¹
5-HT. 10⁻⁹
5-HT. 10⁻⁷
5-HT. 10⁻⁵
Control
Burette number
0
2
4
Marker
Cardiac output
(ml./min)
4 3 2 1 0
Arterial pressure
(mm Hg)
50 40 30 20
Heart rate
(beats/min)
0 100 80 60 40 20 0
Venous pressure
(mm H2O)
50 40 30 20 10 0
V.P.
2 min

Fig. 68

Fig. 69 Another example of the diphasic action of 5-hydroxytryptamine. The first effective concentration (10^{-7}) produced a pure positive chronotropic effect with only slight associated reduction in the amplitude of contraction (decreased pulse pressure). The output and the diastolic pressure rose without any significant rise in the systolic pressure. This was a typical effect at low concentrations of 5-hydroxytryptamine. During the action of 10^{-5} the positive chronotropic effect is associated with a variable degree of change in the amplitude. The onset of effect is quicker and the duration of effect is greater at 10^{-5} than at 10^{-7} as expected. The controls are satisfactory. The drop in the blood pressure on withdrawal of 10^{-5} is not due to the small fall in the venous pressure in changing over the perfusion from burette 1 to the reservoir because a much greater fall in the venous pressure due to flushing back of the fluid of the common perfusion chamber near point 5.15 (F) before the final control produced a very small change showing that the preparation was not very sensitive to changes in the venous pressure.

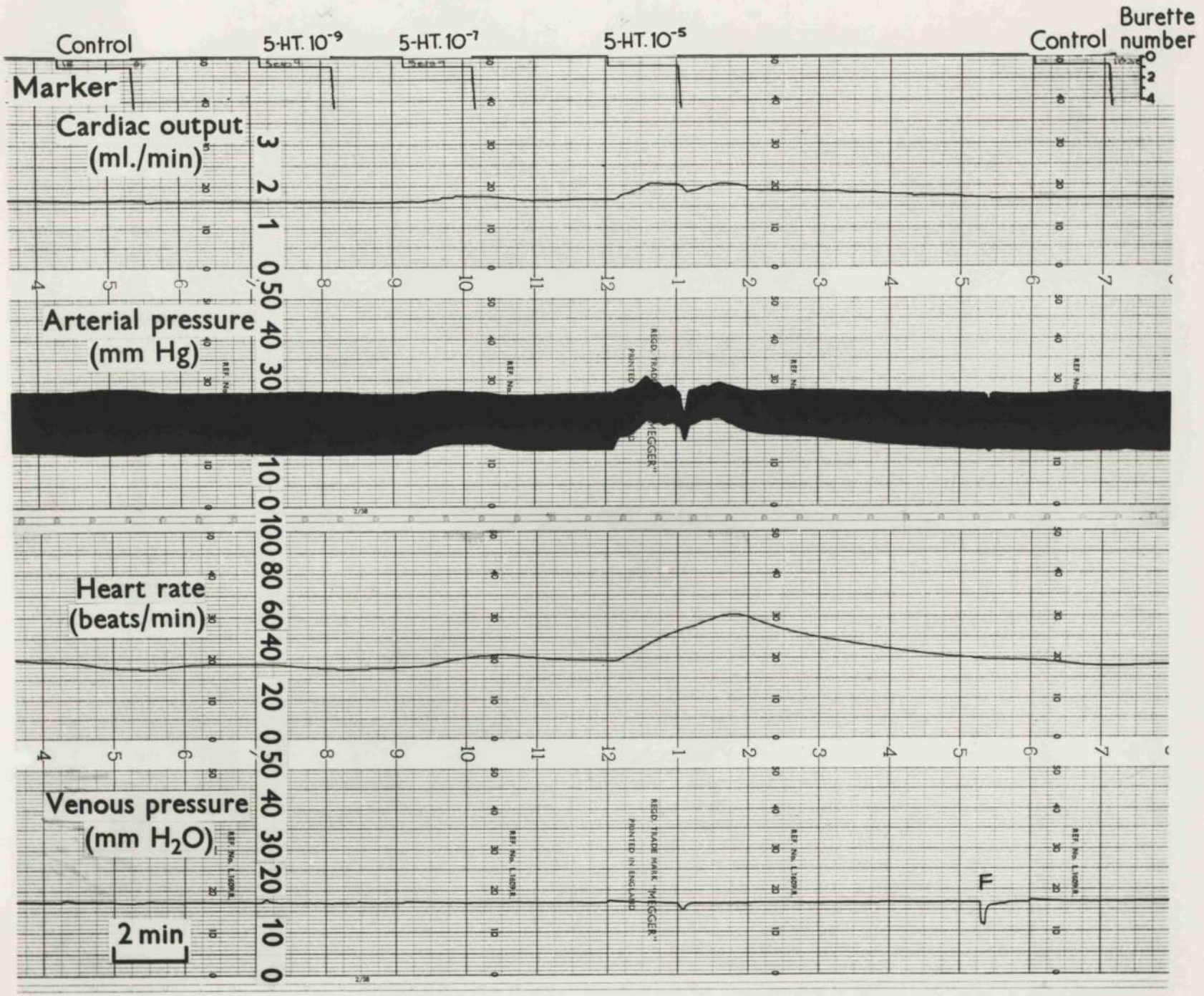

Control
5-HT. 10^{-9}
5-HT. 10^{-7}
5-HT. 10^{-5}
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig. 69

Fig. 70 Graded increase in the inhibitory action of increasing concentrations of 5-hydroxytryptamine. The decrease in the heart rate at 10^{-9} and 10^{-7} is not sufficient to explain the fall in the output and blood pressure because a similar decrease in the rate during the initial control did not produce any change in other parameters. The amplitude of contraction was therefore also inhibited at these concentrations. The inhibition of amplitude at 10^{-5} is very marked. Due to the slow return of heart rate to the normal level after the action of adrenaline which had been tested previously. There is a slow fall in the output and blood pressure in the beginning of the record. This is obviously of no significance. The initial control was satisfactory. The venous pressure was quite stable throughout.

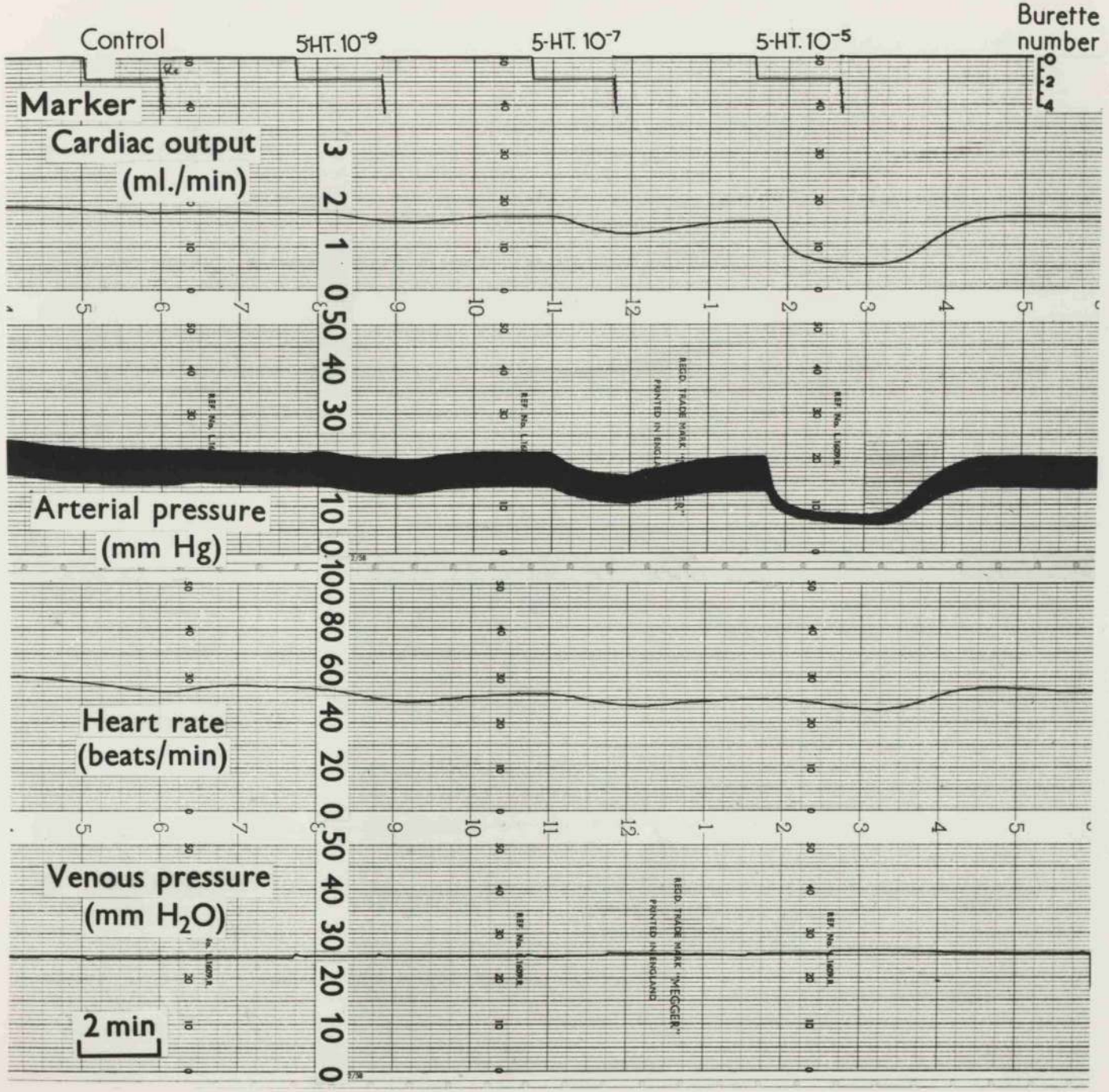

Control
5-HT. 10^{-9}
5-HT. 10^{-7}
5-HT. 10^{-5}
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig. 70

Fig. 71 Concentration-response curves for mean arterial pressure and heart rate obtained by re-plotting the curves of three hearts using a linear scale of actual weight of acetylcholine chloride per ml of each solution

A. curves from Fig. 19b

B. curves from Fig. 19a

C. curves from Fig. 20a

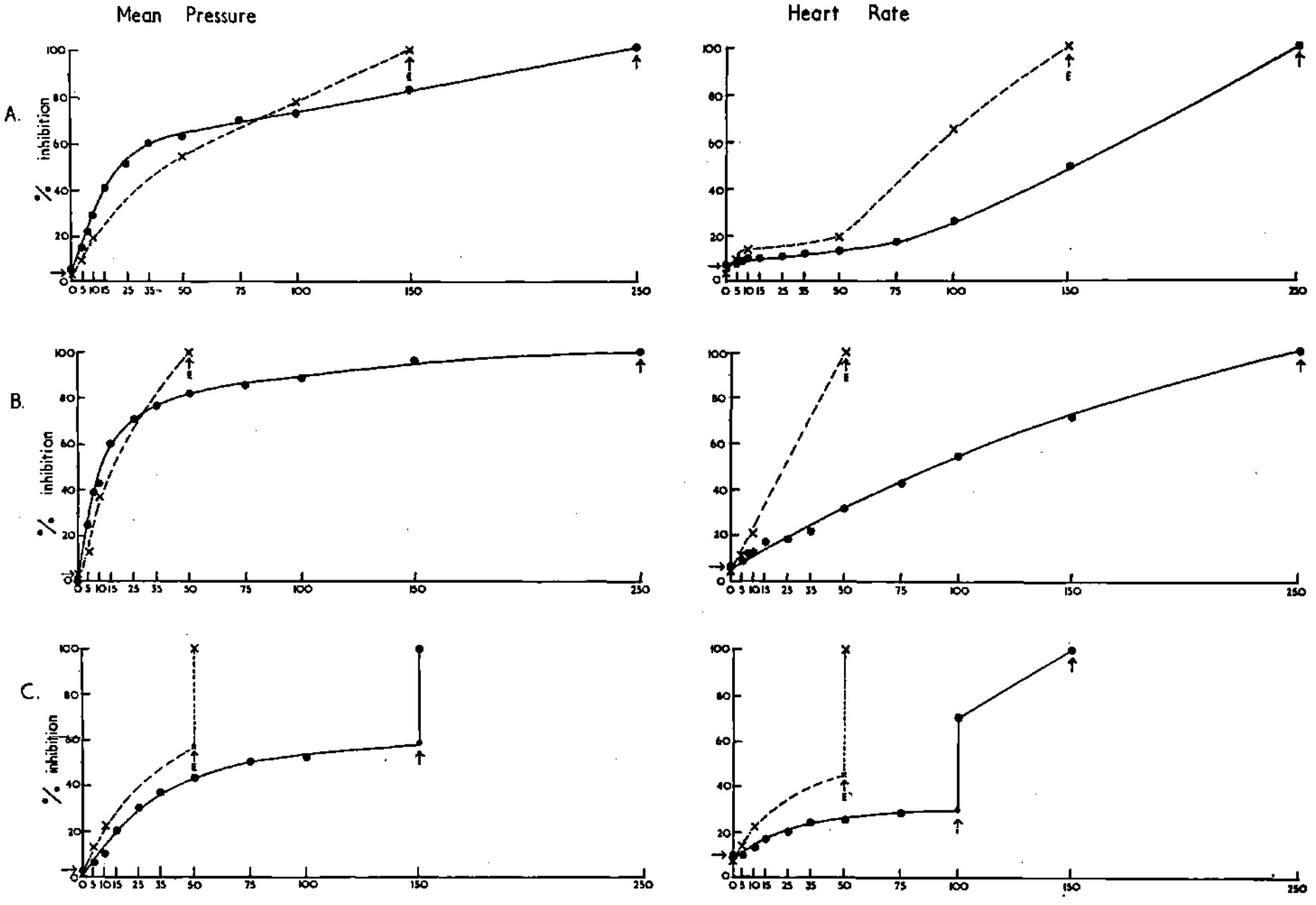

Mean Pressure
Heart Rate
A.
B.
C.
% inhibition
E
0 5 10 15 25 35 50 75 100 150 250
Weight in nanograms of acetylcholine chloride per millilitre of Ringer's solution

FIG. 71

Fig. 72. For description see text.

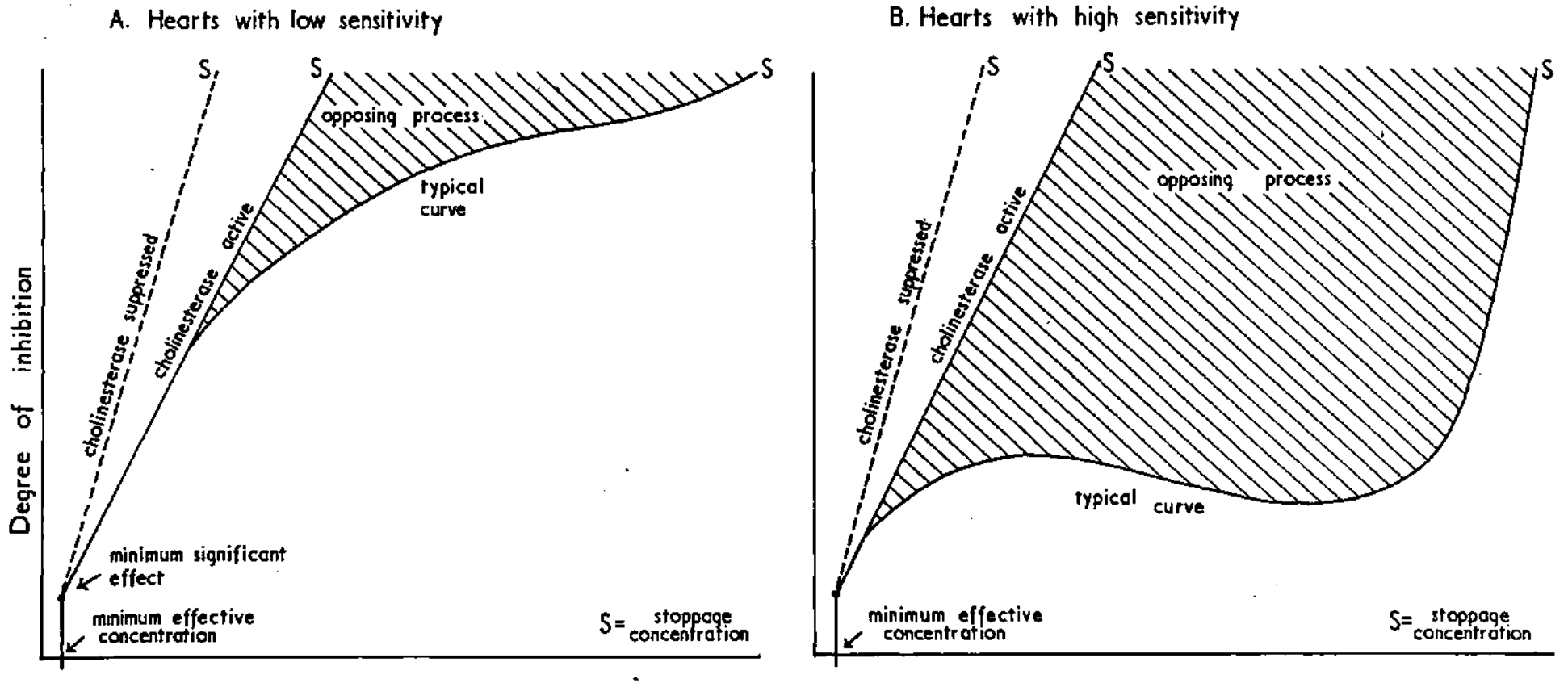

A. Hearts with low sensitivity
B. Hearts with high sensitivity
Degree of inhibition
S
S
S
cholinesterase suppressed
cholinesterase active
opposing process
typical curve
minimum significant effect
mnimum effective concentration
S= stoppage concentration
S
S
S
cholinesterase suppressed
cholinesterase active
opposing process
typical curve
minimum effective concentration
S= stoppage concentration
Increasing concentration of acetylcholine ⟶

FIG. 72

Fig. 73 Grade I effect from the test solution of Portex crystal Vinyl P.V.C. tubing, the solution having been obtained by the method of soaking. The action is inhibitory reducing the heart rate, cardiac output and blood pressure. The venous pressure was stable throughout. Each test is bracketed between controls, the controls from both burette 1 and burette 2 being satisfactory.

Burette number
0
2
4
Control
Crystal vinyl
Control
Crystal vinyl
Control
Control
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2min
3 2 1 0 50 40 30
0 100 80 60 40 20 0 50 40 30 20 10 0
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND

Fig. 74 Grade I effect (inhibitory) with the test solution of 'Portex standard ' P.V.C. tubing, the solution having been obtained by the method of soaking.

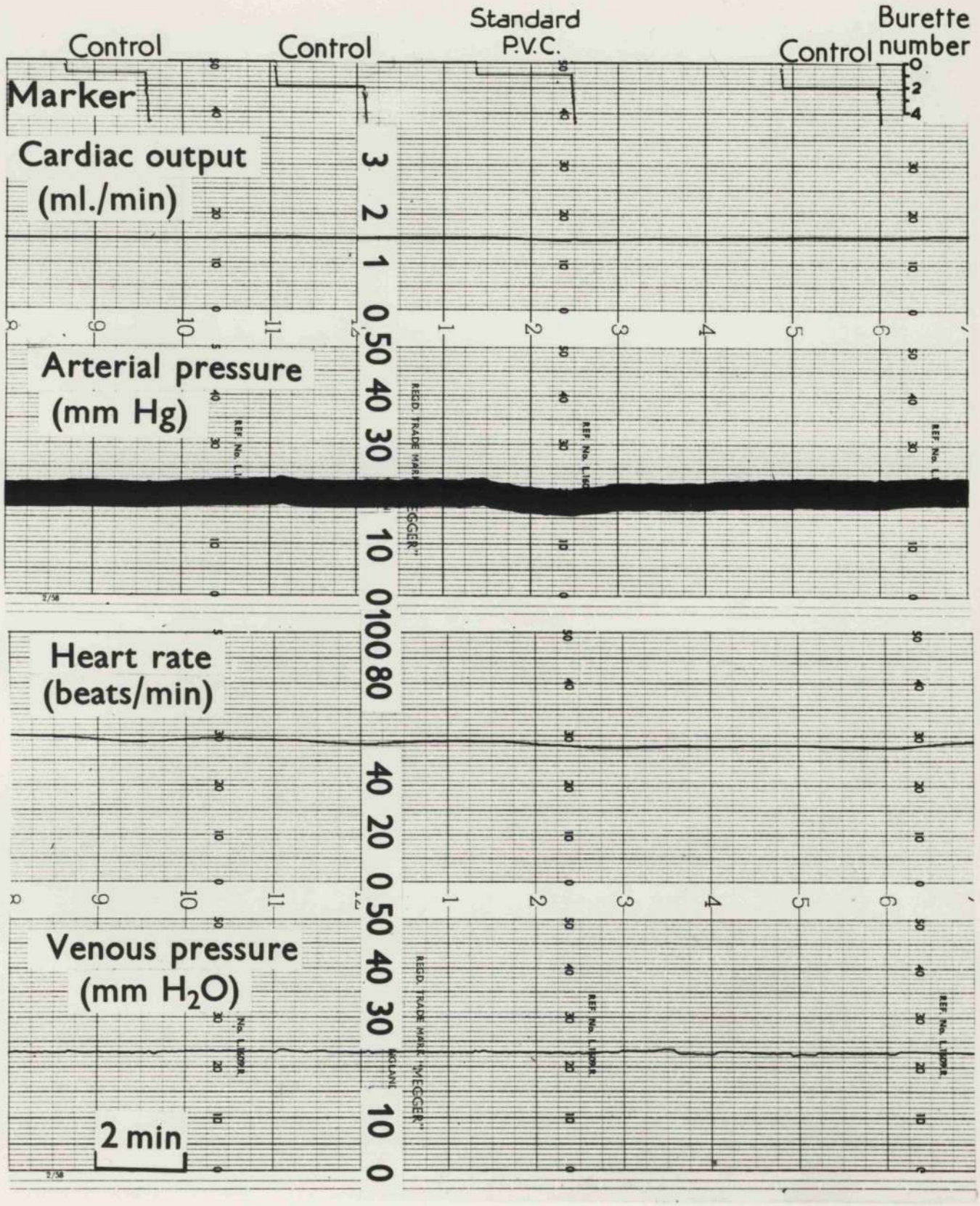

Control
Control
Standard
P.V.C.
Control
Burette
number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H_2O)
2 min

Fig. 74

Fig. 75 Grade II effect from the test solution of 'Portex standard ' P.V.C. tubing, the solution having been obtained by the method of soaking. Minor drifts of the venous pressure pen obviously are of no significance.

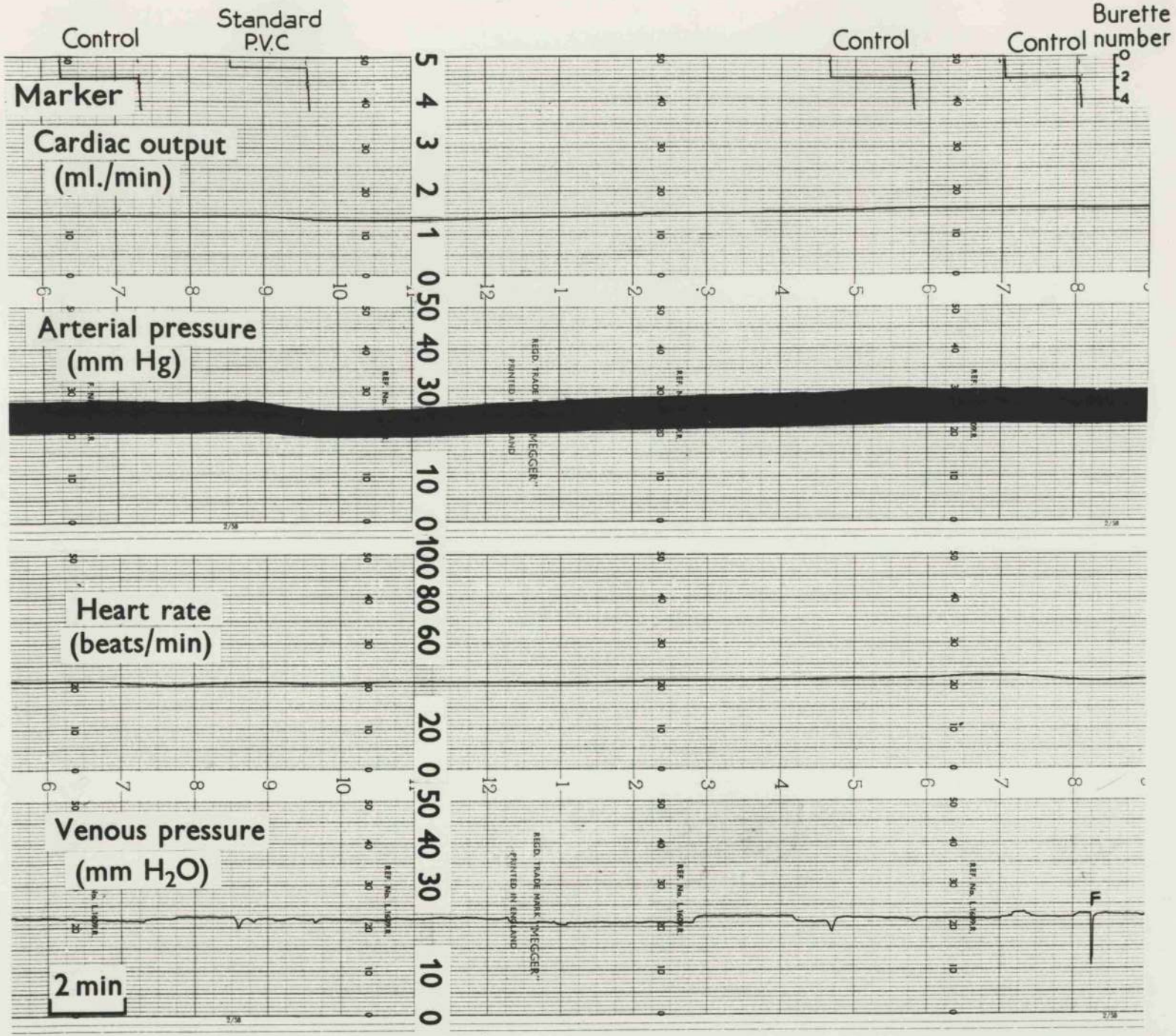

Control
Standard P.V.C
Control
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig. 75

Fig. 76 Grade II effect from the test solution of 'Portex standard ' P.V.C. tubing, the solution having been obtained by the method of boiling. 'A' represents electronic artefact on rate. 'Control (boiling)' means control perfusion with Ringer's solution prepared from the distilled water which had been boiled. (See methods for details of preparation of control and test solutions).

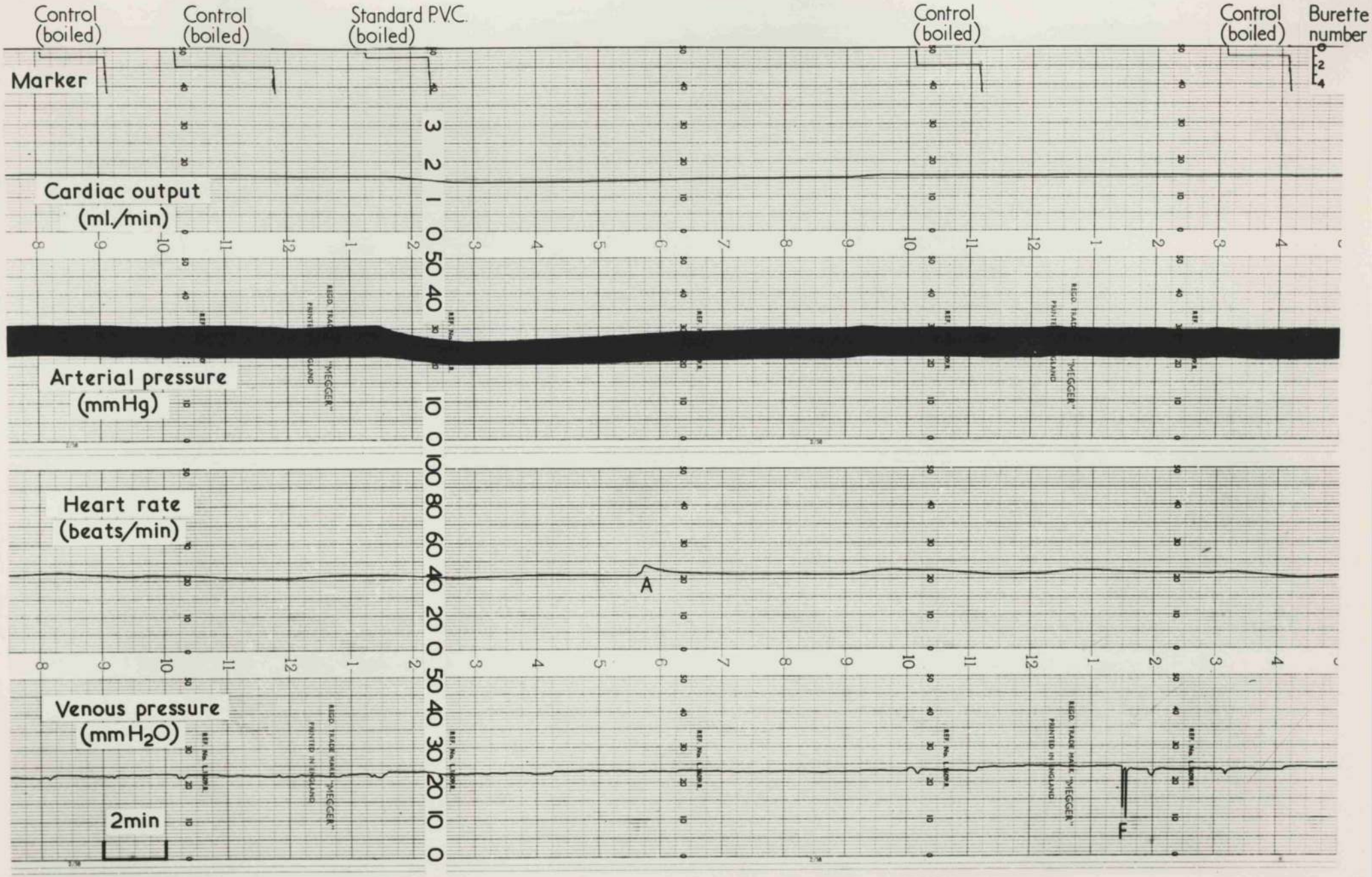

Control (boiled)
Control (boiled)
Standard P.V.C. (boiled)
Control (boiled)
Control (boiled)
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mmHg)
Heart rate (beats/min)
Venous pressure (mm H2O)
A
2min

Fig. 76

Fig. 77 Grade I effect from the test solution of 'X-lon ' P.V.C., the solution having been obtained by the method of soaking. The control solution had been standing in the standpipe connecting tube of burette 2 for some time; this is why the solution was flushed back (F) between points 6.45 and 7.0 before the first control from this burette. The time of change in the output and blood pressure due to the fall in the venous pressure during flushing indicated that the pens were slightly out of alignment; this was corrected subsequently.

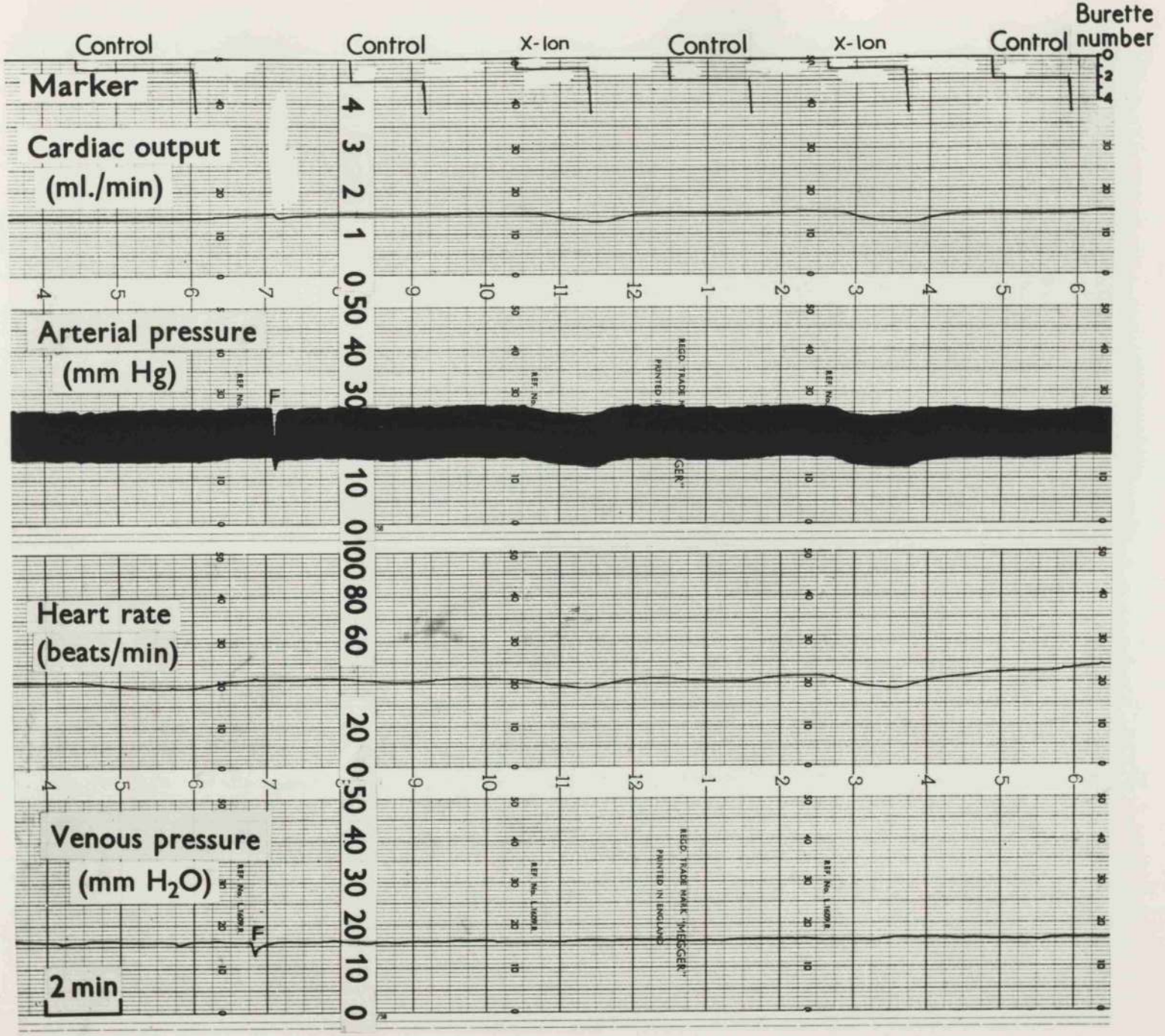

Control
Control
X-Ion
Control
X-Ion
Control
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H_2O)
2 min

Fig. 77

Fig. 78 Grade III effect from the test solution of 'X-lon' P.V.C., the solution having been obtained by the method of soaking. Note the upset in the rhythm. The inhibition of heart rate and amplitude are prominently reflected ih the output and blood pressure. There are also a few extra systoles.

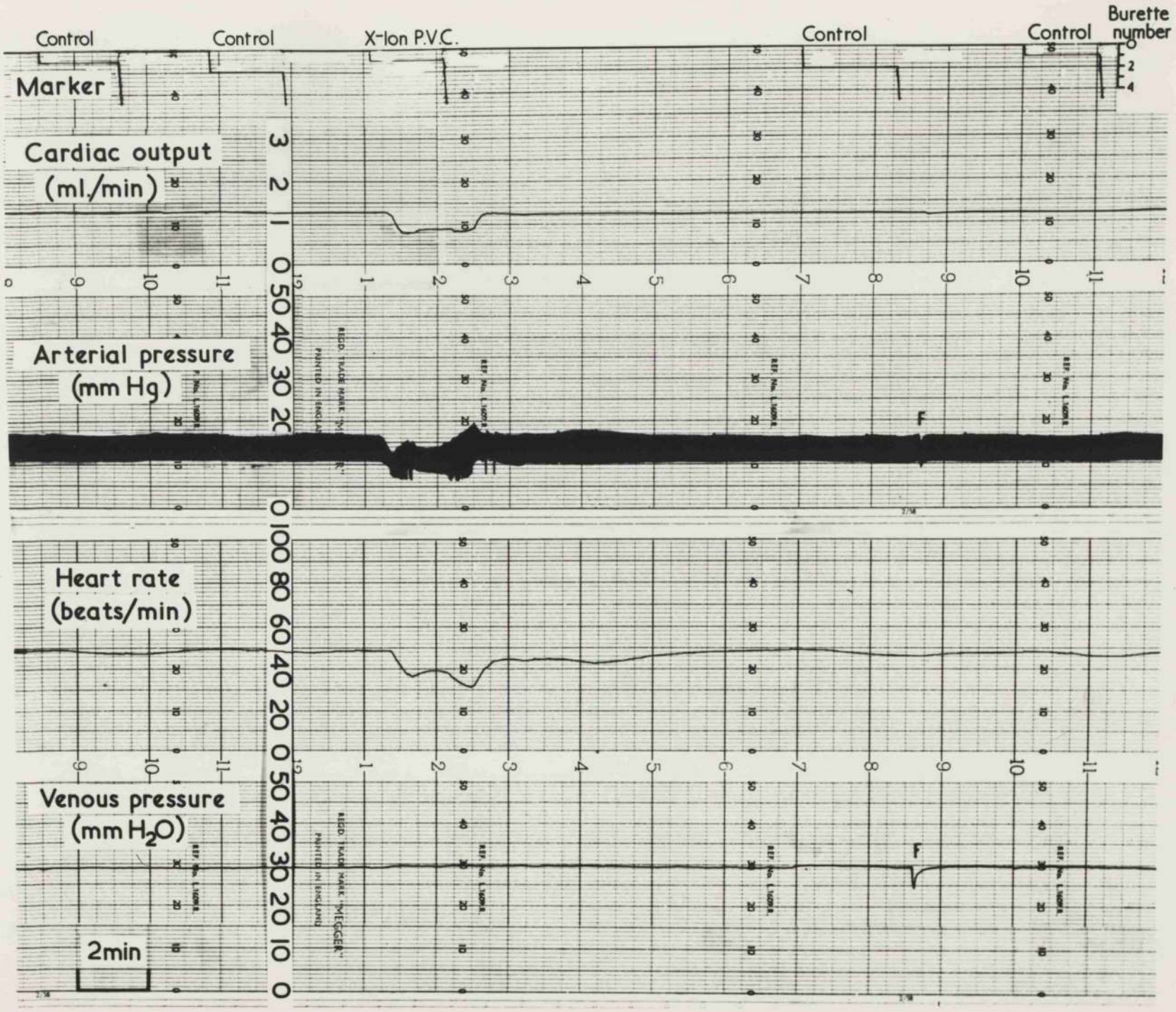

Control
Control
X-Ion P.V.C.
Control
Control
Burette number
0
2
4
Marker
Cardiac output
(ml./min)
3 2 1 0
Arterial pressure
(mm Hg)
50 40 30 20
Heart rate
(beats/min)
100 80 60 40 20 0
Venous pressure
(mm H_2O)
50 40 30 20 10 0
2min
9 10 11 12 1 2 3 4 5 6 7 8 9 10 11

Fig. 79 Grade II effect from the test solution of 'Waterclear P.V.C.' tubing, the solution having been obtained by the method of soaking. Note the delayed effect on the rhythm. 'A' on the output trace indicates artificial change due to the marker pen striking against the output pen at the time of overshoot. The heart was being perfused continuously with control Ringer's solution from burette 1 instead of the usual Ringer's solution from the reservoir because the Ringer's solution in the reservoir had finished. The control and test perfusions were conducted from burette 2.

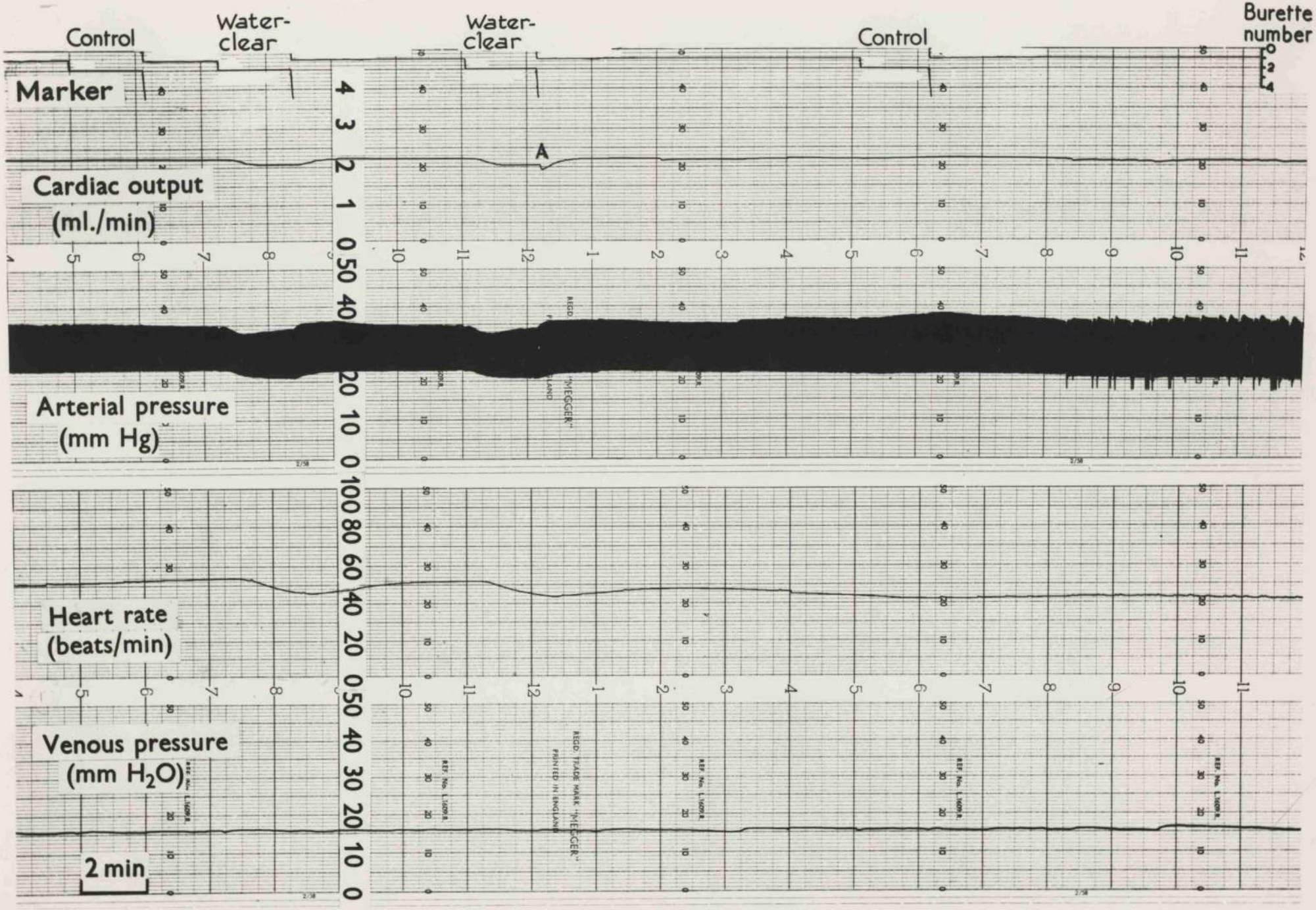

Control
Water-clear
Water-clear
Control
Burette number
Marker
A
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig. 79

Fig. 80 Grade III effect from the test solution of 'Waterclear' P.V.C. tubing, the solution having been obtained by the method of soaking. Note again the delayed effect on rhythm. The upset of rhythm was due to the test solution and not due to control as confirmed subsequently. It never occurred before or afterwards in the entire course of the experiment.

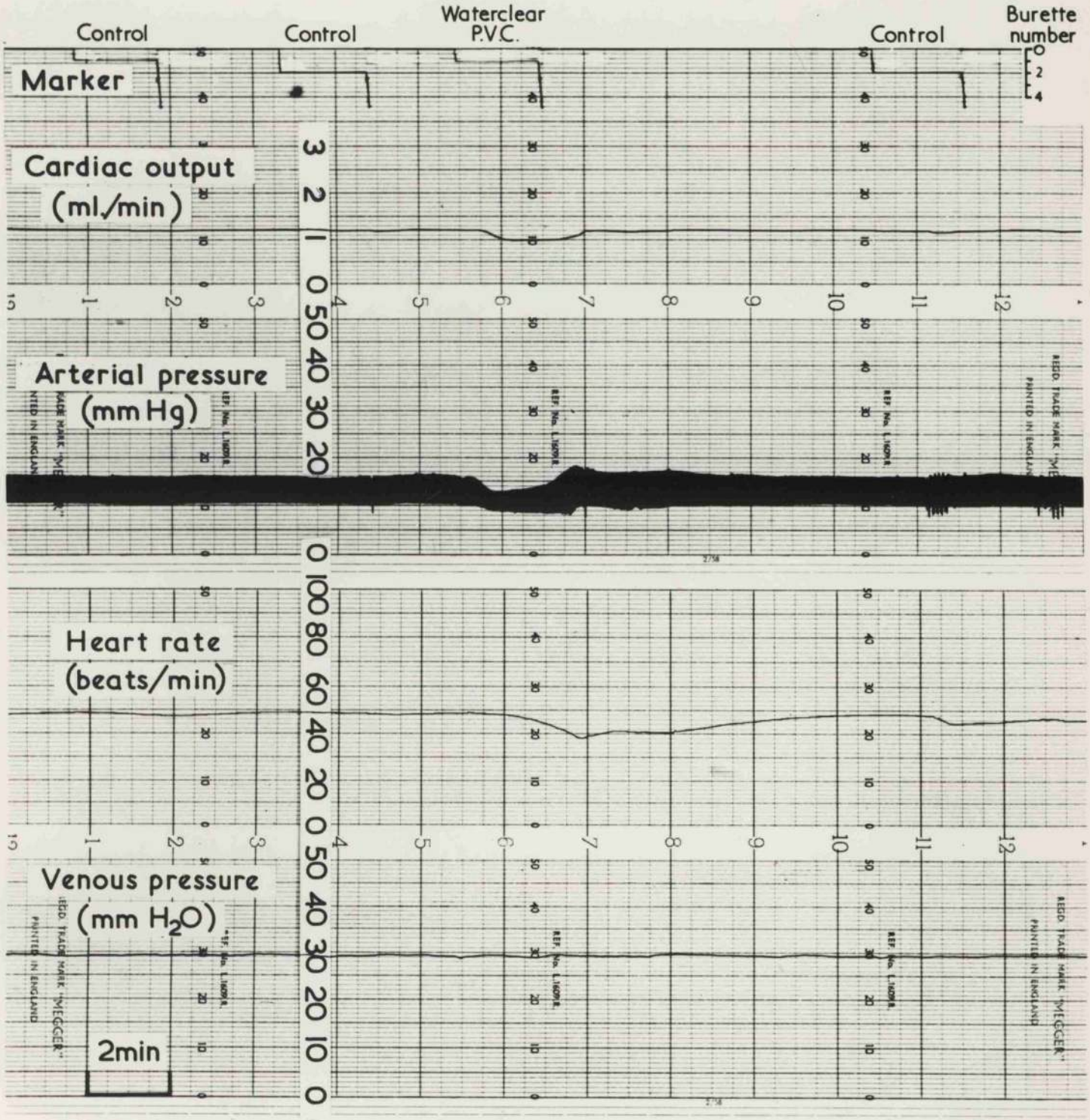

Control
Control
Waterclear P.V.C.
Control
Burette number
0
2
4
Marker
Cardiac output (ml./min)
3 2 1 0
Arterial pressure (mm Hg)
50 40 30 20
Heart rate (beats/min)
100 80 60 40 20 0
Venous pressure (mm H_2O)
50 40 30 20 10 0
2min

Fig. 80

Fig. 81 Grade III effect from the test solution from 'Silicone D.S.R. 551'. Near point 7.30 there was a small accidental rise in the venous pressure (V.P.) while adjusting the reservoir.

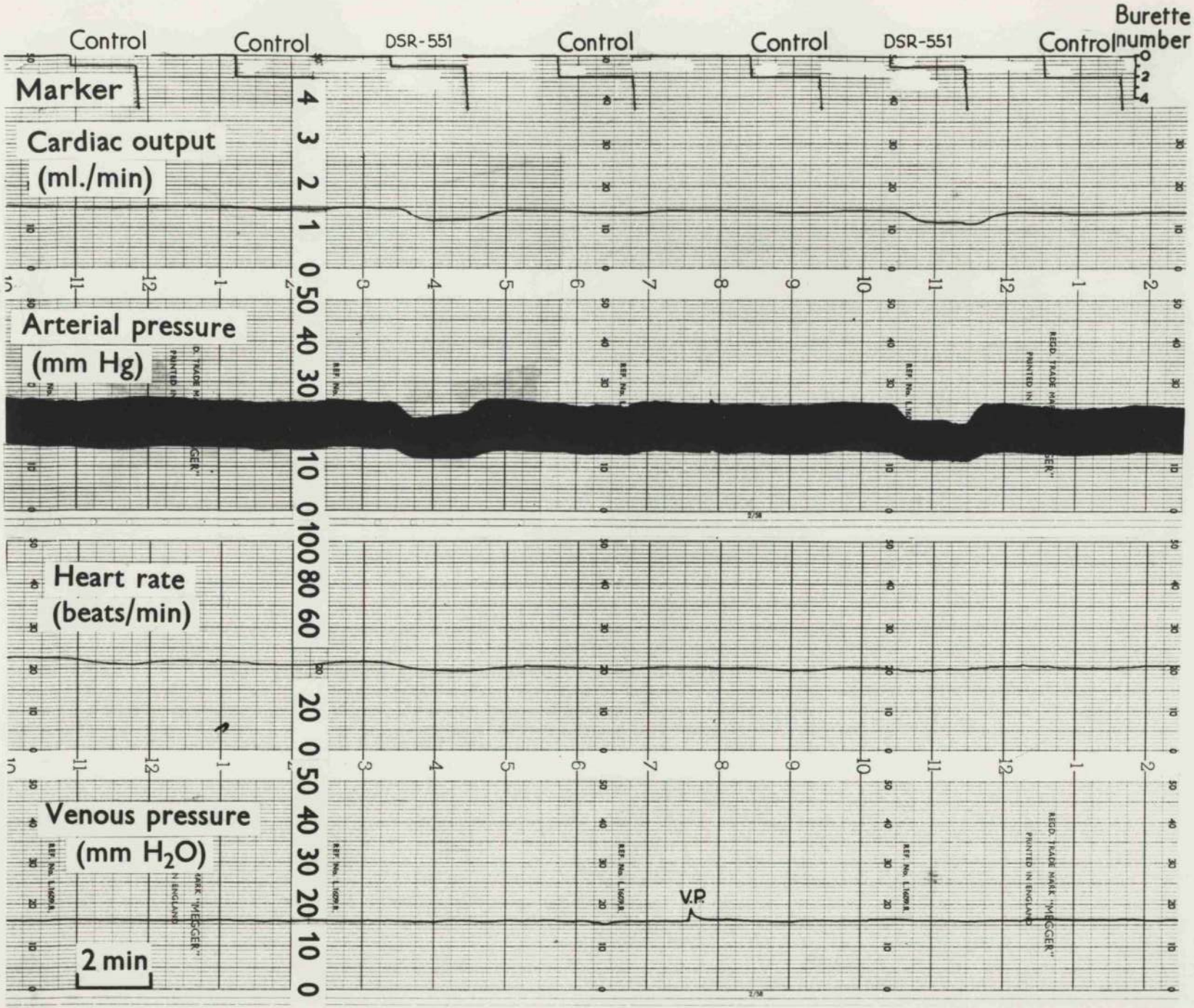

Control
Control
DSR-551
Control
Control
DSR-551
Control
Burette number
0
2
4
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H_2O)
V.P.
2 min

Fig. 81

Fig. 82 Irregularity of rhythm caused by the test solution of 'Portex Crystal Vinyl' P.V.C. tubing.

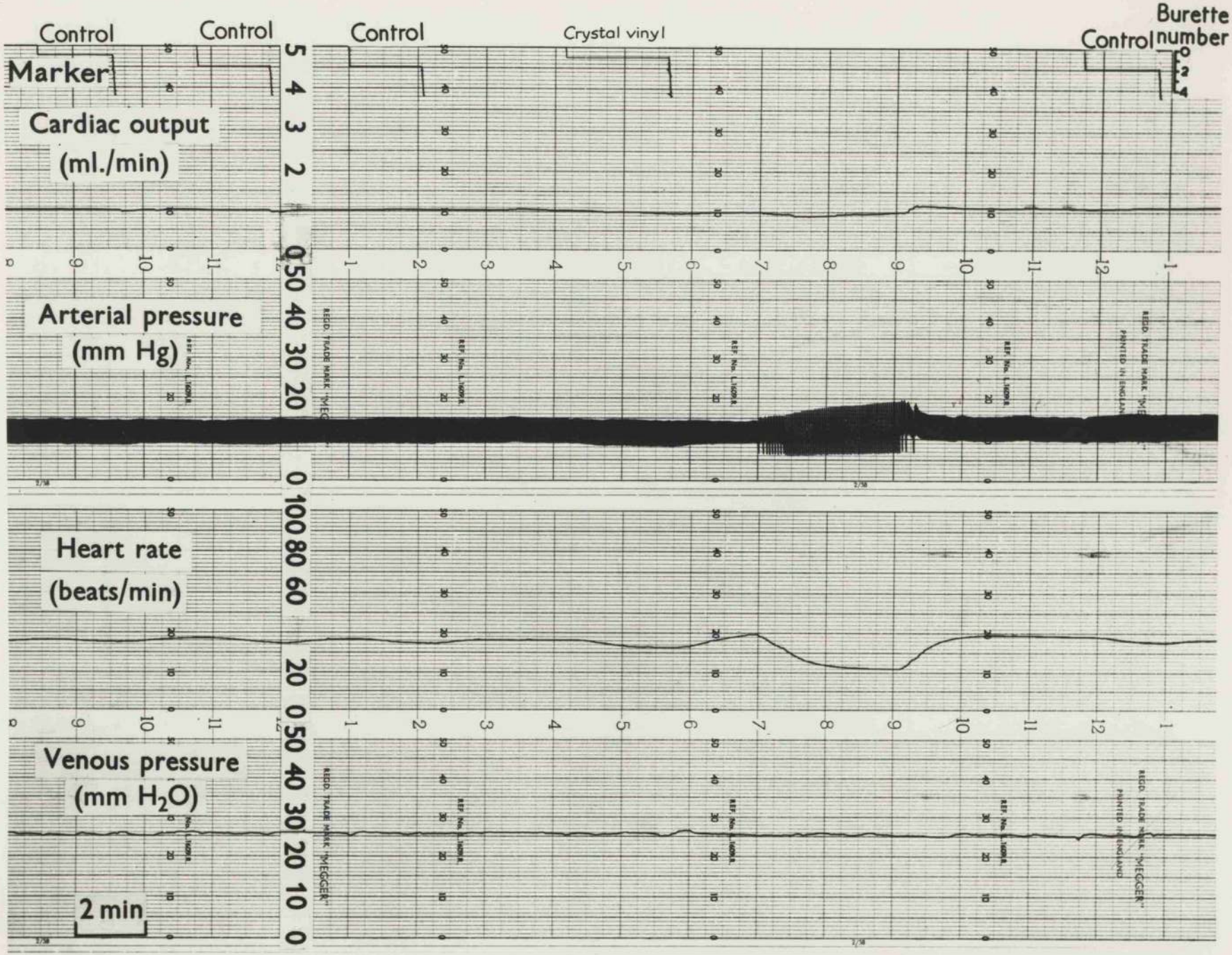

Control
Control
Control
Crystal vinyl
Control
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H_2O)
2 min

Fig. 82

Fig. 83 Irregularity of rhythm caused by the test solution of 'Portex Standard' P.V.C. tubing.

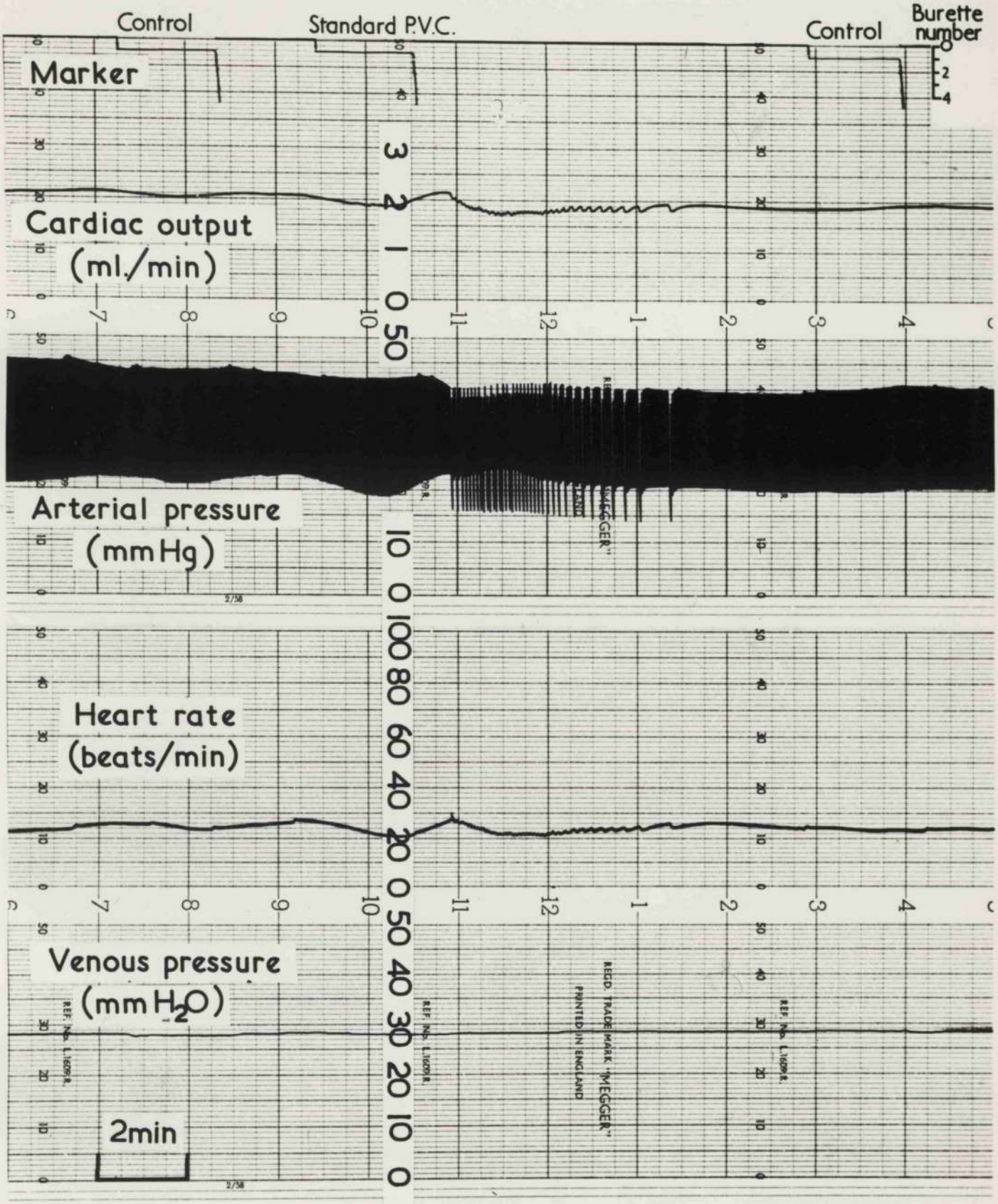

Control
Standard P.V.C.
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mmHg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2min
REED: TRADE MARK "MEGGER"
PRINTED IN ENGLAND

Fig. 83

Fig. 84 Irregularity of rhythm resulting from the test solution of 'Waterclear' P.V.C. tubing. The first test perfusion produced a reduction in the rate associated with a slight increase in the amplitude. The second test perfusion produced a frank inhibitory effect on all the parameters followed by irregularity of rhythm. The effect seems to be cumulative and prolonged.

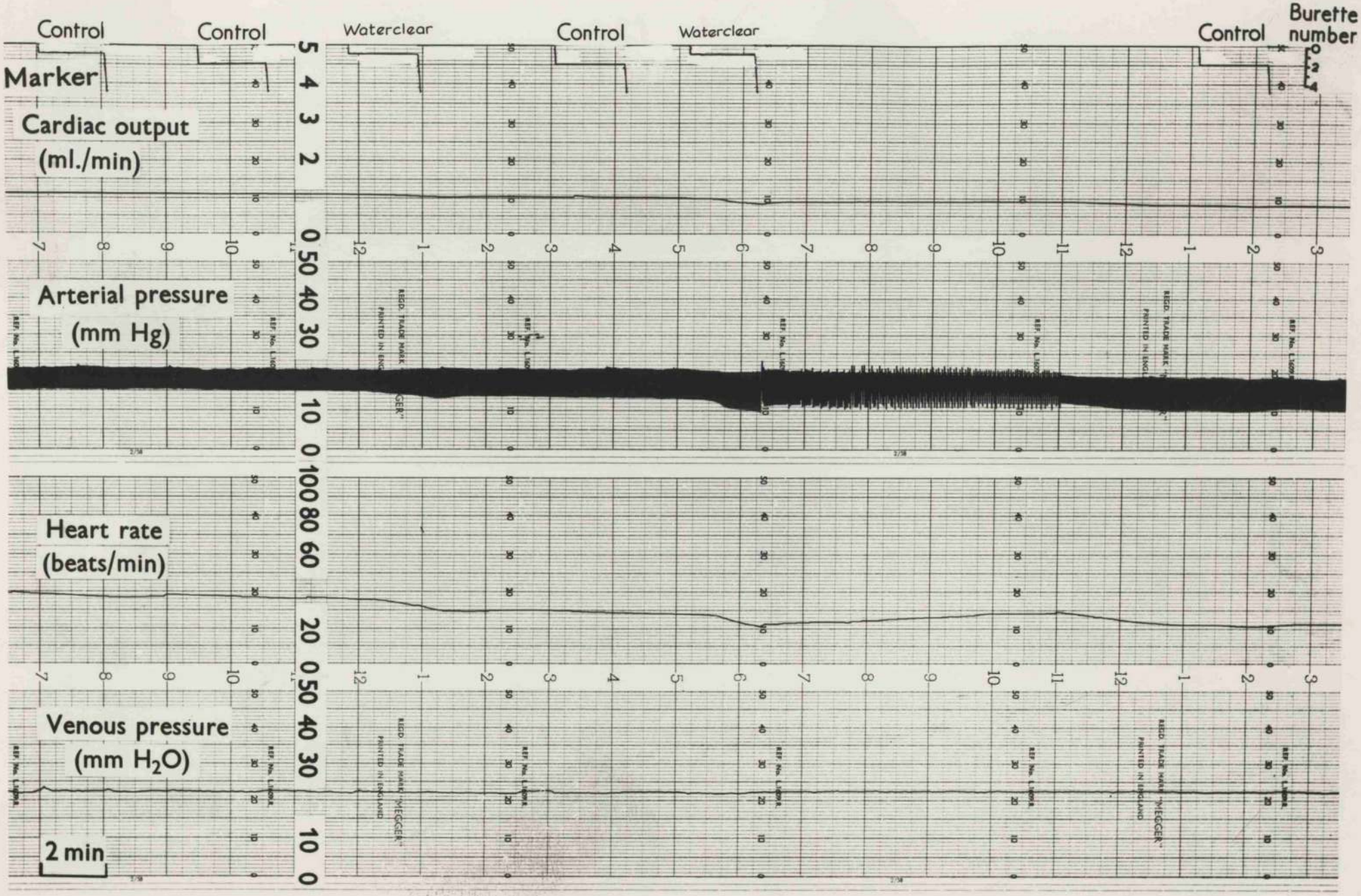

Control
Control
Waterclear
Control
Waterclear
Control
Burette number
Marker
Cardiac output (ml./min)
5 4 3 2
Arterial pressure (mm Hg)
0 50 40 30
Heart rate (beats/min)
10 0 100 80 60
Venous pressure (mm H_2O)
20 0 50 40 30
10 0
2 min

Fig. 84

Fig. 85 Graph showing the grades of effect produced by the test solutions from different kinds of tubing after various intervals of soaking or after $\frac{1}{2}$ hour boiling. The grades of effects shown in this Figure represent the average effect evaluated from two or more test perfusions with the same test solution in each case. The results of experiments on all the 35 frogs are given. In some hearts different grades of effect were obtained from the same kind of tubing after different durations of soaking. These are separately indicated in the legend at the right top corner of the Figure. Numerals in the columns represent durations of soaking in hours.

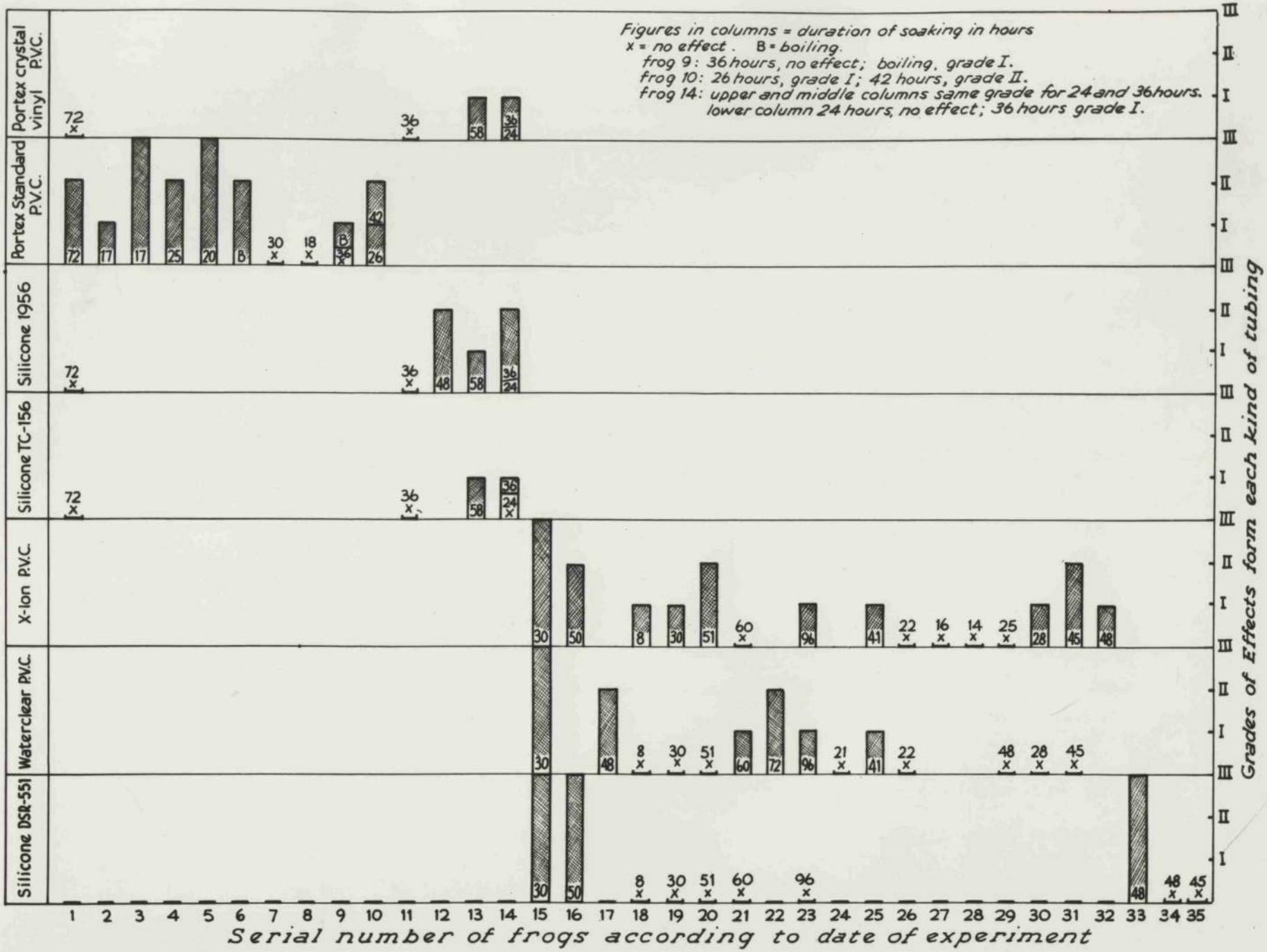

Figures in columns = duration of soaking in hours
x = no effect . B = boiling.
frog 9: 36 hours, no effect; boiling, grade I.
frog 10: 26 hours, grade I; 42 hours, grade II.
frog 14: upper and middle columns same grade for 24 and 36 hours.
lower column 24 hours, no effect; 36 hours grade I.
Portex crystal vinyl P.V.C.
Portex Standard P.V.C.
Silicone 1956
Silicone TC-156
X-lon P.V.C.
Waterclear P.V.C.
Silicone DSR-551
Grades of Effects form each kind of tubing
Serial number of frogs according to date of experiment

Fig. 85

Fig. 86 Comparison of 'Silicone TC-156' and 'Silicone-1956'. The test solution from both kinds of tubing were obtained by soaking the pieces for 24 hours. The test solution of 'Silicone TC-156' showed no toxicity whereas that of 'Silicone-1956' produced a marked biphasic effect on the heart rate and a fall in the blood pressure. The output did not alter possibly because the increase in the heart rate compensated for the reduction in amplitude. There is a junction (J) in the record between points 5.0 and 6.0 indicating that part of the record has been removed to facilitate photographic reproduction.

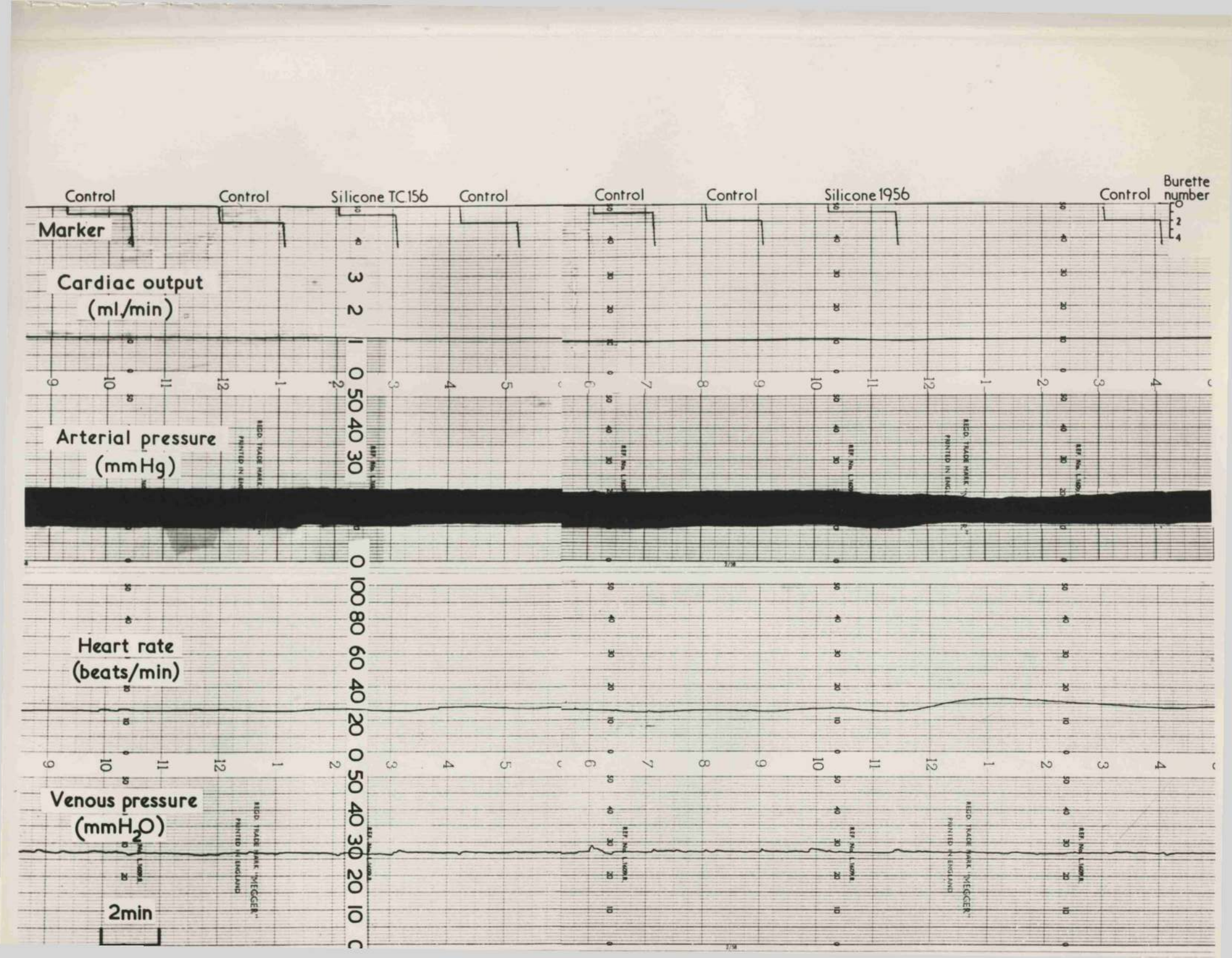

Control
Control
Silicone TC156
Control
Control
Control
Silicone 1956
Control
Burette number
Marker
Cardiac output (ml/min)
Arterial pressure (mmHg)
Heart rate (beats/min)
Venous pressure (mmH2O)
2min

Fig. 87 Extract from the record of same heart as in Fig. 86. The sample of the same test solution from 'Silicone TC-156' obtained after 36hours of soaking, now produced a grade I effect. The effect is similar to that of 'Silicone-1956' after 24 hours of soaking as shown in Fig. 86. A small decrease in the heart rate during controls is of no significance. The effect of toxicity is more prominent on the blood pressure. There is a delayed increase in the heart rate with a few extra systoles.

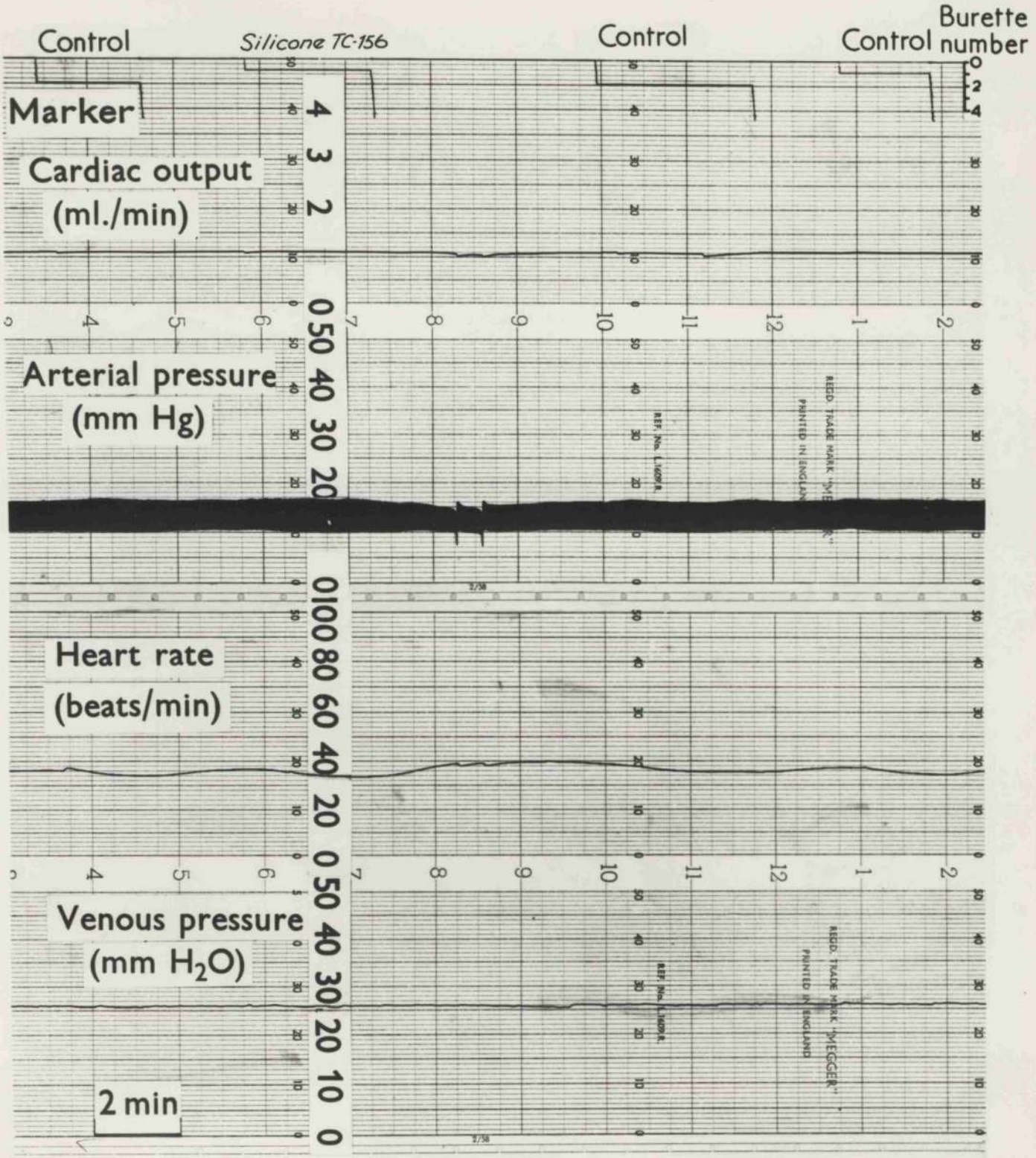

Control
Silicone TC-156
Control
Control
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H_2O)
2 min

Fig. 87

Fig. 88 Extract from the record of same heart as in Figs. 86 and 87. The test solution from 'Silicone-1956' after 36 hours of soaking produced a greater effect than that produced by the test solution of 'Silicone TC-156' (Fig. 87). Also note the biphasic effect on the heart rate and the greater degree of irregularity in rhythm after the perfusion with test solution from 'Silicone-1956'. However it should be appreciated that the test solution from 'Silicone-1956' was perfused for a longer time than that from 'Silicone TC-156'. The important point is that 'Silicone TC-156' did not show any toxicity after 24 hours of soaking but showed toxicity after 36 hours of soaking. 'Silicone-1956' showed toxicity after 24 hours of soaking. Hence 'Silicone TC-156' was slightly better than 'Silicone-1956'.

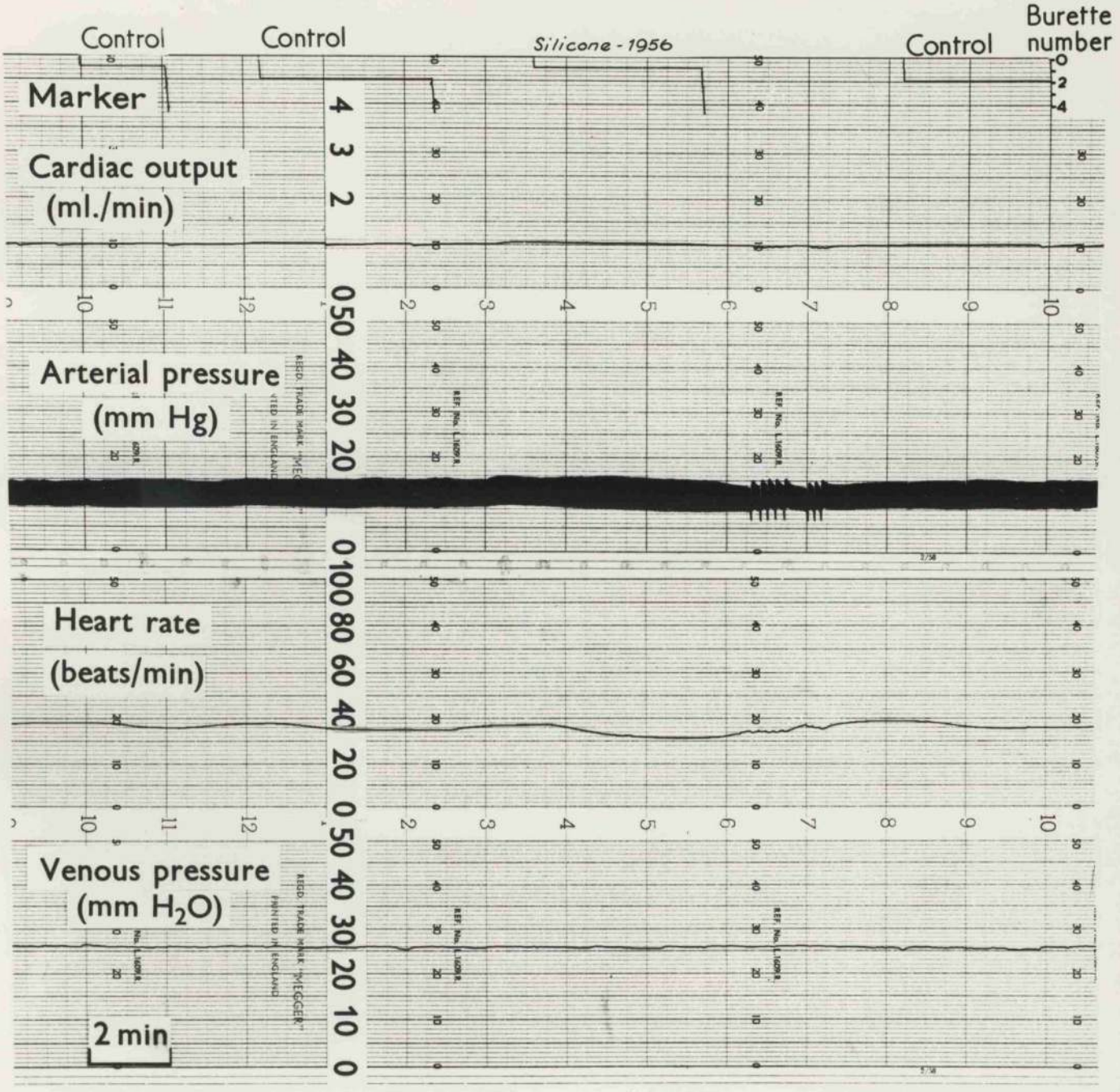

Control
Control
Silicone - 1956
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Fig. 88

Fig. 89 Grade III effect due to the toxicity of 'Silicone DSR-551'.

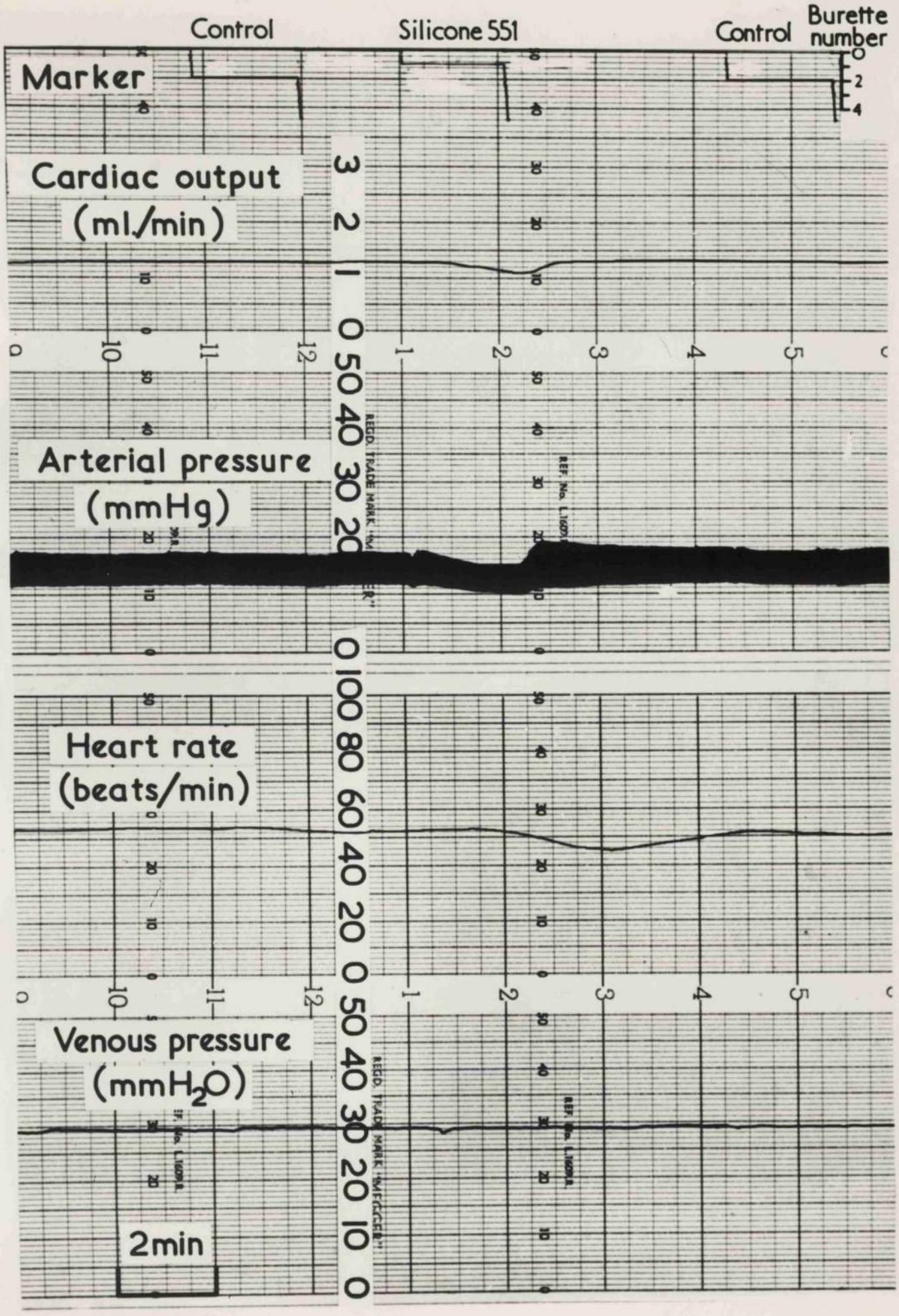

Control
Silicone 551
Control
Burette number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mmHg)
Heart rate
(beats/min)
Venous pressure
(mmH2O)
2min

Fig. 89

Fig. 90 The test solution from 'Waterclear' P.V.C. tubing cleaned several times produced no effect.

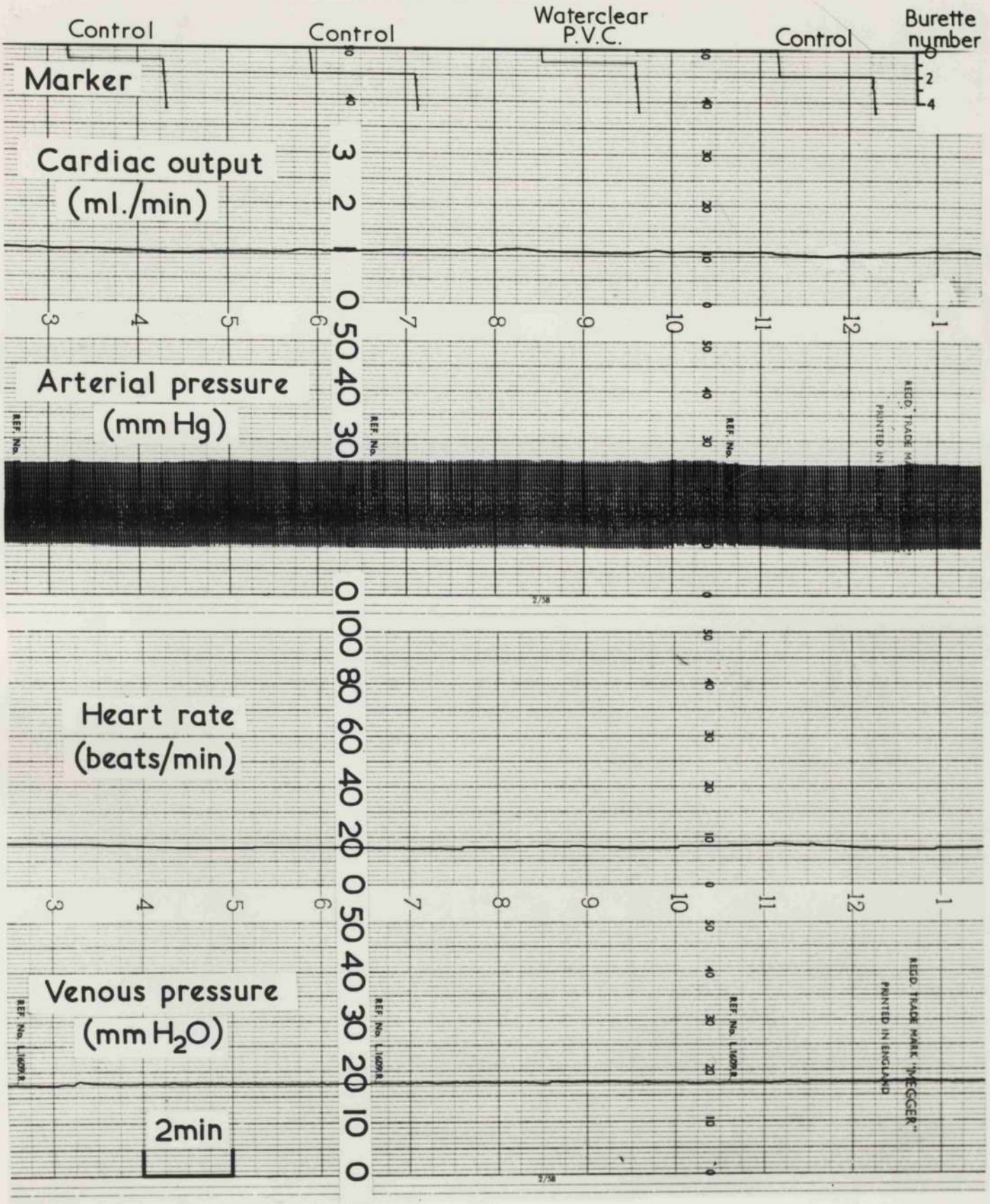

Control
Control
Waterclear P.V.C.
Control
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2min

Fig. 90

Fig. 91 Same heart as in Fig. 90. The test solution from 'X-lon' P.V.C. tubing cleaned several times produced a toxic effect. The gradation of effect from the three types of test solutions indicates that the toxicity was reduced after repeated cleaning. For cleaning procedures see Methods in Part I.

Burette number
0
E2
E4
Control
X-Ion P.V.C. uncleaned
Control
X-Ion P.V.C. cleaned once
Control
X-Ion P.V.C. cleaned repeatedly
Control
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2min
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND

Fig. 92 Comparison of the toxicity of 'X-lon' P.V.C. tubing cleaned several times and not cleaned at all. The test solutions from both cleaned and fresh tubing were obtained after identical duration of soaking. It is clear that the toxicity was reduced after repeated cleaning.

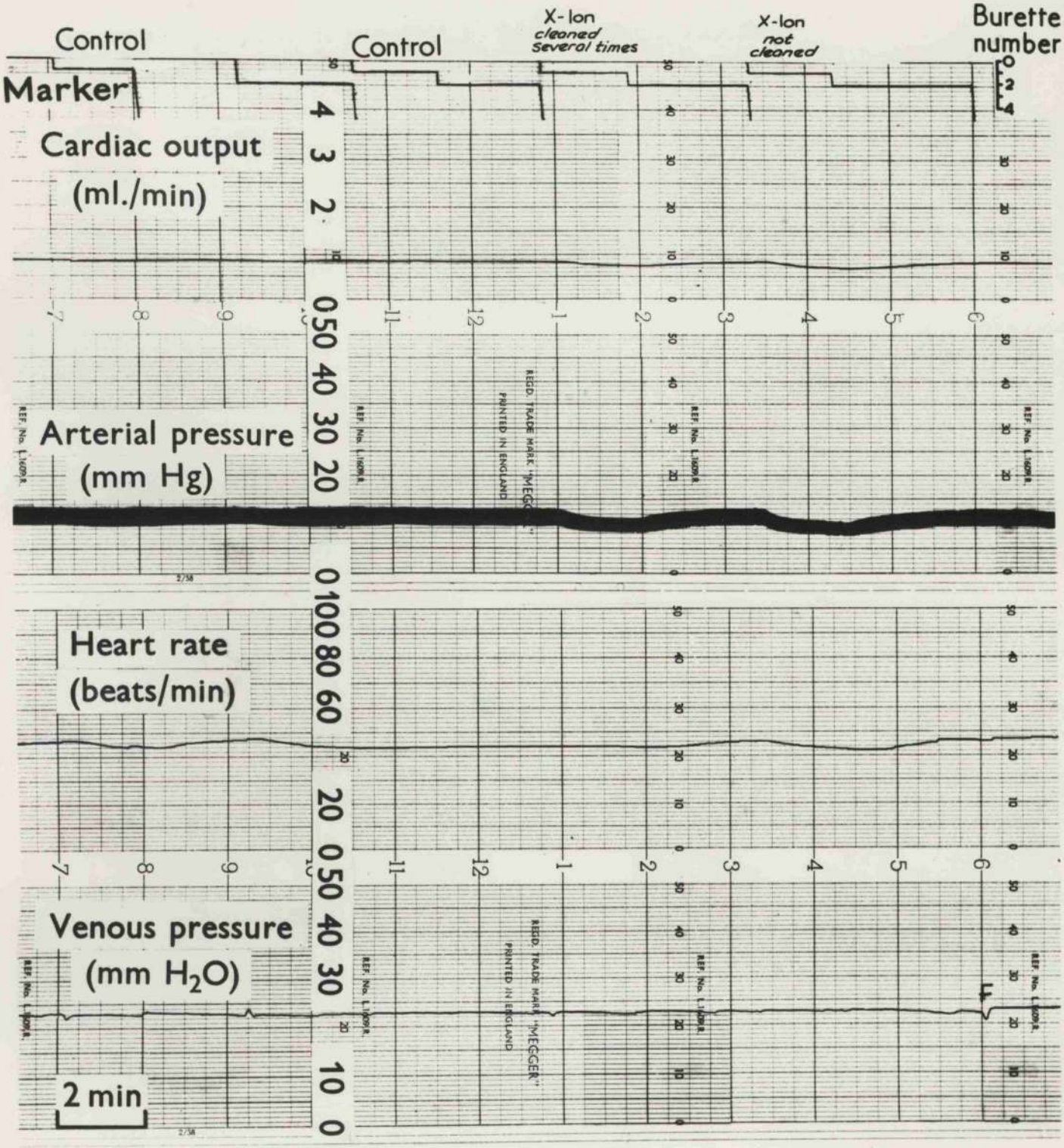

Control
Control
X-Ion
cleaned
several times
X-Ion
not
cleaned
Burette
number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2 min

Fig. 92

Fig. 93 Comparison of toxicity of test solutions from 'X-lon P.V.C' tubing cleaned several times and cleaned once only. The test solution from the tubing cleaned several times produced no effect whereas the test solution from tubing cleaned once only was highly toxic producing a gross irregularity of rhythm.

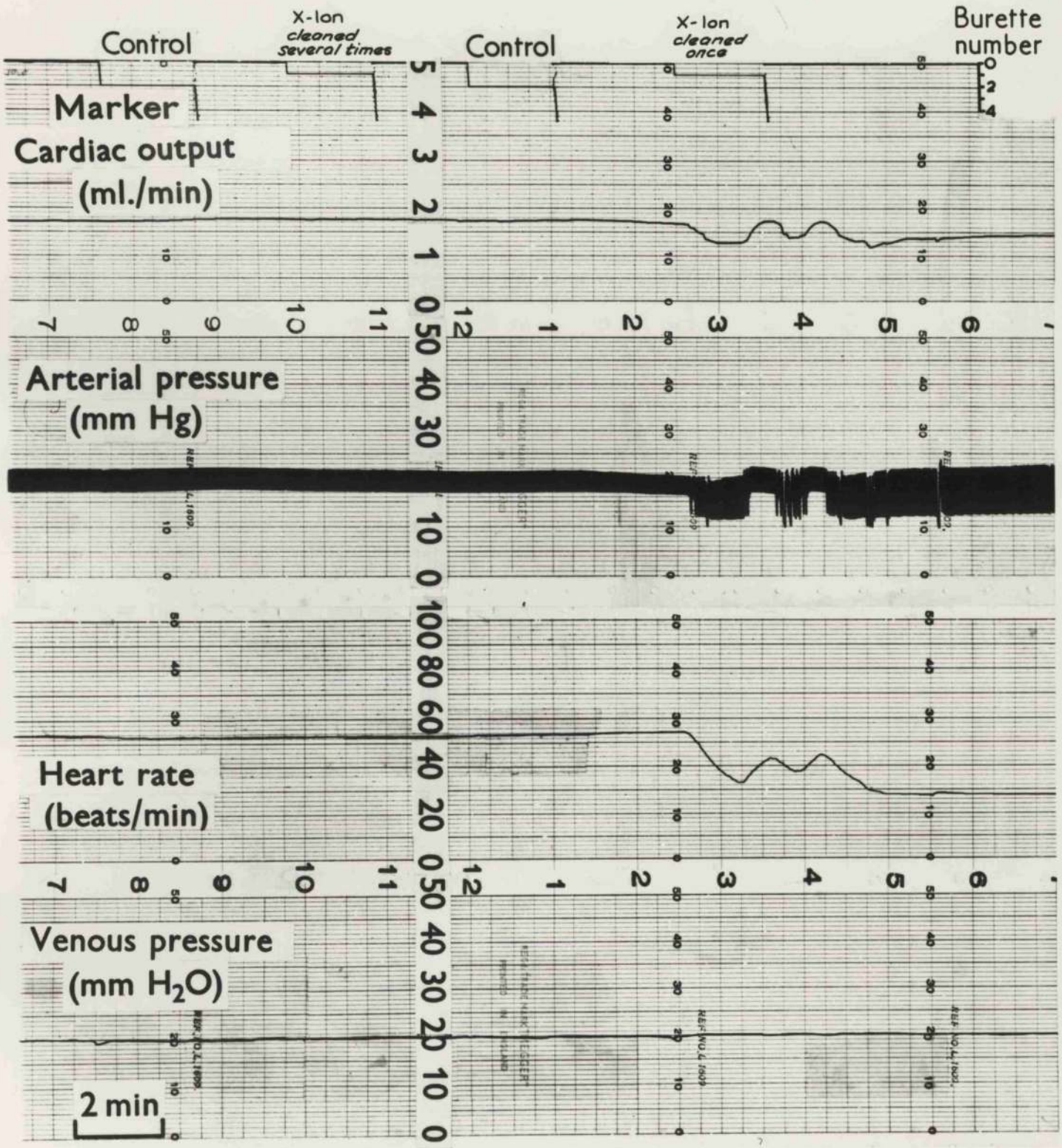

Control
X-lon
cleaned
several times
Control
X-lon
cleaned
once
Burette
number
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2 min

Fig. 93

Fig. 94 Another example of a graded effect from the toxicity of 'X-lon'P.V.C. tubing cleaned several times, cleaned once only and not cleaned at all. The toxicity persisted even after repeated cleaning. In the beginning of the record the heart was being perfused continuously with control Ringer's solution from burette 2 and the control was conducted from burette 1. Near point 10.45 the control Ringer's solution ran out and the perfusion was changed to the reservoir. As there was no significant difference between the Ringer's solution in the reservoir and the control Ringer's solution, the perfusion was continued from the reservoir. The movement of the marker to the position of burette 2 below 'X' indicates that test perfusion with a test solution from 'X-lon' P.V.C. tubing was started from burette 2. Later, when it was realised that the test perfusion should have been conducted from burette 1 which was used for the control, the test perfusion from burette 2 was discontinued and burette 1 was used for the tests.

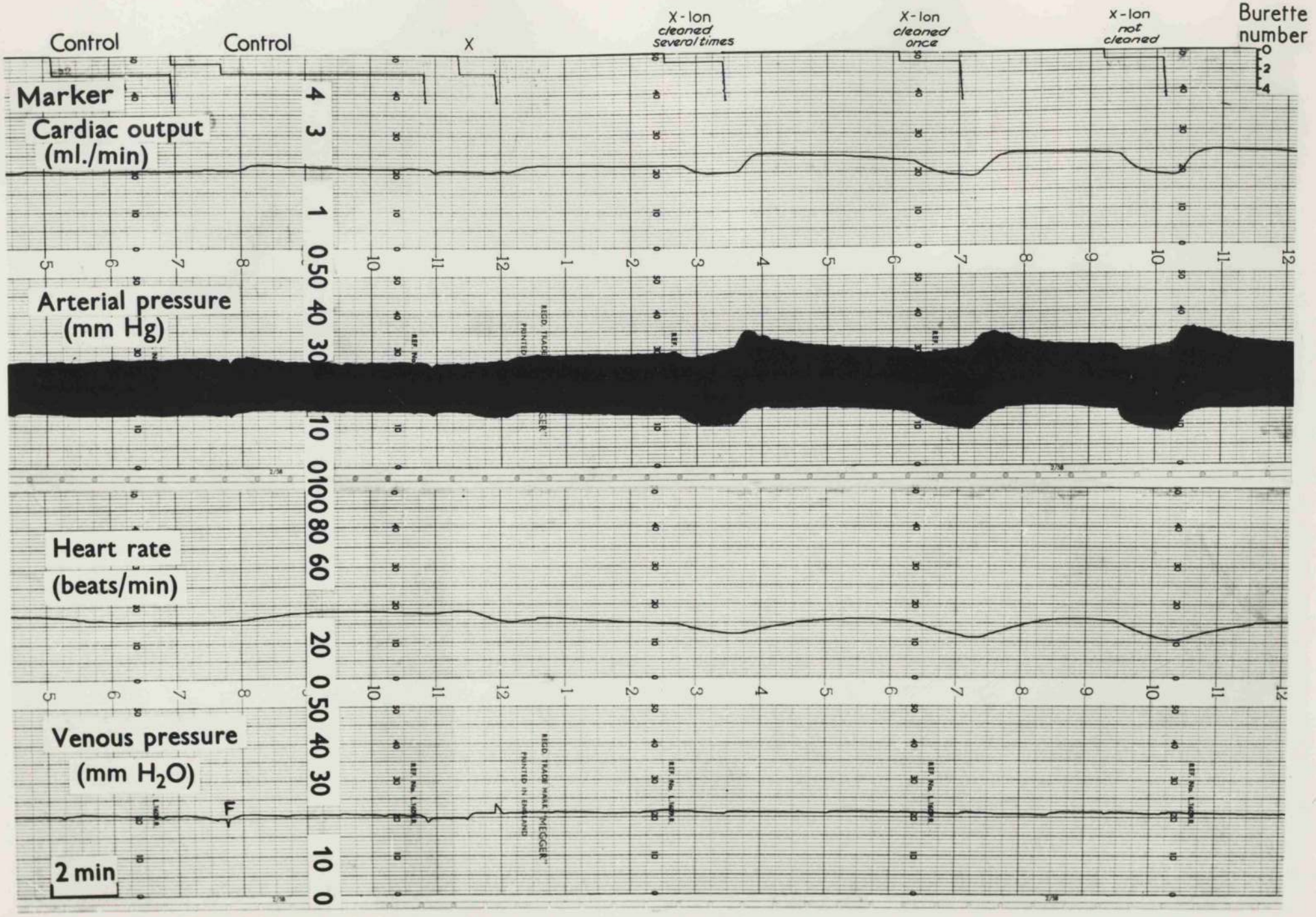

Control
Control
X
X-Ion cleaned several times
X-Ion cleaned once
X-Ion not cleaned
Burette number
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min

Fig. 95 Comparison of the efficiency of the 'Calgon' and 'Bicarbonate' methods of cleaning for the removal of toxicity from the tubing. The test solutions from 'X-lon' P.V.C. tubing cleaned several times by both methods, showed a grade I effect whereas the test solution from 'Silicone DSR-551' tubing cleaned several times by both methods, showed no effect. Hence both methods of cleaning were equally efficient in reducing the toxicity of tubing.

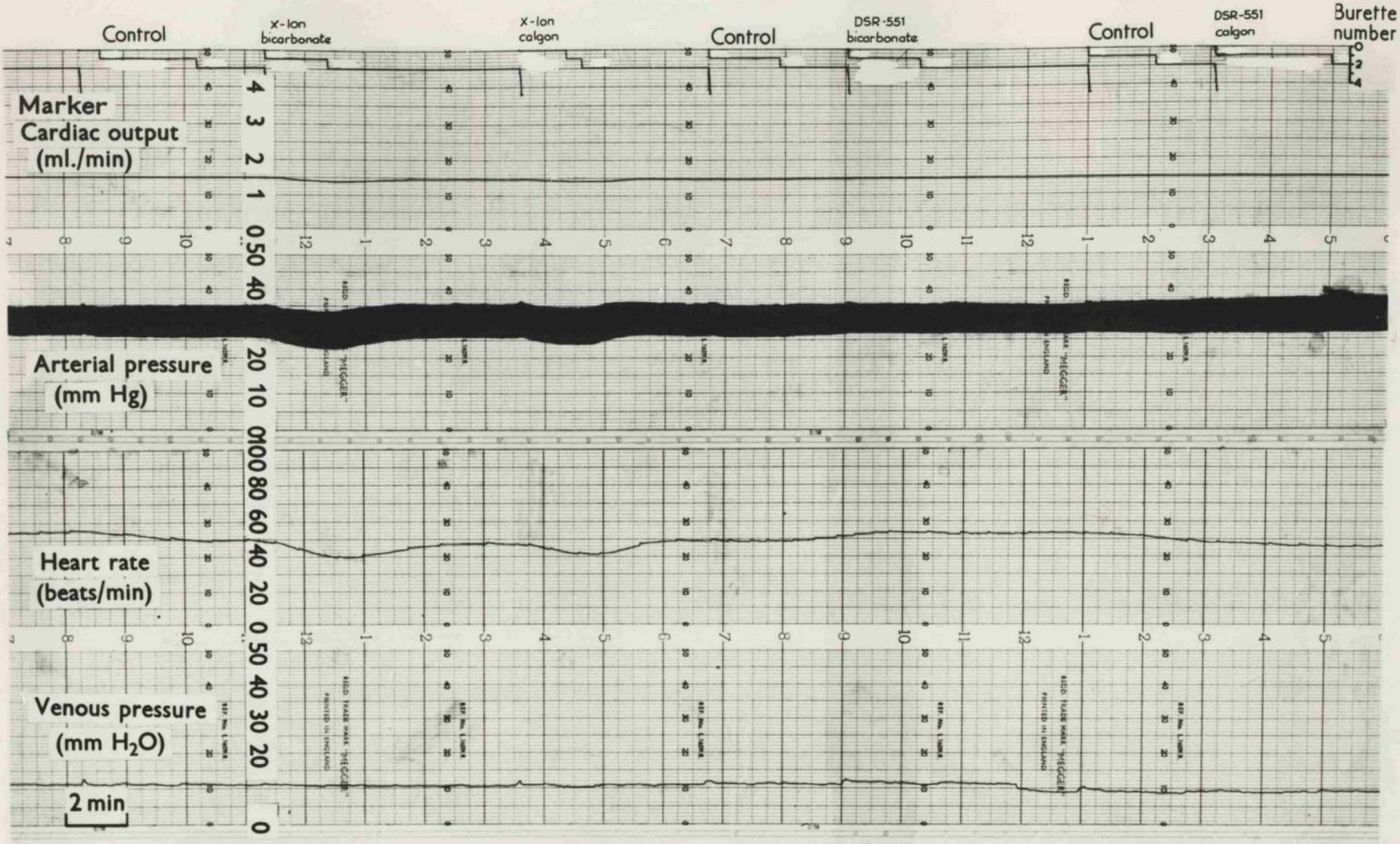

Control
X-Ion bicarbonate
X-Ion calgon
Control
DSR-551 bicarbonate
Control
DSR-551 calgon
Burette number
Marker
Cardiac output (ml./min)
4 3 2 1 0.5 0
Arterial pressure (mm Hg)
40 20 10 0
Heart rate (beats/min)
100 80 60 40 20 0
Venous pressure (mm H_2O)
50 40 30 20 0
2 min

Fig. 95

Table 1.

Frogs used for different purposes.

Hearts used for testing.	number of frogs		
	uninjected	injected with female sex hormones	total
1. Threshold sensitivity to acetylcholine	86	38	124
2. Stoppage concentration of acetylcholine	73	37	110
3. Threshold sensitivity to adrenaline	3	19	22
4. Threshold sensitivity to moradrenaline	3	18	21
5. Threshold sensitivity to 5-hydroxytryptamine	3	14	17
6. Threshold sensitivity to nicotine	2	0	2
7. Effect of changes in the composition of Ringer's solution on sensitivity to acetylcholine	5	0	5
8. Concentration-response relationship	17	0	17

Table 2.

Conc. of Ach	No. of tests	Illustrating figure No.	Mean Value (% inhibition) Rate	B.P.	Maximum variation (% inhibition) Rate	B.P.
1.5×10^{-7}	2	Fig.20b	41	76	3	7
5×10^{-8}	2	Fig.15a	Block	78	Block	3
10^{-8}	2	Fig.15a	11	22	2	1
5×10^{-9}	2	Fig.13c	9	11	2	2
5×10^{-9}	2	Fig.13c	11	17	3	10
10^{-9}	7	Fig.21	13	7	5	3
10^{-9}	3	-	17	20	4	3
10^{-9}	2	-	11	20	7	1
10^{-11}	3	Fig.27	17	10	2	5
10^{-11}	2	Fig.52c	11	27	5	5
10^{-11}	3	Fig.52a	33	28	irregular	11
10^{-11}	3	Fig.54a	46	40	7	5
10^{-13}	7	Figs. 47, 57, 58, 54b	44	30	27	29
10^{-13}	2	Fig.28	58	21	0	0
10^{-15}	3	Fig.54c	22	18	8	10
10^{-15}	2	Fig.55b	13	22	1	5
10^{-17}	3	Figs. 59, 60, 55a	28	51	5	9
10^{-19}	3	Fig. 55c	20	36	9	6

The scatter in the values of percentage inhibition in repeated tests with the same solution.

Table 3.

The incidence of high threshold sensitivity to acetylcholine and the incidence of stoppage at low concentrations of acetylcholine among the hearts of the two sexes.

Sex	Threshold sensitivity at concentration less than 10^{-8} g/ml.			Stoppage at concentrations less than 10^{-7} g/ml.		
	hearts tested	highly sensitive hearts	percentage of highly sensitive hearts.	hearts tested	highly sensitive hearts	percentage of highly sensitive hearts,
Male	45	5	11.1	36	3	8.3
Female	39	7	17.9	36	4	11.1

Table 4

Spontaneous variations in sensitivity to acetylcholine with time.

Date of start	Sex	Duration of previous perfusion	Change in sensitivity	
			+,- or 0	Degree of change
20-12-59	F	24	0	
26-2-60	M	21	+	sensitivity from 10^{-15} to 10^{-17}
13-8-60	M	13	-	sensitivity from 10^{-9} to 10^{-7}
18-8-60	M	13	+	sensitivity at 10^{-7} to stoppage at 10^{-7}
25-8-60	F	24	0	
15-9-60	M	24	-	stoppage from 10^{-8} to 10^{-7}
13-10-60	M	13	-	sensitivity from 10^{-9} to 10^{-7}
26-1-61	M	21	+	sensitivity from 10^{-9} to 10^{-11}

+ increase, - decrease, 0 no change in sensitivity.

Table 5.

Increase in the threshold sensitivity of hearts to acetylcholine after the administration of adrenaline.

Date of start of experiment.	Sex	duration of perfusion in hours.		degree of increase in the sensitivity to acetylcholine.
		before adrenaline	after adrenaline.	
23-1-60	F	24	1.5	from no effect at 10^{-9} to stoppage at 10^{-9}
26-2-60	M	19	1.0	sensitivity from 10^{-17} to 10^{-19}
18-3-60	F	5	0.25	from no effect at $10^{-7.5}$ to effect at $10^{-7.5}$
25-3-60	F	22	1.5	from stoppage at 10^{-9} to sensitivity at 10^{-13}
14-4-60	M	0	2.0	from no effect at 10^{-9} to effect at 10^{-9}
27-10-60	M	18	1.0	sensitivity from 10^{-7} to 10^{-15}
30-12-60	M*	5	1.25	from no effect at 10^{-11} to effect at 10^{-11}
			2.50	sensitivity from 10^{-11} to 10^{-15}
			16.0	sensitivity from 10^{-15} to 10^{-19}
2-2-61	F	4	3.0	sensitivity from 10^{-13} to 10^{-15}

* injected frog (oestradiol 10 mg.)

Table 6.

Threshold sensitivity of hearts to acetylcholine after alterations in the composition of Ringer's solution.

Hearts	alterations in the Ringer's solution.	sensitivity with	
		normal Ringer	modified Ringer
1.	low calcium (75% of normal)	10^{-15}	10^{-15}
	high calcium (150% of normal)	10^{-7}	10^{-7}
2.	low calcium (75% of normal)	10^{-7}	10^{-7}
	low calcium (75% of normal) + sucrose 1 g/l	10^{-7}	10^{-7}
	high calcium (150% of normal)	10^{-7}	10^{-7}
	high calcium (150% of normal) + sucrose 1 g/l	10^{-7}	10^{-7}
3.	normal Ringer without glucose	10^{-7}	10^{-7}
	normal Ringer with glucose	10^{-7}	10^{-7}
	normal Ringer with sucrose in place of glucose	10^{-7}	10^{-7}
	normal Ringer + glucose + sucrose 1 g/l	10^{-7}	10^{-7}
	low calcium (25% of normal) without glucose	10^{-7}	10^{-7}
	low sodium (50% replaced by sucrose)	10^{-7}	10^{-7}
4.	low potassium (50% of normal)	10^{-8}	10^{-7}
	high calcium (150% of normal)	10^{-7}	10^{-7}
5.	low calcium (75% of normal)	10^{-7}	10^{-7}
	low potassium (50% of normal)	10^{-7}	10^{-7}
	high calcium (150% of normal) + low potassium (50% of normal)	10^{-7}	10^{-7}
	low sodium (50% of normal) + low potassium (50% of normal)	10^{-7}	10^{-7}

Table 7

The effect of injections of oestradiol on the weight of frogs: The threshold sensitivity of their hearts to acetylcholine.

serial number of frogs	date of experiment	sex	Weight in grams		change in weight	percentage change in weight	total dose of oestradiol in mg.	threshold sensitivity to acetylcholine
			before injection	after injection				
1	10-11-60	M	...	...	...	...	0.01	10^{-7}
2	11-11-60	M	...	...	...	...	0.01	10^{-7}
3	11-11-60	M	...	...	...	...	0.005	10^{-11}
4	17-11-60	M	17.62	16.62	-1.0	-5.68	0.01	10^{-7}
5	18-11-60	M	23.07	19.57	-3.5	-15.18	0.01	10^{-8}
6	18-11-60	M	12.97	16.57	+3.6	+27.8	0.01	10^{-7}
7	24-11-60	M	17.49	17.45	-0.04	+00.23	0.015	10^{-7}
8	25-11-60	M	5.19	6.74	+1.55	+30.00	0.015	10^{-7}
9	25-11-60	M	22.67	23.45	+0.78	+3.34	0.015	10^{-7}
10	1-12-60	M	18.49	17.94	-0.55	-2.94	0.015	10^{-7}
11	1-12-60	M	...	...	...	...	0.015	10^{-7}
12	9-12-60	M	6.89	8.58	+1.69	+24.50	0.6	10^{-7}

Table 7 (contd.)

13	9-12-60	M	...	...	...	...	0.6	10^{-9}
14	9-12-60	F	9.20	8.91	-0.29	-3.15	0.3	10^{-13}
15	15-12-60	F	32.54	31.91	-0.63	-1.94	4.5	10^{-7}
16	15-12-60	M	29.50	27.38	-2.12	-7.18	4.5	10^{-7}
17	16-12-60	M	...	...	...	...	3.0	10^{-13}
18	22-12-60	M	12.49	12.54	+0.05	+0.40	8.0	10^{-11}
19	23-12-60	M	16.84	14.59	-2.25	-13.34	8.0	10^{-13}
20	30-12-60	M	14.19	21.00	+6.81	+48.0	10.0	10^{-19}
21	12-1-61	M	25.77	26.00	+0.23	+0.9	10.0	10^{-9}
22	13-1-61	F	33.60	34.14	+0.54	+1.61	10.0	10^{-9}
23	8-6-61	F	27.42	25.92	-1.50	-5.47	8.0	10^{-9}
24	8-6-61	F	30.43	32.47	+2.04	+6.69	8.0	10^{-7}
25	15-6-61	F	25.45	26.33	+0.88	+3.46	10.0	10^{-7}
26	15-6-61	M	23.35	23.72	+0.37	+1.59	10.0	10^{-7}
27	22-6-61	F	26.12	26.72	+0.60	+2.30	15.0	10^{-13}
28	22-6-61	M	33.46	33.90	+0.44	+1.31	15.0	10^{-13}
29	22-6-61	M	24.47	24.80	+0.33	+1.35	15.0	10^{-7}

+ increase, - decrease.

Table 8

The effect of injections of progesterone on the weight of frogs:
The threshold sensitivity of their hearts to acetylcholine.

Serial number of frogs	date of experiment	sex	weight in grams		change in weight	percentage change in weight	total dose of progesterone in mg.	threshold sensitivity to acetylcholine
			before injections	after injections				
1	3-2-61	M	20.89	21.17	+0.28	+1.34	1.5	10^{-10}
2	16-2-61	M	16.87	16.52	-0.35	-2.10	4.5	10^{-9}
3	17-8-61	M	19.89	21.30	+1.41	+7.13	3.0	10^{-7}
4	17-8-61	F	20.15	21.68	+1.53	+7.59	3.0	10^{-7}
5	17-8-61	F	15.95	17.72	+1.77	+11.10	3.0	10^{-7}
6	24-8-61	F	15.50	18.17	+2.67	+17.24	5.0	10^{-7}
7	24-8-61	M	18.15	19.27	+1.12	+6.17	5.0	10^{-7}

+ increase, - decrease.

Table 9

Approximate duration between the preparation and use of acetyl--choline solutions in some of the experiments in which low concentrations of acetylcholine (lower than 10^{-7} g/ml) were effective.

date of experiment	duration of standing of solutions	concentration of acetylcholine found to be effective.
5-2-60	24 hours	10^{-13} g/ml
26-2-60	5.5 hours	10^{-15} "
	17.5 hours	10^{-17} "
26-3-60	1.5 hours	10^{-13} "
5-8-60	18.0 hours	10^{-9} "
11-11-60	4.0 hours	10^{-11} "
16-12-60	1.5 hours	10^{-13} "
23-12-60	1.0 hours	10^{-11} "
30-12-60	4.5 hours	10^{-11} "
	2.0 hours	10^{-15} "
14-1-61	1.5 hours	10^{-15} "
	2.0 hours	10^{-19} "
	1.0 hours	10^{-21} "
26-1-61	12.0 hours	10^{-11} "
28-1-61	8.0 hours	10^{-15} "
2-2-61	4.0 hours	10^{-13} "
	5.75 hours	10^{-15} "
3-2-61	3.0 hours	10^{-15} "
	2.0 hours	10^{-19} "

Table 10

Action of increasing concentrations of adrenaline in the hearts of uninjected frogs.

Serial number of frogs	Date	Sex	Concentrations of adrenaline in g/ml.				
			10^{-5}	10^{-7}	10^{-9}	10^{-11}	10^{-13}
1	27-10-60	F		+	0	0	0
2	3-11-60	M	+	+	0	0	
3	2-12-60	M	++	++	--	0	

0 no effect, + stimulation, - inhibition.
Number of plus or minus indicates degree of effect.

Table 11.

Action of increasing concentrations of adrenaline in the hearts of oestradiol injected frogs.

serial number of frogs	date	sex	total dose in mg	concentrations of adrenaline in g/ml.				
				10^{-5}	10^{-7}	10^{-9}	10^{-11}	10^{-13}
1	10-11-60	M	0.01		+++	-	0	0
2	11-11-60	M	0.01		+++	+	+	
3	11-11-60	M	0.005			$^{+}R_{+}$	0	
4	17-11-60	M	0.01		++++	0		
5	18-11-60	M	0.01		+++++	0	0	
6	18-11-60	M	0.01		0	0	0	
7	24-11-60	M	0.015	+++	$^{-}R_{+}$	0	0	
8	25-11-60	M	0.015		++	0		
9	25-11-60	M	0.015	++++++	0	0		
10	1-12-60	M	0.015	++++	--	$^{-}R_{-}$	0	
11	1-12-60	M	0.015	+++	+++			
12	8-12-60	M	0.6		+++	0	0	
13	9-12-60	M	0.6		++	0	0	
14	9-12-60	F	0.3		+++	0	0	
15	15-12-60	F	4.5		++	0	0	
16	15-12-60	M	4.5	++	$^{o}R_{-}$			
17	16-12-60	M	3.0	+++	0			
18	23-12-60	M	8.0	+++	0	0	0	
19	30-12-60	M	10.0		++++S			

+ increase, - decrease in all parameters. 0 no effect.

$^{+}R_{+}$ or $^{-}R_{-}$ increase or decrease of other parameters mainly due to increase or decrease in heart rate respectively.

$^{-}R_{+}$ decrease of other parameters mainly due to increase in rate

$^{o}R_{-}$ decrease in rate with no change in other parameters.

++++S, stimulation followed by stoppage.

Other notations as in Table 8.

Table 12

Actions of increasing concentrations of nor-adrenaline in the hearts of uninjected frogs.

Serial number of frogs	Date	Sex	Concentrations of nor-adrenaline in g/ml.				
			10^{-5}	10^{-7}	10^{-9}	10^{-11}	10^{-13}
1	3-11-60	M	++	+	0	0	
2	4-11-60	M	++++	0	0	0	
3	2-12-60	M	++	-R+	0		

0 no effect, + stimulation.

-R+ fall in other parameters due to increase in heart rate.

Number of plus indicates degree of effect.

Table 13

Actions of increasing concentrations of noradrenaline in the hearts of oestradiol injected frogs.

serial number of frogs	date	sex	total dose of oestradiol in mg	concentrations of noradrenaline in g/ml				
				10^{-5}	10^{-7}	10^{-9}	10^{-11}	10^{-13}
1	10-11-60	M	0.01		++	0	0	
2	11-11-60	M	0.01	+++	++	+	+	
3	11-11-60	M	0.005			+R+	0	
4	17-11-60	M	0.01	++++	+++	0	0	
5	18-11-60	M	0.01		++++	0		
6	18-11-60	M	0.01		++++	0		
7	24-11-60	M	0.015	+++++	0	0	0	
8	25-11-60	M	0.015		0	0		
9	1-12-60	M	0.015	+++	++			
10	1-12-60	M	0.015	+++				
11	8-12-60	M	0.6		++	0	0	
12	9-12-60	M	0.6		+	0	0	
13	9-12-60	F	0.3		++	0		
14	15-12-60	F	4.5		+	0	0	
15	15-12-60	M	4.5	+	+	0		
16	16-12-60	M	3.0	+++++	+			
17	23-12-60	M	8.0	+++	+	0	0	
18	30-12-60	M	10.0		+++S			

+++S stimulation followed by stoppage
other notations as in Table 11.

Table 14

Actions of increasing concentrations of 5-hydroxytryptamine in the hearts of uninjected frogs.

serial number of frogs	date	sex	concentrations of 5-hydroxytrypt--amine in g/ml.				
			10^{-5}	10^{-7}	10^{-9}	10^{-11}	10^{-13}
1	4-11-60	M	0	0	0	0	
2	5-11-60	M	+	0	0		
3	2-12-60	M	+	$\overset{--}{R}_{+}$	0		

notations as in Table 11.

Table 15

Actions of increasing concentrations of 5-hydroxytryptamine in the hearts of oestradiol injected frogs.

Serial number of frogs	Date	Sex	Total dose of oestradiol in mg.	Concentrations of 5-hydroxy-tryptamine in g/ml.				
				10^{-6}	10^{-7}	10^{-9}	10^{-11}	10^{-13}
1	10-11-60	M	0.01	$^{--}$R+-	$^{-}$R-	0	0	
2	11-11-60	M	0.005	$^{-}$R+++	$^{-}$R++	0	0	
3	24-11-60	M	0.015	$^{-+}$R+++	0	0	0	
4	25-11-60	M	0.015	$^{+-+}$R+++	R+	0		
5	1-12-60	M	0.015	$^{+-+}$R++	$^{-}$R+	0	0	
6	5-11-60	M	0.015	$^{++}$R+++	$^{+}$R+	0		
7	8-12-60	M	0.6	---	--	-		
8	9-12-60	M	0.6	+	0	0	0	
9	9-12-60	F	0.3	+	0			
10	15-12-60	F	4.5	+	0	0	0	
11	15-12-60	M	4.5	0	0			
12	16-12-60	M	3.0		---	--	0	
13	23-12-60	M	8.0	$^{-}$R+	0	0	0	
14	30-12-60	M	10.0		$^{-}$R+	0	0	

notations as in Table 11.

Table 16

Threshold sensitivity of the same heart to different substances.

Serial number of frog	Date	Sex	Minimum effective concentration (threshold sensitivity) in g/ml.			
			Acetylcholine	Adrenaline	Nor-adrenaline	5-Hydroxy-tryptamine
1	28-1-60	F	10^{-16}	10^{-11}	not tested	not tested
2	28-10-60	F	10^{-13}	10^{-7}	not tested	not tested
3	3-11-60	M	10^{-7}	10^{-7}	10^{-7}	10^{-5}
4*	11-11-60	M	10^{-11}	10^{-9}	10^{-9}	10^{-7}
5*	8-12-60	M	10^{-9}	10^{-7}	10^{-7}	10^{-9}
6*	16-12-60	M	10^{-13}	10^{-7}	10^{-5}	10^{-9}
7*	23-12-60	M	10^{-11}	10^{-5}	10^{-7}	10^{-5}
8*	30-12-60	M	10^{-10}	10^{-7}	10^{-7}	10^{-7}

*oestradiol injected frogs.

Table 17

Comparison of toxicity of different kinds of tubing in the same heart

Type of tubing	Serial number of frogs															
	1	11	13	14	15	16	18	19	20	21	23	25	26	29	30	31
Portex Standard P.V.C.	++															
Portex Crystal Vinyl P.V.S.	0	0	+	+												
Silicone-1956	0	0	+	++												
Silicone TC-156	0	0	+	+												
X-lon P.V.C.					+++	++	+	+	++	0	+	+	0	0	+	++
Waterclear P.V.C.					+++		0	0	0	+	+	+	0	0	0	0
Silicone DSR-551					+++	+++	0	0	0	0	0					

0 no effect. Number of + indicates grades of effect.

Table 18

Comparison of incidence of toxicity from different kinds of tubing after soaking for 8 to 24 hours.

Kind of tubing	Number of hearts tested	Number of hearts not affected	Number of hearts affected.			
			Total	GradeI effect	Grade II effect	Grade III effect
P.V.C.s						
Portex Crystal Vinyl	1	0	1	1	0	0
Portex Standard	4	1	3	1	0	2
X-lon	4	3	1	1	0	0
Waterclear	3	3	0	0	0	0
Silicones.						
Silicone-1956	1	0	1	0	1	0
Silicone TC-156	1	1	0	0	0	0
Silicone DSR-551	1	1	0	0	0	0

Table 19

Comparison of incidence of toxicity from different kinds of tubing after soaking for 8 to 36 hours.

Kind of Tubing.	Number of hearts tested	Number of hearts not affected	Number of hearts affected.			
			Total	Grade I effect	Grade II effect	Grade III effect
P.V.C.s						
Portex Crystal Vinyl	2	1	1	1	0	0
Portex Standard	7	2	5	2	1	2
X-lon	8	4	4	3	0	1 *
Waterclear	6	5	1	0	0	1 *
Silicones.						
Silicone-1956	2	1	1	0	1	0
Silicone TC-156	2	1	1	1	0	0
Silicone DSR-551	3	2	1	0	0	1 *

* fresh supply, heart of frog 15, sensitive to acetylcholine in very low conc.

Table 20.

Comparison of incidence of toxicity from different kinds of tubing after soaking for 8 to 72 hours or after $^1/_2$ hour boiling.

Kind of tubing	number of hearts tested	number of hearts not affected	number of hearts affected				percentage of affected hearts
			Total	Grade I effect	Grade II effect	Grade III effect	
P.V.C.							
Portex Crystal Vinyl	4	2	2	2	0	0	50.0
Portex Standard	10	2	8	2	4	2	80.0
X-lon	15	5	10	6	3	1*	66.6
Waterclear	14	8	6	3	2	1*	42.8
Silicones							
Silicone-1956	5	2	3	1	2	0	60.0
Silicone TC-156	4	2	2	2	0	0	50.0
Silicone DSR-551	10	7	3	0	0	3*	30.0

* fresh supply. The heart of frog 15 common; same solution used in 2 out of 3 hearts showing grade III effect with 'Silicone DSR-551'

Table 21

Variations in incidence of effect with duration of soaking.

Kind of Tubing.	% of hearts affected by soaking for 8 to 24 hours				% of hearts affected by soaking for 8 to 36 hours				% of hearts affected by soaking for 8 to 72 hours or boiling for $^1/_2$ hour.			
P.V.C.	Total	Grade I	Grade II	Grade III	Total	Grade I	Grade II	Grade III	Total	Grade I	Grade II	Grade III
Crystal Vinyl	100.0	100.0	0	0	50.0	50.0	0	0	50.0	50.0	0	0
Standard	75.0	25.0	0	50.0	71.4	28.5	14.3	28.5	80.0	20.0	40.0	20.0
X-lon	25.0	25.0	0	0	50.0	37.5	0	12.5	66.6	40.0	20.0	6.6
Waterclear	0	0	0	0	16.6	0	0	16.6	42.8	21.4	14.3	7.1
Silicones												
Silicone-1956	100.0*	0	100.0	0	50.0	0	50.0	0	60.0	20.0	40.0	0
Silicone TC-156	0	0	0	0	50.0	50.0	0	0	50.0	50.0	0	0
SiliconeDSR-551	0	0	0	0	33.3	0	0	33.3	30.0	0	0	30.0

* only one heart.

Table 22

Effect of cleaning on the toxicity of tubing.

cleaning of tubing	serial number of frogs in which the heart was tested.															
	24	25		26		27	28	29		30		31		32	34	35
	W	W	X	W	X	X	X	W	X	W	X	W	X	X	S	S
Cleaned several times	0	+	+	0	0	0	0	0	0	0	+	0	++	+	0	0
Cleaned once	+	+		0	+		+++	0	+	0	+	0	++		0	0
Not cleaned	+	+	++	0		++			0		++		+++	++	0	0

W = Waterclear, X = X-lon P.V.C., S = Silicone DSR -551

0 = No effect, + = Grade of effect.

Table 23

Comparison of the two methods of cleaning.

serial frog number	X-lon P.V.C.		Waterclear P.V.C.		Silicone D.S.R.-551	
	calgon method	bicarbonate method	calgon method	bicarbonate method	calgon method	bicarbonate method
21	±	0	+	±	0	0
23	±	+	0	+	0	0
27	±	±	±	±	...	...

0 = no effect, ± = doubtful effect, + = grade I effect.