THE IDENTIFICATION AND ASSAY OF ACETYLCHOLINE AND ADENOSINE TRIPHOSPHATE RELEASED FROM ACTIVE SKELETAL MUSCLE.

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by

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"Science has not solved difficulties, only shifted the points of difficulty".

C. H. Parkhurst

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PREFACE

The work for this thesis was performed at the Boyd Medical Research Institute, the Institute of Physiology and the Department of Biochemistry, University of Glasgow.

The equipment used for continuously recording the venous and arterial pressures, heart rate and cardiac output of perfused frog hearts was developed by Professor I.A. Boyd and Mr. W.R. Eadie over a period of several years and the detals of this equipment are published (Boyd and Eadie, 1961). A description of this apparatus is not intended to be an integral part of this work and only a very brief outline of it will be given here.

After a description of some preliminary results the thesis is divided into two parts for convenience of description.

Part I is concerned with the identification of adenosine triphosphate released from skeletal muscle.

Part II deals with the assay of acetylcholine released from skeletal muscle.

An Appendix describes a technique developed for the separation and purification of acetylcholine which may be contained in tissue perfusates or extracts.

ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor, Professor I.A. Boyd, for the unrestricted use of his laboratory premises and the frog heart perfusion equipment without which much of this work would not have been possible.

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INTRODUCTION

Bioassay techniques are used to test compounds which have either a specific action in vivo or exist in such small concentrations that their estimation is beyond the limits of chemical analysis.

In the case of acetylcholine (ACh), the chemical synthesis and analysis was achieved almost one hundred years ago, but because of the small amounts involved in synaptic and neuromuscular transmission, any investigator wishing to measure the ACh produced at these sites must resort to methods of bioassay. The response of a particular tissue to ACh must be evaluated by comparing it to the response given by a known amount of ACh.

More precisely, the bioassay principle consists of measuring the product of one enzyme system by judging its effect upon another, similar enzyme system. Enzyme reactions are affected by many factors: hydrogen ion concentration, temperature and the availability of energy for the system. These reactions can be accelerated, depressed or even reversed by various interfering substances.

Since a naturally-occurring enzyme system is being used as a "tool" to identify and assess the concentration of a particular compound, it is essential that the limitations of the "tool" are clearly defined. The point was put succintly by Jerne and Wood - "...substantial biological research must precede any attempt to set up a bioassay technique for routine use".

In any quantitative estimation of ACh which may be released at the neuromuscular junction, three main factors have to be considered in a pharmacological approach to the problem. Firstly, the number of nerve impulses passing into the endplate region must be known. Secondly, the diffusion pathway of ACh from the endplate regions to the exterior must offer the minimum of obstruction so that nearly all of the ACh will diffuse into the solution surrounding the preparation. Thirdly, the method of measurement of the released ACh should be reliable, and should not be unduly susceptible to interference from other substances

which might be released from the tissues during stimulation of the muscle. Any one of these unknown substances may interfere directly with the assay procedure, or indirectly by altering the sensitivity of the bioassay preparation to the compound under survey.

A classical example of direct interference in a bioassay procedure was outlined by Dale and Dudley in 1929. While assaying the quantity of the histamine-like substance in extracts of spleen on the cat blood pressure they found a large, but constant, discrepancy in their results. Histamine depresses the blood pressure of the cat. Dale and Dudley found that the spleen extract caused depression of blood pressure which was too great to account for the expected concentration of histamine. They found that an interfering substance, whose action could be blocked by atropine, was causing the excess fall in the cat blood pressure. In other words, their results, obtained with regard to histamine, were falsely high because of the interference of the hitherto unrecognized presence of ACh in the extract.

Other examples of direct interference in bioassays have been noted by Von Euler and Gaddum (1931), Amin, Crawford and Gaddum (1954) and more recently by Lazlo (1963).

An example of indirect interference has been shown by Frommel et al. (1944). They found that the common constituent of tissue extracts, histamine, could sensitize leech muscle, helix heart and frog rectus abdominis preparations to ACh, the increase in sensitivity being analogous to that produced by eserine. Any quantitative estimation of ACh on those preparations in the presence of histamine would be too high, unless the same amount of histamine present in the test solution was deliberately added to the solutions used for comparison.

The present work was initiated in order to investigate the release of ACh from the neuromuscular junction and to produce, if possible, a more accurate correlation of the pharmacological evidence with the electrophysiological evidence of the quantities of ACh which are needed for the natural transmission of the nerve impulse into skeletal muscle. However, the identification in this work of adenosine

triphosphate as a predominant interfering substance released from skeletal muscle has raised the question of whether previous methods of measuring released ACh were specific, or whether unknown substances distorted previous bioassays of ACh liberated from the neuromuscular junction. This opens up the broader issue of whether other substances released from skeletal muscle could also be acting as depolarizing agents on the endplate region.

The Experimental Design

This work was initiated with the intention of measuring the output of ACh from active frog sartorius muscle on the very sensitive frog heart perfusion system of Boyd and Eadie (1961). The ACh released from the muscle would be collected in a surrounding bathing solution and this solution would be assayed for ACh using the frog heart.

The frog sartorius muscle was chosen because (a) the number and situation of the endplates was known, and (b) this muscle has a region at the pelvic end which is devoid of any other motor nerves, and is thus suitable for direct stimulation of the muscle fibres without any inadvertent stimulation of intramuscular motor nerve fibres. This feature of the sartorius muscle provided the opportunity to investigate whether ACh could be released from muscle fibres by direct stimulation.

The output of ACh from a sartorius muscle was not expected to be very great; the following calculation was kindly supplied by Professor Katz:

A frog sartorius muscle contains approximately 1,000 endplates.

If, say, 10^6 molecules of ACh are released per endplate per impulse, 1,000 impulses given to a sartorius muscle would give 10^{12} molecules of ACh. Collected into a 1 ml. volume, this would give a concentration of 3×10^{-10} g/ml. ACh.

About 30% of perfused frog hearts are capable of responding to 10^{-11} g/ml.

or lower concentrations between the months of December and March in Scotland (Boyd and Pathak, 1965). Thus it was theoretically possible to assay the small quantity of ACh which was expected to be released from frog sartorius muscle on "winter" frog hearts. Other conventional preparations used for the bioassay of ACh are either too insensitive, for example frog rectus abdominis muscle (threshold 10^{-8} g/ml., Chang and Gaddum 1933), eserinized leech muscle (threshold 10^{-9} g/ml., Minz 1932), too unstable, for example frog lung (threshold 10^{-16} g/ml., Corsten, 1941), or require a specialized medium to retain their sensitivity to ACh, like clam heart (threshold 10^{-11} g/ml., Welsh and Taub, 1948), whelk heart (threshold 10^{-11} g/ml., Welsh, 1954), and muscle strips of echinodermata (threshold 10^{-9} g/ml. - 10^{-11} g/ml., Bacq, 1939).

Any preparation which requires a special medium would involve conversion of the test solution obtained from the frog sartorius muscle and hence dilution of the contained ACh. Since the concentration expected from the sartorius muscle is so small, no dilution factor could really be afforded in an accurate assay procedure using some of the suitably sensitive preparations previously mentioned.

The perfused frog heart was thus advantageous since the same species was to be used for both the source and the measurement of the ACh. No dilution of the test solution would be necessary and the small concentration of ACh expected would not be diluted beyond the threshold sensitivity of the frog heart.

Although the frog heart can respond to a great many biological compounds, the advantages of its great sensitivity and long-term stability to ACh seemed to outweigh the disadvantages of non-specificity.

HISTORICAL SURVEY

Claude Bernard, while investigating the properties of curare, showed that while curare had no effect on the condition in nerve and muscle fibres, it blocked the transmission of nerve impulses to the muscle. These observations, made in the 1840's, first outlined the special properties which the neuromuscular junction possessed.

Acetylcholine (ACh) was first prepared artificially by Baeyer in 1867. It retained a chemical interest only until Hunt and Taveau in 1909 found that it had a very high biological activity, much more so than choline itself. Three years prior to this Dixon (1906) had demonstrated that inhibition of the heart by vagal stimulation was associated with the liberation of an acetylcholine-like substance in intimate relation to the heart muscle fibres. This work supported the then astonishing suggestion in 1877 by Du-Bois-Reymond who considered that the excitation of a muscle fibre might be due to the release of a chemical stimulant when the impulse arrived at the nerve ending.

In 1907 Langley found that the junctional region of a muscle was highly sensitive to many substances and Reisser and Neuschloss (1921) found that when a gastrocnemius muscle of a frog was exposed to a solution containing ACh it gave a transient contraction, but this only happened when the ACh was applied to the junctional region; the ACh had no effect when applied separately to the nerve or the muscle.

In 1921 Le Heux observed that the action of choline on an isolated loop of intestine was enhanced by the presence of acetates and thought that ACh could be the final activator. It was also in 1921 that Loewi initiated his classical experiments on frog heart perfusates after vagal stimulation and suggested that a substance was released which had the properties of a choline ester.

In 1923 - 1926 several observers (Hess, 1923, Brinkman and Ruiter, 1924 - 1925; Shimidzu, 1926) had obtained evidence of the liberation of an acetylcholine-

like substance from perfused frog skeletal muscles when these were stimulated through the nerve. However, all of them had stimulated a mixed nerve and they did not definitely associate the effect with the motor fibres, or with the transmission of the impulse to the muscle fibres from nerve. Hoet in 1925 observed that the atropine-sensitive rhythm of isolated intestinal muscle was enhanced by strong post mortem stimulation of the vagus nerves before excision of the intestine.

While investigating the identity of the histamine-like constituent of spleen extracts, Dale and Dudley (1929) found a large but constant discrepancy in their results. They used for their bioassay object the blood pressure of the cat under ether or urethane anaesthetic. They found that a surprisingly large proportion of the original effect of the extract was eliminated in the presence of atropine. The activity of this proportion could also be eliminated by raising the pH of the extract. They found also that this same proportion of activity was lost when the extract was brought into contact with "the cells and juices of the whole minced organ".

Dale and Dudley were eventually able to extract chemically fairly constant amounts of ACh from ox and horse spleens. This was the first occasion on which ACh had been found to occur naturally in the animal body.

Earlier, Dale, working with Ewins (1914), had become interested in ACh because it was able to reproduce parasympathetic effects intensely and faithfully in various organs. When these effects were blocked with atropine the ACh in larger doses stimulated ganglion cells in a manner similar to nicotine. This led Dale and Dudley to speculate on the possibility that ACh might occur in the body and be physiologically functional; however, they could not explain the function of large amounts of ACh found in the spleens.

In 1930 Dale and Gaddum examined the evidence for a chemical intermediary in the parasympathetic nerve endings and for its identification as ACh. They concluded that the vasodilator effects of parasympathetic nerves and of sensory fibres stimulated antidromically (and the contractions of denervated muscle accompanying these actions) were due to peripheral liberation of ACh.

In 1933 Adrian wrote - "It is by no means certain that the humoral transmission of the vagal effect differs in kind from the transmission of activity from the motor nerve to striated muscle. An exciting substance liberated at a nerve ending but destroyed within a few thousandths of a second would have little chance of spreading by diffusion and would account well enough for the known properties of the nerve ending. It is equally likely that the more direct kinds of transmission depend, as in nerve fibre, on electric forces disturbing the balanced reactions of surface membranes".

These views were at least one year in advance of any experimental evidence in favour of a chemical transmitter of excitation from motor nerve to skeletal muscle.

In 1934 a preliminary note appeared from Dale and Feldberg stating that they had identified ACh in the perfusate of cat tongue and dog leg muscles in the presence of eserine whilst stimulating the motor nerves. About the same time Feldberg, Minz and Tsudzimura produced strong evidence that ACh was liberated when a nerve impulse reached the adrenal medulla. Also at this time it was shown that ACh was released from ganglia when nervous impulses reached them (Feldberg and Gaddum, 1934; Feldberg and Vartiainen, 1934; Barsoum, Gaddum and Khayyal, 1934). Thus the classical work performed by Dale, Feldberg and Vogt and published in 1936 formed a natural link in the sequence of events leading to the modern conceptions of neuromuscular transmission.

Previously it had been assumed by Frank and his co-workers (Frank and Katz, 1921; Frank, Nothmann and Hirch-Kauffman, 1922) that there was a secondary parasympathetic nerve supply to the skeletal muscle fibres themselves, responsible for maintenance of muscle tone. They regarded the release of ACh from skeletal muscle as perhaps having little connection with motor nerve impulses, since all the fibres of a mixed nerve had been stimulated previously.

Dale, Feldberg and Vogt set out to find whether stimulation of the motor nerve fibres innervating skeletal muscle (excluding antonomic and sensory fibres)

caused the liberation of ACh in appreciable quantities, and, if so, set out to define a site of release. They were aware of the difficulties involved in the identification and possible quantitative estimation of ACh if indeed it was released at the neuromuscular junction. They anticipated that any ACh obtained from a perfused muscle mass would inevitably be greatly diluted. This was in contrast to previous investigations involving ganglia where the small perfusion volume together with the large concentration of synapses helped to raise the ACh level up to easily estimable concentrations.

Another unexpected difficulty arose during the course of their investigation. To ensure successful recovery of ACh they found that perfusion with an eserinized saline solution was necessary. Previously Feldberg (1933) had failed to detect any ACh in the lingual vein when he stimulated the motor hypoglossal nerve of the dog. Franel (1935) also failed to detect ACh in venous blood or in eserinized Locke's solution coming from a perfused limb. Dale et al. (1936) at that time thought it possible that perfusion with saline facilitated in some unknown way the release of ACh from the site of its origin in the blood vessels. Later, Dale supported the work of F.C. MacIntosh (1938) who found that the doses of physostigmine used were insufficient to counteract the blood cholinesterase (see below).

Dale et al. also encountered irregular results at the commencement of their perfusion experiments. This is best reviewed in their own words:- "After the rather prolonged dissection and manipulation the early perfusion samples usually showed detectable amounts of a substance acting like ACh as Hess (1923) observed in his perfusions of frog muscles". Only after half-an-hour of perfusion did this activity disappear. This happened during the perfusion of the cat tongue, cat gastrocnemius muscle, dog gastrocnemius muscle and frog hind limbs. This aspect of the perfusion experiments with saline aroused some criticism at that time. There were suggestions that the release of ACh under such conditions was unphysiological and could perhaps be equated with tissue damage. (Fleisch, Sibul and Kaelin, 1936; Lorente de No,

1938; and more recently, Nachmansohn 1946). However, only after the perfusate showed no ACh-like activity did Dale et al. commence stimulation and collection of ACh.

A disadvantage of saline perfusion, they stated, was the increasing insensitivity of the muscles to motor impulses developed over a long period of time. When perfused for a further period the muscles became oedematous.

In the case of frog hind limbs, during the period of stimulation a rapid decline in twitch was evident in 2 - 3 minutes. The muscle was rested and stimulation again resumed at a frequency of five impulses per second. Fatigue followed more quickly than in the first period of stimulation. They noticed that as the muscle twitch grew weaker, so the amount of acetylcholine-like activity of the perfusate, tested on leech muscle, grew less.

In order to ensure that the ACh was indeed coming from the motor nerve terminals and was not being released directly from active skeletal muscle, Dale paralysed the musculature of a perfused cat tongue with curare and found that stimulation of the motor nerve still had the effect of releasing a similar quantity of ACh into the perfusing fluid.

It is interesting to note at this point the observations of Dale et al. on the vasodilatation occurring during the muscle perfusion. They noted that the venous outflow from perfused muscle was accelerated at the onset of motor nerve stimulation. This was taken as an index of increased vasodilatation. The presence of eserine enhanced this phenomenon. In order to separate the vasodilatation due to muscle "metabolites" from vasodilatation caused by ACh released from the motor nerve terminals, they used curare to block the contraction process. It was found that 90% of the increase in bloodflow was abolished by this procedure, indicating that the vasodilatation observed was principally due to "products of the contractile mechanism".

The tests employed by Dale et al. in 1936 for the identification of ACh have not been improved upon since that time. With concentrations of the order of

10^{-8} g/ml. ACh available it was possible for them to use multiple bioassay techniques.

They established that the unknown substance was:

(a) stable in acid solution but was destroyed in solutions of dilute alkali;

(b) consistently protected from the tissue esterases by eserine;

(c) effective in reducing the blood pressure of a cat; this effect was enhanced by eserine and blocked by atropine;

(d) capable of producing a contraction of leech muscle in the presence of eserine; this action could be blocked by curare.

These tests were taken to indicate that the hitherto unknown compound released from active motor nerve terminals was ACh, or a substance very similar to it.

One of the initial problems of the ACh transmitter theory was whether the ACh could be removed quickly enough to subserve its function as the transmitter of discrete, high frequency nerve impulses into the muscle fibre. Hydrolysis of ACh occurs in many tissues by the enzyme acetylcholinesterase (Stedman, Stedman and Easson, 1932). Marnay and Nachmansohn (1937) showed that the concentration of cholinesterase at the endplates was many thousands of times higher than that found in muscle tissue. They estimated that the ACh could be split by this concentration of cholinesterase within the refractory period of the muscle, i.e. around one millisecond. Histochemical methods have revealed cholinesterase at the motor endplate (Koelle and Friedenwald, 1949), and more recently Couteaux (1958) has shown that it is concentrated on the subsynaptic membrane of the neuromuscular junction, especially in the region of the junctional folds.

In 1938 Gopfert and Schaefer established the existence of an endplate potential using an extracellular recording technique. Kuffler (1942) suggested that the threshold for the initiation of a propagated action potential was about a third of the normal endplate potential height.

In 1950 Fatt and Katz showed that the endplate region of a muscle fibre is the site for many small electrical changes, the spontaneous "miniature" endplate

potentials (m.e.p.p.s). These m.e.p.p.s closely resemble the local potentials which are produced by applying ACh to the endplate region (Fatt and Katz, 1952; del Castillo and Katz, 1955, 1957; Miledi, 1962). It is widely accepted that the m.e.p.p.s are depolarisations produced by the result of packets, or "quanta" of ACh released from the nerve terminal impinging on the endplate receptors. The work of del Castillo and Katz (1954), Boyd and Martin (1956), Liley (1956) and Katz and Miledi (1965) has shown that the endplate potential can be accurately equated to the sum of the m.e.p.p.s which occur over a similar period of time. This suggested that all the quanta of ACh are simultaneously released from the nerve terminal upon the arrival of the nerve impulse.

The theory of quantal release of ACh has been supported by the finding with the electron microscope that the motor nerve terminal is crowded with "vesicles" all of approximately 500Å in diameter (Birks, Huxley and Katz, 1960). The process of release of a quantum of ACh must involve the coalescence of two membranes, the one surrounding the vesicle and the limiting membrane of the nerve terminal. Evidence of this coalescence occurring in a central synapse has been demonstrated by the electron microscope (Gray, 1963).

In 1951 Nastuk applied small quantities of ACh to a single endplate region. He used an electrode of less than 0.5μ external diameter and drove the ACh out of the pipette with a short, single electrical pulse (iontophoresis).
He noted that as the microjet of ACh was directed nearer to the endplate, so the depolarization of the muscle membrane increased. Del Castillo and Katz (1955) successfully applied ACh to both sides of the endplate membrane using the iontophoresis technique and showed that the intracellular application of ACh did not produce an endplate potential.

Two main experimental approaches have been used in an attempt to find out the number of ACh molecules in each quantum; the assay of solutions which have surrounded active muscle preparations and the technique of iontophoresis.

Results obtained from these two approaches are in the same order of

magnitude. Miledi (1961) has shown that it is necessary for 10^5 molecules of ACh to be discharged from a micropipette to produce a depolarization equal to that of a miniature endplate potential. Krnjevic and Mitchell (1961) calculate from their assay of ACh spontaneously released from rat diaphragm that 3.6×10^5 molecules of ACh are contained in each quantum. However, calculations show that if a vesicle of 500 Å in diameter did in fact contain 10^5 molecules, the unlikely concentration of 10 M ACh would be present within the vesicle (del Castillo and Katz, 1956).

METHODS

SETTING UP THE FROG HEART FOR PERFUSION

Both Rana temporaria and Rana pipiens of either sex were used.

After stunning, decapitation and pithing the frog was pinned down in the supine position. The skin of the abdominal and thoracic walls was removed. The abdominal musculature was then removed and the thoracic cavity was entered, avoiding damage to the pericardial cavity. By cutting through the shoulder girdle on both sides a segment of the anterior wall of the thorax was lifted and cut off exposing the heart lying within the pericardial cavity. After tying off any prominent portal veins, the venous cannula was introduced into the exposed posterior vena cava through a small flap in the ventral wall of this vessel. The cannula was securely ligatured into the vein. In order to prevent excessive clot formation at this stage frog Ringer's solution was perfused into the posterior vena cava at a low pressure.

The two aortae were now cannulated, the first cannula leading to a mercury manometer, the other cannula leading to the peripheral resistance unit, the drop counter, and connected to the lead from the first cannula in order to preserve the same pressure in both leads.(Figure 1).

The perfusion pressure was kept between 25 - 35 mm H_2O depending upon the particular heart being perfused.

Calibration of the equipment is dealt with in previous work and is not included as part of this thesis (Pathak, 1962).

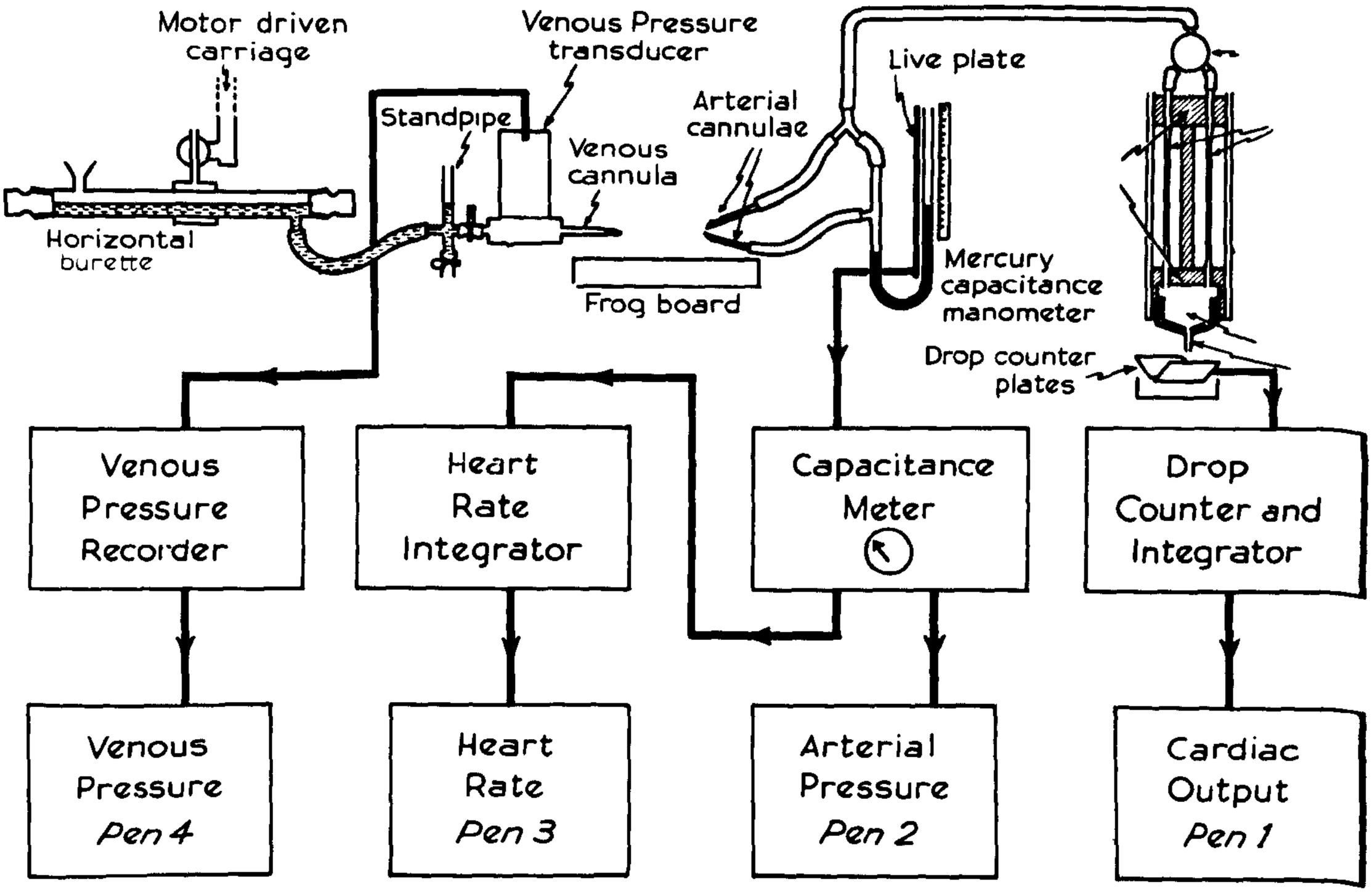


Figure 1.

A block diagram to show the general layout of the frog heart perfusion system of Boyd and Eadie (1961).

At the top left of the diagram the solutions to be tested lie in a horizontal burette which leads into the venous capsule. Because of the small amounts of the test samples in this work a positive pressure system had to be developed. Figure 3 shows the pressure system used in the present work and replaces the horizontal burette scheme depicted here.

DISSECTION OF THE FROG SARTORIUS MUSCLE

The sartorius muscles were obtained from both Rana temporaria and Rana pipiens of either sex. After stunning, decapitation and pithing the frog was laid in the supine position and the skin was removed from the lower left limb and the anterior abdominal wall. The inferior part of the rectus abdominis muscle was pinched up and a V-shaped incision was made into the abdominal cavity. The rectus was then stripped up towards the rib margin, exposing the viscera. The rectum was cut through and by incising the peritoneum and mesentery the viscera was carefully stripped off the posterior abdominal wall towards the diaphragm until this procedure could be followed no further. This revealed the large nerve trunks lying on either side of the spinal column.

Attention was then turned to the tendinous insertion of the left sartorius muscle. The tendon was removed from a point as near as possible to its insertion using fine scissors and forceps under the dissecting microscope. The muscle belly was then freed from underlying structures on the lateral side. This was extended up the whole length of the muscle to its origin at the fascia of the anterior abdominal wall. The proximal part of the muscle belly was then mobilized and the origin of the muscle was then removed from the medial part of the pubis and the lateral abdominal fascia. With the proximal and lateral parts of the muscle thus mobilized, it was possible to turn the muscle over in a medial and downward direction, preserving the lower and medial half; this is the part of the muscle where the nerve enters the muscle belly from the medial side. With the muscle turned down at its origin and at a safe distance from the symphysis pubis, it was then possible to split the symphysis pubis with a strong scalpel blade. The left half of the pubic bone was then turned laterally and the sciatic nerve with its obturator branch was seen to hook round the sciatic notch of the split pelvis, passing into the thigh musculature. The bony projection of the sciatic notch was then modified with coarse scissors and the thigh was pinned out in the laterally-rotated position. The course of the nerve

to sartorius could then be traced backwards to the exposed nerve trunk by splitting the fascia between the muscle planes with a blunt probe under the dissecting microscope. Throughout the procedure frog Ringer's solution was poured over the parts.

The nerve trunks of L2, 3, 4 and 5 were cut as close to the spinal column as possible and then mobilized from the posterior abdominal wall fascia until the main branch to the sartorius muscle was reached. At this point the nerve to sartorius bends at a right angle into the muscle, running across the muscle planes. Great care was necessary under the dissecting microscope to free this final section of nerve. Both nerve and muscle were then completely free from the underlying tissues.

After washing the muscle thoroughly with Ringer's solution while it was still lying on the thigh, the nerve-muscle preparation was then immediately transferred to a container of Ringer's solution, gently shaken for a few moments, then transferred to a 2ml. container of Ringer's solution and left to "equilibrate" for 10 minutes. After this the muscle was then put into the container with 2ml. of Ringer's solution and its nerve trunk laid gently over the stimulating electrodes (see Figure 2). All transfers were done by holding the sartorius muscle by its tendon with fine forceps. This ensured that no muscle fibres were damaged in transit. The nerve trunk was allowed to adhere to the muscle belly by capillary attraction during the transfer process. All Ringer's solutions contained 10^{-6} g/ml. eserine sulphate.

Stimulation of the Muscle

It was found that with a pulse width of 1 millisecond the threshold of stimulation required to make the muscle twitch was usually 0.5 volts. In every case the sartorius muscles were stimulated at 4V with a pulse width of 1 msec at 2 impulses per second for a period of half an hour. The sartorius muscle usually responded to every impulse throughout the period of stimulation. After this half hour period the muscle was left to soak in the 2mL solution for 10 minutes in order

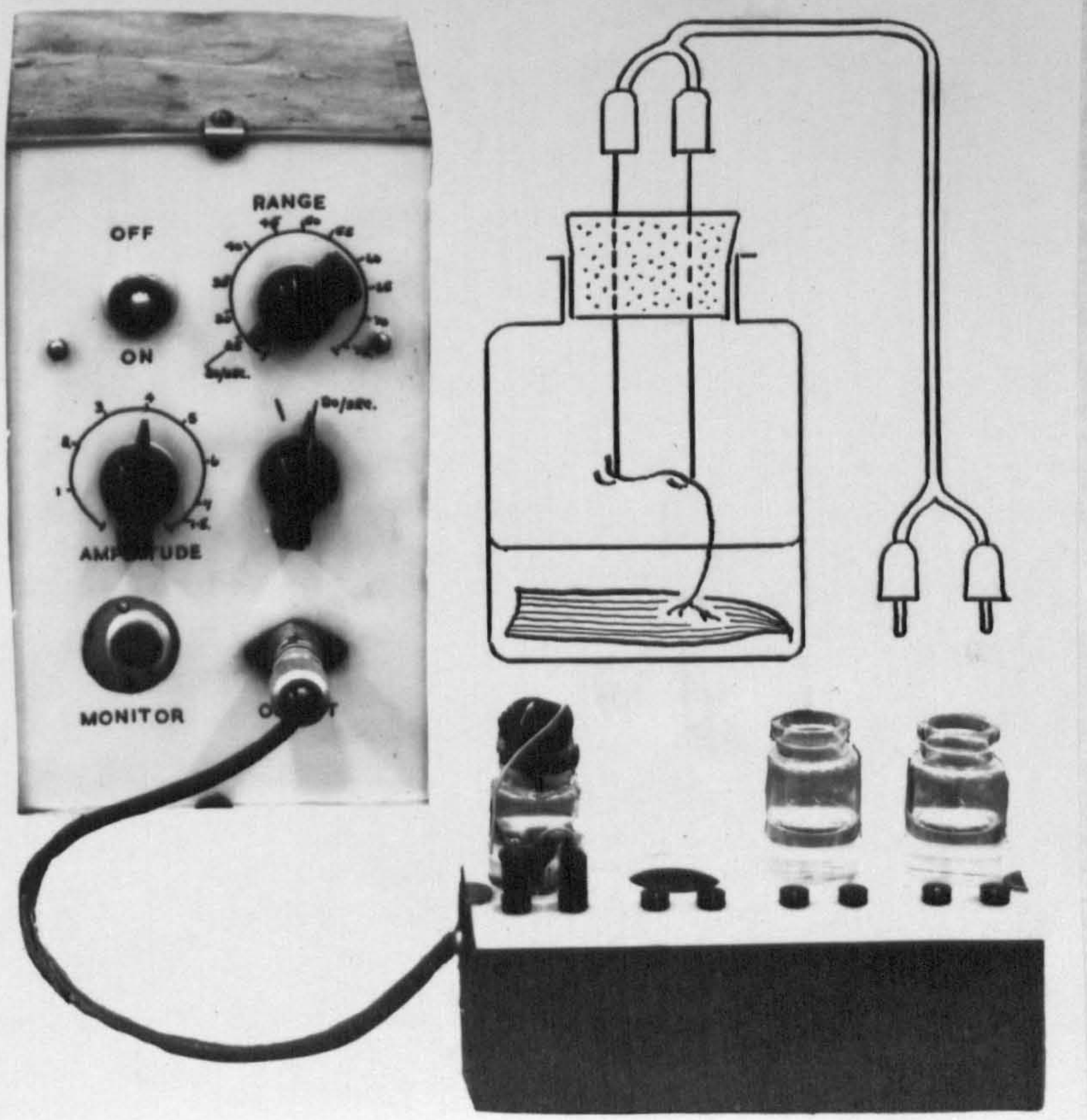


Figure 2.

Apparatus used for the indirect stimulation of the frog sartorius muscle. The photograph shows a pulse generator connected to a bank of four output points connected in parallel. Four stimulated muscle solutions could be obtained at the same time. A muscle is being stimulated from the left hand output point. Two stimulated muscle solutions are seen standing on the right.

The line drawing shows the frog sartorius muscle which lies unsupported in 2 ml. Ringer's solution. The nerve to the muscle lies over platinum electrodes which protrude into the glass container through a cork stopper. The electrical impedance across the nerve does not affect the pulse amplitude. Leads from the electrodes are inserted into the stimulating bank.

The rat hemidiaphragm preparation was also stimulated in this way, oxygenation was effected via a capillary tube (not shown) which passed through the cork stopper. An outlet tube was also present.

to allow time for diffusion of any released acetylcholine into the surrounding bathing solution. The muscle was then removed with fine forceps and the solution was ready for assay on the frog heart perfusion system. This solution will be referred to as the "stimulated muscle solution" throughout the text.

INJECTION OF SOLUTION INTO THE FROG HEART

On account of the small quantity (1ml.) of each test sample to be assayed, it was necessary to devise a system of injection of this volume into the frog heart perfusion system since dilution of the test sample to a suitable volume for the horizontal burette system would dilute the expected ACh in this solution beyond the threshold of the frog heart.

A positive pressure system was adopted (Figure 3). Air pressure in a closed container was matched to the pressure of the normal perfusion Ringer's solution. A 1ml. "tuberculin" syringe containing the test sample was connected between the closed container and the venous pressure capsule (Figure 3), so that the sample would be driven into the heart by a similar pressure to that of the perfusing Ringer's solution.

As a precaution, the closed pressure system was linked by a three-way tap to the atmosphere via a fine needle, and to another closed container which was at a pressure slightly higher than that of the optimum perfusion pressure. By means of this tap the perfusion pressure of the 1ml.sample could be regulated if necessary during the course of the injection.

Procedure : The syringe was filled via a needle with the test sample and fitted between the venous pressure capsule and the air pressure system. The tap from the perfusion reservoir was closed and at the same time the tap allowing the air pressure to reach the syringe was opened. The pressure in the venous capsule was now kept constant at the original perfusion pressure by the three-way tap, momentarily turning towards either the atmosphere or the high-pressure container as required. When the test sample had almost completely entered the venous capsule, the tap from the perfusion resevoir was turned on and the tap between the air-pressure system and the syringe was closed. In practice, small fluctuations in the perfusion pressure occurred (Figure 11) as the test sample was driven from the syringe into the capsule. However, these changes only affected

Figure 3.

System for the Injection of a Test Sample into the Frog Heart Perfusion System.

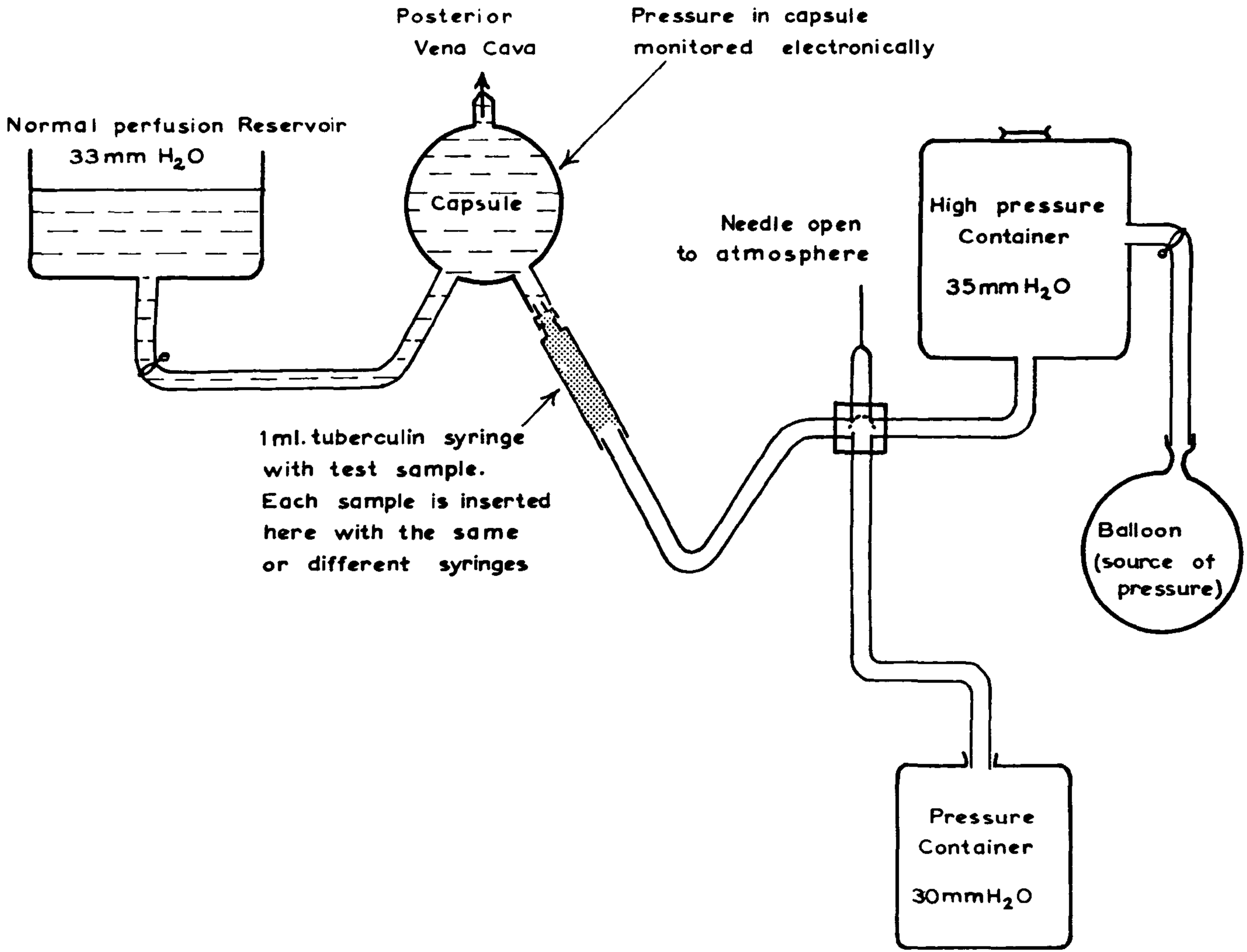
On the left of the diagram is the normal perfusion reservoir which delivers frog Ringer's solution via a capsule into the posterior vena cava of the frog at a constant pressure; here the pressure is 30 mm. H_2O. The pressure is constantly recorded from the capsule. The other conduit into the capsule holds the 1 ml. tuberculin syringe containing the test sample. Attached to the plunger end of the syringe is the tube from the air pressure system.

The syringe is normally connected to the normal pressure container, but can be connected to

(a) atmosphere

(b) high pressure container when necessary.

The pressures in the two containers and in the conduit from the normal perfusion reservoir are all measured by attached water manometers (not shown).


Posterior
Vena Cava
Pressure in capsule
monitored electronically
Normal perfusion Reservoir
33mm H_2O
Capsule
Needle open
to atmosphere
High pressure
Container
35mm H_2O
1ml. tuberculin syringe
with test sample.
Each sample is inserted
here with the same
or different syringes
Balloon
(source of
pressure)
Pressure
Container
30mm H_2O

the cardiac output, heart rate and blood pressure slightly and by the time the sample had reached the heart the pressure from the normal perfusion reservoir was again in operation; thus any effect on the heart caused by the test sample occurred when the perfusion pressure was constant.

Some dilution of the sample in the venous capsule may occur, but it is probable that when the sample is entering the capsule it pushes the Ringer's solution in front of it into the posterior vena cava cannula without much mixing taking place at the interface. Similarly, when the Ringer's solution from the reservoir again flows into the capsule, it pushes the last part of the sample into the cannula.

It has been assumed that the amount of dilution occurring in the venous capsule is the same for all samples injected into the heart.

MEASUREMENT OF POTASSIUM

Measurements were carried out by flame photometry. The sample to be measured was diluted 1 in 50 with distilled water and then measured (using the E.E.L. flame photometer). Six readings were made of each sampel and a mean of those was taken. Similar methods were adopted to measure the concentration of potassium of various Ringer solutions in which the muscles were rested and stimulated. The standard solution of potassium used for comparison was 0.1 μEq/ml. At this order of concentration the response of the flame photometer is directly proportional to the concentrations of K^+ applied.

Example

Standard reading = 39 = 0.1 μEq/ml.

Muscle solution readings = 20. 20. 21. 21. 21. 21. mean - 21.

Concentration of potassium - $\frac{21}{39} \times \frac{50}{1} \times \frac{1}{10} = 2.7$ mM

METHOD OF ESTIMATING CALCIUM CONCENTRATION

The complexiometric titration method was used (Wilkinson, 1957). The dyestuff ammonium purpurate ('Murexide') turns red in alkaline solution in the presence of calcium ions. When the calcium is removed by chelation with ethylene-diamine tetra-acetic acid (E.D.T.A.) the dye returns to its normal blue colour. The end-point of this titration cannot be judged accurately with the eye, so that the use of a photo-electric titrator is necessary.

Reagents

1. E.D.T.A. - A stock solution of 0.36% was diluted 10 times for use.
2. Murexide - A stock solution of 0.6% was diluted 1 in 200 with distilled water.
3. Sodium hydroxide - A decinormal solution was made up from a 2N solution for each batch of test solutions.
4. Calcium standard - A stock di lution of 100 mg Ca++/100 mls was diluted by 10 to give a 2.5 mM solution.

Preparation of Solution for Titration

0.5 ml. of test sample was pipetted into a 10 ml. glass curvette along with 3 ml. of Murexide solution and 6.0 ml decinormal sodium hydroxide solution. An electro-magnetic stirrer was placed in the cuvette before putting it on the titrator platform. The stirring mechanism was switched on.

The burette containing E.D.T.A. was adjusted so that its tip was just below the meniscus of the liquid in the cuvette, taking care not to obstruct the pathway of light through the solution.

A 606 yellow filter was placed in the titrator; the galvanometer reading was adjusted to zero against a blank. The calcium standard solution was then measured, (mean of 3 results, before and after the test samples) followed by the test solutions.

Method of Titration

0.1 ml. amounts of E.D.T.A. were run in until the colour of the solution started to change. Then 0.01 ml. amounts were run in while another observer gauged the movement of the galvanometer spot. The end-point was reached when the spot was not deflected. This was impossible to accomplish with only one person carrying out the titration, since the end-point was reached in effect when the spot failed to move when more E.D.T.A. was run in.

Calculation

Standard calcium solution = 2.5 mM. Test solution = ? mM.

Reading on galvanometer = 1.33 Galvo. reading = 0.58

$$\text{millimolarity of test solution} = \frac{0.58}{1.33} \times 2.5 = 1.09 \text{ mM.}$$

THE ESTIMATION OF PROTEIN

The method described by Lowry et al. (1951) was used. They recommended that this reaction was especially useful in measuring small amounts of mixed tissue proteins, particularly when absolute values were not required.

Reagents

1. 2% Na_2CO_3 in 0.1 N NaOH.
2. 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate.
3. Folin-Ciocalteu phenol reagent diluted to 1 N.
4. Standard solution of bovine serum albumen diluted to a concentration of 50 µg/ml.

Principle

When protein is added to a copper solution in an alkaline medium, a blue colour is developed. This colour change is due almost entirely to the tyrosine and tryptophan content of the protein chains. When the Folin reagent is added to copper-treated protein at pH 10, reduction of the reagent takes place and the blue colour is intensified. At this pH value the reagent is only active for a very short time, so that even a few seconds delay in mixing will reduce the final colour intensity. The colour intensity developed is proportional to the concentration of protein present in the test solution.

Method

50 ml of the alkaline copper reagent is made up as follows :-

49 ml Na_2CO_3/NaOH reagent, 0.5 ml. of $CuSO_4.5H_2O$ and 0.5 ml. of sodium tartrate solution are mixed together. 5 ml of this alkaline copper solution is added to 1 ml. of the solution to be tested for protein. This mixture is allowed to stand for exactly 10 minutes. Then 0.5 ml. of 1 N Folin-Ciocalteu reagent is added and the whole shaken during and immediately after the addition of the reagent. The solution is then left to stand for exactly 30 minutes. During this time a blue colour slowly develops, and this is compared to the blue colour developed

by the standard solution. The comparison was carried out be measuring the absorption of light of the solutions at 625 mμ wavelength on a Zeiss type PMQ 11 spectrophotometer. The standard solution of bovine serum albumen was read twice, once at the beginning of the series and once at the end.

Example

	Reading	
	Before	After
Bovine serum albumen solution =	8	10 (Mean 9)

Test solution = 16

Reading of 9 = 50 μg/ml.

Reading of 16 = $\frac{50}{9}$ x 16 μg/ml. = 89 μg/ml. in test solution.

THE PREPARATION OF APYRASE SOLUTIONS

2 mg of potato apyrase (adenosine - 5' - triphosphatase and adenosine - 5' - diphosphatase, Sigma) crystals were weighted out and placed in a small watch-glass. A stimulated muscle solution was obtained, volume 2 ml. (see Methods section). The apyrase crystals were then brushed carefully off the watch-glass surface into the bottle containing the stimulated muscle solution. These quickly dissolved after gentle shaking of the bottle. The solution was then incubated in a waterbath at 30°C for quarter of an hour. After cooling to room temperature, the solution was then perfused through the frog heart.

On some occasions the stimulated muscle solution was divided into two 1 ml. portions. Then 1 ml. of the solution was incubated with 1 mg of apyrase, the other 1 ml. portion thus being available as a control.

Adenosine triphosphate solutions of suitable concentrations were also made up in frog Ringer's solution and incubated with apyrase in exactly the same manner.

THE FIREFLY LUMINESCENCE PROCEDURE

Materials

Powdered firefly extract (Sigma FLE 50).

Adenosine triphosphate solutions made up in frog Ringer's solution.

Narrow-width glass cuvettes of 0.5 ml. capacity.

Photomultiplier tube with guiding lens system, reflecting cone and covering cannister.

Powerpack and potentiometer.

"Devices" single-channel pen recorder.

The photomultiplier tube is mounted in a light-proof alcove and the whole procedure is performed in a photographic dark room with illumination, when necessary, from a red safety light.

ATP solutions are made up in frog Ringer's solution which match the expected concentration of ATP in the stimulated muscle solution as measured on the frog heart perfusion system (see Figure 1).

The powdered firefly extract is taken out of the deep freeze and allowed to stand until room temperature is attained. It is then reconstituted by pipetting 5 ml. of distilled water into the bottle. With gentle shaking the powder dissolves in a few minutes and insoluble parts of the firefly material are allowed to settle to the base of the container.

0.25 ml. of the supernatant is then pipetted into a narrow-width glass cuvette, the base of which is smeared with "optical glue".

The pen recorder motor is switched on, the speed used is 2.5 mm per second. All light sources are switched off and the red safety light is switched on. With the voltage of the powerpack adjusted to a low value, the copper cannister covering the top of the photomultiplier tube is removed, revealing the reflector

cone.

At this point 0.25 ml. of the stimulated muscle solution is added to the cuvette containing 0.25 ml. of the firefly extract. The reflector cone is now removed from the photomultiplier tube and the cuvette mounted by hand on to the guiding lens. The "optical glue" smeared on the base of the cuvette ensures that a good contact is made with the lens. The reflector cone is then quickly replaced over the top of the tube, enclosing the mounted cuvette. The red safety light is then switched off and the voltage of the powerpack adjusted to 1300 V. After a trace has been obtained on the pen recorder, the high voltage is switched off, the reflecting cone lifted and the cuvette removed. The cone is then replaced and the high voltage is switched on again. This then gives the true base line on the record for a voltage of 1300 V.

From the time when the test solution is added to the firefly extract, till the time when a recording is actually obtained the procedure is carried out as quickly as possible and usually takes about 10 - 15 seconds. This means that the record unfortunately does not encompass the initial 15 seconds of the light emission, so that the initial "flash" of light emitted when ATP first contacts the firefly extract has not been recorded by this method. However, both stimulated muscle solution and a test solution of ATP have similar rates of light signal decay (Figure 36) and the same amplitude of small scintillations, indicating that although the light is probably being reflected in a constant pattern from the inner surface of the relfecting cone, the light-producing reaction is taking place in a random fashion.

COMPOSITION OF RINGER'S SOLUTIONS

Frog Ringer's Solution

	Stock Concentration	Other Components
NaCl	70 g/l (isotonic x 10)	
KCl	9 g/l (isotonic)	
$NaHCO_3$	10 g/l (isotonic)	Glucose 1 g/l
$CaCl_2 6H_2O$	18.8 g/l (isotonic)	Neostigmine 10^{-6} g/ml.
$NaH_2PO_4 2H_2O$	18.72 g/l (isotonic)	

Method

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

400 ml. stock NaCl solution was diluted to 4,000 ml. with distilled water. To this amount was added the other stock solutions:- 100 ml. stock KCl, 90 ml. stock $NaHCO_3$, 60 ml. stock $CaCl_2 6H_2O$ and 30 ml. $NaH_2PO_4 2H_2O$ and the whole shaken thoroughly. 4.28 g of glucose and 4.3 mg of neostigmine were added to give final concentrations of glucose: 1 g/l and neostigmine; 10^{-6} g/ml. pH adjustment to 7.4 (if needed) was made by adding either N HCl or N NaOH. The final concentrations were 109 mM NaCl, 4.3 mM $NaHCO_3$, 3.8 mM KCl, 1.6 mM $CaCl_2$, 0.22 mM NaH_2PO_4.

Kreb's Solution

	Stock Concentration
NaCl	90 g/l
KCl	11.5 g/l
$NaHCO_3$	12.94 g/l
KH_2PO_4	21 g/l
$MgSO_4 7H_2O$	18.54 g/l
$CaCl_2 6H_2O$	24.1 g/l

Method

80 ml. of the stock NaCl solution was diluted to ten with distilled water to 800 ml. To this was added 32 ml. of stock KCl, 8 ml. of stock $NaHCO_3$ and 16.8 ml. of stock KH_2PO_4. The solution was shaken thoroughly and then bubbled with a mixture of 95% O_2 and 5% CO_2 for fifteen minutes. Then 8 ml. of stock $MgSO_4 7H_2O$ and 24 ml. of stock $CaCl_2 6H_2O$ were added.

Locke's Solution

	Stock Concentration
NaCl	250 g/l
$NaHCO_3$	50 g/l
$CaCl_2 6H_2O$	100 g/l
KCl	150 g/l

Method

One litre of solution was made up by mixing 36 ml. stock NaCl, 10 ml. stock $NaHCO_3$, 2.4 ml. stock $CaCl_2 6H_2O$ and 2.8 ml. stock KCl. This was made up to 1,000 ml. with distilled water and 1 g of glucose added. The final concentrations were 154 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 6.0 mM $NaHCO_3$.

Leech Solution

Stock solutions as for Locke's solution.

Method

1,000 ml. of Locke's mammalian solution were made up without adding glucose or eserine. This solution was diluted 1 : 1.4 by adding 400 ml. distilled water. Glucose was added to a concentration of 1 g/litre and eserine, 10^{-5} g/ml. The pH of the resulting solution was lowered to 7.0 by bubbling the solutions for a few minutes with 5% CO_2. The final concentrations were 110 mM NaCl, 4.3mM $NaHCO_3$, 1.6 mM $CaCl_2$, 4 mM KCl.

Cleansing of Glassware

All glassware was cleaned by the Calgon-metasilicate method (Boyd and Pathak, 1964) and sterilized in sealed nylon packages which were opened immediately prior to the use of their contents.

COMPOSITION OF RINGER'S SOLUTION USED (mM)

	$NaCl$	KCl	$CaCl_2$	$NaHCO_3$	KH_2PO_4	NaH_2PO_4	$MgSO_4$
Krebs	119	4.7	2.5	24.8	1.2	-	1.2
Frog	109	3.8	1.6	4.3	-	0.22	-
Locke	154	5.6	2.2	6.0	-	-	-
Leech	110	4.0	1.6	4.3	-	-	-

MAKING UP THE ACETYLCHOLINE SOLUTIONS

Sealed ampoules of 200 mg acetylcholine chloride (powder form) were specially supplied by Roche Products Ltd. Each ampoule of acetylcholine chloride was accompanied by an ampoule of distilled water.

Method

Both ampoules were filed and broken open; 2 ml. of distilled water was transferred by syringe into the ampoule containing 200 mg acetylcholine chloride. The powder dissolved quickly. The solution was then sucked back into the syringe and transferred to a clean, dry 250 ml. graduated cylinder. This was made up to 200 ml. with frog Ringer's solution, giving a concentration of 10^{-3} g/ml. of acetylcholine chloride in frog Ringer's solution.

By transferring 1 ml. of this stock solution of 10^{-3} g/ml. acetylcholine chlordie to a 100 ml. flask and making up to the mark with frog Ringer's solution, a concentration of 10^{-5} g/ml. was obtained. Dilution of 1:100 from this flask resulted in a concentration of 10^{-7} g/ml. From this flask a dilution of 1:10 gave 10^{-8} g/ml., and a dilution of 1:100 gave 10^{-9} g/ml. In this way a range of concentrations from the stock solution of 10^{-3} g/ml. to 10^{-13} g/ml. were obtained.

Intermediate Concentrations

The concentration of 5×10^{-5} g/ml. was prepared by making up 5 ml. of 10^{-3} g/ml. to 100 ml. with Ringer's solution. From this concentration the intermediate concentrations of 5×10^{-7} g/ml., 5×10^{-8} g/ml., 5×10^{-9} g/ml. were made up.

Further intermediate concentrations of 1.5×10^{-9} g/ml., 2.5×10^{-9} g/ml., 3.5×10^{-9} g/ml. and 7.5×10^{-9} g/ml. were made up when required in 100 ml. flasks.

Acetylcholine Solutions for Leech Experiments

The procedure for making up graded acetylcholine chloride solutions for the leech experiments was essentially the same as that for the frog heart experiments. Eserinized leech Ringer's solution was used throughout.

LIST OF DRUGS

Adrenaline :	British Drug Houses Ltd. In sealed 1 ml ampoules of 1 : 1,000 concentration.
Noradrenaline :	Bayer Products Ltd. Supplied as the bitartrate in sealed 2 ml ampoules of 1 : 1,000 concentration.
Adenosine triphosphate :	British Drug Houses Ltd. In powder form as the disodium dihydrogen salt; also supplied by Pabst Laboratories.
Adenosine diphosphate :	Manufactured by Pabst Laboratories.
Adenosine monophosphate :	Manufactured by Pabst Laboratories.
Atropine sulphate :	Solution supplied in 1 ml. sealed ampoules by Evans Ltd., each ampoule containing .6 mg.
Acetylcholine chloride :	The powder was supplied specially by Roche Ltd. in sealed 200 mg ampoules.
Curare :	50 mg d-tubocurarine chloride in 5 ml. solution. Supplied by Duncan Lockhart & Co. Ltd.
Ergotamine tartrate :	Sandoz Products Ltd. Sealed ampoules of 1 ml., each ampoule containing 0.5 mg.
Morphine hydrochloride :	Supplied in powdered form by Burroughes Wellcome & Co.
Neostigmine (eserine) :	Roche Products Ltd. Supplied as neostigmine methyl sulphate in concentrations of 2.5 mg mer ml. in 5 ml ampoules.
Physostigmine sulphate :	Supplied in powder form by Macfarlan Smith Ltd.
Pronethalol :	Supplied as a special sample in powder form by I.C.I. Ltd.

MOLECULAR SIEVE CHROMATOGRAPHY PROCEDURE

Introduction

J. Porath (1959) introduced a crossed-linked dextran gel in order to separate out chromatographically the protein fraction from the blood plasma. His interest lay in the further investigation of the protein and polypeptide molecules. This column separation technique has been adopted commercially ("Sephadex", Pharmacia, Uppsala, Sweden) and there are now available many cross-linked dextran gels capable of separating out large molecules in different ranges of molecular weight.

The type of Sephadex column used was G-25 of particle size 20μ - 80μ in diameter. It has the property of being able to separate molecules of 5,000 molecular weight and less from molecules having a larger molecular weight.

Principle

The Sephadex particles are supplied in dry form and have to be soaked in the solvent liquid before use. After the soaking procedure, the particles swell up, the extent of the swelling depending upon the nature of the solvent and the architecture of the gel matrix. Solute molecules can penetrate the dextran network according to the steric relationships between the molecular structure of the dextran gel and the solute particles. Thus the shape and size of the solute molecules largely determine their penetration, and hence their distribution between gel particles, and solvent lying between these gel particles, is determined. If a substance is able to penetrate the gel matrix completely, it will be maximally retarded in its elution through the column. If a substance has such a large molecular size that it cannot penetrate the gel matrix at all, then no retardation will take place and the substance will be eluted through the column very quickly (Figure 4).

Sephadex columns in general will increase the retardation as the molecular weight of the solute molecule diminishes, but there are some molecules which are

Diagrammatic representation of solute distributions in the Sephadex column.

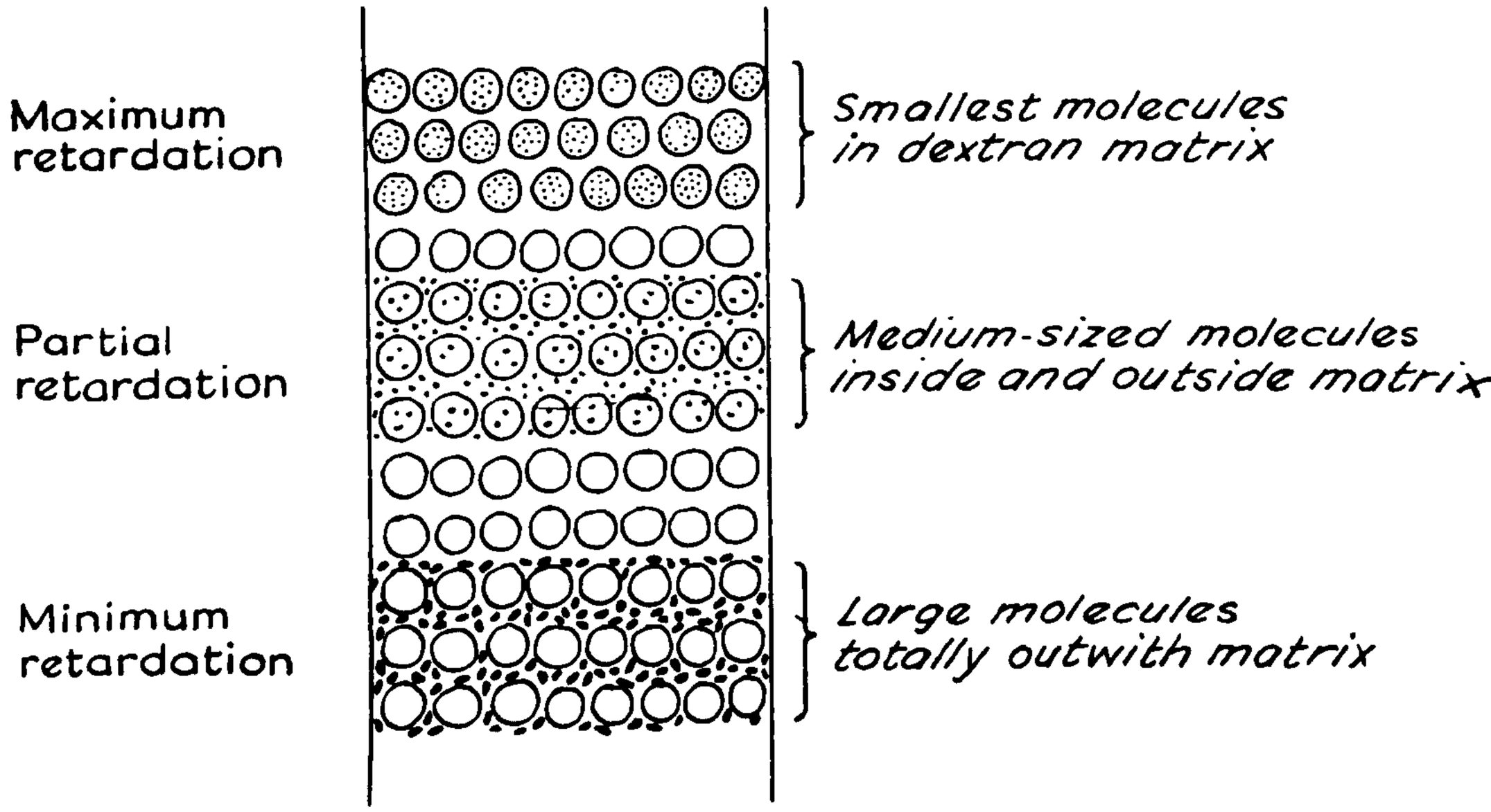


Figure 4. (After Tiselius et al. 1963)

Sephadex particles of cross-linked dextran gel are represented by equal-sized circles. Solute molecules are represented by small, medium-sized and large dots. The size of the gaps (pores) between the cross-linkages in the particles vary according to the type of Sephadex. Sephadex G-25 has a pore size which will just prevent admission of molecules of molecular weight 5000 or over. The shape of the solute molecule will also determine whether penetration into the dextran matrix is possible.

specifically retarded more than they should be according to their molecular weight (e.g. adenosine compounds). These anomalies can be due to one or both of the following:-

1. the shape of the molecule does not fit the gap in the dextran matrix
2. the molecule is highly charged and the gel is repellant.

It is generally better not to elute the column with distilled water, since charged solutes then exhibit inconsistent behaviour. In the present work done with Sephadex G-25, various Ringer's solutions were used for elution.

Construction of Glass Column Container

Three problems naturally arise during the task of setting up a fractionating column.

1. The column material must be packed evenly.
2. The material supporting the base of the column must be rigid, porous to water but not to the column material.
3. The top of the column must be perfectlyhorizontal and preserved as such.

The second requirement was met by the adaptation of a special glass tube. This contained a disc of sintered glass with a suitable pore size which was welded across the lumen of the tube. The glass tube was cut as near to the partition of sintered glass as possible and a spout was fashioned on one side. On to the other side was welded a glass column container of 1.4 cms diameter and length 17 cms.

The third problem was dealt with in the following way. A circle of filter paper (Whatman's No. 1) of slightly smaller diameter than that of the glass tube was allowed to sink gently on to the surface of the column through the fluid above it. This was found to give adequate stability to the column surface when test solutions were added carefully by Pasteur pipette.

Packing the Column

The Sephadex particles are put into a beaker with Ringer's solution and allowed to stand for half-an-hour during which time they swell up.

After vigorous stirring the mixture is poured into the large filter funnel with the connection between the funnel and the glass tube closed (Figure 5). The stirring mechanism is now switched on. This swirls the Sephadex particles around in the funnel, allowing a few of them to descend into the neck of the funnel. The glass column container is now attached to the rubber tubing around the filter funnel neck and the connection is opened. This creates a continuous column of liquid from the sintered glass bottom to the surface of the liquid in the funnel. When the stirrer is switched on, Sephadex particles swirl around in the funnel and descend infrequently into the glass tube. When the stirrer is switched off, the descent of particles down into the tube increases rapidly. The rate of packing and the length of the column could be adequately controlled by this regulating method. This method ensures that the particles are packed in an even fashion so that separations effected by the column can be repeated and standardized.

When the appropriate height was reached (13.5 cm), the rubber connecting tubing was closed off and gently removed from the top of the glass column container. This procedure usually caused some disturbance of the particles at the top of the column, but this settled within a few minutes after the column was transferred to another clamp stand.

As soon as the Sephadex particles settled in a horizontal plane at the top of the column, a disc of filter paper (Whatman's No. 1), cut to fit the internal diameter of the glass tube, was allowed to float down on top of the Sephadex particles. This gave stability to the column top, since the particles are very easily disturbed without this restriction. Every column was packed to a height of 13.5 cm in a tube of internal diameter 1.4 cm.

Fluid was eluted through the column using the hydrostatic pressure developed by the column of Ringer's solution contained in the glass tube above the level of the horizontal plane at the top of the column. The height of this column of solvent varied with each glass container, but was usually of the order of 4 cm.

In order to elute a 2 ml. test sample through the column, the fluid above the

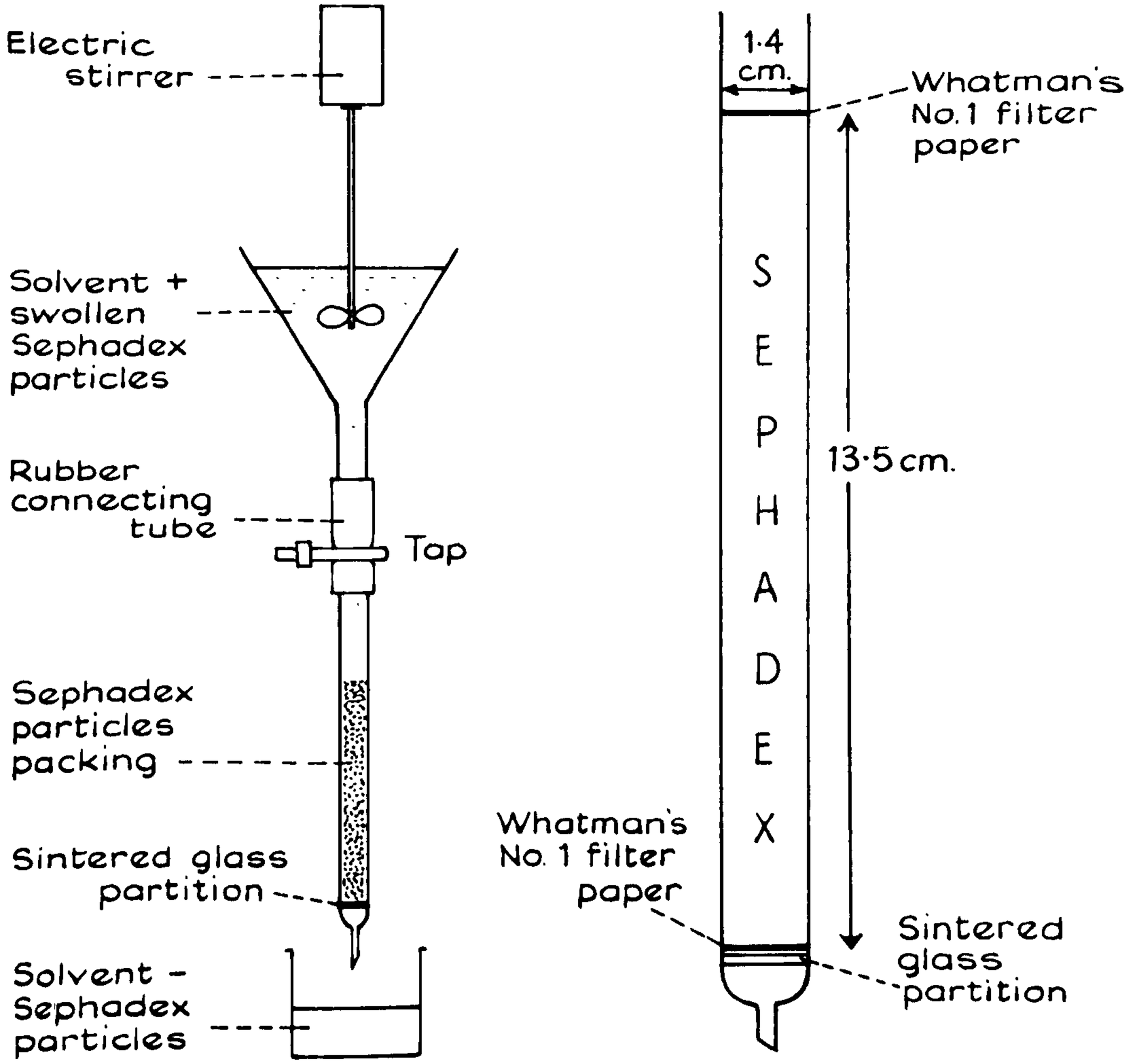


Figure 5. Apparatus for Packing the Sephadex Column

a) The glass tube to contain the Sephadex is inserted into a rubber tube which connects it with the filter funnel containing solvent + Sephadex particles. Solvent - the particles pass through the sintered glass partition into a beaker.

With the rubber connecting tube open and the electric stirrer switched on, Sephadex particles swirl round in the funnel and descent infrequently into the glass tube. When the stirrer is switched off, the descent of particles down into the tube increases rapidly. When the appropriate height of Sephadex was reached (13.5 cm), the connecting tube was then closed and the glass tube removed from the rubber connecting tube.

b) The dimensions of the completed column are shown. Discs of filter paper ensure that the extremities of the column are kept horizontal.

column was allowed to run down through the Sephadex particles until the base of the meniscus just reached the level of the filter paper. Then the 2 ml. sample was carefully pipetted on to the surface of the filter paper by Pasteur pipette. The sample was allowed to run into the Sephadex until the meniscus reached the filter paper level, at which time the Ringer's solution was added carefully until the glass column container was filled completely with the solvent. During the elution and collection of the fractions, the column container was constantly topped up with Ringer's solution.

After collection of the fractions, the elution was stopped by inserting rubber bungs into the basal spout and the top of the glass tube.

Equilibration of the Column

Before each experiment the column was eluted with Ringer's solution , either leech or frog Ringer's solution, for two hours before a test sample was put through. This ensured that the ionic composition of the Ringer's solution was not altered in any way, since over a period of two hours presumably the solute ions completely penetrate the dextran matrix and are distributed evenly between the inside of the particles and the space surrounding them.

Standardizing the Sephadex Column

In order to facilitate the procedure of standardizing the column, the properties of the substance "blue dextran" (Dextran 2000, Pharmacia) were utilized. This substance has a molecular weight of around two million and is therefore eluted through the Sephadex column at a maximum rate. Even when it is present in very small concentrations, blue dextran imparts a distinct blue colour to a solution. The makers recommend a 2% solution for the measurement of void volumes in Sephadex column chromatography. Blue dextran does not react in any way with the column material, although it is said to be slightly adsorbed on to Sephadex G-25 particles. The recommended way to remove this adsorbed blue dextran is to run a solution of serum albumin through the column.

Presuming that the blue dextran does not react in any way with the test

solution, it is convenient to elute a mixture of the two through the column. Adsorption of the blue dextran on to the particles of Sephadex will be counteracted by the protein in the test solution (See protein section).

The volume of the solution surrounding the active sartorius muscle was 2 ml; by a process of trial and error it was found that when a solution of 2 ml. of blue dextran was put through a column of dimensions 13.5 cm x 1.4 cm the blue dextran was eluted into a volume of exactly 3 ml. The passage of the blue dextran through the column is easily followed (See Figure 6).

The volume of fluid required to elute the 2 ml. solution of blue dextran (or any substance which does not enter the pores in the Sephadex particles) through the column is termed the "void volume". Nine columns of height 13.5 cm were packed in distilled water and 2 ml. solutions of blue dextran (approximately 5% concentration) were put through them.

The mean void volume of the nine columns was 11.4 ml. (range 10.75 ml. - 11.75 ml). Thus, for any substance of molecular weight greater than 5,000, it would take approximately 11.4 ml. of Ringer's solution to carry it through the column when it was contained in an initial volume of 2 ml. A newly-packed column was only accepted for use when 2 ml. of blue dextran solution was eluted into a 3 ml. fraction.

Because of the slight variation in the void volume from column to column, it was decided to mix the blue dextran with the test solution and then to follow the course of the blue dextran through the column visually. Only after the column was seen to be clear of the blue dextran were subsequent fractions collected.

The initial 3 ml. fraction containing the blue dextran is termed the "blue dextran fraction", and contains any substances of molecular weight greater than 5,000 that are present in the test solution e.g. protein molecules.

It was for the purpose of isolating ACh from other substances found in the test solution that the properties of the Sephadex column were adopted. The fractions collected after the initial blue dextran fraction were deliberately adjusted

in volume so that most of the ACh could be recovered in one fraction. It was found that a 3 ml. fraction coming 4.5 ml. after the blue dextran fraction contained most of the ACh present in an initial 2 ml. volume eluted through the column.

The behaviour of the other substances in the column was studied to ensure that a column of this dimension could effect a different pattern of elution with substances of different molecular weights. It was convenient to use coloured substances for this study. Figure 6 (coloured plate) shows the various stages in one such experiment. 2 ml. of potassium ferricyanide in a concentration of 20mg/ml. were mixed with 0.1 ml. of blue dextran. This mixture was then put through the column. The fractions collected were those normally collected for the ACh recovery. The estimation of the potassium ferricyanide was made by comparing the intensity of the fraction colours with known concentrations of potassium ferricyanide using a Hawksley colorimeter.

The right hand column of Figure 7 shows the percentage recovery of the substances cupric sulphate, cobalt sulphate and potassium ferricyanide. The left hand column indicates for comparison the percentage recovery of ACh-chloride in the same fractions as measured on the leech muscle from five Sephadex columns.

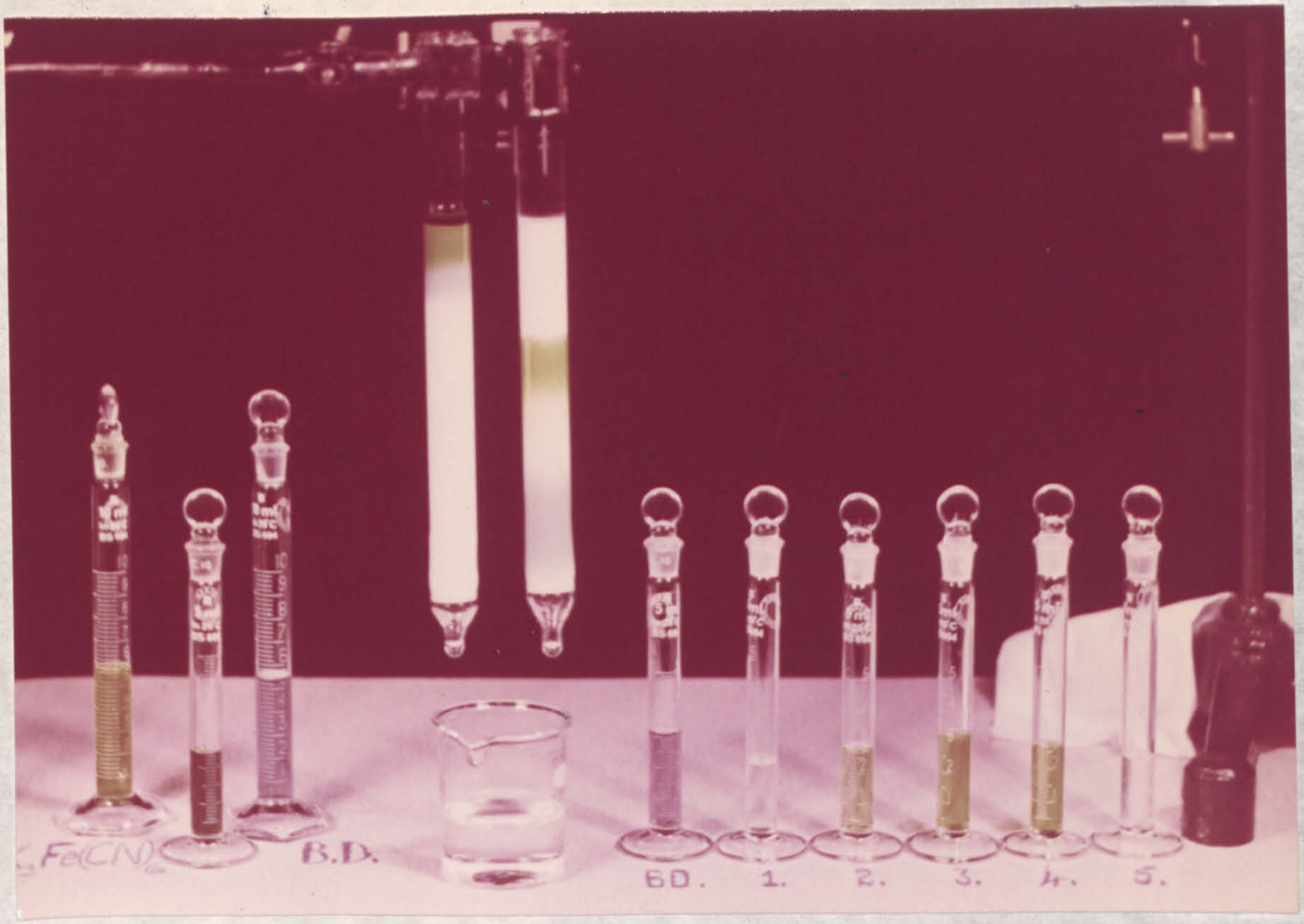


Figure 6.

The separation of a substance of large molecular weight (blue dextran : M.W. 5,000) from a substance of small molecular weight (potassium ferricyanide : M.W. = 329) by the Sephadex column.

On the left of the plate are cylinders containing solutions of potassium ferricyanide (yellow) and blue dextran. The cylinder in the foreground contains a 2 ml. mixture of the two substances. When this mixture is placed at the top of the column and allowed to gravitate through, the blue dextran is seen to advance immediately ahead of the potassium ferricyanide. The second column shows the separation at a later stage. When the subsequent fractions are collected, the blue dextran is eluted into the first 3 ml. and the potassium ferricyanide comes into fractions 2, 3 and 4, most of it being in fraction 3. Neither substance appears in fraction 1.

Figure 7.

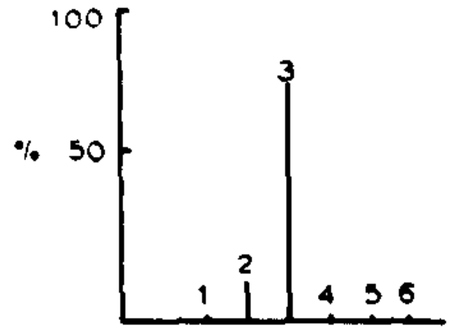
Left Hand Column

The percentage recovery of ACh-chloride in column fractions which were adjusted to contain most of the ACh in fraction 3. Each histogram is constructed from a separate column experiment. The columns were eluted with leech Ringer's solution and the initial ACh solution was made up in the same type of Ringer's solution. Assay of these fractions was done on the leech muscle strip. Note that no acetylcholine appears in fractions 1, 4, 5 and 6.

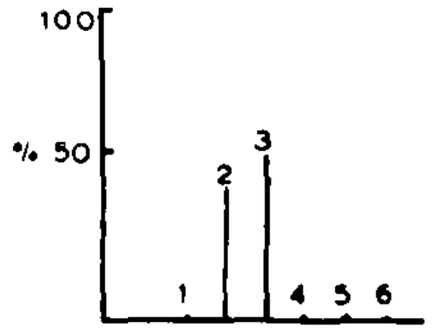
The volume of fractions 1 - 6 was, respectively, 2 ml., 2.5 ml., 3 ml., 2.5 ml., 2 ml., 2 ml..

Right Hand Column

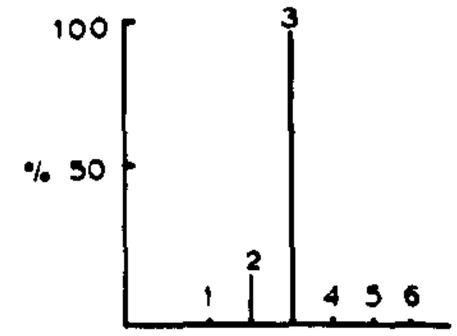
Substances of different molecular weight were put through the column and the same fractions were collected. Note the different distribution of the same substance when distilled water is used as the solvent.



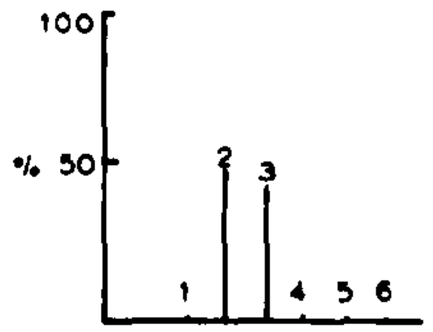
ACh- chloride in leech Ringer



Cupric sulphate (M W 160) in distilled water

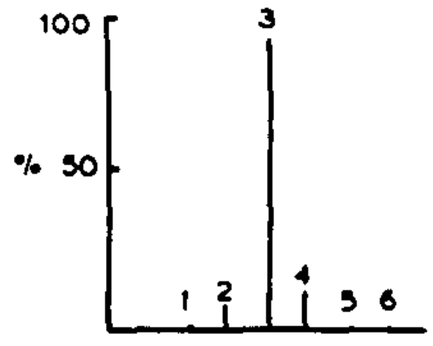


ACh-chloride in leech Ringer

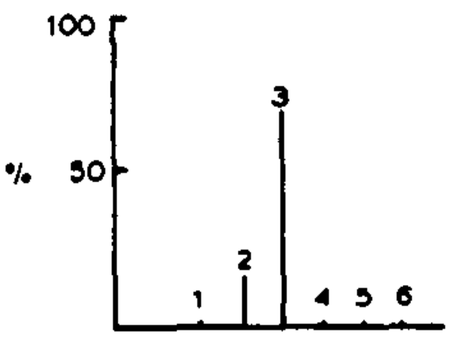


Cobalt sulphate (M W 157) in distilled water

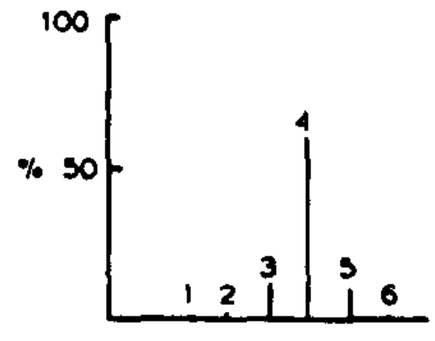
ACh-chloride in leech Ringer



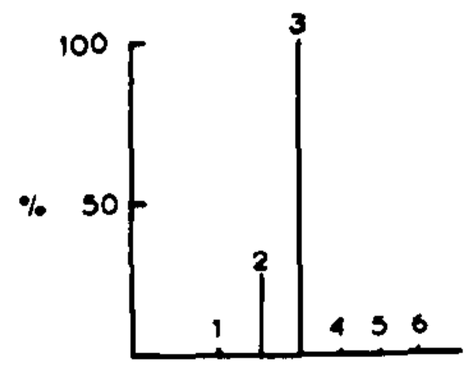
Cobalt sulphate in leech Ringer



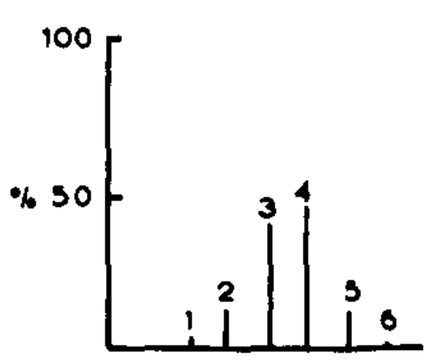
ACh-chloride in leech Ringer



K-ferricyanide (MW 329) in distilled water



ACh-chloride in leech Ringer



K-ferricyanide in leech Ringer

SETTING UP THE LEECH PREPARATION

The method used is a modified version of that used by Murnaghan (1958), who noted that morphine facilitates the quicker relaxation of the leech strip of muscle. The leech muscle was soaked in solutions of morphine before being set up in the bath. This differs from the original method in that Murnaghan set up the leech strip ready to record contractions at the beginning of the morphinization procedure.

A live leech is taken from a tank where the store of leeches were kept in loch water. After crushing the ganglia just posterior to the anterior sucker, the leech is pinned out on a cork board with the ventral side lying upwards. A midline ventral incision is made starting caudally and ending by opening the anterior sucker. Thus the whole of the muscular body wall is eventually pinned flat out with the outside surface next to the cork board. This position displays the grey-white walls of the coelom. Using fine forceps and fine scissors the coelomic wall is completely removed, revealing the dark, chocolate brown inner layer of the body wall musculature. This layer represents collections of large cells containing ingested red blood corpuscles which have migrated from the gut. The dark brown colour is presumably caused by the breakdown products of the haemoglobin pigment within the corpuscles. This layer is very difficult to remove without inflicting some damage upon the actual muscle layer and in practice was never completely removed.

After the process of "cleaning" the inner surface of the body wall, the pinned-out preparation is turned over and pinned out again, this time with the outer surface uppermost. It is convenient to use the natural markings on the dorsum of the leech as a rough guide to the breadth of the strip of muscle removed. On each side of the dorsum of the leech, running longitudinally, are two black lines enclosing a thin yellow line. The strip which lies in between these longitudinal patterns proved to be a suitable breadth. The anterior half of the leech was used

as a rough guide to the length of the strip required, to suit the dimensions of the leech bath.

Having removed this size of segment from the dorsum of the body wall musculature, the strip is then pinned out without any undue tension, on a small cork board and floated upside down in a Ringer's solution containing 5×10^{-5} g/ml. morphine sulphate. After an hour it was then transferred to a Ringer's solution containing 2×10^{-5} g/ml. morphine sulphate (see Table 1) and left to soak for a further hour. The preparation is now transferred to the 2 ml. leech bath. The muscle strip is unpinned and a loop of thread is tied to one end. A long thread is tied to the other. The loop is then passed around the hook of the syringe needle while the long piece of thread is attached to the tension transducer. In this way the strip of leech muscle is suspended in the tubular bath, which is now filled with leech Ringer's solution. After the bath is filled with the Ringer's solution the muscle strip should not touch the sides of the bath (Figure 8).

At this stage the tension of the muscle strip can be adjusted to a suitable value for the particular piece of muscle in the bath by the control attached to the transducer (Figure 8).

The tension developed by the muscle strip will always approach an optimum value, provided that the muscle is not overstretched. If the muscle is suddenly stretched, then it will immediately relax until the original level of tension is reached. If the strip is suddenly shortened passively, the muscle will contract until the original tension value is achieved once again. Amplification is adjusted so that an excursion of 1 mm by the leech muscle will cause a movement of 30 mm on the pen recorder. The tension recording is calibrated by hanging weights on the thread attached to the transducer.

The following procedure is adopted in order to apply a test solution to the muscle strip. The leech bath is drained of fluid by the tap at the base of the needle (Figure 8). A new solution is added by syringe at the top of the bath. During the procedure of changing the solutions in the bath, the leech muscle is

Table 1

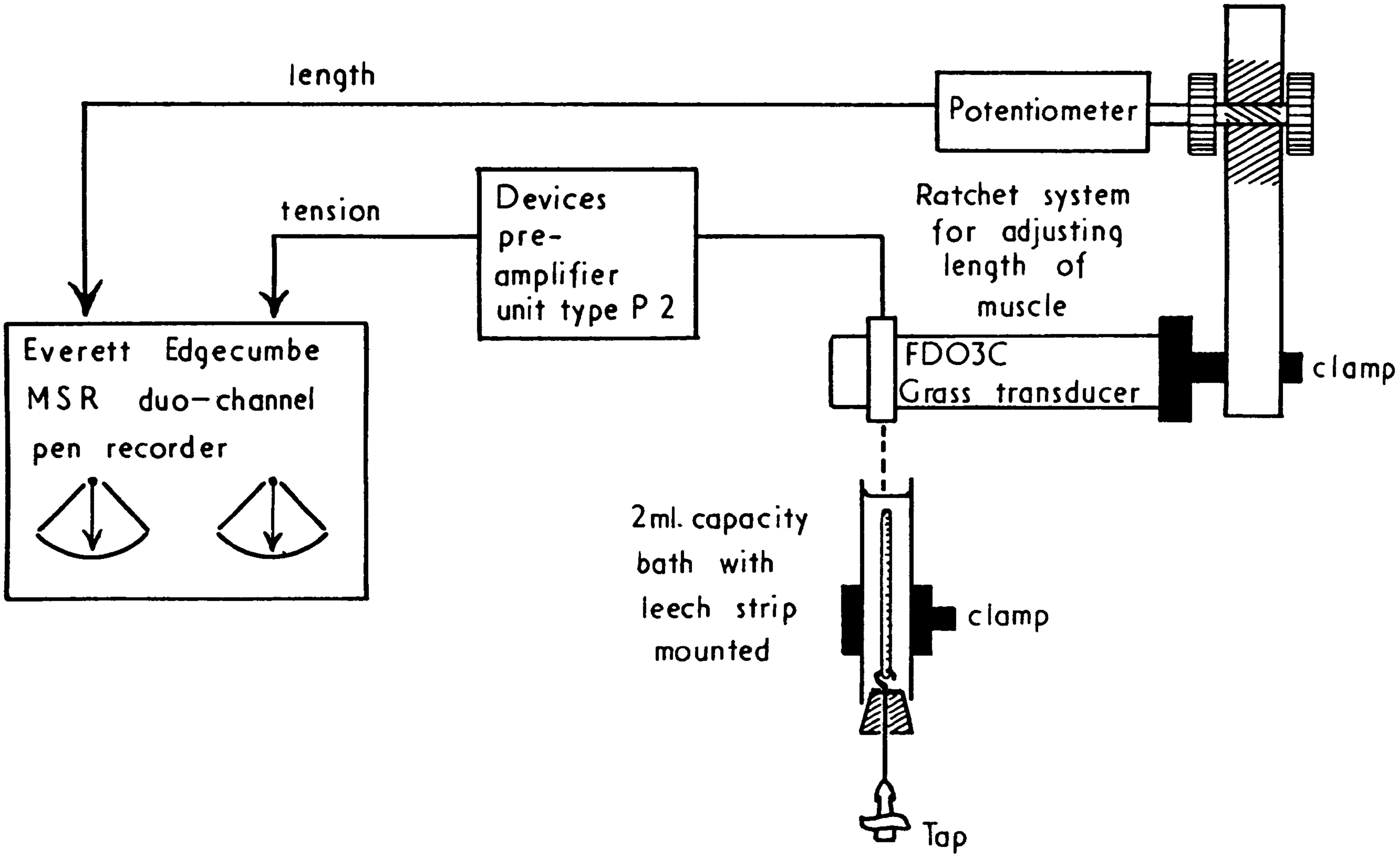
THE PRESENT METHOD COMPARED WITH THAT OF MURNAGHAN

SAME LEECH RINGER'S SOLUTION USED

Present Method	Murnaghan
Strip soaked in morphine sulphate for 1 hour (concentration = 5×10^{-5} g/ml) Strip soaked for another hour in 2×10^{-5} g/ml morphine sulphate and 10^{-5} g/ml eserine sulphate.	Strip soaked in 4 or 5×10^{-5} g/ml morphine sulphate. Left for up to 3 hours. Strip soaked for a further 30 mins. in 4 or 5×10^{-5} g/ml morphine sulphate and 10^{-5} g/ml eserine sulphate solution.
Strip was pinned on to a cork board, being slightly stretched. Board was transferred from one solution to another	Strip was set up in an isotonic fashion in leech bath. The tension being "equivalent to 2 grammes". Then solutions above were put into bath.
Washing between acetylcholine solutions was done with eserinized leech Ringer's solution (10^{-5} g/ml).	Morphine-eserine Ringer's solution was used for washing throughout.
Acetylcholine solutions made up in eserinized Ringer's solution (10^{-5} g/ml).	Test solutions consisted of mammalian-Ringer perfusates of 10^{-5} g/ml eserine diluted 1 : 1.4 before assay.
Each solution allowed to act for 45 secs.	Solutions allowed to act for 2 - 3 mins.
Isometric system, tension measured.	Isotonic system, change of length measured.
Thresholds mostly 10^{-9} g/ml. Sensitivity maintained over 24 hr. period.	Thresholds mostly 10^{-9} g/ml. "In no case did the sensitivity fall off after 5 hours".
Non-oxygenated Ringer's solution. pH = 7.0.	Oxygenated Ringer's solution. pH = 7.3.

Figure 8.

A diagram to show the isometric preparation of leech muscle and associated apparatus which was developed for the assay of ACh. The strip of leech muscle is mounted in a bath of 2 ml. capacity. The distance of the transducer from the bath, and hence the length of the muscle strip, can be adjusted by the ratchet control seen at the right side of the diagram. The length (via a potentiometer) and tension (via pre-amplifier) of the muscle strip can be recorded simultaneously. Once the length of the muscle strip has been set, the ratchet control is not moved throughout the rest of the experimental procedure.


length
Potentiometer
tension
Devices
pre-
amplifier
unit type P 2
Ratchet system
for adjusting
length of
muscle
Everett Edgecumbe
MSR duo-channel
pen recorder
FDO3C
Grass transducer
clamp
2ml. capacity
bath with
leech strip
mounted
clamp
Tap

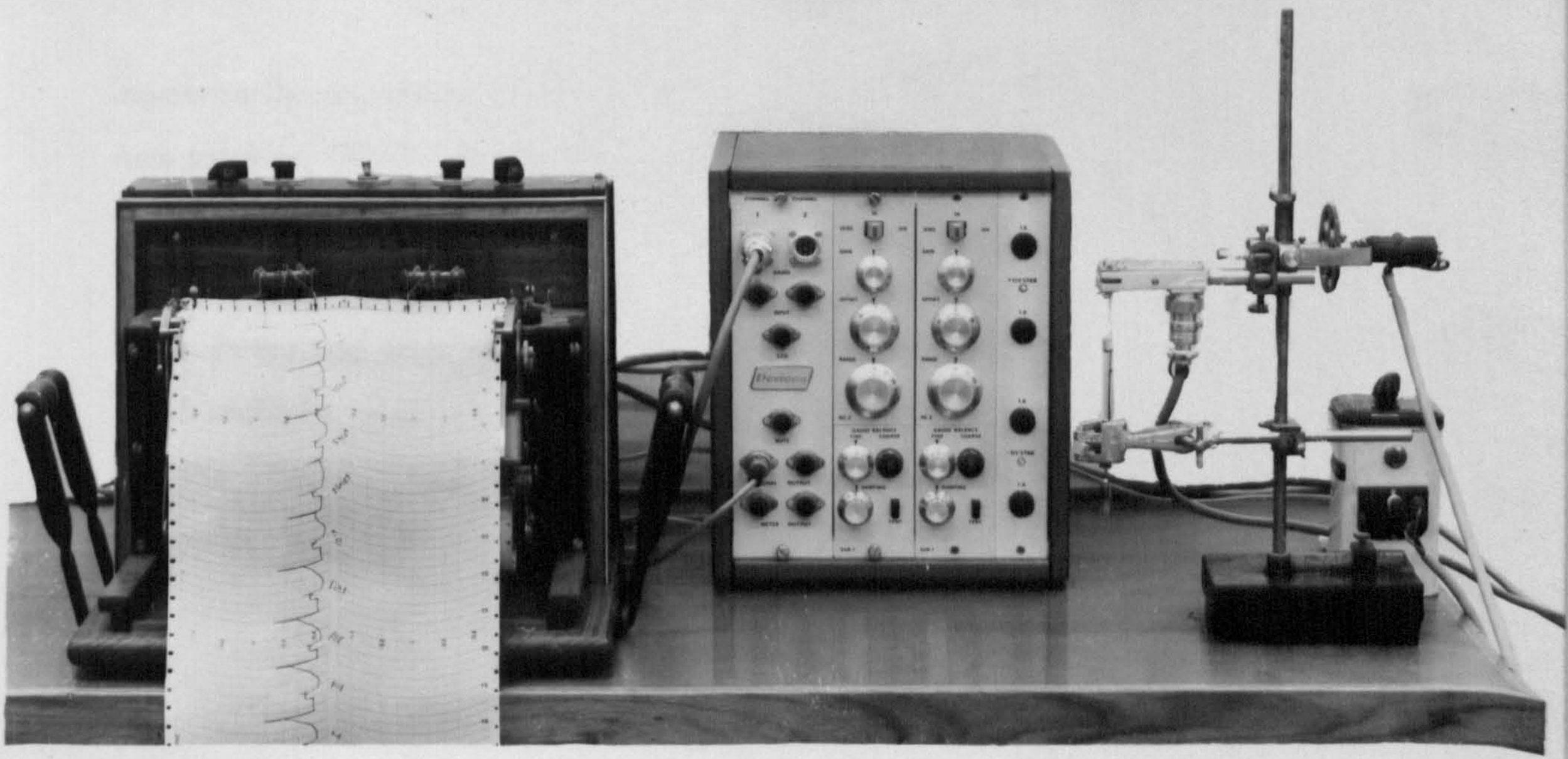


Figure 9. The apparatus used for the estimation of ACh by the isometric leech muscle preparation.

The clamp-stand on the right supports the force displacement transducer (type FTO 3C, Grass Instrument Co.) and the leech muscle bath. The muscle strip is attached to the transducer by a thread. On the other side of the stand is attached the ratchet system for altering the length of the muscle strip. The central piece of apparatus is the Devices preamplifier unit which is linked to a power supply (not seen). The Everett Edgecumbe duo-channel pen recorder, seen on the left, records tension of the leech muscle strip on one channel and length of the strip on the second channel. Amplification of the system was adjusted so that a change in tension of 5 mg could easily be recorded.

momentarily suspended in air and usually sticks to the side of the bath until the new solution fills the bath. The small amount of tension produced by the leech sticking to the bath is always indicated on the record by an unusual, but regular, increment in tension before the effect of each test solution.

Every test solution in this work was applied to the muscle strip for a period of 45 seconds; a clear response could be detected after this short period of time and the effects of ACh solutions could be quickly washed out after this period in contact with the muscle.

RESULTS

THE RESULT OF PERFUSING A STIMULATED MUSCLE SOLUTION THROUGH A FROG HEART

When the solution in which the frog sartorius muscle twitched was perfused through the frog heart, an unexpected finding occurred. Instead of producing the expected slowing of the heart beat, typical of ACh, a sharp stimulatory effect resulted from the solution (Figure 10). The cardiac output, systolic, diastolic and pulse pressure were all increased. In some cases a late increase in the heart rate occurred. In no case, out of forty-one undiluted test solutions perfused through thirty-three frog hearts, was an ACh-like effect seen on the frog heart (Table 2). All solutions stimulated the frog heart. Clearly the effect of any ACh present in the test solution was being completely masked by the effect of other interfering substances.

Figure 10 shows the effect of perfusing a frog heart with a solution in which a sartorius muscle has twitched. The cardiac output (in ml/min), arterial pressure (in mm Hg), heart rate (in beats/min) and the venous pressure (in mm H_2O) are monitored simultaneously. The marker indicates the time taken for a test solution to be perfused into the frog heart. "Sx" indicates the time of perfusion of a stimulated muscle solution into the heart, the "S" referring to the unknown stimulatory substance, or substances, and the "x" indicating that there is an unknown quantity present. The venous pressure recording indicates the pressure at which the heart is being perfused with frog Ringer's solution from a large reservoir. The slight irregularity of the venous, or perfusion, pressure recorded during the time of injection of the test solution into the venous capsule is due to the pressure in the positive pressure system being adjusted manually by tap in order to keep the perfusion pressure during the injection as close to the original perfusion pressure as possible (See Methods).

As soon as the solution reaches the heart there is an initial sharp rise in the systolic pressure, not quite matched by the sharp rise in diastolic pressure. At the

Figure 10.

The effect of perfusing a frog heart with a stimulated muscle solution. Note the sharp rise in the systolic, diastolic and pulse pressures in the arterial pressure recording. At the same time the cardiac output rises very rapidly to reach a peak about one minute later. A further minute later the heart rate reaches a peak value; this effect is slow to pass off. Note the irregularity of the perfusion pressure during the injection. A control injection of Ringer's solution is seen at the right of the trace.

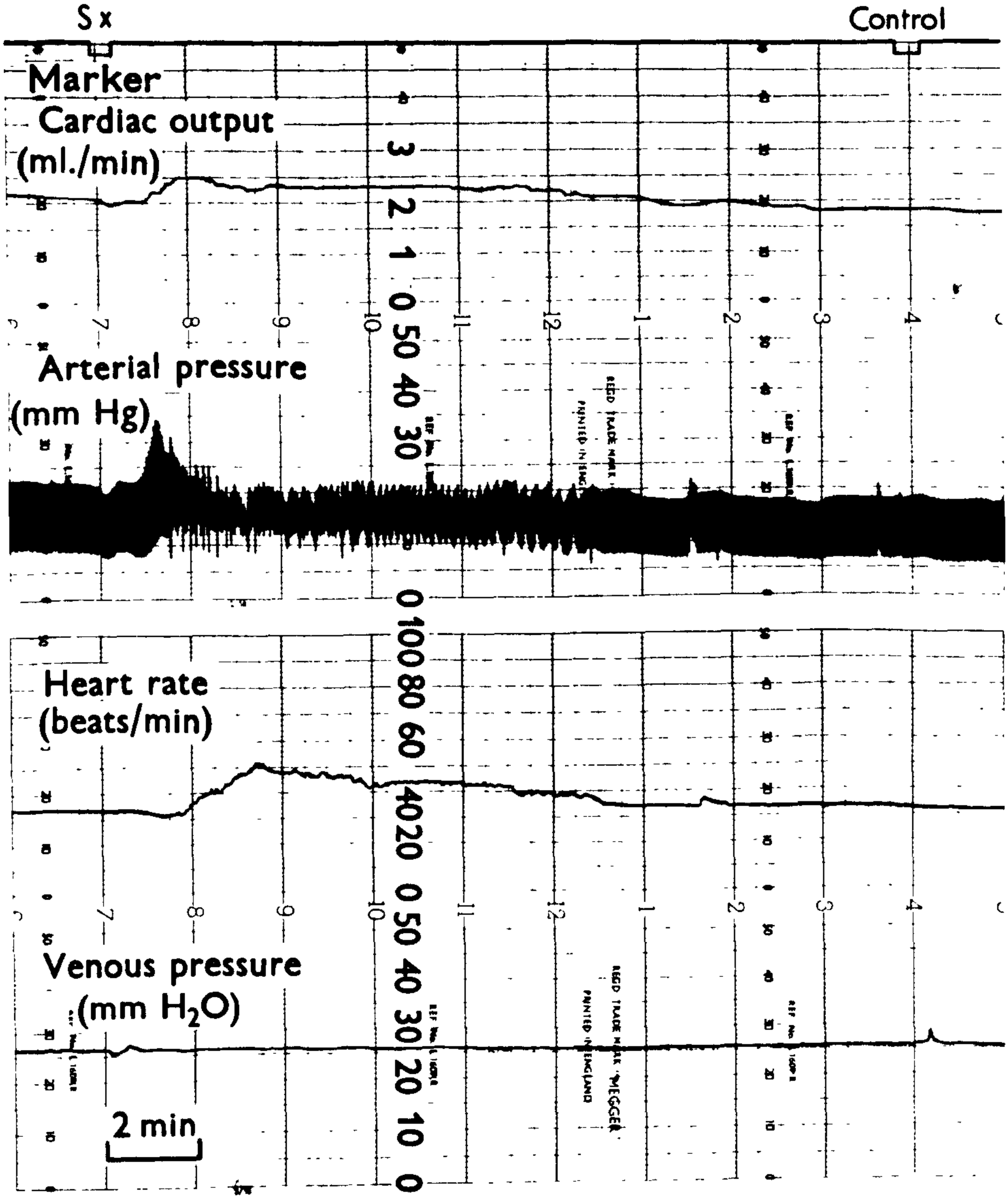

S x
Control
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H2O)
2 min

Figure 11.

The effect of perfusing a solution in which a sartorius mucle has rested for 30 minutes through a frog heart. "Rx" indicates the time of perfusion of the undiluted resting muscle solution. Note the increase in cardiac output and blood pressure. No effect is seen on the heart rate. "Rx" diluted 10 times has no effect on the heart.

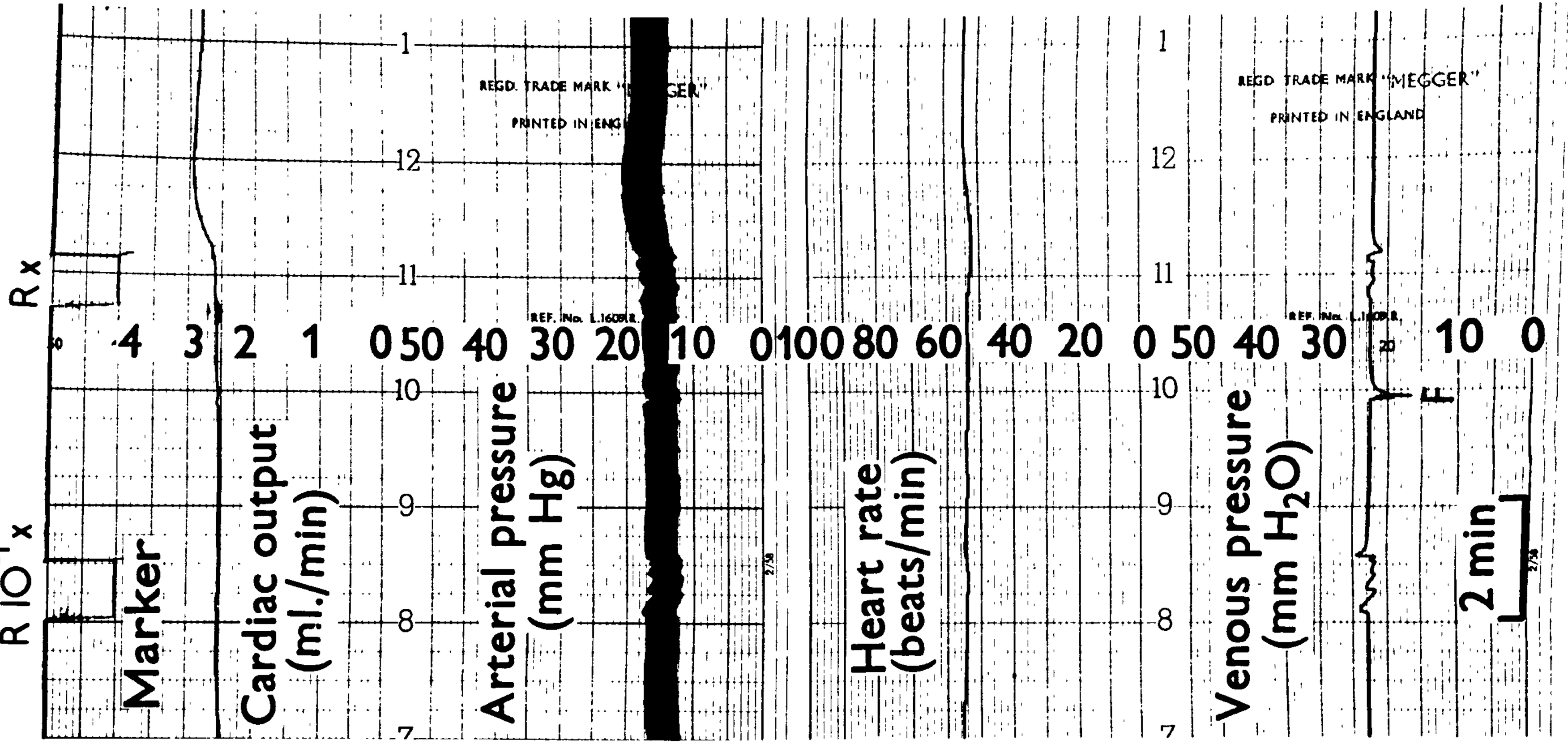

Rx
R IO⁻¹x
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
REGD. TRADE MARK "MEGGER"
PRINTED IN ENGLAND
REF. No. L.1605 R.

Table 2.

THE EFFECT OF RESTING AND STIMULATED MUSCLE SOLUTIONS WHEN PERFUSED THROUGH FROG HEARTS

RESTING MUSCLE SOLUTIONS			STIMULATED MUSCLE SOLUTIONS		
Heart	% Output Increase	% Rate Increase	Heart	% Output Increase	% Rate Increase
1	-13	1	1	25	25 same
	-14	3.5		18	25 muscle
	-13	5	2	15	7
2	0	2.5		6	5
3	- 7	7	3	30	50
	- 2	-12	4	32	heart stopped
4	0	0		100	33
5	11	0	5	24	20
	10	0	6	6	7
	15	0	7	14	28
6	0	0	8	7	10
7	5	33	9	28	-
	4	5		31	2
8	10	20	10	28	25
	7	12		30	14
	24	20	11	12	13
9	0	16		14	4
	0	20	12	20	irregular
	5	4	13	7	23
	7	0	14	20	23
	7	0	15	45	not recorded
10	-60	-40		45	" "
11	3	24	16	38	7
			17	40	24
			18	38	17
			19	23	15
				56	irregular
			20	25	13
			21	32	7
			22	24	7
			23	36	20
			24	56	4
			25	45	12
			26	12	-
			27	25	-
			28	11	24
			29	18	10
			30	35	15
			31	28	-
			32	36	8*
			33	18	15

Mean output rise of 12 = 9%

Six solutions gave a fall in cardiac output (Negative sign)

Five solutions gave no effect

* two pooled solutions

Rate increase was measured as a maximum after injection of the test solution. This usually occurred 2 minutes afterwards.

same time the cardiac output is suddenly increased and reaches a peak value about one minute later. The heart rate increases from 30 beats/min to a peak value of 50 beats/min about two minutes later and only returns to a normal value a further ten minutes later. The heart beat has been rendered irregular for seven minutes by the injection of the test solution, but soon assumes the former regular rhythm. A control injection of frog Ringer's solution shown at the right of the trace is without effect on any parameter.

This particular sartorius muscle was stimulated under isometric conditions and it was noted that some degree of tissue damage was likely to occur at the fixed ends of the muscle. This would contribute unknown products of tissue damage to the surrounding solution which could markedly affect the response of the heart. In order to minimise this risk every subsequent stimulated muscle solution was obtained from a muscle which was allowed to lie in the bathing solution without having any support at the ends of the muscle (Figure 2).

It was important to exclude the possibility that the effect was produced by a sartorius muscle which had only soaked in the bathing solution without stimulation taking place. Figure 11 shows the effect of perfusing a solution in which a sartorius muscle had rested for half-an-hour through a frog heart. "Rx" indicates the time of perfusion of the undiluted resting muscle solution. A slight increase in the systolic and diastolic pressures has occurred, with a simultaneous increase in the cardiac output. No obvious effect is seen on the heart rate. When this solution was diluted ten times, "R 10^{-1} x", the effect was abolished. Out of twenty-three resting muscle solutions perfused through fourteen hearts, five solutions gave no effect, six solutions produced an inhibition of cardiac output and twelve gave a mean increase in cardiac output of 8% (Table 2). Thus the substance, or substances, which cause the stimulation of the frog heart does not cause as great an effect when it comes from a resting muscle solution as it does when it comes from a stimulated muscle solution.

It seems that the effect is produced by a twitching muscle rather than by a

resting one.

The Effect of Curarizing the Preparation

In order to exclude the possibility that the stimulatory effect was produced from substances liberated from nerve terminals, the sartorius muscle was curarized and then stimulated in the usual way via the nerve. Thus the nerve terminals remained active while the muscle did not actually contract. When the resultant stimulated, curarized muscle solution was perfused through the heart, the effect produced was the slight stimulatory one associated with the resting muscle solution (compare Figures 11 & 15). Although the curarization procedure did reduce the interference phenomenon, an ACh-like effect was still not apparent.

It was found that the effect produced by the resting muscle solution could be diluted out more easily than the effect from the stimulated muscle solution. Thus the curarization procedure would allow less dilution of any ACh contained in the stimulated muscle solution. Since any test solution obtained in this way would contain curare, it was important to verify that curare had no effect on the sensitivity of the frog heart to ACh.

Figure 12a shows the response of an insensitive frog heart to graded concentrations of ACh. The threshold response occurred when a concentration of 10^{-9} g/ml. ACh was perfused through this heart. The heart was just arrested by a concentration of 2.5×10^{-8} g/ml. ACh. It was found that a concentration of 5×10^{-6} g/ml. d-tubocurarine was enouch to prevent the isolated sartorius muscle from twitching. Figure 12b shows the response of the same heart to ACh in the presence of 5×10^{-6} g/ml. d-tubocurarine. This concentration of curare was present in both the perfusing Ringer's solution and in the solution containing the graded concentrations of ACh. Again the threshold response is at 10^{-9} g/ml. ACh with cardiac arrest occurring when 2.5×10^{-8} g/ml. ACh is perfused through the heart. Figure 13 shows graphs of the concentration - response relationship of the same frog heart to ACh for cardiac output and heart rate. On the part of the curve useful for bioassay of ACh, from 10^{-9} g/ml. to 5×10^{-8} g/ml. on the abscissa,

(a)

(b)

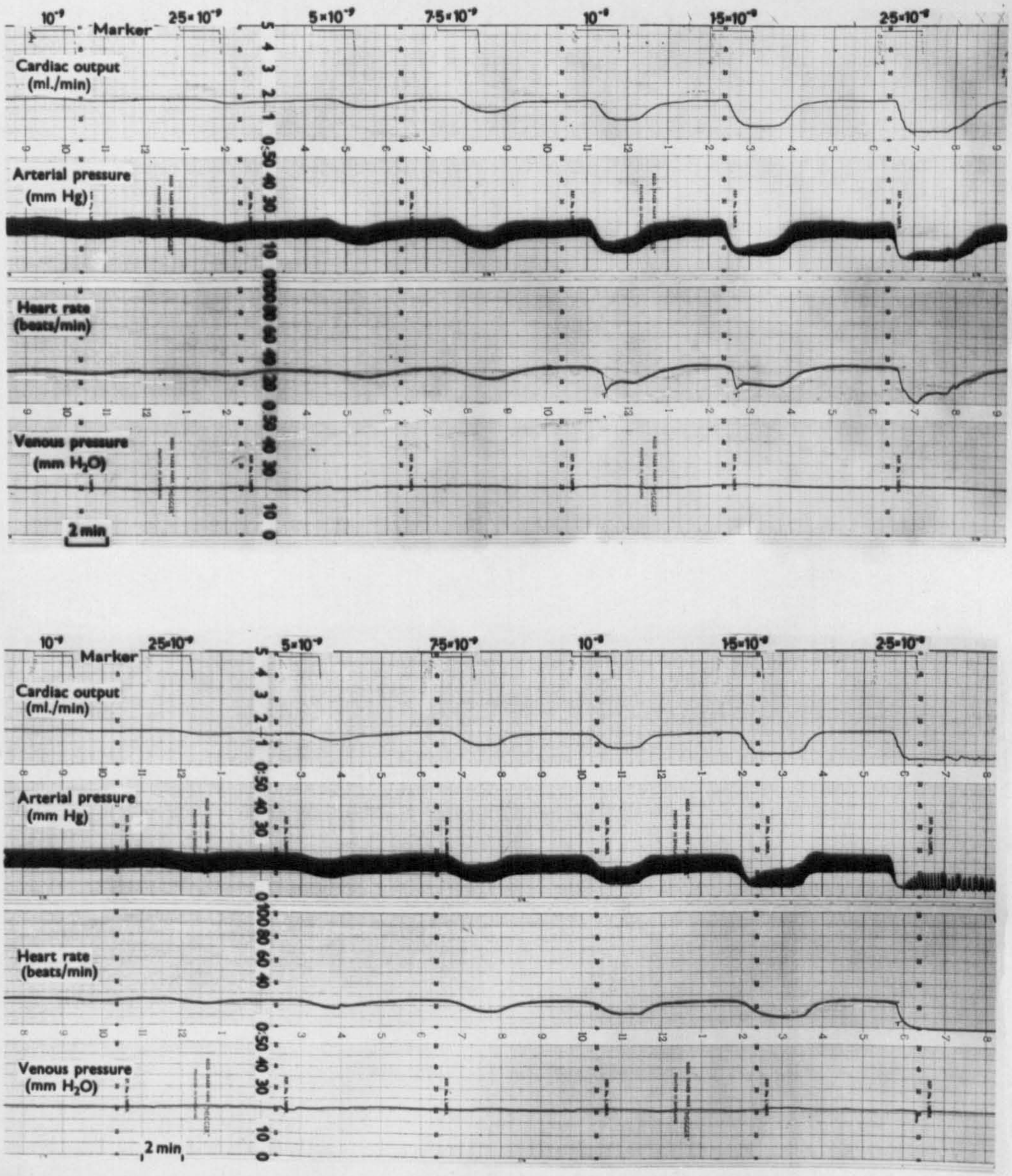


Figure 12.

The response of an insensitive frog heart to graded concentrations of ACh.

(a) Shows the response to graded concentrations of ACh.

(b) Shows the response of the same heart to ACh in the presence of 5×10^{-6} g/ml curare. The response of the frog heart to ACh is unaltered by the presence of curare in the perfusing Ringer's solution.

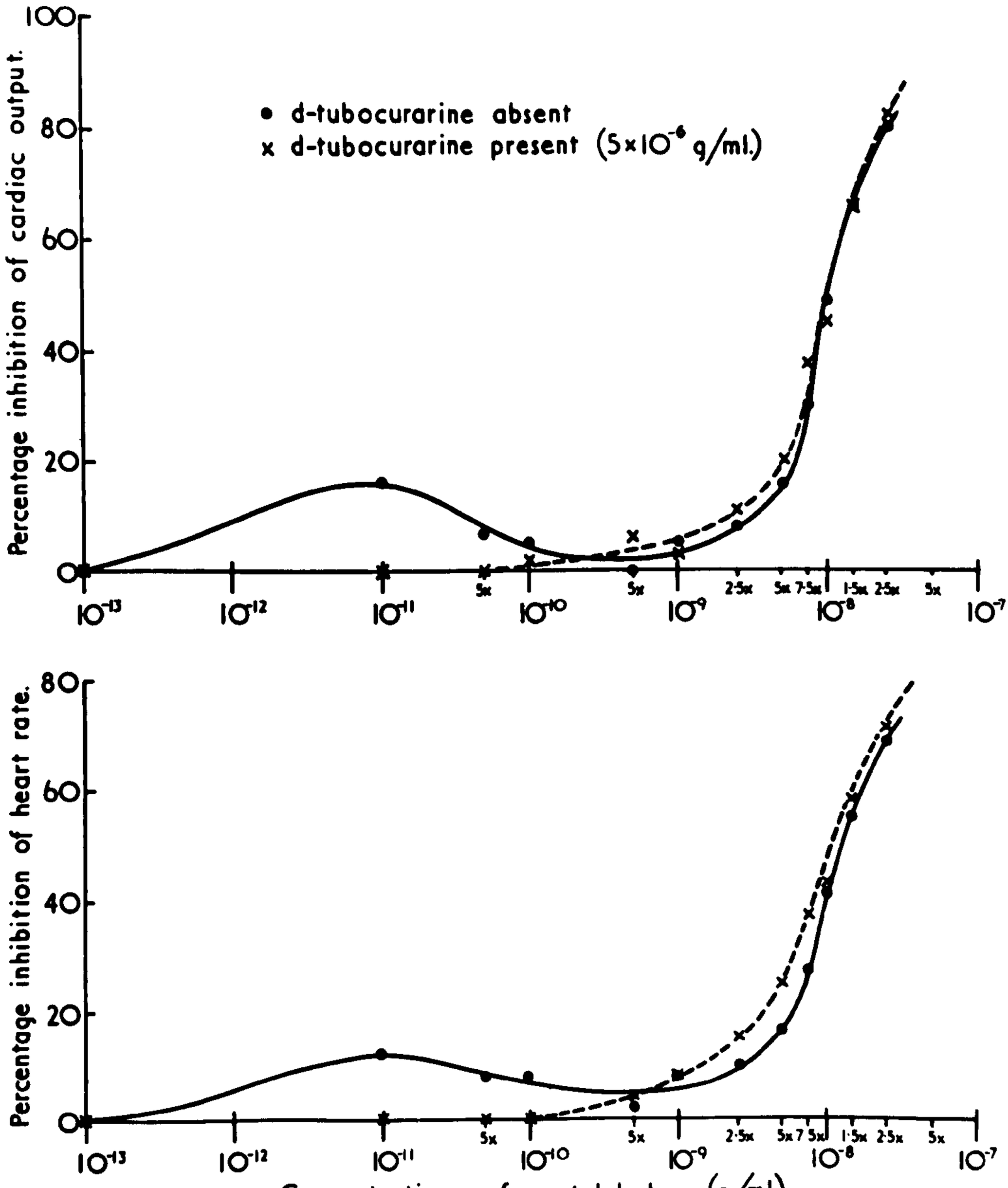


Figure 13.

Graphs of the concentration-response relationship of a frog heart to acetylcholine in the absence and in the presence of curare. There is little difference in the response of the heart to ACh in the presence of curare.

Table 3.

Heart	Before Curare		After Curare	
	Theshold	Arrest	Threshold	Arrest
1.	10^{-9}	2.5×10^{-8}	10^{-9}	2.5×10^{-8}
2.	10^{-9}	5.0×10^{-8}	10^{-9}	5.0×10^{-8}
3.	10^{-9}	--	10^{-9}	2.5×10^{-8}

Concentrations of ACh (g/ml) required to produce threshold responses and cardiac arrest in the absence and presence of curare.

there is little difference in response of the heart to ACh either in cardiac output or in heart rate in the presence of curare. The threshold response of two other hearts was not altered in the presence of this concentration of curare (Table 3).

A PILOT ASSAY ON A VERY SENSITIVE FROG HEART

One experiment was done with an extremely sensitive frog heart in order to find out whether assay of the ACh output from a frog sartorius muscle was possible or not. At a frequency of stimulation of 2/sec for 30 minutes, according to the calculation quoted in the introduction, the theoretical output of ACh from a sartorius muscle should be approximately 10^{-9} g. The muscle was stimulated in a 2 ml. volume, so the concentration of ACh in the surrounding bathing solution should be 5×10^{-10} g/ml.

Result

The response of a very sensitive frog heart to concentrations of ACh in the presence of curare is shown in Figure 14. The threshold response of this heart occurred when a concentration of 2.5×10^{-13} g/ml. ACh was perfused through it. A concentration of 10^{-12} g/ml. ACh stopped the heart. Finely-graded responses are seen between these tests, showing an increasing fall in the heart rate and cardiac output with increasing concentrations of ACh.

The ACh solutions perfused through the heart were made up from Ringer's solution containing 5×10^{-6} g/ml. d-tubocurarine. This does not affect the sensitivity of the heart to ACh (Figure 13).

When a stimulated, curarized muscle solution was perfused through the sensitive frog heart, an effect equivalent to the effect seen when a resting, non-curarized muscle solution was put through the heart was evident. This is shown in Figure 15 (Compare to Figure 11). The solution "Sx" (third test) was obtained from a curarized sartorius muscle which had been stimulated at 2/sec for 30 minutes in 2 ml. of frog Ringer's solution containing 5×10^{-6} g/ml. d-tubocurarine. When this was perfused through the heart it produced an initial slight bradycardia followed by an increase in heart rate and cardiac output, with a rise in systolic and diastolic pressures. When this solution was diluted by half, the slight stimulatory effect disappeared, and an

Figure 14.

The response of a very sensitive frog heart to concentrations of ACh in the presence of curare. A concentration of 2.5×10^{-13} g/ml.ACh produced a threshold response. 10^{-12} g/ml.ACh stopped the heart. All ACh solutions were made up containing a concentration of 5×10^{-6} g/ml. d-tubocurarine.

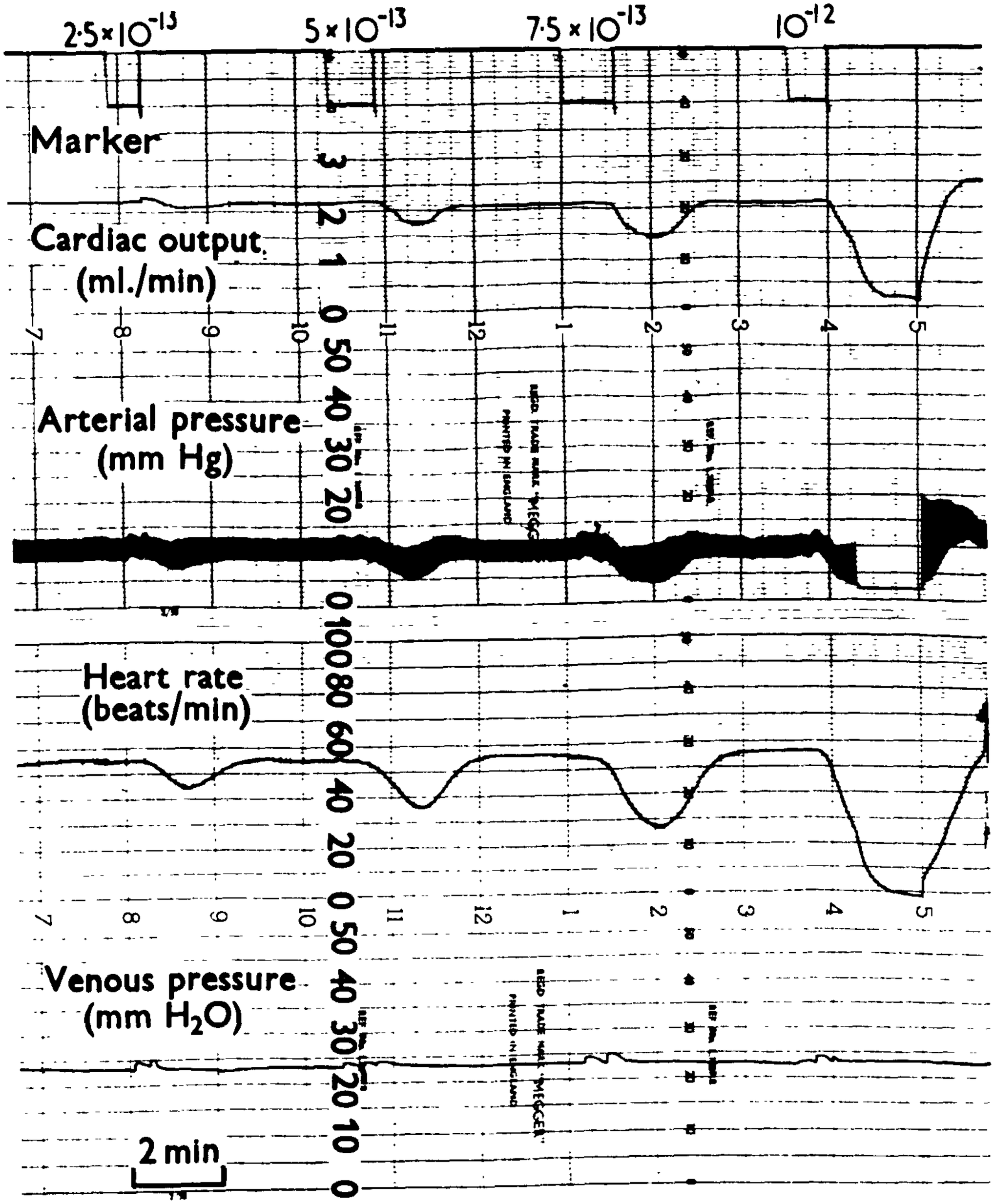
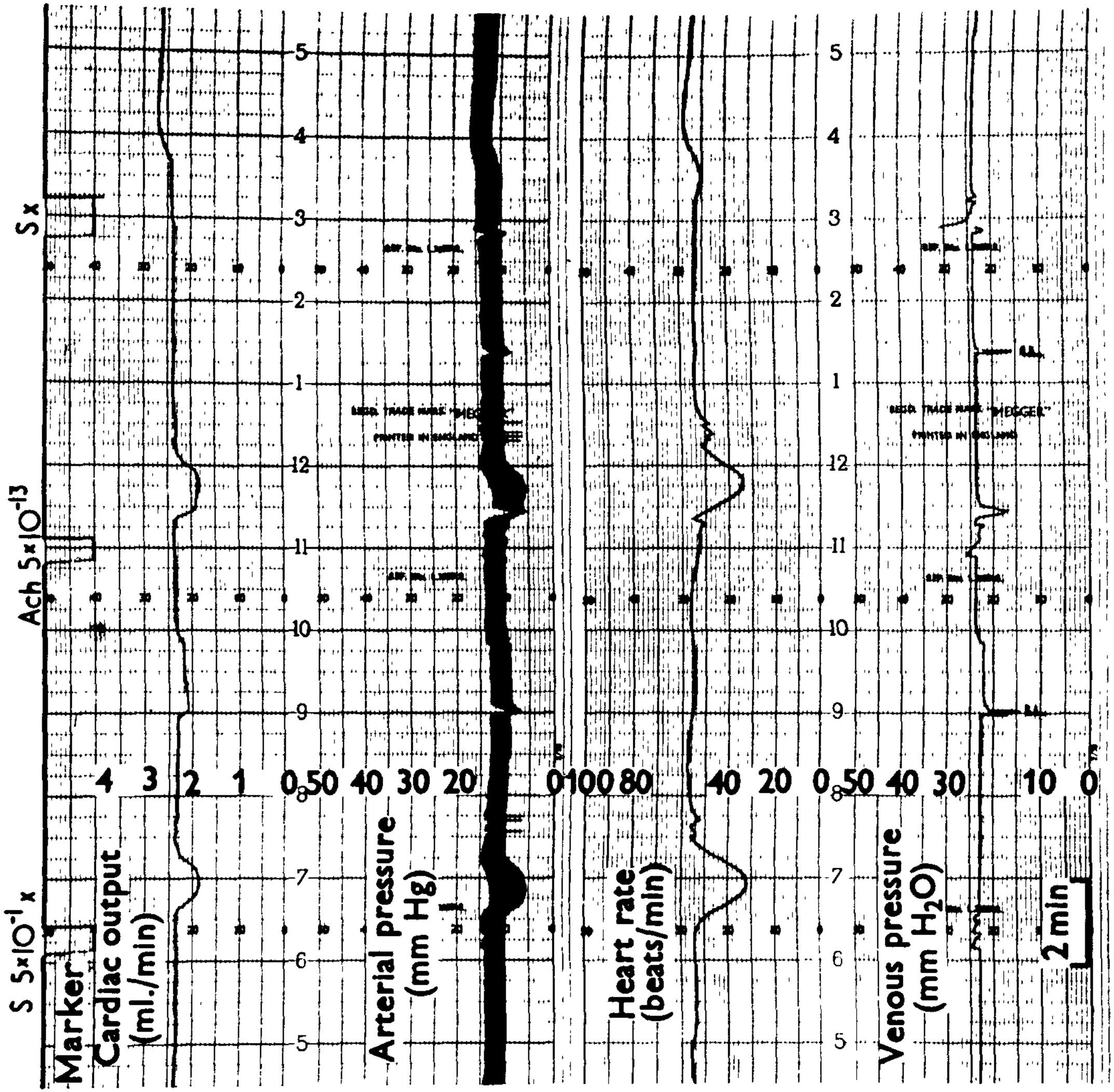

2·5×10⁻¹³
5×10⁻¹³
7·5×10⁻¹³
10⁻¹²
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H₂O)
2 min

Figure 15.

An assay of a stimulated muscle solution on the same frog heart as in Figure 14. "Sx" = solution obtained from a curarized sartorius muscle stimulated indirectly at 2/sec for 30 minutes in 2 ml. Ringer's solution. Note the similarity of the effect of "Sx" to the effect of the resting muscle solution in Figure 11 . "S 5×10^{-1} x" (the solution diluted by half) reveals an ACh-like effect which is matched by a concentration of 5×10^{-13} g/ml. ACh. The sudden fall in the blood pressure which occurred during the effect of this solution was due to a leak in the perfusion system lasting for a few seconds only. Note the drop in the venous, or perfusion at this pressure point also.


Sx
Ach 5×10⁻¹³
S 5×10⁻¹x
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H₂O)
2 min

ACh-like effect was revealed (first test): S 5×10^{-1}. This effect was closely matched to a known concentration of ACh, 5×10^{-13} g/ml. (second test). The stimulated muscle solution when diluted 10 times matched 10^{-13} g/ml. ACh. The standard tests for the identification of ACh were not performed on this solution, partly because of the small volume available and partly because any "manipulation" of the ionic content of such a test solution (e.g. raising the pH to 10, heating the solution to 90°C and restoring to the normal pH and temperature again) might have interfered with the quantitative response of this heart to ACh.

Calculated Output of ACh from the Sartorius Muscle

Test solution diluted by half ≡ 5×10^{-13} g/ml. ACh.

therefore undiluted test solution ≡ 10^{-12} g/ml. ACh.

Muscle was stimulated in 2 ml. volume,

therefore output of ACh from muscle = 2×10^{-12} g.

Test solution diluted 10 times ≡ 10^{-13} g/ml. ACh.

therefore undiluted test solution ≡ 10^{-12} g/ml. ACh.

therefore output of ACh from muscle again = 2×10^{-12} g.

The output per impulse per nerve ending is calculated below:

number of impulses delivered = 3,600 (2/sec for 30 minutes)

amount of ACh released per impulse = 0.6×10^{-15} g.

since there are about 1,000 endplates per muscle,

amount of ACh released/impulse/ending = 0.6×10^{-18} g = 0.5×10^{-19} moles.

This amount is considerably less than that obtained by other workers for mammalian muscle. Krnjevic and Mitchell (1961) calculated an output of 10^{-17} mole/impulse/ending and Krnjevic and Straughan (1963) calulated an output of 10^{-18} mole/impulse/ending using rat diaphragm muscle.

AN ATTEMPTED ASSAY OF ACh RELEASED FROM RAT DIAPHRAGM ON THE PERFUSED FROG HEART

Since the output of ACh had been estimated by other workers from the rat hemidiaphragm preparation (Bulbring, 1946) the author was curious to know whether a solution which had surrounded a twitching hemidiaphragm could produce the same interference phenomenon on the frog heart as the solution obtained from the twitching sartorius muscle.

Result

When a frog heart is perfused with Locke's mammalian solution, its sensitivity to ACh is little altered. Figure 16 shows the comparison of concentration-response curves of a frog heart to ACh for cardiac output and heart rate when it was perfused with frog Ringer's solution and Locke's mammalian solution.

A rat hemidiaphragm was stimulated via the phrenic nerve at twenty impulses per second for twenty minutes in 4 ml of Locke's solution and the resulting solution was injected into a frog heart which was being continuously perfused with Locke's solution. No evidence of an ACh-like effect is seen, even although the hemidiaphragm muscle is expected to release 3.5×10^{-8} g ACh (Krnjevic and Mitchell, 1961). Instead the test solution caused an increase in the force of contraction of the heart with a concomitant bradycardia and a late increase in the heart rate. Since the threshold response of this heart to ACh was 10^{-9} g/ml, and the concentration of ACh in the 4 ml test solution was expected to be 10^{-9} g/ml, clearly an interference phenomenon similar to that encountered with the frog sartorius muscle solutions was taking place (Figure 17) .

Figure 16.

The response of a frog heart to graded concentrations of ACh when perfused with frog and mammalian Ringer's solutions. The upper figure shows the comparison of the percentage inhibition of cardiac output caused by various concentrations of ACh during perfusion of the heart with frog Ringer's solution and Locke's mammalian solution. The lower figure shows the comparison of the percentage inhibition of heart rate. The sensitivity of the heart to ACh is virtually unaffected by perfusion with Locke's mammalian solution.

+ frog ringer
• mammalian ringer

Percentage inhibition of cardiac output.

100
80
60
40
20
0

10^{-10} 10^{-9} 2·5x 5x 7·5x 10^{-8} 1·5x 2·5x 5x 7·5x 10^{-7}

Percentage inhibition of heart rate.

100
80
60
40
20
0

10^{-10} 10^{-9} 2·5x 5x 7·5x 10^{-8} 1·5x 2·5x 5x 7·5x 10^{-7}

Concentration of acetylcholine (g/ml.)

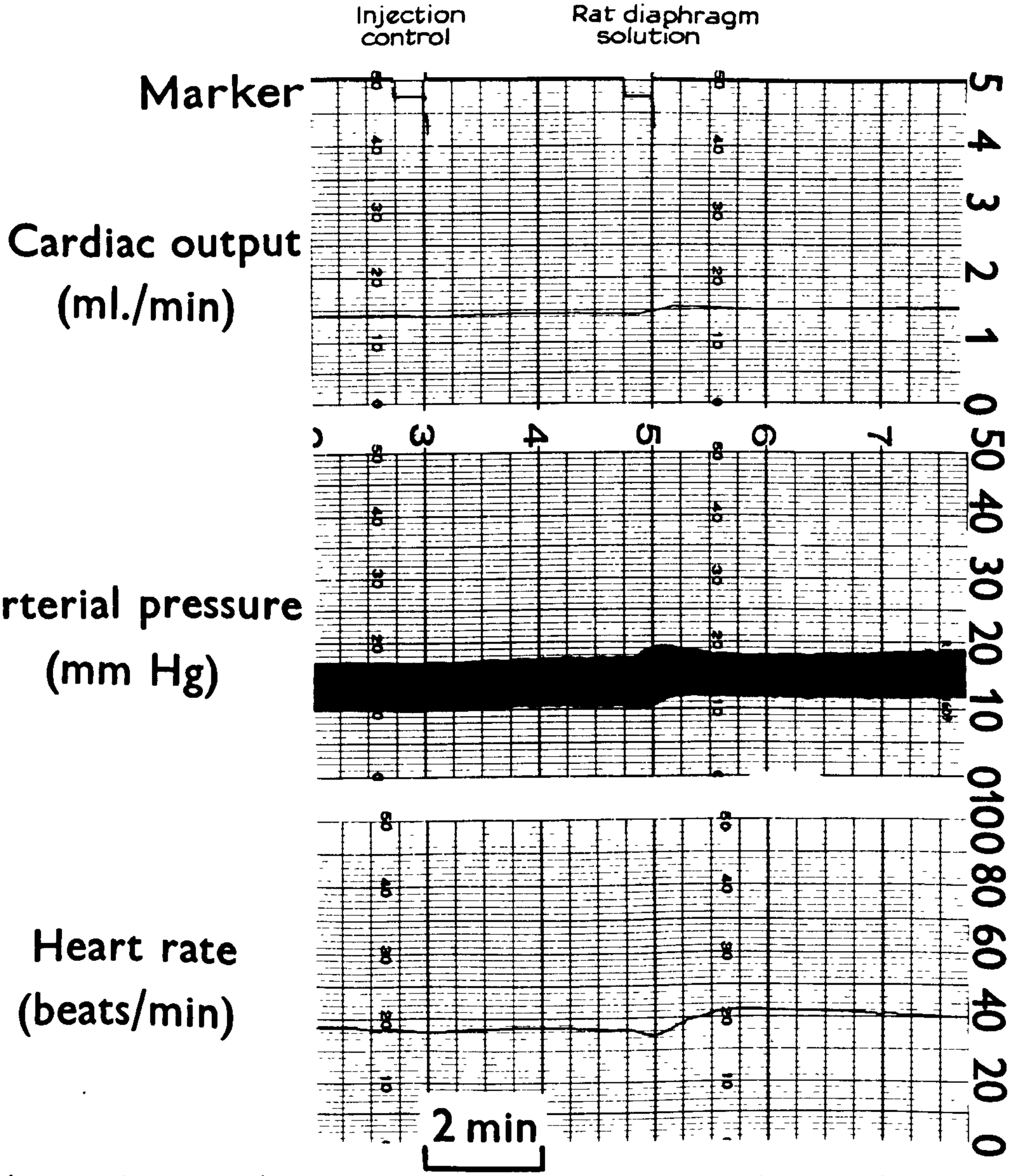


Figure 17.

The result of perfusing a solution in which a rat hemidiaphragm has twitched through a frog heart.

The frog heart is continuously perfused with Locke's mammalian solution. This does not affect the sensitivity of the heart to acetylcholine. The rat hemidiaphragm was stimulated indirectly in 4 ml. Locke's mammalian solution and the resulting solution was perfused through the frog heart.

After a satisfactory control injection, the test solution is seen to cause an increase in the force of contraction of the heart with a concomitant bradycardia and a late rate increase. No evidence of an acetylcholine effect is seen.

An interference phenomenon similar to that encountered with the frog sartorius muscle makes the direct assay of acetylcholine from diaphragm muscle on frog heart impossible.

DISCUSSION

In order to measure the output of ACh from the frog sartorius muscle using the present method, an exceedingly sensitive and stable heart preparation is required. Obviously the sartorius muscle liberates more than ACh into the surrounding bathing solution. In order to detect the ACh present, the frog heart must be sensitive enough to respond to the concentration of ACh in the test solution after the interfering substances have been diluted to an ineffective concentration.

However, in the presence of a depressant substance (ACh) it can never be wholly guaranteed that a stimulatory substance has had its effect completely abolished by dilution ; the only satisfactory way to assay the ACh without this interference would be to elucidate the cause of the stimulation and specifically remove it.

The interference phenomenon of heart stimulation in contrast to the heart inhibition produced by ACh may be the factor responsible for producing such a low result for the output of ACh in comparison to the results of other workers. The result obtained with the rat hemidiaphragm muscle also suggests that a falsely low result for ACh output would be obtained using the frog heart preparation for the assay.

The nature of this particular interference phenomenon is an interesting one. Why should a "metabolic product" liberated from contracting muscle be capable of stimulating the frog heart? All known compounds classified as "metabolites" such as carbon dioxide, pyruvic acid, hydrogen ions, carbonic acid, adenylic acid etc., would be expected to have either no effect or perhaps a depressant effect on the frog heart.

The following section of this thesis describes investigation into the cause of the stimulatory effect, with a view to removing it, thus allowing assay of the ACh released from sartorius muscle to procede without this interference.

PART I

THE IDENTIFICATION OF ADENOSINE TRIPHOSPHATE RELEASED FROM ACTIVE SKELETAL MUSCLE.

INTRODUCTION

When a solution in which a frog sartorius muscle has twitched is perfused through a frog heart, a pronounced augmentation of the heart beat occurs (Figure 10). This section describes the identification of the substance released from active sartorius muscle which was the cause of the heart stimulation.

At the onset of this investigation it appeared that any alteration in the composition of the solution bathing the active sartorius muscle might be responsible for the phenomenon, for example, a change in the hydrogen ion concentration of the solution. Another obvious change in the surrounding bathing solution would be an increase in the concentration of potassium resulting from the efflux of intracellular K^+ from skeletal muscle during the active state. Whether excessive K^+ in the test solution would, in fact, stimulate the frog heart is doubtful, but the possibility had to be excluded.

The frog heart is known to be very sensitive to changes in the Ca^{++} concentration of perfusing Ringer's solutions and MacLean and Hastings (1934) utilized this sensitivity in order to determine the levels of ionized Ca^{++} in the body fluids. They could detect differences in Ca^{++} concentration of 0.1 mM by this method. It was not known whether any change in Ca^{++} concentration would occur in the test solution after stimulation of the sartorius muscle, or, if such a change took place, whether it would cause a stimulatory effect.

Many biologically active substances can become protein-bound if they do not already exist in this form. Also, chemical radicals can become attached to proteins of the simplest nature by "high-energy" bonds. Examples of this have been shown in the investigations of Boyer et al. (1962). They found that the histidine component in a peptide chain could adopt the phosphate radical by way of a high-energy bond similar to those associated with purine or pyrimidine nucleotides. An investigation into the protein content, if any, of the stimulated muscle solution was thus necessary as a preliminary "screening" procedure.

A class of biological compounds which would obviously stimulate the heart are the catecholamines; however, it is unlikely that the adrenergic supply to the blood vessels contained in a single sartorius muscle could release quantities of catecholamines necessary to cause the typical stimulatory effect seen on the frog heart, nevertheless it was necessary to eliminate this possibility.

Other substances which could possibly be liberated from active skeletal muscle into a surrounding bathing solution, such as carnosine, 5-hydroxytryptamine, histamine and plasma contents such as bilirubin were excluded as candidates for causing stimulation of the frog heart by experiments not reported here and in previous work (Pathak, 1962).

Specific investigations into pH changes, potassium changes, calcium changes, protein content and catecholamine content of the test solution now follows.

Changes in Hydrogen Ion Concentration of Fluid Surrounding an Active Sartorius Muscle

In this work the pH of 22 stimulated muscle solutions was measured and the range was found to be 7.2 - 7.9. It has previously been established that alterations in the pH of the perfusing Ringer's solution within this range do not have any direct effect on the frog heart preparation (Boyd and Pathak, 1965).

THE OUTPUT OF POTASSIUM FROM ACTIVE SKELETAL MUSCLE

Ten sartorius muscles were soaked in 2 ml. volumes of Ringer's solution with varying concentrations of K^+. The K^+ concentration did not alter significantly after a 30 minute period of soaking (Figure 18).

Seventeen sartorius muscles were stimulated at 2 impulses per second in 2 ml. volumes of Ringer's solution and the concentration of K^+ in each solution was measured before and after the period of stimulation. An average increase in K^+ concentration in the surrounding bathing solution of 21% was found (Figure 18).

Figure 19 shows the effect of perfusing a frog heart with frog Ringer's solution containing 2.9 mM K^+, a stimulated muscle solution which contained 3.6 mM K^+ and a frog Ringer's solution which contained 3.7 mM K^+. It is clearly seen that the high K^+ Ringer's solution does not stimulate the heart, whilst the test solution, containing a low concentration of K^+, gives a sharp increase of the cardiac output with a concomitant bradycardia. Five other frog hearts were perfused with samples of Ringer's solution containing up to twice the normal amount of K^+ and in no case did a stimulatory effect occur similar to that caused by the test solution.

It is concluded from these experiments that the stimulatory effect is not due to the potassium efflux from active skeletal muscle.

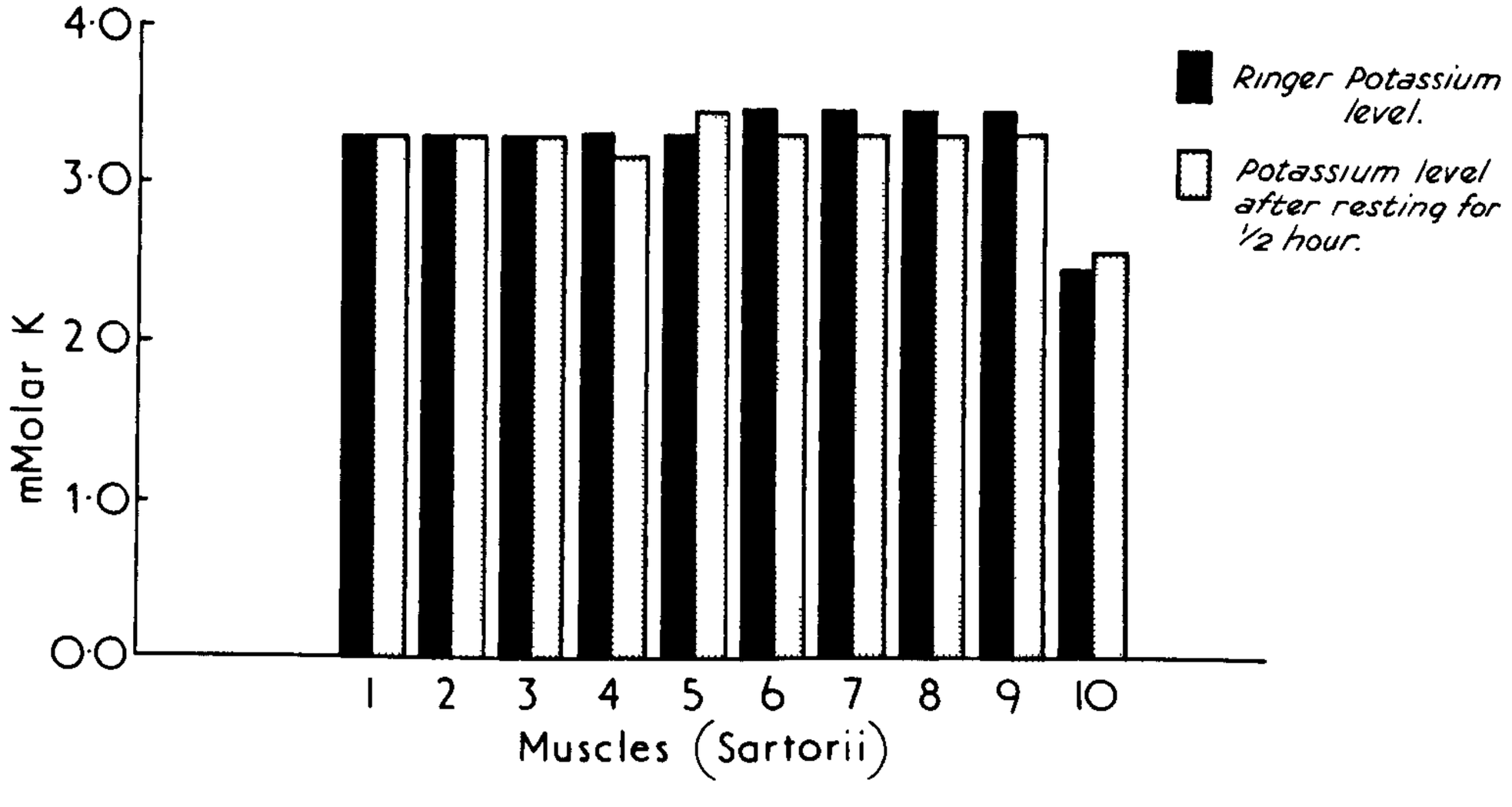
Figure 18.

(a) The potassium concentrations of Ringer's solutions in which frog sartorius muscles were allowed to rest for 30 minutes. Black columns indicate the potassium concentration of the solutions before soaking. The grey columns show the K^+ concentration after soaking the muscle. No change in the K^+ concentration occurs. No difference occurs in the K^+ concentration of the solution after a sartorius muscle rests for 30 minutes.

(b) The K^+ concentrations of Ringer's solutions in which frog sartorius muscles have twitched for 30 minutes. The black column indicates the K^+ concentration of various Ringer's solutions. The grey columns indicate the K^+ concentration after stimulation of the sartorius muscle.

There is an average increase of 21% in the K^+ concentration in 17 experiments.

a)


Ringer Potassium level.
Potassium level after resting for ½ hour.
mMolar K
4·0
3·0
2·0
1·0
0·0
1 2 3 4 5 6 7 8 9 10
Muscles (Sartorii)

b)

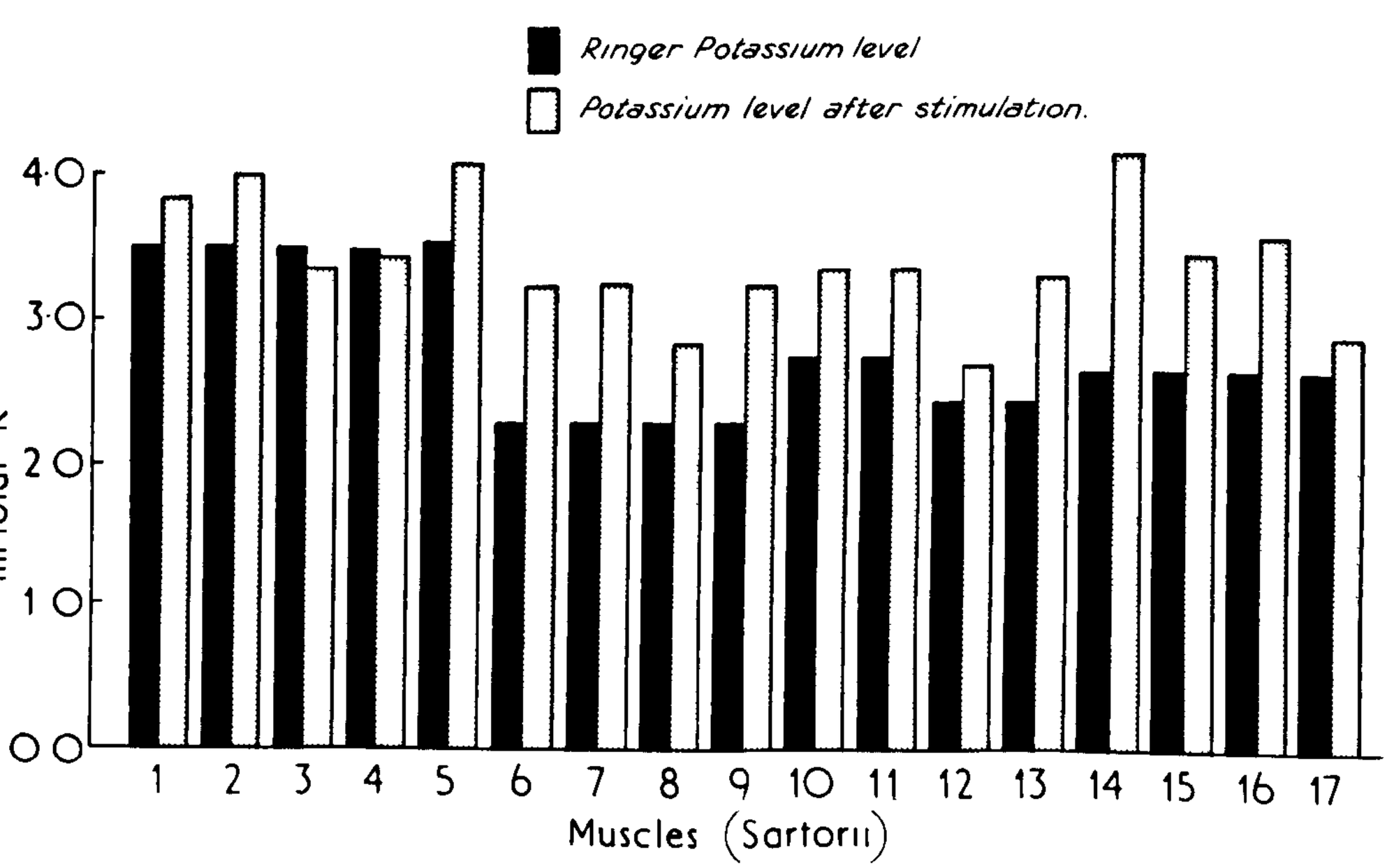
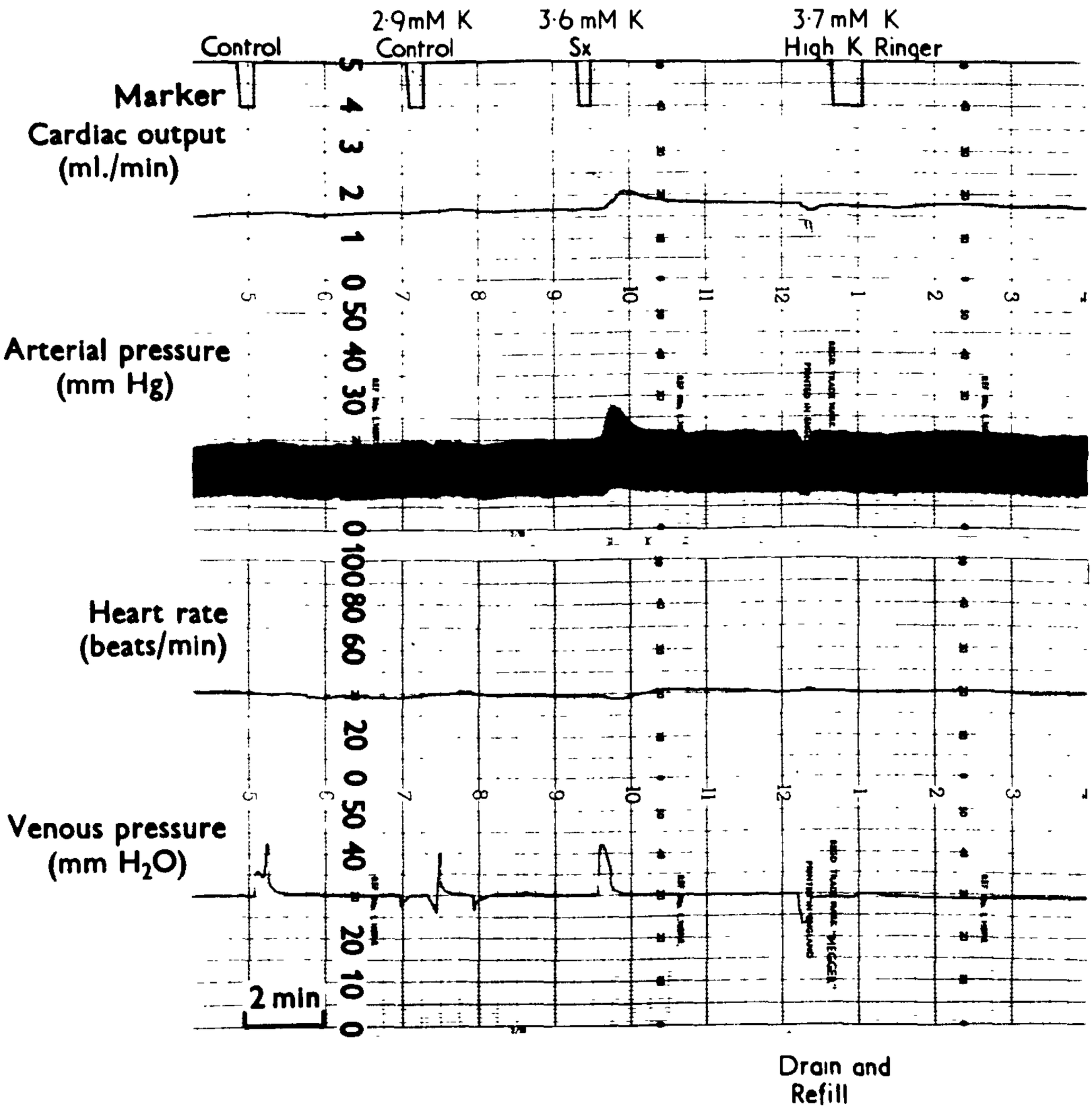

Ringer Potassium level
Potassium level after stimulation.
mMolar K
4·0
3·0
2·0
1·0
0·0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Muscles (Sartorii)

Figure 19.

The effect of perfusing a frog heart with a Ringer's solution containing a high concentration of K^+ and a stimulated muscle solution. The Ringer's solution continually perfusing the heart contained 2.9 mM K^+. When the stimulated muscle solution, "Sx" is perfused through the heart the stimulatory effect is clearly seen. This particular solution had a K^+ concentration of 3.6 mM. When a Ringer's solution containing 3.7 mM K^+ was perfused through the same heart, no effect equivalent to the stimulatory effect was seen. "Drain and refill" indicates flushing of venous capsule in order to wash out any remaining test solution.


Control
2·9mM K
Control
3·6 mM K
Sx
3·7 mM K
High K Ringer
Marker
Cardiac output
(ml./min)
Arterial pressure
(mm Hg)
Heart rate
(beats/min)
Venous pressure
(mm H_2O)
2 min
Drain and
Refill

CHANGES IN CALCIUM CONCENTRATION IN THE SOLUTION SURROUNDING THE ACTIVE SARTORIUS MUSCLE

Considerable data has accumulated from studies on calcium entering skeletal muscle fibres during their contraction. Fenn, Cobb, Manery and Bloor (1938) showed that there is no change in the calcium content of muscle after stimulation. Bianchi and Shanes (1959), however, indicated that although there may be no net alternation in intracellular calcium content, both influx and efflux take place simultaneously during contraction. They also suggested that perhaps the influx of calcium is so small that it cannot be measured accurately, nevertheless they thought it large enough to initiate contraction. According to this postulate, it is conceivable that a significant amount of Ca^{++} could be taken up by an active muscle, eventually depleting the bathing solution of Ca^{++}.

Results

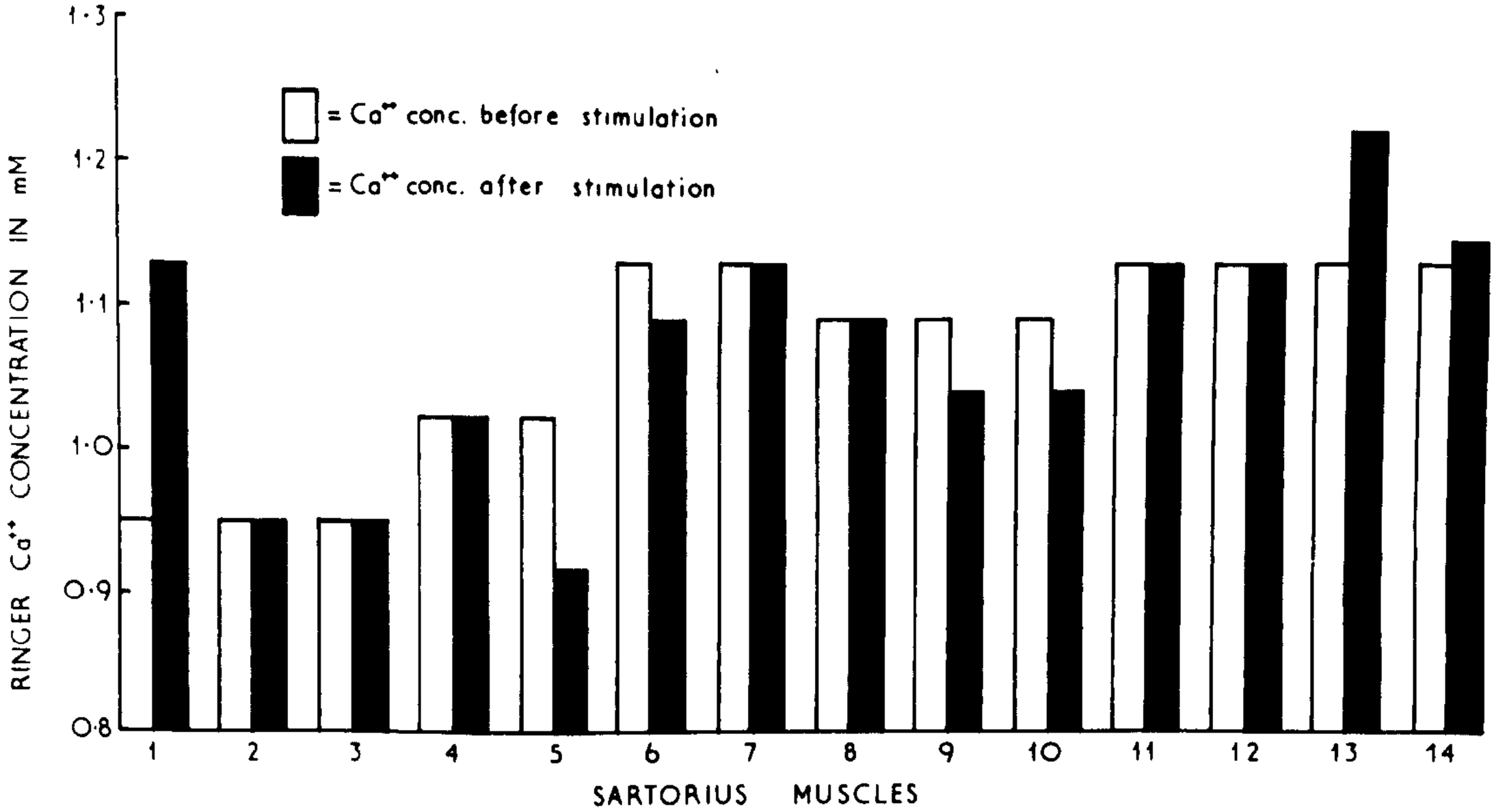
Fourteen experiments were performed where the Ca^{++} concentration of a stimulated muscle solution (2 ml) was measured before and after the period of stimulation (2/sec for 30 minutes). Seven muscles out of the fourteen showed no difference in Ca^{++} concentration after stimulation. The mean concentration of Ca^{++} in Ringer's solutions before stimulation was 1.08 mM; the mean concentration of Ca^{++} in the same solutions after stimulation was also 1.08 mM (Figure 20a).

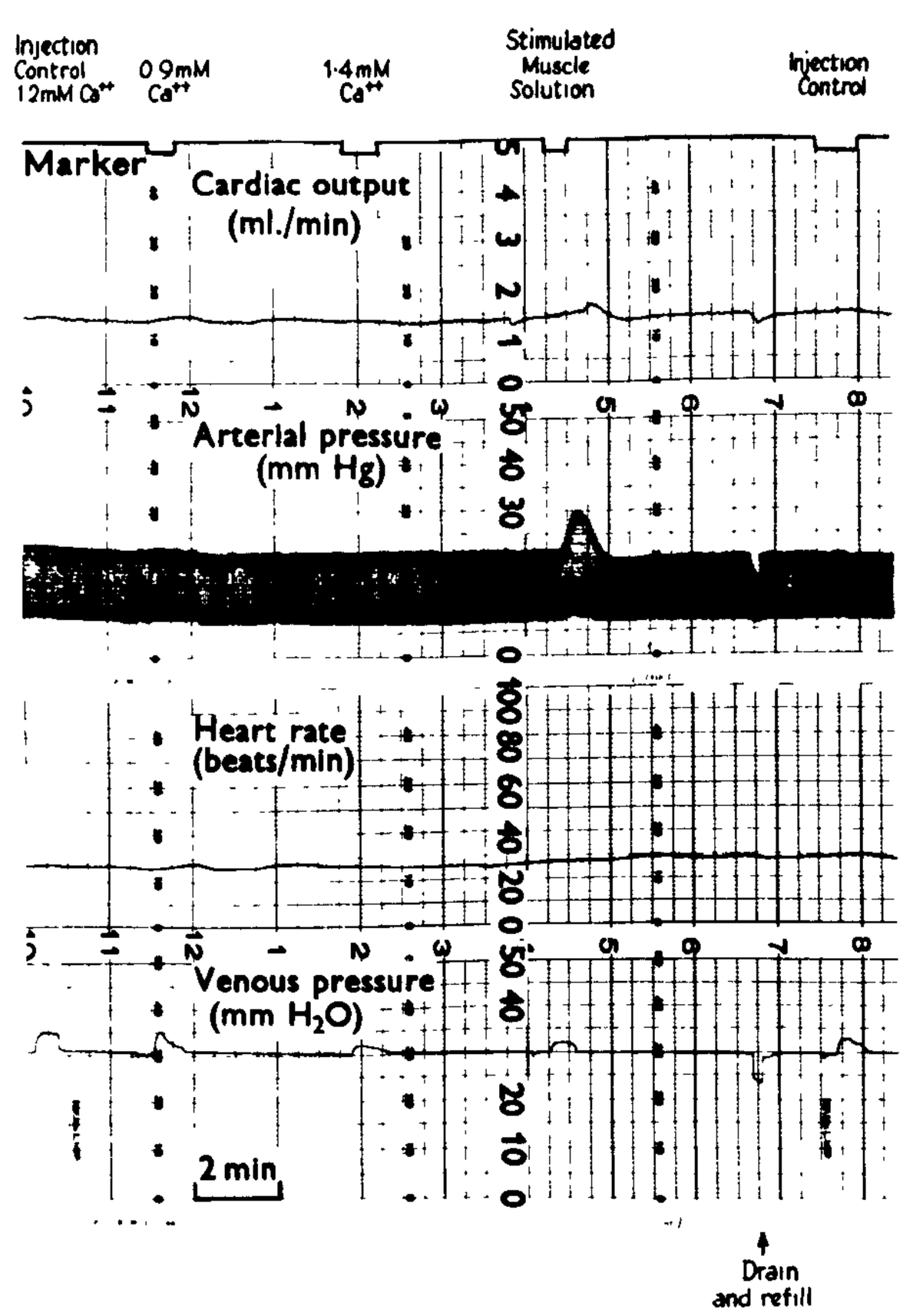
The range of Ca^{++} millimolarity in the solutions after stimulation was 0.92 mM - 1.22 mM. The effects of perfusing Ringer's solutions containing 0.9 mM Ca^{++} and 1.4 mM Ca^{++} through the frog heart are shown in Figure 20b An injection control solution containing 1.2 mM Ca^{++} has no effect. The solution containing 0.9 mM Ca^{++} caused a very slight initial increase in cardiac output and rate, but this was followed by a depression of output and rate. The Ringer's solution containing 1.4 mM Ca^{++} did not have any effect on this heart. A stimulated muscle solution perfused through the heart 4 minutes later gave a typical sharp rise in cardiac output, systolic, diastolic and pulse pressures. This is followed by a rise in rate 2 minutes later. An injection control after flushing

Figure 20.

a) Measurement of the Ca^{++} concentrations of Ringer's solutions in which sartorius muscles were stimulated indirectly.. White columns indicate the Ca^{++} concentrations in solutions before muscle stimulation. Black columns indicate the Ca^{++} concentrations of the bathing solutions after muscle stimulation. Range of concentrations of Ca^{++} after stimulation were 0.92 mM - 1.22 mM.

b) Comparison of the effect of perfusing the Ringer's solutions of 0.9 mM and 1.4 mM Ca^{++} through a frog heart with that of a stimulated muscle solution. No change in blood pressure is produced by the low Ca^{++} solutions. A small increase in the heart rate is produced with the solution containing 0.9 mM Ca^{++}. However no effect on the force of concentration comparable to the effects of a stimulated muscle solution is produced by either the high or low Ca^{++} solutions.


= Ca++ conc. before stimulation
= Ca++ conc. after stimulation
RINGER Ca++ CONCENTRATION IN mM
1·3
1·2
1·1
1·0
0·9
0·8
1
2
3
4
5
6
7
8
9
10
11
12
13
14
SARTORIUS MUSCLES


Injection Control 12mM Ca++
0·9mM Ca++
1·4mM Ca++
Stimulated Muscle Solution
Injection Control
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
Drain and refill

out the venous capsule was satisfactory. The effect of perfusing Ringer's solutions containing differing Ca^{++} concentrations through three frog hearts is shown in Table 4.

The results of this small series can do no more than suggest that, within the sensitivity of the method for measuring Ca^{++} concentration, there is no net loss of Ca^{++} from the bathing Ringer's solution when a skeletal muscle is allowed to contract in it. However, as a cause of the stimulatory effect change of calcium concentration in the Ringer's solution surrounding the sartorius muscle can now be discounted.

Table 4.

Heart	Ca^{++} Conc. in Ringer's solution	% Change in Output	% Change in Rate
1	0.9 mM		- 2%
	1.3 mM	- 8%	- 4%
2	0.9 mM	- 24%	- 1%
	1.3 mM	- 8%	- 2%
3	1.3 mM	- 5%	- 6%

In each case the heart was slowed and the cardiac output tended to fall. Each heart was being continuously perfused with Ringer's solution containing 1.2 mM Ca^{++}.

THE LIBERATION OF PROTEIN FROM ACTIVE SKELETAL MUSCLE

As part of the investigation of the stimulatory effect, the stimulated muscle solution was tested for its protein content. As the sensitive Lowry test for protein was strongly positive, it was decided to study further the liberation of protein from resting and active sartorius muscles; protein itself, or perhaps some protein-linked substance could be the source of the stimulatory effect.

Results

Protein liberation was measured from resting and stimulated muscle every $\frac{1}{2}$ hour over a period of 2 hours. In the resting series there was a decrease in the liberation from 47µg/ml. in the first $\frac{1}{2}$ hour to 16µg/ml. in the fourth $\frac{1}{2}$ hour (Figure 21). In the stimulated series there was a more noticeable decrease in efflux from 72µg/ml. in the first $\frac{1}{2}$ hour to 20µg/ml. in the fourth $\frac{1}{2}$ hour (Figure 21).

A series of measurements of protein liberation was also done where the sartorius muscles were placed in 1ml. Ringer's solution and the container shaken for $\frac{1}{2}$ hour (Table 5). The mean protein liberation from these "agitated" muscles was 90µg/ml.

Protein liberation was also measured every $\frac{1}{2}$ hour from agitated muscles for 2 hours. The protein levels in the solutions became very high, probably indicating that the muscle was macerating.

Subsequently it was possible to separate out the total protein content of the stimulated muscle solution into one fraction using the Sephadex chromatography procedure (Methods). Since the type of column used (G-25) is able to differentiate molecules of molecular weight of 5,000 and over from smaller molecules, the protein which is liberated from the muscle cannot have peptide chains of a molecular weight of less than 5,000; most probably the protein molecules liberated have a much greater molecular weight.

When the column fraction containing the protein was perfused through the frog heart, no augmentation of the heart beat occurred. The fractions from the

column suceeding the protein-containing fraction did not contain any protein when tested by the Lowry technique.

It is clear that the stimulatory effect is not due to protein. It is unlikely that the substance is protein bound, although it is conceivable that it is separated from a protein molecule during the passage of the solution through the Sephadex column.

TABLE 5.

Protein liberation from resting, active and "agitated" sartorius muscles (μg/ml). Sartorius muscles rested in 2ml. Ringer's solution for ½ hour or were stimulated at 2/sec for 30 minutes.

Resting Muscles	Stimulated Muscles	"Agitated" Muscles
50	136	122
91	167	108
78	160	111
50	40	57
85	115	119
67	138	104
50	103	67
60	160	69
68	181	92
78	150	97
59	120	79
74	60	69
90	41	92
67	97	97
58	47	79
57	58	
52		Mean = 90μg/ml
55	Mean = 111	population = 15
28	population = 16	range = 57 - 122
43	range = 40 - 181	
Mean = 63		
population = 20		
range = 28 - 91		

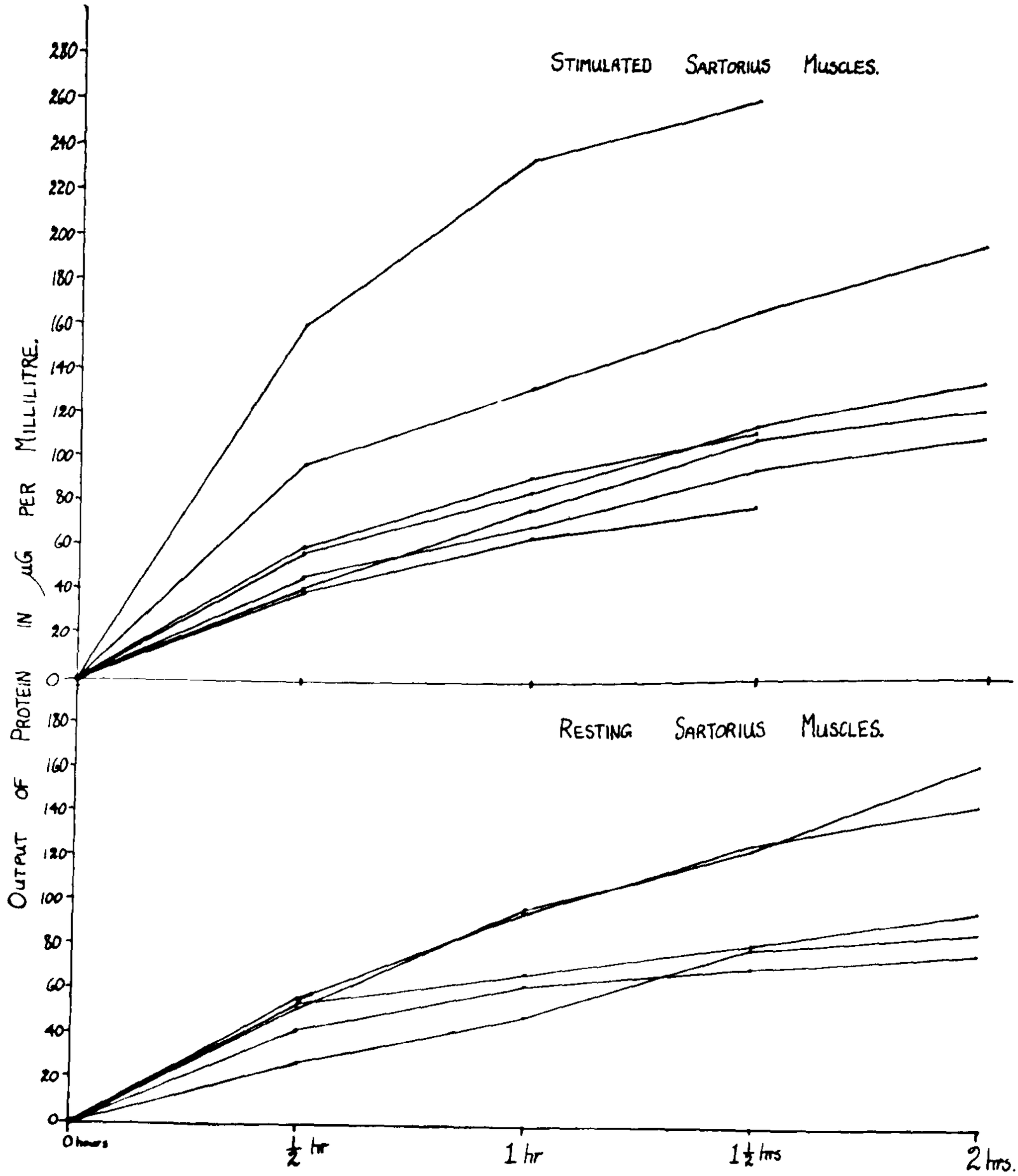
Figure 21. The rate of protein liberation from frog sartorius muscle.

a) Upper diagram shows the amount of protein liberated into a 2 ml. volume from seven stimulated sartorius muscles over a period of two hours. The muscles were twitching at a rate of 2/sec.

b) Lower diagram shows the protein liberation from five sartorius muscles resting in bathing solutions for two hours.

More protein is liberated from active muscles.

a)


Stimulated Sartorius Muscles.
Resting Sartorius Muscles.
Output of Protein in µG per Millilitre.
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0 hours
½ hr
1 hr
1½ hrs
2 hrs.

b)

THE ACTION OF CATECHOLAMINES ON THE FROG HEART

The stimulated muscle solution was obtained by twitching a sartorius muscle in a bathing solution by stimulation of the whole sciatic nerve bundle. Under the supramaximal conditions of stimulation, all the nerve fibres in this large nerve bundle are probably stimulated. This of course includes the efferent post-ganglionic sympathetic nerve supply to the blood vessels of the lower limb, including the blood vessels of the sartorius muscle. These fibres are adrenergic and their stimulation might result in the release of small amounts of catecholamines into the Ringer's solution surrounding the sartorius muscle.

It has long been known that both adrenaline and noradrenaline cause an increase in the force of contraction of the myocardium (Oliver and Schafer, 1895). This is the case when these substances are perfused through the frog heart preparation (Figures 22, 23 and 24).

Results

Using the adrenergic blocking drugs pronethalol and ergot, the following experiment was performed. A frog heart was continuously perfused with Ringer's solution containing 10^{-6} g/ml. pronethalol and 10^{-6} g/ml. ergotamine tartrate (P/E Ringer's solution) in order to block both alpha and beta adrenergic receptors which may both be present in the frog heart (Ahlquist, 1948). The action of the heart was completely unaffected by this procedure. After perfusion of the heart with this Ringer's solution had lasted for an hour or longer, adrenaline (or noradrenaline) was then perfused into the heart. It was found that the action of the catecholamines was blocked by the blocking agents; however, when the stimulated muscle solution was perfused through the same frog heart a few minutes later, its effect on the heart was unaltered.

Figure 22 shows the effect of perfusing a frog heart with 10^{-6} g/ml. adrenaline. The effect is obviously a prolonged stimulatory one; the cardiac

output, blood pressure and heart rate were all raised for 6 minutes after the perfusion of the adrenaline into the heart. After perfusing the heart with P/E Ringer's solution for 1 hour, the effect of 10^{-6} g/ml. adrenaline was almost completely blocked.

Figure 23 shows the effect of perfusing a stimulated muscle solution through the same frog heart as that shown in Figure 22 . There is a sharp increase in the cardiac output and blood pressure. A rise in heart rate is also seen. However, these changes are short-lived, in contrast to the action of the adrenaline on the same frog heart. After perfusion of the heart with P/E Ringer's solution for 2 hours, the effect of the stimulated muscle solution is still apparent. In this experiment three tests were made from one stimulated muscle solution of 2 ml. volume. The test made on this heart after the adrenergic blocking seems to have a smaller effect than those effects previously produced, but this is because a much smaller amount of the test solution was available for perfusion and can be gauged by comparing the lengths of the respective time marker traces, 30 seconds for solutions before the blocking, 15 seconds for the solution after the blocking.

Figure 24 shows the response of a frog heart to a solution of noradrenaline and a stimulated muscle solution before and after adrenergic blockade. The stimulated muscle solution produced a sharp rise and fall of the cardiac output and blood pressure; there was no obvious rise in heart rate. Again this is in contrast to the effects of perfusion with noradrenaline. 10^{-7} g/ml. noradrenaline produced a similar increase in cardiac output to that of the stimulated muscle solution, but this increase was more prolonged. A slight increase in heart rate was produced by both. The effect of perfusing the heart with 10^{-6} g/ml. noradrenaline was profound. An exceedingly large and rapid increase in the blood pressure and cardiac output was accompanied at the same time by a rate increase which had two phases; this effect persisted for 7 minutes. The rapid increase in cardiac output was enough to overcome the drop-counting system of recording.

After perfusion of the heart for 2 hours with P/E Ringer's solution, the effect

Figure 22.

The action of 10^{-6} g/ml. adrenaline on a frog heart before and after the heart was perfused with adrenergic blocking agents.

The vertical dotted line indicates the time during which the heart was perfused for one hour with Ringer's solution containing 10^{-6} g/ml. proethalol and 10^{-6} g/ml. ergotamine tartrate. (P/E Ringer's soltuion). Perfusion of the same concentration of adrenaline after this procedure shows that its action affect had been almost completely blocked.

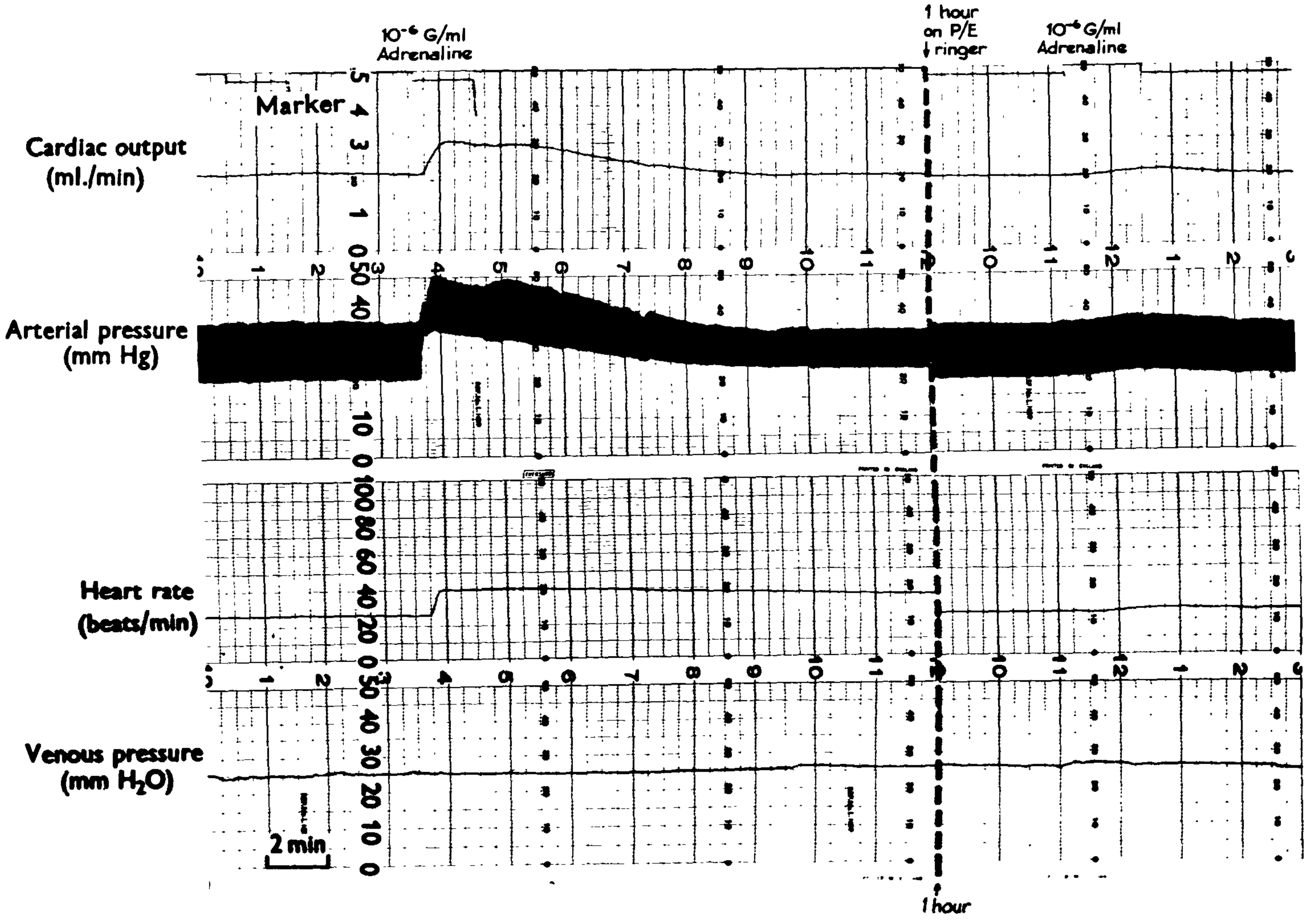

10⁻⁶ G/ml Adrenaline
1 hour on P/E ↓ ringer
10⁻⁶ G/ml Adrenaline
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
1 hour

Figure 23.

The action of a stimulated muscl e solution on the same frog heart as in Figure 22.

The effect of a stimulated muscle solution is seen at the left of the trace. Note the sharp, initial increase in the cardiac output and blood pressure. A rise in the heart rate is also seen. However these changes are short-lived in contrast to the action of the adrenaline on the same heart (Figure 20).

A repeat perfusion of the test solution was done because of the excessively high perfusion pressure during the injection of the first sample. The vertical dotted line indicates the time during which the heart was perfused for two hours with P/E Ringer's solution.

This did not block the action of the test solution. Note that the duration of the third stimulated muscle solution injected is 15 seconds, while the duration of the previous one was 30 seconds.

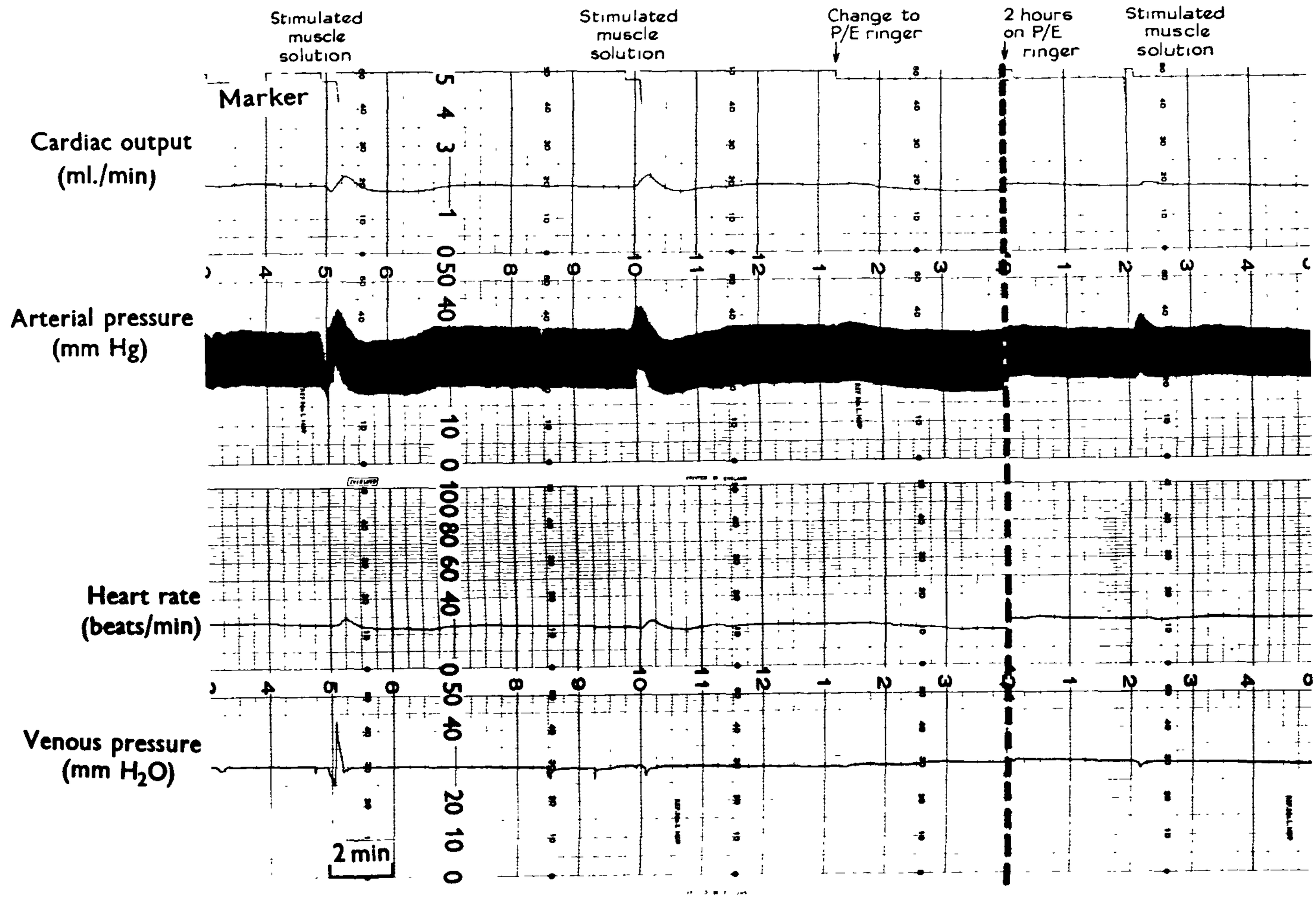

Stimulated muscle solution
Stimulated muscle solution
Change to P/E ringer
2 hours on P/E ringer
Stimulated muscle solution
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min

Figure 24.

The response of a frog heart to noradrenaline and to a stimulated muscle solution. The upper trace shows the effect of perfusing the heart with a stimulated muscle solution. Note the sharp rise and fall of the cardiac output and blood pressure; there is no obvious rise in the heart rate. 10^{-7} g/ml. noradrenaline did not produce a marked stimulatory effect, but 10^{-6} g/ml. produced a very great effect. The cardiac output trace is irregular here because the sudden large increase in the cardiac output overcame the drop-counting system of recording.

The lower trace shows the modified effect of 10^{-6} g/ml. noradrenaline after perfusion of the heart with P/E Ringer's solution for 2 hours. No change occurs in the effect of the same stimulated muscle solution.

The effect of perfusing a solution of 5×10^{-7} g/ml. adenosine triphosphate (ATP) through the heart is seen. Note the similarity of this effect to that of the stimulated muscle solution. The action of ATP is also unaffected by the adrenergic blocking agents.

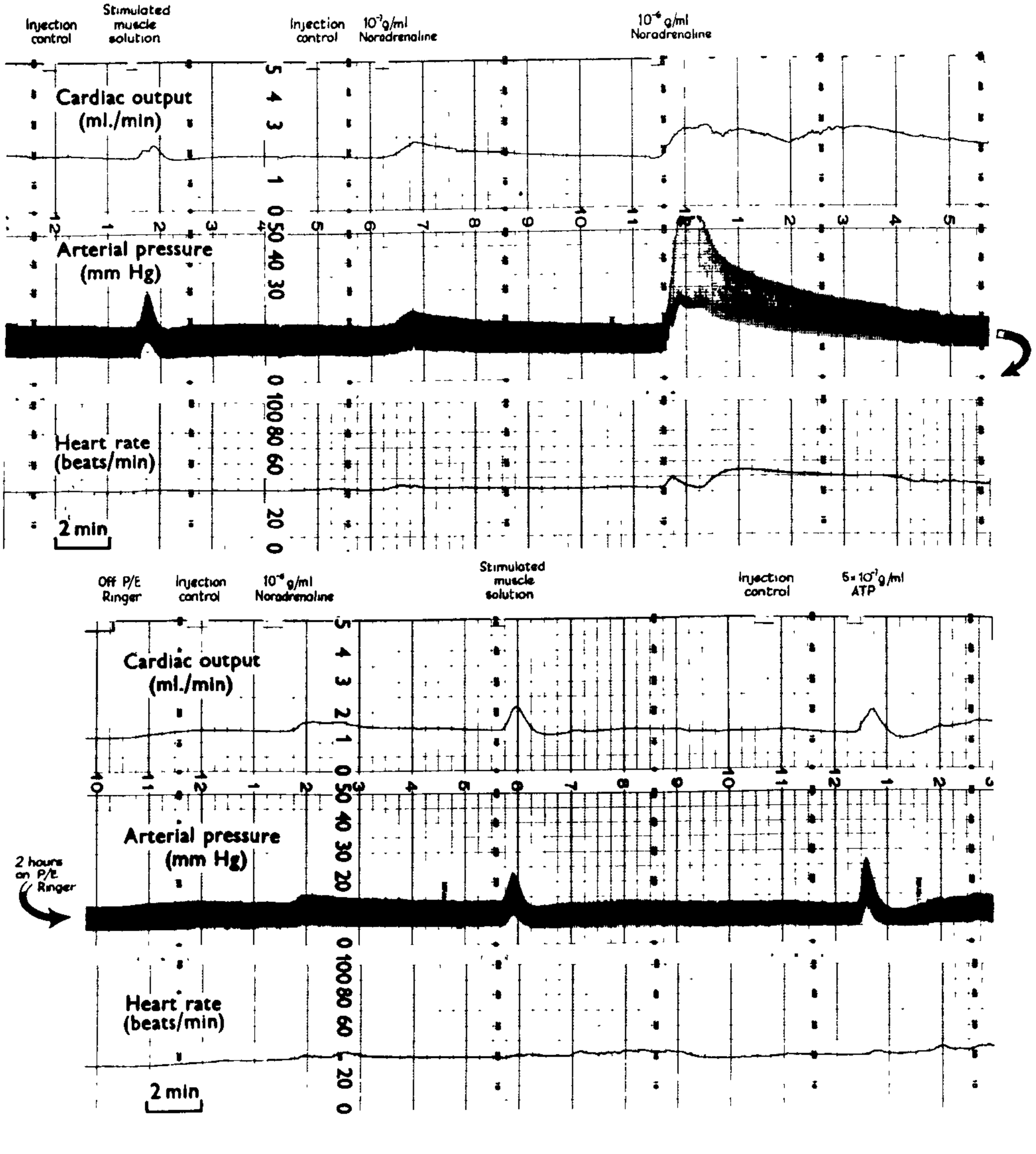

Injection control
Stimulated muscle solution
Injection control
10^{-7} g/ml Noradrenaline
10^{-6} g/ml Noradrenaline
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
2 min
Off P/E Ringer
Injection control
10^{-6} g/ml Noradrenaline
Stimulated muscle solution
Injection control
5×10^{-7} g/ml ATP
Cardiac output (ml./min)
Arterial pressure (mm Hg)
2 hours on P/E Ringer
Heart rate (beats/min)
2 min

Table 6.

THE EFFECT OF ADRENERGIC BLOCKADE ON THE CARDIAC OUTPUT WHEN CATECHOLAMINES AND STIMULATED MUSCLE SOLUTIONS ARE PERFUSED THROUGH THE FROG HEART

HEART	% OUTPUT INCREASE	
	Before Adrenergic Blockade	After Adrenergic Blockade
1.	10^{-7} g/ml. adrenaline 30%	0%
	Test solution -	35%
2.	10^{-7} g/ml. adrenaline -	0%
	Test solution 36%	38%
3.	10^{-7} g/ml. adrenaline -	0%
4.	10^{-7} g/ml. adrenaline 65%	-
5.	10^{-6} g/ml. adrenaline 50%	7%
	Test solution 12%	6% *
6.	10^{-6} g/ml. noradrenaline 200%	21%
	Test solution 64%	53%

* half the amount injected, see Figure 23.

of 10^{-6} g/ml. noradrenaline was greatly modified. Perfusion of the same stimulated muscle solution 5 minutes later showed no change in its effect.

Stimulated muscle solutions were perfused through two other hearts and in neither case was the action of the stimulated muscle solution reduced by the adrenergic blocking agents. (Table 6, hearts 2 and 6).

The evidence from these experiments that the stimulation is not caused by catecholamines can be summarized:-

1. The qualitative effect of adrenaline and noradrenaline on the heart is different from that of the unknown stimulating substance. A prolonged increase in heart rate and cardiac output occurs with the catecholamines (Figures 22, 23, & 24. Their effect is generally longer-lasting, whereas the stimulatory substance causes a sharp, but transitory increase in the cardiac output and rate. With the unknown substance the effect on the blood pressure is also different (Figure 24). There is a sharp, regular increase in the systolic and diastolic pressures, with a slight increase in the pulse pressure at the maximum value of the systolic blood pressure. These effects quickly wear off. With the catecholamines there is an initial sharp increase in blood pressure similar to that caused by the unknown substance, but this increase is sustained and may last for many minutes.
2. In order for the catecholamines to produce a similar effect to the test solution when perfused through the heart, the concentration required must be no less than 10^{-7} g/ml. in the test solution. Thus the isolated frog sartorius muscle, twitching in 2 ml. of the fluid, would have to produce 2×10^{-7} g of catecholamine, while only being stimulated at two impulses per second for 30 minutes.
3. Adrenergic blocking agents block the effects of adrenaline and noradrenaline on the frog heart, but they do not affect the response of

the same frog heart to the unknown stimulatory substance.

From that evidence it can now be stated that the cause of the stimulatory effect on the frog heart is not release of catecholamines from the sartorius muscle.

ADENYLIC ACID DERIVATIVES AND THEIR ACTION ON THE MYOCARDIUM

There is a considerable accumulation of knowledge about the biological actions of adenyl compounds on cardiovascular tissues of many species. Drury and Szent-Gyorgyi (1929) showed that adenylic acid produced heart block in the guineapig and lowered the blood pressure in the dog together with dilating the coronary arteries. Lindner and Rigler (1933) found that extracts from the bundle of His from the calf heart stimulated an hypodynamically-acting frog's heart. On attempting to isolate this compound they found it contained a pentose and a purine base.

Later work of this nature suggested that the biological activity of many of the tissue extracts was really due to their adenosine content (Rigler and Schaumann, 1930; Rothman, 1930). Ostern and Parnas (1932) found that adenosine triphosphate and occasionally muscle adenylic acid increased the strength of the frog heart beat, while adenosine failed to do so; this result was also obtained on frog heart by Lindner and Rigler (1931) and by Drury (1932) on the rabbit heart.

Flossner (1934) found that some nucleic acid derivatives increased while others decreased the amplitude of contraction of perfused frog hearts. At this time most workers agreed that the hearts of experimental animals whether perfused or in the intact animal remained in very good condition when subjected to injection of nucleic acid derivatives. Although these derivatives were known to be powerful coronary vasodilators, it was difficult for some workers to be convinced that the improvement of the heart beat was always due to the action upon the coronary vessels.

In 1950 Green and Stoner studied the action of the adenine nucleotides on the cardiovascular system in some detail. They found that adenosine monophosphate and adenosine triphosphate caused a bradycardia in the perfused isolated rabbit heart- they found that these compounds acted upon the sinu-atrial node, and that their action could be explained by the adenosine content. The amplitude of

contraction they found to be unaffected by adenosine; however, when adenosine triphosphate was perfused through the heart there was an immediate increase in the amplitude of the beat, followed by a depression, which in turn was followed by a sustained increase in the amplitude of contraction. This verified an earlier observation by Dubois-Ferrière (1945) working on perfused frog hearts. Later work has conclusively shown that adenosine triphosphate has an immediate positive inotropic effect on cardiac muscle which is succeeded by a bradycardia or a cardiac arrest (Meyer, 1951; Sekiya, 1953; Kanda et al., 1954; Chevillard and Guerin, 1955; Buday, Carr and Miya, 1961; Versprille, 1963).

Results

In order to test whether other triphosphates were capable of stimulating the frog heart, one experiment was performed where solutions of inosine triphosphate (ITP), uridine triphosphate (UTP), cytosine triphosphate (CTP) and guanosine triphosphate (GTP) were perfused through the frog heart. UTP and ITP had a marked stimulatory effect on the heart but no effect was produced by solutions of CTP or GTP. It is possible that these latter substances had degraded during prolonged storage.

Solutions of the bases uridine, cytidine, inosine and adenosine were also perfused through the heart but no stimulation was produced (Table 7). An interesting finding was that the solution of ITP caused a greater stimulation than that caused by inosine diphosphate on the same heart. Inosine monophosphate did not have any effect on this heart.

Adenosine, in a concentration of 10^{-5} g/ml. caused a 3% fall in cardiac output and a 35% fall in heart rate when perfused through the heart. Figure 25 shows that adenosine monophosphate, even in a very high concentration, did not stimulate the heart. A fall in rate and output is produced by 10^{-6} g/ml. and 10^{-5} g/ml. AMP.

When a solution of ATP was perfused through thefrog heart it produced a similar type of stimulation to that produced by the stimulated muscle solution

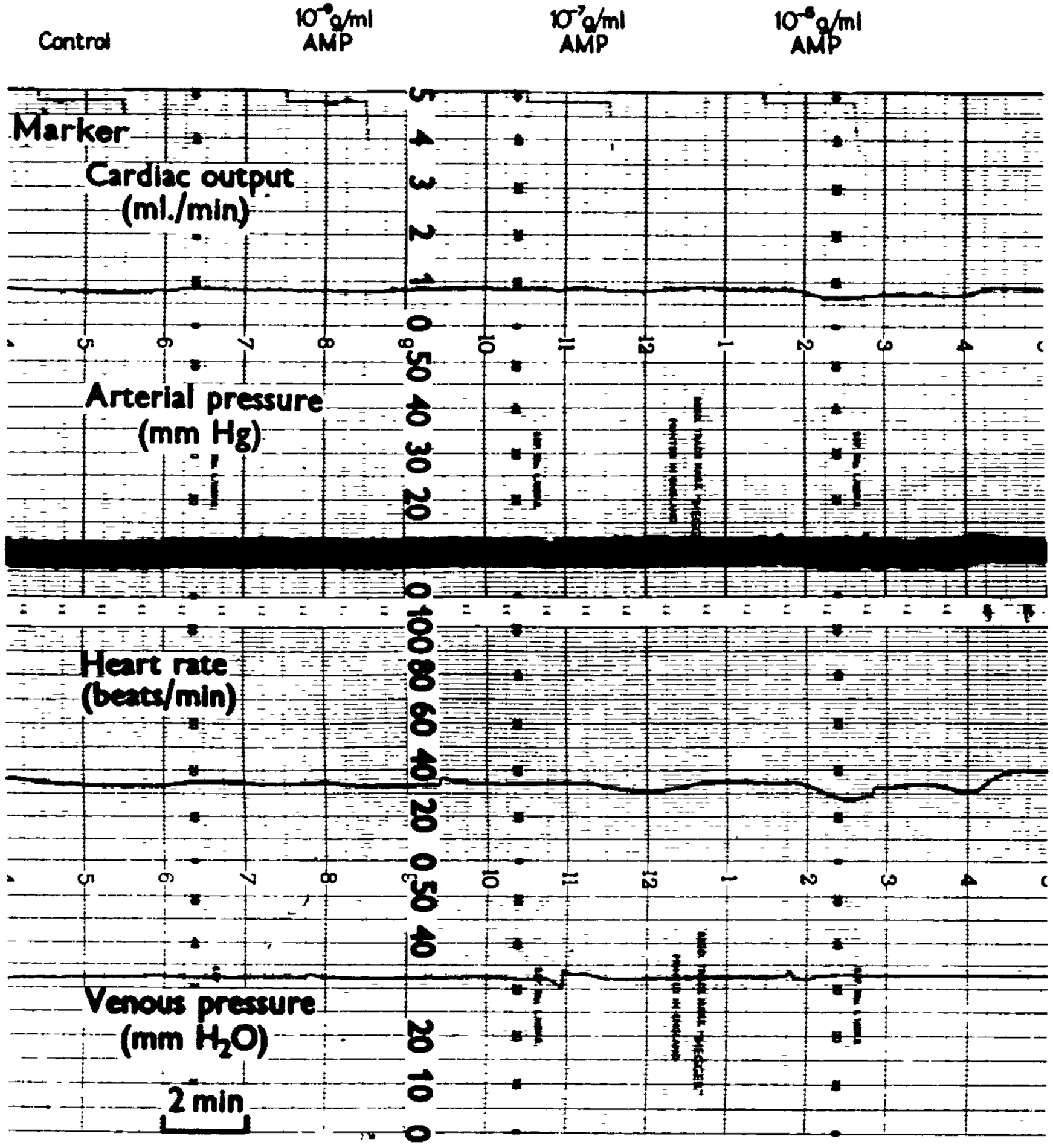
Table 7.

The effect of perfusing various bases and nucleotides through frog hearts.

Hearts	Compound	% Output Change	% Rate Change
1	10^{-5} g/ml. adenosine	-30	-35
	10^{-5} g/ml. AMP	-25	-36
	10^{-5} g/ml. uridine	0	-25
	10^{-5} g/ml. UTP	+220	+34
2	10^{-6} g/ml. inosine	0	0
	10^{-5} g/ml. inosine	0	0
	10^{-4} g/ml. inosine	0	0
	10^{-6} g/ml. IMP	-7	0
	10^{-5} g/ml. IMP	-9	0
	10^{-6} g/ml. IDP	+10	+28
	10^{-6} g/ml. ITP	+20	+31
	10^{-6} g/ml. cytidine	-5	-7
	10^{-6} g/ml. CMP	0	0
	10^{-6} g/ml. CTP	0	0
	10^{-6} g/ml. GTP	0	0
3	10^{-9} g/ml. AMP	0	0
	10^{-7} g/ml. AMP	-5	-12
	10^{-5} g/ml. AMP	-20	-24

Figure 25.

The effect of perfusing adenosine monophosphate through a frog heart. Doses of 10^{-9} g/ml., 10^{-7} g/ml. and 10^{-5} g/ml. are perfused through the heart. No stimulation of the heart is evident. A slight fall in the heart rate is produced by 10^{-7} g/ml. AMP. This fall is accentuated when 10^{-5} g/ml. AMP is perfused into the heart.


Control
10⁻⁹g/ml AMP
10⁻⁷g/ml AMP
10⁻⁵g/ml AMP
Marker
Cardiac output (ml./min)
5 4 3 2 1 0
Arterial pressure (mm Hg)
80 40 30 20 0
Heart rate (beats/min)
100 80 60 40 20 0
Venous pressure (mm H₂O)
50 40 20 10 0
2 min

Figures 28 & 29. There was an immediate rise in systolic, diastolic and pulse pressures, causing a rapid increase in cardiac output. With high concentrations of ATP the heart rate was increased, reaching a peak value 2 minutes later. Usually the stimulated muscle solution produced a greater increase in the heart rate.

A quantitative comparison of the action of ATP and stimulated muscle solutions is given in Table 10.

Discussion

The only substances found which have been able to stimulate the frog heart in a similar fashion to that of the stimulated muscle solution are

a) the catecholamines and

b) the triphosphates of various bases.

The catecholamines as a cause of the effect have already been eliminated; however, it was felt necessary to proceed with further investigation into the possibility of whether the stimulatory substance was adenosine triphosphate, since this is probably the most commonly-occurring of the triphosphates in biological tissue.

Although there is evidence to suggest that ATP is NOT released from skeletal muscle (Hilton and Greengard, 1962), nevertheless the qualitative similarity of the stimulation produced by both ATP and the test solution encouraged further investigation.

COMPARISON OF THE ULTRAVIOLET ABSORPTION OF TEST SOLUTIONS AND SOLUTIONS CONTAINING ADENOSINE COMPOUNDS

Of the compounds which make up the bulk of the muscle structure, only two types have a marked selective light absorption in the ultraviolet range (250mμ - 300mμ), namely the purine derivatives and the proteins. The purine derivatives are 95% adenylic acid derivatives (Caspersson and Thorell, 1942). The purine nucleus in these cases absorbs ultraviolet light strongly at 260 mμ. Absorption of ultraviolet light by protein occurs at 280 mμ (conditioned by tyrosine and tryptophan) but is not so strong as the ultraviolet absorption by the adenylic acid derivatives.

Results

Figure 26 shows the comparison of UV absorption sepctra of two resting muscle solutions, a stimulated muscle solution and a solution containing 10^{-5} g/ml. adenosine monophosphate made up in frog Ringer's solution. The resting solutions were obtained from muscles which had rested for 50 minutes and 120 minutes in 2 ml. of Ringer's solution. The 50 minute resting solution shows a low optical density in the range 250mμ - 280mμ. The 120 minute resting solution has a higher optical density in this range. The optical density for the stimulated muscle solution is markedly raised in this range. The increase in optical density is due to the absorption of UV light by protein; more protein is liberated from stimulated than resting muscles (see Protein Section). The solution containing 10^{-5} g/ml. adenosine monophosphate shows a greatly prounounced, sharp peak of optical density at 260mμ wavelength.

The raised optical density in the range 250mμ - 280mμ caused by protein might mask small peaks of UV absorption resulting from small concentrations of adenosine compounds perhaps present in the test solution. With the Sephadex column technique (p.34 Methods) it was possible to obtain a solution which was capable of stimulating the frog heart and which did not contain any protein

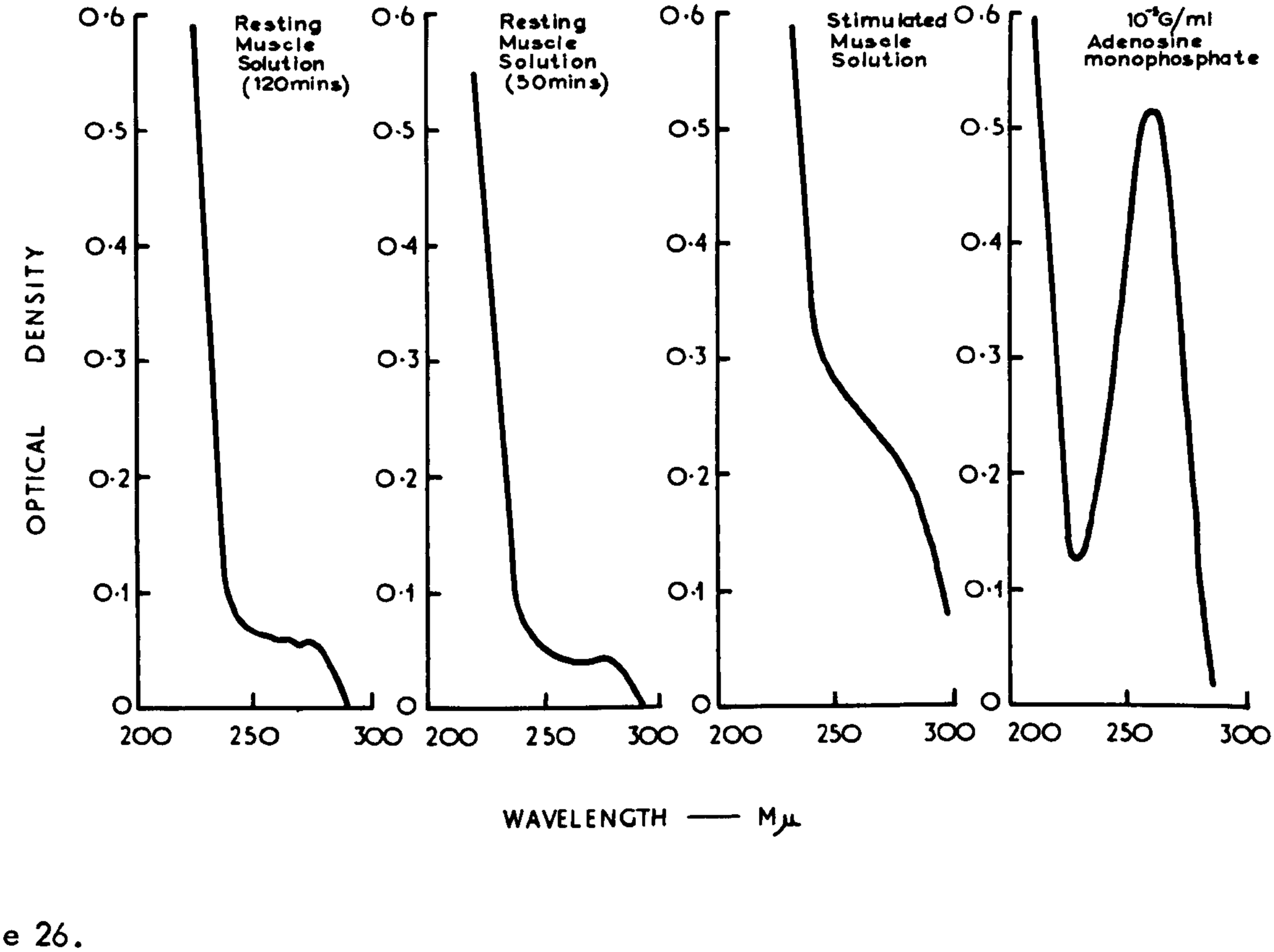


Figure 26.

Comparison of absorption spectra obtained from resting and stimulated muscle solutions with that from a solution of 10^{-5} g/ml adenosine monophosphate.

(a) A solution in which a muscle rested for 120 minutes shows an optical density of 0.06 units between 250 mμ and 280 mμ.

(b) A resting solution of fifty minutes duration gives a lower optical density over the same range.

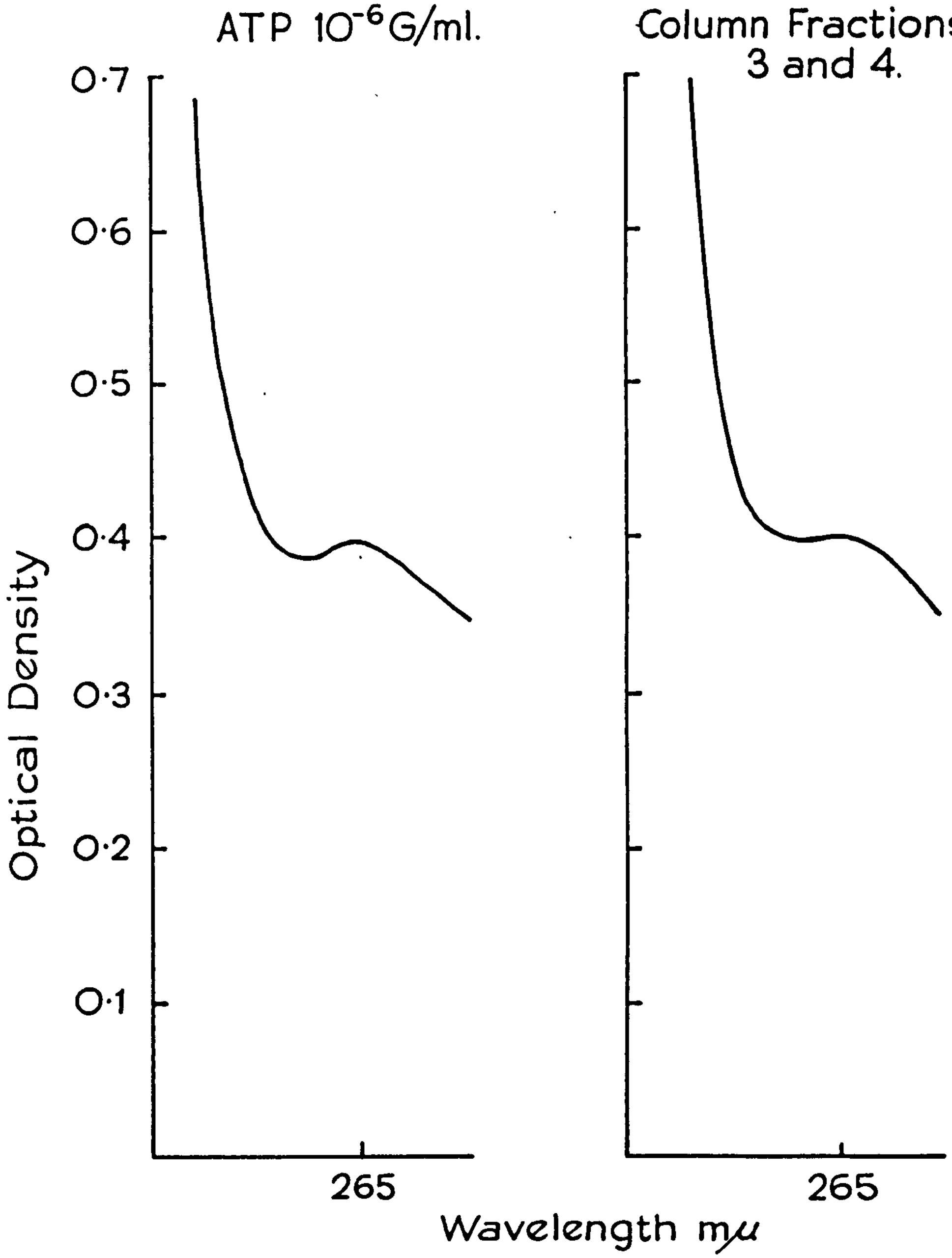
(c) A stimulated muscle solution shows greater absorption over this range.

(d) 10^{-5} g/ml adenosine monophosphate made up in frog Ringer's solution shows a pronounced sharp peak at 260 mμ wavelength.

The increased optical density in the solutions is due to protein liberated from the muscle. More protein is liberated from the stimulated muscle than the resting muscle.

Figure 27.

Comparison of the UV absorption spectra of a solution of 10^{-6} g/ml ATP and a mixture of column fractions 3 and 4 which stimulated the frog heart. Small peaks of UV absorption appear at 265 mμ wavelength.


ATP 10^{-6} G/ml.
Column Fractions 3 and 4.
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Optical Density
265
265
Wavelength mμ

(Figure 27). When the UV absorption spectrum of a mixture of column fractions 3 and 4 (which stimulated the frog heart) was measured, a very slight peak appeared at 265mμ wavelength). This is shown in Figure 27. This suggested that nucleotides were present in the test solution, since these cyclic compounds absorb UV light at approximately this wavelength, depending on the type of solvent in which they are dissolved. Figure 27 shows the UV absorption spectrum of a solution of 10^{-6} g/ml. adenosine triphosphate made up in a similar frog Ringer's solution to that containing fractions 3 and 4. A more pronounced absorption peak is evident at the same position in the spectrum as that of the column fractions 3 and 4.

This preliminary investigation was, of course, not in itself a valid identification procedure, but it is described here because it provided the line of further investigation.

COMPARISON OF THE ACTION OF APYRASE ON THE STIMULATED MUSCLE SOLUTION AND ON ADENOSINE TRIPHOSPHATE

Potato adenosine triphosphatase (ATPase) was first described by Kalckar in 1943. He was able to precipitate a substance out from watery extracts of potato which could split off two groups of phosphate from adenosine triphosphate. He also found that this reaction was activated by calcium ions.

Meyerhof (1945) first suggested the name "apyrase" to describe those adenylpyrophosphatases which act on both ADP and ATP, splitting off two phosphate groups from the latter. A survey of modern literature shows that the two terms "apyrase" and ATPase" are often used interchangeably.

The findings of Eiler and Lee (1951) and Van Thoai (1954) suggested that apyrase split ATP to ADP and that only after this phase had been completed did the formation of AMP take place. Traverso-Cori and Cori (1962) verified this and showed that AMP was not produced in the first 10 minutes of the reaction.

Two enzymes have recently been separated from apyrase (Liebecq, Lallemand and Degueldre-Guillaume, 1963) which act on inosine triphosphate (ITP), guanosine triphosphate (GTP) uridine triphosphate (UTP), cytosine triphosphate (CTP) as well as on ATP. One enzyme converts the triphosphate to the diphosphate, the other enzyme coverts the diphosphate to the monophosphate.

Substrate Specificity of Potato Apyrase

As already indicated by the work of Liebecq et al., the phosphate groups can be split from variously-based nucleotides, so the enzyme is not specific for ADENOSINE triphosphate. These workers also studied the action of potato apyrase on adenosine TETRAphosphate. It was found to be dephosphorylated very slowly by the two fractions of apyrase. A search of the literature has failed to reveal any other energy-rich substance containing phosphate radicals which is acted upon by apyrase. Krishnan (1949) found that no phosphate was liberated from muscle adenylic acid, yeast adenylic acid, sodium pyrophosphate, hexose 1.6 diphosphate

and -glycerophosphate. He showed that even in the crude stage the enzyme showed some degree of specificity towards ATP.

Results

Figure 28 shows the effect of perfusing a frog heart with a solution of 10^{-6} g/ml. ATP. The initial sharp, stimulatory effect on the force of contraction is clearly seen in both the arterial pressure record and in the record of cardiac output. Immediately following this effect is a slight bradycardia. Perfusion of the heart with the same concentration of ATP, having incubated this portion with apyrase for 15 minutes, shows that the initial sharp effect has been abolished. Note that the fall in rate which followed the initial stimulatory effect has not been altered. The pure ATP solution was then perfused through the heart again to show that the sensitivity of the heart to ATP had not been affected in any way by apyrase.

Figure 29 shows the effect of perfusing 0.5 ml. of a stimulated muscle solution through the same frog heart as shown in Figure 28. Once again an initial sharp stimulatory effect on the force of contraction is reflected in both the arterial pressure and cardiac output recordings. In this case a late increase in the heart rate has occurred. When 0.5 ml. of the solution is incubated with apyrase, the sharp stimulatory effect is abolished; the late rise in the heart caused by the stimulatory substance in the test solution has not been abolished by the apyrase.

Ten solutions of ATP were perfused through eight frog hearts before and after incubation with apyrase (Table 8). Eight stimulated muscle solutions were perfused through eight frog hearts before and after incubation with apyrase (Table 9).

In every case the initial stimulatory effect of both the ATP solutions and the solutions in which a sartorius muscle had twitched was abolished. In some cases the sharp increase in cardiac output was supplanted by a gradual rise in output, occurring much later after the perfusion; this was due to a late increase in the heart rate which occurs with solutions which have had insufficient time for incubation with apyrase. It became obvious that this late rate effect which

Figure 28.

The effect of perfusing a solution of 10^{-6} g/ml. adenosine triphosphate before and after its incubation with apyrase through a frog heart.

10^{-6} g/ml. ATP gave an effect qualitatively similar to that of the stimulated muscle solution. There is an immediate increase in the systolic, diastolic and pulse pressures, resulting in a sharp increase in the cardiac output. This is followed by a fall in the heart rate and blood pressure causing a fall in the cardiac output. When 2 ml. of the 10^{-6} g/ml. ATP solution is incubated with apyrase for 15 minutes and the solution perfused through the same heart, the sharp stimulatory effect is abolished. A repeat perfusion of the unincubated ATP solution is done to ensure that the apyrase has not abolished the sensitivity of the heart to adenosine triphosphate. Incubation of ATP solutions without apyrase does not alter their effect on the frog heart.

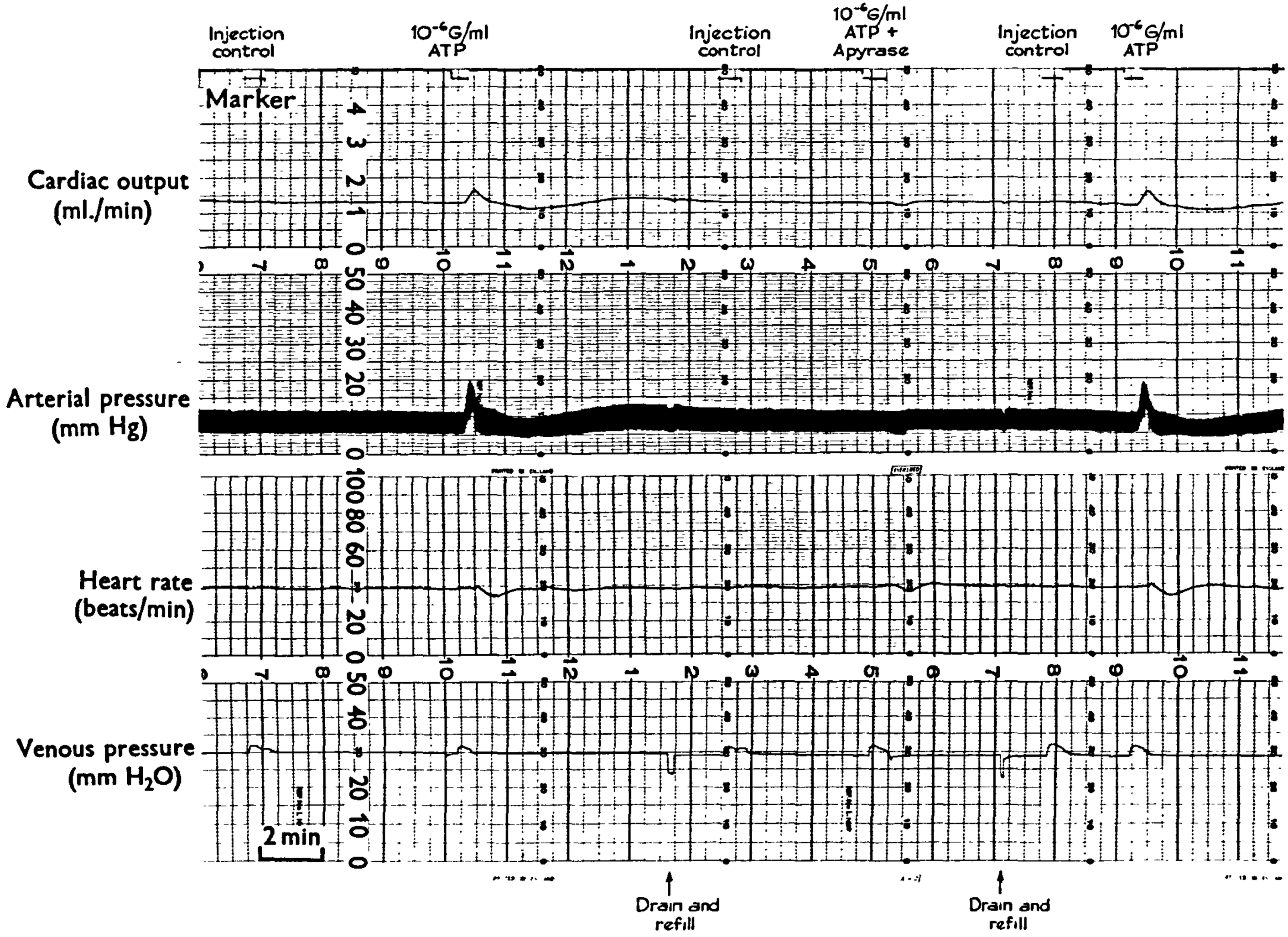
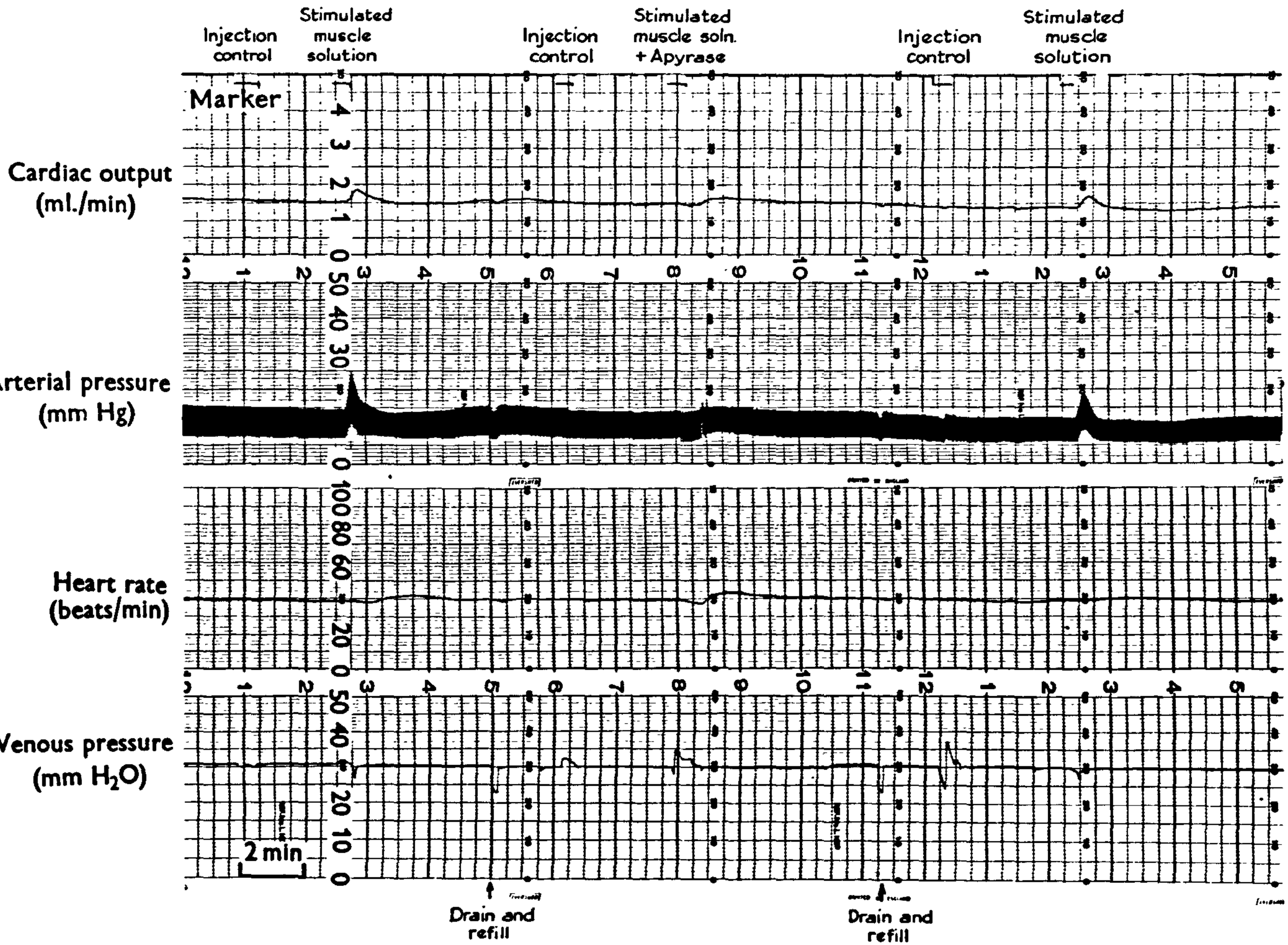

Injection control
10⁻⁶G/ml ATP
Injection control
10⁻⁶G/ml ATP + Apyrase
Injection control
10⁻⁶G/ml ATP
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
Drain and refill
Drain and refill

Figure 29.

The effect of perfusing a stimulated muscle solution before and after its incubation with apyrase through the same frog heart as recorded in Figure 25.

Incubation of the solution with apyrase abolishes the sharp stimulatory effect. A slight rise in heart rate remains. The remainder of the stimulated muscle solution was perfused through the heart in order to show that the heart had not lost its sensitivity to the test solution.


Injection control
Stimulated muscle solution
Injection control
Stimulated muscle soln. + Apyrase
Injection control
Stimulated muscle solution
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min
Drain and refill
Drain and refill

occurred after a test solution was incubated could cause the cardiac output to rise to an even greater level than that level reached when the stimulated muscle solution was perfused through a heart. Figure 30 shows the result of perfusing a frog heart with a solution of 5×10^{-8} g/ml. ATP. This gave the usual stimulatory effect. Also shown is the result of perfusing the heart with solutions of the same concentrations of ATP having been incubated with apyrase (1 mg added to each ml. of ATP solution) for 5, 10 and 15 minutes. The sharp, stimulatory effect is abolished in all three solutions but an arrythmia has been introduced, the rate increase being greater when the incubation period is shorter (Table 8).

Incubating the apyrase with frog Ringer's solution alone for 10 minutes has no effect on either the heart rate or cardiac output when this solution is perfused through the heart.

Clearly some interference phenomenon is produced from the reaction

ATP $\xrightarrow{\text{apyrase}}$ ADP when incubation does not proceed beyond 10 minutes.

In this section it cannot be ruled out that this effect on the heart rate is due to ADP. It was considered unfruitful to perfuse the frog heart with standard preparations of ADP, since commercially prepared ADP is almost certain to contain a minute quantity of ATP. Since the frog heart is especially sensitive to ATP, any contamination of ADP with ATP will distort the frog heart response to perfused commercial ADP. Elaborate purification procedures would be necessary to obtain a pure ADP effect on the frog heart.

Discussion

The potato apyrase used in this work contained 0.9 units per milligramme. One unit will hydrolyse 1μ mole of the substrate per minute at pH 6.5 at 30°C (information supplied by Sigma Company).

Molecular weight of ATP (disodium dihydrogen salt) = 623

1μ mole = 0.623×10^{-3} g/ml. = 6.23×10^{-4} g/ml.

Since the test solutions are incubated with a concentration of 0.9 units of

Figure 30.

The effect of perfusing solutions in which apyrase and ATP were incubated for periods of 5, 10 and 15 minutes. 5×10^{-8} g/ml. ATP gave the usual stimulatory effect. No rate rise occurs. The effect on the frog heart of the same concentrations of ATP incubated with apyrase for different periods is shown. Note the increasing rate effect as the incubation time shortens. Incubating the apyrase with Ringer's solution alone for 10 minutes has no effect on the heart when perfused through it.

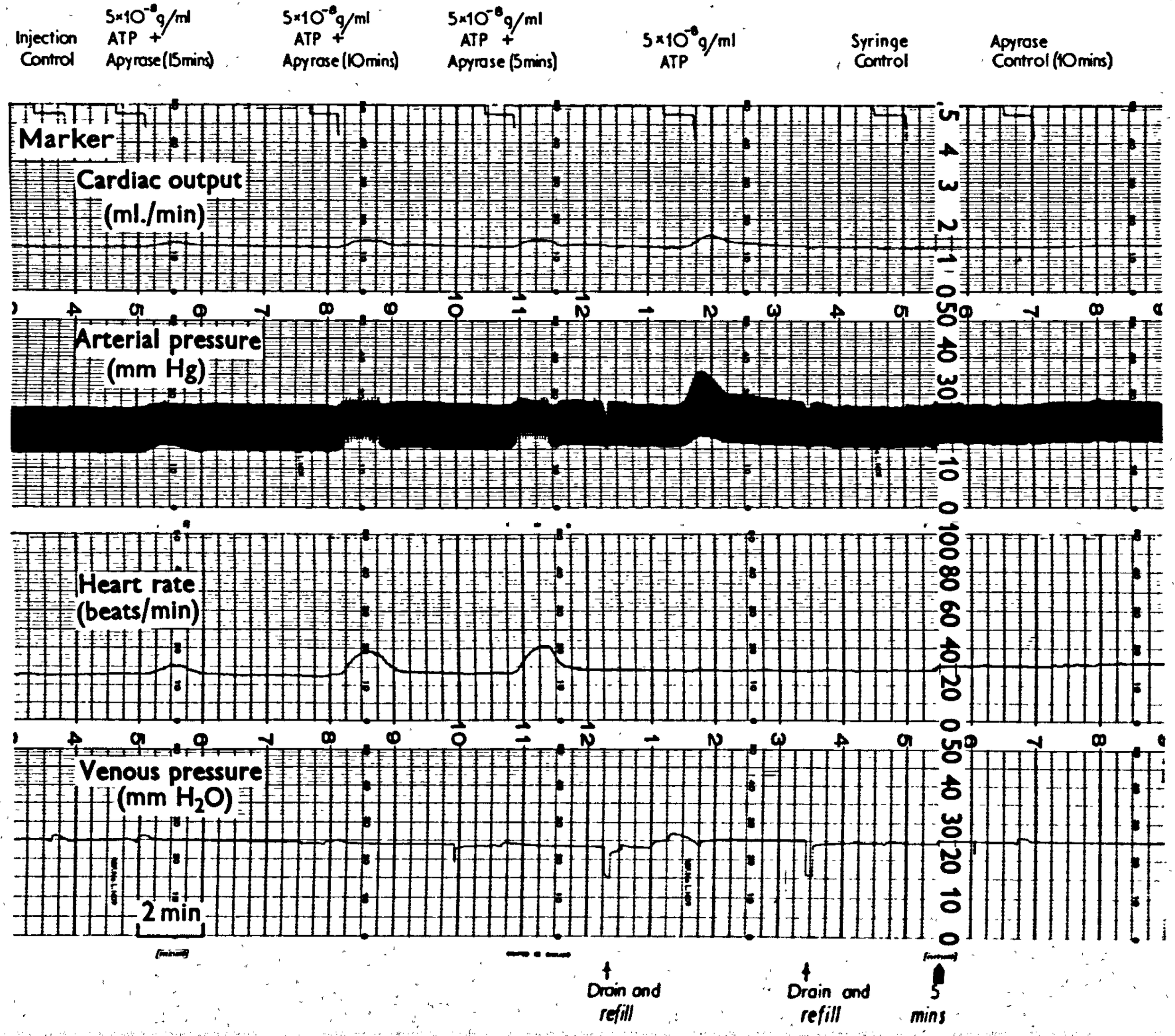

Injection Control
5×10⁻⁸g/ml ATP + Apyrase (15mins)
5×10⁻⁸g/ml ATP + Apyrase (10mins)
5×10⁻⁸g/ml ATP + Apyrase (5mins)
5×10⁻⁸g/ml ATP
Syringe Control
Apyrase Control (10mins)
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H2O)
2 min
Drain and refill
Drain and refill
5 mins

Table 8. The effect of perfusing solutions of ATP which have been incubated with apyrase for varying periods through frog hearts.

Heart	Concentration of ATP	Time of Incubation	% Output Increase	% Rate Increase
1.	10^{-8} g/ml.	--	21	20
	10^{-8} g/ml.	5 minutes	13	40
	10^{-8} g/ml.	10 minutes	10	40
	10^{-8} g/ml.	15 minutes	0	0
2.	5.0×10^{-8} g/ml.	--	24	0
	5.0×10^{-8} g/ml.	5 minutes	16	50
	5.0×10^{-8} g/ml.	10 minutes	16	52
	5.0×10^{-8} g/ml.	15 minutes	8	16
3.	10^{-6} g/ml.	--	60	0
	10^{-6} g/ml.	10 minutes	35	0
4.	10^{-6} g/ml.	--	32	-12
	10^{-6} g/ml.	10 minutes	-4	-7
5.	10^{-6} g/ml.	--	37	0
	10^{-6} g/ml.	10 minutes	0	0
6.	10^{-7} g/ml.	--	50	irreg.
	10^{-7} g/ml.	10 minutes	17	22
	2.5×10^{-7} g/ml.	--	18	0
	2.5×10^{-7} g/ml.	10 minutes	11	17
7.	10^{-8} g/ml.	--	12	0
	10^{-8} g/ml.	5 minutes	14	18
8.	5.0×10^{-8} g/ml.	--	17	0
	5.0×10^{-8} g/ml.	5 minutes	20	20
	10^{-7} g/ml.	--	27	0
	10^{-7} g/ml.	5 minutes	32	30
	10^{-7} g/ml.	10 minutes	18	24

Small concentrations of ATP (not incubated with apyrase) only affect the cardiac output, not the heart rate. When incubated with apyrase, the initial stimulatory effect disappears, but if the time of incubation is insufficient, then a separate rate increase occurs, about one minute later in the perfusion; this is sufficient to increase the cardiac output at the same time.

apyrase per ml., roughly 6×10^{-4} g/ml. of ATP should be hydrolysed per minute. The incubation period required to abolish the effect of a test solution was one of a quarter of an hour at pH 7 at 30°C.

Quantitative comparisons of the effects of ATP and the stimulated muscle solution on the frog heart (Table 10) would suggest that if the stimulatory effect is due to ATP, then it is at a concentration of certainly not more that 10^{-6} g/ml. in the stimulated muscle solution. So there seems to be no theoretical objections to the assumption that all the ATP in both stimulated muscle solutions and pure ATP solutions is hydrolysed.

Apart from the pH being 7 instead of the recommended 6.5, the conditions for the destruction of ATP in the stimulated muscle solution seem to be ideal. The reaction is calcium dependant (Kalckar, 1943) and calcium, of course, is present in the solution; and its concentration remains unaltered throughout the period of stimulation (see calcium section). Liebecq et al. (1963) noted that the activity of potato apyrase was doubled in the presence of plasma proteins. The stimulated muscle solution contains 150 - 200 µg/ml. of protein (see protein section).

The two-stagereaction of apyrase upon ATP is attributed to two components, an adenosine triphosphatase and an adenosine diphosphatase. Since these two components have been separated chromatographically (Liebecq et al. 1963), the possibility that a single enzyme is splitting off the terminal phosphate groups in two steps is remote.

The incubation period for these components seems to be different. Van Thoai (1954) showed that AMP only appeared after incubation of ATP with apyrase for 15 minutes. Traverso-Cori and Cori (1962) showed that all of the ATP is converted to ADP in the first stage and they found that AMP is not produced in the first 10 minutes of the reaction. So it seems that the incubation time required for the adenosine diphosphate component to produce AMP is at least ten minutes, at pH 6.5 and 30°C.

Unfortunately, although the action of each test solution on the frog heart was

Table 9.

The effect of perfusing the SMS through frog heart after incubation with apyrase. Time of incubation in every case 10 minutes.

Heart	Solution	Before Incubation		After Incubation	
		% Output increase	% Rate increase	% Output increase	% Rate increase
1	1	25 *	17+	50 *	--
2	2	18 *	0+	18 *	25
3	3	20 *	21+	36 *	50
4	4	36 *	28+	42 *	30
5	5	37 *	--	65 *	--
6	6	24 *	4+	14 *	15
7	7	40 *	28+	50 *	35
8	8	32 *	6+	16 *	12 *

* Immediately after perfusion

\+ 2 minutes later

* Both occurring 2 minutes after perfusion.

SMS stimulated muscle solution.

In every case the initial stimulatory effect was abolished; unfortunately the late increase in the heart rate resulted in a late rise of cardiac output at the same time. This was due to the fact that each test solution was incubated with apyrase for only 10 minutes.

radically altered after incubation with apyrase, the late increase in the heart rate resulted in a late rise of cardiac output at the same time (Table 9). This effect can be attributed to the fact that each test solution was incubated with apyrase for only 10 minutes; the ideal time of incubation for complete inactivition of ATP is 15 minutes (Figure 27).

The sharp increase in cardiac output before incubation occurred immediately after the period of perfusion into the heart. In every case this effect was abolished by apyrase (not evident from Table 9). After incubation this effect was replaced by a late rise in the heart rate which caused a late rise in the cardiac output.

COMPARISON OF THE BEHAVIOUR OF THE STIMULATORY SUBSTANCE AND ADENOSINE TRIPHOSPHATE IN A SEPHADEX COLUMN

It was possible to perfuse Ringer's solution which had passed through a Sephadex column through a frog heart without disturbing the cardiac rhythm. Thus it became feasible to examine and compare the behaviour of ATP and the unknown stimulatory substance in the column. By perfusing fractions collected from the column through the heart the rate of elution of ATP and the unknown substance through the column could be ascertained.

Results

A 2 ml. sample of stimulated muscle solution was mixed with blue dextran and then eluted through a column run on eserinized frog Ringer's solution. The fractions collected from the column were those standardized for the maximum recovery of ACh (see section on "Behaviour of ACh in the Sephadex Column"). When these fractions were perfused through the frog heart, it was found that the typical stimulatory effect on the heart was produced by fractions 3, 4 and 5. (Figure 31).

The same procedure was carried out for solutions of ATP. A typical result is shown in Figure 32 . Just as with fractions 3, 4 and 5 of the stimulated muscle solution, the column fractions 3, 4 and 5 of the ATP solution stimulated the frog heart.

In order to show that the elution patterns for the unknown substance and ATP were the same, smaller fractions were collected from the column. Figures 33 and 34 show the result of one such experiment. 5×10^{-7} g/ml. ATP were made up in frog Ringer's solution and 2 ml. of this solution was eluted through the column. After the initial 3 ml. fraction containing the blue dextran came through the column, 2 ml. fractions were collected thereafter. These fractions were then perfused through a frog heart (Figure 33) . The stimulatory effect is clearly seen in fractions 3, 4, 5, 6, and 7; a graded pattern of stimulation is

Figure 31.

The effect of perfusing a frog heart with fractions 1 - 6 from the Sephadex column after a stimulated muscle solution had been put through the column. Fractions 1 and 2 do not affect the frog heart. The stimulatory effect appears in fractions 3, 4 and 5 with a peak effect occurring in fraction 4. Fraction 6 and subsequent fractions did not stimulate the heart. The fraction volumes were those used for the collection of ACh from the column (1 = 2 ml., 2 = 2.5 ml., 3 = 3 ml., 4 = 2.5 ml., 5 = 2 ml., 6 = 2 ml.). The venous pressure capsule was drained and refilled between each test solution to ensure that the remnants of one test solution did not interfere with the action produced by the following solution.

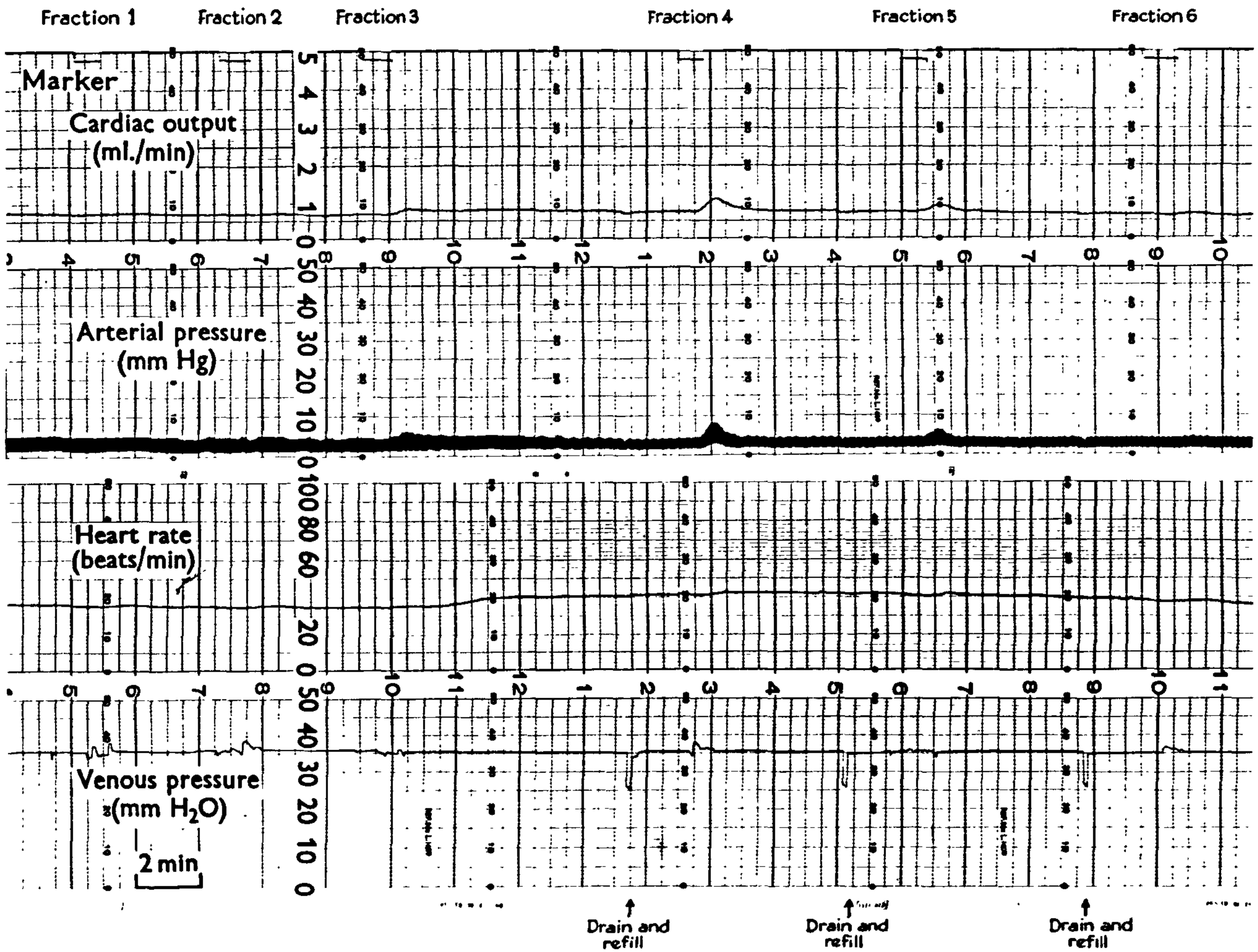

Fraction 1
Fraction 2
Fraction 3
Fraction 4
Fraction 5
Fraction 6
Marker
Cardiac output (ml./min)
5 4 3 2 1 0
Arterial pressure (mm Hg)
50 40 30 20 10 0
Heart rate (beats/min)
100 80 60 20 0
Venous pressure (mm H_2O)
50 40 30 20 10 0
2 min
Drain and refill
Drain and refill
Drain and refill

Figure 32.

10^{-6} g/ml. adenosine triphosphate was made up in frog Ringer's solution and 2 ml. put through the Sephadex column. The usual fractions were collected and perfused through the heart. Fractions 1 and 2 do not stimulate the heart. Fractions 3, 4 and 5 do, with a peak of stimulation in fraction 4. Fraction 6 and subsequent fractions did not stimulate the heart. The effect of perfusing the heart with 10^{-6} g/ml. ATP is seen at the right side of the trace. A control injection of frog Ringer's solution was made between each injection of a test solution.

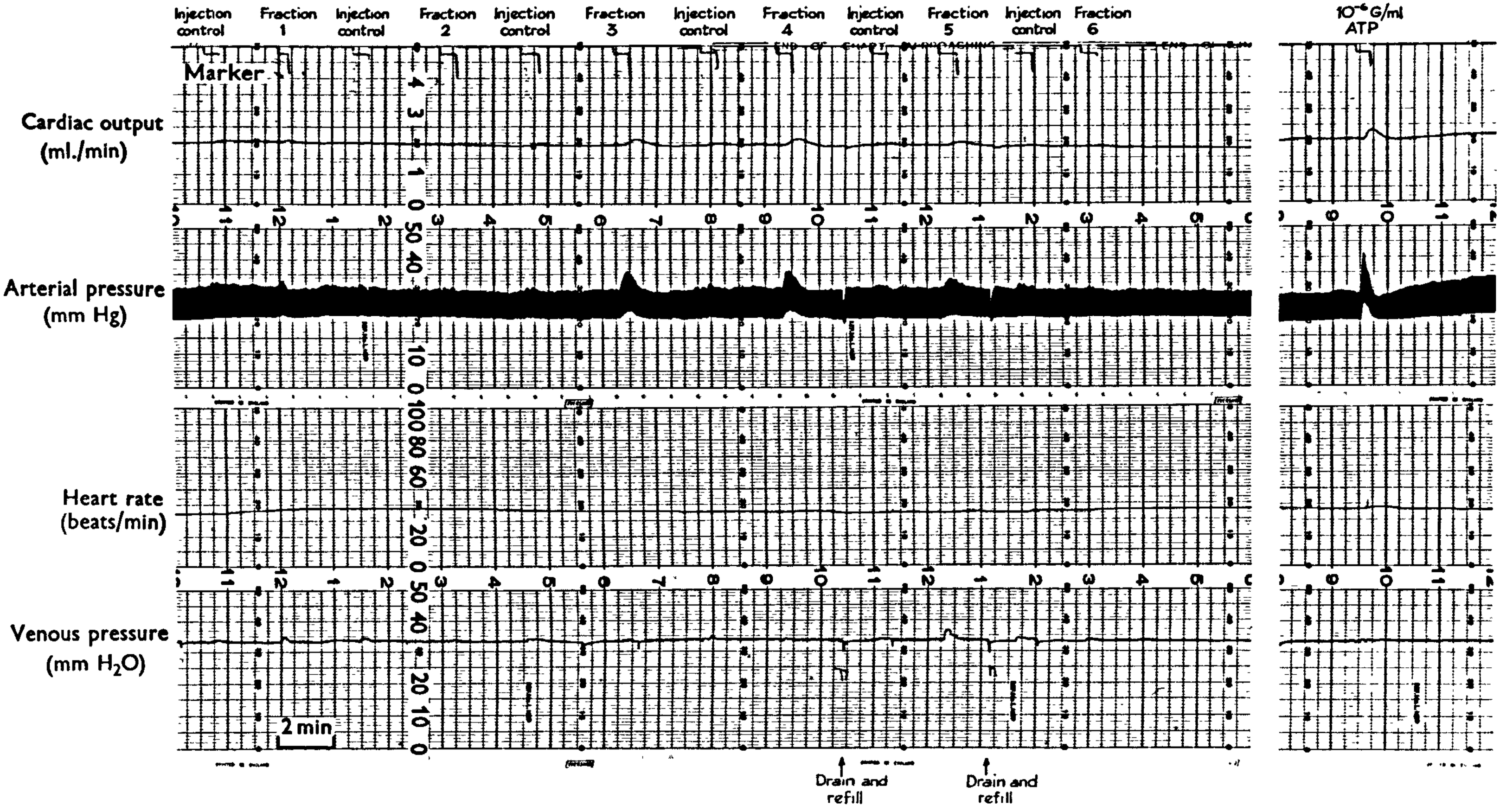

Injection control
Fraction 1
Injection control
Fraction 2
Injection control
Fraction 3
Injection control
Fraction 4
Injection control
Fraction 5
Injection control
Fraction 6
10^{-6} G/ml ATP
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H_2O)
2 min
Drain and refill
Drain and refill

Figure 33.

A solution of 5×10^{-7} g/ml. of adenosine triphosphate was eluted through the same column as was used for the test solution giving the fractions for the previous heart trace. 2 ml. fractions were again collected and perfused through the same frog heart as in Figure 34. The stimulatory effect of the ATP is seen in fractions 3, 4, 5, 6 and 7, with a peak effect in fraction 5. The effect of 5×10^{-7} g/ml. ATP is seen at the end of the trace. "F" indicates the flushing out of the venous pressure capsule.

Injection Control | Fraction 1 | Fraction 2 | Fraction 3 | Fraction 4 | Fraction 5 | Fraction 6 | Fraction 7 | Fraction 8 | Fraction 9 | Fraction 10 | 5×10^{-7} g/ml/ATP

Marker

Cardiac output (ml./min)

5 4 3 2 1 0

Arterial pressure (mm Hg)

150 140 130 120 110 100 90 80 70 60 50 40 30

Heart rate (beats/min)

120 100 80 60 40 20 0

Venous pressure (mm H_2O)

50 40 30 20 10 0

2 min

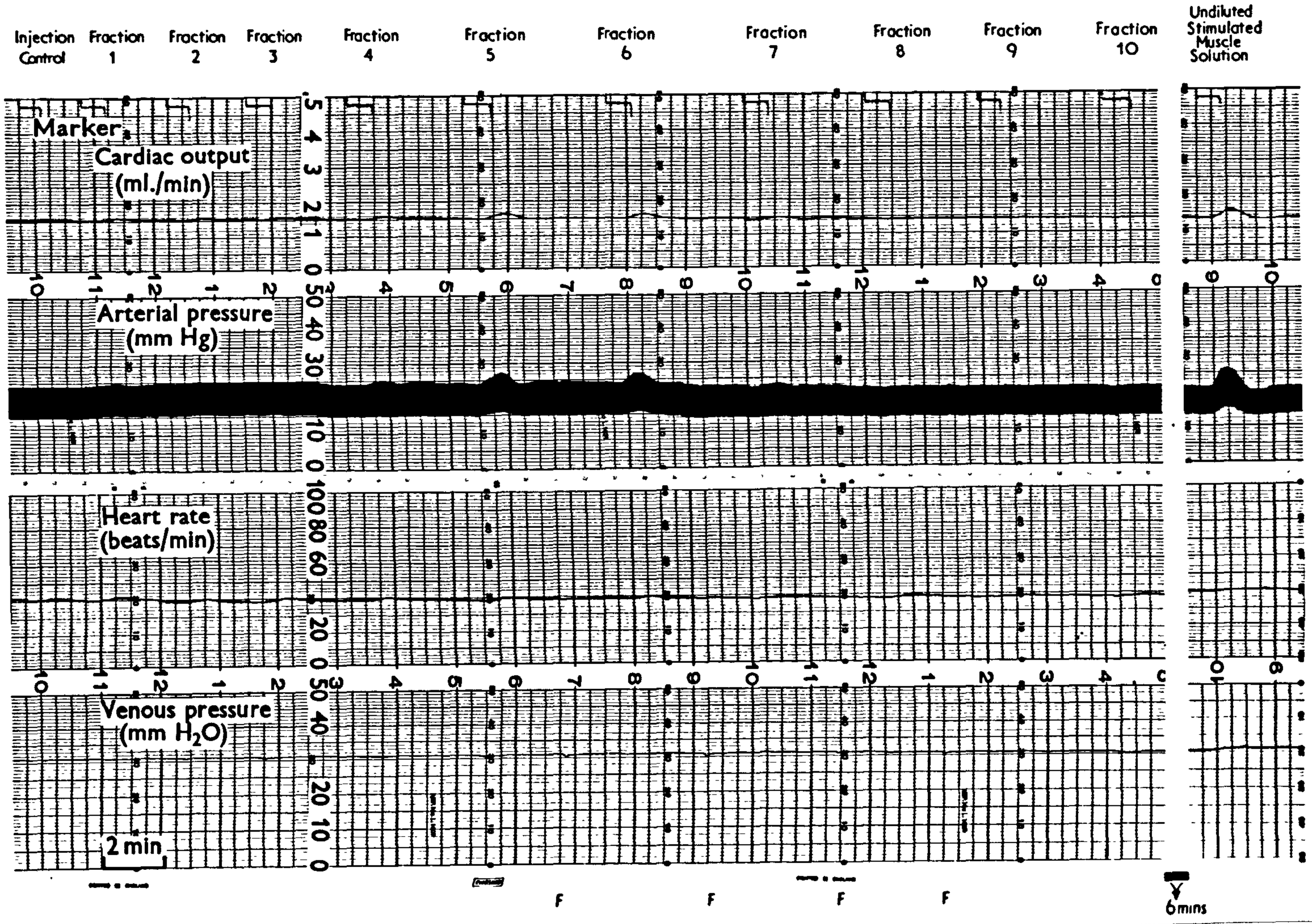
4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2

F F F F F F

Figure 34.

In order to verify that the stimulatory substance in the test solution was eluted through the column into the same fraction as ATP, volumes which were different from the original fractions were collected. In this case 2 ml. fractions were collected throughout.

A stimulated muscle solution was eluted through the same column and 2 ml. fractions were collected and perfused through the same frog heart as in Figure 33. The stimulatory effect is seen in fractions 4, 5, 6 and 7, with a peak effect seen in fraction 5. The effect of the undiluted stimulated muscle solution is shown at the end of the trace. "F" indicates flushing of the venous pressure capsule.


Injection Control
Fraction 1
Fraction 2
Fraction 3
Fraction 4
Fraction 5
Fraction 6
Fraction 7
Fraction 8
Fraction 9
Fraction 10
Undiluted Stimulated Muscle Solution
Marker
Cardiac output (ml./min)
5 4 3 2 1 0
Arterial pressure (mm Hg)
50 40 30
Heart rate (beats/min)
100 80 60 40 20 0
Venous pressure (mm H_2O)
50 40 20 10 0
2 min
F
F
F
F
6mins

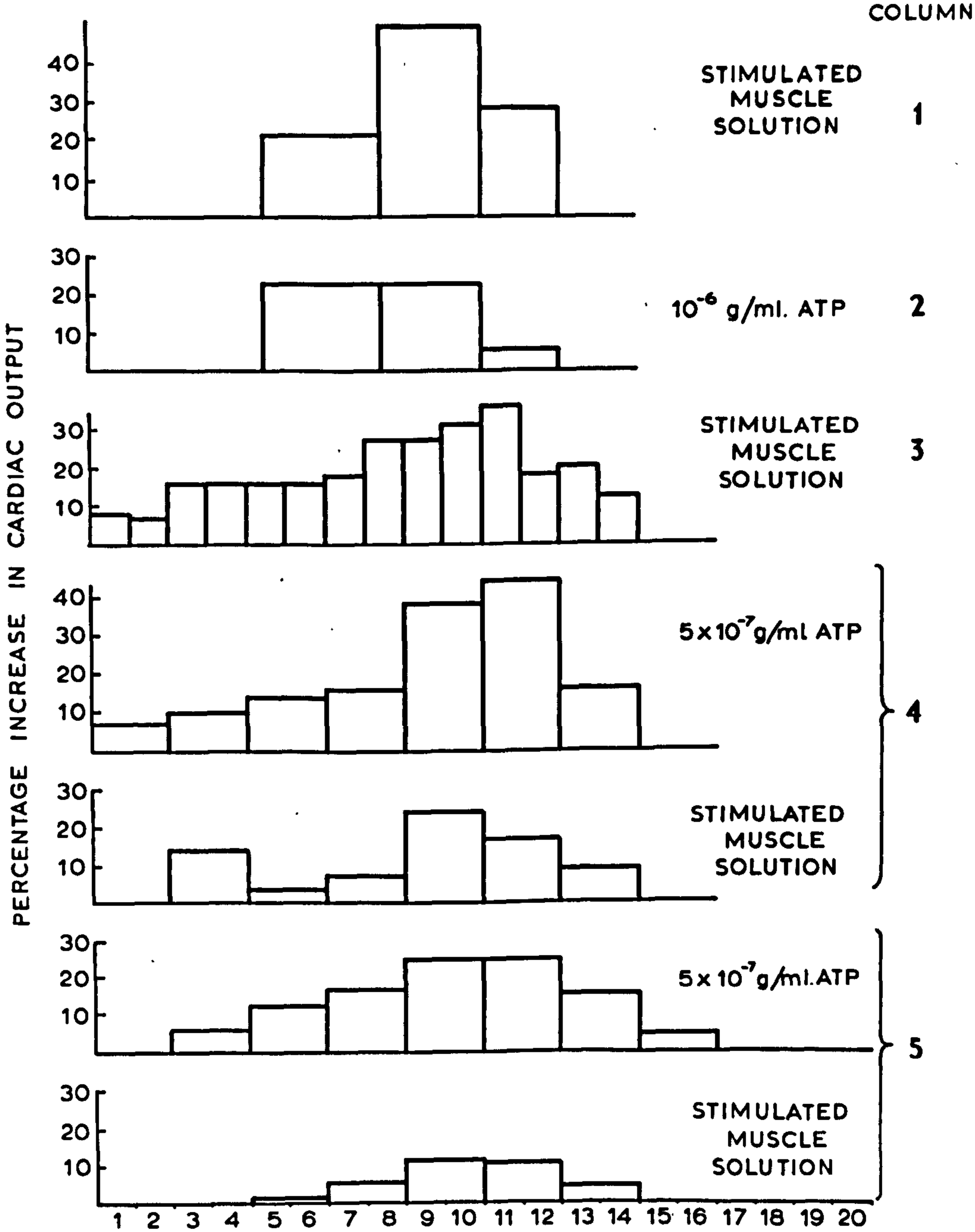


Figure 35.

Comparison of the behaviour of the stimulatory substance and ATP through five Sephadex columns.

seen, with the peak of stimulation occurring in fraction 5.

Stimulated muscle solutions were obtained in the usual way from two frog sartorius muscles. The solutions of 2 ml. each were thoroughly mixed and 2 ml. of this mixture were put through the same column as the ATP solution. Once again 2 ml. fractions were collected from the column. The result of perfusing these fractions through the same frog heart as in Figure 33 is shown in Figure 34 Although the stimulatory effect is not so marked, nevertheless it appears in the same fractions, the peak effect again being in fraction 5.

Figure 35 shows a comparison of elution patterns of both stimulated muscle solutions and solutions of ATP through 5 Sephadex columns. The percentage increase in cardiac output produced by each fraction was used as an indication of the concentration of the ATP and the stimulatory substance. The width of each block represents the size of successive fractions collected, the height represents the percentage increase in cardiac output produced by each fraction. It is clear that the elution pattern obtained from this column chromatography procedure is the same for the unknown substance as it is for adenosine triphosphate.

THE USE OF THE FIREFLY LUMINESCENCE PROCEDURE FOR THE IDENTIFICATION OF THE STIMULATORY SUBSTANCE RELEASED FROM ACTIVE SARTORIUS MUSCLE

When live fireflies (Photinus pyralis) are ground with sand and water, the extract obtained momentarily becomes luminescent; if the grinding is continued the light emission decays rapidly. If adenosine triphosphate (ATP) is added to this extract, a bright flash of light appears immediately and lasts for a considerable time, the intensity and duration of the flash depending on the concentration of the ATP (McElroy, 1947). It was noted by McElroy that with high concentrations of ATP a bright glow is maintained for some time, followed by a slow decay, while with low concentrations of ATP there is an initial burst of light, followed by a prolonged decay in the light signal.

Strehler and Totter (1952) thought that the firefly luminescence phenomenon could be adapted for the assay of ATP in living materials. Using refined light-measuring devices they emphasized that the system could be adapted to the measurement of ATP in very minute quantities. With a quantum-counting photo-multiplier at liquid nitrogen temperatures, they were able to measure concentrations of ATP as low as 10^{-9} g/ml. with an accuracy of $\pm$ 5%. They also emphasized the simplicity of the method, and recommended its use for rapid survey of ATP in many fields of investigation.

Principle of the Method

The linear luminescence response of firefly extracts to added ATP is the basis of this method of ATP analysis. The following reaction is generally agreed upon :

$$ATP + Mg^{++} + O_2 + \text{luciferin-}H_2 + \text{luciferase} = \text{LIGHT}.$$

Mn^{++} ions can replace the Mg^{++} ions and the oxygen content of the air is sufficient to support the reaction. In the presence of the enzyme luciferase the substrate for the reaction, luciferin, is oxidized with the production of light when

oxygen, Mg^{++} ions and ATP are present in excess. The optimum pH for the reaction is 7.8 and the maximum light intensity is observed at 26°C (Strehler and McElroy, 1957).

Specificity of the Reaction

The assay is specific for ATP. However, precursors of ATP will also produce light, provided that the appropriate enzymes for ATP formation are present. The purified enzyme preparation responds only to ATP. Adenosine diphosphate, creatine phosphate, acetylphosphate, inosine triphosphate, uridine triphosphate, guanidine triphosphate and inorganic polyphosphates do not produce light when added to extract of firefly (Strehler and McElroy, 1957).

Results

Five separate stimulated muscle solutions were tested on the firefly extract. On two of these occasions two stimulated muscle solutions were pooled and thoroughly mixed together so that there would be enough of the test sample for estimation on the firefly extract and for perfusion through the frog heart before and after incubation with the enzyme apyrase.

In all three cases the solution caused light to be emitted from firefly extract (Figure 36). In the cases where the solutions had been pooled, the same solution stimulated the heart in a similar manner to ATP, was modified in its action by apyrase, and caused light to be emitted from a firefly extract. When the effect of a pooled solution on the frog heart was matched with a solution of ATP it was found that the same concentration of ATP caused a similar light signal to be emitted from the firefly extract. Figure 37a shows the effect of perfusing graded concentrations of ATP solutions, ranging from 2.5×10^{-8} g/ml. to 7.5×10^{-7} g/ml, through a frog heart. The lowest concentration of ATP causes a sharp, evanescent increase in the systolic blood pressure with no effect on the heart rate, while a concentration of 7.5×10^{-7} g/ml. causes an exceedingly sharp increase in the blood pressure, followed two minutes alter by a gradual increase in heart rate. Graded effects are clearly seen with the intermediate concentrations.

Figure 36.

Comparison of the effects of stimulated muscle solutions and solutions of ATP on extracts of firefly. Deflection upwards from the baseline = light emitted.

(a) A solution of 10^{-8} g/ml. ATP emitted a similar light signal to that emitted from a stimulated solution.

(b) A solution of 5×10^{-8} g/ml ATP gave the same light emission as two pooled test solutions from the same firefly extract.

(c) The light emitted from a solution of 2×10^{-7} g/ml. ATP is shown. The cuvette was removed from the light-measuring device for a few seconds and then replaced. During this time no light was detected. This verified that the light signal was coming from no other source other than the solution. A stimulated muscle solution gave a similar emission of light from the same extract. In each case the voltage delivered to the photo-multiplier tube was 1,300 V.

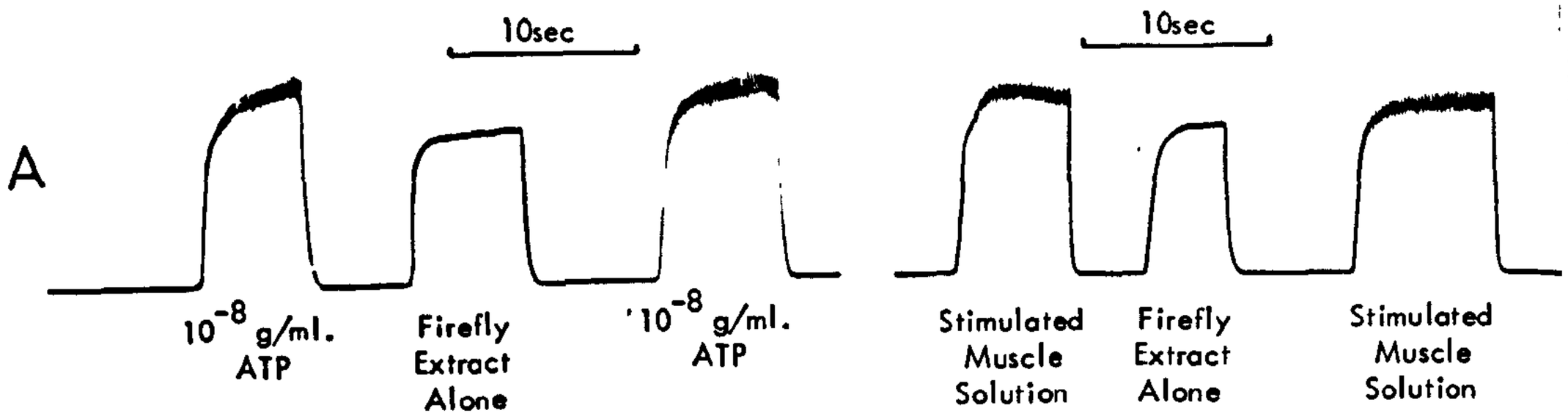

A
10sec
10^{-8} g/ml. ATP
Firefly Extract Alone
10^{-8} g/ml. ATP
10sec
Stimulated Muscle Solution
Firefly Extract Alone
Stimulated Muscle Solution
B
10 sec
Firefly Extract Alone
Stimulated Muscle Solution
10 sec
Firefly Extract Alone
5×10^{-8} g/ml. ATP
C
10 sec
2×10^{-7} g/ml. ATP
Sample Removed and Replaced
Stimulated Muscle Solution

Figure 37a.

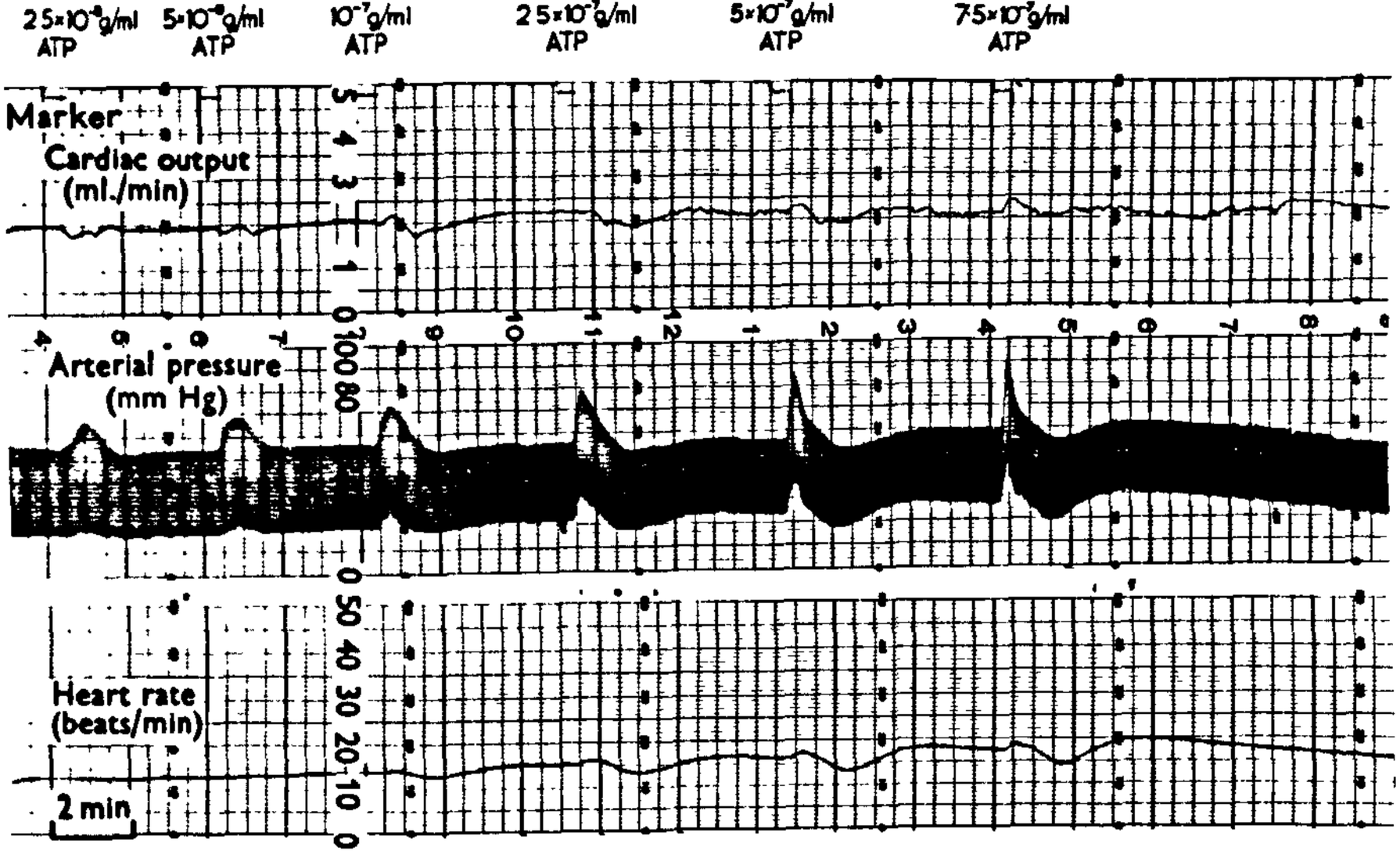
The effect of graded concentrations of ATP solutions when perfused through a frog heart. Note the late rate increase with the higher concentrations of ATP. The output trace is irregular due to a fault in the recording mechanism.

Figure 37b.

A recording of the same perfused heart as in 37a. The stimulated muscle solution has :-

1. been "bracketed" between solutions of ATP,
2. had its effect modified by apyrase,
3. given out a light signal from firefly extract (inset).

(a)


2.5×10⁻⁸g/ml ATP
5×10⁻⁸g/ml ATP
10⁻⁷g/ml ATP
2.5×10⁻⁷g/ml ATP
5×10⁻⁷g/ml ATP
7.5×10⁻⁷g/ml ATP
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
2 min

(b)

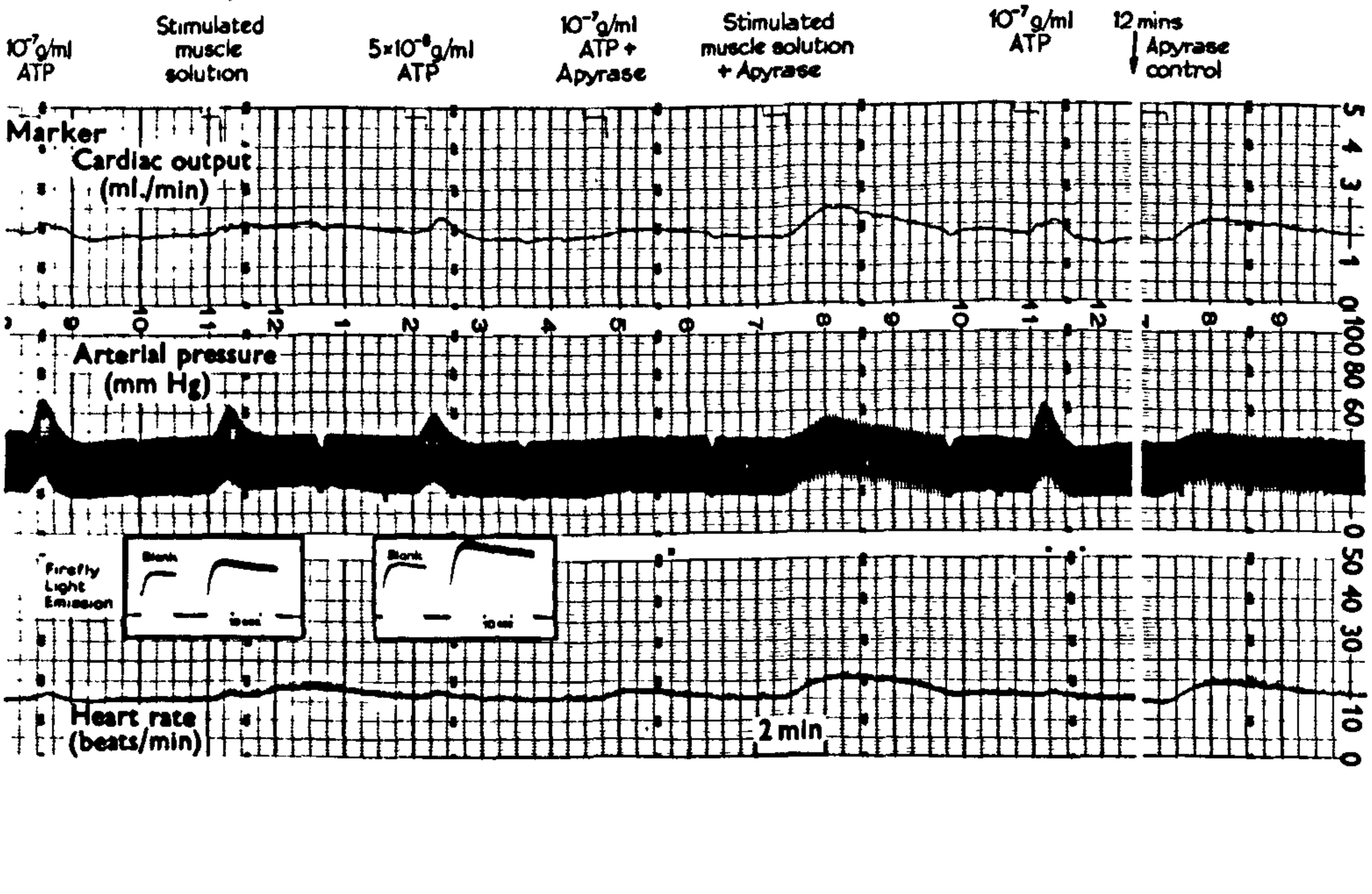

10⁻⁷g/ml ATP
Stimulated muscle solution
5×10⁻⁸g/ml ATP
10⁻⁷g/ml ATP + Apyrase
Stimulated muscle solution + Apyrase
10⁻⁷g/ml ATP
12 mins
Apyrase control
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Firefly Light Emission
Blank
Heart rate (beats/min)
2 min

Figure 37b is a recording of the same heart as in 37a. The effect of a stimulated muscle solution has been "bracketed" between concentrations of 10^{-7} g/ml. and 5×10^{-8} g/ml. ATP. There is a late increase in the heart rate associated with the test solution, but not with the matching ATP solutions (Figure 38). This phenomenon has been noted before (Table 9). After incubation with apyrase, 10^{-7} g/ml. ATP does not give any effect; incubation of the stimulated muscle solution with apyrase caused a modification of the heart's reaction. The sharp increase in blood pressure has been abolished, but the increase in heart rate persisted. That the effect has not been abolished completely (see Figure 29 for example) is probably due to insufficient time for incubation (see discussion of incubation time in "apyrase" section). The 10^{-7} g/ml. solution of ATP was again perfused through the heart in order to establish that the heart still retained its sensitivity to ATP. Apyrase, 1 mg/ml., incubated with frog Ringer's solution was perfused through the heart 12 minutes later. An increase in heart rate and output with cardiac irregularity occurred. This again was probably due to insufficient time for incubation.

Figure 38 shows an enlargement of the lower left quadrant of Figure 37b The inset shows the light emission from firefly tail extract, an upward deflection from the baseline indicating a light signal. The firefly extract alone emitted a small steady signal; when the stimulated muscle solution was added there was an increase in the light emission. This was the same pooled test solution as that perfused through the frog heart shown in this figure. When a concentration of 5×10^{-8} g/ml. ATP was added to another sample of the same firefly extract, a similar increase in the light signal was produced. The amplitude control of the pen recorder was increased for the second record, but there is a proportionately similar increase in the light signal emitted from the test solution and ATP solution than that emitted from the extract alone.

The nature of the light signal would suggest that the light was being emitted from both the test solution and the ATP solution in the same pattern of

Figure 38.

An enlargement of the lower left quadrant of Figure 37b. The frog heart trace "A" shows the qualitative similarity of the effect of the stimulated muscle solution to the effects of two ATP solutions. With the stimulated muscle solution there is an increase in the heart rate approximately 2 minutes later. This is not seen with the ATP solutions.

The inset "B" shows the light emission when the same stimulated muscle solutions and a solution of 5×10^{-8} g/ml. ATP are placed in contact with firefly extract. There is a proportionately similar increase in the light signal emitted from the test solution and ATP solution than that emitted from the extract alone.

10^{-7}g/ml ATP

Stimulated muscle solution

5×10^{-8}g/ml ATP

Marker

A

Cardiac output (ml./min)

Flush capsule

Arterial pressure (mm Hg)

B

Firefly Light Emission

10 sec

Firefly extract alone

Stimulated muscle solution

Firefly extract alone

10 sec

5×10^{-8}g/ml ATP

Heart rate (beats/min)

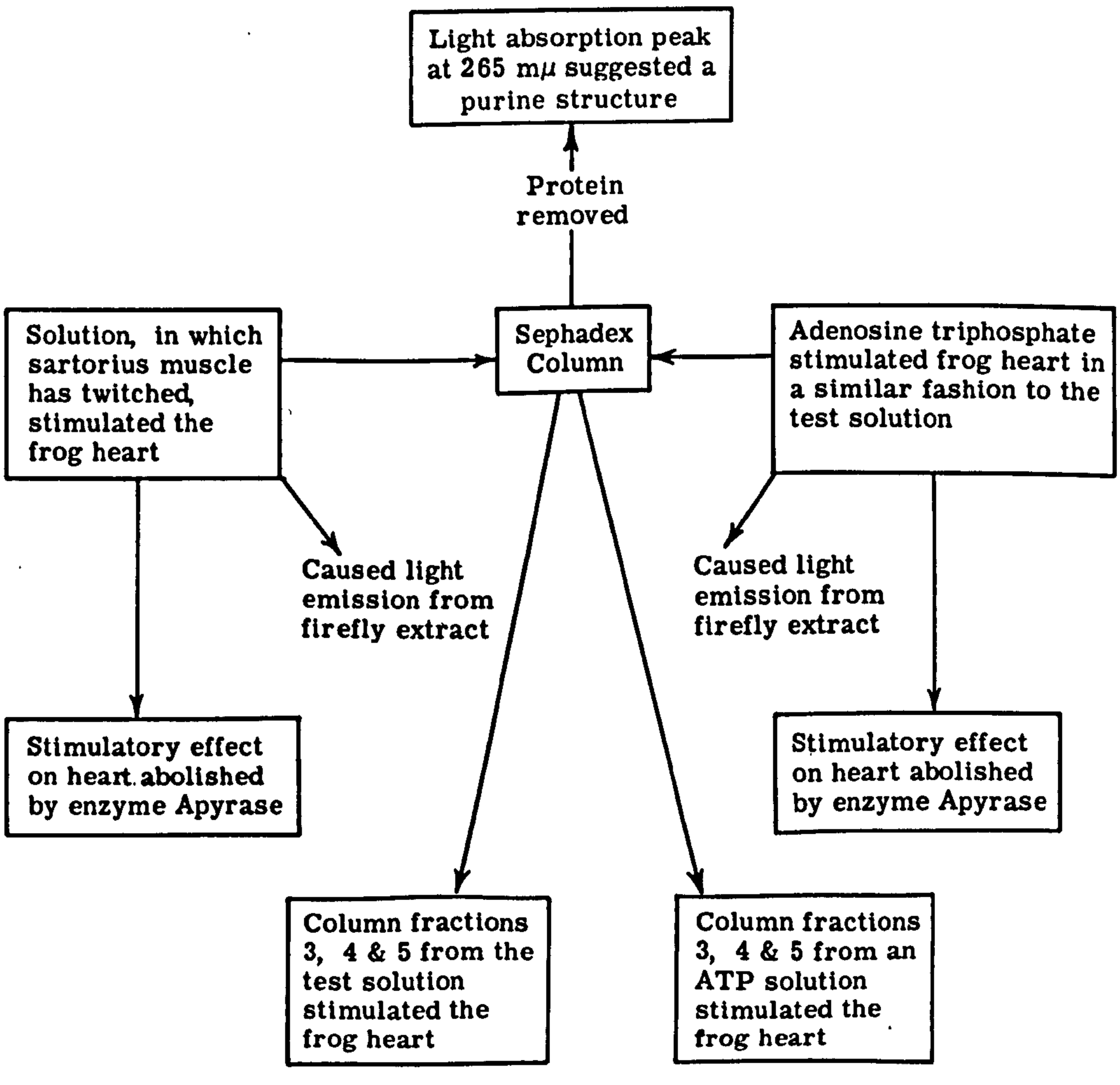
2 min

scintillation.

From these results it is concluded that ATP is present in the test solution and that its presence is the cause of the stimulatory effect on the frog heart.

Figure 39.

DIAGRAM SUMMARIZING THE IDENTIFICATION PROCEDURE OF THE STIMULATING SUBSTANCE RELEASED FROM ACTIVE SARTORIUS MUSCLE



AN ESTIMATE OF THE AMOUNT OF ATP RELEASED FROM ACTIVE SARTORIUS MUSCLE

It was possible to match the stimulatory effect of the test solutions to the effects of known concentrations of ATP on the same frog heart using the initial positive inotropic effect as a measure of the concentration of ATP in the stimulated muscle solution (Figures 28 & 29).

Results

The effects of 13 test solutions were approximately matched to the effects of known concentrations of ATP on 13 frog hearts. Two test solutions were matched to ATP solutions using both the frog heart and the firefly technique; one solution was matched using the firefly technique only. (Table 10).

The mean output of ATP estimated from 14 sartorius muscles was just less than 6×10^{-7} g (range 2×10^{-6} g - 2×10^{-8} g). No accurate estimate of ATP output per gramme of muscle tissue can be given, since the muscles were not weighed beforehand, but a fairly constant size of frog was used. An approximate value can be calculated from the weights of other sartorii weighed later in this work. The average wet weight was 50mg and certainly all muscles used did not weigh more than 100mg; if the lower figure is taken, the output of ATP per gramme of wet muscle is 12μg. Since about 30% of the weight of these muscles is contributed by extracellular fluid (Dydynska and Wilkie, 1963), the output of ATP per g of dry muscle is 17μg (0.027μmole). The intracellular amount of ATP has been estimated at 2 - 4μmole per gramme of dry muscle (Maréchal, 1964) so that there is roughly a 1% loss of intracellular ATP into the solution bathing the sartorius muscle.

It can be seen from Table 10 that in 10 hearts out of 13 the test solution produced an increase in the heart rate. This rate increase usually reached peak value 2 minutes after the initial, sharp stimulatory effect. In six hearts out of 13 the matching ATP solution had no effect on the heart rate, one solution

actually producing a fall in heart rate. In only two cases did the matching ATP solution produce a greater rise in rate than that caused by the stimulated muscle solution. An increase in the heart rate occurring two minutes after the perfusion can be produced with higher concentrations of ATP (Figure 37a). However, the concentrations of ATP required to match the initial stimulatory effect of the test solutions did not usually produce the rate effect caused by the test solutions. It is probable that the test solutions contained other material liberated from active skeletal muscle which acted on the conducting tissues of the heart rather than on the myocardium itself.

Table 10.

MEASUREMENT OF THE AMOUNT OF ATP RELEASED FROM THE SARTORIUS MUSCLE

Heart	% Increase in Cardiac Output		% Increase in Heart Rate
1	test solution	32	7
	2.5×10^{-7} g/ml. ATP	40	4
2	test solution	24	7
	10^{-6} g/ml. ATP	30	-
3	5×10^{-8} g/ml. ATP	27	-
	test solution	36	20
	10^{-7} g/ml. ATP	33	-
	(test solution was equivalent to 5×10^{-8} g/ml. ATP using firefly)		
4	test solution	56	4
	5.0×10^{-7} g/ml. ATP	60	5
5	test solution	45	12
	10^{-7} g/ml. ATP	35	-
6	test solution	12	-
	10^{-7} g/ml. ATP	15	-9
7	test solution	25	-
	5.0×10^{-7} g/ml.	22	-
8	test solution	11	24
	5.0×10^{-8} g/ml. ATP	11	-
9	test solution	18	10
	5.0×10^{-7} g/ml. ATP	21	24
10	test solution	35	15
	5.0×10^{-7} g/ml. ATP	30	10
11	test solution	28	-
	10^{-7} g/ml. ATP	36	-
12	10^{-7} g/ml. ATP	33	3
	test solution (pooled two)	36	8 bracketed
	2.5×10^{-7} g/ml. ATP	49	8
13	test solution	18	15
	5.0×10^{-8} g/ml. ATP	30	3
	(test solution was equivalent to 10^{-8} g/ml. ATP using firefly)		
14	test solution equivalent to 2.0×10^{-7} g/ml. ATP using firefly		

THE SOURCE OF ATP RELEASED FROM SKELETAL MUSCLE

Accidental Sources of ATP

Adenine nucleotide material has been shown to be present in abundance intracellularly (Glick, 1946). The ATP released from skeletal muscle, described here, could have many sources. Damaged muscle cells could release significant amounts of ATP into a surrounding bathing solution. Distortion and rupture of connective tissue cells during the contraction process could also result in some efflux of intracellular ATP into the bathing solution.

Evidence from penetration of the frog muscle cell with micro-electrodes has shown that even after repeated punctures of the same cell, the resting membrane potential remained around 80 mV (Ling and Gerard, 1949). This suggests that the membrane seals itself off in some way after the removal of the microelectrode. The utmost care was taken in the dissection of the sartorius muscle in this work and in every case the muscle was carefully washed and left to soak in a "washing" solution for 10 minutes before the period of stimulation. The contents of any grossly damaged cell would presumably be completely washed out during this procedure.

Another argument against cellular damage as a cause of the presence of ATP in the test solution can be made by citing the results obtained concerning the potassium efflux from the sartorius muscle. Any gross cellular damage would allow large amounts of intracellular potassium to pass into the surrounding bathing solution. In fact no obvious rise in the potassium level in resting muscle solutions was found. The average increase of 25% in potassium in the stimulated muscle solutions is consistent with the normal potassium efflux associated with the propagated action potential (Hodgkin and Horowicz, 1959).

Red blood corpuscles are known to have high concentrations of ATP in their surrounding membranes(Whittam, 1960).If any red blood corpuscles were not initially washed out and became haemolysed during the period of stimulation,

ATP might be detected in the bathing solution.

The only argument presented here against the source of the ATP being ruptured envelopes of haemolysed red blood corpuscles is that the careful washing procedure, coupled with a virtually bloodless dissection, left no obvious collections of RBCs within the muscle bundles. After the period of stimulation the bathing solution was slightly opaque (presumably due to the output of protein from the muscle), but contained no obvious collections of red blood corpuscles.

The Sarcoplasmic Reticulum as a Source of ATP

It is proposed here that the source for the ATP released from the sartorius muscle is the transverse tubular system of the sarcoplasmic reticulum. The nature of this system will now be described briefly.

Observations under the electron microscope (Bennett and Porter, 1953; Porter 1956; Edwards, Ruske, Sonza-Santos and Vellejo-Freire 1956; Porter and Palade 1957) have revealed the presence of an intricate system of internal tubes in skeletal muscle fibres. This system has been given the name "sarcoplasmic reticulum".

Several investigators have suggested that this reticulum may provide a rapid pathway for the inward spread of the depolarization wave from the external membrane during electrical excitation of the muscle fibre.

A summary of the anatomy of this system is given in Figure 40.

H.E. Huxley (1964) and S. Page (1964) have shown in elegant electron microscopic studies that the central element (T-system) of the triad formation in frog muscle fibres is accessible to particles as large as ferritin and colloidal gold (diameters 110 A° and 200 A° respectively, Figures 41 & 42). This gave the first direct evidence of continuity between the lumen of the triad tubules and the extracellular space in frog skeletal muscle.

Endo (1966) has recently investigated the properties of the sarcotubular system in frog skeletal muscle. He found that various fluorescent dyes could diffuse freely

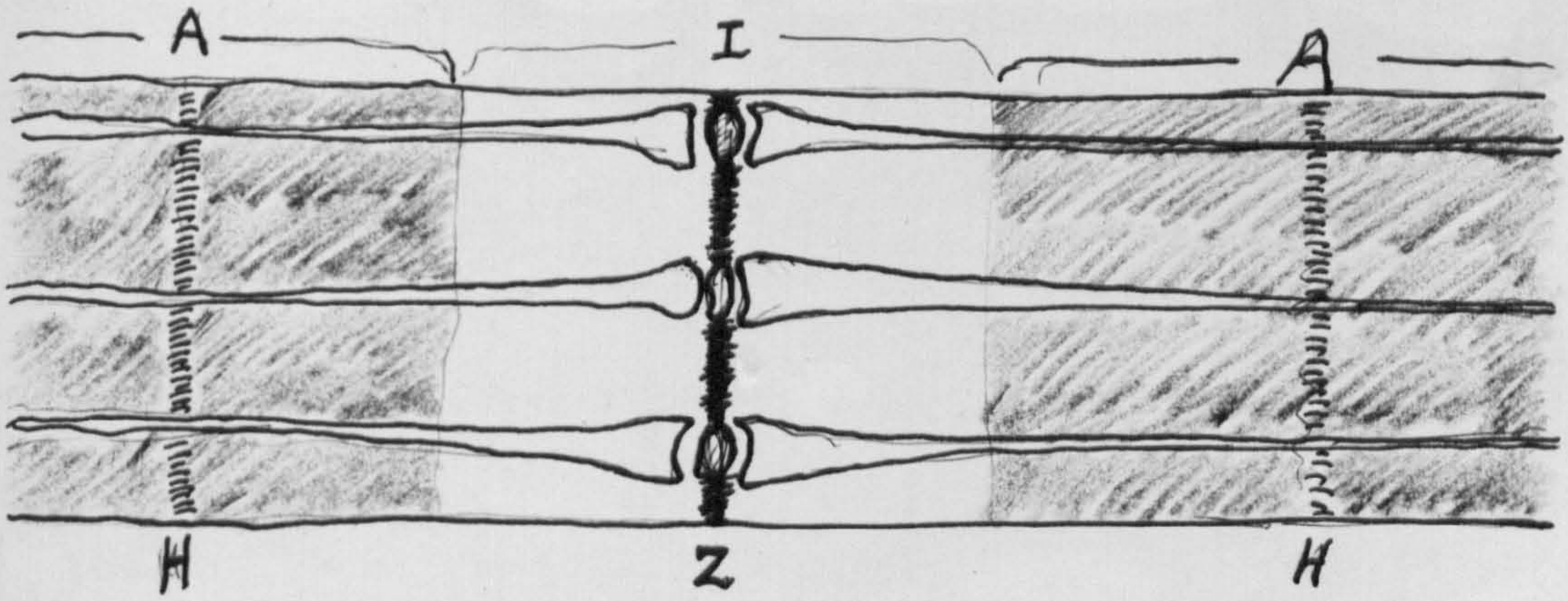
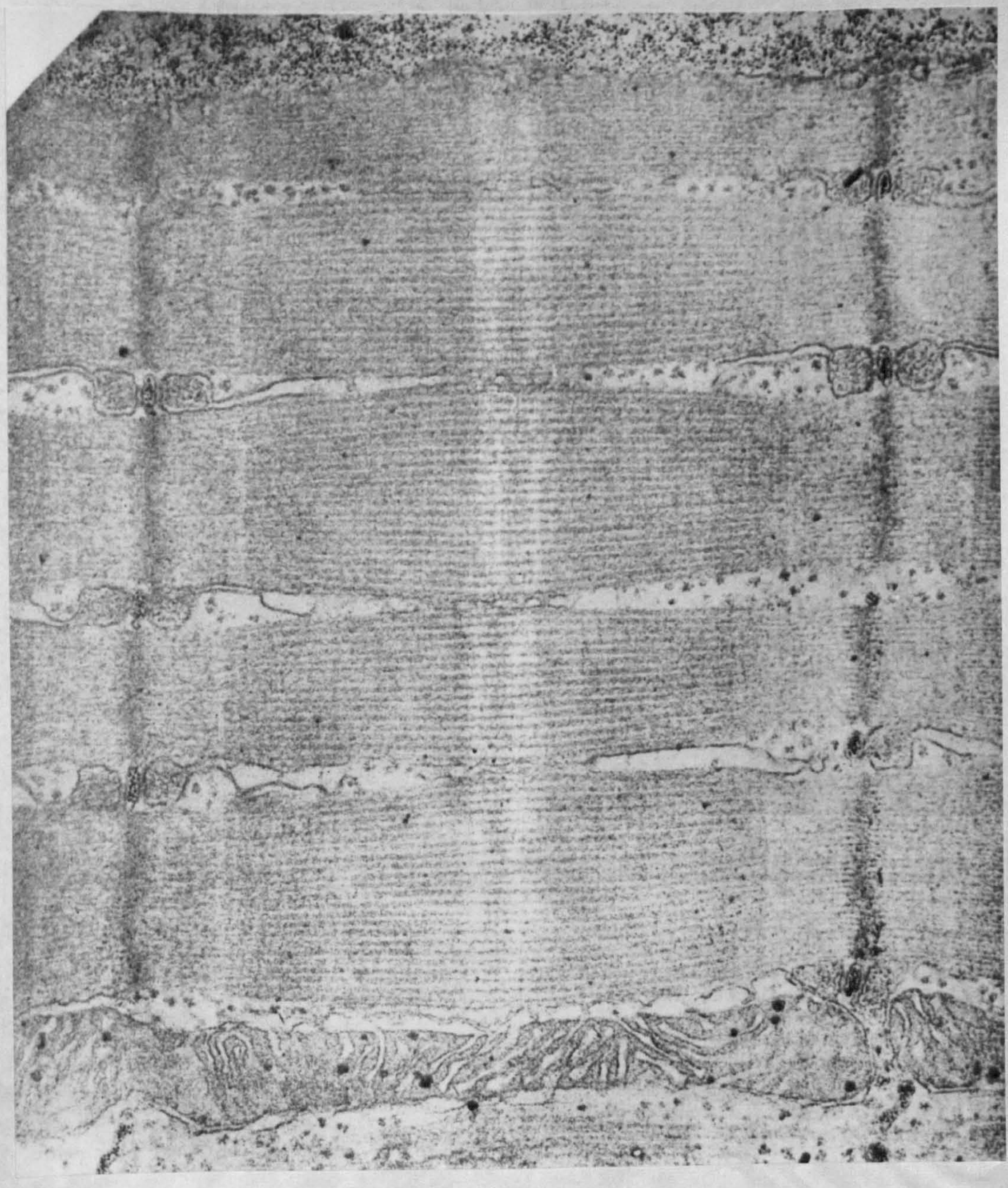


Figure 40.

The system consists of two types of channel which do not communicate directly with each other or with the main part of the muscle sarcoplasm. One part of this system has the form of narrow channels, 200A - 300A in diameter, running alongside each myofibril for nearly all of its length. In the case of the frog sartorius muscle these channels are interrupted at the level of the Z lines. Opposite the I bands the narrow channels open out into a broader terminal region of about 1,000A in width.

The other part of the reticulum has transversely-orientated tubules and is termed the "T-system". These tubules occur at the level of the Z lines in frog sartorius muscle and are possibly continuous for considerable distances across the muscle fibre.

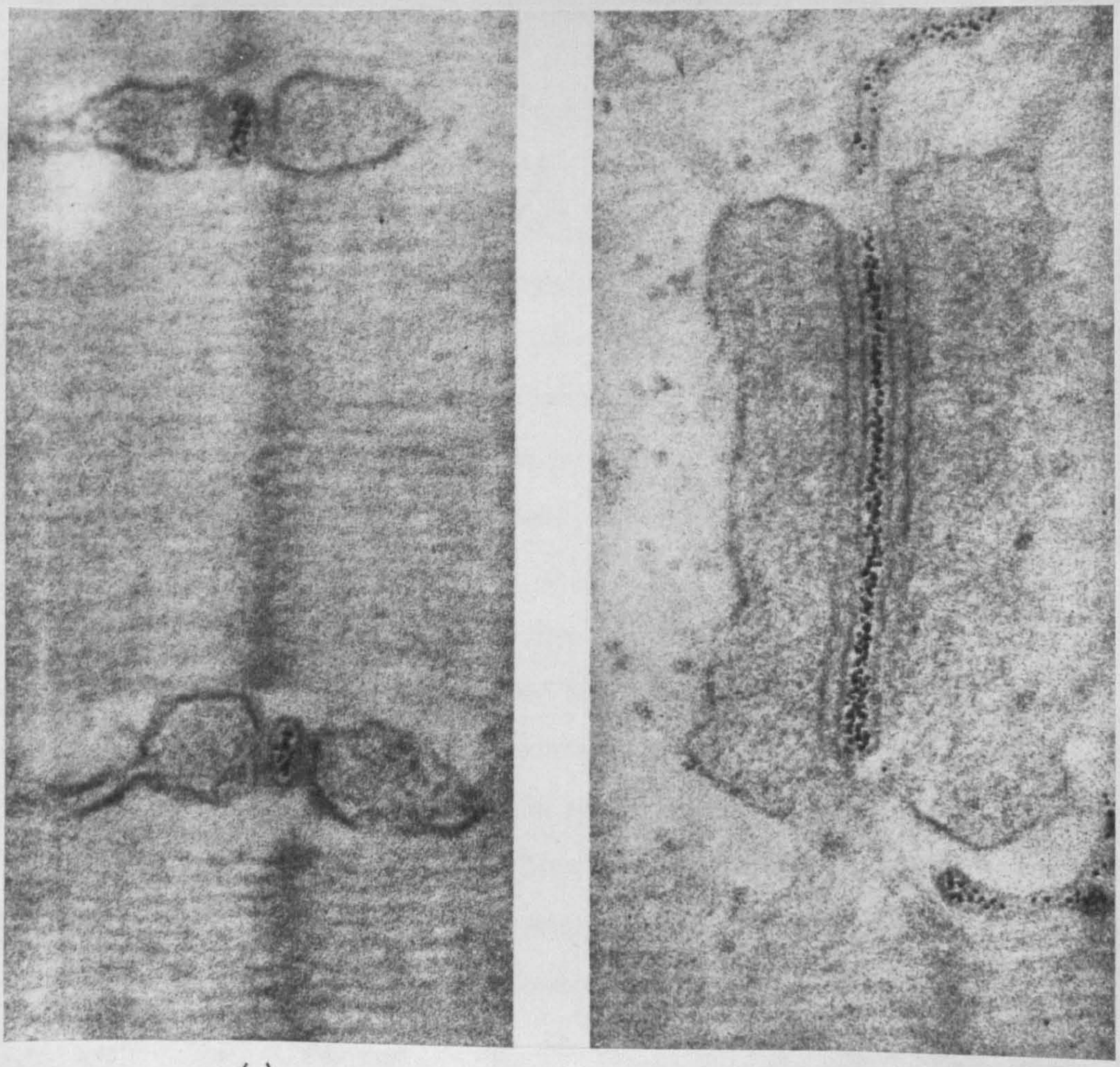
Where the terminal regions of the longitudinal system come into contact with the transverse system, a structure known as a "triad" is formed. Thus a triad consists of a central channel (part of the T-system) flanked on either side by the terminal vesicles of the longitudinal system.



.Figure 38.

A longitudinal section of frog sartorius muscle (x50,000) which had soaked for one hour in a ferritin solution before fixation. The ferritin has only penetrated the T-system of the sarcoplasmic reticulum. Note the relationship of the T-system of tubules to the position of the dark Z line.

(Figures 38 and 39 reproduced by kind permission of Professor H.E. Huxley and the Editor of "Nature".)



(a) (b)

Figure 42a

A higher magnification (x 145,000) of the "triad" structures seen in the previous figure. The granules of ferritin molecules are clearly seen in the lumen of the transverse tubules.

Figure 42b.

A longitudinal section through a transverse tubule at the same magnification. Portions of the T-system can be seen leading away from this triad structure.

(Figure 42a and 42b reproduced by kind permission of Professor H. E. Huxley and the Editor of "Nature").

into and out of a periodically arranged system of tubules which were situated at the level of the Z line. He concluded that the dyes have some access to a component of the triad system. The molecular weight of the dyes used was of the order 600, yet he found in a single muscle fibre preparation that they could diffuse into and out of the fibre within a period of two minutes. By studying the flow rate of these dyes into the fibre he concluded that most of the resistance offered by the tubule to the dye was evenly distributed along the length of the tubule. There did not seem to be any obstruction to the diffusion of the dye at the entrance of the tubule.

Endo estimated that the size of the space into which the dyes were diffusing was 1%- 2% of the fibre volume. This figure lies between the figure of 0.2% calculated for the volume of the central canal of the triad system and the figure of 5% calculated for the whole triad system (Page, 1964; Peachey 1965).

During the process of muscular contraction the T-system must undergo a reduction in its lumen volume. As clear entry and exit of large molecules to and from this system has been demonstrated, it is possible that the nucleotides are squeezed out of the T-system into the extracellular space where they are free to diffuse to the local arterioles. The "pumping" action proposed here implies that the muscle should be able to contract and relax before the most effective release of ATP is achieved. The findings of Hilton (1962) in his human forearm experiments seem to support this hypothesis. No ATP was found in the perfusates of muscles which were not allowed to contract, but in the experiment where they were allowed to contract, a great amount of ATP was detected. This large amount probably has two components; that amount which was released from damaged tissue, since the method employed two needles embedded into the contracting muscle belly, and the "physiological" amount released from muscle which was being allowed to contract, and hence was pumping adenosine triphosphate out from the sarcotubular system.

Other evidence to support this hypothesis is readily available. Many workers

(Caspersson and Thorell, 1942; Engstrom, 1944; Perry, 1952; Hill, 1959 - 1960) have tried to display a special site for ATP within the muscle cell in relation to the actin-myosin complex.

Caspersson and Thorell (1942) used the technique of photographing living skeletal muscle from various species under an ultraviolet microscope. The wavelength was adjusted to 257 mμ so that the high specific absorption of the purine ring would outline the position of the adenylic acids.

They found that in living muscle fibres before exercise a clear picture of sharply differentiated light and dark bands running across the muscle fibres was obvious; after vigorous exercise the living muscle fibre showed that the sharp differentiation between these bands was, in their own words, "more or less smudged out". In resting muscle fibres they found that the substances which strongly absorbed the UV light were most concentrated nearest to the I band of the sarcomere. In muscle which had been fatigued from exercise they found that the A band of the sarcomere showed extremely fine longitudinal striations. These appeared most clearly nearest to the I band and diminished in clarity towards the middle of the A band.

They felt that the only explanation for such a picture was that the strongly absorbing material located near the I band had travelled towards the middle of the A band through a very fine series of longitudinal channels. However, it is proposed here that the material was, in fact, moving in the other direction, that is, from the A band into the I band. The corollary to this view is that the adenine nucleotides are synthesized in the A band and pass along the longitudinal tubules into the I band. At this stage a transfer mechanism for the nucleotides to pass from the longitudinal system to the T-system must be presumed, although no connections between the systems have yet been demonstrated.

A. Engstrom (1944),using a microincineration technique for locating phosphates, showed that the high UV absorption of the isotropic band coincided in position with his newly-found source of phosphate ash. In frog's muscle nearly

one third of the total phosphorus is in the form of adenine nucleotide, so it is likely that Engstrom had outlined one situation of adenine nucleotides in skeletal muscle. S.V. Perry (1952) has shown that about 10% of the total adenine nucleotide in rabbit muscle is bound to the myofibrils and can only be freed by acid. The other 90% of adenine nucleotide is free and occupies the sarcoplasmic space.

D. K. Hill (1959) provided yet another means of approaching the problem of localization of nucleotides in skeletal muscle. Hill developed a method of incorporating tritiated adenine into frog's skeletal muscle. He showed that the labelled material inside the muscle cell was largely adenosine triphosphate. He was able to obtain autoradiograms from freeze-dried and fixed specimens; by staining for the striations at the same time, he could locate more accurately the position of the adenine nucleotides with reference to the sarcomere. He found that about 70% of the nucleotide lay within the I band of the sarcomere and he noted one surprising feature; this nucleotide seemed to have "a transverse freedom of movement within the muscle fibre". He put forward the suggestion that the facility for this transverse diffusion could be provided by the sarcoplasmic reticulum.

Summary and Conclusions

A combined survey of the works considered above reveals the following chain of evidence:-

(a) Adenine nucleotides and, according to D. K. Hill, principally adenosine triphosphate, are found to be concentrated at a certain part of the sarcomere; namely, at the Z line in the isotropic band.

(b) Also lying in this region of the sarcomere is the transverse tubule system of the sarcoplasmic reticulum.

(c) The "transverse freedom of movement" of the adenine nucleotide material described by Hill in the region of the Z line correlates with the experiments of H. E. Huxley, S. Page and

M. Endo who have all demonstrated the free movement of large molecules within the transverse tubular system. These molecules are also able to pass freely between the extracellular space and the transverse tubules.

(d) Caspersson and Thorell noted that the pattern of adenine nucleotide material in the muscle fibres changed after the muscle had been exercised. Whereas before exercise the nucleotides were concentrated in the I bands, after exercising the muscle they found that the A band showed exceedingly fine longitudinal striations, which became clearer when the I band region was approached. They concluded that the material had moved from the I band through a series of fine, longitudinal channels towards the middle of the A band.

To the above list of experimental findings can now be added the evidence from the present work that ATP is released from an active frog sartorius muscle.

Since adenine nucleotide material is present in the region of the Z line and has been shown to have the transverse mobility by D.K. Hill (1960), and since the T-system of the sarcotubular reticulum lies at the position of the Z line, it does not seem unreasonable to assume that the adenine nucleotides are lying in the T tubes. There does not seem to be any obstruction to the extracelluar space from the T-system, so that any ATP lying in the T-tubules would be easily "pumped" out by contracting skeletal muscle.

THE EFFECT OF APYRASE ON THE FROG HEART RESPONSE TO ACh

In order to assay the small amount of ACh released from sartorius muscle on the frog heart preparation, elimination of the stimulatory effect is essential. From the identification procedures used to establish that the stimulatory substance in the solution bathing the muscle is adenosine triphosphate,it appears that the most convenient way of eliminating the ATP effect on the frog heart from the test solution is by incubating it with apyrase for 15 minutes.

The effect of apyrase on the sensitivity of the frog heart to ACh, however, was not known, and therefore the following experiment was designed.

A frog heart was perfused with a finely graded series of ACh concentrations and a concentration-response curve was obtained. The same series of solutions of ACh was then incubated in a waterbath at $30^{\circ}C$ for 10 minutes and a concentration-response relation on the same frog heart was again obtained. A controlled series of incubated ACh solutions alone were perfused through the frog heart and concentration response curves were obtained for the cardiac output and heart rate.

Figure 43 shows the percentage inhibition of the cardiac output and heart rate for the normal ACh series, the ACh series incubated with apyrase and the incubated ACh control series. In this particular heart the threshold concentration of ACh was 2.5×10^{-9} g/ml. This minimal concentration required to inhibit the heart remained the same in the incubated control series, but the threshold concentration required to inhibit the heart after the solutions of ACh had been incubated with apyrase was markedly raised to above 7.5×10^{-9} g/ml. ACh. This has been found in 2 other heart experiments.

Figure 44 shows part of a recording of a perfused frog heart showing the effect of incubating two solutions of ACh with apyrase. The cardiac output is easily seen to be reduced when the solutions of 10^{-8} g/ml. and 1.5×10^{-8} g/ml. of ACh after incubation with apyrase are perfused through the heart. The effect

Figure 43.

The effect of apyrase and incubation on the frog heart's sensitivity to ACh.

(a) The percentage output inhibition (ordinate) is plotted against the concentration of ACh applied to the heart (abscissa).

(b) The percentage rate inhibition (ordinate) is plotted against the concentration of ACh applied to the same heart (abscissa).

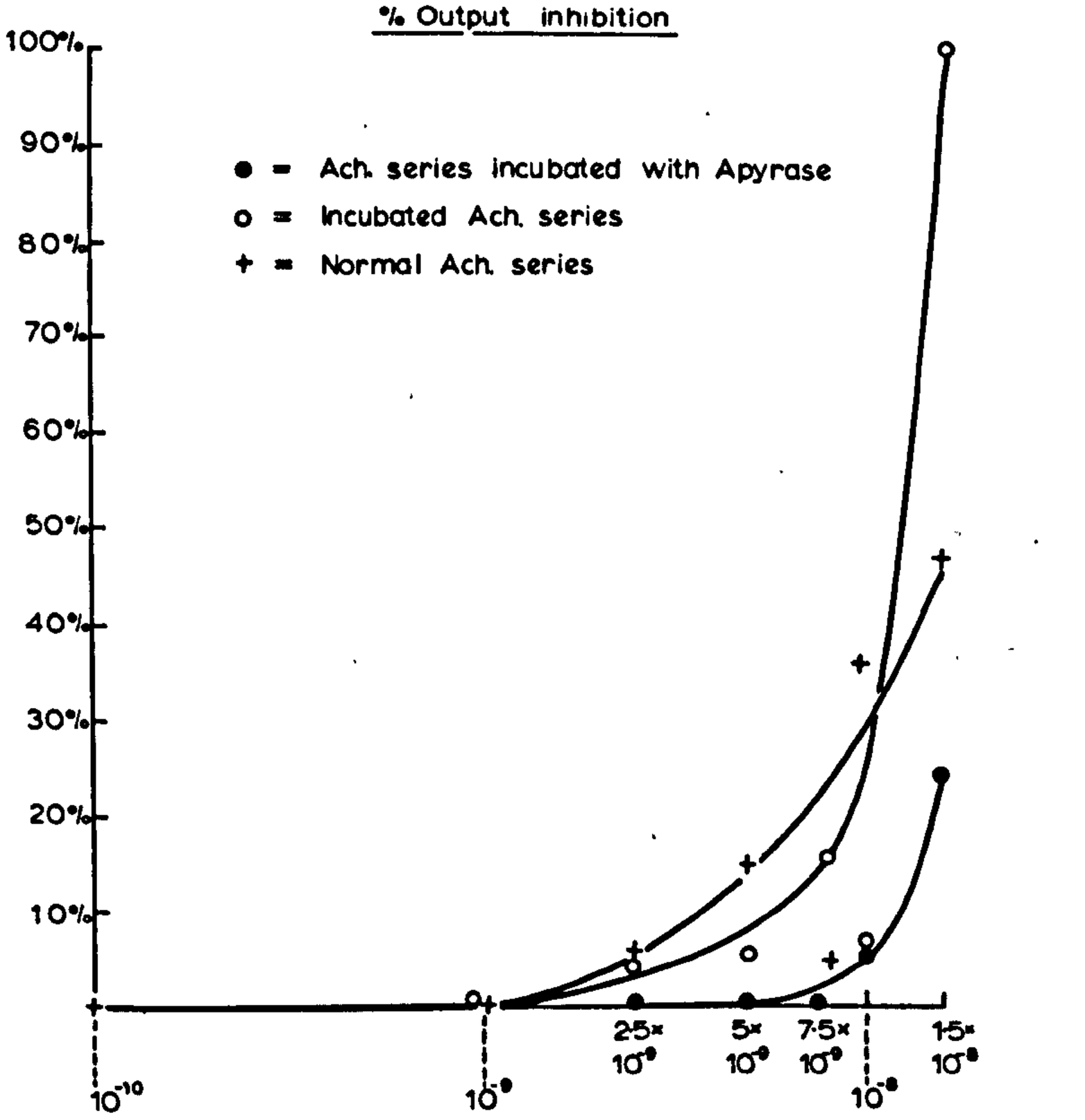
Note that the response of the heart falls when ACh which has been incubated with apyrase is perfused through the heart. This effect could be due to

(1) destruction of ACh by apyrase

(2) alteration of the sensitivity of the myocardium by the apyrase in the perfusate.

It is likely that an ATP-ase such as apyrase will depress the sensitivity of active tissue such as myocardium.

(a)


Effect of Apyrase and Incubation on Heart sensitivity to Ach.
% Output inhibition
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
● = Ach. series incubated with Apyrase
o = Incubated Ach. series
+ = Normal Ach. series
2·5× 10⁻⁹
5× 10⁻⁹
7·5× 10⁻⁹
1·5× 10⁻⁸
10⁻¹⁰
10⁻⁹
10⁻⁸

(b)

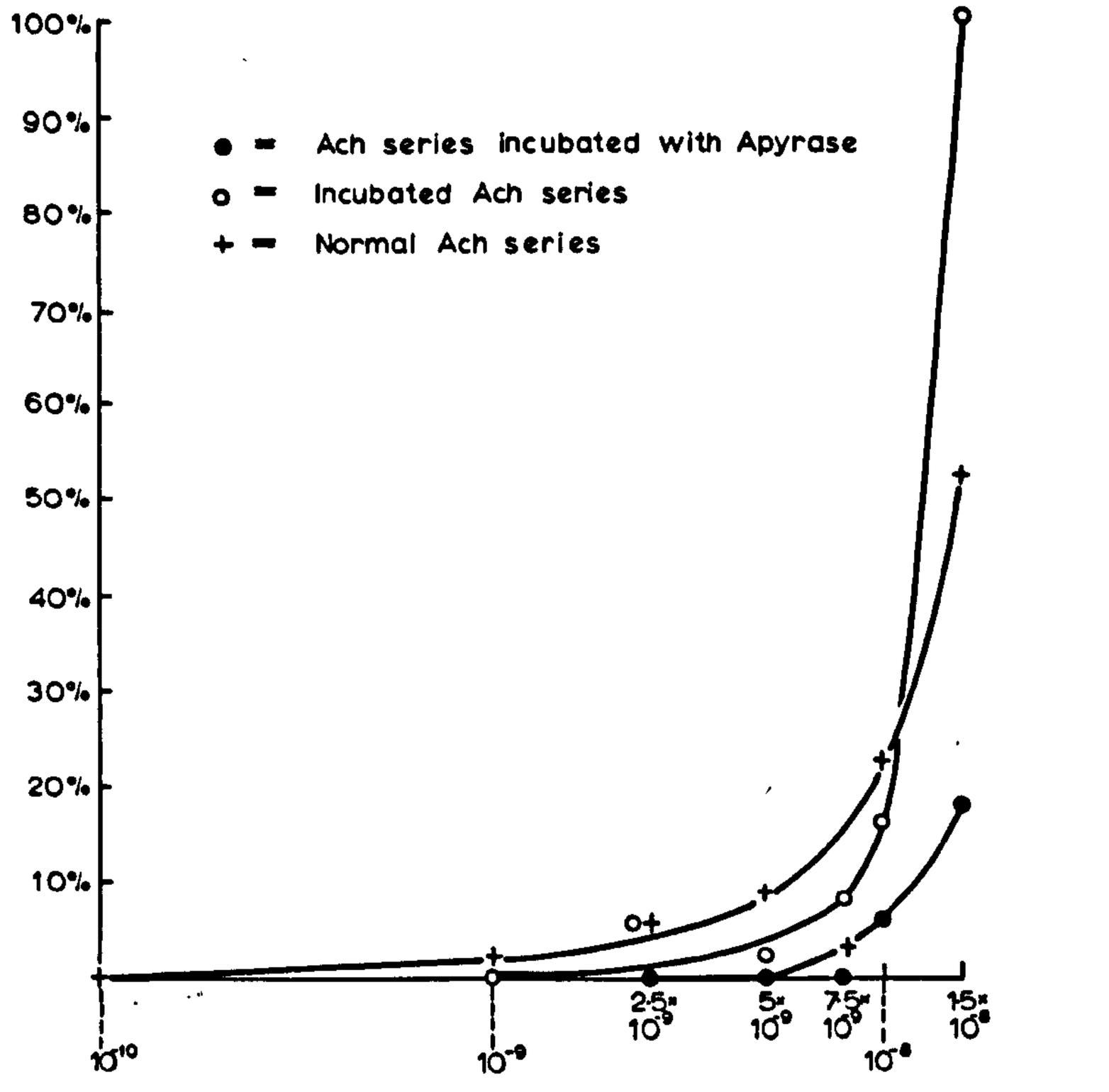

Effect of Apyrase and Incubation on Heart sensitivity to Ach.
% Rate inhibition
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
● = Ach series incubated with Apyrase
o = Incubated Ach series
+ = Normal Ach series
2·5× 10⁻⁹
5× 10⁻⁹
7·5× 10⁻⁹
1·5× 10⁻⁸
10⁻¹⁰
10⁻⁹
10⁻⁸

Figure 44.

The effect on a frog heart of ACh solutions which have been incubated with apyrase.

Two solutions of ACh, 10^{-8} g/ml. and 1.5×10^{-8} g/ml., were incubated with apyrase for 10 minutes, and then perfused through a frog heart. The effect of these solutions can be seen on the left of the trace. The heart rate recording should be ignored in this trace (technical fault). The cardiac output and blood pressure are diminished, but not as much as when the untreated solution of ACh is perfused. The next two perfusions of 10^{-8} g/ml. ACh and 1.5×10^{-8} g/ml. ACh show a clear increase in the inhibition of the heart beat. The last two perfusions show that the incubation procedure alone does not alter the action of ACh in any way. The same amount of output inhibition occurs as with the untreated ACh solutions.

10^{-8}g/ml Ach+apyrase

1.5×10^{-8}g/ml Ach+apyrase

10^{-8}g/ml Ach

1.5×10^{-8}g/ml Ach

Injection control

10^{-8}g/ml Ach-Incubated

1.5×10^{-8}g/ml Ach-Incubated

Marker

Cardiac output (ml./min)

5 4 3 2 1 0

Arterial pressure (mm Hg)

150 100 50

Heart rate (beats/min)

100 80 60 40 20 0

Venous pressure (mm H_2O)

50 40 30 20 10 0

2 min

Drain & refill

Drain & refill

Drain & refill

Drain & refill

Drain & refill

of these solutions of ACh when incubation alone takes place are seen to be the same as the normal series.

Discussion

The three experiments on the frog heart response to ACh were all performed on frog hearts which were very insensitive to the action of ACh. However the apyrase does seem to depress the response of the frog heart to ACh quite substantially, even in these insensitive hearts. This suggests that the effects of incubating smaller amounts of ACh with apyrase would be more profound and a great deal of the advantage of using a sensitive preparation to ACh is wasted. So the problem of assaying ACh released from the frog sartorius muscle on the frog heart is far from being solved.

It is to be noted here that incubation of pure ACh solutions at 30°C for 10 minutes surprisingly does not cause hydrolysis of ACh. Diluted solutions of ACh have been regarded in the past as being very unstable, but this view has to be modified in the light of these findings.

PART II

THE ASSAY OF ACETYLCHOLINE RELEASED FROM THE NEUROMUSCULAR JUNCTION.

THE ISOMETRIC LEECH MUSCLE STRIP AS AN ASSAY PREPARATION FOR ACETYLCHOLINE

INTRODUCTION

The Pharmacology of Leech Muscle

In 1918 Fuehner introduced the leech (Hirudo medicinalis) as a suitable bio-assay object for the estimation of physostigmine (eserine). The complementary actions of acetylcholine and eserine enabled the eserine to be estimated in small quantities in the presence of acetylcholine.

Minz (1932) used the leech muscle to estimate small quantities of acetylcholine. Again the sensitivity of the muscle was increased by having the eserine present.

Feldberg and Krayer (1933) showed that the anterior part of the body wall musculature was 2 to 4 times as sensitive to acetylcholine as the posterior body wall musculature, in the presence of eserine.

All of the above investigations emphasized the difficulties encountered with spontaneous contractions of the preparation and even in cases where spontaneous activity was at a minimum, the problem of adequate, if not complete, relaxation between tests was never really solved. The concentrations of eserine required, together with an apparent seasonal variation to eserine, seemed to raise further difficulties. For instance Feldberg and Krayer noted that in December and January a concentration of 5×10^{-6} g/ml. eserine was "without influence" on the muscle, but in February and March this same concentration of eserine produced powerful contractions so that they had to dilute the eserine to 5×10^{-7} g/ml. in order to obviate this spontaneous activity. Minz noticed that from November to March varying concentrations of eserine (5×10^{-6} g/ml. to 5×10^{-7} g/ml) only caused a slight and gradual increase in tone. He allowed the eserine to exert this effect and found it satisfactory to work on top of this arbitrary baseline.

Variations in the dissection of the leech also seemed to alter the experimental conditions. Fuehner (1918) showed that if the ventral wall of the leech was not removed, thus leaving the main chain of nervous tissue, small spontaneous

contractions are likely. Probably for the same reason Minz advised against the use of a tube of muscle, that is, simply a section taken from the middle of the body, the anterior and posterior suckers being removed. Minz also noted that in these preparations spontaneous activity was initiated by slight mechical stimulation, even when changing the solutions.

The use of the isotonic preparation of the leech for ACh assay became widespread after the realization that ACh was a chemical transmitter at neuronal and myo-neuronal junctions; but MacIntosh and Perry in "Methods in Medical research" (1951) remark that the leech muscle strip is "a valuable object for the identification of ACh and for rouch quantitative work where high sensitivity is required. Intolerably slow when many samples must be tested".

The problem of quicker and more consistent relaxation was approached in one way by M. F. Murnaghan in 1958. He investigated the influence of morphine on the relaxation of the eserinized leech. Paton et al. had shown in 1957 that the addition of morphine to the electrically stimulated guinea-pig ileum had the effect of decreasing the output of ACh. Murnaghan found that a specific concentration of morphine was necessary to:-

1. facilitate relaxation and
2. have no appreciable effect on the sensitivity of the muscle to ACh in the presence of eserine.

Again an isotonic system was used.

In 1961 Szerb devised the micro-leech preparation using a tiny strip of the anterior dorsal muscle and testing it in a bath of volume 0.05 ml. He found that with an isotonic lever system undiluted Locke's solution caused fast spontaneous movements. However, when the muscle strip was mounted isometrically after the optimum tension had been found isotonically, quicker relaxation and a steady baseline were obtained.

Anatomy and Physiology of Leech Muscle

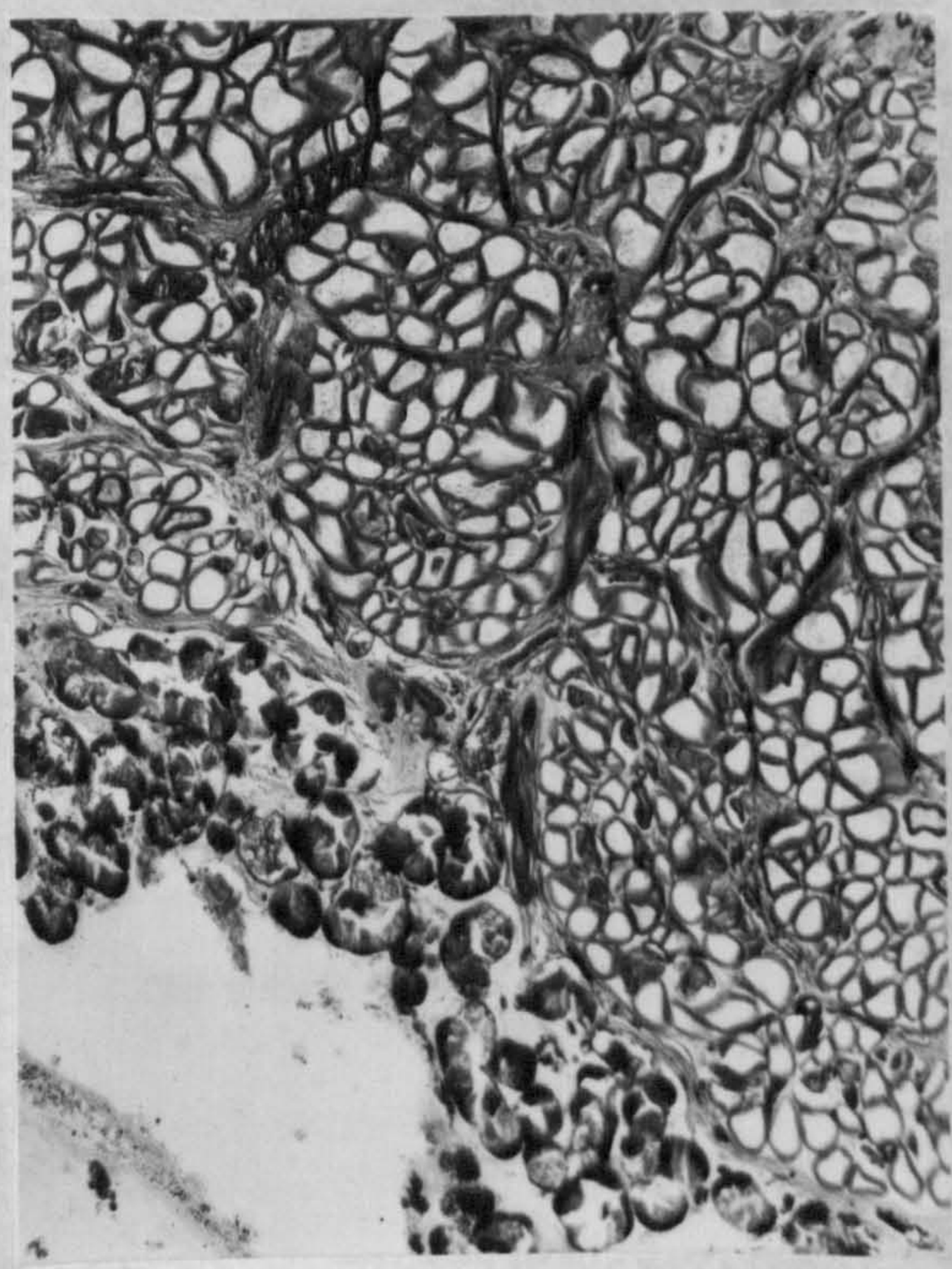
The structure of the leech muscle (Hirudo medicinalis) has recently attracted the attention of electron microscopists and many interesting details have been brought to light by their investigations.

The light microscopy of the muscle fibres of worms was described as early as 1891 by Apathy. However, most of his observations were carried out on Ascaris, only a few results from Hirudo were obtained.

The muscle fibre of Hirudo represents a special type of muscle. Sometimes it is difficult to make a clear distinction between smooth and striated muscle. The locomotory muscles in annelids are designated "helical smooth", showing some features of both smooth and straited muscle. In leeches all muscles appear to be of this type (Mann, 1962).

The muscle coat consists of a circular, an oblique and an innermost longitudinal layer. The longitudinal fibres are very long, 10 cms up to 20 cms, have a helical course around the fibre axis, have a high degree of contractility and are rapid in action. A speed of two contractions per second has been recorded from them. Schwab (1949) states that the individual muscle cells have an extension of 5 - 15 mm. The degree of shortening is said to range from 80% as a maximum to 50% in the normal ambulating leech.

When the muscle is viewed under the low power of a light microscope the transverse section shows large collections of hollow cells which are compressed together (Figure 45). Each cell consists of a cortex of the contractile element which surrounds the sarcoplasmic core in which are situated the nucleus and many mitochondria. The diameter of the cells in a transverse section can vary from 1 - 20 microns. This has been taken to indicate that the very long cell is a tapering one. The contractile cortex contains longitudinal myofilaments. These are arranged into discrete bundles which are seen to have a radial formation in the transverse section. Also present in the transverse section are the so-called "cross filaments" so clearly demarcated in the electron micrographs of Rohlich



100μ (a)

100μ (b)

Figure 45.

(a) Transverse section of leech muscle strip (X 120) showing large collections of hollow muscle cells which are compressed together. The pigmented cells seen at the lower left of the picture are cells bordering the gut which take up ingested red blood corpuscles.

(b) The lower half of the picture shows a longitudinal section of the hollow muscle cells lying just under the epithelium (X 300). In 2 cells the central nucleus can be seen laying in the clear lymph space within the cell.

The lower half shows a collection of glandular cells used for lubricating the leech epithelium.

Stain : methyleneblue.

(Section cut and stained by the Histology division, Physiology Department, University of Glasgow).

(1962). These elements are thought to have either the function of being supportive tissues or of being active contractile parts which serve to prevent excessive thickening of the fibre during its longitudinal shortening.

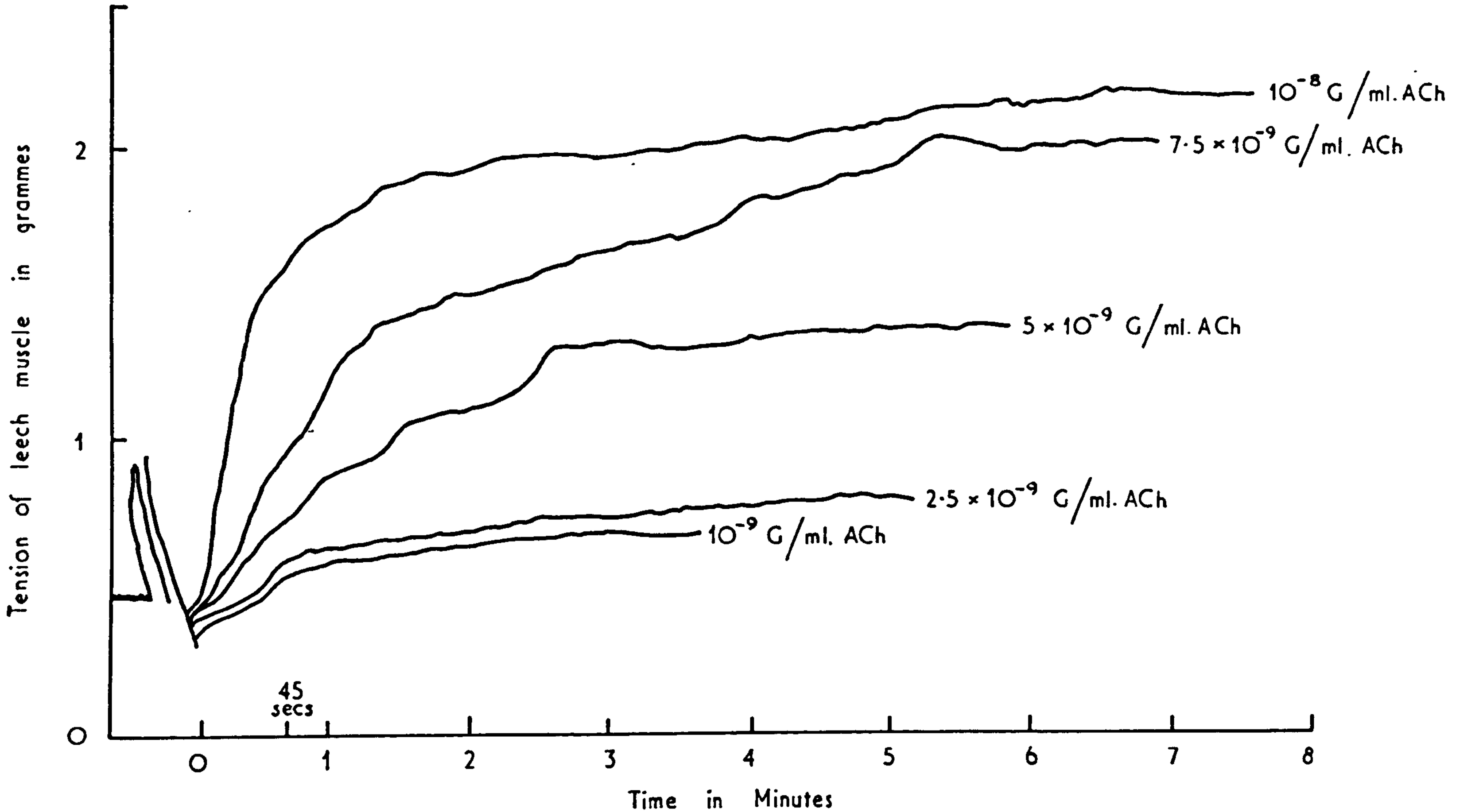
Pucci and Afzelius (1962) have displayed an orderly sarcotubular system in 5 leech species. This system seems to have a close connection with the cell membrane or sarcolemma.

Thus the leech musculature used for the assay of acetylcholine is an advanced and highly organised piece of tissue with the reputation of exhibiting some specificity towards acetylcholine in the presence of eserine without obvious interference from other substances(MacIntosh and Perry, 1951).

RESULTS

When the leech muscle strip was mounted isometrically it developed tension as soon as a suitable concentration of ACh was applied. It was possible to allow ACh solutions to act for as little as 45 seconds on the muscle strip and yet obtain step-wise responses to graded concentrations of ACh. Figure 46 shows the result of allowing each solution of ACh to remain in contact with the muscle until the maximum tension had been achieved. Note the immediate development of tension as soon as the ACh solution was applied to the muscle at zero time. The record was obtained by superimposing the records of 5 responses of the same leech muscle strip to concentrations of ACh ranging from 10^{-9} g/ml. to 10^{-8} g/ml. In each case the records of developing tension are well separated, so that discrimination between each response to a differing concentration of ACh is quite clear, regardless of the time allowed for each solution to be in contact with the muscle strip. After a period of 45 seconds the records are well separated. Test solutions were drained from the bath at this point and the tension of the muscle strip was equated with the concentration of ACh applied. The effect of the ACh on the leech muscle could thus be obtained in 45 seconds, and, with the lower concentrations of ACh, could be washed out in just as short a time. So it was possible to obtain a concentration-response relationship of a leech muscle strip to ACh very rapidly. Figure 47 shows the response of 2 leech muscle strips to graded concentrations of ACh. The effect of each test solution is preceded by a bizarre, but constant, increment in tension; this is due to the leech muscle strip lying up against the side of the bath momentarily just before the test solution of ACh is applied. After allowing the test solution to remain in contact with the leech muscle for 45 seconds, the pen recorder motor is stopped and the test solution drained from the bath. Thereafter the leech muscle is thoroughly rinsed with leech solution until the original baseline tension is obtained. A concentration-response curve is then obtained by plotting the tension developed by the muscle

Figure 46.



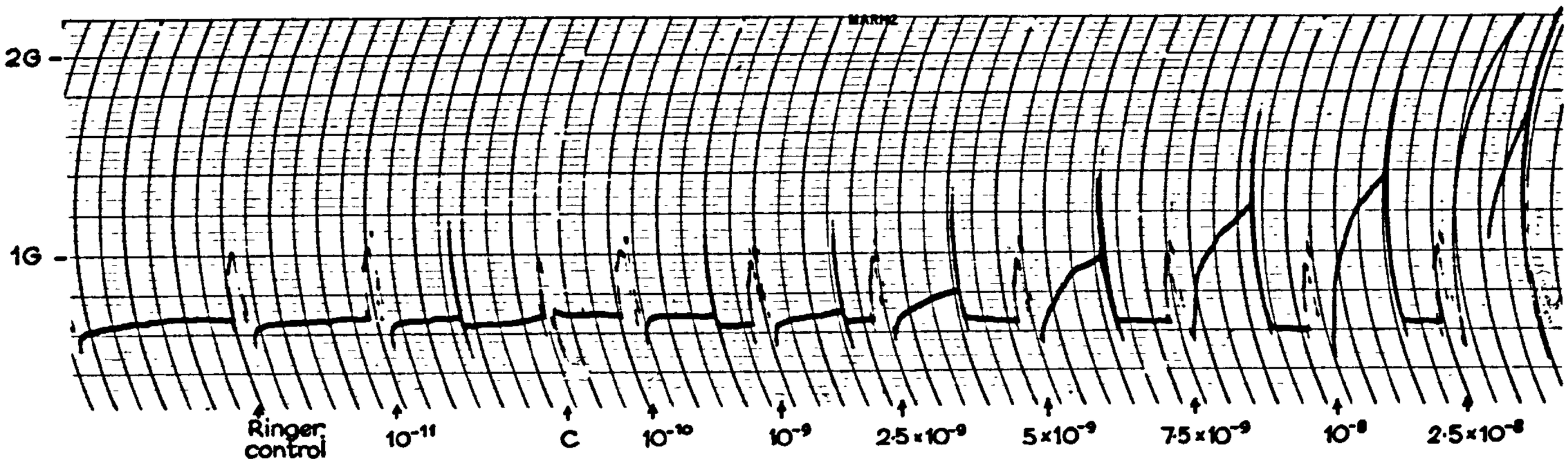
Development of maximum tension of a leech muscle on exposure to increasing concentrations of acetylcholine. (Superimposed records)

Figure 47.

Recordings of the responses of 2 leech muscle strips to graded doses of ACh. The tension in grammes is recorded at the left edge of each trace. Each solution of ACh (g/ml.) is added to the bath at the point indicated by an arrow. C = Ringer's solution. Solutions were applied to the muscle for 45 seconds.

The upper trace shows a response to a threshold concentration of 10^{-9} g/ml. ACh. Responses are seen to graded concentrations up to 2.5×10^{-8} g/ml. With this concentration the tension developed was so great that the pen had to be offset to a lower level on the recording paper.

The lower trace shows the response to ACh of a slightly less sensitive leech muscle. A threshold concentration of 2.5×10^{-9} g/ml. ACh caused this leech muscle strip to develop tension.


2G
1G
Ringer control
10^{-11}
C
10^{-10}
10^{-9}
2.5×10^{-9}
5×10^{-9}
7.5×10^{-9}
10^{-8}
2.5×10^{-8}

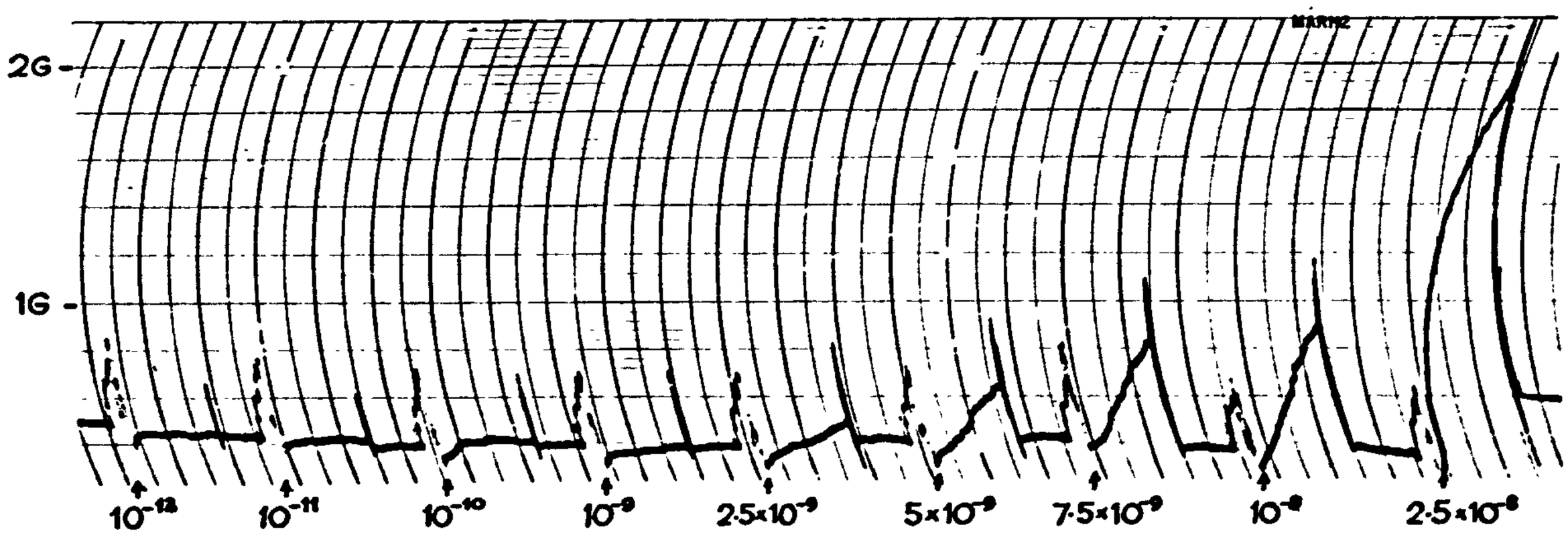
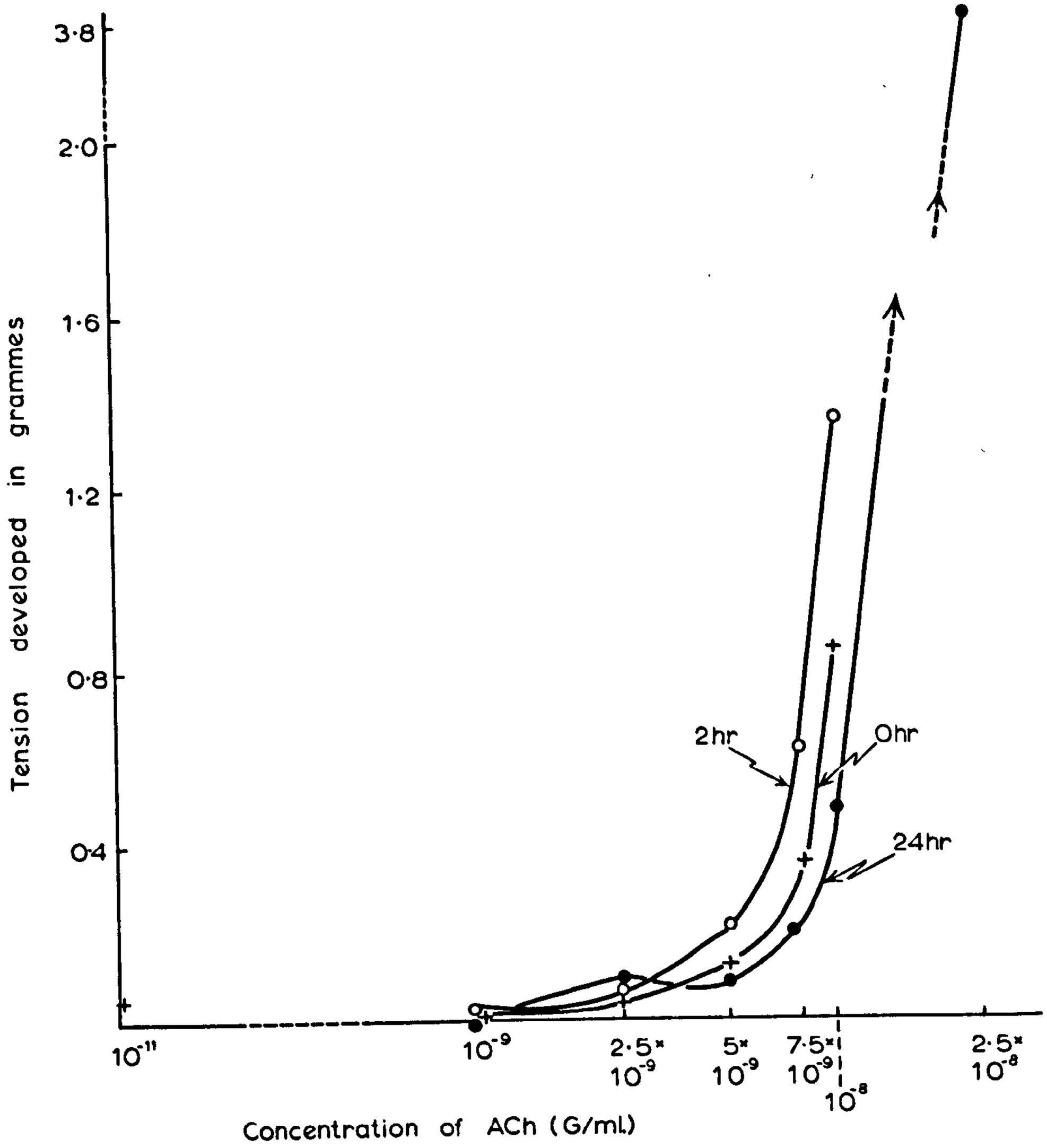

2G
1G
10^{-12}
10^{-11}
10^{-10}
10^{-9}
2.5×10^{-9}
5×10^{-9}
7.5×10^{-9}
10^{-8}
2.5×10^{-8}

Figure 48.

Concentration response curves of a single leech muscle preparation which was kept in the bath of leech Ringer's solution for 24 hours, hr = hours. Concentration response curves to acetylcholine are plotted at 0, 2 and 24 hours. Note that the threshold sensitivity is unaltered, as is the general shape of the response curve after 24 hours.


3·8
2·0
1·6
1·2
0·8
0·4
Tension developed in grammes
2hr
0hr
24hr
10⁻¹¹
10⁻⁹
2·5× 10⁻⁹
5× 10⁻⁹
7·5× 10⁻⁹
10⁻⁸
2·5× 10⁻⁸
Concentration of ACh (G/ml.)

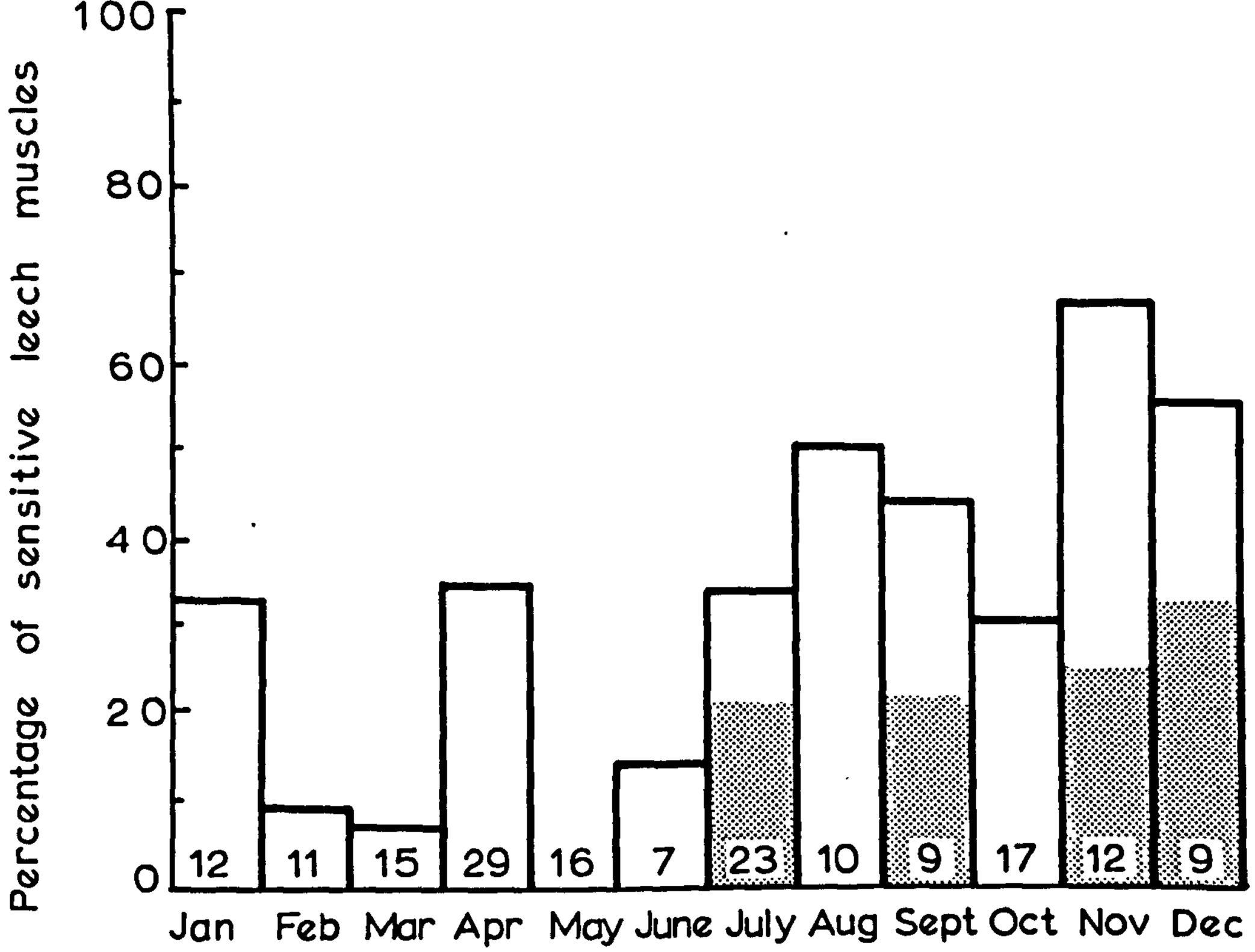


Figure 49.

Histogram showing seasonal incidence of high sensitivity of leech muscle strips to ACh. The number of muscle strips tested in each month is shown at the base of each column. The total height of each column indicates the percentage out of this number responding to 10^{-10} g/ml. ACh or lower concentrations. The height of the stippled columns represent the percentage sensitive to 10^{-11} g/ml. ACh.

strip after 45 seconds against the concentration of ACh producing that tension.

It was found that the sensitivity of the leech muscle to ACh remained constant over many hours, and it is to be noted that no oxygenation of the leech Ringer's solution was necessary. Figure 48 shows concentration response curves obtained from a single leech muscle preparation at 0,2 and 24 hours and the concentration-response curve retains its original shape, which makes the leech muscle strip a useful bioassay object for ACh during any prolonged assay experiment.

Fifty leech muscle strips were used to obtain initial and "24 hour" concentration-response curves. Twenty-five muscles retained their sensitivity, 8 of these actually becoming more sensitive after 24 hours. The other 25 muscles became less sensitive after 24 hours; the greatest loss in threshold sensitivity was from 10^{-11} g/ml. ACh to 10^{-9} g/ml. ACh.

Seasonal Sensitivity

Earlier investigators (Minz, 1932; Feldberg and Krayer, 1933) had noticed a seasonal variation to the anticholinesterase, eserine. It was possible that a seasonal variation to ACh might also exist.

One hundred and seventy leech muscle strips from separate leeches had their response to threshold concentrations of ACh determined in the presence of 10^{-5} g/ml. eserine over the period July, 1964 to December, 1966. The responses of muscles in 1964 were measured isotonically using a Gimbal lever and a smoked drum; all other muscles had their responses measured isometrically. Figure 49 shows the variation of the response to threshold concentrations of ACh for each month of the year. An incidence of low sensitivity appears in May and June, whereupon the sensitivity appears to increase gradually, reaching a peak in November and December, then dropping sharply again in the period February and March.

The Action of Morphine on the Leech Muscle Strip

The method of relaxing the leech muscle with the use of morphine, as advocated by Murnaghan (1958), prompted the initiation of some experiments designed to study the action of ACh and morphine on leech muscle.

Murnaghan cites the work of Paton et al. (1957) who showed that morphine had the effect of depressing the output of ACh in guinea-pig ileum. The rationale behind Murnaghan's original method for relaxing leech muscle was presumably that some form of constant "intrinsic" production of ACh was responsible for the maintenance of the leech muscle tone; .if this intrinsic production of ACh is depressed by morphine, the muscle will lose its tone and relaxation will occur. This whole procedure, of course, was directed at facilitating the management of an ISOTONIC system.

When the leech muscle strip was prepared for an assay procedure by soaking in morphine solutions (see Methods), it was noted that in every case the muscle had almost completely relaxed when set at a constant length in the bath. In three experiments the leech muscle was set up in the bath and a morphine solution was applied to the muscle in the presence of eserine. The alteration in tension was then continuously recorded. Figure 50 (lower trace) shows the effect of applying a solution of 5×10^{-5} g/ml. morphine in leech Ringer's solution to a muscle strip which had a resting tension of 1.5 grammes. The tension fell to 0.5 grammes two minutes after the application of the morphine solution. This rate of tension fall was similar to that found in the other two experiments.

Muscle strips which had been left to soak overnight were found to have relaxed considerably and did not relax further when morphine solutions were applied to them.

The Effect of Curare on the Leech Muscle Strip

Curare selectively blocks the action of ACh on leech muscle (Dale et al., 1936). This blocking action of d-tubocurarine was used in the present work to identify ACh contained in test solutions obtained from active and resting rat skeletal muscle. A study was made of the action of curare alone on the leech muscle strip.

In every experiment used to assay test solutions obtained from rat hemidiaphragm muscles, curare was applied to the leech muscle strip to establish that the action of

test solutions was due to the presence of ACh. In each case the leech muscle was observed to relax quickly after the application of curare.

Figure 51 shows a continuous recording of the tension produced by a leech muscle strip which had been soaked in eserinized leech Ringer's solution overnight. Increasing concentrations of ACh from 10^{-10} g/ml. to 10^{-8} g/ml. cause a steady rise in the muscle tension in spite of vigorous washing between doses. The tension rose from 1.2 grammes to 2.2 grammes. When a solution of 10^{-5} g/ml. curare was put into the bath the tension of the leech muscle strip fell from 2 grammes to 1 gramme in a period of two minutes.

In Figure 50 (lower trace, last test) the application of curare to another leech muscle strip shows the same rate in fall of tension: a fall of 1 gramme over a period of two minutes.

An unexplained finding occurred with two muscle strips; after soaking the muscle overnight in eserinized Ringer's solution, the application of curare did not block the action of ACh.

The Comparison of the Action of Morphine and Curare on a Single Leech Muscle Strip.

In one experiment the action of morphine and curare was compared on the same leech muscle strip. In this case the relaxation caused by morphine proceeded at the same rate as that caused by curare. It had already been noted that after a muscle strip had been allowed to soak overnight and had reached a low level of tension, the application of curare or morphine did not promote further relaxation.

Figure 50 shows a continous recording of the tension developed by a leech muscle strip which had an initial low tension. With the baseline steadily rising, a series of responses to graded concentrations of ACh was initiated. After a concentration of 10^{-8} g/ml. ACh was put into the bath and its effect seen and washed out, the leech muscle strip started to develop a steadily increasing tension. This tension reached a constant value as seen in the lower trace. After a

Figure 50.

Recording of the tension of a leech muscle strip which had an initial low tension. Tension is recorded in grammes on the left edge of the traces. The lower trace is a continuation of the upper trace. D = bath drained and refilled. A graded response to increasing doses of ACh is seen in the upper trace. The lower trace shows the actions of 5×10^{-5} g/ml. morphine and 10^{-5} g/ml. curare. Also note that the response of the muscle to 2.5×10^{-9} g/ml. ACh in the lower trace is the same as that shown in the upper trace (6th test), even though the initial tension of the muscle strip is different.

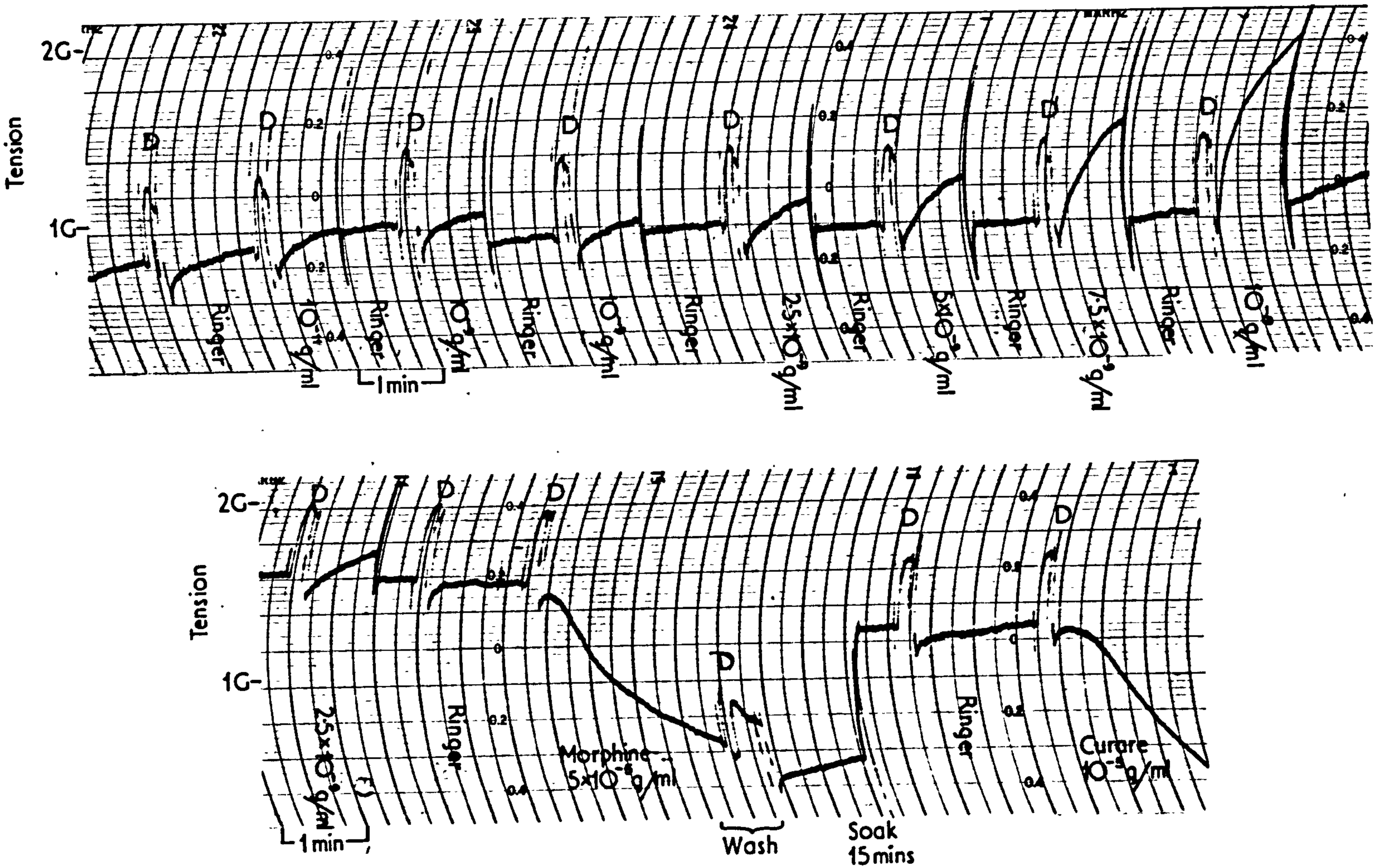
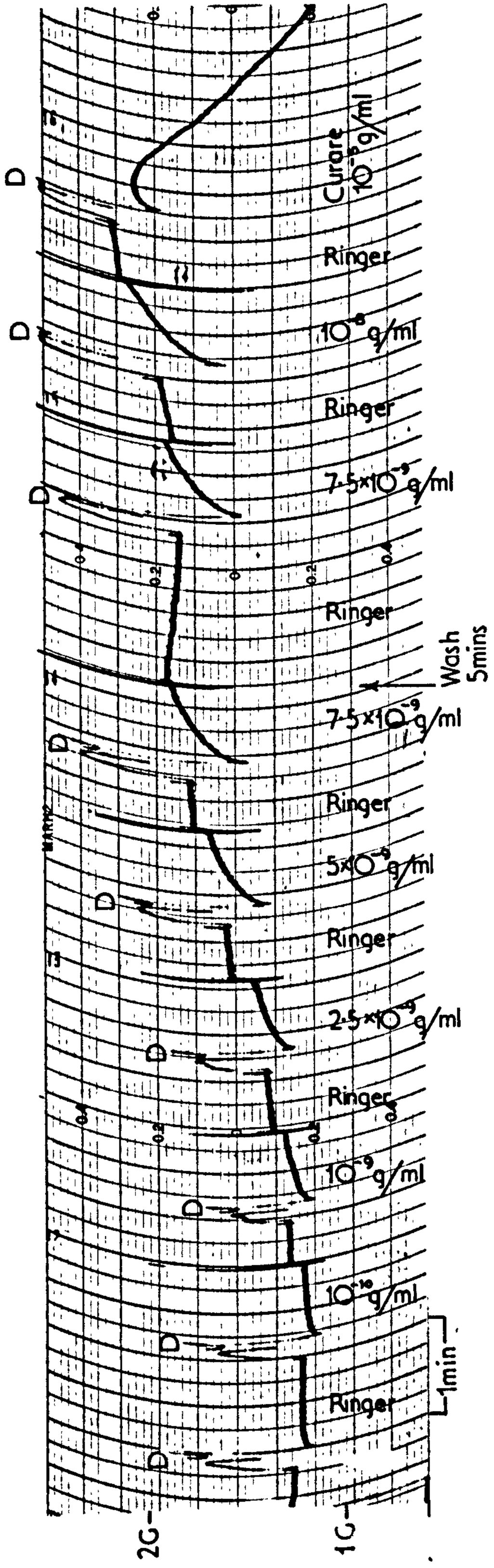

Tension
2G
1G
D
Ringer
10^{-4} g/ml
10^{-3} g/ml
2.5×10^{-3} g/ml
5×10^{-3} g/ml
7.5×10^{-3} g/ml
1 min
Morphine 5×10^{-6} g/ml
Wash
Soak 15 mins
Curare 10^{-5} g/ml

Figure 51.

A continuous recording of the tension produced by a leech muscle strip which had soaked in eserinized Ringer's solution overnight. Tension is marked in grammes on the left of the trace. D = leech bath drained and refilled. The spike after each test solution is where the paper drive was switched to the slow speed (2 cm/hr). Note the steady rise in tension during the applications of ACh, in spite of prolonged washing between solutions. Note the relaxation to the original baseline produced by a solution of 10^{-5} g/ml. curare.


Curare 10^{-5} g/ml
Ringer
10^{-6} g/ml
Ringer
7.5×10^{-9} g/ml
Ringer
Wash 5mins
7.5×10^{-9} g/ml
Ringer
5×10^{-9} g/ml
Ringer
2.5×10^{-9} g/ml
Ringer
10^{-9} g/ml
10^{-10} g/ml
Ringer
1min
2G
1G
Tension

81

satisfactory control with leech Ringer's solution, morphine in a concentration of 5×10^{-5} g/ml. was put into the bath. This caused the expected rapid fall in tension. After two minutes the morphine was drained out of the bath and the muscle strip was washed and then soaked in Ringer's solution for 15 minutes. During this time the muscle strip regained most of its original tension. After a satisfactory control, a solution of 10^{-5} g/ml. d-tubocurarine was put into the bath. This caused a similar fall in the tension of the leech musclestrip to that seen with the morphine solution, i.e. a loss of 1 gramme of tension in a period of two minutes.

DISCUSSION

It was found that the leech muscle strip showed two types of contraction (expressed by development of tension); a "quick" type which is always produced by a concentration of ACh well above the amount causing a threshold response in the leech muscle, and a second type of slow, steady contraction, not obviously initiated by any ACh applied to the muscle strip. The quick type of contraction could produce an increase in tension of 1 gramme in less than 30 seconds, while the slow contraction produced a tension of no more than 100 milligrammes in 1 minute.

It was found that a perfectly satisfactory response curve could be achieved in spite of the simultaneous occurrence of a slow contraction (or a steady increase in "tone"); the slow contraction did not seem to have any effect on the response of the leech muscle to concentrations of ACh well above the amount causing a threshold response, as manifested by the quick type of contraction. Thus any assay can be confidently undertaken on an isometric leech muscle preparation even when the baseline is not a horizontal one.

The interpretation of the response of the leech muscle to "threshold" concentrations of ACh has proved throughout this work to be difficult (Figure 47). In some cases the first solut ion of ACh applied to the muscle produced an increase in tension which was extremely difficult to wash out. Occasionally it was impossible to restore the original tension of the muscle strip (Figure 48). The rate of increase in tension was equivalent to that of the slow contraction and it seemed that the initial small dose of ACh applied to the muscle strip could set off a slow type of contraction which was unaffected by immediate and prolonged washing. This effect could be misinterpreted and taken to mean that a quick contraction had been initiated by a very small concentration of ACh (see the effect of 10^{-11} g/ml. ACh in Figure 50 . However the effect of a quick contraction is easily and rapidly washed out.

The response of the leech muscle to the drugs curare and morphine has suggested

the following theoretical scheme for the behaviour of the leech muscle in the presence of ACh when low concentrations of ACh are applied.

A "store" of ACh is postulated. The nature and situation of this store need not be discussed at this stage, but the main reason for this postulate is that there seems to be a constant source of ACh within the muscle which is available to maintain the muscle tonus. This ACh will be termed "intrinsic" as opposed to "extrinsic" ACh applied to the outside of the muscle. In order to diminish or abolish muscle tone, either the synthesis of intrinsic ACh can be blocked by morphine, or its peripheral action on the muscle fibre can be blocked by curare (Figure 52).

Extrinsic ACh can take two pathways. It can pass into the ACh store if the store is deficient, or it can pass directly to the muscle fibre and cause a quick contraction; or it can pass along both pathways, contributing to the store of intrinsic ACh and causing a quick contraction of the leech muscle fibre.

In a muscle which has fully repleted stores of ACh, all, or nearly all, of the extrinsic ACh contributes to the quick contraction of the leech muscle (Figure On the other hand, if extrinsic ACh is applied to a leech muscle with greatly diminished stores, then all, or nearly all, of it will contribute to the replenishment of these stores (Figure 52) ; if the concentration of ACh applied is a small one, then only after the stores have been repleted will a quick contraction be produced. If the amount of extrinsic ACh is large, only a certain amount of it can be utilized by the stores, the remainder "spills over" causing a quick contraction of the muscle fibres. This should be taken as the true response to threshold concentration of acetylcholine of that particular leech muscle strip. From this point onwards the stores are slowly incorporating applied extrinsic ACh. The baseline may indicate that the muscle tone is still gradually rising, but the increasing doses of extrinsic acetylcholine are now producing a graduated series of quick contractions, thus giving rise to a satisfactory concentration-response relationship.

In an isotonic system where a muscle strip has been allowed to contract fully,

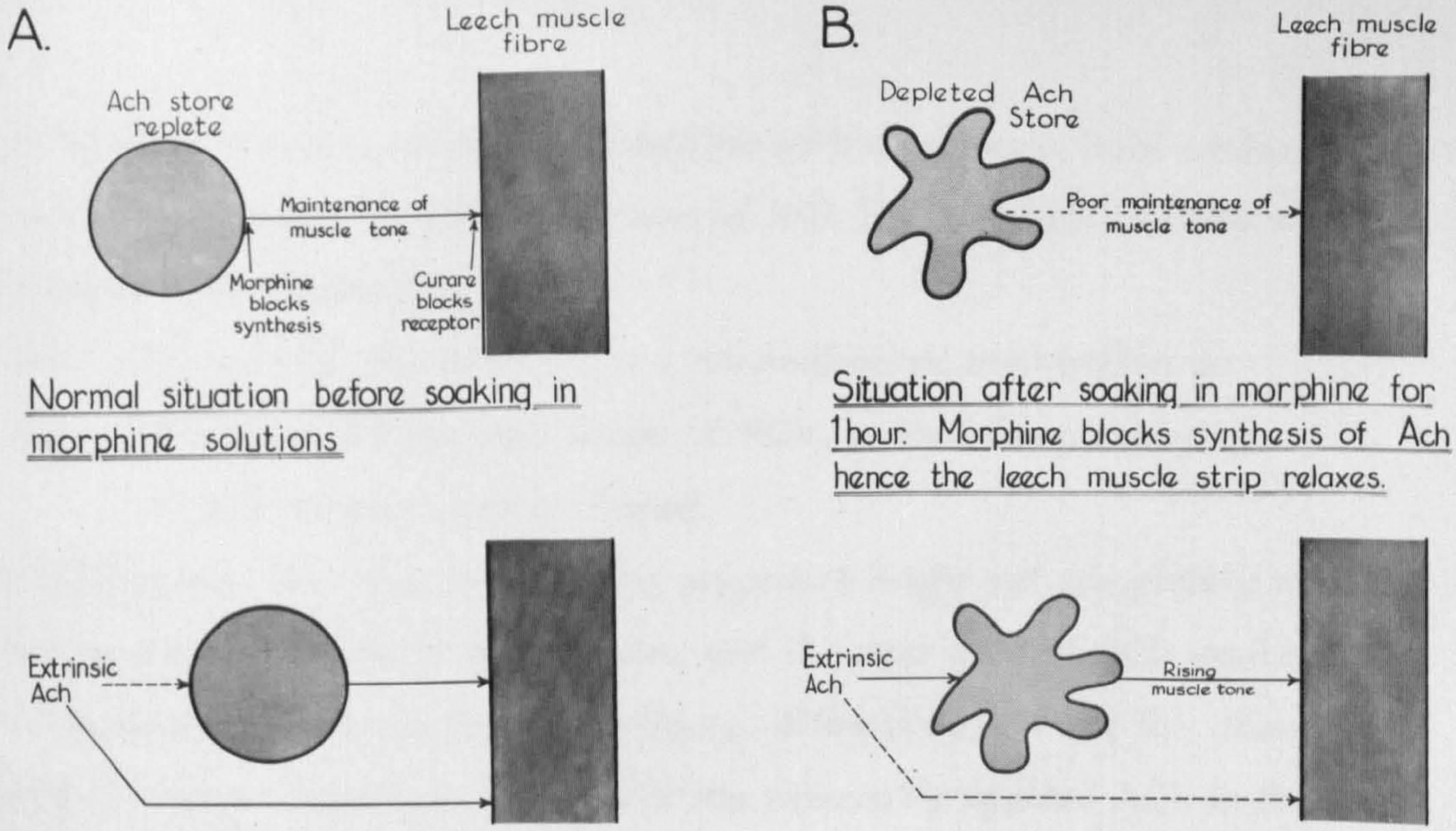


Figure 52. Diagram to represent the "store" hypothesis of ACh in leech muscle.

The leech muscle fibre is represented by an upright rectangle. The ACh store is represented by a shaded circle when replete and a "crenated" circle when depleted of ACh.

A. (Upper diagram) shows that a steady production of ACh from fully repleted stores is responsible for maintaining muscle tone. This process can be blocked by morphine or curare.

A. (Lower diagram) shows the effect of applying extrinsic ACh to the leech muscle in which the stores are fully replete. Muscle tone is fully maintained by ACh from the store; extrinsically-applied ACh is not taken up by the store and has its total effect on the leech muscle directly.

B. (Upper diagram) shows that with a depleted ACh store there is poor maintenance of muscle tone.

B. (Lower diagram) shows that when extrinsic ACh is applied to a muscle with depleted stores of ACh most of it is taken up by the stores, while only a fraction of the applied ACh has a direct effect on the leech muscle fibre.

Directly-acting ACh causes a quick contraction, ACh liberated from the store causes a slow contraction ("tone").

the time taken to return to its original baseline with continuous fluid exchange may be 30 minutes or longer. At threshold doses of ACh it is difficult to know whether this prolonged washing procedure is

(a) diminishing the intensity of a minimal quick contraction or

(b) depleting the intrinsic stores of ACh, so that the underlying muscle tone was being altered.

If the situation was (a), then the washing procedure might not completely wash out the effect of the quick type of contraction, and the next dose of ACh would have its effect superimposed on the previous effect. If the situation was (b), then the depleted ACh stores would take up some of the externally applied ACh in the next solution and the baseline would shift once again, confusing the true threshold response of the muscle to ACh.

With the isometric system, any ACh causing a quick contraction can be washed out of the muscle strip so much more quickly that it becomes obvious when the muscle tone is altering; it has been found that with vigorous washing <u>over a short time course</u> the baseline tension does not alter if the postulated stores of ACh are fully replete. The quick contraction produced by a concentration of ACh just above threshold value can be quickly and easily washed out.

<u>Comparison to the Micro-Leech Preparation</u>

Although both leech preparations are set up isometrically, the micro-leech preparation requires more skill and experience compared to the simple dissection and mounting procedure adopted here.

With the present system the test solution is only allowed to come into contact with the muscle strip for 45 seconds before the washing procedure is started. Probably this means that centrally-situated muscle fibres in the strip do not receive any ACh. If only a small proportion of leech muscle fibres are responding to the ACh, then this system is a pharmacological equivalent of the micro-leech preparation of Szerb (1961).

The micro-preparation was intended for exceedingly small test samples. This

would have the effect of concentrating any ACh released from, say, a cervical ganglion preparation stimulated in 0.1 ml. of fluid. However when other interfering substances are likely to be concentrated as well, no application for this method can be found in assaying ACh output from the neuromuscular junction.

SUMMARY

1. An isometric leech muscle preparation has been devised for the quantitative assay of ACh.
2. The preparation is simple to set up and can maintain a steady tension over a period of 24 hours.
3. The sensitivity of the preparation to ACh can also remain constant throughout a 24 hour period.
4. A seasonal variation to ACh is evident, the most sensitive period occurring during November and December.
5. Two types of contraction seem to occur in leech muscle (in response to ACh). A slow contraction can take place which is postulated to be caused by the production and release of an intrinsic supply of ACh. A fast type of contraction occurs in response to suitable concentrations of ACh applied to the muscle externally.
6. At "suprathreshold" concentrations of ACh the muscle tone (slow contraction) does not interfere with the fast response of the leech muscle strip. Thus a concentration-response relationship can be established despite the simultaneous occurrence of a slow contraction.

ASSAY OF ACETYLCHOLINE RELEASED FROM RAT DIAPHRAGM

INTRODUCTION

The release of ACh had been studied extensively by several workers with the use of the nerve-muscle rat hemidiaphragm preparation (Bulbring, 1946). It was used initially in this work to test the efficacy of the isometric leech muscle as a reliable assay preparation for ACh, since values for the amount of ACh released from rat diaphragm are already well established (Krnjevic and Mitchell, 1961; Krnjevic and Straughan, 1963; Mitchell and Silver, 1963).

The Assay Procedure

Since all test solutions were to be subsequently assayed on the leech muscle preparation, it was convenient to stimulate the muscles in mammalian tissue fluid substitutes which were easily converted by dilution to a Ringer's solution suitable for leech muscle. Eleven hemidiaphragms were stimulated in leech Ringer's solution so that no dilution was necessary. This unphysiological procedure was adopted as a preliminary test for the leech assay technique rather than for the accurate measurement of the ACh release from rat diaphragm muscle.

Where stimulation took place in Locke's solution the conversion to leech solution for assay was accomplished by diluting 1:1.4 with distilled water. It was found at a later stage in the work that the leech muscle did not lose any sensitivity to ACh when suspended in a medium of Krebs' solution which had been diluted 1:1.4 with distilled water. This allowed the hemidiaphragm muscles to be stimulated in continuously-oxygenated Krebs' solution which could then be converted simply to "modified Krebs' solution" for the assay on the leech.

In the unoxygenated series (Table 11) assay of the test solution was performed by matching their effects on the leech muscle with known amounts of ACh - chloride made up in leech Ringer's solution. Where possible, "bracketing" of the test solution between the effects of known concentrations of ACh was carried out and the effect of the test compared to the concentration-response curve obtained at the start of the experiment.

Table of ACh. output from Rat Diaphragm

Rate of stimulation	Volume of Bathing solution	Type of Ringer's solution	Output of ACh. per hemidiaph.
10/sec for 20 min	4 ml	Locke mammalian	2.2×10^{-8} g
			2.2×10^{-8} g
			1.5×10^{-8} g
			1.5×10^{-8} g
			0.8×10^{-8} g
20/sec for 20 min	4 ml	Locke mammalian	2.0×10^{-8} g
			1.4×10^{-8} g
			0.8×10^{-8} g
			0.8×10^{-8} g
			2.8×10^{-8} g
			1.0×10^{-8} g
			2.0×10^{-8} g
			1.0×10^{-8} g
			1.0×10^{-8} g
20/sec for 20 min	4 ml	LEECH	3.0×10^{-8} g
			2.0×10^{-8} g
			2.0×10^{-8} g
			2.0×10^{-8} g
10/sec for 20 min.	2 ml	Locke mammalian	$7. \times 10^{-9}$ g
			7×10^{-9} g
			5×10^{-9} g
			3×10^{-9} g
20/sec for 20 min	2 ml	Locke mammalian	7×10^{-9} g
			7×10^{-9} g
			7×10^{-9} g
			7×10^{-9} g
			7×10^{-9} g
			2.8×10^{-9} g
20/sec for 20 min	2 ml	LEECH	5×10^{-9} g
			5×10^{-9} g
			5×10^{-9} g
			2.5×10^{-9} g
			2.5×10^{-9} g
			4×10^{-9} g
			2.5×10^{-9} g

Unoxygenated Series

Table 11.

Table12.

OUTPUT OF ACh FROM RAT HEMIDIAPHRAGMS INTO KREBS' SOLUTION (ng)

Stimulation took place at 20/sec for 20 minutes followed by a period of 5 minutes soaking. Resting solutions were obtained by soaking the muscle for a period of 25 minutes. Dotted lines connect values successively obtained from the same hemidiaphragm.

Muscle	Resting (2 ml)	Resting $+O_2$(2ml)	Stimulation in 2 ml	Stimulation in 4 ml
1	12. . . .	 7		
2	6. . . .	 18		
3		5. 17		
4	5	. . . . .	. . . . .	 17
5	8. . . .	. . . . . 4. . . .	. . . . .	 15
6	10. . . .	. . . . . 2. . . .	. . . . .	 12
7	12. . . .	 17		
8	11			
9	3			
10	3			
11			8	
12			11	
13			13	
14			11	
15			10	
16			20	
17			22	
18				15
19				23
20				31
21				35
22				24
23				17

In the oxygenated series (Table 12) where the muscles were rested or stimulated in oxygenated Krebs' solution, a more rigid procedure was adopted. An initial concentration-response relationship was obtained for ACh in the leech muscle strip to be used for the assay. The leech muscle lay in Krebs' soltuion diluted 1:1.4 with distilled water. After conversion of the test solution by diluting 1:1.4 with distilled water, its effect was "bracketed" between the effect of two known concentrations of ACh. The test solution was then diluted by half with converted Krebs' solution and again "bracketed" between the effects of known concentrations of ACh. The leech muscle was then soaked in curare for 20 minutes. The test solutions were again applied to the leech muscle strip.

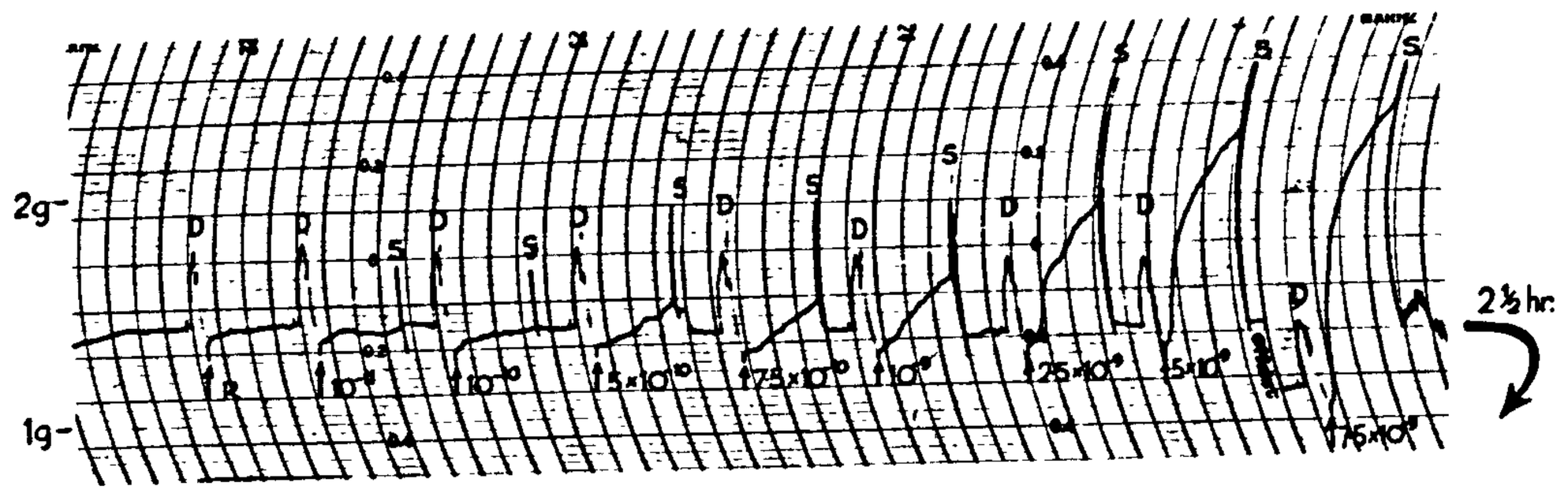
An example of an assay procedure is shown in Figure 53. The leech muscle strip was bathed in Krebs' solution diluted 1:1.4 with distilled water. The rat hemidiaphragm was stimulated at 20 impulses per second in 2 ml. Krebs' solution for 20 minutes. The upper trace of Figure 53 shows the initial concentration-response relationship of the leech muscle to ACh. The threshold response occurred when a concentration of 5×10^{-10} g/ml. ACh was applied to the leech muscle. The lower trace shows the response of the leech muscle to "bracketing" concentrations of ACh and to the test solution after it had been converted to modified Krebs' solution by diluting 1:1.4 with distilled water. The effect of the test solution (T) was "bracketed" between the effects of 5×10^{-9} g/ml. ACh and 2.5×10^{-9} g/ml. ACh. Three minutes later the test solution diluted by half (T/2) gave an effect which was "bracketed" between the effects of 10^{-9} g/ml. and 2.5×10^{-9} g/ml. ACh. After the leech muscle had soaked in a solution of 10^{-5} g/ml. curare for 20 minutes, the response to these solutions was abolished.

A graphic record of the result of this assay procedure is shown in Figure 54 The initial concentration-response curve has been obtained by plotting the tension developed by the leech muscle strip against the concentration of ACh applied (+). The tensions developed by the leech muscle in response to the "bracketing" solutions of ACh lie close to the initial concentration-response curve obtained

Figure 53. An example of a typical assay procedure on the isometric leech muscle preparation.

Upper figure shows the initial concentration response relationship of the leech muscle to ACh. Threshold response occurs at 5×10^{-10} g/ml.

Lower Figure shows the test solution "T" compared with known amounts of ACh. When diluted by half (T/2) its effect lay between 10^{-9} g/ml. and 2.5×10^{-9} g/ml. ACh. Curare blocked the effect of T/2. "D" = bath drained and refilled with a new solution. "S" = paper drive switched to slow speed. Arrow indicates the time of application of the solutions to the muscle.


2g
1g
D
S
R
2½ hr.

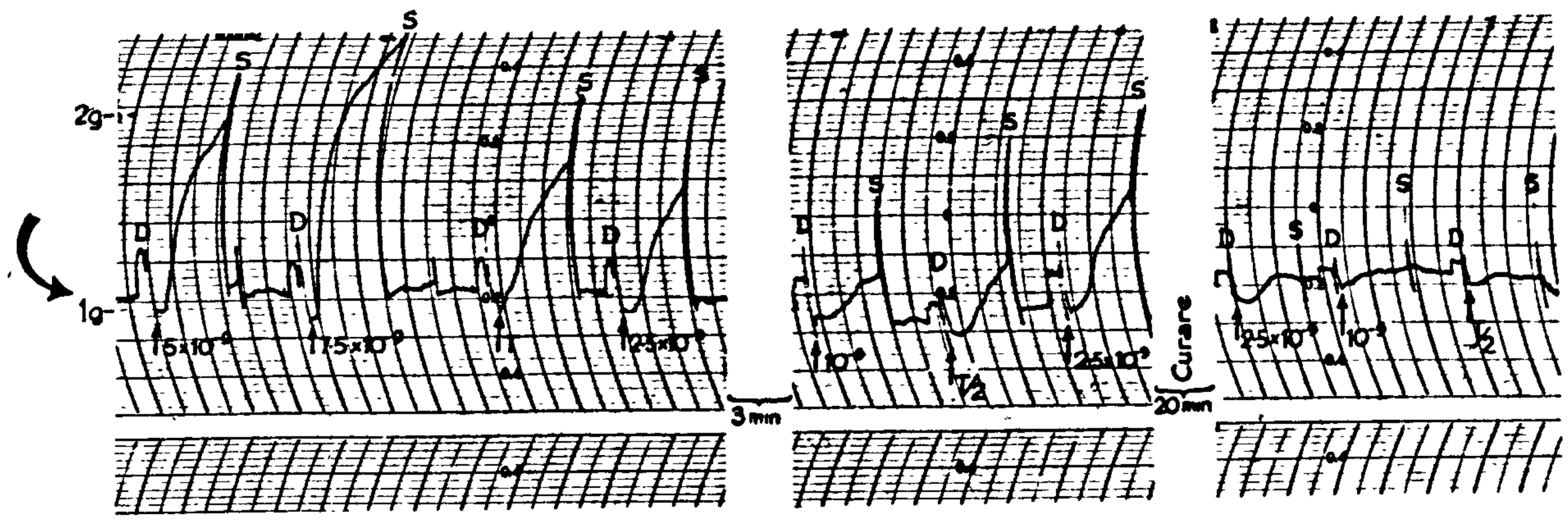
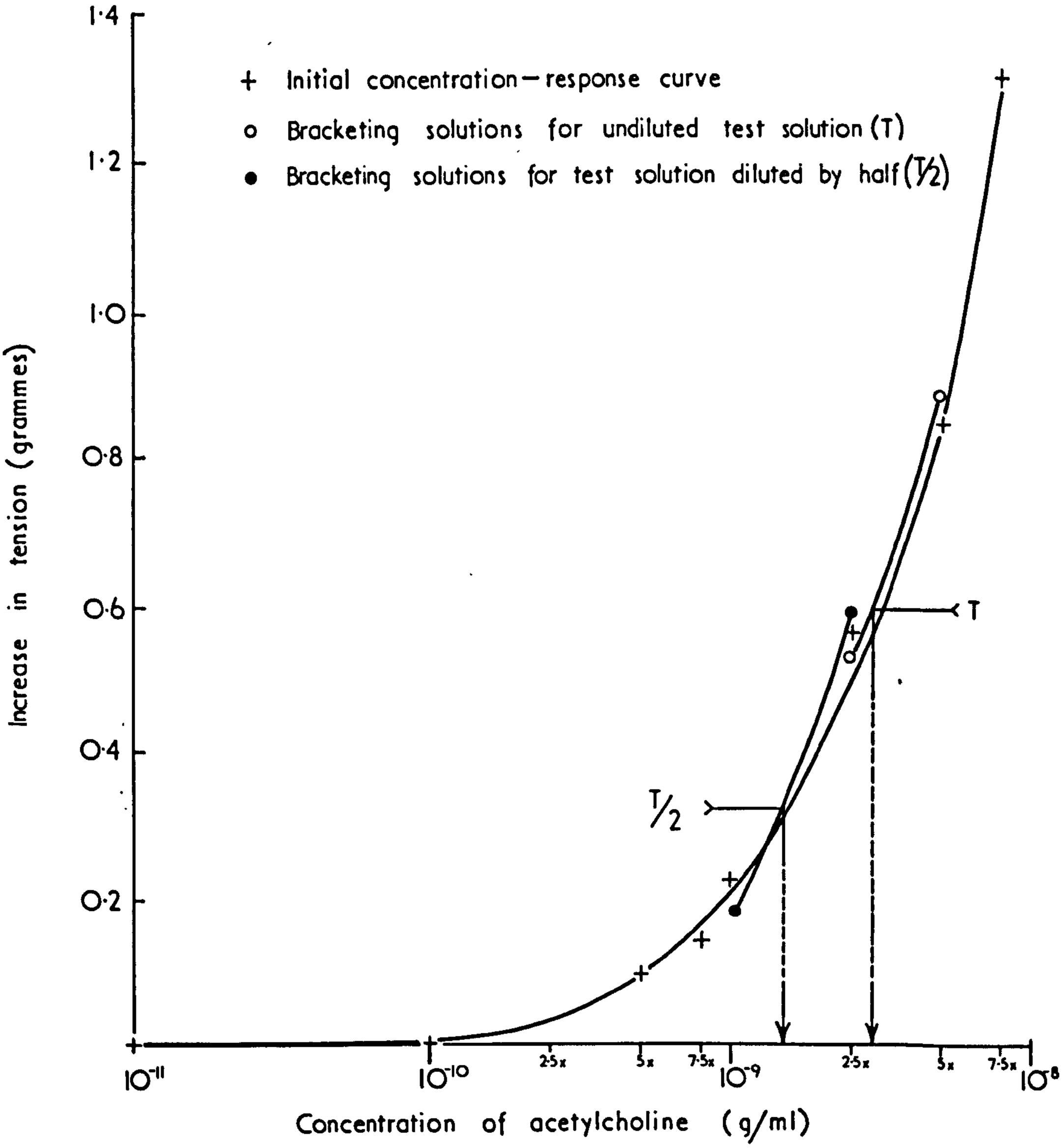

2g
1g
D
S
3 min
Curare
20 min

Figure 54.

Initial concentration-response relation for action of ACh on tension of an isometric leech muscle strip (+). Test solution from rat hemidiaphragm undiluted (T) and diluted by half (T/2) have given tensions equivalent to concentrations of 3×10^{-9} g/ml. ACh and 1.5×10^{-9} g/ml, respectively. Each test "bracketed" between tests with ACh at same time in experiment (0 0, see Figure Diaphragm stimulated at 20 impulses/sec for 20 minutes in 2 ml. Krebs' solution; solutions diluted 1:1.4 before assay.


+ Initial concentration – response curve
o Bracketing solutions for undiluted test solution (T)
● Bracketing solutions for test solution diluted by half (T/2)
Increase in tension (grammes)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
T
T/2
2·5x
5x
7·5x
10⁻¹¹
10⁻¹⁰
10⁻⁹
10⁻⁸
Concentration of acetylcholine (g/ml)

$2\frac{1}{2}$ hours previously.

The test solution (T) was found to be equivalent to 3×10^{-9} g/ml. ACh. The test solution diluted by half gave a response equivalent to 1.5×10^{-9} g/ml. ACh. Since the hemidiaphragm was originally stimulated in a 2 ml. volume which was diluted to 2.8 ml., the output of ACh from the muscle was $3 \times 2.8 \times 10^{-9}$ g, i.e. 8.4 ng.

Identification of ACh

Only one test was used for the identification of ACh. (see General Discussion). When the leech muscle was soaked in a solution of 10^{-5} g/ml. d-tubocurarine for 20 minutes this completely blocked the action of known amounts of ACh applied to the muscle strip. The action of curare on the test solution, however, was not so well defined. In every case the tension of the curarized leech muscle strip had returned to its original baseline after a period of 45 seconds in contact with the test solution; however, in most cases the baseline tension fluctuated immediately after the application of the test solution (Figure 55). An interfering substance invoking an extremely quick contraction of the leech muscle was present in the solution which had surrounded a twitching hemidiaphragm. This finding was present in results reported by Krnjevic and Mitchell in 1961 (see Discussion).

Applications of diluted test solutions to the leech muscle strip aided the identification of the ACh. In each case the test solution diluted by half caused approximately the same effect as half the concentration of ACh which matched the effect of the undiluted test solution (Figure 54). Dilution of the test solution would also reduce the effect of any interfering substances present which might alter the sensitivity of the leech muscle preparation to ACh.

The Effect of Increased Potassium Concentration on the Leech Muscle

In order to exclude potassium as the cause of the residual quick contraction provoked by the test solution when applied to the curarized leech muscle strip, graded concentrations of K^+ in leech solution were applied (Figure 56). Normal

Figure 55. The effect of curare on the action of ACh on leech muscle.

Upper trace: The effect of a solution in which a rat hemidiaphragm was stimulated has been "bracketed" between known concentrations of ACh.

Lower trace: The effect of the same solutions after soaking the leech muscle strip in curare. Note that the effect of the test solution has not been completely blocked.

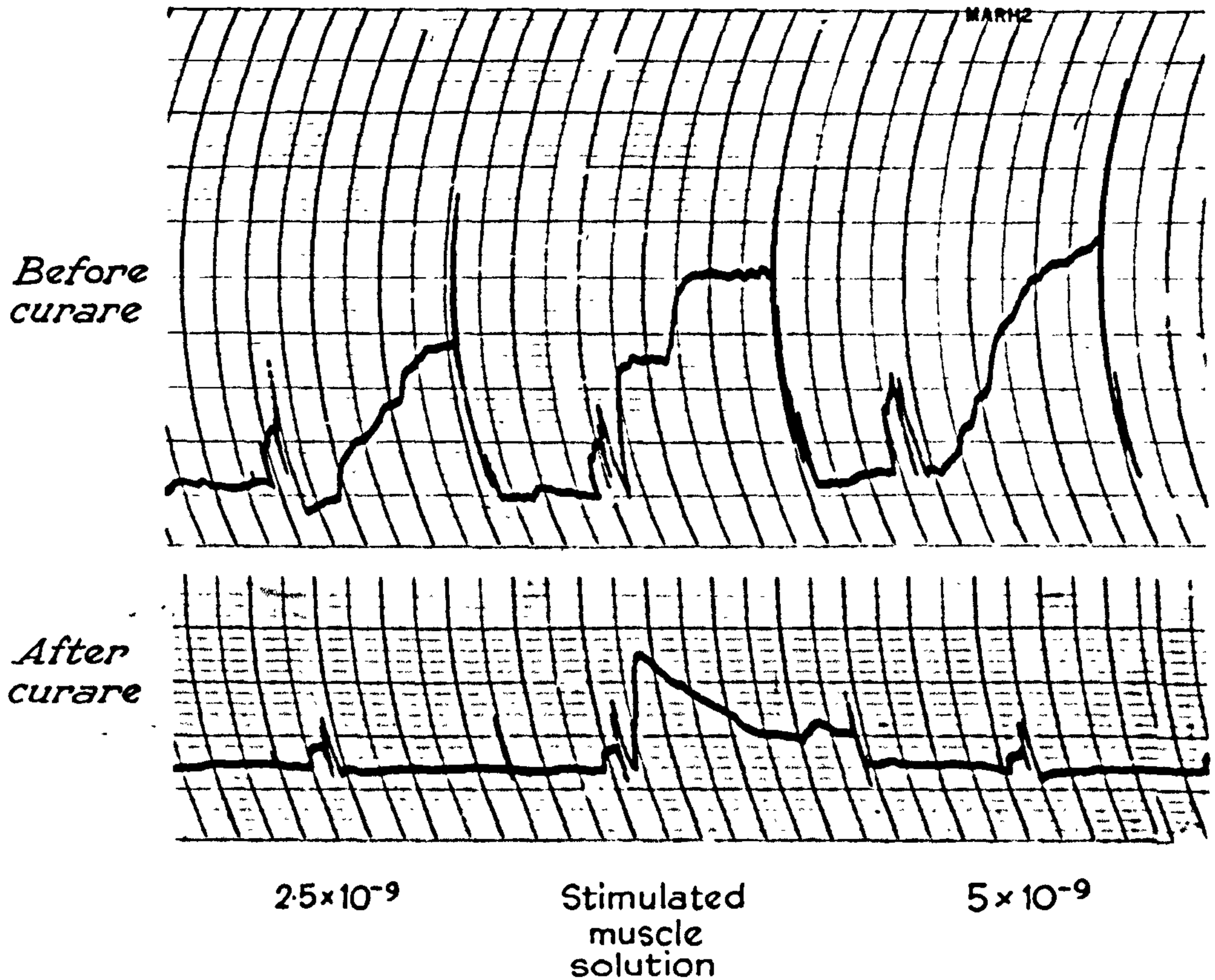
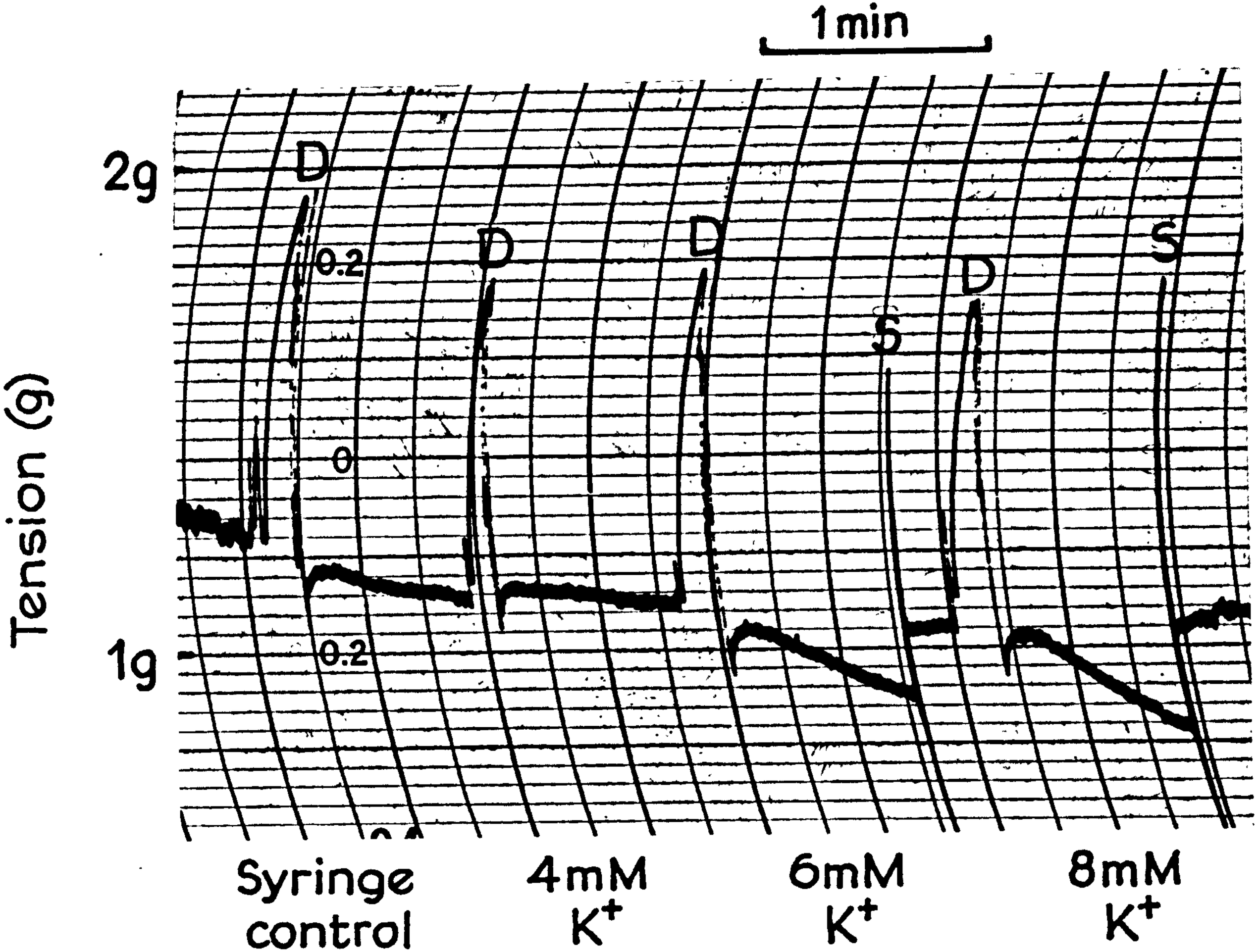

MARH2
Before curare
After curare
2·5×10⁻⁹
Stimulated muscle solution
5×10⁻⁹

Figure 56.

The effect of increasing the concentration of potassium in the Ringer's solution on a leech muscle strip. Solutions are applied to the muscle strip for 45 second periods. "D" = bath drained and refilled with a new solution, "S" = paper drive switched to slow speed.

Ringer's solution containing 4 mM potassium does not affect the muscle strip. Relaxation of the strip is produced by solutions of Ringer containing 6 mM and 8 mM potassium. Thus increased potassium concentration is not the cause of the response of the curarized leech muscle to a stimulated muscle solution.


1min
2g
1g
Tension (g)
D
0.2
0
0.2
D
D
S
D
S
Syringe control
4mM K+
6mM K+
8mM K+

Leech Ringer's solution contained 3mM of K^+. When a concentration of 6mM K^+ was applied to the muscle, relaxation occurred. Further addition of a solution containing 8mM K^+ applied immediately afterwards produced a further relaxation of the leech muscle. While this excluded K^+ as a cause of the early contraction effect, it stressed the importance of excluding substances, released from active skeletal muscle, other than ACh which could distort bioassay estimations (see Discussion).

RESULTS

The ACh output of 53 hemidiaphragm muscles was measured on the isometric leech muscle preparation (Tables 11 and 12). The muscles were stimulated indirectly at 10 impulses/sec or 20 impulses/sec in 2 ml. or 4 ml. of leech solution, Locke's solutions or continuously-oxygenated Krebs' solution. The spontaneous release of ACh from 14 muscles was measured; nine muscles were soaked in 2 ml. of unoxygenated Krebs' solution and five muscles were soaked in 2 ml. of continuously-oxygenated Krebs's solution.

The ACh Release into 2 ml. Volumes of Locke's and Kreb's Solutions

A comparison of the Tables "A" and "B" in Figure 57 shows that the ACh output from hemidiaphragms stimulated at 20/sec for 20 minutes in 2 ml. of Locke's solution is significantly less ($.05>P>.01$) than that released under the same conditions of stimulation into 2 ml. of Krebs' solutions. The mean output into 2 ml. Locke's solution was 6ng while the mean output into Krebs' solution was 14ng. This difference could be attributed to either the type of Ringer's solution surrounding the active muscle or the fact that continuous oxygenation took place in the Krebs' solution during the period of stimulation, whereas the Locke's solution was bubbled with oxygen beforehand, but not during the stimulatory period.

Both Ca^{++} and Mg^{++} ions affect the ACh release at the endplate region, Ca^{++} increasing the output, and Mg^{++} depressing it (Fatt and Katz, 1952). The Ca^{++} concentrations in the Locke's and Krebs' solutions were 2.2 mM and 2.5 mM respectively. This small difference is not likely to reduce the output into Locke's solution. No Mg^{++} was present in the Locke's solution, while 1.2 mM Mg++ was contained in the Krebs' solution. This difference would encourage a greater release of ACh into the Locke's solution than into the Krebs' solution, the reverse of the results obtained.

Figure 57. Output of ACh (ng) from hemidiaphragm muscle stimulated at a frequency of 20/sec for 20 minutes.

"A" Locke's Solution 2 ml. volume	"B" Krebs' Solution 2 ml. volume	"C" Leech Solution 2 ml. volume
7	18	5
7	17	5
7	8	2.5
7	11	2.5
7	13	4
3	11	2.5
	10	
	20	
	22	
population - 6	population - 9	population - 7
range 3 - 7	range 8 - 22	range 2.5 - 7
mean - 6 S.D. ± 1.52	mean - 14 S.D. ± 4.69	mean - 4 S.D. ± 0.98

"D" Locke's Solution 4 ml. volume	"E" Krebs' Solution 4 ml. volume	"F" Leech Solution 4 ml. volume
20	15	30
14	23	20
8	31	20
8	35	20
28	24	
10	17	population - 4
20	15	range 20 - 30
10	17	mean - 24 S.D. ± 4.58
10	12	
population - 9	population - 9	
range 8 - 28	range 12 - 35	
mean - 14 S.D. ± 6.56	mean - 21 S.D. ± 6.70	

It is to be noted here that even less ACh is released into 2 ml. of leech solutions than into 2 ml. of Locke's solution (compare Tables "A" and "C", Figure 57.

The ACh Release into 4ml. Volumes of Locke's and Krebs' Solutions

Tables "D" and "E" of Figure 57 show that the mean output of ACh from hemidiaphragm muscles into 4ml. volumes of Locke's and Krebs' solutions was 14ng and 21ng respectively. This significant difference ($P<.05$) suggests that lack of oxygenation during stimulation of the muscle in the Locke's solution has an important influence upon the release of ACh from the rat diaphragm. However, the mean output of 24ng into a 4 ml. volume of unoxygenated leech solution is similar to that put into oxygenated Krebs' solutions (Table "F", Figure 57) suggesting that some other factor, such as tonicity of the bathing solution, may be operative in the regulation of the release of ACh.

The Comparison of Amounts of ACh Released into 2 ml. and 4 ml. of Locke's Solution

The mean output of ACh into 2 ml. of Locke's solution was 6ng (Table "A"). Under the same conditions of stimulation and oxygenation the mean output into 4 ml. of Locke's solution was 14ng (Table "D"). This difference is highly significant ($P<.01$) and strongly suggests that the volume of surrounding fluid affects the quantity of ACh released from an active hemi-diaphragm muscle.

The Comparison of Amounts of ACh Released into 2 ml. and 4 ml. of Krebs' Solution

Tables "B" and "E" of Figure 57 show the comparisons of ACh release into continuously-oxygenated Krebs' solutions of 2ml. and 4ml. volumes. The mean

amounts released into the 2 ml. and 4 ml. volumes were 14ng and 21 ng respectively. This clear difference ($P<0.1$) again suggests that the greater the volume of fluid surrounding the muscle, the greater is the output of ACh from the muscle, other circumstances remaining constant. This phenomenon could have many explanations (see Discussion); only three are listed at this point.

1. A diffusion gradient for ACh will tend to approach equilibrium more quickly in a smaller volume of surrounding bathing solution. This would result in less diffusion of released ACh into the surrounding solution.
2. Every impulse delivered down the motor nerve might not liberate a normal amount of ACh from the nerve terminal.
3. All of the impulses delivered down the motor nerve may not reach the endplate. This type of nerve block can be produced by anoxic conditions, which might occur more quickly in a small volume of fluid (see Discussion).

In the present work only the existence of the last of these possibilities could be tested. Hemidiaphragms were stimulated at a different frequency and their outputs measured.

ACh Output from Muscles Stimulated at 20/sec and 10/sec into 4 ml. Volumes of Locke's Solution

Hemidiaphragms were stimulated at a frequency of 10/sec and their outputs were compared with those having been stimulated at 20/sec. Figure 58 gives comparative tables for these results. Tables "P" and "Q" of Figure 58 show that the mean outputs of ACh into 4ml. volumes of Locke's solution from muscles stimulated at a frequency of 10/sec and 20/sec were 16ng and 14ng respectively. The mean outputs of ACh into 2 ml. volumes of Locke's solution when the muscles were stimulated at 10/sec and 20/sec were 5.5ng and 6ng respectively (Tables "R" and "S").

From these figures it can be concluded that under these experimental conditions

Figure 58. Output of ACh from hemidiaphragm muscles stimulated at frequencies of 10/sec or 20/sec for 20 minutes.

"P"

Locke's Solution
frequency 10/sec
4 ml. volume

22
15
8
15

population - 5
range 8 - 22
mean - 16 S.D. $\pm$ 5.29

"Q"

Locke's Solution
frequency 20/sec
4 ml. volume

20
14
8
8
28
10
20
10
10

population - 9
range 8 - 28
mean - 14 S.D. $\pm$ 6.56

"R"

Locke's Solution
frequency 10/sec
2 ml. volume

7
7
5
3

population - 4
range 3 - 7
mean - 5.5 S.D. $\pm$ 1.66

"S"

Locke's Solution
frequency 20/sec
2 ml. volume

7
7
7
7
7
3

population - 6
range 3 - 7
mean - 6 S.D. $\pm$ 1.52

Figure 59. The output of ACh (ng) from hemidiaphragms resting or stimulated in 2 ml. Krebs' solution.

Resting Muscle Solutions	Resting Muscle Solutions	Stimulated Muscle Solutions
Oxygenated	Unoxygenated	Oxygenated
7	12	18
17	8	17
4	12	8
5	10	11
2	11	13
	3	10
population - 5	3	11
range 2 - 17	5	20
mean - 7	6	22
	—	—
	population - 9	population - 9
	range 3 - 12	range 8 - 22
	mean - 8	mean - 14

varying the frequency of stimulation from 10/sec to 20/sec does not alter the amount of ACh released from active hemidiaphragm muscles.

The Spontaneous Release of ACh from the Hemidiaphragm

Fourteen hemidiaphragm muscles were soaked in 2 ml. volumes of Krebs' solution for a period of 25 minutes. No significant difference (P>5) was found in the output of ACh between solutions which were not oxygenated and those which were oxygenated (Figure 59). The spontaneous release of ACh from muscles resting in 2 ml. is half of that liberated from muscles stimulated in 2 ml. of Krebs' solution. It is interesting to note that the mean resting release of ACh into 2 ml. volumes of Krebs' solution was 7ng and that the mean output of muscles stimulated at a frequency of 20/sec into 2 ml. of Locke's solution was 6ng (see Discussion).

DISCUSSION

Output of ACh from Stimulated Muscles

The output of ACh into 2 ml. of oxygenated Krebs' solution is probably significantly less than the output into 4 ml.. Also, the output into 2 ml. of unoxygenated Locke's solution is significantly less than the output into 4 ml. The output into leech solution was also found to be greater into the larger volume of surrounding solution. This constant finding could have many causes.

The greater volume of fluid surrounding the muscle will increase the diffusion gradient of ACh so that the smaller volume of bathing solution would impair the diffusion of released ACh from within the musculature into the surrounding solution. The diffusion pathway may a lso be distorted because the hemidiaphragm is unsupported in the surrounding fluid during stimulation.

Unknown substances may be released during muscle activity which interfere with the assay procedure. These interfering "metabolites" would exist in a higher concentration in the 2 ml. solutions and might have a depressant effect on the sensitivity of the leech muscle to ACh. The experiment performed showing that an increase in the K^+ concentration in the solution surrounding the leech muscle causes relaxation of the leech muscle strip is one example of an interfering substance which would have to be considered. It has been established that K^+ comes out of the stimulated muscle into a surrounding bathing solution (see section on K^+ release from sartorius muscle), so that any test solution obtained from a stimulated hemidiaphragm muscle would be likely to have a raised K^+ concentration. When this solution is applied to the leech muscle strip, any contraction caused by ACh present in the solution would be offset by the effect of relaxation produced by the high K^+ concentration. However, in this work the test solutions were diluted by half with modified Krebs' solution so that the higher level of K^+ would be diluted somewhat.

The undiluted stimulated muscle solutions did not have their effects on the

leech muscle completely blocked. The effects of test solutions diluted by half were completely blocked by curare. Although this effect of incomplete blocking was not commented on by Krnjevic and Mitchell (1961), nevertheless their specimen trace of the action of the test solution on an isotonic leech muscle strip clearly shows that the action of the test solution was not completely blocked by curare, while the matching ACh solution had its effect completely abolished. Krnjevic and Mitchell comment:- "when simple solutions of ACh were compared with those in which the muscle contracted, it was found that the latter caused irregular variations in baseline, and also in ACh sensitivity". They suggested that "disturbing factors" were released mainly during contraction of the muscle fibres. A biologically active substance, ATP, has been shown to be released from active skeletal muscle (this Thesis, Part I); however, investigations in the present work have shown that ATP in low concentrations does not affect the leech muscle.

The stimulation of a muscle in 2 ml. volume of solution would presumably induce a state of hypoxia more quickly than stimulation of the muscle in 4 ml., if oxygenation throughout the stimulatory period was inadequate. Krnjevic and Miledi (1957, 1958, 1959) have described a type of failure of neuromuscular propagation occurring during repetitive stimulation of the phrenic nerve. They showed that the most likely sites for this "pre-synaptic block" were either points where the nerve to the muscle branches, or the narrow region just before the terminal expansion of the nerve fibres. They found that this block could be overcome if the stimulation was either stopped or reduced in rate. They concluded that the block was probably caused by anoxia of the intramuscular portion of the motor nerve.

When the muscle is stimulated in a volume of 2 ml., early pre-synaptic block may occur, thus reducing the number of impulses releasing ACh from the nerve terminals. Since the ACh output into 4 ml. of unoxygenated Locke's solution was significantly lower than the output into 4 ml. oxygenated Krebs'

solution, under the present experimental conditions the oxygen content of the Locke's solutions may have been inadequate.

Comparison of the output of ACh from muscles stimulated at 10/sec or 20/sec in 2 ml. or 4 ml. of Locke's solution showed that the output did not significantly increase at the higher frequency of stimulation. This would again suggest that early pre-synaptic block was occurring as a result of hypoxia. If this were so, a frequency of 20/sec would be converted into an effective frequency of, say, 10/sec or less, thus giving a falsely low reading of ACh output for the frequency of 20/sec.

Once again it would have to be considered that the low output of ACh obtained with the high frequency of stimulation might be due to the hypoxic muscle tissue releasing substances into the test solution which would tend to depress the sensitivity of the leech muscle strip to ACh, hence reducing the value obtained for the ACh output in the assay procedure.

A disturbing finding in this work was that the ACh output from resting hemidiaphragm muscles into 2 ml. volumes of Krebs' solution was about the same (7ng) as the output from hemidiaphragms stimulated at 20/sec in 2 ml. volumes of Locke's solution (6ng). (Compare Table "A" in Figure 57 with Figure 59). It must be concluded from this that all, or nearly all, of the impulses were blocked when a hemdiaphragm was stimulated in 2 ml. of Locke's solution at a frequency of 20/sec.

The only set of results obtained here that can be compared with results of other workers are those obtained from stimulating the muscles in 4 ml. volumes of continuously-oxygenated Krebs' solution. Krnjevic and Straughan (1963) found a mean release of 35ng after a period of 20 minutes stimulation at a frequency of 20/sec into a 3 ml. volume at 37°C. The mean release found here using the same frequency of stimulation, over the same period of time, was 21ng (Figure 57 Table "E"). A reduction in output might be expected since the present experiments were performed at room temperature.

Krnjevic and Mitchell (1961) stimulated hemidiaphragm muscles at room temperature for 5 minutes at a frequency of 5/sec and calculated an ACh release of 10^{-17} mole/impulse/nerve ending. If this rate of ACh release is transferred to the present conditions of stimulation (20/sec for 20 minutes), in a rat hemidiaphragm muscle with an estimated 10,000 nerve terminals (Krnjevic and Miledi, 1958), the total output of ACh from the muscle would be 343ng if every impulse reached the nerve endings and the synthesis of ACh was adequate.

The total amount of ACh actually released from the muscle in Krnjevic and Mitchell's experiments is calculated below:

> Stimulation frequency = 5/sec for 5 minutes. Number of impulses = 1,500. If 10^{-17} mole/impulse/nerve ending ACh was released, then the total amount of ACh released in a muscle with 10,000 nerve endings = $1,500 \times 10^{-17} \times 10^{4}$ pmole = 21.4 ng.

This figure agrees with the mean output found in the present work when a muscle was stimulated in 4 ml. Krebs' solution at 20/sec for 20 minutes. From a comparison of the durations of stimulation, it would seem that the bulk of ACh may be released from the muscle in the first 5 minutes of stimulation at a frequency of 20/sec. Thereafter it may be postulated that the rate of synthesis of ACh at the nerve terminal is unable to keep up with the amounts of ACh released initially.

APPENDIX

SEPARATION OF ACETYLCHOLINE FROM OTHER TISSUE PRODUCTS BY MOLECULAR SIEVE CHROMATOGRAPHY

The following section describes the behaviour of small concentrations of ACh in a Sephadex column (see Methods). This method of separation was initially adopted in this work in order to isolate ACh from other substances which may be present in the stimulated muscle solution and which might prevent an accurate estimation of ACh on the frog heart.

Since a very small amount of ACh released into a test solution was anticipated, small concentrations of ACh-chloride were put through the column. In order to detect the ACh in the fractions from the column, a bioassay technique was needed. The isometric leech preparation and the frog heart perfusion system were used for this purpose (see Methods).

Results

By adjusting the volumes of the fractions collected from the column, it was found that about 75% of ACh put into the column could be recovered in a 3 ml. fraction coming 4.5 ml. after the initial blue dextran fraction, i.e. this 4.5 ml. volume contained no blue dextran and very little ACh.

In ten experiments 2 ml. of a solution of ACh-chloride together with 0.1 ml. blue dextran were put through a column which had been packed in, and was continuously eluted with, leech Ringer's solution. The blue dextran fraction was followed through the column and the 3 ml. containing it was collected. The next 2 ml. fraction collected (fraction 1) contained no ACh. The next 2.5 ml. fraction (fraction 2) contained a very small amount of ACh and the following 3 ml. fraction (fraction 3) contained the bulk of the ACh originally put into the column. No ACh was found in any subsequent fraction.

Figure 60 shows an assay of these fractions on the leech muscle preparation. 2 ml. of 2.5×10^{-8} g/ml. ACh-chloride were put into the column. Fraction 1 (F1) did not affect the leech muscle. Fraction 2 (F2) matched the effect of a

concentration of 2.5×10^{-9} g/ml. ACh. Fraction 3 (F3) gave an effect which lay between the effect of 1.5×10^{-8} g/ml. and 10^{-8} g/ml. of ACh. When diluted by half (F3/2), fraction 3 matched the effect of 5×10^{-9} g/ml. of ACh. The percentage recoveries of ACh in fractions 2 and 3 were 12% and 75% respectively.

An example of an assay of the column fractions on the perfused frog heart is shown in Figure 61 . After 2×10^{-7} g/ml. of ACh were mixed with blue dextran and put through the column, the usual fractions were collected. Fraction 1 did not affect the heart. The effect of perfusing fraction 2 through the heart was matched by the effect of 5×10^{-9} g/ml. ACh. Fraction 3 matched the effect of 3.5×10^{-8} g/ml. ACh. The calculated percentage recovery in this case was 6.25% in fraction 2 and 50% in fraction 3.

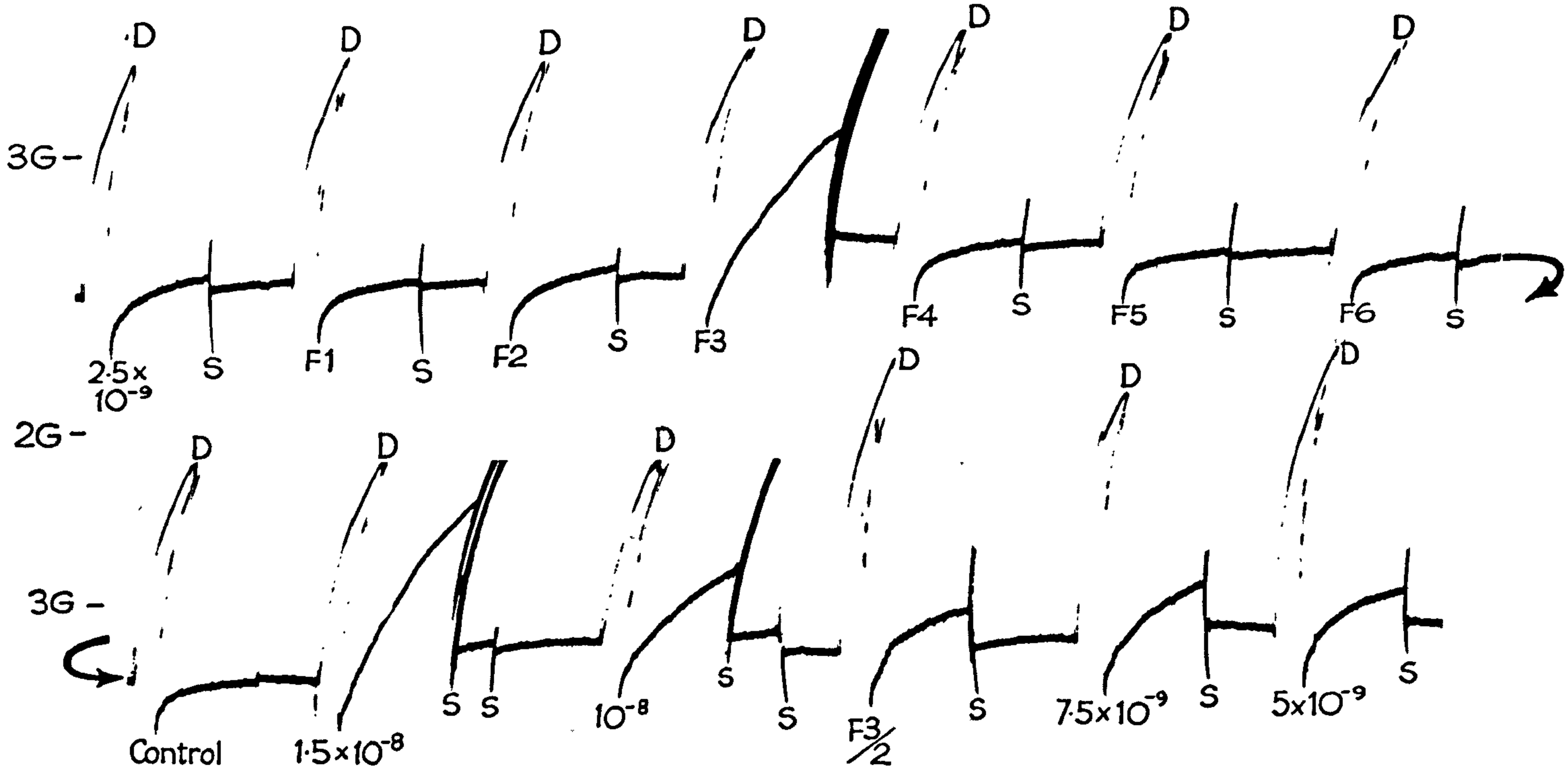
Table 13 shows a compilation of results for the standardization of the behaviour of ACh in the column, using the isometric leech preparation and the frog heart perfusion system for the estimation of the small amounts of ACh in the eluted fractions. It can be seen from this table that there is a wide scatter of results obtained for the estimated amounts of ACh eluted into fractions 2 and 3. This is mainly due to the fact that the estimation of the ACh was performed by direct matching with known amounts of ACh in both the frog heart and the leech preparation. This is not an accurate procedure and further work is needed in order to determine more precisely the distribution of ACh between fractions 2 and 3. However, in no case out of 13 columns tested was ACh detected in any other fractions but 2 and 3, and as such this method can certainly be adopted for complete separation of ACh from substances of very large molecular weight, or a partial separation of ACh from substances of smaller molecular weight.

Figure 60.

The development of tension of a leech muscle strip to concentrations of ACh contained in fractions from a Sephadex column. The lower trace is a direct continuation of the upper one. D = change of solution in the leech bath S = recording motor switched to slow speed. The tension in grammes is shown at the left of the trace. Each test solution is allowed to act for 45 seconds.

Calculation of Percentage ACh Recovery

2 ml. of 2.5×10^{-8} g/ml. ACh are put into the column. Thus the total amount of ACh put into the column = 5×10^{-8} g. Assay of fraction 3 (F3) shows that it contains a concentration of 1.25×10^{-8} g/ml. ACh. Since this fraction has a volume of 3 ml., the total amount of ACh contained in fraction 3 is $3 \times 1.25 \times 10^{-8}$ g $= 3.75 \times 10^{-8}$ g. Percentage recovery in fraction 3 is $\frac{3.75 \times 10^{-8} \times 100}{5 \times 10^{-8}} = 75$. Assay of fraction 2 (F2) shows that it has a concentration of 2.5×10^{-9} g/ml. ACh. Since fraction 2 has a volume of 2.5 ml., the total amount of ACh contained in fraction 2 is $2.5 \times 2.5 \times 10^{-9}$ g $= 6.25 \times 10^{-9}$ g. The percentage recovery in fraction 2 is $\frac{6.25 \times 10^{-9} \times 100}{5 \times 10^{-8}} = 12$.


3G-
D
D
D
D
D
D
D
2·5×10⁻⁹
S
F1
S
F2
S
F3
F4
S
F5
S
F6
S
2G-
D
D
D
D
3G-
Control
1·5×10⁻⁸
S
S
10⁻⁸
S
S
F3/2
S
7·5×10⁻⁹
S
5×10⁻⁹
S

2G -

Figure 61.

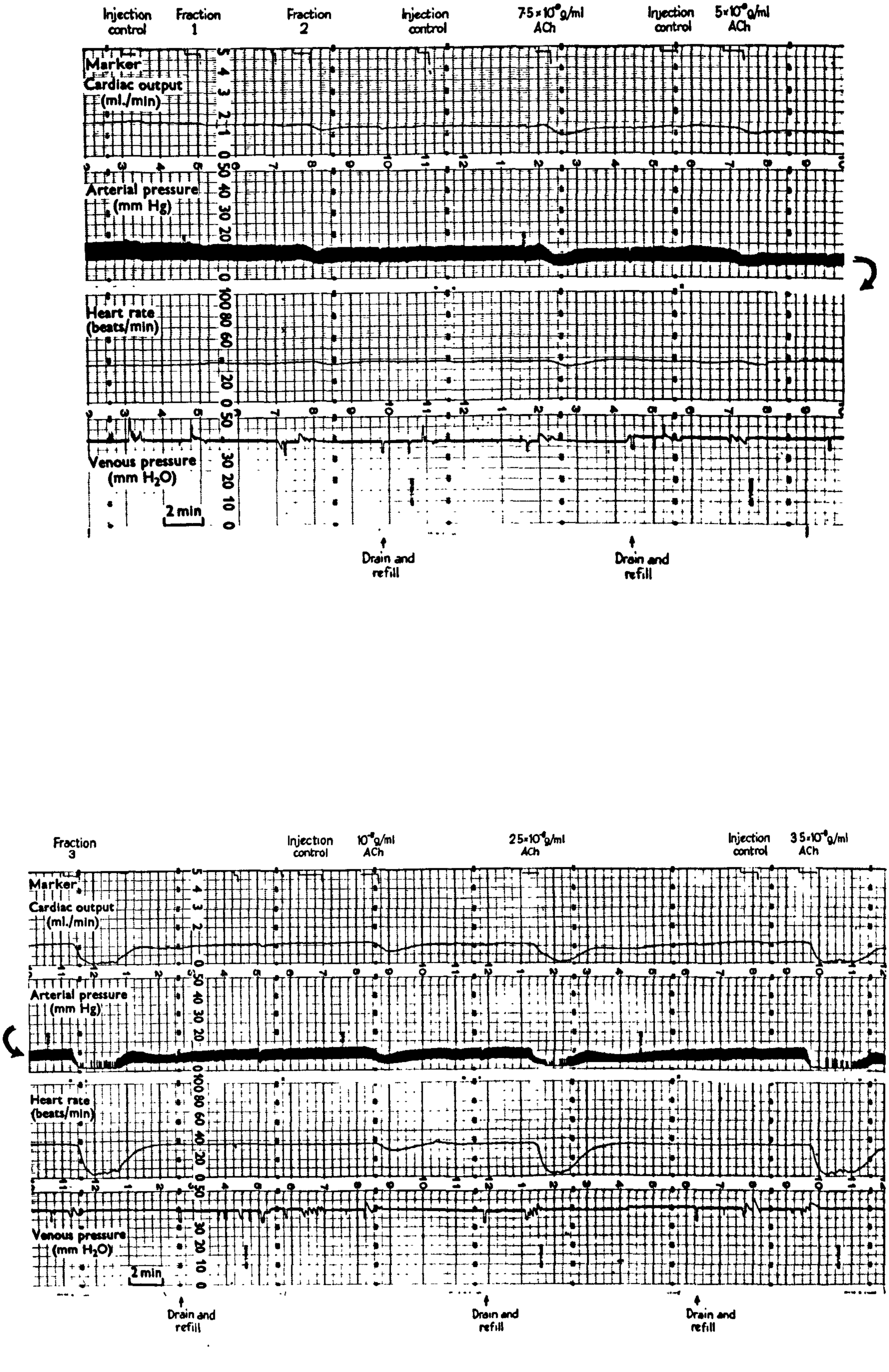
Assay of column fractions for ACh on the frog heart.

The lower trace is a direct continuation of the upper one. An initial concentration of 2 ml of 10^{-7} g/ml. ACh, made up in frog Ringer's solution, was put through a column continuously eluted with frog Ringer's solution. The usual fractions were collected and then perfused through a frog heart.

Fraction 1 had no effect on the heart. Fraction 2 matched the effect of 5×10^{-9} g/ml. ACh. The effect of fraction 3 was matched by a concentration of 3.5×10^{-8} g/ml. ACh.

Calculation of Percentage Recovery

2 ml. of 10^{-7} g/ml. ACh are put into the column. Thus the total amount of ACh put into the column = 2×10^{-7} g. Assay of fraction 3 shows that it contains concentration of 3.5×10^{-8} g/ml. Since this fraction has a volume of 3 ml., the total amount of ACh contained in fraction 3 is $3 \times 3.5 \times 10^{-8}$g = 1.05×10^{-7}g. Percentage recovery in fraction 3 is $\frac{1.05 \times 10^{-7} \times 100}{2 \times 10^{-7}} = 50\%$ Assay of fraction 2 shows that it has a concentration of 5×10^{-9} g = 1.25×10^{-8} g. Percentage recovery in fraction 2 is $\frac{1.25 \times 10^{-8} \times 100}{2 \times 10^{-7}} = 6.25\%$


Injection control
Fraction 1
Fraction 2
Injection control
7·5 × 10⁻⁸ g/ml ACh
Injection control
5 × 10⁻⁸ g/ml ACh
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H₂O)
2 min
Drain and refill
Drain and refill
Fraction 3
Injection control
10⁻⁸ g/ml ACh
2·5 × 10⁻⁸ g/ml ACh
Injection control
3·5 × 10⁻⁸ g/ml ACh
Marker
Cardiac output (ml./min)
Arterial pressure (mm Hg)
Heart rate (beats/min)
Venous pressure (mm H₂O)
2 min
Drain and refill
Drain and refill
Drain and refill

THE PERCENTAGE RECOVERY OF ACh PASSING THROUGH THE COLUMN

A different column was used for each result except where indicated (+)

Table 13.

Solvent and pH	Total Amount of ACh into Column	Amount Recovered	% Recovery Fraction 2	% Recovery Fraction 3	Total Recovery	Method of Measurement
Leech Ringer 7.8	10^{-7} g	10^{-7} g	25.0	75.0	100%	Leech assay
Leech Ringer 7.8+	10^{-7} g	8.9×10^{-8} g	13.0	75.0	88%	Leech assay
Leech Ringer 7.8	2.0×10^{-7} g	1.69×10^{-7} g	13.0	75.0	88%	Leech assay
Leech Ringer 7.8	2.0×10^{-7} g	1.70×10^{-7} g	12.5	75.0	87.5%	Leech assay
Leech Ringer 7.8	5.0×10^{-8} g	4.4×10^{-8} g	12.0	75.0	87%	Leech assay
Leech Ringer 7.8	2.0×10^{-7} g	2.0×10^{-7} g	14.0	90.0	100%*	Leech assay
Leech Ringer 7.8	2.0×10^{-7} g	1.6×10^{-7} g	12.5	67.5	80%	Leech assay
Leech Ringer 7.8	10^{-8} g	10^{-8} g	12.5	90.0	100%*	Leech assay
Leech Ringer 7.8	2.0×10^{-8} g	1.7×10^{-8} g	10.0	72.0	82%	Leech assay
Leech Ringer 7.8	2.0×10^{-7} g	2.0×10^{-7} g	9.5	120.0	100%*	Leech assay
Frog Ringer 7.4 +	2.0×10^{-8} g	2.0×10^{-8} g	1.25 - 3.1	75 - 112	100%	Frog heart assay
Frog Ringer 7.4	2.0×10^{-7} g	10^{-7} g	6.25	50.0	56%	Frog heart assay
Frog Ringer 7.4	10^{-7} g	10^{-7} g	12.5	105.0	100%*	Frog heart assay
Frog Ringer 7.4	10^{-7} g	10^{-7} g	30.0	75.0	100%*	Frog heart assay

\+ same column used.

* more than 100% recovery estimated.

GENERAL DISCUSSION

A PHYSIOLOGICAL ROLE FOR THE RELEASE OF ATP FROM CONTRACTING MUSCLE

ATP as the Mediator of the Local Autoregulation Reflex

In 1880 Gaskell put forward the proposition that local vasodilatation resulted from some product of muscular activity. Anrep and V. Saalfeld (1935) found that potent vasodilator substances appeared in the venous blood emerging from a contracting muscle. Since that time the local regulation of bloodflow through skeletal muscle has been attributed to many products of metabolism. The adenine nucleotides have long been known to be powerful vasodilators (Drury and Szent-Gyorgyi, 1929; Wedd, 1931; Wedd and Drury, 1934; Gillespie, 1934; Fleisch and Weger, 1937; Folkow, 1949; Green and Stoner, 1950; Winbury et al. 1953; Wolf and Berne, 1956) and it was suggested by Rigler (1932) and by Bildings and Maegraith (1937) that ATP possessed vasodilator properties potent enough for its consideration as a normal vasodilator metabolite.. In view of the importance of ATP in muscle metabolism and the close correlation between metabolic activity of skeletal muscle and its bloodflow, ATP has been considered as a potential candidate for the role of mediating the local autoregulation reflex (Folkow, 1949; Hilton, 1959; Folkow and Oberg, 1961; Kjellmer and Odelram, 1965).

Folkow, Haeger and Kahlson (1948) observed that intra-arterial injection of 10^{-7}g of ATP was sufficient to produce a significant vasodilatation in the hindlimbs of a cat. Folkow (1949), investigating the vasodilator action of ATP in various regions, found that the threshold dose for the vasodilator effect on muscular, cutaneous and intestinal vessels of the cat, and coronary vessels of the dog, ranged from 5×10^{-8}g to 10^{-7}g of ATP injected intra-arterially.

The release of adenine nucleotides during muscular contraction has never, up to the present work, been consistently established. Hilton and Greengard (1962) searched for evidence that ATP is released from skeletal muscle. The method that they used to detect the ATP was a fluorimetric one developed by Greengard (1956);

it enabled them to detect concentrations of less than 10^{-7} g/ml ATP. They perfused the gastrocnemius muscle of the cat with Locke's solution and found no evidence of release of ATP during muscular contractions. They tried collecting the fluid which was seeping out of the muscle during stimulation, but again did not detect ATP. They perfused human forearm muscles with Locke's solution in vivo through two needles embedded in the muscle bellies, allowing the muscles to have their own blood supply, and found in one experiment that ATP was present in the perfusate in a concentration of 3×10^{-6} g/ml during the contraction period. However, when the muscles were held under tension with a minimum of contraction taking place, no ATP was found in the perfusate. Hilton attributed this to the fact that less tissue damage was occurring in the isometric preparation and felt reasonably certain that ATP was not released from contracting muscles in amounts which could cause a significant degree of hyperaemia.

It is difficult to estimate the dilution occurring in any perfusion experiments, particularly in one of this type, where the size of the vascular bed is not a constant factor. Also, the greater the vasodilatation, the greater the dilution will be of the vasodilators in the collected perfusates. In the experiments of Hilton and Greengard dilution of ATP beyond the threshold of their method of detection may have occurred. In the present work some frog hearts have been found to be sensitive to concentrations of 10^{-9} g/ml ATP.

ATP as a Humoral Stimulator of the Heart

The stimulating effect of ATP on frog heart shown in this work is in accordance with the findings of Buday, Carr and Miya (1961) who showed that low concentrations of ATP (8×10^{-7} M) produced an increased systolic force in the frog heart with a concurrent bradycardia. This confirmed the earlier investigations on perfused frog hearts (Ostern and Parnas, (1932); Gillespie, (1934); Meyer (1951) and Porro, (1952).).

On intravenous injection ATP is rapidly destroyed, persumably in the lungs (Folkow, 1949). Although ATP is destroyed in the circulation, this destruction does not appear to occur in the blood. Folkow (1949) mixed 1mg of ATP with 1 ml of whole

blood and allowed the mixture to stand for two minutes at 37° C. This procedure did not change the vasodilator properties of the ATP when injected intra-arterially into cat and dog.

If indeed ATP is released into the bloodstream in vivo, from contracting skeletal muscle, it is possible that this is an additional humoral aid to the force of contraction of the myocardium during muscular exercise. The destruction of ATP in the lungs would seem to indicate that no recirculation of the ATP was possible; however the release of ATP into the blood returning to the right atrium from the limb muscle beds ("the muscle pump") may be an important factor in augmenting the force of contraction of the right atrium and so eventually increasing the cardiac output.

THE IDENTIFICATION AND RELEASE OF ACh FROM SKELETAL MUSCLE

Conditions of Stimulation

For the estimation of ACh released per impulse per motor nerve ending, it is obviously of vital importance to ensure that every impulse delivered down the nerve should reach the nerve terminal. The presynaptic block discovered by Krnjevic and Miledi (1957, 1958, 1959) which is shown to occur at high frequencies of stimulation presents a difficult problem. The block can be partial or complete and is thought to be precipitated by anoxia. If ACh release is measured from muscles which have been stimulated indirectly at high frequencies, any calculation of the ACh output based on the assumption that every impulse reaches the nerve terminal is erroneous. Lower frequencies of stimulation would solve this problem, but of course, the amount of ACh produced would never be sufficient for any quantitative estimation.

A convenient method of recording the number of impulses which did reach the nerve terminal is based on the curarized muscle preparation. While the muscle fibres are paralysed, activation of the nerve terminals is still possible and release of ACh is unimpaired. A microelectrode can thus be inserted near to an endplate region. This maneouvre allows monitoring of the endplate potentials to take place without any danger of breaking the microelectrode. Thus release of ACh could be directly related to the number and size of the endplate potentials produced, rather than to the number of impulses delivered down the nerve; the problem of presynaptic block would then be avoided.

Presence of Interfering Substances

Since the bulk of electrophysiological evidence concerning the neuro-muscular junction has been obtained from the post-synaptic region, any attempt to correlate the pharmacological events with electrical events places great importance on the general theory of receptor sites. If a receptor site on the post-synaptic membrane has a molecular configuration suitable for the ACh

molecule, other molecules of similar shape could become attached to the receptor site indiscriminately. An obvious example of this is given by every competitive blocking drug acting at the neuromuscular junction. Whittaker (1963) has given a most comprehensive review of the substances, allied to ACh, which can act as potent depolarizing agents at cholinergic receptor sites. Any parallel release of other, unknown, substances along with ACh at the nerve-muscle junction which could act as alternative depolarizing agents at the same receptor sites cannot be excluded. Since the bioassay systems used to estimate the amounts of ACh are, by definition, cholinergic systems containing ACh receptor sites, the assay procedure by itself may be misleading in that the unknown substances will also combine with receptors as well as the ACh in the test solution. Interference of this sort not only obscures the quantitative estimation of ACh, but also raises the inevitable question of whether it is just ACh which is involved in the natural transmission process.

Identification of ACh

The small amounts of ACh available for bioassay are, of course, insufficient for exclusive chemical analysis. Recourse has thus to be made to other, indirect, methods of identification. Chang and Gaddum (1933) described the following regime:

1. Inhibition of cholinesterase in the test solution (leech muscle, frog heart, denervated skeletal muscle, cat blood pressure etc.) usually increases the response obtained.
2. The presence of blood or tissue extract decreases or destroys the activity of ACh, and this destruction is prevented by cholinesterase inhibitors.
3. ACh is quickly destroyed by hydrolysis in an alkaline medium.
4. Boiling for several minutes in weakly acid solution does not destroy ACh.
5. Atropine, curare and nicotine individually block certain specific

pharmacological actions of ACh, but they do not interfere with the release of ACh at nerve terminals.

6. The responses of various biological indicators to ACh bear a definite quantitative relation to each other, and the chemical mediator being studied must give these same relative values before it can be accepted as ACh.

Any substance capable of attaching itself to the cholinergic receptor site and producing a depolarizarion of the membrane is likely to be acted upon in the same way as ACh by cholinesterase, an alkaline medium or an anticholinesterase. Blocking agents which effectively block ACh will almost certainly have some blocking effect on any substance which can act on the same receptors. A quantitatively similar response on different tissues produced by the chemical mediator being studied could conceivably be mimicked by a related compound; the graded response is a function of the density of the receptors, so that any substance capable of combining with the cholinergic receptor could produce a quantitatively similar response in various biological indicators. The identification of ACh released from rat diaphragm in this work involved only the use of curare. This certainly blocked the action of the substance in the test solutions which caused the leech muscle to contract; the other, conventional, piece of evidence to suggest that ACh was present in these solutions was that the dilution by half of the test solutions was equivalent to half of the ACh solution matching the undiluted test solution; however, again this is a function of the density of the receptor sites in the leech muscle, not a precise identification of ACh contained in any test solution.

The insurmountable problem in the positive identification of very small amounts of ACh would appear to be that the cholinergic receptors in one piece of tissue are used to identify the depolarizing agent released from another tissue which is cholinergically innervated. Less traditional techniques, such as gas chromatography, mass spectroscopy, electron spin resonance and other powerful tools now in

routine use by the physicists may be used in future in order to establish positively that it is ACh which is released from the neuromuscular junction.

Future Work

Although the molecular seive chromatography procedure was standardized for ACh in this work, no immediate use of this technique was made. It is proposed to combine this separation technique with the leech and frog heart assay procedures for the estimation of ACh released from the rat diaphragm. In order to have an accurate estimation of the number of impulses actually reaching the nerve terminal, the rat diaphragm preparation will be curarized and the endplate potentials will be continuously monitored. An accurate correlation of the number of impulses and the amounts of ACh released is needed before other problems of ACh synthesis and the source are investigated.

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Release of adenosine triphosphate from active skeletal muscle

By T. FORRESTER. *Institute of Physiology, University of Glasgow and the Boyd Medical Research Institute, Glasgow*

In a previous communication (Boyd & Forrester, 1965) it was shown that active frog sartorius muscle liberates a substance which has a pronounced stimulatory effect on the perfused frog heart preparation of Boyd & Eadie (1961).

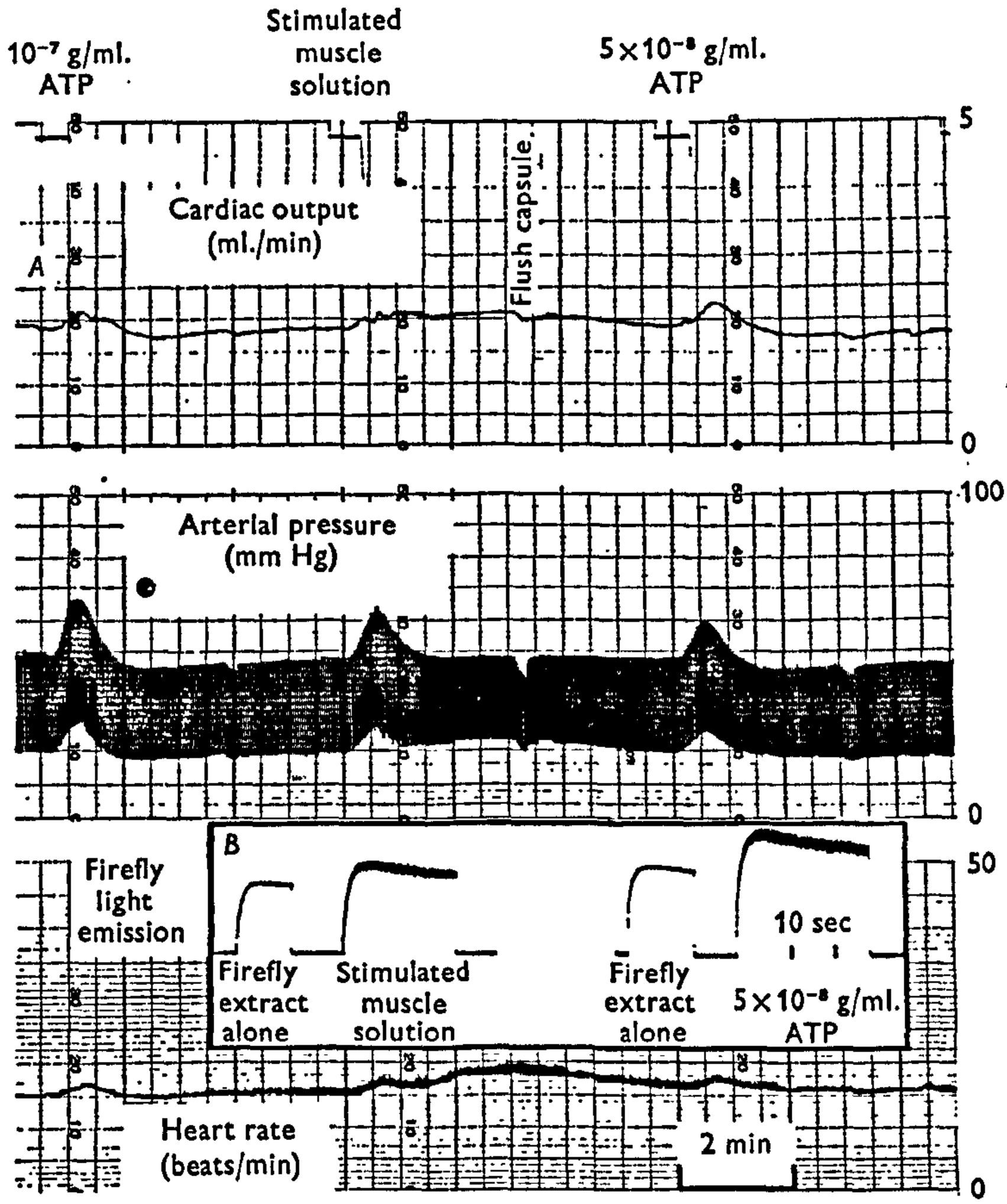

Fig. 1(*a*). The response of a frog heart to perfusion with ATP and with Ringer's solution in which a twitching sartorius muscle was bathed. (*b*) The emission of light from firefly tail extract when exposed to ATP and the stimulated muscle solution used in (*a*) above.

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It has now been established that this stimulatory effect was not due to changes in the potassium or the calcium concentrations in the Ringer's solution bathing the twitching sartorius muscle, nor was it due to release of catechol amines from the muscle, since the effect was not blocked by adrenergic blocking agents.

It appears that the stimulatory effect on the heart is produced by adenosine triphosphate (ATP) released from active muscle. The evidence is as follows:

1. The action of ATP on the frog heart is qualitatively similar to that of the stimulated muscle solution (Fig. 1*a*).

2. In a chromatography procedure the stimulatory substance is eluted through a Sephadex column in the same fractions as adenosine triphosphate.

3. The enzyme 'apyrase' catalyses the reaction ATP–AMP (Kalckar, 1943). Adenosine monophosphate does not stimulate the heart. When a solution of ATP is incubated with apyrase, its stimulatory action on the heart is greatly modified. When a stimulated muscle solution is incubated with apyrase, its stimulatory action is also modified in the same manner.

4. Using a modification of the luciferase method of assaying the ATP. (Strehler & McElroy, 1957) it has been shown that the stimulated muscle solution causes light to be emitted from a firefly tail extract (Fig. 1*b*). The time course of the emitted light from the test solution was similar to that of a known solution of ATP in a similar concentration.

From these results it is concluded that ATP is released from active frog skeletal muscle *in vitro*.

This work was financed in part by an M.R.C. grant. The cost of equipment was defrayed from the D. C. Andrews Fund and the Rankin Fund of the University of Glasgow.

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[*From the Proceedings of the Physiological Society*, 23–24 *September* 1966]
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An isometric leech muscle preparation with stable sensitivity for the assay of acetylcholine

By T. FORRESTER. *Institute of Physiology, University of Glasgow*

The change in length of a strip of muscle from the anterior dorsal wall of the leech (*Hirudo medicinalis*) is commonly used as a specific test for acetylcholine. This has the disadvantage that completion of a test with washing out of acetylcholine may take as long as 30 min, whilst the initial and final lengths of the strip may be completely different. This makes the determination of an accurate concentration–response relation very difficult.

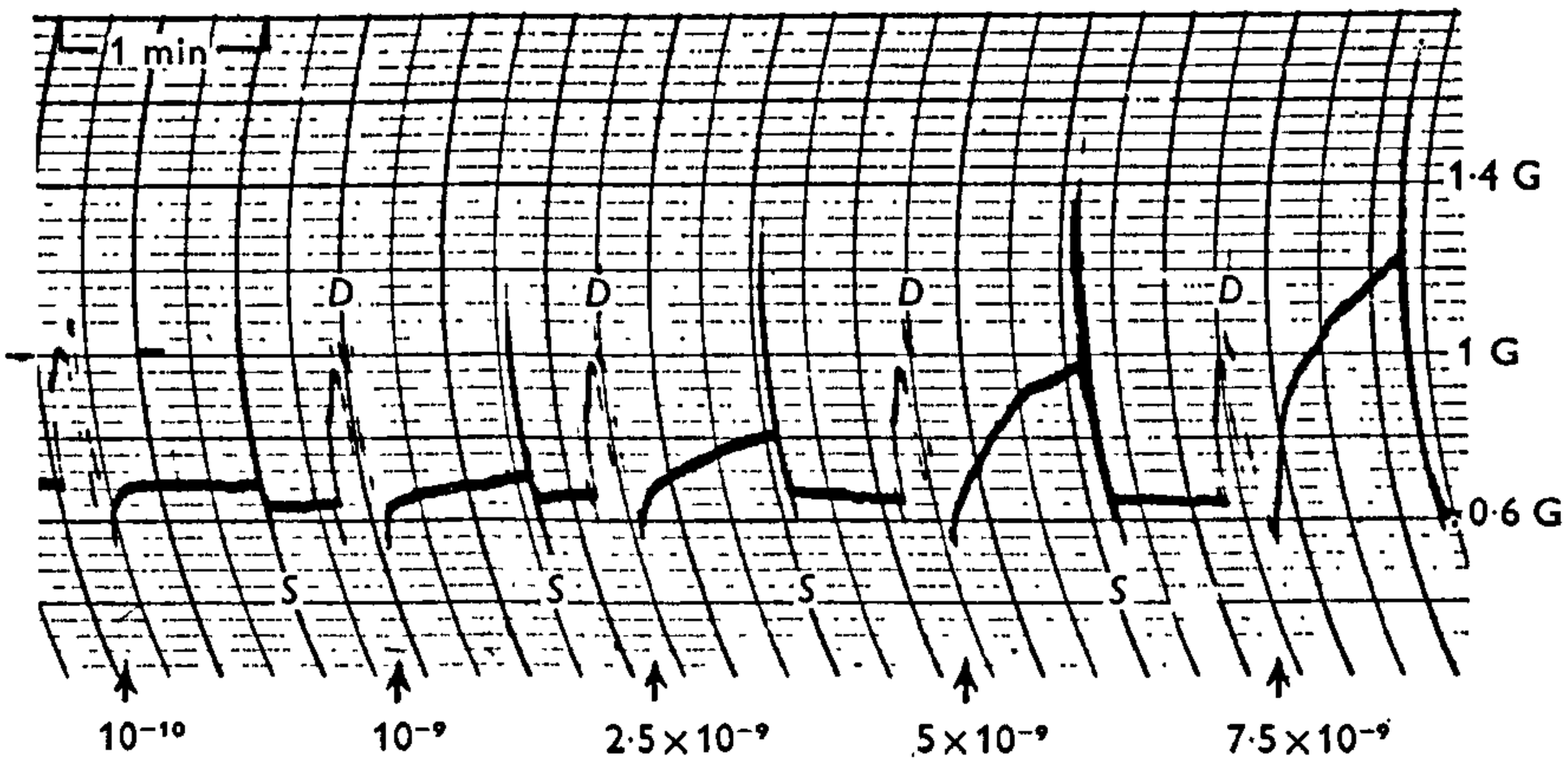

Fig. 1. The tension developed by an isometric leech muscle strip when exposed to graded concentrations of acetylcholine. Note the stability of the base line. Threshold concentration of acetylcholine was 10^{-9} g/ml. which caused an increase in tension of 50 mg in 45 sec. *D*, bath drained and refilled. *S*, paper drive stopped.

With an isometric system the effect of a test solution of acetylcholine can be seen and washed out in less than 1 min, and a constant base line is more easily achieved (Fig. 1). This makes it possible to obtain finely graded responses to small steps of concentration (Fig. 1). Concentration response curves constructed at intervals over a period of 24 hr show that the sensitivity of the leech muscle to acetylcholine does not alter significantly.

The preparation in use at the present time consists of a muscle strip of roughly 40×2 mm mounted in a 2 ml. bath. Tension developed by the muscle strip in response to a dose of acetylcholine is measured by a tension transducer (type FTO 3C, Grass Instrument Co.). Amplification was

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adjusted so that a change of tension of 50 mg could easily be recorded (Fig. 1).

The minimum concentration of acetylcholine causing significant increase in tension in different muscle strips varied from 10^{-11} g/ml. to 10^{-8} g/ml. of acetylcholine. Most preparations had a threshold response at about 10^{-9} g/ml. acetylcholine (Fig. 1).

The specificity, sensitivity and reproducibility of the concentration–response relation of this isometric preparation makes it very suitable for the assay of concentrations of 10^{-9} g/ml. to 5×10^{-9} g/ml. of acetylcholine, a range in which most other conventional procedures are somewhat unreliable.

The identification of acetylcholine. Raising the pH of a test solution, heating to 90° C and readjusting the pH and temperature is said to destroy acetylcholine. However, when this is done with a control solution, the leech muscle relaxes quickly. This effect could mask the action of acetylcholine which was, in fact, still present in the solution. A more satisfactory test consists of soaking the preparation in 10^{-5} g/ml. D-tubocurarine for ten minutes. This abolishes the leech muscle response to acetylcholine and can be reversed by washing for 30 min.

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Purification of chemical transmitter substances by column chromatography

By I. A. Boyd and T. Forrester. *Institute of Physiology, University of Glasgow*

Release of chemical transmitter substances from biological tissue is usually accompanied by the release of many other substances which can interfere with the quantitative estimation of the transmitter.

A column chromatography procedure was developed in an attempt to separate acetylcholine released from active skeletal muscle from other factors, such as protein and nucleotides (Forrester, 1966*a*).

A column of Sephadex (type G-25, Pharmacia, Sweden) was used. This column can separate completely substances of molecular weight 5000 and over from substances of smaller molecular weight. Generally, the smaller the molecule, the more slowly it is eluted through the column.

The dimensions of the column are important, since these determine the extent to which various components of smaller molecular weight in the test solution can be separated. If the column is too large, excessive dilution of the various components occurs which is inconvenient if, for example, they are to be assayed subsequently. If the column is too small, inadequate separation of the components occurs.

A glass tube of internal diameter 14 mm with a sintered glass base was packed with Sephadex G-25 suspended in Ringer's solution to a height of 13·5 cm. A substance with molecular weight greater than 5000, which is visible (e.g. blue dextran), is used to standardize the column. If 2 ml. of a mixture of blue dextran and a known concentration of acetylcholine is put through this size of column, the blue dextran is eluted into a fraction of exactly 3 ml., while almost 100 % of the acetylcholine is recovered in a later fraction of 3 ml. clearly separated from the blue dextran by a fraction containing neither substance.

The separation and recovery of acetylcholine are independent of its concentration; assay of the fluid eluted through the column for acetylcholine on an isometric leech preparation (Forrester, 1966*b*) showed that this method may be used to extract concentrations of acetylcholine as low as 10^{-9} g/ml. from, for example, a tissue perfusate.

In the specific problem for which this technique was developed, the test solution obtained from active skeletal muscle contained protein, acetylcholine released from nerve endings and nucleotides. The chromatography procedure removes the protein so that spectrophotometric identification of nucleotides in later fractions is possible; the liberated acetylcholine is

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recoverable only slightly diluted and almost free of nucleotide material so that its quantitative estimation is facilitated.

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THE IDENTIFICATION AND ASSAY OF ACETYLCHOLINE AND ADENOSINE TRIPHOSPHATE RELEASED FROM ACTIVE SKELETAL MUSCLE.

PRESENTED AS A THESIS FOR THE DEGREE OF Ph.D.
UNIVERSITY OF GLASGOW.
1967

by

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SUMMARY

The assay of ACh released from the neuromuscular junction was attempted using the frog sartorius muscle as the source of ACh and the frog heart preparation of Boyd and Eadie (1961) as the bioassay object. It was theoretically possible to measure the small quantity of ACh (10^{-10} g/ml.) which was expected to be released from the sartorius muscle stimulated at 2 impulses/second for 30 minutes, since 30% of frog hearts are sensitive to concentrations of 10^{-11} g/ml. ACh between the months of December and March (Boyd and Pathak, 1965).

At the onset of the project an unusual finding was encountered. Ringer's solution in which twitching muscles were bathed always produced a marked increase in the force of contraction of the frog heart. Solutions obtained from resting muscles augmented the contractions to a lesser degree. Solutions from indirectly-stimulated muscles paralysed by curare had a similar stimulatory action to solutions obtained from resting muscles. It was also found that a similar stimulatory effect was produced when fluid which had surrounded an active rat hemidiaphragm was perfused through the frog heart.

The stimulatory effect was not due to changes in the potassium or calcium in Ringer's solution bathing the twitching sartorius muscle, nor was it due to release of catecholamines from the muscle, since the

effect was not blocked by adrenergic blocking agents. The stimulatory effect on the heart is produced by adenosine triphosphate released from active skeletal muscle in vitro, the evidence being as follows:-

1. The action of ATP on the frog heart is qualitatively similar to that of the stimulated muscle solution.
2. In a chromatography procedure the stimulatory substance is eluted through a Sephadex column in the same fractions as ATP.
3. The enzyme "apyrase" catalyses the reaction ATP - AMP. Adenosine monophosphate does not stimulate the heart. When a solution of ATP is incubated with apyrase, its stimulatory action on the frog heart is greatly modified. When a stimulated muscle solution is incubated with apyrase, its stimulatory action is also modified in the same manner.
4. Using a modification of the luciferase method of assaying ATP, it has been shown that the stimulated muscle solution causes light to be emitted from a firefly tail extract. The time course of the emitted light from the test solution was similar to that of a known solution of ATP in a similar concentration.

It is suggested that ATP is normally released from active skeletal

muscle *in vivo* and, being a powerful vasodilator, may be the mediator of the local autoregulation reflex. It is further suggested that ATP may be released into the bloodstream *in vivo* in order to augment the force of contraction of the myocardium during muscular exercise.

In order to increase the accuracy of the quantitative estimation of ACh and also to aid in its identification, another method of bio-assay was developed using the isometric leech muscle preparation. This preparation was convenient to set up, showed constant sensitivity and stability over a long period of time and enabled a concentration-response relationship for ACh to be obtained within 30 minutes or less. This encouraged greater accuracy in the quantitative estimation of ACh. A seasonal variation in the sensitivity of the leech muscle to ACh was also apparent, the period of greatest sensitivity being in November and December.

The output of ACh released from active and resting rat hemi-diaphragm muscles was studied using the isometric leech muscle preparation. Results for the resting output of ACh agreed with those of previous workers, but the amounts of ACh recovered from muscles stimulated indirectly wer considerably less than those previously obtained. The total amount of ACh released from the muscle into a solution surrounding the muscle was found to be reduced in a smaller volume of surrounding bathing solution. This evidence, together with the fact that no alteration

in output occurred when the muscles were stimulated at a different frequency, suggests that nerve conduction block was occurring to an unknown degree and that this block was precipitated by anoxia.

The behaviour of small concentrations of ACh was studied in a molecular sieve column chromatography procedure. This was done by eluting Ringer's solution containing ACh through a Sephadex column and assaying the subsequent fractions on the frog heart and the leech muscle. By this method the separation of ACh from other produces of muscle metabolism can be effected, either in solutions which have surrounded active muscles, in tissue perfusates or in tissue extracts.

It is concluded from this work that before any accurate quantitative study of ACh released from the neuromuscular junction can be undertaken, the conditions of stimulation must be rigidly controlled. Also, the release of other substances from active skeletal muscle into a solution bathing the muscle, for example, adenosine triphosphate, may interfere with the assay procedure and so may result in the wrong interpretation of the amounts of ACh actually released from the neuromuscular junction.