

A Study of Cardiac and Ciliary Activity

**Abstract of thesis submitted for
the degree of Doctor of Philosophy**

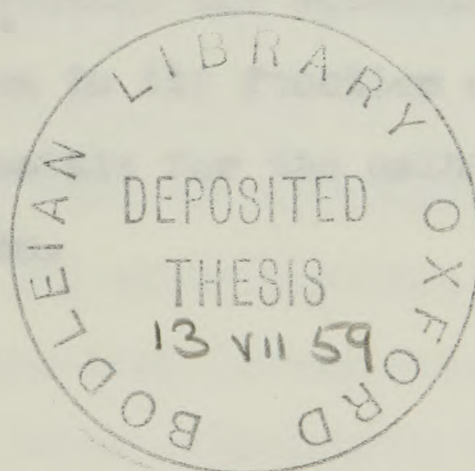
By

A. S. Milton,

St. Catherine's Society,

Oxford

May, 1959



Certain tissues have long been recognized to exhibit apparently normal movements when isolated from the body. Originally this was attributed to the presence of nervous tissue, but Gaskell and Engelmann towards the end of the nineteenth century established that cardiac muscle possessed the power of spontaneous rhythmic activity. It is now generally accepted that the heart beat is myogenic and not neurogenic in origin. Ciliary movement has always been regarded as autonomous and in 1924 Gray drew attention to the fact that the behaviour of cilia under various conditions was similar to that of cardiac muscle.

The natural occurrence and intense biological activity of the catechol amines and acetylcholine led to several workers to suggest that they might act as 'local hormones' controlling tissue activity at the site of production. Evidence has accumulated that acetylcholine, in addition to its function as a nervous transmitter, is responsible for the maintenance of spontaneous tissue movements.

In 1952, Kordik, Bülbbring and Burn made a comprehensive study of the control of the ciliary activity on the epithelium which lined the frog's oesophagus and the rabbit's trachea. They reported the presence of the enzymes concerned with the synthesis and breakdown of acetylcholine, and they demonstrated that both acetylcholine and eserine in low concentrations stimulated activity, whereas in high concentrations these same drugs depressed the activity. Both atropine and tubocurarine were found to depress the activity. Recently Hill (1957) reported that she had been unable to repeat the observations of Kordik et al. on acetylcholine and tubocurarine, and she suggested that the previous results were due to artefacts arising out of the experimental procedure.

As the earlier authors had stated that as a result of their observations they considered the ciliary activity to be controlled by acetylcholine it was thought necessary to undertake further experiments on the effect of tubocurarine and acetylcholine, and to try to account for the differences in the results obtained by Kordik et al. and those obtained by Hill.

A preparation of the frog's oesophagus was used and a very strict experimental procedure was adhered to to avoid the possibility of artefacts arising. Under such conditions it was found that when the membrane was bathed with a bicarbonate Ringer's solution tubocurarine in a concentration of 10^{-6} g/ml caused a marked decrease in activity and acetylcholine in a concentration of 10^{-6} g/ml a marked increase. These results thus confirmed the previous findings of Kordik et al. On reinvestigating the methods which Hill had used it was noticed that in the majority of her experiments the membrane had been bathed with a Ringer's solution buffered with phosphate. Experiments were, therefore, carried out using this Ringer's solution to bathe the membrane and it was found that tubocurarine was in fact without significant effect. Further experiments showed that on the same preparation tubocurarine was without effect in the phosphate Ringer, and then caused a decrease in activity when the Ringer was changed to one containing bicarbonate instead of phosphate.

Experiments showed that the effect was not due to changes in pH, but was probably due to a reduction in Ca^{++} ions caused by the complexing of these ions with the phosphate.

Further experiments were carried out on ciliated epithelium by Bilbring, Burn and Shelley in 1953 using the gill plates of *Mytilus edulis*, an example of a nerve free tissue. They measured both the force and rate of the ciliary beat and observed the effect of acetylcholine, eserine, atropine and tubocurarine on these two activities. They concluded from their results that these cilia were also controlled by acetylcholine. They were able to demonstrate the presence of a 'true' cholinesterase in the tissue, but the one weak link in their chain of evidence concerned the formation of acetylcholine. Though they found quite large amounts of acetylcholine in the gill plates they were only able to find a very low choline acetylase activity which would have been quite unable to maintain the high tissue level of acetylcholine.

The choline acetylase activity of the gill plates has now been reinvestigated. By the use of more accurate and reliable methods a far higher activity has been found. A partially purified preparation of the enzyme showed a twenty fold increase in activity over that

previously observed. The production of acetylcholine was measured over a wide range of temperature. It was found that a steady rate of production occurred over the normal environmental temperature range. The experiments of Bülbring et al. were carried out at 37°C and it was found that at this temperature the rate of synthesis was beginning to fall. Further experiments showed that whereas the enzyme was fairly stable at 20°C it was rapidly inactivated at 37°C.

Gosselin (1959) has recently reported that both 5-hydroxytryptamine and 5-hydroxytryptophan stimulated the ciliary activity on the gill plates of *Mytilus*. He also reported the presence of a 5-hydroxytryptophan decarboxylase. As Blaschko and Hope (1957) failed to detect any amine oxidase activity in the gill plates it was decided to repeat their observations using 5-hydroxytryptamine as a substrate since this had not previously been used.

The results showed that homogenates of *Mytilus* gill plates were able to oxidize 5-hydroxytryptamine but not tryptamine, tyramine or β -phenylethylamine. The reaction was not affected by Marsilid, a potent amine oxidase

inhibitor, but was completely stopped by cyanide. These observations suggested that the enzyme was not an amine oxidase but a phenolase. Further to this idea it was found that 5-hydroxytryptophan was also oxidized.

Experiments on the choline acetylase system and the synthesis of acetylcholine by acetone-dried powders prepared from rabbit atria were carried out in 1949 by Bülbring and Burn. They found that acetylcholine could be synthesized by the acetone powders in the presence of adenosinetriphosphate, citrate and 'boiled brain activator.' They observed that if acetylcholine was added to the reagents at the beginning of the experiment a decreased rate of synthesis resulted. They carried out further experiments in which they used acetone-dried powders which had been prepared from rabbit atria which had ceased to beat after being suspended in an isolated organ bath for many hours. They found that these powders were only capable of synthesizing acetylcholine in very small amounts, and when acetylcholine was added at the start of the experiment an increase in the synthetic rate was observed. A further observation made by Bülbring and

Burn was that if small amounts of acetylcholine were added to a bath containing an atria which had ceased to beat, then the beat was often restarted. A sequel to this observation was made by Burn and Rand (1958) who showed that if a rabbit atria preparation with the vagi intact was cooled until the beat stopped, then stimulation of the vagi restarted the beat.

The experiments now carried out were designed to repeat the observations of Bülbring and Burn using more modern techniques and to attempt to explain the unusual observations that under some conditions acetylcholine depressed, and under other conditions augmented the rate of synthesis. Experiments were also carried out to measure the acetylcholine content of normal atria, and atria which had ceased to beat from exhaustion. Observations were made on the effect of temperature on the rate of synthesis of acetylcholine, and particular attention was paid to that temperature range at which atria ceased to beat when they were cooled.

In the exhausted atria was found to be only 1/10 of the normal. This was thought to account for the low rate

The results obtained showed that the synthesis of acetylcholine was dependent on the availability of acetyl co-enzyme A. This emphasized the point that in any studies on choline acetylase it is essential that the availability of acetyl co-enzyme A does not become rate limiting.

When acetylcholine was added to an incubation fully saturated with acetyl co-enzyme A no significant effect was observed. Further experiments showed, however, that if the acetyl co-enzyme A concentration was reduced then the addition of acetylcholine now caused a decrease in synthesis. Acetone-dried powders from exhausted atria were shown to have the same choline acetylase activity as acetone-dried powders from normal atria, and the addition of acetylcholine to the incubation mixture caused inhibition when the acetyl co-enzyme A concentration was low.

The aceto-co-A-kinase activity of acetone powders from normal and exhausted atria was measured. The activity in the exhausted atria was found to be only 35% of the normal. This was thought to account for the low rate

of synthesis observed by Bülbbring and Burn with exhausted atria as they had relied upon this enzyme to catalyse the formation of acetyl co-enzyme A. Experiments were carried out in which the aceto-co-A-kinase present in the exhausted atria was relied upon to catalyse the formation of acetyl-co-enzyme A, the results showed only a very low rate of synthesis. Under these conditions the addition of acetylcholine to the incubation mixture resulted in an increased synthesis. If the reaction mixture was supplemented with aceto-co-A-kinase a greater rate of synthesis was observed and now the addition of acetylcholine caused a decrease in synthesis. Estimations of the acetylcholine content of both normal and exhausted atria were made and the results indicated a 50% reduction in the exhausted atria.

Experiments on the choline acetylase activity over a wide range of temperature showed that the activity dropped very rapidly below 20°C in the range where atria stopped beating when they were cooled.

Experiments were carried out on rabbit hearts to record the incidence of ventricular fibrillation induced electrically and the relationship between fibrillation, the composition of the perfusing fluid and the temperature.

Armitage, Burn and Gunning (1957) and Kärki (1958) made observations on isolated rabbit hearts perfused using a modified Langendorff apparatus. They induced fibrillation electrically and noted whether the fibrillation continued or whether normal rhythm was restored after the cessation of the electrical stimulus. They recorded the percentage of hearts which remained fibrillating at given ionic concentrations of the perfusion fluid. They showed that a reduction in the K^+ ion, and in the Na^+ ion concentration favoured fibrillation, whereas a decrease in the Ca^{++} ion concentration made fibrillation less likely. The resulting curves plotting percentage fibrillation against ionic composition showed one or two unusual peaks especially when the Ca^{++} ion concentration was varied. In 1958 Goodford found that hearts which remained fibrillating at $37^{\circ}C$ would often revert to a normal rhythm when cooled to $32^{\circ}C$, and he

suggested that at 37°C , which was the temperature used by the previous workers, the heart was in a state of partial anoxia.

Observations have now been made on the persistence of fibrillation at 32°C and 27°C . The results show the same trend as previously found, but the results show a more rational appearance. The results are discussed with a view to the fact that anoxia will both affect the length of the refractory period, and the rate of rise of the action potential.

- W. H. Gaskell, (1897). *J. Physiol.*, 20, 235.
Gaskell, W. H. (1898-1899). *J. Physiol.*, 2, 45.
Gaskell, W. H. (1899). *Philos. Trans.*, 233.
Gaskell, W. H. (1900). *J. Physiol.*, 2, 43.
Gaskell, W. H. (1900). *Methods of Physiology*, Vol. II, p. 169.
Goodford, F. J. (1908). *Brit. J. Pharmacol.*, 11, 140.
Gaskell, W. H. (1909). *Potential measurement*.
Gray, J. (1904). *Proc. Roy. Soc. B*, 26, 95.
Hall, J. B. (1957). *J. Physiol.*, 135, 157.
Hill, H. T. (1958). *J. Physiol.*, 141, 156.
Hodgkin, A. L., Huxley, A. and Tawara, S. (1952).
Brit. J. Pharmacol., 2, 67.

REFERENCES

- Armitage, A. K., Burn, J. H. and Gunning, A. J. (1957).
Circulation Res., 5, 98.
- Blaschko, H. and Hope, D. B. (1957). Arch. Biochem.
Biophys., 69, 10.
- Bülbring, E. and Burn, J. H. (1949). J. Physiol., 108, 508.
- Bülbring, E., Burn, J. H. and Shelley, H. J. (1953).
Proc. Roy. Soc. B, 141, 445.
- Engelmann, T. W. (1882). Pflüg. Arch. ges. Physiol., 29, 425.
- Engelmann, T. W. (1897). Pflüg. Arch. ges. Physiol., 65, 109.
- Engelmann, T. W. (1897). Pflüg. Arch. ges. Physiol., 65, 535.
- Gaskell, W. H. (1880-1882). J. Physiol., 3, 48.
- Gaskell, W. H. (1882). Philos. Trans. 993.
- Gaskell, W. H. (1883). J. Physiol., 4, 43.
- Gaskell, W. H. (1900). Schäfers textbook of Physiology,
 Vol. II, p.169.
- Goodford, P. J. (1958). Brit. J. Pharmacol., 13, 144.
- Gosselin, R. E. (1959). Personal communication.
- Gray, J. (1924). Proc. Roy. Soc. B, 96, 95.
- Hill, J. R. (1957). J. Physiol., 139, 157.
- Kärki, N. T. (1958). J. Physiol., 141, 366.
- Kordik, P., Bülbring, E. and Burn, J. H. (1952).
Brit. J. Pharmacol., 7, 67.

ACKNOWLEDGMENTS

I wish to thank Professor J. E. Sneyd, F.R.S., for the privilege of working in his department and for his constant advice and encouragement while supervising this work.

A Study of Cardiac and Ciliary Activity
advice over a part of this work. This thesis is submitted for the degree of
Doctor of Philosophy
By
A. S. Milton, B. Sc. Ling and Soc.
St. Catherine's Society
Oxford
helping me with my requests about photography and apparatus. May, 1959

I should like to thank Mr. E. A. Sneyd for photographing the figures. Finally I wish to express my appreciation of the patience and sympathy with which Miss Rose Sneyd has helped me during this work.

ACKNOWLEDGEMENTS

I wish to thank Professor J. H. Burn, F.R.S., for the privilege of working in his Department and for his constant advice and encouragement while supervising this work.

I also wish to thank Dr. H. Blaschko for his advice over a part of this work, and for his interest and many helpful ideas. I am also indebted to many other members of the Department for so many interesting discussions about this work. In particular I must thank Mr. H. W. Ling and Mr. O. B. Saxby for their patience in answering and helping me with my many requests about preparations and apparatus.

I should like to thank Mr. E. G. Long for photographing the figures. Finally I wish to express my appreciation of the patience and care with which Miss Foye Hart has typed this thesis.

CONTENTS

Page

PART I. INTRODUCTION

Chapter I. The Theory of Heart Beat	1
Chapter II. Ventricular Fibrillation	12
Chapter III. Acetylcholine and the Heart Beat	27
Chapter IV. Cilia and Ciliary Movement	35
Chapter V. Factors affecting Ciliary Activity	40

PART II. STUDIES ON CARDIAC ACTIVITY

Chapter VI. The relation between temperature and changes in ion concentration on ventricular fibrillation induced electrically.	
Introduction	50
Method	52
Results	56
Discussion	59
Summary	62
Chapter VII. The enzyme systems concerned with acetylcholine synthesis in the atria of the rabbit heart.	
Introduction	63
Method	65
Results	75
Discussion	84
Summary	98

PART III. STUDIES ON CILIARY ACTIVITY

Chapter VIII. The action of tubocurarine
on the ciliary activity of the frog's
oesophagus.

Introduction	99
Methods	101
Results	105
Discussion	110
Summary	113

Chapter IX. Enzymes present in the gill
plates of *Mytilus edulis*.

Choline acetylase

Introduction	114
Methods	115
Results	119
Discussion	120
Summary	123

Oxidation of 5-hydroxytryptamine
and histamine

Introduction	123
Method	125
Results	126
Discussion	128
Summary	130

REFERENCES	131
------------------	-----

CHAPTER I

The Theory of the Heart Beat

At the beginning of the eighteenth century it was generally accepted that the movement of the heart and vessels was due to the continuous action of 'animal spirits' supplied to them by the arteries. The

PART I

well known proposition of the heart beating after the removal was attributed to a reaction of 'animal spirits' remaining in it. There were one or two dissenters in this opinion. **INTRODUCTION** (1657) had suggested that

the heart beat was produced in response to distension of the auricles and ventricles caused by the blood. Ignoring the fact that the heart is a muscular structure Harvey (1578-1634) had evolved the ingenious theory based on the classical conception of the 'pneuma' or 'vital spirit' as the blood entering the heart was immediately rarefied and the resulting expansion and tension of pressure was responsible for the dilation of the heart and the passage of the blood to the lungs and the rest of the body. In 1661 William Harvey (1578-1634) was the first to suggest that the 'animal spirits' were supplied to the heart and vessels by the arteries. The way opened by Harvey's discovery, the heart

CHAPTER I

The Theory of the Heart beat

At the beginning of the eighteenth century it was generally accepted that the movements of the heart and viscera were due to the continuous influx of 'animal spirits' carried to them by the nerves. The well known phenomenon of the heart beating after its removal was attributed to a residue of 'animal spirits' remaining in it. There were one or two dissenters to this opinion. Harvey (1578-1667) had suggested that the heart beat was produced in response to distension of the auricular and ventricular muscle by the blood. Ignoring the fact that the heart is a muscular structure Descartes (1596-1650) had evolved the ingenious theory based on the classical conception of the 'innate heat' of the heart. The blood entering the heart was immediately vaporised and the resulting expansion and increase of pressure were responsible for the dilation of the heart and the passage of the vapour to the lungs and the rest of the body. It was Borelli (1608-1678) who was the first to realise that the 'animal spirits' must be corporeal in nature and suggested 'It may arise by organic necessity, the heart

may move as certain automata move. Or possibly the movement may come from a voluntary effort of which we have ceased to be conscious because it has been repeated so often and so constantly.' (Quoted by Foster, 1901).

Thomas Willis (1621-1666) had a few years earlier suggested that the motions of the heart and viscera were out of control of the Will, because, unlike voluntary movement which was produced by 'animal spirits' manufactured in the cerebrum, the heart and visceral movements were produced by 'animal spirits' formed in the cerebellum. During the eighteenth century this idea that the impulses controlling involuntary movement had a different source from those controlling voluntary movement was gradually extended. Foremost among the workers responsible for this idea were Petit (1674-1750), Winslow (1669-1760) and Whytt (1714-1766). They gradually built up the first comprehensive picture of the autonomic nervous system.

At the same time as these other workers, Haller (1708-1777) was working along different lines. He extended the earlier work of Stensen (1638-1686) and many other workers that the contraction of a muscle was not necessarily dependent on the presence of nerves, but might result as a response to chemical or mechanical stimuli. In his attributing the 'irritability' of muscular tissue to the possession of an inherent force, he became one of the first to suggest that heart movements were myogenic in origin. He believed that the movements of the heart and intestines were entirely due to this force 'vis insita'. 'I also showed that the force was something perpetually living, and that it often broke out into movement though no external stimulus such as could be recognized by us was acting'. (Quoted by Foster, 1901). It had previously been suggested that some constituents of the blood might influence the heart beat, and that the contents of the intestines were responsible for their movements. Heller believed that the stimulus from the blood constituents acted directly on the muscle, and not on the nerve endings as previous workers had suggested.

Although it was well known that the heart of the frog could beat in isolation when no blood was flowing through it, this theory was to survive until well into the nineteenth century.

Thus by the beginning of the nineteenth century the idea that the normal movements of the viscera were due to a continuous influx of some nervous influence had been replaced by the theory that they were due to stimuli acting locally on the nerves or directly on the muscular structure. Heart muscle, which could still go on beating in the absence of blood, was credited with the power of automatic rhythmic contractions.

In the middle of the nineteenth century the discovery of Remak (1844), Ludwig (1848) and Bidder (1852) of groups of ganglion cells in the sinus, the inter-auricular septum and the junctional region between the auricles and ventricles of the heart, caused a revival of the Neurogenic theory. It was first expounded in this new form by Volkmann. The well known experiments of Staⁿnius (1852) led to a close comparison of the regulatory mechanisms of the heart and of the respiratory movements by Rosenthal (1875). Remak's ganglia in the

sinus were supposed to send out impulses to the cardiac muscle at regular intervals in the same way that the cells of the respiratory centre in the medulla sent impulses to the respiratory muscles. The first Stammius ligature between sinus and auricle produced standstill of the auricles and the ventricle by blocking the passage of these impulses. The ventricle was restarted by the second ligature in the auriculo-ventricular groove since this ligature stimulated the rather less easily excitable cells of Bidder's ganglia. The heart beat was due to the automaticity of the ganglia and the blood merely served as a nutrient medium (Lengendorff, 1884).

Doubt was first thrown on this theory by Eckhard (1858) and later by Foster and Dew-Smith (1876) who demonstrated that the ganglion free apex of the frog's heart could be made to beat rhythmically by the passage of a constant current. Bowditch (1871) and Merunowicz (1875) showed that a similar effect could be produced by a nutrient fluid under pressure. The importance of tension in the ventricle walls was emphasized by Ludwig and Luchsinger (1879, 1881) and by Gaskell (1880-1882).

The upholders of the Neurogenic theory claimed that such stimuli bore no relation to those under natural conditions, but were forced to admit that cardiac muscle must be capable of responding to a constant stimulus by a series of rhythmical contractions. For this reason it was suggested that the sinus ganglia sent out a continuous impulse which was relayed to the ventricle by Bidder's ganglia acting as a co-ordinating mechanism (Eckhard, 1876, Marchand, 1878). The observation that in the dying heart the ventricle frequently beat at a different rate from the auricles was caused by this co-ordinating mechanism becoming disorganized and the normal delay between the auricular and ventricular contractions were due to the passage of the impulse through the ganglion cells. Gaskell (1882) demonstrated conclusively that the impulses originating in the sinus could not be continuous since altering the excitability of the ventricle of the frog's heart by warming it or cooling it had no effect on its rate of beat but only altered the amplitude of beat. If the impulses had been continuous and the muscle responded discontinuously, then altering the excitability of the ventricle would have altered both the rate as well as the force of beat. Gaskell stated that 'The

rhythmical contractions of the heart as a whole are caused by discrete impulses which proceed rhythmically from certain motor ganglia to the auricles and ventricles'.

At this stage in the argument various workers, notably Gaskell and Engelmann, began to assemble a body of evidence in favour of the Myogenic theory of the heart beat. This evidence was so conclusive that in spite of severe opposition the theory survives today. In 1882 Engelmann showed that the isolated bulbus arteriosus, and in 1897(a) certain parts of the large veins, would beat rhythmically in isolation although no ganglion cells were present. Gaskell (1883), in his work on the tortoise heart, demonstrated the spontaneous activity of isolated strips of ventricle tissue. Gaskell suggested that the occurrence of ganglion cells in the most easily excitable regions of the heart, in the sinus and the auriculo-ventricular junction was purely fortuitous and a consequence of the mode of development of the heart by the outgrowth and specialisation of the auricles and ventricles from the embryonic tubular heart. He postulated that in addition to ganglia these

regions must contain less specialized tissue retaining the power of spontaneous rhythmical activity as exhibited by the embryonic heart before the development of its nerve supply. The normal wave of contraction originated in the sinus muscle and was conducted rapidly by the more specialized auricular and ventricular muscle, but in the auriculo-ventricular region there was a delay due to its slower passage through the region of less specialized muscle.

The demonstration of the reversal of the heart beat in the skate and tortoise was strong evidence in support of conduction by muscular rather than nervous tissue. Gaskell demonstrated that nervous connections were not necessary for the passage of the contraction wave from auricle to ventricle and that the efficiency with which this took place depended on the amount of intact muscular tissue between the auricle and ventricle. The effect of the second Stannius ligature was attributed to stimulation of the auriculo-ventricular ring. The work of Eckhard (1876), Marchand (1878) and later Kaiser (1894) which showed that removal of Bidder's ganglia prevented conduction of the contraction wave and reduced

the excitability of the region was attributed to damage of the muscle tissue. Gaskell (1900) showed that a single stimulus applied to the muscle of the auriculo-ventricular ring could give rise to a series of contractions, whereas if auricle or ventricle muscle was pricked a single contraction occurred, and stimulation of Bidder's ganglia had no effect. The presence of ganglion cells was attributed to the vagal nerve supply of the heart which was known to have an inhibitory effect when stimulated.

Gaskell and Engelmann used amphibious and reptilian hearts in their experiments. Gaskell's explanation of the heart beat was not at first applied to mammalian hearts, since it was thought that a break in muscular continuity occurred between the auricles and ventricles in the mammal. In 1893, however, Kent showed that strands of muscular tissue and a complex system of branching fibres connected the mammalian auricles and ventricles. Meanwhile the work of Wooldridge (1883), Figerstadt (1884) and of McWilliam (1887) showed that the rhythm of the mammalian heart was essentially the same as that of the cold blooded heart.

Further support for the Myogenic theory was provided by McWilliam in 1887 who showed that the production of fibrillary contractions in the dog's ventricle by a needle thrust as found by Kronecker and Schney (1884) could not be used in support of the Neurogenic theory as ganglia were absent from this spot. Furthermore, the observation by Langley and Dickinson (1890) that very large doses of nicotine did not stop the heart confirmed the supposition that the ganglia were not responsible for the heart beat. In 1890 Badano and Funo showed that the rate of conduction in the heart muscle of a 3rd day chick embryo was much less than previous figures obtained for frog ventricle muscle. Three years later in 1893 Pickering showed that the embryonic chick heart started to beat before it received its nervous supply. Finally, the work of His (1893) and Engelmann (1897b) supported Gaskell's conclusions that the varying rhythmicity of the heart was due to the morphological arrangement of the muscle tissues and not to the presence of nervous elements.

Some opposition to the Myogenic theory arose from the description by Carlson (1905) of the neurogenic heart of *Limulus*, but it was argued that the heart muscle

CHAPTER II

of this species resembled skeletal muscle more closely than vertebrate cardiac muscle in its fundamental properties (Howell, 1917; Krijgsman, 1952). Unlike cardiac muscle it has no apparent refractory period; it can go into tetanus and can also give submaximal contractions.

In 1906 Tawara described the auriculo-ventricular node and Keith and Flack (1907) described the sinu-auricular node in a number of species. With the work of Adam (1906), Elanger and Blackman (1907) and Flack (1910) using methods similar to those of Gaskell, the position of the sinu-auricular node as the 'pacemaker' of the heart became well established and the Myogenic theory of the heart was placed on a firm footing. In 1912, Burrows showed that the rhythmic activity could be maintained for as long as 85 days in tissue cultures from chick embryos and described spontaneous activity in a single cell. This experiment demonstrated beyond all doubt that regular, spontaneous activity could be exhibited by heart muscle in complete independence of nervous influence.

CHAPTER II

Ventricular Fibrillation

Ventricular fibrillation was first described by Ericksen in 1842, and since then it has been the subject of much research. Dawes (1952) pointed out in a review article on experimental cardiac arrhythmias "The literature on the subject is full of theories about the mechanism of cardiac arrhythmias, but hard facts are hard to find." Two competing ideas have been put forward to explain fibrillation. The 'circus movement' theory, originally put forward by Garrey (1914) which involved either a regular circuit of travel by the excitatory impulse with re-entry occurring in a narrowly confined specific pathway, or alternatively entirely random movements; and the 'ectopic forms' theory suggested by Scherf and Terranova in 1949, and Prinzmetal, Corday, Brill, Oblath and Krayner in 1952, whereby the normal pacemaker lost dominance and repetitive disorganized stimulation arose from an ectopic focus.

Garrey based his hypothesis on the following observations. He noted that the ventricles of large hearts would fibrillate easily and continuously, but the ventricles of small hearts often returned to a regular rhythm. He studied pieces of tissue removed from fibrillating ventricles and observed

that the time during which the piece continued to fibrillate was related to its size. Large masses of tissue fibrillated longer than small ones, though this was not the only relevant factor. Bulky pieces fibrillated much longer than narrow strips of the same weight, so that the shape of the piece was important.

If a ring two centimetres wide was cut from the base of the fibrillating ventricle of a turtle heart, the ring continued to fibrillate. The fibrillation still continued if a second cut was made round this cylinder to form a figure of eight, the incision not being completely carried around the ring. A third cut was made across the isthmus so as to open the figure of eight into one large ring. "As soon as this narrowing was completed it was found that the incoordinated fibrillary contractions had resolved themselves into a number of contraction waves which followed each other successively and repeatedly around and around the ring, all progressing in the same direction."

Garrey noticed the spontaneous appearance of conduction blocks in parts of these rings, so that sometimes the impulse travelled on the outside at a given point and then later on the inside of the ring.

Garrey also observed that these blocks affected the contraction waves in one direction only; those moving in the opposite direction passed the region of block and continued their progress around the ring. Garrey concluded that such blocks would prevent the contraction wave from running its usual course, so that the normal coordinated action of the ventricular cells would give place to the confusion conspicuous in fibrillary contractions. He said "The blocks probably result in intramuscular ring-like circuits with resulting 'circus contractions' which are fundamentally essential to the fibrillary process". Porter in 1894 had on weaker evidence proposed a similar idea.

Garrey eliminated the possibility of an ectopic focus by a few simple experiments. He induced fibrillation by electrical stimulation, and then with a ligature or forceps he separated a small piece of muscle from the main fibrillating mass. He observed that the small piece stopped fibrillating and remained quiescent even if it contained the area which had been electrically stimulated.

Garrey stated that the electrical stimulation had not produced a local change which predisposed the muscle to fibrillation.

This theory of the 'circus movement' was developed by Thomas Lewis. Lewis, Feil and Stroud (1918-1920) mapped out the course of the excitation wave in the fluttering atria of dogs by using paired electrical contacts with which they determined the direction of the wave. They were able to show that the course lay around the orifice of the great veins, and that subsidiary 'centrifugal waves' of excitation were thrown off from this main wave to the remainder of the auricles. By establishing that the 'central wave' went around the venae cavae they obtained evidence for the 'circus movement' in vivo. In 1921, Lewis, Drury and Iliescu obtained clinical evidence on patients with auricular flutter and fibrillation. They recorded electrocardiographs in each of three planes perpendicular to each other and showed that the axis of electrical activity in the atria revolved. This revolving was almost uniform in the patients with flutter, but not in patients with fibrillation. They most easily explained their results by assuming that the circular impulse was regular during flutter, but that it sometimes changed its path during fibrillation.

Rest of the auricle, the
ventricles ceased. Cooling the injection site also
had the same effect. Because of these observations Scherf

Andrus and Carter (1930) experimented on dogs using an apparatus which would deliver stimuli to the auricles at any predetermined time during the cardiac cycle. They observed that this stimulus caused the auricles to fibrillate if it was applied shortly after the refractory period, and at a time when the vagus was also being stimulated. A vagomimetic action was also shown to be related to fibrillation by Nahum and Hoff (1940). They applied small pieces of filter-paper soaked in 1:1,000 acetyl- β -methylcholine to the surface of the auricles and observed that fibrillation was easily produced. If it did not occur spontaneously, electrical or mechanical stimulation always caused fibrillation. The fibrillation would last until after the acetyl- β -methylcholine was removed.

Scherf (1947) found that an injection of aconitine into the atria produced a tachycardia lasting 40-60 minutes. However, this tachycardia showed marked differences from the electrically induced effects. He observed that when a ligature was passed round the atria, and tightened so that it separated the site of aconitine injection from the rest of the muscle, the tachycardia ceased. Cooling the injection site also had the same effect. Because of these observations Scherf

could not reconcile his results with the 'circus movement' hypothesis. He suggested that the action of aconitine was to provide a heterotopic focus from which the tachycardia was initiated.

Prinzmetal et al. (1952) also failed to find evidence to support the 'circus movement' theory. They carried out experiments in which they blocked the predicted course of the circus impulse either by burning, cutting, or freezing the muscle; in every case fibrillation continued in the rest of the atrial tissue. Even if the entire right auricle and venae cavae were removed the fibrillation induced by aconitine in the left auricle continued, and they concluded that such observations 'conclusively prove that auricular fibrillation is not perpetuated by a circus or re-entrant phenomenon'.

Much of the work of Prinzmetal was directed to proving that there was not a circus movement round the great veins, and he pointed out that Lewis could not follow the excitation wave completely around the venae cavae. However, Prinzmetal did not necessarily eliminate the possibility of other re-entrant phenomenon. Prinzmetal et al. also studied the question whether there were one

or many foci involved during fibrillation. Using high speed cinematography and electrocardiography they stated that 'although it is possible for two or more foci to exist simultaneously in the auricles, at a given instant only one dominates and all the others remain latent'.

In summarizing it may be said that both the 'circus movement' theory and the ectopic focus theory can explain many of the experimental observations which have been made. However, to obtain a true picture of fibrillation it is necessary to try and discover what changes have occurred in cardiac muscle which predispose it to fibrillate. This approach has attracted far more attention over the past few years, as it has been more and more realized that any physiological phenomenon must eventually be attributed to a biochemical process.

The development of this new approach to fibrillation was recently reviewed by Burn (1956). The original observations were made by Burn, Vaughan Williams and Walker (1955) on the heart-lung preparation of the dog. Electrodes were applied to the tip of the right atria. They found that an infusion of acetylcholine depressed auriculo-ventricular conduction, and also that stimulation

of the right auricle caused fibrillation which continued after the cessation of the stimulation, but stopped when the infusion of acetylcholine was stopped. They also found that fibrillation was stopped by atropine. Eserine caused fibrillation to occur at much lower stimulation rates and evidence was produced which suggested that the action of the eserine was to allow acetylcholine to accumulate in the preparation (Burn and Walker, 1954).

Burgen and Terroux (1953) showed that acetylcholine reduced the duration of the action potential, and this was later reported by Hoffman and Suckling (1953) and by Johnson and McKinnon (1956). These last two also noted that the effect was antagonized by atropine.

Holland (1957) observed that when the rate of loss of potassium from isolated rabbit atria reached a certain critical value, then the atria began to fibrillate. Armitage, Burn and Gunning (1957a) found that potassium was lost from fibrillating ventricle muscle, and that fibrillation could be arrested if the potassium concentration in the perfusion fluid was raised.

Holland and Burn (1957) made further observations on the isolated rabbit atria; they were unable to produce fibrillation when the atria were suspended in normal Locke's

solution at 31°C. If, however, the potassium concentration was reduced to 1.4 mM and acetylcholine was added to the bath, then electrical stimulation caused these atria to fibrillate. The fibrillation continued after the stimulus was withdrawn, but was arrested by increasing the potassium concentration. Removal of the acetylcholine also restored a regular rhythm, and the addition of atropine had the same effect.

Wild and O'Brien (1955) found from studies of the loss of ^{42}K from the ventricle of the turtle heart that the efflux was greatest during the repolarization phase of the action potential. It therefore seemed probable that electrical stimulation affected the potassium flux in such a way as to reduce the duration of the action potential. This hypothesis was strengthened by the observations of Harris and Hutter (1956) who showed that the action of acetylcholine was to double the potassium efflux from the sinus venosus of the turtle heart; and by the work of Holland (1954) who showed that acetylcholine increased the rate of loss of potassium from guinea-pig atria. It appeared as if the fibrillatory action of acetylcholine was mediated by an effect on the ionic potassium flux and thus on the refractory period.

Ventricles behave slightly differently to auricles but the causes of fibrillation appears to closely resemble those of auricles. Thus Armitage, Burn and Gunning (1959a, b) found that raising the potassium concentration in a perfused heart reduced the ease with which fibrillation was produced. Atropine and eserine were, however, without effect on ventricular fibrillation, but this would be expected since Hoffman and Suckling (1953) showed that whilst acetylcholine reduced the duration of the atrial action potential, it did not alter the duration of the ventricle action potential.

Armitage, Burn and Gunning confirmed the observations of Kehar and Hooker (1935), who showed that the concentration of potassium in the perfusion fluid leaving the heart was increased for some minutes after the commencement of ventricular fibrillation.

These results indicated that ventricular fibrillation resembled auricular fibrillation, since changes which reduced the duration of the action potential also caused the ventricle to fibrillate.

Ventricular fibrillation may be readily produced in the dog heart-lung preparation. Unfortunately an immediate fall in the arterial blood pressure occurs, leading to the failure of the coronary flow and the death

of the preparation. One way of maintaining the coronary flow is to maintain the aortic blood pressure in the heart-lung preparation artificially. A more simple method is to perfuse the heart through the coronary vessels by the method of Langendorff (1895). In this method the perfusion fluid is supplied to the aorta at a constant pressure.

Burn, Gunning and Walker (1956), as a result of an investigation of atrial fibrillation in the heart-lung preparation, suggested that there might be a close relation between fibrillation and the movement of ions across the cell membrane. Armitage (1956) and Armitage, Burn and Gunning (1957a, b) initiated ventricular fibrillation in isolated perfused rabbit hearts by applying electrical stimuli. They studied groups of hearts at first under control conditions, and then under different experimental conditions; they recorded for each group the proportion of hearts which fibrillated. They found that when the potassium ion concentration in the perfusion fluid was low, fibrillation occurred easily, sometimes without the necessity of applying electrical stimulation; when the concentration of potassium ions was high fibrillation did not occur at all. They also found that fibrillation

could be arrested by raising the potassium ion concentration of the perfusion fluid.

They investigated the effects of changing the calcium ion, and sodium ion concentrations. They found that the relationship of calcium ions to fibrillation was complex. In twice normal Ca^{++} ions concentration (4.4 mM) there was a high incidence of fibrillation. Such an observation had already been made by Grumbach, Howard and Merrill (1954). At normal, three quarters normal, half normal and a quarter normal calcium ion concentration the incidence gradually got less. However at one eighth normal the proportion of hearts fibrillating rose again. Further reductions in calcium ion concentration diminished the proportion fibrillating until in the absence of calcium hearts did not fibrillate at all. A reduction in the sodium ion concentration to one half caused all the hearts to fibrillate. They suggested that the electrical stimulation was not an essential part of the production of fibrillation, and that the fibrillation was primarily concerned with a disturbance on ion transport, particularly K^+ ion transport.

More recently Kärki (1958) made a more complete study of changes in ion concentration. He found materially the same as Armitage et al., that reductions in potassium ion concentration and sodium ion concentration, and an increase in calcium ion concentration encouraged fibrillation to occur. He found that the effect of increased calcium ions could be neutralized by adding magnesium ions to the perfusion fluid. He also showed that a decrease in chloride ions favoured fibrillation. The main difference between the observations of Armitage et al. and those of Kärki was concerned with the effect of changes in calcium ion concentration. Whereas Armitage et al. had observed a peculiar diphasic effect, Kärki found that there was a gradual fall in the proportion of hearts fibrillating as the calcium ion concentration was reduced. He did not observe any increase when the calcium ion concentration was one eighth normal, though he did record that the percentage fibrillating was slightly higher (33%) at one sixteenth normal than at one eighth normal (30%).

Goodford (1958) investigated the action of metabolic inhibitors on the incidence of ventricular fibrillation. Using the same methods as Armitage he found that metabolic inhibitors caused fibrillation to persist, and also that lack of glucose would precipitate fibrillation. In the course of his work he observed that when a heart was in continuous fibrillation at 37°C , the fibrillation was arrested on cooling to 32°C . At that temperature electrical stimulation did not induce fibrillation, though when the temperature was raised to 37°C , it did so once more. Goodford suggested that this effect might depend on the relation between the amount of oxygen available, and the amount required by the heart. The oxygen which was available was the amount in the perfusion fluid, which was much less than the amount available in blood. Since the oxygen requirement was greater at 37°C than at 32°C it might be that the occurrence of fibrillation at 37°C was aggravated by an insufficient supply of oxygen at that temperature.

CHAPTER III

Dawes (1952) said 'a reasonably strong body of evidence exists to suggest that anoxia of cardiac muscle causes changes which predispose to the development of arrhythmias'. As a result of the observations of Dawes and of Goodford, part of this thesis is concerned with the incidence of fibrillation at temperatures below 37°C , and the relationships between this incidence and the ionic composition of the perfusion fluid.

It has been suggested that the substance responsible for the maintenance of the heart beat is acetylcholine. This idea was advanced by Dawes (1954). Later, Gordon (1956) showed that acetylcholine synthesis occurred both in the atria and the ventricles. It was Katchen (1958) who originally suggested that acetylcholine might be responsible for maintaining the heart beat. Allen and Brown (1954) (1955) and Allen (1956) found an acetylcholine precursor in the heart which liberated free acetylcholine when acidified and warmed. They postulated that this was in some way concerned in maintaining cardiac rhythm.

Such ideas have support from the fact that acetylcholine also has a stimulant action on the heart in certain conditions. For example (1937) and Rothberger and Sachs (1956) showed that low concentrations of acetylcholine or acetylcholine

CHAPTER III

Acetylcholine and the Heart Beat

The best known action of acetylcholine on the heart is an inhibitory one on rate and amplitude of beat. It is perhaps surprising, therefore, that acetylcholine has been suggested as the substance responsible for maintaining the heart beat. Engelhart (1930) and Chang and Gaddum (1933) found acetylcholine present in both the atria and ventricles of the heart, and the first demonstration that the heart could actually synthesize acetylcholine was made by Beznak (1934). Later, Comline (1946) showed that acetylcholine synthesis occurred both in the atria and the ventricles. It was Koshotojanz (1938) who originally suggested that acetylcholine might be responsible for maintaining the heart beat. Abdon and Hammarckjöld (1944) and Abdon (1945) found an acetylcholine precursor in the heart which liberated free acetylcholine when acidified and warmed. They postulated that this was in some way concerned in maintaining cardiac rhythm.

Such ideas have support from the fact that acetylcholine also has a stimulant action on the heart in certain conditions. Thus Sachs (1937) and Rothberger and Sachs (1938) showed that low concentrations of acetylcholine or carbachol

stimulated the isolated atria of the rabbit. Spadolini and Domini (1940) demonstrated a double action of acetylcholine on the isolated guinea-pig heart. In high concentrations, greater than 5×10^{-6} , acetylcholine depressed the heart, but lower concentrations stimulated it.

A number of workers have observed that acetylcholine has a stimulant effect after atropine. Haney and Lindgren in 1945 observed the effect of doses of acetylcholine ranging from 1 mg to 4 mg on the dog heart after denervation and atropinization. They considered that the tachycardia which developed could not be caused by adrenaline released from the adrenal glands since the effect was observed 4 to 7 seconds after injection, and because removal of the adrenals did not alter the response. They suggested that the acetylcholine might be stimulating intracardiac ganglion cells. Hoffmann, Hoffmann, Middleton and Talesnik (1945), McDowall (1946) and McNamara, Krop and McKay (1948) working with isolated mammalian hearts also observed this stimulant action after atropinization. Hoffmann et al. suggested that acetylcholine might be acting by stimulating chromaffin cells in the heart. In support of this McNamara et al. found that the effect of acetylcholine was blocked by

nicotine or dimethyl piperidine. McDowall, on the other hand, showed that acetylcholine continued to stimulate after sufficient nicotine had been given to paralyse autonomic ganglia. He therefore considered that acetylcholine was acting directly on cardiac muscle. Later, Kottegoda (1953) using isolated rabbit atria showed that the stimulant action of acetylcholine and nicotine, seen after atropine had been given, was abolished by hexamethonium. He also suggested that the action was due to the stimulation of ganglia or chromaffin tissue in the atria which liberated an adrenaline-like substance.

In 1949 Burn and Vane were able to show that isolated rabbit atria in which the beat had been arrested by paludrine could be restarted by the addition of acetylcholine to the bath. Vane (1948) had previously found that benzoylcholine and nicotine would not start the beat. However, choline, acetyl- β -methylcholine and carbachol were found to restart the beat. If the acetylcholine were acting by the release of adrenaline from chromaffin cells in the heart, it would be likely that nicotine should restart the beat. In fact it did not which suggested that acetylcholine was acting directly on the atrial muscle in these circumstances. This led to further

experiments on rabbit atria. Bülbbring and Burn (1949) found that after twenty-four to thirty-six hours in Tyrode's solution, isolated atria ceased to beat, and it was found that they could be restarted by the addition of acetylcholine to the bath. These results were confirmed by Holtz and Westermann (1955) who restarted fatigued rat atria with acetylcholine, and also with large amounts of propionyl and butyrylcholine. They found that these effects were blocked by atropine, but not by hexamethonium or pendiomid, indicating that this was a muscarinic action of acetylcholine. Bülbbring and Burn (1949) compared the acetylcholine synthesizing power of restarted exhausted atria with that of atria which had not been restarted and with that of freshly excised atria. A relationship was found between the power to synthesize acetylcholine, and the ability of the atria to contract. The results suggested that the normal rhythmic contractions of the heart were closely linked to the synthesis of acetylcholine. Possibly the local synthesis of acetylcholine in the heart caused a contraction when a certain concentration was reached (Burn, 1950). When the heart was beating normally acetylcholine synthesis must be at an optimum or near

optimum level, so that the addition of acetylcholine depressed the beat. If the level of synthesis was below normal, as in the exhausted atria, then addition of acetylcholine had a stimulant action.

Acetylcholine is rapidly destroyed in the heart by cholinesterases. Their presence in the heart was shown by Plattner and Hintner (1930), Antopal, Glaubach and Glick (1939) and Ord and Thompson (1950). The inhibition of these enzymes by an anticholinesterase such as eserine would be expected to modify the beat, if acetylcholine were responsible for maintaining it. Burn and Kottegoda (1953) found that eserine in concentrations of 10^{-8} and upwards usually slowed the rate of beat in isolated atria. Atria which had been beating for twelve hours showed an increased sensitivity to small doses of eserine. Bilbring, Kottegoda and Shelley (1954) demonstrated a diminished pseudo-cholinesterase activity in atria which had been beating in Locke's solution for twelve hours, and it seemed likely, therefore, that the action of eserine was exerted through the inhibition of cholinesterase.

Evidence that the beating heart produces acetylcholine has been obtained by a number of workers. Runcan (1940) in experiments with the isolated heart of *Rana esculenta*

attached to a Straub cannula found that the amplitude of beat diminished after about ten minutes if the same Tyrode solution remained in contact with the heart. He also found that this effect was abolished by atropine. Runcan suggested that the heart was producing acetylcholine or some similar choline ester without stimulation of the vagal nerves. Genuit (1941) perfused rabbit hearts in order to estimate the amount of acetylcholine liberated during vagal stimulation. He found that the control fluid obtained when the vagi had not been stimulated caused a contraction of the leech muscle, but he thought this was due to a 'resting secretion' of the vagal nerve endings. Spadolini and Giachetti (1953) made observations on the heart of *Bufo vulgaris* similar to those of Runcan and attributed the effects to endogenous formation of acetylcholine by the heart. Burn and Walker (1954) described experiments carried out on the heart-lung preparation of the dog which indicated that acetylcholine was liberated without stimulation of the vagal nerves. The addition of eserine or neostigmine to the blood caused a decrease in heart rate which could be abolished

by atropine. This indicated that there was a small continuous output of acetylcholine. Briscoe and Burn (1954) using the perfused isolated rabbit heart found that an acetylcholine-like substance appeared in the eserinated perfusion fluid. Further experiments by Day (1956) showed that the amount of acetylcholine released by the perfused heart was modified by the rate of beat. Driving the heart electrically at four times the normal rate caused a two and a half times increase in output of acetylcholine.

In 1956, Marshall and Vaughan Williams produced more indications that acetylcholine was concerned in maintaining the heart beat. They found that when isolated atria were cooled to temperatures of between 14° - 20°C , all mechanical activity ceased, but small rhythmical potentials were observed in the region of the pacemaker. When the temperature was increased, these action potentials increased in size and frequency and were propagated across the atria so that contractions developed. This effect could be observed at low temperatures if acetylcholine in a concentration of 10^{-6}

CHAPTER IV

to 10^{-8} g/ml was added to the fluid surrounding the atria. Thus it appeared that the link between the pacemaker and the surrounding tissue, which had been broken by cooling, could be restored by acetylcholine. Burn and Rand (1957) using isolated rabbit atria with the vagal nerves intact showed that atria arrested by low temperatures would start beating when the vagal nerves were stimulated. Philpot and Vaughan Williams (unpublished) found that acetylcholine synthesis was greater in the neighbourhood of the pacemaker than elsewhere.

A more complete investigation of the acetylcholine synthesizing mechanism in rabbit atria has now been made and together with studies on the acetylcholine content of rabbit atria forms a part of this thesis.

CHAPTER IV

Cilia and Ciliary Movement

Although cilia and ciliary mechanisms were first observed and commented on by de Meide in 1683 (Engström, 1951) and by Leeuwenhoek (1632-1723), quoted by Dobell, 1932), it was not until Purkinje and Valentin (1834) described their occurrence in various vertebrate species that ciliary movement became the subject of serious physiological study. Sharpey (1835) and Müller (1840) gave accounts of ciliary movement which indicated that during the eighteenth and early nineteenth centuries a great deal of work had been done on cilia in invertebrates and the Protista, but this was largely descriptive and no very coherent ideas of the mechanism and control of ciliary activity had been advanced. Ehrenberg is quoted as describing the movements of infusorian cilia as being due to minute muscles attached to the bulbous bases of the cilia, and Valentin described the granular striae in certain ciliated cells and epithelium as muscular fibres responsible for ciliary movement. Grant in 1835, after studying the ctenophore Beroë believed that water was pumped in and out of the cilia which were thought to be hollow, and were

thus analogous to the tube feet of echinoderms.

Sharpey (1835), however, considered this view very unlikely since ciliary activity could continue long after death, and in isolated cells. Sharpey's own view was that muscular material probably formed part of the cilia, and this view is much more in agreement with the modern view that the cilium is a contractile structure not unlike a muscle fibre.

It was very early recognized that cilia were not under direct nervous control. As Müller (1840) observed 'Those substances which have a particular action on the nervous system, namely the narcotics, have not the slightest influence on the ciliary motion, which is thus distinguished as a peculiar phenomenon not dependent on nervous energy'. In 1853 Brown-Séquard emphasized that 'The cilia are known to have movements independent of the nervous system.' Nevertheless, during the nineteenth century several workers suggested that at least in some invertebrates the cilia were under nervous control. Apáthy (1897) suggested that the intracellular fibrillar systems apparent in many ciliated cells were nervous in function, though no connections with the nerves could be demonstrated.

The evidence for some measure of nervous control in invertebrates is strong, and since cilia play a very much more important role in them than in vertebrate species this is hardly surprising. Babák (1913) suggested that the periods of quiescence in the activity of cilia on the gills of lamellibranchs indicated some system of nervous control. Copeland (1919, 1922) although he found no definite evidence, obtained clear indications of nervous control in the pedal cilia of certain gastropod molluscs. Grave and Schmitt (1925) described a system of fibres in molluscan gills which they considered were responsible for the co-ordination of the beat. The effects of electrical stimulation were investigated by several workers. G8thlin (1920) demonstrated that the swimming plates of Beroë could be controlled by impulses conducted by the nerve net. An interesting effect which G8thlin noticed was that atropine and chloral hydrate abolished the inhibition caused by electrical stimulation. Nervous control of the foot cilia of the nudibranch mollusc Melibe was suggested by Ayersborg (1923) who described a group of ganglion cells and fibres extending to the base

since in 1900, Lommel had demonstrated that section of the ciliated cells. The only description of nervous effects mediated by nerve fibres actually terminating in the ciliated cells was made by Carter (1926, 1928) in the larvae of the nudibranch Veliger. Electrical stimulation stopped ciliary movement. However, in 1923 Merton demonstrated that electrical stimulation of nerves to the snail's lip could activate previously quiescent cilia.

In the case of vertebrate cilia there is far less evidence for any nervous influence over ciliary movement. In a very striking experiment von Brücke (1917) demonstrated the autonomy of the cilia of the frog's oesophagus. He removed a piece of the epithelium, rotated it through 90° and replaced it. The cilia continued to beat normally at right angles to the surrounding cilia.

No histological evidence has been obtained for the existence of nerve endings in vertebrate ciliated tissue, though it has been reported that nervous stimulation could affect the rate of ciliary beat. Rozansky (1926) reported that the rate of ciliary movement in the dog trachea could be accelerated by vagal stimulation, but it seemed unlikely that this was a natural phenomenon

since in 1908, Lommel had demonstrated that section of both vagi in the dog had no effect on ciliary movement in the trachea. McDonald, Leisure, and Lenneman (1928) showed, however, that stimulation of sympathetic fibres in the frog accelerated the rate of ciliary movement in the buccal cavity, whereas parasympathetic stimulation caused inhibition. Seo (1927) also described accelerator effects on ciliary movement in the frog in response to nervous stimulation. Plattner and Hou (1931) described similar effects, but they attributed this to direct stimulation of the ciliated epithelium and evidently did not believe in any nervous effects.

The evidence suggests that ciliary movement is not dependent on nerve impulses, but that in certain cases it may be modified in response to nervous stimuli. There is thus a close parallel with the heart beat which is also myogenic in origin but which may be quickened in response to stimulation by sympathetic fibres or slowed by parasympathetic impulses.

CHAPTER V

Factors affecting Ciliary Activity

Cilia are extremely sensitive to changes in their environment, amongst the most important are changes in inorganic ions, in pH, in temperature, in the availability of oxygen, and the effects of mechanical stimulation.

The effects of different ions on ciliary activity was investigated by a number of workers, notably Lillie (1902, 1906) and Maxwell (1905). The investigations were continued by Gray (1920, 1922a, b and 1923). He found that a solution containing only one of the four ions, Na^+ , K^+ , Ca^{++} , and Mg^{++} was extremely toxic to the cilia on the gill plates of *Mytilus edulis*. They caused marked disintegration of the cells, even at low pH values. Solutions containing two ions were less detrimental, and solutions containing three ions supported the tissue in a comparatively healthy state for as long as seventy-two hours. But it was only when all four ions were present that the tissue remained normal and healthy as in seawater. Excess magnesium resulted in arrest of the lateral cilia, but this effect could be reversed by raising

the potassium concentration. Potassium was found to be essential for the beat of the lateral cilia, but not for the others. Excess potassium caused tonic contractions of the fronto-lateral cilia while the others were unaffected, though the beat of the lateral cilia was sometimes quickened. Lithium ions were found to slow the beat. Sodium was not only necessary for the maintenance of osmotic pressure, but it was also more effective than any other ion for maintaining the beat. Calcium was found to be essential for the beat of the frontal cilia but was not essential for the others except at low pH values; its lack resulted in an increased sensitivity to hydrogen ions. The effect of lack of calcium and potassium could be alleviated by raising the pH, and this was also effective in counteracting the effect of lithium. In solutions containing tartrates and citrates the bivalent metals magnesium and calcium were probably not present in the ionic state and the cilia behaved as though these metals were absent. The ciliated cells were found to be insensitive to the following ions:- Cl^- , NO_3^- , Br^- , I^- , SO_4^{--} and CH_3COO^- as long as the normal equilibrium of the cations Na^+ , K^+ , Ca^{++} and Mg^{++} was maintained. Similar results have been obtained with

that of varying the osmotic pressure of the solutions (Gaskell, 1932). Goss (1934) found similar results.

heart preparations by Mines (1912), Sakai (1914) and Kohn and Pick (1920).

Ciliary movement is very sensitive to changes in pH. Gray (1922a) showed that in *Mytilus* the cilia when exposed to an acid solution came to rest by gradual slowing of the whole beat, the amplitude of the beat not being affected. He said that the cessation of movement in an acid solution was due to changes which took place inside the cell, and not at its surface. He advanced evidence which suggested that the presence of acid prevented the conversion of chemical energy into kinetic energy. The activity in *Mytilus* would continue between pH values of 5.0 to 9.5 with a maximum rate at 9.0. The cilia were also brought to rest if the osmotic pressure of the external medium exceeded a certain value.

The effect of temperature on ciliary movement was first investigated by Purkinje and Valentin (1834) and it has been confirmed by many workers that cilia are particularly sensitive to changes in temperature (Gray, 1923; Hill, 1957). A rise of 10°C will usually produce a big increase in the rate of beat. The beat is normally arrested at 2°C . These effects may be compared with that of warming or cooling the sinus region of the heart (Gaskell, 1882). Gray (1924) called attention to the

similarity between the temperature coefficient for the frog sinus as calculated by Snyder (1907) and his own figures for the cilia of *Mytilus*. Also the phases of heat rigor obtained by excessive heating resembled those described for the heart.

Gray (1924) measured the oxygen consumption of the gill plates of *Mytilus*. He found that in the absence of oxygen ciliary activity continued only for about one hour. The cilia also stopped beating in the presence of cyanide. When the ciliary activity was depressed by acids the oxygen consumption fell. Increase of temperature increased both the oxygen consumption and the rate of beat. A similar effect had been recorded in 1912 by Evans for the heart.

Ciliated epithelium is extremely sensitive to mechanical stimulation. Kraft (1890) observed the rapid spread of increased activity after stimulation and he realized the importance of trying to interpret the conduction mechanism of ciliated tissues. Seo (1927) believed that the spreading of the wave of excitation took place through a nerve net under the ciliated epithelium of the frog palate, since an incision just below this membrane which separated the epithelium

almost stopped the propagation of the wave. There is little evidence in support of this theory, but the sensitivity to mechanical stimulation is extremely important when the transport of particles is taken as an index of ciliary activity.

Lucas (1932) even claimed that in the absence of mechanical stimulation the cilia of the frog's oesophagus were normally quiescent, and he described the immediate burst of activity which followed the application of lamp black from an atomiser. It has already been mentioned that cardiac muscle is activated by mechanical stimulation and tension.

McDonald, Leisure and Lenneman (1929) were the first to make a real investigation of the effects of the 'autonomic' drugs on ciliary movement. Using the cilia of the buccal cavity of the frog they showed that adrenaline and ephedrine accelerated the transport of particles by the cilia, whereas pilocarpine had an inhibitory effect. Atropine had a mixed action; whereas the effect on the cilia usually stimulatory, particle movement was slowed due to a depression of mucus secretion. Pohle (1931) observed the inhibitory effect of atropine, and also noticed a curious

temperature reversal of the effect of adrenaline; at 16°C a slowing effect was obtained, whereas at 29°C adrenaline accelerated the movement. These results were later confirmed by Shelley (1953) who found that at an intermediate temperature of 22-24°C adrenaline had no effect. Plattner and Hou (1931) made an exhaustive study of the ciliary epithelium of the frog's oesophagus, and observed that acetylcholine in concentrations of 10^{-5} , and 10^{-4} g/ml had a pronounced stimulating effect, also choline in a concentration of 10^{-2} g/ml had a weak effect. Pilocarpine and arecoline were effective stimulants, while eserine though appearing to have no action of its own, was found to potentiate the effect of acetylcholine. Atropine 10^{-5} g/ml abolished all these effects including a weak stimulating effect of adrenaline 10^{-5} g/ml.

Lierle and Moore (1934) reported a detrimental effect of adrenaline on the cilia of the guinea-pig turbinate, but observed a slight stimulating action for ephedrine. However, in 1944, Lierle and Evers demonstrated a slowing effect of ephedrine on nasal cilia.

Boyd (1938) observed a slowing effect with amphetamine on the cilia of the frog's oesophagus, but Proetz (1939) stated that amphetamine had no effect on nasal cilia. Weatherby (1940) reported that nicotine would depress the activity of frog cilia.

Bleich and Klar (1950) observed that penicillin augmented the accelerator effects of acetylcholine and adrenaline on frog cilia and that in its presence eserine, which alone had no effect, had an accelerator effect. Penicillin alone had a slight accelerating effect and all these actions were abolished by atropine.

In 1952, Kordik, Bülbring and Burn made an extensive study of the effects of drugs on the cilia of the frog's oesophagus and the rabbit's trachea. They demonstrated the presence of acetylcholine, and the enzymes choline acetylase and cholinesterase. They observed that in both preparations low concentrations of eserine less than 2×10^{-4} g/ml increased the rate of transport of particles over the ciliated surface, whereas higher concentrations decreased the rate. Acetylcholine 10^{-5} g/ml increased the rate whereas stronger concentrations decreased it. Atropine and d-tubocurarine decreased the rate of transport in both preparations at a concentration of

Cholinesterase was found to be present, and they

10^{-6} g/ml. Adrenaline had a slight accelerating effect on the rabbit trachea. All these effects were reversible. Noradrenaline and histamine were without effect. As the epithelium contained no ganglion cells and very few nerve fibres the authors concluded that the acetylcholine was of non-nervous origin and was acting as a local hormone in promoting the rhythmic movement of the cilia.

Hill (1957) was unable to confirm the effects of acetylcholine and d-tubocurarine on the frog's oesophagus and rabbit's trachea, and she suggested that the results of Kordik et al. were due to artefacts arising from their experimental technique. Later, Burn and Day (1958) repeated and confirmed some of the observations of Kordik et al. A part of this thesis is concerned with a reinvestigation into the effect of acetylcholine and tubocurarine on the frog's oesophagus under varying experimental conditions.

The gill plates of *Mytilus edulis* are nerve-free ciliated structures and were used for further work by Bülbring, Burn and Shelley (1953). Acetylcholine was shown to be present in gill plate extracts, both by biological assay and by paper chromatography. Cholinesterase was found to be present, and they

also reported the presence of the enzyme choline acetylase, though the activity was so low that some doubt was cast upon this by Hebb (1957). The action of acetylcholine, eserine, and d-tubocurarine on the rate of ciliary beat measured both by rate of particle transport and with the use of a stroboscope was found to be similar to that seen in the frog's oesophagus and rabbit's trachea. Atropine, however, was found to act like acetylcholine when given alone, although it diminished the rate when the two substances were applied together. A suggested explanation of this effect was that if atropine blocks the action of acetylcholine by competing for the same receptors, it is conceivable that in some tissues, atropine when given alone might have an acetylcholine like action. The effect of adrenaline was also tested, and was found to accelerate the rate of ciliary movement, though only in rather high concentrations. In 1957 Day produced evidence of a histamine-like substance in extracts of the gill plates of *Mytilus*. Gosselin (1959) has reported that the cilia of the gill plates are accelerated by 5-hydroxytryptamine, and that this effect is potentiated by 5-hydroxytryptophane.

Part of this thesis deals with the choline acetylase system of the gill plates of *Mytilus edulis*, and also an investigation into some of the enzymes concerned with histamine and 5-hydroxytryptamine metabolism.

PART II

STUDIES ON CHOLINE ACETYLASE

CHAPTER VI

The relative between temperature and electrical activity

Investigation of the relationship between temperature and electrical activity

Introduction

Introduction

Cardiac fibrillation can be produced by the

longest propagation of the impulse across the heart

PART II

by electrical stimulation of the myocardium. When

the stimulation is stopped the heart enters a state

of cardiac arrest. The heart enters a state of

STUDIES ON CARDIAC ACTIVITY

Some and others (1957a,b) investigated various factors

which determined whether fibrillation occurred or

not, noting that the amount of K^+ and Ca^{++} ions

in the perfusing fluid. They found for example that

the proportion of hearts which continued to fibrillate

rose as the K^+ ion concentration was raised. Rosen (1958)

made a more complete study of changes in ion concentrations

and Gossard (1958) investigated the action of metabolic

inhibitors. He found that metabolic inhibitors caused

fibrillation to persist and also that a lack of glucose

would precipitate fibrillation. In the course of the

work he observed that when a heart was in continuous

fibrillation at $37^{\circ}C$, the fibrillation would often be

arrested by cooling to $32^{\circ}C$. It was therefore suggested

CHAPTER VI

The relation between temperature and changes in ion concentration on ventricular fibrillation induced electrically

Introduction

Ventricular fibrillation can be produced in the Langendorff preparation of the isolated rabbit heart by electrical stimulation of the ventricles. When the stimulation is stopped the heart either reverts to normal rhythm or fibrillation continues. Armitage, Burn and Gunning (1957a,b) investigated various factors which determined whether fibrillation continued or ceased, among them the amount of K^+ and Ca^{++} ions in the perfusing fluid. They found for example that the proportion of hearts which continued to fibrillate rose as the K^+ ion concentration was reduced. Kärki (1958) made a more complete study of changes in ion concentrations, and Goodford (1958) investigated the action of metabolic inhibitors. He found that metabolic inhibitors caused fibrillation to persist and also that a lack of glucose would precipitate fibrillation. In the course of his work he observed that when a heart was in continuous fibrillation at $37^{\circ}C$, the fibrillation could often be arrested by cooling to $32^{\circ}C$. At that temperature electrical

stimulation did not induce fibrillation, though when the temperature was raised to 37°C it did so once more. Goodford suggested that this effect might depend on the relation between the amount of oxygen available and the amount required by the heart. Since the oxygen requirement was greater at 37°C than at 32°C it might be that the occurrence of fibrillation at the higher temperature was due to an insufficient supply of oxygen at that temperature. Kärki, for example, found that at 37°C no less than 15 out of 28 hearts fibrillated continuously after electrical stimulation although the fluid perfusing the hearts was of the usual ionic concentrations.

These observations suggested that a clearer picture of the effect of changes in ionic concentration would be obtained if experiments were carried out at lower temperatures. The results of such experiments are described.

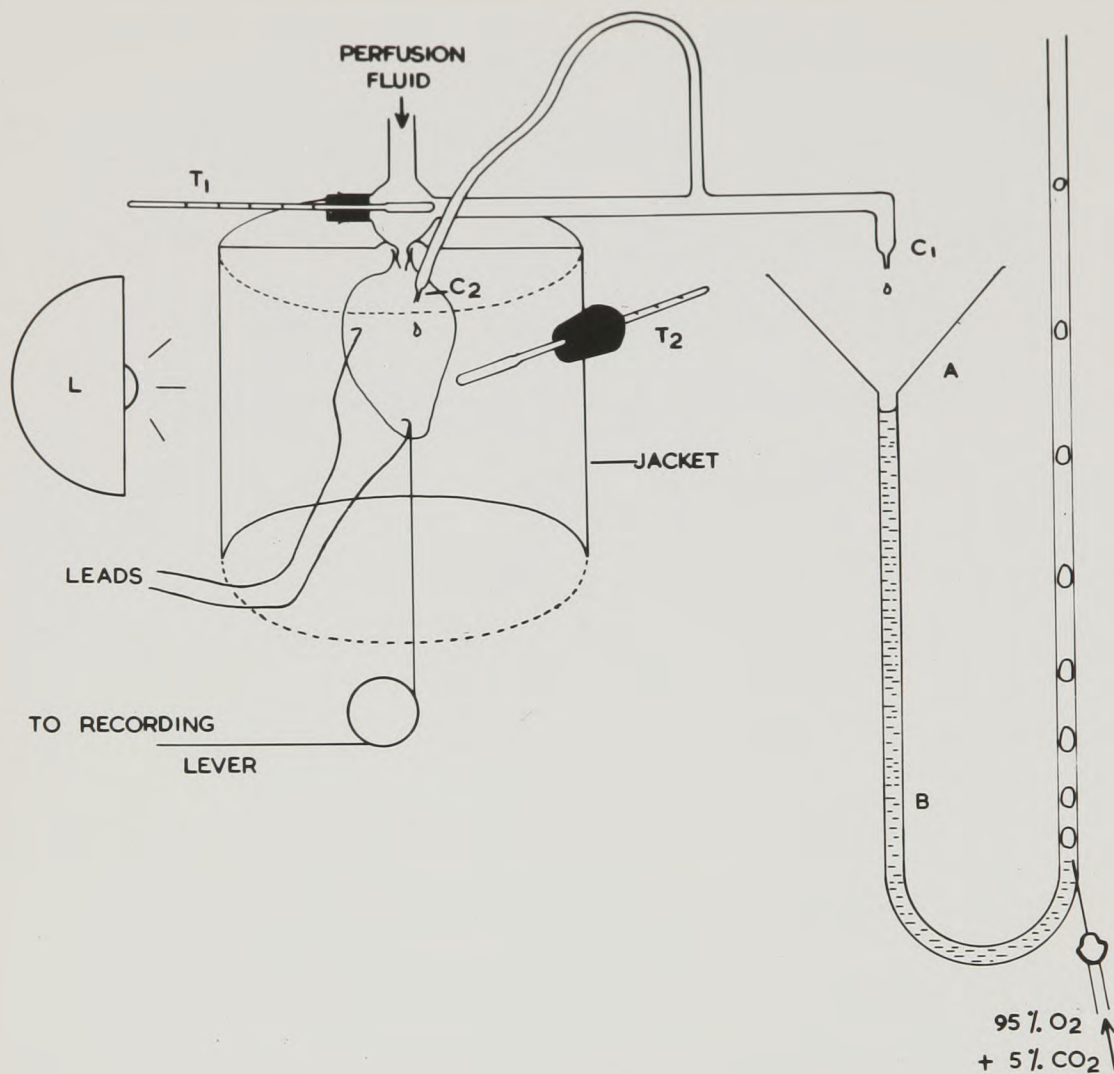


Fig.1. The modification of the conventional Langendorff apparatus used in the study of ventricular fibrillation in the isolated rabbit heart.

T_1 , T_2 = thermometers

L = lamp

C_1 = constriction allowing return of Ringer's fluid to reservoir.

C_2 = spray keeping atrial surface moist.

A, B = air lift return to reservoir.

Method

The method used was similar to that described by Armitage, Burn and Gunning (1957a). Rabbits of either sex weighing between 1.5 to 3.0 Kg were decapitated. The heart was rapidly removed and washed in cold oxygenated McEwen's solution. The surrounding pericardium and fat were carefully cut away to expose the atria. The aorta was cannulated taking precautions to see that neither air bubbles nor the cannula were blocking the coronary vessels. The apparatus used was based on the idea of Langendorff (1895) and is diagrammatically represented in Fig.1.

The temperature of the fluid perfusing the heart was maintained constant despite variations in coronary flow by using the device of Saxby (1956).

The perfusion fluid on reaching the cannula was able either to pass through the aorta into the coronary system, or through the side arm by-passing the heart. This fluid was then returned to the reservoir by an air lift. Some of the fluid in the side arm was not returned to the reservoir but was allowed to pass through a hypodermic needle from which it sprayed over the heart at a rate of approximately 5 ml/min. This

spray was arranged to keep the right auricle particularly well moistened in order to preserve the pacemaker.

The heart was surrounded by a cylindrical perspex jacket from which the front could be detached in order to facilitate the insertion of the E.C.G. leads. This jacket prevented draughts from changing the atmosphere around the heart, so that the air became saturated with water-vapour. By suitably adjusting the position of a lamp situated near the perspex jacket it was possible to maintain the temperature inside the perspex jacket at the same temperature as the fluid entering the heart. The temperature of the perfusion fluid was maintained constant by passing it, after it had left the reservoir, through a coil contained in a water-bath thermostatically controlled to $\pm 0.5^{\circ}\text{C}$. The pressure at which the hearts were perfused was 54 cm of water, and this was maintained by using a Marriotte bottle for the reservoir.

By using a second Marriotte bottle placed at the same level above the heart and attached to the heating coil by a T-piece a different solution could be perfused

through the heart. When such a change was made, the fluid passing through the side arm was not returned to the reservoir until 200 ml had passed through and had been discarded. Since the volume of tubing between the reservoir and the cannula was 100 ml, this ensured that the new perfusion fluid had completely filled the apparatus.

The normal perfusion fluid used was that described by McEwen (1956) and had the following composition:-

(mM) NaCl 132, KCl 5.6, CaCl_2 2.2, NaHPO_4 0.92, NaHCO_3 2.5, glucose 11.1, and sucrose 13.1.

The solution was thoroughly saturated with O_2 95% and CO_2 5%.

The respective mM concentrations were increased or decreased in the appropriate amounts to obtain the test solutions required. In the experiments where the Na^+ ion concentration was reduced to $\frac{1}{2}$ normal, the osmolarity was maintained by adding an equimolar amount of sucrose.

The ventricles were stimulated by platinum electrodes, one piercing the wall of the left ventricle at the apex, and the other near the base midway between the

origins of the right and left coronary arteries. When stimulation was not being applied the electrodes were used as leads to an electrocardiograph (Cossor model 1314). A mechanical record was obtained on a smoked drum by a thread attached to the electrode in the apex of the left ventricle. The stimuli were square wave pulses of 0.75 msec duration and approximately 1 to 2 mA in strength. Stimulation was begun at a rate of about 580/min, and the rate was gradually increased until fibrillation was observed. The maximum rate was 1,200/min. Hearts were stimulated for 5 min.

When the stimulation was stopped, each heart was observed to see whether it would revert to a normal rhythm. Hearts reverting within 15 min were recorded as 'not fibrillating,' while hearts not reverting within this time were recorded as 'fibrillating.'

The hearts were first perfused for 30 min with the normal solution, this was then changed to the solution under test, and at the end of a further 30 min stimulation was applied.

TABLE 1

Proportion of hearts fibrillating and K^+ ion
concentration

K^+ concentra tion mM	37°		32°	
	Proportion	Percentage	Proportion	Percentage
11.2	0/8	0		
5.6	15/28	53	2/8	25
2.8	12/13	90	3/9	33
1.4	10/10	100	6/9	67

Results

Variations in K^+ ion concentration

Observations were made at the normal K^+ ion concentration of 5.6 mM, and at concentrations of one-half, and one quarter of this at 32°C. The results are given in Table 1, together with the results at 37°C obtained by Kärki (1958). The general trend was the same at 32°C as at 37°C, the proportion of hearts which fibrillated increased as the K^+ ion concentration fell. At each concentration, however, the proportion of hearts which fibrillated was much less at 32°C than at 37°C. The difference was particularly noticeable when the K^+ ion concentration was 1.4 mM, because at 37°C many hearts fibrillated spontaneously without stimulation, while at 32°C only 6 out of 9 fibrillated, and then only after stimulation.

A series of six hearts was examined at 27°C; at this temperature it was found that even when the K^+ ion concentration was only 1.4 mM none of the hearts fibrillated. An even more interesting observation was

TABLE 2

Proportion of hearts fibrillating and Ca^{++}
ion concentration

Ca^{++} concentration mM	37°		32°	
	Proportion	Percentage	Proportion	Percentage
8.8	-	-	5/6	83
4.4	12/12	100	3/6	50
2.2	15/28	53	2/8	25
1.1	7/15	47	1/6	16
0.55	3/8	37	-	-
0.275	6/21	30	-	-

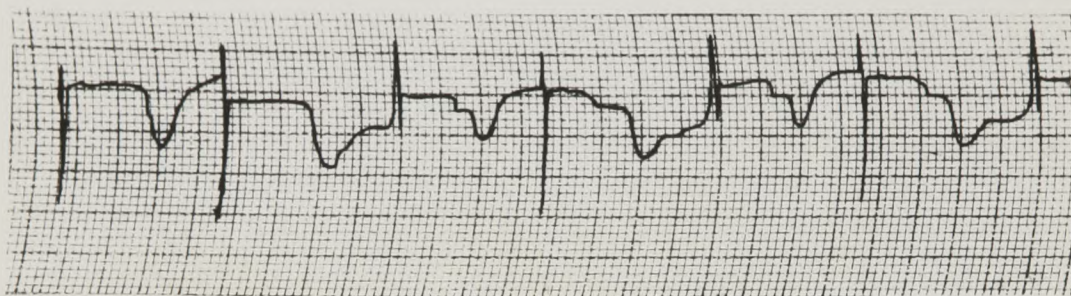
that at 27°C the beat was more regular in 1.4 mM K⁺ ion concentration than in 5.6 mM. This is illustrated in Fig.2.

Variations in Ca⁺⁺ ion concentration

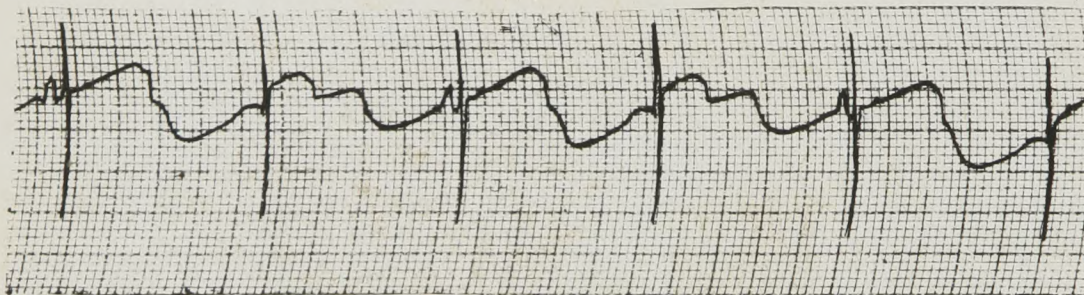
When the K⁺ ion concentration was kept at 5.6 mM and the Ca⁺⁺ ion concentration varied, the proportion of hearts which fibrillated followed a much more reasonable course at 32°C than was found at 37°C by Armitage and his colleagues, and by Kärki. The proportion of hearts which fibrillated was more or less parallel to the Ca⁺⁺ ion concentration, rising to 83% when it was 4 times normal, and falling to 16% when it was $\frac{1}{2}$ times normal. The results are shown in Table 2 and Fig.3.

Variations in Na⁺ ion concentration

When the K⁺ ion and Ca⁺⁺ ion concentrations were kept normal, and the Na⁺ ion concentration was reduced to half, it was found that at 37°C the percentage of hearts which fibrillated rose from 53 to 100. In experiments at 32°C, when a similar reduction in Na⁺ ion concentration was made, the tonicity being maintained by adding sucrose, 4 out of 6 hearts fibrillated, the percentage rising from 25 to 67.



K⁺ion concentration 5.6mM 27°C



K⁺ion concentration 1.4mM 27°C

Fig.2. E.C.G. recorded from ventricle of rabbit heart showing effect of 5.6 mM and 1.4 mM K⁺ at 27°C.

Effect of changes in ionic concentration on normal cardiac rhythm

Observations were also made on the amplitude and rate of beat of the heart. At 32°C the amplitude was larger and often an increase in rate was observed both when the K^+ ion or Na^+ ion concentration was reduced, and also when the Ca^{++} ion concentration was increased.

At 37°C when the hearts were perfused with solution containing 1.4 mM K^+ ions it was usually observed that the amplitude steadily increased together with a slight increase in rate until eventually the heart went into spontaneous fibrillation.

The further observation was made that at 32°C it was more difficult to cause the heart to go into fibrillation on electrical stimulation than at 37°C. This was particularly noticeable in 1.1 mM Ca^{++} ions. Some hearts could be subjected to currents as large as 10 mA without fibrillation occurring.

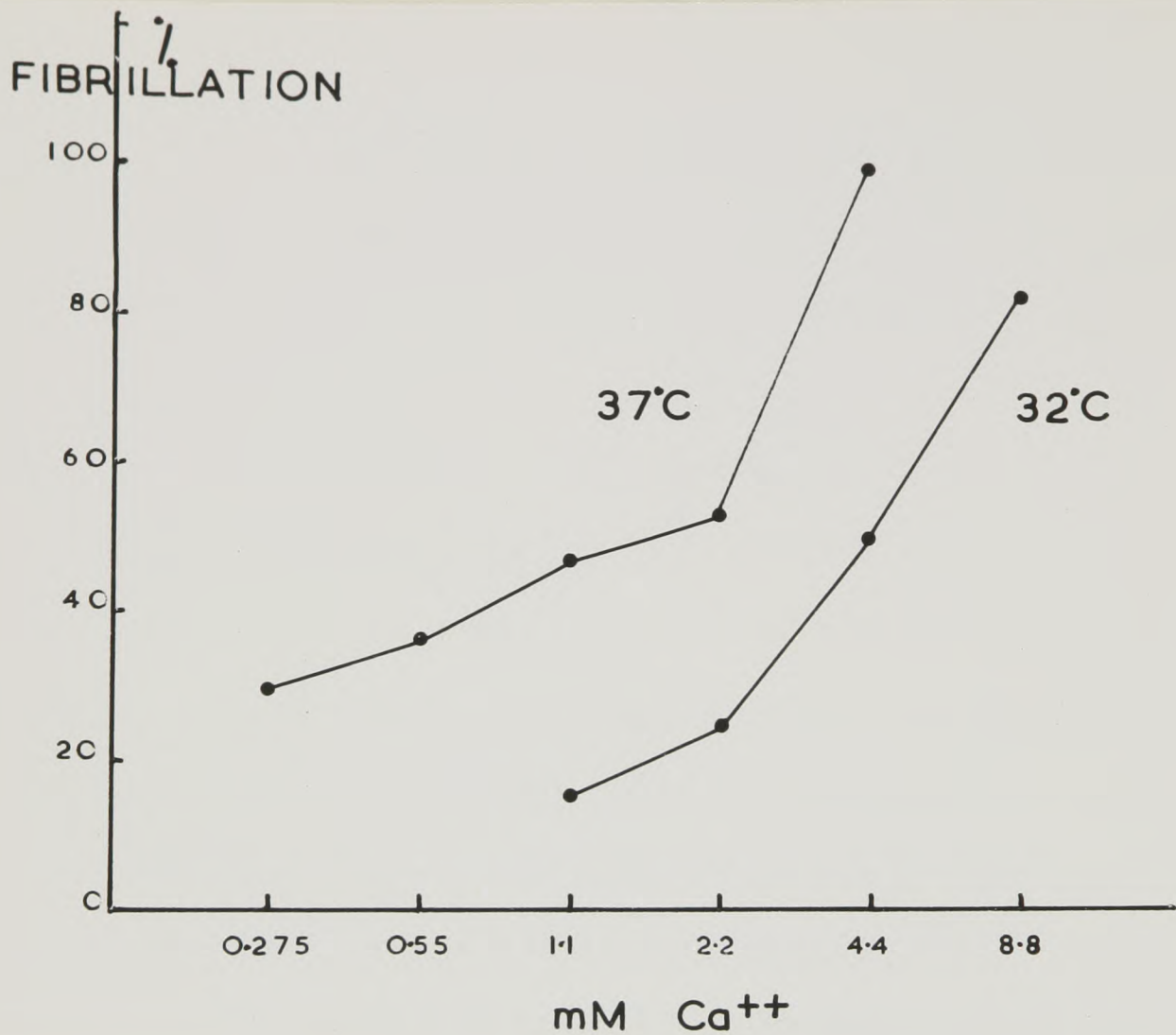


Fig.3. Effect of Ca^{++} ions on fibrillation at 37°C and 32°C. The ordinate shows the percentage of hearts in which fibrillation persisted after it had been induced. The abscissa shows the Ca^{++} ion concentration of the perfusion fluid.

Discussion

The results obtained give general support to the conclusions already reached by Kärki (1958) that a reduction of K^+ ions, and of Na^+ ions favours fibrillation while a decrease of Ca^{++} ions makes fibrillation less likely.

The curve relating the proportion of hearts fibrillating to the Ca^{++} ion concentration which Kärki (1958) obtained, though free from the two peaks described by Armitage et al. (1957a) was of curious shape and suggested that more than one factor was involved. The present observations at $32^{\circ}C$ have lost this complexity and they show that the effect of varying the concentration of Ca^{++} ions affects the proportion of hearts which fibrillate in a roughly linear manner.

The observation that the hearts could not be made to fibrillate at $27^{\circ}C$ even when the K^+ ion concentration was reduced to 1.4 mM is in agreement with results recently published by Covino and Beavers (1958) who found that rabbit hearts would not fibrillate at temperatures below $27^{\circ}C$. However, they considered that this phenomenon was species dependent since they found that fibrillation would occur at low temperatures in the dog heart.

Vaughan Williams (1959) has suggested that an increase in the rate of rise of the action potential will favour fibrillation, whereas a decrease in the rate of rise will mean that the liability to fibrillate will be reduced. Factors which cause an increase in the rate of rise of the action potential are among others an increase in the Ca^{++} ion concentration, a decrease in the K^+ ion concentration, which also increases the resting potential and high temperature. Factors which cause a decrease in the rate of rise are decreased Ca^{++} ion concentration, increased K^+ ion concentration, low temperature, and anoxia. However, the heart will be more likely to fibrillate if the refractory period is shortened (Burn, 1956). Two factors which will shorten the refractory period are anoxia or cyanide. Thus anoxia will have a diphasic effect since it both causes a decrease in the rate of rise of the action potential, and also a shortening of the refractory period. Under conditions of partial anoxia fibrillation will be strongly favoured whereas in complete anoxia no fibrillation will occur as observed by Goodford (1958).

This diphasic effect is probably the cause of the unusual Ca^{++} ion curves of Armitage et al. and of Kärki. At 32°C the factor of anoxia is at a minimum and the tendency to fibrillate will be related directly to the ionic concentration of the perfusion fluid. This is borne out by the experimental results obtained. The reduction in temperature will decrease the likelihood of fibrillation occurring, and is probably the predominant factor at 27°C when it was found that even in very low K^{+} ion concentrations it was not possible to produce fibrillation.

proportion of hearts fibrillating rose as the K^{+} ion concentration fell, yet even with one quarter the normal concentration the proportion was not higher than 67%. The relation of Ca^{++} ion concentration to the proportion of hearts fibrillating changed from biphasic at 37°C to almost linear at 27°C , the proportion increasing as the Ca^{++} ion concentration rose. It was confirmed that a reduction in the K^{+} ion concentration also raised the proportion of hearts which fibrillated.

Summary

Observations have been made on ventricular fibrillation induced by electrical stimulation in the isolated rabbit heart, in extension of the finding that fibrillation was arrested by cooling from 37°C to 32°C . The observations now made suggest that at 37°C the isolated rabbit heart perfused with McEwen's solution is on the edge of anoxia, but that at 32°C the oxygen supply is adequate. At the lower temperature the proportion of hearts fibrillating at any given K^{+} ion concentration was reduced, so that while the proportion of hearts fibrillating rose as the K^{+} ion concentration fell, yet even with one quarter the normal concentration the proportion was not higher than 67%. The relation of Ca^{++} ion concentration to the proportion of hearts fibrillating changed from biphasic at 37°C to almost linear at 32°C , the proportion increasing as the Ca^{++} ion concentration rose. It was confirmed that a reduction in the Na^{+} ion concentration also raises the proportion of hearts which fibrillate. Last, the rate of synthesis was increased.

CHAPTER VII

The enzyme systems concerned with acetylcholine synthesis in the atria of the rabbit heart

Introduction

Studies on the isolated atria of the rabbit heart have shown that in various circumstances the contractions are arrested, and can then be restarted by the addition of acetylcholine. Thus Bülbbring and Burn (1949) described experiments on isolated rabbit atria and on the choline acetylase system present in them. They showed that when isolated atria were allowed to contract for many hours, the beat finally stopped and then could be restarted by the addition of acetylcholine. They found that the ability to synthesize acetylcholine of an acetone-dried powder prepared from atria was greatly reduced when the atria had ceased to beat. They studied the effect of adding acetylcholine to the incubation mixture. When this mixture contained an acetone powder from fresh atria, acetylcholine reduced the synthesis; when, however, the mixture contained an acetone powder from atria which had ceased to beat, then the very low level of synthesis was increased.

Methods

In 1958, Burn and Rand showed that when isolated atria had been cooled until they had ceased to beat, then the beat could be restarted by stimulating the vagi. They suggested that owing to the cooling insufficient acetylcholine was being synthesized to maintain the contractions, and under these conditions stimulation of the vagi released acetylcholine and restarted the beat.

The phenomenon observed by Bülbring and Burn have been re-investigated using more modern techniques, and the effect of temperature on the choline acetylase activity of rabbit atria has been studied. Estimations have been made of the acetylcholine content of rabbit atria under various conditions.

The atria were cut into small pieces and dried in a fine powder. After drying on the filter paper for 5 min the powder was transferred to a desiccator and evacuated over P_2O_5 . After standing at $4^\circ C$ for 24 hr the powder was removed, weighed and suspended in 0.5 ml of cysteine saline solution (5 mg cysteine saline solution/0.5 ml 0.9% NaCl).

The solution was stored at $-20^\circ C$ (for a minimum period of 24 hr). The solution was thawed and centrifuged.

Methods

Enzyme preparation

Acetone powders of rabbit atria were prepared in the following way. Rabbits of either sex of weight between 1.5 to 3.0 Kg were decapitated. The heart was removed and the atria dissected away from the rest of the tissue. The atria were minced and immediately dropped into a large volume (approx. 300 ml) of cold dry acetone (-15°C) contained in a mortar. The acetone was rapidly poured off into a Buchner filter funnel. The tissue was ground, a further volume of acetone added, and then poured off. This operation of grinding and washing was repeated until all the material had been transferred to the filter paper in the form of a fine powder. After drying on the filter paper for 5 min the powder was transferred to a desiccator and evacuated over P_2O_5 . After standing at 4°C for 24 hr the powder was removed, weighed and suspended 40 mg/ml, in cysteine saline solution (6 mg neutralised cysteine hydrochloride/ml 0.9% NaCl).

The solution was stored at -15°C (for a minimum period of 24 hr). The solution was thawed and centrifuged

for 5 min just prior to use.

Co-enzyme A preparation

The co-enzyme A was prepared by a method used by Hebb (personal communication) and is a modified version of that described by Hebb (1955). The procedure was as follows.

10 lb. of brewer's yeast fresh from the vats were added to 6.5 l. of boiling tap-water, so that the temperature remained between 85 and 95°C during the addition. The mixture was allowed to remain at 95°C for 10 min and was then cooled to 20°C. It was centrifuged. Ammonium sulphate 500 g/l. was added to the supernatant and the mixture was extracted with phenol using one part of phenol to eight parts of supernatant. (The phenol solution was prepared by adding 44 ml of boiling water to 1 kg phenol crystals).

The mixture was kept in the dark for 6 to 12 hr. The phenol layer was removed and the phenol extract stored at room temperature for 12 hr. The phenol extract was next shaken with an equal volume of ether (anaesthetic) and the phenol-ether mixture extracted five times with 60 ml aliquots of distilled water. The

water extract (lower layer) was removed after each addition and filtered through glass wool. The aqueous extract was brought to pH 1.7 with 4N-HNO₃, and centrifuged. 10 vol. (500-700 ml approx.) of ice-cold acetone were added to the supernatant and stored overnight at 0 to 2°C. The acetone was poured off and an equal volume of ether poured on to the precipitate. The ether was removed, a fresh volume of ether was then added and also removed.

The precipitate was dissolved in 50 ml distilled water and neutralised with KOH to pH 6.5 to 6.7. The resulting solution was washed five times with 250 ml aliquots of water-saturated collidine, centrifuging after each addition. The solution was washed with pure collidine to remove water taken up during the washings. The collidine was removed and the solution washed twice with 30 vol. dry ether in a separating funnel. The lower layer was collected and filtered through a Whatman No.1 filter.

The ether was removed by placing the solution in a water bath at 65°C and blowing nitrogen gas through it.

The resulting solution was stored at -15°C until required.

The activity of the preparation was assayed using the method described by Kaplan and Lipmann (1948).

Reagents (a)

The incubation medium was composed of the following reagents:

		ml
co-enzyme A	400 units/ml	0.05
KCl	30%	0.04
choline chloride	4%	0.04
MgCl ₂	1.2%	0.08
acetylphosphate	10 mg/ml	0.14
L-cysteine-HCl (neutralised)	30 mg/ml	0.12
phospho-trans-acetylase	2.5 mg/ml	0.10
serine sulphate	1 mg/ml 4% NaCl	0.10
water		0.23
Total		<u>0.90 ml</u>

Dilutions with distilled water were made of the co-enzyme A preparation in the experiments using different concentrations of co-enzyme A. 0.05 ml co-enzyme A (400 units/ml) = 5 units co-enzyme A/mg acetone powder. The acetyl phosphate was obtained commercially and suspended in distilled water.

The phospho-trans-acetylase was also obtained commercially and was suspended in 0.02 M KHCO_3 . All distilled water used for making up solutions was obtained by running tap water through Permutit resin and then distilling the resultant water in an all glass still.

Method of incubation

The reagents were pipetted into suitable glass tubes and incubated for 15 min to initiate the formation of acetyl co-enzyme A, at the required temperature.

After 15 min 0.1 ml of the enzyme preparation was added, the tubes were corked and the incubation allowed to proceed for 1 hr. Control tubes were set up which contained the pre-incubated reagents and 0.1 ml of boiled enzyme. These controls were used for making up the standard acetylcholine solutions.

After exactly 1 hr the reaction was stopped by adding 6 drops B.D.H. Universal indicator, and 0.2 ml $\frac{N}{3}$ -HCl + 1.0 ml $\frac{M}{15}$ phosphate buffer.

Method of acetylcholine estimation

4 ml frog Ringer's solution was added to each tube and the contents boiled for 1 min. After cooling the volume was made up to 10 ml with frog Ringer's solution and stored at -15°C until required.

The acetylcholine content was assayed on the frog rectus muscle preparation. The standard acetylcholine solutions were made up in the control solutions.

Reagents (b)

In the experiments used to compare the activity of normal and exhausted atria the following incubation mixture was used where stated.

		ml
KCl	30%	0.04
choline chloride	4%	0.04
* co-enzyme A		0.05
Na-ATP	20 mg/ml	0.20
MgCl ₂	1.2%	0.08
Na-citrate	6.8%	0.08
Na-acetate	1.36%	0.08
l-cysteine-HCl (neutralised)	30 mg/ml	0.10
** P.L.E.		0.08
Na ₂ HPO ₄	0.25%	0.08
Enzyme		0.10
Water		0.27
	Total	<u>1.00 ml</u>

* The co-enzyme A concentration differed in individual experiments as stated but was in the range 0-400 units/ml.

** P.L.E. refers to pigeon liver enzyme prepared by using the method of Kaplan and Lipmann (1948) with the exception

that the extract was prepared in 0.02 M KHCO_3 instead of 0.02 M NaHCO_3 . In some experiments no pigeon liver enzyme was added and the volume was made up to 1.0 ml with distilled water. There was no pre-incubation, and the incubation was allowed to proceed for 1 hr. The preparation of extracts for assay, and the assay were the same as previously described.

Aceto-co-A-kinase estimation

The method used was similar to that described by Lipmann and Tuttle (1945). The aceto-hydroxamate formed was measured in an E.E.L. photoelectric colorimeter using a green GGR1 (540 m μ) filter.

Acetylcholine content of atria

Atria were removed from rabbit hearts immediately after killing and were suspended in an isolated organ bath containing well oxygenated double dextrose Locke solution at 30°C.

(a) Normal atria. These were allowed to beat for 1½ hr., the Locke solution being changed 30 min after the atria had been set up.

At the end of $1\frac{1}{2}$ hr. the atria were quickly cut down and dropped into ice-cold 0.3 M sucrose solution containing 10^{-5} M eserine.

(b) Cooled atria. These were allowed to beat for $1\frac{1}{2}$ hr, the Locke solution being changed after 30 min. At the end of the $1\frac{1}{2}$ hr. they were slowly cooled to 15°C .

The atria remained at 15°C for 5 min, they were then quickly cut down and dropped into the ice-cold sucrose solution.

Experiments were also carried out where the atria were set up at 30°C and then after 15 min they were cooled very slowly to 18°C . They were allowed to beat at 18°C for 1 hr and then were cooled to 15°C to arrest the beat. After 1 hr at 15°C they were extracted and assayed in the normal way.

(c) Exhausted atria. These were allowed to beat in the Locke solution at 30°C until they stopped. The Locke solution was changed every hour. (They were cooled to room temperature, approx. 15°C , overnight and were rewarmed to 30°C in the morning.

Immediately they stopped beating they were dropped into the ice-cold sucrose solution.

Estimations

The atria were weighed and then homogenized for $1\frac{1}{2}$ min in an all glass homogenizer (100 mg tissue/ml sucrose). The homogenate was then centrifuged for 10 min at 2,000 g in a refrigerated centrifuge at -2°C . The supernatant was decanted off. (At no time after removing the atria from the bath was the temperature allowed to rise above 0°C).

The free acetylcholine content of the supernatant was assayed immediately on the frog rectus muscle preparation using the superfusion technique (Ahmed and Taylor, 1957). 0.5 ml of the supernatant was kept for nitrogen determination and the remainder was brought to pH 4 with dilute acid and heated in a boiling water bath for 10 min. After cooling the solution was neutralised with NaOH and the total acetylcholine assayed on the superfused frog rectus muscle.

0.5 ml of the supernatant was digested for 1 hr with 2.0 ml acid digestion mixture. 0.5 ml eserinated sucrose

was used for the blank. The acid digest was transferred quantitatively to a 10 ml volumetric flask and made up to 10 ml with distilled water.

The nitrogen content was measured using the Micro Kjeldahl Apparatus (Markham, 1942).

The results were expressed as $\mu\text{g ACh/mg N}$ present in the homogenate.

TABLE 3

Effect of Co-enzyme A concentration on
synthesis at 37°C

Co-enzyme A concentration units/mg acetone powder	Exp.1	Exp.2	Exp.3
0.00	< 20	100	58
0.625	150	222	-
1.25	222	317	-
2.50	267	375	-
5.00	233	-	438

Results

Effect of co-enzyme A concentration

Since the synthesis of acetylcholine is known to take place in two stages the first stage being the formation of acetyl co-enzyme A, experiments were carried out to determine the effect of variation in the concentration of co-enzyme A and hence acetyl co-enzyme A on the synthesis of acetylcholine by acetone powders from fresh atria. The results are shown in Table 3. In all three experiments the rate of synthesis rose as the amount of co-enzyme A was increased. The synthesis of acetylcholine which occurred when no co-enzyme A was added was probably due to endogenous co-enzyme A present in the atrial acetone powder.

Effect of acetylcholine on synthesis

Observations were made to see if the finding of Bülbring and Burn (1949) that adding acetylcholine to the incubation mixture caused a decrease in synthesis could be confirmed by the methods described here. The first results failed to demonstrate any effect as shown in Table 4. In one experiment there was a decrease of 3% and in the other an increase of 9%, neither being significant.

TABLE 4

Effect of addition of acetylcholine to the
incubation mixture when 5 units/mg
Co-enzyme A was present. Temp. 37°C

ug Acetylcholine added per g. acetone powder	ACh synthesized/g/hr	
	Exp. 4	Exp. 5
0	263	119
250	256	131

TABLE 5

The relation of Co-enzyme A concentration to
the inhibitory action of acetylcholine on
choline acetylase activity

T. = 37°C

Co.A (units/mg powder)	ACh added μg/g powder	ACh synthesized μg/g/hr	Change
5.00	0	233	-
5.00	275	225	-3.5%
2.50	0	267	-
2.50	275	250	-6.5%
1.25	0	222	-
1.25	275	142	-36%
0.625	0	150	-
0.625	275	75	-50%

As these experiments had been carried out using optimal amounts of co-enzyme A, it was decided to carry out the next experiments in the presence of smaller amounts of co-enzyme A. The amount of co-enzyme A in each experiment was reduced by half of that in the previous experiment and the effect on synthesis of adding 275 μ g acetylcholine/g powder was determined. The results are shown in Table 5 and clearly indicate that inhibition occurs under sub-optimal concentrations of co-enzyme A.

Temperature and choline acetylase activity

The results of five experiments to determine the effect of temperature on the formation of acetylcholine by choline acetylase present in acetone-dried powders from rabbit atria are given in Table 6. In order to compare the experiments, the figures were calculated with reference to the activity at 33°C which was taken as 100, and the resulting values are shown in Table 7. The mean results showed that at 21°C the activity was 43% of that at 33°C, but that at 17°C it had fallen to 21% and at 13°C to 9%. Thus the fall in activity was steep below 21°C.

TABLE 6

The effect of temperature on the synthesis of
Acetylcholine by rabbit atria

Figures show the amount synthesized in
 μg ACh/g acetone powder/hr

Temp. °C	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5
13	-	5	38	19	31
17	-	50	73	62	44
21	125	88	138	81	100
25	131	137	169	119	138
29	175	206	288	150	187
33	238	231	350	194	238
37	300	-	-	-	-
41	323	-	-	-	-

TABLE 7

Choline acetylase activity expressed in relation
to that at 33°C taken as = 100

Temp. °C	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5	Mean
13	-	2.2	10.9	9.8	13.0	9.0
17	-	21.6	22.3	19.5	18.5	20.5
21	52.5	38.1	39.4	41.8	42.0	42.6
25	55.0	59.3	48.3	61.3	58.0	56.4
29	73.5	89.2	82.3	77.3	78.6	78.6
33	100	100	100	100	100	100
37	126	-	-	-	-	126
41	136	-	-	-	-	136

From these results the Q_{10} was calculated for each rise of temperature, and the values are shown in Table 8, together with values for rabbit brain calculated from figures previously published (Milton, 1958). Normal values for the Q_{10} of biological reactions are from approximately 1.0 to 2.5 and this held for choline acetylase activity between 37° to 41°C . Below 37°C , however, the Q_{10} increased in value at a much faster level than is usually encountered, being 5.2 between 17° and 21°C , and 5.9 between 13° and 17°C . The values for choline acetylase in atria were very similar to those obtained for brain at corresponding temperatures.

Effect of acetylcholine on synthesis in cooled atria

In their paper Bülbring and Burn (1949) observed that atria which had beaten for many hours until they stopped could be restarted by the addition of small amounts of acetylcholine. They also observed that the synthesis of acetylcholine by acetone-dried powders prepared from atria which had ceased to beat was very low. When, however, such powders were incubated together with small amounts of acetylcholine, the synthesis was increased. This was in contrast to the effect of

TABLE 8

Q_{10} of choline acetylase from rabbit atria, compared
with figures obtained for choline acetylase from
rabbit brain

Temperature	Atria	Temperature	Brain
13 - 17°C	5.9		
17 - 21°C	5.2	17 - 22°C	5.2
21 - 25°C	3.3	22 - 27°C	3.5
25 - 29°C	3.5		
29 - 33°C	3.2	32 - 37°C	2.5
33 - 37°C	3.1		
37 - 41°C	2.7		

acetylcholine on powders from normal atria where inhibition occurred. Recently Burn and Rand (1959) have shown that if atria are arrested by cooling they may be restarted by stimulating the vagus. An attempt was therefore made to see if a reversal of the action of acetylcholine could be demonstrated in acetone powders prepared from atria which had been arrested by cooling.

The first experiments were carried out using amounts of co-enzyme A which varied from 0 to 2.5 units/mg powder, and synthesis was compared in the presence of 300 μ g acetylcholine/g powder with synthesis in its absence. The results are shown in Table 9. It will be seen that the added acetylcholine had an inhibitory effect which decreased as the co-enzyme A concentration increased. Further observations were, therefore, made in the presence of large amounts of co-enzyme A (5 units/mg). They showed that the synthesis by powder prepared from atria which had been stopped by cooling was practically unaffected by acetylcholine, the results being very similar to those in Table 4. In the next experiments the amount of co-enzyme A was kept constant but low at 0.65 units/mg powder, while the acetylcholine added was varied. The results are given

TABLE 9

The relation of Co-enzyme A concentration to the inhibitory action of acetylcholine on a choline acetylase preparation from atria stopped by cooling.

T. = 37°C

Co.A (units/mg powder)	ACh added μg/g powder	ACh synthesized μg/g/hr	Change
0	0	100	-
0	300	50	-50%
0.625	0	222	-
0.625	300	175	-21%
1.25	0	317	-
1.25	300	275	-13%
2.50	0	375	-
2.50	300	325	-13%

TABLE 10

Relation between amount of acetylcholine added and inhibition of synthesis in atria stopped by cooling. (Co-enzyme A 0.625 units/mg.). Incubation at 37°C

Acetylcholine added per g. powder	Acetylcholine synthesized μg/g/hr	Change %
0	158	-
12.5	154	-2.6
31.2	144	-9.2
62.5	117	-26.4
125.0	54	-65.8

in Table 10 and show quite clearly that no reversal of the inhibitory effect of acetylcholine on synthesis had occurred.

In the above experiments the incubations were carried out at 37°C. Experiments were, therefore, carried out in which powder from cooled atria was incubated at 15°C instead of at 37°C. Again acetylcholine caused inhibition of synthesis in a co-enzyme A deficient system.

Acetylcholine content of normal and cooled atria

As the choline acetylase activity dropped so rapidly below 20°C it was decided to estimate the acetylcholine content of normal atria and atria which had been arrested by cooling.

The normal atria were set up in Locke's solution at 30°C and after 1½ hrs the acetylcholine content was measured. The cooled atria were initially set up at 30°C and after 1½ hrs they were gradually cooled to 15°C. After remaining at 15°C for 5 min the acetylcholine content was measured.

Preliminary experiments using an acid-alcohol extraction procedure suggested that there was not a decrease in the acetylcholine content when the atria were cooled.

TABLE 11

Free and Bound Acetylcholine content of
Normal and Cooled Atria

	$\mu\text{g ACh/mg N in homogenate}$			% Free	% Bound
	Free	Bound	Total		
Normal (32°C)	0.34	0.23	0.57	60.0	40.0
	0.25	0.22	0.47	53.6	46.4
	0.23	0.19	0.42	54.5	45.5
	0.24	0.21	0.45	54.3	45.7
	0.29	0.22	0.51	57.5	42.5
Mean value	0.27	0.21	0.48	56.0	44.0
Cooled (15°C)	0.22	0.30	0.52	42.3	57.7
	0.26	0.30	0.56	46.4	53.6
	0.22	0.36	0.58	38.1	61.9
	0.20	0.30	0.50	39.1	60.9
	0.24	0.28	0.52	44.6	55.4
Mean value	0.23	0.31	0.54	42.5	57.5

This was contrary to expectation. As a result it was decided to measure the total, the free and the bound acetylcholine content of normal and cooled atria. The results of these experiments are given in Table 11.

It will be seen that the total amount of acetylcholine had increased slightly in the cooled atria. However, in the cooled atria the amount of free acetylcholine was lower than in the normal atria. The increase in the total amount was due entirely to an increase in the amount of bound acetylcholine. In the atria which had been at 18°C for 1 hr, then at 15°C for 1 hr, the total acetylcholine content was slightly reduced from normal. There was a marked reduction in the amount of free acetylcholine, with an increase in the amount of bound acetylcholine.

Choline acetylase from atria which had ceased to beat

Acetone-dried powders were prepared from atria which had ceased to beat after being suspended in Locke solution for many hours. The choline acetylase activity was measured and compared with that obtained from normal atria. The results showed that no decline

TABLE 12

Aceto-Co-A kinase activity of acetone powders prepared from normal and exhausted atria. (The results are expressed as colorimeter readings, obtained by using 4 mg acetone powder in 0.1 ml solution. For method see Lipmann & Tuttle, 1945)

	Normal atria	Exhausted atria
	0.50	0.15
	0.55	0.30
	0.50	0.10
average value	0.516	0.183
ratio of values	100/35	

in activity had occurred. In the presence of phospho-trans-acetylase and acetyl phosphate, and in the absence of added co-enzyme A, synthesis still occurred showing that endogenous co-enzyme A was still present.

When acetylcholine was added to an incubation mixture which was deficient in co-enzyme A, inhibition of synthesis occurred as found with normal atria. These results are at variance with the observations of Bülbring and Burn who showed that acetylcholine synthesis was low when they used acetone powders from exhausted atria. They also showed that addition of acetylcholine caused this low rate of synthesis to be increased. However in their experiments the acetyl co-enzyme A was formed from citrate, adenosintriphosphate and 'boiled brain activator.' No enzyme was added, so presumably the reaction was catalysed by aceto-co-A-kinase present in the atrial acetone powders. Experiments were, therefore, carried out to estimate the aceto-co-A-kinase present in acetone powders prepared from fresh atria and from atria which had ceased to beat. The results which are given in Table 12, showed that the enzyme activity had dropped considerably in the acetone powders prepared from exhausted atria.

TABLE 13

Synthetic ability of acetone powder from exhausted
atria, using endogenous and added aceto-Co-A-kinase

Aceto-Co-A-kinase present	ACh added $\mu\text{g/g}$ powder	ACh synthesized $\mu\text{g/g/hr}$	Change
(1) Endogenous	-	35	-
"	15	42	+20%
P.L.E. supplemented	-	80	-
"	15	47	-40%
(2) Endogenous	-	27	-
"	15	38	+40%
P.L.E. supplemented	-	56	-
"	15	34	-40%

As a result of these observations the synthetic ability of, and the effect of added acetylcholine on, acetone powders from exhausted atria was studied using A.T.P., citrate, acetate and aceto-co-A-kinase (see Methods).

When the formation of acetyl co-enzyme A, and hence acetylcholine, was dependent on the aceto-co-A-kinase present in the acetone powders, then the synthesis of acetylcholine was low, and under these conditions the addition of small amounts of acetylcholine to the incubation mixture caused an increase in synthesis, as found by Bülbring and Burn. If, however, the incubation mixture was supplemented with aceto-co-A-kinase by adding 'pigeon-liver enzyme,' then an increased rate of synthesis was observed, and the addition of acetylcholine caused a decrease in synthesis. The results are given in Table 13.

Acetylcholine content of normal and exhausted atria

Since the ability to synthesize acetylcholine was much reduced in exhausted atrial powders it was decided to measure the amount of acetylcholine present in normal and exhausted atria.

TABLE 14

ACh content of atria

Wt. of atria mg	μg ACh extracted from atria			μg ACh/mg N. in homogenate		
	Free	Bound	Total	Free	Bound	Total
Normal	460	0.37	0.92	0.34	0.23	0.57
	616	0.58	1.26	0.25	0.22	0.47
	458	0.23	0.51	0.23	0.19	0.42
	357	0.68	1.25	0.24	0.21	0.45
	396	0.91	1.59	0.29	0.22	0.51
Mean value	457	0.49	1.11	0.27	0.21	0.48
Exhausted	338	0.08	0.37	0.17	0.05	0.22
	401	0.20	0.64	0.19	0.09	0.28
	438	0.18	0.70	0.23	0.08	0.31
	507	0.16	0.51	0.13	0.06	0.19
Mean value	421	0.16	0.56	0.18	0.07	0.25

The results are shown in Table 14. This table shows the amount of acetylcholine extracted from the atria both in the free and bound form. In order to make a direct comparison of observations between atria of widely different weights, the amount of acetylcholine is also given expressed as μg acetylcholine per mg nitrogen present in the homogenate.

These results showed that the total amount of acetylcholine had fallen by 50% in the exhausted atria. Of this reduction, by far the greatest drop had occurred in the bound acetylcholine which had dropped by 67%, whereas the free acetylcholine only showed a drop of 33%.

Discussion

In four different conditions acetylcholine has been found to restart the contractions of atria. Marshall and Vaughan Williams (1956) found that when atria were cooled, the contractions ceased at a temperature below 20°C . In most experiments they were able to record action potentials at the pacemakers, and when these were present, they found that the addition of acetylcholine caused contractions to begin again. Marshall (1957) found that the process of cooling caused a fall in the transmembrane potential and that contractions ceased when it became less than 60 mV. She showed that the addition of acetylcholine caused a rise in the potential and that the restarting of the contractions was due to this rise.

Burn and Rand (1958) showed that atria stopped by cooling could be restarted by the stimulation of the vagi, which was presumably due to the release of acetylcholine.

As previously mentioned, an earlier observation was that of Bülbbring and Burn (1949) who allowed atria to contract in an isolated organ bath until they stopped.

They showed that acetylcholine restarted the beat, and this result was confirmed by Holtz and Westerman (1955). Goodford (1959) determined the intracellular potassium of beating atria and found that when contractions stopped it had fallen to a point at which the transmembrane potential could not have been more than 60 mV. The restarting of the contractions by acetylcholine could therefore be attributed to a rise in the membrane potential.

Briscoe and Burn (1954) found that atrial contractions were also arrested by quinidine, and that again acetylcholine would restart them. When the contractions were arrested by quinidine Armitage (1957) found that they could also be restarted again by reducing the external potassium concentration to one-half. This indicated that the arrest by quinidine was due to a fall in the membrane potential and made it likely that the restarting by acetylcholine was due to the rise in the membrane potential.

Thus in conditions of atrial arrest there is good reason to believe that the action of acetylcholine in restarting the beat was due to the rise in membrane potential which it caused. The atria are known to

contain a system capable of synthesizing acetylcholine (Comline, 1946). The acetylcholine so formed could have as one of its functions the maintenance of a normal trans-membrane potential. Since the formation of acetylcholine occurs in two stages it is necessary in any investigation to realise that the formation of acetyl co-enzyme A which occurs in the first reaction, has a profound effect on the second reaction where the acetyl group is transferred from acetyl co-enzyme A to choline, and is catalyzed by the enzyme choline acetylase. The formation of acetylcholine occurs as follows:

Reaction I.

(a) Acetate + ATP + co-enzyme A $\xrightarrow{\text{aceto-co-A-kinase}}$ acetyl-co-enzyme A + AMP + Pi.

(b) Citrate + ATP + co-enzyme A $\xrightarrow{\text{aceto-co-A-kinase}}$ acetyl-co-enzyme A + AMP + Pi.

(c) Acetylphosphate + co-enzyme A $\xrightarrow{\text{phospho-trans-acetylase}}$ acetyl co-enzyme A + Pi.

Reaction II.

Acetyl co-enzyme A + choline $\xrightarrow{\text{choline acetylase}}$ acetylcholine + co-enzyme A.

In the first experiments the effect of the co-enzyme A concentration in the incubation mixture was studied. Since excess of the enzyme phospho-trans-acetylase was present the acetyl co-enzyme A concentration was approximately proportional to the amount of co-enzyme A present. The results showed very clearly the dependence of acetylcholine synthesis on acetyl co-enzyme A concentration, with a continual increase in synthesis as the co-enzyme A concentration was raised until the system was completely saturated. These results are very similar to those reported by Smallman (1958) for the acetylcholine synthesis by choline acetylase prepared from rabbit brain.

The effect of adding acetylcholine to the incubation mixture was next studied. It was found that under optimal conditions the addition of acetylcholine had no effect on the rate of synthesis. However, in the experiments of Bülbbring and Burn this observation was carried out under sub-optimal conditions, this is illustrated by the fact that their highest rate of synthesis was 75 μ g acetylcholine/g acetone powder/hr, whereas the highest rate recorded here using an identical

source of choline acetylase was 438 μ g acetylcholine/gm acetone powder/hr. It was, therefore, decided to observe the effect of adding acetylcholine to incubation mixtures containing reduced amounts of co-enzyme A, and hence acetyl co-enzyme A. The results showed quite clearly that whereas the addition of acetylcholine had no effect on a system saturated with acetyl co-enzyme A, inhibition occurred which became progressively greater as the acetyl co-enzyme A concentration was reduced.

An explanation of these results can be put forward if it is assumed that the synthesis of acetylcholine occurs on active sites present in the protein molecules of the choline acetylase. To one of these sites acetyl co-enzyme A would be attached; it would combine with choline attached to another site and so form acetylcholine. The acetylcholine so formed would either remain attached to the enzyme or be released. Whether the active site was occupied by acetyl co-enzyme A or by acetylcholine would depend on the concentrations of these substances. When acetyl co-enzyme A was in excess, as under optimal conditions, the effect of adding acetylcholine would be negligible. However, when only small amounts of

acetyl co-enzyme A were available, then an equilibrium would be reached between acetyl co-enzyme A and acetylcholine. In order to maintain this equilibrium the addition of acetylcholine would in effect mean that less acetylcholine would be synthesized.

The effect of temperature on the activity of the choline acetylase catalyzed reaction showed that the activity declined with decrease in temperature in a very similar way to that previously found for the choline acetylase present in rabbit brain (Milton, 1958). The interesting point is that whereas the activity at 21°C was still about 40% of that at 33°C , it fell very rapidly below 21°C being down to 20% at 17°C , and down to 10% at 13°C . Thus in the temperature range where atria cease to beat the choline acetylase activity was greatly affected by temperature. Whereas in the range from 33° to 21°C the Q_{10} was 3.3, in the range 21° to 17°C it was 5.2, and from 17° to 13°C it was 5.9. These are very high Q_{10} values, in fact much higher than those reported for cholinesterase (Wilson and Cabib, 1956).

These observations support the view that the arrest of the atria by cooling is due to the reduction of choline acetylase activity, as a result of which insufficient acetylcholine is formed to maintain the transmembrane potential above 60 mV.

Experiments showed that when the enzyme was prepared from atria which had been stopped by cooling its activity was unaffected, and the addition of acetylcholine to the incubation mixture caused a decrease in synthesis as found in acetone powders from normal atria. Burn and Rand (1958) had suggested that the preparation might behave like the preparation of Bülbring and Burn (1949) from atria stopped by exhaustion where the addition of acetylcholine caused a normally very low rate to be increased. These results gave no indication of such a phenomenon.

In view of these results it was decided to estimate the amount of acetylcholine in both normal atria and in atria which had ceased to beat on cooling. Contrary to expectation the initial results suggested that the cooled atria in fact contained more acetylcholine than the normal atria. However, subsequent experiments showed that the position was more complex than appeared

from estimations of total amounts of acetylcholine. Atria were homogenized in sucrose and then centrifuged. As far as possible the conditions were such that the homogenization would cause rupture of the cells without disintegration of the mitochondria. The centrifugation was such that the cell debris and nuclei were brought down but the mitochondria remained in the supernatant. Estimations were then made of the acetylcholine content of the 'free' acetylcholine present in the resulting supernatant, and the amount of acetylcholine released from a bound state, probably in the mitochondria and microsomes when the supernatant was boiled under acid conditions. The results now showed that the amount of 'free' acetylcholine was reduced on cooling, markedly so after prolonged cooling, and that any increase in acetylcholine content occurred solely in the 'bound' form.

There have been many suggestions that a large part of the acetylcholine of nervous tissue is not in a freely diffusible form but instead is bound in some way to the tissue; and that this binding may involve some kind of sub-cellular particle such as the mitochondrion (Bodian, 1942; Brodtkin and Elliott, 1953). From

Feldberg's (1945) analysis of the problem it appears that the ester enters the bound state as it is produced. Bellamy (1958) and Hebb and Whittaker (1958) suggested that within the cell the ester is in close spatial association with the enzyme of synthesis, that is with choline acetylase. Hebb and Whittaker (1959) showed on fractionation of brain tissues that the intracellular distribution of acetylcholine was similar to that of choline acetylase. They also suggested that both the enzyme and acetylcholine are partly free and partly bound to some part of the sub-cellular structure.

It was for these reasons that estimations of 'free' and 'bound' acetylcholine in the atria were made, with the idea that cooling might interfere with the release of 'bound' acetylcholine into the free form. The results showed that both 'free' and 'bound' acetylcholine existed in the atria, and that on cooling the amount of 'free' acetylcholine was reduced, whereas the 'bound' increased. This concept of interference with acetylcholine release would fit in better with experimental observations, since the atria stopped when cooled when the amount of acetylcholine in the tissue was still high.

Other possibilities exist, one of these is that the large amounts of acetylcholine present in the atria are not concerned with the maintenance of the beat. Philpott (unpublished observations) found that there was a high choline acetylase activity in the region of the pacemaker, and it could be that it is only the choline acetylase in this region that is concerned with the maintenance of the beat.

However, whatever may be the true state of affairs the following two facts have been observed. Firstly that in the temperature range where atria stop beating the choline acetylase activity falls off rapidly, and secondly that on cooling atria the amount of free acetylcholine falls.

Some very puzzling results were obtained by Büllbring and Burn (1949) which showed that the synthesis of acetylcholine was low in acetone powders obtained from atria which had ceased to beat and that when such powders were incubated with small amounts of acetylcholine the synthesis was increased.

The results obtained in the present experiment showed that both the choline acetylase activity and the amount of co-enzyme A was of the same order in acetone powders from both normal and exhausted atria. This could be expected since preparations of both choline acetylase and co-enzyme A are fairly stable. The results showed that under optimal conditions the choline acetylase behaved in the same way whether from normal or exhausted atria, and also that in co-enzyme A deficient systems the addition of acetylcholine caused a decrease in synthesis in both cases.

In the experiments of Bülbring and Burn no enzyme was added to catalyse the formation of acetyl co-enzyme A, and so the acetyl co-enzyme A formed must have been catalyzed by an enzyme present in the atrial acetone powder. This enzyme was presumably aceto-co-A-kinase. When the activity of this enzyme was measured in acetone powders prepared from normal and exhausted atria it was found that the activity of the enzyme in the exhausted atria was only 35% of that found in normal atria. Experiments were carried out in which the aceto-co-A-kinase contained in the exhausted atria was the sole source of enzyme to

A-kinase was present in the exhausted atria.

a very low rate of acetyl co-enzyme A formation.

catalyse the formation of acetyl co-enzyme A. Under these conditions a very low rate of synthesis of acetylcholine was observed as found by Bülbbring and Burn. This experiment also pointed to the fact that the exhausted atria 'in vivo' were not capable of synthesizing acetylcholine at a high rate. The addition of aceto-co-kinase to the reaction mixture in the form of 'pigeon liver enzyme' caused an increase in rate of synthesis, thus confirming the inability of the atrial acetone powder to synthesize sufficient acetyl-co-enzyme A.

The most startling finding was that when acetylcholine was added in small amounts in the absence of added aceto-co-A-kinase to powders from exhausted atria, an increase in synthesis occurred as was described by Bülbbring and Burn. If, however, the incubation mixture was supplemented with aceto-co-A-kinase, then the normal inhibitory action of added acetylcholine was observed.

These results were very difficult to understand, but it seemed possible that the following explanation might account for them. The only difference between the acetone powders obtained from normal and exhausted atria was that in the powders from exhausted atria the aceto-co-A-kinase was greatly diminished. This would result in a very low rate of acetyl co-enzyme A formation.

Smallman (1958) showed that the synthesis of acetylcholine was dependent on the availability of acetyl co-enzyme A in a non-linear relationship. The synthesis of acetylcholine occurred at a very low rate until a certain threshold concentration of co-enzyme A was reached. Above this threshold concentration the rate of acetylcholine synthesis was rapidly increased. Under the aceto-co-A-kinase deficient conditions occurring in experiments with acetone powders from exhausted atria, the acetyl co-enzyme-A concentration might not rise above the threshold value and the rate of synthesis would be very low. When, however, acetylcholine was added to the incubation mixture, the added acetylcholine might have occupied the synthetic sites of the choline acetylase molecules, and as a result there was almost no synthesis. Under these conditions acetyl co-enzyme A accumulated until the concentration rose above the threshold value. When the threshold value was exceeded synthesis continued at an increased rate with the normal competition between acetyl co-enzyme A and acetylcholine for the active sites.

In conclusion it may be said that some evidence has now been produced which supports the hypothesis that one of the functions of the production of acetylcholine

The increased rate of synthesis in the presence of acetylcholine would thus be explained.

The results of the determinations of the acetylcholine content of exhausted atria showed that the total amount of acetylcholine had dropped to 50% of the normal atria value. The results also showed that the amount of 'bound' acetylcholine was very considerably reduced (by 66%). This is in agreement with the enzyme studies, since the decrease in synthetic ability would prevent the high level of 'bound' acetylcholine being maintained. There was also a decrease in the amount of 'free' acetylcholine. It could be that the 'bound' acetylcholine was gradually released to maintain the 'free' acetylcholine concentration. Eventually owing to the decrease in 'bound' acetylcholine due to impaired synthetic ability the 'bound' acetylcholine would be unable to maintain the 'free' acetylcholine. When the 'free' acetylcholine concentration fell so low as to be unable to maintain the transmembrane potential the atria would stop beating. Under these conditions the addition of acetylcholine would restart the beat.

In conclusion it may be said that more evidence has now been produced which supports the hypothesis that one of the functions of the production of acetylcholine

in the atria is to maintain the transmembrane potential.

Summary

The synthesis of acetylcholine by rabbit atria has been studied. Experiments using acetone-dried powders show that the synthesis is very dependent on the availability of acetyl co-enzyme A. In acetyl co-enzyme A deficient systems the addition of acetylcholine causes inhibition. Temperature studies have been made on the enzyme which show a very rapid decrease in activity between 21° - 13° C.

In exhausted atria a decreased aceto-co-A-kinase activity has been observed and it is suggested that this is the cause of the low synthetic ability of exhausted atria previously reported.

Studies on the acetylcholine content of atria show that in both atria stopped by cooling, and in those stopped through exhaustion the amount of free acetylcholine is reduced.

An explanation is given of the various effects observed.

CHAPTER VIII

The action of tubocurarine on the ciliary activity

of the frog's oesophagus

Introduction

Conflicting evidence has arisen from studies on the effect of tubocurarine on ciliary activity in the frog's oesophagus. Kordik, Kolling and Bore (1954)

PART III

STUDIES ON CILIARY ACTIVITY

described experiments in which tubocurarine had no effect on ciliary movement even in concentrations as great as 2 mg/ml. Hill suggested that the results of Kordik et al. were artefacts arising from their experimental methods. As a result of Hill's observations Bore and Jay (1954) made a further study of the action of tubocurarine. They found that tubocurarine in a concentration of 10^{-6} g/ml decreased the ciliary activity in seven consecutive experiments, and in six of these the ciliary activity returned to its initial value when the tubocurarine was removed. They also showed that it was possible to identify tubocurarine solutions of tubocurarine and to distinguish them from Singer's solution by their effect on ciliary activity.

CHAPTER VIII

The action of tubocurarine on the ciliary activity of the Frog's oesophagus

Introduction

Conflicting evidence has arisen from studies on the effect of tubocurarine on ciliary activity in the frog's oesophagus. Kordik, Bühlbring and Burn (1952) found that tubocurarine in a concentration of 10^{-6} g/ml inhibited ciliary activity. However, Hill (1957) described experiments in which tubocurarine had no effect on ciliary movement even in concentrations as great as 2 mg/ml. Hill suggested that the results of Kordik et al. were artefacts arising from their experimental methods. As a result of Hill's observations Burn and Day (1958) made a further study of the action of tubocurarine. They found that tubocurarine in a concentration of 10^{-4} g/ml decreased the ciliary activity in seven consecutive experiments, and in six of these the ciliary activity returned to its initial value when the tubocurarine was removed. They also showed that it was possible to identify unknown solutions of tubocurarine and to distinguish them from Ringer's solution by their effect on ciliary activity.

Methods

The problem was to repeat the experiments of Kordik et al. using tubocurarine in a concentration of 10^{-6} g/ml, and to try and explain the different observations recorded by Kordik et al. and by Hill.

Dissection.

The frog was decapitated removing the upper jaw immediately behind the eye sockets. The lower jaw was left intact. The spinal cord was exposed and the frog was placed dorsal surface upwards on a small cork mat. The skin was slit along the mid dorsal line and the spinal cord and dorsal body wall removed as far back as the sacral loop. The forelegs were removed together with all but the ventral portion of the pectoral girdle. The oesophagus was then cut and the mid dorsal line and the cut continued a short distance into the stomach wall. The oesophagus was pinned out as a roughly rectangular sheet, being kept flat by pins. It was kept as level as possible by placing pieces of cotton wool soaked in Ringer's solution underneath it. In female frogs the ovaries and oviducts were usually removed as these were liable to get in the way. In some experiments the whole of the hindquarters and the viscera were removed.

Methods

The preparation of the frog's oesophagus was similar to that described by Burn and Day (1958); there were, however, a few modifications in the dissection. The frog was decapitated removing the upper jaw immediately behind the eye sockets. The lower jaw was left intact. The spinal cord was destroyed and the frog was placed dorsal surface uppermost on a small cork mat. The skin was slit along the mid dorsal line and the spinal cord and dorsal body wall removed as far back as the sacral hump. The forelegs were removed together with all but the central section of the pectoral girdle. The oesophagus was then cut down the mid dorsal line and the cut continued a short distance into the stomach wall. The oesophagus was pinned out as a roughly rectangular sheet, being kept taut by pins. It was kept as level as possible by placing pieces of cotton wool soaked in Ringer's solution underneath it. In female frogs the ovaries and oviducts were usually removed as these were liable to get in the way. In some experiments the whole of the hindquarters and the viscera were removed.

The rate of ciliary activity was measured by observing particle movement. The preparation was placed inside a perspex box similar to that ^{previously} described. Particles were dropped on to the membrane through a slit in the lid, and the time taken to travel either 0.5 cm or 1.0 cm was measured. This was facilitated by ruling three parallel lines on the lid 0.5 cm apart and observing the particles as they travelled underneath these lines. To avoid errors of parallax a sight was fitted above the lines and which had similar lines 0.5 cm apart ruled on its surface. The particles used were poppy seeds which would pass through a sieve of 40 mesh but were retained by one of 60 mesh (i.e. 0.423 to 0.635 mm in diameter).

The perspex box and all the solutions used were placed throughout the whole of each experiment in a water bath thermostatically controlled at 20°C.

To avoid errors a strict sequence of events was adhered to. The membrane was rinsed with Ringer's solution and exactly 5 min later 10 particles were individually timed. This sequence of events was continued until a steady rate of transport was obtained;

the membrane was then rinsed with Ringer's solution containing the drug under test and exactly 5 min later the particle transport time was measured. A further application of Ringer's solution plus drug could then be made or the membrane rinsed with drug-free Ringer's solution and again particle transport time measured. This alternation of doses of Ringer's solution with Ringer's solution plus drug could be continued for as long as was required.

(2) The preparations usually remained active for about 24 hr, but measurements were made only on newly set up preparations. Particles falling near the cut edges or in the centre of the preparation near the glottis were not included in the results.

(3) Experiments were also carried out using an isolated membrane immersed in Ringer's solution. Under these conditions the membrane was completely dissected from the rest of the frog and pinned out on a piece of $\frac{1}{8}$ inch thick polythene. This was placed in the perspex box and completely immersed in Ringer's solution. In these experiments with a totally immersed preparation particles of garnet of 60 mesh (0.425 mm in diameter) were used as the poppy seeds often floated to the surface of the Ringer's solution.

The average time for each set of 10 particles was calculated in cm/min to give the rate of particle transport.

Five types of Ringer's solution were used. A bicarbonate Ringer as used by Kordik et al.; a phosphate Ringer described by Hill, and three other solutions also used by Hill (personal communication).

The composition of the Ringer's solutions were as follows:

- (1) Bicarbonate Ringer. (mM). NaCl 119, KCl 1.9, CaCl_2 1.1, NaHCO_3 2.4.
- (2) Phosphate Ringer. (mM). NaCl 115, KCl 2.0, CaCl_2 1.8, buffered with Na phosphate 2.0 mM to pH 7.0.
- (3) (mM). NaCl 115, KCl 2.0, CaCl_2 1.8.
- (4) (mM). NaCl 115, KCl 2.0, CaCl_2 1.8, NaHCO_3 10.0.
- (5) (mM). NaCl 115, KCl 2.0, CaCl_2 1.8, NaHCO_3 10.0, buffered with 5% CO_2 .

TABLE 15

Rate of particle transport by cilia of frog

oesophagus (cm/min)

Initial rate	Rate in presence of tubocurarine 10^{-6} g/ml	Rate after removal of tubocurarine
1.07	0.62	0.91
1.16	0.92	1.17
1.39	1.03	1.78
1.98	1.46	1.85
2.29	1.86	2.29
Mean	1.18	1.60

TABLE 16

Effect of acetylcholine 10^{-6} g/ml on rate of
particle transport (cm/min)

Initial rate	Rate in presence of ACh	Rate after removal of ACh	Rate in presence of ACh
0.24	0.45	0.27	0.40
1.24	1.52	1.01	

Results

Using the bicarbonate Ringer's solution the effect of tubocurarine 10^{-6} g/ml was observed. In all experiments, whether using the normal preparation or the totally immersed preparation, a decrease in the rate of particle transport was observed after the addition of tubocurarine. After the tubocurarine had been removed the rate returned to that observed initially. The results of experiments are shown in Table 15. A typical experimental record is shown in Fig.4.

If the initial rate is taken as 100 then the relative rates before, during the presence of tubocurarine, and after its removal may be directly compared. The mean rate during the presence of tubocurarine was 75 per cent of the initial rate, and after removing the tubocurarine it returned to 101 per cent.

Hill was unable to demonstrate the stimulating action of acetylcholine in low concentrations and two experiments were therefore carried out using concentrations of 10^{-6} g/ml. The results, which show this stimulating action quite clearly are given in Table 16.

TABLE 17

Rate of particle transport (cm/min) showing absence
of effect of tubocurarine using phosphate Ringer

Initial rate	Rate in presence of tubocurarine 10^{-6} g/ml	Rate after removal of tubocurarine
1.59	1.57	1.34
2.35	2.39	2.24

The results obtained showed the inhibitory effect of tubocurarine and the stimulating effect of acetylcholine on ciliary activity. The failure of Hill to observe these effects was therefore puzzling and so an attempt was made to try and discover the differences observed. It had been suggested that artefacts had arisen in Kordik's experiments due to the presence of counter currents and the accumulation of mucus. Hill used a membrane totally immersed in Ringer's solution which was claimed to prevent artefacts arising. However, experiments using a totally immersed preparation still showed the inhibitory action of tubocurarine.

A further study of Hill's paper showed one difference which had previously been overlooked. This was that whereas Kordik et al. had used a Ringer's solution containing bicarbonate, Hill used a Ringer's solution containing phosphate in the majority of her experiments. As this appeared to be the only real difference between the experimental procedures, observations were made on the effect of tubocurarine using this phosphate solution. The results of the first two experiments are shown in Table 17. In both these experiments tubocurarine

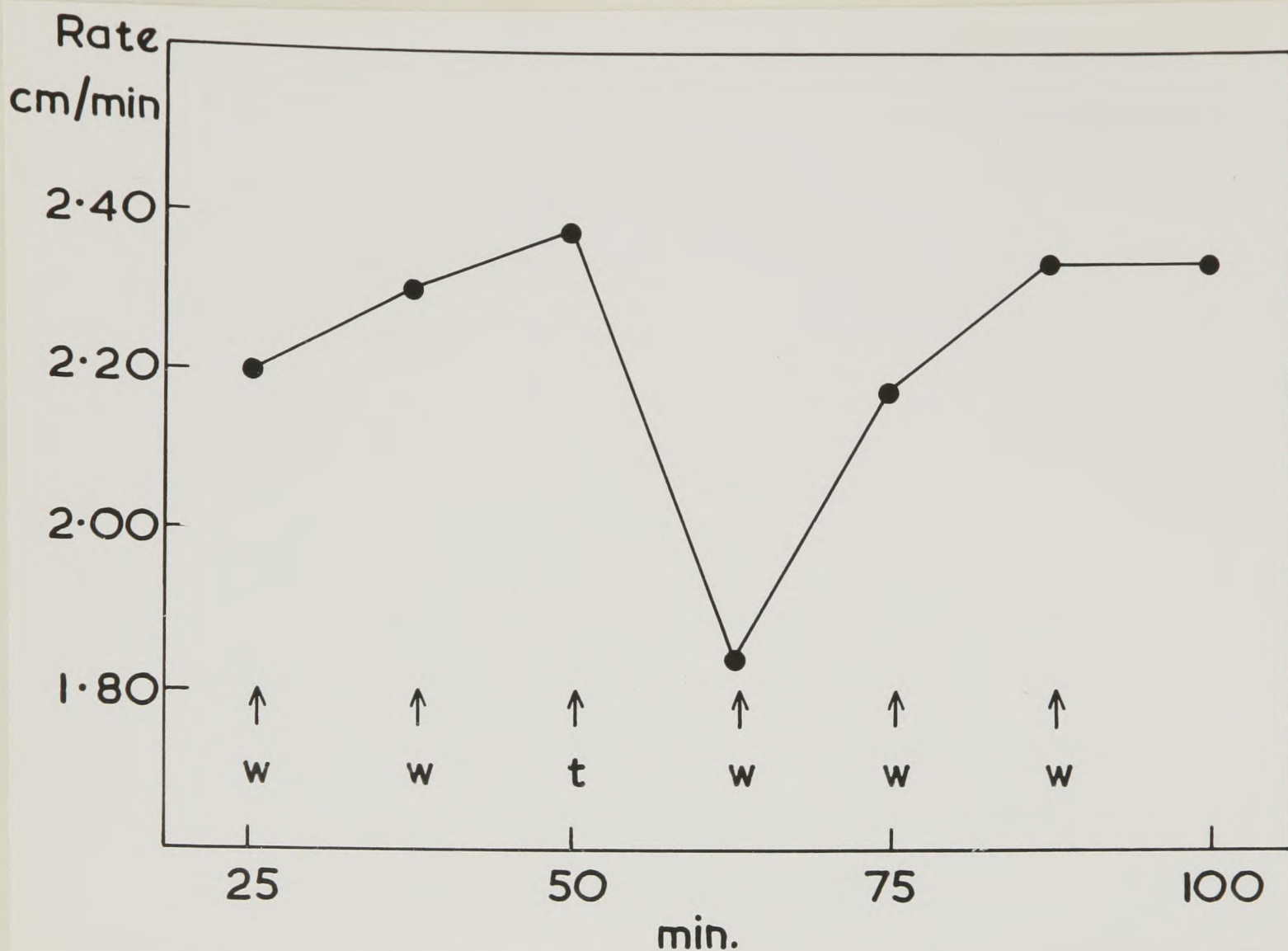


Fig.4. Example of the inhibitory effect of tubocurarine 10^{-6} g/ml on rate of particle transport.

W = membrane bathed with bicarbonate Ringer's solution

t = membrane bathed with bicarbonate Ringer's solution containing tubocurarine 10^{-6} g/ml.

in a concentration of 10^{-6} g/ml had no significant effect.

Experiments were next carried out in which both the Ringer's solution were used for tests on the same preparation. In the first experiment the bicarbonate Ringer's solution was used initially and tubocurarine was observed to exert an inhibitory effect; the Ringer's solution was then changed to that containing phosphate and after equilibration it was found that the tubocurarine no longer exerted an inhibitory effect. In the second experiment the phosphate Ringer's solution was used initially and tubocurarine was observed to have no effect; on changing to bicarbonate Ringer's solution the inhibitory effect of tubocurarine was readily observed. A graph of this experiment is shown in Fig.5.

These results showed that differences occurred when using different types of Ringer's solution. As a result it was decided to carry out further experiments to try and explain how these different effects were exerted.

Effect of pH

The pH of the phosphate Ringer's solution was 7.0, whereas the bicarbonate Ringer's solution varied between

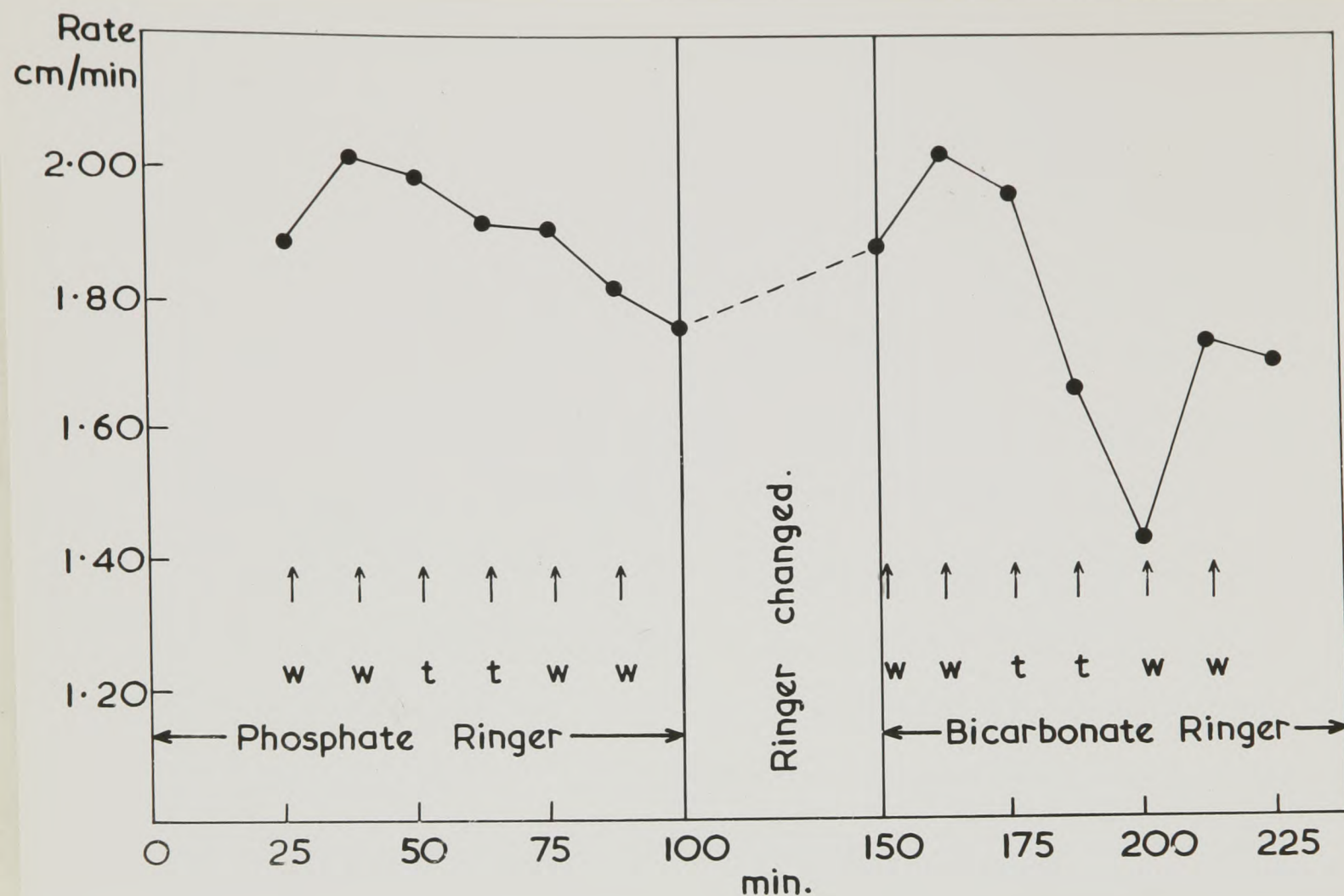


Fig.5. Experimental record showing effect of tubocurarine 10^{-6} g/ml on rate of particle transport on the same preparation when using both phosphate and bicarbonate Ringer's solution.

W = membrane bathed with Ringer's solution.

t = membrane bathed with Ringer's solution containing tubocurarine 10^{-6} g/ml.

7.6 and 8.3 owing to the absence of a buffering system. It should be noted, however, that the pH of frog's blood is about 8.0. Tubocurarine in concentrations of 10^{-4} g/ml and less was found not to alter the pH of either solution.

Experiments were carried out using bicarbonate Ringer's solution which had been brought to pH 7.0. The cilia showed a normal activity, and were still active 20 hr after being set up. Tubocurarine 10^{-6} g/ml showed a marked inhibitory action.

When the pH of the phosphate Ringer's solution was raised to 8.0, visible precipitation of calcium phosphate occurred. The cilia were so depressed and irregular in their rate of beat when using this solution that no measurements were possible.

These observations give no indication that differences in pH were responsible for the different results. However, the effect of calcium precipitation suggested that a reduced Ca^{++} ion concentration due

TABLE 18

Observations using phosphate-free solutions.

Tubocurarine 10^{-3} g/ml. Figures are rate of transport (cm/min)

Ringer solution used	Initial rate	Tubocurarine		Rate after
		3 min after addition	10 min	
(3) (See Methods)	1.78	1.21	1.36	1.89
(3) " "	2.08	2.10	0	0
(4) " "	1.96	1.09	0	0
(5) " "	1.86	1.60	1.28	2.17

to calcium binding with the phosphate might be responsible. In connection with this last observation it was found that Ca^{++} ion free bicarbonate Ringer's solution caused an increase in activity at the start followed by slowing coupled with irregularity. Tubocurarine showed no significant effect under these conditions.

Three other solutions used by Hill (personal communication) were also tested. In all cases tubocurarine caused a decrease in rate of particle transport as shown in Table 18, and in two of the experiments the transport of particles was arrested altogether.

All (1930) found that the presence of phosphate interfered with tissue respiration. This was due to inhibition of the respiration of tissue and was allowed by oxygen was reduced from 10% to 1% when bicarbonate buffer was replaced by a phosphate buffer. All suggested that this might be due to the binding of Ca^{++} ions by the phosphate. The explanation might apply to the whole system and to the whole system.

Discussion

The results obtained confirmed the finding of Kordik, Bülbbring and Burn (1952) that d-tubocurarine in a concentration of 10^{-6} g/ml caused inhibition of the activity of the cilia on the frog's oesophagus. Further investigations showed, however, that this effect depended on the composition of the Ringer's solution used. Inhibition was observed in the presence of a bicarbonate Ringer's solution, but when phosphate was present in the Ringer's solution this inhibitory effect of tubocurarine was no longer observed; however, the inhibitory action of tubocurarine was restored in the same preparation when the phosphate Ringer's solution was replaced by bicarbonate Ringer's solution.

Alt (1930) found that the presence of phosphate interfered with tissue respiration. Thus even the inhibition of the respiration of kidney and liver slices by cyanide was reduced from 98% to 11% when bicarbonate buffer was replaced by a phosphate buffer. Alt suggested that this result might be due to the binding of Ca^{++} ions by the phosphate. This explanation might apply to the cilia since when a Ca^{++} ion free

Ringer's solution was used, after an initial rise in rate, a rapid depression followed and tubocurarine was found to have no significant effect.

Attention has been drawn to the point that the rate of particle transport has been recorded and that this may be affected by mucus secretion. Kordik, Bülbring and Burn (1952) investigated the action of tubocurarine on salivary secretion in the cat and found that it had less than 1% of the paralyzing action of atropine. Nevertheless on the cilia of the frog's oesophagus tubocurarine was rather more potent than atropine in inhibiting ciliary transport. It may also be recalled that Bülbring, Burn and Shelley (1953) observed that tubocurarine slowed the rate of the ciliary beat, as observed by a stroboscope, on the edge of an isolated gill filament of *Mytilus edulis* washed by a flow of sea water. Under these circumstances mucus secretion could play no part in modifying the effects observed.

Hill (1957) stated, as a result of her observations, 'Since ciliary activity was unaffected by tubocurarine it is highly improbable that this drug will produce ciliary depression when given clinically to produce

muscular relaxation during anaesthesia.' In view of the results obtained by Kordik et al. and by Burn and Day as well as those recorded here, this statement may be subject to doubt. Furthermore, the conditions used in experiments with the ciliated epithelium exposed to a moist atmosphere far more approaches conditions found 'in vivo' than where the epithelium is completely immersed.

Summary

Further investigations of the effect of tubocurarine on the transport of particles by the cilia of the frog's oesophagus has confirmed that, when a Ringer's solution containing bicarbonate is used, tubocurarine inhibits the movement in a concentration of 10^{-6} g/ml. If, however, a Ringer's solution containing phosphate is used, tubocurarine is then without effect. The difference in action seems likely to be due to changes in calcium ion concentration caused by the phosphate present in the Ringer's solution.

Through large amounts of work on choline acetylcholine in the tissues (Huxley, 1957) together with an active cholinergic system, only a feeble indication of the presence of a choline acetylcholine system was obtained. In 1957 Huxley stated in a review on choline acetylcholine 'The evidence for the presence of choline acetylcholine might be divided: if it were not that the authors have established beyond doubt that acetylcholine is present in the tissues. The rate of synthesis they found, however, would be quite inadequate to maintain the observed tissue level of acetylcholine. One explanation which would account for their results is that the choline sources may be inactive at 37°C, the temperature at which their measurements were made.' Recently it has been found (Hilton, 1958) that the choline acetylcholine

CHAPTER IX

Enzymes present in the gill plates of *Mytilus edulis*

Choline acetylase

Introduction

It has previously been demonstrated (Bülbring, Burn and Shelley, 1953) that the ciliary movement on the gill plates of *Mytilus edulis* is controlled by acetylcholine. Though large amounts of acetylcholine were found present in the tissue (4 $\mu\text{g/g}$) together with an active cholinesterase system, only a feeble indication of the presence of a choline acetylase system was obtained. In 1957 Hebb stated in a review on choline acetylase 'The evidence for the presence of choline acetylase might be dismissed if it were not that the authors have established beyond doubt that acetylcholine is present in the tissue. The rate of synthesis they found, however, would be quite inadequate to maintain the observed tissue level of acetylcholine..... One explanation which could account for their results is that the *Mytilus* enzyme may be inactive at 37°C, the temperature at which their measurements were made.' Recently it has been found (Milton, 1958) that the choline acetylase

obtained from the brain of Wrasse, a marine fish, which is another example of an animal living at a cold temperature, is most active at 25°C , and is rapidly inactivated at 37°C .

The choline acetylase system has now been reinvestigated.

Methods

Enzyme preparation

The gill plates of *Mytilus edulis* were dissected under sea water at 4°C . They were rinsed twice with cold sea water to remove extraneous mucus which has an inhibiting effect on acetylcholine synthesis. The excess sea water was removed and the gill plates were rinsed once with cold, dry acetone (at -11°C). They were then immediately dropped into a large volume of cold, dry acetone (100 ml acetone/g tissue). The acetone was decanted off, and the gill plates were ground in a mortar, washed with a large volume of cold acetone and the acetone rapidly poured off through a Buchner funnel. The operation of grinding and washing was repeated several times, until all the material had been transferred to the filter-paper in the form of a fine powder. After drying on the filter-paper for 5 min, the powder was

transferred to a desiccator and evacuated over P_2O_5 . After standing at $4^{\circ}C$ for 24 hr, the powder was removed from the desiccator, weighed and suspended, 40 mg/ml, in cysteine saline solution (6 mg neutralized cysteine hydrochloride/ml 0.9% NaCl).

The solution was stored at $-15^{\circ}C$ (for a minimum period of 48 hr). The solution was thawed, and centrifuged for 15 min just prior to use.

Purification of the enzyme

Attempts were made to purify the enzyme using the procedure described by Kumagai and Ebashi (1945) with slight modifications. In the first stage an acetone-dried powder was used instead of freeze-dried material. The last stage was omitted and in the penultimate stage the residue was made up in cysteine-saline solution and dialysed for 24 hr against cysteine saline.

The 2,4,6-trinitrophenyl phosphate was obtained commercially and suspended in distilled water. The 2,4,6-trinitrophenyl acrylate was also obtained commercially and was suspended in 0.02 N KNO_3 . All distilled water used for making up solutions was obtained by running tap water through Permutit resin, and then distilling this water in an all glass still.

Reagents

The incubation medium was composed of the following reagents:

		ml
co-enzyme A	400 units/ml	0.05
KCl	30%	0.04
choline chloride	4%	0.04
MgCl ₂	1.2%	0.08
acetyl phosphate	10 mg/ml	0.14
l-cysteine HCl (neutralised)	30 mg/ml	0.12
phospho-trans-acetylase	2.5 mg/ml	0.10
eserine sulphate	1 mg/ml 4% NaCl	0.10
water		0.13
Total		<u>0.80 ml</u>

The co-enzyme A was prepared as described in Chapter VII. The acetyl phosphate was obtained commercially and suspended in distilled water. The phospho-trans-acetylase was also obtained commercially and was suspended in 0.02 M KHCO₃. All distilled water used for making up solutions was obtained by running tap water through Permutit resin, and then distilling this water in an all glass still.

Method of incubation

The reagents were pipetted into suitable glass tubes and incubated for 15 min at the required temperature, to initiate the formation of acetyl co-enzyme A.

After 15 min 0.2 ml of the enzyme preparation was added, the tubes were corked and the incubation allowed to proceed for 1 hr. Control tubes were set up which contained the pre-incubated reagents, and 0.2 ml of boiled enzyme. These controls were used for making up standard acetylcholine solutions.

After exactly 1 hr the reaction was stopped by adding 6 drops of B.D.H. Universal indicator and 0.2 ml $\frac{N}{3}$ HCl.

Method of acetylcholine estimation

4 ml frog Ringer was added to each tube and the contents boiled for 1 min. After cooling, the volume was made up to 10 ml with frog Ringer and stored at -15°C .

The acetylcholine content was assayed on the frog rectus muscle preparation. The standard acetylcholine solutions were made up in the control samples.

TABLE 19

The effect of temperature on the synthesis of
acetylcholine

Temp. °C	µg ACh/g acetone powder/hr
12	67
17	73
22	91
27	130
32	140
37	123

Results

The effect of temperature on synthesis

The rate of synthesis of acetylcholine was studied over a wide range of temperature. A summary of the mean results of two experiments is shown in Table 19.

The figures illustrate that there is little change in the rate of synthesis at the normal environmental temperature of *Mytilus* (12 to 20°C), but that the rate diminishes at 37°C, presumably as a result of inactivation of the enzyme.

Synthetic ability of the purified enzyme

With a sample of the enzyme purified as described a rate of synthesis of 300 µg ACh/gm material/hr was obtained.

The effect of pre-incubation of the enzyme

The effect of temperature on the enzyme itself was studied. The enzyme preparation was pre-incubated for 1 hr both at 20°C and at 37°C. The treated enzyme was then incubated with the reagents at 30°C in the normal way, and the amount of acetylcholine synthesized was assayed. The results of this pre-incubation are shown in Table 20. The results of this experiment showed quite clearly that the enzyme preparation was relatively stable at 20°C, but that it was rapidly inactivated at 37°C.

TABLE 20

The effect of pre-incubation of the enzyme

	Percentage activity
no pre-incubation	100
pre-incubation at 20°C for 1 hr	95
pre-incubation at 37°C for 1 hr	25

Discussion

In studying the formation of acetylcholine, it is necessary to use a method which is well defined and which will allow the maximum formation of acetylcholine with the minimum formation of other compounds. It is especially important to prevent the formation of acyl co-enzyme A compounds other than acetyl co-enzyme A, and also of analogues of choline and acetylcholine. The method used by Bülbring, Burn and Shelley (1953) suffered from the disadvantage of containing such unspecific reagents as 'boiled brain activator,' and pigeon liver enzyme.

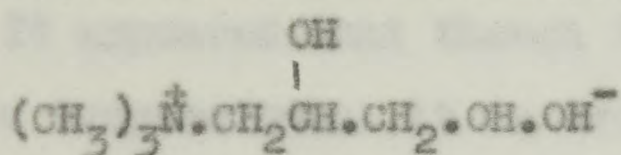
The system which has been used in the present experiments is based on the following two reactions:

- (1) acetylphosphate + co-enzyme A $\xrightarrow{\text{phospho-trans-acetylase}}$
acetyl co-A + PO_4 .
- (2) acetyl co-A + choline $\xrightarrow{\text{choline acetylase}}$ acetylcholine
+ co-A.

It is essential that a system is used which can ensure a continuous and sufficient supply of acetyl co-enzyme A under all conditions, and that this formation of acetyl co-enzyme A does not become rate-limiting especially at low temperatures. Experiments showed that

the system used here fulfilled the necessary conditions.

By excluding citrate, acetate, adenosinetriphosphate and pigeon liver enzyme the formation of other acyl co-enzyme A compounds was prevented, as also was the synthesis of organic acids, which have been shown to inhibit acetylcholine synthesis (Korey, de Braganza and Nachmansohn, 1951). The formation of analogues of choline was also prevented. In 1889 Brieger isolated a toxic substance from *Mytilus* which was said to be trimethylglycerylammonium hydroxide



This substance, an analogue of choline, has been prepared, and when added in equal amounts with choline caused a 20% decrease in acetylcholine synthesis. During the preparation of the acetone powder of the gill plates, the organic acids and many other compounds which are present in homogenates of the gill plates were removed. These considerations indicate that the system used possessed the qualities necessary for the maximum formation of acetylcholine from acetyl co-enzyme A and choline, with the minimum formation of other products.

The results obtained showed that the gill plates of *Mytilus edulis* contain an enzyme which when partially purified was capable of synthesizing acetylcholine in amounts as large as 300 $\mu\text{g/gm}$ material/hr.

The results also showed that a steady rate of acetylcholine synthesis occurred over the environmental temperature range. The optimal temperature was in the region of 32°C , which is the same figure as that quoted by Reisberg (1957) for the choline acetylase present in squid, which is another example of a mollusc.

It appeared that though the enzyme is fairly stable at low temperatures, it is rapidly inactivated at higher temperatures, temperatures at which mammalian choline acetylase is reaching maximum synthesis (Milton, 1958).

In conclusion, therefore, the gill plates of *Mytilus edulis* contain an acetylcholine synthesizing enzyme and this last piece of evidence puts beyond doubt the conclusions arrived at by Bülbbring, Burn and Shelley that the ciliary activity on the gill plates is intimately connected with acetylcholine.

Summary

Previous work has shown that ciliary movement in the gill plates of *Mytilus edulis* is controlled by acetylcholine. This substance was shown to be present in the gill plates together with the enzyme 'true' cholinesterase. The evidence for the presence of a choline-acetylase system was, however, weak.

Experiments show that acetylcholine can be synthesized in appreciable amounts over the environmental temperature range. The experiments also show that whereas the enzyme is stable at the environmental temperature, it is rapidly inactivated at higher temperatures.

An attempt has been made to purify the enzyme.

Oxidation of 5-hydroxytryptamine and histamine

Introduction

There is considerable evidence that 5-hydroxytryptamine is widely distributed among molluscs (Erspamer and Boretti, 1951), and Day (1957) reported a histamine-like substance from the gill plates of *Mytilus edulis*. More recently Gosselin (1959) has reported 5-hydroxytryptamine in the gill plates, and has observed that both 5-hydroxytryptamine and 5-hydroxytryptophan caused an

increase in the rate of ciliary movement. There is, however, very little known about the enzymes concerned with the breakdown of 5-hydroxytryptamine and histamine in *Mytilus* gill plates. Blaschko and Hawkins (1952) were unable to find any amine oxidase activity in *Mytilus*, though more recently Blaschko and Hope (1957) found amine oxidase in the digestive gland, and in the anterior retractor muscle of the byssus. They were unable to show any activity in the gill plates. Blaschko (1941) reported that the cephalopod *Sepia officinalis* did not contain a histaminase.

The majority of the experiments previously carried out on amine oxidase activity had used either isoamylamine, β -phenylethylamine or tryptamine, as substrates. It was therefore decided to use 5-hydroxytryptamine as a substrate for the gill plates, and also histamine which had not previously been tested on the gill plates.

Method

Preparation of gill plate homogenate

The gill plates of *Mytilus edulis* were dissected out at room temperature and weighed. They were homogenised with an equal weight of distilled water at 4°C. When the homogenisation was completed they were dialysed against distilled water at 4°C for 2 hr, the distilled water being changed every half hour. They were then dialysed against $\frac{M}{15}$ phosphate buffer, pH 7.4 for a further 2 hr, changing the buffer every half hour. They were left dialysing against phosphate buffer at 4°C overnight. The homogenate was removed from the dialysing tubes and the final volume measured, and was stored in the deep-freeze at -15°C until required.

Estimation of enzymic activity of homogenate

The enzymic activity was determined manometrically. The main compartment of the manometer flask contained 1.8 ml of the dialysed homogenate. The side bulb contained 0.2 ml of an $\frac{M}{20}$ solution of the substance under test, or 0.2 ml of distilled water. The inner tube contained a filter paper and 0.3 ml N-KOH. The gas phase was O₂ and the temperature was 25°C. After 15 min equilibration, the contents of the side bulbs were tipped into the main compartments.

+ 0.3 ml $\frac{M}{100}$ acetylcholine in the main compartment and

The reaction was followed by taking readings at 0, 5, 10, 15 and 30 min after tipping and then every half hour until the end of the experiment.

Calculation of results

The enzymic activity was expressed as $q\ O_2$, that is $\mu l\ O_2$ taken up per mg fresh weight of tissue per hour. The $q\ O_2$ was calculated from the initial 30 min period, when the rate of the reaction was approximately constant.

Results

(a) Histamine oxidation

No oxygen uptake was observed when histamine was used as a substrate.

(b) 5-hydroxytryptamine

All results are shown in Table 21.

In the first experiment the oxygen uptake using $\frac{M}{20}$ 5-hydroxytryptamine as substrate was observed. The results showed a good oxygen uptake. As a result the effect of Marsilid which is an amine oxidase inhibitor was used in the next experiment. In this experiment the control tube contained 0.24 ml homogenate in the main flask + 0.3 ml water, with 0.3 ml of $\frac{M}{20}$ 5-HT in the side arm and the other flask 2.4 ml homogenate + 0.3 ml $\frac{M}{100}$ Marsilid in the main compartment and

TABLE 21

Oxidation of 5-hydroxytryptamine and
5-hydroxytryptophan by gill plates of
Mytilus edulis

	Side arm	Main Flask	g O ₂
Expt.1	5-hydroxytryptamine	homogenate	0.030
Expt.2	5-hydroxytryptamine	homogenate	0.031
	"	homogenate + Marsilid	0.026
Expt.3	5-hydroxytryptamine	homogenate	0.031
	"	homogenate + KCN	0.000
Expt.4	5-hydroxytryptamine	homogenate	0.038
	tryptamine	"	0.000
	tyramine	"	0.000
	β -phenylethylamine	"	0.000
Expt.5	5-hydroxytryptamine	phosphate buffer	0.000
	"	homogenate	0.039
	5-hydroxytryptophan	phosphate buffer	0.000
	"	homogenate	0.050
Expt.6	5-hydroxytryptamine	phosphate buffer	0.000
	"	boiled homogenate	0.000
	"	homogenate	0.039

0.3 ml $\frac{M}{20}$ 5-HT in the side arm. Equilibration was allowed to proceed for 30 min before tipping. The results showed no difference of oxygen uptake between the flasks containing Marsilid and those without Marsilid.

$\frac{N}{10}$ KCN was used in the third experiment and this completely stopped the oxygen uptake. In the fourth experiment the three amines were examined, namely, tryptamine, tyramine and β -phenylethylamine; none of these showed any oxygen uptake.

The results so far suggested that the enzyme was not an amine oxidase, but perhaps a type of phenolase though specific for 5-HT as it did not oxidise β -phenylethylamine. Further to this in the next experiment both 5-HT and 5-hydroxytryptophan were used, and controls were set up containing phosphate buffer instead of the homogenate. An oxygen uptake occurred both with 5-HT and 5-hydroxytryptophan in the flasks containing the homogenate, but no oxygen uptake occurred in the flasks containing phosphate buffer. This experiment showed that both 5-HT and 5-hydroxytryptophan could be used as substrate. The experiment also showed that

the reaction was not on auto-oxidation.

In the final experiment a sample of the homogenate which had been in a boiling water bath for 20 min was examined using 5-HT as substrate. No oxygen uptake was observed.

Discussion

The results obtained showed quite conclusively that 5-hydroxytryptamine was oxidized by homogenates of the gill plates of *Mytilus edulis*. The control experiments using boiled homogenate, and phosphate buffer in place of the normal homogenate, and which resulted in no oxygen uptake precluded a non-enzymic reaction.

As the reaction was not inhibited by Marsilid but was completely stopped by cyanide, the enzyme was not a true amine-oxidase, but was probably a phenolase. This idea was supported by the observation that 5-hydroxytryptophan was also oxidized. The enzyme was fairly specific as β -phenylethylamine was not oxidized. The results also showed that neither tryptamine or tyramine was oxidized.

These results show for the first time that both 5-hydroxytryptamine and 5-hydroxytryptophan can be oxidized by the gill plates of *Mytilus edulis*. This is of importance after the recent observations by Gosselin on the effect of these compounds on ciliary activity. Of perhaps more importance was the fact that this was an occasion on which a naturally occurring amine was shown to be destroyed in the apparent complete absence of a normal amine oxidase. Preliminary observations indicate that the enzyme is probably present in other organs of *Mytilus*, which also contain an amine oxidase as well. As Welsh (1954) believes that 5-hydroxytryptamine is a neurohormone and transmitter substance in some invertebrates, further work which is outside the scope of this thesis is envisaged to obtain more information about the mode of action of this enzyme.

Summary

The presence of an enzyme which will oxidize 5-hydroxytryptophan and 5-hydroxytryptamine has been observed in the gill plates of *Mytilus edulis*. The enzyme is not inhibited by Marsilid, but is by cyanide, which suggests that it is not an amine oxidase but a phenolase. No histaminase activity could be detected in the gill plates.

REFERENCES

- Abdon, N. O. (1945). Acta Pharmacol. Toxicol., 1, 169.
- Abdon, N. O. and Hammarskjöld, S. O. (1944). Acta physiol. Scand., 8, 75.
- Adam, H. (1906). Pflüg. Arch. ges. Physiol., 111, 607.
- Agersborg, H. P. K. (1923). Quart. J. micr. Sci., 67, 507.
- Ahmed, A. and Taylor, N. R. W. (1957). J. Pharm. Pharmacol., 9, 536.
- Alt, H. L. (1930). Biochem. Z., 221, 498.
- Andrus, E. C. and Carter, E. P. (1930). J. Exp. Med., 51, 357.
- Antopal, W., Glaubach, S. and Glick, D. (1939). Proc. Soc. exp. Biol., N.Y., 42, 280.
- Apáthy, S. (1897). Mitth. zool. Sta. Neapel., 12, 495.
- Armitage, A. K. (1956). D.Phil. thesis, Oxford University.
- Armitage, A. K. (1957). Brit. J. Pharmacol., 12, 74.
- Armitage, A. K., Burn, J. H. and Gunning, A. J. (1957a). Brit. J. Pharmacol., 12, 215.
- Armitage, A. K., Burn, J. H. and Gunning, A. J. (1957b). Circulation Res., 5, 98.
- Babák, E. (1913). Z. allg. Physiol., 15, 184.
- Bellamy, D. (1958). Personal communication.

- Béznak, A. B. L. (1934). J. Physiol., 82, 129.
- Bidder, F. (1852). Arch. Anat. Physiol. Lpz. 163.
- Blaich, W. and Klar, G. (1950). Arch. exp. Path. Pharmac., 211, 67.
- Bleschko, H. (1941). J. Physiol., 99, 364.
- Bleschko, H. and Hawkins, J. (1952). J. Physiol., 118, 88.
- Blaschko, H. and Hope, D. B. (1957). Arch. Biochem. Biophys., 69, 10.
- Bodian, D. (1942). Physiol. Rev., 22, 140.
- Powditch, H. P. (1871). Ber. Sächs. Gesellsch. Wiss., 23, 652.
- Boyd, E. M. (1938). Amer. J. Med. Sci., 196, 44.
- Brieger, L. (1889). Virchows Arch., 115, 483.
- Briscoe, S. and Burn, J. H. (1954). J. Physiol., 126, 181.
- Brodkin, E. and Elliott, K. A. C. (1953). Amer. J. Physiol., 173, 437.
- Brown-Séquard (1853). Experimental Researches, p.109.
H. Bailliere, New York.
- von Brücke, K. T. (1917). Pflüg. Arch. ges. Physiol., 166, 45.
- Bülbring, E. and Burn, J. H. (1949). J. Physiol., 108, 508.
- Bülbring, E., Burn, J. H. and Shelley, H. J. (1953).
Proc. Roy. Soc. B., 141, 445.

Balbring, E., Kottegoda, S. R. and Shelley, H. J.

(1954). J. Physiol., 123, 204.

Burgen, A. S. V. and Terroux, K. G. (1953). J. Physiol.,
120, 449.

Burn, J. H. (1950). Proc. Roy. Soc. B., 137, 281.

Burn, J. H. (1956). Proc. Roy. Soc. Med., 50, 725.

Burn, J. H. and Day, M. (1958). J. Physiol., 141, 520.

Burn, J. H., Gunning, A. J. and Walker, J. M. (1956).
Circulation Res., 4, 288.

Burn, J. H. and Kottegoda, S. R. (1953). J. Physiol.,
121, 360.

Burn, J. H. and Rand, M. J. (1958). J. Physiol., 142, 173.

Burn, J. H. and Vane, J. R. (1949). J. Physiol., 108, 104.

Burn, J. H., Vaughan Williams, E. M., Walker, J. M. (1955).
J. Physiol., 128, 277.

Burn, J. H. and Walker, J. M. (1954). J. Physiol., 124,
489.

Burrows, M. T. (1912). Science 36, 90.

Carlson, A. J. (1905). Amer. J. Physiol., 12, 67 and 471.

Carter, G. S. (1926). J. exp. Biol., 4, 1.

- Carter, G. S. (1928). J. exp. Biol., 6, 97.
- Chang, H. C. and Gaddum, J. H. (1933). J. Physiol., 79, 255.
- Comline, R. S. (1946). J. Physiol., 105, 6P.
- Copeland, M. (1919). Biol. Bull. Wood's Hole., 37, 126.
- Copeland, M. (1922). Biol. Bull. Wood's Hole, 42, 132.
- Covino, B. G. and Beavers, W. R. (1958). J. appl. Physiol.,
13, 422.
- Dawes, G. S. (1952). Pharmacol. Rev., 4, 43.
- Day, S. M. (1956). J. Physiol., 134, 558.
- Day, S. M. (1957). D.Phil. thesis, Oxford University.
- Dobell, C. (1932). Antony van Leeuwenhoek
and his 'little animals' 278, 292. John Bale, Sons
and Danielsson, London.
- Eckhard, C. (1858). Beitr. Anat. Physiol., 1, 147.
- Eckhard, C. (1876). Beitr. Anat. Physiol., 7, 191.
- Elinger, J. and Blackman, J. R. (1907). Amer. J. Physiol.
19, 125.
- Engelhart, E. (1930). Pflüg. Arch. ges. Physiol., 225, 721.
- Engelmann, T. W. (1882). Pflüg. Arch. ges. Physiol., 29, 425.
- Engelmann, T. W. (1897). Pflüg. Arch. ges. Physiol., 65, 109.
- Engelmann, T. W. (1897). Pflüg. Arch. ges. Physiol., 65, 535.
- Engstrom, H. (1951). Acta oto-laryng., Stockh., 39, 364.

- Ericksen, J. E. (1842). London. M. Gazette, 2, 561.
- Erspamer, V. and Boretti, G. (1951). Arch. int. Pharmacodyn., 88, 296.
- Evans, C. L. (1912). J. Physiol., 45, 213.
- Feldberg, W. (1945). Physiol. Rev., 25, 596.
- Flack, M. (1910). J. Physiol., 41, 64.
- Foster, M. (1901). Lectures on the History of Physiology. Cambridge University Press.
- Foster, M. and Dew-Smith, A. G. (1876). J. Anat. Lond., 10, 735.
- Garrey, W. E. (1914). Amer J. Physiol., 33, 397.
- Gaskell, W. H. (1880-82). J. Physiol., 3, 48.
- Gaskell, W. H. (1882). Philos. Trans. 993.
- Gaskell, W. H. (1883). J. Physiol., 4, 43.
- Gaskell, W. H. (1900). Schäfers textbook of Physiology, Vol. II, p.169.
- Genuit, H. (1941). Arch. exp. Path. Pharmacol., 198, 559.
- Goodford, P. J. (1958). Brit. J. Pharmacol., 13, 144.
- Goodford, P. J. (1959). J. Physiol., 145, 221.
- Gosselin, R. E. (1959). Personal communication.
- Göthlin, G. (1920). J. exp. Zool., 31, 403.

- Grant, R. E. (1835). Trans. Zool. Soc. Lond., 1, 9.
- Grave, C. and Schmitt, F. O. (1925). J. Morph., 40, 479.
- Gray, J. (1920). Quart. J. micr. Sci., 64, 345.
- Gray, J. (1922a). Proc. Roy. Soc. B., 93, 104.
- Gray, J. (1922b). Proc. Roy. Soc. B., 93, 122.
- Gray, J. (1923). Proc. Roy. Soc. B., 95, 6.
- Gray, J. (1924). Proc. Roy. Soc. B., 96, 95.
- Grumbach, L., Howard, J. W. and Merrill, V. I. (1954).
Circulation Res., 2, 452.
- Harris, E. J. and Hutter, O. F. (1956). J. Physiol., 133, 58P.
- Hebb, C. O. (1955). Quart. J. Exp. Physiol., 40, 176.
- Hebb, C. O. (1957). Physiol. Rev., 37, 196.
- Hebb, C. O. and Whittaker, V. P. (1958). J. Physiol., 142,
187.
- Hill, J. R. (1957). J. Physiol., 139, 157.
- His, W. (1893). Arb. med. Klin. Lpz., 1, 14.
- Hoffmann, F., Hoffmann, E. J., Middleton, S. and Talesnik,
J. (1945). Amer. J. Physiol., 144, 189.
- Hoffman, B. F. and Suckling, E. E. (1953). Amer. J.
Physiol., 173, 312.
- Holland, W. C. (1954). J. Pharmacol., 111, 1.

- Holland, W. C. (1957). Amer. J. Physiol., 190, 62.
- Holland, W. C. and Burn, J. H. (1957). Brit. Med. J.
1031.
- Holtz, P. and Westermann, E. (1955). Arch. exp. Path.
Pharmak., 225, 421.
- Howell, W. H. (1917). Textbook of Physiology (6th edition),
p.566. W. B. Saunders and Co.
- Johnson, E. A. and McKinnon, M. G. (1956). Nature 178,
1174.
- Kaiser, K. (1894). Z. Biol., 30, 279.
- Kärki, N. T. (1958). J. Physiol., 141, 366.
- Kehar, N. D. and Hooker, D. R. (1935). Amer. J. Physiol.,
112, 301.
- Keith, A. and Flack, M. (1907). J. Anat. Lond., 41, 172.
- Kohn, R. and Pick, E. P. (1920). Pflüg. Arch. ges.
Physiol., 185, 235.
- Kordik, P., Bülbbring, E. and Burn, J. H. (1952).
Brit. J. Pharmacol., 7, 67.
- Korey, S. R. de Braganza, B. and Nachmansohn, D. (1951).
J. Biol. Chem., 189, 705.
- Koshtojanz, G. S. (1938). C. R. Acad. Sci., U.S.S.R., 19,
315.

- Kottegoda, S. R. (1953). Brit. J. Pharmacol., 8, 83.
- Kraft, H. (1890). Pflüg. Arch. ges. Physiol., 47, 196.
- Krijgsman, B. J. (1952). Biol. Rev., 27, 320.
- Kronecker, H. and Schmey, F. (1884). Sitzungsb. Akad. Berlin, 87.
- Kumagai, H. and Ebashi, S. (1954). Nature, 173, 871.
- Langendorff, O. (1884). Arch. Anat. Physiol. Lpz, Suppl. 1.
- Langendorff, O. (1895). Pflüg. Arch. Physiol., 61, 291.
- Langley, J. N. and Dickinson, W. L. (1890). J. Physiol., 11 265.
- Lewis, T., Drury, A. N. and Iliescu, C. C. (1921). Heart, 8, 361.
- Lewis, T., Feil, H. S. and Stroud, W. D. (1918-1920). Heart 7, 191.
- Lierle, D. M. and Evers, L. B. (1944). Laryngoscope 54, 176.
- Lierle, D. M. and Moore, P. M. (1934). Arch. Otolaryng. 19, 55.
- Lillie, R. S. (1902). Amer. J. Physiol., 7, 25.
- Lillie, R. S. (1906). Amer. J. Physiol., 17, 89.
- Lommel, F. (1908). Deutsche Arch. Klin. Med., 94, 365.
- Lucas, A. M. (1932). Proc. Soc. exp. Biol. N.Y., 30, 501.

- Ludwig, C. (1848). Arch. Anat. Physiol. Lpz. 139.
- Ludwig, J. M. and Luchsinger, B. (1879). Zbl. med. Wiss., 17, 404.
- Ludwig, J. M. and Luchsinger, B. (1881). Pflüg. Arch. ges. Physiol., 25, 211.
- McDonald, J. F., Leisure, C. E. and Lenneman, E. E. (1928). Amer. J. Physiol., 85, 395.
- McDowall, R. J. S. (1946). J. Physiol., 104, 392.
- McEwen, L. M. (1956). J. Physiol., 131, 678.
- McNamara, B., Krop, S. and McKay, E. A. (1948). J. Pharmacol., 92, 153.
- McWilliam, J. A. (1887). J. Physiol., 8, 296.
- McWilliam, J. A. (1888). J. Physiol., 9, 167.
- Marchand, R. (1878). Pflüg. Arch. ges. Physiol., 17, 137.
- Markham, R. (1942). Biochem. J. 36, 790.
- Marshall, J. M. (1957). Circulation Res., 5, 664.
- Marshall, J. M. and Vaughan Williams, E. M. (1956). J. Physiol., 131, 186.
- Maxwell, S. S. (1905). Amer. J. Physiol., 13, 154.
- Merton, H. (1923). Pflüg. Arch. ges. Physiol., 198, 1.
- Merunowicz (1875). Ber. Sächs. Gesellsch. Wiss., 251.
- Milton, A. S. (1958). J. Physiol., 142, 25P.

- Mines, G. R. (1912). J. Physiol., 43, 467.
- Müller, J. (1840). Elements of Physiology, Vol. II, 853.
Translated by W. Baly, 2nd edition, London: Taylor
and Walton.
- Nahum, L. H. and Hoff, H. E. (1940). Amer. J. Physiol.,
129, P428.
- Ord, M. G. and Thompson, R. H. S. (1950). Biochem. J., 46,
346.
- Plattner, F. and Hintner, H. (1930). Pflüg. Arch. ges.
Physiol., 225, 19.
- Plattner, F. and Hou, Ch. L. (1931). Pflüg. Arch. ges.
Physiol., 228, 281.
- Pohle, K. (1931). Arch. exp. Path. Pharmac., 159, 452.
- Porter, W. T. (1894). J. Physiol., 15, 121.
- Prinzmetal, M., Corday, E., Brill, I. G., Oblath, R. W.
and Kruger, H. E. (1952). The Auricular Arrhythmias.
Springfield, U.S.A.: Thomas.
- Proetz, A. W. (1939). Arch. Otolaryng. 30, 509.
- Purkinje and Valentin (1834). Arch. Anat. Physiol. Lpz. 391
- Reisberg, R. B. (1957). Yale J. Biol. Med., 29, 403.
- Remak, R. (1844). Arch. Anat. Physiol. Lpz. 463.

- Rosenthal, I. (1875). Bemerkungen über die Thatigkeit der automatischen Nervencentra insbesondere über die Athembewegungen Erlangen. E. Besold.
- Rothberger, G. I. and Sachs, A. (1938). Cardiologia, 2, 71
- Rozansky, N. (1926). Russ. Fiziol. Z., 9, 551.
- Runcan, V. (1940). Arch. exp. Path. Pharmac., 195, 516.
- Sachs, A. (1937). Cardiologia, 1, 74.
- Sakai, T. (1914). Z. Biol., 64, 1.
- Saxby, O. B. (1956). J. Physiol., 133, 4P.
- Scherf, D. (1947). Proc. Soc. Exp. Biol., 64, 233.
- Scherf, D. and Terranova, R. (1949). Amer. J. Physiol., 159, 137.
- See, A. (1927). J. Biophys. Tokyo, 2, cxi.
- Sharpey, W. (1835). Todd's Cyclopaedia of Anat. and Physiol., Vol. 1, 606.
- Shelley, H. J. (1953). D.Phil. thesis, Oxford University.
- Smallman, B. N. (1958). J. Neurochem., 2, 119.
- Snyder, C. D. (1907). Arch. Anat. Physiol. Lpz. 118.
- Spadolini, I. and Domini, G. (1940). Arch. Fisiol., 40, 147.
- Spadolini, I. and Giachetti, A. (1953). Arch. Fisiol., 52, 329.

- Stannius (1852). Arch. Anat. Physiol. Lpz., 85.
- Tawara, S. (1906). Das Reizleitungssystem des
Säugetierherzens. Jena. G. Fischer.
- Vane, J. R. (1948). Brit. J. Pharmacol., 3, 341.
- Vaughan Williams, E. M. (1959). Personal communication.
- Weatherby, J. H. (1940). Proc. Soc. exp. Biol., N.Y.,
45, 284.
- Welsh, J. H. (1954). Fed. Proc., 13, 162.
- Wilde, W. S. and O'Brien, J. M. (1955). United Nations
Publications ix, 1, 12, 704.
- Wilson, I. B. and Cabib, E. (1956). J. Amer. Chem. Soc.,
78, 202.