# ELECTRICAL ACTIVITY AND IONIC GRADIENTS IN CARDIAC MUSCLE, WITH SPECIAL REFERENCE TO THE EFFECTS OF 2-4-DINITROPHENOL

A Thesis submitted for the degree of
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by

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CONTENTS

# FIGURE INDEX

TABLE INDEX

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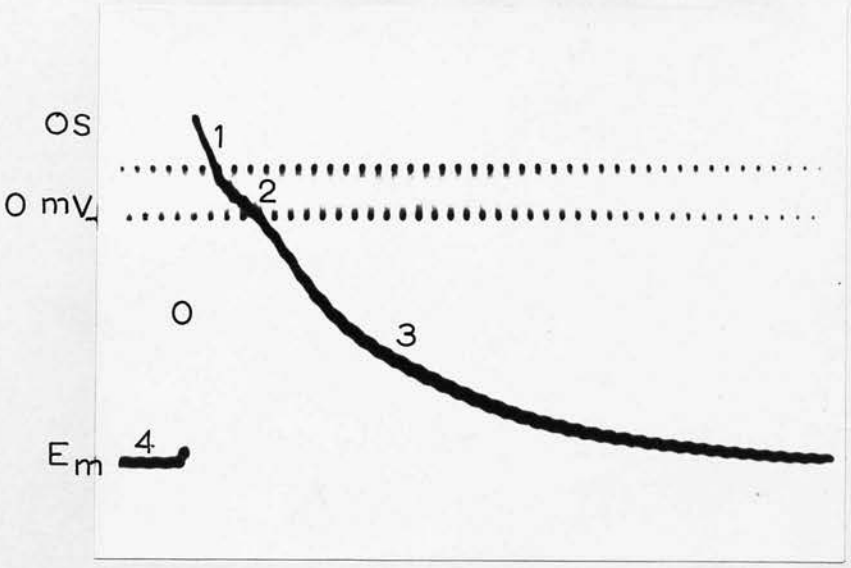
# PREFACE

This Thesis is divided into three parts. In Part I the electrical activity and intracellular ionic contents of sodium, potassium and chloride in the rat auricle have been investigated, to test a hypothesis advanced by Shanes (1958) to account for the action of 2-4-dinitrophenol on the heart. In Part II some observations on arrhythmias produced by 2-4-dinitrophenol in the heart have been extended and investigated. In Part III the results of a few polarisation experiments are presented.

Since the main body of the work has been done and the Thesis written, additional experiments on the efflux of chloride using $Br^{82}$ as tracer have been carried out. The results of these experiments have been added in the results of Part I.

## Notation and Terminology




$E_m$ Resting potential, considered as a positive voltage, in mV.

Overshoot (OS) Potential to which the membrane reverses during the spike, considered as a negative voltage, in mV.

Duration of spike, the time (msec) from the start of repolarisation until the spike is 90% over.

$E_K$, $E_{Na}$, $E_{Cl}$ Equilibrium potentials of K, Na, Cl.

$P_K$, $P_{Na}$, $P_{Cl}$ Ionic permeability to K, Na, Cl.

( ) eg. $(K)_i$ Concentration, eg. of intracellular potassium.

The phases of the action potential are marked on the diagram.

Unless otherwise stated in the text, the line of zero potential across the membrane in the Figures is indicated by the lower edge of the time scale.(usually 50 cps).

# PART I

## THE ELECTRICAL ACTIVITY AND IONIC GRADIENTS IN RAT AURICLE UNDER VARIOUS CONDITIONS

# INTRODUCTION

In 1879 Burdon-Sanderson and Page made a careful study of the electrical events in frog ventricle using a rheotome, with the intention of putting Marey's original work on the refractory period of the heart on a quantitative basis. They found that at 12°C. the refractory period lasted 1.7 seconds and thought that similar, but out of phase, electrical events, occurred at each of the two recording electrodes; each electrical event having the form now known as the monophasic action potential. They thought that the resultant of these out of phase events would give the normal biphasic wave usually recorded; and showed by graphical superimposition that this was so. Later (1883), using a capillary electrometer and falling plate camera, they published photographic records of the electrical activity recorded from frog and tortoise ventricles'. In this paper they showed both the biphasic response obtained from healthy heart, and the monophasic records obtained when the tissue under one of the recording electrodes was killed by heat.

Over the next 40 years there still appears to have been some controversy about the form of the cardiac electrical activity, it being commonly held that it consisted of two separate events, a fast R wave and a much slower T wave, and not of a single

event as in nerve or in muscle. Therefore in 1921 Lord Adrian reinvestigated the recovery processes in frog ventricle as compared to that in nerve, to try to settle the question. For this he used a capillary electrometer and monophasic recording. He stressed the importance of recording between healthy tissue and freshly killed tissue, finding that the quality of the recording deteriorated very quickly with time. His conclusions were that "the only important difference between the form of the response in cardiac muscle and that in skeletal muscle or medullated nerve lies in the fact that in cardiac muscle the rising phase of the response occupies a much smaller fraction of the whole response and the action current remains at its maximum value for a much greater fraction of the whole" and that the action current was largely due to permeability changes of the membrane of the cells.

Since then, by the use of the more refined electrical equipment available and by the introduction of the Ling-Gerard microelectrode (Coraboeuf & Weidmann,1949; Draper & Weidmann, 1951), these observations have been confirmed on all parts of the heart of many animals. The general finding being that the resting potential across the membrane, and the action potential during activity have the same order of magnitude as that in nerve and skeletal muscle, but that the cardiac action potential is

unique in that it lasts for a very long time compared to that of nerve or skeletal muscle.

The action potential in squid axon is now well understood and can be described in terms of a sequence of permeability changes to the sodium and the potassium ions (Hodgkin & Huxley, 1952); and by inference this theory has been applied to mammalian nerve and skeletal muscle. The very prolonged action potentials found in the heart, however, presents a series difficulty to the straightforward application of this theory to the heart. This difficulty is illustrated by the fact that the observed rate of repolarisation of the membrane during the cardiac action potential, is much slower than that predicted from the passive properties of the membrane. Therefore it is not sufficient merely to suppose that there is no increase in potassium permeability during repolarisation in heart cells, but that some additional factor is slowing the repolarisation.

Two kinds of theories have been elaborated to deal with this difficulty. These may be described briefly as the 'permeability' or the 'active transport' theories.

The permeability theory is put in a definite form by Shanes (1958), and supposes that the cardiac action potential is due to a series of permeability

changes to the sodium and potassium ions, analagous to that in squid axon but differing in several important respects. These differences are (a) instead of the brief period of high sodium permeability followed by inactivation as in squid, Shanes supposes that there is a large increase in the sodium permeability at the start of the action potential followed by a long period of gradually declining permeability, so that an appreciable sodium current is still entering the cell during repolarisation of the membrane; and (b), instead of the delayed onset of an increased potassium permeability reaching a high value during repolarisation as in squid, there is a prolonged decrease of the normal potassium permeability followed by a gradual increase to a normal level towards the end of the action potential. Thus the prolongation of the cardiac action potential on this view is due to a balance between the inward sodium current during repolarisation tending to keep the membrane depolarised, and the outward potassium current tending to repolarise the membrane.

This is an attractive theory in that it readily provides an explanation for most of the observed facts on the heart, while at the same time not being too divorced from the situation in the squid axon. There is however no direct evidence for this theory

due to the difficulty in obtaining suitable isolated cells to work on.

The alternative hypothesis, explicitly stated by Hoffman & Suckling (1953) and by Macfarlane (1956) but implicitly held by many others (eg. Webb & Hollander, 1956), is that the long duration of cardiac activity is due to active processes which prolong the action potential. Thus, Macfarlane supposes that there is a transport system moving chloride out of the cell during the action potential, so maintaining the potential across the cell membrane near zero for a long time.

The evidence advanced by Macfarlane in support of this hypothesis will now be discussed. The first piece of evidence is concerned with the temperature coefficient of the various parts of the action potential, with the idea that this would separate chemical and physical processes.

Coraboeuf and Weidmann (1954) measured the temperature changes of the various parts of the action potential of sheep or calf Purkinje tissue in spontaneously beating preparations between 10 and 45°C. and found the following $Q_{10}$ values:- upstroke 1.7, descending limb of the initial spike 1.9, plateau 4.5, final repolarisation 2.6, slow diastolic depolarisation 6.2, and the membrane constants (R & C) 1.5. The important point in

this paper is that the preparation was beating spontaneously, and therefore the rate varied with the temperature and this in itself produces a marked effect on the plateau duration. If the rate is kept constant by driving Purkinje's fibres at a rate of 55 per minute, then (Trautwein, 1953) all the phases of the action potential have a $Q_{10}$ of the order of 1.6.between 25 and 40°C. This then cannot be taken as evidence for the hypothesis that there is active transport during the cardiac action potential. In any case as pointed out by Bayliss (1959, p 59, 111) it is unwise to attach too much importance to the temperature dependence of a process, especially at membranes during periods of low permeability ( p 442-3 ).

The second, and strongest, piece of evidence in support of the 'active transport' hypothesis is that both anoxia and metabolic inhibitors (such as 2-4 dinitrophenol) have a rapid, marked and reversible effect in shortening the cardiac action potential. This important finding has been a challenge to those interested in explaining the cardiac action potential on a permeability basis, and a justification for those who believed that active transport was involved in the action potential directly.

Thus Shanes (1958) attempted to reconcile this

evidence with the permeability theory in the following way; "an important increase in $(Na)_1$ may take place before $(K)_1$ has decreased sufficiently to lower $E_m$ appreciably.....Thus, spike production could be altered by metabolic inhibitors before $E_m$ is changed appreciably" (p 183-184). If this occurred, the sodium current during repolarisation would be decreased more than the potassium current and so the action potential would shorten. It may be noted that to alter the sodium and the potassium gradients by different amounts as required on this theory, it is only necessary to have equal gains and losses of the ions; because of the normal low level of sodium and high level of potassium in the cells. With no evidence to refer to (apart from that on the squid axon with its coupled Na & K transport) this appears to be a reasonable assumption for Shanes to make.

This hypothesis of Shanes provided a starting stimulus, and the rest of this paper is concerned with the testing of this hypothesis and with the consequences which arose from this.

It will be convenient at this stage to stop and review the literature on the various aspects which have just been discussed.

The effects of DNP and anoxia on electrical activity in heart.

Erk and Schaefer in 1944 appear to have been the first workers to study the effects of a pure anoxia on heart action potentials; using good monophasic recording conditions and taking care that the pH of the solutions did not change by using a $N_2$ and $CO_2$ mixture. They found that the replacement of $O_2$ by $N_2$ in the fluid perfusing the heart led, within one to two minutes, to a slowing of the initial part of the action potential (stated in the text but not in fact shown in their records), a shortening and lowering of the whole action potential and a decreased excitability. All these effects were quickly reversed on re-admitting oxygen to the perfusing fluid.

With the introduction of intracellular recording from heart cells, Trautwein et al., in 1954 confirmed these findings by showing that the resting potential and the overshoot were decreased and the action potential shortened by anoxia. They also found that extrasystoles appeared during anoxia in Purkinje's tissue, that the rapidity of changes during anoxia depended on the rate the tissue was driven and that repeated anoxia was more effective than the initial anoxia. In a later paper (Trautwein & Dudel, 1956) they again showed these effects

and also emphasised that often the normal values were overshot on returning to oxygen after anoxia.

In 1955 Marshall using external electrodes on turtle myocardium and Macfarlane & Meares with intracellular recording from frog ventricle showed that 2-4 dinitrophenol (DNP), which was known (Simon, 1953) to block oxidative phosphorylation, produced essentially the same effects as anoxia. Webb and Hollander (1956) extended these observations to rat atria with intracellular electrodes and showed that concentrations of DNP which produced maximum stimulation of respiration in isolated mitochondria from rat heart, shortened the action potential.

Macfarlane (1956) appears to have been the first to suggest that this effect of DNP was consistent with the hypothesis that the long action potential in the heart was due to active transport during the period of the action potential, further suggesting that outward chloride transport would account for the long plateau.

Recently, de Mello (1959) and Lullmann (1959) have repeated this work with intracellular electrodes, without adding any new material.

The effect of DNP and anoxia on the ionic content of heart.

Lemley and Meneely in 1952 studied the effect of anoxia on the water distribution in the hearts of intact rats. As they used the sodium space as an index of the extracellular water, gave no figures for potassium, or the serum of the animals used this paper was useless.

In 1954 Holland and Dunn showed that DNP caused an increased loss of potassium from isolated perfused guinea pig auricles. They did no sodium analysis, the perfusing fluid contained no potassium and no extracellular space measurements were made, so that this paper was of little value.

Hercus, McDowall and Mendel (1955) studied the sodium changes in the right ventricle of rats, both in the intact animals and in perfused hearts, under anoxia. From the graph presented in the paper (p 181) $(Na)_1$ increased from 63 to 74 mequiv/Kg. fibre water after half an hour of anoxia, and did not decrease over the next one and a half hours perfusion in oxygenated Kreb's solution. In the text it is stated that the $(K)_1$ level decreased with the anoxia, but no figures are given. The intracellular levels of the ions were calculated by means of the inulin spaces measured in other rats. This then, although the best paper in the literature, does not help to test Shanes' hypothesis.

The normal ionic gradients in the heart.

While examining the literature for papers on anoxia and metabolic inhibitors, it became clear that there were very few good measurements on the normal gradients of sodium, potassium and chloride in the heart; insufficient to support or to refute the view that the ionic gradients of the ions concerned were sufficient to give the observed membrane potentials. A detailed analysis of the more recent papers will be given below, but the main points will be summarised here:-

1. No paper was found in which the ionic gradients and the electrical potentials were measured in the same hearts, or in the same laboratory by the same workers.

2. Many of the papers quoted in reviews (Shanes, 1958 p 72, Cranefield & Hoffman, 1958) were found on examination to be inadequate in that very gross assumptions had been made about the extracellular space. These assumptions fell into the following groups; (a) Extracellular space measured by the sodium space or by the chloride space, thus assuming that these ions were extracellular. (b) The extracellular space calculated on a Donnan basis, by letting $(K)_o = (Cl)_i$ and thus assuming that both the potassium andthe chloride ions were passively distributed about the cell membranes.

3. In several papers, the authors relied on the work of others for a measurement of the extracellular space. The main objection to this is that the space obtained by different workers differs very markedly, presumably due to differences in the breed of the animals, the length of the perfusion time, the degree of the blotting of the hearts and so on. A good example of this is shown by the inulin spaces of rat heart quoted in the literature; whole heart (largely the ventricles) 28% (Bleachen & Fisher); ventricle, 22% (Hercus, McDowall & Mendel, 1955); right ventricle of young rats, 17% (Kuhns, 1954); right ventricle, 15%, left ventricle, 9% (Barclay, Hamley and Houghton, 1959). With such a range to choose from, any space can be used which gives the desired result.

No exhaustive review of the literature was attempted. In the following account the main emphasis has been placed on the review articles, and on the recent papers on the subject.

Some collected data for sodium, potassium and chloride in heart is given by Lohman and Weicher (1934) from the earlier literature. Fenn in 1936 reviewed the earlier literature (mainly on skeletal muscle), and used the chloride space as an estimate of the extracellular space to calculate the intracellular concentrations of the ions. This

was an important advance on those workers before him in this century, who had ignored the inhomogeneous nature of tissues in the consideration of the ionic contents. Manery (1954), in an important review covering the previous ten years, after discussing the ionic content of heart concluded; "In the case of both cardiac muscle and the smooth muscle of the gastointestinal tract no firm conclusions can be drawn, even about the electrolyte distribution; another field in electrolyte research obviously awaits investigation." Unfortunately this clarion call was not taken up by the succeeding workers in the field.

The paper by Hercus et al. in 1955 gives values for the sodium and the potassium content, and the inulin space of rat ventricle under various conditions, and is one of the few good analytical papers found. Unfortunately it is difficult to determine the purpose of the investigation.

1956 saw several papers on the electrolyte composition of the heart. Gerther et al. measured the Na, K, Cl and the water content of rabbit heart under various conditions and calculated the intracellular concentrations of these ions (with due corrections for Donnan effects as suggested by Manery) by assuming that Cl was all extracellular. The method of Cl analysis was by using hot conc.

$HNO_3$ and 'excess' $AgNO_3$, the actual amount added not being stated. Reiter studied the effect of frequency, strophanthin etc. on the Na and the K content of rat ventricle, using the value of inulin space from Kuhns (1954). The values of $(Na)_1$ and $(K)_1$ which he obtained are similar to those found in the present experiments.

Robertson and Peyser also in 1956 tried to find a correlation between the sucrose space and the electrolyte concentrations of the cat's heart. They were unsuccessful. The value of $E_K$ calculated from their results is 84 mV, the $E_{Na}$ 73 mV and the $E_{Cl}$ 63 mV. These results may be incorrect because 1. The sucrose was given one day previously and 2. the animals were nephrectomised when the sucrose was given; so that the sucrose could have entered the cells in this time and the electrolyte state would almost certainly have been abnormal.

In 1957 Johnson published a good paper on the sodium exchanges in the frog ventricle. He showed that the sucrose space was constant (for 2 hours) after an initial period of about five minutes perfusion with the sucrose solution, and gave values for the intracellular levels of sodium and for the sodium fluxes. His perfusing fluid was a Ringer's

solution diluted with an isotonic sucrose solution, so that the actual values of the intracellular Na obtained (14 mequiv/Kg f.w.) were probably low. He found that about 30% of the total sodium was in the cells, a figure similar to that found in the present experiments.

In 1957 Rayner and Weatherall studied the effects of digoxin and ouabain on the potassium movements in rabbit auricles, and later (1959) the effect of ACh. on the potassium movement on rabbit auricles. They measured the inulin space, getting the surprisingly large value of 44%. As support for this they included some sodium efflux curves, which could really be made to fit almost any value of extracellular space. The method used for the space determination consisted in soaking hearts in an inulin solution for an hour, and then in an inulin free solution for a further hour, with analysis of the inulin in both the solutions. Two objections may be made to this method:- 1. the initial inulin solution was made by adding inulin to Tyrode, so that the osmotic pressure of the solution was increased; and 2. these authors seem not to have carried out tissue blanks for reducing substances and, if this is done for rat heart (see results) a correction of the order of 15% has to be applied to obtain the true sucrose space. As their

outsoaking was some three times as long as that in the present experiments, this figure must be regarded as a minimum one. Even so, this reduces their space to 37%. Consistent with this view is the fact that although sodium analyses were done, no figures are given in the papers and analysis of rabbit auricles by the author (Weatherall) give insufficient sodium to fill the extracellular space qoted by Rayner and Weatherall.

Goodford (1959) perfused rabbit atria for varying periods with Ringer-Locke solution and observed a continuous loss of potassium with time. He used the figure of 44% obtained by Rayner and Weatherall to calculate the intracellular levels of potassium at the various times, undeterred by changes in the water content of the tissue with the passage of time (up to 48 hours). He then concluded that as the calculated $E_K$ when spontaneous activity had ceased, was 60 mV, ( the same value as that found by Marshall in 1957 when cooled auricles stopped beating) this was the critical level for the stopping of spontaneous activity.

This concludes the review of the literature on the ionic content of heart.

This work started with the intention to carry out Na and K analysis and electrical recording from the same hearts (a) to obtain data both on the ionic gradients and the membrane potentials under the same conditions and (b) to follow the changes in the ionic gradients and the electrical activity during metabolic inhibition with DNP, specifically to test the hypothesis proposed by Shanes and (C) to see if it would be possible to test between the two general hypotheses for the prolongation of the action potential in the heart.

The results obtained from this work showed quite clearly (Lamb, 1960) that Shanes' hypothesis was incorrect; the cells lost much more K than they gained Na under DNP, leading to equal changes in the equilibrium potentials. It was still possible to explain the great shortening in the action potential on the basis of the alteration in the gradients of the K and the Na ions, and an attempt was made to do this in this paper. The basis for this was the demonstration by Weidmann (1956) and by Délège (1959) that a diminution of the K and the Na gradients, by changes in the extracellular levels of the ions, led to a shortening of the action potential. It was soon realised however, that this would not do, for quantitatively the changes in the ionic gradients

with DNP were insufficient to account for the large alterations in the action potentials.

Therefore, in view of these difficulties, the attempt to explain the phenomenon in these terms was abandoned.

The clue to the next part of the investigation was provided by the fact that there was a much greater loss of potassium by the hearts, than a gain of sodium. Because of this it was decided to measure the chloride content of the rat auricles under the same conditions as previously studied. As an additional check on the size of the extracellular space as previously determined (Lamb, 1960), the sucrose space of the tissue was also measured.

A review of the literature at this stage showed that chloride had been neglected as an ion, both in analysis and in electrical measurements. The older analytical literature, eg. Lohman and Weicher (1934), and Clark et al. (1938) showed that the chloride content of heart was higher than that of skeletal muscle, but this was attributed (Clark) to the greater extracellular space of the heart.

Apart from the papers already reviewed the folloing two papers were of interest; Barclay, Hamley and Houghton (1959) give figures for the chloride, sodium, sucrose and the inulin spaces of the rat heart. The figures for the sucrose and the

sodium spaces are similar to those found in the present experiments, but the chloride space and hence the chloride content is much higher. Both the inulin and the sucrose spaces show very large standard errors.

The most important paper in the literature is undoubtedly that of Hutter and Noble (1959), who showed that it was probable on electrical grounds 1. that the chloride equilibrium potential was less (ie. more positive) than the membrane potential in the hearts of the sheep and the cat; and 2. that the $P_{Cl}$ was low compared to the $P_k$ in the resting membrane.

In the experiments described in section Ia of the results, it was found that the chloride content of the cardiac cells was some six times higher than would be expected, if the chloride was distributed across the membrane passively. This meant that some active process was required to maintain this chloride within the cells, and also confirmed the work of Hutter and Noble (1959).

By the substitution of other anions for chloride in the Tyrode perfusing the heart, it has been shewn that chloride, despite it's low resting permeability, contributes an appreciable amount of current during certain phases of repolarisation in the cardiac action potential.

A new hypothesis for the action of DNP on the heart

At this stage of the investigation, the main facts on the action of DNP on the action potential of the heart were as follows; 1. shortening of the action potential due to an increased rate of repolarisation; 2. decrease in the resting potential and the overshoot of the action potential; 3. decrease of the excitability, with slowing of the upstroke velocity and of the conduction velocity; 4. a slight increase of the $P_K$ (rabbit auricle, Weatherall, 1960).

The main puzzling features were 1. the great shortening of the action potential, with only a small increase of the $P_K$ (30%); 2. the 10 mV fall in the resting potential at a time when the $P_K$ was raised and the $E_K$ had only dropped by 5 mV; this would be unlikely to be due to an increase in the $P_{Na}$ as the upstroke velocity was decreased and the overshoot greatly diminished despite only a 5 mV drop in the $E_{Na}$. 3. the low excitability fo the hearts, despite high ionic gradients and a fairly good resting potential (70 mV).

A hypothesis which fits all these facts was conceived about this time. This hypothesis supposes that under DNP or anoxia there is a progressive and fairly large increase in the chloride permeability of the membrane, which is reversible on removal of the DNP or the anoxia.

The effect of this is 1. to move the resting potential further away from the $E_K$ and towards the $E_{Cl}$, which is still not passively distributed; 2. hasten repolarisation of the action potential towards this new resting potential and hence to reduce the overshoot and shorten the action potential 3. slow the upstroke velocity, (a) because the membrane is being clamped near the $E_{Cl}$ and (b) because the $E_m$ is lowered and hence (Weidmann, 1955) reducing the inward sodium current on excitation. Eventually this increase in the $P_{Cl}$ is great enough to stabilise the membrane at its resting value and hence to make the heart inexcitable.

For the adequate testing of this hypothesis it will be necessary to use a tracer technique and measure the alteration of the $P_{Cl}$ during treatment with DNP. This has not been done, because of lack of equipment and time. Another deduction from this hypothesis is that in the absence of chloride ions in the medium (eg. Cl replaced by a large anion), the effect of DNP or of anoxia should be much reduced. The evidence that this is so is presented in Part Ib of the results.

This new evidence would make it seem likely that (a) the action of DNP or anoxia on heart muscle can be explained in terms of the alteration of the permeabilities of chloride and potassium, and (b)

that the action potential of heart muscle, like that of skeletal muscle and nerve is due to a series of permeability changes and is not due to any active transport of ions during the action potential.

## METHODS.

### General Experimental Procedure.

The animals used in this work were rats (Wistar albino Glaxo) of various ages and weights (usually 5 month females), fed an ordinary diet and not starved before the experiment.

Each animal was anaesthetised with an intraperitoneal injection of 1-2 mls. of 25 % urethane, the chest opened, the heart removed and put into a Tyrode's solution at c. 6°C. The left auricle was then dissected free in a paraffin bath, also at 6°C., and then transferred to the experimental bath. The time interval between removing the heart from the animal and placing it in the experimental bath was about 1 minute.

The auricle was pinned against stimulating electrodes in the floor of the experimental bath with a light glass rod, perfused with oxygenated Tyrode at 35°C., and driven once per second from a valve stimulator.

During the experiment intracellular records were recorded from the auricle, and at the end of the experiment the auricle was removed, blotted and placed in a weighed vessel. This was then weighed to get the wet weight, dried at 60 to 100°C. to constant weight (30 minutes) and weighed again for the dry weight.

The time for which the auricle was perfused varied with the experiment, as indicated in table I. In the experiments with DNP, perfusion with the DNP solution was continued until the action potentials from the auricle had shortened to a minimum value. This took a mean time of 15 minutes. In the recovery experiments from DNP, perfusion was then continued for a further 15 minutes with normal Tyrode.

At the end of a series of experiments the 35 mm. film was removed from the camera, developed etc., and the action potentials measured by projection onto a calibration grid.

Perfusion Apparatus.

The experimental bath was made of a dental impression wax, and had a volume of 10 mls. The depth of the fluid in the bath was kept at 5 mms. by suction from a water pump. Mounted in the floor of the bath was a perspex block to allow for bottom lighting, and two Ag/AgCl. stimulating electrodes. Mounted in the sides of the bath were two spiral Ag/AgCl. electrodes, the indifferent electrodes for the DC amplifiers and the inlet and the outlet tubes.

Perfusion was by a gravity feed, continuous flow technique; the Tyrode starting in a 2 litre glass flask oxygenated by a 95% $O_2$ 5% $CO_2$ mixture flowing through a rubber tube immersed in an

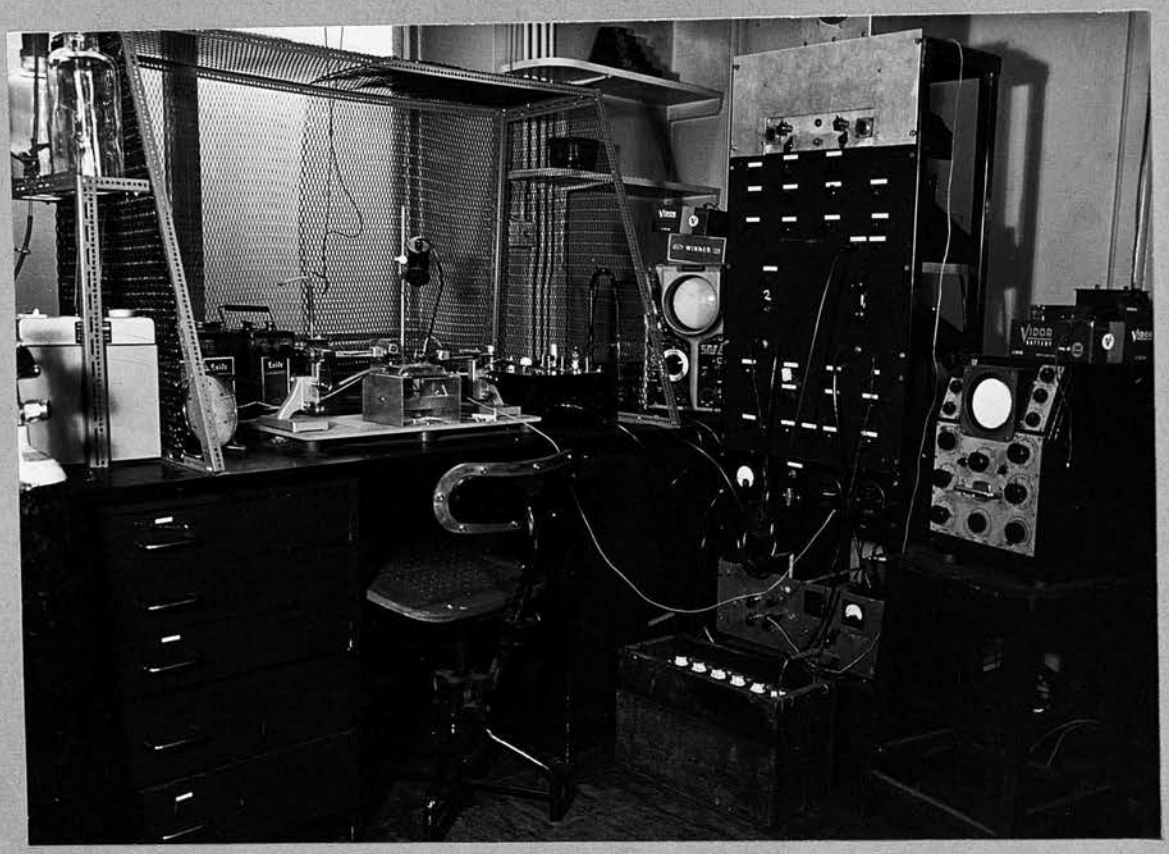


Fig. 1

General view of the equipment. On the extreme left is shewn the thermostatically controlled bath, with the perfusion bottles above it. On the right is the electronic equipment. In the centre of the cage is the platform with the perfusion bath and manipulators on it, and the cathode follower box to it's side. Behind the perfusion bath are the batteries for the Scalamp meter, and above it the lamp.

Fig. 2.

Close-up of the perfusion bath. Fluid entered from the left and was sucked out at the right. The coils at the back of the bath are the bath electrodes. The 2 wires coming in on the left are for the thermocouple. The auricles were pinned down with the glass rod shewn. The electrodes were held in the rubber tube, shewn above (right) the bath, on the left are extra stimulating electrodes. Behind the bath is the cathode follower valve, with connecting wire.

electrically heated, thermostatically controlled, bath at 45°C. (Grant) and then through a dropper into a short length of tube feeding the bath. The perfusion system was triplicated as far as the dropper, the fluid required being selected at the inlet to the dropper by means of polythene clips. The time taken for a new perfusion fluid to reach the bath was of the order of 10 seconds.

The bath temperature was continuously monitored by means of a copper-constantin thermocouple immersed in the bath alongside the auricle, the reference couple being immersed in the thermostatically controlled bath. The output of the thermocouple was displayed on a Scalamp galvanometer (R. 24 ohms), set beside the bath. The thermocouple was calibrated against a mercury thermometer, and the calibration was found to stay constant over the six month period of these experiments. By variation of the flow rate and/or mixing two streams of Tyrode at different temperatures, the bath temperature could be set in the range 10 to 37°C., with a time lag of a minute or two.

The Microelectrodes.

The electrodes used for the intracellular recording were either pulled by hand from suitable pieces of Monax tubing, or by means of a machine from 1.25 mm. outside diameter Pyrex tubing.

It was found that the most satisfactory results were obtained when no initial cleaning of the glass was done. The electrodes were then filled by boiling under reduced pressure for about half an hour, and then stored at room temperature in the 3 M. KCl. solution. Electrodes for use were selected on their resistance, measured by means of a 20 megohm shunting resistance placed in parallel with the electrode and the calibrator box (Donaldson, 1958, p 544, fig. 35.6a). Electrodes of 20-40 megs. were found to be the most suitable for these experiments. No measurements of tip potential were carried out.

The electrodes were held in one of the following ways; 1. The Woodbury technique (1956, with Brady), using 25$\mu$ silver wire and the electrode stuck to it by capillarity, for vigorously beating hearts; 2. by means of a floppy rubber tube for the majority of the experiments; and 3. by means of a cathoded wire spring for the polarisation experiments. In each case the electrode carrier was mounted on a perspex block held on a brass rod, and mounted in a Prior micro-manipulator.

In order to keep the whole system rigid, the manipulators, the bath and the input cathode follower were mounted on a piece of $\frac{3}{8}$" steel plate 2' by 10". This plate was mounted on six door stops on

the bench. This arrangement was found to be satisfactorily stable, and allowed the electrode to remain in the tissue for a considerable time while changing fluids and walking around the room.

Electrical Equipment.

The electrical equipment used was conventional, and consisted of push-pull cathode followers feeding a balanced DC amplifier, the output being displayed on a Nagard oscilloscope and on one beam of a Cossor oscilloscope. The other beam of the Cossor displayed a 50 c.p.s. time marker from the mains. This tube was photographed onto 35 mm. paper by a Cossor camera.

The valves in the cathode followers were 6SB7s, triode connected. The one fed by the electrode was mounted horizontally near the bath , connection being made by 2 inches of silver wire. The other cathode was mounted a foot away, within the cage. The input grid current of the valve used was $2 \times 10^{-11}$ amps. (In the experiments in which polarising currents were passed through the membrane, see later, the apparent value of the resistance facing the electrode, $V_o/I_o$, was 55 Kohms; which means that this value of grid current would alter the $E_m$ by only $10^{-3}$ mV.)

The DC amplifier consisted of three stages of amplification in a step-up design. The first two

stages were connected as 'long-tailed pairs' (Donaldson, 1958, p 181), and a large amount of negative feedback was applied between the anodes of the output stages and the cathodes of the penultimate stages. Gain balance was obtained by variable potentiometers in the cathodes of the cathode-followers, and static balance by means of (a) a battery and bridge circuit between the cathode follower and the DC amplifier and (b) a cathode balancing device in the first stage of the amplifier (Donaldson, 1958, p 190-191). This arrangement allowed the gain of the amplifier to be varied, without unbalancing the amplifier. No accurate measurement of the input timeconstant could be made, for lack of a suitable relay, but the response at 30 Kc.p.s. was only about 50% down on that at 1 Kcps. To give an audible indication of the position of the sweep, the final anode voltage of the amplifier was connected to a 'whistling device', consisting of a neon across a 100 pF. condenser in series with a 20 meg. resistor. This was then fed to a normal audio amplifier.

The voltage calibrator unit was built with 2 Muirhead Decade resistance units (tens & hundreds of ohms), a 1.5 V.gas lighter battery and a 100$\mu$A meter for setting the circulating current. The output was checked against a Cambridge mV meter and

the 100 $\mu$A meter calibrated accordingly. The calibrator was inserted into the circuit between the bath electrode and the other cathode follower grid.

Stimulation was from a conventional valve stimulator unit through an isolating transformer to the tissue. It was usually found that minimum hum was obtained when one side of the secondary of the stimulating transformer was earthed.

A small bench screen 4 by 3 by 2½ feet was built around the bath and the cathode follower, and the shielding was completed by a plate under the bench. The whole equipment was earthed to the water mains by means of a stout cable. Despite all afforts to suppress the interference from the electrically heated bath, it was found necessary to switch it off when photographs were taken, as there was an irregular hum from it.

Solutions.

The Tyrode solution used had the following basis composition (millimoles per litre) :-
$Na^{+}$; 157.2; $K^{+}$, 2.68; $Ca^{++}$, 2.34; $Mg^{++}$, 0.49; $Cl^{-}$, 142.4; $HCO_3$ , 20.0; Phosphate, 0.32; Glucose, 1.0 g per litre.

For the experiments on the chloride substitution, the NaCl in this basic Tyrode was replaced by appropriate amounts of NaI, sodium methyl sulphate or sodium benzene sulphonate, according to their molecular weights.

The 2-4 dinitrophenol used was supplied by BDH as the sodium salt (M.W. 207), each 100 g of the salt had an added 66 g of water. 18 mg. of the reagent was dissolved in 5 mls. of normal NaOH with gentle heating, and ½ a ml. of this solution was then added to 400 mls. of the Tyrode. The final concentration of the DNP was thus 13$\mu$ moles/litre. This amount of NaOH added to the Tyrode produced no change in the pH.

All the solutions used in these experiments were freshly prepared from stock solutions (where appropriate) on the day of the experiment.

Chemical Analysis.

Sodium and Potassium.

The method used was a wet-ashing technique, modified from that described by Rayner & Weatherall (1957). After the auricle had been dried etc. as already described, 0.3 mls. of a mixture of equal amounts of concentrated sulphuric and nitric acids was added, and gentle heat applied to the vessel by holding it over a low Bunsen flame in the hand. When cool, the contents of each vessel was then diluted with ion free distilled water to 10 mls. (In some of the early experiments the dilution was to 25 mls. and in some of the later ones to 15 mls) A control tube was always run with each batch, containing all the reagents except the tissue.

The sodium and the potassium content of each solution was then estimated in duplicate on an EEL flame photometer, against standards containing 2 & 3 gamma of Na and K respectively. If the duplicates were not within 2% further readings were taken. The standards were made from BDH volumetric solutions and stored in polythene bottles.

Addition of 0.3 mls of the mixture of the nitric and sulphuric acids to the standard produced no interference effect. No recovery experiments were done.

Chloride.

The method used for the analysis was suggested by P. Croghan (see Ramsay, Brown and Croghan, 1955), and is an electrometric method.

Initial experiments on the recovery of the chloride from the tissue, showed that the greatest recovery was obtained when the hearts were soaked in dilute nitric acid in the cold, that some 50% was lost by ashing at 500°C. for ½ an hour and that some 20% was lost by charring at 300°C. for ½ an hour. Curiously enough, recovery experiments with NaCl in a crucible by itself showed no loss at 300°C. The reason for this discepancy is not known.

After blotting the auricles, they were placed in crucibles ⅞" in diameter and height and then dried at 60°C. for ½ an hour. To each crucible

was added 0.3 mls. of a 5% solution of nitric acid and the hearts allowed to stand overnight (18 hrs) in the refrigator. Next day the crucibles were removed and allowed to reach room temperature, and then the chloride estimated directly in the crucible.

The apparatus used for the titration consisted of a siver wire with a ball on the end, an Agla micrometer syringe filled with N/20 $AgNO_3$ with a long glass extension of the nozzle containing a platinum wire embedded in its wall, and a millivoltmeter. The silver wire and the syringe were mounted on a movable arm, so that they could be lowered into the crucible together. The electrical potential developed between the silver and the platinum wires was fed to the cathode follower of the main amplifier and then to a 42 A meter. This meter was calibrated up to 500 mV.

For the actual measurement of the chloride content of the auricle, the silver wire and the end of the extension on the syringe were lowered into the solution in the crucible and the voltage read on the meter. (This gave a rough indication of the chloride content). $AgNO_3$ was then added from the Agla syringe in appropriate amounts, and the contents of the crucible stirred with a fine glass rod until the voltage reading was constant. As the endpoint was approached smaller and smaller quantities of $AgNO_3$

were added, until at the endpoint the addition of one unit of $AgNO_3$ (about a hundredth of the total required for the titration) gave a deflection of about 30 mV on the meter.

During the analysis of each batch of hearts, the chloride content of (a) blanks of the nitric acid solution and (b) 5$\mu$ litre quantities of N/10 NaCl (also made from BDH volumetric reagents) was measured. The latter gave values about 1-2% from the expected, but the chloride content of the auricles was taken as the end ppint of the $AgNO_3$ titration.

Iodide.

This was analysed by the same method used for chloride. For the titration the meter was reset with a FSD of 1000 mV, the titration then taking place between 900 and 300 mV. At the endpoint addition of one unit of $AgNO_3$ (ie. $10^{-8}$ equiv.), produced a voltage swing of 80 mV. No standard solution of NaI was used for calibration purposes, but 5$\mu$L. samples of the NaI Tyrode were analysed, withinresults within 2% of those expected.

It was not found possible to analyse the same heart for sodium, potassium and chloride, so that a so that a separate series of hearts were analysed for Na and K, and for Cl. Analysis for Na & K after Cl analysis, gave normal values for Na but values for K some 10-20% low.

Sucrose Spaces.

An isotonic (9.64 g%) solution of sucrose was prepared and kept in the refrigerator. Normal Tyrode (but with the glucose absent) was diluted by the addition of 100 mls of the sucrose solution to 900 mls of Tyrode, giving a final sucrose concentration of about 1%.

Auricles were perfused with this solution in the usual way for 20 to 25 minutes (Johnson 1957), then removed and blotted and placed in 5 ml crucibles. They were then dried at 60°C. for ½ an hour and weighed as usual. To each crucible was added ½ a ml. of water, and the hearts allowed to soak in this for ½ an hour.

The sucrose content of this fluid was then measured by the method of Heyrovsky (1956), after the auricle had been removed. (The method consists of adding b-indolyl acetic acid and conc. HCl and then incubating the mixture in a water bath at 35°C. for 70 minutes. The resulting colour was read on a Hilga Spectrophotometer at 5300 Å.)

With each batch of hearts were run (a) a reagent blank, (b) two heart blanks (ie. auricles not perfused with sucrose), and (c) three 5 µL. samples of the perfusing fluid. The reagent blank was used to set the zero of the spectrophotometer (and was always small), the other tubes being read against it.

From the wet and the dry weights of the hearts the water content was worked out. After correction of the sucrose content of each heart by subtracting the tissue blank (this correction was quite high, reducing the apparent sucrose content by 15%), the sucrose space of each auricle was computed, and the result expressed as a percentage of the wet weight. Care was taken in choosing the dilutions to ensure that the sucrose content was within the linear range of the method.

No analyses for ionic content of these hearts was done.

Sodium Efflux Experiments.

The hearts were washed with an isotonic solution of choline chloride at a low temperature for varying periods, and then analysed for their sodium content.

The choline chloride solution contained 170 mequiv. choline chloride, 2.35 m.equiv. calcium chloride and 500 gamma atropine sulphate per litre.

The choline chloride solution was contained in a 2 litre bottle some 3 feet above the bench and gravity fed via a narrow rubber tube to a polthene tube inserted into a test tube. The end of this polythene tube was expanded, by cutting it open and ironing it flat, and the auricle was pinned to it by means of a steel needle tip. The test tube

and the last part of the rubber delivery tube were immersed in a 2 litre beaker filled with ice-cubes in water, so that the temperature of the auricle was maintained at about $6^{\circ}C$.

Just before perfusion the auricle was opened by a slit down the side, pinned to the polythene platform and then perfused at a flow rate of 20-30 mls. per minute. After the perfusion the auricle was dried and analysed in the usual way for sodium. During the perfusion, the excess fluid was removed from the test tube by a water pump connected to a side arm.

Weighing.

The standard deviation of 34 repeat weighings of the same test tube was 0.2 mgs., so that the S.D. of a weighing involving two weighings (eg. the wet or dry weight of an auricle) will be $0.2 \times 2^{\frac{1}{2}}$ = 0.3 mgs. As the mean weights of the dried auricles were of the order of 3-5 mgs., this means that a single dry weight may show an error of 20% and a mean (ie. a group) dry weight an error of 6% due to the weighing alone. Therefore although the results have been expressed in m.equivs./Kg. dry weight as well as in other ways, such results are only useful in a qualitative way. The results used for the calculations are those expressed per wet weight, the resulting errors due to the weighing then being

of the same order as those of the analysis.

Design of the Experiments.

Owing to the scope of the original experiments being extended, the first series of experiments with DNP on auricles were not planned statistically. The later experiments with choline washouts of hearts treated with DNP etc. were designed around a randomised block design (Mather, 1951). The experiments on the effect of DNP on the chloride content of auricles, were designed in that two auricles perfused with Tyrode and two with Tyrode containing DNP were treated consecutively and then analysed at the same time. The rats for this experiment were chosen in a random manner.

# RESULTS.

## Basic Analytical Data.

Table I shows the basic analytical data for the left auricles of the rats used in this work. Each line represents data largely obtained on the same hearts. The data is presented both as m.equiv/Kg. dry and wet weights but, as discussed in the method's section, all further calculations were done on the wet weight figures.

## Extracellular Space.

In order to express these results in terms of the concentrations of the ions in the intrafibre water and hence obtain the ionic gradients, it is necessary to measure the extracellular space. Two methods have been used to do this:-

1. the Sodium Efflux Method.

This method was suggested by A.M.Shanes and (separately) by O.Hutter in conversation, and was adapted from a method used by Krnjevic (1955) for the sciatic nerves of cats.

Table II shows the results of washing 15 auricles for various times with choline chloride at 6°C. These results are also shown in Fig.3 plotted as the log. of the sodium content left in the auricle (m.equiv/Kg. wet wt.) against time of washing, together with the sodium content of normal rat auricles.

## TABLE I.

Basic analytical data for the left auricle of rat hearts. The data in each line (apart from some of the electrical measurements) were obtained on the same auricles. The figures in column 1 in brackets are the number of auricles. The figures in the other columns (thus ± 3.8) are the standard errors of the means. T stands for Tyrode, D for Tyrode containing 13 u-mole of 2-4 dinitrophenol.

| Treatment (minutes) | Wet wt. mg. | Water % | Potassium | Sodium | Chloride | Potassium | Sodium | Chloride | Electrical Data | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | m-equiv./kg. wet wt. | | | m-equiv./kg. dry wt. | | | $E_m$ (mV) | OS (mV) | Duration m.sec. |
| 1 T (8) | 22.7 | 78.1 | 85.4±3.8 | 60.3±4.6 | | 391±17.7 | 270±20.6 | | 80±1.4 | -28±1.1 | 55±4.7 |
| 15 T (7) | 14.3 | 79.3 | 74.1±1.2 | 59.8±2.1 | | 360±23.6 | 290±22.4 | | 80±0.9 | -32±2.5 | 49±2.3 |
| 30 T (4) | 13.6 | 78.1 | 70.9±1.36 | 64.7±1.21 | | 325±10.9 | 297±13.9 | | 81±0.9 | -27±2.8 | 52±3.8 |
| 50 T (2) | 14.0 | 76.3 | 62.9 | 65.6 | | 268 | 280 | | 80±1.3 | -21±4.1 | 45±6.6 |
| 100T (3) | 16.0 | 78.0 | 40.1 | 67.6 | | | | | | | |
| 15 D (11) | 23.3 | 79.5 | 62.1±3.7 | 63.2±4.3 | | 303±18.1 | 308±20.6 | | 70±3.0 | 0±3.6 | 6±1.2 |
| 15D + 15T (7) | 19.9 | 79.2 | 72.7±3.1 | 67.6±3.7 | | 349±15.2 | 325±16.6 | | 78±2.5 | -16±2.4 | 35±3.4 |
| 0 T (2) | 24.6 | 79.3 | | | 62.5 | | | 302 | | | |
| 15 T (9) | 16.0 | 80.1 | | | 61.5±1.6 | | | 310±16.2 | | | |
| 15 D (10) | 16.6 | 80.8 | | | 52.5±1.4 | | | 268±12.5 | | | |
| 33 T (6) | 14.6 | 79.0 | | | 55.6±1.3 | | | 265±9.1 | | | |
| 15 D + 15 T (4) | 11.7 | 80.2 | | | 50.3±2.3 | | | 256±18.4 | | | |

TABLE II.

Rat auricles perfused with choline chloride solution at 6° C. The figures in brackets in column 1 are the numbers of auricles used.

| Perfusion Time (minutes) | | Na content m-equiv./kg. wet wt. |
|---|---|---|
| 2 | (1) | 23.2 |
| 3 | (1) | 22.6 |
| 6 | (8) | 20.0 |
| 10.3 | (3) | 14.8 |
| 15 | (1) | 11.7 |
| 20 | (1) | 8.53 |

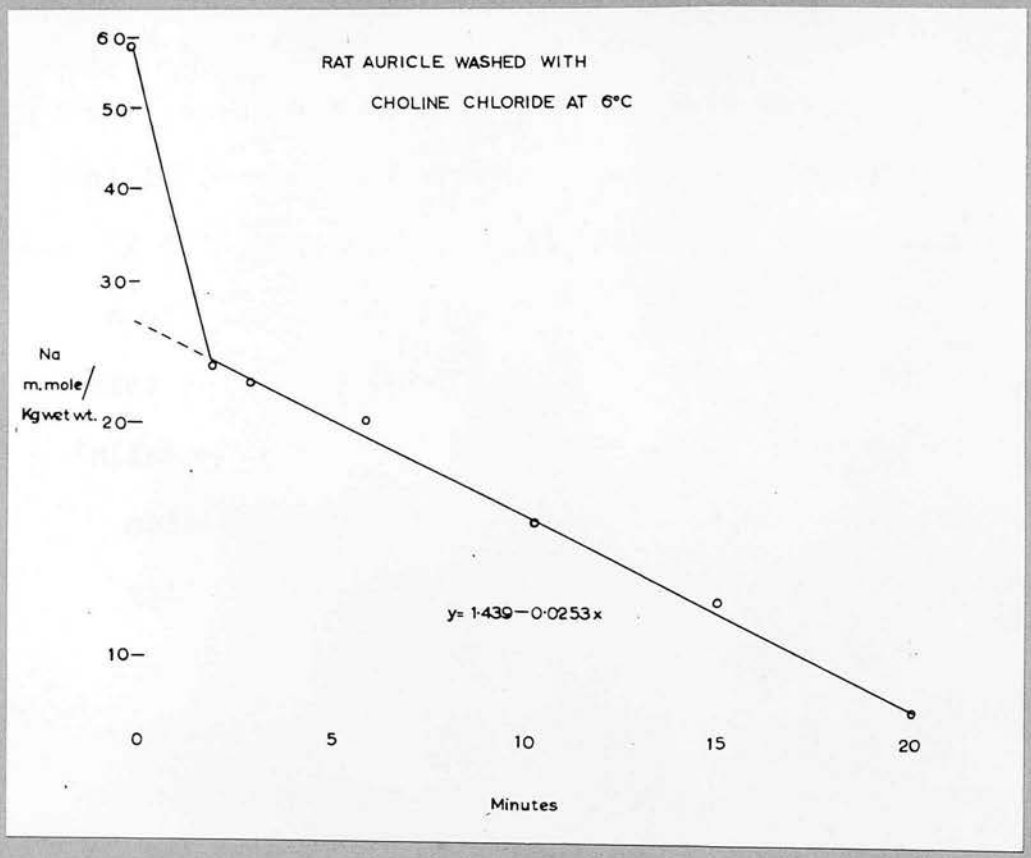



Fig. 3

Sodium efflux curve from rat auricle into choline chloride at 6°C. The auricles were washed for the times shown on the X axis, and then the sodium content shown on the Y axis measured. The equation shown is the regression equation for the slow part of the curve.

It can be seen that there are at least two components to this graph, a fast initial component (poorly represented in this experiment) and a slow component. This slow component is exponential in form and fits the equation $y = 1.439 - 0.0253\ X$ very well (r is 0.95). It has been assumed (see Shanes, 1957, p 127) that the initial part of this graph represents the sodium leaving the extracellular spaces, and the slow exponential part the sodium crossing the cell membranes. It was originally pointed out by Mr A.F.Huxley that this slow component of the curve consistently overestimates the intracellular content of sodium, because the intercellular sodium level is never zero due to the diffusional delay of the sodium out of the tissue. At the membrane this means that the influx of Na is never reduced to zero. This argument applies even more forcibly to the initial component of the curve, when the sodium activity between the cells is very high. The result of this is that the intercept of the equation on the Y axis has to be dropped to give a true estimate of the amount of the sodium in the cells at the start of the perfusion. The amount of the correction required was shewn by Dainty and Krnjevic (1955) to be proportional to the ratio of the rate constants of the slow and the fast components of the curve (ie $K_s/K_f$, where $K_f$ is obtained by subtracting the slow from the fast part of the curve).

If it is assumed that the fast component of this curve is correctly represented by the two points on this graph, then the correction to be applied is 25% (see Appendix). The final answer given by this method is an extracellular space of 24.8 g/100 g wet weight.

For the purpose of this analysis the following assumptions have been made:-

1. that the fast component of the curve can be deduced from two points. This is unlikely, but the true slope of $K_f$ is unlikely to be less steep than it is and this means that the correction will be smaller, and hence the true value of the space will lie somewhere between 21% (uncorrected value) and 25% (corrected value).
2. that efflux curves obtained with choline chloride washing are comparable to those obtained with sodium tracers(used by Dainty in the theoretical analysis for the correction). This assumption has not been corroborated, but Krnjevic (1955) did obtain similar efflux curves of sodium into sucrose as Dainty and Krnjevic (1955) obtained with $Na^{24}$ into $Na^{23}$ Tyrode, with cat nerve.
3. that the concentration of sodium in the interspaces is similar to that in the perfusing fluid, that is no correction has been applied for any Donnan effect in the interspaces.

2. The second method used to estimate the extracellular space was by measurement of the Sucrose Space.

To check the extracellular space obtained by the previous method and to obtain some idea of the variability of the space, sucrose spaces were estimated in ten auricles.

Sucrose was chosen because it has been found to give consistently higher 'spaces' than inulin in various tissues (frog muscle, Tasker et al., 1959; heart, Barclay, Hamley and Houghton, 1959), presumably because of its smaller molecular weight and therefore the ease with which it can be washed into and out of the rather small spaces between cells. It was assumed that equilibrium between the Tyrode and the extracellular space was reached in twenty minutes (Johnson, 1957) and that the sucrose would all diffuse out in half an hour.

The result for these ten auricles was an extracellular space of 25.1 ± 0.7 g/100 g wet weight in auricles with a mean wet weight of 14.2 ± 0.9 mgs. and a water content of 77.8 ± 0.4%. This shows a satisfactory agreement with the result obtained with the sodium efflux experiments, and increases the confidence in the concept of the "extracellular space".

Despite attempts to standardise the blotting procedure, it can be seen that there are quite

large variations in the water content of hearts done at various times, eg. 'sucrose space' hearts and 'chloride' hearts are 77.8 and 80.1 respectively. It was felt that this variability was probably a reflection of changes in the extracellular water; and it was decided to take the sucrose space measurement (in round figures) as meaning that the intracellular water was 53 g/100g wet tissue for hearts with a total water of 78 g/100g wet tissue. Thus hearts with a mean water content of 80%, were considered to have an extracellular space of 27% and the correction to be applied to obtain the intracellular levels of the ions altered accordingly.

Hearts treated with DNP appear to show some small increase in the water content (eg. hearts analysed for chloride with and without DNP after 15 minutes perfusion). The figures of Hercus et al. (1956) for rat ventricle and anoxia also show this increase, which their space measurements showed to be confined to the intracellular phase. In the calculations with these hearts the difference between the water content with and without DNP has been attributed to a change in the intracellular water content.

In calculation of the amount of each ion in the extracellular space and hence the correction to be applied to the total content to get the intracellular level, no correction has been made for any Donnan

effect; so that the ions have been considered to be at the same concentration in the interspaces as in the perfusing fluid.

(a) Results in normal auricles.

1. The ionic gradients.

In table III are presented the normal intracellular levels of Na, K and Cl, calculated as previously described.

The main points shown in this table are:-

a. $(K)_i$ shows an initial large fall in the first few minutes, followed by a more gradual decline in value with time.

b. $(Na)_i$ also shows an initial fall and then rises steadily, apart from the last observation at 100 minutes. It is known (Fisher, personal communication) that rat hearts swell with time ( when perfused ) and so any estimate of $(Na)_i$ at 100 minutes cannot with much confidence be based on a space measurement made at 20 minutes.

c. $(Cl)_i$ appears to decline somewhat with time. Little reliance can however be placed on the initial value for $(Cl)_i$, being based on only two observations, so that it can only be said to be of the same order as the rest.

These results for K and Na are of the same order as those in the literature for rat heart, where proper measurement of the extracellular space has

## TABLE III.

Intracellular ionic concentrations during perfusion with Tyrode's solution. All figures are expressed as m-equiv./kg. fibre water. s.e. ± m.e.

| Perfusion Time (minutes) | Sodium | Potassium | Chloride | Sodium + Potassium |
|---|---|---|---|---|
| 1-2 | 39.4 ±2.6 | 160 ±7.0 | 47.0 + | 199.4 |
| 15 | 34.9 ±4.0 | 139 ±2.3 | 43.2 ±3.0 | 173.9 |
| 30 | 47.8 ±2.3 | 133 ±2.6 | 35.1 ±2.6 | 180.8 |
| 50 | 54.7 | 117 | - | 171.7 |
| 100 | 52.0 | 74.3 | - | 126.3 |

+ No perfusion, only two experiments.

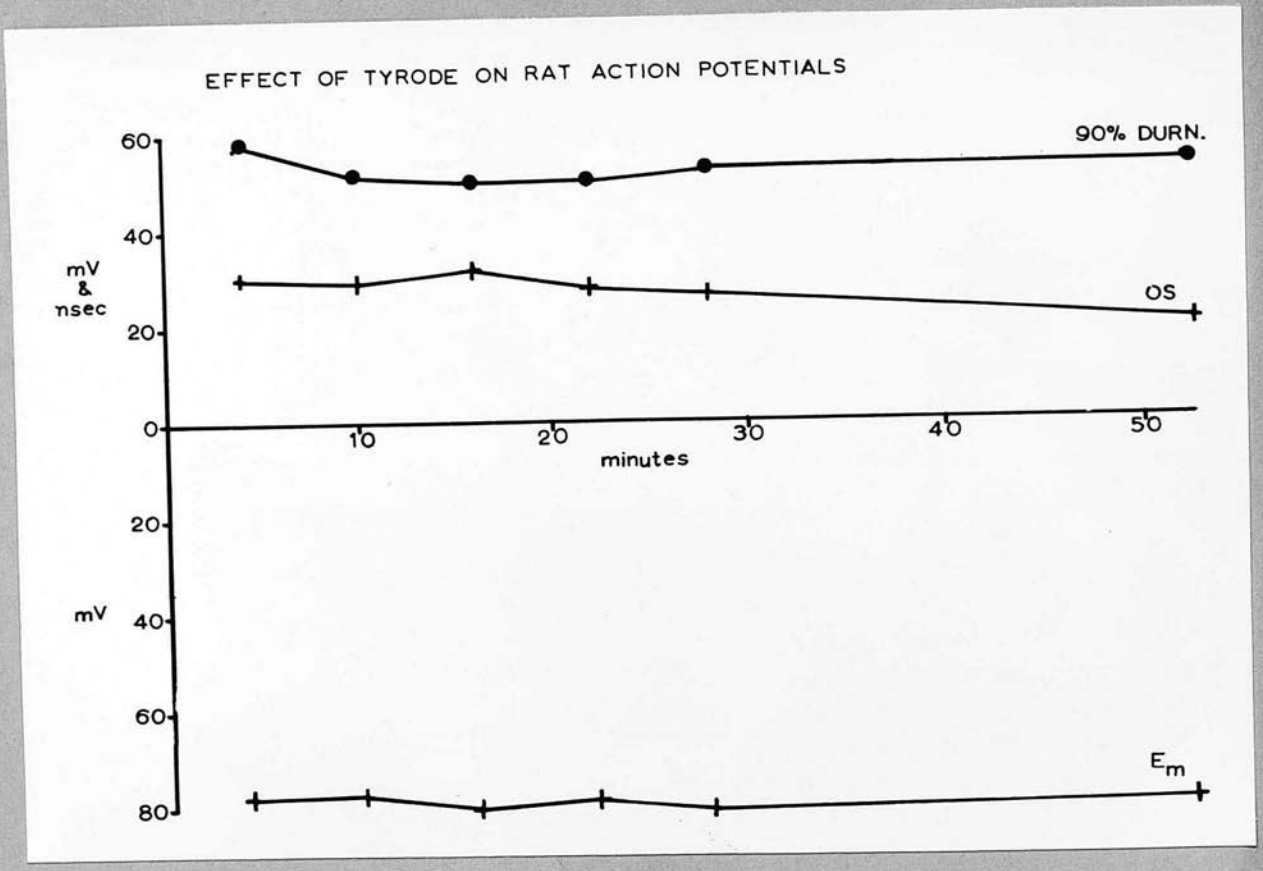
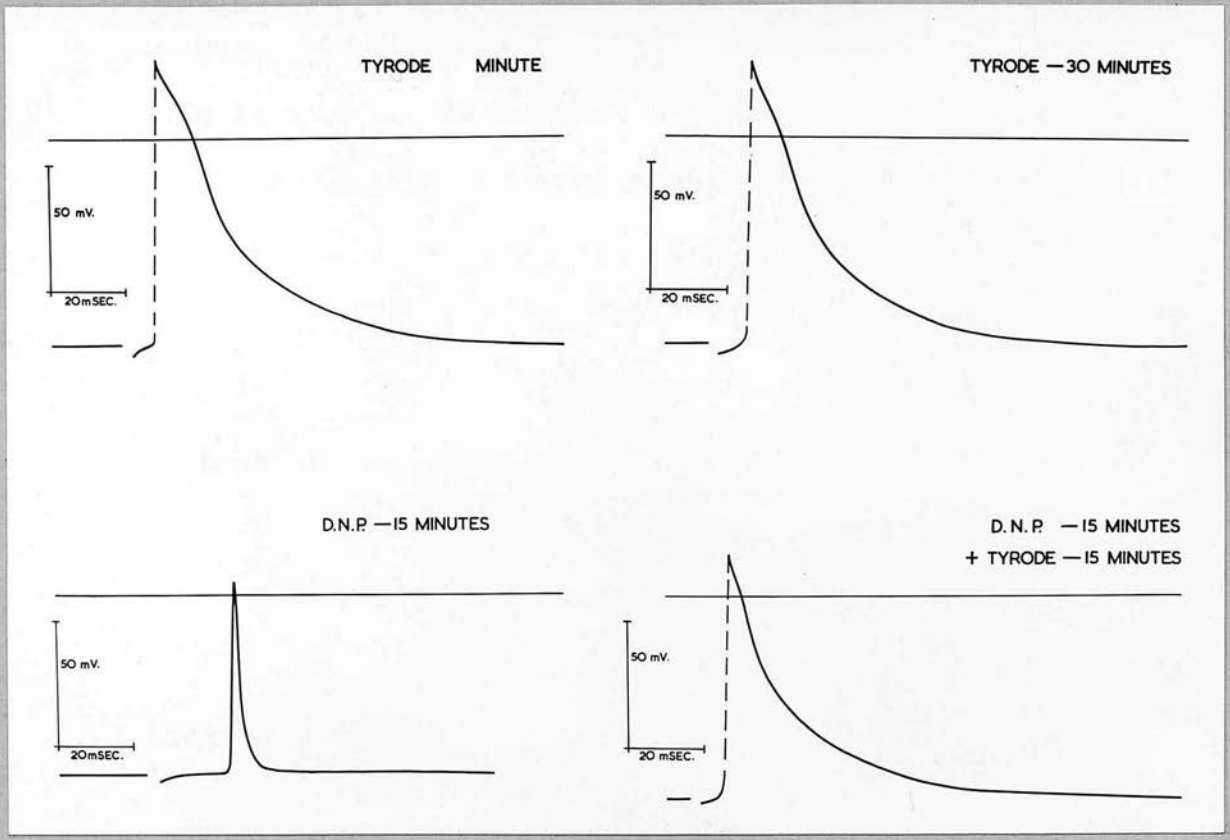



Fig. 4.

Changes in the values of the resting potentials, the overshoots and the durations of the action potentials of rat auricle on perfusion with Tyrode at 35°C. The values shewn are the means of all the figures obtained for each 6 minute period.




## Fig. 5

Typical action potentials (tracings) recorded from rat auricle after the treatments indicated (the first action potential was after 15 minutes Tyrode). The action potentials were chosen because they had the characteristics of the mean values in Table I.

been made. In Hercus et al. (1955), Reiter, (1956), Barclay et al. (1959), the sodium spaces of the latter recalculated to $(Na)_i$, using their sucrose space. The recalculated figures of Barclay et al. for $(Cl)_i$, are much higher than those found here. The decrease of $(K)_i$ with time was observed by Rayner and Weatherall (1957) and Goodford (1959) for rabbit auricle, and by Hercus et al. (1955) for rat ventricle; the latter workers also showed the $(Na)_i$ increase with time, when perfused.

2. Action Potentials.

In figure 4 are shown the values of the resting potentials, the overshoots and the durations of actions potentials recorded from auricles perfused with normal Tyrode. In table I are shown the actual values of these parameters at the times of the chemical analyses. In all the values of duration quoted in the paper (unless otherwise stated), the value of duration is that measured from the top of the action potential to 90% of repolarisation. Typical action potentials for the first few minutes of perfusion and after half an hour's perfusion, are shown in Fig. 5.

It can be seen that there is no change in the resting potential or the duration with perfusion, but there is a tendency for the overshoot to decrease.

## TABLE IV.

The ionic gradients and observed potentials of rat auricle after 15 minutes perfusion with Tyrode's solution.

| | Sodium | Potassium | Chloride |
|---|---|---|---|
| Ionic concentration in cells (m-equiv./kg.) | 35 | 139 | 43 |
| Ionic concentration in Tyrode (m-equiv./kg.) | 157.2 | 2.68 | 142.4 |
| Equilibrium potential (mV) | -40 ±3 | 105 ±0.5 | 32 ±3 mV |
| Resting potential (mV) | | 80 | |
| Overshoot (mV) | -32 | | |

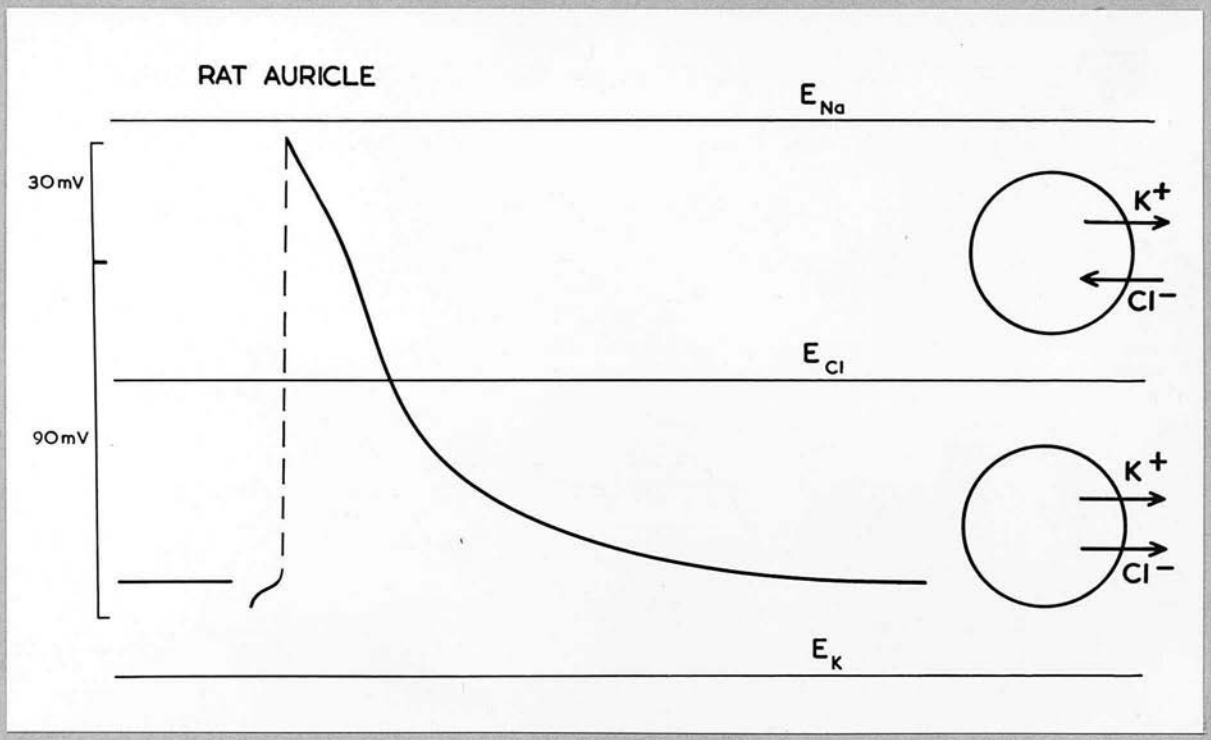



Fig. 6

Typical action potential from rat auricle after 15 minute's perfusion with Tyrode, with the equilibrium potentials for sodium, potassium and chloride measured at the same time, superimposed on it. To the right is shown the way K and Cl may move during repolarisation.

3. Comparison of Ionic Gradients and Action Potentials.

If an ion is passively distributed across a membrane, then the work done in transporting an ion across the membrane is zero (ie. the electrochemical gradient is zero). Such an ion, at equilibrium, ought to conform to the relation:-

$$\frac{(Na)_o}{(Na)_i} = \frac{(K)_o}{(K)_i} = \frac{(Cl)_i}{(Cl)_o} = \exp.(VF/RT) \quad \ldots\ldots\ldots(1)$$

where V is the internal potential, F is Faraday's constant, R is the gas constant and T the absolute temperature. At 35°C. this reduces to the form

$$E = 61 \log.\frac{(Na)_i}{(Na)_o} \text{ or } \frac{(K)_i}{(K)_o} \text{ or } \frac{(Cl)_o}{(Cl)_i} \quad \ldots\ldots\ldots(2)$$

E in this case is known as the equilibrium potential, because at this value of potential there is no tendency for a net movement of ions to occur.

It is now possible to determine if any of the ions conform to this equation and hence are passively distributed. In Table IV and in Fig. 6 are shown the calculated equilibrium potentials for Na, K and Cl and the resting potential and overshoot from rat auricle, after 15 minutes perfusion with Tyrode. From this data the following conclusions can be drawn:-

(a) Neither Na, K or Cl are passively distributed across the auricular cell membrane, (see Appendix I) and therefore their gradients have to be maintained by active

transport systems.

(b) The potassium equilibrium potential is greater than the resting potential and the sodium equilibrium potential greater than the overshoot. These facts are consistent with the hypothesis developed for squid nerve, that the resting potential is determined by the potassium ion and the upstroke and the overshoot by the sodium ion.

Table V shows the equilibrium potentials and the electrical data for auricles perfused with normal Tyrode for other times. It can be seen that the results are qualitatively the same as those discussed above. It may also be pointed out that there is a larger percentage change in $E_{Na}$ than in $E_K$ with perfusion, and that correspondingly the overshoot drops more than the resting potential with time of perfusion.

4. Further evidence that chloride is actively maintained in heart cells.

The new finding of interest is that heart cells contain more chloride than expected, and this will now be discussed in more detail. (see Appendix I).

Fig. 7 shows a cell with a resting potential of 80 mV suspended in a medium containing, in addition to the other ions, 142 m.equiv. of chloride. If the ion is in equilibrium and chloride moves freely through the membrane, apart from the constraint placed on it by virtue of its charge, then the inward

TABLE V.

The equilibrium potentials of sodium, potassium and chloride and the observed potentials in rat auricle, after perfusion with Tyrode's solution for various times. (± SE – wherever)

| Perfusion Time (minutes) | $E_{Na}$ (mV) | $E_K$ (mV) | $E_{Cl}$ (mV) | $E_m$ (mV) | O.S. (mV) |
|---|---|---|---|---|---|
| 1-2 | -37 ± 6 | 109 ± 0.5 | 29 | 80 | -28 |
| 15 | -40 ± 3 | 105 ± 0.5 | 32 ± 3 | 80 | -32 |
| 30 | -32 ± 2 | 104 ± 0.5 | 37 ± 3 | 81 | -27 |
| 50 | -28 | 100 | - | 80 | -21 |

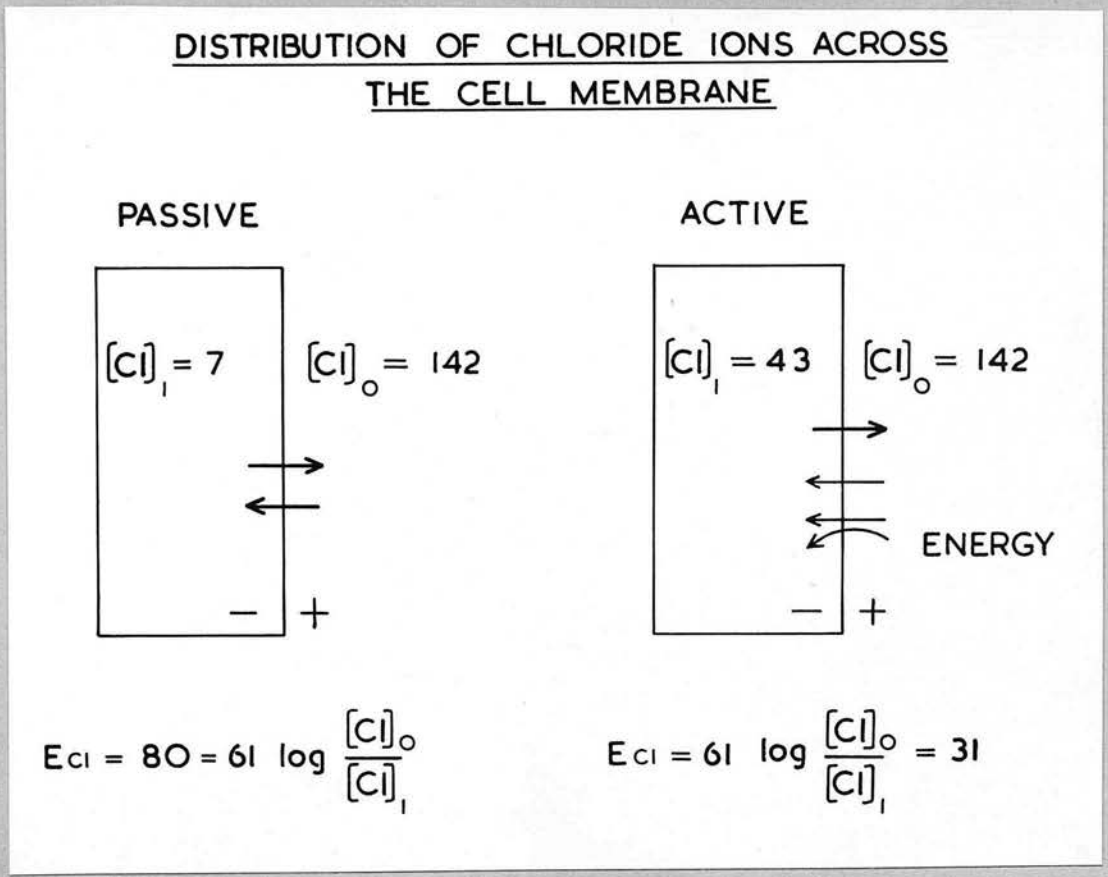



Fig. 7.

Schematic representation of 2 cells in which chloride is passively and actively distributed across the membrane. In passive distribution the passive fluxes are equal. In the active distribution the passive fluxes are unequal, and an additional inward transport of chloride is required. Below are shewn the Nernst equations with the equilibrium potentials for the 2 situations in them.

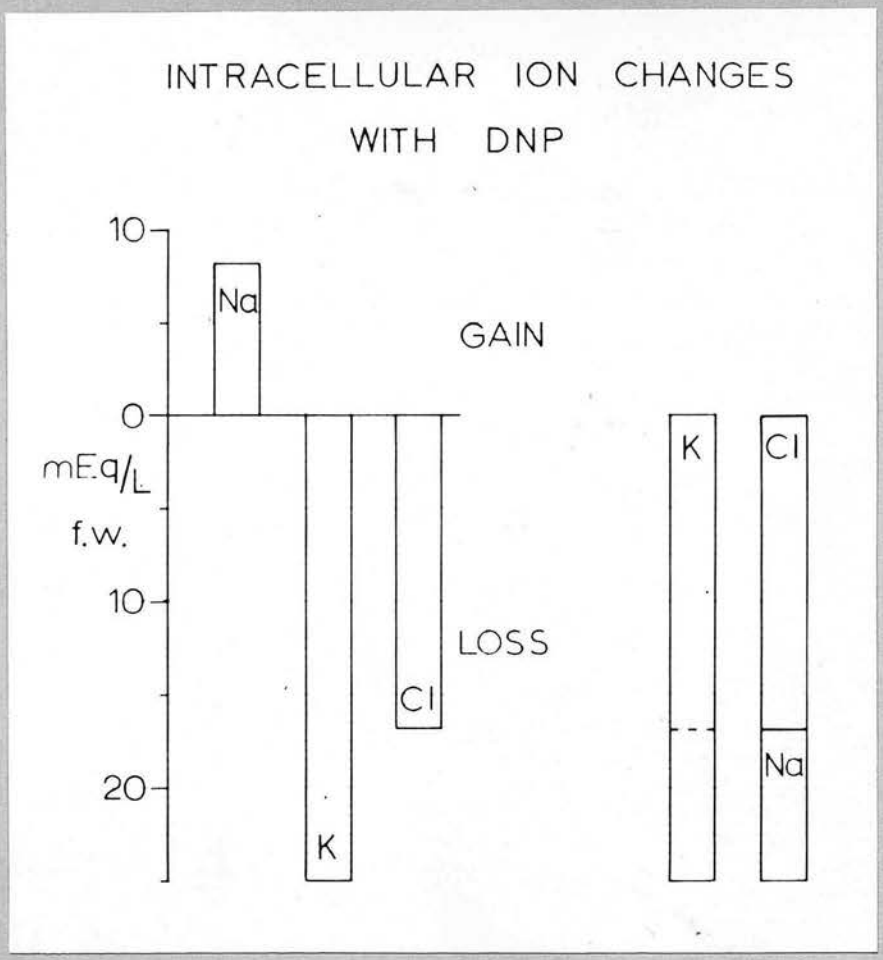



Fig. 8.

On the left are shewn the gains and losses of ions by the cells during 15 minutes treatment with DNP. On the right these have been fitted together to show that electrical neutrality is preserved. The cells thus exchange some K for some Na, and then lose KCl.

and outward fluxes are equal. From this condition the intracellular concentration of chloride can be calculated (assuming equal activities) from the Nerst Equation (2);

$$E_{Cl} = 61 \log. \frac{(Cl)_o}{(Cl)_i}$$

The result obtained is an intracellular value for chloride of 7 m.equiv./Kg. fibre water.(Fig. 7).

The situation actually found in these auricular cells by analysis, is a value of 43 m.equiv/Kg.fw. This then means that the passive fluxes are no longer in equilibrium (Fig. 7), the efflux exceeding the influx. To maintain this system in equilibrium active transport of chloride into the cells is required (Fig. 7).

A consequence of this distribution is that metabolic inhibitors ought to cause a loss of chloride, by stopping the active transport system. Similarily, any procedure which increases the $P_{Cl}$ also might be expected to lead to a loss of chloride. In Fig. 8 it is shown that DNP causes a fall of $(Cl)_i$ of 17 m.equiv/Kg.fw in 15 minutes ($p<0.001$), and so this supports the concept that chloride is actively maintained within these heart cells. In passing, it may be noted that this experiment does not in fact provide evidence that DNP does block the active transport of chloride, because an increase in $P_{Cl}$ would give the same result; it merely provides

good evidence that chloride is not passively distributed.

One possibility which was overlooked during the main body of this work was that the electrical activity of the action potential itself might lead to the accumulation of chloride by the cells, due to the fact that the membrane potential was not always at the resting potential. To test this possibility experiments were done with 3 pairs of auricles, perfused with an iodide Tyrode, one of each pair being driven at 60/minute and the other left quiescent. At the end of 30 - 40 minutes' perfusion the driven hearts contained 60.4 m-equiv/kg wet weight of iodide (actual values 58.2, 61.8 & 61.3), whereas the quiescent hearts contained 56.9 m-equiv/kg wet wt. (actual values 59.0, 52.3 & 59.3); a value not significantly different from the driven auricles. This conclusion is supported by the tracer experiments quoted below.

5. $Br^{82}$ efflux experiments.

Preliminary experiments have been done with quiescent rat auricle at 37°C, using $Br^{82}$ as a tracer for chloride. In these experiments the auricles were soaked in a $Br^{82}$ Tyrode for 30 minutes, and then the efflux of the $Br^{82}$ into normal Tyrode followed, by passing the auricles through a series of tubes containing inert Tyrode. The activity in each tube and that remaining in the auricle was then estimated by the gamma activity in a Scintillation Counter & scaler. In each experiment two auricles were

so treated, one in Tyrode and one in Tyrode containing 13 uM of DNP.

The results of the first ~~three~~ four such experiments show good agreement, and indicate that 1. the intracellular chloride in these auricles is substantially lower than calculated by direct analysis, and is 17·9 ± 1·2 (SE) m-equiv/kg fibre water†; 2. DNP causes this intracellular chloride to decrease to about the value expected for passive distribution ; 3. the amount of total chloride in the extracellular space is too high to be accomodated in the space available, at the concentration in the Tyrode; and 4. the efflux of chloride from the cells under DNP is some three to four times as fast as that from normal cells.

These observations seem to support the hypothesis that there is more chloride in the cells than can be accounted for on the basis of passive distribution around the membrane potential, and suggest that active transport is required to keep this extra chloride there. They also support the hypothesis that DNP increases the chloride permeability of the cells. These new observations in conjunction with the chemical analysis, raise the question of where the excess chloride in the tissue is being held. It seems possible to suppose that chloride is being bound in the extracellular space, in hearts immersed in Tyrode's solution, but clearly more experiments

† see appendix IV

## 6, The chloride transport system

On the assumption that there is some system which transports chloride into the cells to maintain the electrochemical gradient away from zero, a few experiments were done to try and characterise it.

In the first group of experiments the auricles were perfused with an Iodide Tyrode for 30 to 60 minutes, and it was found that the iodide content of the auricles was of the same order as the expected chloride content (58.8 ± 0.5 m-equiv/kg wet wt., n = 7).

In a second group of experiments the auricles were perfused with Tyrode's solution with no external potassium, and it was found that the chloride content declined to the same value as when treated with DNP (52.7 ± 0.7 m-equiv/kg wet wt.).

These results are consistent with the hypothesis that there are parallel 'pumps' at the heart cell membrane, one exchanging K for Na as in squid axon and one transporting KCl into the cell. More rigorous testing requires the use of tracers to determine the unidirectional fluxes.

7. The consequences on the action potential of this distribution of chloride.

In Fig. 6 is shown a normal action potential from rat auricle together with the equilibrium potentials for Na, K and Cl, after 15 minutes perfusion with normal Tyrode. To the right of the figure are shown the way in which the ions may move during repolarisation of the membrane during the action potential. Potassium can leave the fibre during the whole process, but chloride can only enter during the initial half of repolarisation if the ions are to move down their electrochemical gradients. This means that chloride can only contribute current to repolarise the membrane until $E_{Cl}$ is reached, and then will tend to hinder repolarisation (in effect KCl will leave the fibre).

A series of experiments were done to test this, by replacing chloride with the more permeable ion iodide (Hutter and Noble, 1959) or with the impermeable anion benzene sulphonate (P.Croghan personal communication); thus studying the effect of an increased or a decreased anion effect on the action potential.

(a) Large anion for chloride.

Representative action potentials from a typical experiment on rat auricle are shown in Fig.9. 9a shews a normal action potential recorded from the edge of the auricle, 13 minutes after being placed

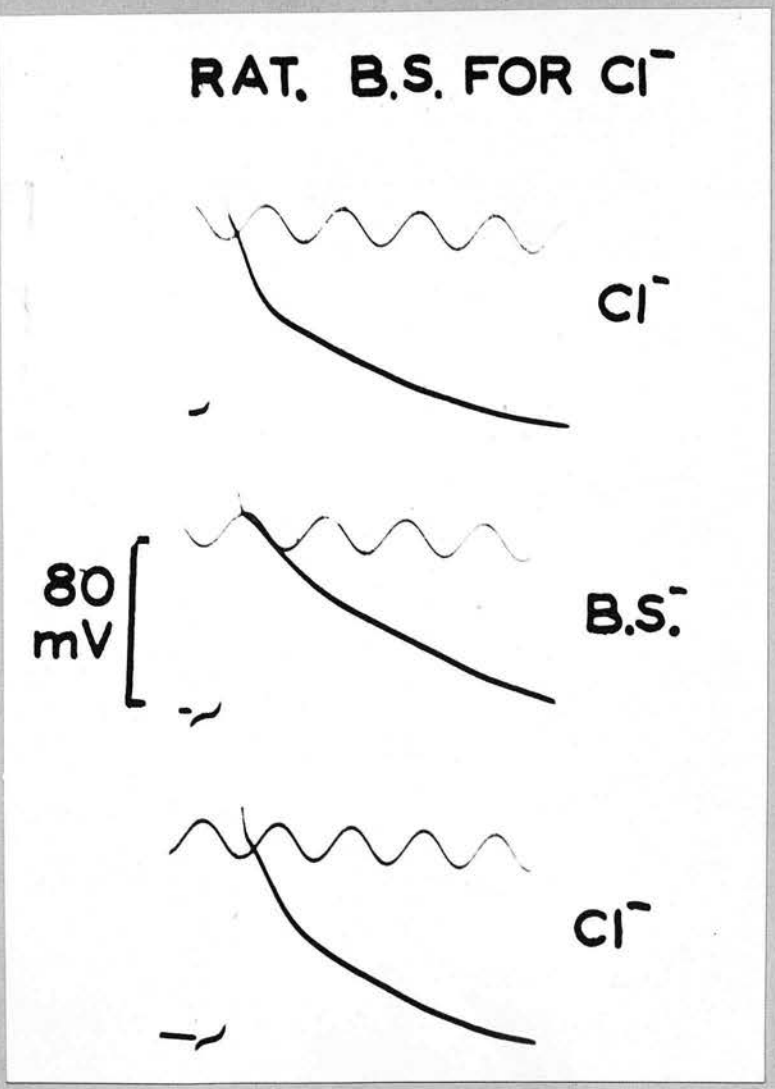



Fig. 9

Rat auricle, substitution of benzene sulphonate ion for chloride ion in perfusing Tyrode. Phase 2 of of the action potential slowed from 4.7 to 2.0 V/sec and recovers to 3.3 V/sec. Time interval between first and second record 35 minutes, and between second & third 5 minutes, all same area. Time scale 50 cps.

in normal Tyrode. Phase 2 of the action potential has a repolarisation rate of 4.7 V/second and ends with a marked inflection at about 30 mV; approximately the chloride equilibrium potential. Phase 3 of the action potential has a repolarisation rate of about 0.8 V/sec. for most of it's course.

Fig. 9b shows an action potential recorded from the same area some 35 minutes after replacing the normal Tyrode with a benzene sulphonate Tyrode. There has been a marked slowing of phase 2, which now has a repolarisation rate of 2 V/sec. and the inflection before phase 3 is almost gone. The rate of repolarisation of phase 3 is slightly greater, so that the overall effect has been to keep the total duration of the action potential the same.

Fig. 9c shows a record from the same area, after the auricle had been back in normal Tyrode for 5 minutes. The repolarisation rate of phase 2 is now increased to 3.3 V/sec. and the inflection has reappeared at 30-40 mV. Phase 3 is being repolarised at a faster rate than the other two, and the whole action potential is shorter.

In this experiment phase 1 of the action potential was not much affected by the removal of the chloride, but frequently this procedure led to a marked blunting of the whole top of the action potential (Fig.17c). The reason for this difference is not known.

(b) Iodide for chloride.

The effect of this substitution was most clearly seen in an experiment with sheep auricle, although similar but less marked results were obtained with rat auricle and ventricle.

Fig. 10 shows serial records removed from this experiment with sheep auricle. In 10a the auricle has been in normal Tyrode for 1 hour (driven at 60/minute), and a normal action potential results. In Fig. 10b the auricle has been in an iodide Tyrode for 20 minutes, with a marked change in the configuration of the initial part of repolarisation. Phase 2 of the action potential has now disappeared, phase 1 carrying on to an inflection at about 20 mV and then continuing at a slower rate of repolarisation in phase 3.

In Fig. 10c the auricle has been replaced in normal Tyrode , with the re-appearance of phase 2, and in 10d the auricle has once more been returned to an iodide Tyrode with the disappearance of phase 2 again. (10c & d are from the same cell, separated by two minutes. The Tyrode in 10d had the $(K)_o$ increased to 5.4 m.equiv/Kg.)

The actual rates of repolarisation of phase 2 are as follows; a, 0.25; b, 1.9; c, 0.35; and d, 1.6 volts per second. The overall duration of the action potentials during these procedures was not much altered.

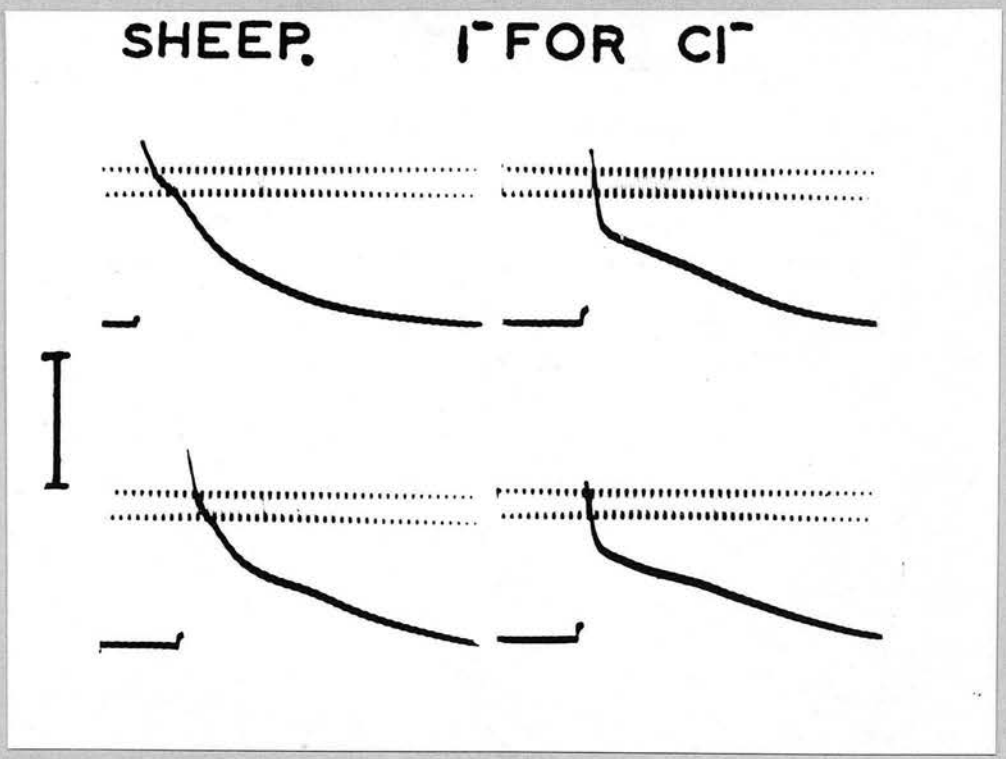



Fig. 10

Sheep auricle, substitution of iodide for chloride in the perfusing Tyrode. Top left (a) chloride, top right (b) iodide for 20 mins, bottom left (c) chloride for 5 mins, and bottom right (d) iodide for 2 minutes (same cell, $(K)_o$ increased to 5.4 m-equiv/l). Records obtained in that order in the same area. Time scale 50 cps. Voltage scale 80 mV.

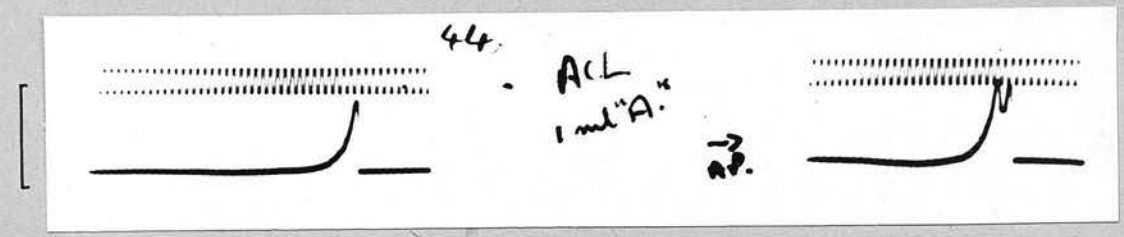



Fig. 11.

Sheep auricle, perfused with Tyrode with a $(K)_o$ of 5.4 m-equiv/l, and in which half the chloride was replaced by iodide. Left, stimulus artefact only, auricle inexcitable. Right, after addition of ACh. (to a final concentration in bath of about $5 \times 10^{-3}$ moles) a small action potential appeared. Time, 50cps. Voltage, 80 mV.

In the experiments with rat auricle and ventricle it was found that (contrary to that of Hutter and Noble, 1959) replacing the chloride in the Tyrode with iodide never produced inexcitability. It was thought that this was due to (a) using a heart from a small animal where the $P_K/P_{Cl}$ ratio was probably higher than in the sheep and calf hearts used by Hutter and Noble and (b) using a Tyrode with a low external potassium, so that the potassium gradient was higher than normal.

These points were investigated by perfusing a rat auricle with Tyrode solution containing various levels of potassium, and also increasing the external potassium in the Tyrode perfusing the sheep auricle shown in Fig. 10.

In the rat the $(K)_o$ had to be increased to 10.8 m.equivs. before the auricle became inexcitable (at an $E_m$ of 48 mV), whereas the sheep auricle became inexcitable at an external potassium of 5.4 m.equiv. This would seem to provide some support for the suggestions made above.

In the sheep it was found that the heart became inexcitable with a $(K)_o$ of 5.4 m.equivs. and only half of the chloride replaced with iodide and under these conditions some excitability returned when ACh. ($10^{-3}$M.) was added to the bath. Fig. 11 shows the poor action potentials under these circumstances, presumably due to the increased $P_K$

TABLE VI.

Intracellular ionic levels in rat auricle treated with 2-4 dinitrophenol (13 μ-mole). Results are expressed as m-equiv./kg. fibre water.

| Perfusion Time (minutes) | Sodium | Potassium | Chloride | Sodium + Potassium |
|---|---|---|---|---|
| 15 DNP | 43.0 | 114 | 26.4 | 157.0 |
| 15 DNP + 15 Tyrode | 52.0 ± 7 | 133 ± 5·7 | 23.9 | 185.0 |

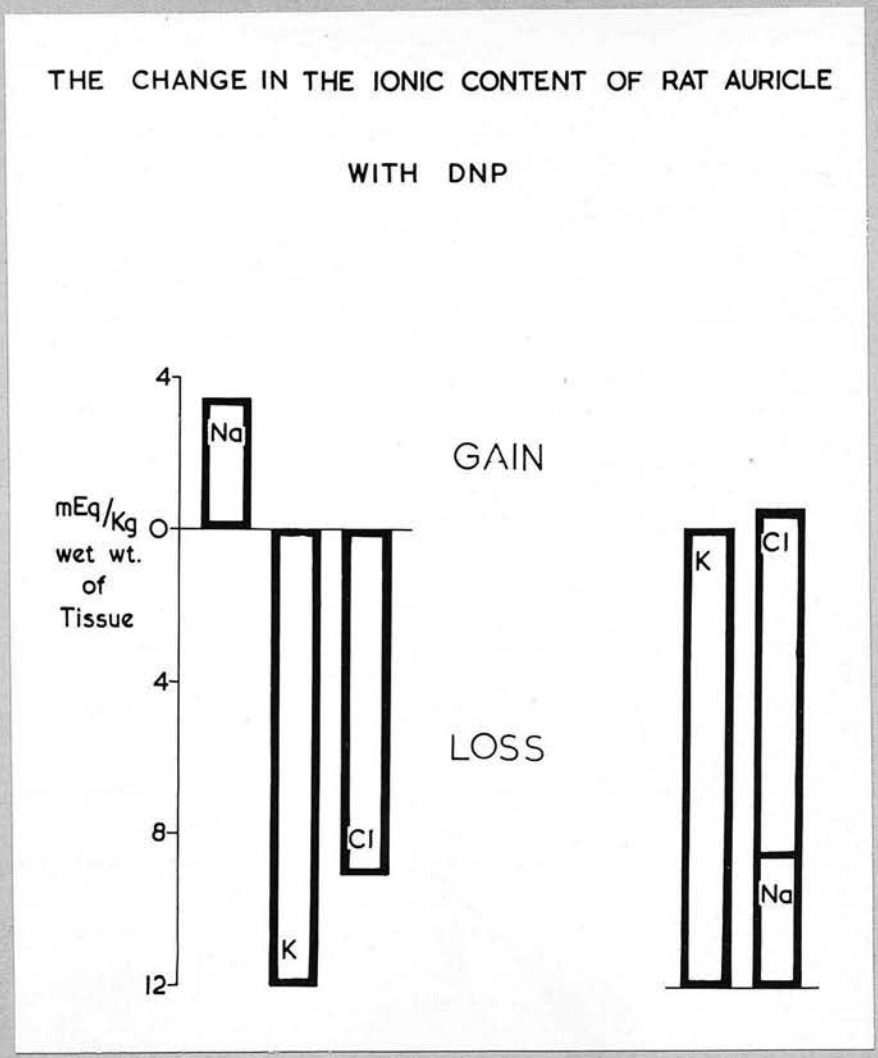



Fig. 12.

On the left is shown the effect of 15 minutes DNP. treatment on the ionic content of rat auricle. On the right these changes have been fitted together to show that electrical neutrality is preserved.

which ACh is known to produce (see Hutter, 1957, for references).

The results in this section may be summarised by saying that the effects of alteration in the anions is most marked in phase 2 of the cardiac action potential. That this coincides with the p period of low permeability of the action potential is discussed later.

(B) The results in auricles treated with DNP.

1. Ionic levels.

The intracellular levels of the ions are shown in Table VI, after 15 minutes of DNP treatment and also after 15 minutes of DNP and 15 minutes of recovery in Tyrode. By comparison with Table III it can be seen that DNP has produced a drop of $(K)_i$ of 25 ($p < 0.05$), a drop of $(Cl)_i$ of 16.8 ($p < 0.001$) and a rise of $(Na)_i$ of 8.1 m.equiv/Kgfw (Na change not significant at 5% level).

Therefore the overall affect of DNP on the ionic levels in the cells is to cause a loss of 17 m.equivs of KCl and an exchange of 8 m.equivs of K for 8 m.equivs of Na per Kg. fibre water. This is shewn in Fig. 12 as changes per Kg. wet weight and in Fig. 8 as changes per Kg. fibre water.

After 15 minutes of recovery in normal Tyrode after DNP the $(K)_i$ level has returned to normal, whereas the $(Na)_1$ level has shown a further increase

(all probabilities calculated on a wet wt. basis)

TABLE VII.

Sodium content of rat auricles washed with choline chloride for 6 minutes at 6°C. Column 1 indicates the initial treatment of the auricles before the choline washing and also (in brackets) the number of auricles treated.
T indicates Tyrode perfusion, and DNP, Tyrode + DNP perfusion.

| Treatment (minutes) | Sodium content m-equiv./kg. wet wt. | m-equiv./kg. dry wt. |
|---|---|---|
| 1-3 T (8) | 20.0 | 108.5 |
| 30 T (3) | 21.8 | 113 |
| 53.5 T (2) | 23.6 | 128 |
| 13 DNP (3) | 27.5 | 134 |
| 13 DNP + 15 Tyrode (3) | 28.8 | 139 |

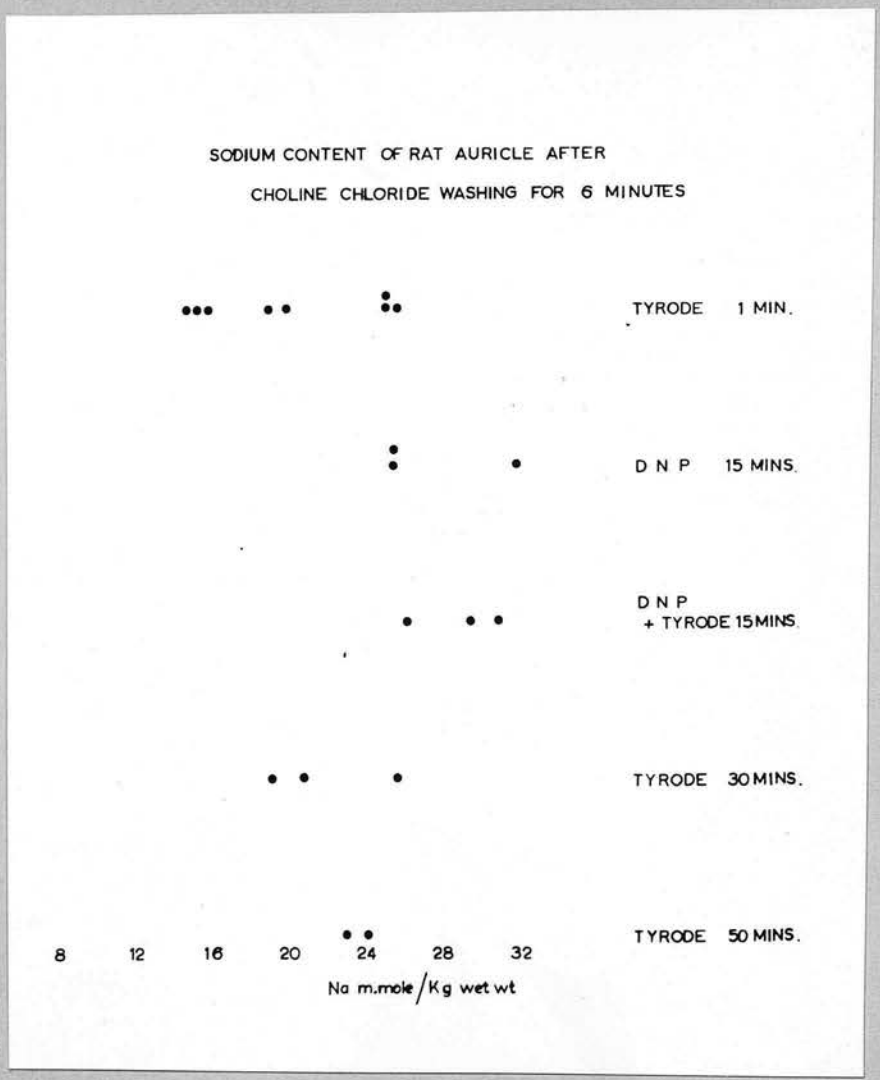



Fig. 13

Each spot represents one auricle. Each auricle was treated as indicated on the right of the figure and then perfused with choline chloride at 6°C. for 6 minutes and finally analysed for the residual sodium content.

and the $(Cl)_i$ level a further decrease.

Under DNP the sum of the main cations appears to approach that of the perfusing Tyrode, from the higher value found within the cells normally.

At the time of doing these experiments it was considered important to make sure that $(Na)_i$ was changing in the way indicated in Table VI, and not for example decreasing again after recovery from DNP. To check this point a further series of experiments were carried out. In these each auricle, after the usual perfusion treatment, was washed with a choline chloride solution at $6^{\circ}C.$ for 6 minutes to remove a large part of the extracellular sodium and therefore to make any intracellular changes more obvious. The results of these experiments are shown in Table VII and Fig. 13, in which the sodium content of each auricle in m.equivs/Kg. wet weight is plotted against the treatment. The same sodium trend appears as in the main table, and so it is considered that $(Na)_i$ is behaving as in Table VI under the various treatments.

2.Electrical events.

In Fig. 14 is shown the mean values for the resting potentials, the overshoots and the duration of all the action potentials recorded from the experiments with DNP. As the time for shortening varied between experiment to experiment, the durations of all the experiments have been readjusted to the

overall mean of 15 minutes. Thus the time to reach 50% shortening has been readjusted to 7½ minutes irrespective of the actual duration of the experiment (the decrease in the duration is linear with time).

The main points presented in this graph are:-

(1) When treated with DNP the resting potential declines steadily from 80 mV to 70 mV, the duration decreases steadily from 55 msec. to 6 msec. and the overshoot falls from 28 mV to zero. All these changes are significant at the 2% level.

(2) On removal of the DNP the resting potential continues to decline for a few minutes and then recovers to 78 mV. The overshoot returns to 20 mv and the duration to 35 msec. These changes are also all significant at the 2% level. ( the level of significance between the DNP level of the $E_m$ and the recovered level of the $E_m$ is only 10%, but in each of 8 experiments the $E_m$ dropped under DNP and then recovered after removal of DNP).

In Fig. 5 are shown representative action potentials recorded from the auricles during these experiments (tracings made from enlargements) at the various times indicated. These action potentials were selected as having the mean measurements shewn in Table I. These pictures merely emphasise the profound shortening produced by DNP, with the concomitant slowing of the upstroke velocity and slowing of the conduction velocity.

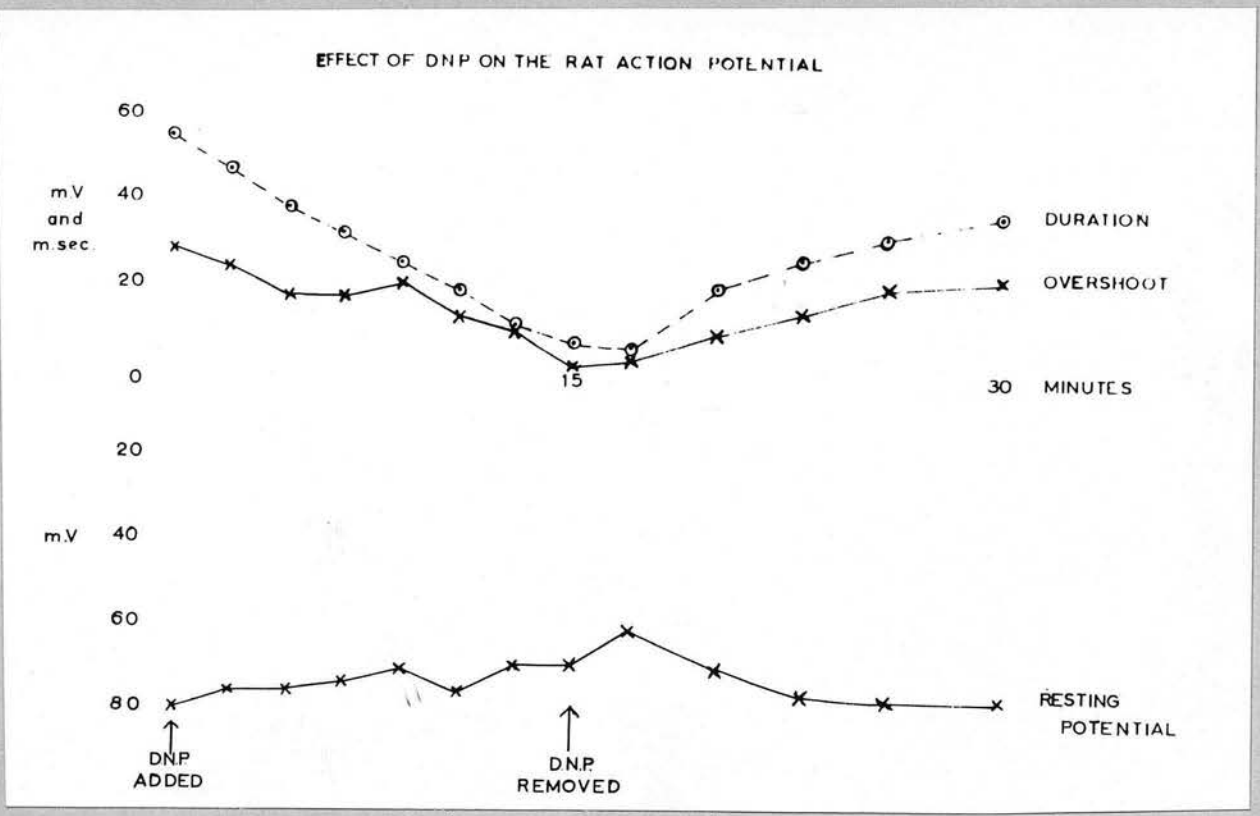



Fig. 14.

Changes in the resting potentials, the overshoots & the duration of action potentials of rat auricle, under DNP treatment and recovery. The points on the graph are the means of all the experiments recorded. During DNP treatment the points are the means over 2 minute intervals, and during recovery over 3 minute intervals.

TABLE VIII.

Changes in intracellular ionic concentrations and action potentials with 2-4 dinitrophenol treatment, to test Shanes' hypothesis. Potential changes are indicated as towards (-) or away from (+) zero potential across the membrane.

| | Sodium | Potassium | Chloride |
|---|---|---|---|
| Change in intracellular ionic concentration (m-equiv./kg. fibre water) | +8 | -25 | -17 |
| Change in equilibrium potential (mV) | -5 | -5 | +13 |
| Change in overshoot (mV) | -32 | | |
| Change in resting potential (mV) | | -10 | |

DNP causes a shortening of the action potential from 45 to 6 m.sec. (measured at 90% repolarisation).

3. Test of Shanes' hypothesis for the action of DNP.

This hypothesis supposed that the effect of DNP would be to cause an important increase in $(Na)_i$ before $(K)_i$ had decreased sufficiently to cause an appreciable drop in $E_m$; and thus to decrease the sodium current during repolarisation of the membrane and so to shorten the action potential.

In Table VIII the relevant data to test this hypothesis is presented. It is clear that the gain in sodium by the cells is much less than the loss of potassium, leading to equal changes in the equilibrium potentials of sodium and potassium. Also, after DNP the action potential recovers to a considerable extent although $E_{Na}$ has decreased further ( and hence should have led to a greater shortening on Shanes' theory) while the $E_K$ has returned to normal.

Therefore it can be concluded that this theory is probably incorrect.

Another possibility which was considered (Lamb, 1959), was that the $E_K$ drop by itself might be sufficient to shorten the action potential; the increase of $E_K$ after the DNP then causing the lengthening of the action potential during recovery. The basis of this attempt was the finding by Weidmann (1956) that if the $E_K$ is dropped by an increase in the external potassium, then the action potential shortens and the $E_m$ drops. Quantitatively

however, this hypothesis is untenable for Weidmann found that the $E_K$ had to be dropped by about 50 mV to produce the effect, compared to the drop in the $E_K$ with DNP of only 5 mV. This was checked directly in these auricles in one experiment in which $E_K$ was decreased by 5 mV, by an increase in the $(K)_o$ from 2.7 to 3.3 m.equivs/litre. Also, of course, it is well known that $(K)_i$ decreases with time of perfusion and yet produces no shortening of the action potential.(Table I).

Other puzzling features shown in Table VIIIare

a. a drop of $E_m$ of 10 mV with DNP when $E_K$ only dropped by 5 mV and $P_K$ was probably increased (Weatherall, 1959).

b. the enormous drop in the overshoot from -32 to 0 mV, with only a 5 mV change in the $E_{Na}$ and a small increase in the $P_K$.

4. Chloride hypothesis for the action of DNP.

As discussed in the introduction this impasse was surmounted when it was realised (a) that chloride was actively maintained within the cells and (b) that under these conditions an increase in the $P_{Cl}$ would resolve most of the difficulties.

Electrical experiments were then performed to test this hypothesis as best as possible with the means at hand. The following results were obtained;

(a) An auricle treated with DNP in normal Tyrode for about an hour was placed in a benzene sulphonate

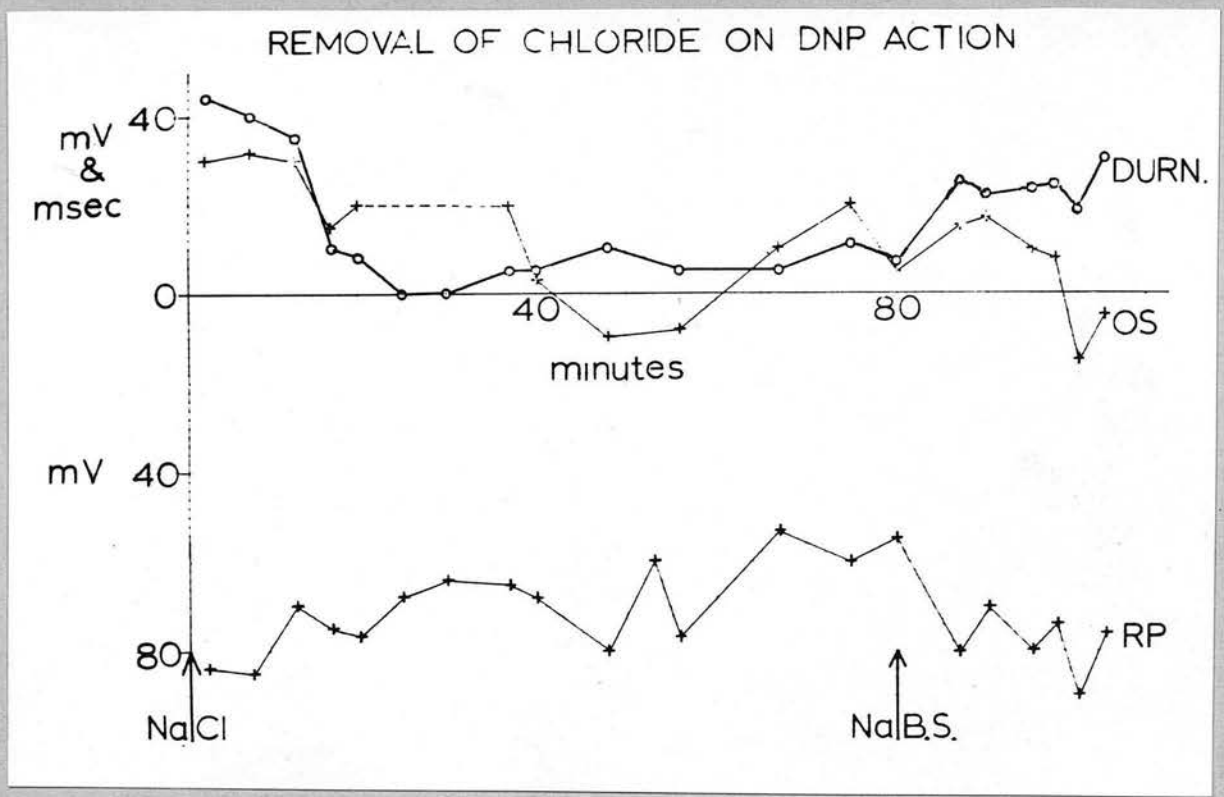



Fig. 15

In this experiment the auricle was placed in a Tyrode solution cotaining 13 mole of DNP at zero time. This caused a progressive fall in the OS & the duration and in the resting potential. When the chloride in the Tyrode was replaced by benzene sulphonate (with the same amount of DNP present) there was an increase in the duration of the action potentials and the resting potentials returned to 80 mV.

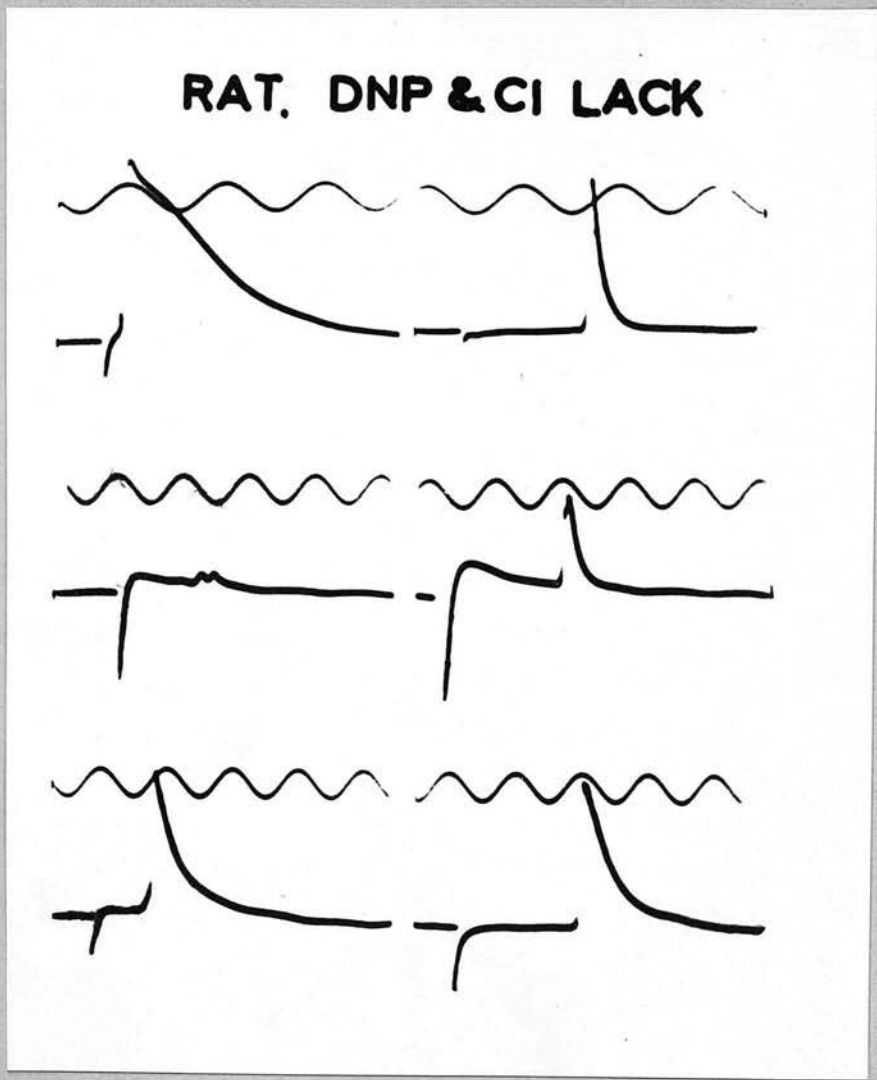



Fig. 16

Representative action potentials from the experiment shown in Fig. 15. Top (a & b), normal action potential & shortened A.P. in DNP Tyrode. Middle (c & d), poor A.P.s after DNP treatment for 30-60 mins. Bottom (e & f), typical A.P.s after chloride in Tyrode replaced by benzene sulphonate with same DNP content. Time scale, 50 cps. (note change in sweep velocity between top and other rows). Voltage, 80 mV.

Tyrode containing DNP (same conc.). The action potential recovered from it's shortened state of about 6 msec and $E_m$ of 70 mV, to a duration of about 20 msec and an $E_m$ of 80-90 mV. Fig. 15 shows the graph produced from this experiment, and Fig. 16 representative action potentials taken from the film (note the change in time scale between the first two action potentials and the rest). The action potentials shortened from 45 msec to 6 msec (a & b) with DNP and remained about this level for the next hour. Over a large part of the auricle (central region) no action potentials could be obtained (c) with rather poor action potentials near the edges (d). At 80 minutes the NaCl in the Tyrode was replaced by benzene sulphonate and in a few minutes† action potentials could be obtained all over the heart, and these action potentials had a duration of about 20 msec. By comparison of the stimulus artefacts (retouched) in 16 c and d, with e and f it can be seen that the excitability has increased as well. (See also Fig 31)

(b) Auricles treated with DNP in a benzene sulphonate or methyl sulphate Tyrode, only shortened to 20 msec with no decrease in the excitability and (usually) no fall in the resting potential (Fig. 17 e & f).

(c) Similar results were obtained with rat ventrcle or auricle with $N_2$ and $CO_2$ (Fig. 17d).

† no records were taken : the first few minutes

(d) In one auricle perfused with a benzene sulphonate Tyrode for an hour, the action potential shortened as usual to about 20-25 msec. On changing to DNP in normal Tyrode the heart rapidly became inexcitable, with poor action potentials. (Unfortunately no photographs of the end of this experiment were obtained.)

At this stage it was considered that sufficient evidence of this nature had been obtained to support the hypothesis, and that more conclusive evidence would be sought later by the use of appropriate tracers.

5. Calculation of the increase of $P_{Cl}$ necessary to account for the effect.

If this is the correct explanation of the action of DNP, then a rough idea of the order of the increase in $P_{Cl}$ can be obtained by calculation on the Goldman equation. By substitution of the values of $E_m$, $(K)_i$ and $(Cl)_i$ after 15 minutes of DNP treatment (and assuming that sodium contributes little to the membrane potential, as before) then;

$$70 = 61 \log. \frac{114\ P_K + 142\ P_{Cl}}{2.7\ P_K + 26\ P_{Cl}}$$

which gives a ratio of $P_K/P_{Cl}$ of 3 to 1.

If there is an increase of $P_K$ of 30%, as found by Weatherall (1959) for the rabbit, then the true ratio of $P_{Cl}$ with the resting $P_{Cl}$ is 4 to 1. This means that the effects of DNP on rat auricle can be explained by an increase of $P_K$ of 30%, & of $P_{Cl}$ of 400%.

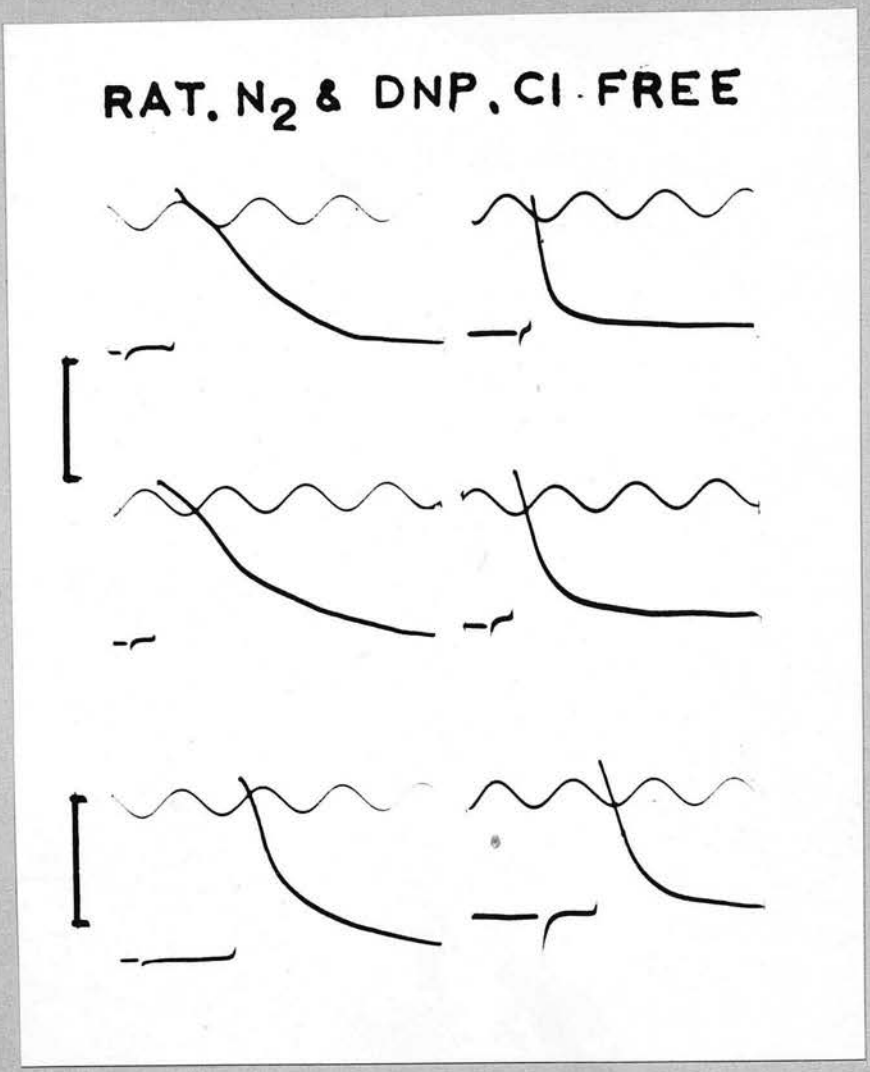



Fig. 17

Top (a & b), normal and DNP treated rat ventricle. Middle (c & d), same ventricle in benzene sulphonate Tyrode, with $O_2$ and with $N_2$ presnet. Bottom (e & f), rat auricle in DNP for 1 hour with methyl sulphate and benzene sulphonate respectively substituted for the chloride. Time scale, 50 cps. Voltage, 80 mV.

## DISCUSSION.

### 1. The measurement of the extracellular space.

In single celled tissues estimations of the intracellular concentrations of the ions can be made directly by (1) expressing the axoplasm (squid) and analysis, or by (2) analysis of the whole tissue (single muscle fibres, Shaw, 1955). The main problem in these tissues is one of the small amount of the material available.

In multicellular tissues, on the other hand, the main difficulty is that some correction has always to be applied for the ions held in the spaces between the cells. To do this some measure of the intercellular space has to be made, and an assumption about the concentrations of the ions in this space made.

The ideal way of measuring this space would be to add a substance to the perfusate which would diffuse quickly throughout the extracellular space, but would not enter the cells the extracellular fat. No such substance exists, and consequently all such methods suffer from the disadvantage that the results vary with the substance used, and also with the time of perfusion of the substance.(Manery, 1954)

In the narrow channels of the extracellular space it is likely that the physical dimensions of the foreign molecules used, are of importance for purely mechanical reasons. Thus, of the saccharides

used for the estimation of the extracellular space, it has been found that those with small molecules such as sucrose and rafinose produce consistently higher spaces than large molecules such as inulin. (Barclay, et al. 1959; Fisher,R.B., personal communication). It is reasonable to suppose that these larger spaces are more nearly the true ones.

The other difficulty, that of the length of perfusion, does not appear to be so serious, as Johnson (1957) showed that the sucrose space of frog heart stayed constant from 5 to 120 minutes of perfusion.

On the whole then, it seems reasonable to assume that the space measured with sucrose after 20 minutes perfusion, approximates to the true extracellular space.

The other main method for the measurement of the extracellular space, is to make use of the fact that ions diffuse from between the cells faster than they cross the cell membranes, and so efflux curves from multicellular tissues have two main components representative of these two different kinds of ion movement.

This method is only practicable if the time to cross the membrane is slow compared to the time to diffuse from between the cells, thus leading to easily distinguishable components on the efflux curve. As the $Q_{10}$ of membrane permeability is

greater than that of free diffusion, a lowering of the temperature helps the situation by slowing membrane diffusion (Shanes, 1957, and personal communication). In the analysis of such a curve, account must be taken of the complex situation at the membrane and a suitable correction applied. This correction has been worked out for the sodium ion by Dainty (Dainty and Krnjevic, 1955 and personal communication).

The close agreement of the results obtained by these two methods, around 25%, suggests that the true value of the extracellular space is around this level; and also suggests that the two methods used are reliable indices of the extracellular space by themselves.

Manery (1954) quotes figures both for the theoretical distribution of electrolytes between plasma and the extracellular space, and for those experimentally determined. These corrections are of the order of 3% of the total ions in the extracellular space. Tt was felt that as these corrections were worked out (a) for skeletal muscle and (b) using plasma or blood and not the Tyrode used here, it was not worthwhile to apply these corrections in this analysis; so that the calculations were done on the assumption that ions in the plasma and the extracellular space were at the same concentration.

2. Normal auricles.

It has been shown that under all conditions studied the $E_K$ was greater than the $E_m$, and the $E_{Na}$ greater than the overshoot. These results are in accord with the ionic hypothesis developed for the squid axon, that is the ionic gradient for potassium is sufficient to account for the resting potential being a state in which the potassium ion is dominating the membrane and the sodium gradient is sufficient to account for the action potential as being a state in which the membrane approaches the condition of being dominated by the sodium ion.

The results for sodium and potassium differ from those in the squid in that (a) the $E_K$ is far removed from the $E_m$ and it is not possible to hold the view that potassium is passively distributed about the resting potential; and (b) the sodium gradient is much less than in the squid of in sartorius (Conway, 1957) due to a higher intracellular level of sodium. This lower sodium gradient may be related to the slower rate of rise of the cardiac action potential, than in squid or sartorius.

The main new evidence about ions in the heart is that the present evidence suggests very strongly that chloride is actively maintained within the cells, so that the chloride equilibrium potential is far removed from the resting potential. Consistent with this view is the work of Hutter and Noble (1959)

showing that chloride contributes little to the resting permeability of the membrane, for if the transport number of chloride was twice that of potassium as in frog skeletal muscle (Hodgkin and Horowicz, 1959), then the $E_{Cl}$ would dominate the membrane potential instead of the $E_K$. This effect can be shewn if the chloride is replaced by the more permeable anion iodide. The actual value of the contribution of the chloride ion to the resting permeability calculated from the present results, shows a satisfactory agreement with that obtained by Hutter & Noble by a different method.

This situation in the heart is in marked contrast to that found in most tissues so far investigated. Thus in frog skeletal muscle both analytical data (Conway, 1957) and electrical data (Hodgkin & Horowicz, 1959) indicates that chloride is passively distributed around the resting potential with a high permeability. A similar situation appears to occur in the skeletal muscle of carcinus (Shaw, 1955), in red cells (see Davson, 1959 for references), in squid axon (Hodgkin, 1958 p 3). In motoneurones (Eccles, 1959) the results obtained have been interpreted to mean that the $E_K$ is at 90 mV, and the $E_{Cl}$ coincident with the $E_m$ at 70 mV. For a discussion of results in myelinated nerve fibres see the Appendix.

There is evidence for active transport of

chloride in the salivary glands (Lundberg, 1958), in the gastric mucosa (Hogben, 1951) and at the tonoplast membrane in Nitella (MacRobbie & Dainty, 1958). In the frog skin chloride movements are usually passive (see Davson, 1959 for references & a general discussion on frog skin) but when frogs are depleted of chloride, active uptake of this ion occurs (Jorgensen, Levi & Zerahn, 1954).

Some of the consequences of this distribution of chloride in the heart will now be discussed:-

(1) At rest the $E_m$ will be determined by the equilibrium potentials and the relative permeabilities of the potassium and the chloride ions. This serves to explain the wide divergence of the $E_m$ from the $E_K$ in this tissue at rest, and the fact that replacement of the chloride in the Tyrode by a large anion usually leads to an increase in the $E_m$ (Hutter and Noble, 1959; and personal observations). From the results of Draper and Weidmann (1951),(in which the NaCl in the Tyrode was replaced by saccharose) it appears that in Purkinje's tissue the situation may be different, for removal of Na and Cl from the medium led to no consistent change in the $E_m$.

(2) During the action potential chloride is only available to carry current to repolarise the membrane until the chloride equilibrium potential is reached. Quantitatively it has been shown (Fig. 9) that the amount of current carried during this

part of the action potential by chloride, is more important than might have been expected from it's resting permeability of 10% of the total. A probable explanation of this is as follows; phase 2 of the action potential was shown by Weidmann (1951) to be a period of low permeability in Purkinje's tissue (P reduced to a third of normal) and this probably applies to action potentials form the rest of the heart. Most of this reduction in permeability must be due to a change in $P_K$ and if it is assumed that $P_{Cl}$ is unchanged then, during this period $P_{Cl}$ and $P_K$ will become comparable in value so that any alteration in the chloride contribution will have a marked effect.

In Fig. 9 the rate of repolarisation of phase 2 with chloride present is 4.7 V/sec and without chloride is 2 V/sec. If it is assumed that there are no interactions when chloride is removed and that the effect of sodium is negligible, then the $P_{Cl}$ is 1.4 times the $P_K$ at this period of the action potential. This means that if $P_{Cl}$ has remained unchanged, then $P_K$ has been reduced to about a tenth of it's resting value during phase 2 of the action potential.

(3) During the latter part of the action potential (phase 3) efflux of KCl will occur and tend to slow the repolarisation rate, so that chloride may contribute directly to the prolonged time course of the action

potential. This hypothesis is attractive in that it could explain the fact that the time course of repolarisation of phase 3 of the action potential is much slower than can be accounted for on the time constant of the membrane at the same time. (When measuring the time constant of the membrane the ions in the membrane carry current in a direction appropriate to their charge, so that this gives a measure of the total permeability of the membrane; however during the action potential the ions in the membrane show a net movement down their electrochemical gradients.)

This hypothesis cannot however be pressed, for although removal of most of the chloride (95%) from the Tyrode usually leads to some increase of the rate of repolarisation, this increase appears to be insufficient to explain all the slowing. This need not exclude the hypothesis altogether, for removal of chloride certainly leads to a gross change in the initial part of repolarisation and may well upset the normal permeability relationships very severely. It would probably be desirable to repeat the polarisation experiments in the complete absence of chlofide, recording action potentials at the same time, to obtain comparable figures for the time constant of the membrane and the rate of repolarisation of the action potential in the same cells. Although not known for certain, it seems likely that

the short term experiments for chloride depletion done by Hutter and Noble (1959) might give misleading results. This is because it is easiest to impale a fibre, measure the time constant of the membrane, change the solution and measure it again. There is normally so much chloride in the cells however, that perhaps half an hour is required to deplete them of it and it is usually not possible to stay in the same cell for this length of time. Moving to another cell complicates matters by introducing another variable.

(4) One experiment was carried out with the right auricle of a rat, in which the chloride content was reduced by soaking the auricle in a benzene sulphonate Tyrode for one hour. At the end of this time the auricle was spontaneously active at a rate of 260/minute. A large quantity of adrenaline (c. 1 mg.) was then added to the bath. This produced an enormous increase in the vigour of the beat but only increased the rate to 330/minute. This may mean that adrenaline and ACh act on the pacemaker of the heart by varying the rate of change of the prepotential slope to or from the equilibrium potentials of chloride and potassium.

(5) Finally it should be pointed out that this distribution of chloride is important in a negative way. If chloride were passively distributed around the $E_m$ with a high permeability, it would not be

possible for heart cells to have the prolonged action potentials found, unless there was a decrease in the chloride permeability during the action potential.

The main experimental findings on the cardiac action potential are as follows; (those concerned with the $E_m$ only have been omitted. The resistance measurements are from Weidmann, 1951 and were made on Purkinje's tissue)

(a) The upstroke (phase 0) is a period of low resistance, is dependent on the external sodium level (Draper & Weidmann, 1951; Délèze, 1959) and is a period of rapid rate of change of the membrane potential.

(b) The height of the overshoot is related to the sodium gradient, and varies with the logarithm of the external sodium according to the Nerst equation (Draper & Weidmann, 1951).

(c) The initial part of repolarisation (phase 1) starts as a region of low resistance, rapidly returns to a normal resistance with a rate of repolarisation comparable to the normal time constant of the membrane, and ends (phase 2) as a period of high resistance with a very slow rate of repolarisation. During phase 2 the anion present in the perfusate has a marked influence on the rate of repolarisation suggesting that anion and potassium permeabilities are of the same order.

(d) During the later part of repolarisation (phase 3), the membrane resistance is normal (Weidmann, 1951; & results Part III), there is an efflux of potassium (Wilde, 1957) and the rate of change of the membrane potential is less than expected for the time constant of the membrane.

These findings support the hypothesis that the cardiac action potential occurs due to the following sequence of events:-

(1) There is an initial increase of $P_{Na}$, of a smaller order than that found in squid axon, leading to the upstroke of the action potential and moving the membrane potential towards $E_{Na}$. Inactivation of $P_{Na}$ then occurs as in squid.

(2) The membrane potential then returns towards the resting level due to K and Cl movements down their electrochemical gradients at their resting permeability values, this constituting phase 1.

(3) A decrease in the potassium permeability starts, leading to a slowing of the repolarisation rate (phase 2), a higher membrane resistance and a situation where alterations in the anion contribution to repolarisation can have a marked influence.

(4) The potassium permeability starts to return to normal again leading to a more rapid rate of repolarisation, but as chloride also now leaves the cells with potassium the rate of repolarisation never reaches that to be expected from the time constant of the membrane.

This hypothesis differs from that proposed by Shanes (1958) only in that there is no continuation of an increased sodium permeability into the repolarisation part of the action potential. The main difficulty about this theory, is that it is more difficult to account for the effect of alteration of external sodium on the duration of the action potential (Draper & Weidmann, 1951; Déléze, 1959) than on Shanes' theory. One possible loophole is that the effects of sodium changes are secondary to changes in the upstroke or the overshoot.

If the heart action potential can be explained on a permeability basis, then it seems likely that species differences are due to (a) alterations in the permeabilities of the basic ions concerned and (b) differences in the ratios of the permeabilities of the ions concerned. The simplest hypothesis which might cover this situation is to suppose that, given there is a basic series of permeability events in the heart action potential, the longer the action potential the lower the potassium permeability, with fairly constant sodium and chloride permeabilities throughout the entire series of hearts.

Deductions from this hypothesis are:-

(1) hearts with long action potentials should have a resting time constant greater than hearts with short action potentials (potassium largely determines the $E_m$ at rest and therefore contributes most to the

time constant of the resting membrane). From the few figures in the literature (Purkinje's tissue excluded, because rather different in many respects) this appears to be the case. Trautwein & Dudel (1958) using dog atrium found a time constant of 20 msec compared to the 2-4 msec in the present experiments (Part III).in the rat. Dog atrial action potentials usually last some 150-200 msec (Brooks et al.,1955 p 104), compared to 50 msec of the rat atrium, so that the ratios of the time constants of these auricles is of the same order as the ratios of the durations of the action potentials. Trautwein and Dudel also found that treatment with ACh reduced the time constant to 2 msec (when the highest doses were used), and Hoffman and Suckling (1953) showed that the maximum shortening of the action potential produced by ACh was to a duration of about 50 msec. Under ACh treatment then (which probably causes an increase in the $P_K$ - see Hutter, 1957 for references) the dog auricular actiom potential assumes the same shape and duration as the rat auricular action potential, and has a similar time constant.

(2) The $P_K/P_{Cl}$ ratio should change as the duration of the action potential changes. There is no direct evidence for this at the moment, but it would seem to be significant that it is much easier to make the sheep auricle inexcitable, by substitution

of part of the chloride with iodide, than the rat auricle. This suggests that the ratio of the potassium to the chloride permeabilities is lower in the sheep than in the rat heart.

(3) It might be expected that if the ratios of the potassium and chloride permeabilities varied in a systematic manner as suggested, then the resting potential of the cells ought to be highest in the small animals and lowest in the large animals. This does not appear to be described in the literature (Eg. see Cranefield & Hoffman, 1958), but on the other hand there is no systematic study of this point in the literature, with account of the equilibrium potentials of the ions conserned.

The evidence to hand then does lend some support to this simple hypothesis of the species differences of the cardiac action potential, but is insufficient to allow of any great confidence in it. Further work is required to settle this matter.

3. The chloride transport system.

The results obtained supports the hypothesis that chloride is transported into the cells as a neutral complex in conjunction with potassium, but of course does not exclude other hypothesis such as that chloride ions exchange across the cell membrane with bicarbonate ions.

At first sight it is rather surprising that

iodide is transported by the cells at least as well as chloride, as this presumably means that the carrier system is non-specific enough not to differentiate between anions of this different shell size. It appears however that a similar situation occurs in the gastric mucosa, and that bromide is trnsported preferentially to chloride (Heinz et al., 1954, who also review the earlier literature on bromide and iodide trnsport in the gastric mucosa). In salivary glands Lundberg (1958) found that bromide was transported as well as chloride but that iodide, thiocyanate and nitrate were transported very poorly, so that the transport mechanism could distinguish between bromide and iodide. It is not known if this specificity is due to the actual locus on the carrier molecule, or to the channels leading to the carrier system.

It would probably be worthwhile to investigate the specificity of the carrier system in the heart, in order to find a small anion of a similar order of permeability to chloride which was not actively accumulated. With such an anion instead of chloride it should be possible to determine the part this distribution of chloride plays in the long action potential in the heart.

4. Auricles treated with 2-4 dinitrophenol.

The marked and rapid effect of DNP and anoxia on the cardiac action potential has seemed a sufficient justification for the beliefs of those who consider that the cardiac action potential is an active process, without further proof. On the other hand Shanes (1958) has pointed the way to a possible explanation of this phenomenon within the framework of the hypothesis developed for the squid axon, also without any proof.

In the results section it has been pointed out that the predictions made by Shanes' hypothesis do not fit the facts and therefore it has been dismissed. The hypothesis advanced by Macfarlane that the prolongation of the action potential in the heart is due to active transport (? chloride), and that DNP shortened the action potential by stopping this transport cannot be so readily dismissed and will now be discussed.

It is generally considered that active transport systems, like other energy requiring functions of cells, are driven by the energy obtained when ATP is broken down (see Hodgkin & Keynes, 1955 and Caldwell & Keynes, 1957 who seem to have established this for squid axon). This will be assumed to apply to the active transport of ions in the heart in the following section.

Grauer (1957) showed that the ATP levels in the

rat ventricle showed a continuous fall from the 4$^{th}$ to the 11$^{th}$ minutes, when the intact animals were deprived of oxygen by clamping the trachea. Four minutes coincides with the disappearance of the glycogen stores from rat heart (Grauer's statement and Wittels, Recrir & Frank 1959). It can be assumed that when oxidative phosphorylation ceases (due to the withdrawal of oxygen), ATP is produced from glycogen by anaerobic pathways until the glycogen stores are depleted and from then onwards the available ATP diminishes, to reach a minimum (almost zero) value at 11 minutes.

DNP, when used in concentrations which produce maximum increase in respiration, inhibits oxidative phosphorylation during the tricarboxylic acid cycle (Simon, 1953; note that glycolytic phosphorylation is also probably inhibited). From figufes given by Webb and Hollander (1956) for rat mitochondria at 30$^{o}$C. maximum stimulation of oxygen uptake is in the range of 1-10$\mu$M, so that it appears probable that ATP production is being inhibited at this concentration. However, no direct figures could be found in the literature for the heart, but Cross et al. (1949) using kidney and liver mitochondria and Mudge (1951) on rabbit kidney slices showed that DNP produced maximum inhibition of inorganic phosphate uptake and of potassium and sodium accumulation respectively at a concentration where

maximum stimulation of respiration occurred. This concentration in both cases was 50 $\mu$M at a temperature of 25°C. As the present experiments were carried out at 35°C. it is likely that at the concentration of 13 $\mu$M used oxidative phosphorylation was being adequately blocked. (In one experiment at 20°C. ATP at 13 $\mu$M had no effect during one hour's exposure).

At this concentration of DNP then, it would seem reasonably to assume that the ATP content of rat heart under DNP was similar to that under anoxia. If this is so then concomitant with the disappearance of ATP from the heart the ionic levels decrease and the action potential shortens. This appears to fit the active transport hypothesis fairly well. However, these results also fit the hypothesis that there is a large increase in the chloride permeability, during this metabolic inhibition.

As a prelimnary test between these hypothesis experiments were done in which chloride was replaced in the perfusate with a large anion. The effect of this was quite striking and showed that many of the effects of DNP and anoxia were abolished if chloride was not present in the perfusate. Thus, after one hour's perfusion with DNP or with a glucose free solution in which the oxygen was replaced by nitrogen, the action potential was only reduced to a duration of 20-30 msec instead of the expected 6 msec.

After this time in DNP or a nitrogen Tyrode, there would probably be no ATP left in the cells, but the action potential was still long compared to nerve or skeletal muscle.

Under these conditions the action potential was however shorter than normal, and this might be held to show some evidence for active transport. Recently Weatherall (1960) presented evidence that DNP causes an increase in the potassium permeability of some 30% in rabbit auricle; and this would explain the residual shortening of the action potential in chloride free media, at least qualitatively.

These conclusions will need to be checked by suitable efflux experiments with chloride (or more probably bromide) tracers, but calculation from the the electrical data suggests that an increase in the chloride permeability of the order of four times would account for the observed effects on the resting potentials.

Thus it seems probable that the rapid effect of DNP on heart action potential's cannot be taken as evidence that the prolongation of the action potential is due to active causes during the action potential itself; and as this is really the only piece of evidence that cardiac action potentials are active, then this theory must be dismissed for lack of any evidence.

The net changes of ions shown in this work do not enable any conclusions to be drawn as to whether the active transport systems are in fact being stopped by the DNP. For this, unidirectioan flux measurements are required. Few of these appear to have been carried out, but recently Weatherall (1960) reported that DNP did not reduce the potassium influx in rabbit atria. Conn and Wood (1956) on the other hand, found a 20% depression of the potassium uptake under anoxia in dog ventricle. No figures are available about sodium and chloride movements. Thus, no conclusions can be drawn about this matter at the moment, but it is clear that energy is required to transport ions against their electrochemical gradients and in the system (squid axon) which allows of easiest investigation, a lack of ATP leads to stoppage of the active transport systems (Hodgkin and Keynes, 1955).

The reason for the increase in the $P_{Cl}$ under DNP or anoxia is unclear. Possibilities are that (a) the normal permeability state of the membrane requires energy and that removal of all the ATP leads to an alteration of this state; (b) the normal $P_{Cl}$ is maintained by the ratios of chloride of other ions across the membrane. This latter is unlikely, for the electrical activity returns towards normal after DNP at a time when the $(Cl)_i$ is still low, and there is no evidence for the former scheme.

Finally it may be noted that this hypothesis may be applicable to other tissues than the heart. Thus Schoepfle and Bloom (1959) found that DNP or cyanide led to a progressive loss of excitability of single myelenated fibres, with a high resting potential. This they explained as a progressive loss of activity of the sodium carrier mechanism, but the facts could equally well be fitted by an increase in the chloride permeability tending to clamp the membrane at the resting level.† In general it is probable that a mechanism of this sort would only stop the excitability in cells in which the increase in the sodium permeability during the action potential is not of a very high order. This would probably account for the failure in heart and myelenated nerve fibres but not in the squid (Hodgkin & Keynes, 1955) under DNP treatment.

† In the Appendix (II) it is pointed out that the ionic distribution in myelenated nerve is probably the same as in the heart, and this makes it even more likely that an increase in $P_{Cl}$ is occuring. It would be at least worthwhile to repeat the DNP experiments in chloride free solutions, to see if the effects of DNP were reduced.

# PART II

## EXPERIMENTALLY PRODUCED ARRHYTHMIAS IN AURICLES AND VENTRICLES

## INTRODUCTION.

During the experiments with DNP on rat auricle described in Part I, it was frequently observed that the application of a single supra-threshold stimulus to the auricle during repolarisation of an action potential led to repetitive activity in the auricle. This phenomenon was investigated in a separate series of experiments and the results will be presented in Part II of the thesis.

Sir Thomas Lewis (1925) appears to have been the first person to propose the hypothesis that fi fibrillation could be started by an extrasystole just after the refractory period of a normal beat. He shows such an event in an E.C.G. taken from one of his patients.

Eccles and Hoff in 1934 used an extrasystole as an antidromic stimulus, to send back into the pacemaker to try and infer some of the properties of the pacemaker. They found that if the extrasystole was early enough in the relative refractory period and the vagus was beeing stimulated at the same time ( and hence the A.R.P. was shortened ), then this second stimulus gave rise to repetitive firing in 50% of their cats. From this they set up a hypothesis that the S.A. node was being fractionated by the second stimulus and so giving rise to the fast rhythms.

In 1934 Andrus and Carter found much the same

thing in dog's auricle. The second stimulus never gave rise to fibrillation if the vagus was unstimulated, but always did during vagal stimulation. They found that the A.R.P. had to be shortened to about a third to a half to produce the effect. They also observed that stimulation of an auricular appendage led to the first beat of the repetitive activity with such a small time delay, that it seemed unlikely that the effect could be due to a re-entrant phenomenon around the base of the auricles.

Wiggers and Wegria in 1939 reviewed the literature on this phenomenon and showed that ventricular fibrillation could be produced at will in the dog ventricle by a single stimulus applied (via electrodes sewn into the wall) during the "vulnerable period" of the heart cycle in late systole. They then defibrillated the ventricles by the application of a DC potential between two metal plates also attached to the ventricular wall. The curious feature about this work is that a single stimulus to an apparently normal ventricle produced the fibrillation. In a later paper (Moe et al., 1941) this effect is again mentioned and in this paper they say that the extra stimulus needs to be several hundred times the normal threshold to produce the effect. This probably means that the ventricular tissue below the stimulating electrodes was made

abnormal by the stimulating current, and certainly they had no evidence that it was normal.

With the introduction of intracellular recording from heart cells Hoffman and Suckling (1953) repeated the earlier observations (although apparently unaware of them) and showed that the application of a single stimulus at the end of repolarisation of the action potential of dog auricle treated with ACh, led to fast repetitive activity at rates of 700-800 beats per minute. Surface records taken at the same time gave pictures very similar to that in clinical flutter.

Professor Burn and his colleagues have produced a series of papers (see Burn, 1957 for references) in which they showed that isolated rabbit atria fibrillated if the external potassium was reduced to about 1.4 m.equiv/litre, ACh was added and electrical stimulation was applied at 500-800 pef minute. Their hypothesis for this effect was that a critical potassium flux had to be exceeded before fibrillation was produced.

Later Klein and Holland (1958) showed that during fibrillation the potassium efflux was 50% greater than during the application of the ACh plus the effect of rapid stimulation by itself (these measured separately) and considered that this proved the hypothesis. No attempts were made to produce fibrillation in other ways and study the

potassium fluxes then.

The assumptions which they made about this flux were (a) ACh with and without fibrillation produces the same increase in the potassium flux, which seems reasonable; and (b) that the potassium required to repolarise the membrane at a driven rate of 1200/minute was the same as that required to repolarise the membrane during fibrillation at the same rate, and hence the extra potassium flux was the cause of the fibrillation. This seems very improbable, because it is impossible that normal auricles driven at 1200/minute could actually follow at this rate, and therefore a comparison is being made between a heart driven at say 400/minute ( by stimuli) and one at 1200/minute (During fibrillation). So that if the usual ionic hypothesis (Hodgkin, 1958) applies to heart, this invalidates the argument straightaway because of the extra potassium required to repolarise the added 800 beats per minute.

Recently Sekul and Holland (1959) have been measuring $Cl^{36}$ and $Ca^{45}$ exchanges in atrial fibrillation produced by ACh and rapid stimulation, and somehow come to the conclusion that the fibrillation is due to an increased sodium flux. The reasoning which they use to arrive at this conclusion is not clear from the paper, and in any case the above argument appears to invalidate the work.

Burn and his colleagues were also able to show that the ventricles could be made to fibrillate when the potassium content of the perfusate was decreased, and fast stimuli applied. Under these conditions there was little increase in the K flux and the hypothesis proposed for this situation was an alteration of the calcium on the membrane. One interesting finding of this work was that the addition of ATP to the medium always either returned the fibrillation to normal or converted it to a tachycardia. With the idea that ATP is concerned with the binding of calcium at the membrane, they applied DNP to some of the hearts and found that this caused fibrillation.

A recent paper of some interest is that of Matsumura and Takaori (1959) who showed that if aconitine is dropped onto a ventricle, fibrillation is produced and this is accompanied by shortened action potentials (recorded by intracellular electrodes) regularily repeated.

The results presented in this part of the thesis show that, when the action potential of auricular or ventricular muscle is shortened in a variety of ways, it is possible to set up fast repetitive activity by means of an extra stimulus applied just outside the A.R.P. of the action potential of the normal stimulus. This supports the hypothesis that this repetitive activity is merely a consequence of

the shortening of the action potential and is not necessarily related to changes in any specific ionic permeability.

On this basis it is possible to set up a hypothesis for the clinical conditions of flutter and fibrillation in auricles and ventricles, under the usual conditions which produce it.

## METHODS.

In general these were the same as those described in Part I. In some of the earlier experiments with ACh, mouse auricles were used, and the experimental bath was similar to that of Draper and Weidmann, 1951. The ACH used was in the range of $3.5 \times 10^{-3}$ to $3.5 \times 10^{-8}$ moles/litre in the bath.

The stimuli used in these experiments were locked to the time base, the first one being kept in a constant position and the second one (used as a test stimulus) brought towards the first, into the relative refractory period.

The records were photographed either on still film or onto moving film in a Cossor camera. When the latter method was used, there was frequently a certain amount of hum on the record from the camera motor. With the former method, it was usually not possible to obtain a record of the entire response to the two stimuli, and many records were missed in this way.

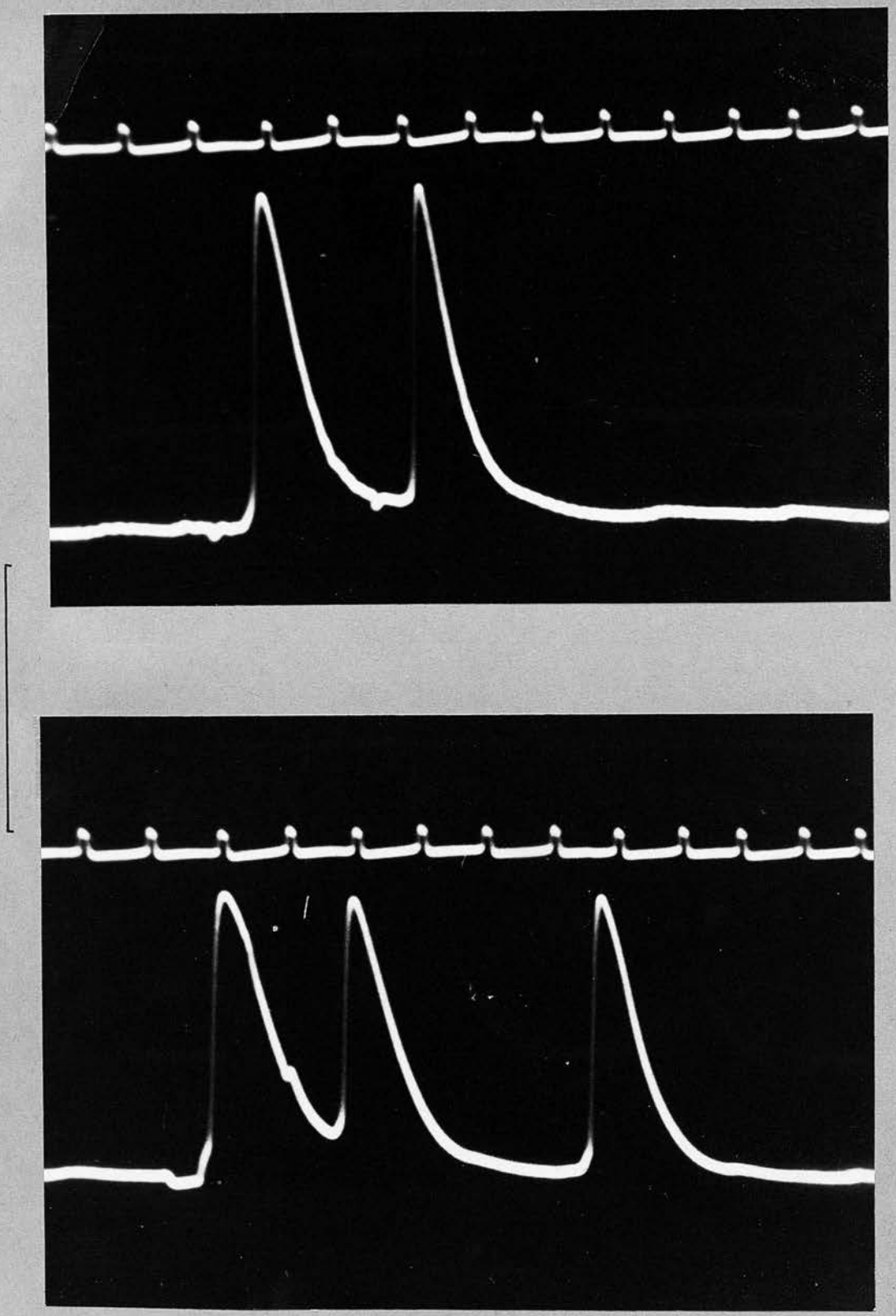


Fig. 18

Mouse auricle with ACh. in the bath. Top (a), stimuli separated by 25 msec. Bottom (b), stimuli separated by 18 msec, extra late beat appears. Time scale 10 msec. Voltage scale, 80 mV.

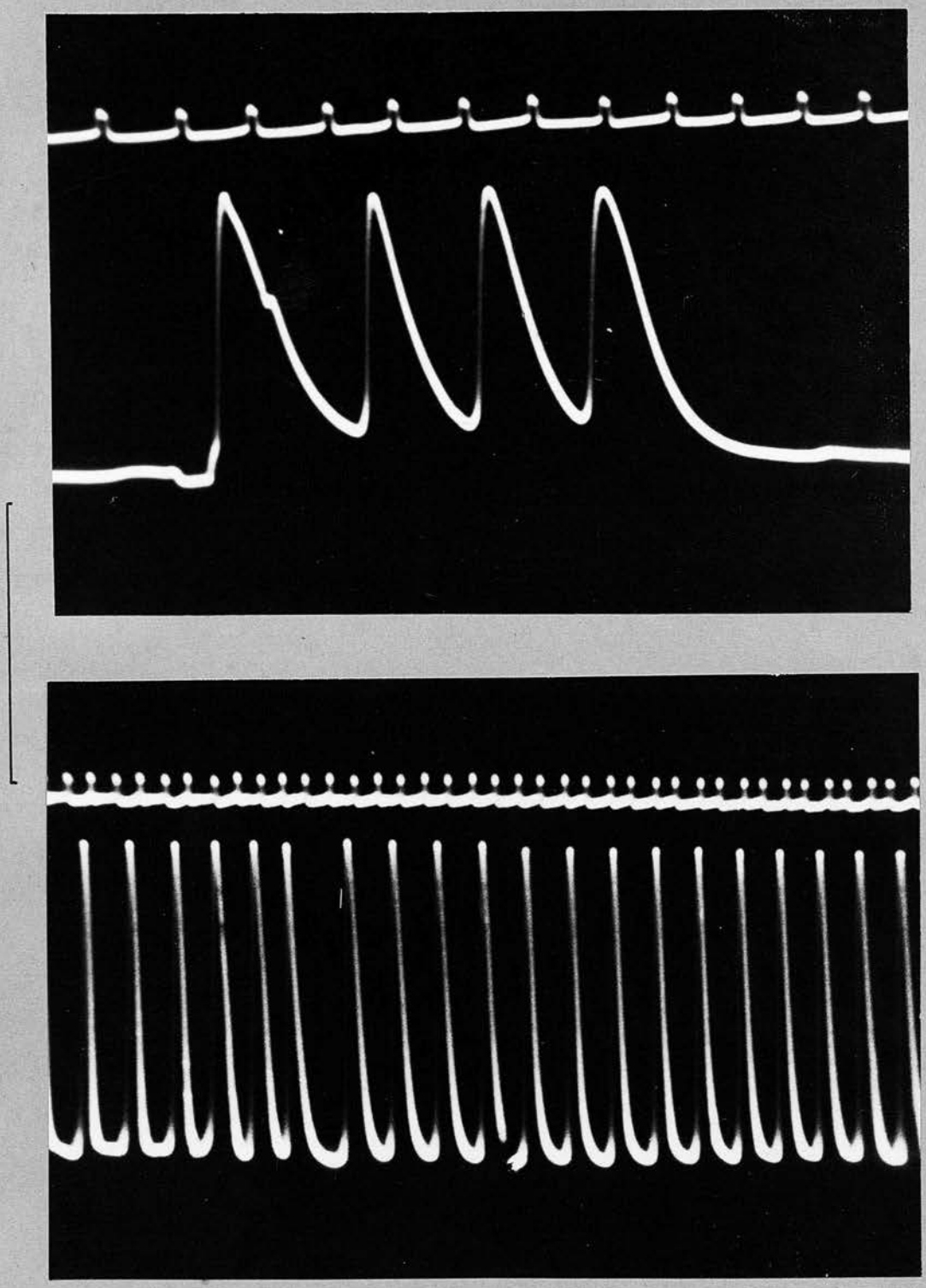


Fig. 19

Same mouse auricle as Fig. 18, also with ACh present. Top (a), 2 stimuli giving 4 beats. Stimulus separation, 14 msec. Bottom (b), repetitive activity set up by moving the second stimulus slightly nearer. Time scale, 10 msec. Voltage scale, 80 mV.

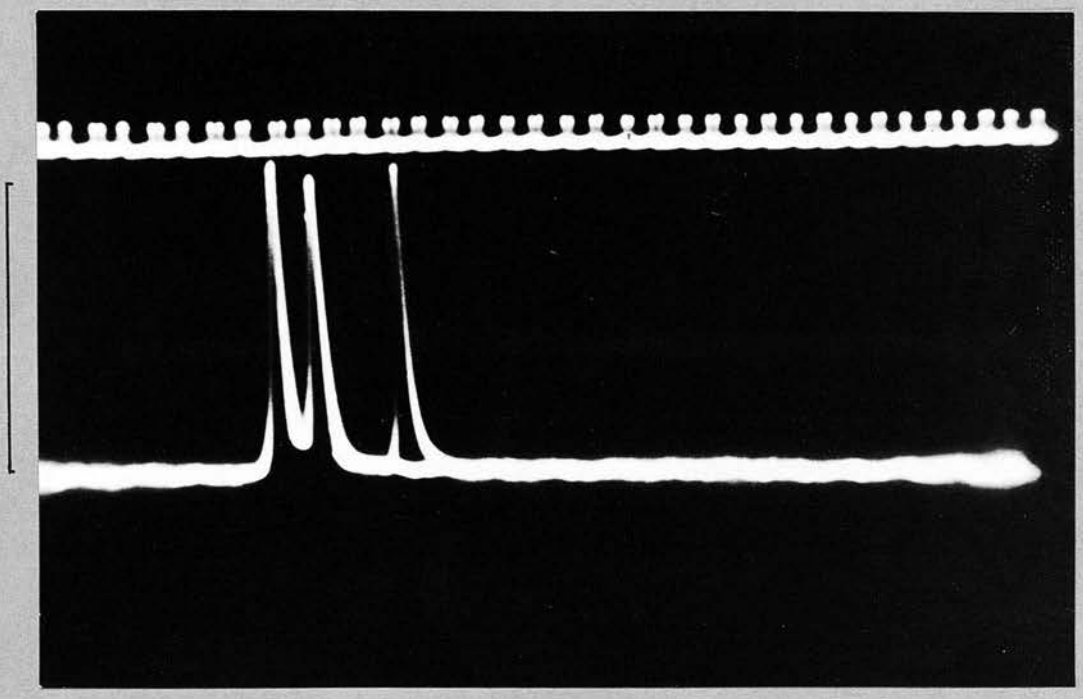


Fig. 20

Mouse auricle with ACh added. Two sweeps photographed with stimulus separation left constant, extra late beat appears on one sweep only. Time scale, 10 msec. Voltage scale, 80 mV.

## RESULTS.

It was very soon found that the phenomenon of late and early beats described by Eccles and Hoff (1934) could be obtained from hearts under a variety of conditions, whether or not the pacemaker was present. Fig. 18 shows two photographs taken from recordings from the right auricles of a mouse, with ACh added to the bath. In 18a the two stimuli are separated by 25 msec and each produces a normal action potential. In 18b the second stimulus has been moved nearer (18 msec) and now the second stimulus has given rise to a normal action potential (but note the increased latency) and also an extra late action potential. In 19a (from the same experiment) the second stimulus has been moved closer to the first (14 msec), the latency of the second beat has increased slightly and two extra beats have appeared. The first of these extra beats is now in an early position compared with that in 18b, corresponding to Eccles and Hoff's finding. In Fig. 19b is shewn the effect (on a much slower sweep speed) of moving the second stimulus slightly nearer still; the auricle is now showing repetitive activity at a high rate (3,100/min).

The actual timing of the stimuli for these effects was found to be critical in any one preparation, as is shown in Fig. 20, but varied fairly widely in different preparations and at different times.

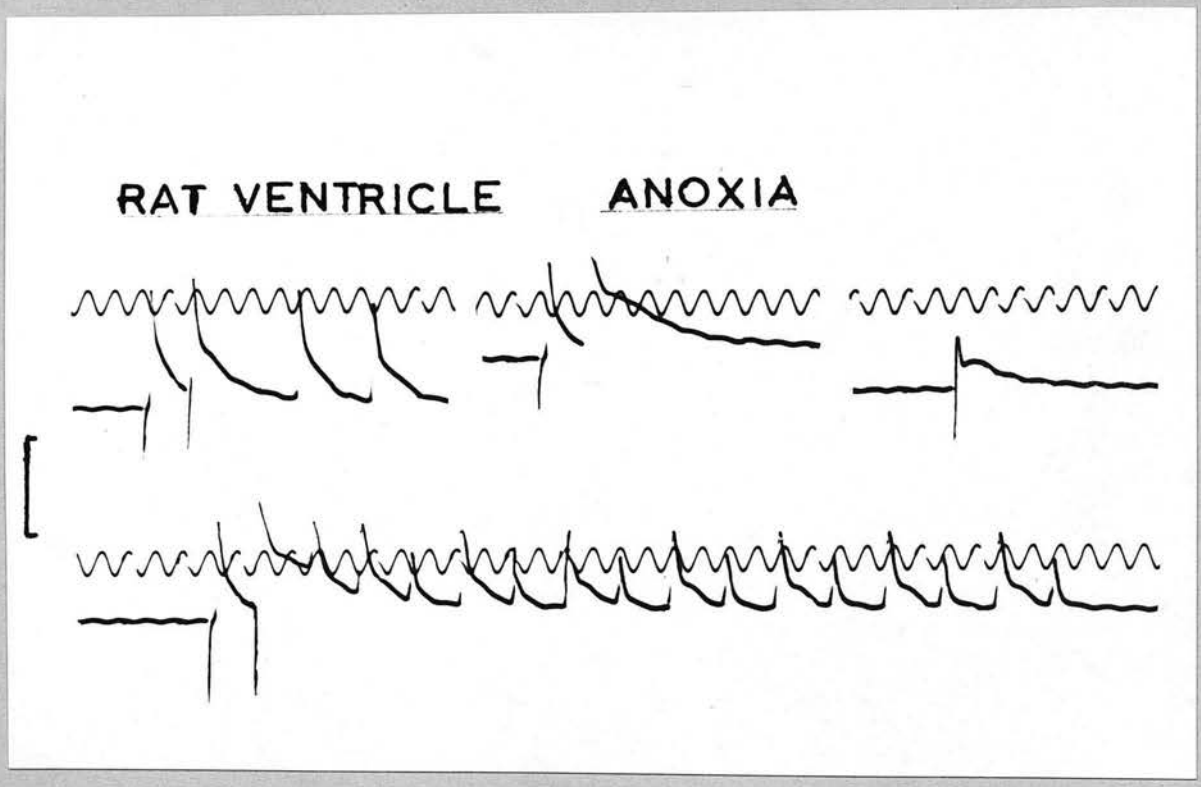



Fig. 21

Rat ventricle, normal Tyrode with the $O_2$ replaced by $N_2$ about 8 minutes before these records taken. Top left, 2 stimuli (separated by 30 msec) leading to 4 action potentials. Bottom, a few minutes later the same stimulus separation leading to a series of rather irregular action potentials. Top middle, the second stimulus needs to be very large to produce a response as the excitability decreases. Top right, a single stimulus only produces a local response. $O_2$ was then re-admitted and the excitability returned. Time, 50 cps. Voltage, 80 mV. A.P.s in order obtained.

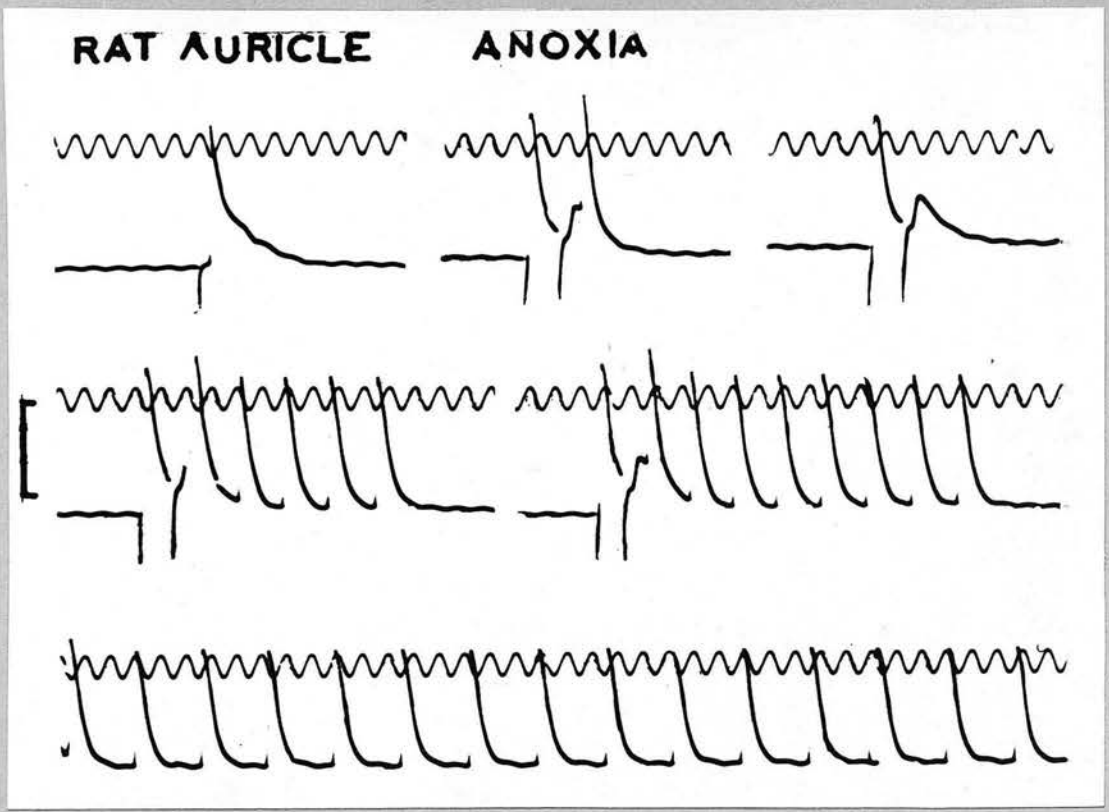



Fig. 22

Rat auricle, normal Tyrode with the $O_2$ replaced by $N_2$. Top left, normal A.P. Then anoxia until A.P. failed, rest of records in order during recovery. Top middle, 2 stimuli at 29 msec apart give 2 responses. Middle row, left, 2 stimuli at 26 msec give 6 action potentials. Middle row, right, 2 stimuli at 23 msec apart give 9 A.P.s. Bottom, prolonged activity produced by 2 stimuli a few minutes later(about the same separation). Top right, action potential recovering it's normal length and 2 stimuli at this separation only gives 1 response. Time, 50 cps. Voltage, 80 mV.

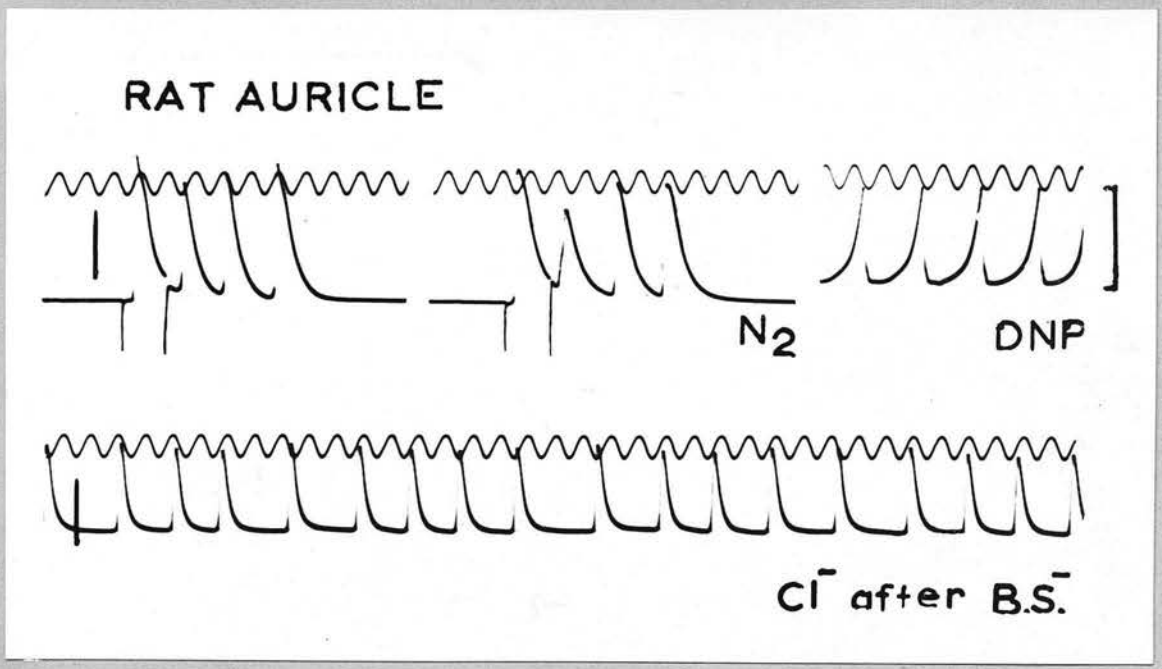



Fig. 23

Rat auricle, fast rhythms produced in various ways. Top left & bottom, auricles soaked in benzene sulphonate Tyrode and then replaced in normal Tyrode, 2 stimuli produced 4 and a whole series of action potentials at different settings of the stimuli. Top centre, 2 stimuli and anoxia. Voltage scale for these records on left, 50 mV. Top right, DNP on auricle, part of fast rhythm produced by 2 stimuli. Voltage scale, 80 mV, and record reads from right to left. All time scales, 50 cps.

This sequence of events was found very commonly in auricles (both right and left) of mice and rats when treated with ACh. Occasionionally as the test stimulus was moved closer to the first stimulus, the single late beat became a single early beat; but more commonly when it became early it was followed by others, as shown in 19. The lengths of these repetitive rhythms set up by two stimuli varied enormously, from short ones of a few action potentials to long ones of several hundred action potentials lasting several minutes.

When the auricles or ventricles of rats were treated with DNP or anoxia, the action potential shortened (as previously described) from a normal duration of 50 msec to ne of only a few msec, with a decrease in the excitability. In such hearts the application of two stimuli (as described above) before treatment or during the severe shortening produced by the treatment gave rise to two responses only, with no repetitive firing. If these two stimuli were applied during the intermediate condition when the action potential duration was 20-30 msec and the excitability of the tissue was still high, then a similar series of events occurred as with ACh. These repetitive rhythms could be obtained either during the shortening produced by DNP or anoxia, or during the lengthening after the removal of DNP or the re-admittance of oxygen, but

never during the height of their action; presumably because of the low excitability of the preparation at this time. Fast rhythms produced in these various ways are shown if Figs. 21, 22 and part of 23.

In a few experiments carried out with Tyrode containing no potassium, it was found that the action potential shortened considerably after about half an hour's perfusion. At this time repetitive activity could be obtained by 2 stimuli. (Fig. 24).

In auricles soaked in benzene sulphonate and then replaced in normal Tyrode the action potential shortened and then returned to normal, presumably as the chloride was first all extracellular and then was accumulated in the cells. Under this shortening long runs of repetitive activity could be set up with 2 stimuli.(Fig. 23). This was a phenomenon more frequently observed when the heart was first placed in an iodide Tyrode than in a chloride Tyrode, presumably because of the greater permeability to iodide than to chloride (Hutter & Noble, 1959).

The repetitive activity, once set up, did not appear to show any differences dependent on the mode of it's origin, that is the records obtained by DNP treatment were not different from those obtained by ACh etc. Usually the repetitve activity was regular, at rates of 1200 to 2000 per minute (rat).

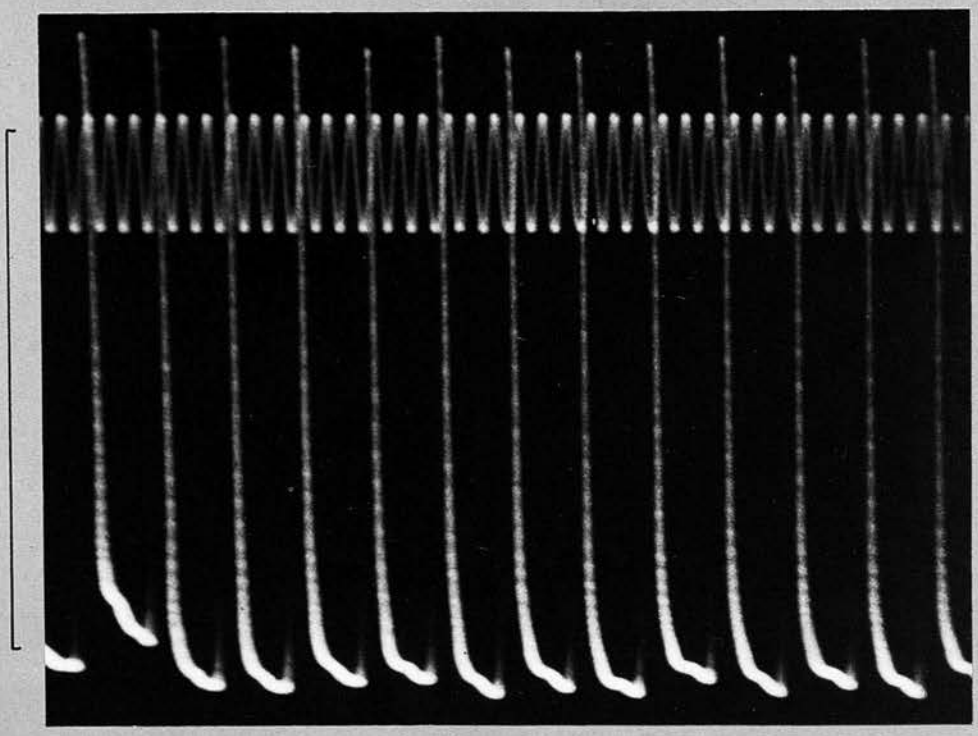


Fig. 24.

Repetitive activity set up in a rat auricle by two stimuli, during the shortening produced by perfusion with a Tyrode solution containing no K. Perfused for 30 minutes. Time scale, 50 cps. Voltage, 80 MV.

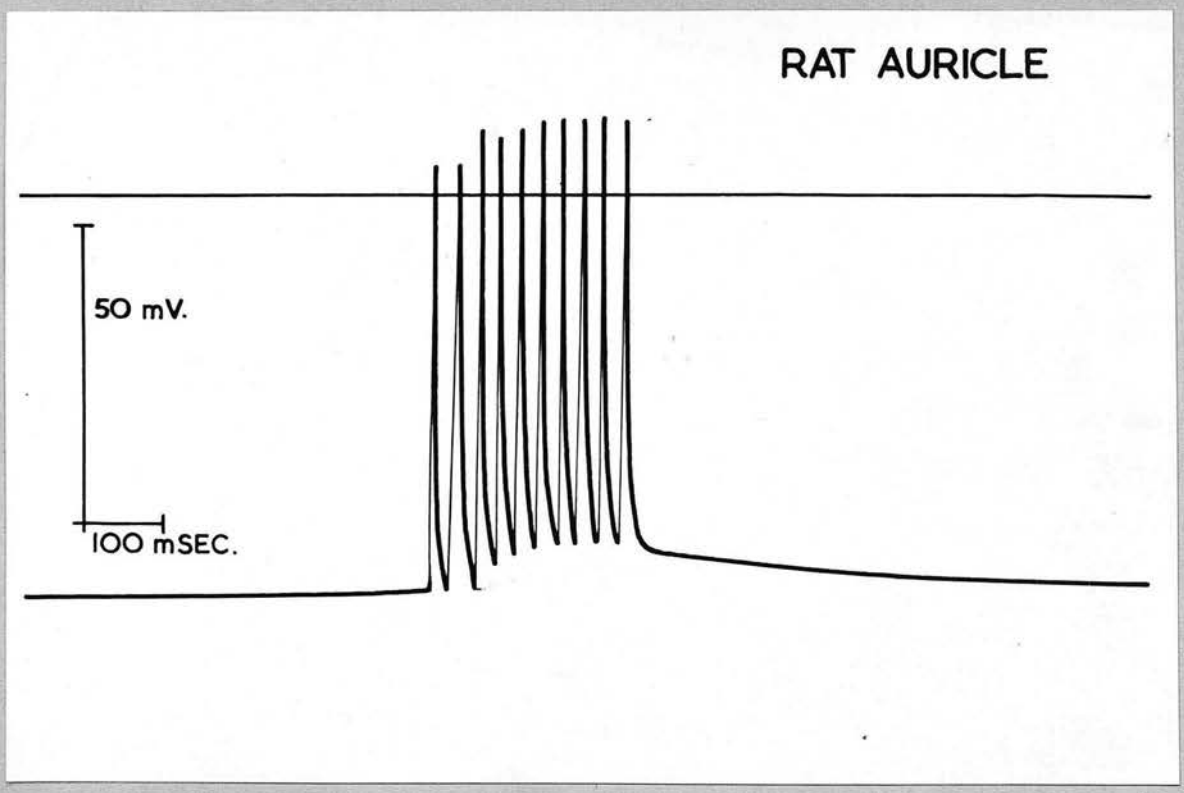



Fig. 25

Repetitive activity in rat auricle treated with DNP. (tracing from enlargement). The stimulus in this case was a burst of impulses from a multivibrator, ending after the first action potential. This figure was used to calculate a diffusion barrier some 137 $A^{\circ}$ from the active layer. The upper line is through zero potential across the membrane.

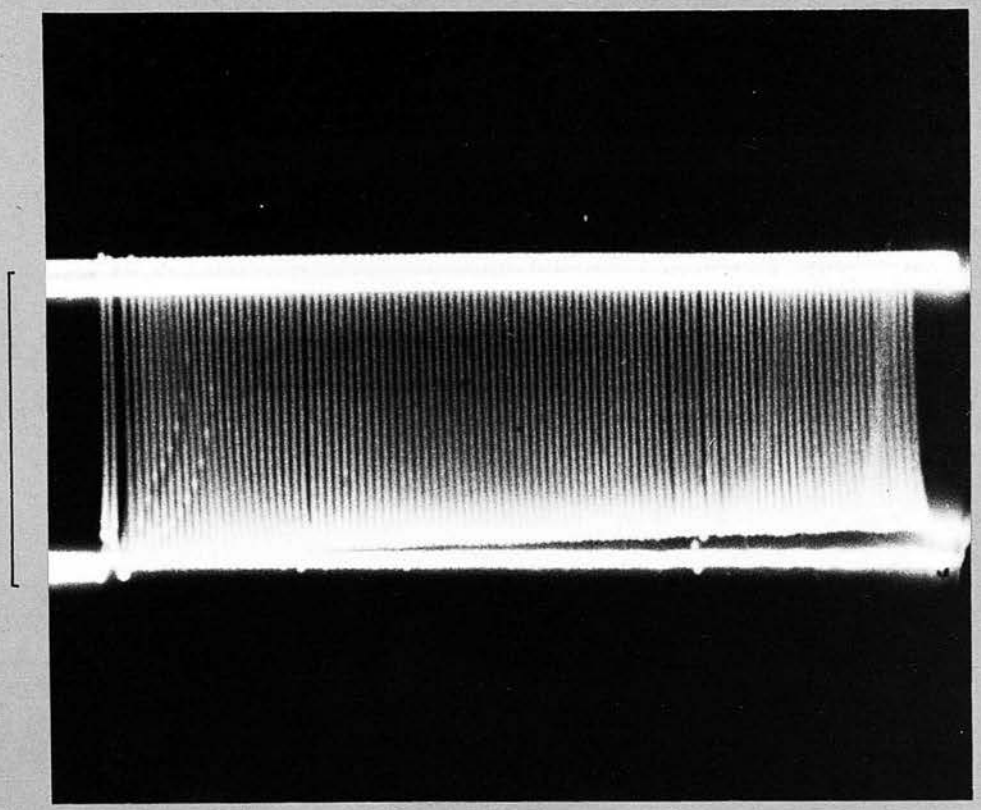


Fig. 26

Mouse auricle treated with ACh. Repetitive activity set up with 2 stimuli. Note that the film was exposed for two sweeps. The baseline (ie $E_m$) rose steadily during the activity, and then declined afterwards (end of first sweep and during second sweep) to it's resting level. Voltage scale, 80 mV. Time scale, action potentials separated by about 15 msec.

It was frequently observed during these experiments that the baseline tended to rise during the repetitive activity and then return to normal at the end. Due to rather inadequate photographic equipment it was found rather difficult to get good records of this effect, but Fig. 25 shows a tracing made from such a record. In this case the fast rhythm was set up with a burst of impulses from a multivibrator in a rat auricle treated with DNP. It can be seen that both the resting potential and the overshoot rise in an exponential fashion to a limiting value, and then decline afterwards. Sometimes the baseline continues to rise steadily during the activity and then declined afterwards (Fig. 26), the reason for this is unknown and will not be discussed further.

It seemed probable that this was a similar phenomenon described and investigated by Hodgkin and Frankenhauser (1956) in the squid axon, and the results obtained have been treated in a similar manner. No quantitative studies were made of the effect on the phenomenon of changing $(K)_o$, as done by Hodgkin and Frankenhauser, to establish that the baseline change behaved as a potassium electrode; but it was observed in the experiments with $(K)_o$ 0 that the basline change was much greater than in a normal Tyrode.

A full analysis of Fig 25 was carried out (see

the Appendix for the details) and an outline of this will now be given.

After the first impulse the basline has risen by 3.5 mV. This corresponds to an increase in $(K)_o$ from 2.7 to 3.5 m.equiv according to the data given by Vaughan Williams (1959) for rabbit auricle. (It was decided to use an experimentally derived relationship between the $E_m$ and $(K)_o$ rather than the Nernst equation, because at these low levels of external potassium the Nernst equation is not a good fit and also it has been shewn in Part I that the $E_m$ is determined by potassium and chloride.)

This value of $(K)_o$ has to be corrected for the effect of leakage of potassium away from the membrane. This correction involves the use of the rate of decay of the effect at the end of the series of impulses, and raises the value of $(K)_o$ to 3.9 m.equiv/litre.(The actual rise near the active membrane due to one impulse is 1.17 m.equiv/litre).

Now Rayner and Weatherall (1959) found a value of 1.6 pmoles/$cm^2$ for the potassium efflux from rabbit auricle per second. If this flux applies to rat atria under the conditions of these experiments, then the apparent "space" near the active membrane from which diffusion is limited is 137 $A^o$.

If each cell were surrounded by a "space" of this order, then the distance between cells would be about 260 A , which corresponds fairly well to the

anatomical separation of 90-200 $A^{o}$ found by Sjostrand (1958) between the cells in mouse heart muscle.

No very thorough investigation of this effect was undertaken, but it was observed that the baseline rise was not present in all the cells sampled. The reason for this is not known, but it may be that the phenomenon can only be obtained from those cells lying below the outer layer of cells and therefore surrounded on all sides by the rather narrow intercellular spaces.

## DISCUSSION.

The phenomenon of fast repetitive activity can be obtained in auricular tissue with ACh, and in auricular and ventricular tissue with DNP, anoxia, low external potassium, with local application of aconitine (Matsumura & Takaori, 1959) and in normal Tyrode after benzene sulphonate. The most obvious common feature possessed by all these conditions is that the action potential is shortened, but others may of course exist.

One of these has been proposed, from time to time, by Burns and his colleagues (for references, see the introduction) who state that a critical potassium flux must be exceeded before fibrillation can start. The evidence for this view will now be examined (their own evidence will be ignored, for the reasons given in the introduction):-

It is now almost certain that the main action of ACh on auricular tissue is to increase the potassium permeability, the evidence for this being 1. an increased net loss of potassium (Lehnartz, 1936) 2. an increased efflux of potassium (Hutter, 1957) 3. movement of the $E_m$ towards the $E_K$ value (Trautwein & Dudel, 1958) all with the addition of ACh.

Recently Weatherall (1960) has presented evidence that there is a 30% increase in the $P_K$ under DNP; Conn (1956) on the other hand found no

evidence of an increased $P_K$ with anoxia, but did find an increased potassium efflux which he attributed to an increase in the rate of diffusion from the extracellular space to the blood stream. If the hypothesis proposed in Part I for the action of these agents is correct, then the main alteration is an increase in the $P_{Cl}$ rather than the $P_K$.

No evidence is available on the action of aconitine on the membrane, and as previously discussed normal Tyrode after benzene sulphonate Tyrode probably shortens the action potential because the chloride is then available to carry repolarisation current during the whole of the repolarisation process.

It has been stated by Délèze (personal communication) that Weidmann has evidence for an decrease in the $P_K$ when the external potassium is reduced, but this has not yet been published.

Thus the evidence for Burns' hypothesis is far from complete and it is easier at this stage to accept the simpler view that the shortening by itself is sufficient to cause the arrhythmia.

The curious observation of Wiggers & Wegria (1939) that the ventricles of a dog could be made to fibrillate by a single stimulus applied to normal tissue requires some comment, as it is the only such report in the literature. The most likely explanation for this is that the stimulated tissue was not normal due to the high currents used.

Certainly they had no evidence that these areas of their hearts were normal, and in the present experiments where the state of the action potentials were recorded, it was never possible to set up arrhythmias in hearts with normal action potentials.

One of the basic questions arising in the production of these fast rhythms is whether the repetitive activity is arising in a single cell or in a section of the tissue due to a miniature circus movement involving several or all the cells.

The first type of hypothesis implies that the sodium carrier system is able to be activated before the cell membrane is repolarised and hence can be excited by the repolarisation potential and so give repetitive activity in this way. Such a focus of activity could then drive the rest of the tissue in the usual way.

Segers (1941) obtained marked afterpotentials (negative, ie. depolarisations) in hearts treated with solutions containing increased amounts of calcium, and it was thought (suggestion of D. Whitteridge) that the repetitive activity might be starting from these negative afterpotentials. An attempt was made to test this hypothesis by varying the calcium levels in the perfusate, and noting any change in the incidence of the fast rhythms. In 5 experiments with mouse auricle, in which the calcium level was raised or lowered by a factor of 3, there

was no change in the incidence of the rhythms as compared to that in normal Tyrode with ACH. It should be pointed out that it is now considered that these experiments did not adequately test the hypothesis.

The alternative hypothesis, that a re-entry phenomenon is occurring, requires that the action potentials in the tissue be out of phase, once the fast rhythm is set up. This could be determined by suitable multielectrode recording, but has not so far been done. Calculation of the time required for the impulse to travel round the auricle, using the conduction velocity of 0.5 metre/second obtained by Draper & Mya-Tu (1959) and a conduction path of 1.5 cms, gives a figure of about 30 msec. This agrees with the action potential duration required to set up these fast rhythms under DNP or anoxia. This observation then, fits this second type of hypothesis.

It is not really clear on either hypothesis what the function of the second stimulus is, for on either hypothesis one would have thought that repetitive activity could arise with one stimulus together with shortening of the action potential. A paper by Dipalma (1955) may be significant in this respect in that he showed that in cat atrium the latency of a premature beat may be reduced to zero, so that this may be the function of the extra

stimulus. The significance of this is not clear however.

From previous workers and the results obtained in the present work it seems clear that the conditions required for repetitive activity in myocardium are (a) a shortening of the action potential and hence of the ARP. and (b) application of a second stimulus at an appropriate period during the repolarisation of the action potential.

Under experimental conditions in atria in a bath with ACh present or with vagal stimulation, a suitably placed second stimulus gives rise to activity which consists of regularily spaced action potentials at fast rates, which may be obtained anywhere in the auricle. With external recording of this event, the records so obtained have a saw-toothed waveform (Hoffman & Suckling, 1953), and look similar to those obtained in the clinical condition of auricular flutter. The clinical condition then, may simply be due to a fast repetitive activity in an auricle whose action potentials have been shortened by excessive vagal activity.

In fibrillation of auricles and ventricles, the muscle shows inco-ordinated electrical and mechanical activity all over, with no obvious sign of the regularily repeated events seen in these fast rhythms. A hypothesis which fits this situation is to suppose that a small area of the myocardium

is affected by the precipitating agent, eg. DNP, anoxia, aconitine etc., which produces a shortening of the action potential and thus makes it vulnerable to an extra stimulus sent in from the surrounding tissue. If such an extra stimulus is available this tissue responds by becoming a fast pacemaker. Now, the rest of the myocardium being normal, cannot respond at the same rate as this pacemaker but follows it at it's own rate and hence in an irregular manner. The overall gross picture then, is one of incoordinated electrical and mechanical activity.

Evidence obtained from the literature to support this hypothesis is as follows:-

1. Many studies (eg. see Scher & Young, 1957) have shewn that excitation in ventricular muscle spreads outwards from the cavity towards the surface of the heart, so that in any block of tissue the whole thickness of the ventricular wall is subjected to lateral excitation over a period of 30 or so msecs (in dogs, x 2 this in man). This is almost an ideal situation for excitation of a piece of anoxic tissue and it's conversion to an ectopic pacemaker. As there is usually a slowing of the conduction velocity in partially necrotic tissue this aids the production of this process, by spreading out the time sequence of the stimuli.

2. Brooks et al. (1955) in their book show pictures

of action potentials obtained when electrodes were pushed at fibrillating ventricles of a dog. Generally these showed irregular distorted action potentials, consistent with the view that a cell with a normal action potential was being overdriven. Now and then, however, they found regular repetitive activity at a fast rate, consistent with the view that these cells were part of the pacemaker for the fibrillation. They did not seem to appreciate the significance of their finding and were merely disappointed that they did not always find this regular activity. If the hypothesis outlined above is correct then one would expect to hit the pacemaker rather infrequently with an electrode, but would usually record normal if rather irregular action potentials the rest of the time. (The pictures referred to are on p 136, with a note in the text that a paper on this was to be published in the American J. Physiology. No such paper appears to have been published, and a letter to them on this subject has produced no reply so far.)

3. If the hypothesis is correct then one might expect that there would be a regular series of contractions around the pacemaker (but so fast that a contracture effect was obtained to the naked eye) with irregular quiverings elsewhere. This in fact was described by Hoffa & Ludwig in 1850 (quoted by Garrey, 1924) in the first published description of

ventricular fibrillation, induced by electrical stimulation. " They described a limited region of 'tetanus', or rather a condition of persisting contraction immediately about the electrodes; but in the rest of the heart contractions were recorded which were weak and absolutely irregular." (Garrey, 1924, p 215).

4. Garrey also summarises a great deal of evidence which all shows that fibrillating agents, including anoxia, produce fibrillation when applied locally to the ventricle but not when the whole ventricle is exposed to them. This is consistent with the view that out of phase pulses are required from normal tissue to set off the ectopic pacemaker, in an abnormal piece of tissue.

5. Garrey also showed that fibrillating ventricles reverted to normal rhythms when the size of tissue was reduced, this being consistent with this hypothesis.

There seems then to be some evidence for the hypothesis that the basic factor in fibrillation in the whole heart is due to this phenomenon of fast repetitive activity in a part of it. This hypothesis will need to be tested further, by experiments in which intracellular recordings are made from fibrillating ventricles. If it can be shewn that ectopic pacemakers are always produced by these agents when applied locally, and that repetitive activity can be recorded from them, then this should

greatly strengthen the hypothesis. It would also be desirable to try and distinguish between the re-entry and single celled theories of the origin of these fast rhythms, possibly by the use of multi-recording techniques.

# PART III

## POLARISATION EXPERIMENTS

## INTRODUCTION.

The polarisation experiments were undertaken to measure some of the electrical characteristics of the membrane of the rat auricular cells, under the conditions of these experiments.

The most extensive work done on this problem in the past has been that of Weidmann and his co-workers (see Weidmann, 1956 for references), using intracellular electrodes and Purkinje's tissue. Some measurements were made by Trautwein, Kuffler & Edwards (1956) on frog ventricle with external electrodes, giving values of Rm of 200-300 ohms and of Cm of 3.0 uF per $cm^2$ (recalculated to 30 uF by B. Katz). Trautwein & Dudel (1958) obtained some figures for the normal dog auricle in the course of an analysis of the action of ACh.

## METHODS.

For the polarising experiments a square wave stimulator was built, the square waves being obtained by short circuiting the output of the battery unit with a Carpenter Type relay (ex W.D.). The current flow through the current electrode could be continuously monitored by the voltage developed

across a 100 Kohm. wirewound resistor, through a second cathode follower and D.C. amplifier and then displayed on the second beam of the Cossor tube. Calibration of the current was achieved by means of another calibrator box, also made from Muirhead decade resistors.

A switch was incorporated in the square wave stimulator circuit so that (1) the current electrode could be switched to act as a voltage electrode, and (2) the current electrode could be disconnected for clibration purposes.

Some difficulty was experienced in getting suitable electrodes for passing the relatively high ($10^{-7}$ A) currents required for the polarisation and numerous changes of electrodes had to be made during the experiments.

The procedure used for these experiments was either (1) introduce two electrodes fro voltage recording and then switch one to pass current and see if electrotonic potentials could be picked up by the other, or (2) introduce the voltage electrode and then advance the current electrodes towards the tissue (with current pulses on it all the time) and a. watch for the change in the shape and the size of the current pulses as the current electrode touched the surface of the tissue (& it's resistance changed) and b. watch for the appearance of electrotonic potentials on the voltage trace.

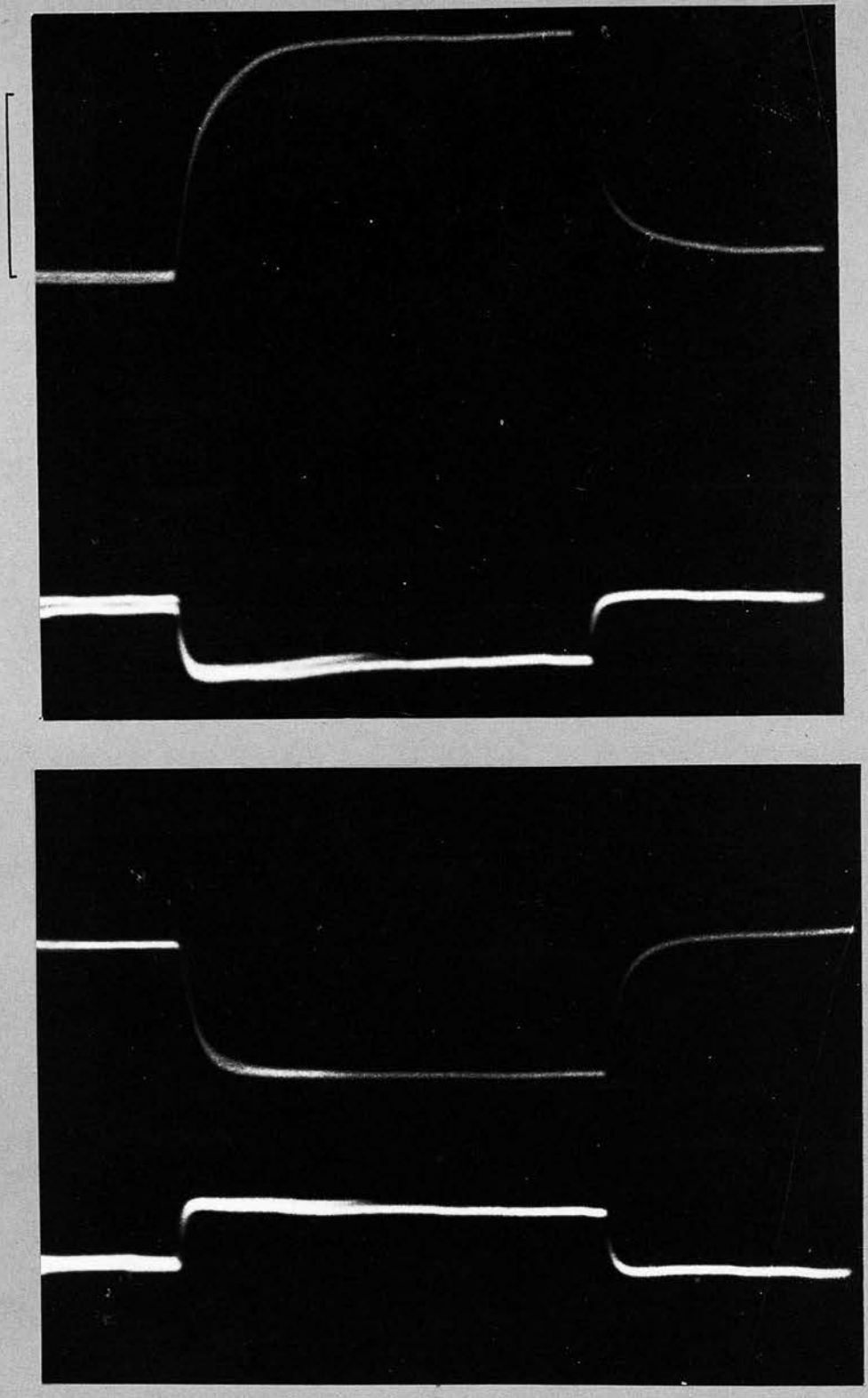


Fig. 27

Rat auricle, 2 intracellular electrodes. In each record the top trace is the response of the tissue to the current passed into the cell (monitored on the bottom trace). Top record, depolarisation. Bottom record, hyperpolarisation. Current cal. $3.5 \times 10^{-7}$ A. Voltage calibration, 20 mV.

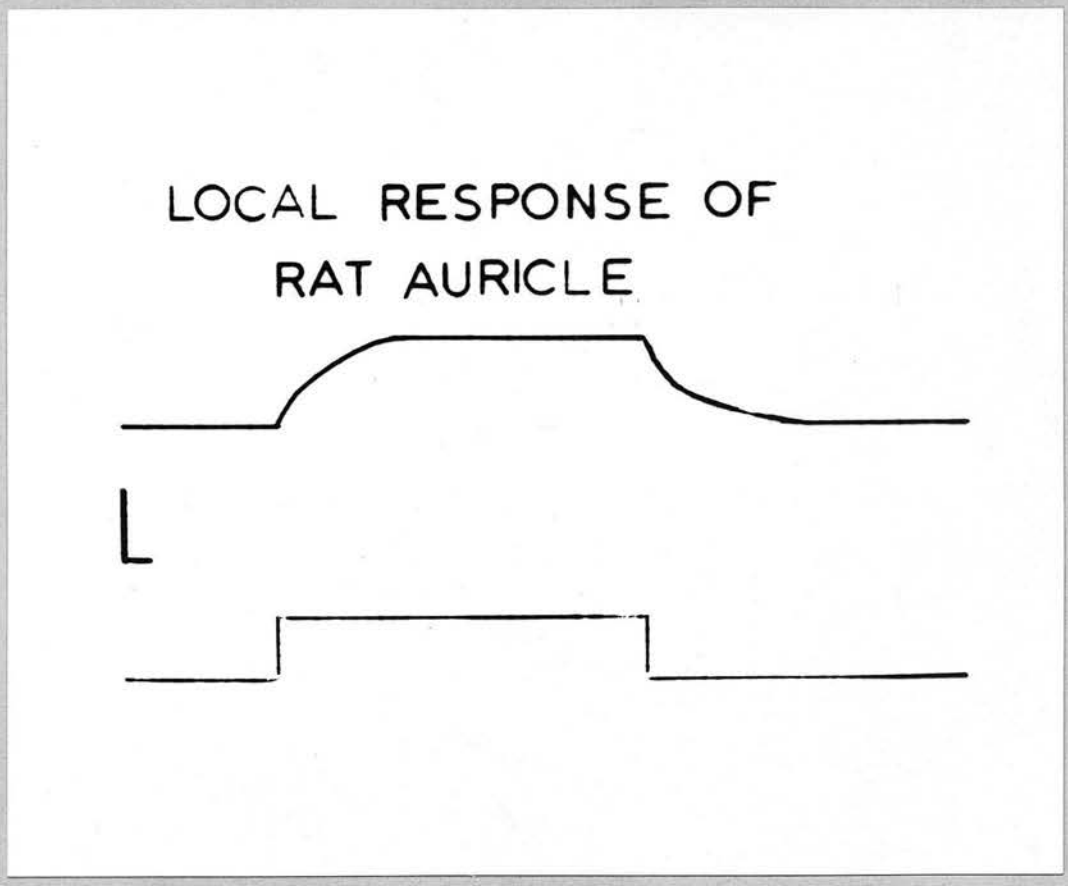



Fig. 28

Curve obtained by the graphical subtraction of the hyperpolarisation response from the depolarisation response, shown in Fig. 27. Lower trace shows the applied stimulus (idealised). Scales, vertical 10 mV; horizontal 2 msec.

TABLE IX.

Membrane characteristics for rat auricle, obtained with hyper-polarising pulses. Z calculated by dividing observed voltage change by applied current. The figures in brackets in column 1 indicate the number of observations made. Distance between electrodes 150 microns.

| Resting potential mV | Time constant on m.sec. | Time constant off m.sec. | Z $x10^4$ ohms |
|---|---|---|---|
| 75 (8) | 4.0 | 4.3 | 4.4 |
| 76 (5) | 2.2 | 2.3 | 4.3 |

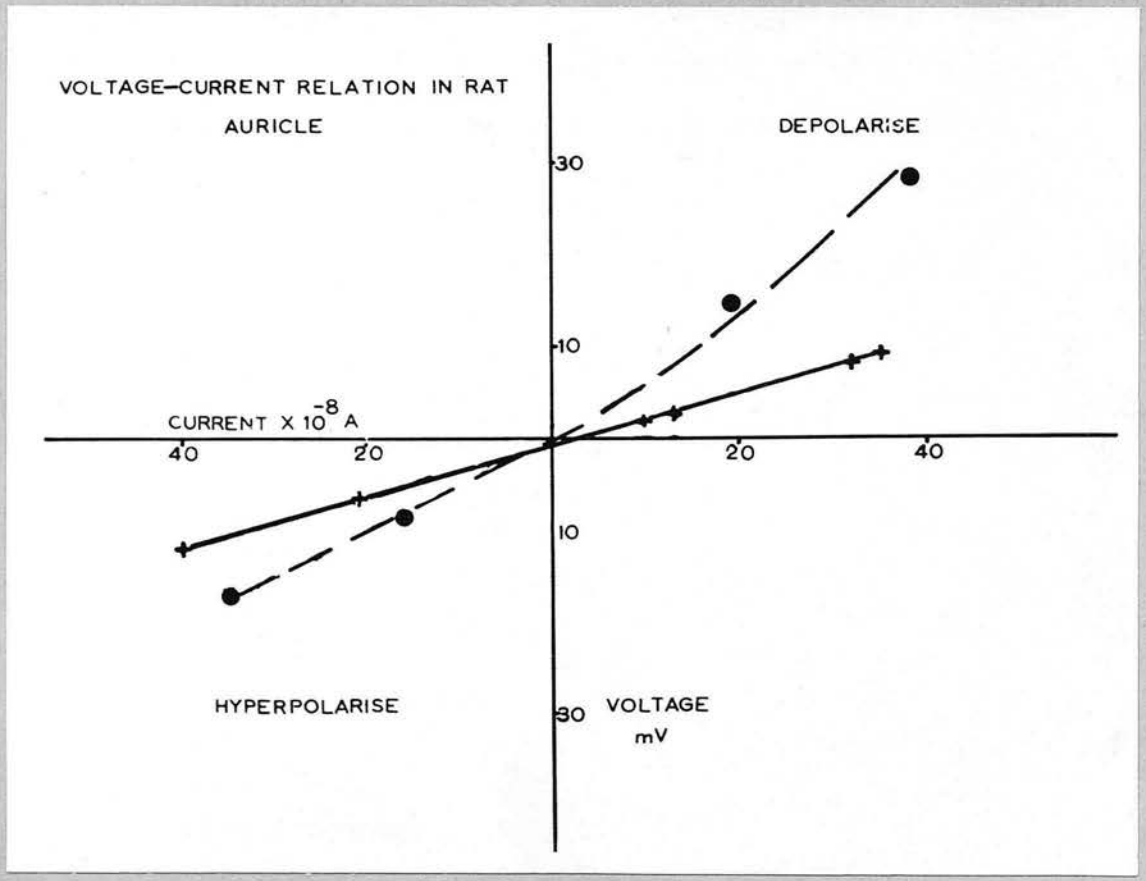



Fig. 29.

Changes in the $E_m$ caused by the application of polarising current in rat auricle. Each line represents the values obtained from one impalement. (Only two are shown, for clarity). In one of these the membrane response is non-linear on depolarisation, presumably due to a local response. Zero on the voltage scale represents the membrane potential, 76-80 mV.

## RESULTS.

### 1. Polarisation Experiments.

Fig. 27 shows typical electrotonic potentials produced by subthreshold pulses of current applied across the cell membrane by an intracellular electrode. In this experiment the same current was applied to hyperpolarise and to depolarise the membrane. It can be seen that the membrane response was greater on depolarisation, presumably due to the local response of the membrane. (This could be verified by repeating the experiment in a sodium free medium). The hyperpolarisation response was subtracted from the depolarisation response graphically and the difference, the local response of the membrane, is shown in Fig. 28.

Table IX presents the results taken from the records of this experiment, for two voltage recording positions. All the points used to calculate these figures are shown in Fig. 29. This figure shows the asymmetrical response of the membrane to applied currents in two of the experiments.

A general feature of these experiments was that high currents ($2 \times 10^{-7}$ A) were required to polarise the membrane, (as compared to sartorius) and that the value of resistance " facing the electrode " was very low (40,000 ohm) for cells of this size. It is very likely (Weidmann, 1952) that this is due to the short circuiting effect of the syncytium.

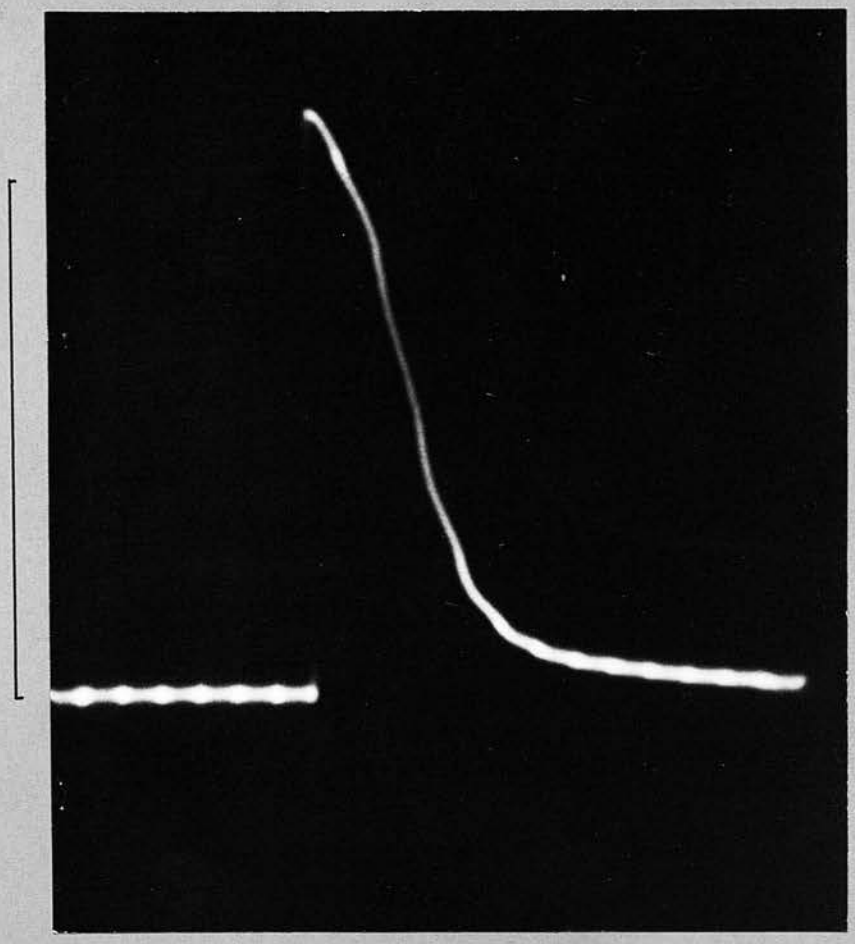


Fig. 30.

Rabbit auricular action potential, record taken during the polarisation experiments. The duration of this action potential (90%) was 100 msec, An exponential (with resting membrane CR) through the top of the action potential would last for about 40 msec. The voltage scale is 80 mV. and the blobs on the record are separated by 20 msec.

TABLE X.

Membrane characteristics for beating rabbit auricle, obtained with hyper-polarising pulses. Z calculated by dividing observed voltage change by applied current. Figures in brackets in column 1 indicate the number of observations made. All data obtained from same intracellular recording. Distance between electrodes 200 microns

| Position in cycle | Resting Potential | Time Constant | | Z $\times 10^4$ |
|---|---|---|---|---|
| | | On. | Off. | |
| Diastole (5) | 70 | 2.5 | 3.0 | 1.3 |
| Repolarisation (4) | 30 | - | - | 1.2 |

One successful experiment was performed on a spontaneously beating rabbit auricle. The results (Table 10) show that when the membrane has repolarised to 30 mV the resistance (Z) is not very different from that during diastole. This agrees with the results of Weidmann (1951) on Purkinje's tissue.

In Fig. 30 is shown an action potential from this heart about the same time the results in Table 10 were obtained. It shows quite clearly that the the rate of repolarisation of the membrane during the action potential (phase 3) is much slower than can be accounted for on the passive resting properties of the membrane.

## DISCUSSION.

The application of cable theory to cells demands that the cell be circular in cross section and have an infinite length. This situation can be approached in Purkinje's tissue, in which single cells run for appreciable distances without cross connections. In other parts of the heart the anatomical structure of the syncytium make it's application impossible, and therefore use can only be made of the time constant and the impedance of the membrane.

The present experiments are elementary and give only a rough indication of these quantities. The main difficulty was in seeing the electrodes to get them close enough. The experiments do confirm, on this tissue, that the action potential repolarises at a slower rate than the resting membrane(see Part I).

## SUMMARY.

1. Experiments have been performed to test a hypothesis proposed by A. M. Shanes to account for the action of 2-4-DNP and anoxia on the heart.

2. These experiments consisted of measurements of the intracellular concentrations of Na, K & Cl in rat auricle, with simultaneous recording of the electrical activity with intracellular electrodes. These measurements were made on auricles which were perfused with Tyrode or with Tyrode containing DNP, and driven at 1/second.

3. The extracellular space of the rat auricle was also measured. The value obtained by a sodium efflux technique was 24.8 %, and by the sucrose space was 25.1%.

4. After 15 minutes' perfusion with normal Tyrode, the intracellular levels of the ions are:- Na, 35; K, 140; and Cl, 43 m-equiv/kg fibre water, and the resting potential is 80 mV, the overshoot 32 mV and the duration (90%) 50 msec.

5. Auricles treated with Tyrode containing 13 umole/l. of DNP gain 8 m-equivs Na, and lose 25 m-equivs K and 17 m-equivs Cl per kg fibre water in 15 minutes. This also produces a drop in the resting potential of 10 mV, abolishes the overshoot and decreases the duration to 5 msec.

6. These results are consistent with the ionic hypothesis for the resting and action potentials, as developed for squid axon.

7. A new feature is that there is much more chloride in the cardiac cells than can be accounted for on the basis of passive distribution around the resting potential.

8. In experiments in which the chloride in the Tyrode was replaced with iodide, it was found that the cells accumulated the iodide to the same extent as chloride.

9. Possible hypothesis for this chloride distribution are that chloride is actively maintained within the cells or that it is passively distributed around some potential less than the resting potential. This latter hypothesis is possible because the auricles were driven in these experiments, but is made unlikely because quiescent auricles take up iodide to the same extent as driven auricles.

10. It therfore appears likely that there is an active transport system in heart which carries chloride into the cells.

11. Substitution experiments indicate that chloride only contributes a small proportion of the resting conductance, but about 50% of the conductance during certain parts of the action potential (phase 2).

12. A hypothesis for the action of DNP is proposed, as it is shewn that Shanes' hypothesis is not correct. This new hypothesis supposes that there is a large increase in the permeability to chloride under DNP and anoxia. Substitution experiments support this hypothesis, by showing that the effects of DNP and anoxia are much reduced if chloride is replaced by large anions. in the perfusing Tyrode.

13. It has been shown that, during the shortening produced by ACh, DNP and anoxia on the cardiac action potential, it is possible to set up sustained repetitive activity at rates of 1200-2000 per minute.by the application of a single extra stimulus during repolarisation of the action potential.

14. A hypothesis for the clinical conditions of flutter and fibrillation is proposed and discussed, with this phenomenon as it's basis.

15. A few measurements have been made of the time-constant of rat and rabbit atrial fibres with two intracellular electrodes. These show the presence of a local response on depolarisation, with no sign of delayed rectification. The time constants obtained were of the order of 2-4 msec.

16. Some evidence is presented showing the presence of a barrier to free diffusion some 130 $A^{o}$ from the active membrane of heart cells.

# APPENDIX

II The ionic distribution in other tissues.

The distribution of ions in myelenated nerve has been studied by Krnjevic (1955) and by Shanes (1957).

Krnjevic estimated the extracellular space by a sodium efflux method and was largely interested in the sodium and the potassium distribution. In order to reduce the $(Cl)_i$ to a low enough level to be passively distributed at an $E_m$ of 70 mV (with this value of space) this author had to assume that there was a large quantity of chloride in the inter-spaces of the nerve, thus making this phase highly hypertonic.

Shanes (1957, p 131) using desheathed toad sciatic nerves found an $E_K$ of 115, an $E_{Cl}$ of 38 and an $E_{Na}$ of -25 mV (the extracellular space was estimated by sodium efflux and also by sucrose, agreeing to 10%). As the resting potential of these fibres is known to be about 70 mV (Huxley & Stampfli, 1951), this means that none of these ions are passively distributed and a rather similar situation is present as in heart.

In rabbit leucocytes, Wilson & Manery (1949) found an $E_K$ of 82 mV, an $E_{Cl}$ of 12 mV and an $E_{Na}$ of -20 mV (worked out from their figures). The potential across the membrane (unknown) can only fit one of these and therfore the other two ions must be actively transported.

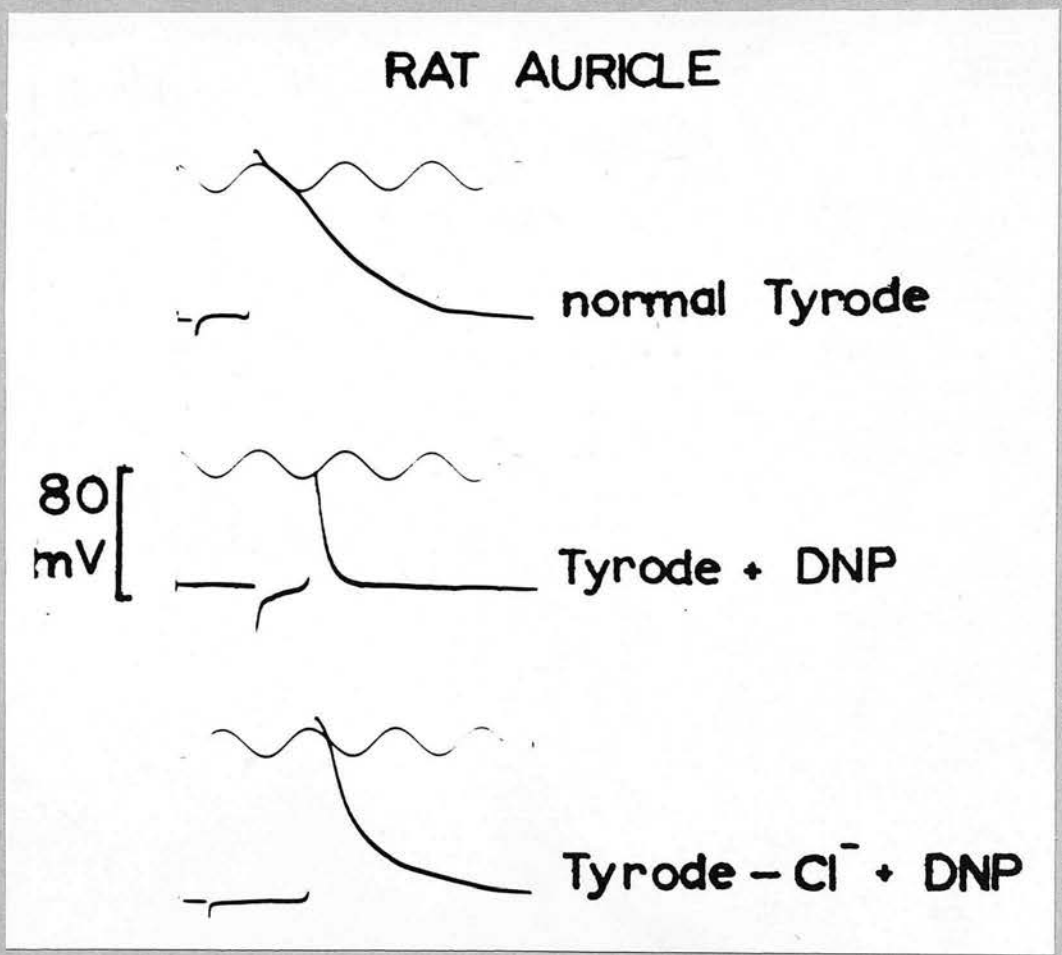



Fig. 31

Rat auricle, effect of DNP with and without chloride present. Top, normal action potential. Middle, normal Tyrode with DNP 13 moles. Bottom, same auricle in benzene sulphonate Tyrode and DNP 13 moles. Time scale, 50 cps. Voltage scale, 80 mV.

It seems, then, as though the picture of the ionic concentrations in the heart cells is not unique but is that found in several other tissues in the body. It should however be ppinted out (a) that in myelenated nerve the concentration of the ions in the actual myelin is unknown and therefore there is uncertainty about the intracellular concentrations of the ions, and (b) that in the leucocyte the membrane potential is unknown and therefore the electrochemical gradients cannot be determined. At the moment, then, it is only in the heart that the electrochemical gradients can be determined with any accuracy, and so this provides the best tissue to study this particular distribution of chloride.

III Extracellular space by sodium efflux.

The ratio of the rate constants of the slow and the fast parts of the curve (Fig. 3), $K_s/K_f$ is 0.142, which is almost the same found by Dainty & Krnjevic (1955). The correction applied was 25%, the same as that used by these authors.

From the regression equation, the intercept of the slow component on the Y axis is 1.439; which is equal to 27.5 m-equiv of Na per kg wet weight. This value was corrected to 28.6 m-equiv/kg wet wt. because of the waterlogging of the tissues which occurs with the choline washing. This corrected value was then reduced by 25% (see above) to give a final value of 21.4 m-equiv/kg wet wt. as the sodium in the cells at the start of the choline perfusion. As the total sodium in the tissue is 60.3 m-equiv/kg wet wt., that in the extracellular spaces is 38.9 m-equiv/kg wet wt. and the intracellular sodium constitutes about 35% of the total. (Johnson, 1957 found a figure of 31% for frog ventricle). If the sodium in the interspaces is at a concentration of 157.2 m-equiv/l (as in the Tyrode) then the size of the extracellular space is 24.8% (of wet wt.).

III Fall in resting potential during repetitive activity.

This analysis was carried out on the record shewn if Fig. 25.

The definitions and the measurements used for this analysis are as follows:-

t the time to the end of the first impulse at the start of the baseline rise (ie. between the 2nd & 3rd impulses in Fig.25) and equal to 30 msec.

$\tau$ the time constant at the end of the series of impulses, equal to 80 msec.

$(K)_o$ the normal level of external K, 2.7 mequ/l

$(K)_o^t$ the actual value of the external k at the end of time t, ie at the end of the first impulse.

At the end of the time t, there has been a rise in the baseline of 3.5 mV (ie. $E_m$ nearer zero potn.). This corresponds to a value of $(K)_o^t$ of 3.5 m-equiv/kg from the experimentally derived relationship for rabbit auricle, by Vaughan Williams (1959). Therefore the actual rise in the potassium near the active membrane - $(K)_o^1$ - is 0.8 m-equiv/l per impulse.

This value of $(K)_o^1$ is low because of the constant leakage away from the membrane, and a correction must be applied dependent on the time constant of the decay at the end of the series of impulses ( ).

The true value of the rise in the potassium level near the active membrane, in the absence of any leakage, is $(K)_o^1 \exp. t/\gamma$ per impulse. This gives a value of 1.17 m-equiv/litre.

Rayner & Weatherall (1959) found a value of 1.6 pmoles/cm$^2$ (ie. $10^{-12}$moles) as the efflux of potassium per impulse in rabbit auricles. If it is considered that a similar figure applies to the rat, then an efflux of this amount per impulse leads to an increase in the potassium near the active membrane of 1.17 m-equiv/l. and so the "space" into which the potassium is flowing can be calculated. If this space is the same in one plane as the membrane then the depth is given by:-

$$\frac{1.6 \times 10^{-12}/cm^2}{1.17 \times 10^{-6}/cm^3} = 137\ A^o$$

As alredy discussed this agrees reasonably well with the anatomical separation of the cells, and there is also some evidence that only the cells deep to the surface layer give this effect.

IV. The chloride content of heart cells.

Straightforward chemical analysis of rat auricles after soaking in Tyrode for 15 minutes, leads to an intracellular value (after correction for the chloride in the extracellular space) for chloride of about 40 m-equiv/kg fw., instead of the expected value of 7 for passive distribution about the $E_m$. On the other hand analysis of $Br^{82}$ efflux curves into normal Tyrode, leads to a result of 18 m-equiv /kg fw. Thus both methods give values greater than the expected value, but differ from each other to a considerable extent.

The efflux method is inherently the more accurate of the two, because it gives a direct estimate of the intracellular fraction of the chloride and is insensitive to the size of the extracellular space. It does have the disadvantage that one is assuming that the cells are treating bromide in the same way as chloride, and is not accumulating the bromide differentially.

The analytical method on the other hand is very sensitive to changes in the size of the extracellular space, and to any change of the concentration of an ion in that space, and is therefore less reliable for that reason.

It is probable, therefore, that the efflux method gives the more reliable estimate of the intracellular chloride. If this is so, then the $E_{Cl}$ is 67 mV (SE $\pm$2 mV), a value very significantly lower than the 80 mV of the normal $E_m$.

These analyses are helped by the effects of DNP on the chloride content of the auricles. For if chloride is passively distributed around the $E_m$ then, as the $E_m$ falls with DNP, the $(Cl)_i$, and therefore the total chloride, should rise. DNP, however, causes a significant fall of the chloride in the auricle, and so this supports the hypothesis that the $E_{Cl}$ is less than the $E_m$. It should be pointed out, however, that DNP may be altering the amount of chloride held in the extracellular space, and so this evidence is not final. The prelimnary experiments with DNP on $Br^{82}$ efflux also show that the intracellular chloride is reduced to about 7 m-equiv/kg fw; and if this turns out to be correct, then this is very strong evidence for the view that the $E_{Cl}$ is less than the $E_m$ in the normal cell.

V. Discussion of the electrophysiological findings in the light of the passive and active hypotheses for the distribution of chloride.

Hutter and Noble (1959) originally observed that replacing the chloride in Tyrode with the more permeant ion Iodide in sheep auricle, led to a fall in the $E_m$ and inexcitability. When these experiments were repeated with rat auricles no effect was observed with $(K)_o = 2.5$, but a drop in the $E_m$ & inexcitability when the potassium was raised to 10.8 mM. The easiest explanation of this phenomenon is to suppose that the $E_I$ and $E_{Cl}$ are less than the $E_m$ and that iodide stabilises the membrane at the $E_{Cl}$

It is difficult to see how this observation fits the hypothesis that chloride is passively distributed around the membrane potential, because one would expect that iodide would also become passively distributed and then not contribute to the membrane potential. It is unlikely that this is a toxic manifestation of iodide, as 1. the auricles recovered in normal Tyrode very quickly and 2. no ill effects were observed with the same iodide concentration but the $(K)_o \simeq 2.5$ instead of 10.5 mM.

The experiments showing that chloride carries a large part of the current during phase 2 of the action potential, cannot differentiate between chloride distributed around 67 mV or around 80 mV, because both are far removed from the potential during the plateau of the action potential.

DNP causes a drop in the excitability of heart cells and a decrease in the $E_m$ of some 10 mV. If the chloride of the Tyrode is replaced with benzene sulphonate either before or during the DNP treatment this fall in the excitability or decrease in the $E_m$ does not occur. These facts are difficult to reconcile with passive distribution of chloride around the $E_m$, because a passive ion should have no effect on the $E_m$; but are explicable if 1. the $E_{Cl}$ is less than the $E_m$ and 2. an increase in the $P_{Cl}$ occurs with DNP.

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