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# THE EFFECTS OF ANOXIA ON THE ISOLATED RAT PHRENIC-NERVE-DIAPHRAGM PREPARATION

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It has been postulated that anoxia directly and selectively impairs the active transport of sodium across the muscle fibre membrane (Awad & McDowall, 1952; Awad, 1953), and this was supposed to account for the behaviour of a phrenic-nerve-diaphragm preparation when it was made anoxic. The hypothesis was derived in part from the reported spontaneous occurrence of a neuromuscular block some time after a preparation had been allowed to recover from anoxia. This spontaneous failure was reversed by lowering the sodium concentration and enhanced by increasing it. High sodium concentrations were also reported to decrease the resistance of a preparation to anoxia, whilst low concentrations increased it.

The effects of anoxia were reinvestigated in order to examine further the relationship between anoxia and active sodium transport. The first effect of anoxia is to produce a neuromuscular block (Awad & McDowall, 1952; Awad, 1953; Ellis & Beckett, 1954) and this part of the earlier descriptions has been confirmed and extended to include quantitative estimates of some of the parameters involved. However, the spontaneous failure referred to above was never seen, and the response of a preparation to anoxia could not be shown to depend on the sodium concentration. The effects of anoxia can reasonably be explained without assuming any specific effect on the sodium transport mechanism.

An account of this work has previously been submitted to the University of London in part fulfilment of the requirements for the degree of Doctor of Philosophy.

## METHODS

The technique used involved continuously repeated maximal electrical stimulation of an isolated segment of rat diaphragm, either directly or through the attached phrenic nerve, at a frequency of 14–16/min, and has been described in detail in a previous paper (Paul, 1960).

Recording, when isometric, was effected by means of a movable anode valve (RCA 5734) and DC amplifier, with display by a pen-writing oscillograph. Maximum twitch tension was obtained when the muscle was held under a resting tension of about 5 g.

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Anoxia was produced by substituting a mixture of 5% $CO_2$ in nitrogen for the usual aerating mixture of 5% $CO_2$ in oxygen. This will subsequently be referred to as $N_2$-anoxia.

Increases in potassium concentration were made by adding suitable amounts of a molar solution of potassium chloride to the bath. The dilution of the other ions so produced was never more than 1%. For experiments with lowered potassium or altered sodium concentrations the bath solution was replaced with one specially prepared. Where appropriate the osmotic pressure was maintained with sucrose.

## RESULTS

### *The response to anoxia*

When an indirectly stimulated phrenic-nerve-diaphragm preparation is subjected to $N_2$-anoxia it responds in the characteristic way illustrated in Fig. 1. From the point at which $N_2$-anoxia was begun three stages can be seen. The first stage is short, and during this period no effect can be detected on the twitch height. This probably represents the time taken for the oxygen content of the bath solution and the tissue to be substantially replaced by nitrogen. The second stage is marked by a progressive increase in the twitch height, and is followed by the third stage during which the twitch declines rapidly until the muscle fails to respond to indirect stimuli. Increasing the stimulus strength does not elicit a contraction, but the muscle still responds to direct stimulation, although the

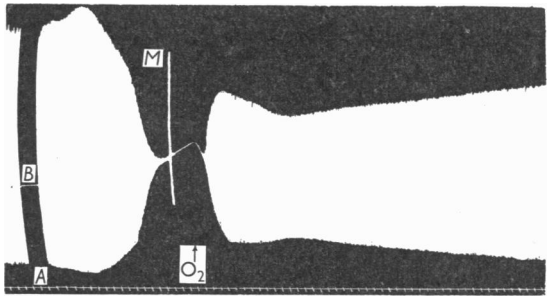


Fig. 1. Response of a rat phrenic-nerve-diaphragm preparation stimulated indirectly and maximally at 15/min to $N_2$-anoxia, and its subsequent recovery with oxygen. *A*, beginning of $N_2$-anoxia; *M*, response of the directly stimulated muscle; $O_2$, reintroduction of oxygen to the bath. Time marker, 1 min. Isotonic recording; *B*, base line, with muscle contraction upwards.

twitch obtained is not usually as big as that obtained before anoxia with indirect stimulation (see below, effects of anoxia on muscle fibres). It has previously been demonstrated that this failure of indirect excitability is not due to a block of conduction along the phrenic nerve (Awad, 1953) and the block must therefore be at the neuromuscular junction. Ellis & Becket (1954) reached the same conclusion. Action potentials can in fact be recorded from the phrenic nerve after indirect excitability has been lost.

The mean durations of the stages of anoxia in a series of forty-six experiments were, stage 1, 1·5 min ± 0·1 (S.E. of the mean); stage 2, 2·1 min ± 0·1; stage 3, 9·4 min ± 0·4. The time taken for a neuromuscular block to develop ($t_{NMB}$) is the sum of these three stages, and was 12·9 min ± 0·5.

Alterations in $t_{NMB}$ indicate alterations in the resistance of the preparation to anoxia if the experimental conditions remain the same.

There was a considerable variation in the individual observations, but when two preparations were made from the same diaphragm by using both left and right sides, under similar experimental conditions both preparations gave similar response times even though the individual twitch heights were often quite different. Two preparations from the same animal could therefore be used to compare the effects of different experimental conditions on the response to anoxia, one hemi-diaphragm being used as a control of the other. In this way it was found that neither eserine in concentrations of $2 \times 10^{-5}$ g/ml. nor calcium concentrations of 10–12 mM (between 4 and 5 times normal concentration) have any effect on the $t_{NMB}$.

All the results described so far were obtained with an isotonic recording system. Parallel experiments were done using an isometric recording technique, and the response was similar in all respects. The results of a typical experiment are shown in Fig. 2. Of particular interest is the demonstration that the increase in the twitch response during stage 2 is a genuine increase in the tension developed by the muscle.

The $t_{NMB}$ might be expected to depend on the rate at which oxygen is removed from the bath solution, and during $N_2$-anoxia this is determined mainly by the flow of nitrogen through the solution. Anoxia can be produced most rapidly by stopping the aeration and immediately emptying the bath, refilling it with an air-free solution, or with one previously equilibrated with nitrogen, and this in fact results in a shorter $t_{NMB}$, the first stage being negligible whilst the second and third stages are normal. Slow anoxia can be produced by simply cutting off the gas supply, in which case $t_{NMB}$ is considerably lengthened. In a group of eight such experiments $t_{NMB}$ was 53–150 min. For much of this time no effect on the twitch height was recorded, and obviously the oxygen dissolved in the bath is sufficient to maintain activity for some time. The rate of utilization of oxygen by the tissue might, in these circumstances, be expected to control $t_{NMB}$ to some extent, and on increasing it, by either doubling the rate of stimulation or by doubling the external work load, $t_{NMB}$ was approximately halved. An interesting observation in these experiments with slow anoxia was the absence of the increase in twitch height before a neuromuscular block occurred.

When the twitch height was recorded isotonically, approximately 50% of the muscles subjected to anoxia developed a contracture like that

shown in Fig. 1. Normally anoxia was not continued beyond the point at which a neuromuscular block developed, but a few experiments were done in which anoxia was prolonged, and in these cases the contracture continued to build up whether the muscle was stimulated or not, and eventually reached a maximum level which was some 2–3 times the normal twitch height.

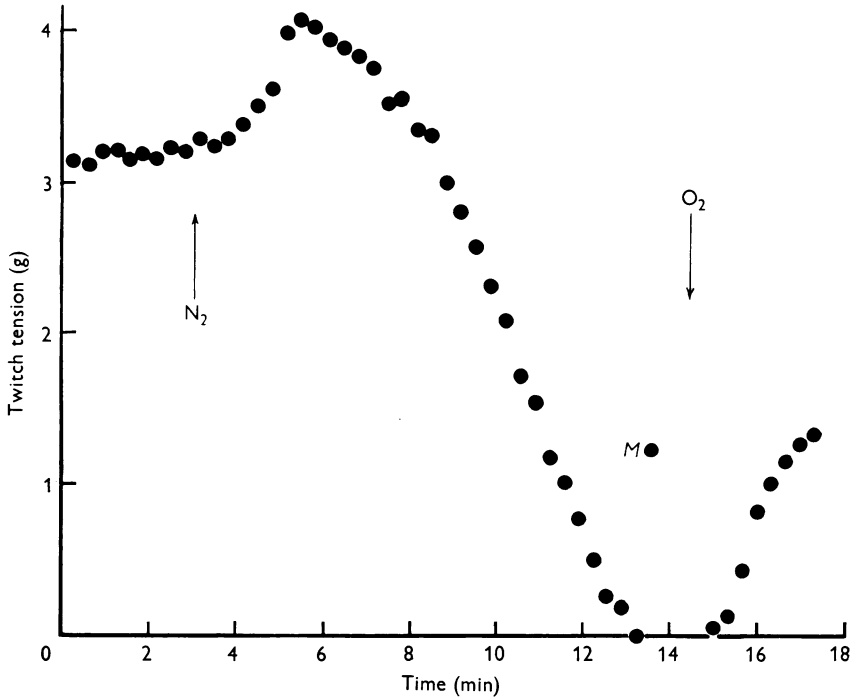


Fig. 2. Response to $N_2$-anoxia recorded isometrically. Each point represents the mean tension of 5 consecutive twitches. The muscle was indirectly stimulated at 15/min with maximal shocks. The muscle was held under a resting tension of 4·8 g throughout the experiment. $N_2$, beginning of $N_2$-anoxia; *M*, response of the directly stimulated muscle; $O_2$, reintroduction of oxygen to the bath.

When a contracture was obtained before a neuromuscular block occurred, the response of the directly stimulated muscle was often smaller than when no contracture was observed. There was no apparent correlation between the degree of contracture and the response of the directly stimulated muscle but a distinction could be made between preparations showing a contracture and those not doing so by comparing the directly elicited response with an arbitrary level of 2/3 the pre-anoxic twitch height. Compared to this level, 78 % of muscles with a contracture had given a smaller direct response, whilst 85 % of muscle without a contracture had given a greater direct response.

No contracture was observed when the twitch height was recorded isometrically, which suggests that this type of muscle shortening has only a limited capacity for performing work, as the muscle was normally held under a resting tension of about 5 g. It can be prevented from occurring under isotonic conditions by loading the lever; 5 g placed 2 cm from the lever fulcrum was normally sufficient. With this load an 'isotonic' twitch was still obtained.

*Effects of $N_2$-anoxia on the muscle fibres*

To assess effects of anoxia other than on the neuromuscular junction, neuromuscular transmission was eliminated by repeating the experiment on a curarized preparation. The response of a preparation which had been curarized with a concentration of $2 \times 10^{-5}$ g/ml. D-tubocurarine and was stimulated directly is shown in Fig. 3. The first two stages were similar in duration to those obtained from an indirectly stimulated preparation (cf. Fig. 1), but the muscle continued to respond to direct stimuli for some

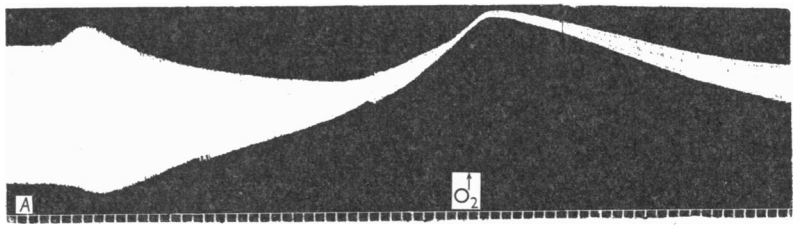


Fig. 3. The response of a curarized preparation to $N_2$-anoxia recorded isotonically, with muscle contraction upwards. The muscle was stimulated directly with maximal shocks at 15/min. *A*, beginning of $N_2$-anoxia; $O_2$, reintroduction of oxygen to the bath. Time marker, 1 min.

40 min before direct excitability was lost. In every similar experiment where a curarized muscle was made anoxic a contracture developed before the loss of direct excitability, provided that the twitch was recorded isotonically. Again no contracture occurred under isometric conditions. The appearance of a contracture seems to be associated with the effects of anoxia on the muscle fibre, and does not appear to be linked to the failure of neuromuscular transmission. During the period in which indirect excitability disappears, the muscle fibres will also be affected to some extent, and this explains why the response of the muscle stimulated directly after a neuromuscular block has appeared is usually smaller than the pre-anoxic twitch height. Furthermore, as the appearance of a contracture indicates that the muscle fibres are being affected, it is not surprising that the direct response is smaller when a contracture has been recorded.

*Recovery with oxygen*

The above results show that there are two factors to be considered in the anoxic response, the effect on the neuromuscular junction and the effect on the muscle fibres. It is therefore convenient to describe the recovery of a curarized preparation first, as muscle fibres only have to be considered.

*Recovery of a curarized preparation from $N_2$-anoxia.* Reintroducing oxygen into the bath brings about a recovery of direct excitability and a decline in the contracture. It can be seen from Fig. 3 that these two take place together. The initial rate of recovery is about 1 % per minute of the pre-anoxic twitch height, and this rate only gradually declines as maximum recovery is approached, which in curarized preparations amounts to about 55 % of the pre-anoxic twitch, and is reached after about 1½ hr.

*Recovery of a normal preparation from $N_2$-anoxia.* Reoxygenation of the bath solution relieves the neuromuscular block and restores the response to indirect stimulation. There is a time lag between restoring the oxygen supply and the reappearance of the twitch, analogous to the first stage of anoxia. The twitch normally appears within a minute of reoxygenation. Isometric recording gives a better measure of this delay, and in the experiment of Fig. 2 the delay was $> 44$ sec $< 48$ sec, the interval between successive stimuli being 4 sec.

The recovery of indirect excitability occurs in two stages. The rate of recovery is high initially and it then slows quickly and considerably. This is shown in Fig. 1. It will also be seen from this figure that the contracture is also reversed by oxygen, and this begins well before the twitch reappears. The decline of the contracture and the consequent alteration of the base line sometimes produces a pronounced hump in the trace, as shown in the figure. Normally the contracture begins to decline after about 10 sec reoxygenation, and there is no apparent link with the reversal of the neuromuscular block.

During recovery from $N_2$-anoxia 40 % recovery (as a percentage of pre-anoxic twitch height) was always obtained in under 10 min, the mean values being 42·2 % ± 3·5 (S.E. of 9) and 6·1 min ± 1·1 (S.E. of 9), and over the next 50 min recovery continued at a much slower rate, such that after 30 min it was 65·1 % ± 2·7 (S.E. of 17) and after 60 min 77·2 % ± 3·7 (S.E. of 11). The rate of recovery over the initial rapid phase varied in different preparations from 2·6 %/min to as much as 23 %/min. During the recovery period up to 30 min the *maximum* recorded rate was only 1·2 %/min, and over the next 30 min (to 1 hr recovery) it was never greater than 0·6 %/min. Maximum recovery rarely reaches 100 % of the pre-anoxic twitch height or tension, and in a second series of experiments it was found to be 77 % ± 5 (S.E. of 9) and was obtained in 64·5 min ± 4·7 (S.E. of 9).

Comparing the recovery of normal and curarized preparations, two points can be made. First, the rapid recovery phase only occurs after a neuromuscular block, and secondly, the rate of recovery of a curarized preparation is the same as the rate of recovery in the slow second stage of a normal preparation. This strongly suggests that the rapid recovery phase is due to the recovery of neuromuscular transmission, whilst the slower component is due to recovery of muscle fibres that had been affected by anoxia. To confirm this, some preparations were curarized after the peak of the rapid recovery had been passed. The response then obtained by direct stimulation of the muscle was equal in size to that previously obtained by indirect stimulation, showing that the ultimate restraint on recovery is a partial loss of direct excitability.

*Effects of further periods of anoxia*

Subjecting a preparation to $N_2$-anoxia for a second time after allowing it to recover from the first period produces a qualitatively similar response to that of the first period, but stages 1 and 3 are shorter in duration. The preparation recovers on reoxygenation in a similar fashion to that described above, and with some further reduction in the twitch height. When a second period of anoxia was begun 1 hr after recovery from the first period had been initiated (this interval being just sufficient for maximum recovery) stage 1 decreased from 1·5 min $\pm$ 0·1 (S.E. of 23) to 1·3 min $\pm$ 0·1 and stage 3 from 10·2 min $\pm$ 0·8 (S.E. of 23) to 1·7 $\pm$ 0·7. Both these decreases were significant (stage 1, $P = 0{\cdot}02$–$0{\cdot}01$; stage 2, $P < 0{\cdot}01$; by $t$ test).

Seventy-five per cent of the muscles made anoxic for the second time develop a contracture before a neuromuscular block, and the onset of contracture is more rapid than during the first period. The mean difference was about 2 min, which is just significant ($P = 0{\cdot}05$–$0{\cdot}02$).

A small number of preparations were subjected to a third period of anoxia and no particular difference was found between the response times of the second and third periods of anoxia except for a small decrease in the duration of stage 3, which was not significant. Again the preparations recovered when oxygen was reintroduced into the bath, but with a further reduction in final twitch height. These results show that the initial period of anoxia produces impairment of the preparation in two ways; the resistance to further periods of anoxia is reduced, and the twitch height after recovery is decreased. Subsequent periods of anoxia only reduce the twitch height further, having little effect on the resistance to anoxia.

*Effects of alterations in stimulation rate*

The rate of stimulation does not alter the response to anoxia appreciably until it is increased to about 1/sec. Figure 4 shows the response to anoxia of two hemi-diaphragms from the same animal when stimulated at various frequencies. The only deviation from the expected response was when stimulating at 64/min, when the $t_{NMB}$ was reduced by nearly 3 min. This reduction may have been due to the loss of potassium from the fibres and the subsequent decrease in membrane potential occurring at high rates of stimulation (Creese, Hashish & Scholes, 1958), which might summate with the similar effects of anoxia described by Creese, Scholes & Whalen (1958). The fact that a transient rise in twitch height was seen on increasing the stimulation rate to 64/min lends support to the suggestion that a substantial amount of potassium does accumulate in the interfibre spaces. No such increase in twitch height was seen when the stimulation rate was raised to 32/min only.

*Effects of potassium*

Anoxia causes the diaphragm to lose potassium (Creese, 1954) and increasing the external potassium concentration results in a lowered tissue potassium content (Paul, 1960). Therefore it was not surprising to find that the $t_{NMB}$ was shortened by increasing the external potassium concentration, and that decreasing the external potassium concentration had the opposite effect.

Potassium also affects the increase in twitch height during stage 2. The increase in height was less when the potassium concentration was increased, although no consistent effect was seen on lowering the potassium concentration, the peak height sometimes being increased but at other times unaltered. Figure 5 shows the effect of increasing the potassium concentration on both the pre-anoxic twitch height, and on the maximum height recorded during stage 2. When a small increase in potassium concentration no longer produced an increase in the pre-anoxic twitch height then no increase was observed during stage 2 either. This suggests that the increase in stage 2 is due to an increased potassium concentration in the extra-fibre spaces presumably due to losses from the fibres themselves.

The stage 2 response disappears or is very much reduced if anoxia is carried out at a lower temperature than normal. At 25° C no increase in twitch height is seen, and $t_{NMB}$ is very much longer than at 37° C. The absence of stage 2 is not due to any effect of temperature on the augmenting action of potassium, the response to increased potassium concentration being unaltered at 25° C.

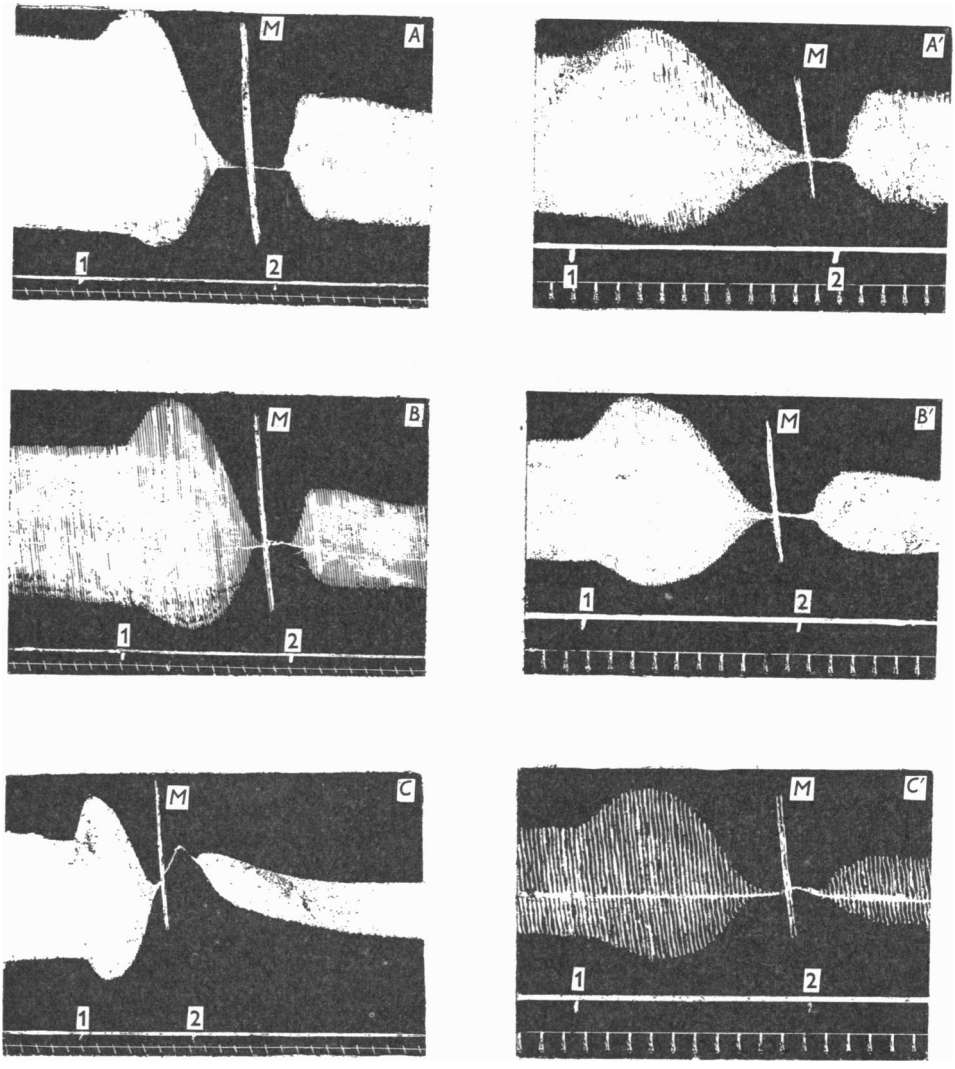


Fig. 4. The effect of variations in the stimulation frequency on the response to $N_2$-anoxia recorded isotonically. *A*, *B*, and *C* are the responses of one hemidiaphragm and *A'*, *B'* and *C'* are the responses of the other, both having been prepared from the same diaphragm. In each case $N_2$-anoxia was begun at signal 1, $O_2$ reintroduced at signal 2, and the muscle stimulated directly at *M*. Time marker, 1 min. Rates of stimulation were, *A*, 16/min, *B*, 8/min, *C*, 64/min; *A'*, 16/min, *B'*, 32/min, *C'*, 6/min. Stimulation was maximal and indirect.

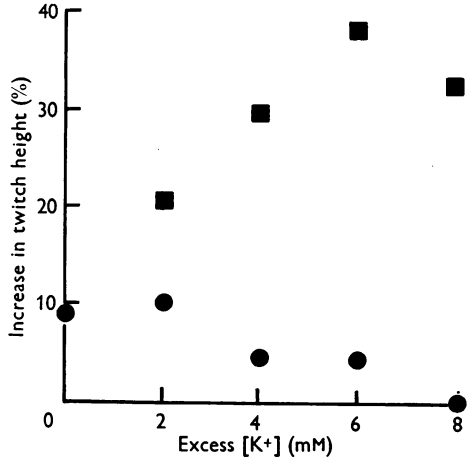


Fig. 5. The effect of excess [$K^+$] on the pre-anoxic twitch height, and on the maximum height recorded during stage 2 of the response to $N_2$-anoxia. The potassium concentration was raised, and its effect on the twitch height recorded for a few minutes before beginning the $N_2$-anoxia. ■, increase (%) in twitch height due to $K^+$ before anoxia. ●, increase (%) of the $K^+$-augmented twitch height during stage 2 of the response to $N_2$-anoxia.

### *The effects of sodium*

Varying the concentration of sodium in the bath between the limits 100–170 mM does not adversely affect the response of a diaphragm preparation. Indeed, between 100 and 125 mM sodium the twitch height usually increases. Below 100 mM the twitch height is reduced, and with less than 60 mM sodium both indirect and direct excitability are lost. Increasing the sodium concentration above 170 mM also depresses the twitch height, but this can be duplicated by sucrose and is apparently an osmotic effect.

Within the range 100–170 mM the sodium concentration did not affect $t_{NMB}$ in the manner reported by Awad (1953). The only positive effect was a rather poorer recovery than expected with 170 mM sodium in the bath.

## DISCUSSION

Anoxia abolishes both indirect and direct excitability in the rat diaphragm, but the development of a neuromuscular block spreads through the muscle more rapidly than the loss of contractability of the fibres. The first visible sign of anoxia is an increase in twitch tension developed by the muscle. This is an effect on the muscle fibres, and it was found to be complementary to the effect of potassium in increasing the twitch tension. It is probably caused by a leakage of potassium from the cells such as that

demonstrated to occur as a consequence of anoxia (Creese, 1954; Calkins, Taylor & Hastings, 1954). It has been suggested that the effect is analogous to post-tetanic potentiation (R. J. S. McDowall, personal communication) which is easily obtained in this preparation. Presumably this potassium leakage will take place at a rate depending on the speed at which oxygen is removed from the bath solution, and when this is rapid potassium loss will also be rapid, with potassium tending to accumulate in the extrafibre spaces, producing the observed increase in twitch tension. When the muscle is made anoxic more slowly, e.g. when the oxygen supply is simply turned off, the potassium leakage will then be slow and potassium might not accumulate in the extrafibre spaces to the same degree, with the result that no increase in twitch tension occurs. Ling & Gerard (1949) have demonstrated that potassium leakage from cells does not affect their membrane potential if it does not accumulate in the extrafibre spaces.

The increased twitch effect also disappears at 25° C. In this case the cells might be able to maintain their internal potassium concentration for a longer period, owing to a general slowing of metabolism tending to conserve anaerobically useful metabolites. The primary effect of anoxia must be to interfere with the normal aerobic metabolism of the cells. Ever since the work of Araki (1891) it has been recognized that anoxia is accompanied by the formation of large quantities of lactic acid, and more recently Calkins *et al.* (1954) have shown that rat diaphragm produces much lactic acid during anoxia, especially in the early stages, indicating that a reserve of material that can be metabolized anaerobically does exist.

As anoxia becomes more severe the twitch tension declines and indirectly stimulated preparations develop a neuromuscular block. How the block is produced is not known, but as neither eserine, which delays the destruction of acetyl choline after release, nor high calcium concentrations, which increase its release (del Castillo & Stark, 1952; Hutter & Kostial, 1954) have any effect on the $t_{NMB}$, it seems unlikely that impairment of acetylcholine release can be solely responsible, as it would be necessary to suppose that acetylcholine production stopped suddenly at a single end-plate. This appears unlikely in view of the work of Fatt & Katz (1952) showing the end-plate potential to be the summed result of a large number of acetylcholine quanta reacting with an equal number of sensitive sites.

Anoxia precipitates the presynaptic block associated with intermittent conduction that develops at high rates of stimulation (Krnjević & Miledi, 1959). The times given for the initiation of the effects of anoxia and for recovery with oxygen are similar to the times for stage 1 and for recovery reported in this paper, bearing in mind the forty-fold difference in stimulation rate. The effect of low temperature is also similar, preparations retaining their indirect excitability for longer during anoxia at low rates of

stimulation in both types of experiment. However, it is not certain that a presynaptic block does occur at the very low rate of stimulation used in this investigation, and there was no evidence of intermittent conduction during anoxia, the twitch height decreasing quite smoothly to zero.

A second possibility is that as the membrane potential falls during anoxia (Creese, Scholes *et al.* 1958) it may become too low for an impulse to be transmitted, but with the large electrical impulse still able to excite the muscle directly. In the frog the neuromuscular transmitter lowers the membrane potential of the end-plate to 10–20 mV negative (del Castillo & Katz, 1954), and, assuming a similar situation in rat diaphragm, as the falling membrane potential approaches this critical value, local current flow between the end-plate when depolarized by the transmitter and the adjacent areas of the fibre membrane may be inadequate to initiate a propagated action potential. In some fibres the membrane potential approaches zero after prolonged anoxia (Creese, Scholes *et al.* 1958) and it is probable that these fibres lose their direct excitability.

Direct excitability can remain when anoxia has abolished neuromuscular transmission because some muscle fibres of curarized preparations recover before neuromuscular transmission would be expected to reappear in a normal preparation. If the return of excitability depends on repolarization of the membrane, then these muscle fibres can be directly stimulated at a lower membrane potential than that necessary for neuromuscular transmission.

A hypothesis has been advanced that accounts for the effects of anoxia in terms of intracellular accumulation of sodium (Awad & McDowall, 1952; Awad, 1953), and was derived to account for a spontaneous neuromuscular block that appeared some time after a preparation had recovered from anoxia, its appearance being accelerated by high sodium concentrations and delayed or prevented by low sodium concentrations (Awad & McDowall, 1952). The hypothesis was extended by Awad (1953), who also attributed the primary anoxic neuromuscular block to accumulation of sodium within the cells. These observations could not be repeated during the present experiments; the spontaneous neuromuscular block did not appear even after 8 hr recovery.

It is likely that anoxia is accompanied by an intracellular accumulation of sodium, as the active elimination of sodium from the cells is supported by their metabolism, but this will be a consequence of the general metabolic stress and not a specific effect on active sodium transport. In fact, at low temperatures the muscle is more resistant to anoxia whilst at the same time sodium accumulates at a faster rate than at 37° C (Creese, 1954). If the intracellular sodium concentration was the deciding factor in the response to anoxia, this is the opposite of what might be expected to occur.

The failure of the twitch height to regain the pre-anoxic level during recovery must mean that some fibres are permanently damaged by anoxia. Dean (1940) found that a proportion of the potassium lost by a frog muscle during anoxia was not regained on reoxygenation, and he also concluded that some fibres fail to recover. A second effect of anoxia is to reduce the resistance of the remaining active fibres to subsequent exposure to anoxia. This is not progressive with each period of anoxia as is the increasing number of fibres failing to recover, but is almost completed during the first period of anoxia. It may be that certain enzyme systems are disrupted during anoxia and cannot be restored to normal on reoxygenation as the tissue is isolated. Interference with some enzyme systems has been demonstrated during anoxia. Greig & Gorier (1943) showed that cocarboxylase was dephosphorylated during anoxia, and destruction of enzymes during anoxia has been postulated by Fuhrman, Fuhrman & Field (1950) and Merrill, Lemley-Stone & Meneely (1957) in the rat myocardium. If an enzyme system is affected by anoxia the ability of the tissue to carry on anaerobic metabolism may be impaired also and its resistance to anoxia decreased.

The contracture that is associated with the effects of anoxia on the muscle fibres has been previously reported by Creese, Hashish *et al.* (1958) and it appears to be similar to other contractures such as those produced by potassium, drugs and constant electric currents (Kuffler, 1946) in that it arises in conditions where the membrane potential is very much reduced. It was also found by Kuffler that direct excitability was lost as the contracture developed and this is true of the rat diaphragm also. The appearance of the contracture is no doubt due to the membrane potential being reduced to the level at which the events leading to shortening of the contractile elements are initiated (see Katz, 1950).

## SUMMARY

1. The effects of anoxia produced by 5 % $CO_2$ in $N_2$ have been studied on the isolated rat phrenic-nerve-diaphragm preparation.

2. Anoxia first produces a rise in twitch tension, and this is followed by a loss of both indirect and direct excitability.

3. The rise in the twitch tension appears to be similar to that produced by an increase in the external potassium concentration.

4. Loss of direct excitability spreads more slowly than the neuromuscular block, and is accompanied by a contracture if the external load is small.

5. Preparations recover on reoxygenation, recovery of indirect excitability spreading more rapidly than that of direct excitability.

6. The loss of excitability during $N_2$-anoxia was accelerated by increased external $K^+$ concentration and by stimulation at rates greater than 1/sec; slowed by decreased external $K^+$ concentration and lowered temperature; but unaltered by variations in external sodium concentration and by stimulation at rates up to 30/min.

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