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THE EFFECTS OF OSMOTIC PRESSURE CHANGES ON THE SPONTANEOUS ACTIVITY AT MOTOR NERVE ENDINGS

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Fatt & Katz (1952) described a particularly striking effect on the frequency of the spontaneous miniature end-plate potentials (e.p.p.'s) of frog muscle, which resulted from changing the osmotic pressure of the external fluid. In one experiment, for example, a 50% increase in osmotic pressure resulted in a 45-fold rise in the frequency of the random potentials. A converse experiment was cited in which a 50% decrease in osmotic pressure reduced the frequency by a factor of more than 30.

Since the normal e.p.p. may be considered as a large, synchronized burst of miniature e.p.p.'s evoked by the nerve impulse (del Castillo & Katz, 1954 *a*, *b*), the study of an agent which induces large changes in the frequency of these random discharges was of interest. The nature of this osmotic effect has so far remained obscure, and in the present experiments an attempt has been made to determine if its size and time course are related to properties of the solute molecules and to the permeability of the nerve endings. The procedure was, therefore, to increase the molarity of the external fluid by adding substances like glycerol and ethanol, which are known to penetrate cell membranes easily, or alternatively like sucrose which may not penetrate at all (cf. Overton, 1902).

METHODS

Intracellular recording from the end-plate regions of fibres of the m. ext. long. dig. IV of frogs (*R. temporaria*) was used in all experiments (Fatt & Katz, 1951; del Castillo & Katz, 1954*a*). The nerve-muscle chamber was fitted with a convex lens across which the muscle was lightly stretched. This arrangement served to restrict the movement of muscle fibres which accompanied draining and refilling of the chamber. Thus, in some experiments it was possible to change the bathing fluid several times without withdrawing the microelectrode. In most experiments, however, readily identifiable fibres were chosen and the microelectrode was removed during changes of the fluid. Fibres having low initial frequencies of spontaneous potentials were selected for most experiments to facilitate counting experimentally increased rates and to reduce the error due to coincident potentials.

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All solutions contained prostigmine, 10^{-6} g/ml. In some experiments the calcium concentration of the Ringer's solution was reduced and magnesium was added, equal alterations being made to both the isotonic and hypertonic solutions. The sodium concentration was then altered to maintain the appropriate tonicity. The normal isotonic solution contained 116 mM-Na, 2·1 mM-K, 1·8 mM-Ca and 121·7 mM-Cl. The osmolarity of this solution is 0·225, and percentage changes in osmotic pressure refer to this value. NaCl, sucrose, glycerol and ethanol were the solutes employed for increasing osmotic pressure.

RESULTS

Effects on the frequency of the miniature e.p.p.'s

NaCl *and sucrose.* No difference was discernible in the present experiments between the effects of sucrose and NaCl, if equal increments in the osmolar concentrations of each were employed. The results obtained with these two solutes will be presented together. There was, however, sufficient variation within and between experiments to have disguised small disparities. Fig. 1 (circles) shows the results of an experiment in which the external fluid was replaced by one made 25 % hypertonic by the addition of sucrose (to a sucrose concentration of 0·056 m). The change from isotonic to hypertonic solution was made at zero time and the return to isotonic fluid took place 68 min later. Within 5 min of the increase in tonicity, the miniature e.p.p. frequency had increased by a factor larger than 10 and remained at the higher level for over an hour. The return to isotonic solution was followed by recovery to approximately the original frequency. In most cases, however, the 'hypertonic frequency' did not remain as constant as this. 'Hypertonic frequency' will be used as an abbreviation for 'frequency of the miniature e.p.p.'s in hypertonic solution'; 'isotonic frequency' will have the corresponding meaning.

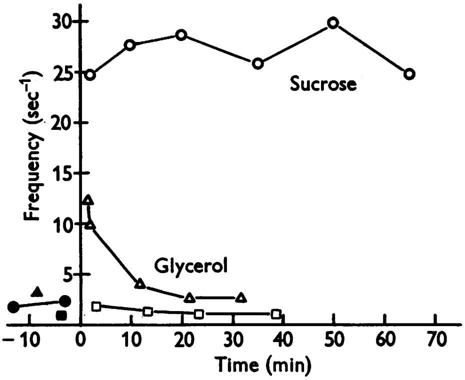

Fig. 1. The effect of hypertonic solutions on the frequency of the spontaneous miniature e.p.p.'s. Ordinate: average frequency in number of discharges per second. Abscissa: time in minutes after changing to the hypertonic solution. ○, test solution was 25 % hypertonic with added sucrose; □, △, test solution was 25 % hypertonic with glycerol: the three trials were performed sequentially in the order, □, ○, △. Solid symbols indicate isotonic medium: the solid circles represent the post-hypertonic control for the first glycerol trial, and the solid triangle the post-hypertonic control for the sucrose trial.

Fig. 2*a* and *b* show examples of the variation in 'hypertonic frequency' that was encountered. It was in these two experiments that the largest changes in frequency during the test period were found. The fact that the variations were sometimes so large can be at least partially explained as follows. A given increase in osmotic pressure seems to cause the frequency to increase by a certain multiple rather than by a given amount. The evidence for this contention will be considered below; but if that is true, it will be clear that any incidental frequency drift will be magnified during the period of increased discharge rate in the hypertonic solution. Consideration of a particular case should clarify this point. In the experiments of Fig. 2*a*, the lowest frequency measured after the return to isotonic solution was over twice the original 'isotonic frequency' (11 and 4·9/sec, respectively). One might therefore have expected a gradual rise in discharge rate during the hypertonic period of as much as 100%. The observed increase was about 65%.

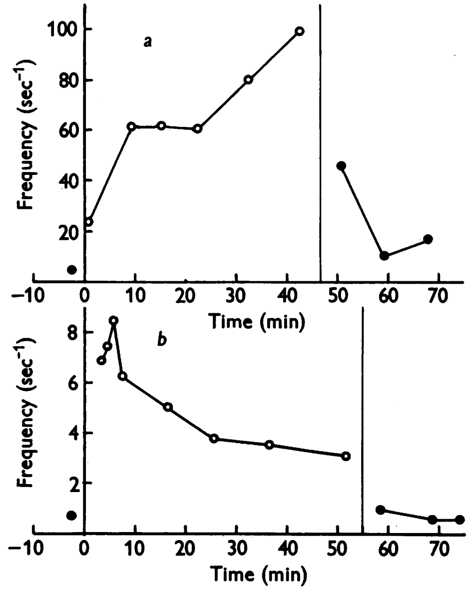

Fig. 2. Variations with time in the effect of hypertonic solutions on the frequency of the spontaneous discharges. In both experiments (on different muscles) the test solution was 26% hypertonic with sucrose. Ordinates, frequency of random potentials per second; abscissae, time after changing to hypertonic solution. At the vertical line, in each case, the preparation was returned to isotonic fluid: ○, hypertonic fluid; ●, isotonic fluid.

If the frequencies are plotted logarithmically, a better presentation is obtained; for then any constant, incidental drift produces a constant slope without being unduly magnified during the hypertonic period. In Fig. 3 *a* and *b* are plotted the results from nine experiments (on eight different preparations) in which the test solution was 25 or 26% hypertonic, using NaCl or sucrose.

The ordinates are the logarithm of the ratio of discharge frequency (f) to the initial 'isotonic frequency' (f_0); the abscissae, time in minutes. The initial controls in isotonic solution are not given since the value on the ordinate for each of them is zero. In Fig. 3b are shown the post-hypertonic control values for those experiments in which measurements were successfully made after returning to isotonic medium. The actual time at which the preparation was returned to the isotonic fluid is not shown but is given in each case as t = 0.

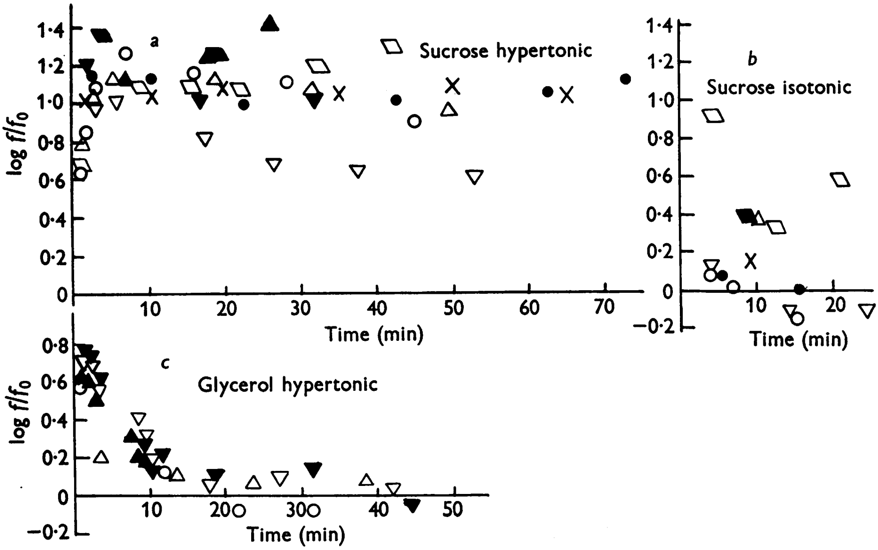

Fig. 3. Composite results of fourteen experiments. Ordinates, log ratio of discharge frequency to initial 'isotonic frequency'; abscissae, a and c, time in minutes after the change to hypertonic solution; b, after return to isotonic medium. a, nine experiments in which sucrose or NaCl was used to make the test solution 25 or 26% hypertonic; b, post-hypertonic controls for seven of the nine experiments shown in a. The actual time at which the preparation was returned to isotonic solution is not shown, but is given in each case as zero time. In four of the experiments in a and b (solid symbols), both control and test solutions contained 1·35 mM-Ca and 5·8 mM-Mg. c, test solution 25% (○, △) or 50% (▲ ▼ ▽) hypertonic with glycerol.

The use of the ratio f/f_0 simplifies comparisons between experiments and permits a test of the idea that a given hypertonicity multiplies the discharge frequency by a certain factor. The scatter of f/f_0 is large when measured at times after the application of the hypertonic solution greater than about 15 min. Values range from 4·1 to 26. If, however, one makes the comparison at some earlier time, there is considerable constancy among the different experiments. Measured within the period 5–10 min after the change to hypertonic solution, the values of f/f_0 were between 11 and 14 in seven of the nine experiments. (In the other two f/f_0 was in the vicinity of 20.) This degree of variation may be compared with the 27 times range of the initial 'isotonic frequencies' (f_0), from

0·18 to 4·9/sec. The increased scatter in f/f_0 at later times might be expected, for the chances of incidental disturbance must increase as time goes on. The presence of such incidental variations would not be surprising since the frequency of the miniature e.p.p.'s is quite labile, being considerably affected by such factors as temperature, stretch on the muscle, mechanical irritation and polarization level of the nerve endings. It may also be mentioned that on repetition of the experiment (△) more consistent results were obtained on the end-plate which showed the largest deviation from the others (▽). Thus it seems reasonable to conclude that this osmotic phenomenon involves a mechanism which, in effect, multiplies the 'isotonic frequency' by a certain factor.

A few additional experiments were made with other concentrations of NaCl and sucrose. In an experiment with 13% hypertonic solution (sucrose), all six values of 'hypertonic frequency' (over a period of 50 min) fell within $4 \times (f_0 \pm 1$ S.E.). In several experiments 50% hypertonic solutions were used and initial values for f/f_0 in the vicinity of 40–50 were obtained. These figures are not very reliable, however, since the counting error was large at these high frequencies and the subsequent time course of the effect was quite variable. Summarizing the results with NaCl and sucrose, a 25% increase in osmotic pressure typically evoked a 10–15 times increase in discharge frequency. Furthermore, although quite large variations were found in the 'hypertonic frequency', the latter always remained considerably above the original 'isotonic frequency' throughout the test period.

Glycerol and ethanol. The results were quite different, however, when either glycerol or ethanol was the added solute. In Fig. 1, the upper curve was obtained with sucrose, and the two lower curves (□, △) show the effect of adding glycerol. The three trials were made on the same end-plate, one with glycerol being made before, and one after, the sucrose trial. In each case the osmolarity of the test solution was 0·281 (25% hypertonic). In three other experiments, using test solutions made 50% hypertonic with glycerol, similar results were obtained. In each of them, after reaching a value of 4–6 during the first 2 min in the hypertonic solution, f/f_0 fell to less than 2 in about 10 min. After about 15 min, values of f/f_0 were all within the range of 0·85–1·3, and no single value was significantly different from unity. The five glycerol trials are represented in the semilogarithmic plot of Fig. 3*c*.

Several experiments were also made using ethanol to increase the osmotic pressure of the Ringer's solution. In three successful experiments (using 27, 50 and 55% hypertonic media) f/f_0 was not significantly different from unity within 5 min of the introduction of the test solutions. In several other experiments, maintained, or steadily increasing, augmentations in the discharge rate were observed in the ethanol solutions. In these cases, however, the frequency either failed to decrease upon returning to the isotonic solution, or this control value was not successfully obtained.

Effects on the size (*quantum content*) *of the e.p.p.*

In view of the large increase in spontaneous discharge frequency evoked by hypertonic NaCl and sucrose solutions, several experiments were made to determine if a corresponding increase in the size or quantum content (del Castillo & Katz, 1954*a*) of an e.p.p. response to a nerve impulse could also be detected. In Table 1 the results of five such experiments are shown. The test solution in each case was 25 % hypertonic. NaCl was the added solute in the first two experiments, sucrose was used in the last three. The Mg and Ca concentrations of the solutions were adjusted to give small values for *m* (the numbers of 'quanta' in the e.p.p.; del Castillo & Katz, 1954*a*), the adjustments being identical for both test and control solutions in any one experiment. The values of *m* were obtained by dividing the mean amplitude of the e.p.p. response by the mean amplitude of the spontaneous discharges, as measured in each solution. None of the differences, either between the amplitude of the e.p.p.'s or between the values of *m*, as determined in the two solutions, is statistically significant. It should be observed, however, that the S.E. given after each value is rather large. This is in part due to the wide fluctuation in the size of the e.p.p. observed at low values of *m* (del Castillo & Katz, 1954*a*). Thus, any small changes in *m* evoked by the hypertonic solutions would not have been detected. It is clear, however, that any change which may occur must be very much smaller than the concomitant change in the frequency of the spontaneous potentials. The frequency increased by a factor of 10–15, whereas the average change in *m* for the five experiments was an increase of about 8 %. It might be objected that the use of solutions with added Mg and reduced Ca could have prevented any large effect of increased osmotic pressure on the value of *m*. While this cannot be ruled out, it can be shown that similar alteration in these ions has no appreciable effect on the osmotic increase in spontaneous discharge rate. In four of the nine experiments represented in Fig. 3*a* and *b* (solid symbols), both isotonic and hypertonic solutions contained $\frac{3}{4}$ of the normal amount of Ca and 5·8 mM-Mg. In addition, one experiment

TABLE 1. The effect of hypertonic solutions on the average size and quantum content of the end-plate response to nerve stimulation (mean ± S.E.)

All test solutions 25 % hypertonic, by the addition of NaCl in expts. 1 and 2, and of sucrose in expts. 3–5. Expts. 4*a* and *b* were performed on the same fibre.

	Isotonic		Hypertonic	
Expt.	Size of e.p.p. (mV)	*m*	Size of e.p.p. (mV)	*m*
1	4·45±0·35	3·01±0·61	4·20±0·50	3·04±0·42
2	3·67±0·18	10·4 ±0·79	4·06±0·24	9·9 ±0·76
3	2·69±0·16	4·07±0·30	2·49±0·20	4·01±0·38
4*a*	3·86±0·28	3·30±0·25	3·60±0·19	3·79±0·26
4*b*	3·22±0·23	3·01±0·28	3·39±0·30	3·49±0·38
5	2·64±0·25	3·72±0·41	2·84±0·21	4·58±0·41

was performed using solutions with half the normal Ca and 14 mM-Mg. A value of f/f_0 greater than 10 was still obtained in this case with a 25% hypertonic test solution (sucrose).

DISCUSSION

The large augmentation of the frequency of the spontaneous miniature e.p.p.'s evoked by disproportionately small increases in osmolarity (using NaCl and sucrose) has been verified, and found to be fairly reproducible and reversible. With considerable variation among experiments, the average tendency was for the high frequency to be maintained or to fall slightly during hypertonic test periods ranging from 19 to 72 min, but only if sucrose or NaCl was the added solute. The comparatively small and transient effects seen during the application of glycerol and ethanol can readily be explained by assuming that these molecules enter the nerve terminals much more rapidly than sucrose or NaCl. That this is a reasonable assumption is, of course, supported by similar conclusions on the relative permeabilities of many cells to these substances (cf. Heilbrunn, 1952). The difference between the effects obtained with the two pairs of solutes indicates that the frequency increase depends upon permeability and probably on a volume change of the nerve terminals or of some structures within them, rather than, for example, on an increased rate of collision between non-specific solute molecules and the surface membranes of these structures. Further, it shows that the method employed is a feasible one for measuring the relative permeabilities of the motor nerve terminals to certain substances.

Perhaps the most interesting aspect of the increase of discharge rate in hypertonic solutions is its magnitude. A 40–50-fold rise in frequency resulted from increasing the osmotic pressure by 50%; and one can estimate that a 5–10% increase in osmotic pressure would double the discharge rate. There is not enough known of the anatomical basis of the quantal release of ACh to give a satisfactory explanation of this phenomenon, but certain types of speculation can be rejected. For example, suppose that some species of molecule, A, in solution within the nerve terminal must collide with an ACh quantum in order to release the latter. Then the osmotic loss of water from the terminal would increase the concentration of both A and the ACh quanta and raise the frequency of collisions. Such a mechanism would be insufficient, by itself, to explain the observed magnitude of the frequency increase, however. Even if half the internal water were lost from the nerve terminal (which would happen with an ideal osmometer in a 100% hypertonic solution), the frequency would tend to increase only by a factor of 4.

There is a type of mechanism which would allow very large frequency increases to occur, limited only by the number of available ACh quanta. Suppose that some property of the ACh release unit must have a value above a certain threshold in order for the unit to escape. Then if changes in osmotic pressure were to alter either the threshold level or the mean value of the specified property within the population of units, large numbers of the latter could then become available for release. The postulated property, for example, could be the diameter of a particle containing the ACh quantum (see del Castillo & Katz, 1956) and the threshold, the size of pores in the membrane. If the particles were bounded by semipermeable membranes, their variation in size with changes in osmotic pressure would be in the proper direction to explain the frequency increase in hypertonic solutions.

Another type of explanation might be sought in relation to the small rise in the resting potential of the motor nerve fibres which is to be expected in hypertonic solutions, owing to the effective increase in the concentration of internal K ions. This idea depends upon the observation that polarization of the nerve terminals can influence the spontaneous discharge rate (del Castillo & Katz, 1954*c*). The explanation seems unlikely, however, in view of several points of difference between the polarization and osmotic effects. Whereas a decrease in osmotic pressure reduces the discharge frequency (Fatt & Katz, 1952), either cathodic or anodic polarization (of sufficient intensity) evokes an increased rate of firing. The increase in discharge frequency accompanying cathodic polarization is inhibited by added Mg, but the osmotic increase in frequency seems not to be. The anodic polarization effect is apparently of quite a different character from the osmotic increase in firing rate, occurring quite suddenly and in bursts as the current strength was increased. Furthermore, the anodic effect was associated with a 'facilitation' of the e.p.p. response which, again, is not the case with the osmotic phenomenon. It seems as if the osmotic effect operates at a place in the pre-synaptic apparatus different from the site of action of polarizing currents.

SUMMARY

1. The effect was studied of increases in the osmotic pressure of the external fluid on the frequency of the spontaneous miniature end-plate potentials and on the size of e.p.p. responses at frog end-plates.

2. Several solutes were used to raise osmotic pressure. With sucrose or NaCl large, prolonged increases in the frequency of the random discharges were observed. A 25% increase in osmotic pressure typically evoked a 10–15-fold rise in discharge rate. The factor of increase tended to fall within these limits, although the initial rates observed in Ringer's solution varied over a 27-fold range.

3. When glycerol or ethanol were the added solutes, the increase in osmotic pressure produced only small and transient rises in the frequency of the miniature e.p.p.'s.

4. No corresponding increase in the size or quantum content of e.p.p. responses was observed when NaCl or sucrose was added to the Ringer's solution.

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