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EFFECT OF FREQUENCY OF ELECTRICAL STIMULATION ON THE CONCENTRATION OF INTERMEDIARY METABOLITES IN MAMMALIAN NON-MYELINATED FIBRES

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The present experiments are concerned with the effects of electrical stimulation on the concentrations of phosphate esters in non-myelinated nerve fibres. Phosphate esters of living cells serve as an intermediary step in the conversion of energy derived from the oxidation of foodstuffs into a form which the cells can use to carry out their function. For peripheral nerves this concept has been discussed by Gerard (1955). It has been difficult, however, to obtain evidence in support of the view from experiments performed on myelinated nerve fibres. For instance, Gerard & Tupikova (1939) apparently obtained decreases in the levels of creatine phosphate (CrP) and adenosine triphosphate (ATP) in these nerve fibres in the frog only with severe conditions of stimulation. The difficulty of demonstrating such decreases in myelinated nerve fibres as a result of electrical stimulation can be understood by considering the absolute size of the ion movements during impulse conduction, since the estimated change in sodium concentration per impulse is extremely small. On the other hand, in small non-myelinated fibres such as the C fibres of vertebrates, the estimated change in sodium concentration per impulse is several thousand times greater. These fibres, then, should have an enormously greater energy dissipation per impulse, in the form of downhill ion movements, than do myelinated A fibres, and should, therefore, require correspondingly greater energy to restore the ionic gradients which existed before the impulse conduction. Accordingly, a nerve bundle containing C fibres should provide a more suitable preparation than myelinated nerve fibres for the study of the role of ATP in the energetics of impulse conduction.

We have therefore examined the effect of nerve stimulation on the level of

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CrP and ATP, as well as some other metabolites, in nerve bundles from the rabbit vagus, since these are composed predominantly of such non-myelinated C fibres (Evans & Murray, 1954). Analysis of these compounds in the relatively small amount of tissue available in such nerve bundles was made possible by the development of highly sensitive analytical methods based on the combined use of enzymic and fluorimetric techniques (Greengard, 1956, 1958 and unpublished experiments). Our results show that the CrP and ATP content of these fibres is readily depleted by short periods of activity, suggesting that CrP and ATP serve as reservoirs of energy for the active restoration of the ion gradients existing before impulse conduction. A short account of this work has been published in the Proceedings of the Physiological Society (Greengard & Straub, 1957).

METHODS

Preparation and stimulation of nerve bundles. Adult lop-eared rabbits were anaesthetized with urethane given as a 25% (w/v) solution into the marginal ear vein (1·6 g/kg). The experiments were carried out at 21–24° C. From the two cervical vagi corresponding pieces were removed, cleaned under a dissecting microscope, cut to equal length (4–5 cm), mounted on platinum electrodes and shown to conduct impulses by monitoring the compound action potential. One vagus served as unstimulated control and the other was stimulated at 15 or 50/sec with stimuli supramaximal for C fibres. After a 15 sec period of stimulation the nerve, while still being stimulated, was plunged into a centrifuge tube containing 1·5 ml. of 0·1 M triethanolamine buffer at pH 8·1 which had been pre-heated to 100° C in a boiling water-bath. This procedure was used to eliminate any post-tetanic recovery period. After 40 sec the tube was cooled rapidly to 0° C and the contents were homogenized by means of a glass pestle ground to fit the lower part of the tube. Next, 1·5 ml. of alcohol-free chloroform was added to the homogenate, the tube stoppered with a ground-glass stopper, shaken vigorously for 3 min and centrifuged at 3000 *g* for 10 min. The clear supernatant was then removed for analysis of the intermediary metabolites. These procedures for the fixation and extraction of the nerves by heating at a neutral pH and treatment with chloroform are, with minor modifications, those previously described by Greengard, Brink & Colowick (1954). These procedures have two advantages over methods using precipitants such as trichloroacetic acid. First, the nature of extraction is more gentle and, second, there is less interference with the subsequent estimations of the intermediary metabolites, since these estimations are based on the use of suitably purified enzymes, the activity of which can be seriously inhibited by precipitants such as trichloroacetic acid.

Estimation of the intermediary metabolites. The clear supernatant was analysed for the following nine intermediary metabolites: CrP, ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), glucose-6-phosphate, 1,3-diphosphoglycerate, phosphoenolpyruvate, pyruvate and α-ketoglutarate, by the methods of enzymic fluorimetry. Two portions of different amounts were used for each estimate to be carried out on the supernatant from a nerve extract. The results of the two analyses usually agreed within 1–2%. When there was a discrepancy of greater than 4%, the results were discarded, and the estimation was repeated.

The principles of these methods as well as the analytical procedures used for the estimation of ATP, ADP, phosphoenolpyruvate, glucose-6-phosphate and pyruvate have already been described (Greengard, 1956, 1958). Some improvements in the assay procedures for these compounds as well as the methods used for the estimation of CrP, AMP, α-ketoglutarate and 1,3-diphosphoglycerate will be described in detail elsewhere.

CrP was estimated by the addition of creatine phosphate-ADP transphosphorylase to the system used for the assay of ATP; AMP was estimated by the addition of myokinase to the system used for the assay of ADP; α-ketoglutarate and 1,3-diphosphoglycerate were estimated by using glutamic dehydrogenase and 3-phosphoglyceraldehyde dehydrogenase, respectively.

Estimation of nerve mass. Although the analytical procedures used for estimating the amounts of metabolites in nerves were accurate, the manner of calculating the concentrations of metabolites presented a problem. The vagus nerve bundle, after cleaning and draining excess water had, on the average, a wet weight of about 9 mg. Both because of the possible damage caused by partially drying the vagus nerve with its delicate C fibre population, and because of the large percentage error involved in making a wet weight estimation of this small amount of tissue, a different procedure was adopted. For the present purpose the absolute weight of the nerve bundles was not of prime importance, but rather the relative amounts of tissue used for the control and stimulated bundles; therefore their length was used as a measure of the mass.

The average weight of the vagus nerves was found to be $2{\cdot}0 \pm 0{\cdot}1$ mg/cm length in eleven nerves prepared solely for the purpose of this measurement. Therefore, in order to express the values obtained for the phosphate compounds in mμmole/mg wet weight and thereby to facilitate comparison with results obtained by other workers, the experimentally determined concentrations of these compounds (mμmole/cm length) were multiplied by 0·50.

In the preliminary account published in the Proceedings of the Physiological Society (Greengard & Straub, 1957) not only the length of the nerve bundles but also their protein content was taken as the basis for calculating their relative mass. The results obtained on the effect of stimulation on the concentrations of intermediary metabolites were essentially the same when using the protein content of the nerve bundles as when using their length as the basis for calculation. However, since there was a doubt as to the reliability of the protein estimations, these results have been omitted from the present paper.

RESULTS

Of the nine different compounds examined, glucose-6-phosphate, 1, 3-diphosphoglycerate, phosphoenolpyruvate and α-ketoglutarate could not be detected in the nerve bundles whether extracted at rest or during electrical stimulation. If these substances had been present in a concentration higher than 0·03 mμmole/mg wet weight they should have been detected with the methods of estimation used.

Pyruvate was found in the nerve bundles. Its mean concentration in the unstimulated bundles was $0{\cdot}22 \pm 0{\cdot}02$ mμmole/mg wet weight, and, in the nerve bundles extracted during electrical stimulation, was $0{\cdot}23 \pm 0{\cdot}03$ mμmole/mg wet weight. Thus activity produced no significant change in the concentration of pyruvate.

The results obtained for CrP, ATP, ADP and AMP from nerve bundles extracted at rest are given in Tables 1 and 2. Table 1 gives the absolute mean values for 8–9 nerve bundles, and Table 2 gives the ratios of CrP, ADP and AMP to ATP. The figures in brackets in Table 2 are the ratios obtained from the values of Table 1; the other figures are the means of the ratios calculated for 16 individual nerves including the eight of Table 1.

When the nerves were extracted during electrical stimulation, and provided the frequency of stimulation was sufficiently high, the content of CrP and ATP decreased as is shown in Table 3. For each nerve extract the sum of the CrP plus ATP and the value for ATP were directly estimated; the value for CrP was obtained by difference.

The results given in Table 3 show that on electrical stimulation at 50/sec

there was a highly significant decrease by about 16 and 18% in CrP and ATP, respectively. With a stimulation frequency of 15/sec there was no change in the CrP level; the ATP level increased by 8% but this increase is not statistically significant.

TABLE 1. Concentrations of CrP, ATP, ADP and AMP, in resting cervical vagus nerves of rabbit, expressed as mμmole/mg fresh weight ±S.E.M. Means of eight, and in the case of CrP of nine experiments

CrP	1·96±0·28
ATP	2·11±0·24
ADP	0·28±0·02
AMP	0·08±0·01

TABLE 2. Concentrations of CrP, ADP and AMP, in resting cervical vagus nerves of rabbit, expressed as mole/mole of ATP±S.E.M. Means of sixteen experiments

CrP/ATP	0·94±0·07	(0·93)
ADP/ATP	0·14±0·01	(0·13)
AMP/ATP	0·04±0·01	(0·04)

TABLE 3. Effect of electrical stimulation on the levels of CrP and ATP in rabbit cervical vagus nerves. The mean values are the concentration in the stimulated nerves as percentage of that in the resting contralateral nerves. The values have been calculated with respect to equal lengths of resting and stimulated nerves. The data were analysed by the t test and the probability of obtaining t by chance is given by P

Stimulation frequency (per sec)	No. of expts.	CrP + ATP		ATP		CrP	
		Mean±S.E.	P	Mean±S.E.	P	Mean±S.E.	P
15	5	105·6±6·1	0·4 – 0·5	107·9±4·0	0·1 – 0·2	101·5±9·9	0·8 – 0·9
50	8	82·7±3·0*	<0·001	82·0±2·4	<0·001	84·4±4·3	0·001 – 0·01

* Nine experiments.

The decreases in CrP and ATP with a stimulation frequency of 50/sec represent minimal values for the phosphate changes in the nerve fibres because of dilution by the surrounding connective tissue, the phosphate content of which is unlikely to take part in these changes. Since the maximal physiological frequency of firing of non-myelinated autonomic nerve fibres is about 10/sec (Folkow, 1952; Douglas & Ritchie, 1957), our results with stimulation at a frequency of 15/sec accord with the expectation that resynthesis is capable of coping with energy demands at physiological frequencies, at least during short periods of activity.

In nerves which were extracted during electrical stimulation at a frequency of 50/sec the ADP and AMP content was found to be unchanged in six experiments, but in two it increased sufficiently to account for a great part of the break-down of ATP. The failure to obtain an increase in ADP and AMP in the other experiments is explicable in terms of further degradation of these esters. The results of the two positive experiments were practically identical and the results of one of these are given in Table 4. The ADP increased by 1·97 and the AMP by 0·18 mμmole upon stimulation, whilst the ATP decreased by 2·66 mμmole. The total adenosine nucleotide content was almost unchanged;

it agreed within 4% in the stimulated and resting nerves. Although the absolute concentration changes for ADP and ATP were of the same order, the percentage change in ADP, on account of its lower concentration in the resting nerve, was much greater than in ATP.

TABLE 4. Recovery of ATP as ADP and AMP in cervical vagus nerve stimulated at 50/sec for 15 sec. The resting values were obtained from an equal length of the contralateral nerve. Values expressed as mμmole per nerve

	Rest	Stimulated	Absolute change	Content of stimulated nerve (as % of that of resting nerve)
ATP	12·95	10·29	−2·66	79·4
ADP	0·32	2·29	+1·97	716·0
AMP	0·13	0·31	+0·18	238·0
Total	13·40	12·89	−0·51	96·2

DISCUSSION

Our results show that, in non-myelinated small C fibres, short periods of activity, provided the frequency of stimulation is sufficiently high, cause a decrease in CrP and ATP content. A concomitant increase in ADP and AMP could only be demonstrated in two out of eight experiments. However, the problem of the pathway of ATP break-down is complicated by the possibility of further degradation of the initial break-down products. For example, if ADP is the initial break-down product, it might quickly be dephosphorylated to AMP, which might then be deaminated to inosine monophosphate. This could explain the fact that, in the majority of our experiments, the loss of ATP was not associated with an increased concentration of ADP and AMP.

The changes in CrP and ATP content regularly observed in non-myelinated nerves during activity are unlikely to be associated with the action potential itself, since during the action potential the movements of Na^+ ions into and of K^+ ions out of the axons are down electrochemical gradients. Thermodynamically, therefore, these movements require no source of chemical energy (Hodgkin, 1951). However, after the impulse the nerve fibres are left with an excess of Na^+ and a deficit of K^+ and, since the flow of Na^+ out of the axons and of K^+ back into the axons, necessary to restore the nerve fibres to their initial states, requires movement of these ions against their electrochemical gradients, it is in this recovery process that chemical energy must be utilized (cf. Hodgkin & Keynes, 1955).

Recently Caldwell & Keynes (1957) have shown that, in giant axons previously poisoned with cyanide, injection of ATP into the axons results in an increased extrusion of Na^+. On the assumption that ATP is similarly involved in the extrusion of sodium ions which have entered nerve fibres during an impulse, the changes in phosphate esters of C fibres observed in the present experiments can readily be related to the ion movements involved per impulse.

In a non-myelinated nerve fibre, the average increase per impulse in sodium concentration ΔC, which equals the average decrease per impulse in potassium concentration, is given by the expression

$$\Delta C = \frac{Q \times A}{V} = \frac{Q \times \Pi d l}{\frac{1}{4}\Pi d^2 l} = \frac{4Q}{d},$$

where ΔC is the concentration change in mole/ml.,

Q is the influx of sodium or efflux of potassium in mole/impulse.cm^2 surface area,

A is the surface area of the fibre in cm^2,

V is the volume of the fibre in cm^3,

d is the diameter in cm, and

l the length of the fibre in cm.

We have estimated that for C fibres, $Q = 5 \times 10^{-12}$ mole/cm^2.impulse at 24° C (Greengard & Straub, 1958), in fair agreement with the values obtained for giant axons. Thus

$$\Delta C \text{ (mole/ml.)} = \frac{20 \times 10^{-12}}{d \text{ (cm)}}.$$

By expressing ΔC in mM and d in microns, we obtain the numerical relationship

$$\Delta C \text{ (mM)} = \frac{0{\cdot}2}{d\ (\mu)}.$$

Thus for a fibre of 0·5 μ diameter ΔC = 0·4 mM; that is, there will be an 0·4 mM increase in sodium concentration and an equal decrease in potassium concentration for each impulse. Our estimates of ATP and of CrP for these fibres were 2·1 and 2·0 m-mole/kg respectively. Thus, if no resynthesis were to take place, and if one molecule of ATP were utilized for each sodium ion extruded, then recovery from about five impulses would deplete the entire store of ATP and recovery from five additional impulses would deplete the CrP reservoir as well. Two considerations which suggest that a somewhat larger number of impulses than that just calculated might be required to deplete the phosphate esters of the nerve fibres are the following. First, on thermodynamic grounds, it is possible that as many as four or five sodium ions might be extruded per molecule of ATP.

The free energy change, ΔG, for the extrusion of 1 g.ion of Na is given by the expression

$$\Delta G = RT \ln \frac{[\mathrm{Na_o}]}{[\mathrm{Na_i}]} + FE_r$$

where R is the molar gas constant,

T the absolute temperature, $[\mathrm{Na_o}]$ and $[\mathrm{Na_i}]$ the outside and inside concentrations of sodium, F Faraday's constant and E_r the resting membrane potential.

R = 1·986 cal/degree and F = 23,050 cal/mole.V. In the conditions of our experiments, T = 296° absolute and $[\mathrm{Na_o}]$ = 154 mM. Assuming the values $[\mathrm{Na_i}]$ = 40 mM (Krnjevic, 1955) and

E_r = 0·080 volts, we calculate that ΔG = 2635 cal. Burton (1958), citing the work of T. Benzinger, R. Hems, K. Burton & C. Kitzinger (in preparation), has recently given as the most reliable estimate for the standard free energy of hydrolysis of ATP a value of −8400 calories. Taking into account the concentrations obtained in the present experiments, ATP 2·1 and ADP 0·3 mM, and using a value of 7·0 mM for inorganic phosphate, found for several other nervous tissues (Rossiter, 1955), the free energy of hydrolysis of ATP in these fibres becomes −12,450 cal. Thus one Na^+ ion extruded per molecule of ATP would represent an efficiency of about 21 % and 4·7 Na^+ ions extruded per molecule of ATP an efficiency of 100 %.

Secondly, if only 60 % of the nerve bundle were intracellular space (Manery & Hastings, 1939), the average intracellular concentration of ATP and of CrP would be nearly twice as high as the value in m-mole/kg whole nerve. Even taking these two considerations into account, however, the calculations for the ion movements involved per impulse in C fibres indicate that not more than a few dozen impulses would be adequate, in the absence of resynthesis, to deplete the known high-energy phosphate reservoirs of non-myelinated nerve fibres.

Calculation of the ion movements per impulse in large myelinated fibres such as the A fibres of the frog sciatic nerve explains why it is so much more difficult to detect, in these nerves, a decrease in CrP and ATP content as a result of stimulation. First, Hodgkin (1951) has estimated that, owing to the presence of the insulating myelin layer, a myelinated fibre needs to exchange only about 1/300 as much sodium in order to conduct an impulse as a non-myelinated fibre of equal diameter. Secondly, in non-myelinated fibres the change in Na^+ and K^+ concentration per impulse is, presumably, inversely proportional to the fibre diameter, so that a 0·5 μ non-myelinated fibre should have a 20 times greater change in concentration of Na^+ and K^+ per impulse than a 10 μ non-myelinated fibre. Therefore it would appear that the average increase per impulse in Na^+ concentration in a 10 μ myelinated fibre would be only about $1/300 \times 1/20 = 1/6{,}000$ of that in an 0·5 μ non-myelinated fibre. If the 10 μ myelinated fibre were representative of the fibres in the frog sciatic nerves used in the experiments of Gerard & Tupikova (1939) and the 0·5 μ non-myelinated fibre representative of the fibres in rabbit vagus nerves used in the present experiments, then the rate of energy dissipation per unit volume of axon would have been 3000 times greater in our experiments, in which the frequency of stimulation was 50/sec, than in the experiments of Gerard & Tupikova in which the frequency of stimulation was 100/sec. Therefore, in our experiments the rate of utilization of chemical energy in restoring the ionic gradients should have been enormously greater, and with it the possibility of demonstrating a decrease in the content of high-energy phosphate esters, since such a decrease depends upon utilization of these esters at a greater rate than they can be resynthesized. Obviously, when the rate of resynthesis of these phosphate esters keeps up with their break-down no decrease in their content can be expected. This probably happened when the C fibres were stimulated at a frequency of 15/sec and no decrease of CrP and ATP was observed.

It is interesting, in this connexion, that our nerve fibre preparations maintained unimpaired spikes for the entire 15 sec period of stimulation at the lower frequency of stimulation, but invariably showed partial failure of the compound action potential when stimulated at 50/sec for 15 sec. This failure can be explained by the decrease in the level of high-energy phosphate, following which the nerve fibres could be expected to accumulate sodium and lose potassium to the point at which they are no longer able to conduct impulses. In our experiments the time which elapsed between the onset of stimulation and the fixation of the nerve bundles was 15 sec. The observed decrease of CrP and ATP in these experiments shows that the utilization of the energy associated with the break-down of these compounds must have occurred within these 15 sec. This is not surprising since a quick utilization of chemical energy would be expected from the results on giant axons in which extra sodium injected into the axons began to be extruded with a time lag of at most a few seconds (Hodgkin & Keynes, 1956), and also from the results on the positive after-potential in mammalian C fibres which follows the action potential within a few hundred milliseconds and which is probably a sign of increased extrusion of sodium ions (Greengard & Straub, 1958).

SUMMARY

1. The effect of electrical stimulation on the concentrations of intermediary metabolites has been studied in the rabbit cervical vagus nerve, which is composed primarily of C fibres.

2. Glucose-6-phosphate, 1,3-diphosphoglycerate, phosphoenolpyruvate and α-ketoglutarate could not be detected in either resting or stimulated nerves, the limit of sensitivity of the analytical methods being 0·03 mμmole/mg wet weight.

3. Pyruvate was present in about 0·2 mμmole/mg wet weight in both the resting and stimulated nerve bundles.

4. The concentration of CrP as well as of ATP in resting nerve bundles was about 2·0 mμmole/mg wet weight. During stimulation at 50/sec for 15 sec there was a decrease of about 17% in these two esters.

5. In two out of eight experiments the decrease of ATP could be accounted for by the formation of ADP and AMP.

6. The relative ease of depleting CrP and ATP in C fibres, compared with A fibres, is explained in terms of greater energy requirements for reversing the much larger changes in concentrations of sodium and potassium per impulse calculated to occur in the C fibres.

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