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ON THE METABOLIC BASIS OF NERVOUS ACTIVITY

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SUMMARY

1. A study has been made of the metabolic substrates that can support the active extrusion of sodium ions from mammalian non-myelinated nerve fibres. The post-tetanic hyperpolarization obtained in chloride-free Locke solution, which reflects the electrogenic component of the sodium pump, was used as the index of metabolic activity.

2. When glucose was removed from the Locke solution there was no immediate change in the size of the post-tetanic hyperpolarization.

3. However, glucose is essential for metabolism in nerve fibres; for when a competitive inhibitor, deoxy-D-glucose, was added to the Locke solution the post-tetanic response was much reduced or abolished. Larger concentrations of deoxy-D-glucose were required in the presence of glucose than in its absence.

4. This effect of deoxy-D-glucose could be reversed by glucose, fructose, pyruvate and acetate.

5. The depressant effect of deoxy-D-glucose was enhanced by oxaloacetate and by malate.

6. Malonate, a competitive inhibitor of the conversion of succinate to fumarate, reduced or abolished the post-tetanic hyperpolarization.

7. This effect of malonate could be overcome by glucose and by pyruvate.

INTRODUCTION

During the passage of an action potential in nerve sodium ions enter the axoplasm and potassium ions leave as a result of the passive flow of these cations down their electrochemical gradients. The ionic balance, which has been thus disturbed, is restored during the subsequent period of recovery by a process that necessarily involves the expenditure of energy. This process has been extensively studied in recent years in mammalian non-myelinated C fibres, which are particularly well-suited for such an

examination because of the large amount of nerve membrane per gram of nerve (Keynes & Ritchie, 1965). The operation of this mechanism is associated with an increased oxygen consumption of the fibres (Ritchie, 1967; Rang & Ritchie, 1968*a*), a recovery heat production (Howarth, Keynes & Ritchie, 1968), and a hyperpolarization of the fibres (Ritchie & Straub, 1957; Rang & Ritchie, 1968*b*). Greengard & Straub (1962) have used the post-tetanic hyperpolarization to study the various substrates that support the metabolism associated with sodium extrusion in nerve. The accuracy of their experiments was, however, necessarily limited by the fact that they were using mammalian sympathetic non-myelinated fibres, bathed in Locke solution of normal composition, and the post-tetanic hyperpolarizations recorded were small (usually 2–3 mV). Recently, Rang & Ritchie (1968*b*) have shown that in mammalian non-myelinated fibres the post-tetanic response is greatly increased (up to 35 mV) when the chloride ions in the solution are replaced by impermeant anions such as isethionate. This effect was attributed to the operation of an electrogenic sodium pump that is normally short-circuited by the chloride ions in the medium. It seemed worth while, therefore, to re-examine some of the points studied by Greengard & Straub (1962) in their original experiments using the much larger post-tetanic hyperpolarization measured in chloride-free solution.

METHODS

Adult rabbits (5–6 kg) were killed by injection of air into an ear vein. Each cervical vagus was rapidly removed, de-sheathed under a dissecting microscope (× 40), and mounted in a sucrose-gap apparatus (Stämpfli, 1954; Straub, 1957; Armett & Ritchie, 1960) to record the resting and action potentials. The solution bathing the nerve could be rapidly changed with relatively little electrical artifact. The electrical potentials were recorded by means of calomel electrodes connected to a cathode follower, using an oscilloscope to monitor action potentials, and a fast potentiometric recorder (Varian 2000) to record the slow potential changes that followed a period of electrical activity.

The Locke solution contained (mM): sodium isethionate, 154; K_2SO_4, 2·8; $CaSO_4$, 5·0; Tris (hydroxymethyl) aminomethane (Tris, brought to pH 7·2 with sulphuric acid), 2·5; D-glucose, 5·0.

The post-tetanic responses were nearly always recorded after stimulating at a frequency of 30/sec for 5 sec, using supramaximal stimuli of 0·5 or 1 msec duration applied through platinum electrodes surrounding the nerve about 4 mm from the Locke–sucrose recording interface. In the experiments of Fig. 3 the frequency of stimulation was 50/sec.

All experiments were carried out at room temperature (20–24° C). Wherever possible means ± S.E. are given.

RESULTS

The effect of removing glucose

One of the main findings of Greengard & Straub (1962) was that removing the glucose from the Locke solution rapidly decreased the size of the post-tetanic hyperpolarization in about half of their preparations. This finding suggested an immediate dependence of the sodium pump on an external supply of glucose in the sympathetic C fibres that they studied. No such

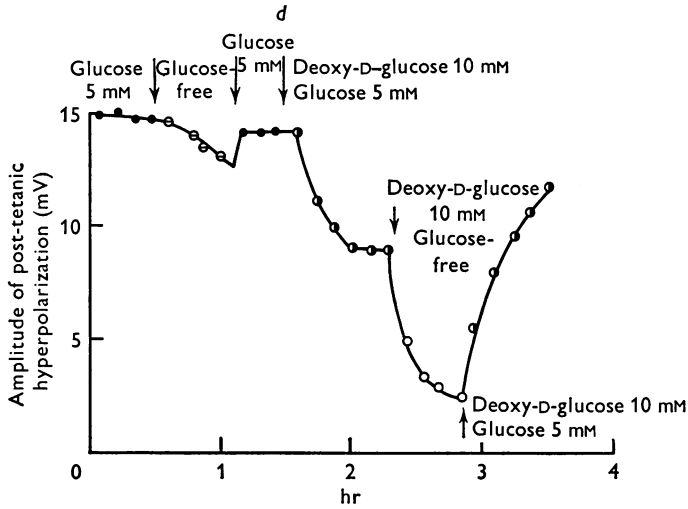

Fig. 1. The effect of deoxy-D-glucose on the amplitude of the post-tetanic hyperpolarization of the non-myelinated fibres of a rabbit de-sheathed vagus nerve. The Locke solution contained: ●, glucose 5 mM; ⊖, no glucose; ◑, deoxy-D-glucose 10 mM and glucose 5 mM; ○, deoxy-D-glucose 10 mM in the absence of glucose. *d* marks the beginning of exposure to deoxy-D-glucose.

dependence on glucose was found in the present experiments on vagus nerves. In ten tests in which the Locke solution was replaced by a glucose-free Locke solution, exposure of the fibres for about ½ hr never produced more than a small change in the size of the post-tetanic response (a decrease of 6 ± 3 %); nor was there any marked change when some of these preparations were exposed to the glucose-free solution for a further ½ hr. The diminution of the response on changing to glucose-free solution in the experiment in Fig. 1 was about the largest fall that was obtained in these experiments; in most experiments no change at all was detected. There was thus no evidence of any great and immediate dependence on glucose in these fibres. Nevertheless, a tight coupling between active ion transport and an external supply of glucose does exist in vagal non-myelinated fibres, as will be described below.

The effect of metabolic inhibitors

Deoxy-D-glucose. One possible explanation for the relative insensitivity of vagal non-myelinated fibres to a lack of an exogenous supply of glucose is that they possess an endogenous carbohydrate supply. Therefore, it was decided to use the metabolic inhibitor deoxy-D-glucose in order to evaluate the importance of such a supply for active ion transport. This compound is probably the most specific inhibitor of glycolysis currently available. It affects several enzymic steps in glycolysis, but its main site of action

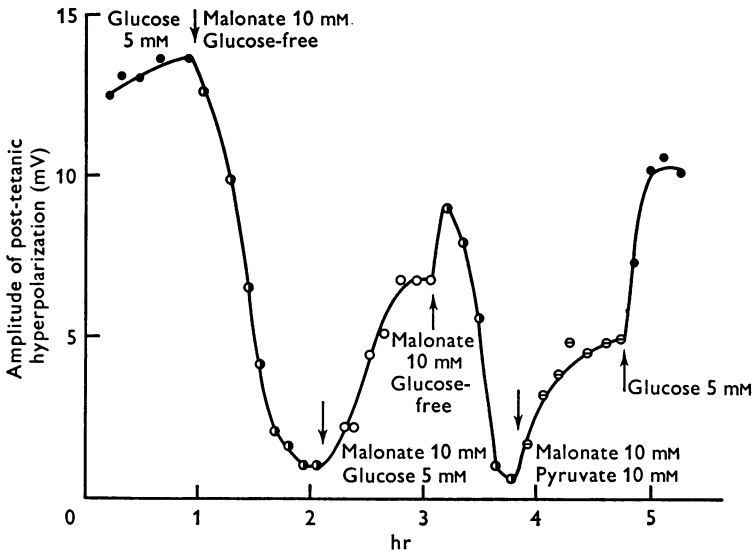

Fig. 2. The effect of malonate on the amplitude of post-tetanic hyperpolarization of the non-myelinated fibres of a rabbit de-sheathed vagus nerve. The Locke solution contained: ●, glucose 5 mM; ◑, malonate 10 mM; but no glucose; ○, malonate 10 mM and glucose 5 mM; ⊖, malonate 10 mM and pyruvate 10 mM.

appears to be the enzyme phosphohexoisomerase, which converts glucose-6-phosphate to fructose-6-phosphate (Webb, 1966). When nerves in Locke solution containing the normal amount of glucose (5 mM) were exposed to deoxy-D-glucose in a concentration of 10–50 mM (◑, Fig. 1) there was a profound fall in the size of the post-tetanic response, which began within 3–5 min after the inhibitor was added and continued to develop during the next hour; in seven experiments after 30 min the response had fallen to $0{\cdot}71 \pm 0{\cdot}09$ of its original value. This indicates that before adding the drug, the nerve fibres had indeed been using glucose (or glycogen) as the energy source for maintaining active ion transport.

Malonate. Malonate is an inhibitor in the tricarboxylic acid cycle that

competes with succinate by forming a complex with the enzyme succinic acid dehydrogenase and so prevents succinate from being converted to fumarate; this is an example of true competitive inhibition, which can be overcome by giving enough succinate. In keeping with this biochemical effect, malonate was found to be a very effective inhibitor of the post-tetanic response (◑, Fig. 2); in three experiments in the presence of glucose it reduced the response to $0{\cdot}20 \pm 0{\cdot}02$ of its original value; in three other experiments in the absence of glucose the corresponding value was $0{\cdot}23 \pm 0{\cdot}11$.

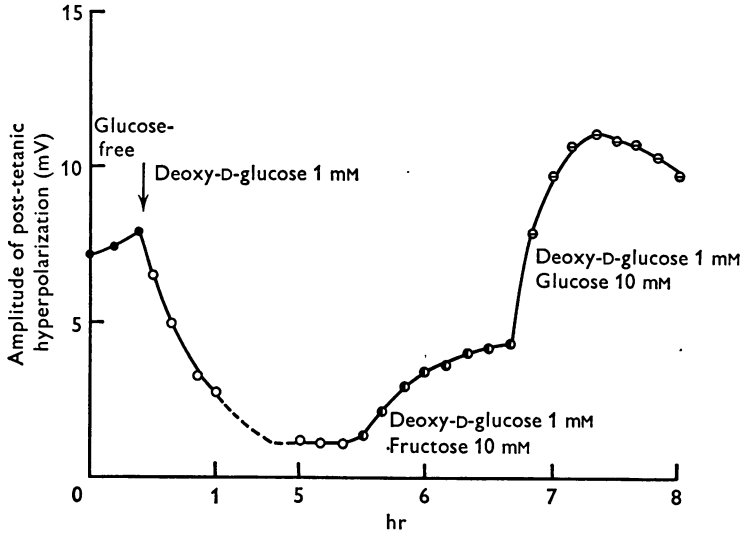

Fig. 3. The effect of fructose and of glucose on the amplitude of post-tetanic hyperpolarization of the non-myelinated fibres of a rabbit de-sheathed vagus nerve treated with deoxy-D-glucose. The Locke solution contained: ●, glucose-free; ○, deoxy-D-glucose 1 mM and no glucose; ◐, deoxy-D-glucose 1 mM and fructose 10 mM; ⊖, deoxy-D-glucose 1 mM and glucose 10 mM.

The effect of various substrates in restoring ion pumping in fibres poisoned with deoxy-D-glucose or malonate

D-Glucose. In keeping with the competitive nature of the inhibition by deoxy-D-glucose (Webb, 1966), it was found in the present study that the inhibition by deoxy-D-glucose is affected by the amount of glucose in the medium bathing the nerve. Thus, whereas concentrations of deoxy-D-glucose as high as 10–50 mM were required to reduce the post-tetanic response (Figs. 1, 4, 5) in the presence of glucose (to $0{\cdot}71 \pm 0{\cdot}09$ of its original value in 30 min, seven experiments), concentrations as low as 0·5 or 1 mM produced a large reduction in the size of the post-tetanic hyper polarization (Fig. 3) in the absence of glucose (to $0{\cdot}50 \pm 0{\cdot}06$ of its original

value, eleven experiments). Also in keeping with the competitive nature of the inhibition, nerves would be expected to be more sensitive to a reduction of the external glucose concentration in the presence of deoxy-D-glucose. The experiment of Fig. 1 shows that this is the case. In this experiment a 30 min exposure of fresh nerve to glucose-free Locke solution reduced the post-tetanic response by less than 15%. However, after the same nerve had been exposed to deoxy-D-glucose in a concentration of 10 mM (from the arrow marked *d* until the end of the experiment), which produced a reduction of about 40% in the post-tetanic response when 5 mM glucose was present in the Locke solution, removing the glucose from the external medium promptly produced a further large fall (about 45%) in the amplitude of the post-tetanic response.

Glucose (○, Fig. 2) similarly overcame the depression of the post-tetanic response produced by malonate, again in keeping with the known competitive nature of the inhibition produced by this agent; the increased glucose concentration presumably led to an increase in the succinate in the nerve.

The sensitivity to removing glucose of a preparation that had been previously exposed to deoxy-D-glucose (or to malonate) is in striking contrast to the lack of effect of removing the glucose in a fresh preparation never exposed to an inhibitor. Thus, removal of glucose from six inhibitor-treated preparations reduced the response by $0{\cdot}49 \pm 0{\cdot}10$ of its original value in about $\frac{1}{2}$ hr; the corresponding reduction in eleven fresh preparations was $0{\cdot}06 \pm 0{\cdot}03$.

Fructose. Fructose (10 mM) was also found to be able to reverse the inhibition of the post-tetanic response that followed exposure of nerves to deoxy-D-glucose in the absence of glucose; thus in four experiments in which exposure to deoxy-D-glucose had reduced the size of the response to $0{\cdot}11 \pm 0{\cdot}02$ of its original value, fructose (10 mM) restored it to $0{\cdot}31 \pm 0{\cdot}09$. This effect, which means that the C fibres used contain a fructokinase, was less marked than that produced by the same concentration of glucose. This is illustrated in Fig. 4, which shows that recovery of the post-tetanic response, which was only partial in the presence of fructose, became much more complete when an equimolar amount of glucose replaced the fructose.

Pyruvate and acetate. Deoxy-D-glucose acts rather specifically on hexokinase and phosphohexoisomerase (Webb, 1966), the initial steps in glucose break-down. It therefore ought to be possible to reverse the depressant effect of deoxy-D-glucose on active ion transport, not only by adding glucose but also by adding metabolites of glucose that are lower down in the metabolic pathway. Five experiments with pyruvate and three with acetate showed that this is indeed the case. Thus, pyruvate (○, Fig. 4) and acetate (⊖, Fig. 4) in a concentration of 10–20 mM rapidly restored, at least partially, the metabolic response of C fibres poisoned with 1–20 mM

deoxy-D-glucose. Pyruvate restored the response from $0{\cdot}23 \pm 0{\cdot}10$ to $0{\cdot}51 \pm 0{\cdot}08$ of its original value; acetate increased the response of the poisoned preparation from $0{\cdot}31 \pm 0{\cdot}01$ to $0{\cdot}56 \pm 0{\cdot}13$ of its original value. Neither pyruvate nor acetate had any effect on fresh vagus nerves in normal Locke solution. This latter result is in contrast to the results of Greengard & Straub (1962), who found using sympathetic C fibres that both these substances increased the post-tetanic hyperpolarization of their preparations in normal Locke solution, which again suggests that in sympathetic C fibres metabolism may be limited by the availability of exogenous

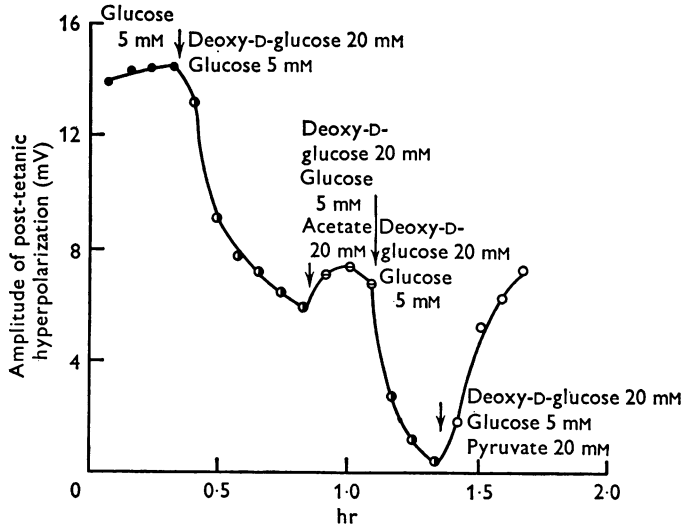

Fig. 4. The effect of acetate and of pyruvate in restoring the amplitude of the post-tetanic hyperpolarization of the non-myelinated fibres of a rabbit de-sheathed vagus nerve poisoned with deoxy-D-glucose. The Locke solution contained: ●, 5 mM glucose; ◑, deoxy-D-glucose 20 mM and glucose 5 mM; ⊖, deoxy-D-glucose 20 mM, glucose 5 mM, and acetate 20 mM; ○, deoxy-D-glucose 20 mM, glucose 5 mM, and pyruvate 20 mM.

glucose, in contrast to the metabolism of vagal fibres used in the present experiments, in which endogenous stores of carbohydrate seem sufficient to maintain active ion transport.

Pyruvate is less potent than glucose in restoring metabolism in nerves poisoned with deoxy-D-glucose. Thus in three experiments where deoxy-D-glucose had reduced the size of the response to $0{\cdot}18 \pm 0{\cdot}12$ of its original value pyruvate (10 mM) produced an increase in the response of $0{\cdot}19 \pm 0{\cdot}04$ of its original value, whereas glucose (10 mM) increased the response by $0{\cdot}52 \pm 0{\cdot}09$.

Pyruvate also reversed the depression of the post-tetanic response produced by malonate (Fig. 2); in six experiments in which malonate (10 mM) had reduced the response to $0{\cdot}21 \pm 0{\cdot}05$ of its original value, pyruvate

(10 mM) increased it to 0·56 ± 0·10. This effect was not as marked, however, as that produced by glucose (possibly because glucose enters the cell more readily than pyruvate); thus in three experiments in which malonate had reduced the size of the response to 0·18 ± 0·12 of its original value, pyruvate (10 mM) increased the size by 0·19 ± 0·04, whereas glucose (10 mM) increased it by 0·52 ± 0·05.

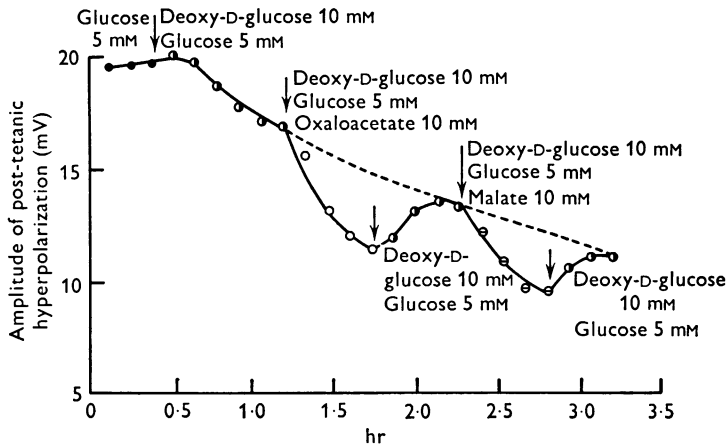

Fig. 5. The effect of oxaloacetate and of malate on the amplitude of the post-tetanic hyperpolarization of the non-myelinated fibres of a rabbit de-sheathed vagus nerve poisoned with deoxy-D-glucose. The Locke solution contained: ●, glucose 5 mM; ◑, deoxy-D-glucose 10 mM and glucose 5 mM; ○, deoxy-D-glucose 10 mM, glucose 5 mM, and oxaloacetate 10 mM; ⊖, deoxy-D-glucose 10 mM, glucose 5 mM, and malate 10 mM.

Oxaloacetate, malate and related substances. Neither oxaloacetate nor malate (10 mM) had much effect on nerves bathed in normal Locke solution. However, in nerves that had been exposed to deoxy-D-glucose (0·5–10 mM) in concentrations sufficient to reduce, but not abolish, the post-tetanic hyperpolarization, oxaloacetate (three experiments) and malate (three experiments) in a concentration of 10 mM further reduced the size of the post-tetanic hyperpolarization (Fig. 5), by 0·33 ± 0·04 and 0·17 ± 0·03 of its original value in the fresh nerve respectively. This reduction was reversed on removing these substrates from the medium (Fig. 5). Other components of the tricarboxylic acid cycle (isocitric acid, 10 mM; α-ketoglutaric acid, 10 mM) neither restored nor further reduced the size of post-tetanic responses that were depressed by deoxy-D-glucose (perhaps because of an inability to penetrate into the axoplasm). Nor had succinic acid (10 mM) any effect on the depression of the post-tetanic response produced by malonate (three experiments). These compounds by themselves produced little or no effect on the action potential.

DISCUSSION

Gasser (1950, 1955, 1956, 1958) has emphasized the fact that there are differences in the electrical responses of different kinds of mammalian C fibres (see also Armett & Ritchie, 1963). The present experiments show that the C fibres from fresh untreated rabbit vagus nerve appear to be also metabolically different from those of C fibres from sympathetic nerves of the same animal. For example, sympathetic C fibres are markedly sensitive to glucose, pyruvate, and acetate, whereas vagal C fibres are not; furthermore, exposing C fibres to malonate, which inhibits succinic acid dehydrogenase and thus prevents the proper functioning of the tricarboxylic acid cycle, increases the size of the post-tetanic response of the sympathetic fibres but not of the vagal C fibres. However, after vagal C fibres have been exposed to a metabolic inhibitor, deoxy-D-glucose, in a concentration that impairs metabolism only slightly so that the post-tetanic response is only just reduced, they begin to resemble the sympathetic C fibres. Thus, under these conditions, vagal C fibres become sensitive to the glucose concentration of the medium, so that removal of the external glucose leads, as in normal sympathetic C fibres (Greengard & Straub, 1962), to a rapid fall in the post-tetanic response. Such vagal nerves, poisoned by deoxy-D-glucose, further resemble sympathetic nerves in that pyruvate and acetate each increase the size of the post-tetanic response even in the presence of glucose. Moreover, both vagal C fibres treated with deoxy-D-glucose and sympathetic C fibres were inhibited by intermediates of the citric acid cycle such as oxaloacetic and malic acids. However, an important difference remains between the two types of fibres. For even in the deoxy-D-glucose-treated vagus nerves malonate reduced the size of the response, which is what normally would have been expected of an inhibitor of the tricarboxylic acid cycle, whereas this compound was found by Greengard & Straub (1962) to increase the size of the post-tetanic hyperpolarization of sympathetic nerves. This finding in the present experiments casts some doubt, therefore, on the hypothesis of Greengard & Straub (1962) that some acetylated compound is rate limiting for the active extrusion of sodium ions, at least in vagal C fibres. This is important because of the role that one acetylated compound, acetylcholine, has been postulated to play in nervous conduction (Nachmansohn, 1959).

The marked sensitivity to external glucose of the C fibres studied by Greengard & Straub (1962) indicates that these preparations were metabolically in quite a different state than were those used in the present experiments. The reason for the difference in metabolic sensitivity between vagal and sympathetic C fibres is not clear. One explanation for the fact that vagal fibres can be made to resemble sympathetic fibres by treatment

with low concentrations of deoxy-D-glucose is that the sympathetic fibres have lower stores of glucose (or glycogen) than do vagal fibres. Alternatively, the enzymes of glycolysis may be less active in the sympathetic fibres. Such differences in intermediary metabolism may possibly be explained by the fact that Greengard & Straub (1962) used bundles of C fibres from the rabbit sympathetic trunk, which is much smaller and much more difficult to dissect than the vagus nerves used in the present experiments. Whatever the explanation is, however, the fact that deoxy-D-glucose, through preventing glycolysis, interferes with the system for active transport of ions in C fibres makes it a most useful reagent. Thus, it should now be possible, by using bundles of fibres from the rabbit vagus nerve treated with deoxy-D-glucose, to map all the possible sources of energy available to the axons for supporting the active transport of sodium and potassium ions.

Schoepfle (1967), studying the effect of deoxy-D-glucose on single myelinated nerve fibres, has reported only slight, delayed, effects of this inhibitor on these fibres. Since Schoepfle's results might be considered to cast doubt on the efficacy of deoxy-D-glucose as an inhibitor of the metabolism of nerve axons, a few points about his study should be mentioned. First, Schoepfle studied the maximum rate of change of membrane potential during the impulse, which would not be expected to have any but the most indirect relationship to metabolism. Secondly, he measured the time for this parameter to decay to 95% of its control value (i.e. to decay by 5%), which demands a great deal of the accuracy of biological measurements. Thirdly, no data were given for the time required for this parameter to fall by 5% in nerves not exposed to deoxy-D-glucose. Fourthly, Schopfle reported a lack of ability of lactate, pyruvate, fructose, and glucose to provide protection against deoxy-D-glucose inhibition, but under circumstances where there was, seemingly, no demonstrable inhibition by deoxy-D-glucose (see his Fig. 2). Schoepfle's results, therefore, do not invalidate the present conclusions on the role of glycolysis in nervous function and its interruption by deoxy-D-glucose. Indeed, the relatively slight effects that Schoepfle obtained in myelinated fibres emphasize the importance of using a preparation of small fibres for metabolic studies. For, as has been pointed out many times (Ritchie & Straub, 1957; Greengard & Straub, 1962; Ritchie, 1967; Howarth *et al.* 1968; Rang & Ritchie, 1968*a*), the extra metabolism associated with the nerve impulse ought to be much more pronounced in a preparation of small fibres, particularly one in which the ionic imbalance occurs all along the length of the fibre, than in a preparation of fibres that are large and which, being myelinated, allow the ionic interchange between the axoplasm and external environment to occur only at the very restricted region of the nodes.

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