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SPECTROPHOTOMETRIC MEASUREMENTS OF METABOLIC RESPONSES IN ISOLATED RAT BRAIN CORTEX

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SUMMARY

An approach is described for the determination of respiratory intermediates in isolated tissue by means of dual-wavelength absorption spectroscopy. Brain slices were studied by this method and the following results were obtained:

1. Electrical pulses evoke a cyclic transition from a relatively more oxidized to a more reduced phase in NAD(P)H and the cytochromes. Cessation of the pulses allows the level of respiratory intermediates to return to their previous steady state.

2. 30 mM K^+ causes the levels of NAD(P)H to go in a direction opposite to that of electrical pulses indicating that these two forms of stimulation should not be equated.

3. Tetrodotoxin blocks the response of NAD(P)H and the cytochromes to electrical stimulation, but does not effect the steady state levels of these intermediates. It was concluded that the respiratory response to pulses is caused by the depolarization of excitable membranes.

4. A slice deprived of glucose does not respond to electrical stimulation. On addition of 5 mM glucose the level of NAD(P)H oscillates, indicating a dynamic equilibrium in the various metabolic intermediates. Substrate slowly restores the pulse response in the levels of NAD(P)H.

INTRODUCTION

Cerebral metabolism has often been measured *in vitro*, but despite the rapidity of nerve conduction and the briskness of brain respiration *in vivo*, optical methods have rarely been employed to follow the metabolic reactions that accompany brain activity, despite the fact that McILWAIN[1] has demonstrated that isolated brain cortex can be stimulated by electrical pulses to a 3-fold increase in aerobic respiration. CHANCE AND SCHOENER[2] have correlated *in vivo* changes in the fluorescence of reduced pyridine nucleotides [NAD(P)H] and changes in the electroencephalogram with anoxia. NAD(P)H in neurones of the crayfish stretch receptor increased with various metabolic inhibitors, but did not show change with maintained stretch[3]. AUBERT *et al.*[4] demonstrated changes in fluorescence and absorption in the electric organ of *Electrophorus* with electrical stimulation.

CUMMINS AND MCILWAIN[5] have shown that electrical pulses applied to brain slices result in a rapid loss of K^+ and uptake of Na^+, which is reversible on cessation of the electrical pulses. A preliminary report by CUMMINS[6] has shown that the respiratory intermediates of brain tissue can be easily measured, and that electrical pulses cause an increase in reduced pyridine nucleotides. In this paper, we wish to report, in detail, a dual-wavelength approach for the measurement of respiratory intermediates of isolated tissue. Electrical pulses and 30 mM K^+, as demonstrated by this approach, evoke opposite responses in the respiratory intermediates of brain slices.

METHODS

Rats were decapitated and the brain rapidly excised. Slices 0.33 to 0.36 mm were cut from each hemisphere with a razor blade and guide and placed in oxygenated Krebs–Ringer solution in the manner described by MCILWAIN AND RODNIGHT[7]. The normal concentration of bathing solution was: 146 mM Na^+, 5 mM K^+, 1.2 mM Mg^{2+}, 1.0 mM Ca^{2+}, 26 mM HCO_3^-, 128 mM Cl^- and 10 mM glucose. Throughout the experiment, the medium was gassed with CO_2–O_2 (5:95, v/v), and maintained at 37°.

The apparatus of black plastic which holds the slice in the spectrophotometer (Perkin–Elmer Model 356) is shown in Fig. 1. The slice is placed in the well of the plastic holder on top of a supporting nylon screen. Another screen is placed on top of the slice, the two parts of the plastic holder are squeezed together, and the assembly held in place by a slot within the chamber. Medium is poured on either side of the tissue and the chamber is placed in the spectrophotometer against the center of the photomultiplier tube. Quartz windows at either end of the chamber and an oval hole in the tissue holder allows both wavelengths of light to pass through the slice onto the photomultiplier tube. The light path through the chamber was 3.8 cm, and the weight of the tissue slice in the oval window was about 25 mg.

The slice is maintained by pumping oxygenated Krebs–Ringer at 37° from an external jacketed reservoir to either side of the tissue holder. Electrical pulses are

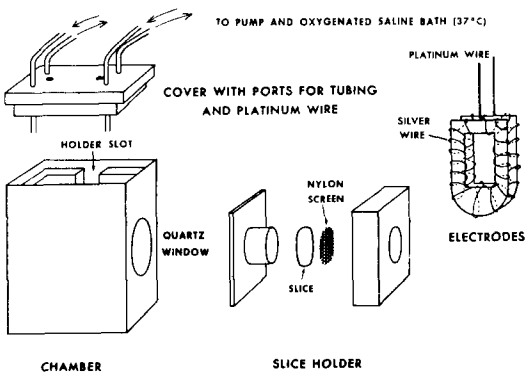

Fig. 1. Chamber designed to hold and maintain tissue slice for spectral measurements on respiratory components in the Perkin–Elmer Model 356 Dual Beam Spectrophotometer. The isolated tissue is placed in the slice holder and supported with nylon screen. The assembly is placed in the slot within the chamber and maintained by pumping oxygenated saline from an external bath. The slice may be subjected to electrical pulses by placing the electrode holder, wound with silver wire, on either side of the slice. Leads from a Grass stimulator are then attached to the Pt wire which extends through the cover.

generated by means of a Grass stimulator (10 V, 100 cycles/sec) and monitored by an oscilloscope. The pulses were applied by means of silver electrodes wound around a hole in a plastic holder (see Fig. 1). The electrodes were placed in the chamber on either side of the slice. The Perkin–Elmer 356 was used in the dual-wavelength mode and the intensities of both wavelength bands were adjusted optimally by means of optical attenuators. Holes were drilled in the cover to accommodate the electrode wires and the input and output tubing. The total volume of media in the system, including chamber, tubing, and external reservoir, was 270 ml. Changes in the solution bathing the slice or addition of inhibitors were made in the external reservoir and pumped to the slice. Before recordings were made, pyridine nucleotides in the tissue were allowed to reach a steady state level. This process was temperature dependent and usually took 15–20 min. Unless otherwise stated, the absorbance was set at 0.03 full scale.

RESULTS

Fig. 2 compares the response in the level of NAD(P)H (340–380 nm) of brain and liver slices to electrical pulses and K^+ (30 mM). Liver tissue (Curve A) is unresponsive to electrical pulses, but addition of K^+ to a concentration of 30 mM caused a small lowering and then a rise in the level of NAD(P)H. Brain tissue (Curve B) was quite responsive to electrical pulses and to 30 mM K^+. The application of electrical pulses to brain caused a very rapid (under 1 sec) and repeatable change to a more oxidized state of the nucleotides followed by a rapid (10–20 sec) and more extensive reduced phase. Once the pulses are turned off, the level of NAD(P)H returned to the original steady-state value. The effect of 30 mM K^+ on brain tissue was to send the levels of

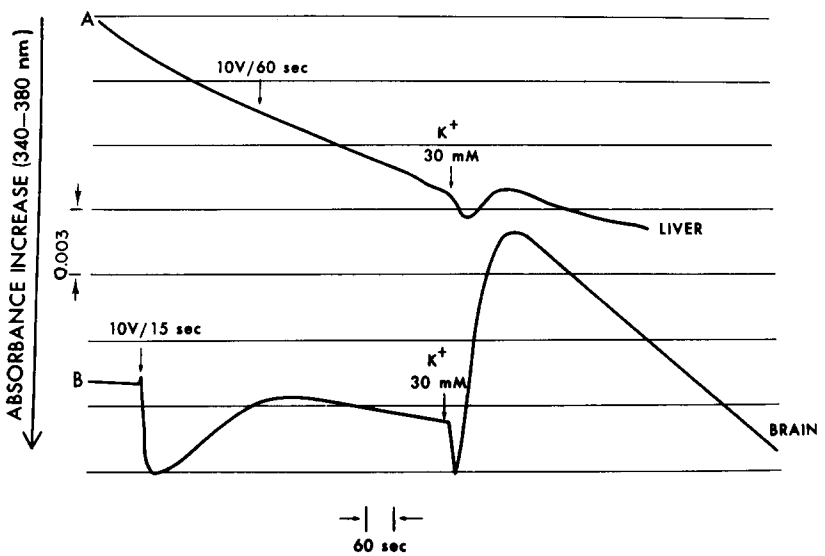

Fig. 2. Comparison between the effect of electrical pulses and 30 mM K^+ on the level of NAD(P)H in isolated brain and liver tissue. Both liver (Curve A) and brain (Curve B) slices were prepared, maintained, and pulses applied as described in text. 30 mM K^+ was added to the external reservoir. NAD(P)H was measured as the difference between 340 and 380 nm. Relative absorbance is 0.003 unit between the horizontal lines.

NAD(P)H in a direction opposite to electrical pulses. The addition of 30 mM K^+ evoked in brain tissue first an increase in reduced pyridine nucleotide, followed by an extensive and prolonged increase in the level of oxidized pyridine nucleotide [NAD(P)$^+$]. The comparisons in Fig. 2 indicate both different excitable responses on the part of liver and brain to electrical pulses, and a difference in the mechanism by which 30 mM K^+ stimulates respiration, as compared to electrical pulses.

The effect of various durations of electrical pulses on the same slice is shown in Fig. 3. Fifteen sec of electrical pulses evoked nearly as much reduced pyridine nucleotide as pulses of longer duration. Cessation of pulses of 15 sec and 2 min caused the trace in the level of NAD(P)H to return to the original baseline. The initial oxidative phase of the pulse response was only occasionally as extensive as seen in Fig. 3, but the greater response was repeatable in a particular slice; the plateau part of the recovery curves was also dependent on the preparation. Continuous pulses caused

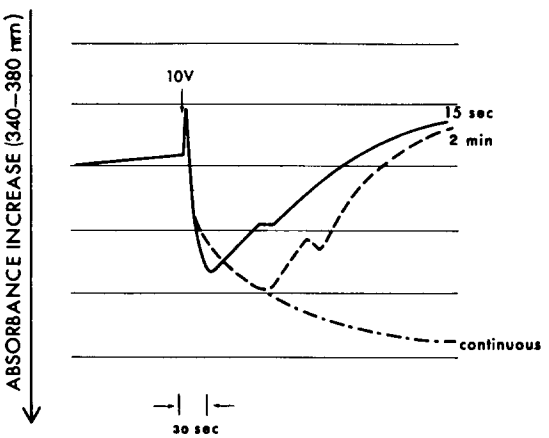

Fig. 3. Effect of electrical pulses applied for 15 sec (———), 3 min (– – – –) and continuously (— · —) on the levels of NAD(P)H in isolated brain tissue. A single slice was used for this experiment. Relative absorbance is 0.003 unit between horizontal lines.

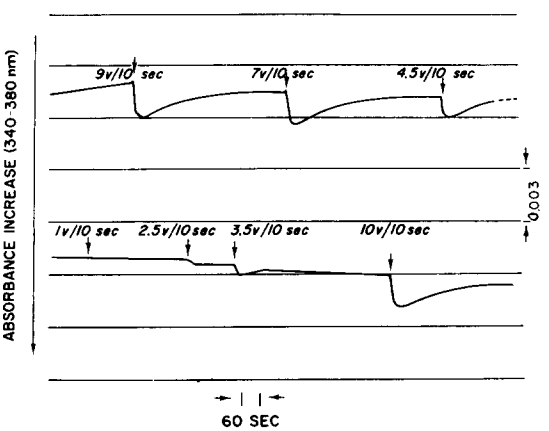

Fig. 4. Effect of various pulse voltages on the extent of NAD(P)H formation. A voltage was applied to the slice and allowed to return to the original baseline before the next voltage was applied.

pyridine nucleotides to remain at an elevated steady state; however, this level is only 25 % higher than that obtained with 15 sec of pulses. It would seem then that electrical pulses are effective within a few seconds in causing a near maximal stimulation of the tissue, and resulting in extensive reduction of NAD(P)H.

Fig. 4 shows that a brain slice was responsive to successive short (10 sec) electrical pulses over a period of 30 min and that after each stimulation, the trace returns to the original baseline. In this experiment, the voltage was varied from 1 to 10 V. The relative response of NAD(P)H in this experiment was plotted against the voltage of the electrical pulse. Fig. 5 shows that the voltage response curve was sigmoid shaped with an optimum voltage at 7 V, and half maximum response at 4.5 V. This curve is very similar to that obtained by McIlwain and Joanny[8] for the effects of various stimulating voltages on brain slice respiration. The magnitude of voltage (7 V) is an indication of the electrolytic energy needed to depolarize the various membranes in brain and cause metabolic responses such as the changes in respiratory intermediates.

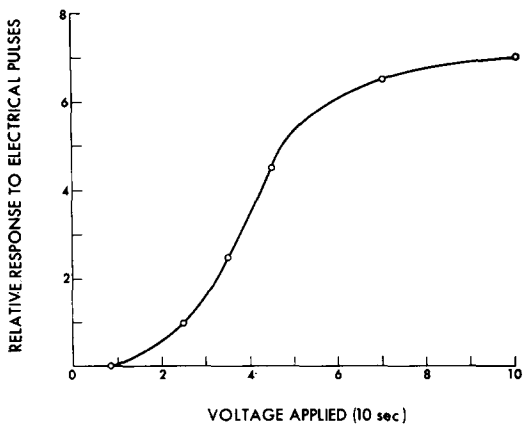

Fig. 5. Response in the level of NAD(P)H to the pulse voltage. Relative absorbance units were obtained from Fig. 4. Voltage was applied for 10 sec.

The level of pyridine nucleotides was influenced by the addition of various metabolic inhibitors. An example of the effect of a metabolic inhibitor is shown in Fig. 6, where the addition of 10^{-3} M ethylbarbital caused a very rapid buildup in the amount of NAD(P)H followed by a slight recovery phase. The pile-up of reduced nucleotides has been shown to be concerned with mitochondrial respiration due to the inhibition of mitochondrial NADH dehydrogenase[9]. The addition of azide ($5 \cdot 10^{-4}$ M) caused an additional increase in NAD(P)H aud may inhibit cytochromes in those pathways not completely affected by ethylbarbital.

Fig. 7 demonstrates that other intermediates in the respiratory chain can be affected by electrical pulses. Curve A compares the response in cytochromes that was measured by the difference between 410 and 430 nm, to changes in NAD(P)H (Curve B), and shows that electrical pulses evoke an extensive transition of the cytochrome chain from a more oxidized to a more reduced form, similar to that obtained with pyridine nucleotides. This effect is preceded by the small oxidative transition similar to Curve B for pyridine nucleotides. The cytochrome trace returns to the original

baseline on cessation of pulses; however, it takes longer than the NAD(P)H experiment, indicating concentration differences between NADH and the cytochromes. This effect may be due to the fact that the cytochrome chain or a process coupled to it is rate limiting.

Fig. 7 also shows that the addition of tetrodotoxin blocks response to electrical pulses of the respiratory intermediates. Tetrodotoxin has been shown to be a "membrane type" inhibitor which blocks the movement of Na^+ within the neuronal mem-

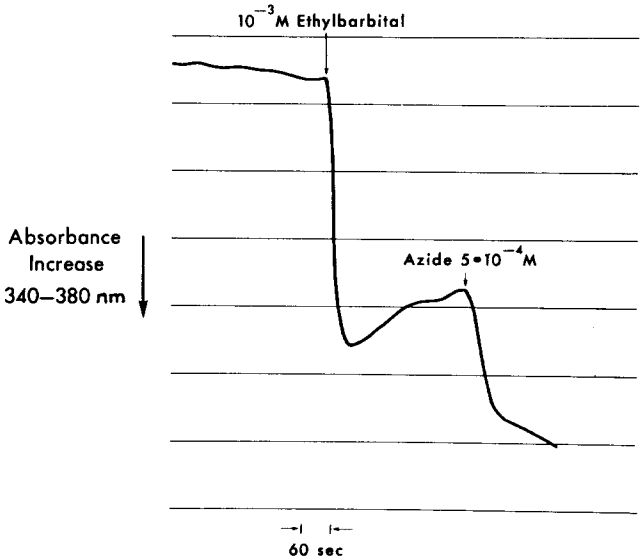

Fig. 6. Effect of two metabolic inhibitors on the level of NAD(P)H. Ethylbarbital (10^{-3} M) and sodium azide ($5 \cdot 10^{-4}$ M) were added to the external reservoir. Relative absorbance equals 0.003 unit between the horizontal lines.

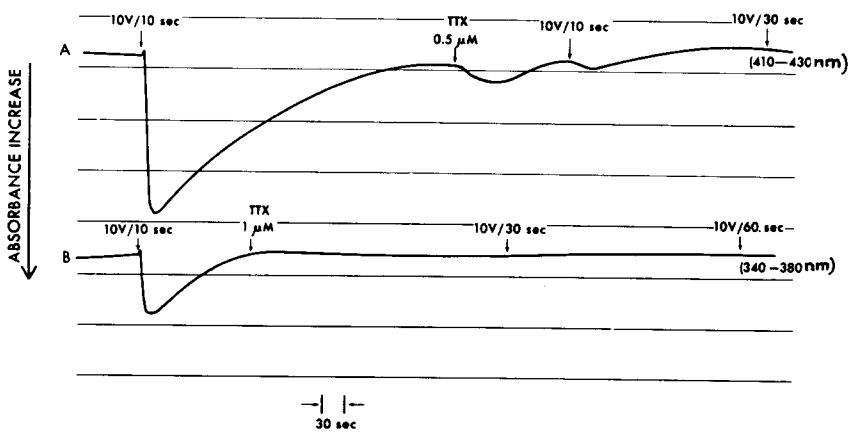

Fig. 7. Comparison between the effects of electrical pulses and tetrodotoxin (TTX) on the level of reduced pyridine nucleotides (Curve B) and on the cytochrome system (Curve A). NAD(P)H was measured as the difference between 340 and 380 nm, and the γ-cytochrome region was measured as the difference between 410 and 430 nm. Different slices were used for Expts. A and B.

brane[10]. The normal steady state level of NAD(P)H (Curve B) and the cytochromes (Curve A) is not affected by tetrodotoxin, whereas metabolic inhibitors change the level of NAD(P)H (see Fig. 6). This result would indicate that the changes brought about by electrical pulses in isolated brain tissue are initiated by excitation of neuronal membranes. The resultant changes in respiratory intermediates would then be due to an increased demand for energy from the metabolic processes that are associated with the depolarization of neurones.

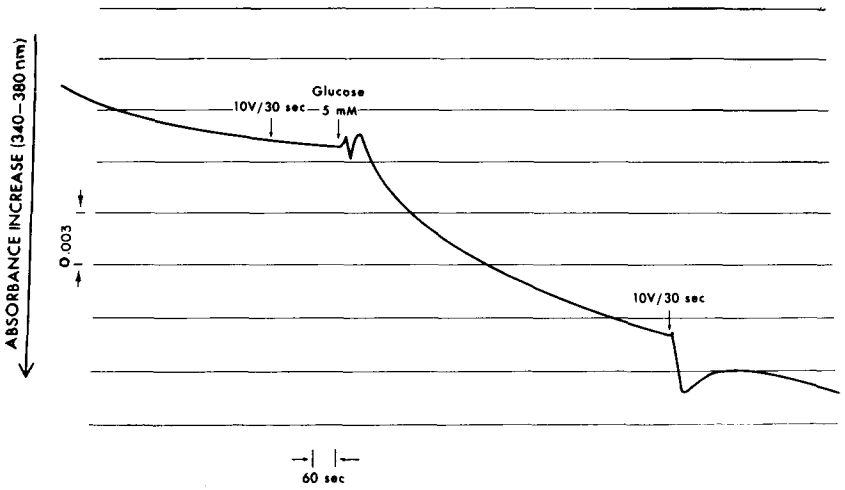

Fig. 8. Oscillation in level of reduced pyridine nucleotides induced by glucose. The brain was "starved" by omitting glucose from the Krebs–Ringer medium and the oscillation induced by adding glucose (5 mM) to the supporting bath.

Glucose, as shown in Fig. 8, was needed in order to obtain a response in the level of NAD(P)H to electrical pulses. There was no effect of electrical pulses without glucose; and in the absence of glucose, the tissue does not attain a stable level of NAD(P)H. Addition of 5 mM glucose evoked an oscillation in the level of NAD(P)H. Typically, there were several oscillations each of longer frequency after which the tissue seeks a higher steady state level of NAD(P)H. Fifteen min after the addition of glucose, the response to electrical pulses was restored. A likely explanation for oscillations in the level of NAD(P) would be that a key metabolic intermediate becomes limiting and has to be regenerated during the period of each oscillation. The length of time for the recovery response indicates that a number of metabolic interactions are involved in reconstitution of tissue excitability.

DISCUSSION

The application of electrical pulses to isolated brain tissue evokes a biphasic response in the absorption spectra of NAD(P)H and the cytochromes. The curve resembles that obtained by AUBERT *et al.*[4] for the fluorescence of NAD(P)H in *Electrophorus electricus*. It is noteworthy that similar conditions of electrical pulses are required to obtain good stimulation. The brain slice response is faster than that obtained from *Electrophorus* and this in part may reflect the temperature at which the two dif-

ferent preparations are maintained. However, the two preparations differ in the degree of reductive response of the cytochrome system[11] to stimulation, since a relatively large amount of cytochrome becomes affected in brain tissue. In addition, the greater importance of the respiratory chain of brain mitochondria is indicated by similar biphasic time courses for both cytochromes and NAD(P)H (see Fig. 7). The stimulated response of rat brain cortex slices initiates a more actively stimulated metabolism compared to fluorescent studies on squid axon[12] and the stretch receptor organ of the crayfish[3]. The magnitude of the response of cortex may be due to the large number of nerve endings in the slice which may constitute a high percentage of the stimulatory response. In the nerve endings, the proximity of synaptic vesicles which concentrate ATP[13], to mitochondria may cause an unusual metabolism for pathways involving adenine nucleotides when compared to results on isolated mitochondria.

CUMMINS AND MCILWAIN[5] demonstrated that electrical pulses cause rapid movements of Na^+ into and the K^+ out of brain slices. These studies showed that the ion movements began within 10 sec of the initiation of electrical pulses and that much of the increased metabolic activity probably was determined by the need of the tissue to maintain normal levels of K^+. In the studies presented in this publication, the recovery curves for the respiratory intermediates after the current was turned off, are similar to those previously determined for K^+ uptake in brain slices. This data and the fact that tetrodotoxin inhibits the response of the respiratory chain to electrical pulses, but does not affect the unstimulated slice indicate that the Na^+ current due to membrane depolarization initiates the train of respiratory events. Many workers (*e.g.* TREVOR AND CUMMINS[14]), have shown that the activity of (Na^+–K^+)-stimulated ATPase in brain to be very high, and that this enzyme could utilize ATP to pump Na^+ out of brain tissue at a rate that would pace the increased respiratory activity.

These studies may shed some light on those mechanisms which determine rates of energy reactions in brain. MCILWAIN[1] has shown that on application of pulses to brain slices, the inorganic phosphate rises and phosphocreatine falls to a plateau level in about 5 sec; on cessation of pulses these two intermediates return within 60 sec to their original value. These rates are similar to those reported here for the reduced phase of formation of pyridine nucleotides. The studies of MCILWAIN[1] showed that ATP levels go through a transient (2–3 sec) lowering and return to their normal value, a time course somewhat slower than shown in our studies for the initial shift to a more oxidative state. One may speculate that on application of electrical pulses, cellular changes in adenine nucleotides induce ultrastructural alterations in brain mitochondria similar to those observed in isolated mitochondria[15]. The reactions concerned in the morphological transition, and the levels of ADP *plus* inorganic phosphate, could cause the transition from a relatively more oxidized to a more reduced phase of the respiratory chain which is seen after application of electrical pulses to brain slices. Our data show that the cytochromes also become relatively more reduced, which may indicate that changes in the concentration of adenine nucleotides may slow the rate of oxidative phosphorylation. This interpretation would fit well with CHANCE's[16] description of a switching of control operations between mitochondrial respiration and glycolysis. The mechanism of this switching could be due to a glycolytic enzyme which displays hysteretic properties as has been discussed by FRIEDEN[17]. A hysteretic enzyme mechanism may be necessary for example to avoid turning on adaptive enzyme systems.

The dynamic equilibrum of the respiratory intermediates of brain is emphasized by the oscillations that occur on the addition of glucose to a starved tissue (Fig. 8). Oscillations, similar in frequency, have been observed for conformational changes in mitochondria[18], and have been noted in perfused heart preparations[19]. The conditions for mitochondrial oscillation are dependent on the composition of the media, but it is noteworthy that they could be initiated by phosphate and dampened by the addition of ADP[18]. The slow rise in the level of NAD(P)H following the oscillation on addition of glucose could be explained by the restoration of ATP to its normal level, and electrical pulses would only be effective, for example, when a sufficient level of ATP is attained in the synaptic vesicles[13]. The oscillations could reflect both the substrate-linked association of NAD(P)H to metabolic control reactions of glycolysis and to mitochondrial respiration that are present in the complex cellular systems of isolated brain cortex.

Elevated K^+ concentrations have also been shown to stimulate respiration in brain slices. The fact that addition of 30 mM K^+ produces just the opposite response to that observed with electrical pulses in terms of the oxidized–reduced states of NAD(P)H, would argue against anoxia in the slice as accounting for the large production of NAD(P)H on electrical stimulation. Since the oxygen uptake increase observed with elevated K^+ and electrical stimulation are of the same order of magnitude, different combinations of metabolic control mechanisms must be operative with the two stimuli. It is reasonably certain that the system is tightly coupled in the case of electrical stimulation, since in spite of the increased oxygen uptake under these conditions, reduced pyridine nucleotide concentration increases. It is not clear that the reverse is true with K^+ stimulation. OKAMOTO AND QUASTEL[20] have reported that the elevation of K^+ lowers ATP and presumably increases the amount of ADP available to mitochondria. With electrical pulses some activation of turnover rates in the glycolytic and tricarboxylic acid pathways has occurred, and a set of circumstances may be operating to limit the rate of electron transport with electrical pulses that does not occur in elevated K^+ concentrations. It is also possible that the complex behavior of K^+ towards glycolytic rate reported by ELLIOTT AND BILODEAU[21] may account for the increased oxidation of NAD(P)H by making entry of substrate into the mitochondria rate limiting.

Both electrical and potassium stimulation are involved in mechanisms which promote the formation of cyclic adenosine 3′,5′-monophosphate (cyclic AMP). Electrical stimulation[22] and high K^+ (ref. 23) increase the formation of cyclic AMP after 30 sec, and within 15 min a level of cyclic AMP many times that of the endogenous level is reached. Since both electrical pulses and elevated K^+ concentrations result in a depolarization of the excitable membrane, presumably the increased rate of cyclic AMP formation is related to this phenomena. However, since the level of NAD(P)H responds in opposite manner with the two stimuli, it is evident that cyclic AMP does not play the deciding role in the regulation of metabolic rate in brain tissue of the rat. Since both elevated K^+ and electrical pulses increase the release of neurotransmitter[24], it is possible that the increase in cyclic AMP level is more closely related to the turnover of neurotransmitter substances, as has been suggested[25].

This brings up something of the dilemma in the study of metabolic activity of brain tissue. There has been a tendency in the past to equate the effects of high K^+ and electrical stimulation on metabolism of nervous tissue. The present study of

course completely dispels that notion. However, the following problem remains. It is possible that K^+ stimulation represents only a selective stimulation of energy-utilizing systems of nervous tissue. For example, both electrical pulses and elevated K^+ result in an increase in neurotransmitter release as mentioned above. However, K^+ stimulation has no effect on Na^+ turnover in brain slices[26], whereas electrical pulses increase turnover of Na^+ (ref. 5). Consequently, one might presume that the response to electrical pulses is modulated by the character of ionic distributional changes. On the other hand, elevated K^+ would be expected to affect metabolic rate in nonexcitable tissues as well as neurons, since it would depolarize both types of cells and stimulate the Na^+–K^+ transport system which is apparently present in all cells. The effect of electrical pulses on this same system should occur only in cells which are electrically perturbable. It is possible, therefore, that the metabolic response of the glial elements in the brain slice predominate under conditions of high K^+. Hopefully, further analysis of the kinetic inputs into the metabolic response of the tissue as a whole will lead to the resolution of this problem.

ACKNOWLEDGEMENTS

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