Effects of Membrane Depolarization on Nicotinamide Nucleotide Fluorescence in Brain Slices

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1. Simultaneous measurement of tissue NAD(P)H fluorescence and respiration was used to elucidate some of the early chemical changes after depolarization of the membranes of cerebral-cortical cells. Depolarization was effected by the application of a train of short-duration voltage pulses across a slice of guinea-pig cerebral cortex. The pulses cause a biphasic change in fluorescence corresponding to an early (within 1 s) oxidation and later (about 10 s) reduction of nicotinamide nucleotide. The major portions of both these redox changes are unrelated to the accompanying increase in slice respiration of about 75%. 2. The early oxidation requires Ca^{2+} and phosphate in the bathing medium and appears to be largely due to an early mitochondrial uptake of these ions. 3. The ensuing reduction occurs in the cytosol, as judged by its almost complete elimination in the presence of exogenous pyruvate. It becomes markedly attenuated with increasing time of incubation. This and the ability of exogenous 6-*N*-2'-*O*-dibutyryl cyclic AMP to cause a reduction in the absence of electrical pulses suggest that it results from a cyclic AMP-mediated activation of glycogenolysis. 4. Further results, obtained in the presence of exogenous pyruvate, indicate that in addition to the above effects a net transfer of NADH from the mitochondrial to cytosolic space (presumably via a shuttle mechanism) is activated by the electrical pulses. 5. The lack of any sizeable (more than 2% of basal fluorescence) fluorescence change associated with the respiratory increase caused by the pulses, and the fact that any change which may be associated with it corresponds to a small reduction of mitochondrial nicotinamide nucleotide, show that a simple state 4 $\rightarrow$ state 3 mitochondrial transition cannot account for the increased respiration. The results suggest, rather, a co-ordinated control of respiration, at more than one site.

Reversible depolarization of cells in cerebral-cortical slices may be effected by a train of short-duration voltage pulses applied across the tissue (Hillman *et al.*, 1963). Because such depolarization is an important component of cortical function much interest has centred upon the biochemical (Kakiuchi *et al.*, 1969; McIlwain & Bachelard, 1971) and physical (Keeseey *et al.*, 1965; Okamoto & Quastel, 1970; Lipton, 1973) events associated with such pulses.

In the present report some insight into the early events accompanying such depolarization has been gained by measuring its effect upon the tissue fluorescence associated with reduced nicotinamide nucleotide in slices of guinea-pig cerebral cortex. This experimental approach was introduced in the late 1950's (Duysens & Ames, 1957; Chance & Jobsis, 1959) and has since been applied to many preparations, including those of nervous tissue (Brauser *et al.*, 1970; Jobsis *et al.*, 1971; Landowne & Ritchie, 1971*a,b*). In the work reported here, measurements of tissue reflectance and respiration have been made together with the fluorescence measurements. This

was done both to eliminate the possibility of artifact and to assess the relationship of nicotinamide nucleotide redox changes to the long-studied increase in tissue respiration associated with membrane depolarization in this tissue (McIlwain, 1951).

Large cyclic changes in the NADH/NAD⁺ ratio occur; these are similar to those observed spectrophotometrically by Cummins & Bull (1971). Rather surprisingly, these changes are largely unrelated to the increased respiration. They apparently result from movements of Ca^{2+} and phosphate ions and from alterations in intracellular concentrations of cyclic AMP.

Methods

The preparation of the tissue, the tissue chamber in which the experiments were performed and the measurement of reflectance and respiration are all described in detail elsewhere (Lipton, 1973). Briefly, slices were prepared as described by McIlwain & Rodnight (1962) by using a cutting guide after decapitation of the guinea pigs. The top slice was mounted between two horizontal silver-wire grids in the chamber. Unless otherwise indicated the slice was

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superfused with oxygenated buffer (composition given below) at 35°C. The solution in the region of the slice was vigorously stirred with a magnetic stirrer (Cole-Parmer) to keep the slice from becoming anoxic.

An O₂ electrode placed downstream from the slice allowed for measurement of respiration by constant-flow respirometry. Superfusate was pulled across the slice very slowly (0.2 ml/min) by an LKB 1200 peristaltic pump. A stepwise change in respiratory rate leads to a gradual change in the O₂ partial pressure recorded by the O₂ electrode. The time for the full change in rate to be recorded depends on the superfusate flow rate (which was low because of the small tissue mass), the mixing and the distance of the electrode from the tissue. This time was minimized but was still 4 min. Exponential voltage pulses, height 10 V, decay time 0.5 ms, were applied between the grids by a Grass SD9 stimulator at about 60/s. Reflectance and fluorescence from the slice were measured simultaneously and the method was based on that developed by Chance *et al.* (1962) for reflectance microfluorimetry. The methodology was extended to allow simultaneous measurement of fluorescence and reflectance in a way similar to that described by Jobsis *et al.* (1971). A prism beam-splitter was inserted into the path of the light emitted from the tissue, thus allowing it to impinge on suitably filtered 'reflectance' and 'fluorescence' phototubes. The former was filtered by Wratten no. 2A, 16 and 35 filters transmitting only above 620 nm and the latter was filtered by a Wratten no. 2A and an interference filter transmitting between 445 and 455 nm. Tissue was illuminated by a 1000 W mercury arc filtered by a Corning 5874 water-cooled filter. This has two transmission 'windows', one in the u.v., with a maximum at 365 nm and the other in the red, above 600 nm.

The compositions of buffers used were as follows: (all concentrations in mM): standard buffer: Na⁺ = 157, K⁺ = 5.0, Ca²⁺ = 0.75, Mg²⁺ = 1.0, Cl⁻ = 153, SO₄²⁻ = 1.0, HPO₄²⁻ = 5.0, pH 7.4; glucuronate buffer: Na⁺ = 156, K⁺ = 5.0, Ca²⁺ = 0.75, Mg²⁺ = 1.0, glucuronate = 156, SO₄²⁻ = 4.25, HPO₄²⁻ = 1.0, pH 7.4.

All buffers contained 10 mM-glucose.

All the reagents were B and A analytical grade except for sodium glucuronate, which was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Results

Effects of electrical pulses

Although depolarization of the cell membranes of cerebral-cortical slices may be effected by several chemical agents, that induced by electrical pulses has the advantage of being readily reversible (Hillman *et al.*, 1963). The present paper is thus confined to experiments in which such pulses were used for de-

polarization. Fig. 1 shows a typical set of the reflectance, fluorescence and respiratory responses to pulses applied continually for 4 min. The apparent gradual nature of the increase in respiration is, as described in the Methods section, an artifact of the flow system. The true time-course of this increase could not be obtained from these measurements. The latter do, however, show the steady-state respiration after at least 4 min of pulses.

The reflectance response has been fully described (Lipton, 1973) and results from cellular swelling. The roman numerals I-V in Fig. 1 describe five well-defined phases of the fluorescence response. These will be referred to as phase P₁ etc. There is an early

Fig. 1. Slice response to electrical pulses

Reflectance (top trace), fluorescence (middle trace) and respiration (bottom trace) of guinea-pig cerebral-cortical slice before, during and after the continual application of electrical pulses across the slice. Reflectance and fluorescence both increase in the downward direction as denoted by the arrow, and the bar next to the ordinate denotes 10% of the basal value of each parameter. 'Reflectance' denotes the intensity of light reflected in the red region of the spectrum (above 620 nm) and 'fluorescence' denotes light emitted between 445 and 455 nm after excitation centred at 365 nm. Respiration increases in the upward direction as denoted by the arrow, and the bar next to the ordinate denotes 50% of the basal respiration. The vertical arrows in the body of the figure denote the onset (upward) and termination (downward) of the electrical pulses. The above description of the terms and conventions applies to all ensuing figures except Fig. 9. In this and all other experiments, unless specifically noted, the first set of pulses was applied between 25 and 35 min of setting up the slice. In this experiment basal respiration = 11.3 μ l of O₂/h per mg dry wt.

Table 1. *Magnitudes and kinetics of the different phases of the fluorescence response to electrical pulses*

The phases of the response are defined in Fig. 1. The table shows the average ($\pm$ s.e.m.) for the first responses from 10 different preparations selected at random. All the responses occurred in standard buffer. The magnitude of response is the maximum amplitude of the particular response and is expressed as a percentage of the basal (pre-stimulation) fluorescence of the sample. For phase P_{II} the half-time is the time from the onset of the pulses until $\frac{1}{2}$ the maximal response has occurred. For phases P_{IV} and P_V it is the time from turning off the pulses until $\frac{1}{2}$ the maximal response has occurred.

Phase	Magnitude of response (%)	Half-time
P _I	7.5 $\pm$ 1.2	—
P _{II}	23 $\pm$ 4	23 $\pm$ 4 s
P _{IV}	14 $\pm$ 3	36 $\pm$ 8 s
P _V	11 $\pm$ 3	5.4 $\pm$ 1.5 min

fluorescence decrease of about 8% (phase P_I) followed by a far larger increase (phase P_{II}) which leads to a plateau (phase P_{III}) about 25% more fluorescent than the non-stimulated state. The recovery, after the pulses were turned off, begins with a rapid fluorescence decrease (phase P_{IV}) which is followed, after a plateau lasting about 2min, by a far slower decrease (phase P_V) of about the same magnitude as phase P_{IV}. The lack of symmetry between the forms of the responses to initiation ('on') and termination ('off') of the pulses is apparent. The 'on' response shows both an oxidation and a reduction phase whereas the 'off' response shows only oxidation phases. In addition the overall 'off' response is far slower than the 'on' response.

This five-phase response was always observed in the standard bathing medium and the magnitudes of phases P_I, P_{II}, P_{IV} and P_V as well as the kinetics of the latter three are summarized for 10 different preparations in Table 1. Owing to their rapidity the kinetics of phase P_I were analysed in a separate set of experiments (described below) in which a cathode-ray oscilloscope was used.

It is interesting to compare phase P_{II} with the magnitude of the fluorescence increase produced by complete tissue anoxia. This was about 60% of the basal fluorescence, and approximately one-half of it was abolished by the addition of maximal amounts of pyruvate to the superfusate (P. Lipton, unpublished work; five experiments).

Fig. 2. *Early kinetics of slice response to electrical pulses*

Oscilloscope tracings of the reflectance and fluorescence responses to electrical pulses applied across the slice. Pulses were applied continually, beginning at the times denoted by the stimulus arrows. (a) and (b) denote experiments carried out on two different preparations.

Early kinetics

Fig. 2 is an oscilloscope trace of the early fluorescence and reflectance responses to pulses. Two experiments on different time-scales are shown. The top trace emphasizes phase P_I and the bottom trace shows the temporal relationship between phase P_I and the beginning of phase P_{II}. The pulses were applied throughout the periods shown on the traces. Phase P_I starts within 1 s of beginning the pulses. It becomes half-maximal about 2 s later in the top trace and about 4 s later in the bottom trace. The time-course of the reflectance decrease is appreciably slower in both traces, strongly indicating that phase P_I is not an artifactual consequence of the reflectance change. The plateau between phases P_I and P_{II} in the lower trace indicates that the onset of Phase P_{II} does not affect the magnitude of phase P_I. In this sense, at least, the two phases of the pulse response are independent.

Effect of glucuronate buffer

To test the above inference that phase P_I does not result from the reflectance change, pulses were applied while the slice was superfused by glucuronate buffer.

Fig. 3. Effect of glucuronate buffer on slice response to electrical pulses

Reflectance, fluorescence and respiration responses of the slice to pulses applied first in the presence of standard buffer and then in the presence of glucuronate buffer. The discontinuity in the tracing is inserted for space considerations and extends over the period of equilibration with the glucuronate buffer. Superfusate was changed at the time denoted by the vertical bar by substituting glucuronate buffer in the reservoir supplying the chamber at that time. This buffer was equilibrated to the same temperature and O_2 partial pressure as that of the preceding (standard) buffer before being put in the reservoir. This procedure was applied in all cases in which solutions were changed during experiments. In this experiment basal respiration = $12.2 \mu\text{l}$ of O_2/h per mg dry wt.

In this buffer the reflectance changes are eliminated whereas the respiratory increase is unaffected (Lipton, 1973). Fig. 3 shows one such experiment. Although it completely eliminated the reflectance change the glucuronate buffer caused only a 35% decrement in the early fluorescence decrease. In six such experiments, in all of which the reflectance change was completely eliminated, phase P_I was decreased by $30 \pm 6\%$ (S.E.M.). Thus, whereas this percentage of phase P_I may well be a reflectance artifact, the larger portion of the response represents a true diminution of fluorescence. Fig. 3 shows that phase P_{II} declined with time. This occurred irrespective of the anion in the buffer and will be considered below in the Results section.

In the following experiments the pulse response was studied with respect to its possible biochemical bases.

Effect of Ca^{2+} salts

The pulse response in a Ca^{2+} -free superfusate was investigated in five identical experiments, one of which

Fig. 4. Effect of Ca^{2+} removal on slice response to electrical pulses

Reflectance, fluorescence and respiration responses of the slice to pulses applied in the presence, absence and then presence of calcium in the superfusing medium. Ca^{2+} -free superfusate was provided, at the time shown, by rinsing and then filling the reservoir with pre-equilibrated Ca^{2+} -free buffer. The composition of this buffer differed from that of the standard buffer only in that it contained no $CaCl_2$. In this experiment basal respiration = $11.5 \mu\text{l}$ of O_2/h per mg dry wt.

is shown in Fig. 4. In Fig. 4 the second response was elicited in the Ca^{2+} -free buffer, and both phase P_I and the reflectance change are clearly far smaller than in either the prior or ensuing response, both of which occurred in standard buffer. Again, phase P_{II} declined continually with increasing time. Table 2 summarizes these experiments. Phase P_I in the absence of Ca^{2+} is compared with its magnitude in the presence of Ca^{2+} . The latter is tabulated for depolarizations before and after that in which Ca^{2+} was absent in order to negate, as far as possible, the effect of the duration of incubation. Phase P_I in the absence of Ca^{2+} was attenuated by 60% compared with the average of these two magnitudes. In contrast with the effects on Phase P_I and reflectance, the removal of Ca^{2+} salts from the bathing medium did not attenuate the respiratory increase produced by the pulses nor, indeed, did it affect the basal respiration of the slice. This last is in contrast with results of others (Whittam *et al.*, 1965; Bourke & Tower, 1966) and the basis for the difference may be the far shorter incubation in the absence of Ca^{2+} in the present experiments. If this is the basis it indicates that internal, rather than external, Ca^{2+} depletion leads to the increased respiration observed by the other workers.

Table 2. Effect of Ca²⁺ removal on slice fluorescence, reflectance and respiratory responses to electrical pulses

Columns (1), (2) and (3) are average values from five experiments (±s.e.m.) of the responses to successive 4–5 min series of electrical pulses. The data in column (2) are average responses in standard buffer without Ca²⁺. In each experiment these responses were obtained after those in column (1) and before those in column (3). Both the latter were obtained in standard buffer. The percentage decrement was calculated for each experiment and its average ±s.e.m. from the five experiments is shown in the final column of the table. It is defined as:

100 × (1 - (response in Ca²⁺ free-buffer) / (average of the two responses in standard buffer))

	Magnitude of response (% of basal fluorescence)			Percentage decrement
	(1) +Ca ²⁺	(2) -Ca ²⁺	(3) +Ca ²⁺	
Fluorescence (phase P _i)	6.0 ± 0.9	2.3 ± 0.5	4.9 ± 0.7	58 ± 6
Reflectance	4.2 ± 0.3	1.6 ± 0.2	3.6 ± 0.3	59 ± 5
Respiration	65 ± 8	55 ± 7	63 ± 8	14 ± 9

Fig. 5. Effect of P_i removal on the slice response to electrical pulses

Reflectance, fluorescence and respiration responses to electrical pulses applied first in the absence of superfusate phosphate, then in its presence and then again in its absence. The slice was set up in phosphate-free Hepes buffer. In this buffer the 5mM-sodium phosphate was replaced by 5mM-Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl)ethanesulphonic acid]. The pH was maintained at 7.4. Then 45min elapsed until the first bout of pulses shown here. At the first vertical mark 5mM-sodium phosphate (pH7.4) was added to the superfusate in the reservoir. At the second vertical mark the reservoir was again filled with the phosphate-free buffer containing Hepes. In this experiment basal respiration = 10.8μl of O₂/h per mg dry wt.

Effect of P_i

Because the movement of Ca²⁺ across biological membranes is often largely dependent on P_i, the effect of this anion on the pulse response was investigated. As shown in Fig. 5, phase P_i was markedly attenuated in phosphate-free buffer although the latter had no effect on the respiratory increase. In contrast with the effect of Ca²⁺, the reflectance response was in no way dependent on phosphate. Another aspect of phosphate dependence differed from that of the Ca²⁺ dependence and accounts for the choice of the sequence of media exemplified by Fig. 5. Thus, whereas addition of phosphate to the previously phosphate-free buffer led to a quite rapid restoration of the full response, the opposite manoeuvre, i.e change of superfusate from normal to a phosphate-free buffer, led to a very slow decline in phase P_i [phase P_i becoming half-maximal in about 40min]. This will be considered in the Discussion section. Table 3 summarizes the results of this set of experiments. The absence of phosphate from the superfusate decreased phase P_i by about 70%.

Effect of pyruvate

Exogenous pyruvate oxidizes cytosolic NADH via the lactate dehydrogenase system and has thus been used by several groups of workers to assess the portion of NADH fluorescence resulting from cytosolic nicotinamide nucleotide (Scholz *et al.*, 1969; Brauser *et al.*, 1970). It was so used here in two separate protocols to assess the portion of the response to the pulses owing to changes in the cytosolic NADH. In Fig. 6, 20mM-pyruvate was added during the

Table 3. *Effect of the absence of P_i on slice fluorescence, reflectance and respiratory responses to electrical pulses*

Columns (1) and (2) are average values from four experiments ($\pm$ S.E.M.) of the responses to successive 4–5 min series of electrical pulses. The data for column (1) were obtained about 45 min after the slices were set up in a phosphate-free Hepes buffer (pH 7.4, see legend to Fig. 5) superfusate. The data for column (2) were obtained 30 min after addition of 5 mM-sodium phosphate buffer (pH 7.4) to this superfusate. The percentage decrement was calculated for each experiment and its average ($\pm$ S.E.M.) is shown in the final column of the table. It is defined as:

$$100 \times \left(1 - \frac{\text{response in phosphate-free buffer}}{\text{response in standard buffer}} \right)$$

	Magnitude of response (% of basal fluorescence)		Percentage decrement
	(1) –Phosphate	(2) +Phosphate	
Fluorescence (phase P _{II})	1.3 $\pm$ 0.5	4.8 $\pm$ 0.7	71 $\pm$ 8
Reflectance	4.8 $\pm$ 0.5	5.4 $\pm$ 0.4	11 $\pm$ 7
Respiration	68 $\pm$ 9	62 $\pm$ 9	–10 $\pm$ 7

Fig. 6. *Effect of pyruvate added during the slice response to electrical pulses*

Pre-equilibrated sodium pyruvate was added to the superfusate reservoir to give a final concentration of 20 mM. The pyruvate was added at the vertical mark while the electrical pulses were being applied. In this experiment basal respiration = 10.9 μ l of O₂/h per mg dry wt.

response to the pulses and produced a rapid decrease in fluorescence, almost to the basal value, indicating that much of the NADH produced by the stimulus was in the cytosol at this stage. In the three such ex-

Fig. 7. *Slice response to electrical pulses in the presence of pyruvate*

The reflectance, fluorescence and respiration responses of the slice to pulses applied in the presence of standard buffer + 20 mM-sodium pyruvate. The pre-equilibrated pyruvate was added at the time shown by the vertical mark. In this experiment basal respiration = 12.2 μ l of O₂/h per mg dry wt.

periments phase P_{II} was decreased by an average of 90%. The other protocol is illustrated by Fig. 7. Pyruvate was added to unstimulated tissue after a response in standard buffer. There was no fluorescence change, indicating that the nicotinamide nucleotide in the cytosol was highly oxidised. Pulses were then initiated. Based on the above results it was expected that phase P_{II} would be markedly attenuated, which it was. In addition to this, however, the overall response to the pulses was distinguished by a long oxidation phase after the attenuated phase P_{II}. It is notable that this oxidation was not reversed when the pulses were turned off and that it reached a

Fig. 8. Effect of prolonged incubation on slice response to pulses

Standard buffer was the superfusate throughout this experiment. Pulses were applied for 4–5 min at intervals of about 20 min over a period of about 70 min. In this experiment basal respiration = $12.7 \mu\text{l}$ of O_2/h per mg dry wt.

Fig. 9. Summary of effect of duration of incubation on slice response to electrical pulses

This graph summarizes five separate experiments of the type shown in Fig. 8 in which bouts of pulses were applied at different times during prolonged incubation. The magnitude of each response ($P_I = \Delta$; $P_{II} = \bullet$; respiration = $\blacksquare$) is expressed as a percentage of its value in the first response of that particular experiment (time = 0) and this is correlated with the time during the experiment at which the response was elicited. The individual experiments are not differentiated in this representation.

maximum after prolonged stimulation. A similar pattern was obtained in all four experiments in which this protocol was used. In both protocols the 'off' response in the presence of pyruvate consisted of a small rapid oxidative phase, indicating that the mitochondria in the stimulated tissue are slightly more reduced than in the unstimulated tissue.

Neither the reflectance nor the respiratory responses were affected by the presence of pyruvate. The small decrease in reflectance immediately after the addition of pyruvate, shown in Fig. 7, probably results from a cellular swelling owing to entry of sodium pyruvate.

Duration of incubation

In preliminary experiments, in which the slices had been superfused for a long time before a series of pulses were applied, only a very slight phase P_{II} was observed. Only when the pulses were applied earlier did a sizeable phase P_{II} emerge. That these observations were due to a decay of phase P_{II} with time is indicated by the experiment in Fig. 8. This shows the effect of increased incubation time on the pulse response. It is clear that, whereas the respiratory response and phase P_I remain unattenuated, phase P_{II} is severely decreased in successive intervals of pulses. The reflectance response diminished somewhat with time but not nearly as dramatically as did phase P_{II} . Fig. 9 summarizes five such experiments. It shows the magnitudes of phases P_I and P_{II} and the respiratory increase as a function of time. The magnitudes are expressed as percentages of their values in the first

response of the pertinent experiment. The conclusions from the experiment shown in Fig. 8 are borne out. These results are important not only because they show that the state of the tissue determines the nature of the response but also because they show that the respiratory response is not dependent on phase P_{II} . Thus the respiratory increase is unchanged throughout the experiment whereas phase P_{II} goes from its maximal value to near zero.

The results also show that the normal phase P_{II} is not due to portions of the tissue becoming anoxic during the increased respiration associated with the pulses. Thus phase P_{II} is eliminated in the final stimulus although both basal respiration and the increased respiration associated with the pulses are essentially unchanged.

Effect of dibutyryl cyclic AMP

Depolarization by electrical pulses and by other agents increases the cyclic AMP content of cerebral cortex slices (Kakiuchi *et al.*, 1969). To see if part, or all, of the nicotinamide nucleotide response might result from this increase the effect of 6-*N*-2'-*O*-dibutyryl cyclic AMP in the superfusate was investigated. Fig. 10 shows the results of one of three experiments. Nicotinamide nucleotide fluorescence increased markedly with successive additions of the cyclic AMP derivative. Respiration and reflectance were, however, unaffected. The other two experiments gave similar results, average percentage fluorescence

Fig. 10. Effect of dibutylr cyclic AMP on slice reflectance, fluorescence and respiration

The sodium salt of pre-equilibrated 6-*N*-2'-*O*-dibutylr cyclic AMP (Bu_2cAMP) was added to the superfusate reservoir at the times shown by the vertical marks. The additions led to successive concentration increments of 2 mM.

increases with respect to basal fluorescence being 2.5, 10, 10, 5 and 1 after successive increments of 2 mM-dibutylr cyclic AMP.

Discussion

Membrane depolarization in guinea-pig cortical slices, induced by electrical pulses, leads to a biphasic change in nicotinamide nucleotide fluorescence. This change indicates an early nucleotide oxidation, which begins within 1 s, and a larger reduction, which is half-maximal about 20 s after the pulses begin and which is maintained during (4 min of) membrane depolarization. Repolarization, after removal of the pulses, is associated with a biphasic reoxidation of the nicotinamide nucleotide. The magnitudes of the two phases of this reoxidation are about equal. The fast one has a half-time of about 20 s and the slow phase, beginning after about a 2 min plateau, a half-time of about 4 min.

The early nicotinamide nucleotide oxidation and subsequent reduction have been noted in other nervous-system preparations *in vitro* during either activity or sustained depolarization. These preparations include mammalian peripheral nerve (Landowne & Ritchie, 1971*a,b*) and sympathetic ganglia (Brauser *et al.*, 1970), rat brain slices (Cummins & Bull, 1971) and frog dorsal-root ganglia (Rodríguez-Estrada, 1967). Although light-scattering artifacts were not considered in any of the above work the qualitative similarity between the results in the different preparations as well as their similarity to the results reported here, in which light-scattering artifacts were eliminated and after which the 'oxidation' was only attenuated by 30–40%, indicates that the oxidation-reduction cycle does occur as a result of membrane

depolarization in several vertebrate nervous-system preparations. In addition, Rosenthal & Jobsis (1971) have reported experiments in the intact cortex of the cat which indicate that the response of that tissue to repeated stimuli of the kind used in the present report is similar to that of the brain slice. Thus after 7 s of stimulation (the longest they reported), nicotinamide nucleotide began a reduction phase after an early oxidation. This indicates that the slice may well be a useful model for studying the metabolic effects of steady potential changes in cortex.

Except for Landowne & Ritchie (1971*a,b*) working with the mammalian c fibres, little work has been done to elucidate the bases for the nicotinamide nucleotide responses reported above. Brauser *et al.* (1970) showed that pyruvate completely blocked the nicotinamide nucleotide reduction in the sympathetic ganglion leaving a large monophasic oxidation. This suggests that the cytosolic nicotinamide nucleotide became reduced whereas the mitochondrial coenzyme became oxidized during the activity.

Nicotinamide nucleotide redox state and respiration

The results indicate strongly that the major portions (over 60%) of the nicotinamide nucleotide response to the pulses are not specifically associated with the large increase in respiration that also occurs. Thus phase P_I was decreased by 60% after removal of Ca^{2+} salts or phosphate from the superfusate with no associated diminution of the respiratory increase. Also, phase P_{II} was almost completely abolished after prolonged incubation of the slice and there was, again, no associated attenuation of the respiratory increase. The nature of any nicotinamide nucleotide change which actually is associated with the increased oxidative metabolism was not directly established. The results in the presence of sodium pyruvate did, however, indicate that there was a small steady-state mitochondrial reduction associated with this increase. Thus in both protocols in which pyruvate was used there was a small and rapidly occurring oxidation of NADH when the pulses were turned off (Figs. 6 and 7). Assuming that cytosolic nicotinamide nucleotide was maintained at a steady oxidized amount by this concentration of pyruvate [this is strongly indicated by unpublished results (P. Lipton) showing that 20 mM-pyruvate caused a maximal oxidation of nicotinamide nucleotide during anoxia] these experiments show that mitochondrial nicotinamide nucleotide was slightly reduced in the steady state by the electrical pulses. This small reduction is inconsistent with the notion that activation of respiration results from the action of increased concentrations of ADP or P_I acting at mitochondrial oxidative phosphorylation sites. This would lead to a more oxidized mitochondrial nicotinamide nucleotide state (Chance & Williams, 1956). The result is consistent with the

suggestion of Kini & Quastel (1959) that the increased respiration owing to high K^+ concentrations results from activation of pyruvate dehydrogenase. It is also consistent with results reported by Krebs (1970) on the activation of respiration in heart muscle as a result of an increased work-load. A doubling of respiration led to a very small (3%) increase in the mitochondrial NADH/NAD⁺ ratio as judged by the ratio of β -hydroxybutyrate/acetoacetate. Krebs (1970) suggested multiple mitochondrial regulatory sites associated with the increased ATP utilization. This is clearly a possibility in the brain-slice system.

Origin of phase P_1

In agreement with results of Nakazawa & Quastel (1968), removal of Ca^{2+} from the superfusate did not significantly affect the pulse-induced increase in respiration, indicating that the membrane depolarization is not affected by the absence of Ca^{2+} . The fact that phase P_1 is rapidly decreased by 60% after Ca^{2+} removal is, then, evidence that Ca^{2+} entry rather than the depolarization itself leads to the major portion of phase P_1 in normal media. Such a Ca^{2+} entry has been observed by several workers (Lolley, 1963; Bull & Trevor, 1972). The present results indicate that this entry occurs within less than 1 s of membrane depolarization. The known effect of Ca^{2+} on the nicotinamide nucleotide redox state of isolated mitochondria (Chance, 1965) is consistent with the assumption that this Ca^{2+} -dependent portion of phase P_1 is due to increased mitochondrial uptake of Ca^{2+} resulting from the increased cellular Ca^{2+} concentration. A similar conclusion was drawn by Landowne & Ritchie (1971a) about the early oxidation phase in peripheral nerve. The absence of an effect of the Ca^{2+} uptake on respiration may be due either to its small size or to its transitory nature (Lolley, 1963).

The long decay time of phase P_1 after removal of P_1 from the superfusate (Fig. 5) strongly indicates that it is intracellular, rather than extracellular P_1 , that is required for the full response. Thus the decay of the response after removal of superfusate Ca^{2+} was complete within 10 min. The magnitude of phase P_1 attenuation in the absence of phosphate is approximately equal to that in the absence of Ca^{2+} suggesting that the ions are required for the same process. The most likely basis for the P_1 requirement is, then, that the movement of this anion across the mitochondrial membrane is necessary for the Ca^{2+} uptake. This is not true in isolated mitochondria where, for example, acetate will suffice as an accompanying anion (Lehninger, 1970) or, indeed, where limited Ca^{2+} uptake can occur in the absence of any accompanying anion (Chance, 1965). It may well be true, however, *in situ*, where acetate is limited and where Ca^{2+} -H⁺ exchange may be prevented.

The origin of the portion of phase P_1 which is independent of Ca^{2+} and of P_1 was not investigated in this study.

Origin of phase P_{II}

Concentrations of pyruvate that maximally oxidize nicotinamide nucleotide during anaerobiosis (P. Lipton, unpublished work) almost completely abolish phase P_{II} , whether added during phase P_{II} or before the application of electrical pulses. This is strong evidence that phase P_{II} represents reduction of nicotinamide nucleotide in the cytosol (Scholz *et al.*, 1969; Bucher *et al.*, 1972). This agrees with the conclusions of Brauser *et al.* (1970) in the rat cervical ganglion and of Landowne & Ritchie (1971b) in the peripheral nerve about the cellular location of the stimulus-induced nicotinamide nucleotide reduction. This 25% increase of fluorescence which represents phase P_{II} is about equal to the cytosolic fluorescence increase that occurs during anaerobiosis. This latter is determined as equal to the maximal attenuation of fluorescence on addition of pyruvate during complete anaerobiosis (P. Lipton, unpublished work).

Although increased utilization of exogenously supplied glucose is thought to account for the increased slice respiration brought about by the pulses (McIlwain, 1966), increased net glycogenolysis (LeBaron, 1955) is the most likely source of the large reduction of cytosolic NAD⁺. Thus the disappearance of phase P_{II} with increasing duration of incubation, in the absence of any decrease in basal or stimulated respiration, would be accounted for by assuming a gradual decrease in slice glycogen with time. It could not be accounted for if the origin of phase P_{II} was the increased glycolysis associated with the increased respiration. The rapid turnover and small pool size of cerebral glycogen (McIlwain & Tresize, 1956; Coxon, 1970) make this quite plausible, though it was not investigated experimentally.

Electrical pulses are known to elevate slice cyclic AMP content very rapidly (Kakiuchi *et al.*, 1969). The observation (Fig. 10) that exogenous dibutyryl cyclic AMP causes a reduction of slice NAD⁺ is consistent with the possibility that this increased slice cyclic AMP content is causing phase P_{II} . This would, in turn, be consistent with the conclusion that the latter results from an increase in the rate of glycogenolysis as cyclic AMP is known to activate this process in many tissues including cultured rat astrocytes (Opler & Makman, 1972). Although Kakiuchi & Rall (1968) found no effect of histamine or noradrenaline on phosphorylase activity in brain slices and so concluded that cyclic AMP does not affect glycogenolysis in this tissue, they neither tested for glycogenolysis directly nor excluded the possibility of compartmentation of the cyclic AMP increase resulting from the neurohumoral agents. This last might well make it

inaccessible to the bulk of the tissue phosphorylase. A glycogenolytic origin for the equivalent of phase P_{II} in stimulated nerve fibres was inferred by Landowne & Ritchie (1971b).

Because their origin was not investigated phases P_{IV} and P_V , the 'off' phases of the response, will not be considered here. It is, however, notable that they are far slower than the 'on' phases of the response. According to Hillman *et al.* (1963) the membrane potential returns to basal values about 2.5 min after removal of the pulses. This is approximately the same time-period during which phase P_{IV} occurs but is far sooner than phase P_V . Thus phase P_{IV} may be associated directly with the repolarization but phase P_V apparently denotes a recovery process which persists after the repolarization is complete.

Oxidation in the presence of pyruvate

Although most aspects of the effect of superfusate pyruvate have been considered with reference to phase P_{II} and are interpretable in a straightforward manner, this is not so for the prolonged and irreversible phase of nicotinamide nucleotide oxidation during stimulation in the presence of pyruvate (Fig. 7). The oxidation is probably occurring in the cytosol, via the lactate dehydrogenase system (Scholz *et al.*, 1969). The question, then, concerns the source of the reduced nicotinamide nucleotide which is slowly gaining access to this system. Its most likely origin is the mitochondrial space, the only large repository of nicotinamide nucleotide aside from the cytosol. The observation may be explained by assuming that the membrane depolarization in some way activates the movement of NADH from the mitochondrion to the cytosol. Because of the impermeability of the mitochondrial membrane to NADH (Lehninger, 1951), a possible mechanism for this movement is via a reducing equivalent 'shuttle' involving the dicarboxylic acids (Bucher & Klingenberg, 1958; Shrago & Lardy, 1966; Williamson *et al.*, 1969). Such a movement might not be observed by this technique in the absence of exogenous pyruvate as the 'NADH' so transferred would not be oxidized in the cytosol. In the presence of pyruvate, however, a fluorescence decrease would be expected, corresponding to the cytosolic oxidation of the NADH transferred. This would be irreversible, the pyruvate essentially acting as a sink for the transferred NADH.

The proposed tentative explanation for the different aspects of the fluorescence response have not taken into account the extreme cellular heterogeneity of the tissue of the slice. Because this is very great it has been deemed wisest at this stage to attempt to account for the events on the implicit assumption that each phase, at least, occurs entirely in one cell type. This may very well be untrue.

The results indicate that there are profound changes in cellular energy metabolism resulting from membrane depolarization in this tissue. The similarity between the fluorescence changes here and those reported in the brain *in situ* during repetitive stimulation indicate that it may be possible to study metabolic events in the latter through this far more accessible preparation. From various parameters of the fluorescence changes Rosenthal & Jobsis (1971) concluded that the fluorescence response *in situ* was associated with the slow or steady potential shifts that follow from the direct cortical stimulation. This is consistent with their similarity to the changes in the slice preparations, which also probably represent changes owing to steady depolarization (Hillman *et al.*, 1963).

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