Biochem. J. (1980) **192**, 741–751
Printed in Great Britain

The regulation of pyruvate oxidation during membrane depolarization of rat brain synaptosomes

Walter T. SCHAFFER and Merle S. OLSON
Department of Biochemistry, The University of Texas Health Science Center, San Antonio, TX 78284, U.S.A.

(*Received 23 June 1980/Accepted 4 August 1980*)

Studies were performed to elucidate factors involved in the regulation of pyruvate dehydrogenase activity in rat brain synaptosomes during membrane depolarization. Addition of 24mM-KCl to synaptosomes resulted in increases in rates of O_2 consumption (90%) and [1-^{14}C]pyruvate decarboxylation (85%) and in the active/total ratio of extractable pyruvate dehydrogenase (90–100%) within 10s. Neither pyruvate (10mM) nor dichloroacetate (10mM) affected the activation state of the enzyme complex. Also, the activation state of pyruvate dehydrogenase was unaffected by addition of 1mM-octanoate, L-(−)-carnitine, 3-hydroxybutyrate, glutamate, citrate, lactate, L-malate, acetate, acetaldehyde or ethanol. Removal of Ca^{2+} by using EGTA lowered the active/total ratio to about 70%, although the rate of O_2 consumption and pyruvate decarboxylation was unaffected. Rates of pyruvate decarboxylation in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in the presence and absence of NaF and EGTA demonstrated a linear correlation with changes in the activity of the enzyme complex. This observation indicated that a change in the activation state of pyruvate dehydrogenase from 90 to 100% active could result in a 27% increase in the rate of pyruvate decarboxylation. It is suggested that the pyruvate dehydrogenase complex is an important site for the regulation of substrate utilization in rat brain synaptosomes. Further, the phosphorylation/dephosphorylation system and direct feedback-inhibitory effects on the enzyme complex both play a significant role in rapidly adapting pyruvate decarboxylation to changes in the requirements for mitochondrial energy production.

Kini & Quastel (1959) demonstrated that the rate of [1-^{14}C]pyruvate decarboxylation was enhanced after elevation of the K^+ concentration in brain slice incubations. This observation strongly implicated pyruvate dehydrogenase as a major locus for regulation of substrate oxidation in brain. Additional evidence was provided by the activation of pyruvate dehydrogenase observed in rat cortical slices incubated under depolarizing conditions for 10min (Kovachich & Haugaard, 1977). Since nearly 100% of the energy requirements of mammalian brain are supplied by aerobic glycolysis, it is not surprising that pyruvate dehydrogenase is an important regulatory enzyme (Sokoloff, 1972). The intricate regulatory mechanisms for this enzyme complex, which include feedback regulation and covalent modification by a kinase/phosphatase interconversion system, make pyruvate dehydrogenase an ideal candidate for this role (for review see Randle *et al.*, 1978; Ngo & Barbeau, 1978*a*). Although purified pyruvate dehydrogenase from rat brain is apparently similar to the enzyme complex derived from other tissues regarding regulation by pyruvate (Stacpoole & Berry, 1971; Booth & Clark, 1978), ATP/ADP (Portenhauser & Wieland, 1972), NADH/NAD^+ or acetyl-CoA/CoA (Batenburg & Olson, 1975; Pettit *et al.*, 1975; Ngo & Barbeau, 1978*a*,*b*) and Ca^{2+} (Denton *et al.*, 1972; Pettit *et al.*, 1972; Jope & Blass, 1975; Booth & Clark, 1978), there are indications that regulation of brain pyruvate dehydrogenase *in vivo* may be considerably different from that in other organs. For example, even under conditions of prolonged starvation, Siess *et al.* (1971) demonstrated that brain pyruvate dehydrogenase was not inactivated; a marked departure from the regulation of pyruvate dehydrogenase in other tissues. Although the brain enzyme need not adapt to different substrates in the well fed animal, pyruvate dehydrogenase must be regulated under conditions of the extreme variability in energy demands resulting from intermittent periods of quiescence and excitation. Theoretically, adaptation to

0306-3283/80/110741-11$01.50/1

fluctuations in energy demands could be met either via feedback inhibition or interconversion. The purpose of the present study was to investigate the relative contribution of either regulatory mechanism to rapid changes in the rate of pyruvate decarboxylation subsequent to membrane depolarization of a functional neuronal system.

Experimental

Materials

[1-^{14}C]Pyruvate, Aquasol and phenylethylamine were purchased from New England Nuclear. Serum stoppers and centre wells were obtained from Kontes. Ionophore A23187 was a generous gift from the Eli Lilly Laboratories and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. P. G. Heytler of the E. I. DuPont de Nemours Company. Ficoll (mol.wt. 400000) was obtained from Sigma.

Synaptosome preparation

Cerebra from six to eight adult Sprague–Dawley rats (200–250g) were removed rapidly and placed in cold isolation medium consisting of 0.32M-sucrose, 1mM-EDTA, 0.6mg of defatted dialysed bovine serum albumin/ml (Chen, 1967) and 10mM-Tris/HCl, pH7.40. The inclusion of albumin in the isolation medium was found to increase the magnitude of the KCl-induced respiratory response. In all other respects synaptosomes were prepared as described by Booth & Clark (1978). After the final centrifugation synaptosomes were resuspended in isolation medium to a final protein concentration of 20–30mg/ml estimated by using the biuret procedure (Layne, 1957) with bovine serum albumin as standard.

Mitochondrial contamination of synaptosomes

To estimate the contamination of free mitochondria and broken synaptosomes in this preparation, the rotenone-insensitive NADH–cytochrome *c* reductase (EC 1.6.99.3) activity of the preparation was compared with the activity of the mitochondrial pellet obtained from the flotation gradient (Lai *et al.*, 1977). Considering all of the protein of the pellet to be that of mitochondria, the mitochondrial contamination in the synaptosomal fraction was found to be less than 8%.

Incubations and estimation of functional integrity

The functional integrity of the preparation was tested before each experiment by measuring the increase in the rate of the O_2 consumption after depolarization with KCl. Synaptosomes suspended in isolation medium (0.2ml) were added to 1.8ml of incubation medium consisting of 130mM-NaCl, 4mM-KCl, 1.1mM-$CaCl_2$, 1mM-$MgSO_4$, 5mM-Na_2HPO_4, 10mM-glucose or 1mM-sodium pyruvate, and 20mM sodium Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid], pH7.4, in a 25ml Erlenmeyer flask. The flask was rotated at 110rev./min in a gyratory water bath (New Brunswick Scientific Co.) at 37°C for 15min. After the incubation period, the suspension was transferred to a water-jacketed stirred oxygen-electrode chamber, where a basal rate of O_2 consumption was measured. If the rate of respiration was not increased by at least 90% over the basal rate after adding 10μl of 4M-KCl, the preparation was discarded. The mean increase in the rate of respiration after depolarization of 17 preparations was 137% greater than the unstimulated rate. Similar additions of either 4M-NaCl or 4M-LiCl had no stimulatory effect on the rate of respiration. Additionally, mitochondria removed from the pellet of the Ficoll gradient showed low basal rates of O_2 consumption (e.g. less than 1ng-atom/mg of protein per min) after glucose or 1mM-pyruvate and exhibited no enhancement of respiration on addition of KCl. Because of the small amount of contamination with free mitochondria and the low rates of mitochondrial O_2 consumption, the contribution of extra-terminal mitochondria to the results presented was ignored.

Experiments were performed within 1h after preparation of synaptosomes, since the respiratory response to KCl depolarization declined after 3h. In some experiments reported in the present study, depolarization was achieved without changing the osmolarity of the suspensing medium. To accomplish iso-osmotic depolarization, 1ml of the suspension was added to 1ml of prewarmed Hepes buffer in which a portion of the NaCl was replaced by KCl and the $CaCl_2$ concentration was 1.0mM. For respiratory studies iso-osmotic dilution was performed in the oxygen-electrode chamber, whereas for other experiments the incubations were diluted into 25ml flasks in the gyratory water bath.

Pyruvate decarboxylation

At the end of the incubation period 1ml of the synaptosomal suspension was added to a flask containing 1ml of dilution medium with 1mM-[1-^{14}C]pyruvate (250d.p.m./nmol). The flask was sealed with a rubber serum stopper equipped with a plastic centre well. At the end of the second incubation period, 0.5ml of 18% (w/v) $HClO_4$ was injected into the sealed flask to stop the reaction and liberate $^{14}CO_2$. The $^{14}CO_2$ was trapped by adding 0.3ml of phenylethylamine to the centre well. The flask was agitated at room temperature for 1h before the centre well was removed to a scintillation vial containing 10ml of Aquasol and the radioactivity was estimated. The rate of [1-^{14}C]pyruvate decarboxylation, reported as nmol/min per mg of protein, was corrected for changes in the specific

radioactivity of the pyruvate in the dilution medium caused by pyruvate in the initial incubation. Corrections also were made for quenching of scintillation data and for non-enzymic decarboxylation of ^{14}C-labelled pyruvate by measuring the rate of $^{14}CO_2$ production in the absence of synaptosomes.

The rates of [1-^{14}C]pyruvate decarboxylation reported in the present study were not corrected for decarboxylation reactions other than pyruvate dehydrogenase. Since both 'malic' enzyme (EC 1.1.1.40) and pyruvate carboxylase (EC 6.4.1.1) have been demonstrated in brain (Salganicoff & Koeppe, 1968) and because α-oxoglutarate was observed to accumulate under incubation conditions employed in the present study (results not shown), the rate of [1-^{14}C]pyruvate decarboxylation as measured by $^{14}CO_2$ production must be interpreted with care. Estimates of the proportion of the overall rate of [1-^{14}C]pyruvate decarboxylation due to pyruvate dehydrogenase in the perfused liver and heart were 85 and 95% respectively. Although the actual amount of ^{14}C entering the tricarboxylic acid cycle from [1-^{14}C]pyruvate was not determined in these synaptosome preparations, it is unlikely that the amount of decarboxylation of administered [1-^{14}C]pyruvate not due to pyruvate dehydrogenase could exceed 10–15% (Scholz *et al.*, 1978; Olson *et al.*, 1978).

Activation state of the pyruvate dehydrogenase complex

The activation state of the pyruvate dehydrogenase complex was measured by using a rapid freezing technique coupled to [1-^{14}C]pyruvate decarboxylation essentially as outlined by Taylor *et al.* (1975). For measurement of the portion of the enzyme complex in the active form, 0.75ml of the incubation was added to a tube, kept in ice, containing 0.15ml of an activating solution, containing 0.268M-Mops (4-morpholinepropanesulphonic acid), 5mM-dithiothreitol, 33mM-dichloroacetic acid, 7mM-$CaCl_2$ and 66mM-$MgCl_2$, pH 7.0, in addition to 0.1ml of normal rabbit serum and 0.1ml of stopping solution (0.25M-NaF and 20mM-EDTA). After this addition, the tube was immediately frozen in a solid-CO_2/acetone bath. For measurement of the total pyruvate dehydrogenase activity, 0.75ml of the incubation mixture was added to a tube kept at 37°C, containing 0.15ml of activating solution, 0.1ml of rabbit serum and sufficient carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to give a final concentration of 5μM. After a 10min incubation, 0.1ml of stopping solution was added and the suspension was rapidly frozen in the solid-CO_2/acetone bath. For the assay, the active and total pyruvate dehydrogenase extracts were thawed sequentially and 0.75ml was added without delay to 25ml Erlenmeyer flasks kept at 37°C containing 0.75ml of assay medium containing 8mM-[1-^{14}C]pyruvate (100d.p.m./nmol), 4mM-NAD^+, 2mM-dithiothreitol, 0.4mM-CoA, 5mM-oxalacetic acid, 0.04% (w/v) Lubrol WX, 0.8mM-thiamin pyrophosphate and 2mM-$MgCl_2$, pH 6.0. The flasks were sealed with a rubber serum stopper equipped with a plastic centre well, incubated for 5min and stopped with 0.5ml of $HClO_4$, as described for measurement of synaptosomal pyruvate decarboxylation. Scintillation counting and data correction also were performed as described before.

Decarboxylation of [1-^{14}C]pyruvate was found to be linear during the period of measurement and proportional to the synaptosomal protein concentration present in the assay. In no case, even under incubation conditions known to cause maximal activation of the complex, was the active portion of the enzyme significantly greater than the total extractable enzyme activity. Also, the stopping conditions employed for treatment of active extracts were judged to be adequate for inactivation of the interconversion system as evidenced by the lack of increased enzyme activity in thawed extracts incubated for 15min at 4°C.

Results

The effect of KCl on O_2 consumption, pyruvate decarboxylation and the activation state of pyruvate dehydrogenase

An experimental system useful for characterizing the mechanism by which a stimulatory event on the neuronal membrane is relayed to enzymes regulating mitochondrial energy generation must possess certain characteristics. Rat brain synaptosomes prepared by the procedure noted above provided such a system as they are (*a*) derived from nervous tissue, (*b*) essentially free of non-excitable cells and free mitochondria and (*c*) capable of appropriate respiratory stimulation on excitation or K^+-mediated depolarization.

Increasing the K^+ concentration without changing the osmolarity of the suspending medium, as shown in Fig. 1, resulted in increased rates of O_2 consumption (*a*) and pyruvate decarboxylation (*b*). Since maximal rates of respiration and pyruvate decarboxylation were found to occur at 24mM-KCl, this concentration was employed for depolarization in subsequent experiments, even though considerably higher concentrations are necessary in brain slices (Kini & Quastel, 1959; Elliot & Bilodeau, 1962).

Increases in the rate of O_2 consumption of at least 90% compared with the unstimulated rate were found to occur within 10s after depolarization (results not shown). Whether this rather slow response, compared with transmissive events, represents a real lag in the metabolic response to

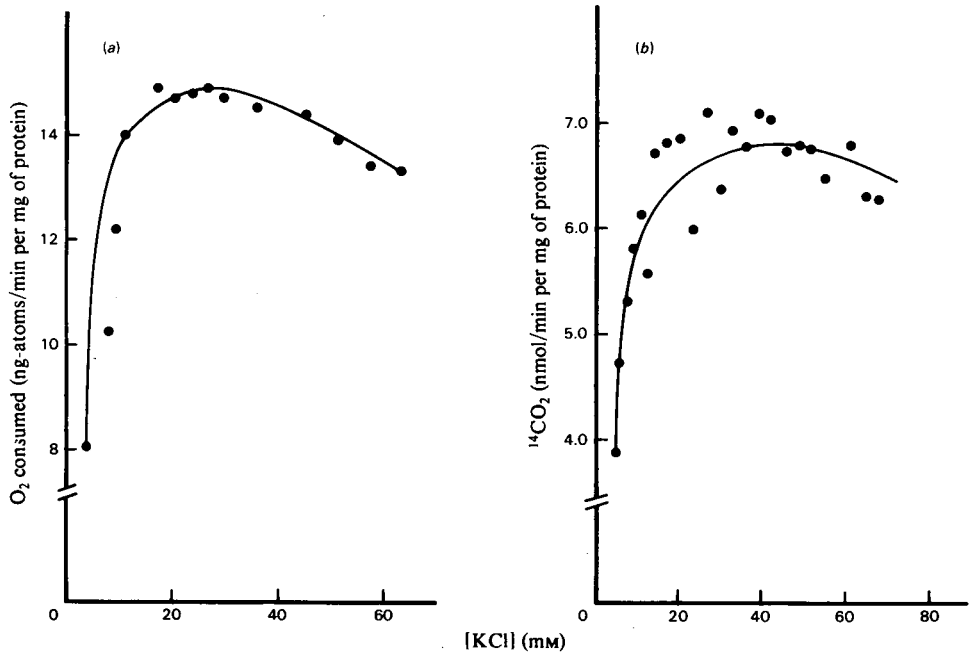

Fig. 1. *The rate of O_2 consumption (a) and the rate of [1-^{14}C]pyruvate decarboxylation (b) as a function of iso-osmotic KCl concentration*

Pre-incubated synaptosome suspension (1 ml) was added to 1 ml of diluting medium containing sufficient KCl (replacing an equal amount of NaCl) to give the indicated final concentration. For (*a*), synaptosomes were diluted in the oxygen-electrode chamber and the rate of O_2 consumption was followed for at least 6 min. In (*b*) the dilution medium contained 1 mM-[1-^{14}C]pyruvate (250 d.p.m./nmol) and dilution was carried out on the gyratory water bath as described in the Experimental section.

increased energy demands or reflects temporal limitations of the oxygen-electrode system is presently unknown. However, it was also found that pyruvate decarboxylation increased to 85% greater than the unstimulated rate within 30 s after dilution of the initial incubation with a high-KCl medium (Fig. 2*a*). After 30 s the rate of decarboxylation began to decline and by 2 min approached the rate for non-depolarized synaptosomes, which did not change over this same time period. This temporal variability in pyruvate decarboxylation contrasts with the prolonged linearity (at least 10 min) of iso-osmotic K^+-stimulated respiration (results not shown). In 4 mM-KCl and at 30 s after depolarization, the rate of decarboxylation was 2-fold higher than could be predicted by the rate of respiration, assuming all decarboxylation occurred at pyruvate dehydrogenase and 5 reducing equivalents were produced by complete oxidation of each pyruvate decarboxylated. By 3 min after dilution with depolarizing medium, however, the rate of production of reducing equivalents derived from the rate of $^{14}CO_2$ production exactly accounted for O_2 consumption.

Measurements of the activation state of the pyruvate dehydrogenase complex (Fig. 2*b*) indicated that the interconversion system may activate the complex quickly enough after depolarization to account for the observed increase in $^{14}CO_2$ production and respiration. The active portion of the enzyme increased from 90% of the total extractable activity in the original incubation in the presence of 4 mM-KCl, to 100% within 10 s after increasing the KCl to 24 mM. Also, the enzyme complex remained fully activated after 2 min, even though pyruvate decarboxylation had decreased, apparently by feedback inhibition (Fig. 2*a*). Although the depolarization-induced change in pyruvate dehydrogenase activity was small in comparison with changes in respiration and $^{14}CO_2$ production, the rapidity of the activation of the complex suggested an important function in adaptation of substrate supply to the increased energy demands associated with an excited state in nerve terminals.

The effect of pyruvate on the activation state of pyruvate dehydrogenase

Because glycolysis is greatly accelerated after stimulation of neutral tissue (McIlwain, 1963; DeBelleroche & Bradford, 1972) and the substrate

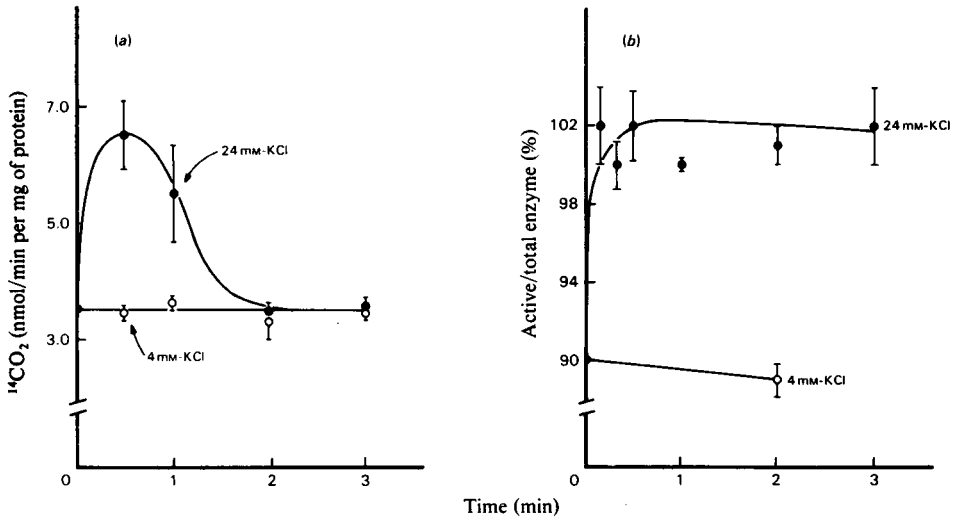

Fig. 2. *The rate of [1-^{14}C]pyruvate decarboxylation (a) and the percentage pyruvate dehydrogenase in the active form (b) as a function of time after dilution with depolarizing and non-depolarizing medium*

Pre-incubated synaptosomes (1 ml) were added to 1 ml of diluting medium containing sufficient KCl (replacing an equal amount of NaCl) to give either 4 or 24 mM-KCl. In (*a*) the dilution medium contained 1 mM-[1-^{14}C]pyruvate (262 d.p.m./nmol). The incubations were stopped at the indicated time and $^{14}CO_2$ was collected as described in the Experimental section. The values shown are means ± S.D. for three determinations of the rate of decarboxylation averaged over the time period after the preceding time point. In (*b*) samples were taken for determination of pyruvate dehydrogenase activity at the indicated times (see the Experimental section). Pyruvate (1.0 mM) was the substrate in both the pre-incubation and in the diluent.

pyruvate inhibits pyruvate dehydrogenase kinase (Siess *et al.*, 1971; Portenhauser & Wieland, 1972; Hucho *et al.*, 1972; Dennis *et al.*, 1978*a*), the effects of an increased rate of pyruvate production on the activation state of pyruvate dehydrogenase were investigated. Addition of pyruvate to the incubation medium was found to stimulate the rate of O_2 uptake by 5–10% compared with glucose-supported rates for synaptosomes incubated in medium containing either 4 mM- or 24 mM-KCl (results not shown). The saturable effect of pyruvate on respiration (e.g. the concentration at which the respiratory stimulation was half-maximal was 50 μM) contrasted markedly with the effect of pyruvate concentration on the activation state of pyruvate dehydrogenase (Fig. 3). In the absence of added substrate, the enzyme was fully active. Addition of pyruvate resulted in a decrease in the active/total ratio with a minimum observed near 0.75–1.0 mM, followed by a slight increase in the activation state at higher pyruvate concentrations. Even at 10 mM-pyruvate the complex was only 93% in the active form in contrast with the nearly complete reactivation seen in the perfused heart (Dennis *et al.*, 1978*a*) and in liver mitochondria (Portenhauser & Wieland, 1972). Consistent with the inability of pyruvate to completely reactivate synaptosomal pyruvate dehydrogenase and the belief that halogenated carboxylic acids inhibit kinase activity by interacting with a

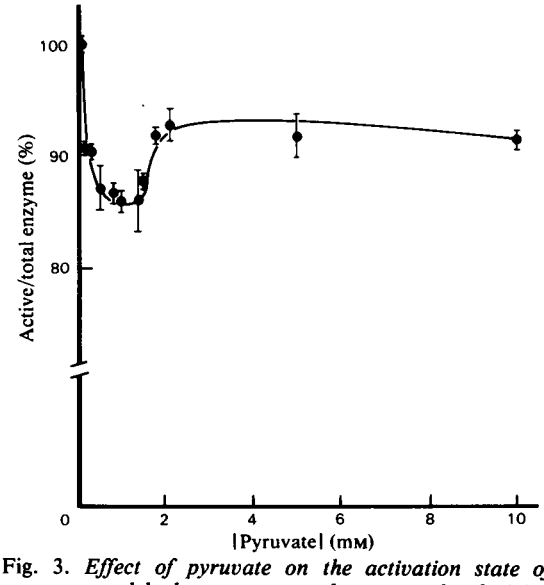

Fig. 3. *Effect of pyruvate on the activation state of pyruvate dehydrogenase under non-depolarizing conditions*

Samples for determination of active and total pyruvate dehydrogenase activity were removed from incubations after 14 min at 37°C in the gyratory water bath in the presence of the indicated final concentration of pyruvate. Each value is the mean ± S.D. for from four to eight determinations on at least two different preparations.

pyruvate-binding site on the kinase (Whitehouse *et al.*, 1974) was the observation that dichloroacetate, as high as 10mM, also failed to activate pyruvate dehydrogenase above 95% active/total activity in synaptosomes (results not shown), whereas 10μM was sufficient for activation in rat heart (Whitehouse *et al.*, 1974). Therefore, it appears that increased rates of glycolysis subsequent to membrane depolarization would have only a small effect on the activation state of pyruvate dehydrogenase.

Because 1mM-pyruvate provided the greatest amount of inactivation, this concentration was used in the remainder of the present studies. Also, the use of exogenous pyruvate alone obviated variations in the pyruvate supply caused by alterations in the rate of glycolysis. Additionally, in incubations of non-depolarized synaptosomes in the presence of 1mM-pyruvate, the active portion of pyruvate dehydrogenase was consistently near 90% of the total enzyme activity. Incubations in media containing 10mM-glucose provided variable active/total ratios (90–100%), perhaps because of differences in cytoplasmic volume. The 15min initial incubation period also was found to be ideal, not only for the establishment of linear respiratory rates (results not shown), but also for establishing the greatest measured difference in the activation state between unstimulated and KCl-stimulated synaptosomes. Cold synaptosomes, fresh from preparation, were found to have $68.3 \pm 2.5\%$ of the enzyme complex in the active form (results not shown). Incubation for 5min at 37°C in medium containing 4mM-KCl resulted in complete activation of the enzyme. After 15min the enzyme was 90% active and further incubation resulted in activation to 98% by 25min.

The effect of other oxidizable substrates on the activation state of pyruvate dehydrogenase

Since the influence of pyruvate as an inhibitor of synaptosomal pyruvate dehydrogenase kinase appeared to be minimal in comparison with other tissues (Portenhauser & Wieland, 1972; Dennis *et al.*, 1978*b*), it seemed imperative to investigate the effects of other oxidizable substrates on the activity of the enzyme complex. It was found that the activation state was unchanged after 15min incubations in the presence of 1mM-pyruvate and a variety of other substrates (octanoate, 3-hydroxybutyrate, glutamate, citrate, lactate, malate, acetate, acetaldehyde and ethanol) also at 1mM. The active portion of the enzyme complex (20.73 ± 0.72nmol/min per mg of protein) was about 90.0% of the total activity (22.98 ± 0.69nmol/min per mg protein) in all incubations (i.e. three to six determinations for each substrate). These results and observations by Jope & Blass (1976) with whole-brain mitochondria and brain slices, indicate striking differences between brain and other organs in which octanoate, acetate and 3-hydroxybutyrate have marked effects on the activation state of pyruvate dehydrogenase (Evans *et al.*, 1963; Garland *et al.*, 1964; Wieland *et al.*, 1971; Portenhauser & Wieland, 1972; Taylor *et al.*, 1975; Dennis *et al.*, 1978*a*,*b*; Scholz *et al.*, 1978). Consistent with a lack of effect of these substrates is the observation that brain function is supported almost entirely by aerobic glycolysis (Sokoloff, 1972).

The effect of Ca^{2+} and EGTA on O_2 consumption, pyruvate decarboxylation and the activation state of pyruvate dehydrogenase

The influx of Ca^{2+} into nerve terminals during depolarization has been implicated in the control of several neuronal processes, including neurotransmitter release and axonal conduction (Katz & Miledi, 1967; Swanson *et al.*, 1974; Goddard & Robinson, 1976; Krueger *et al.*, 1977). In view of these findings and the fact that Ca^{2+} is known to be important in the activation of the multienzyme complex catalysed by pyruvate dehydrogenase phosphatase (Pettit *et al.*, 1972; Denton *et al.*, 1972; Jope & Blass, 1975; Davis *et al.*, 1977; Booth & Clark, 1978), studies were performed to determine the Ca^{2+} sensitivity of the synaptosomal enzyme.

At low KCl concentrations, increasing the concentration of the Ca^{2+}-selective chelator, EGTA, caused a gradual increase in the respiratory rate (Fig. 4*a*). However, under depolarizing conditions there was a marked respiratory decrease near an EGTA concentration of 0.85mM in a medium containing 1mM-Ca^{2+}. The EGTA concentration necessary to produce the respiratory decrease was dependent on the total Ca^{2+} concentration in the incubation. For example, more Ca^{2+} required additional EGTA. Furthermore, adding Ca^{2+} to a suspension of depolarized synaptosomes, previously depleted of metal cation by 0.85mM-EGTA, reconstituted the respiratory rate to non-depleted values. A similar addition of Mg^{2+} was ineffective, indicating that the respiratory decrease was a reversible effect of Ca^{2+} depletion and not a non-specific effect of the chelator itself. Not surprisingly, chelator-induced changes in O_2 consumption were similar to changes in the rate of [1-^{14}C]pyruvate decarboxylation (Fig. 4*b*) at both low and high KCl concentrations. However, these changes were not similar to changes in the activation state. In low KCl medium there was a progressive decrease in the active/total ratio to about 70%, as the EGTA concentration was increased to around 6mM (Fig. 5). A similar effect was observed at 24mM-KCl (results not shown), except that in the absence of chelator and at 0.85mM-EGTA the complex was fully in the active form (Table 1). At 0.85mM-EGTA, there was a marked discrepancy between changes in the rate of pyruvate decarboxylation and the activation state of

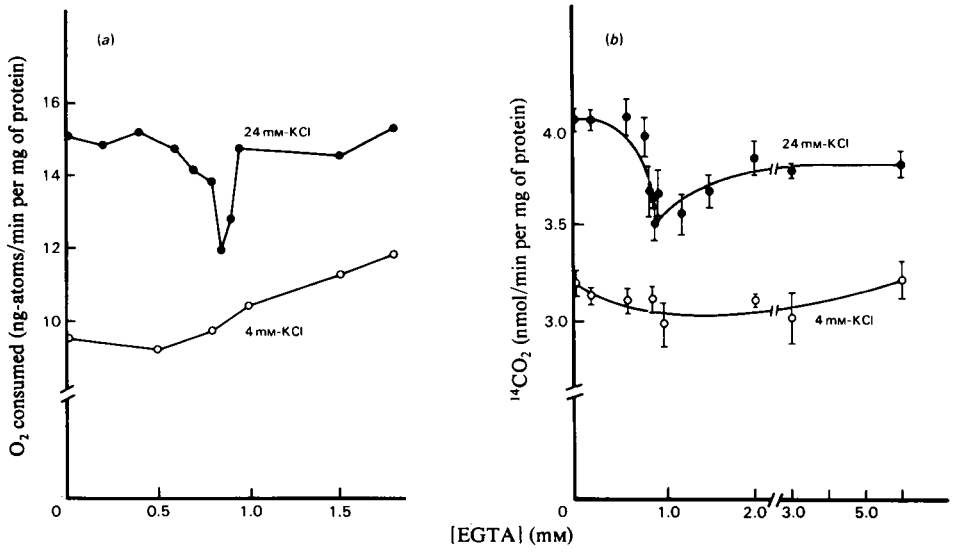

Fig. 4. *The effect of EGTA on the rate of O_2 consumption (a) and [1-^{14}C]pyruvate decarboxylation (b)*
Preincubated synaptosomal suspension (1 ml) was added to 1 ml of iso-osmotic diluent containing 1 mM-$CaCl_2$, either 4 or 24 mM-KCl and sufficient EGTA to give the indicated final concentration. In (*a*), dilution was carried out in the oxygen-electrode chamber and the rate of O_2 removal was monitored for at least 6 min. The values shown are individual rates obtained from a single preparation, although similar respiratory profiles were observed for 10 different preparations. In (*b*) pre-incubated synaptosomes were added to dilution medium containing 1 mM-[1-^{14}C]-pyruvate (527 d.p.m./nmol) and the incubation was terminated after 5 min. Values shown are means ± S.D. for three to six determinations on three different preparations.

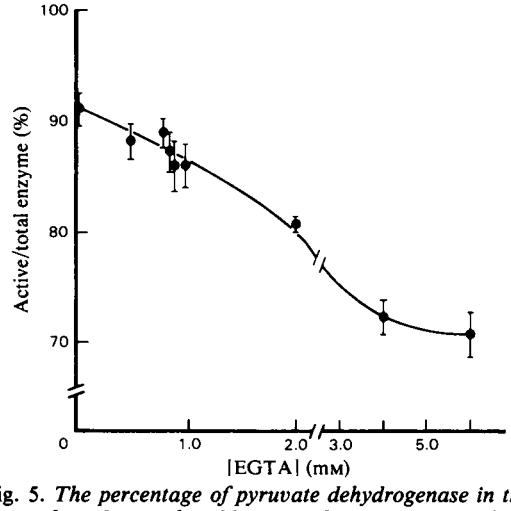

Fig. 5. *The percentage of pyruvate dehydrogenase in the active form 2 min after dilution with iso-osmotic medium under non-depolarizing conditions containing sufficient EGTA to give the indicated final concentration*
Values are means ± S.D. for four to six determinations on two preparations.

the enzyme complex, presumably mediated by feedback inhibition. The addition of the bivalent cation ionophore A23187 abolished the differences in the active/total ratio observed at high and low KCl (Table 1). This effect of ionophore A23187 is consistent with the idea that mitochondrial Ca^{2+} becomes available for chelation in the presence of the ionophore (Walajtys *et al.*, 1974). This concept also is supported by the observation of high rates of respiraton (20 ng-atoms of O_2/min per mg of protein) under depolarizing and non-depolarizing conditions in the presence of ionophore A23187 and 1 mM-Ca^{2+}, presumably due to Ca^{2+} cycling (Pfeiffer *et al.*, 1976). The addition of EGTA in the presence of the ionophore caused a progressive decrease in respiration (e.g. at 0.85 mM-EGTA, 12 ng-atoms/min per mg of protein and at 2 mM-EGTA 8.6 ng-atoms/min per mg of protein; results not shown), perhaps reflecting the drop in enzyme activity or decreased Ca^{2+} cycling. Because of the apparent disparities in the rate of pyruvate decarboxylation and the extractable pyruvate dehydrogenase activity at 0.85 mM and at higher

EGTA concentrations, in the absence of ionophore A23187, it seemed appropriate to investigate the capacity for regulation of pyruvate decarboxylation at a low and constant active/total pyruvate dehydrogenase ratio.

The rate of O_2 consumption and pyruvate decarboxylation under conditions of maximal enzyme inactivation

In an effort to completely inactivate pyruvate dehydrogenase phosphatase, synaptosomes were incubated under conditions reported to result in conversion of 97% of the liver multienzyme complex into the inactive form (Leiter *et al.*, 1978). After a 15 min initial incubation, synaptosomes were incubated for an additional 5 min under non-depolarizing conditions in the presence of 20 mM-NaF, 5 μg of ionophore A23187/ml and 2 mM-EGTA (Table 2). In the absence of phosphatase inhibitors, the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, stimulated respiration 3-fold and pyruvate flux nearly 2-fold (Table 3). Addition of $CaCl_2$ under these conditions was without measurable effect (results not shown). In the presence of ionophore A23187 and EGTA or NaF with ionophore A23187 and EGTA the rate of respiration was changed only slightly, whereas the rate of pyruvate decarboxylation was lowered 30%

Table 1. *Effect of EGTA, ionophore A23187 and depolarization on the activation state of pyruvate dehydrogenase*

A 1 ml portion of pre-incubated (15 min, 37°C) synaptosomes was added to a 25 ml Erlenmeyer flask containing 1 ml of iso-osmotic medium containing KCl, EGTA and/or ionophore A23187 to give the indicated final concentrations. In all cases total Ca^{2+} was 1.0 mM. After 2 min, samples were taken for determination of both actual (active) and total pyruvate dehydrogenase activity. Values reported are ratios of actual activity to the total activity and represent means ± S.D. for six to nine measurements of four different synaptosomal preparations.

			100 × Active/total pyruvate dehydrogenase activity		
Addition	[KCl] (mM)	EGTA (mM) ...	0	0.85	2.00
None	4		91.5 ± 1.5	91.4 ± 2.3	83.4 ± 0.6
	24		103.1 ± 2.5	101.5 ± 2.1	91.5 ± 3.1
Ionophore A23187 (5 μg/ml)	4		99.4 ± 3.3	87.8 ± 0.4	76.9 ± 4.5
	24		102.0 ± 1.6	86.5 ± 1.6	79.8 ± 1.2

Table 2. *Effect of uncoupler and $CaCl_2$ on O_2 consumption, pyruvate decarboxylation and the activation state of pyruvate dehydrogenase in non-depolarized rat brain synaptosomes under conditions favouring pyruvate dehydrogenase phosphatase inhibition*

After synaptosomes were pre-incubated 15 min with 1 mM-pyruvate as substrate at 37°C, 1 ml of the incubation was added to 1 ml of iso-osmotic medium under non-depolarizing conditions containing NaF, EGTA, and ionophore A23187, where indicated, to give final concentrations of 20 mM, 2 mM and 5 μg/ml respectively. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or $CaCl_2$ was added 1.5 min later to give final total concentrations of 5 μM and 3 mM respectively. Measurement of the rate of O_2 consumption was carried out as described in the Experimental section and the values shown are means for two determinations. Measurement of the rate of pyruvate decarboxylation was initiated after 100 s dilution and stopped 200 s later. Values are means ± S.D. for three determinations. Active/total pyruvate dehydrogenase activity was determined as described in the Experimental section on samples removed from the incubation 5 min after dilution. Each value is the mean ± S.D. for six measurements on two different preparations.

Addition	O_2 consumption (ng-atoms/min per mg of protein)	Pyruvate decarboxylation (nmol/min per mg of protein)	Active/total pyruvate dehydrogenase (%)
None	8.1	2.68 ± 0.06	89.5 ± 2.8
+Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone	25.8	4.86 ± 0.08	99.7 ± 1.2
+EGTA + ionophore A23187	8.6	1.82 ± 0.04	73.9 ± 2.7
+EGTA + ionophore A23187 + carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone	19.40	3.44 ± 0.11	87.7 ± 1.4
+EGTA + ionophore A23187 + $CaCl_2$	15.98	3.66 ± 0.17	85.0 ± 1.9
+EGTA + ionophore A23187 + NaF	8.30	1.88 ± 0.03	74.1 ± 2.0
+EGTA + ionophore A23187 + NaF + carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone	11.96	2.49 ± 0.03	76.5 ± 1.0
+EGTA + ionophore A23187 + NaF + $CaCl_2$	12.86	3.31 ± 0.11	78.7 ± 1.3

Table 3. *Effect of uncoupler and $CaCl_2$ on O_2 consumption, pyruvate decarboxylation and the activation state of pyruvate dehydrogenase in rat brain synaptosomes under conditions favouring pyruvate dehydrogenase phosphatase inhibition*

After synaptosomes were pre-incubated for 15 min at 37°C, 1 ml of the incubation was added to 1 ml of iso-osmotic medium under polarized conditions containing NaF, EGTA and ionophore A23187 to give final concentrations of 20 mM, 2 mM and 5 μg/ml respectively. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or $CaCl_2$ was added 1.5 min, later to give final total concentrations of 5 μM and 3 mM respectively. Measurement of the rate of O_2 consumption was carried out as described in the Experimental section and the values shown are means for two determinations. Measurement of the rate of pyruvate decarboxylation was initiated 100 s after dilution and stopped 200 s later. Values are means ± S.D. for three determinations. Active/total pyruvate dehydrogenase activity was determined as described in the Experimental section on samples removed from the incubation 5 min after dilution. Each value is the mean ± S.D. for six measurements on two different preparations.

Addition	O_2 consumption (ng-atoms/min per mg of protein)	Pyruvate decarboxylation (nmol/min per mg of protein)	Active/total pyruvate dehydrogenase (%)
None	8.1	2.68 ± 0.06	89.5 ± 2.8
+Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone	25.8	4.86 ± 0.08	99.7 ± 1.2
+EGTA + ionophore A23187	8.6	1.82 ± 0.04	73.9 ± 2.7
+EGTA + ionophore A23187 + carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone	19.40	3.44 ± 0.11	87.7 ± 1.4
+EGTA + ionophore A23187 + $CaCl_2$	15.98	3.36 ± 0.17	85.0 ± 1.9
+EGTA + ionophore A23187 + NaF	8.30	1.88 ± 0.03	74.1 ± 2.0
+EGTA + ionophore A23187 + NaF + carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone	11.96	2.49 ± 0.03	76.5 ± 1.0
+EGTA + ionophore A23187 + NaF + $CaCl_2$	12.86	3.31 ± 0.11	78.7 ± 1.3

and about 25% of the total enzyme complex was inactivated. In incubations containing NaF as well as EGTA and ionophore A23187 the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or $CaCl_2$ had only marginal effects on the activation state, whereas respiration increased 44 and 55% respectively for carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and $CaCl_2$ additions. Similar enhancements of $^{14}CO_2$ production were observed. In the absence of NaF, ionophore A23187 and EGTA were only partially effective in preventing reactivation of the complex by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and $CaCl_2$. The rate of O_2 consumption increased nearly 90% for both additions and increases in $^{14}CO_2$ production were of a similar magnitude. In all cases, within the error of measurement, the rate of pyruvate decarboxylation was sufficient to account for the rate of O_2 consumption. Additionally, in the presence of NaF, ionophore A23187, EGTA and $CaCl_2$ the rate of pyruvate decarboxylation was sufficient to account for the rate of respiration normally observed at 24 mM-KCl, even though 20% of the enzyme was inactivated.

The Effect of changes in the activation state on rates of pyruvate decarboxylation in the presence of carbonyl cyanide p-trifluoromethoxyphenylhydrazone

In the presence of uncoupling concentrations of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone it was found that changes in the active/total ratio were reflected in the rate of O_2 consumption and pyruvate decarboxylation (Table 2). Indeed, linear regression analysis of the percentage active/total versus the rate of pyruvate decarboxylation in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone with or without phosphatase inhibitors indicates high correlation ($r = 0.996$), consistent with the hypothesis that the uncoupler completely relaxes feedback inhibition and enzymic activity *in situ* is a function of the activation state. Such an analysis provided evidence that a 10% change in active as a percentage of total enzyme from 90 to 100% could result in a 27% increase in the potential rate of pyruvate decarboxylation (from 3.80 to 4.83 nmol/min per mg of protein). Therefore, in spite of the small magnitude of the change in the activation state a rather large change in the metabolic flux through the pyruvate dehydrogenase reaction was observed.

Discussion

Studies reported in the present paper indicate that synaptosomal pyruvate dehydrogenase is of primary importance in regulating the rate of oxidizable substrate admission to intra-terminal mitochondria (Figs. 1 and 2), supporting observations made with brain slices (Kini & Quastel, 1959). The synaptosomal system was also shown to possess a pyruvate dehydrogenase interconversion system that rapidly

adjusts the activation state of the enzyme complex, to satisfy the increased energy demands of membrane depolarization (Fig. 2*b*). The response to KCl depolarization supports observations in brain slices, except that activation in synaptosomes occurs at least 60 times faster and at lower KCl concentrations (Kovachich & Haugaard, 1977). Whether this difference in the response to KCl depolarization can be attributed to the large proportion of non-excitable glial cells in brain slices is unknown (Diamond *et al.*, 1964). In other respects, synaptosomal pyruvate dehydrogenase compared favourably with other brain preparations. The total extractable enzyme activity was midway between values for brain slices (7.7nmol/min per mg of protein) (Kovachich & Haugaard, 1977) and whole-brain mitochondria (51–70nmol/min per mg of protein) (Jope & Blass, 1975; Booth & Clark, 1978). The percentage active/total pyruvate dehydrogenase of cold synaptosomes fresh from preparation (68.3%) (results not shown) and after incubation in the presence of a Ca^{2+} chelator (Tables 1 and 2) agreed well with values from whole-brain mitochondria (60–84%) (Jope & Blass, 1976; Booth & Clark, 1978). After an initial incubation at low KCl concentrations, however, the activation state of the synaptosomal enzyme was considerably higher (Table 1 and Fig. 2*b*) than in cortical slices from young male rats (76%) (Kovachich & Haugaard, 1977), perhaps again reflecting tissue heterogeneity. The high active/total ratio observed under non-depolarizing conditions precluded a large change in the activation state after depolarization. However, the change to total activation after depolarization was consistent and rapid, suggesting that despite being small, the observed interconversion was important.

Consideration of the possible mechanisms by which the acceleration of pyruvate oxidation subsequent to membrane depolarization is mediated by the interconversion system suggested marked differences in regulation of the synaptosomal enzyme compared with other tissues. For example, although low pyruvate concentrations had an apparent activating effect on pyruvate dehydrogenase kinase (Fig. 3) and high pyruvate concentrations afforded some activation of the complex, the effects were much smaller than have been observed in the isolated perfused rat heart (Dennis *et al.*, 1978*a*,*b*). Similar minimal effects of pyruvate concentration on the brain enzyme have been reported for brain slices (Jope & Blass, 1976). However, there is evidence that 2mM-pyruvate transiently protects the whole-brain mitochondrial complex from inactivation in the presence of exogenously added ATP and oligomycin (Booth & Clark, 1978). Also, Siess *et al.* (1971) have shown inhibition of pig brain pyruvate dehydrogenase kinase activity by pyruvate. In both cases, there was no exogenously added Ca^{2+}, so that phosphatase activity may have been minimal.

Synaptosomal pyruvate dehydrogenase differed from the enzyme complex derived from other tissues in the lack of response to alternative oxidizable substrates. The lack of inactivation in the presence of octanoate, 3-hydroxybutyrate and acetate at concentrations known to be inhibitory in heart and liver are consistent with the observed lack of change in the activation state in whole brain after prolonged periods of starvation (Siess *et al.*, 1971). Whether the inability of alternative substrates to lower the active/total ratio reflects variations in the response of the interconversion system to changes in mitochondrial acetyl-CoA/CoA or NADH/NAD^+ or whether alternative substrates are simply not metabolized quickly enough remains unknown.

It seems clear that changes in Ca^{2+} concentrations subsequent to membrane depolarization (Katz & Miledi, 1967) may be of great importance in activating pyruvate dehydrogenase. Addition of EGTA under depolarizing (Table 1) and non-depolarizing conditions (Fig. 4) caused a marked decrease in extractable activity, consistent with observations of pyruvate dehydrogenase phosphatase activation by Ca^{2+} (Denton *et al.*, 1972). The lack of inactivation of O_2 consumption and pyruvate decarboxylation at high EGTA concentrations (Fig. 3) further implicates the importance of Ca^{2+} in changes in the activation state. However, it appears that the enzyme complex from synaptosomes is incapable of inactivation below 70% in the presence of 6mM-EGTA (Fig. 4) or under conditions known to inactivate 97% of the liver mitochondrial enzyme (Leiter *et al.*, 1978) (Table 2). This observation suggests an additional departure in the regulation of the synaptosomal enzyme complex from other tissues.

The addition of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to synaptosomal incubations (Table 2) represents an attempt to evaluate pyruvate dehydrogenase activity *in situ* under conditions in which inhibition by feedback effects should be minimal. Based on a linear regression analysis for carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone treated incubations (see the Results section), a change from 90 to 100% active enzyme would correspond to a potential 27% increase in pyruvate decarboxylation. Although it is not at all clear why a 10% change in extractable enzymic activity should result in a nearly 30% change in the capacity to oxidize pyruvate within intra-terminal mitochondria, these results suggest that changes in the activation state may be very important in regulating pyruvate flux. Extrapolation to 89.5% activity (Table 2), indicates that a maximum of 3.75nmol of pyruvate could be decarboxylated/min per mg of protein in the

presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. In fact, under non-depolarized conditions (Table 2) 2.68nmol/min per mg of protein was observed, indicating a 28% decrease from the theoretical maximal rate of decarboxylation, presumably by feedback inhibition. If non-depolarized rates of $^{14}CO_2$ production (Table 2) increased by 85% of the original rate on depolarization (Fig. 2), the rate in 24mM-KCl would be 4.96nmol of $^{14}CO_2$/min per mg of protein or very similar to the maximal rate of pyruvate decarboxylation observed in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Table 2).

Similar reasoning suggests that by 2min after KCl addition (Fig. 2) 44% of the total pyruvate dehydrogenase activity *in situ* was suppressed by feedback inhibition.

In summary, the pyruvate dehydrogenase complex has been shown to be of primary importance in adapting the rate of pyruvate oxidation to changes in energy requirements associated with membrane depolarization in isolated nerve terminals. This adaptation necessaily occurs via both relaxation of feedback inhibition, as well as interconversion of the enzyme complex to the active form. Also, the activation process subsequent to membrane depolarization probably is mediated by Ca^{2+} influx.

We thank Dr. David Otto for help with the manuscript and Mr. J. Bernal for his expert technical assistance. This research was supported by Grant HL-24654 from the National Institutes of Health.

References

Batenburg, J. J. & Olson, M. S. (1975) *Biochem. Biophys. Res. Commun.* **66**, 533–540

Booth, R. F. G. & Clark, J. B. (1978) *J. Neurochem.* **30**, 1003–1008

Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173–181

Davis, P. F., Pettit, F. H. & Reed, L. J. (1977) *Biochem. Biophys. Res. Commun.* **75**, 541–549

DeBelleroche, J. S. & Bradford, H. G. (1972) *J. Neurochem.* **19**, 585–602

Dennis, S. C., Padma, A., DeBuysere, M. S. & Olson, M. S. (1978*a*) *J. Biol. Chem.* **253**, 7369–7375

Dennis, S. C., DeBuysere, M. S., Scholz, R. & Olson, M. S. (1978*b*) *J. Biol. Chem.* **253**, 2229–2237

Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163

Diamond, M. C., Krech, D. & Rosenzweig, M. R. (1964) *J. Comp. Neurol.* **123**, 111–120

Elliot, K. A. C. & Bilodeau, F. (1962) *Biochem. J.* **84**, 421–428

Evans, J. R., Opie, L. H. & Reynold, A. E. (1963) *Am. J. Physiol.* **205**, 971–976

Garland, P. B., Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 409–427

Goddard, G. A. & Robinson, J. D. (1976) *Brain Res.* **110**, 331–350

Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W., Reed, L. J. (1972) *Arch. Biochem. Biophys.* **151**, 328–340

Jope, R. & Blass, J. P. (1975) *Biochem. J.* **150**, 397–403

Jope, R. & Blass, J. P. (1976) *J. Neurochem.* **26**, 709–714

Katz, B. & Miledi, R. (1967) *J. Physiol.* (London) **192**, 407–436

Kini, M. M. & Quastel, J. H. (1959) *Nature* (*London*) **184**, 252–256

Kovachich, G. B. & Haugaard, N. (1977) *J. Neurochem.* **28**, 923–927

Krueger, B. K., Forn, J. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 2764–2773

Lai, J. C. K., Walsh, J. M. Dennis, S. C. & Clark, J. B. (1977) *J. Neurochem.* **28**, 625–631

Layne, E. (1957) *Methods Enzymol.* **3**, 447–454

Leiter, A. B., Weinberg, M., Isohashi, F., Utter, M. F. & Linn, T. (1978) *J. Biol. Chem.* **253**, 2716–2723

McIlwain, H. (1963) *Biochem. J.* **55**, 618–624

Ngo, T. T. & Barbeau, A. (1978*a*) *J. Neurochem.* **31**, 69–75

Ngo, T. T. & Barbeau, A. (1978*b*) *Can. J. Neurol. Sci.* **5**, 231–238

Olson, M. S., Dennis, S. C., Routh, C. A. & DeBuysere, M. S. (1978) *Arch. Biochem. Biophys.* **187**, 121–131

Pettit, F. H., Roche, T. E. & Reed, L. J. (1972) *Biochem. Biophys. Res. Commun.* **49**, 563–571

Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 575–582

Pfeiffer, D. R., Hutson, S. M., Kauffman, R. F. & Lardy, H. A. (1976) *Biochemistry* **15**, 2690–2697

Portenhauser, R. & Wieland, O. (1972) *Eur. J. Biochem.* **31**, 308–314

Randle, P. J., Sugden, P. H., Kerbey, A. L., Radcliff, P. M. & Hutson, N. J. (1978) *Biochem. Soc. Symp.* **43**, 47–67

Salganicoff, L. & Koeppe, R. E. (1968) *J. Biol. Chem.* **243**, 3416–3420

Scholz, R., Olson, M. S., Schwab, A. J., Schwabe, U., Noel, C. & Braun, W. (1978) *Eur. J. Biochem.* **86**, 519–530

Siess, W., Wittman, J., and Wieland, O. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 447–452

Sokoloff, L. (1972) in *Basic Neurochemistry* (Siegel, G. J., Albers, W. R., Katzman, R. & Agranoff, B. W., eds.), Little Brown and Co., Boston, MA

Stacpoole, P. W. & Berry, M. N. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 767

Swanson, P. D., Anderson, L. & Stahl, W. L., (1974) *Biochim. Biophys. Acta* **356**, 174–183

Taylor, S. I., Mukjerhee, C. & Jungas, R. L. (1975) *J. Biol. Chem.* **250**, 2028–2035

Walajtys, E. I., Gottesman, D. P. & Williamson, J. R. (1974) *J. Biol. Chem.* **249**, 1857–1865

Whitehouse, S., Cooper, R. H. & Randle, P. J. (1974) *Biochem. J.* **141**, 761–774

Wieland, O., von Runke, H. & Loffler, G. (1971) *FEBS Lett.* **15**, 295–298