Reviews

Cerebral ischemia revisited: New insights as revealed using in vitro brain slice preparations

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Summary. The elucidation of the pathophysiological mechanisms of cerebral ischemia/hypoxia dictates the use of experimental models which mimic this disabling brain condition. In vivo experimental models have been available for many decades and are responsible for the bulk of, though incomplete, knowledge we have about these mechanisms. Since study in isolation of each postulated mechanism is impossible in vivo, the need for an in vitro experimental model has intensified in recent years. Consequently, rat and guinea pig hippocampal slice preparations have emerged as the models of choice. This review attempts to highlight some of the results obtained using brain slices in the study of cerebral ischemia/hypoxia and compare them to those obtained in vivo. Both the biochemical and the physiological correlates of energy metabolism, ion homeostasis, neurotransmission and neuromodulation of this brain condition are reviewed. The agreements, and especially the disagreements, between the in vivo and in vitro findings are emphasized. Details are given of the possible roles of both lactic acid, Ca^{2+} and excitotoxins in the neuronal damage inflicted by cerebral ischemia/hypoxia. Recent attempts to protect brain slices against experimental cerebral ischemic/hypoxic damage are also reviewed here briefly.

Key words. Cerebral ischemia/hypoxia; hippocampal slice; lactic acid; excitotoxins; glutamate; NMDA; calcium ions; neuronal protection.

Introduction

Cerebral ischemia, or stroke, is primarily a reduction in the cerebral blood flow to a level inadequate for normal brain function. It is the third leading cause of death in the United States behind heart disease and cancer [55]. Clinically, this syndrome is characterized by a number of neurological deficits that have a rapid onset and 24-h progression. The brain's demand for energy is the highest of all organs; while its weight is approximately 2% of the total body weight it consumes 20% of the body's resting oxygen consumption [55]. Oxygen and glucose are the primary substrates for aerobic energy metabolism in the brain. Interruption of blood flow results in immediate shortage in oxygen supplies followed by diminishing glucose levels.

Thus, a cerebral ischemic insult quickly depresses ATP (and other high-energy phosphates) levels to the point where ion homeostasis cannot be maintained and normal cerebral functions are completely shut down. Yet, the brain can withstand certain lengths of ischemic insult before any dysfunction is apparent. How long the brain can survive without energy supply is still under debate, although the experts agree that it is short and can be measured by minutes, and hence, the urgency in renewing blood circulation of stroke victims. This short period of resistance to ischemia is the only time in which the physician has the opportunity to reperfuse the brain in an effort to prevent any neurological deficit.

During the last decade many studies on cerebral ischemia were aimed at prolonging this period of resistance by intervention after the onset of the insult. Others attempted to protect the brain against this syndrome prophylactically. Yet, our understanding of the mechanisms involved in the production of ischemic brain damage is not complete. Ongoing research in many laboratories around the world aims at elucidating these mechanisms. In the present review we examine some recent insights and promising results obtained in in vitro studies on cerebral ischemia, and highlight a few unexpected findings and future expectations.

In vivo vs in vitro

To start with, the investigator of cerebral ischemia has to make a choice as to the model system he or she should use. There are many model systems to choose from and the final decision depends mainly on the questions the experimenter tries to answer. Most cerebral ischemia in vivo models employ rodents such as the rat and the gerbil. They all attempt to interrupt blood flow to the brain employing methods ranging from decapitation [69], cerebrospinal fluid (CSF) compression [62], cardiac arrest [33], drowning and bleeding [57], to arterial inflow occlusion [34], 4-vessel occlusion [76] and positive acceleration (centrifugation) [36]. While each of these models has its own advantages and disadvantages, the 4-vessel occlusion in the rat and the ligation of the two common carotids in the gerbil have become the most popular models in the study of cerebral ischemia. In contrast, until recently not much effort has been put into the development of in vitro models, since most of the established investigators in the field considered such models to be inadequate due to their complete lack of blood circulation. However, if one would, for reasons of simplicity, consider blood vessels

and blood components merely as delivery vehicles for substrates of energy metabolism and as a washout system of waste products, then the advantages inherited in in vitro systems could justify their use. Two such systems emerged in the last decade, brain slice preparations and cell cultures. Of the two, the former appears to be the preferred system in the study of cerebral ischemia/hypoxia. Thus, the present review will deal mainly with results obtained from studies which employ brain slices. Nevertheless, since the bulk of our knowledge on the topic has originated from studies which employ in vivo models of cerebral ischemia, one cannot avoid citing them time and again. In vitro systems in general, and brain slice preparation in particular, offer many advantages over in vivo techniques. First and foremost, there is the total control by the investigator over the extracellular environment of the tissue under study, including the ionic, hormonal and gaseous components, and temperature. Secondly, in vitro systems offer an immediate and direct access to the extracellular compartment, due to the lack of a blood-brain barrier, an access limited only by the rate of diffusion. The third advantage stems from the direct visualization of the brain region under study, a very important consideration when placement of electrodes and other probes is required. Fourth, the brain slice(s) under study is available for functional as well as biochemical and morphological analyses, and last but not least, anesthetics are not required, an important consideration when ischemia and its outcome are concerned (see below). Along with these advantages, one should be aware of the major limitations of brain slice preparations. These are: 1) severed inputs and outputs which lead to some distinct differences from the intact brain; 2) an absence of behavioral output; 3) lack of blood-borne components whether beneficial or detrimental.

Nonetheless, the above-mentioned advantages of brain slice preparations lured many investigators to use them in the study of the mechanisms underlying ischemic brain damage [19, 24, 42–45, 47, 48, 67, 69, 84, 92, 104, 117, 118] and brain resuscitation and pharmacological protection [2, 3, 6, 7, 13, 31, 52, 68, 78, 85, 90, 91, 94–99, 101, 102, 112, 117].

Correlates, parameters and measurements in cerebral ischemia/hypoxia

A model system of cerebral ischemia must allow the measurement of at least one of three major correlates, namely, a physiological or functional, a biochemical, and/or a structural correlate. Many of the different models make it available to measure all three correlates within one experiment. However, frequently the one correlate being measured depends on the investigator's training, experience and preference. Thus, the physiologist will measure function, the biochemist will deal with metabolic changes and the morphologist will examine structural abnormalities. As with many fields of life sciences, it is the biochemist who at the end provides most of the details, although without the contributions of his colleagues, the physiologist and the morphologist, the picture cannot be completed. The biochemical correlate of cerebral ischemia can be divided into two major metabolic changes: those affecting energy metabolism and changes in the metabolism of neurotransmitters and neuromodulators. Function can be measured either at the behavioral level or at the cellular level. In this review, neither behavioral changes nor morphological alterations following cerebral ischemia will be discussed. All the measurements performed using in vivo models of cerebral ischemia, excluding those involved with blood flow, can be made using in vitro models. Nevertheless, despite hundreds of published studies, the investigator of cerebral ischemia who uses an in vitro system in his studies continues to face skepticism and suspicion. Consequently, much of the research done in the first few years following an introduction of a new in vitro model for cerebral ischemia is aimed at justifying it as a model by comparing results with those obtained from the established, classical in vivo models. Although, in general, a good correlation does exist between data obtained using both approaches, it is when the results do not match that the in vitro approach is being questioned.

Before discussing the biochemical and the functional correlates in detail, a brief description of the preparation and maintenance of brain slices is due. To date, most of the studies on cerebral ischemia/hypoxia employing brain slice preparations have been done using either rat or guinea pig hippocampal slices. To maintain brain slices, two different types of incubation chambers exist, the interface type and the submerged type. Although both have produced similar results, certain inherited differences between the two may lead one to choose the interface over the submerged type, or vice versa [83]. The lack of blood supply in brain slices force their users to rely on diffusion for reaching adequate levels of oxygen and other nutrients within the tissue, hence, the high concentration of O_2 (95%) and glucose (10 mM) used in slice experiments to maintain 'normal' physiological and biochemical functions. For the same reason the thickness of brain slices does not usually exceed 500 μm. Other components which make up the slice environment closely resemble those found in natural cerebrospinal fluid (CSF) and mimic their concentrations [84]. The perfusion rate of this artificial CSF (ACSF) assures an ample supply of its components with adequate removal of waste products.

The biochemical correlate

Energy metabolism and metabolites. It was demonstrated more than a quarter of a century ago that the levels of adenosine triphosphate (ATP) quickly diminish upon ischemia/hypoxia [51]. The ischemic reduction in ATP levels is a direct result of the inability of anaerobic glycolysis, and other alternative ATP forming processes, to fill-in for the quickly-diminishing oxidative phosphorylation.

Although calculations can show that the brain's ATP pools are small and would be completely consumed within 2 min of the onset of cerebral ischemia[51], brain function usually recovers after 5–10 min of ischemic insult and in certain instances (see below) even after 60 min of oxidative phosphorylation shutdown[69]. One is led to conclude that despite its inefficiency, glycolysis provides enough ATP to at least delay the appearance of 'the last gasp'. When glycolysis diminishes so do the chances of the brain for recovery. What is required for glycolysis to be sustained under ischemic/hypoxic conditions and thus, to provide a minimal but important ATP production? For one, a continuous supply of glucose. Since the ischemic insult halts the supply of this substrate, the maintenance of glycolysis depends entirely on glucose reserves and to some extent on glycogen storage. Jilek has shown[37] glycogen to be the source of substrate for glycolysis in young animals, while at the same time demonstrating the inability of adult animals to use glycogen stores for this purpose. Jilek postulated that the higher resistance of young animals to ischemia is the result of their ability to use glycogen for glycolysis during the ischemic episode[37]. Nevertheless, the rate of glycolysis increases severalfold upon ischemia (Pasteur effect) which in essence should enhance the consumption of the limited pools of glucose and shorten the life of the ischemic brain. In light of the above information one may rationalize on the possibility that hyperglycemic animals would withstand cerebral ischemia better than hypoglycemic ones. This possibility was tested by preloading experimental animals with glucose and then exposing them to ischemia[42, 60]. Histological examination of their brains revealed greater damage than their starved counterparts. In addition, significantly larger amounts of lactic acid were accumulated in the brains of hyperglycemic animals upon ischemia[41, 59]. Based on these findings, a hypothesis has been put forward postulating lactic acidosis to increase the degree of ischemic brain damage[41, 60, 61, 78, 79, 105, 106]. Paradoxically, better recovery from complete rather than severe incomplete ischemia has been observed[62, 63, 81], results which could be explained by an increase in lactic acid to a higher level in severe incomplete ischemia than in complete ischemia[49, 64, 82]. However, the acidosis (decreased pH) concomitant to lactate accumulation cannot by itself account for the increased ischemic injury[55]. While lactate accumulation is relatively uniform all over the ischemic brain, the ischemic injury is heterogenous[55]. Moreover, lowering the cellular pH by increasing PCO_2 induced only minor morphological changes[22]. Our own studies, using in vitro hippocampal slices, indicate lactic acid to have no detrimental effect on hypoxic neuronal tissue even at concentration of 20 mM (pH = 5.5)[97]. Although these results do not support the above hypothesis, they are in full agreement with our finding that increased levels of glucose in the bathing medium of brain slices protect them against hypoxic damage[19, 98]. The ability of a glucose load to improve the ischemic/hypoxic outcome in vitro is in accordance with our general knowledge of energy metabolism; i.e. under hypoxic conditions in which oxidative phosphorylation rates are nil, glycolysis rates are increased severalfold to consume all available glucose reserves (Pasteur effect). The larger these reserves are, the longer glycolysis is maintained and, although inefficient, the minimal amounts of ATP synthesized via this pathway should prolong the survival of the brain under ischemic/hypoxic conditions. Moreover, since oxygen and glucose are the two most important substrates for energy metabolism, the lack of both should be more harmful than the lack of either one alone, a postulate supported by our results[25, 26]. Thus, one has to look for an alternative explanation for the increase in ischemic damage in hyperglycemic animals.

One of the strongest arguments against the use of brain slices in the study of cerebral ischemia is their lower levels of ATP, total adenylates, creatine and phosphocreatine (PCr) compared to the in vivo levels. These metabolite levels are roughly 50% of their in situ levels[117]. Thus, one could be led to assume the in vivo brain to be more resistant to oxygen lack than brain slices, an assumption which is wrong. This discrepancy may be explained by the in vitro values of the energy charge and the ratio PCr/ATP which are maintained near those found in vivo[117]. Such values suggest that the primary high energy compounds, ATP and PCr, are maintained in appropriate proportion although their absolute level has been compromised[117]. Lactate is another metabolite of which brain slices produce more than the in vivo brain[117]. Lactate production has been used as a reliable measurement of diminishing oxygen supplies and enhanced rates of anaerobic glycolysis. Whether the higher levels of lactate in the brain slice are the result of lack of oxygen at the center of the slice, or a reflection of decapitation ischemia from which slices may be unable to completely recover, is still to be determined. Nevertheless, despite the heightened lactate magnitude, brain slices exhibit what appear to be normal synaptic responses to both oxygen presence and absence.

As mentioned before, the acidosis resulting from augmented lactic acid production has been postulated to produce the observed cerebral ischemic damage[23, 41, 60, 61, 79, 80, 105, 106]. One may argue that since brain slices are maintained at physiological pH at all times, an increase in lactate level should have no ill effect on normal function of the isolated brain tissue. Before dealing with this issue (see 'The physiological correlate' below) let us examine the response of the immature brain to hypoxia and increased lactic acid production. Lactic acid has long been regarded as the end product of glycolysis, and as such incapable of supporting energy metabolism. The higher tolerance of immature animals to hypoxia[32] was assumed to stem from supposedly predominant anaerobic glucose consumption and high production of lactic acid[11, 12].

This assumption was proved to be wrong when more recent experiments indicated conclusively that the perinatal brain derives no energy from anaerobic glycolysis under physiological conditions[115]. Other studies have shown that the tolerance of immature animals to hypoxic insult depends not on their ability to form energy anaerobically, but rather on the low cerebral metabolic activity relative to adults[20,114,115]. Consequently, the ability of the immature brain to utilize lactate as an energy substrate has been demonstrated. It has been suggested to contribute to the immature brain's tolerance to the known deleterious effects of hypoglycemia and hypoxia[115]. The inability of mature animals to utilize lactate as an energy substrate is due to the blood-brain barrier impermeability to this substance, and is mistakenly interpreted by many as the brain's inability to metabolize lactate for energy production.

Monoamine, catecholamine and amino acid neurotransmitters. Many other brain metabolite levels have been traced for changes upon ischemia. In the case of the monoamine and catecholamine neurotransmitters the reports are conflicting as to whether their levels increase or decrease during ischemia. Gamma-amino butyric acid levels increase gradually with longer periods of ischemia[22,56,113], while glutamate levels are either unchanged[22,113] or are slightly depressed during cerebral ischemia[56]. Nevertheless, recent findings have attributed to glutamate (and aspartate) a key role in the mechanism leading to ischemic/hypoxic brain damage (see below).

Neuromodulators. Taurine is another amino acid which attracted the interest of researchers in the field of ischemia/hypoxia. Although not a neurotransmitter, taurine is believed to have an important neuromodulatory role by attenuating Ca^{2+} movements across the neuronal membrane[27]. Similarly to glutamate, total cerebral taurine levels do not change upon ischemia/hypoxia; however, its intra- to extracellular distribution changes as was evident by the manifold increase in the extracellular level[25,26]. A possible role for taurine in the higher resistance of immature animals to ischemia/hypoxia has been suggested[96] and will be discussed in more detail later. Among other neuromodulators, cyclic AMP has been shown to accumulate during cerebral ischemia[10]. The increase in this neuromodulator occurs during the first minute after the onset of the ischemic insult followed by a return to baseline values[53,54,109]. In contrast, cyclic GMP levels decrease with ischemia in a much slower rate than the one observed with cyclic AMP[77,109]. The importance of these changes in determining the extent of an ischemic insult is unknown.

The physiological correlate

The physiological or functional correlate can best be measured by electrophysiological means, and brain slice preparations have made these measurements easier to execute and interpret than in vivo. Since brain slices, by the nature of their preparation, are devoid of neuronal inputs from other brain areas, any response recorded in these slices must be evoked either by spontaneous changes within the sliced tissue itself or by an external stimulus. The tight control over the environmental conditions on one hand, and the source of the tissue for these studies (pure bred, young adult animals, supplied by a reliable breeder) on the other, hold to a minimum spontaneous or unexpected changes within the tissue. Thus, any response recorded is the result of changes created by the experimenter in the slice environment. For quantitative and statistical reasons, most cerebral ischemia investigators who use either rat or guinea pig hippocampal slices, prefer to record the response of a whole population of neurons rather than that of a single cell. Such responses are usually evoked by applying an electrical stimulus to axons emerging from one group of neurons forming a monosynaptic connection with the dendrites of another group. In the hippocampal slice preparation two major monosynaptic circuits have been used: the CA3 axons, better known as the Schaffer collaterals which synapse with the dendrites of the CA1 pyramidal cells,

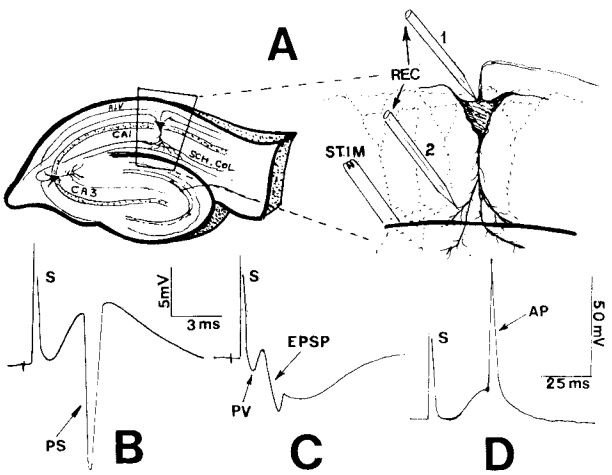

Figure 1. A schematic representation of a rat hippocampal slice preparation(*A*). The boxed-in area on the left shown enlarged on the right. Alv = alveus; CA1, CA3 = Cornu Ammonis, field 1 and field 3; SCH.COL = Schaffer collaterals of stratum radiatum; REC = recording electrodes 1 and 2; STIM = stimulating electrode. *B* shows a trace of a population spike (PS) evoked by orthodromic stimulation of the Schaffer collaterals and recorded by recording electrode 1 from the pyramidal cell body layer of area CA1. *C* is a trace of an excitatory postsynaptic potential (EPSP) and a prevolley (PV) evoked by orthodromic stimulation and recorded by recording electrode 2 in the dendritic region of area CA1. *D* is a typical intracellular record of an action potential (AP) evoked in a single pyramidal cell in area CA1. In all traces S = stimulus artifact. All the experiments described here were performed in a dual, linear-flow incubation chamber[93] using rat hippocampal slices and electrophysiological methods[94,95,97–102]. Extracellular recordings of population responses (population spike) in the stratum pyramidale of the CA1 region, evoked by stimulating the Schaffer collaterals, were used as a measurement of neuronal function. Population spike could be evoked at the beginning of each experiment in all slices placed in the chamber. A slice in which a population spike of 3 mV or greater could not be evoked following hypoxic and reoxygenation periods, or any other treatment, was considered to be nonfunctional. Stimulus intensity was always 2 × threshold (3–5 V) which produced a maximal population spike amplitude. Temperature in all experiments, unless indicated otherwise, was 34 ± 0.5 °C.

and fibers of the perforant path which synapse with the granule cells in the dentate gyrus. Figure 1 illustrates the possible placements of both stimulating and recording electrodes and the corresponding responses one may record. The preferred responses recorded in the study of ischemic/hypoxic insults in vitro are the extracellular ones since they represent responses of a large number of neurons, usually of similar characteristics. The easiest to record and analyze are responses (population spikes) of either pyramidal cells in area CA1 or of granule cells in the dentate gyrus of the hippocampus. These two different areas within the same brain structure also represent two cell populations which differ in their resistance to hypoxia, the CA1 layer being one of the most hypoxia-sensitive neuronal populations in the brain[18]. Nevertheless, studies on hypoxia in vitro which make use of the more laborious, intracellular recordings are an indispensible source of information as to the mechanisms involved in neuronal membrane response to oxygen lack. The first visible change one can trace upon omission of oxygen from the slice environment is a fall in the population spike amplitude. The reduction in amplitude starts within 2 min after the exchange of nitrogen for oxygen and 2–3 additional minutes are required for complete disappearance of the evoked response[93]. However, if the hypoxic insult is less intensive (75% N_2/20% O_2/5% CO_2 mixture instead of 95% N_2/5% CO_2), changes in the population spike amplitude could be delayed and, in some occasions, not noticeable at all during the duration of the insult[84]. Figure 2 displays such responses.

Intracellular recordings show similar behavior[24], indicating that different neurons, and even groups of neurons, within one region of a given brain structure may exhibit differential sensitivity to oxygen lack[84]. Nevertheless, the best electrophysiological, quantitative estimate of neuronal damage following hypoxic or ischemic insult in vitro is the measurement of recovery of neuronal function upon reoxygenation. Thus, most in vitro studies use protocols which are divided into three major periods: a) a preincubation period, b) an insult period, and c) a recovery period. The effect of the insult period can be measured in many slices after the recovery period by comparing the neuronal function recorded in them to that of the preincubation period. Continuous monitoring of neuronal function during all three periods can usually be made in one or two slices in a given experiment. This approach allows the accumulation of large enough numbers which can then be treated statistically.

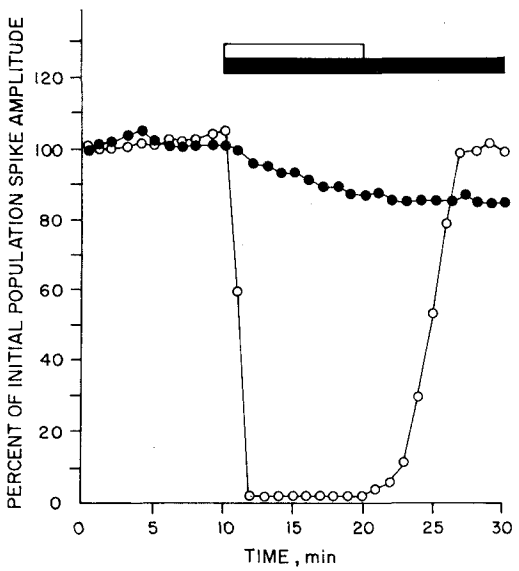

Figure 2. Changes in the amplitude of population spikes (see fig. 1 B) upon exposure of hippocampal slices to 20-min hypoxia (75% N_2/20% O_2/5% CO_2). In one slice (○—○), the amplitude fell to less than 10% of its baseline level within few minutes of the onset of hypoxia. In the other (●—●), only slight change is noticeable. For technical details see legend to fig. 1 and Reid et al.[84].

Percentage of rat hippocampal slices showing neuronal function after 45 min of perfusion (35 min pre-hypoxia and 10 min hypoxia) with different glutamate receptor ligands and 30 min reoxygenation, washout period. Each slice in a given experiment was tested at the end of the reoxygenation period for presence of a population spike (> 3 mV) by stimulating it orthodromically and recording its response from the CA1 stratum pyramidale layer. Statistical analysis was done using X^2-test for significant differences.

Ligand	Number of slices (recovered/total)	Recovered (%)
None (control)	35/41	85
Glu (1 mM)	11/30[a]	37
Asp (1 mM)	12/49[a]	24
NMDA (10 μM)	12/74[a]	16
NMDA (10 μM) + APV (20 μM)	13/27[b,c]	48
NMDA (10 μM) + APV (50 μM)	11/14[c]	79

[a] Significantly different from control ($p < 0.0005$). [b] Significantly different from control ($p < 0.005$). [c] Significantly different from 10 μM NMDA ($p < 0.0005$).

Energy metabolism and metabolites. Kass and Lipton[44] have shown that the reduction in the amplitude of the population spike and its eventual complete disappearance upon hypoxia is the direct result of reduced ATP levels. By preincubating slices with 25 mM creatine for 2 h they tripled the phosphocreatine (PCr) levels. This treatment increased ATP concentration[117] which allowed a significantly higher recovery rate from hypoxia than without it. Concomitant measurements of ion distributions showed a decrease in sodium and calcium influx and potassium efflux upon hypoxia after pretreatment with creatine, indicating a prolonged Na-K pump activity and delayed neuronal damage[43,44]. As mentioned above, the degree of recovery of the population spike after hypoxic or ischemic insult has been used as an estimate of the degree of neuronal damage. The standard hypoxic insult chosen by most brain slice users is 10 min of 95% N_2/5% CO_2 atmosphere. Under these conditions, 80%–90% of the slices exhibit recovery of their orthodromic CA1 population spike to its pre-hypoxic amplitude. Once such a standard has been established, it is easy to determine the effect of additional factors on the recovery rate of neuronal function from hypoxia. For

instance, if a given factor is known to worsen the outcome of the hypoxic insult, a fall in the standard recovery rate should be observed. If one expects a beneficial effect of a given treatment on hypoxic neuronal tissue, the standard hypoxic insult can be prolonged (to reduce the recovery rate) and by doing so allow the beneficial effect to be expressed. Our own studies[19,98,99] along with those of Okada and his colleagues[67,68] have made use of this principle in showing the worsening effect of hypoglycemia and the beneficial effect of hyperglycemia on recovery of neuronal function from hypoxia. These results are in contradiction with the known in vivo deleterious effect of hyperglycemia on ischemic cerebral tissue[41,59]. Notwithstanding, they highlight the very reason for which in vitro systems have been used, namely, to separate and isolate one factor and its effect from others. Okada[67] correlated his electrophysiological results with biochemical measurements by demonstrating that the tissue levels of ATP and PCr are falling faster in the absence of both oxygen and glucose than in the absence of either one alone. Moreover, a recovery of these metabolites to pre-hypoxic levels was observed in slices exposed for at least 60 min to either oxygen or glucose lack. Perfusion of slices with ACSF depleted of both oxygen and glucose for more than 10 min prohibits full recovery of ATP levels. Thus, maintaining high glucose levels in hypoxic tissue should attenuate the fall in ATP and PCr levels and improve the post-hypoxic outcome. Such were the results reported by us[98]. This unexpected finding raised our doubts as to the validity of the hypothesis that postulates lactic acidosis enhancement of neuronal damage upon ischemia. Again, one can design an experimental protocol, using the brain slice preparation, to assess the role of lactic acid in hypoxic neuronal damage[97]. Figure 3 demonstrates clearly that at least in vitro lactic acidosis does not adversely affect the outcome of hypoxic insult on neuronal function. Moreover, there is an indication of a beneficial effect of intermediate levels of lactate on hypoxic neuronal tissue. While lack of detrimental effect of lactic acid on hypoxic tissue in vitro supports some in vivo studies to this effect[22,55], it undoubtedly raises further questions as to the role of lactic acid in brain energy metabolism. Lactate has long been regarded as the end product of anaerobic glycolysis and, as such, incapable of supporting energy metabolism. Even after the demonstration by Vannucci and his colleagues[115] that the perinatal brain is capable of utilizing lactate as energy substrate, the prevailing notion is that the mature brain cannot. Our recent study[100] clearly dispels this notion by demonstrating the ability of rat hippocampal slices to maintain normal neuronal function with lactate as a sole energy fuel. Furthermore, it appears as if lactate, when available, is preferred over glucose both by the perinatal brain[115] and the adult one (fig. 4). This phenomenon may be explained, at least in part, by the need to first invest two moles of ATP for the phosphorylation of one mole of glucose before it can be utilized glycolytically. Since ATP is at deficit under the experimental conditions (lack of energy substrate), the preference in utilizing lactate over glucose, upon their introduction, should be the right choice, at least thermodynamically. That is because lactate can directly enter the tricarboxylic acid cycle via pyruvate, a pathway which requires no investment of ATP. Such also are the conditions immediately after an episode of cerebral ischemia and at the beginning of recirculation. One may conclude therefore, that accumulation of lactate during cerebral ischemia could be beneficial to the brain upon recirculation and that the enhanced ischemic damage in hyperglycemic animals may be evoked by other mechanism(s) not directly related to lactic acidosis[107].

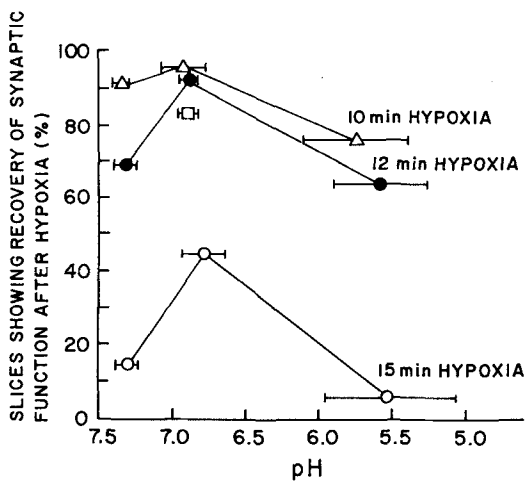

Figure 3. The rate of recovery of neuronal function (population spike) after exposure of rat hippocampal slices to various lengths of hypoxia (95% N_2/5% CO_2) and various degrees of lactic acidosis. The decreased pH did not worsen the outcome of hypoxia as compared to control (pH = 7.4). If at all, lactic acid (10 mM, ph 6.6–6.9)appeared to improve the recovery rate at all three periods of hypoxia. The effect of HCl at the same pH range (□) was not different from that of lactic acid. For more details see fig. 1 and Schurr et al.[97].

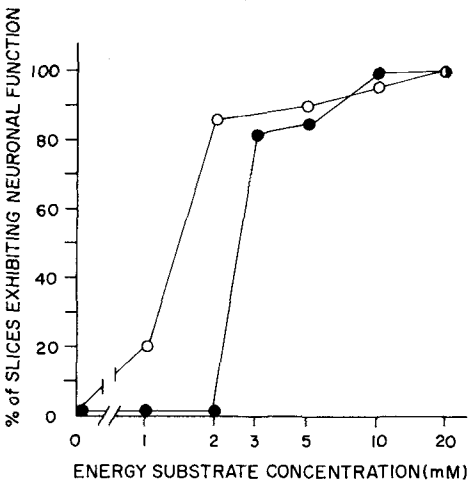

Figure 4. The recovery of neuronal function in the presence of either lactate or glucose after a long exposure to ACSF without glucose. Slices appear to recover better in the presence of 2 mM lactate than in the presence of 2 mM glucose. For more details see fig. 1 and Schurr et al.[100].

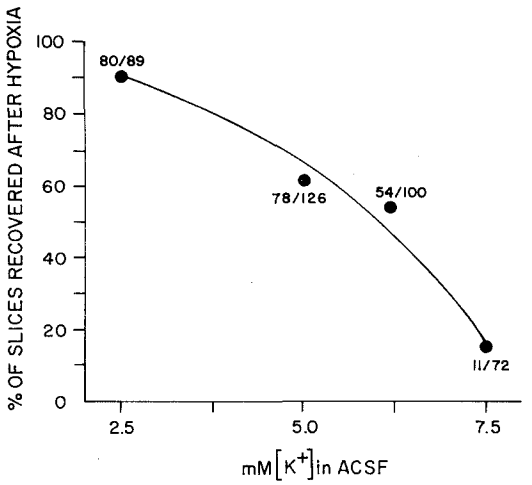

Figure 5. The influence of increasing $[K^+]_e$ on the recovery rate of neuronal function in rat hippocampal slices from 10-min hypoxia (95% N_2/5% CO_2). The higher was the $[K^+]_e$ the lower was the recovery rate. The ratio above each data point is the number of slices showing neuronal function/total number of slices. For more details see fig. 1 and Reid et al.[85].

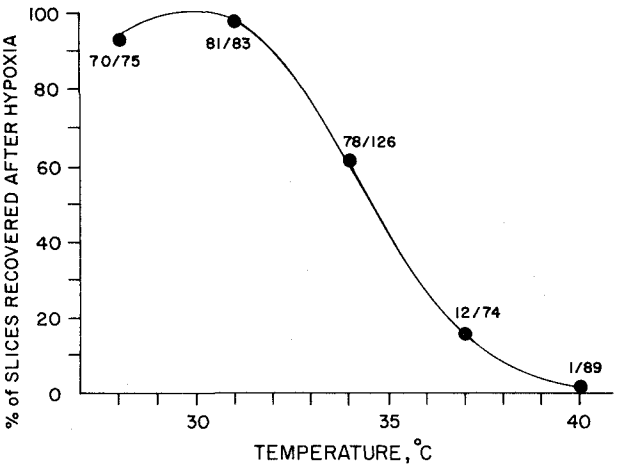

Figure 6. The effect of increasing temperature on the recovery rate of neuronal function in rat hippocampal slices from 10-min hypoxia. The higher was the temperature, the lower was the recovery rate. For other details see fig. 5 and Reid et al.[85].

In determining the outcome of cerebral ischemia/hypoxia, certain ions and their homeostasis and several amino acid neurotransmitters and neuromodulators are not less important than energy substrates and metabolites.

Ion changes and homeostasis. Hypoxic depolarization of nerve cells is a general phenomenon which blocks synaptic transmission and is believed to be driven by increased K^+ conductance[28,29]. It has been shown by Reid and his colleagues[85] that the extracellular level of K^+ can affect the outcome of an hypoxic insult in the hippocampal slice preparation (fig. 5).

There is a correlation between the rise in extracellular K^+ ($[K^+]_e$) upon acute cerebral ischemia in vivo (hypoxic depolarization)[28] and the decrease in recovery rate from hypoxia in vitro with increased $[K^+]_e$. Moreover, the same conditions that delay or enhance the in vivo increase in $[K^+]_e$[28] have shown to increase or decrease, respectively, the recovery rate of neuronal function after hypoxia in vitro. Thus, the glycolytic inhibitor iodoacetic acid and hypoglycemia enhanced the increase in $[K^+]_e$ in vivo[28] and decreased the recovery rate of neuronal function in hippocampal slices[98]. Hyperglycemia and hypothermia delayed the increase in $[K^+]_e$[28] and improved the recovery rate of neuronal function in vitro after hypoxia[67,85,91,98] (see fig. 6).

The hypoxic depolarization mentioned above is usually preceded by a transient hyperpolarization[31] which has been suggested to be the outcome of a reversible depression of Ca-current[45] triggered by a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$)[45]. During reoxygenation, lack of recovery of neuronal function has been correlated with inhibition of active K^+ transport[104], inhibition induced by Ca^{2+} influx during the hypoxic depolarization[28–30,42,104,105]. Thus, it is generally accepted today that Ca^{2+} influx and overload are the initial processes responsible for hypoxic cell damage. While the exact mechanism by which calcium exerts its damaging effect is unknown, several treatments which reduce or block Ca^{2+} influx may improve the outcome of ischemia/hypoxia. The hippocampal slice preparation has been an excellent screening system for potential protective treatments against hypoxic brain damage including that of calcium blockers[31,42]. Modulating calcium fluxes across biological membranes is a function putatively attributed to the sulfur amino acid taurine[46,50]. Moreover, young animals are known for their higher tolerance to lack of oxygen and higher taurine brain levels than adults, both which decline with maturation[21,40,108,110,111]. Not surprisingly, we were able to show that pretreatment of hippocampal slices with taurine increases their resistance to hypoxia[101]. However, suppressing Ca^{2+} influx by employing calcium blockers does not appear to provide the desired protection against hypoxic damage. This is mainly due, as increasing evidence shows, to the opening of alternative channels permeable to Ca^{2+} during the hypoxic episode, channels which are insensitive to calcium blockers (see below).

Amino acid neurotransmitters and excitotoxins

For more than 50 years evidence has mounted supporting the role of glutamate (Glu) and aspartate (Asp) as endogenous excitatory neurotransmitters. Over the past two decades, an increased body of information has also accumulated on the neurotoxicity of these and similar dicarboxylic amino acids[70–72,74,87]. Consequently, a trend has recently emerged in which the pathophysiology of CNS trauma, cerebral palsy, epilepsy, Huntington's chorea, Alzheimer dementia, schizophrenia and other conditions and diseases is attributed to excitotoxins[15,73,103]. Cerebral ischemic/hypoxic damage has also

been suggested to originate from the neurotoxicity of Glu and Asp[8, 15, 17, 37, 38, 73, 88, 89, 103]. Whereas the evidence for a neurotoxic role of Glu and Asp is rather convincing, conflicting data have been published concerning the mechanism(s) by which these amino acids exert their excitotoxic effects[14, 66, 75]. The involvement of Ca^{2+} in the neurotoxicity of Glu and Asp is under debate. Recently we completed a study on the relationship between the excitotoxins, Ca^{2+} and hypoxic neuronal damage. The results obtained in this study confirm the postulated interrelations between Ca^{2+} and the excitotoxins. They also indicate that Ca^{2+} is the principal factor, while the excitotoxins have a secondary role in the ensuing neuronal damage after hypoxia. When hippocampal slices were preincubated with increasing levels of Glu for 45 min (35 min pre-hypoxia + 10 min hypoxia), the percentage of slices which showed recovery of neuronal function following reoxygenation decreased accordingly (fig. 7). Asp (1 mM) exerted a similar enhancement of hypoxic damage. However, when slices were perfused with 0 mM Ca^{2+}-ACSF for 45 min (35 min prior to, plus 10 min during the hypoxic period), neuronal function recovered in essentially all of them after 30 min reoxygenation, regardless of Glu or Asp concentration used (fig. 7). It is important to realize that neuronal function gradually disappears upon depletion of Ca^{2+} from the ACSF. During this depletion the tissue exhibits epileptic-like activity[4, 5] until a population spike can no longer be evoked. However, upon repletion of Ca^{2+} the neuronal function recovers immediately and fully. Single representative experiments depicting the effects of hypoxia combined with Glu, and those of hypoxia combined with Glu in the presence or absence of Ca^{2+}, are shown in figures 8 A and 8 B, respectively. These results indicate that Ca^{2+} rather then Glu (or Asp) is the principal factor involved in the inability of neuronal function to recover after reoxygenation. Moreover, a minimal, critical concentration of Ca^{2+} has to be present extracellularly for Glu to exert its adverse effect upon hypoxia. We calculated the critical Ca^{2+} concentration to be 1.3 mM (fig. 9). Contrary to Glu and Asp, their analogue N-methyl-D-aspartate (NMDA)[116] depressed synaptic function under normoxic conditions at concentrations as low as 50 µM, whereas 10 µM of NMDA was sufficient to induce hypoxic neuronal damage equal to that induced by 3000 µM Glu (table and fig. 8 C). The NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV)[116] reduced, in a dose-dependent fashion, the NMDA-en-

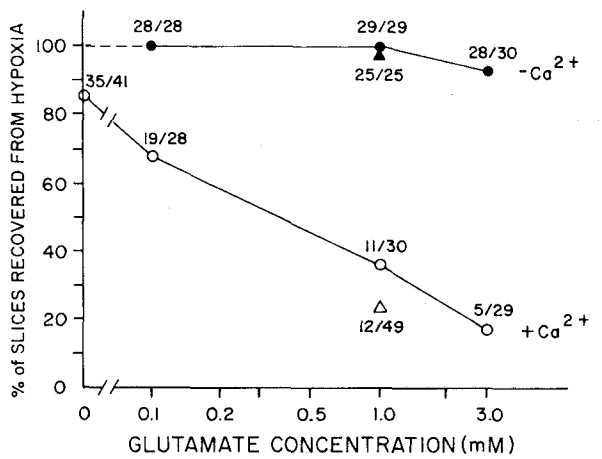

Figure 7. Percentage of rat hippocampal slices showing synaptic function after 10 min hypoxia in the presence or absence of Ca^{2+}. Glu (circles) at 0.1, 1.0 or 3.0 mM, or Asp (triangle) at 1.0 mM, were perfused, following 15 min of baseline recording, through the ACSF for 35 min prior to, and during the 10-min hypoxic period. They were washed out during the reoxygenation period (30 min). The ratio above each point is the number of slices showing neuronal function/total number of slices.
Hypoxia was produced by changing the standard gas mixture of 95% O_2/5% CO_2 to 95% N_2/5% CO_2. To eliminate Ca^{2+}, a no Ca^{2+}-ACSF was supplied following the baseline period and through the hypoxic episode. Reperfusion with normal ACSF was begun with the reoxygenation period. For more details see fig. 1.

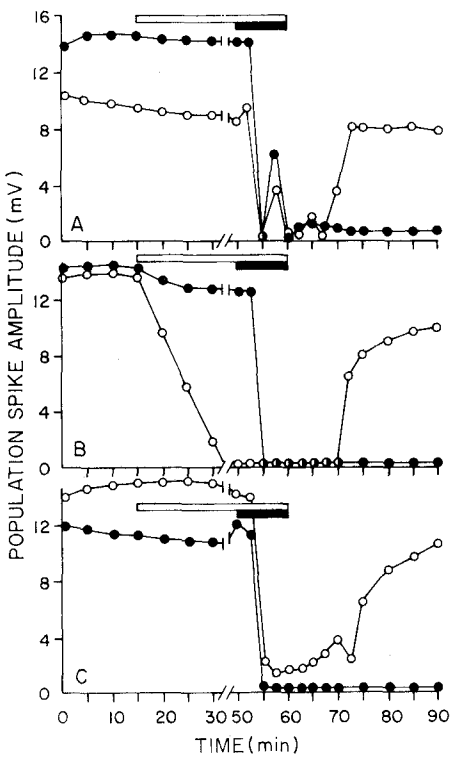

Figure 8. Changes in population spike amplitude over time recorded (every minute, but only every 3rd to 5th record is shown) from one hippocampal slice in each compartment of the dual chamber during three separate representative experiments, as affected by the following treatments: *A* 10-min hypoxia (filled bar) combined with 1 mM Glu perfusion, in one compartment of the dual chamber (●), for 45 min (35 min prior to hypoxia and 10 min during hypoxia, open bar). The control compartment (○) was exposed to 10-min hypoxia only. After hypoxia the control slice showed recovery of the population spike whereas the Glu-treated one did not. *B* 10-min hypoxia (filled bar) combined with 3 mM Glu and 0 mM Ca^{2+}-ACSF perfusion, in one compartment of the dual chamber (○), for 45 min (open bar). The control compartment (●) was perfused for 45 min with 3 mM Glu in normal ACSF (2.5 mM Ca^{2+}) and was exposed to 10-min hypoxia. Only the slice that was perfused with 0 mM Ca^{2+}-ACSF recovered its neuronal function after hypoxia. *C* 10-min hypoxia (filled bar) combined with 45-min perfusion of 10 µM NMDA (open bar) with (○) or without (●) 50 µM APV. Only the slice that was perfused both with NMDA and APV recovered its neuronal function after hypoxia. For other experimental details see figs 1 and 7.

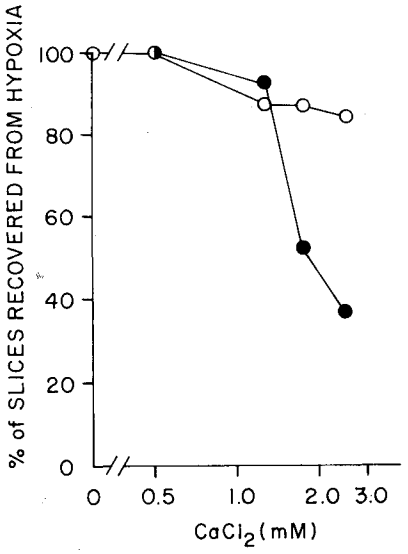

Figure 9. The dependence of Glu-enhanced hypoxic damage on the concentration of Ca^{2+} in the ACSF perfusing rat hippocampal slices. Slices were perfused for 15 min with normal ACSF (baseline) before changing to ACSF containing 1 mM Glu in one compartment of the dual chamber and 0, 0.5, 1.25, 1.75 or 2.5 mM Ca^{2+}-ACSF in both compartments. 35 min later slices were exposed to 10-min hypoxia. Upon reoxygenation the perfusate in both compartments was changed back to normal ACSF. As the concentration of $CaCl_2$ in the ACSF increased, the percentage of Glu-treated slices (●) showing recovery of neuronal function from hypoxia decreased. Control slices (○) showed no hypoxic damage or a slight damage at Ca^{2+} concentration of 2.5 mM. Other details as in figs 1 and 7.

hanced hypoxic damage (table and fig. 8C). Nevertheless, Ca^{2+} depletion from the ACSF completely abolished the enhancement of hypoxic damage by NMDA, as all NMDA-treated slices (12/12) recovered their neuronal function after 30 min reoxygenation. This outcome is similar to the one found with Glu (and Asp) in the absence of Ca^{2+}.

These results could explain what appear to be contradictory conclusions in two recent publications. It has been suggested that Glu becomes neurotoxic via the NMDA receptor upon reduction in intracellular energy levels[65], while another study claims that NMDA antagonists lack a protective potential against hypoxic damage in the hippocampal slice preparation[2]. Our findings support the notion that Glu and Asp become neurotoxic only after the intracellular energy levels have been reduced (hypoxia), since under normoxic conditions neither Glu nor Asp showed any neurotoxicity. As to the conclusion that NMDA receptors are not involved in neuronal hypoxic damage[2], our results indicate that there is no direct involvement of NMDA receptors in such damage, although their activation, which requires the presence of both an NMDA agonist and Ca^{2+}, can enhance the damage inflicted by hypoxia. The reported lack of APV protection against hypoxia[2] is probably due to the fact that any Glu secreted during hypoxia from hippocampal slices is washed away by the continuous perfusion of the tissue and never reaches high enough levels to enhance hypoxic damage. In addition, the Ca^{2+} concentration in the ACSF used in that study was below its critical level. By adding the excitatory amino acids or their analogue to the medium we were able to produce neurotoxicity, but only upon hypoxia. Under these circumstances, protection by APV could be shown (table and fig. 8C). Nevertheless, these results clearly indicate that Glu, Asp and NMDA are innocuous when Ca^{2+} is reduced or completely omitted from the perfusion medium. Therefore, one may conclude that hypoxic damage to neuronal tissue, at least in vitro, is the result of Ca^{2+} influx followed by its intracellular overload. NMDA agonists may enhance both Ca^{2+} influx and its overload, and thus, the hypoxic damage, since NMDA receptor channels allow Ca^{2+} entry[9,16,35,58]. However, Ca^{2+} influx and its intracellular accumulation appear to be the major processes leading to hypoxic neuronal damage since it occurs even in the absence of NMDA-receptor agonists and, in which case, could not be blocked by an NMDA antagonist. Thus, in the case of the excitotoxins, as with other cases, brain slice preparations confirm some of the in vivo findings and the ideas put forward to explain them, yet, they allow the fine tuning not possible in vivo and the separation of the grain from the chaff.

Protection against ischemic/hypoxic brain damage. A treatment that improves the outcome of cerebral ischemia/hypoxia can be a direct result of our better understanding of the mechanisms leading to this outcome. With the present partial understanding of these mechanisms, we can point at partial successes in improving the outcome of cerebral ischemia. Brain slice preparations are becoming the system of choice in protection-prevention studies. We have already mentioned the improvement in recovery from hypoxia seen after pretreatment with glucose[67,69,98], pretreatments that increase PCr levels in the tissue[42,68,117], preincubation with taurine[101], hypothermic conditions[67,85,91] and perfusion with an NMDA antagonist (see this review). Pretreatment with local anesthetics produces protection against hypoxic neuronal damage[7,52,95] either by depressing metabolism, or by preventing the large transient increase in membrane permeability to Na^+ upon depolarization[86] and its concomitant Ca^{2+} influx, or both.

Barbiturates have been shown to protect hippocampal slices against hypoxic damage[3], probably by suppressing metabolism, while chloropromazine provided similar protection by delaying Ca^{2+} influx induced by spreading depression[6]. The possibility that oxygen-free radicals, formed upon reoxygenation following hypoxia, are involved in the production of membranal damage has been tested in the guinea pig hippocampal slice preparation[78]. Consequently, it has been shown that pretreatment with drugs such as methylprednisolone, indomethacin and allopurinol, all known to interfere either with free radical generation or lipid peroxidation, is protective against hypoxia[112]. Rat hippocampal slices pretreated with glutamine were more resistant to hypoxic insult than their

untreated controls[102]. As mentioned earlier, young animals exhibit higher tolerance to lack of oxygen than their adult counterparts. They also show adaptability to hypoxia[1,37] not found in mature animals. The drop in tolerance, and especially in adaptability to hypoxia with aging, could be related to loss of neuronal tissue plasticity. Nevertheless, recently we were able to show that hippocampal slices prepared from brains of adult rats are capable of increasing their resistance to hypoxia by adaptation[94].

Concluding remarks

The availability, at last, of in vitro model systems for the study of cerebral ischemia offers its investigators new insights into some of the mechanisms involved in this crippling brain condition. While in general, the data generated by these systems agree with the knowledge already gained in vivo, other results are less agreeable, forcing one to reassess what was considered to be common knowledge. On the road to better understanding of the mechanisms leading to brain damage upon cerebral ischemia and the search for means to minimize it, no approach should be discounted. The advantages of the in vitro approach in general, and those of brain slice preparations in particular, already have contributed significantly to our understanding of some of these mechanisms. In addition, these advantages make in vitro assessment and screening of potential treatments or prevention of the outcome of cerebral ischemia both faster and more economical. Finally, the excellent opportunity that the use of intact brain tissue in vitro offers in decoding other CNS functions and processes, such as neuronal plasticity, memory and brain aging, should ensure the continuous use of this approach.

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