The effect of $[Ca^{2+}]$ and $[H^+]$ on the functional recovery of rat brain synaptosomes from anoxic insult *in vitro*

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The energy status (as measured by the ATP/ADP ratio), oxidative metabolism ($^{14}CO_2$ output) and neurotransmitter synthesis ($[^{14}C]$ acetylcholine production) by rat brain synaptosomes utilizing $[U-^{14}C]$ glucose has been studied. The ability of anoxia *in vitro* to permanently alter these parameters was investigated with reference to external $[Ca^{2+}]$ and $[H^+]$. It has previously been shown that anoxic damage to synaptosomal preparations is only apparent when their metabolism is stimulated by veratridine [Harvey, Booth & Clark (1982) *Biochem. J.* **206**, 433–439]. It is concluded that low $[Ca^{2+}]$ ameliorates, and high $[H^+]$ exacerbates, the damage sustained by veratridine-stimulated anoxic synaptosomes. The combined effects of low pH, anoxia and veratridine stimulation on synaptosomal metabolism most closely approximated to the irreversible damage to brain metabolism observed during acute hypoxia *in vivo* [Booth, Harvey & Clark (1983) *J. Neurochem.* **40**, 106–110]. Suitably treated synaptosomal preparations may therefore be usefully employed as models to study impaired neurotransmitter synthesis *in vivo*.

Rapidly prepared minimally contaminated synaptosomes (Booth & Clark, 1978) exhibit membrane potential changes on depolarization (Scott & Nicholls, 1980; Hargittai *et al.*, 1982) that are comparable with the action potential observed in whole-nerve preparations (Hodgkin & Huxley, 1952). They have been used in studies on the effects on the brain of graded hypoxia (Ksiezak & Gibson, 1981b) and hypoglycaemia (Harvey *et al.*, 1982).

The cerebral effects of mild hypoxia *in vivo* seem to be mediated at least partly at the cholinergic synapse (Gibson & Blass, 1976; Gibson & Duffy, 1981) and irreversible alterations in the oxidative and acetylcholine-synthetic capacity of synaptosomes prepared from hypoxic animals have been observed experimentally (Booth *et al.*, 1983). However, the application of a simple anoxic insult *in vitro* to synaptosomal preparations does not produce the same effects seen with synaptosomes prepared from animals subjected to hypoxic episodes *in vivo*

(Rafalowska *et al.*, 1980a,b; Harvey *et al.*, 1982). This probably reflects the complexity of the situation *in vivo* and suggests that additional parameters other than simple anoxia might be involved. The purpose of this paper is to investigate the effects of two possible parameters that may also be a part of the hypoxic syndrome *in vivo*, namely $[Ca^{2+}]$ changes and alterations in $[H^+]$.

A 10^3 – 10^4 -fold transmembrane gradient of $[Ca^{2+}]$ is maintained both by the passive permeability of the plasma membrane to Ca^{2+} and active Ca^{2+} extrusion processes. The relative importance of the Ca^{2+} -activated ATPase and the Ca^{2+}/Na^+ exchange as extrusion processes is still the subject of controversy (Åkerman & Nicholls, 1981). It is clear, however, that increases in membrane Ca^{2+} permeability, induced by, for example, ionophore A23187, result in cell death (Kaiser & Edelman, 1977). Since synaptic function requires controlled Ca^{2+} influx (Blaustein, 1975) the nerve ending may experience irreversible damage as a result of the high internal $[Ca^{2+}]$ under conditions (hypoxia and ischaemia) where energy-dependent Ca^{2+} -efflux mechanisms are impaired. Both in the liver (Mittnacht *et al.*, 1979) and in the heart (Shen & Jennings, 1972) Ca^{2+} has been implicated as an active agent in irreversible ischaemic damage.

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Brain cells are very well protected against pH variation, and acidosis *in vivo* occurs principally as a result of endogenous lactate production (Siesjö, 1982). Synaptosomes tolerate 4–6 mM-lactate as substrate under buffered aerobic conditions (Pastuszko *et al.*, 1981, 1982), although the effects of high lactate concentrations (e.g. 20–35 $\mu\text{mol/g}$ wet wt.) that occur under certain conditions [hyperglycaemic–ischaemic transitions (Rehncrona *et al.*, 1980)] have not been investigated. Previous work (Pastuszko *et al.*, 1981) indicates that low pH in itself has no irreversible effect on synaptosomal K^+ homeostasis during aerobiosis or anoxia. In the present paper the effects that $[\text{Ca}^{2+}]$ decrease and $[\text{H}^+]$ increase may have on the ability of the synaptosome to maintain its energetic status to synthesize acetylcholine have been investigated. This has been assessed by studying the effects of these parameters on the damage to these processes known to occur in synaptosomes undergoing stimulated anoxia *in vitro*. From these studies an understanding of the parameters involved in the damage caused by hypoxic insult should become available.

Materials and methods

Synaptosomes

Forebrains from fed adult male rats (180–200 g) were used to prepare synaptosomes by the method of Booth & Clark (1978). These were washed once in modified Krebs–Henseleit medium {136 mM-NaCl / 5.6 mM-KCl / 16.2 mM- NaHCO_3 / 1.2 mM- MgCl_2 / 1.2 mM- NaH_2PO_4 / 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4} and resuspended in the same medium, which was also used for synaptosomal incubations.

Incubations

Preliminary incubations to assess O_2 uptake and adenine nucleotide content were undertaken in a Clark-type oxygen electrode chamber. During these incubations, some synaptosomes were subjected to 10 min anoxia. Secondary incubations to measure $^{14}\text{CO}_2$ and ^{14}C acetylcholine production from D-[U- ^{14}C]glucose were performed in sealed Erlenmeyer flasks. Final concentrations of glucose (1 mM), choline (100 μM), neostigmine (200 μM) and Ca^{2+} (2.2 mM) were as described previously (Harvey *et al.*, 1982). Omission of Ca^{2+} , addition of veratridine or alteration of pH was the same in the secondary incubation as in the primary one, with some exceptions (see the Results section). Veratridine was supplied as veratrine (an alkaloid mixture; Sigma Chemical Co., Poole, Dorset, U.K.). A ratio of one part veratridine to four parts veratrine was assumed (Scott & Nicholls, 1980) and veratridine additions were made as 400 μM - (final concentration) veratrine (approx. 100 μM -veratridine).

Alteration of the final pH (to 6.2) was made in the primary incubation by addition of the synaptosomal suspension at pH 7.4 to an equal volume of Krebs–Henseleit medium adjusted to pH 3.0. In the secondary incubation, the excess medium into which synaptosomes were diluted was adjusted to pH 6.2 directly. These adjustments were made by addition of 2 M-HCl. Ca^{2+} (2.2 mM) was present in all incubations performed at low pH.

Assays

These were as described by Harvey *et al.* (1982), except that some samples for nucleotide analysis were extracted in trichloroacetic acid (6%, w/v, final concentrations) and assayed by the method of Knox & Jurand (1981) by using a reverse-phase high-pressure-liquid-chromatographic column after extensive washing with water-saturated diethyl ether. $^{14}\text{CO}_2$ was trapped by hyamine, ^{14}C acetylcholine was extracted by the method of Fonnum (1969) and characterized by acetylcholinesterase, and proteins were measured by the method of Gornall *et al.* (1949).

Results

Incubations in which Ca^{2+} was omitted from the incubation medium yielded the data in Table 1. Table 2 gives results in which incubations were conducted at low pH. This Table also contains data from incubations in which some synaptosomes were allowed to 'recover' from low pH by a secondary incubation in Krebs–Henseleit saline at pH 7.4. No change was observed in the structural integrity and stability of synaptosomes in the pH range 5.7–7.4 as assessed by the measurement of occluded lactate dehydrogenase activity (M. Messripour, unpublished work). Only changes that were significantly different ($P < 0.05$) by Student's *t* test are noted.

Effects of Ca^{2+} omission and low pH in the absence of veratridine (unstimulated synaptosomes)

In aerobic conditions, the omission of Ca^{2+} increases oxidative metabolism (O_2 uptake and $^{14}\text{CO}_2$ output) as noted by Lefresne *et al.* (1977), whereas there is a small decrease in ^{14}C acetylcholine synthesis. There is no change in energy state as determined by $[\text{ATP}]/[\text{ADP}]$, although Ca^{2+} omission does increase the total measurable synaptosomal (ATP + ADP) content (Table 1).

In the absence of Ca^{2+} , 10 min of anoxia *in vitro* causes a marked decrease in the subsequent $^{14}\text{CO}_2$ output, compared with the equivalent aerobic incubation (Table 1). This contrasts with the response when Ca^{2+} is present in the incubation, when $^{14}\text{CO}_2$ output is similar in both aerobic and post-anoxic conditions. When Ca^{2+} is omitted, the decrease in

Table 1. *Effect of Ca²⁺ on synaptosomal energy and neurotransmitter metabolism*

Synaptosomes were incubated in Krebs–Henseleit saline either in the presence or in the absence of 2.2 mM-Ca²⁺ (final concentration). O₂ uptake, adenine nucleotide levels, ¹⁴CO₂ and [¹⁴C]acetylcholine production from [U-¹⁴C]glucose have been measured as indicated in the methods section. Veratridine (100 μM final concentration) has been added to the incubation where indicated and some synaptosomes have been subjected to a 10 min anoxic period *in vitro*. All values are means ± s.d. for the numbers of determinations shown in parentheses and are expressed as per mg of synaptosomal protein (except [ATP]/[ADP]). Abbreviations used; ND, not determined; N.S., not significant (i.e. *P* > 0.05). Statistical significances have been calculated by Student's unpaired, two-tailed *t* test: *0.05 > *P* > 0.01; ***P* < 0.01. Significances on the left hand side below each column are with respect to the unstimulated aerobic value (± Ca²⁺); those on the right hand side below each column are with respect to the presence and absence of Ca²⁺ under the same conditions. Values in the presence of Ca²⁺ are taken from the work of Harvey *et al.* (1982).

		O ₂ uptake (nmol/min per mg of protein)	[ATP]/[ADP] ratio	[ATP + ADP] (nmol/mg of protein)	¹⁴ CO ₂ (nmol/min per mg of protein)	[¹⁴ C]Acetylcholine (pmol/min per mg of protein)
Aerobic	+Ca ²⁺	3.42 ± 0.98 (20)	1.45 ± 0.58 (11)	2.59 ± 0.70 (12)	0.91 ± 0.12 (9)	11.0 ± 2.2 (12)
	−Ca ²⁺	4.93 ± 0.78 (44)	1.62 ± 0.83 (18)	3.29 ± 0.91 (19)	2.38 ± 0.38 (22)	9.4 ± 1.9 (20)
		**	N.S.	*	**	*
Aerobic + veratridine	+Ca ²⁺	6.48 ± 1.08 (29)	0.77 ± 0.23 (6)	3.02 ± 0.77 (6)	3.53 ± 0.13 (9)	5.5 ± 0.9 (9)
	−Ca ²⁺	7.81 ± 0.71 (21)	0.89 ± 0.1 (9)	2.28 ± 0.97 (9)	3.91 ± 0.99 (9)	3.8 ± 1.6 (6)
		**	N.S.	N.S.	**	*
Post-anoxic	+Ca ²⁺	ND	1.01 ± 0.45 (9)	1.99 ± 0.47 (9)	1.07 ± 0.21 (9)	9.6 ± 3.4 (8)
	−Ca ²⁺	ND	1.72 ± 0.83 (12)	2.37 ± 0.70 (12)	1.76 ± 0.26 (12)	8.6 ± 1.9 (10)
			N.S.	N.S.	**	N.S.
Post-anoxic + veratridine	+Ca ²⁺	ND	0.41 ± 0.35 (4)	2.63 ± 0.50 (4)	2.17 ± 0.43 (9)	3.5 ± 1.5 (11)
	−Ca ²⁺	ND	0.28 ± 0.26 (6)	1.51 ± 0.29 (7)	3.04 ± 0.38 (9)	4.1 ± 1.8 (9)
			**	**	**	**
			N.S.		**	N.S.

Table 2. *Effects of increased [H⁺] on synaptosomal energy status and acetylcholine synthesis*

Synaptosomes were incubated in Krebs–Henseleit saline, pH 6.2, in the presence or in the absence of veratridine (100 μM). Some synaptosomes were subjected to a 10 min period of anoxia followed by a subsequent incubation in Krebs–Henseleit saline at either pH 6.2 or 7.4. Other conditions were as described in the legend to Table 1. Results are expressed as means ± s.d. for the numbers of determinations shown in parentheses (see Table 1). Statistical significances are indicated below each measured parameter. Those on the left-hand side are with respect to 'aerobic, pH 6.2' data. Those on the right-hand side are with respect to the equivalent incubation at pH 7.4 (the 'Ca²⁺' data in Table 1). Abbreviations used: ND, not determined; N.S., not significant (i.e. *P* > 0.05). *, 0.05 > *P* > 0.01; **, *P* < 0.01.

Primary incubation conditions	O ₂ uptake (nmol/min per mg of protein)	[ATP]/[ADP] ratio	[ATP + ADP] (nmol/mg of protein)	Secondary incubation conditions	¹⁴ CO ₂ output (nmol/min per mg of protein)	[¹⁴ C]Acetylcholine synthesis (pmol/min per mg of protein)
Aerobic, pH 6.2	2.78 ± 0.66 (29)	1.09 ± 0.22 (5)	2.59 ± 1.33 (6)	Aerobic, pH 6.2	1.02 ± 0.30 (11)	5.1 ± 1.4 (12)
	**	N.S.	N.S.		N.S.	*
				Aerobic, pH 7.4	0.89 ± 0.19 (11)	7.9 ± 2.5 (5)
					N.S.	*
Aerobic, pH 6.2 + veratridine	2.58 ± 0.37 (10)	0.64 ± 0.13 (5)	0.82 ± 0.12 (5)	Aerobic, pH 6.2 + veratridine	1.52 ± 0.77 (7)	2.63 ± 1.65 (6)
	N.S.	**	*		N.S.	**
Post-anoxia, pH 6.2	ND	0.94 ± 0.24 (4)	2.51 ± 0.13 (4)	Aerobic, pH 6.2	0.96 ± 0.28 (12)	5.1 ± 2.3 (12)
		N.S.	N.S.		N.S.	N.S.
			*	Aerobic, pH 7.4	0.77 ± 0.09 (9)	7.2 ± 2.1 (4)
					N.S.	N.S.
Post-anoxia, pH 6.2 + veratridine	ND	0.72 ± 0.32 (6)	0.59 ± 0.15 (6)	Aerobic, pH 6.2 + veratridine	0.57 ± 0.13 (6)	1.32 ± 1.21 (6)
		N.S.	*		**	*

¹⁴CO₂ output observed after anoxic insult is not paralleled by similar reductions in [ATP]/[ADP] and [¹⁴C]acetylcholine synthesis.

Incubations carried out at pH 6.2 in the presence of Ca²⁺ showed no change in [ATP]/[ADP] or ¹⁴CO₂ output relative to those at pH 7.4, although O₂

uptake and [^{14}C]acetylcholine synthesis were decreased. An anoxic interlude caused no additional change in any parameter. Thus unstimulated synaptosomes are no more vulnerable to anoxia at pH 6.2 than they are at pH 7.4 (Tables 1 and 2).

In synaptosomes initially incubated at pH 6.2 but 'rescued' into pH 7.4 buffer for the secondary incubation, there were no further changes in $^{14}\text{CO}_2$ output. [^{14}C]Acetylcholine synthesis increased, but not to the equivalent pH 7.4 values. Thus, low pH itself reduces [^{14}C]acetylcholine synthesis and this reduction is only partly reversible by secondary incubation at pH 7.4.

Effects of Ca^{2+} omission and low pH in the presence of veratridine (stimulated synaptosomes)

Veratridine has previously been shown to increase the Na^+ conductance of neuronal plasma membranes (Ohta *et al.*, 1973), causing increased synaptosomal respiration (Scott & Nicholls, 1980) and neurotransmitter release (de Belleruche *et al.*, 1982). Under aerobic conditions, veratridine has similar effects on synaptosomes, whether Ca^{2+} is present or absent: namely, O_2 uptake and $^{14}\text{CO}_2$ output are increased, [ATP]/[ADP] and [^{14}C]acetylcholine synthesis are decreased (Table 1). The effects of Ca^{2+} omission and the addition of veratridine are approximately additive in the case of the stimulation of O_2 uptake and the decrease of [^{14}C]acetylcholine synthesis, but not for [ATP]/[ADP] or $^{14}\text{CO}_2$ (Table 1).

Although veratridine stimulation during anoxia has qualitatively similar effects on synaptosomal metabolism whether or not Ca^{2+} is present, the omission of Ca^{2+} reduces the extent of damage incurred during a stimulated anoxia/reoxygenation cycle. Indices of damage may be calculated (Harvey *et al.*, 1982) and for all three aspects of metabolism examined, the indices in the absence of Ca^{2+} are closer to unity than the equivalent controls (Table 3).

Low pH (6.2) completely suppresses the stimulation of O_2 uptake, partly suppresses the stimulation of $^{14}\text{CO}_2$ output but does not affect the reductions in [ATP]/[ADP] and [^{14}C]acetylcholine synthesis observed in veratridine-stimulated aerobic synaptosomes (Table 2). An anoxic interlude causes no further change in [ATP]/[ADP], although $^{14}\text{CO}_2$ and [^{14}C]acetylcholine production are further decreased. Indices of damage for low-pH incubations accordingly show (Table 3) that damage to $^{14}\text{CO}_2$ output and [^{14}C]acetylcholine synthesis is more severe at low pH, whereas damage to energy status is not.

Linear regression analysis

All data in Tables 1 and 2, and data from Booth *et al.* (1983) (synaptosomes from hypoxic rats) were

Table 3. *Indices of damage (C) under various conditions of stimulated anoxia and hypoxia*

C is calculated as [(post-anoxic value with veratridine)/(post-anoxic value without veratridine)]/[(aerobic value with veratridine)/(aerobic value without veratridine)].

Condition	Value of C for:		
	[ATP]/[ADP] ratio	$^{14}\text{CO}_2$ output	[^{14}C]Acetylcholine synthesis
Stimulated anoxia			
With Ca^{2+} , pH 7.4*	0.77	0.52	0.72
No Ca^{2+} , pH 7.4	0.81	0.85	1.18
With Ca^{2+} , pH 6.2	1.30†	0.40	0.50
Hypoxia <i>in vivo</i>			
With Ca^{2+} , pH 7.4‡	0.90	0.67	0.38

* Taken from the results of Harvey *et al.* (1982).

† A value >1 is obtained owing to large changes in (ATP + ADP) content (see Table 2).

‡ Taken from the results of Booth *et al.* (1983).

Fig. 1. *Correlation of acetylcholine synthesis with $^{14}\text{CO}_2$ output*

The data are taken from Tables 1 and 2 of the present paper and certain datum points (1, 2 and 3) have been calculated from data in Ksiezak & Gibson (1981a) (1, unstimulated aerobic synaptosomes; 2, unstimulated anoxic synaptosomes; 3, dinitrophenol-stimulated aerobic synaptosomes). It should be noted that the $^{14}\text{CO}_2$ output for point (3) is 6.7 nmol/min per mg of synaptosomal protein and is off the scale. O, Incubations under various conditions in the presence of veratridine; ●, incubations under the same conditions in the absence of veratridine.

analysed: O₂ uptake, [¹⁴C]acetylcholine synthesis and [ATP]/[ADP] ratio were examined as functions of ¹⁴CO₂ output. O₂ uptake was found to be a single function of ¹⁴CO₂ output ($O_2 = 1.419 \text{ CO}_2 + 1.636$) with a strong correlation coefficient ($r = 0.936$; $P < 0.001$) (results not shown). There was no good correlation of either acetylcholine synthesis or [ATP]/[ADP] with ¹⁴CO₂ output unless 'veratridine-added' data were treated separately from those of unstimulated preparations (Figs. 1 and 2). Data are included that have been calculated from the work of Ksiezak & Gibson (1981a) (Fig. 1) and Ksiezak & Gibson (1981b) (Fig. 2).

It is evident that, in the absence of veratridine, acetylcholine synthesis is not well correlated with ¹⁴CO₂ output ($r = 0.363$; $P > 0.2$). In the presence of veratridine, acetylcholine synthesis correlates reasonably well ($r = 0.785$; $P < 0.05$) with ¹⁴CO₂ output, although for a given metabolic rate acetylcholine synthesis is lower than in unstimulated preparations. Addition of veratridine reduces the energetic ([ATP]/[ADP]) status of synaptosomes essentially irrespective of other conditions, as shown by the poor correlation ($r = 0.248$; $P > 0.5$) between [ATP]/[ADP] and ¹⁴CO₂. In the absence of

veratridine, [ATP]/[ADP] appears to be much more closely correlated with general metabolic activity as measured by ¹⁴CO₂ output.

Discussion

The overall [ATP]/[ADP] ratio has been used in the present work to monitor the energy status of the synaptosomes. However, measurements of phosphocreatine in these preparations indicate a qualitatively similar response to veratridine stimulation and/or anoxia to that of the [ATP]/[ADP] ratio. Phosphocreatine concentrations [nmol·(mg of synaptosomal protein)⁻¹; means ± s.d. (n = no. of determinations)] in synaptosomal preparations derived from animals subjected to hypoxic insult were not significantly affected by a subsequent anoxic episode [2.3 ± 0.2 ($n = 5$) in controls; 2.4 ± 0.2 ($n = 4$) in the post-anoxic group], but synaptosomes from normoxic rats stimulated with veratridine showed a 34% decrease in the phosphocreatine levels [2.2 ± 0.3 ($n = 12$) in controls; 1.4 ± 0.1 ($n = 6$) in controls + veratridine]. The [ATP]/[ADP] ratio of synaptosomal preparations has been found to be lower than that of whole brain (Booth & Clark, 1979; Ksiezak & Gibson, 1981b) under similar conditions. This may reflect the high activity of the (Na⁺ + K⁺)-stimulated ATPase in the synaptosome and the higher specific activity of mitochondrial enzymes.

The effects of omission of Ca²⁺ on oxidative metabolism are largely mediated via its interaction with Na⁺ homeostasis (Lolley & McIlwain, 1964), causing increased (Na⁺ + K⁺)-stimulated ATPase activity. Effects may also occur at the level of the regulation of key enzymes in the tricarboxylic acid cycle, since it has been shown in heart and adipose-tissue mitochondria that pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase are all activated by Ca²⁺ concentrations in the μM range (McCormack & Denton, 1980; Denton *et al.*, 1980). Schaffer & Olson (1980), however, were unable to demonstrate a change in synaptosomal pyruvate decarboxylation on the addition of EGTA. In most of the conditions studied (Table 1) acetylcholine synthesis is lower in the absence of Ca²⁺, presumably due to the Ca²⁺ dependence of acetylcholine release (Katz & Miledi, 1970). It is clear that the effects of Ca²⁺ on the damage caused by anoxia to synaptosomal metabolism are complex but may be clarified by proposing two main effects of Ca²⁺. First, the synaptosomal metabolic rate as judged by O₂ consumption and CO₂ output appears to vary inversely with respect to the Ca²⁺ concentration (Table 1; Lefresne *et al.*, 1977; Ksiezak & Gibson, 1981a,b). Thus lower Ca²⁺ concentrations stimulate synaptosomal metabolism, rendering it more

Fig. 2. Correlation of [ATP]/[ADP] ratio with ¹⁴CO₂ output

The data are taken from Tables 1 and 2 of the present paper and certain datum points (1 and 2) are calculated from data in Ksiezak & Gibson (1981b) (1, unstimulated aerobic synaptosomes; 2, unstimulated anoxic synaptosomes). ○, Incubations under various conditions in the presence of veratridine; ●, incubations under various conditions in the absence of veratridine.

susceptible to anoxic insult. Secondly increased $[Ca^{2+}]$ may stimulate free-radical generation (Dempoulous *et al.*, 1980; Cadenas & Boveris, 1980) and also phospholipase A_2 activity (Waite, 1969; Pfeiffer *et al.*, 1979). Hence the Ca^{2+} concentration in the synaptosome is very critical to the damage it experiences from an anoxic insult, since these two effects of Ca^{2+} are working in opposite directions. Thus when synaptosomal metabolism is already stimulated by veratridine, the presence of high Ca^{2+} concentrations tends to exacerbate the effects of anoxic damage due to the high metabolic rate (Table 3).

It seems unlikely that the effects of low pH are due entirely to interference with the plasma-membrane Na^+ -conductance channel (Woodhull, 1973). Although this would explain the absence of veratridine stimulation of oxidative metabolism at pH 6.2, it would not account for the fall in the $[ATP]/[ADP]$ ratio (since low pH in itself does not cause such a fall; see Table 2). This reduction in energy status suggests that cytosolic ATP utilization is increased as a result of increased Na^+ cycling, but that low pH reduces the ability of intrasynaptic mitochondria to respond to this change. When synaptosomes that have been stimulated by veratridine are subjected to an anoxic insult at a low pH (6.2), the damage indices (Table 3) of all parameters deviate more extensively from unity than do those from similar synaptosomes insulted at a higher pH (7.4) and approach damage indices seen with synaptosomes derived from animals subjected to hypoxic insult *in vivo* (Booth *et al.*, 1983).

It has been shown (Molenaar & Polak, 1976; Suszkiw & O'Leary, 1982) that newly synthesized acetylcholine is preferentially released during depolarization of brain slices and synaptosomes. The acetyl group of acetylcholine is derived from glucose without dilution (Browning & Schulman, 1968; Lefresne *et al.*, 1977), i.e. no endogenous pools substitute as precursors for that moiety. This is in contrast with the choline moiety, where ample endogenous pools may substitute in cortical synaptosomes (Kessler & Marchbanks, 1979) and in the preparation used in the present work (S. A. K. Harvey, R. F. G. Booth & J. B. Clark, unpublished work). Thus, a rationale can be constructed for the dependence of cholinergic function on ongoing carbon metabolism. In addition, in the present work, $100\mu M$ -choline has been added to the incubations, which, together with the endogenous pools, makes it likely that choline 'transport' into the synaptosomes is extremely rapid. Also in this synaptosomal preparation (Clark *et al.*, 1982) the maximal activity of the choline acetyltransferase is in considerable excess ($\geq 10^2$) of the observed rate of acetylcholine synthesis. This leaves provision of cytosolic acetyl-CoA as the most likely locus of

damage; since the pyruvate dehydrogenase complex activity is not damaged by hypoxia (Ksiezak, 1976), the entry/exit of metabolites across the mitochondrial membrane involved in the provision of cytosolic acetyl-CoA and/or the regeneration of acetyl-CoA in the cytosol may be considered as likely targets at which the damage process might be instigated. The data in Figs. 1 and 2 are consistent with this suggestion and may be rationalized in the following manner. In the absence of veratridine stimulation, various treatments (low pH, hypoxia *in vivo* but not anoxia *in vitro*) affect the ability of the mitochondrion to export acetyl groups via translocators or the regeneration of cytosolic acetyl-CoA. Thus acetylcholine synthesis under these conditions is more sensitive to perturbations in the oxidative metabolism (as measured by $^{14}CO_2$ output) than is the synaptosomal energy status (as measured by $[ATP]/[ADP]$) (Figs. 1 and 2). In the presence of veratridine, the sensitivity of both acetylcholine synthesis and energy status ($[ATP]/[ADP]$) decrease with respect to oxidative metabolism ($^{14}CO_2$ output). Shifts in mitochondrial membrane potential caused by the stimulation of synaptosomal metabolism by the addition of such agents as veratridine or uncouplers may give rise to partial dissociation of acetyl-group export from oxidative metabolism (cf. Ksiezak & Gibson, 1981a).

It is now well established that the preliminary reversible effects of hypoxia *in vivo* are rapidly followed by irreversible brain damage and death if the atmospheric partial pressure of O_2 is ≤ 40 mmHg (Siesjö, 1978). Under these conditions the arterial O_2 partial pressure is approx. 22 mmHg, and brain tissue concentrations of O_2 and lactate are very low (< 5 mmHg) and very high [$> 32\mu mol \cdot (g \text{ wet wt.})^{-1}$] respectively (Siesjö, 1978). It might be anticipated therefore that the application of such conditions, i.e. anoxia and low pH, *in vitro* to synaptosomal preparations would lead to similar damaging effects on synaptosomal metabolism, as seen in synaptosomes isolated from rats subjected to hypoxic insult *in vivo* (Booth *et al.*, 1983). The effects reported in the present paper on the more severe inhibition of acetylcholine synthesis (as assessed by damage indices) caused by anoxic and low pH insult relative to the effects on oxidative metabolism ($^{14}CO_2$ output) and energy status ($[ATP]/[ADP]$) are consistent with similar observations made on synaptosomes derived from rats subjected to hypoxia *in vivo* [O_2 concentration 38 mmHg for 25 min (Booth *et al.*, 1983)]. This suggests that isolated synaptosomal preparations may, under the appropriate incubation conditions, be used as a model for the effects of hypoxia on synaptic function and that such a system, as described here, may be profitably used in the investigation of mechanisms of protection against hypoxic damage.

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