The effects *in vitro* of hypoglycaemia and recovery from anoxia on synaptosomal metabolism

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Synaptosomes from several regions of the rat brain were found to exhibit half-maximal rates of $^{14}\text{CO}_2$ output and [^{14}C]acetylcholine synthesis from D-[U- ^{14}C]glucose at glucose concentrations approx. 50-fold lower than those required by the brain *in situ*. However, synaptosomal acetylcholine synthesis was found not to be directly proportional to substrate oxidation as measured by $^{14}\text{CO}_2$ output. When synaptosomes had been exposed to anoxia *in vitro*, their metabolic indices ($^{14}\text{CO}_2$ and [^{14}C]acetylcholine synthesis, and adenine nucleotide levels) were found not to be significantly different from control aerobic values, unless they had been subjected to veratridine depolarization. This is in accord with previous findings that neither the absolute metabolic rates nor the vulnerability to hypoxic damage exhibited by brain *in situ* is reflected by brain slices *in vitro*, unless these are stimulated by depolarization. The use of synaptosomes as a model for synaptic damage *in vivo* is discussed.

Synaptosomes are known to exhibit many of the energetic and neurotransmitter characteristics of the synaptic ending *in situ* (De Robertis *et al.*, 1962; Whittaker, 1969; Jones & Bradford, 1971). In the whole organism, it has been postulated that the synapse might be the primary locus of neurological failure during hypoxia (Härkönen *et al.*, 1969; Dolivo, 1974). It might therefore be expected that hypoxia or hypoglycaemia *in vivo* would alter the characteristics of derived synaptosomes, or that synaptosomes from control animals might be vulnerable to treatments *in vitro* that mimic hypoxia or hypoglycaemia.

It has been shown that the energy metabolism of synaptosomes from hypoxic rats is little different from that of synaptosomes from normoxic rats, as assessed by O_2 uptake and ATP/ADP ratios (Rafalowska *et al.*, 1980), and that anoxia *in vitro* has no irreversible effect on the ion homeostasis of synaptosomes maintained at pH 7.4 (Pastuzcko *et al.*, 1981). These results are compatible with the extensive evidence that neurological failure *in vivo* occurs before cerebral energy failure (see e.g. Siesjö, 1978), and suggest that observation of parameters such as neurotransmitter synthesis might be more relevant.

The early experiments of Quastel *et al.* (1936) demonstrated that optimal synthesis of the neuro-

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transmitter acetylcholine by rat brain cortex slices was dependent on the presence of glucose and O_2 . Recently, it has been demonstrated that acetylcholine synthesis is extremely vulnerable to a lack of O_2 or to reduced substrate supply, both *in vivo* (Gibson & Blass, 1976; Gibson & Duffy, 1981) and *in vitro* (Gibson *et al.*, 1975; Ksiezak & Gibson, 1981b). However, there is little information in the literature regarding the recovery of acetylcholine synthesis by synaptosomes after metabolic stress *in vivo* or *in vitro*. This information is obviously of interest as a model for neurological recovery.

The synaptosomal preparation used in the present work shows minimal contamination with either free mitochondria or endoplasmic reticulum and is metabolically competent (Booth & Clark, 1978). The purity of the preparation has been confirmed by electron-microscope studies (Booth & Clark, 1978; Deutsch *et al.*, 1981) and the synaptosomes generate membrane potentials comparable with the synapse *in situ* (Deutsch *et al.*, 1981). Further, the synaptosomes exhibit high-affinity choline uptake and are enriched in choline acetyltransferase (Harvey, 1981).

Materials and methods

Synaptosomes

Adult male rats of 180–200 g were used. They were permitted food and water *ad libitum*. Synaptosomes were obtained from the forebrains of these

animals by the method of Booth & Clark (1978). The synaptosomal pellet was washed once in Krebs–Henseleit–Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (136 mM-NaCl, 5.6 mM-KCl, 16.2 mM-NaHCO₃, 1.2 mM-MgCl₂, 1.2 mM-NaH₂PO₄, 10 mM-Hepes, pH 7.4) and was resuspended in the same buffer containing 2.2 mM-CaCl₂.

Incubations

Synaptosomes were incubated at 37°C in Krebs–Henseleit/Hepes buffer containing 2.2 mM-CaCl₂. Other additions were (final concentrations): glucose (1 mM, unless otherwise specified), choline (100 µM), physostigmine (100 µM) or neostigmine (200 µM). Where radioactive glucose was added it was of high specific radioactivity (approx. 300 Ci/mol) and uniformly labelled, from The Radiochemical Centre, Amersham, Bucks., U.K. Final specific radioactivity was 1–5 µCi/mmol.

Experiments in which ¹⁴CO₂ and [¹⁴C]acetylcholine production were measured were performed in Krebs–Henseleit/Hepes buffer with D-[U-¹⁴C]-glucose in a stoppered Erlenmeyer flask containing 500 µl of hyamine in the centre well. This incubation was stopped after 20–30 min by injection through the rubber cap of 1 ml of trichloroacetic acid (3.3%, w/v, final concentration) containing an internal standard of [³H]acetylcholine. Flasks were then incubated for a further 30 min.

In some experiments the O₂ consumption of synaptosomes (8–10 mg of protein/ml) was first measured using a Clark-type oxygen electrode. Of the stirred suspension 250 µl was then transferred from the oxygen electrode chamber into HClO₄ (6% final concentration) for evaluation of ATP, ADP and phosphocreatine, and a further 500 µl was transferred into a 9-fold excess of Krebs–Henseleit/Hepes buffer in an Erlenmeyer flask for measurement of ¹⁴CO₂ and [¹⁴C]acetylcholine.

While in the oxygen electrode chamber, some synaptosomes were made anoxic by allowing them to respire until there was no further change in O₂ tension. After 10 min of anoxia, 500 µl of synaptosomal suspension was immediately taken for incubation in an Erlenmeyer flask as described. The rest of the suspension was re-oxygenated by stirring with exposure to air for 2 min before quenching with HClO₄. These synaptosomes were designated 'post-anoxic' to distinguish them from 'aerobic' synaptosomes, which were removed from the oxygen electrode chamber while their rate of O₂ consumption was constant.

HClO₄ extracts were neutralized by the addition of 5 M-K₂CO₃ in 0.5 M-TEA buffer followed by centrifugation to remove precipitated KClO₄. Trichloroacetic acid extracts were neutralized by repeated washing with diethyl ether followed by

the addition of sodium phosphate buffer (10 mM), pH 7.2.

Measurement/characterization of metabolites

ATP, ADP and phosphocreatine. ATP and phosphocreatine were measured by the method of Lamprecht *et al.* (1974) and ADP by the method of Jaworek *et al.* (1974). Alternatively, ATP and ADP were measured by a slight modification of the h.p.l.c. method described by Booth & Clark (1979). Good agreement was obtained between the two methods of measurement.

¹⁴CO₂ and [¹⁴C]acetylcholine. ¹⁴CO₂ was absorbed into 500 µl of 1 M-hyamine hydroxide in methanol over a period of 30–60 min after quenching of the incubation with trichloroacetic acid. [¹⁴C]-Acetylcholine was isolated from the neutralized extract by the method of Fonnum (1969). After extraction into 10 mg of sodium tetraphenylboron in 1 ml of heptan-3-one, choline esters were back-extracted into 100 µl of 2 M-HCl, which was then washed with 1 ml of heptan-3-one. The acidic aqueous phase was then neutralized, divided in two, and both portions were re-extracted with tetraphenylboron/heptan-3-one after treatment of one with purified acetylcholinesterase. [¹⁴C]Acetylcholine was calculated as the reduction in the extractable radioactivity as a result of acetylcholinesterase treatment. Alternatively, the acidic aqueous phase was vacuum-dried in the presence of solid NaOH or Ca(OH)₂ and the methanol-soluble component of the residue was submitted to high-voltage electrophoresis by the method of Potter & Murphy (1967).

Isolated radioactive fractions were quantified by scintillation counting in 10–15 ml of scintillation cocktail [25% (v/v) Triton X-100 in toluene, containing 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre]. Counting was performed on a Packard 2425 scintillation counter in ³H and ¹⁴C channels using the external-standard mode. Quench correction was made using quench curves predetermined by counting both ³H and ¹⁴C standards.

Absolute quantities of ¹⁴CO₂ and [¹⁴C]acetylcholine were calculated on the basis that their specific radioactivities were respectively one-sixth and one-third of that of glucose substrate (Grewaal & Quastel, 1973). Proteins were measured by the method of Gornall *et al.* (1949).

Results

Hypoglycaemia in vitro

When ¹⁴CO₂ output or [¹⁴C]acetylcholine synthesis is plotted as a function of glucose concentration, both parameters show simple kinetics with single values for apparent $V_{\max}$ and K_m (Fig. 1,

legend). The low apparent K_m values observed mean that 1 mM-glucose is optimal, and that the V_{max} values presented here can be directly compared with data obtained by Ksiezak & Gibson (1981b), who used synaptosomes made by the method of Booth & Clark (1978) and incubated them in 1.5 mM-glucose. The V_{max} value for the synthesis of [14 C]acetylcholine presented here is in good agreement with the value of 14 pmol/min per mg of synaptosomal protein obtained by Ksiezak &

Gibson (1981b), although the $^{14}\text{CO}_2$ output obtained by those workers was higher (approx. 3 nmol/min per mg of synaptosomal protein) than that apparent here, which was 0.99 nmol/min per mg of synaptosomal protein.

When [14 C]acetylcholine synthesis is plotted against $^{14}\text{CO}_2$ output using data obtained with whole-forebrain synaptosomes, a non-linear curve results (Fig. 1). Qualitatively similar results are found (Table 1) with synaptosomes prepared from either cortex or striatum. In Table 1, [14 C]acetylcholine synthesis expressed as a percentage of $^{14}\text{CO}_2$ output decreases as glucose concentration is reduced. These results confirm the data shown in Fig. 2 and suggest that the production of [14 C]acetylcholine from [U- 14 C]glucose is more vulnerable than $^{14}\text{CO}_2$ output to a reduction in the substrate concentration.

Anoxia in vitro

Fig. 2 shows the changes that occur in the rate of synaptosomal O_2 uptake and [14 C]acetylcholine synthesis during and after a period of anoxia. It is clear that during the anoxic period [14 C]acetylcholine synthesis from D-[U- 14 C]glucose falls by approx. 75%, and that on re-oxygenation a full recovery of both the rate of O_2 consumption and acetylcholine synthesis occurs. The failure of anaerobiosis to completely eliminate acetylcholine synthesis was noted by Quastel *et al.* (1936). The full recovery of [14 C]acetylcholine synthesis after reoxygenation implies that no irreversible damage has been done to the ability of the synapse to synthesize acetylcholine by 10 min anoxia, and contrasts with the inability of the organism to regain neurological competence after prolonged interruption of O_2 supply.

It has been noted that in Krebs–Henseleit medium glycolytic flux during anaerobiosis is sufficient to maintain synaptosomal membrane potentials close to control values (Scott & Nicholls, 1980) and to reduce K^+ loss from synaptosomes (Pastuszko *et al.*, 1981). It seems therefore that simple anaerobiosis

Fig. 1. [14 C]Acetylcholine synthesis as a function of $^{14}\text{CO}_2$ output

The bars represent S.E.M. for $5 \leq n \leq 20$, where n is the number of determinations. Values associated with points are concentrations of glucose substrate (mM). When the appropriate Lineweaver–Burk plots were constructed (not shown), apparent kinetic parameters were as follows. For $^{14}\text{CO}_2$ production, $V_{max} = 0.99$ nmol/min per mg of protein, $K_m = 80 \mu\text{M}$; for [14 C]acetylcholine production, $V_{max} = 12.5$ pmol/min per mg of protein, $K_m = 150 \mu\text{M}$.

Table 1. Effect of glucose concentration on synaptosomes from different brain regions

Results are means $\pm$ S.E.M. for the numbers of determinations shown in parentheses. Regional dissection was essentially as described by Glowinski & Iversen (1966). Experimental protocol was as described in the Materials and methods section. When the ratios (B/A) for 50 μM - and 1 mM-glucose are compared by unpaired Student's t test, P values are: whole-forebrain synaptosomes, 0.02; cortical synaptosomes, 0.06; striatal synaptosomes, 0.07.

Concentration of glucose	Origin of synaptosomes	$^{14}\text{CO}_2$ output (nmol/min per mg of protein) (A)	[14 C]Acetylcholine synthesis (pmol/min per mg of protein) (B)	$\frac{B}{A} \times 100$
50 μM	Whole forebrain	0.48 ± 0.04 (7)	3.6 ± 0.3 (7)	0.8 ± 0.04 (7)
1 mM		1.00 ± 0.18 (20)	11.8 ± 1.7 (20)	1.1 ± 0.07 (20)
50 μM	Cortex	0.69 ± 0.04 (5)	5.1 ± 1.5 (4)	0.9 ± 0.15 (4)
1 mM		1.05 ± 0.09 (11)	10.9 ± 1.8 (9)	1.3 ± 0.09 (10)
50 μM	Striatum	0.62 ± 0.05 (6)	12.8 ± 2.3 (6)	1.6 ± 0.12 (6)
1 mM		0.95 ± 0.13 (11)	18.7 ± 2.6 (9)	1.9 ± 0.08 (7)

Fig. 2. O_2 uptake and $[^{14}C]$ acetylcholine synthesis by synaptosomes during anoxia and postanoxic recovery. Synaptosomal O_2 uptake and $[^{14}C]$ acetylcholine synthesis were measured before, during, and after a 10 min period of anoxia. Synaptosomes in the buffer medium (see the Materials and methods section) were incubated at $37^\circ C$ with stirring. O_2 uptake was measured using a Clark-type electrode, with 1 mM-unlabelled glucose as substrate. After 10 min anoxia, the O_2 content of the medium was raised by the addition of $50 \mu g$ of catalase and 265 nmol of H_2O_2 . A separate vessel was not used for postanoxic incubation. $[^{14}C]$ Acetylcholine (ACh) synthesis during any given period was measured by adding high-specific-radioactivity D-[U- ^{14}C]glucose at the beginning of the period, and stopping the reaction with trichloroacetic acid containing $[^3H]$ acetylcholine at the end. The numbers of determinations and s.e.m. bars are shown; '100%' or initial aerobic characteristics are $3.42 \pm 0.22 \text{ nmol}$ of O_2/mg of protein per min (s.e.m.; $n = 20$) and $11.0 \pm 0.6 \text{ pmol}$ of ACh/mg of protein per min (s.e.m.; $n = 12$).

in vitro is insufficient to damage synaptosomal metabolism. This is confirmed by the data in Tables 2 and 3, which show the metabolic characteristics ($^{14}CO_2$ and $[^{14}C]$ acetylcholine synthesis) and energy metabolites (ATP, ADP and phosphocreatine) of synaptosomes under various conditions. It can be seen that acetylcholine synthesis is not significantly reduced during postanoxic incubations, and that $^{14}CO_2$ output, like O_2 uptake (see Fig. 2), may increase. When the postanoxic state is compared with the aerobic control (Table 3) there is a significant decrease in the ATP content, but no significant decrease in the phosphocreatine, ADP concentration or ATP/ADP ratio. This suggests that there is no major perturbation to the synaptosomal energy status after an anoxic episode. There is, however, a significant decrease in the total adenine nucleotide pool (ATP + ADP), which is not the case when the synaptosomes are incubated in other conditions (Table 3).

A similar phenomenon has been described in the ischaemic/non-ischaemic transition for the perfused heart (Hutchinson *et al.*, 1981) and may be associated with an attempt by the cell to maintain ATP/ADP ratios under adverse conditions by removing a proportion of the adenine nucleotide pool as bound polymer.

Synaptosomal metabolism in the presence of veratridine

The alkaloid veratridine increases the Na^+ conductance of the plasma membrane of excitable cells (Ohta *et al.*, 1973). This results in a stimulation of the plasma-membrane (Na^+/K^+)-dependent ATPase, an increased rate of hydrolysis of cytosolic ATP, and thus a stimulation of mitochondrial respiration (Scott & Nicholls, 1980). Table 2 shows that under aerobic conditions veratridine significantly increases both O_2 uptake and $^{14}CO_2$ production, changes that are consistent with an increase in mitochondrial respiration. Table 3 shows that in the aerobic state the presence of veratridine significantly increases synaptosomal ADP content with a concomitant

Table 2. *Metabolic characteristics of synaptosomes subjected to anoxia in vitro with and without veratridine*
Results are expressed as means $\pm$ s.e.m. for the numbers of determinations shown in parentheses. The experiments were carried out by the protocol outlined in Fig. 1 and the Materials and methods section. Statistical comparisons are carried out with respect to the aerobic controls: **, $P < 0.005$; *, $P < 0.05$. n.s. = not significant. O_2 uptake in the presence of veratridine ($100 \mu M$ final concn.) was significantly higher ($6.84 \pm 0.2 \text{ nmol/min per mg}$ of synaptosomal protein) (mean $\pm$ s.e.m.; $n = 29$) than in its absence (3.42 ± 0.22 ; $n = 20$).

Incubation	$^{14}CO_2$ output (nmol/min per mg of protein)	$[^{14}C]$ Acetylcholine synthesis (pmol/min per mg of protein)
Aerobic	0.91 ± 0.03 (19)	11.0 ± 0.6 (12)
Aerobic + veratridine ($100 \mu M$)	3.53 ± 0.04 (9)**	5.5 ± 0.3 (9)**
Postanoxic	1.07 ± 0.07 (9)*	9.6 ± 1.2 (8) n.s.
Postanoxic + veratridine ($100 \mu M$)	2.17 ± 0.14 (9)**	3.5 ± 0.5 (11)**

Table 3. *Energy metabolites of synaptosomes subjected to anoxia in vitro with and without veratridine*

Results are expressed as nmol/mg of synaptosomal protein (mean $\pm$ S.E.M.) for the numbers of determinations shown in parentheses. All statistical comparisons are made with respect to the aerobic control: **, $P < 0.005$; *, $P < 0.05$; n.s., not significant. For details of the aerobic/anoxic protocols, see Fig. 1 and the Materials and methods section.

Incubation	Metabolite concn. (nmol/mg of synaptosomal protein)				
	ATP	ADP	Phosphocreatine	ATP/ADP	ATP + ADP
Aerobic	1.46 $\pm$ 0.10 (13)	1.09 $\pm$ 0.15 (12)	2.18 $\pm$ 0.28 (12)	1.45 $\pm$ 0.17 (11)	2.59 $\pm$ 0.20 (12)
Aerobic + 100 μ M-veratridine	1.40 $\pm$ 0.16 (7) n.s.	2.05 $\pm$ 0.30 (6)**	1.44 $\pm$ 0.13 (6) n.s.	0.77 $\pm$ 0.09 (6)*	3.02 $\pm$ 0.31 (6) n.s.
Postanoxic	1.04 $\pm$ 0.11 (9)*	1.11 $\pm$ 0.15 (9) n.s.	1.74 $\pm$ 0.26 (3) n.s.	1.01 $\pm$ 0.15 (9) n.s.	1.99 $\pm$ 0.16 (9)*
Postanoxic + 100 μ M-veratridine	0.64 $\pm$ 0.10 (4)**	2.00 $\pm$ 0.37 (4)**	0.73 $\pm$ 0.34 (4)*	0.41 $\pm$ 0.18 (4)*	2.63 $\pm$ 0.25 (4) n.s.

Table 4. *Relative effects of veratridine on synaptosomes from normoxic rats incubated with and without anoxia in vitro*

In this Table similar parameters are compared in the presence and absence of veratridine for both postanoxic (a) and aerobic (b) synaptosomes. To compare the relative effect of the postanoxic state with the normal aerobic state, whilst allowing for the effect of veratridine in both situations, the parameters C (a/b) is used. * Indicates a ratio of two values that are significantly different at the $P < 0.05$ level; ND, not determined.

Ratio of values	ATP/ADP	O ₂	¹⁴ CO ₂	[¹⁴ C]Acetylcholine
Postanoxic synaptosomes (veratridine/no veratridine) (a)	0.41*	ND	2.03*	0.36*
Aerobic synaptosomes (veratridine/no veratridine) (b)	0.53*	2.00*	3.89*	0.50*
C (= a/b)	0.77	ND	0.52	0.72

reduction in the ATP/ADP ratio, which is consistent with increased cytosolic ATP utilization. No significant change was observed in the content of ATP, phosphocreatine or (ATP + ADP) under these conditions. However, the presence of veratridine under these conditions caused a 4-fold increase in ¹⁴CO₂ output but a 50% reduction in [¹⁴C]acetylcholine synthesis (Table 2). The 'stimulus-coupling' of depolarization/repolarization to neurotransmitter synthesis makes the veratridine-dependent decrease in acetylcholine synthesis seem paradoxical. However, Grewaal & Quastel (1973) observed that, although the amount of acetylcholine released from slices of cerebral cortex was increased in the presence of protoveratrine, total acetylcholine synthesis was reduced.

The combined effect of anoxia and veratridine

Although veratridine itself may alter parameters affected by anoxia, the combined effects of veratridine and anoxia on the synaptosome may be used as an approximation to a model for studying the effects of anoxia on a working (i.e. depolarizing/polarizing) nerve ending. Table 2 shows that, in the presence of veratridine, ¹⁴CO₂ production is significantly reduced by a period of anoxia, in contrast with the unstimulated incubations. Similarly, veratridine stimulation reduces the postanoxic recovery of [¹⁴C]acetylcholine synthesis, the rate of which is only about 30% of the control aerobic rate. Table 3 indicates that, relative to the unstimulated incubations, the postanoxic recovery of energy metabolites in the presence of veratridine was severely impaired,

with significant decreases in the content of ATP and phosphocreatine, and in the ATP/ADP ratio. These changes again occur without a significant change in the (ATP + ADP) content.

The inclusion of veratridine throughout the incubation means that in addition to stimulating the metabolism of the synaptosomal preparation under aerobic conditions, it is also rendering the synaptosomes more susceptible to the anoxic insult. It has been demonstrated that more profound changes in synaptosomal membrane potentials occur during stimulated anaerobiosis than during aerobic stimulation or unstimulated anaerobiosis (Scott & Nicholls, 1980). Table 4 gives an index of the effects of veratridine on aerobic synaptosomes (= b) and on those that have been exposed to anoxia (= a). Therefore the ratio C (= a/b) is an index of any irreversible damage occurring during stimulated anoxia. A parameter that is altered by veratridine alone, but that is not irreversibly altered by stimulated anoxia, would yield C = 1, since its alteration by veratridine would be the same whether or not there had been an intervening anoxic period. It can be seen that on this basis, substrate oxidation as measured by ¹⁴CO₂ output suffers a greater irreversible change than either energy status or acetylcholine synthesis, which are altered to approximately the same extent.

Discussion

The magnitude of the apparent K_m values reported here for acetylcholine synthesis and glucose oxidation are comparable with others found in the

literature for synaptosomal glucose metabolism, e.g. $240\mu\text{M}$ for glucose uptake (K_m ; Diamond & Fishman, 1973) and $440\mu\text{M}$ for acetylcholine synthesis (approximate saturating concentration; Lefresne *et al.*, 1978). These results contrast with the rather high K_m for glucose uptake into whole brain (5–7 mM; Bachelard, 1971; Bachelard *et al.*, 1973), although in brain slices a low- K_m component for hexose uptake has been observed (Fletcher & Bachelard, 1978). These differences probably reflect the influence of the blood/brain barrier.

The differences in the apparent K_m values of this preparation for glucose for $^{14}\text{CO}_2$ production and [^{14}C]acetylcholine synthesis mean that parallel decreases in these parameters do not occur (Fig. 1), in contrast with the results of other workers using pyruvate as substrate for brain minces (Gibson *et al.*, 1975). However, although $^{14}\text{CO}_2$ production and [^{14}C]acetylcholine both share common pathways through glycolysis and the pyruvate dehydrogenase complex, $^{14}\text{CO}_2$ output is additionally dependent on the tricarboxylic acid-cyclic activity, whereas [^{14}C]acetylcholine synthesis depends also on the rates of formation, transport and utilization of an acetyl group carrier (see, for example, Tuček, 1978). It has recently been demonstrated that, under different conditions, the output of $^{14}\text{CO}_2$ from pyruvate dehydrogenase complex, $^{14}\text{CO}_2$ from the tricarboxylic acid cycle and [^{14}C]acetylcholine synthesis can be altered to different extents (Ksiezak & Gibson, 1981a). The results presented here confirm that $^{14}\text{CO}_2$ production and [^{14}C]acetylcholine synthesis may be disproportionately altered.

It has been known for some time that brain slices do not exhibit a metabolic rate comparable with that *in vivo* unless stimulated by electrical pulses (McIlwain, 1953) or high K^+ depolarization. Our data suggest that similarly synaptosomes do not provide a good model for synaptic function *in vivo* unless continuously depolarized.

It has been demonstrated that the effect of veratridine in this type of experiment is not merely to uncouple intrasynaptosomal mitochondria through elevation of cytoplasmic Na^+ concentration (Scott & Nicholls, 1980). This is consistent with previous data (Booth & Clark, 1979), which showed that synaptosomes treated with uncoupler suffer a loss of (ATP + ADP), whereas veratridine-treated synaptosomes show no such loss.

Veratridine exacerbates the consequences of anoxia *in vitro*, but its relative effects (Table 4) are that the postanoxic recovery of $^{14}\text{CO}_2$ production is impaired to a greater extent than either the ATP/ADP ratio or [^{14}C]acetylcholine synthesis as assessed by the values of the parameter (C). However, neurological function *in vivo* is more vulnerable than energy metabolism to irreversible changes resulting from ischaemia (Nördstrom *et al.*, 1976).

This may be in part due to the insufficiency of our simple anoxic model *in vitro* using synaptosomes stimulated with veratridine but may also be a reflection of the more complex situation relating to ischaemia *in vivo* where additional factors such as limitation of substrate supply, reduced pH, increased cytosolic $[\text{Ca}^{2+}]$ and loss of K^+ may cause additional damage.

The use of $^{14}\text{CO}_2$ output to assess the metabolic status of cholinergic synaptosomes needs justification because of the possibility that reductions in $^{14}\text{CO}_2$ and [^{14}C]acetylcholine synthesis occur not only because of reduced metabolic flux but as a result of increased dilution of metabolite pools with endogenous substrates. It is generally accepted that acetylcholine is synthesized from exogenous glucose without apparent dilution (Browning & Schulman, 1968; Lefresne *et al.*, 1973).

The degree of dilution that occurs with respect to $^{14}\text{CO}_2$ can be assessed by calculating the apparent respiratory quotients ($^{14}\text{CO}_2$ output/ O_2 uptake) from data produced from aerobic incubations. The apparent respiratory quotients are 0.28 ± 0.01 (mean $\pm$ S.E.M., $n = 18$) in the absence of veratridine, and 0.54 ± 0.01 (mean $\pm$ S.E.M., $n = 6$) in its presence. Two factors are responsible for an apparent respiratory quotient less than unity: dilution of radioactive substrate, and the accumulation of radioactive intermediates (Von Korff & Kerpelfronius, 1975) and related compounds (Van den Berg, *et al.*, 1969, Clarke *et al.*, 1975). It has been demonstrated (Ksiezak & Gibson, 1981a) that the fractional accumulation of radioactive compounds represents 0.74 and 0.45 of the metabolic flux through the pyruvate dehydrogenase reaction in unstimulated and dinitrophenol-stimulated synaptosomes respectively. This implies that, whether veratridine is present or not, the accumulation of radioactive intermediates would be sufficient to account for the apparent respiratory quotients shown above and that dilution phenomena need not be invoked.

From these studies it is clear that the veratridine-stimulated synaptosome may be a more useful model for assessing damage to the nerve ending than the unstimulated synaptosomal preparation. Kovachich (1980) in studies on the effects of high-pressure O_2 concentrations on brain slices also found that the presence of veratridine provided a better approximation to the system *in vivo*. The nature of the irreversible damage caused by anoxia in these experiments is such that significant deficits are observed in both energy metabolism (as reflected by ATP/ADP ratios) and [^{14}C]acetylcholine synthesis and more particularly in general catabolic activity as reflected by $^{14}\text{CO}_2$ production from labelled glucose. It seems unlikely that these effects are due to simple dilution phenomena. However, using the veratridine-stimulated synaptosome as our model, further experimentation

is necessary to study the effects of other phenomena associated with anoxic insult *in vivo*, e.g., pH changes, Ca^{2+} and K^{+} alterations, in order to allow us to differentiate between the relative sensitivities of these metabolic systems to hypoxic insult *in vivo*.

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