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[^{14}C]Acetylcholine synthesis and [^{14}C]carbon dioxide production from [U-^{14}C]glucose by tissue prisms from human neocortex

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1. [^{14}C]Acetylcholine synthesis and $^{14}CO_2$ production from [U-^{14}C]glucose has been measured in tissue prism preparations from human neocortex. 2. Electron micrographs of prisms from human and rat neocortex show that both contain intact synaptic endings with evenly-distributed vesicles and normal-appearing mitochondria, but only poorly preserved cell body structure. 3. Synthesis of [^{14}C]acetylcholine in prisms from rat neocortex is similar to estimates for turnover *in vivo*. Synthesis in prisms from human neocortex is 18% of that in rat tissue and 64% of that in tissue from baboon neocortex for incubations performed in 31 mM-K^+. 4. Investigations of prisms prepared from rat brains stored at 37°C after death revealed that synthesis of [^{14}C]acetylcholine in the presence of 31 mM-K^+ was greatly decreased within 30 min of post-mortem incubation, whereas synthesis at 5 mM-K^+ and production of $^{14}CO_2$ at both K^+ concentrations were only significantly affected after longer periods. Changes were similar in neocortex and striatum. Thus human autopsy material is unlikely to be suitable for use with this system. 5. Investigations using animal models suggest that [^{14}C]acetylcholine synthesis and $^{14}CO_2$ production are not affected by surgical or anaesthetic procedures. 6. Neither [^{14}C]acetylcholine synthesis nor $^{14}CO_2$ production in human prisms was significantly changed with age between 15 and 68 years. 7. Samples from patients with the dementing condition Alzheimer's disease showed a significant decrease in [^{14}C]acetylcholine synthesis to 47% of normal samples and a significant increase of 39% in production of $^{14}CO_2$.

Advances in the understanding of central-neurotransmitter systems and the demonstration in Parkinsonism of a clear defect in dopaminergic neurons has led to increasing attempts to detect neurotransmitter abnormalities in other neurological diseases.

Hitherto, assessment of neurotransmitter function has relied largely on measurements of synthetic enzymes and of the concentrations of transmitters and metabolites in tissue samples (from autopsy brains) and body fluids. The synthesizing ability of fresh human tissue samples has rarely been measured. In the present paper we describe a sensitive method for the determination of acetylcholine synthesis in tissue prisms prepared from human grey matter. Such measurements are applicable to the examination of small amounts of neocortex from biopsy samples taken for diagnostic purposes from patients with certain neurological disorders (in particular dementia).

The *in-vitro* system chosen was based on that used in rat studies by Gibson and his co-workers (Gibson *et al.*, 1975; Gibson & Blass, 1976*a*) in which the synthesis of acetylcholine from radiolabelled carbohydrate substrates was measured in a tissue prism preparation of whole brain (i.e. mechanically sliced in two directions). They showed, using both the method *in vitro* (Gibson *et al.*, 1975; Gibson & Blass, 1976*a*) and related studies *in vivo* (Gibson & Blass, 1976*b*), that conditions that affect the metabolism of glucose have a closely related effect on the synthesis of acetylcholine, even though acetylcholine synthesis utilizes only a small proportion of the total glucose metabolized. Therefore, in the present study of human samples, [U-^{14}C]-glucose was used as substrate, allowing a parallel estimate of total glucose metabolism (from $^{14}CO_2$ production) together with the synthesis of [^{14}C]-acetylcholine. The method was modified, in investigations with rat tissue, to improve the linearity

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of response with time and to facilitate the removal of unreacted radioactive glucose and metabolites, thus making it more suitable for small samples. The method has been applied to the investigation of changes in glucose metabolism and [^{14}C]acetylcholine synthesis in a human dementing condition, Alzheimer's disease, in which decreased choline acetyltransferase activity is now a well-established finding (Bowen, 1980).

Previous attempts to examine the effect of aging on the cholinergic system in normal human brain, using estimation of choline acetyltransferase activity in autopsy samples, have produced contradictory results (see the Discussion section). Since studies in animals suggest that choline acetyltransferase activity is not the rate-limiting step in synthesis (Tuček, 1978; Marchbanks & Wonnacott, 1979), we have determined synthesis directly in biopsy material from patients of various ages. Metabolism of the transmitter depends on neuronal activity (Tuček, 1978), so incubations were performed in both low (5mM) and high (31mM) K^+ concentrations, thus measuring acetylcholine synthesis in resting and stimulated conditions. Cerebral glucose consumption has been reported to be decreased in normal aging (Dastur *et al.*, 1963), but this may be due to increased blood concentrations of ketone bodies and their utilization by the brain (Gottstein *et al.*, 1970). Thus, in the present study the effect of age on total glucose metabolism is reported for the system *in vitro*, which is free from such external complicating influences.

Experimental

Materials

[U-^{14}C]Glucose (>230Ci/mol) and [1-^{14}C]-acetylcholine were from The Radiochemical Centre, Amersham, Bucks., U.K. The glucose was diluted appropriately with cold glucose (BDH Chemicals, Poole, Dorset, U.K.). Ammonium reineckate was also from BDH Chemicals. Hyamine hydroxide (1M in methanol) was from Fisons Scientific Apparatus, Loughborough, Leics., U.K.; paraoxon, acetylcholine bromide, choline chloride and acetylcholinesterase (type VI-S from electric eel) were from Sigma Chemical Co., Poole, Dorset, U.K.; Bio-rex 9 (200–400 mesh) and AG 1 (X8; 100–200 mesh) ion-exchange resins were from Bio-Rad Laboratories, Richmond, CA, U.S.A.; and Aquasol was from New England Nuclear, Boston, MA, U.S.A.

Tissue samples

Rat brains were obtained from adult (>3 months) male Porton Wistar animals after stunning and decapitation. Immediately on removal, brain tissue was placed in ice-cold, modified Krebs–Ringer phosphate buffer (141mM-NaCl/5mM-KCl/1.3mM-$CaCl_2$/1.3mM-$MgSO_4$/10mM-Na_2HPO_4, adjusted to pH 7.4 with HCl), containing 2.5mM-glucose and freshly gassed with 100% O_2, and transferred to the cold room (4°C). Control human samples were apparently normal neocortex with underlying white matter (13 from temporal lobe, six from frontal and one from parietal regions) taken to allow removal of tumours (six craniopharyngiomas, seven meningiomas, five gliomas, one pituitary tumour and one epidermoid cyst). These were placed in ice-cold modified Krebs–Ringer buffer as soon as was practicable after removal (usually less than 2min). Samples of diseased brain were from biopsy material (eight from temporal lobe and five from frontal regions) taken at diagnostic craniotomy and were treated as for controls. Histopathology of adjacent tissue samples revealed the presence of senile plaques and neurofibrillary degeneration, confirming that these patients had Alzheimer's disease. Baboon neocortical samples were from animals killed by cardiac arrest under anaesthesia. The brain was rapidly removed and a coronal slice (approx. 2cm thick) was transferred to ice-cold modified Krebs–Ringer buffer.

Preparation of tissue prisms and incubations

Grey matter (including all cortical layers and free of meninges) was dissected from the required regions and mechanically chopped (McIlwain tissue chopper: Mickle Laboratories Engineering Co., Gomshall, Surrey, U.K.) at 0.3mm intervals in two directions separated by 45°. The resulting prisms were placed in 5ml of cold buffer, separated by trituration through a wide-bore disposable pipette tip and allowed to settle. Excess buffer (3.5ml) was removed and the volume was re-adjusted to 10ml with fresh buffer. After gassing (100% O_2 for 30s) pre-incubation was performed for 45min in a stoppered 50ml conical flask at 37°C in a shaking water-bath with regassing (30s) every 15min. The flask was then transferred to ice and excess buffer was removed.

Incubations were performed in 25ml conical flasks in a final volume of 3ml containing 0.1ml of tissue suspension (usually 1.0–2.0mg of protein), modified Krebs–Ringer phosphate buffer, 2mM-choline, 40μM-paraoxon and 2.5mM-[U-^{14}C]-glucose (2.8μCi/flask for rat and baboon experiments and 11.2μCi/flask for human studies). Flasks were oxygenated for 30s and sealed with 'subaseal' stoppers from which was suspended a small glass vial containing a glass fibre (Whatman GFA) filter disc. Incubations were performed at 37°C and were concluded by injection of 0.2ml of 1M-Hyamine on to the filter disc, followed after 15s by injection into the reaction mixture of 0.5ml of cold 1.4M-$HClO_4$ containing 35mM-acetylcholine as carrier. Flasks were left on ice for at least 15min before removal of

stoppers. Filter discs were transferred to glass scintillation vials and the small vial rinsed with Aquasol to a final volume of 12ml. To neutralize the Hyamine 0.3ml of 1M-HCl was added and radioactivity was measured after 24h in a Beckman LS100C liquid-scintillation counter.

The reaction mixture was transferred to conical centrifuge tubes and the flasks were washed with 0.5ml portions of 0.2M-$HClO_4$ containing 5mM-acetylcholine. The tissue was separated by centrifugation at 800*g* for 10min and the resulting pellet was washed with 1ml of 0.2M-$HClO_4$ containing 5mM-acetylcholine. The pooled supernatant fractions and the pellets were frozen (−20°C) and the [^{14}C]acetylcholine content of the supernatant was determined within 3 days. The protein contents of the pellets were determined by the method of Lowry *et al.* (1951). Homogenization of tissue before separation from the acidified media was not performed, as this led to higher but variable results. This additional radioactivity behaved differently from authentic acetylcholine under a variety of conditions, and was extractable into the organic phase of a chloroform/methanol partition (Folch-Pi *et al.*, 1957), suggesting it may have been lipid.

As initial additions and gassing of flasks were performed on ice, values were corrected for blanks, which were incubated for 2min. (Test flasks were therefore incubated for the desired time plus 2min.) These blanks provided adjustment for initial temperature changes and for small amounts of radioactivity subsequently isolated with the acetylcholine fraction, which were independent of incubation time and tissue content, but could not be removed by prior re-purification of the substrate. Test incubations were performed in quadruplicate and blanks were usually in triplicate.

Determination of [^{14}C]acetylcholine synthesis

Acetylcholine (as well as any other quaternary ammonium compounds) was precipitated by addition of 3.5ml of saturated ammonium reineckate (adjusted to pH2 with $HClO_4$). Tubes were left cold for at least 5min and centrifuged at 800*g* for 10min. The pellet was resuspended in 2.5ml of saturated ammonium reineckate and layered with a Pasteur pipette on a further 2ml of saturated ammonium reineckate containing 10% glycerol. The tubes and pipette were rinsed with 1ml of ammonium reineckate that was added to the upper layer and centrifugation was performed at 800*g* for 10min. Resuspension and layering was repeated and the pellet was then washed with 3.5ml of ammonium reineckate. The resultant pellet was mixed thoroughly with 2ml of methanol (adjusted to pH4.5 with glacial acetic acid) containing 75mg of Bio-rex 9 resin/ml to absorb the reineckate ion and release the bound compounds. The resin suspension was added to a small glass column containing 1ml of resin/methanol mixture and the eluate was collected in a precounted scintillation vial containing 12ml of Aquasol. Tubes and columns were washed with 2 × 0.5ml of acidified methanol and the effluent was collected in the same vial. Radioactivity was determined using a Beckman LS100C liquid-scintillation counter, which was adjusted to provide maximum (efficiency)^2/background values for these mixtures because of the low levels of radioactivity in some vials.

For estimation of the hydrolysis by acetylcholinesterase of radioactivity in the final acidified-methanol effluent of selected samples, 0.9ml fractions (or 1.4ml fractions from human samples) were dried under N_2 and incubated for 2h at 37°C in 0.3ml of 0.02M-Na_2HPO_4 buffer (pH7.0), containing 0.1M-NaCl and 0.02M-$MgCl_2$ and 40 units of acetylcholinesterase. (Duplicate samples without enzyme were run in parallel to allow an estimate of the extent of hydrolysis.) At the end of the incubation 1.7ml of water was added and the total placed on columns (0.6cm × 12cm) of AG 1-X8 resin in water to remove released groups. Effluents were collected in vials and the column and tubes were washed with water (2 × 2ml), which was also collected. Determinations of radioactivity were performed using Aquasol scintillation mixture in a Beckman LS100C liquid-scintillation counter.

For direct estimates of the amount of acetylcholine produced by prisms, the incubation was concluded by the addition of carrier-free $HClO_4$ and the flask contents were transferred as for radioassay. Acetylcholine was extracted from the tissue-free supernatant as described by Marchbanks & Israel (1971), except that the final aqueous extract was freeze-dried. Acetylcholine was determined by bioassay with guinea-pig ileum, essentially as described by Boura *et al.* (1954), and quantified by comparison with acetylcholine standards similarly extracted from incubation mixtures.

Post-mortem studies in rats

Male Porton Wistar rats (average age 8 months) were killed by decapitation after stunning. Heads were maintained at 37°C for required periods after which the brain was removed into ice-cold modified Krebs–Ringer buffer. Striatum and neocortex were dissected at 4°C for preparation of tissue prisms.

Effects of anaesthesia in rats

Anaesthesia was induced with 50mg of thiopentone/kg body wt. and the animal was intubated. After approx. 1h, suxamethonium (1mg/kg body wt.) was injected and anaesthesia was maintained with 1% (v/v) halothane in O_2/N_2O (1:1, v/v). After 6min the rat was injected with 0.2mg of tubocurarine/kg body wt. and killed by decapitation after

a further 30min. An untreated control animal was also killed and the brain tissue was processed simultaneously.

Unilateral ischaemia in baboons

Partial tissue ischaemia was induced by occlusion of the middle cerebral artery under anaesthesia (Symon *et al.*, 1979). Local tissue blood flow was determined by the hydrogen-clearance technique (Pasztor *et al.*, 1973). Prisms were prepared from grey matter of the perisylvian region and the combined parasagittal and intermediate frontal temporal convexity regions of both the control and ischaemic hemispheres.

Results

Assay method

Plates 1(*a*) and 1(*b*) show typical electron micrographs of tissue prisms of neocortex from rat and human brain respectively, at the conclusion of the pre-incubation period. Although there is an almost total absence of recognizable cell bodies, many nerve endings are seen containing evenly-distributed vesicles and normal-appearing mitochondria. Attached postsynaptic membrane may be seen in some cases, but there is little evidence of other postsynaptic structure. The electron micrographs indicated morphological similarity of the preparation from the two species. As only small amounts of human brain were available, detailed experiments were performed on rat preparations and the procedure was examined to a more limited extent with human material. Typical time-course results for synthesis of [^{14}C]acetylcholine and $^{14}CO_2$ production by a prism preparation from rat neocortex are presented in Fig. 1(*a*). Production of [^{14}C]-acetylcholine by the prisms was markedly increased in the presence of high concentrations of K^+ (31mM) and under these conditions was linear for at least 80min. As found by previous workers (Browning & Schulman, 1968; Lefresne *et al.*, 1973) synthesis in the presence of 5mM-K^+ decreased at later time points. Synthesis to 60min was essentially linear and therefore this time was chosen for subsequent incubations. The rate of $^{14}CO_2$ production showed some increase with time, suggesting that in this case there was dilution of the label in intermediate pools. However, the deviation from linearity at 60min was not great, and thus $^{14}CO_2$ determinations at this point provided an indication of overall glucose metabolism. Although approximately three times as much [^{14}C]acetylcholine was produced by prisms from the striatum similar time courses were found in the presence of 5mM- and 31mM-K^+ to those from neocortex.

Results obtained from tissue cooled at the conclusion of pre-incubation were the same as those where tissue was used without cooling. As it was more convenient to set up incubation flasks on ice, this was done routinely. In the absence of the pre-incubation period initial rates of acetylcholine synthesis at both 5 and 31mM-K^+ were increased, resulting in non-linearity over 60min incubations. It has been shown that slices undergo a number of changes in environment due to the movement of ions during the first 20min of incubation (Franck, 1972) and this may account for the observed effect of pre-incubation.

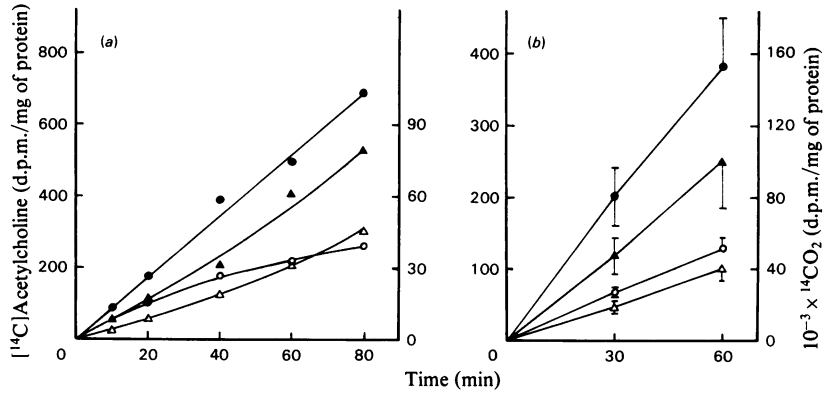
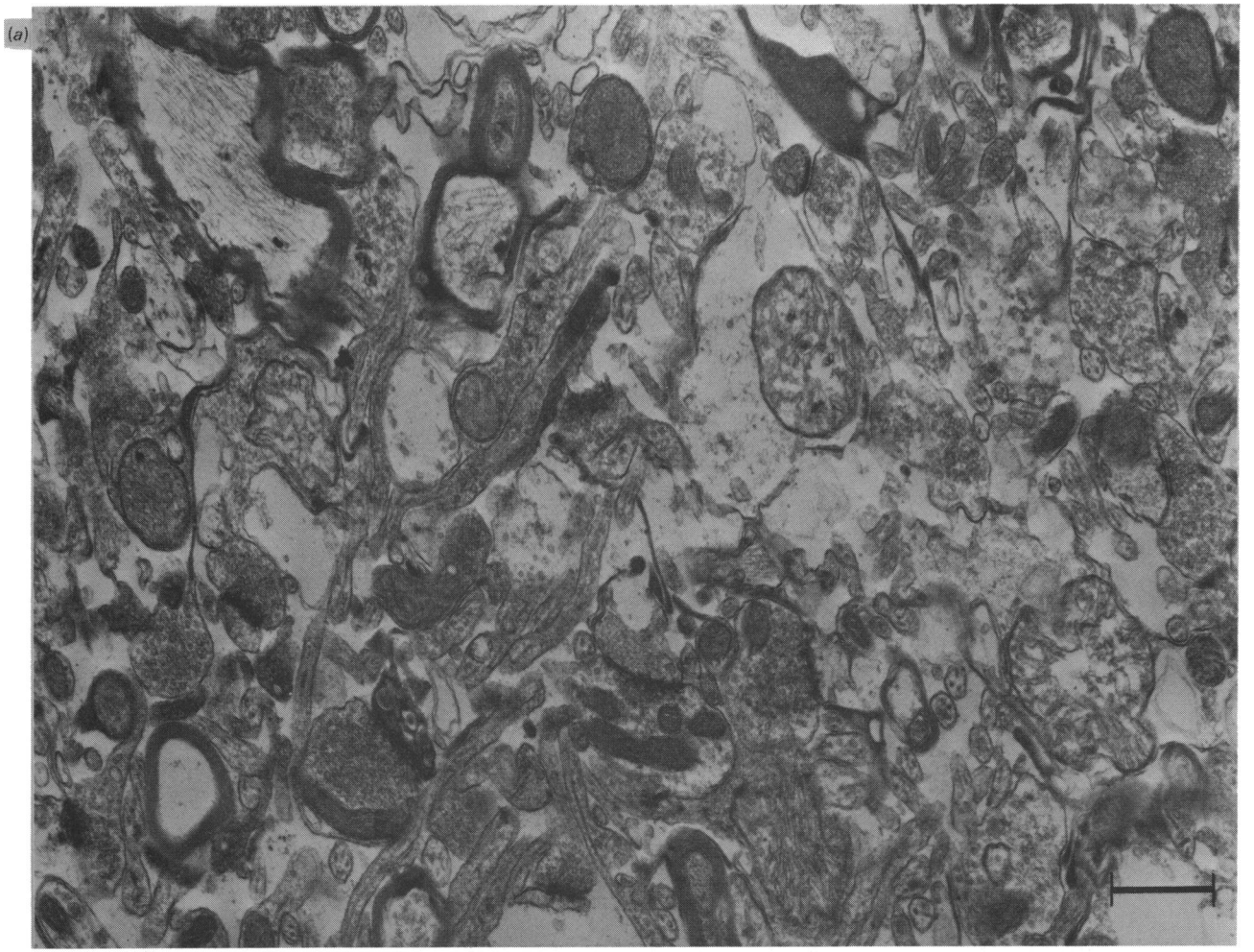


Fig. 1. *Time course of production of* [^{14}C]*acetylcholine* (○ *and* ●) *and* $^{14}CO_2$ (△ *and* ▲) *in tissue prisms from* (*a*) *rat and* (*b*) *human brain*

(*a*) shows a typical time course for a preparation of rat neocortex. Incubation mixtures contained 2.8μCi of glucose/flask, and either 5mM-K^+ (open symbols) or 31mM-K^+ (filled symbols). (*b*) shows means ± S.D. for 30 and 60min incubations of four separate human preparations. Incubation mixtures contained 11.2μCi of glucose/flask and either 5mM-K^+ (open symbols) or 31mM-K^+ (filled symbols).



EXPLANATION OF PLATE 1

Electron micrograph of prisms prepared from (*a*) *rat neocortex and* (*b*) *human neocortex*

Prisms were removed after pre-incubation and fixed for 30 min at room temperature in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M-phosphate buffer, rinsed in phosphate buffer and treated with 1% OsO_4 in phosphate buffer for 1 h. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and viewed in a JEOL 100S electron-microscope. Scale markers represent 1 μm.

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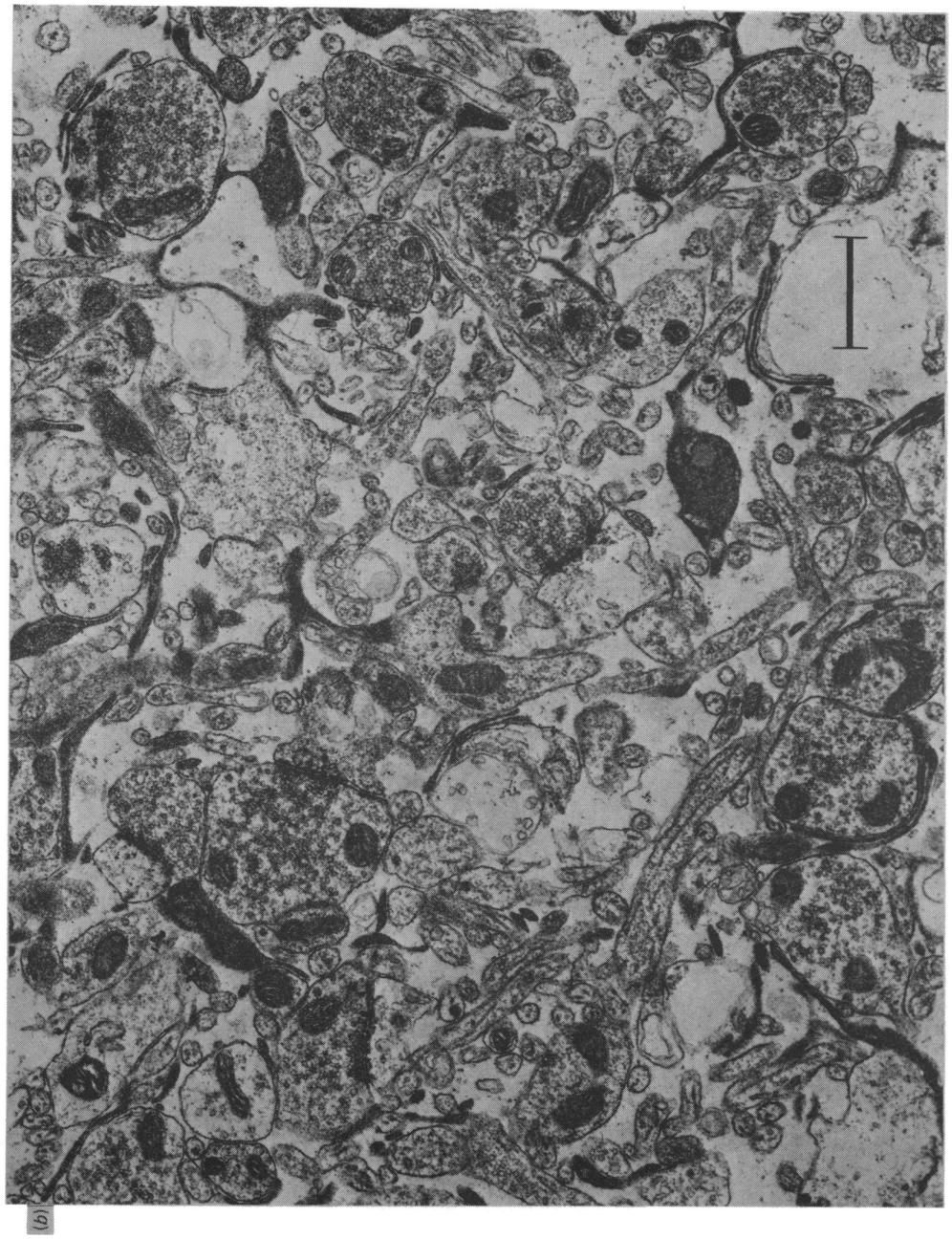
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(b)

Recovery of authentic radiolabelled acetylcholine added to the incubation mixture was better than 90%. Acetylcholinesterase hydrolysis of the material collected in the final acidified methanol fraction was (after correction for blank values) $91 \pm 3.4\%$ in the samples that had been incubated with 31 mM-K^+ and $88 \pm 4\%$ where 5 mM-K^+ had been used. Authentic radioactive acetylcholine was hydrolysed 98% under the same conditions. However, when the authentic material was treated by the normal isolation procedure before hydrolysis, this value was decreased to $91 \pm 4\%$, suggesting that at both K^+ concentrations over 90% of the radioactivity in the final acidified methanol fraction was in acetylcholine. This contrasts with the findings of others where reineckate precipitation has been used to isolate acetylcholine after incorporation of radiolabelled glucose *in vivo* (Gibson *et al.*, 1978). In their studies 30–50% of the isolated labelled material was not hydrolysed by acetylcholinesterase.

Studies by other investigators using slice and prism preparations from rat brain have indicated that there is little or no dilution of glucose label by intermediate pools in the synthesis of acetylcholine (Browning & Schulman, 1968; Lefresne *et al.*, 1973; Gibson & Blass, 1976*a*; Quastel, 1978). If this is assumed to apply in the present study, it is possible to express the synthesis of acetylcholine in mass units, producing an average value for rat neocortex prisms in the presence of 31 mM-K^+ of 36.89 ± 5.20 pmol/min per mg of protein. In five incubations direct estimates of acetylcholine production were obtained by bioassay and found to be $100.5 \pm 16.7\%$ of the results from radioassay of the same samples, confirming that the radiolabel does not undergo substantial dilution in the system described here.

Effect of post-mortem time

Klemm & Kuhar (1979) and Dodd *et al.* (1979) have raised the possibility of measuring metabolically complex functions *in vitro*, even after extended post-mortem periods. As the examination of human material would be facilitated if autopsy samples were suitable, the effect of a short post-mortem delay (up to 2 h) was investigated in rat brain (Figs. 2 and 3). Both the cerebral cortex and striatum were examined to determine whether post-mortem changes varied between brain substructures. It is known that these two regions are differentially affected by the pathological process in Alzheimer's disease (Bowen *et al.*, 1976*a*). In both regions, even after only 30 min post-mortem incubation, marked changes were evident in the response of [^{14}C]acetylcholine synthesis to 31 mM-K^+. By contrast, synthesis of [^{14}C]acetylcholine in the presence of low K^+ was apparently unaffected and overall glucose metabolism changed by only a small

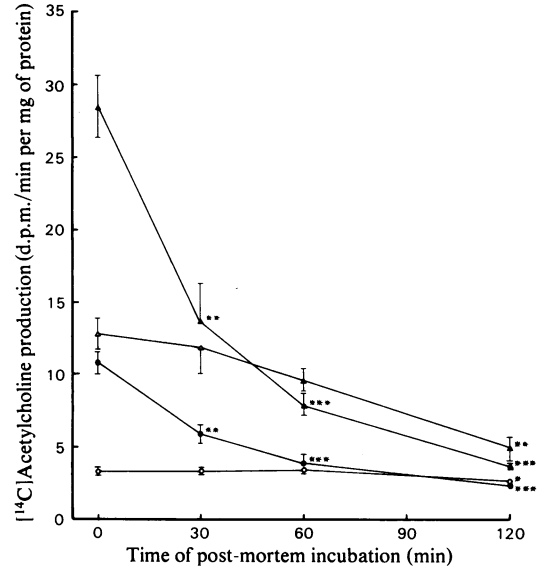

Fig. 2. *The effect of post-mortem time on [^{14}C]acetylcholine synthesis from [U-^{14}C]glucose*

Prisms were prepared from striatum (△ and ▲) and neocortex (○ and ●) of brains maintained in the skull at 37°C for indicated times after decapitation. Incubation media-contained 5 mM-K^+ (open symbols) or 31 mM-K^+ (filled symbols). All points are means ± S.E.M. for independent determinations on three or four animals. Results significantly different from the zero-time incubation point are indicated: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's *t* test).

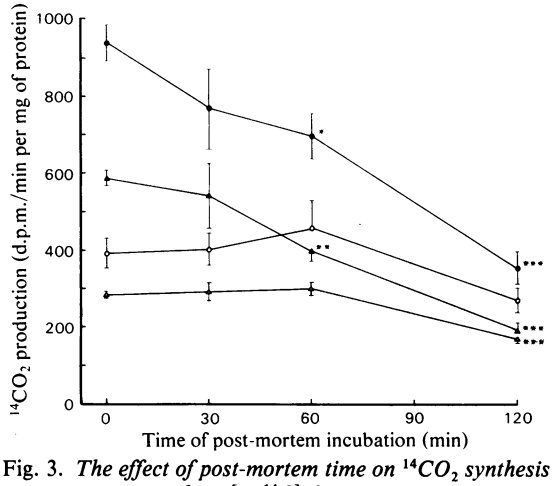

Fig. 3. *The effect of post-mortem time on $^{14}CO_2$ synthesis from [U-^{14}C]glucose*

Prisms prepared from striatum (△ and ▲) and neocortex (○ and ●) are the same as those described in the legend to Fig. 2. Incubation media contained 5 mM-K^+ (open symbols) or 31 mM-K^+ (filled symbols). Results significantly different from the zero-time pre-incubation point are indicated: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's *t* test).

(non-significant) amount at this time point. After 60min the response of [^{14}C]acetylcholine synthesis to elevated K^+ had completely disappeared in both regions, yet significant changes in the synthesis at 5mM-K^+ occurred only after 120min. After 120min the response of $^{14}CO_2$ formation to K^+ depolarization had also disappeared. Because of the instability to post-mortem changes of both measurements, the investigation of human samples was restricted to fresh material obtained at craniotomy.

Assay of human brain neocortex

Fig. 1(*b*) shows the response to time of $^{14}CO_2$ production and [^{14}C]acetylcholine synthesis in human samples. At both 5mM-K^+ and 31mM-K^+ the results are consistent with the time course obtained with preparations from rat neocortex. Acetylcholinesterase treatment of radioactivity in the final acidified methanol fraction of four incubations (at 31mM-K^+) from a human sample resulted in hydrolysis of 82.6 ± 21.1% of the product. (The greater variability compared with rat samples is a reflection of the smaller number of counts in samples from incubations of human tissue.)

As the human material was obtained during operations and was subject to surgical manipulation before removal, animal models were used to investigate changes that may have resulted from the anaesthetic regimen or from short-term ischaemia possibly occurring during the removal of the tissue. The comparison of two rats treated to mimic a typical anaesthetic procedure with untreated controls assayed simultaneously is shown in Table 1. Virtually identical results were obtained for [^{14}C]-acetylcholine synthesis and $^{14}CO_2$ production in both 5mM- and 31mM-K^+. Samples removed from baboons that had undergone unilateral ischaemia under anaesthesia also showed no significant differences from material removed from the non-ischaemic hemisphere (Table 2). Samples were taken from areas most affected by the decreased blood flow (perisylvian region), and an area of lesser decrease (parasagittal and intermediate region). It is noteworthy that the control values for these two cortical regions do not differ significantly from each other.

A further possible complication in the investigation of human samples arises from the nature of the normal material available. In all cases investigated in

Table 1. *Effect of anaesthesia on production of* [^{14}C]*acetylcholine and* $^{14}CO_2$ in rat neocortex

Rats were treated with thiopentone (50mg/kg body wt.) and suxamethonium (1mg/kg body wt.) and anaesthesia maintained with 1% (v/v) halothane in O_2/N_2O (1:1, v/v). After 6min tubocurarine (0.3mg/kg body wt.) was injected and the animals were killed by decapitation after a further 30min. Control animals underwent no treatment before decapitation and the tissue was processed in parallel with that from treated animals. Results presented are the means of quadruplicate determinations for two animals in each group.

	[^{14}C]Acetylcholine (d.p.m./min per mg of protein)		$^{14}CO_2$ (d.p.m./min per mg of protein)	
	5mM-K^+	31mM-K^+	5mM-K^+	31mM-K^+
Treated	3.49; 3.05	9.79; 9.30	295; 350	719; 822
Control	3.44; 2.68	10.18; 8.83	271; 354	593; 850

Table 2. *Effect of unilateral ischaemia on* [^{14}C]*acetylcholine synthesis and* $^{14}CO_2$ *production in baboon neocortex*

Baboons were subjected to unilateral ischaemia by occlusion of the middle cerebral artery. After 30min, the animal was killed and a brain slice was transferred to ice-cold buffer. Grey matter was dissected from the perisylvian, the parasagittal and the intermediate frontal parietal convexity regions of both the ischaemic and control hemispheres. Prisms were prepared for measurement of [^{14}C]acetylcholine synthesis and $^{14}CO_2$ production as described in the Experimental section. Blood-flow results are those obtained within 5min of occlusion. Before occlusion all the values were very similar to those for the control hemisphere. Flows significantly different from pre-occlusion values are indicated: $^*P < 0.05$; $^{**}P < 0.001$ (Student's *t* test). Values are means ± S.D. for five animals.

	Perisylvian region		Parasagittal and intermediate region	
	Control hemisphere	Ischaemic hemisphere	Control hemisphere	Ischaemic hemisphere
Cerebral blood flow (ml/100g per min)	52 ± 20	11 ± 5**	53 ± 21	32 ± 15*
[^{14}C]Acetylcholine (d.p.m./min per mg of protein)				
5mM-K^+	1.11 ± 0.21	1.02 ± 0.22	1.16 ± 0.09	1.11 ± 0.28
31mM-K^+	2.86 ± 0.68	3.05 ± 0.86	3.10 ± 0.74	3.44 ± 0.76
$^{14}CO_2$ (d.p.m./min per mg of protein)				
5mM-K^+	232 ± 59	236 ± 55	260 ± 70	254 ± 67
31mM-K^+	528 ± 116	514 ± 145	527 ± 147	469 ± 140

the present study, the tissue was removed to allow access to a tumour or for associated decompression of the skull, and was separate from the primary site of tumour growth. In some cases where adjacent samples were examined histologically, no obvious abnormalities were seen. However, it is not possible to exclude completely the presence of abnormal material or changes due to compression damage in some samples. A comparison of results for samples separated into three major groups according to the associated tumour type (meningioma, glioma and craniopharyngioma, which have different growth and invasion properties) did not reveal significant differences (one-way analysis of variance) for any of the measurements made. This is consistent with the overall results being a good indication of values for normal tissue.

Effect of aging in humans

There was no significant correlation (linear regression analysis) between age and [^{14}C]acetylcholine synthesis in the presence of either 5 mM- or 31 mM-K^+ (Fig. 4). Similarly, $^{14}CO_2$ production was not significantly changed with age (Fig. 5). In neither case was there a significant difference between samples from the temporal lobe and those from the other regions.

[^{14}C]Acetylcholine synthesis and $^{14}CO_2$ production in Alzheimer's disease

A preliminary investigation with this assay system

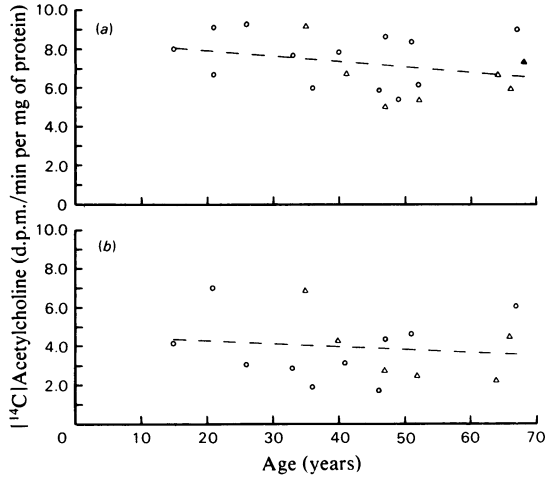

Fig. 4. *Relationship between age and the production of [^{14}C]acetylcholine by human neocortical tissue prisms incubated in (a) 31 mM-K^+ and (b) 5 mM-K^+*

Results are presented for samples from temporal (○), frontal (△) and parietal (▲) lobes. There is no significant correlation between age and [^{14}C]acetylcholine synthesis at either K^+ concentration (31 mM, $r = -0.30$; 5 mM, $r = -0.12$, by linear regression analysis).

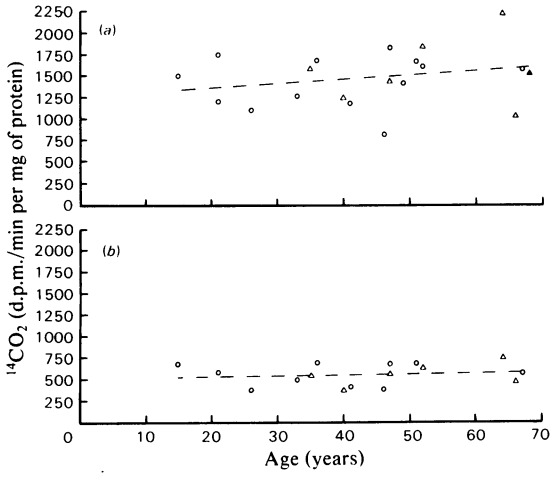

Fig. 5. *Relationship between age and the production of $^{14}CO_2$ by human neocortical tissue prisms incubated in (a) 31 mM-K^+ and (b) 5 mM-K^+*

Results are presented for samples from temporal (○), frontal (△) and parietal (▲) lobes. There is no significant correlation between age and $^{14}CO_2$ production (31 mM-K^+, $r = 0.23$; 5 mM-K^+, $r = 0.14$, by linear regression analysis).

Table 3. *[^{14}C]Acetylcholine synthesis and $^{14}CO_2$ production in biopsy samples from Alzheimer's-disease patients*

Neocortical samples from patients with Alzheimer's disease were obtained at diagnostic craniotomy. Grey matter was dissected and synthesis of [^{14}C]acetylcholine and $^{14}CO_2$ was measured as described in the Experimental section. Control neocortex was material removed to allow surgical procedures. Results presented are means ± S.D. of values obtained for incubations at 31 mM-K^+. Results significantly different from control are indicated: $^*P < 0.01$ (Wilcoxon rank test). Essentially the same proportional differences between control and Alzheimer's-disease samples was found for incubations in 5 mM-K^+.

	n	[^{14}C]Acetylcholine (d.p.m./min per mg of protein)	$^{14}CO_2$ (d.p.m./min per mg of protein)
Control	20	7.3 ± 1.4	1479 ± 328
Alzheimer's disease	13	3.4 ± 1.2*	2059 ± 352*

(Sims *et al.*, 1980) showed a significant decrease in [^{14}C]acetylcholine synthesis in Alzheimer's disease. This finding is confirmed in the present study for which the data are obtained from larger control and Alzheimer's-disease groups. Furthermore, these results indicate that the production of $^{14}CO_2$ by prisms from Alzheimer's-disease patients is significantly higher than in control material (Table 3).

Discussion

Suitability of assay

Morphological examination of the tissue prisms reveals that, as previously reported for adult rat cerebellum slices (Garthwaite *et al.*, 1979, 1980), these are primarily preparations of intact synaptic endings in the presence of disintegrated cell structures. As with conventional preparations of synaptosomes, the prisms provide a useful means of determining acetylcholine synthesis free from the influence of the cell body. Tissue prism systems combine some advantages of synaptosomes and slices, in that the preparation procedure is rapid, is almost entirely performed in the presence of physiological buffers and requires little manipulation of the tissue, but allows multiple sampling from small specimens.

The amount of acetylcholine synthesized in the system may be determined by assuming that there is no dilution of radiolabel in the production of acetylcholine from [U-^{14}C]glucose. The similarity of mass determinations thus calculated to measurements obtained directly by bioassay confirms the validity of this assumption for rat neocortical prisms. Table 4 shows the average acetylcholine production estimated from radioassay of rat tissue and compares this with values similarly calculated for human and baboon preparations. The value for synthesis by rat neocortex samples are close to values previously reported for neocortical slices (Tower & Elliot, 1952; Browning & Schulman, 1968; Grewaal & Quastel, 1973). The values also agree closely with estimates of the acetylcholine turnover rate in rat neocortex of 11–17pmol/min per mg of protein (Malthe-Sorenssen *et al.*, 1978; Wood *et al.*, 1980). Similarly, our results for acetylcholine synthesis by striatum (when converted into mass units) fall within the range found for turnover in striatum (Weiler *et al.*, 1979).

The lower synthesis of [^{14}C]acetylcholine in human compared with rat neocortical prisms (Table 4) would appear to be a genuine species difference.

Table 4. *Species comparison of acetylcholine synthesis*
Results have been converted into mass units based on the assumption that label from [U-^{14}C]glucose is not diluted in the production of acetylcholine. Results are means ± S.D. with the numbers of samples in parentheses.

	Acetylcholine (pmol/min per mg of protein)	
	5 mM-K^+	31 mM-K^+
Human	3.66 ± 1.56 (16)	6.82 ± 0.29 (20)
Baboon	4.12 ± 0.81 (5)	10.62 ± 2.31 (5)
Rat	12.29 ± 1.13 (7)	36.89 ± 5.20 (7)

Neither low rates of blood flow, which possibly occur as tissue is excised, nor the anaesthetic regimen used for diagnostic craniotomy affected the synthesis in animal model systems. Estimates of neocortical neuronal densities indicate a similar difference between humans and rats (Brizzee *et al.*, 1964; Brizzee & Ordy, 1979; Cragg, 1975; Huttenlocher, 1979) to that observed for acetylcholine synthesis. It is of further interest that similar differences for human, baboon and rat occur for glutamate decarboxylase activity (Chalmers *et al.*, 1970; Bayoumi & Smith, 1973; Bowen *et al.*, 1976*b*; Spillane *et al.*, 1977), the rate-limiting enzyme mediating 4-aminobutyrate synthesis (Roberts & Kuriyama, 1968). This suggests that the relative synthetic potential for these two neurotransmitters (which may in part be a reflection of the relative cell number of each type) changes similarly with ascent of the mammalian phylogenetic scale.

Tower & Elliot (1952) have compared the synthesis of acetylcholine in human, rat and rhesus monkey using bioassay determinations of acetylcholine in hand-cut slices (containing 5–20-fold the material used for incubation in the present study). The values for rhesus monkey compared closely with those obtained in the present study for baboons and the results for rat were also very similar in the two investigations. Acetylcholine synthesis in human samples (tissue obtained close to sites of epileptogenic activity) were approximately half those reported in the present paper. Both studies observed a smaller stimulation from increasing K^+ concentration with incubations of human samples than with those from the other two species.

Post-mortem changes

In contrast with the rapid post-mortem effects observed in the present investigation, studies on choline uptake (Klemm & Kuhar, 1979), amino acid and somatostatin release and metabolic activity (Dodd *et al.*, 1979) in rat brain synaptosomes prepared after extended post-mortem storage indicate at least a partial resistance of these systems to post-mortem changes. In general, in the previous studies, tissue was maintained at room temperature, or below, during the post-mortem period. The relevance of this to the human situation is questionable (Bowen *et al.*, 1976*a*), as the superficial temperature of human brain is in excess of 25°C, even at 4h after death (Perry *et al.*, 1977; Spokes & Koch, 1978). Thus, in the present study, the model chosen involved the preparation of tissue prisms from rat brains maintained for short post-mortem periods at 37°C.

Under these conditions the response of synthesis of [^{14}C]acetylcholine to increased K^+ was rapidly diminished by post-mortem delay before processing the tissue. The large changes in this value found

30min post mortem seemed not to be due to a deterioration in overall glucose metabolism in the synaptic endings, as no significant change in the $^{14}CO_2$ production was found. Furthermore, this lack of change and the normal levels of [^{14}C]acetylcholine synthesis at low K^+ concentrations indicate that the decreased response of [^{14}C]acetylcholine to high K^+ was not due to the destruction of a subpopulation of cholinergic nerve endings nor to changes in metabolic pool sizes. A decline in the production of [^{14}C]acetylcholine in the presence of 5mM-K^+ only became evident with a similar decrease in CO_2 production, consistent with the findings of other studies (Gibson *et al.*, 1975) of a close dependence of acetylcholine synthesis on energy metabolism.

Effect of age

The data for [^{14}C]acetylcholine synthesis of human samples clearly demonstrate the absence of large age-related changes and suggest that presynaptic cholinergic function is stable to at least around 70 years. Previous attempts to examine the cholinergic system in normal aging, using estimation of the synthesizing enzyme choline acetyltransferase, have produced contradictory results. Thus the present study is consistent with findings of no significant change between the ages of 20 and 95 in cortex (Spokes, 1979), and similar observations in the temporal lobe between 50 and 92 years (Bowen *et al.*, 1979) and in frontal neocortex between 50 and 93 years (Bowen *et al.*, 1976*a*; White *et al.*, 1977). By contrast, other workers (McGeer & McGeer, 1976; Perry *et al.*, 1977; Davies, 1979) have reported large unexplained decreases in neocortical choline acetyltransferase activity with age.

The lack of significant change in $^{14}CO_2$ production with age indicates that the potential for oxidative carbohydrate metabolism is maintained into senescence. Unlike acetylcholine synthesis, which is localized predominantly in the synaptic endings, glucose metabolism is associated with many parts of brain cells. Therefore, the loss of some structural integrity in the tissue prism preparation, as revealed by electron micrographs, probably produces loss of some oxidative capacity. Hence, although the results clearly indicate no major change affecting overall glucose metabolism of specific intact structures, the possibility of aging changes associated with structures damaged in processing cannot be completely eliminated. Studies showing a relationship between acetylcholine production and carbohydrate metabolism in prisms (Gibson *et al.*, 1975; Gibson & Blass, 1976*a*) and the confirmation *in vivo* of this (Gibson & Blass, 1976*b*) indicate the usefulness of measurements of glucose metabolism in prism systems.

Changes in Alzheimer's disease

The examination of biopsy samples from Alzheimer's-disease patients confirms the previous observation (Sims *et al.*, 1980) of a gross malfunction of [^{14}C]acetylcholine synthesis in this disease. This is most readily explained as reflecting a loss of function in cholinergic nerve endings. Among the alternative explanations, the possibility must be considered that the apparent decrease is due to a dilution of the label resulting from increases in intermediate pool sizes in the pathway from [U-^{14}C]glucose to [^{14}C]acetylcholine. This explanation is difficult to reconcile with other evidence of damage to the cholinergic system in Alzheimer's disease, including decreases in choline acetyltransferase activity (see Bowen, 1980, for review) and post-mortem acetylcholine content (Richter *et al.*, 1980), especially as the activity of choline acetyltransferase correlated with the acetylcholine synthesis in a series of samples from Alzheimer's-disease patients and controls (Sims *et al.*, 1980). In addition to the defect of [^{14}C]acetylcholine synthesis the larger sample size of the present study has revealed a significant increase in $^{14}CO_2$ production. A number of possible explanations may account for this observation, including a change in the size or turnover rate of associated metabolic pools (e.g. amino acids), a change in the total number of active mitochondria, the uncoupling of some mitochondria, or increased activity of an alternative pathway of glucose oxidation. Thus further work is required to determine the full importance of this finding and the relationship, if any, to the cholinergic malfunction.

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