

Effects of Arachidonic Acid on Glutamate and γ -Aminobutyric Acid Uptake in Primary Cultures of Rat Cerebral Cortical Astrocytes and Neurons

Albert C. H. Yu, Pak H. Chan, and Robert A. Fishman

Brain Edema Research Center, Department of Neurology, School of Medicine,
University of California, San Francisco, California, U.S.A.

Abstract: The effects of arachidonic acid on glutamate and γ -aminobutyric acid (GABA) uptake were studied in primary cultures of astrocytes and neurons prepared from rat cerebral cortex. The uptake rates of glutamate and GABA in astrocytic cultures were 10.4 nmol/mg protein/min and 0.125 nmol/mg protein/min, respectively. The uptake rates of glutamate and GABA in neuronal cultures were 3.37 nmol/mg protein/min and 1.53 nmol/mg protein/min. Arachidonic acid inhibited glutamate uptake in both astrocytes and neurons. The inhibitory effect was observed within 10 min of incubation with arachidonic acid and reached approximately 80% within 120 min in both types of culture. The arachidonic acid effect was not only time-dependent, but also dose-related. Arachidonic acid, at concentrations of 0.015 and 0.03 μ mol/mg protein, significantly inhibited glutamate uptake in neurons, whereas 20 times higher concentrations were required for astrocytes. The effects of arachidonic acid were not as deleterious on GABA uptake as on glutamate uptake in both astrocytes

and neurons. In astrocytes, GABA uptake was not affected by any of the doses of arachidonic acid studied (0.015–0.6 μ mol/mg protein). In neuronal cultures, GABA uptake was inhibited, but not to the same degree observed with glutamate uptake. Lower doses of arachidonic acid (0.03 and 0.015 μ mol/mg protein) did not affect neuronal GABA uptake. Other polyunsaturated fatty acids, such as docosahexaenoic acid, affected amino acid uptake in a manner similar to arachidonic acid in both astrocytes and neurons. However, saturated fatty acids, such as palmitic acid, exerted no such effect. The significance of the arachidonic acid-induced inhibition of neurotransmitter uptake in cultured brain cells in various pathological states is discussed. **Key Words:** Arachidonic acid—Glutamate— γ -Aminobutyric acid—Neurons and astrocytes in cell cultures. Yu A. C. H. et al. Effects of arachidonic acid on glutamate and γ -aminobutyric acid uptake in primary cultures of rat cerebral cortical astrocytes and neurons. *J. Neurochem.* 47, 1181–1189 (1986).

Polyunsaturated fatty acids (PUFAs), especially arachidonic acid (20:4) and docosahexaenoic acid (22:6), are rapidly released following ischemia, electroconvulsive seizures, and various pathological insults (Bazan and Tureo, 1980; Gardiner et al., 1981; Rehncrona et al., 1982; Tang and Sun, 1982; Yoshida et al., 1982). These fatty acids in vitro also are active in the induction of cellular (cytotoxic) brain edema (Chan and Fishman, 1978, 1985; Chan et al., 1983a, 1985); in vivo they cause both cellular and vasogenic edema. In brain slices, 20:4 and other PUFAs caused swelling associated with increased Na^+ and decreased K^+ content (Chan et al., 1979). Such effects may well

be attributed to alterations in cell membrane integrity and failure of Na^+ , K^+ -ATPase activity which would induce the functional changes associated with cellular edema accompanying cerebral ischemia and other pathological insults. The special vulnerability of neurons and glia in the cellular edemas also requires elucidation.

Glutamate and γ -aminobutyric acid (GABA) are known to be the major amino acid neurotransmitters in the brain (for review, see Di Chiara and Gessa, 1981). Increased release of glutamate due to enhanced neural activity is associated with a broad range of brain insults that contribute to the formation of brain

Received November 20, 1985; accepted April 15, 1986.

Address correspondence and reprint requests to Dr. P. H. Chan at Department of Neurology, M-794, University of California, San Francisco, CA 94143, U.S.A.

Abbreviations used: AIBA, α -aminoisobutyric acid; 20:4, arachidonic acid; dBcAMP, dibutyrylcyclic AMP; 22:6, docosahexaenoic

acid; FCS, fetal calf serum; GABA, γ -aminobutyric acid; GFAP, glial fibrillary acidic protein; GAD, glutamic acid decarboxylase; MEM, modified Eagle's minimum essential medium; 18:1, oleic acid; 16:0, palmitic acid; PUFAs, polyunsaturated fatty acids; TCA, tricarboxylic acid.

edema (see Benveniste et al., 1984; Hansen, 1985; Kimelberg and Ransom, 1986). Baethman et al. (1980) have shown that the perfusion of high concentrations of glutamate into the ventricular system of cats leads to cerebral edema. Using brain cortical slices, it has been demonstrated that glutamate greatly increases intracellular brain swelling (e.g., Pappius and Elliott, 1956; Banay-Schwartz et al., 1974; Chan et al., 1979), a process that may mainly involve astrocytes (Møller et al., 1974), but equimolar GABA had no effect (Chan et al., 1979). Glutamate also induced swelling in C₆ glioma cells (Kempinski et al., 1982), retinal Müller cells (Casper et al., 1982), astrocytes from various brain regions (Van Harreveld and Fikova, 1971), and astrocytes in primary culture (Kimelberg and Ransom, 1986). These observations indicated that the accumulation of extracellular glutamate has cytotoxic effects on brain cells (Olney, 1983). Malfunction of the normal uptake and/or deactivation mechanisms of glutamate would result in intrasynaptic accumulation and thereby induce cellular injury. Therefore, it is important to understand the uptake mechanisms for these amino acid neurotransmitters.

The transport and metabolism of glutamate and GABA at the cellular level in brain have recently attracted considerable interest. Several investigators have demonstrated that glutamate and GABA, after neuronal release, are removed by accumulation into the adjacent astrocytes and reuptake into neurons (McLennan, 1976; Höslí and Höslí, 1978; Hertz, 1979; Yu and Hertz, 1982; Schousboe and Hertz, 1983). A high-affinity uptake mechanism for glutamate and GABA has been demonstrated in various *in vitro* preparations of cortical tissue (for reviews, see Hertz, 1979; Schousboe and Hertz, 1983). Using brain slice and synaptosomal preparations, Chan et al. (1983b) demonstrated that 20:4 (0.5 mM) caused a significant reduction in the high-affinity uptake of GABA and glutamate. However, uptake of α -aminoisobutyric acid (AIBA), the nonmetabolized amino acid, was not affected. These data have suggested that 20:4 and glutamate may have a synergistic effect on inducing cellular edema. However, the specificity of the effect of 20:4 on the deactivation mechanism, i.e., uptake into astrocytes and reuptake into neurons, of glutamate and GABA is not clear and requires further elucidation. We now use primary cultures, composed of a highly purified cell population (Hertz, 1979; Kimelberg, 1983; Schousboe and Hertz, 1983), to study directly the effects of PUFAs on neurotransmitter uptake in astrocytes and neurons.

MATERIALS AND METHODS

Astrocyte cultures

Primary cultures of cerebral cortical astrocytes were prepared as described by Booher and Sensenbrenner (1972), Yu et al. (1982), and Hertz et al. (1985). Newborn Sprague-Dawley rats (Simonsen, Gilroy, CA, U.S.A.) were used in-

stead of mice. Cerebral hemispheres were removed aseptically from the skulls and freed of the meninges. Subsequently, the two hemispheres were split and the olfactory bulbs, basal ganglia, and hippocampal formation were removed. This left the neopallium, i.e., the portion of cortex dorsal and lateral to the lateral ventricle. The cleaned neopallia were cut into small cubes ($\approx 1 \text{ mm}^3$) in a modified Eagle's minimum essential tissue culture medium (MEM) (Hertz et al., 1985) with 20% horse serum (K. C. Biological, Lenexa, KS, U.S.A.) or fetal calf serum (FCS) (from Sterile System, Logan, UT, U.S.A.). The tissue was disrupted by vortex-mixing for 1 min as described by Bullaro and Brookman (1976) and the suspension was passed through two sterile nylon Nitex sieves (from L. and S. H. Thompson, Montreal, P.Q., Canada) with pore sizes of 80 μm (first sieving) and 10 μm (second sieving). A volume of cell suspension equivalent to one-tenth of brain was placed in a 60-mm Falcon tissue dish (Becton Dickinson, Oxnard, CA, U.S.A.). Fresh MEM supplemented with 10% FCS was added to the dish to a final volume of 3 ml. All cultures were incubated at 37°C in a 95%:5% (vol/vol) mixture of atmospheric air and carbon dioxide with 95% humidity. The culture medium was changed after 3 days of seeding and subsequently two times per week. After 2 weeks, the cultures reached confluency and were grown in the additional presence of 0.25 mM dibutyryl cyclic AMP (dBcAMP) (Sigma, St. Louis, MO, U.S.A.). The cultures were used for the study of free fatty acid effects on amino acid uptake after they were over 4 weeks old.

Neuronal cultures

Primary cultures of cerebral cortical neurons were prepared in principle as described in Yavin and Yavin (1974), Dichter (1978), Yu and Hertz (1982), Yu et al. (1984), and Hertz et al. (1985). Brain hemispheres of 16- to 17-day-old Sprague-Dawley rat embryos were used. The neopallium, freed of meninges, was carefully dissected and cut into small cubes ($\approx 1 \text{ mm}^3$) and trypsinized for 2–3 min using 0.2% trypsin 1:250 (Gibco) in Puck's saline at room temperature. After the addition of 10% horse serum to inhibit the trypsin, the tissue was triturated with a 10-ml pipette. The resulting cell suspension was centrifuged for 5–6 min at 900 g. The pellet was suspended in serum-free MEM with a glucose concentration of 30 mM instead of 7.5 mM and the suspension was filtered through a Nitex mesh with a pore size of 80 μm . An amount of cell suspension corresponding to one brain per three dishes was seeded in 60-mm Falcon tissues dishes, which had been coated with D-polylysine by overnight exposure to 12.5 $\mu\text{g}/\text{ml}$ of D-polylysine in water (Yu et al., 1984). After 15–20 min of incubation at 37°C, unattached cells (mostly nonneuronal cells) were removed together with the medium, which was replaced with glucose-enriched MEM plus 5% horse serum (K. C. Biologicals, Lenexa, KS, U.S.A.). The cultures were incubated at 37°C in 95%:5% (vol/vol) mixture of atmospheric air and carbon dioxide (95% humidity). After 4–5 days of culturing, 40 μM cytosine arabinoside was added to the cultures to curtail astrocytic growth (Dichter, 1978). Twenty-four hours later, the cultures were refed with fresh medium without the cytotoxic agent and used for biochemical studies between the ages of 12 and 14 days.

Uptake studies

Uptakes of glutamate and GABA were determined as previously described (Yu and Hertz, 1982; Yu et al., 1984). For

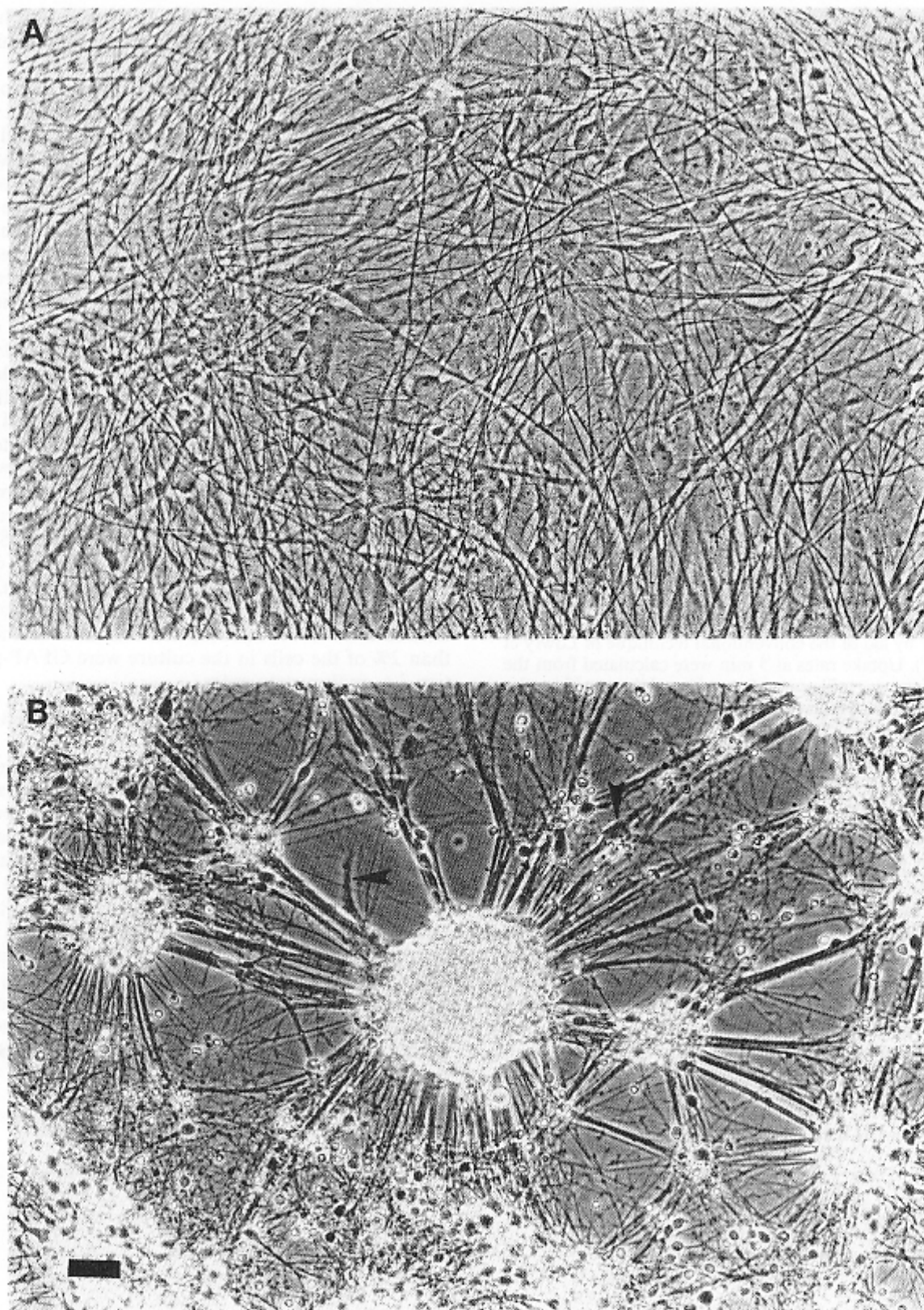


FIG. 1. Phase-contrast micrographs of primary cultures. **A:** Astrocytes obtained from newborn rats and grown for 32 days in culture. **B:** Cerebral cortical neurons obtained from cerebral hemispheres of 17-day-old rat embryos and grown for 14 days in culture with cytosine arabinoside treatment between days 4 and 5. Arrows indicate contaminating astrocytes. The magnification is the same for the two figures and bar equals 40 μm .

measurement of the effects of various fatty acids on the uptake of these neurotransmitters, cultures were washed two times with 37°C serum-free modified MEM, then preincubated in a similar MEM containing the desired amount of fatty acid, i.e., 3 ml of the desired millimolar of the fatty acid per culture which contained 400–500 µg protein in the case of astrocytes and 800–1,000 µg protein in the case of neurons. The time of preincubation varied from 10 to 120 min. Corresponding control cultures were incubated for the same time period without exposure to the fatty acid. Since glutamine in MEM may partly hydrolyze to glutamate during medium storage, glutamine was added immediately before the use of the medium. At the end of the preincubation period, either [U-¹⁴C]glutamate or [U-¹⁴C]GABA (Amersham, Arlington Heights, IL, U.S.A.) was added directly to the culture. The final concentration of the amino acid was 50 µM with a radioactivity of 0.1 µCi/ml. The uptake incubation lasted for exactly 5 min, a time short enough to ensure that the uptakes occurred at close to their initial rates (Hertz et al., 1978; Yu and Hertz, 1982). Another advantage of the short uptake incubation period is that it minimizes the loss of accumulated amino acid as carbon dioxide, a metabolic process that may be quite pronounced in the case of glutamate (Yu et al., 1982; Hertz et al., 1983; Yu and Hertz, 1983). After the preincubation period, the cultures were rapidly washed twice with ice-cold MEM. One milliliter of 1 M sodium hydroxide was added and radioactivity and protein were determined in the dissolved cultures, the former using a Beckman LS7000 scintillation counter and the latter by aid of the conventional technique of Lowry et al. (1951). Uptake rates at 5 min were calculated from the radioactivity per milligram of protein and the specific activity in the incubation media (Yu and Hertz, 1982; Yu et al., 1984).

RESULTS

Culture morphology

Figure 1A shows the rat cerebral cortical astrocytes in culture for 32 days. These cultured cells show a development similar to that reported by Kimelberg (1983) and Hertz et al. (1985). The cells responded to dBcAMP by losing their epithelial-like structure and developing processes stretching in multiple directions (Fig. 1A). The staining of glial fibrillary acidic protein (GFAP) with anti-GFAP serum demonstrated that >90% of the cultured cells were GFAP-positive (Yu, Chan, and Fishman, unpublished data). The cultures

were viable in vitro for at least 6 months, and had a stable protein content of 400–500 µg/60-mm culture dish, after 3 weeks of culturing.

Figure 1B shows the rat cerebral cortical neurons at 14 days in culture. The cells grown in the culture were those that had attached to the polylysine-coated surface before the first change of medium (refer to Materials and Methods). The developmental differentiation of the cells in the culture is very similar to that reported with mouse cells (Yu et al., 1984). The culture consisted mainly of neuronal-like cells linked together by a dense network of processes. At an early age, most cells appeared as single cells, but some aggregation occurred, with the cells forming small clusters as the culture aged (Fig. 1B). High glutamate decarboxylase (GAD) level and high potassium-induced release of GABA were observed in these rat cultures (Yu, Chan, and Fishman, unpublished data). These are two of the many markers used to demonstrate the GABAergic characteristics of the neurons (review, Hertz et al., 1985). These cultures could be maintained in vitro between 17 and 21 days. The protein content was 800–1,000 µg/60-mm culture dish. Some glial cells were found in some cultures even after cytosine arabinoside treatment (arrows in Fig. 1B), but their presence did not affect the interpretation of the data because GFAP staining demonstrated that fewer than 2% of the cells in the culture were GFAP-positive, i.e., mature astrocytes.

Effect of arachidonic acid on glutamate and GABA uptake in astrocytes

Uptake of glutamate and GABA was measured in primary cultures of rat astrocytes. The concentration of glutamate or GABA in the incubation medium was 50 µM, i.e., a concentration well above the Michaelis-Menten constant (K_m) in both astrocytes and neurons (Hertz et al., 1978; Yu and Hertz, 1982), and also higher than the extracellular concentrations of those amino acids in the brain (Hamberger et al., 1983). Under this condition, the uptake rate of glutamate in astrocytes was 10.4 ± 2.4 (SEM; $n = 65$) nmol/mg protein/min, a rate that is comparable to that reported in mouse astrocytes (Hertz et al., 1978). The rate for GABA uptake was found to be 0.125 ± 0.006 (SEM; $n = 20$) nmol/mg protein/min.

TABLE 1. Changes in the uptake rate of glutamate in primary culture of rat cerebral cortical astrocytes after 30 and 90 min exposure to various concentrations of 20:4

Time of exposure (min)	Control (0 mM 20:4)	Concentrations of 20:4 (mM)				
		0.005	0.01	0.025	0.05	0.1
30	100 ± 5.1	113 ± 6.8	102 ± 3.8	89 ± 8.3	79 ± 7.1 ^a	51 ± 2.7 ^c
90	100 ± 2.2	98 ± 8.5	85 ± 5.4	88 ± 1.3 ^b	55 ± 4.6 ^c	24 ± 4.6 ^c

Data are expressed in percentage of the uptake rate in control cultures ± SEM. Results are averages of four to six experiments independent from the data shown in Figure 2. Rate of glutamate uptake (5 min) in control cultures = 10.4 nmol/mg protein/min.

^a $p < 0.025$; ^b $p < 0.005$; ^c $p < 0.0005$, compared to control, using Student's *t* test for statistical analysis.

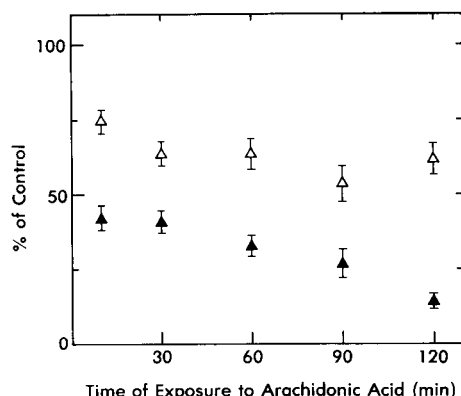


FIG. 2. Rates of uptake of [U - ^{14}C]glutamate, expressed as percentages of control values, in primary cultures of astrocytes as a function of exposure time to 0.05 mM (Δ) and 0.1 mM (\blacksquare) 20:4. The uptakes were measured at 5 min. The concentration of glutamate was 50 μ M. Results are means of 7–14 experiments and SEM values are shown by vertical bars.

Table 1 shows the changes in the 5-min uptake rate of glutamate in astrocytes after 30 and 90 min exposure to various concentrations of 20:4. Data were expressed as a percentage of the uptake rate in the corresponding control cultures. At concentrations of 0.05 and 0.1 mM, 20:4 (i.e., equivalent to 0.3 and 0.6 μ mol/mg protein) significantly inhibited the uptake of glutamate ($p < 0.005$) at 90 min. Doses below 0.025 mM (i.e., equivalent to 0.15 μ mol/mg protein) did not exert a significant effect. At 30 min exposure, the effect on glutamate uptake caused by 0.1 mM 20:4 was significantly stronger than that caused by 0.05 mM, $p < 0.005$. At 90 min exposure, the uptake of glutamate in astrocytes inhibited by 0.1, 0.05, and 0.025 mM was 76%, 45%, and 18%, respectively, indicating the inhibition caused by 20:4 was dose-related.

Figure 2 shows a time-course study of the changes in glutamate uptake in astrocytes as a function of time of exposure to 0.05 and 0.1 mM 20:4. At 0.05 mM,

20:4 caused a 25% inhibition of glutamate uptake within the first 10 min of exposure and reached a maximum of 50% after 90 min. At 0.1 mM, 20:4 inhibited the uptake by 50% within 10 min of exposure and reached almost 85% at 120 min.

Similar experiments were done with GABA uptake in the cultures. Results (data not shown) indicated that GABA uptake in astrocytes was not inhibited by doses of 20:4 up to 0.1 mM even after 2 h of incubation.

Effects of 20:4 on uptake of glutamate and GABA in neurons

The uptake of glutamate and GABA was measured in rat cerebral cortical neurons in culture. The rates were 3.37 ± 0.427 ($n = 24$) nmol/mg protein/min and 1.53 ± 0.049 ($n = 20$) nmol/mg protein/min, respectively. These rates were averaged from the control cultures which were compared with cultures preincubated with fatty acid.

Table 2 shows that the uptake of glutamate was inhibited by all the doses of 20:4 studied after exposure for both 30 and 90 min. The degree of inhibition was clearly dose-related. At both time periods, the inhibition of glutamate uptake increased as the concentrations increased and each increment was statistically significant from the previous concentration, with $p < 0.0005$. 20:4 also inhibited GABA uptake, but to a lesser extent than glutamate uptake. Lower doses of 20:4 (0.01, 0.05 mM; i.e., equivalent to a 0.03, 0.15 μ mol/mg protein; note: neuronal culture protein content is two times higher than that of astrocyte culture) did not affect GABA uptake at either 30 or 90 min. Only with 0.1 mM (i.e., equivalent to 0.3 μ mol/mg protein) was the GABA uptake significantly reduced.

Figure 3 shows the effects of 0.1 mM 20:4 on glutamate and GABA uptake in neurons as a function of time. This time-course study was done to obtain a clear comparison between the sensitivity of the glutamate and GABA uptakes at a similar concentration

TABLE 2. Changes in the uptake rate of [U - ^{14}C]glutamate and [U - ^{14}C]GABA in 14-day-old primary cultures of rat cerebral cortical neurons after 30 and 90 min exposure to various concentrations of 20:4

Time of exposure (min)	Control (0 mM 20:4)	Concentrations of 20:4 (mM)			
		0.005	0.01	0.05	0.1
Glutamate					
30	100 ± 1.8	89 ± 2.2 ^b	83 ± 3.8 ^d	52 ± 3.7 ^e	37 ± 2.0 ^e
90	100 ± 1.8	76 ± 1.9 ^e	88 ± 3.9 ^e	45 ± 2.9 ^e	23 ± 2.1 ^e
GABA					
30	100 ± 6.4	97 ± 1.9 ^a	91 ± 5.4	92 ± 3.2 ^a	88 ± 3.6 ^c
90	100 ± 7.8	86 ± 7.0 ^a	95 ± 4.7 ^a	77 ± 1.7 ^c	50 ± 3.5 ^e

Values are in percentage of the control uptake rate \pm SEM. Results are averages of 6–15 experiments. Rate of glutamate uptake (5 min) in control cultures = 3.4 nmol/mg protein/min. Rate of GABA uptake (5 min) in control cultures = 1.5 nmol/mg protein/min.

^a Not significant; ^b $p < 0.05$; ^c $p < 0.01$; ^d $p < 0.005$; ^e $p < 0.0005$, compared to control group, using Student's t test for paired statistical analysis.

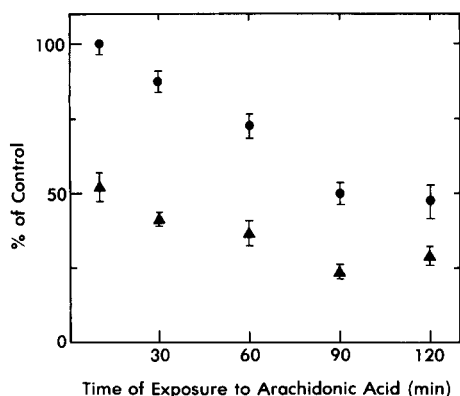


FIG. 3. Rates of uptake of [U-¹⁴C]glutamate (▲) and [U-¹⁴C]GABA (●) as percentages of control values, in primary cultures of cerebral cortical neurons as a function of time of exposure to 20:4 (0.1 mM). The concentrations of both amino acids were 50 μM. The uptakes were measured at 5 min. Results are means of 6–15 experiments; vertical bars indicate SEM.

of 20:4. Although Table 2 shows that lower doses of 20:4 inhibit glutamate uptake, 0.1 mM was chosen because lower doses did not demonstrate a distinct inhibition of GABA uptake (Table 2). Results indicated, as in astrocytes, that glutamate uptake was very sensitive to 20:4 and was inhibited by almost 50% after 10 min of exposure. The inhibitory effect also increased with time of incubation and reached over 70% at 90 min. GABA uptake did not respond as did glutamate uptake to 0.1 mM of 20:4. No inhibition of GABA uptake was seen in the first 10 min of exposure. However, a slight inhibition was shown at 30 min and thereafter, until it reached a maximum inhibition of 50% at 90 min.

Effects of other fatty acids on glutamate uptake in astrocytes

As 20:4 exerted no inhibition of GABA uptake in astrocytes, only the effects of fatty acids on glutamate uptake were studied. Results in Table 3 showed that 0.05 mM (i.e., equivalent to 0.3 μmol/mg protein) palmitic acid (16:0), at both 30 and 90 min exposure, did not affect the uptake of glutamate. Oleic acid (18:1) caused a slight but significant ($p < 0.05$) inhibition at both incubation periods. This effect was less

than that of 20:4 and was not increased with time, as the inhibition at 30 min was not significantly different from that at 90 min. At a concentration of 0.05 mM, 22:6 (i.e., equivalent to 0.3 μmol/mg protein) significantly inhibited the glutamate uptake at both 30 and 90 min of exposure. The effect on the glutamate uptake seemed to be stronger than that of 20:4 at similar concentration. Time-course studies of 22:6 (results not shown) also demonstrated a rapid inhibitory effect of this fatty acid on the glutamate uptake. None of the fatty acids studied had an observable effect on the GABA uptake in astrocytes.

Effects of fatty acids on glutamate and GABA uptake in neurons

The effects of 0.1 mM (i.e., equivalent to 0.3 μmol/mg protein) of 16:0, 18:1, and 22:6 on glutamate and GABA uptake in cerebral cortical neurons were measured (Table 4). A concentration of 0.1 mM fatty acid was chosen for this study because it caused a major inhibition of GABA uptake (refer to Table 2). Only data from the 90-min exposure of the neurons to the fatty acids were shown because this period was long enough to observe the inhibitory effect.

16:0 inhibited neither GABA nor glutamate uptake in neurons, as had been seen in astrocytic cultures. 18:1 affected glutamate uptake to the same degree as 20:4. The effect of 22:6 was greater than that of 20:4. 18:1 had a weaker effect on GABA uptake in neurons than did 20:4 or 22:6. However, the effect was significant, $p < 0.05$. Again, 22:6 exerted a greater inhibition on GABA uptake than a similar concentration of 20:4.

DISCUSSION

The morphology and biochemistry of cells in culture from rat cerebral cortex were very similar to those prepared from mouse (Schousboe and Hertz, 1983; Yu et al., 1984; Hertz et al., 1985) and rat (e.g., Kimmelberg, 1983). As shown in astrocytic cultures, over 90% of the cells were GFAP-positive. As in neuronal cultures, there was high GAD activity and K⁺-induced Ca²⁺-dependent release of GABA. Astrocytes and neurons accumulate glutamate very rapidly, thereby indicating the importance of this mechanism

TABLE 3. Effects of fatty acids on the uptake of [U-¹⁴C]glutamate in primary cultures of rat astrocytes

Time of exposure (min)	Fatty acids (0.05 mM)				
	Control	16:0	18:1	20:4	22:6
30	100 ± 5.3	99 ± 15.9 ^a	73 ± 0.43 ^b	63 ± 4.5 ^c	59 ± 1.2 ^d
90	100 ± 3.5	122 ± 4.3 ^a	74 ± 0.48 ^b	53 ± 6.2 ^d	33 ± 5.2 ^d

Controls were cultures without exposure to any fatty acids. Values are in percentage of the uptake rate of the control ± SEM. Data for 20:4 are from experiments independent from those shown on Table 1 and Fig. 2. For rate of uptake of glutamate, refer to Table 1. Results are averages of three to seven experiments.

^a Not significant; ^b $p < 0.05$; ^c $p < 0.25$; ^d $p < 0.0005$, using Student's *t* test for statistical analysis.

TABLE 4. Effects of fatty acids (90 min exposure) on the uptake of [U - 14 C]glutamate and [U - 14 C]GABA in primary cultures of rat cerebral cortical neurons

	Control	Fatty acids (0.1 mM)			
		16:0	18:1	20:4	22:6
Glutamate	100 \pm 2.0	93 \pm 17.3 ^a	26 \pm 6.6 ^c	29 \pm 0.7 ^c	6 \pm 0.5 ^c
GABA	100 \pm 5.7	112 \pm 3.3 ^a	81 \pm 4.9 ^b	47 \pm 1.4 ^c	24 \pm 3.5 ^c

Controls were cultures without exposure to any fatty acids. Values are in percentage of the uptake rate of the control cultures \pm SEM. Concentrations of glutamate and GABA were 50 μ M. Results are from three to six experiments independent from data shown in Fig. 2 and Table 2.

^a Not significant; ^b $p < 0.05$; ^c $p < 0.0005$, using Student's t test for statistical analysis.

in removing extracellular glutamate which is known to cause neural damage at high concentrations (Hertz, 1979). Astrocytes take up glutamate at a rate that is approximately a hundred times higher than GABA uptake; this supports the hypothesis that astrocytes are more involved in deactivating glutamate than GABA (Hertz, 1979). On the other hand, neurons take up more GABA than astrocytes which agrees with the observation described by Schousboe and Hertz (1983).

In brain slices, 20:4 and other PUFAs induced cell membrane perturbation and caused a reduction in the uptake of neurotransmitter glutamate and GABA as well as a reduction of Na^+ , K^+ -ATPase activity (Chan et al., 1983b). These events lead to a shift in cations and water and ultimately development of cellular edema. Similarly, the inhibition of glutamate uptake observed now in primary cultures of astrocytes and neurons by PUFAs supports the observations made in brain slices. It also demonstrated that the 20:4 effect on the inhibition of glutamate uptake involved neurons and astrocytes. The lack of an effect of 20:4 on GABA uptake in astrocytes but not in neurons indicates that the inhibition of GABA uptake observed in brain slices might be a neuronal phenomenon. These findings are in agreement with the observation in synaptosomal preparations that the Na^+ -dependent synaptosomal amino acid uptake system is specifically inhibited by low concentrations of unsaturated but not saturated fatty acid (Rhoads et al., 1982, 1983; Chan et al., 1983b). Our data further indicate that neurons are more sensitive to the deleterious effects of 20:4 than astrocytes. Concentrations of 0.005 mM 20:4 caused no inhibition of glutamate uptake in astrocytic cultures, but significantly inhibited glutamate uptake in neurons (Tables 1 and 2). This observation may, in part, be due to the difference in the membrane properties of neurons and astrocytes, thus probably accounting for the vulnerability of neurons to various kinds of insults, such as convulsive seizures, spreading depression, and hypoxia, where PUFAs are released and excessive amounts of the glutamate are accumulated (e.g., Benveniste et al., 1984). The lack of inhibition of GABA uptake in astrocytes exposed to a high concentration of 0.1 mM 20:4 may also be explained by

a lack of sensitivity of the cells to 20:4. It is possible that the effect of 20:4 on functional integrity of cell membranes is very specific. This hypothesis is further supported by the lack of inhibition of AIBA uptake in cultures of astrocytes (data not shown). Moreover, the inhibition of neuronal GABA uptake may be a unique property of GABAergic neurons. Further studies with higher concentrations of 20:4 on astrocytic cultures were not pursued because GABA, unlike glutamate, does not cause cell swelling (Chan et al., 1979; Bourke et al., 1983).

Uptake of glutamate and GABA was affected only by PUFAs, especially 20:4 and 22:6, but not by saturated fatty acids such as 16:0. The effect of 22:6, at a similar concentration, was always greater than that of 20:4. This finding agrees with those observed in brain slices (Chan et al., 1983a,b) and also reconfirms that PUFAs play a role in the neurotransmitter uptake in brain cells. Furthermore, it implies that the degree of unsaturation of fatty acids may be important for the inhibition.

The effect of PUFAs on neurotransmitter uptake was very rapid and also dose-dependent. It has been shown in vivo that during ischemia the level of 20:4 in brain increased and reached a level of 5 nmol/mg protein (Yoshida et al., 1982; Rehncrona et al., 1982). This concentration of 20:4 was estimated without consideration of the possibility of compartmentation of 20:4 which exists in situ. During pathological insults, the concentration of 20:4 together with that of other PUFAs in the extracellular space or in certain subcellular structures (e.g., synaptosomes) would be much higher than this reported value. Concentrations of 20:4 used in this study (0.015–0.6 μ mol/mg protein) were 3–120 times higher than the concentrations observed in the in vivo pathological conditions. However, the concentration is underestimated since other PUFAs are also involved.

As 20:4 affects Na^+ , K^+ -ATPase (Chan et al., 1983b), the inhibition of amino acid uptake may be a secondary effect related to the failure of energy metabolism and/or ion transport (Erecińska et al., 1984). The inhibition may also involve the intermediate metabolites of 20:4 and free radicals. It has been shown that PUFAs caused a transient formation of superox-

ide radicals and lipid peroxides in brain slices (Chan and Fishman, 1985). Similar observations were made in cultured cells (Chan et al., 1986). Furthermore, it has also been shown in cultured astrocytes using hyperbaric oxygen as a source of oxygen free radicals that glutamate uptake was significantly inhibited (Yu et al., 1985).

PUFA inhibited glutamate uptake would lead to extracellular accumulation of this excitotoxic compound. The increase in extracellular glutamate under anoxia observed by Benveniste et al. (1984) may be partly due to the deficiency in the uptake system. It has been shown that a high concentration of extracellular glutamate can induce depolarization of astrocytes (Bowman and Kimelberg, 1984). This mechanism would also open up ion channels which leads to an uptake of water and astrocytic swelling (Kimelberg and Ransom, 1986). Recent studies by Simon et al. (1984) and Rothman (1985) have shown that excitatory amino acid plays an important role in neuronal cell death of hippocampus. The toxic effects caused by failure of the glutamate uptake system may also be metabolically related. Astrocytes accumulate a major part of extracellular glutamate (Hertz, 1979; Hertz et al., 1983) and convert it to α -ketoglutarate and subsequently to carbon dioxide and succinyl CoA as metabolic substrate (Yu et al., 1982; Hertz et al., 1983; Yu and Hertz, 1983). Neurons take up extracellular glutamate as one way to replenish the loss of this compound during neurotransmission (Yu, 1980). Therefore, the inhibition of glutamate uptake induced by PUFAs in astrocytes and neurons would cause a deficiency in the supply of metabolic fuel to astrocytes in the form of glutamate, and would also be detrimental to the replenishing mechanisms in neurons.

In summary, PUFAs affect glutamate uptake in both astrocytes and neurons. Neurons in cultures were more sensitive to PUFAs than astrocytes in terms of amino acid uptake. The inhibitory effect of PUFAs on the amino acid uptake was quite specific, as only glutamate uptake was severely inhibited. This observation suggests the inhibition was not due to a general alteration in membrane integrity caused by 20:4. Its relationship to the effect of 20:4 on free radical formation and calcium uptake alteration needs to be clarified.

Acknowledgment: We thank Dr. Larry Eng for the supply of the anti-GFAP serum. We also thank Dr. Sylvia F. Chen, Ms. Susan M. Longar, and Ms. Sheryl Colwell for help in preparation of the manuscript. This work was supported by NIH Grant NS-14543.

REFERENCES

- Baethmann A., Oettinger W., Rothenfuber W., Kempfski O., Unterberg A., and Geiger R. (1980) Brain edema factors: current state with particular reference to plasma constituents and glutamate, in *Advances in Neurology*, Vol. 28: *Brain Edema: Pathology, Diagnosis, and Therapy* (Cervós-Navarro J. and Fersat R., eds), pp. 171–195. Raven Press, New York.
- Banay-Schwartz M., Gergeley A., and Lajtha A. (1974) Independence of amino acid uptake from tissue swelling in incubated slices of brain. *Brain Res.* **65**, 265–276.
- Bazan N. G. and Tureo E. B. R. (1980) Membrane lipids in the pathogenesis of brain edema—phospholipids and arachidonic acid, the earliest membrane components changed at the onset of ischemia, in *Advances in Neurology*, Vol. 28: *Brain Edema: Pathology, Diagnosis, and Therapy* (Cervós-Navarro J. and Fersat R., eds), pp. 197–205. Raven Press, New York.
- Benveniste H., Drejer J., Schousboe A., and Diemer N. H. (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* **43**, 1369–1374.
- Booher H. and Sensenbrenner M. (1972) Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and brain in flask cultures. *Neurobiology* **2**, 97–105.
- Bourke R. S., Kimelberg H. K., Daze M., and Church G. (1983) Swelling and ion uptake in cat cerebrocortical slices: control by neurotransmitters and ion transport mechanisms. *Neurochem. Res.* **8**, 5–24.
- Bowman C. L. and Kimelberg H. K. (1984) Excitatory amino acids directly depolarize rat brain astrocytes in primary culture. *Nature* **311**, 656–659.
- Bullaro J. C. and Brookman D. H. (1976) Comparison of skeletal muscle monolayer cultures initiated with cells dissociated by the vortex and trypsin. *In Vitro* **12**, 564–570.
- Casper D. S., Trelstad R. L., and Reif-Lehrer L. (1982) Glutamate-induced cellular injury in isolated chick embryo retina: Müller cell localization of initial effects. *J. Comp. Neurol.* **209**, 79–90.
- Chan P. H. and Fishman R. A. (1978) Brain edema: induction in cortical slices by polyunsaturated fatty acids. *Science* **201**, 358–360.
- Chan P. H. and Fishman R. A. (1985) Brain edema, in *Handbook of Neurochemistry* (Lajtha A., ed), pp. 153–174. Plenum Press, New York.
- Chan P. H., Fishman R. A., Lee J. L., and Candelise L. (1979) Effects of excitatory neurotransmitter amino acids on swelling of rat brain cortical slices. *J. Neurochem.* **33**, 1309–1315.
- Chan P. H., Fishman R. A., Caronna J., Schmidley J. W., Prioleau G., and Lee J. (1983a) Induction of brain edema following intracerebral injection of arachidonic acid. *Ann. Neurol.* **13**, 625–632.
- Chan P. H., Kerlan R., and Fishman R. A. (1983b) Reductions of γ -aminobutyric acid and glutamate uptake and ($\text{Na}^+ + \text{K}^+$)-ATPase activity in brain slices and synaptosomes by arachidonic acid. *J. Neurochem.* **40**, 309–316.
- Chan P. H., Fishman R. A., Longar S., Chen S., and Yu A. (1985) Cellular and molecular effects of polyunsaturated fatty acids in brain ischemia and injury, in *Progress in Brain Research*, Vol. 63 (Kogure K., Hossmann K.-A., Siesjö B. K., and Welsh F. A., eds), pp. 227–235. Elsevier Science Publishers B. V. (Biomedical Division), Amsterdam.
- Chan P. H., Chen S., Yu A. C. H., and Fishman R. A. (1986) Super-oxide formation induced by arachidonic acid in astrocytes, in *Abstracts of the 17th Meeting of the American Society for Neurochemistry*, p. 281.
- Di Chiara G. and Gessa G. L., eds (1981) *Advances in Biochemical Psychopharmacology*, Vol. 27: *Glutamate as a Neurotransmitter*. Raven Press, New York.
- Dichter M. A. (1978) Rat cortical neurons in cell culture: culture methods, cell morphology, electrophysiology and synapse formation. *Brain Res.* **149**, 279–293.
- Erecińska M., Nelson D., Wilson D., and Silver I. A. (1984) Neurotransmitter amino acids in the CNS. I. Regional changes in amino acid levels in rat brain during ischemia and reperfusion. *Brain Res.* **304**, 9–22.
- Gardiner M., Nilsson B., Rehnström S., and Siesjö B. K. (1981) Free fatty acids in the rat brain in moderate and severe hypoxia. *J. Neurochem.* **36**, 1500–1505.

- Hamberger A., Berthold C. H., Karlsson B., Lahmann A., and Nystrom B. (1983) Extracellular GABA, glutamate and glutamine measured *in vivo*—perfusion-dialysates from the rabbit hippocampus, in *Glutamine, Glutamate and GABA in the Central Nervous System* (Hertz L., Kvamme E., McGeer E. G., and Schousboe A., eds), pp. 473–492. Alan R. Liss, New York.
- Hansen A. J. (1985) Effect of anoxia on ion distribution in the brain. *Physiol. Rev.* **65**, 101–148.
- Hertz L. (1979) Functional interactions between neurons and astrocytes I. Turnover and metabolism of putative amino acid transmitters. *Prog. Neurobiol.* **13**, 277–323.
- Hertz L., Schousboe A., Boechler N., Mukerji S., and Federoff S. (1978) Kinetic characteristics of the glutamate uptake into normal astrocytes in culture. *Neurochem. Res.* **3**, 1–14.
- Hertz L., Yu A. C. H., Potter P. L., Fisher T. E., and Schousboe A. (1983) Metabolic fluxes from glutamate and towards glutamate in neurons and astrocytes in primary cultures, in *Glutamine, Glutamate and GABA in Central Nervous System* (Hertz L., Kvamme E., McGeer E. G., and Schousboe A., eds), pp. 327–342. Alan R. Liss, New York.
- Hertz L., Juurlink B. H. J., and Szuchet S. (1985) Cell cultures, in *Handbook of Neurochemistry. Vol. 8*, 2nd edit. (Lajtha A., ed), pp. 603–661. Plenum Press, New York.
- Höslí L. and Höslí E. (1978) Action and uptake of neurotransmitters in CNS tissue culture. *Rev. Physiol. Biochem. Pharmacol.* **81**, 135–188.
- Kempski O., Gross U., and Baethmann A. (1982) An *in vitro* model of cytotoxic brain edema: cell volume and metabolism of cultivated glial- and nerve-cells, in *Advances in Neurosurgery* (Driesen W., Brock M., and Klinger M. eds), pp. 254–258. Springer-Verlag, New York.
- Kimelberg H. K. (1983) Primary astrocyte cultures—a key to astrocyte function. *Cell. Mol. Neurobiol.* **3**, 1–16.
- Kimelberg H. K. and Ransom B. R. (1986) Physiological and pathological aspects of astrocytic swelling, in *Astrocytes* (Federoff S. and Vernadakis A., eds). Academic Press, New York (in press).
- Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951) Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
- McLennan H. (1976) The autoradiographic localization of L-[³H]-glutamate in rat brain tissue. *Brain Res.* **115**, 139–144.
- Møller M., Lund-Andersen H., Møllgård K., and Hertz L. (1974) Concordance between morphological and biochemical estimates of fluid spaces in rat brain cortex slices. *Exp. Brain Res.* **22**, 299–314.
- Olney J. W. (1983) Excitotoxins: an overview, in *Excitotoxins* (Fuxe K., Roberts P., and Schwarcz R., eds), pp. 82–96. Macmillan, London.
- Pappius H. and Elliott K. A. C. (1956) Water distribution in incubated slices of brain and other tissues. *Can. J. Biochem. Physiol.* **34**, 1007–1022.
- Rehncrona S., Westerberg E., Akesson B., and Siesjö B. K. (1982) Brain cortical fatty acids and phospholipids during and following complete and severe incomplete ischemia. *J. Neurochem.* **38**, 84–93.
- Rhoads D. E., Kaplan M. A., Peterson N. A., and Raghupathy E. (1982) Effects of free fatty acids on synaptosomal amino acid uptake systems. *J. Neurochem.* **38**, 1255–1260.
- Rhoads D. E., Osburn L. D., Peterson N. A., and Raghupathy E. (1983) Release of neurotransmitter amino acids from synaptosomes: enhancement of calcium-independent efflux by oleic and arachidonic acids. *J. Neurochem.* **41**, 531–537.
- Rothman S. M. (1985) The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J. Neurosci.* **5**, 1483–1489.
- Schousboe A. and Hertz L. (1983) Regulation of glutamatergic and GABAergic neuronal activity by astroglial cells, in *Dales' Principle and Communication Between Neurons* (Osborne N. N., ed), pp. 131–141. Pergamon Press, Oxford.
- Simon R. P., Swan J. H., Griffiths T., and Meldrum B. S. (1984) Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* **226**, 850–852.
- Tang W. and Sun G. Y. (1982) Factors affecting the free fatty acids in rat brain cortex. *Neurochem. Int.* **4**, 269–273.
- Van Harreveld A. and Fikova E. (1971) Light- and electron-microscopic changes in central nervous tissue after electrophoretic injection of glutamate. *Exp. Mol. Pathol.* **15**, 61–81.
- Yavin E. and Yavin Z. (1974) Attachment and culture of dissociated cells from rat embryo cerebral hemispheres on polylysine coated surface. *J. Cell Biol.* **62**, 540–546.
- Yoshida A., Abe K., Busto R., Watson B. D., Kogure K., and Ginsberg M. D. (1982) Influence of transient ischemia on lipid-soluble antioxidants, free fatty acids and energy metabolites in rat brain. *Brain Res.* **245**, 307–316.
- Yu A. C. H. (1980) Uptake of glutamine and glutamate in cultured neurons. M. Sc. Thesis, University of Saskatchewan, Saskatoon, Saskatoon, Canada.
- Yu A. C. H. and Hertz L. (1982) Uptake of glutamate, GABA and glutamine into a predominantly GABAergic and a predominantly glutamatergic nerve cell population in culture. *J. Neurosci. Res.* **7**, 23–35.
- Yu A. C. H. and Hertz L. (1983) Metabolic sources of energy in astrocytes, in *Glutamine, Glutamate and GABA in the Central Nervous System* (Hertz L., Kvamme E., McGeer E. G., and Schousboe A., eds), pp. 431–439. Alan R. Liss, New York.
- Yu A. C. H., Schousboe A., and Hertz L. (1982) Metabolic fate of ¹⁴C-labeled glutamate in astrocytes in primary cultures. *J. Neurochem.* **39**, 954–960.
- Yu A. C. H., Hertz E., and Hertz L. (1984) Alteration in uptake and release rates for GABA, glutamate, and glutamine during biochemical maturation of highly purified cultures of cerebral cortical neurons, a GABAergic preparation. *J. Neurochem.* **42**, 951–960.
- Yu A. C. H., Chan P. H., Fishman R. A., and Chen S. F. (1985) Arachidonic acid effect on amino acid uptake in cultured brain cells, in *Abstracts of the 16th Meeting of the American Society for Neurochemistry*, p. 197.