Metabolism of Polyamines by Cultured Glioma Cells

EFFECT OF ASPARAGINE ON γ -AMINOBUTYRIC ACID CONCENTRATIONS

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The activity of ornithine decarboxylase (EC 4.1.1.17) increased in confluent cultures of glioma C₆BU-1 cells 3 h after adding a complete serum-containing medium, and was maximal 5 h later. The activity of *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) increased soon after addition of the complete medium to the cells, and reached its peak after 11 h. The activity of diamine oxidase (EC 1.4.3.6) also increased soon after adding complete medium and was maximal 8 h later, when the activity of ornithine decarboxylase reached its peak. The increase in the activity of *S*-adenosyl-L-methionine decarboxylase was accompanied by changes in cellular spermidine and spermine concentrations, whereas the increase in the activity of diamine oxidase was followed by the accumulation of γ -aminobutyric acid, which was detected both in the cells and in the medium. Asparagine enhanced the utilization of radioactive putrescine by glioma cells suspended in buffered-salt/glucose solution and increased intracellular and extracellular γ -aminobutyric acid concentrations. Radioactive putrescine was converted into spermidine and spermine by glioma cells after addition of a serum-containing medium, but not after adding buffered-salt/glucose solutions, in the presence or absence of asparagine. The kinetics of ornithine decarboxylase 'induction' and the half-life of the enzyme differed in cells incubated with buffered asparagine solutions and serum-containing media.

The naturally occurring polyamines and the diamine putrescine have been implicated in cellular growth and differentiation (Cohen, 1971; Bachrach, 1973). It has been well-established that their cellular concentrations fluctuate during the various phases of growth and development (Russell & Durie, 1978). One explanation for the elevated putrescine concentration, observed in growth, is the increased activity of ornithine decarboxylase (EC 4.1.1.17) (Russell & Snyder, 1968), whereas the increase in polyamine concentrations is explained by the activation of *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) (Russell, 1973).

As in other systems, the concentrations of brain polyamines increase during growth and development. This has been demonstrated for the brain of the chick embryo (Caldarera *et al.*, 1969; Sobue & Nakajima, 1978), fish and mouse (Seiler, 1973), human foetus (Sturman & Gaul, 1974) and chick embryo retina (DeMello *et al.*, 1976). The catabolism of putrescine in mammalian brain has also been studied, and γ -aminobutyric acid has been identified as the product of putrescine oxidation in

the brain of the rat (Seiler *et al.*, 1971; Konishi *et al.*, 1977), mouse (Seiler & Knödgen, 1971) and fish (Seiler *et al.*, 1973). In this respect, putrescine catabolism in the brain resembles that reported for bacteria (Bachrach, 1960).

The availability of cultured neoplastic neural cells offers new possibilities for the study of the mechanism of polyamine biosynthesis and degradation by various neural cell types under defined experimental conditions. In previous studies, I reported (Bachrach, 1975) that the induction of ornithine decarboxylase in cultured neuroblastoma and glioma cells is mediated by cyclic AMP. The induction of *S*-adenosyl-L-methionine decarboxylase in cultured glioma and neuroblastoma cells has also been described (Bachrach, 1977). Chen & Canellakis (1977) demonstrated that asparagine increases the activity of ornithine decarboxylase in cultured neuroblastoma cells. Asparagine, or its metabolites, also stimulate diamine oxidase activity in various cells (Quash *et al.*, 1976). Asparagine may thus exert two opposing effects on cellular polyamine metabolism, namely, stimulation of

synthesis (by activating ornithine decarboxylase) and enhancement of degradation (by activating diamine oxidase).

The present paper deals with the effect of a serum-containing medium and of asparagine in buffered-salt/glucose solutions on the activity of the biosynthetic decarboxylases and diamine oxidase in cultured glioma cells. The metabolism of polyamines during the various stages of the growth of glioma cells is also described.

Materials and Methods

Materials

Reagents. [1,4-¹⁴C]Putrescine dihydrochloride (sp. radioactivity 59.9 Ci/mol), *S*-adenosyl-L-[carboxy-¹⁴C]methionine (sp. radioactivity 7.5 Ci/mol) and DL-[1-¹⁴C]ornithine (sp. radioactivity 59 Ci/mol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. [*Me*-³H]Thymidine (sp. radioactivity 13 Ci/mmol) was obtained from the Atomic Energy Commission, Nuclear Research Centre, Negev, Israel.

Cells. Rat glioma clone C₆BU-1 cells were grown as previously described (Bachrach, 1975) in Dulbecco's modified Eagle's minimum medium (DMEM medium; supplied by Grand Island Biological Co., Grand Island, NY, U.S.A.; catalogue no. H-21), supplemented with 5% foetal calf serum. For enzymic assays, cells were grown in 100mm plastic dishes in the presence of 10ml of medium. In the metabolic studies, the radioactive material was added to 2.0ml quantities of medium in 50mm plastic dishes. All plates were incubated in a humidified atmosphere of CO₂/air (1:19). In some experiments, buffered-salt/glucose solution [0.02% (w/v) CaCl₂; 0.04% (w/v) KCl; 0.02% (w/v) MgSO₄·7H₂O; 0.68% (w/v) NaCl; 0.22% (w/v) NaHCO₃; 0.014% (w/v) Na₂HPO₄·H₂O; 0.1% (w/v) glucose] was added to the cells. This solution was supplemented with 10mM-asparagine, where indicated.

Methods

Determination of decarboxylase activities. After removing the growth medium from the cells, they were washed with ice-cold phosphate-buffered saline solution [0.8% (w/v) NaCl; 0.02% (w/v) KCl; 0.115% (w/v) Na₂HPO₄; 0.02% (w/v) KH₂PO₄, pH 7.2]. To each plate, 1.0ml of EDTA buffer (0.025M-Tris/HCl buffer, pH 7.2, containing 2.5mM-dithiothreitol, 0.50mM-EDTA and 25μM-pyridoxal phosphate) was added. Cells were then scraped off the plates with a rubber policeman and frozen and thawed twice. After centrifugation at 4500g for 10min, 0.25ml of the supernatant fluid was incubated at 37°C with either [1-¹⁴C]ornithine (50μl, 4μCi/ml, sp. radioactivity 59 Ci/mol) or

S-adenosyl-L-[¹⁴C]methionine (50μl, 4μCi/ml, sp. radioactivity 7.5 Ci/mol) in tubes (100mm × 16mm) equipped with rubber supporting polyethylene centre wells (Kontes Glass Co., Vineland, NJ, U.S.A.). After incubation at 37°C for 45min, 0.2ml of Hyamine hydroxide (Packard Instrument Co.) was injected into each centre well and the tubes were incubated for an additional 15min. The reaction was stopped by injecting 0.2ml of 3% (v/v) HClO₄ into the reaction mixture and the tubes were agitated for 15min to release the CO₂. Centre wells were then removed, placed into scintillation vials and counted for radioactivity. Proteins were assayed by the method of Lowry *et al.* (1951).

Determination of diamine oxidase activity. The activity of this enzyme was determined by the method of Kusche *et al.* (1973) as follows. Cells from confluent cultures were scraped off the plates, suspended in 0.2M-phosphate buffer, pH 7.2, and frozen and thawed twice. Cellular material was sedimented by centrifugation at 4500g for 10min, and finally suspended in 10ml quantities of phosphate buffer. For assay of diamine oxidase activity, 0.3ml quantities of the cellular preparations were incubated at 37°C with 50μl quantities of [1,4-¹⁴C]putrescine (3μCi/ml, sp. radioactivity 60μCi/mmol). After 30min, reaction was stopped by the addition of 0.1ml of 14% (v/v) HClO₄. The oxidation products were then extracted with 3.0ml quantities of 0.35% (w/v) 2,5-diphenyloxazole (Packard) in toluene, supplemented with 0.5ml of alkaline buffer (saturated NaHCO₃ solution, brought to pH 12.2, by adding 2M-NaOH). The suspension was then shaken for 5min, centrifuged and frozen at -70°C. The liquid organic phase was then poured off and placed in a scintillation vial containing 3.0ml of toluene, supplemented with 0.35% (w/v) 2,5-diphenyloxazole. The radioactivity was finally determined in a Packard scintillation counter.

Incorporation of [³H]thymidine into cellular DNA. To confluent cultures of glioma cells, [*Me*-³H]thymidine (0.1ml, 2.5μCi, sp. radioactivity 13 Ci/mmol) was added along with 2.0ml of growth medium. After incubation at 37°C for 30min, incorporation was stopped by removing the medium. The confluent monolayers were then washed with 30ml quantities of 5% (v/v) HClO₄, which removes free radioactive thymidine from the cells adhering to the plastic dishes. Cells were dissolved in 0.5ml quantities of 1.0M-NaOH and radioactivities and protein concentrations were determined.

Separation of polyamines. Cells were scraped off the plates and suspended in water. Polyamines were then extracted with HClO₄ (final concentration 3%, v/v) dansylated and assayed by the method of Seiler & Wiechmann (1967). Portions of the dansyl derivatives were placed on silica t.l.c. plates

(Eastman Chromagram sheets) and separated by t.l.c. with ethyl acetate/cyclohexane (2:3, v/v) as solvent. The plates were then placed next to films (Kodak Noscreen) and radioactive spots were detected after exposure for 2–4 days. Radioactive materials were then scraped off the plates, placed in scintillation vials and counted for radioactivity.

The separation of γ -aminobutyric acid from the polyamines was achieved by t.l.c. of the dansyl derivatives, with the following solvents consecutively: benzene/cyclohexane/methanol (85:15:2, by vol.); diethyl ether/cyclohexane (9:10, v/v). Radioactivities on fluorescence were determined in the extracts of the various spots, as described above.

Results

Induction of decarboxylases and diamine oxidase in glioma cells

The activities of ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase increase in confluent cultures of glioma cells after adding fresh medium (containing DMEM medium and serum). It may be seen (Fig. 1a) that the activity of ornithine decarboxylase increases after a lag period of approx. 3 h and reaches its peak at 8 h after addition of medium to the cells. The increase in *S*-adenosyl-L-methionine decarboxylase follows a different time course: the activity increases slowly a short time after adding the fresh medium and is maximal after 11 h. In addition to the biosynthetic decarboxylase, a diamine oxidase is also induced in glioma cells.

Fig. 1(b) shows that the activity of this enzyme increases soon after addition of medium to the confluent cultures and after 6–8 h is three to four times higher than at zero time. Fig. 1(b) also shows that confluent glioma cultures incorporate little radioactive thymidine into cellular DNA. Incorporation increases slowly after adding fresh medium, maximal values being attained approx. 14 h after addition.

Effect of asparagine on ornithine decarboxylase activity

When confluent glioma cells are treated with asparagine in a buffered-salt/glucose solution, an increase in ornithine decarboxylase activity is observed even 1 h after incubation at 37°C (Fig. 2). This activity remains increased throughout an incubation of 20 h, unlike the activity of ornithine decarboxylase induced by complete medium. The latter increases after a lag period of 3 h and returns to basal values after approx. 15 h (Fig. 2).

A buffered-salt/glucose solution without asparagine has only a slight effect on the activity of ornithine decarboxylase: the activity increases only

Fig. 1. The activity of biosynthetic decarboxylase and diamine oxidase in glioma cells

To confluent cultures of C₆BU-1 glioma cells, fresh complete medium was added. (a) The activity of ornithine decarboxylase (●) and of *S*-adenosyl-L-methionine decarboxylase (○) was determined at various times. (b) The activity of diamine oxidase (○) and the incorporation of [³H]thymidine into cellular DNA (▲) were determined.

Fig. 2. Effect of asparagine and complete medium on the activity of ornithine decarboxylase

To confluent cultures of glioma cells were added either buffered-salt/glucose solutions (□), buffered-salt/glucose and 10mM-asparagine (Δ) or DMEM medium with 5% calf serum (●). After incubation at 37°C, the activity of ornithine decarboxylase was determined.

after incubation for approx. 6 h (Fig. 2). The activity of *S*-adenosyl-L-methionine decarboxylase is not affected by asparagine in buffered-salt/glucose solution, being 55 and 59 pmol/mg of protein at zero time and after 2–4-h incubation respectively.

Effect of asparagine on the half-life of ornithine decarboxylase

Numerous studies indicated that the half-life of ornithine decarboxylase is extremely short, ranging from 15 to 30 min. One possible explanation for the persistence of ornithine decarboxylase activity (Fig. 2) could be an increase in its stability. To test this possibility, the activity of ornithine decarboxylase was determined after adding cycloheximide (20 µg/ml) to glioma cells, with addition of either complete medium or buffered-asparagine solutions. It may be seen (Fig. 3) that the half-life of ornithine decarboxylase is approx. 20 min in cells incubated with complete medium, compared with 60 min in cells fed with buffered-asparagine solution

Intracellular polyamine concentrations

It is to be expected that the increase in the activity of the biosynthetic decarboxylase should be followed by an increase in cellular polyamine concentrations. Indeed, putrescine concentrations increase after addition of complete medium to the cells and are maximal after 6 h (Fig. 4). Thereafter, the concentrations decrease but increase again in a biphasic manner after approx. 18 h. The decrease in putrescine concentrations at 6 h after addition of complete medium is explained by: (a) formation of γ -aminobutyric acid; (b) synthesis of spermidine and spermine.

Fig. 4 clearly shows that the concentration of γ -aminobutyric acid increases after addition of complete medium to glioma cells. A significant increase in cellular γ -aminobutyric acid is observed after 9 h, 1–2 h after attaining maximal diamine oxidase activity (cf. Fig. 1b). The cellular concentration of the polyamines, spermidine and spermine also increase after addition of medium to the cells. This increase paralleled the increase in the activity of the biosynthetic decarboxylases (cf. Fig. 1a).

Metabolism of putrescine

In addition to the synthesis of putrescine from ornithine, glioma cells are also capable of metabolizing putrescine added to the growth medium. Glioma cells were grown in 50 mm plastic dishes. After 4 days [1,4-¹⁴C]putrescine (0.5 µCi, sp. radioactivity 59.9 Ci/mol) was added to 2.0 ml of fresh medium. At various times, medium was removed, freeze-dried and analysed for polyamines. Radioactive material was also extracted from the cells with 0.5 ml of 3% (v/v) HClO₄ and similarly analysed. Fig. 5(a) illustrates the disappearance of [¹⁴C]putrescine from

Fig. 3. *Stability of ornithine decarboxylase*
The decay of ornithine decarboxylase activity was determined after adding cycloheximide (20 µg/ml) to glioma cells to which was added either DMEM medium with 5% calf serum (●) or buffered-salt/glucose solution supplemented with 10 mM-asparagine (Δ).

Fig. 4. *Polyamine concentrations in glioma cells*
To confluent cultures of C₆BU-1 glioma cells fresh complete medium was added. After incubation at 37°C, polyamines were extracted from the cells, dansylated and separated by t.l.c. Fluorescent material was scraped off the respective spots, extracted with ethyl acetate and assayed fluorimetrically. Symbols: □, spermine; ▲, γ -aminobutyric acid; ○, spermidine; ●, putrescine.

the growth medium and the formation of radioactive γ -aminobutyric acid. It may be seen that the concentration of γ -aminobutyric acid is maximal when cells are treated with asparagine in a buffered-salt/glucose solution (Fig. 5a).

Fig. 5. Uptake and metabolism of [^{14}C]putrescine by glioma cells suspended in various media

To confluent cultures of glioma cells were added either buffered-salt/glucose solution, buffered-salt/glucose plus 10mM-asparagine or DMEM medium supplemented with 5% foetal calf serum. Radioactive putrescine was then added to all cultures and polyamines were determined in (a) growth medium or (b) glioma cells. Symbols: O, putrescine; Δ , γ -aminobutyric acid; $\bullet$, spermidine; $\blacktriangle$, spermine.

Conversion of putrescine into polyamines

The conversion of radioactive putrescine into polyamines and γ -aminobutyric acid is illustrated in Fig. 5(b), which shows that spermine is synthesized from radioactive putrescine only in the presence of complete medium. In this medium, radioactive putrescine accumulated in the cells for 2h. Thereafter, it is converted into spermidine, spermine and γ -aminobutyric acid. Intracellular putrescine concentrations are relatively high in cells incubated in buffered-asparagine solutions. Under these experimental conditions cellular γ -aminobutyric acid increases markedly, whereas that of spermidine remains relatively low. This observation confirms the suggestion that asparagine affects diamine oxidase activity.

Discussion

The metabolism of polyamines in mouse neuroblastoma cells was first described by Kremzner *et al.* (1975), who found that γ -aminobutyric acid is formed from putrescine in the presence of a serum-containing medium. Sobue & Nakajima (1977) extended these studies for glioma cells and

reported that the synthesis of spermidine from putrescine was closely related to the proliferation of the cells, whereas γ -aminobutyric acid was formed at a later stage of the cell cycle. Our experiments (Fig. 1a) show that the peak of ornithine decarboxylase activity precedes that of *S*-adenosyl-L-methionine decarboxylase. It is also apparent that, by adding fresh medium to confluent cultures of glioma cells, a diamine oxidase is induced (Fig. 1b). These findings are consistent with the observations of Baylin *et al.* (1978), who reported the association of diamine oxidase and ornithine decarboxylase in rapidly proliferating epithelium. A similar correlation between diamine oxidase and ornithine decarboxylase has also been described by Quash *et al.* (1979), who found that in rat kidney cells grown *in vitro* diamine oxidase activity increased after addition of complete medium, reaching maximal values after 24h. The activity of diamine oxidase 24h after addition of complete medium was 50% higher in cells transformed by avian sarcoma virus. At this stage of growth, ornithine decarboxylase activity was also increased in the transformed cells (Don *et al.*, 1975).

According to Seiler & Al-Therib (1974), the oxidation of putrescine in the brain is not catalysed by a diamine oxidase. They found that the diamine is first converted into the monoacetyl derivative, which is further metabolized by a monoamine oxidase. In our study we used the procedure employed for the assay of diamine oxidase activity (Kusche *et al.*, 1973) and found an increase in enzyme activity after adding complete medium to confluent glioma cells (Fig. 1b). Other experiments (results not shown) also demonstrated that asparagine in buffered-glucose solution caused a 90% increase in diamine oxidase activity of glioma cells after incubation for 5h. It is most likely that this diamine oxidase is responsible for the formation of γ -aminobutyric acid from putrescine (Figs. 4 and 5). The findings that asparagine enhances the formation of γ -aminobutyric acid from putrescine (Fig. 5) is best explained by the activation of diamine oxidase by 2-oxosuccinamate, which is an intermediate of asparagine decarboxylation (Quash *et al.*, 1976, 1979). Asparagine, or its metabolites, not only activate diamine oxidase, but also lead to an increase in ornithine decarboxylase activity (Chen & Canelakis, 1977). It has been suggested that the decrease in cellular polyamines by incubating cells in a serum-free medium supplemented with asparagine may decrease ornithine decarboxylase antienzyme activities, thus facilitating the activation of ornithine decarboxylase (Costa, 1978). In addition, asparagine was found to stabilize ornithine decarboxylase, thus leading to an apparent increase in its activity (Chen & Canelakis, 1977; Costa, 1978). Our results (Fig. 3) are in line with these observations and demonstrate the stabilization of or-

nithine decarboxylase by adding buffered-asparagine solutions to glioma cells. It is also evident from Fig. 2 that the activation of ornithine decarboxylase by asparagine differs from that triggered by a serum-containing medium by the following kinetic properties. (1) In the presence of asparagine the increase in ornithine decarboxylase activity is an early event and occurs even after 1 h, unlike the activation by serum. (2) In the presence of asparagine, high activities of enzyme are sustained for a longer period of time (Fig. 2). Similar results have been reported by Costa (1978).

Other studies (Costa & Nye, 1978) also stressed the difference between ornithine decarboxylase activation by serum and buffered asparagine. The paradoxical effect of asparagine and its ability to enhance both the degradation and synthesis of putrescine may be explained by a recent finding (McCann *et al.*, 1979) that γ -aminobutyric acid increases the activity of ornithine decarboxylase. If this finding is general then the primary effect of asparagine may be the activation of diamine oxidase. This may lead to the accumulation of γ -aminobutyric acid, which, in turn, enhances putrescine synthesis.

Further studies are required to gain a better understanding of the mechanisms by which asparagine affects the activity of ornithine decarboxylase and of diamine oxidase. It is, however, clear that this amide affects the biosynthesis and degradation of putrescine and thereby may control cellular functions.

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