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Metabolic Fate of [U-^{14}C]-Labeled Glutamate in Primary Cultures of Mouse Astrocytes as a Function of Development

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Abstract. The metabolic fate of [U-^{14}C]-labeled glutamate in astrocytes grown in primary cultures for 1–3 weeks in the absence or presence of dibutyryl cyclic AMP was followed using dansylation with ^{3}H-dansyl chloride and subsequent thin layer chromatography of the dansylated amino acids. No indication was found of classical metabolic compartmentation, i.e., the specific radioactivity of glutamine (^{14}C/^{3}H ratio) never exceeded that of its precursor, glutamate. In accordance with a relatively late maturation of glutamine synthetase activity the rate of formation of labeled glutamine was much faster in 3-week-old than in 1-week-old cultures. The opposite was found for aspartate formation, but under all conditions incorporation of radioactivity into aspartate was pronounced.

The activities of glutamate-metabolizing enzymes in astrocytes are known to undergo pronounced alterations during development and differentiation [2, 4, 6, 12, 17]. Thus, the activity of glutamine synthetase (GS) shows a marked but relatively slow increase in parallel with the increase in GS activity in whole brain during postnatal ontogenesis, whereas that of glutamate dehydrogenase (GLDH) after a rapid early increase remains unaltered or even declines, and glutamate-oxaloacetate transaminase (GOT) activity shows a continuous but very slow increase. In agreement with the neuronal localization of glutamate decarboxylase [cf. ref. 13], astrocytes in primary cultures are devoid of any glutamate decarboxylase activity [*Wu, Hertz and Schousboe,* unpublished experiments; cf. also ref. 20].

Information about enzyme activities does not necessarily allow conclusions concerning the actual rates of metabolic conversion in the living cells. In the present work the studies of alterations in enzyme activities during the development of astrocytes in primary cul-

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tures were therefore supplemented with investigations of the metabolic fate of glutamate, determined by incubation with uniformly labeled [^{14}C]-*L*-glutamate and subsequent determination of specific radioactivities of the amino acids found to be labeled, i.e., glutamine and aspartate. This was done by dansylation of tissue extracts with [^{3}H]-dansyl chloride [11], chromatographic separation of the dansylated amino acids, and determination of ^{14}C/^{3}H ratios. Since dansyl chloride reacts equally well with ^{14}C and ^{12}C amino acids the ^{14}C/^{3}H ratios in the individual spots are a direct expression of the specific radioactivities regardless of possible variations in dansylation efficiency [9, 18]. Furthermore, since specific activities were measured rather then the absolute amounts of incorporated radioactivity, release or further metabolic conversion of the amino acids will not interfere with the interpretation of the results unless occurring from subcompartments which show especially fast labeling.

Materials and Methods

Methods

Cultures of astrocytes were prepared as described by *Hertz* et al. [5]. The parts of the cerebral hemispheres above the lateral ventricles were dissected out of the brains of newborn Swiss mice and grown either for 1 or 3 weeks in a tissue culture medium (modified Eagle's MEM) with serum or for 2 weeks in such a medium and subsequently for a 3rd week in the additional presence of 0.25 m*M* dibutyryl cyclic AMP (dBcAMP), a procedure known to evoke a distinct morphological differentiation of the cells.

The specific radioactivities of glutamate, glutamine and aspartate were determined utilizing the general procedures described by *Osborne* [11], *Minchin and Beart* [9], and *Voaden* et al. [18] and the modifications introduced by *Bullaro* [1]. Each culture was incubated in an atmosphere of 5% CO_2 in air for between 2 and 30 min in serum free tissue culture medium to which 50 μM [U-C^{14}]-*L*-glutamate (in general 1.3 μCi/ml) had been added. To ensure that hydrolysis of glutamine in the medium did not increase its content of glutamate, glutamine (2 m*M*) was not added until immediately before the experiments. After the incubation the cultures were rinsed three times with ice-cold phosphate-buffered saline [16] and subsequently another three times with Tris solution (1 m*M*; pH 7.4), scraped off the culture dishes in a Tris/trichloroacetic acid (1 m*M* Tris, 0.4 *M* TCA) solution, left in this solution at room temperature for at least 30 min, and centrifuged for 5 min at 12,000 *g*. Protein was determined in the pellets using the conventional Lowry technique [8] and supernatants were extracted six times with water saturated diethyl ether, excess of which subsequently was blown off with nitrogen or air. The pH of the solution was adjusted to 8.0 with 0.05 *M* potassium hydroxide and stored at –70°C until further processing.

A volume of supernatant equivalent to an amount of tissue containing 100 μg protein was lyophilized to dryness and redissolved in 20 μl 0.05 *M* potassium bicarbonate, adjusted to pH 10.0. For dansylation, which took place at 37°C, in subdued light, and lasted for 30 min, 4 μl of the bicarbonate solution was added to a foil-covered 400-μl Beckman polyethylene tube together with 2 μl 29.7 m*M* [^{3}H]-dansyl chloride in acetone, containing 2 μCi of the isotope (supplied in toluene which was removed by lyophilization), and 10 μl acetone. Samples of 1 μl of the dansylation products (i.e., corresponding to 1.25 μg of the original tissue protein), were chromatographed in triplicate, and in dim light, in two dimensions on a 5 × 5 cm polyamide sheet in three different developing solutions, i.e. (v/v): (1) water:formic acid (100:3); (2) benzene:acetic acid (9:1); and (3) ethyl-acetate:methanol:acetic acid (20:1:1), the two latter of which were used in the same (second) dimension. In some cases 6- to 10-pmol samples of dansyl aspartate, dansyl glutamate or dansyl glutamine were spotted on the back of the sheets to ensure correct identification of the dansylated reaction products from the tissue extract.

After the chromatographic separation, individual spots were scraped off the sheets with a spatula under ultraviolet light and dispersed (using a sonifier) in 200 μl glacial acetic acid. ^{14}C and ^{3}H activities were measured simultaneously using a Beckman LS9000 liquid scintillation spectrometer, and ^{14}C/^{3}H ratios were calculated. With the specific radioactivities used of glutamate in the incubation medium and of dansyl chloride in the dansylation mixture, the different spots after varying incubation periods contained between 50 dpm and 8,000 dpm of ^{14}C and between 800 dpm and 22,000 dpm of ^{3}H.

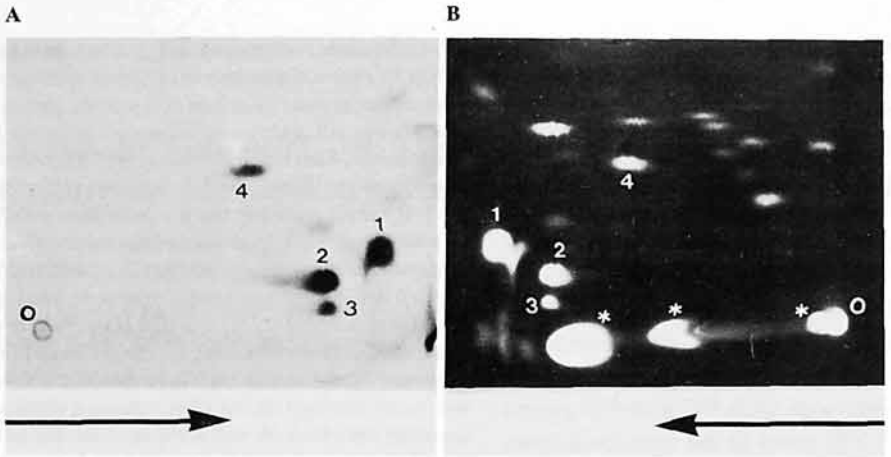

Fig. 1.A Autoradiogram of dansyl-amino acids from a culture incubated for 30 min with [U-^{14}C]-glutamate (13 μCi/ml), dansylated with nonradioactive dansyl chloride and exposed for 1 day. Note that radioactivity virtually is confined to four amino acids: (1) glutamine; (2) glutamate; (3) aspartate; (4) condensation product of glutamate. **B** Photograph of corresponding chromatogram (mirror image) viewed under ultraviolet light. Note large amounts of amino acids 1–4. Other amino acids are not indicated but can be identified, e.g., from *Osborne* [11]; the limit of detectability is about 1 pmol which, with a tissue amount $\simeq$ 10 μg, corresponds to 0.1 μmol/g wet weight. Dansyl waste products are shown by *. In both **A** and **B** directions of first dimension are indicated by arrows and origins by 0.

In a few cases the extract from the cultures were dansylated with nonradioactive dansyl chloride and the resulting chromatograms autoradiographed, using a Kodak Xomat-R XR1 film.

Materials

Plastic 60-mm tissue culture dishes were purchased from Falcon, Oxnard, Calif.; Nitex® nylon mesh (used in preparation of cultures) from L. & S.H. Thompson & Co., Montreal, P.Q., Canada; vitamins and amino acids (for medium preparation), dBcAMP and dansyl amino acids from Sigma Chemical Co., St. Louis, Mo.; Schleicher & Schuell F-1700 micropolyamide sheets from Schleicher & Schuell Inc., Keene, N.H.; [U-^{14}C]-*L*-glutamate (specific radioactivity 250 mCi/mmol) and [^{3}H]-dansyl chloride (specific radioactivity 10–40 Ci/mmol) from New England Nuclear, Montreal, P.Q., Canada. Serum was obtained from selected horses.

Results

A typical autoradiogram of the dansylated amino acids from approximately 10 μg wet weight (1.25 μg protein) of a 3-week-old culture incubated for 30 min in a medium with 50 μM [U-^{14}C]-glutamate is shown in figure 1A and the corresponding chromatogram in figure 1B. It can be seen that the cells contain a multitude of different amino acids in relatively large amounts (cf. figure legends) and that only glutamate, aspartate and glutamine contain a substantial amount of radioactive label. For this reason only these three amino acids were studied in detail.

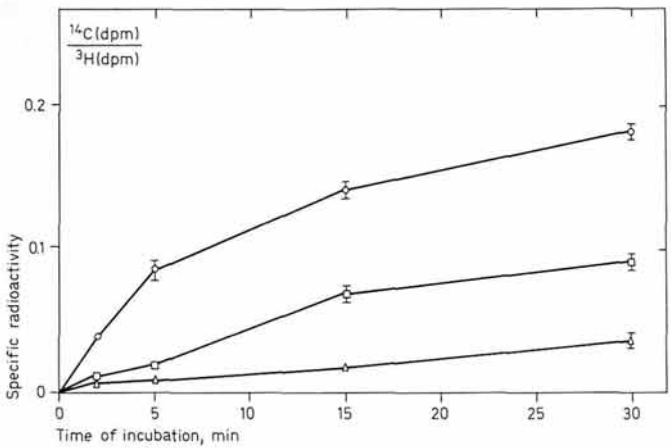

Fig. 2. Specific radioactivities [$^{14}C/^{3}H$ (dpm) ratios] of glutamate (0), glutamine (Δ) and aspartate (□) in 1-week-old primary cultures of astrocytes shown as a function of incubation time with 50 μM [U-^{14}C]-*L*-glutamate.

The specific activities of glutamate, glutamine and aspartate, expressed as $^{14}C/^{3}H$ (dpm) ratios (cf. 'Introduction') in 1-week-old cultures after incubation with labeled glutamate for 2–30 min are shown in figure 2 and the corresponding values obtained in 3-week-old cultures in figure 3. In 3-week-old cultures which had not been treated with dBcAMP, the specific radioactivity of glutamate increases rapidly and has almost reached a constant level already after 5 min (fig. 3). A rather similar course was followed in dBcAMP-treated cultures of the same age (results not presented), whereas the increase in radioactivity is considerably slower in 1-week-old cultures (fig. 2). The difference between 1- and 3-week-old cultures is consistent with a much lower rate for glutamate uptake at 1 week of age (cf. 'Discussion') and approximately similar glutamate contents at both ages, as evidenced by roughly similar ^{3}H radioactivities per glutamate spot (results not presented). The specific radioactivity of glutamine increases also much more slowly in 1-week-old cultures (fig. 2) than in 3-week-old cultures, grown in the absence of dBcAMP (fig. 3), probably partly reflecting the slower equilibration of its precursor, glutamate and partly reflecting the lower glutamine synthetase activity at the early age (cf. 'Discussion'). Another possibility, i.e., that the difference in $^{14}C/^{3}H$ ratios at the different ages could be due to different pool sizes of glutamine is ruled out by the finding that the ^{3}H radioactivities per spot (≃ 10 μg wet weight) were identical (results not presented). After treatment with dBcAMP the incorporation of ^{14}C into glutamine decreases again (fig. 3).

In contrast to glutamate and glutamine, the specific radioactivity of aspartate follows almost similar courses at the two ages, with a slightly slower rise in 1-week-old cultures (fig. 2) than in 3-week-old cultures (fig. 3), probably due to the slower rise of the specific radioactivity in glutamate. Cultures treated with dBcAMP showed at least as much incorporation into aspartate as those which had not been treated (results not presented). In spite of the almost similar courses for aspartate labeling under all conditions, the rate of

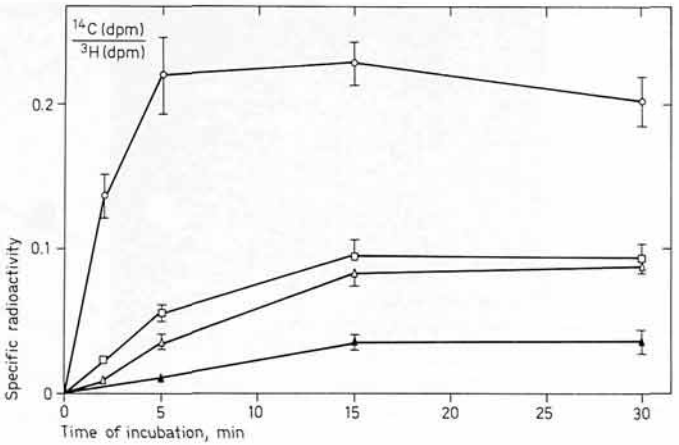

Fig. 3. Specific radioactivities [$^{14}C/^{3}H$ (dpm) ratios] of glutamate (0), glutamine (△ and ▲) and aspartate as a function of incubation time in 3-week-old primary cultures of astrocytes grown in the absence (0, △ and □) or presence of 0.25 m*M* dBcAMP (▲) during the last week and incubated as in figure 2.

conversion of glutamate to aspartate must be considerably higher in 1-week-old cultures than in 3-week-old untreated cultures since the aspartate pool was almost three times larger at the early age (2,688 dpm ± 452 (SEM) ^{3}H per spot versus 977 dpm ± 138 ^{3}H per spot).

Discussion

Three points should be emphasized in the present study: (1) the absence of classical metabolic compartmentation at all stages of cellular differentiation, as shown by a normal precursor-product relationship between glutamate and glutamine, i.e., higher specific radioactivity of glutamate than of glutamine at a time when both amino acids seem close to equilibrium; (2) the good correlation between alterations in enzyme activities and in actual metabolic fluxes during postnatal development, and (3) the considerable conversion of glutamate to aspartate.

The normal precursor-product relationship is in agreement with a similar observation by *Nicklas and Browning* [10] in C-6 glioma cells but at variance with findings by *Rose* [15] in bulk-separated glial cells, probably due to cellular cross-contamination of the latter. The metabolic compartmentation observed in peripheral ganglia [9, 14] and in rat retina [18] can probably also be accounted for by the heterogeneous cellular composition of these structures, in spite of the fact that the precursor used is mainly accumulated into glial cells. Absence of metabolic compartmentation in a cellularly homogeneous preparation is obviously in agreement with the concept that the metabolic compartmentation observed in the brain in vivo reflects the cellularly mixed composition of this organ.

The slower labeling of both glutamate and glutamine in 1-week-old than in 3-week-old cultures is consistent with the considerably lower glutamate uptake rate and GS activity in the younger cultures [4]. The increase in glutamine synthesis and GS activity with age might suggest that the relative importance of glutamine synthesis increases with increasing cellular differentiation. This point of view is,

however, not supported by the decreased GS activity and glutamine formation in morphologically differentiated cultures, obtained after treatment with dBcAMP, a treatment which also enhances several aspects of functional maturation [3, 17]. The GLDH activity, in contrast, decreases during maturation of astrocyte cultures [4], which might explain the specially rapid conversion of glutamate to aspartate in 1-week-old cultures. It is, however, unknown whether this reaction occurs as an oxidative deamination, catalyzed by GLDH, or as a transamination, catalyzed by GOT, an enzyme increasing in activity with age and present at high activities (at least ten times higher than those of GLDH) in both 1-week-old and 3-week-old cultures [4].

By whatever route aspartate is labeled from radioactive glutamate, the rate of the process was pronounced. This does not necessarily indicate a net metabolic degradation of glutamate, but might be explained by an isotopic exchange reaction between α-ketoglutarate and glutamate catalyzed by the transaminase [cf. ref. 7]. The rapid labeling might, on the other hand, equally well indicate that glutamate is utilized by the astrocytes as a metabolic substrate, a situation comparable to the use of glutamine as an energy source by fibroblasts and intestinal cells [19, 21]. It is an indication in this direction that the oxygen consumption by cultured astrocytes seems to be better maintained in a glucose-free medium containing glutamate or glutamine than in a corresponding medium without any of these amino acids [*Hertz and Hertz*, unpublished experiments]. Unequivocal demonstration of utilization of glutamate as a metabolic substrate would be of functional interest both for the understanding of reactions involved in energy metabolism in astrocytes and because it would establish that synthesis of glutamine, although undoubtedly occurring at a considerable rate at all ages, is only one among several metabolic routes along which glutamate is degraded in astrocytes.

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