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# ONTOGENETIC DEVELOPMENT OF GLUTAMATE METABOLIZING ENZYMES IN CULTURED CEREBELLAR GRANULE CELLS AND IN CEREBELLUM *IN VIVO*

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The ontogenetic development of the enzymes phosphate activated glutaminase (PAG), glutamate dehydrogenase (GLDH), glutamic-oxaloacetic-transaminase

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(GOT), glutamine synthetase (GS), and ornithine-δ-aminotransferase (Orn-T) was followed in cerebellum in vivo and in cultured cerebellar granule cells. It was found that PAG, GLDH, and GOT exhibited similar developmental patterns in the cultured neurons compared to cerebellum. PAG showed, however, a more pronounced phosphate activation in the cultured granule cells compared to in vivo. The activity of GS remained low in the cultured neurons compared to the increasing activity of this enzyme found in vivo. On the other hand Orn-T exhibited an increase in its specific activity in the cultured cells as a function of time in culture in contrast to the non-changing activity of this enzyme in vivo. Compared to cerebellum the cultured neurons exhibited higher activities of GLDH, GOT, and Orn-T whereas the activity of PAG was only slightly higher in the cultured cells. The activity of GS in the cultured neurons was only 5–10% of the activity in cerebellum in vivo. It is concluded that cultured cerebellar granule cells represent a reliable model system by which the metabolism and function of glutamatergic neurons can be conveniently studied in a physiologically meaningful way.

## INTRODUCTION

From in vivo as well as tissue culture studies of glutamate mediated neurotransmission it is clear that a complex metabolic interaction between neurons and astrocytes is required for normal function of this process (cf. Ref. (12)). A considerable number of studies employing primary cultures of astrocytes have focused on a characterization of the astrocytic metabolism of glutamate (13, 45). Less emphasis has been placed on corresponding studies on primary cultures of glutamatergic neurons although activities of some of the enzymes involved in glutamate metabolism have become available recently (28).

From studies of evoked release of glutamate from cultures of cerebellar granule cells it has become evident that such cultures may be suitable as a model system for glutamatergic neurons (7, 8, 10, 21, 23). On this basis it seems appropriate to investigate in this type of neurons the activities of key enzymes involved in the metabolism of glutamate. This might allow conclusions as to which enzymes might be responsible for the synthesis of transmitter glutamate. In order to allow such conclusions it seemed important to be able to assess the validity of these cultured neurons as model systems for their in vivo counterparts. Accordingly, the development of the activities of these enzymes was determined both in the cultured cells and in cerebellum.

Preliminary accounts of parts of the present study have been published previously (19, 20).

## EXPERIMENTAL PROCEDURES

*Materials.* NMRI mice (newborn to 28-day-old animals) were obtained from the SPF animal quarters in the Panum Institute. Plastic tissue culture dishes were purchased from NUNC A/S (Denmark) and horse serum from Gibco/Biocult Lab., Ltd. (Scotland). Cytosine arabinoside, poly-L-lysine, trypsin, DNAse, amino acids, and vitamins were obtained from Sigma Chemical Company, St. Louis, enzymes and coenzymes from Boehringer, Mannheim, FRG. All other chemicals were of the purest grade available from regular commercial sources.

*Tissue Culture.* Cerebellar granule cells were cultured essentially as described by Messer (25), Yu and Hertz (44), and Drejer et al. (7), but with some minor modifications introduced by Meier and Schousboe (24). Cerebella were taken from 7-day-old mice and placed in Puck's solution. The tissue was cut in 0.5 × 0.5 mm cubes by a McIlwain tissue chopper, and exposed for 10 min to 0.025% (w/v) trypsin (37°C) followed by trituration in a DNAse solution (0.004%, w/v) containing a trypsin inhibitor (0.03%, w/v) from soy beans (39). The cells were suspended ($6 \times 10^6$ cells/ml) in a modified Eagle's minimum essential medium (11) containing 10% (v/v) horse serum, 1 mM glutamine, 24.5 mM KCl, 30 mM glucose, 7 μM *p*-aminobenzoic acid and 100 mU/L insulin. The cell suspension was subsequently inoculated ($3 \times 10^7$ cells/25 $cm^2$ flask) into poly-L-lysine coated (36) NUNCLON® culture flasks. After 48 hr in culture, 50 μM cytosine arabinoside which prevented glial outgrowth (25) was added and 24 hr later this medium was changed to an analogous medium without the mitotic inhibitor. The cells were cultured for 2–14 days with further exposure to cytosine arabinoside between days 7 and 8.

*Preparation of Homogenates.* Cerebella were isolated from the brains of mice at the different ontogenetic stages (newborn to 28-day postnatal) and cultures were scraped off the plastic tissue culture flasks after removal of the culture medium and rinsing the cultures with a 150 mM TRIS-HCl buffer pH 7.3. Homogenates were prepared from these tissue samples in different buffers depending upon the enzyme in question. For glutamate dehydrogenase (GLDH, EC 1.4.1.3) and glutamic-oxaloacetic-transaminase (GOT, EC 2.6.1.1) homogenates were prepared in 0.05 M potassium phosphate, 0.2 mM pyridoxal phosphate, 1 mM 2-aminoethylisothiouronium bromide hydrobromide (AET) and 0.1 mM EDTA; pH 7.2 and subsequently sonicated for 2 × 10 sec at 0°C using a Branson sonifier (50 watts). For ornithine aminotransferase (Orn-T, EC 2.6.1.13) homogenates were prepared in a similar manner in an analogous buffer, which contained in addition 0.25% (v/v) Triton X-100 and for determination of glutamine synthetase (GS, EC 6.3.1.2) activity homogenates were prepared in 0.05 M imidazol-HCl buffer; pH 7.2 and subsequently sonicated. For phosphate activated glutaminase (PAG, EC 3.5.1.2) homogenates were prepared in 0.1 M TRIS-HCl buffer, pH 7.4, using a small Potter-Elvehjem homogenizer equipped with a hand operated piston.

*Enzyme Assays.* The activity of GLDH was measured at 27°C according to the method of Schmidt (33), GOT activity was assayed employing the method of Bergmeyer and Bernt (2) with the modifications described by Schousboe et al. (35). Orn-T activity was determined employing the o-aminobenzaldehyde method of Peraino and Pitot (29). The activity of PAG was determined by a slight modification (17) of the fluorometric method of Curthoys and Lowry (5). This method agreed favorably with the method of Kvamme and Svenneby (15), but is much more sensitive. GS activity was determined as described by Berl (3) employing creatine phosphate/creatine kinase as the ATP generating system (34). All enzyme activities except that of GLDH were determined at 37°C.

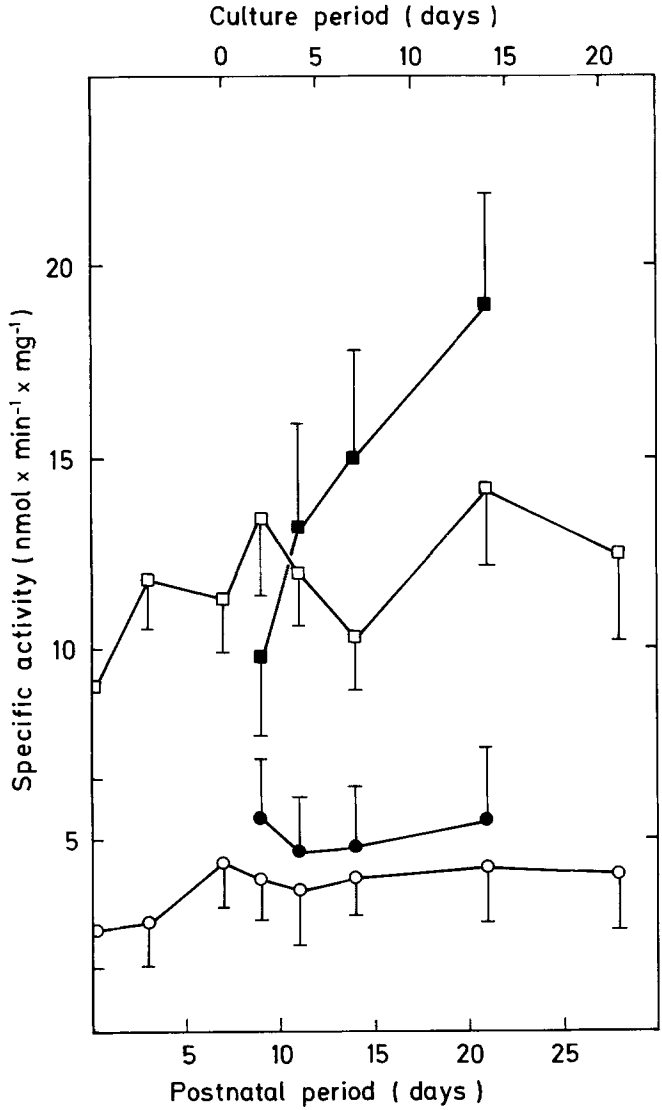


FIG. 1. Specific activity (nmol $\times$ $min^{-1}$ $\times$ $mg^{-1}$ protein) of phosphate activated glutaminase assayed at 5 mM phosphate (circles) and 20 mM phosphate (squares) as a function of development. Open symbols indicate the enzyme activity in cerebellum in vivo and closed symbols indicate the enzyme activity in cultured cerebellar granule cells. Lower abscissa gives number of days after birth and the upper abscissa gives the number of days in culture after the inoculation of cells into the culture flasks corresponding to 7 days after birth.

Results are averages of 4–6 experiments with SEM indicated by vertical bars.

TABLE I

ACTIVITIES OF GLUTAMATE METABOLIZING ENZYMES IN GLUTAMATERGIC AND GABAERGIC NEURONS IN CULTURE

| Enzyme | Enzyme Activity (nmol × $min^{-1}$ × $mg^{-1}$) | |
|---|---|---|
| | Glutamatergic neurons | GABAergic neurons |
| PAG | 19.0 ± 2.9 (6) | 18.8 ± 2.1 (5) |
| GLDH | 62.8 ± 7.3 (4) | 25.4 ± 0.9 (5) |
| GOT | 1657 ± 147 (5) | 280 ± 14 (5) |
| Orn-T | 3.1 ± 0.3 (4) | 5.3 ± 0.4 (5) |

Maximal activities of PAG, GLDH, GOT and Orn-T in cultured cerebellar granule cells (glutamatergic neurons) taken from Figures 1, 2, 3, and 5. For comparison the corresponding activities in 2-week-old cultures of cerebral cortex interneurons (GABAergic neurons) have been given. These values are taken from Larsson et al. (18). The activities have been determined at 37°C except for GLDH which was assayed at 27°C. PAG activity was determined at a phosphate concentration of 20 mM. Values represent means ± SEM with numbers of experiments in parentheses.

Protein contents were measured by the method of Lowry et al. (22) using bovine serum albumin as the standard.

## RESULTS

### *Developmental Profiles of Enzyme Activities In Vivo and in Culture*

*PAG.* The development of the activities of PAG in cerebellum in vivo and in cerebellar granule cells in culture is shown in Figure 1. It is seen that PAG activity at the low phosphate concentration (5 mM) exhibits similar developmental profiles in the cultured neurons and in cerebellum. At the high phosphate concentration (20 mM), PAG in granule cells exhibits an increase in specific activity as a function of time in culture and the activity reached after two weeks in culture is given in Table I. Moreover, it can be seen from Figure 1 that also the phosphate activation of the enzyme increases with time in culture. This contrasts the in vivo situation where very little increase in enzyme activity as well as phosphate activation was observed as a function of age. At the earlier stages of development the enzyme had the same activity in the cultured cells and in vivo, whereas at later developmental stages the PAG activity in the cultured granule cells at high phosphate concentration appears to be slightly higher than in vivo.

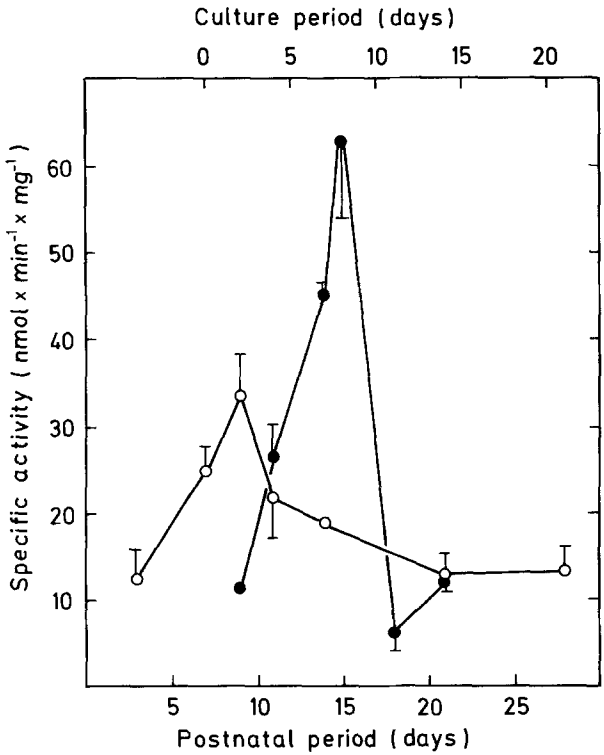


FIG. 2. Specific activity (nmol $\times$ $min^{-1}$ $\times$ $mg^{-1}$ protein) of glutamate dehydrogenase as a function of development in cerebellum in vivo (○) and in cultured cerebellar granule cells (●). Upper and lower abscissa indicates days in culture and in vivo, respectively as explained in Figure 1.

Results are averages of 3-6 experiments with SEM indicated by vertical bars if they extend beyond the symbols.

*GLDH.* Figure 2 shows the development of the activity of GLDH in cultured granule cells compared with cerebellum in vivo and Table I gives the maximal activity in the cultured cells. It is seen that the enzyme exhibits a maximum in its specific activity both in vivo and in the cultured cells. The peak of activity occurs somewhat earlier in vivo (9 days postnatally) than in the cultured cells (8 DIV, corresponding to 15 days postnatally). It is also seen that cultured cells have significantly ($P < 0.02$) higher GLDH activity at the peak than whole cerebellum.

*GOT.* The activity of GOT in cerebellum as well as in cultured granule cells as a function of age is seen in Figure 3. The specific activity of the enzyme increases 5 to 10 fold during development both in vivo and in culture. After 14 days in culture the granule cells had 2-3 times as high

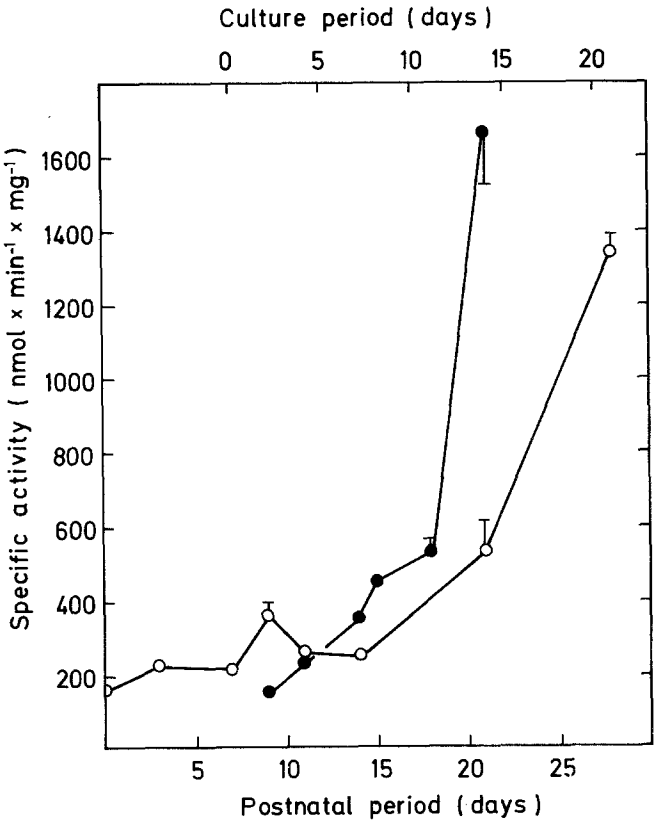


FIG. 3. Specific activity (nmol × $min^{-1}$ × $mg^{-1}$ protein) of glutamic-oxaloacetic-transaminase as a function of development in cerebellum in vivo (○) and in cultured cerebellar granule cells (●). Upper and lower abscissa indicates days in culture and in vivo, respectively as explained in Figure 1.

Results are averages of 3-5 experiments with SEM indicated by vertical bars if they extend beyond the symbols.

activity as cerebellum at the corresponding postnatal stage (21 days), but only slightly higher than the activity in cerebellum at 28 days. The activity in 2-week-old cultures has also been given in Table I.

*GS.* The activity of glutamine synthetase in cerebellum together with the activity in the cultured granule cells as a function of age is shown in Figure 4. It is seen that the specific activity of the enzyme shows a sharp increase during the period 1–2 weeks after birth. The activity in the cultured neurons shows a moderate decrease as a function of the culture period and at all ages the activity in the cultured neurons is low compared to the activity in cerebellum in vivo.

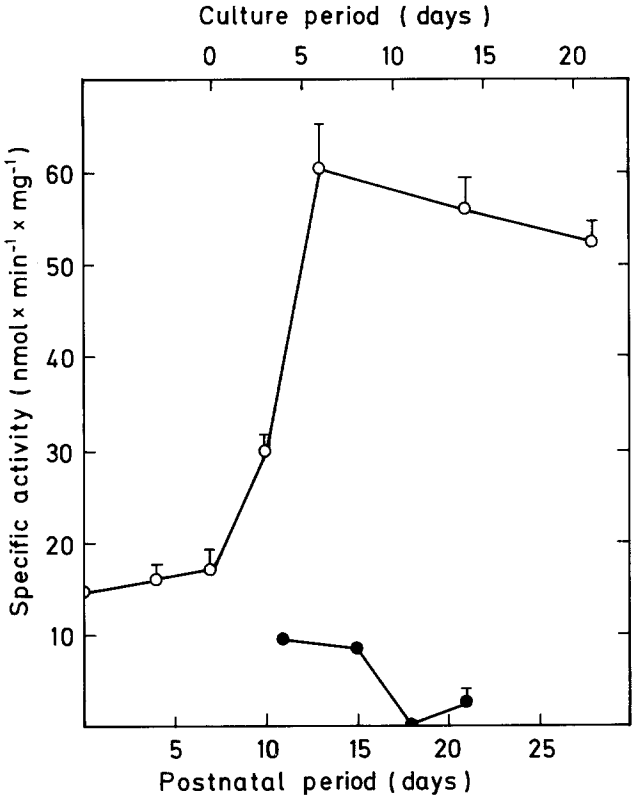


FIG. 4. Specific activity (nmol × $min^{-1}$ × $mg^{-1}$ protein) of glutamine synthetase as a function of development in cerebellum in vivo (○) and in cultured cerebellar granule cells (●). Upper and lower abscissa indicates days in culture and in vivo, respectively as explained in Figure 1.

Results are averages of 3-5 experiments with SEM indicated by vertical bars if they extend beyond the symbols.

*Orn-T.* Figure 5 shows the developmental profiles of the specific activity of Orn-T in cerebellum and cultured granule cells. No significant changes are seen in the activity of Orn-T in vivo as a function of development. In contrast to this the cultured cerebellar granule cells exhibit an increase in Orn-T activity as a function of the culture period. After 14 days in culture the activity of this enzyme in the cells (Table I) is slightly but statistically significantly ($P < 0.05$) higher than in whole cerebellum of the corresponding age (21 days).

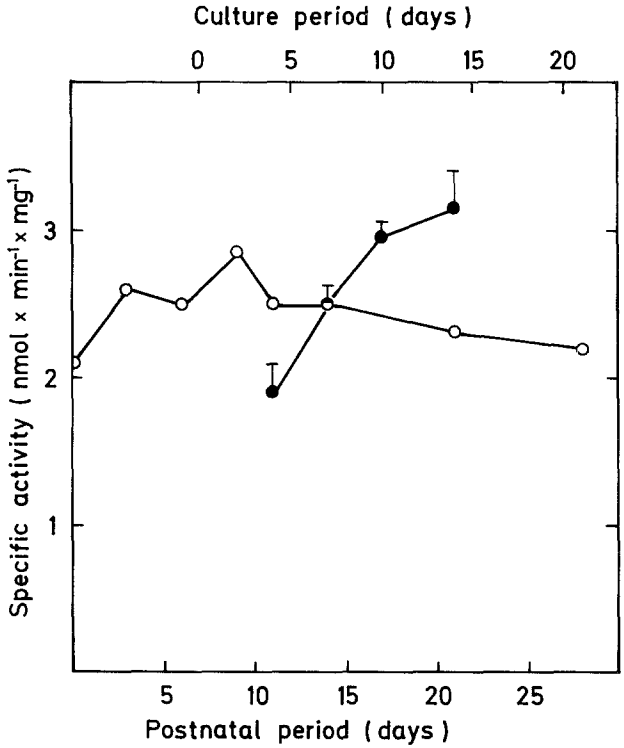


FIG. 5. Specific activity (nmol × $min^{-1}$ × $mg^{-1}$ protein) of ornithine-δ-aminotransferase as a function of development in cerebellum in vivo (○) and in cultured cerebellar granule cells (●). Upper and lower abscissa indicates days in culture and in vivo, respectively as explained in Figure 1.

Results are averages of 3–7 experiments with SEM indicated by vertical bars if they extend beyond the symbols.

## DISCUSSION

It has previously been shown that cultured cerebellar granule cells release glutamate in a calcium-dependent manner upon stimulation with potassium (7, 8, 10, 21). The present study shows that the development of the activities of key glutamate metabolizing enzymes with the exception of GS in such cultured granule cells corresponds to the developmental patterns of these enzymes in cerebellum in vivo. It may be particularly interesting that GLDH exhibits a peak in its activity both in vivo and in the cultured cells during the period of neurite formation (cf. Ref. (27)).

This observation is not in agreement with results of Rothe et al. (32) who in vivo found a continuous but slight increase in the GLDH activity, but is in accordance with the observation that GLDH activity in cerebellum is enriched in the granule layer (31). The decrease in GLDH activity in vivo during the postnatal period 9–14 days could partly be explained by the development of glial cells (which contain much less GLDH than neurons (18)) during this period as reflected by the increase in the activity of GS (Figure 4), which is located in astroglial cells (26). The corresponding decrease in the cultured cells can, however, not be explained by the presence of glial cells since the GS activity in the cultures at all times was very low indicating that the cultures are only marginally contaminated with astrocytes. This confirms previous observations by Currie (6). Also the PAG activity assayed at a high phosphate concentration increases rapidly in the cultured cells. At a low phosphate concentration no corresponding developmental increase occurs. This means that the phosphate activation of the enzyme almost triples in the cultures between day 2 and 14, whereas no corresponding increase in phosphate activation occurs in cerebellum in vivo (Figure 1) or in cultured cerebral cortex interneurons (18) which represent a model for GABAergic neurons (43).

On the assumption that the cultured granule cells express characteristics of their in vivo counterparts a comparison of the activities of glutamate-synthesizing enzymes in the granule cells with the corresponding enzyme activities in GABAergic neurons might give information about the relative importance of these enzymes for synthesis of transmitter glutamate. In order to facilitate this comparison Table I shows in addition to the enzyme activities in the cultured granule cells the activities in cultured GABAergic cerebral cortex interneurons (18). Different enzymes such as PAG (4, 14), Orn-T (40, 41) and GOT (1) have recently been suggested as regulatory enzymes for synthesis of neurotransmitter glutamate. In addition GLDH could be important for the synthesis of transmitter glutamate. The finding that Orn-T showed only a moderately higher activity in the granule cells than in cerebellum, suggests that this enzyme is not important for synthesis of glutamate. This is in agreement with the finding by Drejer and Schousboe (9) that only GABAergic neurons possess Orn-T which exhibits a low $K_m$ value for its substrate, ornithine. Accordingly, this enzyme may rather be important for regulation of synthesis of GABA than of glutamate (37, 42). In contrast to this both GLDH and GOT exhibited higher activities in the granule cells than in the GABAergic neurons. This may accordingly suggest that any one or a combination of these enzymes could be of particular importance for the regulation of the synthesis of transmitter glutamate. The high GLDH activity disagrees with a recent report by Patel et al. (28) in which the GLDH activity was

found to be lower in granule cells than in astrocytes. A high GLDH activity in neurons is, however, in keeping with conclusions by Weil-Malherbe and Gordon (38) and Quastel (30) that glutamate dehydrogenase is associated primarily with the neurons. Whether or not the GLDH catalyzed reaction proceeds towards the production of glutamate or 2-oxoglutarate (as in astrocytes (13, 45)) remains to be established. Preliminary studies of the oxidation of [1-$^{14}$C]glutamate in granule cells via the tricarboxylic acid cycle suggest, however, that glutamate is only poorly oxidized in the granule cells (13). The high GOT activity in the granule cells compared with the GABAergic neurons may support the recent conclusion based on immunohistochemical studies (1) that GOT is associated with glutamatergic nerve endings. The high PAG activity in the cultured granule cells compared with cerebellum in vivo agrees with a similar finding by Patel et al. (28) and with the somewhat higher activity of the enzyme in synaptosomes than in astrocytes (16). The finding that the phosphate activation of the enzyme is more pronounced in the granule cells (Figure 1) than in GABAergic neurons (18) may suggest a special role for this enzyme in glutamatergic neurons.

Future studies of possible differences in the regulatory mechanisms of these enzymes combined with determinations of metabolic fluxes for glutamate and glutamine in cultured GABAergic and glutamatergic neurons may allow further conclusions as to the enzymes responsible for the regulation of amino acid neurotransmitter synthesis. In this context it appears particularly attractive to study mechanisms for PAG and Orn-T since results by Kvamme et al. (16), Wong et al. (41), Yoneda et al. (42) and Drejer & Schousboe (9) suggest that subspecies of these enzymes may exist. Primary cultures of glutamatergic neurons may well prove useful in such future studies since the developmental pattern of these neurons in culture strongly indicate that this preparation can be used to obtain results which in a meaningful way reflect the in vivo situation.

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