The Uptake of γ -Aminobutyrate by Organotypic Cultures of Chick Spinal Cord

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1. Explants of spinal cord from 10-day chick embryos maintained for up to 16 days in culture rapidly accumulated γ -amino[^3H]butyrate when incubated at 25°C or 36°C in a medium containing 50 nM γ -aminobutyrate. The mechanism of the uptake process has many of the properties of an active-transport system: it is Na^+ -dependent, temperature-sensitive, inhibited by ouabain, and displays saturation kinetics. The apparent K_m for γ -aminobutyrate is 1.7×10^{-5} M, and $V_{\max}$ is 33 pmol/min per g. 2. The rate of accumulation of γ -amino[^3H]butyrate in cultures between the ages of 3 and 16 days was remarkably constant and was not related to the morphological maturity of the spinal-cord explants. 3. The present demonstration in spinal-cord explants of an active transport system for γ -aminobutyrate, already established for non-cultured nervous tissue, means that nervous-tissue culture can provide a convenient model for studying uptake processes in the central nervous system.

Nervous tissue maintained *in vitro* normally undergoes morphological development in terms of myelination and synaptogenesis (Crain, 1966; Bunge *et al.*, 1967; Murray, 1965). Further, physiological and biochemical differentiation has been shown to occur in nervous tissue cultured over various periods of time (Crain, 1966; Hild & Tasaki, 1962; Lehrer *et al.*, 1970; Werner *et al.*, 1971; Seeds, 1971). Thus nervous tissue in culture exhibits many of the characteristics of nervous tissue *in vivo*.

Possibly one of the most characteristic biochemical properties of nervous tissue is its ability rapidly to accumulate neurotransmitter substances. Uptake mechanisms have been shown for γ -aminobutyrate (Elliott & Van Gelder, 1958; Siskin & Roberts, 1964), for glutamate and glycine (Logan & Snyder, 1971), for 5-hydroxytryptamine (Chase *et al.*, 1969) and for the catecholamines (Snyder *et al.*, 1969). If similar uptake processes could be demonstrated in cultured nervous tissue this would add validity to the study of the central nervous system *in vitro*. There is considerable evidence supporting the view that γ -aminobutyrate is an inhibitory neurotransmitter in the central nervous system (Krnjević, 1970). Consequently the present investigation was undertaken to determine if there is an active uptake system for γ -aminobutyrate in cultured organotypic explants of chick spinal cord, and if so, whether it could be related to the morphological development of the cultures. Spinal cord was chosen for this investigation, since several studies have measured the development of neurotransmitter enzymes in cultures identical with those described in the present paper. Tunnichliff

& Kim (1973) observed an increase in the activity of both L-aromatic amino acid decarboxylase and catechol O-methyltransferase that paralleled the morphological maturation of the explants *in vitro* measured as myelination and synaptogenesis. In addition, choline acetyltransferase and acetylcholinesterase activities have been shown to increase over a 2-week period in cultures similar to those employed in the present study (Kim *et al.*, 1972; T. Oh, personal communication).

Methods

Tissue culture

The spinal cord was taken from 10-day-old White Leghorn chick embryos, freed of meninges in Hanks' balanced salt solution (Hanks & Wallace, 1949) and sectioned in slices of $1\text{ mm}^2 \times 0.5\text{ mm}$. Three explants were placed on each collagen-coated cover-slip ($11\text{ mm} \times 22\text{ mm}$), which was then put into a test tube to which was added 0.7 ml of culture medium [equal parts of horse serum, medium 199 (Grand Island Biological Co., Grand Island, N.Y., U.S.A.), Hanks' balanced salt solution and supplementary glucose at 600 mg/100 ml of medium]. The test tubes were left in a static position for 24 h at 36°C before being placed in a roller-drum turning at 10 rev/h. The culture nutrient medium was renewed once a week, and sample explants were examined regularly under a light-microscope with a Zeiss 40 $\times$ oil-immersion objective. After 13 days of culture *in vitro*, 10-20 cultures were fixed and stained by a modified

Bodian method (Kim, 1970) for determination of their morphological development.

Uptake of γ -amino[^3H]butyrate by cultured explants

This method is essentially that of Iversen & Neal (1968). After the explants had been maintained in culture for the required period of time (3–16 days) cultures on six cover-slips were placed in a 10 ml staining jar together with one that had been immersed in boiling water for 1 min to act as a tissue blank. The cultures were then washed three times with 10 ml of Krebs–Ringer buffer (see below). The explants were incubated in 5 ml of oxygenated Krebs–Ringer buffer under an atmosphere of O_2 at 25°C for 15 min. The buffer was then aspirated from the staining jar and replaced by 5 ml of identical buffer at 25°C except that it contained 50 nM γ -amino[^3H]butyrate. The incubation was continued under O_2 for a further 15 min. The reaction was stopped by aspirating off the incubation medium and washing the cultures three times with 10 ml of Krebs–Ringer buffer. Each cover-slip was transferred to a counting vial and 2 ml of 95% (v/v) ethanol added. After the ethanol had been evaporated off by using a 90°C water bath, 0.5 ml of the tissue solubilizer NCS (obtained from Amersham–Searle, Arlington Heights, Ill., U.S.A.) and 1 ml of water were added. Digestion of the cultures was allowed to proceed for 12 h. Finally 10 ml of Bray's (1960) scintillation fluid was added before counting of the ^3H radioactivity in a Nuclear–Chicago Isocap/300 liquid-scintillation counter.

The results are expressed on a per-culture basis, 'per culture' referring to the three explants on each cover-slip. The wet weight of nervous tissue was 2 ± 0.36 mg whenever random samples of three explants were weighed.

Materials

γ -Amino[2,3- ^3H]butyrate (specific radioactivity 1 Ci/mmol) was obtained chromatographically pure from New England Nuclear Corp., Boston, Mass., U.S.A.

Incubation medium. Krebs–Ringer buffer contained the following: 118.5 mM-NaCl; 4.75 mM-KCl; 1.8 mM- CaCl_2 ; 1.2 mM- MgSO_4 ; 16 mM-sodium phosphate buffer, (pH 7.4); D-glucose, 1 g/l.

Results

Morphological development of the cultures

Explants that were examined under the microscope after 13 days culture *in vitro* after fixing and staining showed considerable maturity as regards myelination and overall appearance (Plate 1). Thus we felt justified in employing 13-day cultures for most of the following experiments.

Uptake and metabolism of γ -amino[^3H]butyrate as a function of time

The time-course of γ -aminobutyrate uptake by 13-day cultures at 25°C in the presence of 50 nM γ -amino[^3H]butyrate is illustrated in Fig. 1. The rapid uptake was linear with time up to 30 min, and then the rate of accumulation of γ -amino[^3H]butyrate gradually decreased during the final 90 min, when the tissue/medium ratio was 50:1 (nCi of γ -amino[^3H]butyrate/g of tissue : nCi of γ -amino[^3H]butyrate/ml of medium). A very similar pattern of uptake was observed with 7-day explants.

In one experiment 13-day cultures from 15 cover-slips were incubated for 15 min at 25°C in the presence of 50 nM γ -amino[^3H]butyrate. The cultures were homogenized in 100 μl of 0.1 M-HCl and 50 μl of 10% (w/v) trichloroacetic acid was added. After centrifugation, the supernatant was evaporated to dryness in a freeze-drier and the residue redissolved in 10 μl of 80% (v/v) ethanol. This extract was applied to a thin-layer plate with a cellulose support and the chromatogram developed in a solvent system of ethanol–5.1 M- NH_3 (7:3, v/v). Finally the ^3H -labelled spots were located by dividing the plate into a grid before scraping off the cellulose, extracting the radioactive intermediates with 1 ml of methanol and then adding 15 ml of 0.3% (w/v) 2,5-diphenyloxazole and 0.1% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene and counting the radioactivity in a Nuclear–Chicago Isocap/300 liquid-scintillation counter.

Fig. 1. Time-course of γ -aminobutyrate uptake

For details see the Methods section. Each point corresponds to the mean of six determinations. The vertical bars represent the S.E.M. The tissue/medium ratio is measured as (nCi of γ -amino[^3H]butyrate/g of tissue)/(nCi of γ -amino[^3H]butyrate/ml of medium).

EXPLANATION OF PLATE I

Morphological development of explants after 13 days in culture

For details see the Methods section. The bars indicate a scale of $30\mu\text{m}$. (a) Four large anterior motor neurons; (b) myelinated fibres; (c) a multipolar anterior motor neuron.

γ -Aminobutyrate was identified on the plate by comparing its R_F value with that of authentic γ -amino- $[^3\text{H}]$ butyrate. We found that over 90% of the total radioactivity was in the form of γ -amino $[^3\text{H}]$ -butyrate.

In another preliminary experiment 13-day cultures were incubated in the presence and absence of amino-oxyacetic acid at a concentration of $10\mu\text{M}$. This compound has been used in γ -aminobutyrate-uptake studies since it is known to inhibit γ -aminobutyrate- α -oxoglutarate transaminase (EC 2.6.1.19) (Wallach, 1961), the enzyme responsible for γ -aminobutyrate catabolism. We found that amino-oxyacetic acid had no effect on γ -aminobutyrate uptake and therefore we did not use this compound in any further experiments.

Temperature and γ -amino $[^3\text{H}]$ butyrate uptake

In order to study the effects of temperature on the uptake process, 13-day cultures were incubated in the presence of γ -amino $[^3\text{H}]$ butyrate at 4°C , 15°C , 25°C and 36°C for up to 20 min. We observed a direct relationship between the rate of accumulation and the temperature of the incubation medium (Fig. 2).

The calculated activation energy of the uptake system was only 15070J/mol (Fig. 3) when calculated from plots of $\log v$ against the reciprocal of absolute temperature.

Fig. 2. Effects of temperature on γ -aminobutyrate uptake

For details see the Methods section. Each point corresponds to the mean of six determinations. The vertical bars represent the S.E.M.

Effects of Na^+ and γ -aminobutyrate

Incubations of 13-day cultures were carried out in Krebs-Ringer buffer that contained, in addition to 50nm - γ -amino $[^3\text{H}]$ butyrate, 50nm -Tris-HCl buffer. The Na^+ content of the medium was regulated by the addition of various proportions of NaCl. The Cl^- content was kept constant by adding appropriate amounts of choline chloride. In this way the Na^+ content was altered for both the first 15 min incubation and for the incubation in the presence of labelled γ -aminobutyrate. The rate of γ -amino $[^3\text{H}]$ butyrate uptake increased with an increasing concentration of Na^+ in the incubation medium (Fig. 4).

Some 13-day cultures were also incubated at 25°C for 15 min in a medium containing 50nm - γ -amino- $[^3\text{H}]$ butyrate together with various amounts of non-radioactive γ -aminobutyrate to give final amino acid concentrations from $1\mu\text{M}$ to $100\mu\text{M}$. The values of uptake obtained were found to lie on a straight line when plotted by the method of Lineweaver & Burk (1934) (Fig. 5). The apparent K_m for γ -aminobutyrate uptake was $17 \times 10^{-5}\text{M}$ and the $V_{\max}$ was calculated as 33pmol/min per g (assuming that one culture weighs 2mg).

Effects of metabolic inhibitors

Cultures were preincubated in the presence of $10\mu\text{M}$ -ouabain for 15 min and incubated for a further 15 min in the presence of the inhibitor together with 50nm - γ -amino $[^3\text{H}]$ butyrate. The ouabain produced a 26% inhibition of uptake (Table 1). However, under

Fig. 3. Activation energy of γ -aminobutyrate accumulation

These values are plotted from the values obtained in Fig. 2 at 20 min.

Fig. 4. γ -Aminobutyrate uptake as a function of Na^+ concentration in the medium

For details see the text. Each point corresponds to the mean of six determinations. The vertical bars represent the S.E.M. The Na^+ content was varied by the addition of different amounts of NaCl . The Cl^- content was kept constant by the addition of appropriate amounts of choline chloride.

Fig. 5. Lineweaver-Burk plot of the rate of uptake against γ -aminobutyrate concentration

For details see the text. Each point corresponds to the mean of six determinations. The amino acid content of the medium was regulated by adding non-labelled γ -aminobutyrate to give a final concentration range of $1\text{ }\mu\text{M}$ – $100\text{ }\mu\text{M}$.

anoxic conditions where the Krebs-Ringer buffer had been pre-gassed with N_2 and the incubation done under N_2 , no inhibition of uptake was observed (Table 1).

Further, in an experiment where cultures were incubated under hyperbaric O_2 at a pressure of 620 kPa (90 lb/in^2) for 15 min, no differences were observed in the rate of γ -aminobutyrate uptake compared with controls incubated at ambient atmospheric pressure (Table 1).

γ -Aminobutyrate uptake in relation to age of cultures

Explants that had been cultured for 3, 7, 10, 13 and 16 days were incubated for 15 min in the presence of 50 mM - γ -amino[^3H]butyrate at 25°C . The rate of uptake of γ -aminobutyrate showed no statistical difference between any of the age groups (Table 2).

Discussion

Many reports exist on the active uptake of putative neurotransmitters by the central nervous system. However, the present paper reports for the first time kinetic data on such a transport system in nervous-tissue culture. Independent studies by Lasher & Zagon (1972) and Sotelo *et al.* (1972) indicate that tissue cultures of rat cerebellum have the ability to accumulate γ -amino[^3H]butyrate, but no kinetic data were

Table 1. Effects of ouabain, anoxia and hyperbaric oxygen on γ -aminobutyrate uptake

Uptake is expressed as pmol/culture ($\pm\text{S.E.M.}$). Six cultures were used for each determination. For details see the Methods section. Concentration of ouabain was $10\text{ }\mu\text{M}$. Anoxic conditions were produced by pre-gassing the incubation medium with N_2 and carrying out the experiment under a flow of N_2 , whereas the control was done under a flow of O_2 . The pressure of hyperbaric oxygen was 620 kPa (90 lb/in^2).

Treatment	Uptake of γ -aminobutyrate	
	(pmol/culture)	(% of control)
Control	0.848 ± 0.012	100
+Ouabain	0.625 ± 0.009	73.7
Anoxia	0.838 ± 0.085	98.8
Hyperbaric oxygen	0.845 ± 0.015	99.6

Table 2. Uptake of γ -aminobutyrate in cultures aged 3–16 days

Uptake is expressed as pmol/culture ($\pm\text{S.E.M.}$). Ten cultures were used for each determination. For details see the Methods section.

Age of cultures (days)	...	3	7	10	13	16
Uptake of γ -aminobutyrate		0.902 ± 0.017	0.854 ± 0.026	0.877 ± 0.022	0.911 ± 0.010	0.865 ± 0.011

included in either report. There have been several studies showing that nervous tissue can accumulate γ -aminobutyrate from an external medium (Elliott & Van Gelder, 1958; Blasberg & Lajtha, 1965; Weinstein *et al.*, 1965; Iversen & Neal, 1968). Under our experimental conditions, spinal-cord explants cultured for 13 days displayed a pronounced ability to take up γ -amino[^3H]butyrate from a medium containing a low concentration of the amino acid (50 nM) at 25°C. Moreover, we could find no evidence of metabolism of the labelled substrate for 15 min after incubation. The system in our spinal-cord explants that accumulates the γ -aminobutyrate from the medium exhibits many characteristics of an active-transport process. In addition to a γ -aminobutyrate tissue/medium ratio of 50:1 after a 2 h incubation, it shows saturation kinetics, Na^+ -dependency and temperature-sensitivity and is inhibited by ouabain. It could be argued that the increase in radioactivity that we find in the cultures after incubation with γ -amino[^3H]butyrate is due to an exchange of labelled amino acid with the endogenous pool. Yet the concentration of γ -aminobutyrate in spinal cord is between 0.4 and 2.3 $\mu\text{mol/g}$ (Davidoff *et al.*, 1967) compared with the concentration in the external medium of only 50 pmol/ml. Consequently even an exchange of a molecule of γ -amino[^3H]butyrate for a molecule of endogenous γ -aminobutyrate must entail active uptake.

In spite of having shown that cultures of spinal-cord tissue are able to accumulate γ -aminobutyrate by a process displaying many properties of an active uptake system, we could not demonstrate that there was a relationship between the morphological maturity of the explants and the rate of uptake (Table 2). There was, instead, a remarkable consistency in the amount of γ -aminobutyrate accumulated by cultures from 3 days to 16 days of age. We do know that the cultures had undergone considerable morphological development *in vitro* in terms of myelination, by the 13th day (Plate 1). Moreover, in cultures identical with those employed in the present experiments, we have shown that synapse formation occurs by the 7th day (Tunnicliff & Kim, 1973). Since γ -aminobutyrate has been strongly implicated as an inhibitory synaptic transmitter (Krnjević, 1970), it could reasonably be expected that the rate of uptake of this amino acid by nervous tissue would increase in parallel with an increase in the development of synapses. This would be in line with various reports describing increased neurotransmitter enzyme activity in cultured nervous tissue (Seeds, 1971; Werner *et al.*, 1971; Kim *et al.*, 1972). That we find no similar increase in the uptake process of γ -aminobutyrate, which is probably the main inactivation mechanism for this neurotransmitter (Iversen & Neal, 1968), might indicate that this amino acid does not have as important a function in spinal cord as it appears to

have in the brain. Indeed, Curtis & Johnston (1970) have suggested that glycine has the major role of inhibitory transmitter in the spinal cord. Further, we have measured L-glutamate decarboxylase (EC 4.1.1.15) activity in maturing cultures of spinal cord and found no evidence of an increase in the enzyme over a 21-day period even though the activity of other neurotransmitter enzymes did increase (Tunnicliff & Kim, 1973). Iversen & Johnston (1971) have also demonstrated that spinal cord possesses an active transport system for γ -aminobutyrate, yet these observations do not necessarily imply that γ -aminobutyrate is an inhibitory transmitter in spinal cord. There have been previous occasions where workers have found a lack of relationship between γ -aminobutyrate function and uptake. Bowery & Brown (1972) claimed that sympathetic ganglia have the ability to accumulate γ -aminobutyrate even though they contain virtually no endogenous γ -aminobutyrate, L-glutamate decarboxylase or γ -aminobutyrate- α -oxoglutarate transaminase (Salvador & Albers, 1959; Nagata *et al.*, 1966).

The present results indicate that nervous-tissue culture offers possibilities for the study of neurotransmitter uptake processes in the central nervous system, even though they do not support the idea of γ -aminobutyrate playing a major role in spinal-cord function.

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