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Subcellular Localization of γ-Aminobutyrate Transaminase and Glutamate Dehydrogenase in Adult Rat Brain

EVIDENCE FOR AT LEAST TWO SMALL GLUTAMATE COMPARTMENTS IN BRAIN

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The subcellular localizations of γ-aminobutyrate transaminase (EC 2.6.1.19) and glutamate dehydrogenase (EC 1.4.1.2) in brain tissue of adult rats were compared with each other and with those of NAD^+–isocitrate dehydrogenase (EC 1.1.1.41) and monoamine oxidase (EC 1.4.3.4; kynuramine as substrate). Crude mitochondrial fractions from brain tissue were centrifuged in continuous sucrose density gradients. γ-Aminobutyrate transaminase and glutamate dehydrogenase were always found at a higher density than NAD^+–isocitrate dehydrogenase and monoamine oxidase. When centrifuged for 1 h at $53000 g_{av.}$, there was a slight difference between the distribution profiles of glutamate dehydrogenase and γ-aminobutyrate transaminase. This difference was larger when the centrifugation time was only 15 min. It is concluded that there are subpopulations of brain mitochondria with differing proportions of γ-aminobutyrate transaminase and glutamate dehydrogenase. The results are discussed in relation to evidence obtained with labelled precursors *in vivo* that there are at least two small glutamate compartments in adult brain.

Compartmentation of glutamate metabolism in brain has been observed by many authors (for reviews, see Berl & Clarke, 1969; Balázs *et al.*, 1973; Van den Berg, 1973; Van den Berg *et al.*, 1975). Various models, differing in detail but basically similar, have been proposed to account for the incorporation patterns found for a number of precursors into glutamate and related amino acids in mammalian brain. A model was proposed to account for data obtained with specifically labelled glucose and acetate in the mouse brain, consisting of two tricarboxylic acid cycles connected by a flow of γ-aminobutyrate in one direction and a flow of glutamine in the opposite direction (Van den Berg *et al.*, 1969; Van den Berg & Garfinkel, 1971). The carbon skeleton of γ-aminobutyrate is converted into succinate by the combined action of γ-aminobutyrate transaminase and succinic semialdehyde dehydrogenase (EC 1.2.1.16) and the amino group is transferred to α-oxoglutarate. The entry of γ-aminobutyrate is therefore directly coupled with the production of glutamate. As there is no change in the concentration of the combined tricarboxylic acid-cycle intermediates or related compounds in the steady-state situation that applies to all these experiments, the glutamate formed must be removed. In the model proposed this glutamate is converted into glutamine and transported to the site of synthesis of γ-aminobutyrate. That there is indeed this coupling of γ-aminobutyrate degradation with glutamate–glutamine synthesis has been shown by an analysis of the effects of amino-oxyacetic acid and ammonia on the concentrations of amino acids in brain (Van den Berg & Matheson, 1975).

The compartmentation of glutamate metabolism in brain was initially proposed on the basis of experiments with labelled glutamate (Berl *et al.*, 1961) and [^{15}N]ammonium acetate (Berl *et al.*, 1962*a*,*b*). ^{15}N from [^{15}N]ammonium acetate was found to be preferentially incorporated into the amino group of glutamine compared with the amino group of glutamate. This was observed both in the cat brain (Berl *et al.*, 1962*a*) and in the rat brain (Tsukada, 1966). Since ammonia is incorporated into an amino group by the action of glutamate dehydrogenase, this enzyme can be expected to play a role in the formation of glutamate in the compartment in which glutamine is made in large amounts. This compartment is generally called the small glutamate compartment, i.e. a metabolic region or space in the brain in which precursors are incorporated more extensively into glutamine than into glutamate.

γ-Aminobutyrate has been shown to be a precursor of this small glutamate compartment, since with

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this precursor there is a greater and a more rapid incorporation into glutamine than glutamate (Baxter, 1968; Balázs *et al.*, 1970). γ-Aminobutyrate transaminase is, of course, the first enzyme involved in the degradation of γ-aminobutyrate in the brain.

When crude mitochondrial fractions from adult brain are subjected to density-gradient fractionation in sucrose density gradients, it has been observed that glutamate dehydrogenase and γ-aminobutyrate transaminase sediment at higher densities than citrate synthase (EC 4.1.3.7), NAD^+-isocitrate dehydrogenase, monoamine oxidase and glutaminase (EC 3.5.1.2) (Salganicoff & De Robertis, 1965; Van Kempen *et al.*, 1965; Neidle *et al.*, 1969; Blokhuis & Veldstra, 1970). Evidently, the mitochondria containing two of the enzymes characteristic of the small compartment can be partially separated from the bulk of the brain mitochondria.

There are data available suggesting that the small compartment in brain is metabolically unhomogeneous. It has been shown, for example, that γ-aminobutyrate and propionate, both precursors of the small compartment on the basis of their labelling pattern of glutamate and glutamine, do not label aspartate to the same extent (Baxter, 1968; Balázs *et al.*, 1970; O'Neal *et al.*, 1966); γ-aminobutyrate labels aspartate to a greater extent than does propionate. Further, there is theoretically no need for glutamate dehydrogenase to play a role in the metabolism of γ-aminobutyrate. If it can be shown that glutamate dehydrogenase and γ-aminobutyrate transaminase are not present in the same proportion in the same subpopulation(s) of brain mitochondria, this finding could lend support to the hypothesis that the small compartment is metabolically heterogeneous. Therefore we made a detailed investigation of the rate of sedimentation of mitochondrially bound glutamate dehydrogenase and γ-aminobutyrate transaminase from adult brain. We found that these two enzymes have indeed different rates of sedimentation, indicating that the mitochondria in which they are located differ in one or more properties determining sedimentation behaviour. In addition, a comparison was made of the sedimentation behaviour of these enzymes between crude mitochondrial fractions derived from the cerebrum with those of the cerebellum to see whether the degree of heterogeneity is dependent on the anatomical structures from which these mitochondria are derived.

Materials and Methods

Chemicals

All chemicals were of analytical grade. Solutions were made in demineralized glass-distilled water. Sucrose solutions were made immediately before use and adjusted to pH 7.0–7.4 with solid Tris.

Methods

Preparation of the homogenates. Rats (male Wistar, 2–3-months-old, obtained from the Department of Pharmacology, University of Leiden) were killed between 9 and 10 a.m. by decapitation, and two cerebral hemispheres or five cerebella were removed, weighed and homogenized in ice-cold 0.32 M-sucrose (20 vol./g wet wt.) in a Potter–Elvehjem homogenizer (Braun, Melsungen, Germany; model C, Teflon pestle, clearance 0.25 mm), operated at 1000 rev./min.

Primary fractionation. Brain homogenates were separated into four primary fractions by the differential centrifugation method of De Robertis *et al.* (1962). The homogenate, usually 12 ml, was centrifuged for 10 min at $810g_{av.}$ at 4°C in an Eispyrouette (Phywé, Göttingen, Germany). The pellet was washed once to give the crude nuclear fraction. The combined supernatants were centrifuged for 20 min at $11\,500g_{av.}$ to give the crude mitochondrial fraction; the resulting supernatant was centrifuged for 60 min at $92\,000g_{av.}$ in a Spinco L centrifuge to give the crude microsomal fraction and the final supernatant (cytosol).

Sucrose-density-gradient centrifugation. Sucrose density gradients were prepared by the procedure of Peterson & Sober (1959). For 1 h centrifugation experiments a two-step gradient was used, 2 ml over the range 0.8–1.0 M-sucrose and 36 ml over the range 1.0–1.6 M-sucrose. For 15 min centrifugation experiments a 38 ml linear gradient of 0.8–1.4 M-sucrose was used.

The crude mitochondrial fraction obtained by differential centrifugation was homogenized by hand in 0.8 M-sucrose (5 vol./g of original tissue). The gradient was loaded with 0.75 ml of this crude mitochondrial suspension, followed by 0.5 ml of 0.32 M-sucrose. This whole procedure was carried out in less than 15 min at 4°C. Centrifugation was carried out in a Spinco L2-65 or L2-65B centrifuge with the SW 27 rotor for 15 min or 1 h at 20 000 rev./min ($53\,000g_{av.}$) at a temperature of 3–4°C. This velocity was chosen to give hydrostatic pressures and gravitational fields similar to those used previously (Van Kempen *et al.*, 1965). The hydrostatic pressures in the gradient are probably below the values that would result in damage to the particles (Wattiaux *et al.*, 1971; Reijnierse, 1973). At the end of a centrifugation run 18 fractions of 2 ml each and the top remaining fraction were collected from each gradient with a peristaltic pump fitted with a glass capillary tube, starting in the high-sucrose region. The sucrose concentration in each of the fractions was measured with an Abbe refractometer.

Enzyme assays. Tissue preparations, except for monoamine oxidase, were pretreated with 0.5% (v/v) Triton X-100 for 30 min at 4°C before assay.

(1) Lactate dehydrogenase. This was assayed as described by Kornberg (1955). The 3.0ml incubation mixture included 100mM-potassium phosphate buffer, pH7.4, 71μM-NADH, 333μM-sodium pyruvate and a suitably diluted enzyme preparation. The oxidation of NADH was followed at room temperature (about 20°C) by measuring the E_{340} decrease on a Gilford 2400 spectrophotometer.

(2) Glutamate dehydrogenase. This was measured by a modification of Kammeraat's (1966) method. The reaction was followed in the direction of glutamate synthesis by measuring the decrease in NADH. The incubation mixture of 1.5ml consisted of 100mM-potassium phosphate buffer, pH7.7, 0.1% bovine serum albumin, 50μM-NADH, 0.6mM-α-oxoglutarate, 50μM-ADP, 40mM-$(NH_4)_2SO_4$, 3mM-Amytal and a suitably diluted enzyme preparation. The pH values of the $(NH_4)_2SO_4$ and α-oxoglutarate solutions were adjusted with alkali to 7.7 and that of Amytal to a pH between 9.5 and 10.0. Standards containing 10 or 50μM-NADH and water to replace α-oxoglutarate were always included. The reaction was allowed to proceed for 30min at 25°C and was stopped by heating for 5min at 100°C. After cooling, the tubes were centrifuged at 1400g for 15min. The supernatants were incubated for 10min at 20°C and the NADH remaining was measured fluorimetrically with an Aminco–Bowman spectrophotofluorimeter. The excitation and emission wavelengths were 346nm and 462nm respectively (both uncorrected). If more than half of the NADH had disappeared the reaction was repeated with a more diluted enzyme preparation.

(3) NAD^+–isocitrate dehydrogenase. This was measured with a modification of Goebell & Klingenberg's (1964) method. The reaction was in the direction of α-oxoglutarate formation; the NADH formed was measured fluorimetrically as described for glutamate dehydrogenase. The 1.5ml incubation mixture consisted of 100mM-potassium phosphate buffer, pH7.6, 3mM-NAD^+, 8mM-$MgCl_2$, 3mM-ADP, 3mM-trisodium D,L-isocitrate and a suitably diluted enzyme preparation. Standards containing 2.5 or 25μM-NADH replacing isocitrate were always included. The reaction was allowed to proceed for 30min at 37°C.

(4) γ-Aminobutyrate transaminase. This was assayed by a modification of a procedure described earlier (Van Kempen, 1964; Van den Berg & Van Kempen, 1964). The quinaldine derivative formed by the reaction of succinic semialdehyde with 3,5-diaminobenzoic acid was measured fluorimetrically in an Aminco–Bowman spectrophotofluorimeter. The excitation and emission wavelengths were 406nm and 507nm respectively (both uncorrected). The 1.0ml incubation mixture consisted of 50mM-Tris–HCl buffer, pH8.2, 250mM-γ-aminobutyrate, pH8.2, 250mM-α-oxoglutarate, pH8.2 and a suitably diluted enzyme preparation. Blanks contained enzyme solutions inactivated by heating for 5min at 100°C. Standards contained different amounts of succinic semialdehyde replacing the enzyme solution. The reaction proceeded for 45min at 37°C and was stopped by heating for 5min at 100°C. To each sample was then added 1.5ml of a solution made by mixing 2vol. of 250mM-3,5-diaminobenzoic acid, adjusted to pH6.0 with K_2CO_3, with 1vol. of 500mM-potassium phosphate buffer, pH5.8. After mixing, the tubes were incubated for 60min at 60°C and, after cooling, centrifuged at 1400g for 15min. A 0.2ml sample of the supernatant was added to 1.8ml of a 10mM-potassium phosphate buffer, pH6.0. The fluorescence was then measured. Succinic semialdehyde was synthesized by the method of Langheld (1909*a*,*b*), as modified by Kammeraat & Veldstra (1968). Pure succinic semialdehyde synthesized by the procedure of Schenck (1953) was used as an absolute standard. This was a gift from Dr. G. M. J. Van Kempen, Endegeest, Oegstgeest, The Netherlands.

(5) Monoamine oxidase. Monoamine oxidase was measured by a modification of the method of Kraml (1965). 4-Hydroxyquinoline, the product of spontaneous cyclization of the aldehyde formed by the oxidative deamination of kynuramine, was measured fluorimetrically. The 1.5ml incubation mixture consisted of 33mM-sodium borate–HCl buffer, pH8.2, 0.2mM-EDTA, 50μM-kynuramine and enzyme solution. Controls contained water replacing kynuramine. Standards contained the buffer, EDTA and different 4-hydroxyquinoline concentrations. After incubation for 30min at 37°C 0.5ml of 4M-NaOH was added. After another incubation of 10min in a 20°C water bath, the fluorescence was measured in an Aminco–Bowman spectrophotofluorimeter at excitation and emission wavelengths of 318nm and 386nm respectively (both uncorrected).

Presentation of data

Activities were expressed as percentages of the total recovered activities. As there are always slight differences between gradients prepared on different occasions, we have used the following procedure to average the results of duplicate experiments. For each of the gradients analysed the percentage activity for each enzyme measured was plotted against sucrose concentration. The points were connected by straight lines. The curves obtained were divided into intervals of 0.025M-sucrose, starting at 1.0M. At these intervals the percentage activity was read for each of the curves and the means were obtained; for each curve the S.E.M. values are given. If there was a large change in the slope within a 0.025M-sucrose interval, the procedure

was repeated for a 0.0125 M-sucrose interval. All the enzymes represented in the figures were assayed in the same gradient. This is essential for the proper interpretation of small systematic differences in sedimentation patterns of particles in density gradients. Ideally one would analyse the data quantitatively by using methods devised for the analysis of properties of particles sedimenting in a gravitational and density field (de Duve *et al.*, 1959). However, such an analysis is only possible when the particles studied are reasonably homogeneous, which in the present case they are not. Instead of this type of quantitative analysis we are therefore very much dependent on visual-pattern analysis.

Results

Distribution of γ-aminobutyrate transaminase and glutamate dehydrogenase in density gradients of crude mitochondrial fractions from the cerebral hemispheres (centrifugation time 1 h)

The distribution profiles of γ-aminobutyrate transaminase, glutamate dehydrogenase, lactate dehydrogenase, NAD^+-isocitrate dehydrogenase and monoamine oxidase obtained when crude mitochondrial fractions of the cerebral hemispheres were centrifuged for 1 h at 53000$g_{av.}$ are presented in Fig. 1. As shown by the distribution of lactate dehydrogenase, the nerve endings (synaptosomes) are clearly separated from the bulk of the mitochondria. This is commonly observed in this type of gradient (Johnson & Whittaker, 1963; Salganicoff & De Robertis, 1965; Van Kempen *et al.*, 1965). None of the four mitochondrial enzymes (glutamate dehydrogenase, γ-aminobutyrate transaminase, NAD^+-isocitrate dehydrogenase and monoamine oxidase) showed an identical sedimentation profile, confirming previously reported findings (Salganicoff & De Robertis, 1965; Neidle *et al.*, 1969; Blokhuis & Veldstra, 1970). Glutamate dehydrogenase and γ-aminobutyrate transaminase were found to sediment at higher sucrose densities than NAD^+-isocitrate dehydrogenase and monoamine oxidase, and glutamate dehydrogenase was found at a somewhat higher density than γ-aminobutyrate transaminase (Fig. 1*b*). As it has been observed that these last two enzymes showed an almost identical distribution at a longer centrifugation time (Salganicoff & De Robertis, 1965), we decided to study the distribution of these two enzymes at a centrifugation time of only 15 min.

Distribution of mitochondrial enzymes after a centrifugation time of 15 min

The distribution profiles for the same enzymes found after centrifuging in gradients (0.8–1.4 M-sucrose) for 15 min are presented in Fig. 2. Most of the nerve endings (lactate dehydrogenase) have not yet entered the gradient and formed a peak. Most of the activity of the four mitochondrial enzymes

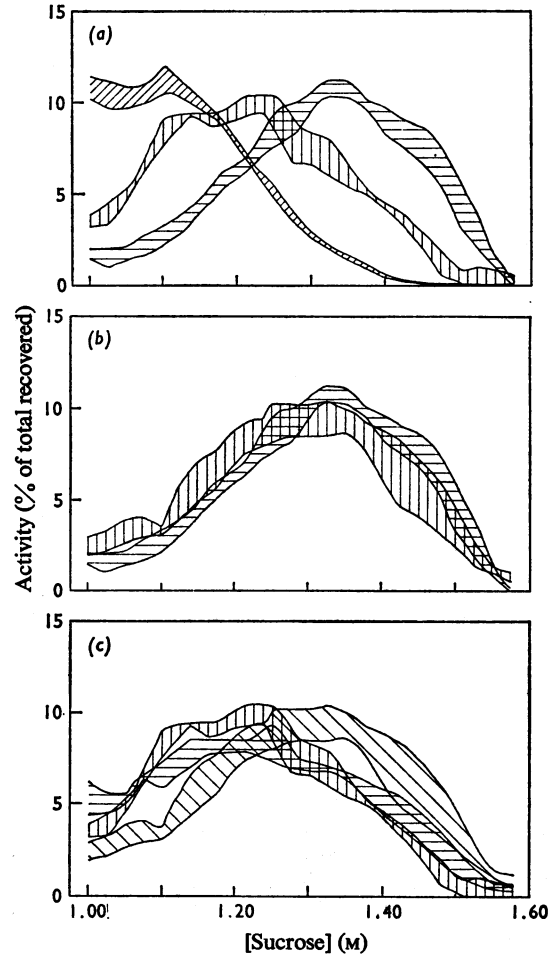

Fig. 1. *Distribution of NAD^+-isocitrate dehydrogenase, glutamate dehydrogenase, γ-aminobutyrate transaminase and monoamine oxidase in sucrose density gradients after 1 h centrifugation*

Crude mitochondrial fractions from adult rat cerebrum were centrifuged for 1 h at 53000$g_{av.}$. The averaged results of three separate experiments are represented, as described under 'Methods'. The average total recoveries were: NAD^+-isocitrate dehydrogenase, 87%; glutamate dehydrogenase, 95%; lactate dehydrogenase, 96%; γ-aminobutyrate transaminase, 101%; monoamine oxidase, 78%. (*a*) NAD^+-isocitrate dehydrogenase, ▥; glutamate dehydrogenase, ▤; lactate dehydrogenase, ■. (*b*) Glutamate dehydrogenase, ▤; γ-aminobutyrate transaminase, ▥. (*c*) NAD^+-isocitrate dehydrogenase, ▥; monoamine oxidase, ▤; γ-aminobutyrate transaminase, ■.

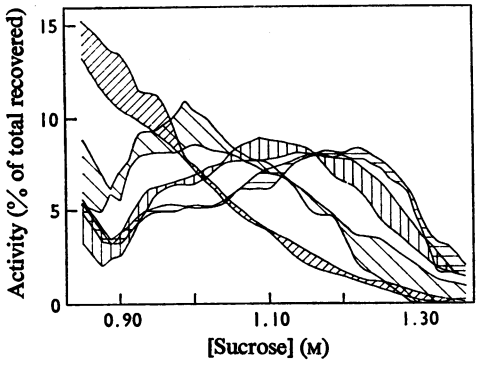

Fig. 2. *Distribution of NAD⁺–isocitrate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase and γ-aminobutyrate transaminase in sucrose-density gradients after 15 min centrifugation*

Crude mitochondrial fractions from adult rat cerebrum were centrifuged for 15 min at 53000g_{av}. The averaged results of three separate experiments are presented. The average total recoveries were: NAD⁺–isocitrate dehydrogenase 68%, glutamate dehydrogenase 87%, lactate dehydrogenase 99% and γ-aminobutyrate transaminase 76%. Explanation of the symbols: NAD⁺–isocitrate dehydrogenase, ■; glutamate dehydrogenase, ▤; lactate dehydrogenase, ■; γ-aminobutyrate transaminase, ▥.

has already penetrated the gradient, the distribution curves observed still being fairly flat. However, glutamate dehydrogenase and γ-aminobutyrate transaminase were already separated from NAD⁺–isocitrate dehydrogenase and monoamine oxidase. The separation between glutamate dehydrogenase and γ-aminobutyrate transaminase was larger with these centrifugation conditions than that found with centrifugation of longer duration. Evidently, mitochondria enriched in glutamate dehydrogenase sedimented at a higher rate than mitochondria enriched in γ-aminobutyrate transaminase.

Distribution of the enzymes in preparations derived from the cerebellum

A few experiments were carried out with crude mitochondrial preparations from the cerebellum with centrifugation time of 1 h. The results are presented in Fig. 3. Generally, the distribution curve for each enzyme was sharper in the cerebellar than in the cerebral preparations, suggesting a smaller degree of heterogeneity for the cerebellum-derived particles in which these enzymes are localized. As in the cerebrum, glutamate dehydrogenase and γ-aminobutyrate transaminase were observed to be separated from NAD⁺–isocitrate dehydrogenase and monoamine oxidase. There was some indication of a

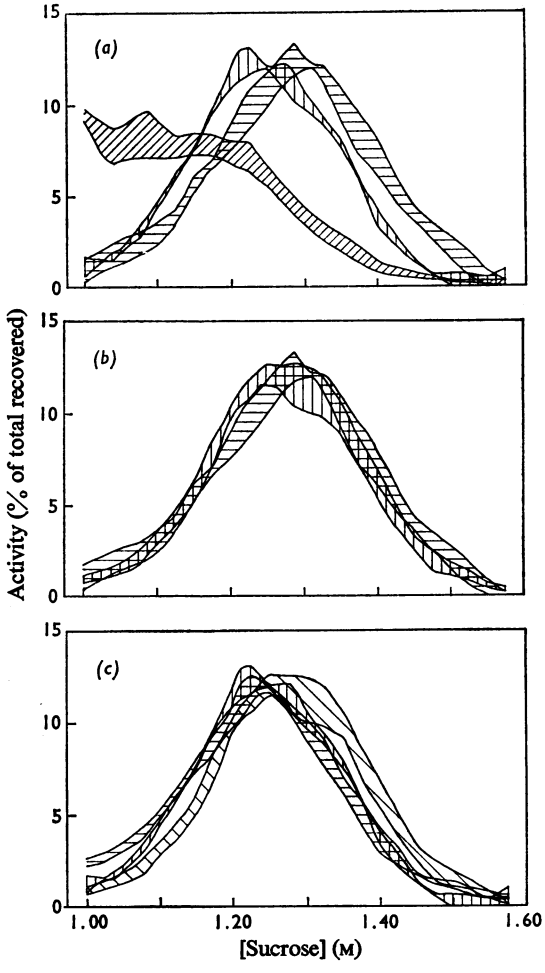

Fig. 3. *Distribution of NAD⁺–isocitrate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase, γ-aminobutyrate transaminase and monoamine oxidase in sucrose density gradients of preparations from adult rat cerebellum after 1 h centrifugation*

Crude mitochondrial fractions from adult rat cerebellum were centrifuged for 1 h at 53000$g_{av.}$. The averaged results of three separate experiments are presented. The average total recoveries were: NAD⁺–isocitrate dehydrogenase 88%, glutamate dehydrogenase 92%, lactate dehydrogenase 102%, γ-aminobutyrate transaminase 99% and monoamine oxidase 90%. (*a*) NAD⁺–isocitrate dehydrogenase, ▥; glutamate dehydrogenase, ▤; lactate; dehydrogenase, ■. (*b*) Glutamate dehydrogenase, ▤; γ-aminobutyrate transaminase, ▥. (*c*) NAD⁺–isocitrate dehydrogenase, ▥; monoamine oxidase, ▤; γ-aminobutyrate transaminase, ■.

difference in distribution between glutamate dehydrogenase and γ-aminobutyrate transaminase, but this was less than that observed in the cerebrum.

Discussion

Heterogeneity of mitochondria from brain

Since De Robertis *et al.* (1962) and Gray & Whittaker (1962) showed with density-gradient-centrifugation methods that the crude mitochondrial fraction from brain was extremely heterogeneous with respect to its particle composition, a great deal of attention was paid to a characterization of myelin, nerve endings (synaptosomes) of many types, mitochondria and lysosomes. Most attention was given understandably to a detailed investigation of various properties of nerve endings, much less to various properties of mitochondria.

Enzymes bound to mitochondria sediment in sucrose density gradients at a higher density than enzymes characteristic of nerve endings. In most studies sedimentation profiles for mitochondrial enzymes have only been obtained for one centrifugation condition, but in many of these studies discontinuous gradients were used with obviously unsatisfactory resolution. Nevertheless, the data obtained suggested the existence of at least two subpopulations of mitochondria, one characterized by the presence of a relatively large amount of glutamate dehydrogenase, γ-aminobutyrate transaminase and acetyl-CoA synthase and the other by a relatively large amount of the classical citric acid-cycle enzymes (Salganicoff & De Robertis, 1965; Van Kempen *et al.*, 1965; Neidle *et al.*, 1969; Blokhuis & Veldstra, 1970). When two enzymes show the same distribution at one centrifugation time this does not necessarily indicate that these enzymes are present to the same extent in the same particles. This is shown by our data on glutamate dehydrogenase and γ-aminobutyrate transaminase. There is almost total overlap between the two enzymes after 1 h of centrifugation, but with centrifugation for 15 min it is clear that the distributions are not identical.

This separation can only signify that glutamate dehydrogenase and γ-aminobutyrate transaminase are present in different proportions in subpopulations of mitochondria that differ in one or more factors which influence sedimentation in a sucrose density gradient. As the separation between the two enzymes is largest in the beginning of the sedimentation, one can assume the weight of the particles to be one of the important factors. On the basis of available evidence it is impossible to answer the question whether there are a limited number of more or less discrete subpopulations of mitochondria each characterized by the presence of only one of these enzymes in addition to other obligatory mitochondrial enzymes or whether there exist an almost endless spectrum of mitochondria differing gradually in the proportion of enzymes and other constituents. As most of the distribution curves for particle-bound mitochondrial enzymes do not have a shape to be expected for a homogeneous particle, it can be anticipated that there is an extensive heterogeneity of brain mitochondria, the degree of heterogeneity depending on the source of the particles, as can be seen from a comparison of the distribution curves of cerebral and cerebellar mitochondria.

Mitochondrial heterogeneity and the existence of more than one small glutamate compartment in the adult brain

In the introduction it was noted that the relative labelling of aspartate and glutamate was not the same for propionate and γ-aminobutyrate as precursors. The difference in sedimentation behaviour between glutamate dehydrogenase and γ-aminobutyrate transaminase argues for the possibility either that these differences are related to the existence of different metabolic patterns within the small compartment, or for the existence of more than one small glutamate compartment in brain. As argued in the introduction, there is no theoretical necessity for glutamate dehydrogenase to play a role in the metabolism of γ-aminobutyrate. Therefore it need not be in the metabolic compartment, or space, where γ-aminobutyrate is converted via the tricarboxylic acid cycle into glutamine. From the data reported in the present paper, it seems that glutamate dehydrogenase has a restricted localization in the brain. The role of this enzyme in ammonia formation or removal has received considerable attention over the last 40 years or more (see Weil-Malherbe, 1974). Elsewhere we have reported observations on the effect of acute ammonia on the metabolism *in vivo* of glutamate and related amino acids (Matheson & Van den Berg, 1975; Van den Berg & Matheson, 1975). A fuller characterization of the glutamate dehydrogenase and γ-aminobutyrate transaminase containing subpopulations of brain mitochondria is clearly desirable.

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