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UPTAKE AND METABOLISM OF EXOGENOUS γ-AMINOBUTYRIC ACID BY SUBCELLULAR PARTICLES IN A SODIUM-CONTAINING MEDIUM*

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Abstract—A morphologically heterogeneous "mitochondrial" fraction was prepared from a sucrose homogenate of whole mouse brain. This particulate fraction, which contains endogenous γ-aminobutyric acid (γABA), was suspended for 10 min in an ice-cold buffered solution which contained Na^+ ions and ^{14}C-γABA. During this interval there was a Na^+-dependent binding of ^{14}C-γABA to the particles. The suspension was then incubated at 29°. This resulted in a rapid decrease in the total γABA content of the suspending medium and the particles and accumulation of non-volatile radioactive metabolites of γABA in the particles, and the liberation of $^{14}CO_2$ from the suspension. The major radioactive metabolites were CO_2 and aspartate. Glutamate and glutamine were found to a lesser extent. A purified microsomal fraction studied under the same conditions as the crude mitochondrial preparation rapidly released its γABA into the suspending medium at 29° and did not metabolize γABA.

When K^+ was substituted for Na^+ in the test system, the particles of the mitochondrial suspension released γABA into the suspending medium, and some metabolism of endogenous γABA occurred. However, there appeared to be no entry of radioactive γABA into the particles. When the particles were suspended in 0·25 M sucrose, results were obtained similar to those seen in the K^+-containing suspension with the exception that there was no decrease in the γABA content of the system.

STUDIES of the Na^+ ion-dependent binding of ^{14}C-γ-aminobutyric acid (γABA) to subcellular fractions of brain homogenates *in vitro*[1–4] led to the observation that some fractions could metabolize the ^{14}C-γABA rapidly at temperatures above 4°. The present study was undertaken to obtain information about the metabolism of ^{14}C-γABA and of the endogenous γABA which is in the particles of these preparations, and to investigate the possibility of a relationship between this metabolism and the Na^+ ion-dependent binding.

MATERIALS AND METHODS

A "mitochondrial" fraction which has been shown to be morphologically heterogeneous and a microsomal fraction which is predominantly composed of vesicular smooth-surfaced endoplasmic reticulum[3] were prepared from 0·25 M sucrose homogenates. These fractions, which contain endogenous γABA,[2,3] were suspended in a

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buffered solution of NaCl to which 2-^{14}C-γABA (specific activity 4·0 mc/mmole; radiopurity >99%) was added. The 2-^{14}C-γABA was obtained from Isotope Specialties Co., Burbank, Calif. The final concentrations were: 0·2 M NaCl; 0·05 M Tris-HCl buffer, pH 7·3; and 2-^{14}C-γABA, 0·75 μg/ml of suspension. On the average, the radioactive mitochondrial and microsomal suspensions contained 3·5 mg and 2·2 mg protein/ml suspension, and the particles represented less than 3% of the volume of the suspension. These preparations have been previously described in studies of the low-temperature binding of radioactive γABA.[2]

All experiments were performed in 500-ml Erlenmeyer flasks containing 100 to 200 ml of the suspension, which was aerated by a continuous stream of water-saturated air. After the addition of the 2-^{14}C-γABA the preparations were kept at 0° to 4° for 10 min to permit binding of the isotope.

The flasks were then transferred to a shaker and incubated at 29° with constant shaking and aeration. At intervals during incubation, 12-ml aliquots of the suspension were removed from the flasks. Aliquots were centrifuged in precooled centrifuge tubes at 0° to 4° for 15 min at 15,000 *g*. The particle-free supernatants and the pellets were analyzed for radioactivity[2] and γABA. The particle-free supernatant was decanted and its volume measured. The pellet was resuspended in slightly less than 6 ml of chilled H_2O. The pH of both the particulate suspension and the supernatant was adjusted to 4 with HCl, and the preparations were heated for 10 min in a boiling water bath. The supernatant and particulate samples were then brought to a volume of 15 ml and 6 ml respectively. Aliquots of 40 μliters each were used for the assay of radioactivity in a scintillation counter as described previously.[2] Results are reported as pellet and supernatant counts per minute per milliliter of original incubation mixture. Previous work has shown that no more than 3% of supernatant[2] radioactivity is held in the pellet. A suitable correction factor was applied to the radioactivity of the pellet to correct for this entrainment.

The sum of the radioactivities of the pellet and supernatant corresponded to measurements made on the unfractionated sample. A decrease of total radioactivity in the system during incubation was observed. Additional radioactivity was lost during acidification and heat inactivation. Radioactivity in the supernatant could also be decreased by bubbling nitrogen through the solution. In several instances it was shown that virtually all of this lost radioactivity could be recovered by passing the air leaving the flask through a solution of $Ca(OH)_2$ or Hyamine. The losses of radioactivity are, therefore, assumed to be in the form of $^{14}CO_2$.

For the measurement of γABA, the inactivated preparations were filtered through a glass-wool filter to remove coagulated protein, and the filter washed with water. If sufficiently washed, no radioactivity was found to remain associated with the proteins on the filter. Filtrates and washings were combined and adjusted to pH 8 to 8·3 and the samples evaporated to dryness at 40° to 50° with air passing over them. The dried samples were resuspended in 2 ml H_2O. The radioactivity in 40-μliter aliquots was again measured to calculate the mechanical losses of γABA due to routine transfers and filtrations. With careful washing and transfer the losses can be kept down to the 5% level. Aliquots of 0·4 ml were used for the enzymatic determination of γABA.[5] The values were calculated as micrograms of pellet or supernatant γABA per milliliter of original suspension.

In some experiments, the radioactive metabolites of 2-^{14}C-γABA were isolated

and the radioactivity determined separately as follows. Aliquots (20-40 μliters) of the final samples were spotted on Whatman 3MM paper. Electrophoresis was carried out, in a Savant high-voltage electrophoresis tank, with 8% formic acid and a potential gradient of 30 V/cm for 2 hr. A good separation was obtained of γABA from all other radioactive components. To separate completely glutamic acid, aspartic acid, and glutamine, a second electrophoretic run was performed at right angles to the original one, using only that portion of the paper containing the overlapping spots of these amino acids. The second dimension was run at pH 4 with a formic acid–pyridine buffer (17 ml formic acid+24 ml pyridine in 4 liters of H_2O) and a potential gradient of approximately 20 V/cm for 1 hr. Amino acid spots were visualized on paper with the trinitrobenzene-1-sulfonic acid spray reagent.[6] To facilitate location of the spots from samples containing very low levels of amino acids, 10 μg of each amino acid was co-electrophoresed with the extracts as carriers. The radioactivity of the amino acids was measured in a liquid scintillation spectrometer after combustion of the spots to $^{14}CO_2$. This analytical method has been described in detail.[6]

The above procedure yielded a 95% to 105% recovery of the radioactive γABA and metabolites in the pellet samples. Analysis of the supernatant samples gave less satisfactory results. The recoveries were generally from 60% to 80%. This was attributable to the high salt content of the concentrated supernatant samples, which resulted in considerable streaking during electrophoretic separation. In most experiments a definite trend was noted in which the recoveries in the supernatant samples increased slightly as a function of time of incubation of the suspension. One suggestion is that there may have been a selectively poor recovery of the ^{14}C-γABA and that, as the ^{14}C-γABA content decreases through metabolism, the total recovery increases. In the experiments in which electrophoresis and combustion were used, the radioactivity was determined in an ethanolamine–methanol system.[6]

RESULTS

Incubation at 29° in air (standard experiment)

The results of a typical experiment are shown in Fig. 1 in which radioactivities and γABA contents of suspending fluid and particles from 1 ml of suspension are plotted as a function of the time of incubation at 29°.

The ability of "crude mitochondrial" preparations to metabolize γABA is clearly established by the decrease of the γABA content in the whole suspension (Fig. 1B). Approximately 80% of the γABA initially present was metabolized within 210 min. That a considerable amount of the γABA was degraded to volatile products is indicated by the progressive, almost linear decrease of the total counts of the system (Fig. 1A). Approximately half the radioactivity disappeared in a gaseous form, presumably as CO_2 (see Methods). The relatively smaller loss of radioactivity than of γABA by the system suggested that there was a preferential metabolism of endogenous γABA and/or that nonvolatile metabolites of γABA accumulated in the system. If γABA metabolites accumulated, the radioactivity at any stage of the incubation, whether measured in the suspending fluid or in the particles, could not be attributable solely to ^{14}C-γABA.

The γABA content of the suspending fluid (Fig. 1B) decreased rapidly at the beginning, but no further changes occurred with incubation beyond 60 min. The radioactivity of the suspending fluid (Fig. 1A) decreased to approximately 40% of

the initial values within 30 to 60 min. Subsequently, however, a rather slow continuous rise occurred until the end of the incubation. This late increase of radioactivity without a concomitant increase of γABA content in the suspending fluid suggested that there was a continuous release from the particles of radioactive nonvolatile γABA metabolites, a process which conceivably might have occurred from the beginning of these experiments. If so, the rate of decrease of the ^{14}C-γABA in the suspending medium would be greater than indicated by the decrease in supernatant radioactivity (Fig. 1A).

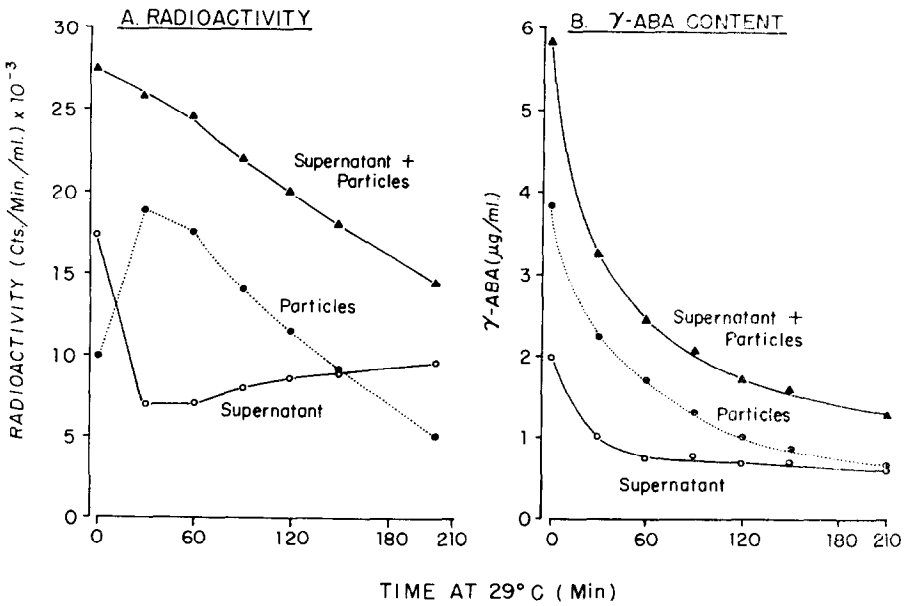

FIG. 1. Radioactivity and γABA content of a "mitochondrial" suspension as a function of time of incubation at 29° in 0·2 M NaCl containing 0·05 M Tris buffer, pH 7·3. The incubation at 29° was preceded by 10 min at 0°.

The simultaneous disappearance of both γABA and radioactivity from the medium in the initial 60 min of incubation could be attributable either to metabolic degradation of γABA in the suspending fluid or to uptake and subsequent metabolism in the particles. It was also considered possible that γABA was metabolized to nonvolatile products and that the latter, rather than γABA, were taken up by the particulate matter. The marked increase in the first 30 min of the radioactivity of the particles (Fig. 1A), while the γABA content was decreasing, would be compatible with such a possibility.

γABA is metabolized in the particles.

The following experiment was performed to determine whether or not the metabolism of γABA could occur in the supernatant fluid independently of the particles. A freshly prepared saline suspension, mixed with ^{14}C-γABA at 0° for the usual 10 min, was divided into two aliquots and the particulate matter removed from one of them by a 15-min centrifugation at 15,000 *g* in the cold, while the other aliquot was kept at 0°.

The particle-freed medium and the whole suspension were then incubated at 29°. The whole suspension gave results similar to those shown in Fig. 1. The particle-freed suspending fluid showed no decrement whatsoever, either in radioactivity or in γABA content. The lack of γABA metabolism in the absence of the particles was further confirmed by electrophoretic analysis which showed the radioactivity in the fluid to be associated only with γABA. The inability of the suspending fluid to metabolize γABA showed that in the initial 30-60 min period of the standard experiment there must have occurred a large transfer of the γABA from medium to particles.

Metabolism of ^{14}C-γABA gives labeled aspartate, glutamate, glutamine, and CO_2

A typical experiment is shown in Fig. 2 in which partition of the nonvolatile radioactive constituents was made by high-voltage electrophoresis. Only four labeled substances were detected: γABA, aspartic acid, glutamic acid, and glutamine. Good agreement between the total radioactivity of the original particles before electrophoresis and the total counts recovered from the corresponding electropherograms indicated that little, if any, radioactivity was found in substances other than the four amino acids listed above. At time zero at 29°, approximately 15% of the radioactivity of the particles was in the form of aspartate (Fig. 2), indicating that the initial 10-min preincubation of the flasks on ice was inadequate to inhibit metabolism completely. This is in contrast to the previously reported[2] inability to detect any significant formation of metabolites at low temperatures. This discrepancy may be due to the increased sensitivity of the present detection methods and/or a difference in the adequacy of the temperature controls. Aspartic acid was the major ^{14}C-containing metabolite in the particles at all times except during the initial 15 min, when ^{14}C-γABA was still present in relatively large amounts. Radioactivity in glutamine was low, and glutamic acid contained, at most, 13% of counts in the particles. Because of salt interference, aspartic and glutamic acids could not be separated clearly in

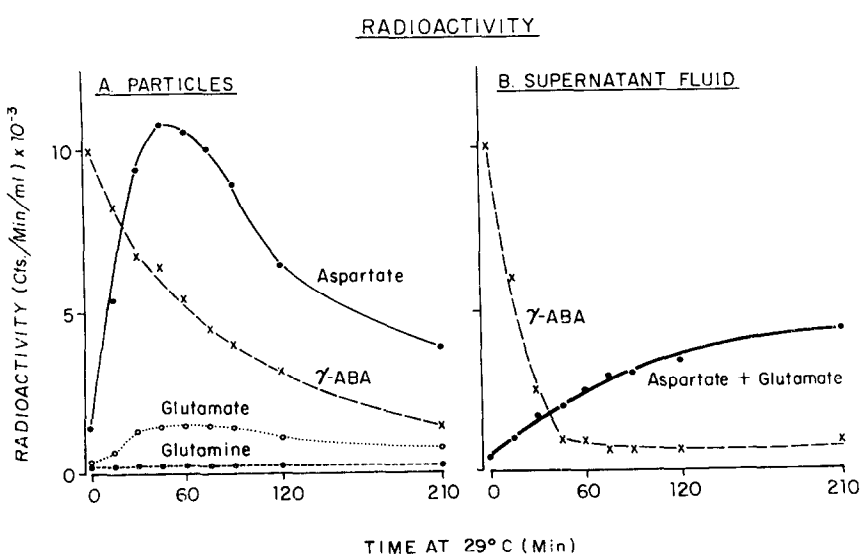

FIG. 2. Levels of 2-^{14}C-γABA and its radioactive metabolites in the particles and in the supernatant fluid of a mitochondrial suspension as a function of time of incubation. Conditions as in Fig. 1.

the samples of supernatant fluid. Therefore, in the latter case the results for the two substances are reported together. Radioactive glutamine was not detected in the samples of fluid, but small amounts may have been included in the aspartate–glutamate areas.

From the data in Fig. 2 it is seen that the ^{14}C-γABA was taken up rapidly by the particles from the fluid and that *in the particles* metabolites of γABA were formed. The fall in content of ^{14}C-γABA in the supernatant fluid (Fig. 2B) was much more rapid than the decrease of ^{14}C-γABA in the particles (Fig. 2A). The release of ^{14}C-metabolites into the medium occurred from the beginning of the incubation at 29° (Fig. 2B). This explains the findings that the amount of γABA in the fluid (Fig. 1B) was still decreasing at a time when the radioactivity stopped decreasing (Fig. 1A), and that subsequently the radioactivity in the supernatant fluid increased progressively while the γABA content remained at a constant low level. There was a maximal accumulation of ^{14}C-aspartic acid in the particles at 60 min (Fig. 2A) and a decline thereafter. The loss of radioactivity from ^{14}C-aspartic acid of the particles (Fig. 2A) could only partially be accounted for by the exit of aspartic acid from the particles into the medium (Fig. 2B). The results of another experiment (Fig. 3) suggest that

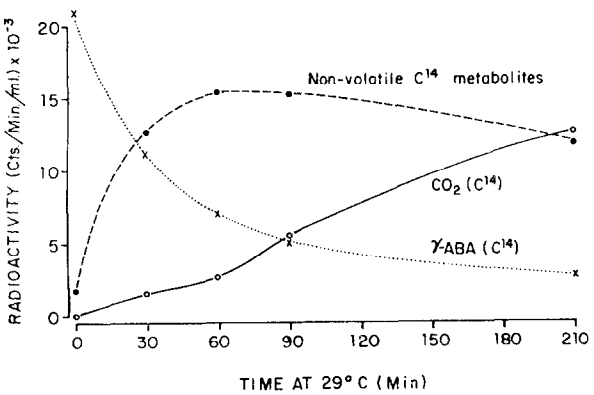

FIG. 3. Radioactivity of mitochondrial suspension in the form of γABA, CO_2, and nonvolatile metabolites as a function of time of incubation. Conditions as in Fig. 1.

$^{14}CO_2$ formation occurred, at least in part, from ^{14}C-aspartic acid which was formed from ^{14}C-γABA and accumulated during the first 60 min of the incubation at 29°, since the rate of formation of $^{14}CO_2$ after 60 min was greater than the rate of decrease of ^{14}C-γABA in the whole system. In this experiment (Fig. 3) the sum total of the radioactivities recovered appears to increase over the time interval of the experiment. This is related to a trend of obtaining better recoveries from the suspending fluid samples at the later time intervals of incubation (see Methods).

Metabolic experiments in a Na^+-free medium

Previous experiments had led to the proposal of a model in which Na^+ ions are required for the binding and carrier-mediated diffusion (in and out) of γABA in brain subcellular particles at 0°–4°.[4] It was logical to postulate that a Na^+-dependent binding of γABA to the particles and transport into the particles would be required to

take place prior to the metabolism of exogenous γABA at 29°. Thus it would be expected that in the absence of Na^+ there would be a decreased movement of isotope into the particles and a decreased metabolism of the isotopically labeled γABA.

That Na^+ is required for the metabolism of exogenous γABA at the elevated temperature under our experimental conditions was shown in the following experiment. Pellets obtained from sucrose homogenates by the usual procedure were resuspended in buffered KCl (0·2 M KCl, 0·05 M Tris HCl buffer) or in 0·25 M sucrose, and the 2-^{14}C-γABA was added. The suspensions were incubated at 0° for 10 min and then at 29° for 180 min. At various time intervals the suspending media and particles were assayed for γABA and radioactivity. The results of both the KCl and the sucrose experiments are shown in Fig. 4. Significant amounts of radioactivity did not become associated with the particles in either experiment at any time (Fig. 4A), indicating

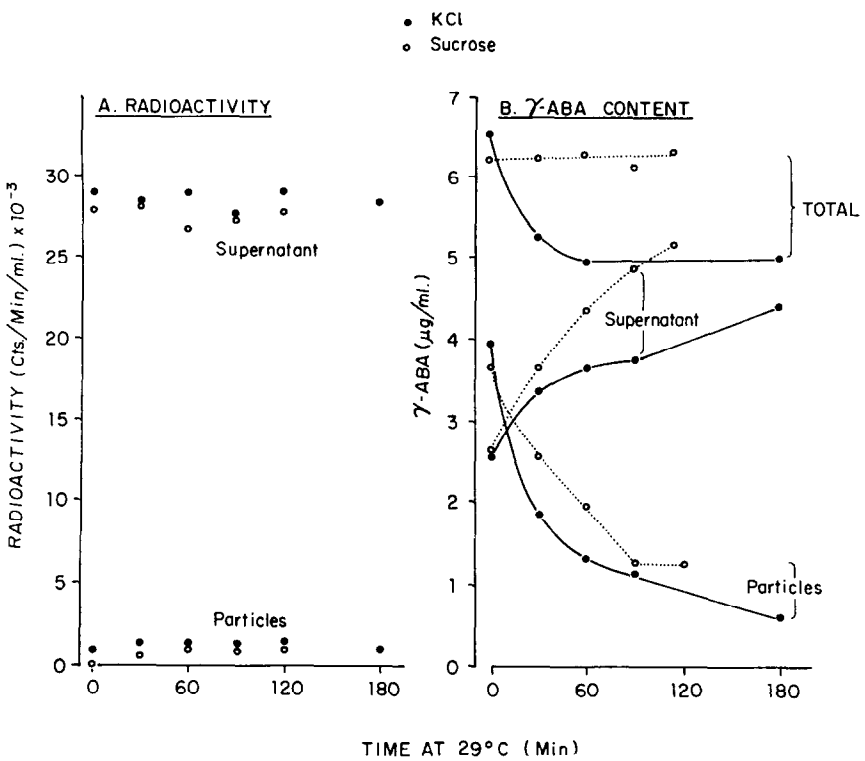

FIG. 4. Radioactivity and γABA content of a mitochondrial suspension as a function of time of incubation at 29° in 0·2 M KCl or 0·25 M sucrose containing 0·05 M Tris buffer, pH 7·3. The incubation at 29° was preceded by 10 min at 0°.

that there was no binding during the 10 min at 0° and that no uptake of free γABA had occurred during the entire experiment. The γABA content of the particles (Fig. 4B) decreased rapidly and progressively in both preparations, much in the same way as in the NaCl-containing suspensions discussed in previous sections. However, in contrast with the latter type of preparation the γABA contents of the KCl and sucrose supernatants (Fig. 4B) increased markedly from the beginning of the experiment until the end, indicating that large amounts of γABA were released from

particles to the medium. The KCl and sucrose preparations differed from each other in that the release of γABA into the KCl medium was considerably less than in sucrose. The difference may be attributed to there being less γABA available for release in a KCl medium, owing to intraparticulate metabolism of γABA in the KCl as contrasted to the lack of metabolism in sucrose (Fig. 4B). Controls which were run in the presence of NaCl showed the previously demonstrated pattern of isotope uptake and metabolism to $^{14}CO_2$.

Metabolic experiments with microsomal particles

Microsomal suspensions, predominantly composed of smooth-surfaced endoplasmic reticulum, can presumably carry out carrier-mediated diffusion of γABA at 0°.[3] A microsomal pellet was prepared as previously described and a standard metabolic experiment performed. It is seen from the results in Fig. 5 that no metabolism of γABA had

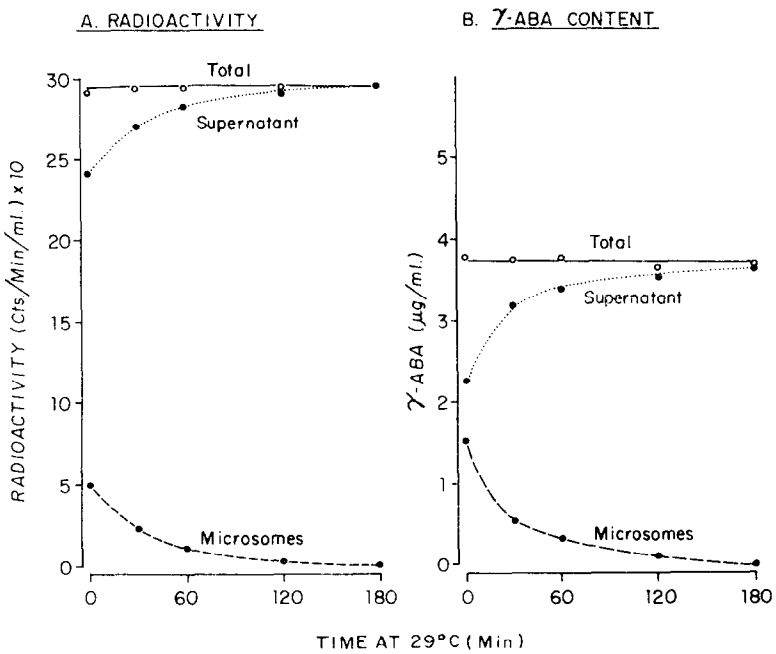

FIG. 5. Radioactivity and γABA content per milliliter of a microsomal suspension as a function of time of incubation. Conditions as in Fig. 1.

taken place. The total radioactivity and γABA content of the system remained constant throughout the period of incubation. The decrease in radioactivity of the pellet and concomitant increase in the supernatant showed that the ^{14}C-γABA taken up during the 10-min preincubation at 0° was released during the incubation at 29° (Fig. 5A). Likewise, the endogenously contained γABA of the microsomal pellet was released to the medium (Fig. 5B). The above experiment showed that the microsomal elements which are in the "crude mitochondrial" pellet[3,4] not only did not metabolize γABA but also probably served as a source of γABA for those components of the pellet in which γABA metabolism took place.

DISCUSSION

Previous studies of the Na^+ ion-dependent binding of γABA to crude mitochondrial preparations at 0°[4] led to the proposal that the binding was to a carrier which mediates the diffusion of γABA across the membrane of the various subcellular particles found in this mitochondrial preparation. In the present investigation it was demonstrated that in the presence of Na^+ the particles of a crude mitochondrial preparation rapidly depleted the suspending medium of γABA and metabolized this γABA in addition to metabolizing the intraparticulate γABA. It was also established that the microsomal elements, which are among the components of the crude mitochondrial fraction,[3] released their γABA to the suspending medium. It can therefore be concluded that some of the intraparticulate γABA was transferred via the suspending fluid from nonmetabolizing to metabolizing particles. When KCl was substituted for NaCl there was some metabolism of intraparticulate γABA, and release of γABA, but no net uptake. Thus, the occurrence of intraparticulate metabolism of γABA in the absence of Na^+ ions did not support the rapid net movement of γABA into the metabolizing particles.

The finding that 2-^{14}C-γABA is degraded to CO_2 and the report that the transamination of γABA to succinic semialdehyde occurs in mitochondria,[7] indicate that the mitochondria are the actively metabolizing particles. Particles which are metabolically inactive with respect to γABA metabolism, such as microsomes and those nerve-ending fragments that may be devoid of mitochondrial inclusions, probably release their γABA into the medium, from which it can be taken up into the mitochondria. In view of the heterogeneity of cellular sources from which brain mitochondria are derived (neuronal, glial, endothelial), it would be difficult to determine whether mitochondria from all the sources can carry out γABA metabolism or whether this capacity is restricted to certain types of mitochondria. Since it has been demonstrated that γABA transaminase is found in the free mitochondria of brain homogenates, and that the mitochondrial inclusions within the nerve-ending particles may have little or no transaminase,[7] it seems likely that the Na^+-dependent uptake and metabolism of γABA may be a property of mitochondria from sources other than nerve endings. The above considerations do not rule out the possibility that nerve endings may have the ability to take up γABA.

The finding of a rapid intraparticulate conversion of ^{14}C-γABA to ^{14}C-aspartic acid indicates that sufficient endogenous α-ketoglutarate was available to enable γABA to be transaminated, so that the ^{14}C-succinic semialdehyde formed from the γABA could be readily converted to succinate[8] and then to oxalacetate. It is likely that under our experimental conditions the supply of acetyl CoA was small enough so that at any time only a portion of the available oxalacetate was converted to citrate and oxidized to CO_2 via the tricarboxylic acid cycle, while the remainder was transaminated with the abundant endogenous supply of glutamate to form aspartic acid. Metabolic studies of 1-^{14}C-γABA during incubation with mouse brain minces showed that the largest amount of radioactivity, with the exception of γABA, was found at all times in aspartate.[9] Other studies [10] with mitochondrial fractions in the presence of Na^+ (40 mM Na phosphate buffer) have demonstrated that exogenously added γABA is metabolized to CO_2.

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