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A CARRIER-MEDIATED TRANSFER MODEL FOR THE ACCUMULATION OF ^{14}C γ-AMINOBUTYRIC ACID BY SUBCELLULAR BRAIN PARTICLES*

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Abstract—An analysis of the Na^+-dependent accumulation of ^{14}C-γABA by subcellular brain particles indicates that the isotope moves sequentially from the suspending medium into a rapidly equilibrating pool and then into a slowly equilibrating pool. The total γABA content of the rapidly equilibrating pool increases with increasing Na^+ concentration. The larger the rapidly equilibrating pool the more rapidly isotope moves into and out of the slowly equilibrating pool. The total γABA content of the slowly equilibrating pool decreases as a function of time, and the rate of decrease is increased by increasing the size of the rapidly equilibrating pool.

The above findings are interpreted as evidence for a carrier-mediated diffusion process in which Na^+ is required to activate the binding of γABA to the carrier. The significance of the Na^+ activation is discussed in terms of a model for active transport.

THE occurrence of large quantities of γ-aminobutyric acid (γABA) in the vertebrate central nervous system (CNS)has resulted in an extensive interest in the physiological and biochemical significance of this compound.[1] One of the interesting observations was that subcellular fractions obtained from sucrose homogenates of mouse brain can accumulate ^{14}C-γABA at 0° in 0·2 M NaCl.[2] Results obtained with some other tissues suggests that this phenomenon may be restricted to the CNS.[3] Subsequent studies demonstrated the existence of considerable quantities of endogenous γABA in the particulates of "mitochondrial" and "microsomal" fractions. Suspension of the "mitochondrial" particles in saline containing ^{14}C-γABA resulted in a loss of endogenous particulate-held γABA. This was more than compensated for by a binding of γABA from the suspending medium, which resulted in a net increment of total γABA content of the particles.[4] It was not ascertained whether the Na^+-dependent accumulation of ^{14}C-γABA represents an irreversible incorporation, an equilibrium binding, or a combination of both. The present study demonstrates the existence of rapidly and slowly equilibrating pools of ^{14}C-γABA. The findings reported in this paper will be presented within the framework of a model which is described below.

The cell membrane of the presynaptic nerve-ending particles and the membranes of the vesicular fragments of the endoplasmic reticulum contain mobile binding sites. Na^+ ions are required to activate the binding of γABA to these mobile sites. Upon

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the removal of Na^+ ions, γABA molecules are released (the binding site is inactivated). The γABA bound on the outer surface of the membrane equilibrates rapidly with the γABA which is in solution in the external medium. The γABA bound on the inside of the membrane equilibrates rapidly with γABA which is in solution within the particles. The binding sites are partially restricted to one side or the other side of the membrane by a barrier. The frequency with which binding sites traverse the barrier at 0° to 4° is small in comparison to the frequency with which binding sites exchange γABA molecules with the solution. The *rapidly equilibrating pool* of bound γABA would represent the carrier-bound γABA which exists on the exterior surface of the membrane. Free γABA in solution within the particle and bound γABA internal to the barrier would be the *slowly equilibrating pool.* Prior to the introduction of the centrifugally prepared particles into a Na^+-containing medium there would be large quantities of free γABA within the particles. The Na^+ ions activate the carrier sites on both sides of the barrier, permitting the endogenous γABA and ^{14}C-γABA to move back and forth across the membrane. In the present experiments a greater degree of saturation of the carriers with γABA on the inside of the membrane than on the outside results in a net movement of γABA across the membrane and out of the particle.

METHODS

Preparation of particles

Whole mouse brains were homogenized in 9 parts (w/v) of 0·25 M sucrose. This and all subsequent procedures were carried out at 0°–4°. The following description is for the preparation of particles from a 14-ml aliquot of the sucrose homogenate. Depending upon the amount of material required, multiples of this volume were prepared. The homogenate was centrifuged in a refrigerated International centrifuge at 1,500 *g* (10 min). An 11-ml aliquot of the supernatant was centrifuged at 15,000 *g* (15 min) in the no. 40 rotor of a Spinco centrifuge. The maximal amount of supernatant was removed with a syringe. Electron microscopy revealed such pellets to be morphologically heterogenous, containing numerous nerve-ending particles, mitochondria, and some vesicular and nonvesicular membrane fragments.

Binding procedure, saline suspension (Method 1)

The 15,000-*g* pellet from one tube was resuspended in a solution of NaCl and Tris–HCl buffer, pH 7·3, to a final volume of 17 ml. One-tenth ml of an aqueous solution of 2-^{14}C-γABA was added. Unless otherwise stated, the isotope was added immediately after suspension of the particles in the buffered saline. The final concentrations were 0·2 M NaCl, 0·05 M Tris–HCl, pH 7·3, and approximately 0·75 μg 2-^{14}C-γABA per ml of suspension (specific activity 2·71 mc/mmole).

Sucrose–saline suspension (Method 2)

The particles of one tube were resuspended in 3·4 ml of 0·25 M sucrose. The sucrose suspension was then introduced into a tube containing 13·7 ml of the required mixture of salts, buffer, and isotope, so that the final concentrations were the same as in Method 1 but also containing 0·05 M sucrose.

Determination of supernatant and particulate ^{14}C-γABA

The suspension prepared by either of the above methods was incubated at 0°–4° for the appropriate time intervals. The particles were removed from the supernatant

by centrifugation of a 10-ml sample at 15,000 *g* for 15 min. The total elapsed time from the start of centrifugation to removal of the supernatant was 20 min. In one set of experiments a shorter centrifugation time was required. The procedure adopted was to use the no. 40 Spinco rotor, set it at maximal speed (40,000 rpm), and permit it to accelerate for 5 min at which time it had attained a speed of 23,000 rev/min. At 5 min the rotor was decelerated and at 10 min the supernatant was decanted.

The particle-free supernatant was decanted thoroughly. The sides of the tube were dried with a cotton swab, and the pellet was resuspended in ice-cold water to a given volume. Three 40-μl aliquots of the supernatant and resuspended pellet were introduced into scintillation counting vials which contained 1 ml of Hyamine. Fifteen ml of counting fluid was added (3 g 2, 5-diphenyloxazole, plus 100 mg 1, 4-bis-2(5-phenyloxazolyl)-benzene in 1 liter of reagent grade toluene). These samples and appropriate blanks[4] were counted two or three times for 5 min in a Packard Tri-carb scintillation counter.

γABA Determinations

The remaining supernatant and resuspended pellets were adjusted with HCl to pH 4–5 and immersed in a boiling-water bath for 5 min. The sample was filtered through glass wool and the filtrate adjusted to pH 8–8·3 and dried under an i.r. lamp with a good air flow. Water (2 ml) was added to the dried sample. Turbidity was removed by centrifugation and 0·4-ml aliquots of the clear supernatant were assayed enzymatically for γABA.[5] In this reaction the conversion of NADP to NADPH is followed spectrophotometrically. The supernatant samples prepared as above were slow to react because of the high salt concentration (approximately 1 M), and as long as 2 hr were required for completion of the reaction in some instances. Stable maximal readings and valid data were obtained by this method, as shown by recoveries of known amounts of added γABA. Aliquots (40 μl) of the aqueous solution were monitored for radioactivity. From the amount of γABA and radioactivity in the solution and given the radioactivity of the original sample, the amount of γABA in the original sample was calculated. Once having obtained values for the supernatant and pellet, corrections were made for a 3% entrainment of supernatant counts and γABA in the pellet.[4]

Unless otherwise specified the results are expressed as radioactivity and γABA content of the pellet and supernatant fluid obtained from 1 ml of the radioactive suspension.

^{14}C-γABA was not metabolized under the conditions of these experiments, all of the radioactivity being present as γABA.[4]

Protein determination

Protein determinations made by the method of Lowry *et al.*[6] showed a protein content of 2·8 to 3·2 mg protein per ml of the radioactive suspension in the various experiments. Approximately 90% of the protein is associated with the particles.

RESULTS AND DISCUSSION

Evidence for the rapidly equilibrating pool

If γABA is on a binding site which is in true equilibrium with the supernatant, and the volume of supernatant is increased without altering the concentration of the

various constituents of the supernatant, the ratio of total bound γABA to the concentration of γABA in the supernatant should remain constant. If the original suspension contains isotopically labeled γABA and the added supernatant is not labeled, the isotope should redistribute until the specific activities of the bound γABA and the supernatant are the same. At this time the ratio of equilibrating pellet counts (P_c) to supernatant concentration (S_c) of counts should assume the original value found prior to the dilution ($P_e/S_c = K_1$). If for some reason there is x amount of isotope sequestered within the particles and if it is not affected by the dilution of the supernatant, then a constant ratio would be obtained only if the x counts were substracted from the total counts in the pellet (P_t). Then the relationship $(P_t - x)/S_c = K_1$ would obtain. The model which was outlined in the introduction suggests that the latter situation would be simulated if the rate of transfer across the barrier were so slow that it could be disregarded. If this rate were not negligible, with the decrease in the specific activity of the γABA in the supernatant by the dilution there would be a

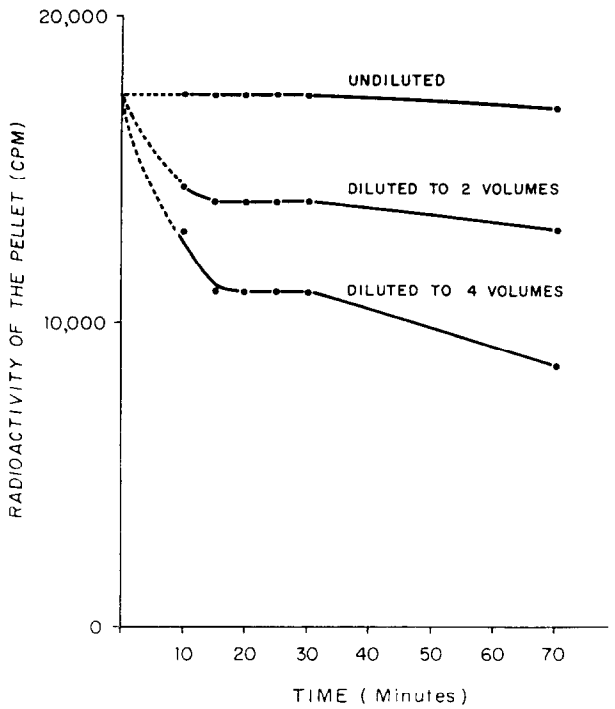

FIG. 1. Dilution of radioactive suspension with nonradioactive supernatant. Forty min after the start of incubation in 2-^{14}C-γABA, nonradioactive supernatant is added to increase the supernatant volume 2 and 4 times. At various time intervals after the dilution the particles are sedimented. The stated times are the intervals between dilution and removal of supernatant from the sedimented particles.

decreased probability of a labeled molecule entering the particle and a decreased probability of the re-entry of a labeled molecule which had left the particle. If the latter conditions prevailed the quantity of labeled γABA sequestered in the particles would decrease with decreasing specific activity of the γABA in the supernatant, but the total quantity sequestered should remain the same.

Particles were suspended in buffered saline (see Method 1); 2-^{14}C-γABA was added to one aliquot and to a second aliquot an equivalent amount of nonradioactive γABA was added. After 30-min incubation at 0° the nonradioactive suspension was centrifuged rapidly (see Methods, for rapid centrifugation procedure) and the supernatant fluid was used to dilute aliquots of the radioactive suspension, thus altering the volume and specific activity of the supernatant, but not the composition. Aliquots of undiluted, twofold, and fourfold diluted radioactive suspensions were centrifuged at 5-min intervals. The radioactivity and γABA content of each pellet and supernatant were determined.

The counts remaining in the pellet as a function of time and supernatant volume are shown in Fig. 1. With increasing dilution more radioactivity was lost from the pellet and the values were constant between 15 and 30 min. Between 10 and 30 min the concentrations of supernatant γABA (2·45 μg/ml) and the total pellet content of γABA (4 μg) were the same for the three dilutions. Between 30 and 70 min, counts were lost from the pellets, more at the higher dilutions, and there was a slight decrease in γABA content.

The data for the 15- to 30-min intervals lend themselves to the analysis suggested in the introduction to this experiment (see Table 1). The ratios of total pellet counts (P_t) to supernatant concentration (S_c) for the one-, two-, and four-fold dilutions were

TABLE 1. INFLUENCE OF DILUTION OF SUPERNATANT FLUID ON DISTRIBUTION OF RADIOACTIVITY AT 20 MINUTES

	Radioactivity (cpm)		
	Undiluted	Twofold dilution	Fourfold dilution
Observed values			
Total pellet cts (P_t)	17,500	14,000	11,000
Total supernatant cts (S_t)	22,500	26,000	29,000
Supernatant cts/ml (S_c)	22,000	13,000	7,250
P_t/S_c	0·77	1·08	1·51
P_t, calculated values			
No sequestration	17,500	11,200*	6,500*
Counts sequestered, 7,900	17,500	13,550*	11,000
Counts sequestered, 9,200	17,500	14,000	11,800*

* Calculated values from formula 2 (see text).
Supernatant concentration of γABA was 2·45 μg/ml and total γABA content of pellet was 4 μg at all dilutions.

0·77, 1·08, and 1·51. Therefore, the initial redistribution indicated that all the particle counts were not re-equilibrating. If all the isotope were re-equilibrating, the ratio should have remained at 0·77 for all dilutions. The theoretically expected number of counts in the pellet for the different dilutions with no sequestration are shown in Table 1. The number of counts released by dilution were less than theoretically expected. If part of the pellet counts x were sequestered, then the ratio should be $(P_t - x)/S_c$. The value for x can be obtained by the formula:

$$\frac{P_{t_1} - x}{S_{c_1}} = \frac{P_{t_2} - x}{S_{c_2}} \tag{1}$$

in which the distribution of counts for two dilutions is required to solve for x. Introducing the values for the one- and four-fold dilutions the value for x was calculated to be 7,900 cpm. When the values for the one- and two-fold dilutions were used in the calculation, the sequestered counts were calculated to be 9,200 cpm. Thus the number of sequestered counts is dependent on dilution and, as suggested in the model, there would appear to be a slowly equilibrating pool.

If it is assumed that the isotope is sequestered and not affected by dilution, the theoretical distribution of isotope for any increase in volume can be calculated from the following formula*

$$S_t = \frac{K_2 V}{K_1 + V} \tag{2}$$

The total counts in the supernatant (S_t) can be predicted for any multiple of the original volume (i.e. $V = 2$ or 4 in the present experiment) if the values for the two constants (K_1 and K_2) are given. K_2 represents the total nonsequestered counts; K_1 is the ratio of nonsequestered isotope in the pellet to the concentration of isotope in the supernatant fluid.

By applying formula 1 to the undiluted and fourfold diluted samples, the value of 7,900 cpm was calculated for the sequestered counts. With this value and formula 2, a predicted value of 13,550 cpm was obtained for the total pellet counts for the twofold dilution. This is in good agreement with the observed value of 14,000 cpm. By means of the observed distributions for the undiluted and twofold diluted samples, the sequestered counts were calculated to be 9,200 cpm. A predicted value of 11,800 cpm was obtained for the fourfold dilution, which compared favorably with the observed value of 11,000 cpm.

An estimation of the size of the rapidly equilibrating pool can be made in the following way. If 7,900 or 9,200 counts are in the slowly equilibrating pool, then out of the 17,500 bound counts in the original suspension there would be 9,600 (17,500–7,900) or 8,300 (17,500–9,200) counts in the rapidly equilibrating pool. By definition the rapidly

* The derivation of the formula follows: If γABA is bound and in equilibrium with a given concentration of γABA in the supernatant, then an increase in the volume of the system with supernatant that has the same concentration of γABA as the supernatant of the suspension will leave the ratio of total bound γABA to supernatant concentration of γABA unaltered. If isotope is present initially but no isotope is added when the volume is increased, the isotope will redistribute until the specific activities of bound and free γABA are the same. At this time the ratio of counts also will have the original value. This can be expressed as:

$$\frac{P_e}{S_t/V} = K_1 \tag{A}$$

where P_e is the equilibrating counts of the pellet, S_t is total supernatant counts, and V is the supernatant volume.

The total counts in the system will remain a constant (K_2) and

$$P_e = K_2 - S_t \tag{B}$$

by substituting for P_e in equation A

$$\frac{K_2 - S_t}{S/V} = K_1 \tag{C}$$

$$S_t = \frac{K_2 V}{K_1 + V} \tag{D}$$

Thus given K_1 and K_2, S can be determined for any increase in supernatant volume.

equilibrating pool would have the same specific activity as the supernatant. Thus, given the assumed number of counts in the rapidly equilibrating pool and the specific activity of the supernatant (91,800 cpm/μg), values of 1·05 (using 9,600 cpm) or 0·90 μg (using 8,300 cpm) of γABA are contained in the rapidly equilibrating pool. The total pellet γABA was 4 μg. In terms of our model this would mean that approximately one molecule was bound to a carrier on the external surface of the barrier for every three inside. It is on the basis of this estimate of the relative pool sizes and the observed rapidity of exchange between the 2·45μg pool of free γABA and the 0·9 to 1·05 μg of rapidly equilibrating bound γABA that the concept of a barrier has been invoked for the model. The relationship of the two pools to each other will become apparent in the following experiments.

After 30-min incubation of the particles in 2-^{14}C-γABA, the rapidly equilibrating pool (which makes up approximately one fourth of the total pellet γABA) contained approximately one half the total counts in the pellet. This threefold difference in the specific activity of the two pools was useful in a subsequent experimental analysis of the model.

Role of Na^+

The model predicts that if particles contain ^{14}C-γABA in the rapidly and slowly equilibrating pools, resuspension of the particles in a Na^+-free medium should result in all the counts being lost from the rapidly equilibrating pool; and the counts in the slowly equilibrating pool should be trapped because of inactivation of the carriers. This assumes that no residual Na^+ is present in the system. Resuspension in NaCl plus a high concentration of nonradioactive γABA should chase out the bound isotope from the rapidly equilibrating pool. Thus, both procedures should result in the same initial drop in bound counts. However, in the case of resuspension in Na^+ plus nonradioactive γABA, the carriers would be operative, and isotope would continue to be lost from the slowly equilibrating pool. Resuspension in KCl plus nonradioactive γABA should result in the same *initial* loss as KCl alone or NaCl plus nonradioactive γABA. Resuspension in NaCl alone should result in a loss of γABA from the particles because of the absence of γABA in the external fluid. The decreased saturation of the rapidly equilibrating pool in the latter instance should result in an increased net movement of γABA out of the slowly equilibrating pool. The entrance of γABA of low specific activity from the slowly equilibrating pool into the external medium would dilute out the bound counts of the rapidly equilibrating pool.

To test the above predictions particles were permitted to bind γABA for 30 min (Method 1), sedimented, and the supernatant decanted. The particles were then resuspended to the original volume in one of the following media: (a) 0·2 M NaCl, 0·05 M. Tris–HCl, pH 7·3; (b) 0·2 M NaCl, 0·05 Tris–HCl (pH 7·3), 200 μg γABA/ml; (c) 0·2 M KCl, 0·05 M Tris–HCl, pH 7·3; (d) 0·2 M KCl, 0·05 M Tris–HCl (pH 7·3), 200 μg γABA/ml. At 5, 30, and 90 min after resuspension, the particles were sedimented (15,000 *g*, 15 min) and counts in the supernatant fluid and particles determined. Losses of radioactivity from the pellet as a function of time are shown in Fig. 2. The time intervals are those at which the supernatant was decanted. The zero time values were obtained at the time of resuspension of the particles in the four different media. In conformity with the model, the initial losses of bound counts were the same in NaCl + γABA, KCl alone, and KCl + γABA, but significantly less in NaCl. The

subsequently greater rate of loss in NaCl + γABA than in KCl + γABA is explicable in terms of the greater number of carriers available in the presence of NaCl to convey isotope out of the slowly equilibrating pool. If all Na^+ were removed, the KCl and KCl + γABA curves should have overlapped. However, the experimental procedure results in a residual NaCl concentration of approximately 0·006 M NaCl in the KCl resuspension, and this may well account for the greater rate of loss in the medium containing KCl and γABA. The factors which contribute to the greater rate of loss in the Na^+ resuspension have been discussed in the preceding part of this section.

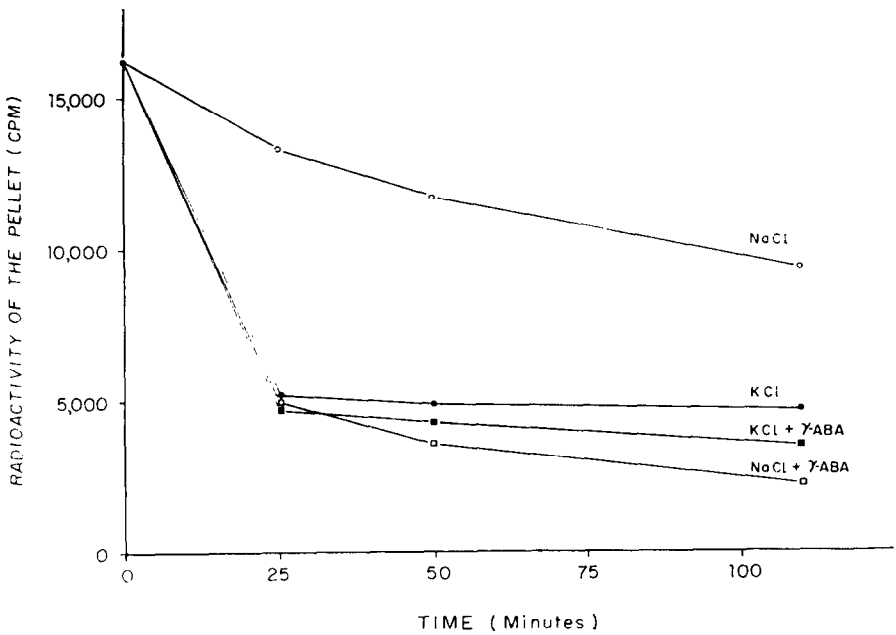

FIG. 2. Release of counts from particles as a result of resuspension in different media. Resuspension media are: 0·2 M NaCl; 0·2 M KCl; 0·2 M KCl + 200 μg γABA/ml; 0·2 M NaCl + 200 μg γABA/ml.

Direct evidence for the postulated effect of decreased Na^+ concentration on the rapidly equilibrating pool

In a preceding experiment it was suggested that at the end of a 30-min incubation the rapidly equilibrating pool would contain approximately one fourth of the γABA and one half of the isotope. Thus, if the size of the rapidly equilibrating pool could be decreased by inactivating the postulated Na^+-dependent binding sites, there should be a significant drop in the specific activity of the particles. In the following experiment the inactivation was achieved by diluting the suspension with KCl and comparing the results to those obtained by dilution in NaCl.

Particles were suspended in buffered saline (Method 1) and 2-^{14}C-γABA was added. After a 30-min incubation at 0°–4° one aliquot was diluted with four volumes of buffered NaCl (0·2 M NaCl, 0·05 M Tris–HCl, pH 7·3) giving a final concentration of 0·2 M Na^+. A second aliquot was diluted with four volumes of buffered KCl (0·2 M KCl, 0·05 M Tris–HCl, pH 7·3) to give a final concentration of 0·04 M Na^+ and 0·16 M K^+. The two diluted aliquots and an aliquot of the undiluted suspension were

centrifuged immediately at 15,000 *g* (15 min.) Twenty min after the dilution the supernatants were removed from the pellet.

The specific activities of the original pellet and supernatant were 12,500 and 4,600 cpm/μg γABA respectively (Table 2). Dilution in Na^+ would be expected to decrease the saturation of the rapidly equilibrating pool and thereby increase the net rate of flux out of the slowly equilibrating pool. The dilution in Na^+ released 0·46 μg of γABA. The specific activity of the released γABA was 4,300 cpm/μg, which is in close agreement with the specific activity of the particles prior to dilution. This indicates that

TABLE 2. THE EFFECT OF DILUTION IN NaCl AND KCl ON THE RELEASE OF RADIOACTIVITY AND γABA FROM THE PARTICLES

	Radio-activity (cpm)	γABA (μg)	γABA (cpm/μg)
Original suspension			
Supernatant	28,000	2·2	12,500
Pellet	16,500	3·6	4,600
Released from pellet Fivefold dilution, final salt conc., 0·2 M NaCl	2,000	0·46	4,300
Released from pellet Fivefold dilution, final salt conc., 0·04 M NaCl + 0·16 M KCl	8,000	0·68	11,700

approximately the same percentage of γABA was lost from the two pools. Dilution in KCl lowers the Na^+ concentration and would be expected to decrease the number of binding sites of the rapidly equilibrating pool. γABA released from the rapidly equilibrating pool should have the same specific activity as the supernatant (12,500 cpm/μg). The decrease in the number of binding sites also would decrease the carrier-mediated release of γABA of low specific activity which is in the slowly equilibrating pool. The dilution in KCl resulted in a release of 0·68 μg γABA, which had a specific activity of 11,700 cpm/μg.

A method for estimating the approximate sizes of the rapidly and slowly equilibrating pools

In the subsequent experiments the sizes of the rapidly and slowly equilibrating pools were estimated in the following manner. Five minutes prior to centrifugation of a radioactive suspension, a 2-ml aliquot was removed and placed in a tube that contained 20 μl of an aqueous solution of γABA (40 mg/ml) so that the final concentration was 400 μg/ml. The two samples were centrifuged simultaneously at 15,000 *g* (15 min). The supernatants were decanted immediately. Only radioactivity in the supernatant was determined in the case of the 2-ml sample containing added γABA (chase sample). The usual analysis of distribution of isotope and γABA content was carried out on the pellet and supernatant of the other sample (standard sample). The supernatant counts of the chase sample minus the supernatant counts of the standard sample represented the counts removed from the binding sites of the rapidly equilibrating

pool. The total counts in the pellet minus the chased counts were taken to represent the counts in the slowly equilibrating pool. By definition the supernatant and rapidly equilibrating pool were taken to have the same specific activities. By determination of the specific activity of the supernatant of the standard sample and knowing the number of counts in the rapidly equilibrating pool, the size (amount of γABA) of the pool could be calculated. The remaining counts and quantity of γABA of the pellet were assigned to the slowly equilibrating pool. In this procedure there was a small overestimate in the size of the rapidly equilibrating pool, and an underestimate in the size and specific activity of the slowly equilibrating pool, because the chase not only liberates virtually all the counts from the rapidly equilibrating pool but also some from the slowly equilibrating pool. The higher the specific activity of the slowly equilibrating pool the greater the errors would be.

The effect of Na^+ concentration on the rapidly and slowly equilibrating pools

The model states that an increasing Na^+ concentration should increase the number of active carrier sites. For the slowly equilibrating pool the net result would be an acceleration of the rates at which counts enter, at which the specific activity increases, and at which the size of the pool decreases. The last should occur only if the carrier sites were less saturated on the external surface than on the internal surface of the membrane. The γABA released from the slowly equilibrating pool would be distributed between the supernatant and the rapidly equilibrating pool. Thus the specific activity of the supernatant should drop more rapidly with an increased Na^+ concentration. The total amount of γABA found in the supernatant will depend on how great the increment in binding sites might be with increasing concentrations of Na^+.

The particles were resuspended in a small volume of 0·25 M sucrose (see Method 2) and aliquots were added to tubes containing buffered salt solutions and ^{14}C-γABA. The three final suspensions contained 0·2 M KCl, 0·1 M KCl + 0·1 M NaCl, and 0·2 M NaCl. At 20 and 80 min after resuspension in the salt solutions, aliquots were centrifuged along with 2-ml γABA chase tubes as described in the preceding section. The supernatant fluid was decanted at 40 and 100 min respectively.

In conformity with the model (see Table 3) increasing Na^+ concentration resulted in an accelerated rate of change in the specific activity of the supernatant, which may

TABLE 3. SPECIFIC ACTIVITY AND γABA CONTENT OF SUPERNATANT AND THE SPECIFIC ACTIVITY AND RADIOACTIVITY OF THE SLOWLY EQUILIBRATING POOL AS A FUNCTION OF TIME AND ELECTROLYTE COMPOSITION

	Time (min)	0·2 M KCl		0·1 M KCl + 0·1 M NaCl		0·2 M NaCl	
		(cpm/μg γABA)	(μg γABA)	(cpm/μg γABA)	(μg γABA)	(cpm/μg γABA)	(μg γABA)
A	40	17,000	2·50	16,500	2·27	15,000	2·09
Supernatant	100	15,000	2·94	13,000	2·59	11,500	2·43
		(cpm/μg γABA)	(cpm)	(cpm/μg γABA)	(cpm)	(cpm/μg γABA)	(cpm)
B	40	0	0	500	1,800	850	2,900
Slowly equilibrating pool	100	0	0	1,250	3,470	1,700	4,580

For all the above samples total γABA was 6·19 μg/ml of suspension, total radioactivity was 44,500 cpm/ml of suspension, and the specific activity of the suspension was 7,200 cpm/μg γABA.

be interpreted to be a reflection of the greater number of carrier sites available for translocation of γABA across the barrier in both directions. In spite of this the total supernatant γABA decreased with increasing Na^+ concentration (Table 3, A). This is attributable to the increased number of active carrier sites in the rapidly equilibrating pool. Agreement with the model is also seen in the finding that with increasing Na^+ concentration there was an increase in the number of counts in the slowly equilibrating pool (Table 3, B). The specific activity values also show the expected trend. The total γABA in the pellet and the size of the two pools as a function of time and Na^+ concentration are shown in Fig. 3. Over the time interval which was studied the total

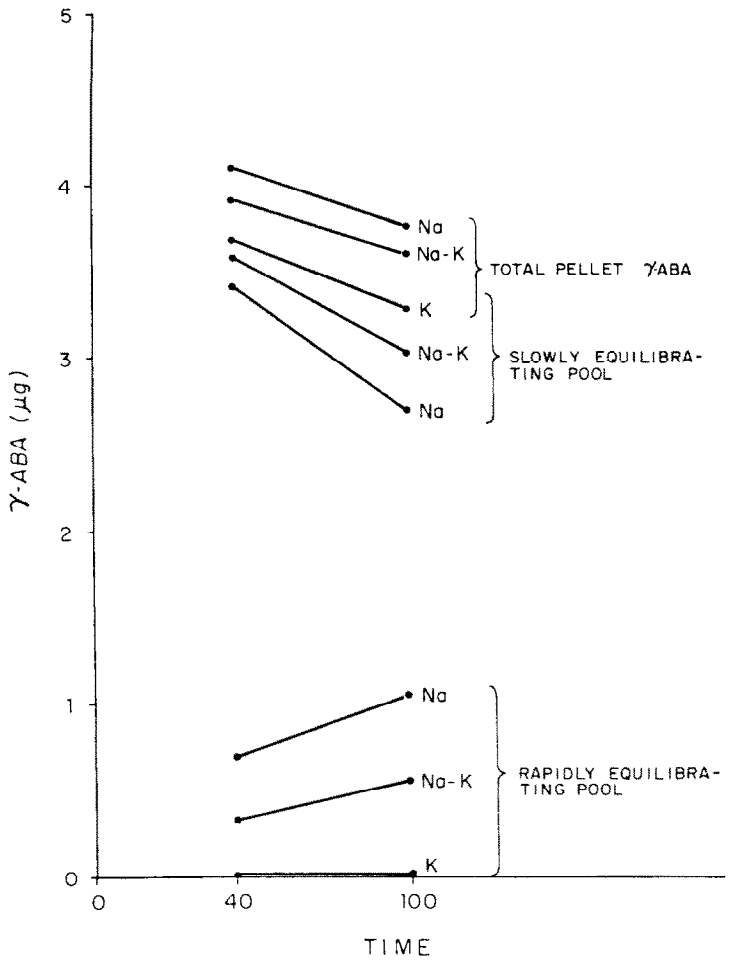

FIG. 3. Total pellet γABA and the size of the rapidly and slowly equilibrating pools as a function of time and Na concentration: Na = 0·2 M NaCl; Na − K = 0·1 M NaCl + 0·1 M KCl; K = 0·2 M KCl.

γABA decreased in an almost parallel fashion for the three conditions. The higher the Na^+ concentration the greater the total amount of γABA associated with the particles. The analysis in terms of the two pools demonstrated the predicted increase in the rate of loss from the slowly equilibrating pool as a function of Na^+ concentration. This

general trend has already been inferred from the changes in the specific activity of the supernatant. The rapidly equilibrating pool increased in size with Na^+ concentration and with time. Theoretically this time-dependent increase may be a result of any one of three factors or a combination of them: (1) an increased binding may occur because of a greater degree of saturation of the binding sites; (2) a slow rate of activation of the binding sites may have been detected because the Na^+ and isotope were added simultaneously; (3) as the specific activity of the slowly equilibrating pool increases, any release from this pool in the chase tube would tend to increase the calculated value for the γABA content of the rapidly equilibrating pool.

Changes in pool size as a function of time

It was of interest to examine the time-dependent changes in pool size, specific activity, and radioactivity of the rapidly and slowly equilibrating pools after the exposure of particles to labeled γABA. Thirty min after suspension of the particles in saline, 2-^{14}C-γABA was added to the suspension. If there is a time-dependent activation of the binding sites by Na^+, as suggested in the previous section, the prior exposure of particles to Na^+ might be expected to minimize this possible variable. At

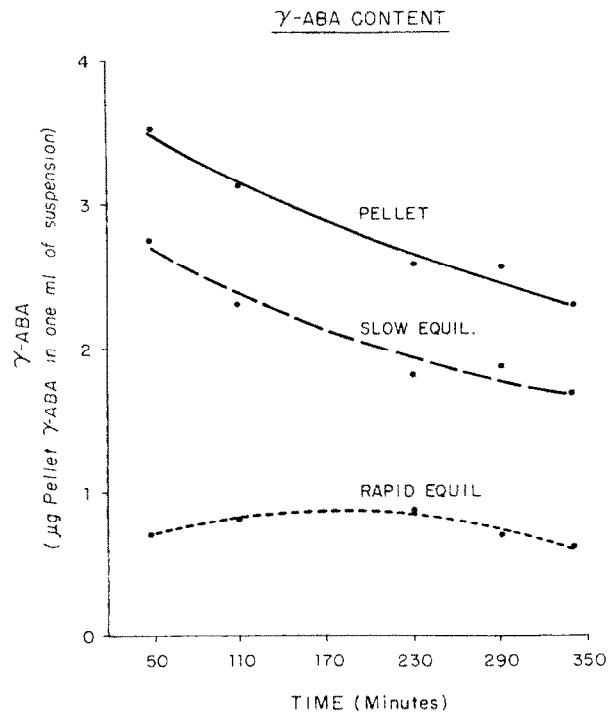

FIG. 4. γABA content of rapidly and slowly equilibrating pools of the pellet and the total γABA content of the pellet as a function of time.

30-min and 1-hr intervals thereafter, aliquots were centrifuged and analyzed for radioactivity, γABA content, and specific activity of the rapidly and slowly equilibrating pools of the particles. The time values of Figs. 4–6 represent the times at which the supernatant fluid was decanted after the addition of the isotope to the suspension.

One ml of the suspension (particles and supernatant) contained 5·16 μg of γABA, an activity of 35,000 cpm/ml, and a specific activity of 6,750 cpm/μg γABA. In Figs. 4–6 are shown the changes that occurred in the total pellet. The total γABA content of the pellet (Fig. 4) decreased with time. The accumulation of counts in the pellet (Fig. 5) went through a maximum at approximately 110 min and decreased thereafter. The

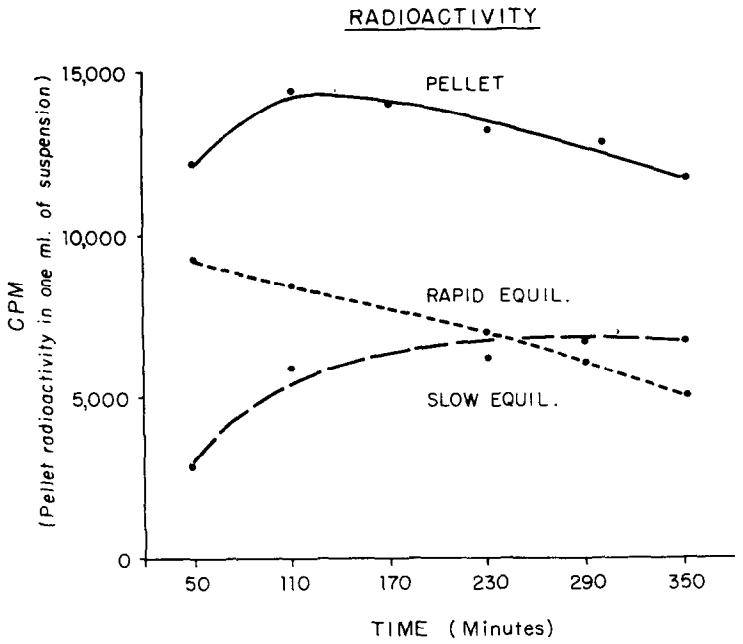

FIG. 5. Radioactivity of the rapidly and slowly equilibrating pools of the pellet and the total radioactivity of the pellet as a function of time.

specific activity of the pellet increased (Fig. 6) up to 170 min and remained constant to the end of the experiment. This constant specific activity represents a balance of the changes occurring in the two pools (Fig. 6). The decreasing specific activity of the rapidly equilibrating pool is offset by the increasing specific activity of the slowly equilibrating pool in such a manner that the specific activity of the pellet remains essentially constant.

The rate of loss of γABA in the slowly equilibrating pool was similar to that found for the loss of total γABA (Fig. 4) from the pellet. The incorporation of radioactivity into the slowly equilibrating pool (Fig. 5) reached a constant level at 170 min. The specific activity of this compartment increased rapidly at first and then more slowly (Fig. 6). This presumably should continue until the specific activities of the two pools are the same.

The specific activity of the rapidly equilibrating pool (Fig. 6) decreased with time because of the slow exchange with the slowly equilibrating pool. The total counts in the rapidly equilibrating pool decreased as a function of time (Fig. 5). The γABA content (Fig. 4) of the rapidly equilibrating pool rose slightly and then declined. The increase could possibly be attributed to an initial increased saturation of the binding

sites and the decrease to a slow inactivation of binding sites in the membrane. The latter phenomenon was more apparent in experiments carried out for a longer period of time. It can be concluded that the above trends were in qualitative agreement with those that would have been predicted from the model.

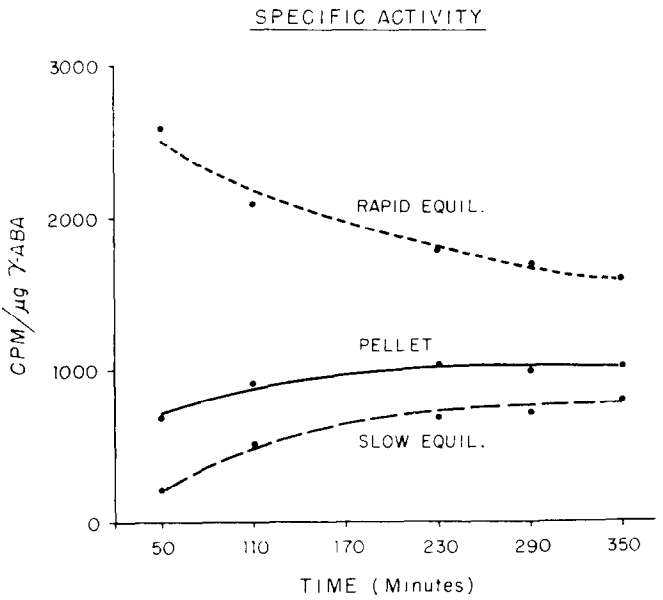

FIG. 6. Specific activity of the rapidly and slowly equilibrating pool of the pellet and the specific activity of the pellet as a function of time.

DISCUSSION

The carrier-mediated transfer model presented in this paper is essentially similar to the one formulated by Wilbrandt and Rosenberg,[7] with one difference—the introduction of the concept of a barrier which must be traversed by the carrier.

It is generally agreed that a carrier-mediated transfer across membranes is a basic prerequisite for active transport.[7, 8] Evidence will be presented in the future, which indicates that the subcellular particles used in this investigation can carry out Na^+-dependent active transport at 28°.

There is a Na^+ requirement for active transport of various sugars[9] and amino acids[10–12] by brain slices,[10] Ehrlich ascites cells, [10] rat intestine,[9] and red blood cells.[12] An analysis of the kinetics of the Na^+-dependent active transport of glycine by pigeon red blood cells has resulted in the conclusion that the effect of Na^+ is to govern the binding of glycine.[12] Our results support the possibility that a similar requirement exists for the binding of γABA by subcellular particles of mouse brain.

One of the logical extensions of the present model is that if a Na^+ gradient could be maintained across the membrane, active transport would ensue even at 0°–4°. Let us assume that the carrier is free to move across the barrier at a given rate: when the carrier is in a region of high Na^+ concentration it would tend to bind γABA, and in a region of low Na^+ concentration it would tend to release it. This would result in a net

translocation of γABA across the barrier, even if the internal and external concentrations of γABA were the same. Accumulation of internal sodium would result in the ability of the carrier to bind γABA on the inside of the membrane and to carry it out as well, which is essentially the situation presumed to exist in the case of the present experiments. In the living cell the energy-dependent Na^+ pump could serve as a regulator for the concentrative abilities of the cell. This could also explain the inhibition of active transport by such compounds as ouabain and dinitrophenol[7, 8] which interfere with the electrolyte balance of the cell. These compounds would not necessarily be expected to interfere with the binding ability of the carrier. In agreement with this idea we have found that the carrier-mediated transfer of γABA is not altered by ouabain or dinitrophenol at 0°–4° but that at elevated temperatures (28°) the active transport of γABA is profoundly inhibited by these substances. These studies will be published subsequently.[13]

In the present experiments evidence has been presented which fits the hypothesis of a carrier-mediated diffusion. Additional support for our model would be a direct demonstration of the existence of spheroidal membrane structures in the subcellular fractions that bind γABA, the ability of the isotope to enter a pool of nonbound endogenous γABA within the spheroidal structure, and to demonstrate that the slowly equilibrating pool is sensitive to osmotic factors. The following information applies to these points.

If the "mitochondrial" fraction is permitted to incorporate isotope, and the particles are then sedimented and resuspended in a saline-free sucrose medium, the expectation is that the binding sites will be inactivated, and the isotope that has entered the slowly equilibrating pool will be trapped in solution inside the particle with endogenous γABA which occupies this space. Subfractionation of such material on a sucrose density gradient has shown[14] that the ^{14}C-γABA and endogenous γABA are associated with a subfraction which is predominantly composed of presynaptic nerve-ending particles, and on a protein basis these particles are the major component of this "mitochondrial" fraction. It has also been reported[2] that pre-exposure of a crude "mitochondrial" fraction to hypotonic conditions results in a decreased binding of the isotope. This would be accounted for in terms of our model by the release of γABA into the suspending medium with a concomitant decrease in the size of the slowly equilibrating pool and increase in γABA content of the suspending fluid. This would decrease the probability of the isotope's entering or being retained in this pool and decrease binding to the rapidly equilibrating pool. Unpublished results[15] from our laboratory indicate that the previously reported binding to microsomal fractions[4] can be related to the vesicular component of the microsomal fraction, which has been interpreted to be resealed fragments of the endoplasmic reticulum. These particles also constitute a carrier-mediated transfer system.

In view of this information and the fact that a heterogeneous mitochondrial fraction was utilized in the present investigation, the conclusion is that the properties of the mitochondrial fraction are predominantly a reflection of events associated with the nerve-ending particles which, on a protein basis, may well represent over 50% of the total material of the mitochondrial fraction.[14] The possibility that there may be some binding of isotope to unsealed fragments of membrane or that there may be some endogenous γABA which is bound to some unspecified site would in no way change the present interpretation, since neither of these two possibilities could conceivably

alter the interpretation, which is solely dependent on the unique relationships of the two pools to each other. The possibility of other binding events would result only in a false estimate of the absolute size of the two pools.

One other point that might bear emphasis is that the two pools cannot readily be conceived of as two classes of binding sites in which there is a site–site migration. Such a hypothesis would lead to the predicament of having to explain why the size of the slowly equilibrating pool decreases with time and why the rate of decrease increases as the number of activated carriers is increased.

ADDENDUM

At the time of submission of this manuscript Vidaver presented direct evidence for the relationship of the Na^+ gradient to glycine transport by the pigeon red blood cell: G. A. Vidaver, *Biochemistry* **3**, 795, (1964), and *ibid.*, p. 803.

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