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Binding of Radioactive Sodium, Potassium, and Bromide in Guinea Pig Brain Homogenates.* (26745)

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Binding of various electrolytes in the brain has been described over the years since Fremy in 1841 first noted the presence of fixed mineral substances in brain tissue. Thudichum found potassium to be the chief inorganic constituent present in protagon and demonstrated that calcium is attached to cephalin. Koch and Pike(7) showed that cephalin contains more potassium than sodium, but that in lecithin the reverse is true.

Christensen and Hastings(3) found that cephalin has the ability to combine with sodium and potassium with equal affinity in amounts increasing with pH. At neutrality they found that 0.5-0.6 equivalents of alkali were bound per mole of cephalin.

Folch(4) reported 30% excess of cations over anions in brain, accounting for this "anion deficit" by the binding of approximately this proportion of brain cations to cerebron sulfuric acid and phosphatidyl serine. Twenty per cent of the brain sodium (10 mEq) was bound to phosphatidyl serine.

Stone and Shapiro(13) filtered homogenates of brain and muscle under negative pressure at 3-4°C and found that 20% of brain and muscle potassium did not diffuse through cellophane, while 100% of sodium was freely diffusible. Bergen, Stone and Hoagland(2) used the ultrafiltration technic of Stone and Shapiro to demonstrate binding of about 30% of rat brain potassium not influenced by adrenalectomy. Their results confirmed earlier reports of a slowly exchanging brain potassium compartment which develops with age in rats. On the other hand, Leiderman and Katzman(9) demonstrated a significant difference in radio-potassium uptake by the brains of normal and adrenalectomized rats. They(8) found 20 mEq of radio-potassium per kilogram of wet brain to be non-exchangeable in normal adult rats, as determined by comparison of brain and plasma specific activities up to 72 hours after intraperitoneal injection. This non-exchangeable potassium compartment was not present in rats younger than 35 days and was abolished by adrenalectomy in adults.

Shaw and Simon(12) found that the potassium in toad nerve and muscle, allowed to die naturally or poisoned with iodoacetate or cyanide, did not equilibrate with the surrounding medium. They postulated potassium binding to explain this effect.

Shanes and Berman(11) studied diffusion out of toad sciatic nerve. They found that a small but significant proportion of radioactive sodium did not come to diffusion equilibrium with the surrounding medium.

Pappius and Elliott(10) noted that potassium content of rat cerebral cortex slices in a Warburg apparatus under anaerobic conditions failed to come to equilibrium with the medium when potassium concentration of the medium was high. They suggested that this disequilibrium might be due to binding of a large portion of intracellular potassium.

Recapitulation of the reported literature seems to indicate that there is probably

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"bound" potassium in brain tissue. The location or chemical moiety involved in the binding is suggested only by Folch's work, and the metabolic and/or chemical conditions of the binding are far from well-defined at this stage in our knowledge.

The term "binding" has been used by the investigators cited with several different though related meanings, with reference to observations that the electrolytes were: "retained by tissue," "combined with elements," "non-exchangeable," "slowly exchangeable," or perhaps "only present in unexpectedly high concentration." These differences in definition have probably accounted for most of the discrepancies reported by different workers.

The present study grew out of our previous study(5) of the diffusion of radioactive ions out of brain tissue slices. In this earlier paper, only the beginning of the diffusion process was considered. In the present investigation the later parts of the diffusion curves, at or near equilibrium, have been studied.

Radioactive tracers. Hypotonic or isotonic solutions of radioactive sodium ($Na^{24}Cl$) potassium ($K^{42}Cl$) or bromide (KBr^{82}) were used in these experiments, the first 2 as solutions in hydrochloric acid, the latter in water.

The solutions were brought to approximate neutrality prior to use. To adjust for their high radioactive concentrations, and for convenience in injection, the lots were diluted with normal saline.

General methods and tissue preparations. Unselected healthy adult male guinea pigs were employed in all experiments where brain tissue was required. In those experiments requiring radioactive brain tissue, each guinea pig was injected intraperitoneally with 200-400 μc of isotope solution, fed and watered *ad lib.*, and sacrificed after a delay to allow for distribution of the tracer: 12 hours for Na^{24} and 24 hours for K^{42} or Br^{82}. The uptakes of these tracers were determined in a series of experiments by sacrificing the animals 1½, 3, 6 or 9 hours after intraperitoneal injection.

All brain tissue was prepared as follows: The animal was rapidly decapitated, the brain was removed and divided longitudinally by a median cut through hemispheres and brain stem. Brain stem, basal ganglia, and choroid plexi were removed, then the pia was stripped with fine forceps, leaving the cortex and some white matter, but free of macroscopically obvious blood. At the same time, the liver was removed and small pieces cut from it. The strips of tissue were pressed firmly between sheets of absorbent paper to remove as much blood as possible. The tissue pieces were then transferred to a tared homogenizer tube and weighed. Standard mammalian Ringer's solution was added, and the mixture homogenized with a Potter-Elvehjem tissue grinder with Teflon pestle. Ringer's solution was added to the homogenate to bring the tissues to final dilution of 1:20.

Thoroughness of homogenization was evaluated by microscopic examination of the sedimented plug obtained by centrifuging samples of homogenate. Smears and paraffin sections of the sediment showed many intact nuclei but no intact cells. Irregular granular material with an occasional red cell or torn length of capillary were also noted.

The experimental procedures carried out were of two general categories: (1) equilibrium dialysis, and (2) centrifugation followed by analysis of the supernatant and sediment.

Dialysis experiments. For the dialysis experiments 20 ml of the dilute homogenate was dialyzed against 400 ml of isosmotic solution through cellophane sausage tubing. Dialysis was continued for 2 hours at room temperature.

Samples from the dialysis bag and the external solution were transferred to tared planchettes, weighed, and dried with a spreading agent. Radioactivity was determined either with a Scott type Geiger-Muller counter or a Tracerlab scintillation counter with 1.29 g/cm^2 lead shielding to remove beta emission.‡ Radioactive decay, self ab-

‡ We would like to thank Dr. K. G. Scott and staff of the Radioactivity Center, Univ. of California, San Francisco, for their very kind assistance.

sorption, and coincidence corrections were carried out as appropriate.

The materials to be dialyzed were prepared according to the following procedures.

Procedure 1: ("Blank" in Fig. 2). The dialysis bag was filled with a modified Ringer's solution to which was added a small drop of radioactive tracer, but no brain homogenate.

Procedure 2: ("Control" in Fig. 2). The dialysis bag was filled with homogenized non-radioactive brain to which was added a small drop of radioactive tracer. This procedure was the same as Procedure 1, except for addition of non-radioactive brain homogenate.

Procedure 3: ("Supernatant" in Fig. 2). The dialysis bag was filled with supernatant obtained by centrifugation of homogenized radioactive brain from a tracer-injected animal. See "Centrifugation Experiments."

Procedures 4 and 5: (NOT CONVULSED in Fig. 2). The dialysis bag was filled with homogenized radioactive brain from a tracer-injected animal, either not premedicated ("No Drug") or injected with sodium pentobarbital prior to sacrifice ("Pentobarbital").

Procedures 6-7-8: (CONVULSED in Fig. 2). The dialysis bag was filled with radioactive brain homogenate from a tracer-injected animal convulsed with one of 3 agents ("Cocaine," "Electric Shock," or "Metrazol"). The basic procedure here was the same as with Procedures 4 and 5 above.

An additional series of control dialyses was done with homogenized radioactive liver tissue from tracer-injected animals.

Centrifugation experiments. Centrifugation experiments were carried out on brain tissue from tracer-injected animals either after convulsions (cocaine, electric shock, or metrazol), or sacrificed without premedication. Thirty ml portions of dilute brain homogenate, prepared as described previously, were centrifuged for 15 minutes at 3,000 rpm. Samples of supernatant and sediment were weighed and dried, and radioactivity was determined. (Fig. 3). The supernatant was also studied by dialysis (see above, procedure 3).

Development of binding. A series of dialysis experiments were carried out with intervals of 1½, 3, 6, and 9 hours between injection and sacrifice, in order to define the rate at which the non-exchangeable electrolyte fractions develop. The technics were exactly the same as in the control dialysis studies, except for the variation in post-injection intervals.

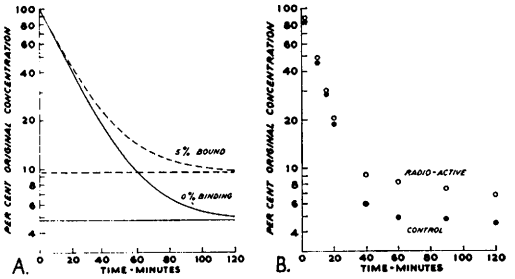
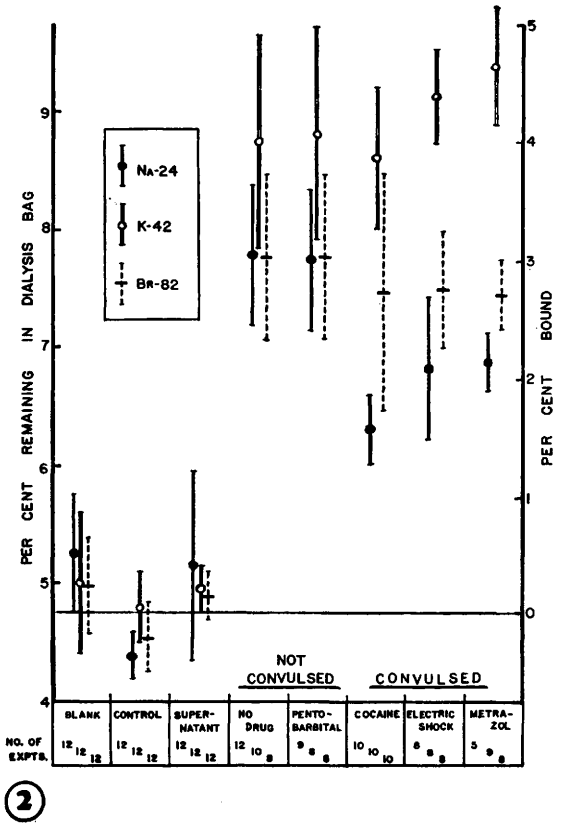


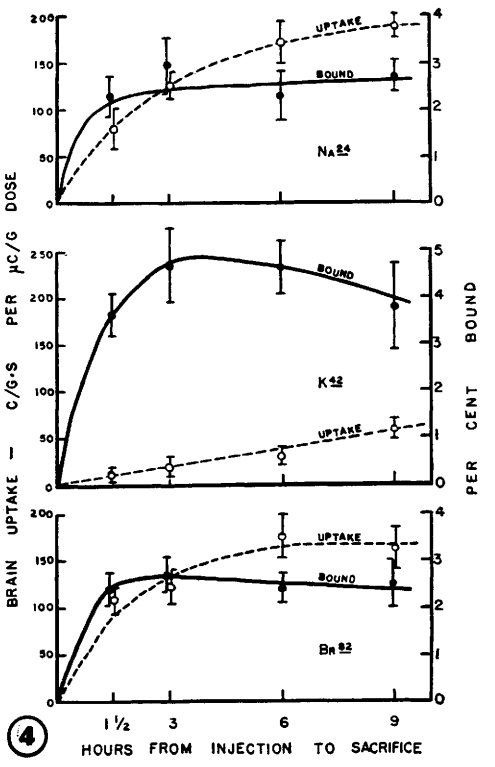
FIG. 1A. Theoretical dialysis curves based on experimental method described.

FIG. 1B. Experimental curves of the same type as theoretical curves of 1A, using radioactive bromide as tracer. Points indicate radioactivity found inside the dialysis bag after dialysis for various periods. See text for additional details.

Results. 1. *Dialysis experiments.* At equilibrium, assuming free permeability, radioactive tracer should be evenly distributed throughout the 20 cc inside the bag and the 400 cc outside the bag. Thus, if there is no fixation ("binding") of the radioactive tracer inside the dialysis bag, concentrations of both inner and outer solutions should be 4.76% of the concentration inside the bag at the beginning of dialysis, as in the lower theoretical curve of Fig. 1A. If 5% binding were present, the curve would approximate the upper theoretical curve of Fig. 1A. Fig. 1B shows the results of a series of preliminary experiments with radiobromide, which confirmed that binding actually was present. Similar results were obtained from preliminary studies with potassium and sodium. Comparison of columns 2 and 3 of Fig. 2 with column 1 immediately indicates the absence of significant binding of Na^{24}, K^{42} or Br^{82} *in vitro* or in the centrifuged supernatant. From the latter observation it appears that these isotopes are combined essentially, if not entirely, in the centrifugable portion of the homogenates (see also "Centrifugation Experiments").

It is to be emphasized that binding is re-





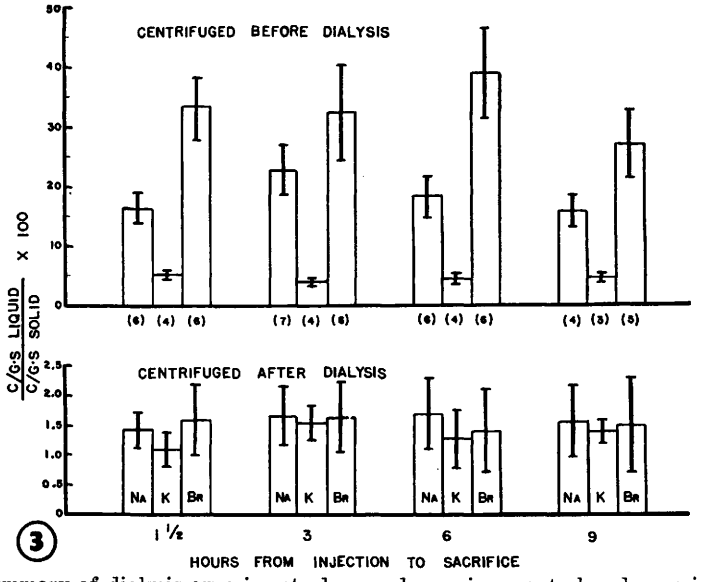


FIG. 2. Summary of dialysis experiments done under various control and experimental conditions. As described in text, values given for each set of data are recorded as percent of original radioactivity retained inside the cellophane dialysis bag at the end of dialysis (left scale) or percent of original brain radioactivity bound (right scale). Mean values are indicated, with stand. deviations.

FIG. 3. Centrifugation studies as described in the text. Means and stand. deviations are indicated. Parenthetical numbers represent numbers of experiments performed.

FIG. 4. Radioactive uptake and binding in the brain. The same animals were used for both determinations. Uptake, as indicated, was measured as proportion of inj. dose, while binding, as described elsewhere, was recorded as % of radioactivity originally present in the brain. Experimental points are means, and stand. deviations are indicated.

TABLE I. Effect of Convulsions on Binding. "Control" data are derived from columns 4 and 5 of Fig. 2, while "Convulsed" data derive from columns 6, 7, and 8. Values are reported as % of radioactivity present in the brain. Parenthetical numbers indicate number of experiments in the respective groups. Ranges given are stand. dev., and "p" was calculated using the "t" test.

	Controls, % bound	Convulsed, % bound	Significance of diff. "p"
Na^{24}	3.1 ± .6 (21)	2.0 ± .4 (23)	<.01
K^{42}	4.1 ± .9 (18)	4.3 ± .5 (27)	.4
Br^{82}	3.1 ± .7 (16)	2.8 ± .6 (26)	.1

ported here in percent of radioactivity *present in the brain.* There appears to be no obvious relation between these percentages and injected dose, at least in the dosage ranges employed here. Amount of binding can also be reported in mEq/kg wet brain in the case of sodium and potassium, but this is not possible or relevant with bromide, which is present only in tracer amounts.

The use of proportionality values (percentages) rather than chemical equivalents or other units appears to be justified by the observation that only such proportionality values make obvious the striking similarity between the binding of the 3 tracer elements studied, 2 of them normal brain constituents and the third a foreign tracer substance. No other investigator of whom we are aware has considered the possibility that such physiologically dissimilar substances might react in such similar ways in the brain. Possibly this has been missed because the similarities never appear with the usual methods of displaying the data.

Columns 4 and 5, Fig. 2, indicate that about 3% of sodium (1.7 mEq/kg wet brain), 3% of bromide, and 4% of potassium (3.5 mEq/kg) are bound *in vivo,* as measured by this technic.

Convulsions caused by cocaine, metrazol or electric shock (columns 6-7-8, Fig. 2) effected a significant decrease of the bound sodium: 50% (0.8 mEq/kg) in the case of cocaine, and 30% (0.5 mEq/kg) with metrazol or electric shock. No change in binding of potassium or bromide was noted in these convulsed animals. These data are summarized in Table I.

2. *Centrifugation experiments.* The results of the centrifugation experiments are given in Fig. 3. The guinea pigs used in these experiments were treated as described previously, samples of homogenate being taken for dialysis as well as for centrifugation. Centrifugation was carried out both before and after 2 hours' dialysis. The bars represent quotients obtained by dividing the calculated activity of the dry centrifuged sediment into the activity of the centrifuged supernatant. These quotients bear no straightforward relationship to percentage of isotope binding, but serve as a useful basis for comparison of different groups of experiments: the smaller the value of the quotient, the greater the proportion of bound isotope in the centrifuged sediment, and the less the proportion in the supernatant.

Analysis of the centrifuged homogenate before dialysis (Fig. 3) indicated that, of the 3 electrolytes, bromide was present in the sediment in smallest amount, sodium was next, and potassium was present in the greatest proportion.

After 2 hours of dialysis, on the other hand (Fig. 3, bottom), the proportions of sodium, potassium, and bromide in the sediment diminished, and became identical within the limits of experimental error.

3. *Development of binding.* The curves of Fig. 4 indicate that binding of all 3 ions studied occurred very rapidly, reaching nearly full value within 1½ hours after injection. This figure also shows the lack of relation between uptake and development of binding.

4. *Liver controls.* From every third or fourth animal a section of liver tissue was taken and dialyzed in precisely the same manner and at the same time as the brain tissue from the same animal. This was done as a further check on the method and to demonstrate possible differences between brain and a non-nervous tissue. Table II indicates that there is no significant binding of radio-sodium or bromide in liver homogenates. There is, however, questionably significant binding of K^{42} at about 40% the level observed in brain.

Discussion. The concept of binding is

TABLE II. Absence of Binding in Homogenized Liver. Ranges given are stand. dev.; "p" values were calculated by "t" test.

	% remaining in dialysis bag at 2 hr	Apparent % bound	Significance of binding, "p"
Na^{24}	5.6 ± 1.3 (12)	.9	.4
K^{42}	6.5 ± 1.0 (12)	1.8	.1
Br^{82}	5.1 ± .8 (14)	.5	.4

rather inexact at times, and criticism can justifiably be leveled against any approach so far employed in study of the subject. The major methods which have been used in the past are: 1. Analysis of tissue extractives, 2. Diffusion procedures employing semipermeable membranes, 3. Centrifugation of tissue homogenates, 4. Measurement of ionic activities by physical or chemical methods (*e.g.*, membrane electrodes), 5. Influx or outflux studies, and 6. Nuclear resonance technic.

In the present experiments, methods 2 and 3 were used. Demonstration that a tissue extractive contains a certain amount of an ionic species does not prove that this combination is physiologically important, nor that there is any actual combination whatsoever *in vivo*. Similarly, failure to show a bound ionic fraction by a dialysis method does not negate the possibility that there may be in the intact cell a loose combination of the ion, of great physiological importance.

Not only must the rate at which an ion complex can dissociate be considered, but also the site of binding within the cell may be of basic importance. Bartley and Davies (1) showed that mitochondria could maintain concentration gradients of sodium, potassium, magnesium, and phosphate under a variety of conditions. They obtained maximum ratios of internal concentration to external concentration at 0°C in absence of substrate enzymatic co-factors, such as ATP or DPN, when concentration in the external medium was very low.

Our observations for Na^{24}, K^{42} and Br^{82} suggest that the bound fractions are not present in mitochondrial or microsomal fractions, as defined by other workers. The centrifugation methods used here would leave these fractions in the supernatant, and no binding was observed in these centrifuged supernatants. (Cf Fig. 2, third column).

Age may be a significant variable, as indicated by Katzman and Leiderman's(8) demonstration of a non-exchangeable potassium compartment in rat brain, developing with age. We have carried out studies on the effect of age on binding as we have defined it, which will be reported later.

Our results are in contrast with those of Bergen, Stone, and Hoagland(2), who used a dialysis technic similar to ours and found about 30% of adult rat brain K^{42} to be non-diffusible through cellophane. The reason or reasons for this difference in results are not presently clear to us. Degree of homogenization and possible species differences remain to be investigated.

The absence of binding of sodium in guinea pig liver is in partial disagreement with data of Griswold and Pace(6) on rat liver. They found, using flame photometry following perfusion and differential centrifugation, that sodium and potassium were retained (7%-18%) in "mitochondrial" and "microsomal" fractions of minced liver.

The effect of convulsions on bound Na^{24} is most interesting. The decrease in the bound sodium (Fig. 2) must represent an actual loss from the bound compartment during convulsions, rather than a simple dilution effect such as might result from development of brain edema, since if the 50% change were a simple dilution effect, total brain sodium would have had to rise to the 200% level, which was impossible under these experimental conditions.

There was no significant increase or decrease in the binding of potassium or bromide during convulsions. This lack of change may mean that potassium and bromide are more strongly bound than sodium. More likely, however, it simply implies that any changes which occur effect both bound and unbound fractions similarly, so that the 2 remain in equilibrium and the relative proportions do not change.

The results of experiments on the dialysis of centrifuged supernatant and the studies on pre- and post-dialysis centrifugation support each other in demonstrating that the

bound fractions are in the solid, sedimentable component of the brain homogenate. These observations, the similar percentages of binding of the 3 elements, the similarity of the time courses of development of their binding in the brain, and the lack of binding in liver constitute considerable evidence of parallel behavior of physiologically dissimilar ionic species (Fig. 2, bottom of Fig. 3, and binding curves of Fig. 4). These data suggest that the binding may depend upon some unique characteristic of brain tissue, rather than being a function of the specific physiological characteristics of the individual elements.

Myelin is the most obvious unique constituent of brain. It has been demonstrated by electron micrography to have a layered structure. We should like to suggest that the occurrence of similar proportions of binding of the 3 dissimilar elements studied here may depend upon the colloidal nature of the myelin membrane. On the other hand, the diminution of binding of only one of these elements (sodium) as a result of convulsive activity suggests that the quantitatively similar binding of the 3 may be coincidental.

Further studies of a more specifically chemical nature are presently under way to evaluate these possibilities.

Summary. 1. Dialysis of guinea pig brain homogenates from animals given intraperitoneal injections of radioactive tracers revealed that 3%-4% of brain sodium (1.7 mEq/kg wet brain), bromide and potassium (3.5 mEq/kg) were "bound", *i.e.*, did not pass the dialysis membrane. 2. Centrifugation studies indicated that these "bound" fractions were contained in the sedimentable portion of the homogenates, and therefore are not mitochondrial or microsomal, as these terms are usually defined. 3. Similarities in amount and development of "binding" of these 3 elements, and in the effects of centrifugation, as well as the absence of comparable binding in liver suggested that this binding may be related to the presence of myelin. 4. The level of bound sodium decreased 50% as a result of convulsions, with no concomitant change in bromide or potassium. 5. The significance of these observations was discussed.

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