MAGNESIUM DEFICIENCY AND EXCITABILITY IN THE RAT:
AN EXAMINATION OF SELECTED BIOCHEMICAL AND
PHYSIOLOGICAL EVENTS RELATING MAGNESIUM
STATUS TO BEHAVIOR

by
Douglas Robinson Buck

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DEDICATION

To

John and Marjorie Buck
And
Ed and Marge Geiger
ACKNOWLEDGMENTS

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I thank the members of my graduate committee. They have given important theoretical and procedural suggestions, and have reviewed the several articles which have been written, adding clarity of thought and otherwise improving the quality of the work.

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Douglas R. Buck
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The effect of Mg status on the behavior of rats, as determined by nonspecific excitability level and audiogenic seizure susceptibility, was investigated. Also, selected biochemical and neurological mechanisms mediating the chain of events from dietary magnesium deficit to the hyperexcitability symptoms were examined. Weanling rats fed a low magnesium (10 ppm) diet for 14 days had reduced serum, cerebrospinal fluid and brain magnesium concentrations, and increased brain (Na⁺ K⁺)-ATPase activity. They exhibited increased NEL and became highly susceptible to audiogenic seizures. Through dietary manipulation and intraperitoneal and cerebral intraventricular injections, it was possible
selectively to alter either serum or cerebrospinal fluid magnesium concentrations.

Both nonspecific excitability level and audiogenic seizure susceptibility responded inversely to cerebrospinal fluid magnesium concentration, but not to serum magnesium, unless the latter was very high. In this instance, nonspecific excitability level but not seizure activity was depressed.

Brain serotonin concentration was elevated in 150-200 g rats fed a low magnesium diet for 21 days, compared with rats fed a control diet (0.73 µg/g fresh brain cf. 0.56 µg/g).

The magnesium deficient rat may serve as an excellent model for epilepsy research. The animal can often be revived following seizure, thus enabling the study of drug interactions while using a small number of subjects. Preliminary findings indicate that the most promising subjects are female rats between 3 and 5 weeks old at the start of feeding and who are fed a low magnesium diet 17 to 21 days.

A procedure for determining (Na\(^+\) K\(^+\))-ATPase activity in rat brain homogenates, without requiring isolation or purification, is described. Computer modeling techniques have yielded expressions for equating enzyme activity to sodium, potassium, magnesium or calcium concentrations in the reaction media. A thorough statistical treatment of the data is presented.
GENERAL INTRODUCTION
GENERAL INTRODUCTION

Magnesium deficiency in mammals is a well known cause of hyperexcitability, as reflected in seizure prone-ness and other symptoms. This fact is extensively documented in the literature with respect to a number of species including man. Most of the research has aimed at substantiating the seriousness of the problem. Some has aimed at identifying magnesium deficiency effects on the composition of various organs and tissues. However, little has been done to delineate the chain of events leading from the nutritional insult to the characteristic behavioral manifestations of it. The investigations reported and discussed herein have attempted to do this.

This dissertation comprises five separate parts which were prepared and written with publication in mind. The research is original and was conducted principally by the author, although others assisted. Arthur W. Mahoney and Deloy G. Hendricks share credit for any publications that result, and when reference is made to authors the plural is used.

The general theme is set by the thesis title. The original intent was to conduct research which would shed light on the mechanisms responsible for altering behavioral excitability due to altered magnesium status. The first
three parts are directed to the general theme. Behavioral excitability was determined by measuring nonspecific excitability level and audiogenic seizure susceptibility. Magnesium status was usually altered by dietary manipulation, although cerebral intraventricular injections were also used.

In conducting this research it seemed logical first to determine if altered magnesium status causes its characteristic behavioral effects by affecting some peripheral mechanisms or strictly by its action on the central nervous system. Then it seemed needful to clarify the relationships among dietary magnesium deficiency, serum magnesium concentrations, cerebrospinal fluid concentration, nonspecific excitability level, and audiogenic seizure susceptibility. Next, since \((Na^+ K^+)\)-ATPase occupies a key role in the central nervous system as the enzyme responsible for converting chemical energy into electrical potential, it seemed important to consider it. Finally, it seemed necessary to consider the effects of altered magnesium status on brain neurotransmitters.

While undertaking any research, ideas and information are continually presenting themselves. Some of these are only tangentially related to the central theme, yet they are often worth developing and explaining so as not to become lost and that others may benefit. Such was the rationale behind development of the fourth and fifth parts. While gathering behavioral data on the magnesium deficient rat,
the prospect presented itself that here might be a useful model of epilepsy. The fourth part directs itself to preliminary research characterizing the model. Finally, to answer some of the questions related to the central theme, a procedure for determining \((\text{Na}^+ \text{K}^+)\)-ATPase had to be perfected in our laboratory. In the process it seemed beneficial to use this opportunity to explain the statistical precision of the procedure and to develop a series of equations which might be useful for predictive purposes.
PART I

EFFECT OF MAGNESIUM DEFICIENCY ON NONSPECIFIC EXCITABILITY LEVEL (NEL) AND AUDIOGENIC SEIZURE SUSCEPTIBILITY
INTRODUCTION

In a recent review and case report (121), it has been surmised and reported that marginal magnesium deficit, not severe enough to elicit seizures, may, in fact be responsible for a number of complaints mistaken for psychoneurosis. Other reports (77, 120) reflect the concern. They recognize that magnesium deficiency may be a practical problem for human beings.

Well-known and nearly universal symptoms of magnesium depletion are seizure susceptibility and hyperexcitability. Literature acknowledging the relationship between the mineral deficiency and these symptoms has been reviewed (91, 121, 149). Those suffering malnutrition from gastrointestinal problems (47, 97) and alcoholics (30, 136) seem especially susceptible. The symptoms have been observed in rodents (2, 14, 43, 76, 104, 118), rabbits (79), dogs (101, 144), ruminants (10, 38), fowl (9), primates (146), and man (33, 47, 57, 59, 95, 116). Assessment of seizure susceptibility has often been done by exposing magnesium deficient animals, usually rats, to a loud noise and counting the number thrown into seizure (7, 25, 43, 56, 103). Assessment of hyperexcitability apart from seizure susceptibility in animals has usually been done by describing how they react in their cages to routine laboratory activity or to the presence of the
researcher. In man, it has been done by self-report or clinical observation.

Although existing literature provides considerable descriptive information about heightened excitability due to magnesium deficiency and some quantitative information about seizure susceptibility, little has been done to quantitate the relationship between magnesium deficiency and other behavioral measures of excitability. It was our purpose to do this.

Lát (81) and Lát and Gollová-Hémon (82) have identified a phenomenon they call Nonspecific Excitability Level (NEL). NEL refers to the level of activity of an organism as measured by large vertical and horizontal movements such as rearing and locomotion. It bears a relationship to hippocampal slow wave (theta) activity (82). Estimating NEL is done by (a) counting certain spontaneous movements such as rearing and locomotion an organism makes during an initial period in a novel environment; or (b) measuring the rate of habituation of these responses. Although NEL may be altered by drug (62), hormonal (81, 82) and dietary (48, 81, 82) treatments, the effect of magnesium deficiency has not hitherto been assessed.

To assess the effect of magnesium on NEL, and to ascertain and clarify the relationships between NEL and audiogenic seizure susceptibility, we set the following objectives: (1) to see if magnesium deficiency causes an
increase in NEL which is reversible; (2) to compare NEL with audiogenic seizure susceptibility; and, (3) to define the relationships between the two behavioral measures and serum and CSF magnesium. To attain these objectives, three experiments were designed. The first was to assess the effects that length of time on a magnesium deficient diet had on NEL. The second was to see if these effects could be reversed by dietary rehabilitation. The third was to assess the effects of injecting deficient animals with magnesium.

**METHOD**

Male weanling Sprague-Dawley rats were fed control diet for at least a day and allowed to become acclimated before experiments were commenced. They were housed in stainless steel cages with wire fronts and bottoms. Diet was provided in glass, and deionized water was provided in polyethylene containers having rubber stoppers and stainless steel lick spouts. Temperature of the animal room was maintained between 20 and 23°C. Lights were on daily from 0700 hrs to 1900 hrs. Diet consisted of 20% casein, 5% sucrose, 53% corn starch, 10% corn oil, 2% vitamin mix and suggested minerals for growth (ICN Pharmaceuticals) consisted of, per kg, the following: 4.5 g vitamin A, 0.25 g vitamin D, 5 g α-tocopherol, 45 g ascorbic acid, 5 g inositol, 75 g
choline chloride, 2.25 g menadione, 5 g p-aminobenzoic acid, 4.5 g niacin, 1 g riboflavin, 1 g pyridoxine-HCl, 1 g thiamine-HCl, 3 g Ca pantothenate, 20 mg biotin, 90 mg folic acid and 1.35 mg vitamin B-12. The magnesium deficient diet was found by analysis to contain 55 mg of Mg per kg diet. Control diet was prepared by adding 400 mg Mg per kg as MgCO₃ to the deficient diet.

Nonspecific excitability level (NEL) was determined by a modification of Lát and Gollavá-Hémon's procedure which was by counting the number of responses made by each rat during the first two minutes after it was placed in a chamber consisting of a glass enclosure 11 cm wide x 22 cm long x 23 cm high with a wire mesh bottom. The responses, counted by two observers, were: the number of times the rat moved from one corner to another, the number of times its nose was raised above the body, the number of rearing responses (counted also as nose-raise responses), the number of grooming responses, the number of fecal pellets excreted, and whether or not it urinated. These responses were respectively weighted: 1, 1, 1, 2, 1 and 2, and added to obtain an activity total for each.

Audiogenic seizures were induced in susceptible animals by the method of Patton (102). Our device utilized two school bells producing 115 dB inside a metal chamber. Rats were exposed to the noise for 1½ minutes or until tonus occurred. Death from seizures was usually prevented by
chest massage until normal respiration was regained. CSF was collected from the cisterna magna in Yale 26 gage \( \frac{1}{2} \) in needles with the points slightly bent to prevent clogging during insertion. Blood was collected by inserting heparinized capillary tubes into the retro-ocular capillary bed.

In Experiment 1, 54 rats were randomly assigned to nine groups of six and housed two per cage. Group one was terminated at the onset. Groups 2 through 8 were fed the magnesium deficient diet; one of these groups was selected to be terminated every other day, the last being terminated on the 14th day of the experiment. Group 9 was fed control diet throughout and was terminated on the 14th day. NEL was measured between 10:30 and 11:00 a.m. on only the group of rats being terminated. These were then tested for audiogenic seizure susceptibility. CSF and serum were collected just before killing.

In Experiment 2, 20 rats were paired according to initial NEL and were individually housed. Both groups were fed magnesium deficient diet for eight days. Blood was taken and serum magnesium level determined. Final adjustments were made in group matchings so that between-group magnesium levels were identical. One group was then fed control diet four days while the other remained on deficient diet. Rats were pair fed during this period. Terminal NEL for each rat was compared with initial NEL. This was done because blood sampling (or giving IP injections) increased
within group variability. Subtracting initial from final NEL reduced this and made it possible to obtain statistical significance in some cases where it was not otherwise obtainable. CSF and serum were collected just before termination.

In Experiment 3, 24 rats were matched according to initial NEL and assigned to three groups of eight. They were individually housed. Two groups were fed magnesium deficient diets. The third group was fed control diet. All were fed ad libitum for five days, and pair fed thereafter. On the 12th day, the first group was injected with MgCl$_2$ (0.1 ml of 5% MgCl$_2$·6H$_2$O per 20 g rat weight). The second and third groups were injected with equiosmolar NaCl. NEL was determined beginning three minutes postinjection and compared with initial NEL. Assessment of audiogenic seizure susceptibility was made beginning 5½ min postinjection. CSF and blood were collected at about 8 min postinjection.

Any CSF containing cells, and blood were centrifuged for 10 min and the clear CSF or serum collected. All samples were diluted with 0.1 N HCl (70:1 dilutions for CSF, 50:1 dilutions for serum) and analyzed by atomic absorption spectroscopy for magnesium.

Comparing the number of rats susceptible to audiogenic seizures in one treatment group with the number susceptible in another was made by Chi Square analysis. Other data comparisons were made against least significant
differences computed by analysis of variance, or by t-tests.

RESULTS

The effect of duration of magnesium deficiency on CSF and serum magnesium concentration is shown in Figure 1. Serum magnesium concentration decreased rapidly from 1.87 mEq/1 to 0.91 mEq/1 by Day 2, and to 0.63 mEq/1 by Day 6 where it essentially remained for the remainder of the experiment. CSF magnesium concentration decreased from 1.86 mEq/1 initially to 0.94 mEq/1 by Day 14. The rate of this decrease was more gradual than for serum magnesium concentration. When CSF magnesium concentration was 1.44 mEq/1 or less, namely on Days 8, 12 and 14, NEL was significantly greater, and susceptibility of some rats to audiogenic seizures was observed (Figures 2 and 3).

The effect of duration of magnesium deficiency on NEL is shown in Figure 2. Significant (p<0.05) increases in NEL from Day 0 were observed by Day 8. On Days 12 and 14, significant increases in NEL of these deficient groups were also demonstrated when compared with the NEL of a control group terminated on Day 14. The classic magnesium deficiency symptoms of vasal dilation appeared after 5 days. From Day 8 on, deficient animals seemed more irritable than controls. When placed in the activity chamber, they would remain very active for the full two minutes. Control animals tended to be active at first while exploring their new environment.
Figure 1. CSF and Serum Magnesium Concentration vs. Length of Time Rats were Fed a Magnesium Deficient Diet. ▲, CSF Mg of rats fed magnesium deficient diet. ○, serum Mg of rats fed deficient diet. △, CSF Mg of rats fed control diet. ○, serum Mg of rats fed control diet. LSD 0.01 for serum Mg = 0.51 mEq/L. N = 6, all groups. (a) Significantly lower (p<0.05) than CSF Mg concentration of rats terminated on Day 0. (b) Significantly lower than CSF mg of rats fed control diet terminated on Day 14. (T-tests were performed on the CSF data using the pooled variance (0.0225). The number of rats in each group from which clear CSF was successfully collected were: 5, 5, 6, 3, 3, 4, 4 and 5 for Days 0, 2, . . . , 14; and, 4 for rats fed control diet terminated on Day 14.)
Figure 2. Nonspecific Excitability Level (NEL) of Rats Fed a Magnesium Deficient Diet. ▲, rats fed magnesium deficient diet. ●, rats fed control diet. N = 6, all groups. LSD 0.05 = 13.3. (a) Significantly greater than NEL of rats terminated on Day 0. (b) Significantly greater than NEL of rats fed control diet terminated on Day 14. $R^2$ for the relationship between NEL and CSF mg concentration = 0.72. $R^2$ for the relationship between NEL and serum Mg concentration = 0.28.
Then they would settle down and be more content to pursue a single mode of behavior such as sniffing, grooming or resting. One rat in the deficient group terminated on Day 12, and two in the group terminated on Day 14 went into mild spontaneous seizures while in the chamber.

The effect of duration of magnesium deficiency on audiogenic seizures susceptibility is shown in Figure 3. Although younger rats sometimes ran wildly about the cage when subjected to a 115 dB bell, none developed tonus until Day 8. By Day 14, all deficient rats developed tonic or tonic-clonic seizures in response to the bell. The relationship between seizure susceptibility and NEL from a group by group comparison was strong (R² = 0.76; p<0.001).

The effects of dietary rehabilitation are shown in Figure 4. Rats rehabilitated for four days with control diet had terminal CSF and serum magnesium concentrations of 1.89 and 2.38 mEq/1, respectively, as compared with 1.22 and 0.84 mEq/1 for the deficient group. NEL was significantly reduced among the rehabilitated rats. None of the rehabilitated rats was susceptible to audiogenic seizures.

NEL was significantly lower for magnesium deficient rats injected with MgCl₂ than for deficient rats injected with saline (Figure 5), but they were still susceptible to audiogenic seizure. Serum magnesium was greatly elevated as a result of the MgCl₂ injection, while CSF magnesium increased only slightly. Compared with a control group,
Figure 3. Percent of Rats Fed a Magnesium Deficient Diet that Seized when Exposed to a 115 dB Bell. ▲, rats fed magnesium deficient diet. ●, rats fed control diet. N = 6, all groups. Bell was on for 1 min or until tonus occurred. (a) Chi Square is significant (p<0.05). R² for the relationship between percent susceptible to audiogenic seizure and CSF Mg concentration = 0.83. R² for the relationship between percent susceptible to seizure and serum Mg concentration = 0.17.
Figure 4. Effect of Magnesium Rehabilitation on NEL and Audiogenic Seizure Susceptibility. All comparisons are significant (p<0.05). Rats were assigned to two groups according to initial NEL and were fed Mg deficient diet 8 days. N = 10, both groups. One group was then rehabilitated four days. Seizures included animals running wildly about the cage. Respective t-values for NEL, serum and CSF Mg concentrations = 2.45, 12.08 and 7.06. Chi Square for comparing the percent susceptible to audiogenic seizure = 7.80.
Figure 5. Comparison of Several Parameters Among Three Treatment Groups in Experiment 3. Two groups of rats were fed Mg deficient diet 12 days. A third group was fed control diet 12 days. N = 8, all groups. One deficient group was injected with MgCl₂ (0.1 ml of 5% MgCl₂·6H₂O per 20 gm rat weight). The other deficient group and the control group were injected with equiosmolar NaCl. NEL was determined at 3 min postinjection; audiogenic seizure susceptibility was determined at 5½ min postinjection; and CSF and blood were collected at about 8 min postinjection. (a) Values indicated by similar letters differed significantly (p<0.05). Respective LSD 0.05 for NEL, CSF and serum Mg concentrations = 14.2, 0.18 mEq/l and 0.65 mEq/l. Respective Chi Square values for comparisons between the percent susceptible to audiogenic seizure of two deficient groups and the control group = 5.86 and 4.88.
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<td>NaCl</td>
<td>NaCl</td>
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<td>-15.8</td>
<td>4.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-7.1</td>
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<tr>
<td>Final - Initial</td>
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<tr>
<td>Percent Seizures</td>
<td>87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
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<td>Serum Mg (mEq/L)</td>
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<td>1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>CSF Mg (mEq/L)</td>
<td>1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;ab&lt;/sup&gt;</td>
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those injected with magnesium exhibited significantly more seizure susceptibility, higher serum magnesium concentration, and lower CSF magnesium concentration.

DISCUSSION

Under the conditions of our experiments, magnesium deficiency in weanling rats causes an increase in NEL which is reversible. Differences in NEL appear after 8 days on a deficient diet and are clearly evident by 12 days (Figure 2). Extreme deficiency, however, tends to reduce NEL by inducing spontaneous seizures in the activity chamber; while seizing, rats are unable to move about. Elevated NEL, as a result of magnesium deficiency, is reduced by dietary rehabilitation (Figure 4).

There is a strong relationship between NEL and audiogenic seizure susceptibility when magnesium intake is controlled by diet ($R^2 = 0.76$; Figures 2 and 3); increased NEL occurs concurrently with increased audiogenic seizure susceptibility. Deficient animals, rehabilitated on a control diet, show both reduced NEL and audiogenic seizure susceptibility (Figure 4). Changes in NEL do not coincide with changes in audiogenic seizure susceptibility, however, when deficient rats are injected IP with magnesium raising the serum magnesium concentration to 6.6 mEq/l, provided CSF magnesium concentration remains low (1.4 mEq/l) (Figure 5). Under these conditions those injected with magnesium show
reduced NEL while maintaining seizure susceptibility.

NEL appears to be a more sensitive estimate of generalized CNS excitability than audiogenic seizure susceptibility during the earlier stages of magnesium deficiency. Significant increases in the index were observed by Day 8, while increases in seizure susceptibility were not evident before Day 12 (Figures 2 and 3). This opens the possibility that some kind of activity measure might be devised which could assess the effects of marginal magnesium deficit before the more serious symptoms of hallucinations, delerium tremens and convulsions develop.

Discomforture from retro-ocular puncture or IP injection tends to reduce the effectiveness of NEL as an excitability index by increasing within group variability. Some rats, normally highly excitable, just sit. Others move about more. The adverse effects of this discomforture may be minimized by comparing final NEL values with the initial.

With dietary manipulation of magnesium intake, NEL and audiogenic seizure susceptibility appear to reflect changes in CSF magnesium concentration (Figures 1, 2 and 3). Differences in serum magnesium concentration in rats fed magnesium deficient diet are evident in two days (Figure 1) (23, 26). A decrease in CSF magnesium concentration occurs more slowly. We demonstrated this decrease after eight days and found a concurrent increase in NEL. These observations and those of others (25, 26, 29, 44, 107) show that hyperexcita-
bility relates to CSF magnesium but not to serum magnesium concentration. Lát and Gollová-Hémon (82) found that NEL is a good indicator of generalized CNS activity as reflected in hippocampal slow wave activity. Our data suggest that NEL may be a good indicator of increased CNS activity due to magnesium deficiency.

Very high serum magnesium (6.57 mEq/l) lowers NEL, even though audiogenic seizure susceptibility of magnesium-deficient rats may remain high due to low CSF magnesium concentration (Figure 5). Under these conditions NEL appears to be a poor indicator of CNS activity. It has been suggested (23, 60, 121, 149) that a block due to elevated serum magnesium concentration may occur at the neuromuscular junction. This would reduce the effects of efferent impulses upon the musculature. Thus, even though CNS activity may remain high, a behavioral activity index would yield low values.

Possible neurological mechanisms (54, 80, 110) leading to the behavioral effects of magnesium deficiency are: (1) increased ease of transmission across synapses of the CNS; and, (2) axonal effects resulting in lowered depolarization thresholds. Both mechanisms serve to make nerve cells more sensitive to stimulation from whatever source and more susceptible to premature firing. That the nervous system becomes overly responsive to stimulation as a result of magnesium deficiency has been amply demonstrated, although
the precise connection between the nutritional insult, CNS excitability and behavior has yet to be elucidated. Our data, showing that NEL increases as a result of magnesium deficiency, suggest, along with the observations of other researchers (48, 81, 82), that altered intake of some nutrients plays an important role in CNS excitability. A note of caution is urged in assuming that an activity index reflects CNS excitability in all cases, however, since some treatments (Experiment 3) may dampen the activity response while considerable CNS excitability remains.

To summarize: Magnesium deficiency in weanling rats causes an increase in NEL. This effect is reversible by diet. There is a strong relationship between NEL and audiogenic seizure susceptibility except when serum magnesium concentration is very high. Both NEL and audiogenic seizure susceptibility relate inversely to CSF magnesium concentration except in the case of very high serum magnesium. In this instance, NEL is depressed while seizure susceptibility remains. Possible mechanisms explaining the effects of magnesium on NEL and audiogenic seizure susceptibility have been briefly discussed.
PART II

MAGNESIUM EFFECTS ON SPONTANEOUS ACTIVITY, CATION CONCENTRATIONS AND (Na\(^{+}\) K\(^{+}\))-ATPase
INTRODUCTION

A well-established relationship exists between magnesium status and excitability in mammals (27, 120, 149). Possible mechanisms explaining the relationship have been considered (7, 27, 28, 121).

A standard method for quantifying the hyperexcitability phenomenon caused by Mg deficiency has involved testing the audiogenic seizure susceptibility of small animals (7, 26, 29, 42, 43, 56, 103, 104). This procedure, however, is traumatic for the animals, and also poses statistical problems when small groups are compared. We therefore sought for a more benign and sensitive method.

The method chosen was adapted from Lát's spontaneous activity procedure (81, 82). He and Gollová-Hémon (82) found that spontaneous locomotion and rearing responses bear a close relationship to hippocampal slow wave (theta) activity and may be said to reflect non-specific central nervous system excitability in rats. Recently, we (15) reported that an 8-day dietary Mg deficit produced an increase in this spontaneous activity in weanling rats. The increase coincided with audiogenic seizure susceptibility when serum Mg concentration was normal or low, further indicating that this behavioral technique is a useful tool for estimating
generalized nervous system excitability.

The first objective of the current research was to confirm our earlier finding (15) that magnesium deficiency causes increased spontaneous activity in rats, and also to see if excess dietary magnesium reduced the activity. Other objectives were to gain further insight into the chain of events which links Mg status with central nervous system excitability and the possible pathological symptoms such as hyperactivity, by: (a) monitoring serum, CSF and brain cation concentrations; and, (b) determining (Na\(^+\) K\(^+\))\text{-ATPase} activity in brain. (Na\(^+\) K\(^+\))\text{-ATPase} is strongly implicated as the electrogenic cation pump which maintains electrical gradients in nervous tissue by ejecting Na from the cell (119, 127, 128). If this enzyme fails to function, Na gradients are not maintained, burst thresholds are lowered and hyper-excitability and eventually death ensue.

**METHOD**

Two sets of experiments were designed and carried out to attain the aforementioned objectives. Male, weanling Sprague-Dawley rats were used throughout. They weighed 48 ± 3 gm at the onset of the first set and averaged 42 ± 2 gm at the start of the second. Ambient temperature in the animal room was 25°C. Lights were on daily from 0800 hrs to 2000 hrs. The Mg deficient diet, to which measured amounts of Mg were added for the control and high Mg diets,
contained 0.0055% residual Mg. This base diet consisted of 20% casein, 5% sucrose, 53% corn starch, 10% corn oil, 2% vitamin mix and suggested minerals for growth (99), without Mg. The vitamin fortification mixture (ICN Pharmaceuticals) consisted of, per kg, the following: 4.5 gm vitamin A, 0.25 gm vitamin D, 5 gm α-tocopherol, 45 gm ascorbic acid, 5 gm inositol, 75 gm choline chloride, 2.25 gm menadione, 5 gm p-amino benzoic acid, 4.5 gm niacin, 1 gm riboflavin, 1 gm pyridoxine-HCl, 1 gm thiamin-HCl, 3 gm Ca pantothenate, 20 gm biotin, 90 mg folic acid and 1.35 mg vitamin B-12.

In Experiment 1, 42 rats were matched according to their initial spontaneous activity and randomly assigned to 4 groups of 10, with 2 extras. These groups were fed deficient, control (400 mg Mg added per kg diet) and high (2.5 gm Mg added per kg diet) Mg diets for two weeks. A fourth group was fed high Mg diet 12 days and a deficient diet 40 hours. In Experiment 2, 46 rats were matched by weight and assigned to 3 groups of 10, with extras. These were fed deficient, control and high Mg (4 gm Mg added per kg diet) diets, also for 2 weeks. Extras were fed Mg deficient diet and used to replace other deficient animals that died. Rats in both experiments were fed ad libitum for 5 days. Thereafter, those fed Mg supplemented diets were pair fed to their squad mates fed the deficient diet.

On the final day, spontaneous activities were assessed for all rats in Experiment 1 by counting responses
made during the first two min. in a glass test chamber 11 x 22 x 23 cm high. Responses counted were: the number of times the rat moved from one corner to another; the number of times it raised its head above the rest of its body; the number of rearing responses, grooming responses and fecal pellets excreted; and, whether or not it urinated.

After anesthetizing the animals with ether, CSF was collected in Yale BD needles. Blood was obtained by retro-ocular puncture. The head was decapitated and brain excised. Brain homogenates were prepared using 1 part brain and 9 parts deionized water. Three 2 ml aliquots of the homogenates from each rat in Experiment 2 were quickly frozen and stored at -80°C for ATPase analysis. Five ml of homogenate from each animal in both experiments were digested by adding 5 ml of 37% HCl and allowing the mixture to stand at room temperature for two days. 150 μl of the digested brain homogenate was pipetted in duplicate into test tubes containing 3 ml of deionized water and 0.3 ml chloroform. After shaking and brief centrifugation each aliquot was analyzed for Mg, K and Na by atomic absorption or flame emission spectrophotometry. Cerebrospinal fluid containing cells and the blood were centrifuged and clear CSF and serum collected. Cerebrospinal fluid Mg and K were analyzed after diluting the CSF 70:1 with 0.1 N HCl. Serum Mg and K were analyzed in 50:1 dilutions. Serum and CSF Na were both analyzed in 500:1 dilutions.
ATPase assays were conducted on recently thawed 10:1 brain homogenates. Reaction medium for activating (Na$^{+}$ K$^{+}$)-ATPase contained, per liter, the following: 150 mM Na$^{+}$, 40 mM K$^{+}$, 3 mM Mg$^{2+}$, 3 mM ATP$^{-}$, and 36.5 mM TRIS (pH = 7.4). Sodium and K were omitted from the medium inhibiting (Na$^{+}$ K$^{+}$)-ATPase and 0.1 mM ouabain was added. TRIS was added to keep osmolarity constant. Substrate, 300 µl, was pipetted into incubation tubes and kept in an ice-water bath. Reactions were initiated by adding 20 µl of homogenate and placing the tubes in an oscillating water bath at 37°C. Incubations were terminated after 4 min. by adding 0.5 ml of 10% TCA and replacing the tubes in the ice-water bath. Tubes were centrifuged for 10 min. and 0.5 ml of the supernatant withdrawn for analysis. The phosphate released during incubation was determined in a final volume of 2.7 ml by the method of Gomórrí (53) with 1% MgCl$_2$·6H$_2$O added to help stabilize color. Residual brain phosphate was also determined. Differences between values obtained from incubation in the medium activating (Na$^{+}$ K$^{+}$)-ATPase and those from the medium inhibiting it were used as a measure of (Na$^{+}$ K$^{+}$)-ATPase activity. Differences between the values obtained from incubation in the medium inhibiting (Na$^{+}$ K$^{+}$)-ATPase and residual phosphate were used as a measure of other ATPase activity. Protein was determined by the Lowry et al. (88) method, using serum albumin standard.

Data comparisons were made against least significant
difference values (LSD) calculated by analysis of variance. Usually a randomized complete block design was employed with rats on the same pair feeding squad forming one block. One rat in the deficient group in Experiment 1 was missing, and one brain homogenate of a control animal in Experiment 2 was lost. Substitutions were made for the missing data, with the number of degrees of freedom correspondingly reduced and appropriate bias corrections made.

RESULTS

The effect of feeding diet containing different levels of Mg for two weeks on spontaneous activities of growing rats is shown in Figure 1. Spontaneous activity increased significantly in the deficient animals compared with the other groups. There was no appreciable decrease in the activity of rats fed a high Mg diet.

The effects of diet containing different levels of Mg on serum and CSF cation concentrations are shown in Table 1. There were sizable differences in serum Mg concentrations due to feeding with deficient, control and high Mg diets. Rats fed a high Mg diet 12 days had normal serum values after they were fed a deficient diet for 40 hrs. Serum Ca and Na concentrations were greater in the deficient groups. There were no significant differences in K concentrations. Cerebrospinal fluid Mg concentrations were lower in the deficient than in the other groups. Rats fed high
Figure 1. Nonspecific Excitability Level (NEL) of Rats Fed Different Levels of Mg for 2 Weeks. (a) Significantly higher than the other groups.
Table 1. Serum and CSF Cation Concentrations (mEq/l) of Rats Fed Diets Containing Different Levels of Magnesium for Two Weeks.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>n</th>
<th>Mg</th>
<th>Ca</th>
<th>Na</th>
<th>K</th>
<th>n</th>
<th>Mg</th>
<th>K</th>
<th>Na</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>9</td>
<td>0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>137.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.87</td>
<td>5</td>
<td>1.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.84</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>1.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>135.9</td>
<td>5.84</td>
<td>6</td>
<td>1.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.51</td>
<td></td>
</tr>
<tr>
<td>High Mg</td>
<td>10</td>
<td>2.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>134.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.45</td>
<td>4</td>
<td>1.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.22</td>
<td></td>
</tr>
<tr>
<td>High 12 days Defic 40 hrs</td>
<td>10</td>
<td>1.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>136.6</td>
<td>6.60</td>
<td>6</td>
<td>1.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.65</td>
<td></td>
</tr>
<tr>
<td>LSD (p&lt;0.05)</td>
<td></td>
<td>0.32</td>
<td>0.27</td>
<td>2.8</td>
<td>(1.15)</td>
<td></td>
<td>0.18</td>
<td></td>
<td>(0.68)</td>
</tr>
<tr>
<td>LSD (p&lt;0.01)</td>
<td></td>
<td>0.44</td>
<td>0.37</td>
<td>-</td>
<td>-</td>
<td></td>
<td>0.25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>10</td>
<td>0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>137.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.94</td>
<td>6</td>
<td>1.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.32</td>
<td>150.0</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>1.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>135.2</td>
<td>5.81</td>
<td>6</td>
<td>1.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.28</td>
<td>148.3</td>
</tr>
<tr>
<td>High Mg</td>
<td>10</td>
<td>2.72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>134.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.81</td>
<td>6</td>
<td>1.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.22</td>
<td>148.2</td>
</tr>
<tr>
<td>High 12 days Defic 40 hrs</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LSD (p&lt;0.05)</td>
<td></td>
<td>0.35</td>
<td>0.28</td>
<td>1.9</td>
<td>(0.69)</td>
<td></td>
<td>0.14</td>
<td></td>
<td>(0.24)</td>
</tr>
<tr>
<td>LSD (p&lt;0.01)</td>
<td></td>
<td>0.47</td>
<td>0.38</td>
<td>-</td>
<td>-</td>
<td></td>
<td>0.19</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Values having different superscripts are significantly different.
Mg diet had normal CSF Mg values. There were no significant CSF Na and K concentration differences.

The effect of diet on whole brain cation composition is shown in Table 2. Brain Mg concentration was lower in the deficient groups. Sodium concentration tended to be marginally higher in the deficient groups. The increase in brain K in the Mg deficient group observed in Experiment 1 was not verified in Experiment 2.

The activity of brain \((Na^+ K^+)-ATPase\) was higher in both the deficient and high Mg groups compared with the control (Table 3). There were no significant differences in brain weight, percent protein in brain, total protein, or activity of other ATPases.

**DISCUSSION**

**Spontaneous Activity:** The increase in spontaneous activity as a result of Mg deficiency (Figure 1) is consistent with our previous findings (15). Spontaneous locomotion, rearing and head raising behavior, as a measure of excitability, appears to reflect central nervous system activity (15, 81, 82). In Mg deficiency, increased spontaneous activity coincides with increased seizure susceptibility provided CSF Mg concentration is normal or low (15); it does not reflect central nervous system excitability or CSF Mg concentration when serum Mg is greatly elevated (to 6.7 mEq/l), presumably because neural discharges from the
Table 2. Brain Cation Concentrations (mEq/kg fresh tissue) of Rats Fed Diets Containing Different Levels of Magnesium for Two Weeks.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Experiment 1</th>
<th></th>
<th></th>
<th>Experiment 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mg</td>
<td>Na</td>
<td>K</td>
<td>n</td>
<td>Mg</td>
</tr>
<tr>
<td>Deficient</td>
<td>9</td>
<td>13.6\textsuperscript{a}</td>
<td>56.0\textsuperscript{a}</td>
<td>97.9\textsuperscript{a}</td>
<td>10</td>
<td>12.2\textsuperscript{a}</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>14.6\textsuperscript{b}</td>
<td>54.2\textsuperscript{b}</td>
<td>96.8</td>
<td>9</td>
<td>13.3\textsuperscript{b}</td>
</tr>
<tr>
<td>High Mg</td>
<td>10</td>
<td>14.7\textsuperscript{b}</td>
<td>54.3\textsuperscript{b}</td>
<td>96.6\textsuperscript{b}</td>
<td>10</td>
<td>13.0\textsuperscript{b}</td>
</tr>
<tr>
<td>High 12 days Defic 40 hrs</td>
<td>10</td>
<td>14.4\textsuperscript{b}</td>
<td>53.8\textsuperscript{b}</td>
<td>96.4\textsuperscript{b}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LSD (p&lt;0.05)</td>
<td>0.7</td>
<td>1.2</td>
<td>1.2</td>
<td>0.4</td>
<td>(2.0)\textsuperscript{1}</td>
<td>(1.1)</td>
</tr>
<tr>
<td>LSD (p&lt;0.01)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a,\textsuperscript{b}Values having different superscripts are significantly different.}

\textsuperscript{1}_F was not significant.
Table 3. Weight, Protein and ATPase Activity of Brain in Rats Fed Diets Containing Different Levels of Magnesium for Two Weeks.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
<th>Activity of Other ATPases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain Wt (gm)</td>
<td>Brain Wt (gm)</td>
<td>Percent Brain Protein</td>
<td>Total Brain Protein (gm)</td>
<td>(Na K)-ATPase Activity^1</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td></td>
<td>(gm)</td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>9</td>
<td>10</td>
<td>12.5</td>
<td>0.196</td>
<td>0.123^a</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>9</td>
<td>12.6</td>
<td>0.198</td>
<td>0.109^b</td>
</tr>
<tr>
<td>High Mg</td>
<td>10</td>
<td>10</td>
<td>12.5</td>
<td>0.191</td>
<td>0.122^a</td>
</tr>
<tr>
<td>High 12 days</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defic 40 hrs</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSD (p&lt;0.05)</td>
<td>(0.062)</td>
<td>(0.048)</td>
<td>(0.3)</td>
<td>(0.007)^2</td>
<td>0.009</td>
</tr>
<tr>
<td>LSD (p&lt;0.01)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.012</td>
</tr>
</tbody>
</table>

^a,b Values having different superscripts are significantly different.

^1ATPase activity is expressed as μM phosphate released per min per mg tissue protein.

^2F was not significant.
central nervous system are blocked at the neuromuscular junction. Present data (Figure 1, Table 1) suggest that serum Mg concentration may rise as high as 2.47 mEq/l without blocking these efferent discharges. A possible sedative effect of this modest increase has not, however, been tested with low CSF Mg concentration.

Although we have observed an increase in spontaneous activity in Mg deficient rats, Caldwell et al. (17) have reported a decrease in activity as measured by a vibration counter for longer periods. The relationship between excitability, emotionality and lability, which may explain this discrepancy in behavior, has been discussed (82). Magnesium deficient rats seem to make more sporadic large movements when first placed in a novel environment. They also accommodate more slowly. Control animals tend longer to pursue a single course of action. After settling down they may, for example, explore one corner of the chamber and "dig" for a while before grooming themselves or moving elsewhere. In the process they make more small movements. Conditions under which activity is monitored may also make a difference. Our spontaneous activity measurements were made in a brightly lit chamber during the day, after arousing the normally quiet or sleeping animals.

Cations: The effects of Mg deficiency on cation concentrations in serum, CSF and brain observed in this experiment concur generally with published data. Magnesium
concentration in serum is related to dietary Mg intake and can fluctuate over a wide range below and above normal (Table 1)(26). Cerebrospinal fluid Mg concentration decreases if serum Mg falls considerably (Table 1)(26, 29), but does not rise above normal if rats are fed high Mg diets or given single IP injections containing moderate amounts of Mg (Table 1)(26). On the other hand, a rise has been reported in the dog when serum values were kept very high (5 mEq/1) for several hours (100). The Mg concentration of the CSF is kept constant because plasma ultrafiltrate contains less Mg (0.9 - 1.2 mEq/1) than CSF (5, 22) and an active carrier that is saturated at normal serum Mg concentrations transports Mg from serum to CSF (5, 51, 100). Brain Mg equilibrates fairly rapidly with CSF (26, 29) and a decline in total brain Mg follows its decrease in CSF (Table 1)(26, 29).

Serum Ca concentration increases in Mg deficiency in the rat (Table 1)(2, 24, 45, 90, 93, 153), but not in other species (2, 115). It does not appear to decrease appreciably with high serum Mg (Table 1)(45).

Serum Na concentration increases as a result of Mg deficiency in the rat (Table 1)(50, 92, 93, 153). However, it does not increase in the dog or monkey (144, 146). The elevation in the rat is due to raised aldosterone concentration in blood (50, 131) which stimulates renal Na resorption. Cerebrospinal fluid Na concentration is maintained higher.
than both plasma ultrafiltrate and serum Na (Table 1) (5), presumably by an active pump (5). The small increase in serum Na concentration due to Mg deficiency is not reflected appreciably in the CSF (Table 1), although this does not negate the possibility that small within group variability would permit identifying a difference.

Excitable tissues, including muscle (45, 50, 90, 92, 93, 151, 153) and heart (45, 93), show elevated Na and reduced K concentrations in Mg deficiency. This is consistent with the view that an electrical gradient exists between intracellular and extracellular spaces in all excitable tissues, with intracellular Na concentration maintained at a lower level than extracellular Na, and a K gradient lying in the opposite direction. Mg deficiency causes a lowering of these gradients in muscle and heart; Na leaks into the cells, and K moves out. Unfortunately, Na and K analyses provide no indication that this is the case in brain tissue. We (Table 2) have found a slight increase in brain Na due to Mg deficiency, but others (93) have found no increase.

The reason that brain does not exhibit the expected Na response is open to question. A possibility is that the central nervous system is in such a state of activity normally that the increment of added flux due to Mg deficiency is relatively slight. A change in intracellular Na concentration might go undetected by whole brain analysis, especially if affected cells contribute little towards
modifying the total Na pool or if there are mechanisms acting to offset the tendency. One mechanism acting to offset the expected rise in intracellular Na concentration might involve the increased availability of brain (Na⁺ K⁺)-ATPase (Table 3). With more of this enzyme available for active transport, Na gradients would tend to be normalized in spite of Mg deficiency. Another mechanism might involve increased brain Ca (22). Increased brain Ca in Mg deficiency acts in concert with reduced brain Mg at the synapse, where Ca stimulates and Mg inhibits acetylcholine release, to increase excitatory post-synaptic potentials and contribute to overall excitability. However, Ca acts on all nerve axons to reduce Na influx (72), and neurons mediated by other transmitters may actually undergo a net increase in intracellular Na. Thus overall brain Na may not change much.

Loss of K from the intracellular space likewise cannot be inferred by CSF or whole brain analyses (Tables 1 & 2) (93). There have been no differences in serum K concentrations observed in the rat (Table 1) (90). A decrease has been reported in the dog and monkey (144, 146).

ATPase: Increased (Na⁺ K⁺)-ATPase activity in whole brain homogenates (Table 3) suggests that, relative to other proteins, more enzyme is available for active transport. The increased available (Na⁺ K⁺)-ATPase may reflect a protective compensatory reaction to offset the hyperexcitability due to ionic changes. This "metabolic adaptive reaction"
(133) is not without precedent. Increased (Na\(^+\) K\(^+\))-ATPase has been observed in experimental brain injury (86), from hypoxia (133), electric shock (20) and dosing with pentylenetetrazol (Metrazol) (8, 111). All of these can lead to convulsions and other symptoms of hyperexcitability. Conversely, decreased (Na\(^+\) K\(^+\))-ATPase has been observed in uremia (96), a condition which leads to hypoexcitability. Exceptions to the rule seem to be agents which act directly on (Na\(^+\) K\(^+\))-ATPase. Ouabain, zinc, copper (37) and cobalt (65), which can also cause convulsions, lead to reduced (Na\(^+\) K\(^+\))-ATPase activity. These substances are powerful inhibitors of the enzyme and may permanently destroy its activity or be present in the reaction medium during analysis. Notwithstanding these exceptions, the general inference is still tenable: that a cellular mechanism, which responds to need, acts to increase or decrease the amount of (Na\(^+\) K\(^+\))-ATPase available for active transport.

The details of this mechanism are unknown. Certainly, genetic de-repression responding to changes in ATP, ADP or some other messenger is one possibility, at least during the growth phase. Altered exposure of active sites on the (Na\(^+\) K\(^+\))-ATPase molecule by some membrane bound factor (46) or an interaction between a particle bound and soluble fraction (117) are other possibilities. An ATPase response observed in whole brain homogenates or in the microsomal fraction alone poses more questions than it answers.
CONCLUSIONS

Magnesium deficiency leads to increased spontaneous activity as determined by the number of large movements rats make when placed in a novel environment (Figure 1) (15). In attempting to gain further insight into the chain of events that links magnesium status with central nervous system excitability, as reflected in this and other pathologies, we have monitored serum, CSF and brain cation concentrations, and determined (Na⁺ K⁺)-ATPase activity in brains of weanling rats.

The relationships between Mg status and cation concentrations have been explored. Magnesium deficiency led to decreased serum, CSF and brain Mg, and decreased serum Ca concentrations. Mg excess led to an increase in serum Mg, but no other significant changes in cation concentrations. A marginal, and not clearly demonstrable, increase in brain Na, and no change in brain K, suggest that at least some brain cells do not undergo a loss of K and gain of Na. A possible explanation for this, implicating increased (Na⁺ K⁺)-ATPase (Table 3) and increased brain Ca (22), has been developed. Elevated brain Ca may act to reduce Na influx into all neurons, including inhibitory neurons. Increased burst thresholds of these neurons would, of course, lessen their modulating effect upon excitatory neurons. This effect, together with heightened acetylcholine release at the synapses of excitatory neurons, would tend to
accentuate the overall excitability level.

The increased $(\text{Na}^+ \text{K}^+)-\text{ATPase}$ in Mg deficiency suggests that there is a cellular attempt being made to counter the increased excitability caused by the deficiency, by increasing the quantity or activity of this enzyme. Its increased activity due to Mg excess is difficult to interpret at this point, especially when no changes in cation concentrations are observed; but, it also opens the possibility that some kind of insult is occurring which is being countered.
PART III

EFFECTS OF CEREBRAL INTRAVENTRICULAR MAGNESIUM INJECTIONS AND A LOW MAGNESIUM DIET ON NON-SPECIFIC EXCITABILITY LEVEL, AUDIOGENIC SEIZURE SUSCEPTIBILITY AND SEROTONIN
INTRODUCTION

In an earlier report (15) we presented data indicating that the mechanism whereby Mg deficiency acts to induce hyperexcitability lies in the central nervous system rather than peripherally. We did this by showing that audiogenic seizure susceptibility occurs in rats if cerebrospinal fluid (CSF) Mg concentration is low, even when serum Mg concentration is much greater than is otherwise considered normal (6.6 mEq/l compared with 2.1). Our present objective was to confirm this contention by injecting Mg directly into the brain while keeping the serum Mg concentration constant. We then assessed the effects of cerebral intraventricular (c.i.v.) Mg injections on Nonspecific Excitability Level (NEL) and audiogenic seizure susceptibility.

Our second objective was to determine the effects that these injections might have on brain neurotransmitters. There is scanty information published concerning this matter. Itokawa et al.(67) found a marginal but not clearly significant decrease in the amount of serotonin in brains of rats fed a low Mg diet for 25 days; however, the trend was not evidenced by later research (68). Essman (42) found decreased brain serotonin levels in mice fed a low Mg diet 14 days.

To help clarify the serotonin issue and to determine if Mg status relates to other neurotransmitters, we analyzed
the effects of c.i.v. Mg injections on brain serotonin, norepinephrine and dopamine. Intraventricular injections permit the relationships between Mg status and neurotransmitter concentrations to be studied free of long-term dietary effects and complicating interactions involving peripheral and transport mechanisms.

METHOD

Animal Care: Sprague-Dawley male rats were used in all experiments. They were housed individually in stainless steel cages and fed deionized water from plastic containers having stainless steel lick-spouts. The basic diet consisted of 58% dextrose, 20% casein, 10% corn oil, 2% vitamin mix (15) and 4% mineral mix (99) containing 0.75% calcium as carbonate, 0.6% phosphorus as monosodium phosphate and 0.27% potassium as chloride. Cellulose (6%) was added to make up to 100%. The low Mg diet, by analysis, contained less than 10 mg Mg per kg. Control diet was made by adding 400 mg Mg per kg as MgCO₃ to the low Mg diet. Ambient temperature in the animal room was 25°C and lights were on daily from 0800 to 2000 hours.

Subject Preparation and Intraventricular Injections: Permanent cerebral intraventricular (c.i.v.) canulations were done on 150-200 g rats in a manner similar to that of Mabel et al. (89). Canulae (15 mm long) were prepared from 22 ga disposable needles with the heads clipped off; they
had a small copper wire flange soldered 5-6 mm from the point. The rat was anesthetized with pentabarbital and placed in a stereotaxic instrument. The skull was bared, 5 small holes were drilled and 4 small anchor screws inserted. The canula was inserted through the fifth hole, located 1.3 mm lateral at the rostral edge of bregma. Normal saline, pumped through the canula during insertion at 10 µl per min by a syringe pump attached via 0.023 inch polyethylene tubing, was pressure-monitored by a transducer. A drop in pressure indicated proper placement of the canula in the lateral ventricular, usually 3.0-3.5 mm deep. Dental acrylic was poured about the canula, flange and anchor screws, and allowed to dry. The skin was sutured over it. An insect pin, covered with petroleum jelly, was inserted the full length of the canula to stopper it and prevent clogging.

Permanently canulated rats were fed the low Mg diet 21 days, then given 10 min injections containing 0, 2, 6, 17 or 50 µg Mg in 80 µl of artificial cerebrospinal fluid (CSF) (Table 1). This was done between 1300 and 1700 hrs. Injections were given by a syringe pump at a rate of 8 µl per min, after the rat was lightly anesthetized with ether, the insect pin was removed, and the lead tubing was attached. They were tested 10 min following the injections.

Acute canulations were performed on weanling rats (50-60 g) after they were fed the low Mg diet 14 days. The canula was inserted 1 mm lateral and 1.3 to 2.0 mm deep in
Table 1. Composition of Artificial CSF*

<table>
<thead>
<tr>
<th>Approximate Ion Concentrations (µM/1)</th>
<th>µg Mg per 80 µl Injection (by analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg++</td>
<td>Na+</td>
</tr>
<tr>
<td>0</td>
<td>150</td>
</tr>
<tr>
<td>1</td>
<td>149</td>
</tr>
<tr>
<td>3</td>
<td>147</td>
</tr>
<tr>
<td>10</td>
<td>140</td>
</tr>
<tr>
<td>30</td>
<td>120</td>
</tr>
</tbody>
</table>

*All mixtures were made from chloride salts and 0.05% albumin buffer, and were adjusted to pH 7.4 with dilute NaOH.

the manner described. These rats, lightly anesthetized with ether, were given 5 min injections containing 0 to 25 µg Mg in 40 µl of artificial CSF. They were tested 15 min later, after the canula was withdrawn and when the effects of anesthesia had worn off.

CSF was withdrawn from the cisterna magna and analyzed as described previously (15).

Behavioral Testing: Non-specific Excitability Level (NEL) and audiogenic seizure susceptibility were measured as described previously (15).

Monoamine Analysis: Rats used for monoamine analysis were killed by the near freezing method of Takahashi and Aprison (138). The brain was excised in a cold box (-5°C) and stored under liquid nitrogen. Monoamine extraction was done.
at 0°C by a modification of the method of Shea and Aprison (123). Each brain was homogenized with 25 ml cold (15/85, v/v) 1 N formic acid/acetone for 1½ min in a Virtis blender at 30,000 rpm. After centrifugation at 8000 x G for 10 min, the supernatant was removed. The pellet was resuspended in 10 ml of the formic acid/acetone mixture. After recentrifugation the supernatant was removed and combined with the other fraction. Three 10 ml aliquots of the combined supernatants were removed, shaken for 10 min with 8/1 chloroform/heptane and centrifuged for 5 min. The lipid phase was aspirated away. One ml of the aqueous phase was transferred to another test tube and dried at 37°C under a stream of nitrogen. The sample tube was tightly capped and stored at -35°C for up to 1 week.

The N₂ dried tissue extract was solubilized in 1 ml of 0.005 N HCl. Five ml of 0.25 M pH 8.1 carbonate buffer were added, and the mixture was passed through a fritted funnel. Four ml were placed on a double column apparatus as described by Karasawa et al. (72). The appropriate eluants were analyzed spectrofluorometrically by the procedures outlined by these authors. Internal standards, made with 1 part 0.005 N HCl and 5 parts 0.25 M carbonate buffer, and spikes were carried through the same steps as the samples.

RESULTS

The results of 10 min injections of artificial CSF,
containing different amounts of Mg, into the lateral ventricles of 5 groups of Mg-deficient, permanently canulated rats, are shown in Figure 1. Results pertaining to a sixth group, not injected, are also shown.

Magnesium concentration of the CSF reflected the amount of Mg injected. Non-specific excitability level (NEL) was decreased slightly with the 0 Mg injection, appeared to be normal when 6 µg of Mg was injected, and was decreased drastically with the larger doses. Few of the injected animals were seizure prone.

Because the above rats were apparently too old to seize consistently if injected, we sought to test c.i.v. Mg injection-effects on younger animals. However, since the skulls of younger animals grow rapidly, permanent canulations were impractical and we had to resort to performing acute canulations. Figure 2 shows the results of 5 min c.i.v. injections of artificial CSF into the lateral ventricles of Mg deficient rats via acute canulations. The 25 µg Mg injection significantly lessened the seizure severity of these animals.

The effects of c.i.v. Mg injections and low Mg diet on NEL and brain monoamine concentrations in permanently canulated rats are shown in Table 2, with statistical data shown in Table 3. Compared with 2 µg injections, 50 µg Mg injections reduced NEL. Serotonin concentration was greater in the brains of rats fed the low Mg diet than of dietary
Figure 1. C.i.v. Mg Injection-effect on CSF Mg Concentration, NEL and Audiogenic Seizure Susceptibility. 150-200 g male rats were permanently canulated and fed low Mg diet 21 days. They weighed 217 ± 26 g on the final day. Their serum Mg concentration was 0.78 ± 0.29 mEq/l and did not differ among groups. There were 5 rats per injected group, 20 in the non-injected group. (a,b,c) Values having different superscripts were significantly different (p<0.05). Treatment differences were compared against least significant differences calculated from the pooled variances; 0.399, 144 and 1.51 respectively, for CSF Mg concentration, NEL and Seizure Severity Score. (40% of the non-injected rats exhibited rapid running or more severe seizures.)
Figure 2. C.i.v. Mg Injection-effects on NEL and Audiogenic Seizure Susceptibility. Weanling rats were fed low Mg diet 14 days and were injected with 0 or 25 μg Mg via acute canulations. They weighed 91 ± 13 g on the final day. N = 10 and 9 for the 2 groups. aThis group had a significantly lower Seizure Severity Score than the 0 Mg injected group. t = 2.67 (p<0.02). (50% of the 0 Mg injected group exhibited rapid running or more severe seizures.)
Table 2. Effects of C.i.v. Mg Injections and Low Mg Diet on NEL and Brain Monoamine Concentration (µg/g fresh tissue). Values in the table are group means. Figures in parentheses refer to the number of observations per group.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body Weight (g)</th>
<th>Brain Weight (g)</th>
<th>Parameter Measured</th>
<th>C.i.v. Mg Injection 2 µg</th>
<th>50 µg</th>
<th>Diet Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Mg</td>
<td>237±26</td>
<td>1.69±0.09</td>
<td>NEL</td>
<td>26 (8)</td>
<td>0.9 (8)</td>
<td>13 (16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5HT (µg/g)</td>
<td>0.66 (8)</td>
<td>0.82 (7)</td>
<td>0.73 (15)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NE (µg/g)</td>
<td>0.37 (8)</td>
<td>0.39 (8)</td>
<td>0.38 (16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DA (µg/g)</td>
<td>0.68 (6)</td>
<td>0.79 (6)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.73 (12)</td>
</tr>
<tr>
<td>Control</td>
<td>263±23</td>
<td>1.75±0.08</td>
<td>NEL</td>
<td>35 (4)</td>
<td>0.4 (4)</td>
<td>18 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5HT (µg/g)</td>
<td>0.53 (4)</td>
<td>0.58 (4)</td>
<td>0.56 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NE (µg/g)</td>
<td>0.35 (4)</td>
<td>0.35 (4)</td>
<td>0.35 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DA (µg/g)</td>
<td>0.73 (4)</td>
<td>0.68 (4)</td>
<td>0.70 (8)</td>
</tr>
<tr>
<td>Injection Means</td>
<td></td>
<td></td>
<td>NEL</td>
<td>29 (12)</td>
<td>0.8 (12)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15 (24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5HT (µg/g)</td>
<td>0.61 (12)</td>
<td>0.73 (11)</td>
<td>0.67 (23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NE (µg/g)</td>
<td>0.36 (12)</td>
<td>0.38 (12)</td>
<td>0.37 (24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DA (µg/g)</td>
<td>0.70 (10)</td>
<td>0.74 (10)</td>
<td>0.64 (20)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significantly more 5HT than in brains of the dietary controls.

<sup>b</sup>Significantly lower activity than that of the 2 µg Mg injected group.

<sup>c</sup>Significantly more DA than in brains of rats fed a deficient diet injected with 2 µg Mg and those fed control diet injected with 50 µg Mg.
Table 3. Results of General Least Squares Analysis of the NEL and Brain Monoamine Data Presented in Table 2.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Pooled Standard Deviation</th>
<th>2 µg Mg Injection vs. 50 µg Mg P</th>
<th>Low Mg Diet vs. Control Diet F P</th>
<th>Interaction F P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEL</td>
<td>10</td>
<td>47.9 &lt;0.01</td>
<td>0.92 ns</td>
<td>1.19 ns</td>
</tr>
<tr>
<td>5HT</td>
<td>0.15 µg/g</td>
<td>2.64 ns</td>
<td>7.38 &lt;0.02</td>
<td>0.68 ns</td>
</tr>
<tr>
<td>NE</td>
<td>0.04 µg/g</td>
<td>0.64 ns</td>
<td>4.13 ns</td>
<td>1.17 ns</td>
</tr>
<tr>
<td>DA</td>
<td>0.07 µg/g</td>
<td>1.20 ns</td>
<td>1.01 ns</td>
<td>7.96 &lt;0.20</td>
</tr>
</tbody>
</table>

*The denominator for calculating F ratios for NEL and NE was based on 20 df; for 5HT, it was based on 19 df; and, for DA, 16 df.*
controls. There was a significant dopamine interaction; the 50 µg Mg injected group fed the low Mg diet had the most of this neurotransmitter.

**DISCUSSION**

C.i.v. injections of 17 µg Mg or more, compared with injections of 6 µg or less, decreased the excitability of our subjects as assessed by NEL or audiogenic seizure severity (Figure 1 and 2, Table 3). This was observed while serum Mg concentration remained unchanged, confirming our earlier hypothesis that the primary effect of Mg status on excitability is due to its action on the central nervous system and not upon the musculature. The only exception is when serum Mg concentration is high enough to produce a curare-like block (15).

The injection process alone reduced excitability (Figure 1), possibly due to increased ventricular pressure, a phenomenon known to have behavioral consequences (89). For this reason, inclusion of sham-injected controls is necessary when undertaking c.i.v. injection experiments.

There was an interesting qualitative difference between c.i.v. Mg injection-effects on NEL and on audiogenic seizure severity. A 0 µg Mg injection reduced both NEL and seizure severity; however, a 6 µg Mg injection reversed this effect on NEL but not on seizure severity; and, all groups of rats receiving c.i.v. injection displayed less seizure
activity regardless of the Mg content. This loss of seizure activity due to the injection process forced us to use younger subjects which are much more seizure prone. However, even with the younger rats, only 50% were susceptible to seizure, and the seizure severity score was low (Figure 2). This compares poorly with results from other non-c.i.v. injection experiments in which most of the rats seized and the seizure severity score was high (4.1)(15, 16).

The role of serotonin in moderating behavior is well established and has been reviewed (13, 32). Brain serotonin levels have an inverse relationship to arousal and spontaneous activity. Behavioral effects resulting from serotonin depletion include hyperactivity and seizure proneness. Those resulting from increased serotonin levels include sedation and depressed activity. In two recent reports, Green et al. (55) demonstrated an inhibitory effect of c.i.v. serotonin injections on the spontaneous motor activity of rats, and Trimble et al. (143), showed that administration of 5-hydroxytryptophan (a serotonin precursor) to baboons completely blocked photogenic seizures.

Based on our findings that brain serotonin concentration was increased in rats fed a low Mg diet, and the trend of c.i.v. injections to increase serotonin, we conclude that serotonin may play a contributive role in mediating excitability. Our results lay in the opposite direction of Essman's (42) who found that dietary Mg depletion reduced
brain serotonin levels. However, his experiments were performed on younger subjects made acutely deficient in 16 days. From our activity data (Figures 1 and 2) (15, 16), we observed that older rats fed a deficient diet longer than 14 days grow progressively less excitable and their CSF Mg concentration is not drastically reduced. Thus, if the arousal hypothesis applies, increased serotonin in the brains of the younger Mg deficient rats compared with controls may be expected, whereas it would not be expected in the brains of older animals such as used by us and Itokowa et al. (68). The increased serotonin in the brains of the older animals may be a protective adaptive response, aimed at reducing the damaging effects of hyperexcitability.

While the serotonin response to c.i.v. Mg injections may contribute towards reduced excitability, we cannot infer from our data that this is the only modulating effect. Other mechanisms may operate. Magnesium is known, for example, to compete with a calcium mediated release of the neurotransmitter acetylcholine at the synapse of isolated preparations (66, 74) although the significance of this in the living mammal is yet to be understood.
PART IV

PRELIMINARY REPORT ON THE MAGNESIUM DEFICIENT RAT AS A MODEL OF EPILEPSY
INTRODUCTION

Epilepsy among men and women of all ages is a widespread health problem with incidences of 0.3 - 70 per 1000 (70). As a result, extensive research efforts are aimed at devising appropriate models with which to test the effectiveness of various treatments. Models frequently used for this purpose (106) involve convulsant drug (such as pentylene tetrazol) injections, cobalt implantation, drug withdrawal, the use of audiogenic seizure-prone strains of mice, electro-shock and the "kindling" method (49). Each model has its advantages and disadvantages, and its unique applications.

In working with young rats, we noticed that they become hyperactive within a few days when fed a low Mg diet and, shortly thereafter, highly susceptible to audiogenic seizures (15). Rats showing tonic seizure intensity during one trial may often be revived for subsequent tests. The effects of Mg deficiency appear quite stable and predictable over a few days. These facts, and the possibility of controlling the severity of deficiency by dietary means, have led us to conjecture that the young Mg-deficient rat may be an ideal test model for evaluating the effectiveness of symptom-reducing therapy for epilepsy. Reported here are preliminary findings of such research.
MATERIALS AND METHODS

Sprague-Dawley outbred rats from Simonsen Laboratories in Gilroy, California, were used in all experiments. They were housed individually in stainless steel cages unless otherwise reported. They were fed a powdered diet in glass containers, containing 20% casein, 58% dextrose, 10% corn oil, 2% vitamin mix (15) and 4% mineral mix containing 0.75% calcium as carbonate, 0.6% phosphorus as monosodium phosphate, 0.27% potassium as chloride, and other minerals in recommended amounts for rats (99). Cellulose (6%) was added to make up to 100%. The low Mg diet was found by analysis to contain less than 10 parts per million of magnesium. Control diet was made by adding 400 mg Mg per kg as carbonate to the low Mg diet. Ambient temperature in the animal room was 25°C and lights were on daily from 0800 to 2000 hrs. Testing was done between 1300 and 1700 hrs. Rats within treatment groups were time-matched to minimize circadian effects. Injections were given IP 45 min prior to testing. All injections were 0.2 ml per 100 g body weight. Other procedures such as handling and weighing the rats prior to testing were standardized as much as possible.

Seizures were induced by exposing the rats to noise generated by two school bells in a metal chamber creating a sound intensity of 115 decibels (102). Seizure severity was rated on a scale of 0 to 5. Zero signified no seizure; 1 = rapid running; 2 = clonic, characterized by full body con-
vulsions but with the animal usually on its feet; 3 = tonic flexion, characterized by the animal on its side, rigid and kicking; 4 = tonic extension, characterized by rigidity and extended legs; and, 5 = lethal seizure. Nonspecific excitability level (NEL) was determined according to a modified procedure of Lát and Gollová-Hémon (82) in a glass (thin-layer chromatography) chamber, 11 x 22 x 23 cm, inverted over a wire mesh floor. One cm wide tape divided the floor into six equal (approx. 5 x 7 cm) sectors. A rubber band was stretched horizontally around the chamber, equidistant from the floor at all points, at the height of a resting animal. A rearing response was counted when the rat raised both paws off the floor and its nose above the band. A nose-raise response was counted when the rat raised its nose above the band. A locomotion response was counted when the rat moved both front paws from one floor sector into another. A grooming response was counted when the rat licked its paws, washed its face or cleaned its body for 8 sec. If grooming continued for another full 8 sec it was counted again. The number of fecal pellets excreted was also counted. All responses occurring with a 2 min period were counted, starting within 10 sec after the rat was placed in the chamber. Responses were weighted 1, 1.5, 0.5 and -1 for rearing, nose-raise, locomotion and grooming respectively. The first fecal pellet excreted from each rat was counted 4, the second was counted 3, the third,
2 and the fourth, 1. Additional pellets were not counted.

Additional information regarding the specifics of each experiment is given in conjunction with the figures and tables, as data are presented in the "results" section. This information includes the sex and age of the rats, the drugs used, brief descriptions of the experiments and the nature of the statistical analyses.

RESULTS

The effects of diphenylhydantoin, phenobarbital, d-amphetamine sulfate, l-dopa and magnesium chloride on NEL and audiogenic seizure severity of 5-wk-old Mg-deficient male rats are shown in Figures 1 and 2. Amphetamine caused an increase in NEL compared with saline, while l-dopa and magnesium chloride caused a decrease, on the first day of testing (Figure 1). Diphenylhydantoin and phenobarbital protected against seizure, but amphetamine, l-dopa and magnesium chloride did not. All rats injected with amphetamine on the first day of testing died. Figure 2, which shows the results of two days of testing, reflects the same trends as Figure 1. Rats who survived an initial day of testing were less seizure-prone when tested the next day.

The effects of diphenylhydantoin and phenobarbital on 8-wk-old Mg-deficient female rats is shown in Figure 3. Phenobarbital caused an increase in NEL. Both diphenylhydantoin and phenobarbital reduced seizure severity,
Figure 1. Effect of DPH, PhB, Amph, DOPA and MgCl₂ injections on NEL and Audiogenic Seizure Severity of 5-wk-old Mg-deficient Male Rats. Weanling male rats were fed a low Mg diet 14 days and tested for NEL and audiogenic seizure severity. DPH = diphenylhydantoin (100 mg/kg), PhB = phenobarbital (32 mg/kg), Amph = d-amphetamine sulfate (3 mg/kg), DOPA = L-dopa (100 mg/kg), MgCl₂ = magnesium chloride (60 mg/kg), NaCl = normal saline-injected Mg-deficient rats, CONT = saline-injected diet controls (pair fed to a deficient group). Deficient rats weighed 102 ± 9 g on the final day. Diet controls weighed 112 ± 8 g. Serum Mg concentrations = 1.06 ± 0.31 mEq/l for deficient rats, and 6.78 ± 1.85 mEq/l for the MgCl₂ injected. N = 4 for each drug injected group, 20 for the saline injected deficient, and 8 for diet controls. Least significant differences for comparing a drug injected group with the saline injected deficient were 19 (p<0.05) and 24 (p<0.01) for NEL, and 1.3 (p<0.05) and 1.7 (p<0.01) for seizure severity scores. These were calculated from pooled variances, 260.8 and 1.282, respectively, for NEL and seizure severity.
Figure 2. Effect of DPH, PhB, Amph, DOPA and MgCl₂ on NEL and Audiogenic Seizure Severity of 5-wk-old Mg-deficient Male Rats (Cont.). Data presented here showing that the Mg-deficient rat model may be adapted to several experimental designs were obtained from a modification of the experiment reported in Figure 1. Rats were assigned to ten groups of four. Five groups were injected with the respective drugs on one day (Day 1). All ten groups were then tested for NEL and audiogenic seizure severity. Five groups thus tested on Day 1, on the next day (Day 2) were injected with drugs and retested. The first number at the base of each column represents the Day 1 response. The second represents the Day 2 response. Numbers at the head are Day 1 plus Day 2 averages (N = 8). Data were analyzed using a randomized complete block statistical design. Two MgCl₂ injected rats died and the missing data were estimated, with appropriate degrees of freedom reduced and bias corrections made. For NEL: Drug F (4/32) = 4.25, p<0.05; Days F (1/32) = 0.89, N.S. For seizure severity: Drug F (4/32) = 8.34, p<0.01; Days F (1/32) = 8.35, p<0.01. (a) Least significant difference (p<0.05).
LSD_{0.05}^a = 22

LSD_{0.05} = 1.6

<table>
<thead>
<tr>
<th>INJECTION</th>
<th>NEL</th>
<th>SEIZURE SEVERITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td>25</td>
<td>1.1</td>
</tr>
<tr>
<td>PhB</td>
<td>35</td>
<td>1.3</td>
</tr>
<tr>
<td>Amph</td>
<td>44</td>
<td>1.0</td>
</tr>
<tr>
<td>DOPA</td>
<td>55</td>
<td>3.5</td>
</tr>
<tr>
<td>MgCl_2</td>
<td>21</td>
<td>3.7</td>
</tr>
<tr>
<td>INJECTION</td>
<td>19</td>
<td>2.5</td>
</tr>
</tbody>
</table>

DPH = Phenobarbital, PhB = Phenytoin, Amph = Amphetamine, DOPA = Dopamine, MgCl_2 = Magnesium Chloride
Figure 3. Effects of DPH and PhB on NEL and Audiogenic Seizure Susceptibility of 8-wk-old Mg-deficient Female Rats. 5-wk-old female rats were fed low Mg diet for 21 days; assigned to 3 groups; injected with DPH (75 mg/kg), PhB (32 mg/kg) or saline; and, tested. Results of this testing are shown on the left side of the graph (Day 1). N = 5, all groups. F(2/12) for NEL = 7.8; p<0.01. LSD (p<0.05) = 24. F(2/12) for seizure severity = 32; p<0.01. LSD (p<0.05) = 1.0. On the day following this testing (Day 2), two pair of rats from each group were injected with substances other than they received on Day 1. Results of this testing are shown on the right side of the graph. (Injections in parentheses indicate the substances injected the previous day.) N = 2, each group. F(5/6) for NEL = 3.7, NS. F(5/6) for seizure severity = 4.6; p<0.05. LSD (p<0.05) = 2.4.
phenobarbital more than diphenylhydantoin.

Diphenylhydantoin and phenobarbital dose effects on 6-wk-old female rats are shown in Table 1 and displayed pictorially in Figure 4. Neither drug influenced NEL until the dose exceeded 15 mg/kg. At 30 mg/kg, phenobarbital elevated NEL. NEL remained high until the dose reached 85 mg/kg, after which sedation occurred. At concentrations of 60 mg/kg and greater, diphenylhydantoin depressed NEL in a linear fashion. Seizure severity was lessened by both drugs when the concentration exceeded 15-20 mg/kg.

Age and sex comparisons of audiogenic seizure severity are shown in Table 2. Younger males were more susceptible to seizure than older males, even though the older animals were fed the low Mg diet longer. Six to 8-wk-old females, fed a deficient diet 17-21 days, displayed mixed susceptibility to seizure. The females could usually be revived following seizure, however, while many of the younger males died. An occasional young male fed a control diet exhibited seizure activity.

DISCUSSION

From these preliminary findings, the Mg-deficient rat appears to be an excellent prospect as a model for evaluating the effects of anti-epileptic treatments on the central nervous system. Preparation of the subjects for testing is easy. Susceptibility to seizure may be regulated
Table 1. Diphenylhydantoin and Phenobarbital Dose Effects on NEL and Audiogenic Seizure Susceptibility of 6-wk-old Mg-deficient Female Rats.

<table>
<thead>
<tr>
<th>Injection</th>
<th>Dose (mg/kg)</th>
<th>Log Dose</th>
<th>N</th>
<th>NEL</th>
<th>Percent Seizures</th>
<th>Seizure Severity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier</td>
<td>0</td>
<td>-</td>
<td>7</td>
<td>47±13</td>
<td>50</td>
<td>1.6±1.8</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>-</td>
<td>7</td>
<td>51±11</td>
<td>43</td>
<td>2.0±2.5</td>
</tr>
<tr>
<td>DPH</td>
<td>5</td>
<td>0.70</td>
<td>4</td>
<td>67±19</td>
<td>50</td>
<td>1.4±2.4</td>
</tr>
<tr>
<td>DPH</td>
<td>10</td>
<td>1.0</td>
<td>3</td>
<td>49±14</td>
<td>67</td>
<td>2.3±2.1</td>
</tr>
<tr>
<td>DPH</td>
<td>20</td>
<td>1.3</td>
<td>3</td>
<td>45±9</td>
<td>0*</td>
<td>0.0</td>
</tr>
<tr>
<td>DPH</td>
<td>30</td>
<td>1.5</td>
<td>3</td>
<td>61±9</td>
<td>0*</td>
<td>0.0</td>
</tr>
<tr>
<td>DPH</td>
<td>60</td>
<td>1.8</td>
<td>3</td>
<td>43±9</td>
<td>0*</td>
<td>0.0</td>
</tr>
<tr>
<td>DPH</td>
<td>100</td>
<td>2.0</td>
<td>3</td>
<td>36±11</td>
<td>0*</td>
<td>0.0</td>
</tr>
<tr>
<td>DPH</td>
<td>200</td>
<td>2.3</td>
<td>3</td>
<td>21±12*</td>
<td>0*</td>
<td>0.0</td>
</tr>
<tr>
<td>PhB</td>
<td>5.5</td>
<td>0.74</td>
<td>4</td>
<td>69±19</td>
<td>50</td>
<td>1.3±1.9</td>
</tr>
<tr>
<td>PhB</td>
<td>15</td>
<td>1.2</td>
<td>2</td>
<td>51±20</td>
<td>0*</td>
<td>0.0</td>
</tr>
<tr>
<td>PhB</td>
<td>30</td>
<td>1.5</td>
<td>3</td>
<td>79±17*</td>
<td>33</td>
<td>0.3±0.6</td>
</tr>
<tr>
<td>PhB</td>
<td>55</td>
<td>1.7</td>
<td>3</td>
<td>79±18*</td>
<td>0*</td>
<td>0.0</td>
</tr>
<tr>
<td>PhB</td>
<td>85</td>
<td>1.9</td>
<td>3</td>
<td>33±30</td>
<td>33</td>
<td>0.8±1.4</td>
</tr>
<tr>
<td>PhB</td>
<td>170</td>
<td>2.2</td>
<td>2</td>
<td>0*</td>
<td></td>
<td>0.0</td>
</tr>
</tbody>
</table>

Least Significant Differences for comparing treated groups with saline or carrier injected groups (p<0.05)

*Significantly different from saline or carrier injected group.

Weanling female rats were fed low Mg diet for 17 days. They weighed 96 ± 10 g on the final day. Carrier consisted of 40% propylene glycol, 10% ethanol and 50% water. Drug dilutions were made with this carrier. Percent seizure comparisons were made using Chi Square tests with Yates Correction. Least significant differences were calculated from pooled variances, 260.2 and 2.556, respectively, for NEL and seizure severity scores. Four non-injected rats who were not weighed and handled as much as the carrier or saline injected, all seized. Their seizure severity score = 3.0 ± 2.3.
Figure 4. Dose Response Curves Showing the Effects of DPH and PhB on NEL and Audiogenic Seizure Susceptibility of Weanling Mg-deficient Female Rats. (See Table 1 for experimental details.)
Table 2. Age and Sex Differences in Audiogenic Seizure Susceptibility of Mg-deficient Rats.

<table>
<thead>
<tr>
<th>Age¹</th>
<th>Sex</th>
<th>Days Fed Diet</th>
<th>N</th>
<th>Body Weight² (g)</th>
<th>NEL</th>
<th>Percent Seizures</th>
<th>Seizure Severity Score</th>
<th>Percent Lethal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weanling</td>
<td>Males</td>
<td>14 (Cont)³</td>
<td>19</td>
<td>103±10</td>
<td>39±15</td>
<td>95</td>
<td>4.1±1.4</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>17</td>
<td>18</td>
<td>99±11</td>
<td>49±12</td>
<td>61</td>
<td>1.9±2.0</td>
<td>11</td>
</tr>
<tr>
<td>5 wk old</td>
<td>Males</td>
<td>21</td>
<td>20</td>
<td>212±25</td>
<td>36±16</td>
<td>40</td>
<td>1.5±1.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>21 (Cont)⁵</td>
<td>6</td>
<td>147±15</td>
<td>32±9</td>
<td>100</td>
<td>4.0±0.6</td>
<td>17</td>
</tr>
<tr>
<td>Adult</td>
<td>Males</td>
<td>31</td>
<td>10</td>
<td>434±78</td>
<td>22±11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

¹Age at initiation of feeding.
²Body weight on the final day.
³Mg supplemented controls.
by diet. Appropriate subjects may be revived for retesting. And seizure susceptibility is clearly due to its action on the central nervous system, not upon peripheral systems. This is evidenced by the fact that elevated serum Mg alone does not lessen seizure severity (Figures 1 and 2), nor does depressed serum Mg directly induce seizure susceptibility (15, 26). Development of the symptoms occurs only after the concentration of Mg in the cerebrospinal fluid falls.

The two common anti-epileptic drugs employed, diphenylhydantoin and phenobarbital, yielded the desired and expected results by reducing the incidence and severity of seizures. This occurred when doses exceeded 15-20 mg/kg (Table 1). We identified no significant interaction effects due to alternate treatments of these drugs (Figure 3), although the small number of subjects per experimental unit suggests caution in ruling out the possibility at this point.

By careful manipulation of age, sex and length of time fed a low Mg diet, it is possible to produce a group of subjects consistently susceptible to audiogenic seizures, but who may be revived for subsequent testing. We feel, from data presented in Table 2 and from general experience, that the 4 to 5-wk-old female rat fed a low Mg diet for 21 days may be the best model. Older male rats are not consistently susceptible. Younger males do not survive the trauma of seizure.

The mechanism by which Mg deficiency induces seizure
susceptibility is unknown, although it may act by removing the competitive inhibition of the calcium facilitated release of transmitter at acetylcholine mediated neurons (66, 74, 110). That l-dopa and amphetamine do not lessen seizure severity (Figures 1 and 2) suggests that elevated amounts of the catecholamine transmitters are not involved in the etiology of Mg-deficiency symptoms, since l-dopa leads to the production of dopamine, and amphetamine to the buildup of nor-epinephrine in the synaptic cleft (31). A possible role of serotonin, on the other hand, cannot be ruled out. Decreased brain serotonin exacerbates, while increased serotonin protects against pentylene tetrazol induced seizures (141, 142).

Furthermore, diphenylhydantoin and phenobarbital, which obviously protect against seizures, are known to induce elevated brain serotonin (11); and Mg deficiency results in lowered serotonin levels in the brain of the young rat (42).

The young Mg-deficient rat, in addition to exhibiting seizure susceptibility, also displays elevated NEL before seizure susceptibility becomes too great and if diet administration does not exceed 14 days (15). These conditions were not met in the experiments reported here (Table 2). However, it seems certain that older rats or rats fed a low Mg diet longer than 14 days do not manifest heightened NEL.

Drug effects on NEL were mixed. The expected excitatory effect of amphetamine on NEL was observed (Figure 1). Large doses of diphenylhydantoin and phenobarbital
produced sedation (Table 1, Figure 4). Moderate doses of phenobarbital, on the other hand, elevated NEL (Table 1, Figures 3 and 4), although the heightened activity is not normal. The heightened activity suggests that some neural systems, perhaps those mediated by norepinephrine, are activated by this drug in the Mg-deficient rat. In one experiment (Figure 3) we observed that five out of 10 rats injected with 32 mg/kg phenobarbital began drinking, and a couple began eating, shortly after injection. Norepinephrine and other monoamine neurotransmitters are believed to elicit these responses (122).
PART V

A PROCEDURE FOR PREDICTING (Na$^+$ K$^+$)-ATPase ACTIVITY FROM RAT BRAIN HOMOGENATES
INTRODUCTION

The \((Na^+ K^+)-activated ATPase\) enzyme system \((Na^+ K^+)-ATPase\) has been strongly implicated as the cation pump which maintains resting potentials in nervous tissue \((119, 127, 128)\). With its unique ability to convert chemical energy into electrical potential by causing ionic movement, it is a major link between biochemical processes and electrical functioning of the nervous system.

Proper excitability in nervous tissue requires that this enzyme be amply but not excessively active. A direct correlation has been established between excitability and intracellular sodium, and epileptic seizures result when intracellular sodium levels increase at the expense of potassium \((154)\). Some epileptics have low potassium levels in the cerebrospinal fluid (CSF) \((147)\). Magnesium deficiency \((24, 78, 144)\) and "in vivo" inhibition of \((Na^+ K^+)-ATPase\) \((36, 37)\) have been implicated in seizures. Reynolds \((109)\) and Viukari \((148)\) have contended that epilepsy and schizophrenia are antagonistic disorders, that one results when resting potentials are depressed, the other when they are elevated from their normal levels.

The effectiveness of the \((Na^+ K^+)-ATPase\) pump system depends upon a number of factors, among them being the availability of the substrate, ATP. The availability of ATP
in turn depends upon the functioning of the glycolytic pathway, the tricarboxylic acid cycle and oxidative phosphorylation. The concentrations of ions in the CSF and intracellular fluid also influence pump activity. Because so many factors converge here, the enzyme pump system seems to be one good place to begin investigating physiological disorders of the nervous system.

Only a few procedures assessing the activity of (Na⁺ K⁺)-ATPase have been described in detail (12, 69, 75, 105, 139) with little discussion of variability, and several of these require the use of expensive automated equipment.

Due to the key position of (Na⁺ K⁺)-ATPase in mammalian physiology and the need for a detailed description of a simple assay procedure, this research was undertaken. The objectives of the research were: (1) to present in some detail a rapid, dependable method for determining (Na⁺ K⁺)-ATPase activity using equipment commonly found in the research laboratory, (2) to identify and discuss sources of variation and suggest how precision may be improved, and (3) to develop programmable expressions enabling the prediction of enzyme activity in preparations where activity does not strictly follow Michaelis-Menten kinetics.

Since little difference has been found between the properties of the membrane bound and purified enzyme (108), it is felt that complex extraction and centrifugation techniques are unnecessary for many medical, pharmacological
and nutritional problems involving \((\text{Na}^+ \text{ K}^+)\)-ATPase analysis. For this reason a simplified procedure for assessing \((\text{Na}^+ \text{ K}^+)\)-ATPase activity in rat brain homogenates is described.

**METHODS**

Fourteen Sprague-Dawley male, weanling rats were fed standard laboratory diet for 4½ weeks. Their weights averaged 176 g on the final day. Brain homogenates were prepared using 1 part brain and 9 parts deionized water. The brain homogenates from 7 rats were pooled, partitioned into 3 ml aliquots, quickly frozen in a dry ice methanol bath and stored at -85°C for use throughout the experiment. Brain homogenates of the remaining 7 rats were divided into 2 aliquots each, similarly frozen and stored, and used to determine enzyme variability among animals. Standard Lowry protein analyses \((88)\) were run in triplicate on pooled and individual homogenates using human serum protein standard. The final results of the \((\text{Na}^+ \text{ K}^+)\)-ATPase assay were expressed per mg tissue protein.

The \((\text{Na}^+ \text{ K}^+)\)-ATPase assay was modified from that of Bonting et al.\((12)\). EDTA and \(\text{CN}^-\) were deleted from the reaction media. EDTA interfered with precision and \(\text{CN}^-\) was not necessary since the small amount of respiration taking place was not measurable under the conditions of the experiment. The 4 min incubation period at 37°C employed by
Reisin and Keps (114) was used since this gave a linear relationship between enzyme concentration and activity.

Reaction media for both activating and inhibiting the enzyme were prepared fresh daily in amounts adequate to complete each step of the experiment (Table 1). In all cases TRIS buffer was used to bring osmolarity to 450 ± 20 mEq/l at pH 7.35.

Substrate, 300 µl, was pipetted into triplicate incubation tubes and kept in an ice-water bath. Reactions were initiated by adding 20 µl of recently thawed homogenate and placing the tubes in an oscillating water bath at 37°C. Incubations were terminated after 4 min by adding 0.15 ml of 10% TCA and replacing the tubes in the ice-water bath. Tubes were centrifuged for 10 min and 0.5 ml of the supernatant withdrawn for analysis. The phosphorus released during incubation was determined in a final volume of 2.7 ml by the method of Gömörri (53). MgCl₂ · 6H₂O was added to a final concentration of 1% to stabilize color. Automatic diluters and pipetters were used throughout, except in preparing reaction media. We found that up to 70 aliquots were a convenient number to assay at one time. Differences between the values obtained from the media activating the enzyme and those from media inhibiting the enzyme (Table 1) were used as a measure of the (Na⁺ K⁺)-ATPase activity for each concentration of the substance in question. The entire procedure was replicated for each variable substance on
Table 1. Concentrations of Substances Used in Reaction Media.

<table>
<thead>
<tr>
<th></th>
<th>Reaction Medium for Activating the Enzyme</th>
<th>Reaction Medium for Inhibiting the Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>ml/20ml*</td>
</tr>
<tr>
<td>Na⁺</td>
<td>150</td>
<td>2.88</td>
</tr>
<tr>
<td>K⁺</td>
<td>40</td>
<td>0.80</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>3</td>
<td>0.60</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>ATP⁻</td>
<td>3</td>
<td>6.00</td>
</tr>
<tr>
<td>TRIS</td>
<td>36.8</td>
<td>0.74</td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.1</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*ml of stock solutions used to make 20 ml of reaction medium. Concentrations of stock solutions of sodium, potassium and TRIS were 1 M, magnesium was 0.1 M, and ATP and ouabain were 0.01 M. Disodium ATP was used throughout.

Minerals were varied one at a time during each step of the experiment, except when determining the effect of potassium on enzyme activity in which 50 mM Na⁺ instead of 150 mM was used to keep osmolarity from being excessive.
separate days. Curve fitting was done by general least squares analysis.

Concentrations in the reaction media were selected to bracket the physiological range of CSF and whole brain concentrations of the respective ions. Approximate literature values for CSF and whole brain concentrations of Na\(^+\), K\(^+\), Mg\(^{++}\) and Ca\(^{++}\) for small animals were, respectively, 155, 3, 2 and 3 mM/L for CSF (3, 34), and 48, 100, 6 and 1 mM/L for whole brain (3, 4, 132).

RESULTS

Pooled vs. Individual: The purpose of comparing pooled vs. individual rat brain homogenates was twofold: (1) to see if there was a sizable variability among animals, and (2) to find out whether the (Na\(^+\) K\(^+\))-ATPase activity of the pooled sample, which was used in all subsequent steps of the experiment, fell within an acceptable range of the mean of the homogenates from the individual animals. The results are presented in Table 2. The enzyme activity of the pooled homogenate was higher than, but within 1 SD of the mean activity of the individual homogenates.

Variability: An analysis of variance of the data showed that variability among animals was greater than the other contributing factors, and that variability between replications (days) was greater than among determinations (Table 3). An efficiency analysis of the mean square
### Table 2. Comparison of (Na⁺ K⁺)-ATPase Activity of Pooled and Individual Rat Brain Homogenates (10:1).

<table>
<thead>
<tr>
<th></th>
<th>Pooled</th>
<th>Individual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Mean activity (µmoles phosphate per min per mg tissue protein)</td>
<td>0.208</td>
<td>0.204</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>-</td>
<td>0.021</td>
</tr>
</tbody>
</table>

### Table 3. Analysis of Variance of (Na⁺ K⁺)-ATPase Data from 7 Individual Rat Brain Homogenates in 2 Replications Each with 3 Determinations per Replicate.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Estimate of MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals</td>
<td>6</td>
<td>0.01021</td>
<td>0.00170</td>
<td>0.00128</td>
</tr>
<tr>
<td>Replications/animals</td>
<td>7</td>
<td>0.00299</td>
<td>0.00043</td>
<td>0.00027</td>
</tr>
<tr>
<td>Determinations/reps.</td>
<td>28</td>
<td>0.00444</td>
<td>0.00016</td>
<td>0.00016</td>
</tr>
</tbody>
</table>
estimates revealed that precision could be improved 50% by increasing the number of replications from 2 to 3, 4% by increasing the number of determinations from 3 to 4, and 39% by increasing the number of replications to 3 and decreasing the number of determinations to 2.

**Effect of Sodium:** The effect of sodium on enzyme activity is shown in Figure 1. The relationship was logarithmic up to 150 mM \((r^2 = .99)\). The equation for the data is:

\[
\text{Activity (µMP/min)} = 0.096 \log(\text{mM Na}^+) - 0.049
\]

**Effect of Potassium:** The effect of potassium on \((\text{Na}^+ \text{ K}^+)\)-ATPase activity is shown in Figure 2. This ion did not have as pronounced an influence as did sodium, although at low values (10 mM or less) its effect is considerable. At 40 mM and above, there was no significant increase in activity with concentration. The relationship was not logarithmic and curve fitting was necessary to explain the data. The equation arrived at is:

\[
\text{Activity (µM P/min)} = 0.050 (1-e^{-0.216(\text{mM K}^+)}) + 0.006
\]

**Effect of Magnesium:** The effect of magnesium on \((\text{Na}^+ \text{ K}^+)\)-ATPase activity is shown in Figure 3. The equation representing the data is:

\[
\text{Activity (µM P/min)} = 0.019 (1-e^{-1.71(\text{mM Mg}^{++})}) + 0.022
\]

The data show considerable dependence of enzyme activity on magnesium concentration up to 3 mM. Further
Figure 1. Effect of Sodium on (Na⁺ K⁺)-ATPase Activity. Average deviation of points from the regression = 0.014. R sq. = .99. See text for equation and Table 1 for media composition.

Figure 2. Effect of Potassium on (Na⁺ K⁺)-ATPase Activity. Concentrations of other substances in reaction medium for enzyme activation were 3 mM ATP, 3 mM Mg++] and 50 mM Na⁺. Average deviation from the regression = 0.012. R sq. = .66. See text for equation.
increases in magnesium had no measurable effect.

**Effect of Calcium:** Increases in calcium at low concentrations had a considerable inhibitory effect on \((\text{Na}^+ \text{ K}^+)\)-ATPase activity (Figure 4). The relationship was essentially an inverse function:

\[
\text{Activity (µM P/min)} = \frac{0.043}{(\text{mM Ca}^{++}) + 0.28} + 0.023
\]

Small increases in calcium concentration beyond 2 mM had little effect on enzyme activity.

**DISCUSSION**

The first objective of this research was to describe a rapid, dependable technique for predicting \((\text{Na}^+ \text{ K}^+)\)-ATPase activity from rat brain homogenates. Results suggest that rather extended experiments can be undertaken to measure \((\text{Na}^+ \text{ K}^+)\)-ATPase activity using pooled rat brain homogenates from several animals and equipment available in most laboratories. Well over 12 separate runs were made from the pooled homogenates of 7 rat brains, permitting 3 determinations at each of several concentrations of the mineral being varied. Final volumes of the samples ready for colorimetric determination were large enough to permit easy reading on a standard grating spectrophotometer.

The second objective was to analyze the data statistically to identify possible sources of variation. The pooled standard deviation for \((\text{Na}^+ \text{ K}^+)\)-ATPase activity was
Figure 3. Effect of Magnesium on (Na\(^+\) K\(^+\))-ATPase Activity. Average deviation from the regression = 0.014. R sq. = .96. See text for equation and Table 1 for media composition.

Figure 4. Effect of Calcium on (Na\(^+\) K\(^+\))-ATPase Activity. Average deviation from the regression = 0.012. R sq. = .96. See text for equation and Table 1 for media composition.
0.021 µmoles phosphate per mg tissue protein, suggesting that moderate, but not small, differences in activity due to treatment (pharmacological, dietary, etc.) would be identifiable.

A large source of error was due to day-to-day variation, which is most likely attributed to uneven makeup of the reaction media, slight pH differences and other procedural inconsistencies. We purposely made the reaction media from scratch each day so as to determine the importance of these error sources. Of course, apparent precision may be improved by storing samples until all could be assayed concurrently or by making up reaction media ahead of time including all ingredients except ATP which could be added on the day the assay is to be run.

An efficiency analysis of the data showed that precision may be improved considerably by increasing the number of replications, but only slightly by increasing the number of determinations. Without replication, identification of smaller differences between control and experimental groups or samples would be possible since this replication's error factor is large. However, without replication, there is no way to check the values obtained.

The third objective was to develop programmable expressions enabling the predication of enzyme activity. Equations enabling prediction of \((Na^+ K^+)\)-ATPase activity knowing the concentrations of sodium, potassium, magnesium
and calcium are presented in the previous section.

Mineral effects on \((\text{Na}^+ \text{ K}^+)\)-ATPase, usually from microsomal fractions, have been well established by previous research (1, 41, 58, 87, 98, 108, 112, 113, 124-126, 129, 130, 140). Our results with whole brain homogenate generally concur with these findings. The relationship between sodium concentration and \((\text{Na}^+ \text{ K}^+)\)-ATPase activity up to 150 mM sodium was logarithmic in the presence of 40 mM potassium, having a \(K_m = 24.5\) mM. According to some researchers (41, 87, 108, 112, 124) activity diminishes in the presence of high sodium, especially in the presence of low potassium. Our data show that, in the presence of 40 mM potassium, activity may be falling off at 175 mM sodium. This peaking effect is thought to be attributed to sodium competing for a potassium activation site while not sharing in the activation there (41, 87), but there are other possibilities (87, 98, 112, 124). Also, the affinity of one site for sodium relative to potassium can increase with ATP concentration (112, 129, 130).

The relationship between potassium concentration and \((\text{Na}^+ \text{ K}^+)\)-ATPase activity was not logarithmic, and required the use of curve fitting techniques to predict it. Albers and Koval (1) have suggested that potassium fills a catalytic function at one site and a regulatory function at another. Both sodium and potassium compete for the latter site (1, 87). When potassium concentration exceeded 25-40 mM in the presence of high sodium in our experiment, differential
effects of activation and regulation were lost. It has been shown that inhibition caused by a high potassium concentration is reduced in the presence of a high sodium concentration (108, 112, 125). The degree of fit between predicted and actual values was poorer for potassium than for the other cations.

The greater dependence of \((\text{Na}^+ \text{K}^+)\)-ATPase activity on magnesium concentration up to the concentration of ATP is well known and an Mg-ATP complex has been implicated (58, 125, 126).

Calcium has an inhibitory effect on \((\text{Na}^+ \text{K}^+)\)-ATPase activity (113, 125, 126, 140). Inhibition may be expressed as in inverse function of calcium concentration except at very low concentrations. At low calcium concentrations in our experiment, predicted activity did not coincide with the actual. It has been traditionally held that calcium complexes with ATP to form Ca-ATP, which reversibly competes with Mg-ATP. More recently, however, calcium has been reported to compete at sodium and potassium activation sites and cause inhibition there as well (113).

The maximum \((\text{Na}^+ \text{K}^+)\)-ATPase activity for rat brain homogenates as determined under the conditions of the method presented here was \(0.204 \pm 0.021\) µmoles phosphate per min per mg tissue protein, which indicates that this is a fairly sensitive procedure.

The expressions developed in this report for pre-
dicting (Na\(^+\) K\(^+\))\text{-ATPase} activity apply to substrate and cation concentrations in the ranges specified, and cannot be expected to apply if concentrations are very large or small. Use of different concentrations may necessitate changes in the equations or require the development of more sophisticated expressions. However, since the apparent activity of the (Na\(^+\) K\(^+\))\text{-ATPase} enzyme complex does not strictly follow Michaelis-Menten kinetics (112), mathematical modeling may be a pragmatic tool for defining its activity relative to the concentrations of substances in the reaction medium.

Some disorders of the nervous system can probably be traced to irregularities in (Na\(^+\) K\(^+\))\text{-ATPase} activity, either directly or as they influence other pathways which make substrate and various cofactors available to the enzyme. Copper and zinc toxicities, for example, may poison the enzyme (37). The procedure as herein described may provide an efficient method for determining that activity.
GENERAL SUMMARY AND CONCLUSIONS
SUMMARY

The original intent of the investigations presented in this dissertation was to shed light on the mechanisms responsible for altered behavioral excitability in mammals due to dietary Mg deficit. The chain of events that leads from the dietary insult to symptoms of hyperexcitability such as increased spontaneous activity or decreased seizure threshold, is very complex. Only a few selected biochemical and physiological steps have been considered and discussed in the dissertation. The more important findings are summarized below. These results have also been integrated into a short theoretical treatise entitled "Magnesium deficiency-induced hyperexcitability," which views the "state-of-the-art" of known events relating the dietary deficit to it. There is much yet to be done, for many facts are still to be identified, properly understood, and weighted as to their relative importance or influence on mediating behavior under the myriad of conditions to which a living organism is subjected in the course of its life. A final section, "Suggestions for future research," completes the work.

Magnesium deficiency in weanling rats caused an increase in nonspecific excitability level (NEL) and in audiogenic seizure susceptibility (Part I). These behavioral
effects were apparent after eight days of Mg restriction and could be reversed by dietary rehabilitation. Serum Mg declined rapidly from $1.87 \pm 0.10 \text{ mEq/l}$ to $0.91 \pm 0.24 \text{ mEq/l}$ in two days. Cerebrospinal fluid (CSF) Mg decreased gradually from $1.86 \text{ mEq/l}$, becoming significantly lower $(1.44 \pm 0.23 \text{ mEq/l})$ after eight days. When deficient rats were injected IP with Mg chloride, raising the serum Mg concentration to $6.6 \text{ mEq/l}$, NEL decreased to normal while audiogenic seizure susceptibility remained. Both NEL and audiogenic seizure susceptibility in rats reflect central nervous system Mg concentration, except when serum Mg concentration is very high. Very high serum Mg concentration lowers NEL but does not reduce audiogenic seizure susceptibility caused by low CSF Mg.

In a second series of experiments (Part II), the effects of dietary Mg deficiency and excess on NEL, cation concentrations and $(\text{Na}^+ \text{K}^+)-\text{ATPase}$ were determined, also in weanling rats. Magnesium deficiency led to increased NEL; decreased serum, CSF and brain Mg concentrations; increased serum calcium; increased serum sodium; and, increased brain $(\text{Na}^+ \text{K}^+)-\text{ATPase}$ activity. Magnesium excess led to no significant changes in spontaneous activity, an increase in serum Mg concentration, no other significant changes in cation concentrations, and increased $(\text{Na}^+ \text{K}^+)-\text{ATPase}$ activity. Results are integrated with the literature and their significance in providing understanding about mechanisms relating to
neural excitability is discussed.

Cerebral intraventricular injections of 17 and 50 µg Mg in artificial CSF progressively depressed the NEL of permanently canulated 150-200 g rats fed a low Mg diet for 21 days (Part III). Compared with control diet, the low Mg diet caused an increase in brain serotonin (0.73 µg/g fresh tissue compared with 0.56 µg/g). Weanling rats fed the low Mg diet for two weeks, then injected with 25 µg Mg via acute canulations displayed reduced audiogenic seizure susceptibility in contrast to sham-injected rats.

To test the feasibility of the Mg deficient rat as a model of epilepsy (Part IV), young or weanling rats were fed a low Mg diet for at least two weeks and injected IP with several drugs. Diphenylhydantoin and phenobarbital strikingly reduced the audiogenic seizure susceptibility of the Mg deficient rats. Amphetamine and l-dopa had no significant effect. Diphenylhydantoin depressed NEL. Phenobarbital elevated NEL at moderate doses and led to sedation at high doses. Amphetamine elevated NEL. L-dopa and Mg (as chloride) depressed NEL. The most promising subjects for the model appear to be female rats between three and five weeks old at the start of feeding. These subjects, if thrown into seizure by a loud noise, may be revived for subsequent testing, thus affording the opportunity for assessing drug interactions and to design meaningful experiments using few animals.
A procedure that gives quick, accurate and quantitative predictions of \((\text{Na}^+ \text{ K}^+)\)-ATPase activity from rat brain homogenates using standard laboratory equipment is described (Part V). The experimental design employed in testing the procedure comprises two replications per sample with three determinations. Sources of variation are discussed. The error factor for determinations was slight. The error factor for replications was more extensive. Precision may be improved 50% by increasing the number of replications to three but only slightly by increasing the number of determinations per replicate. The advantage of using pooled instead of individual homogenates is discussed. The mean value for \((\text{Na}^+ \text{ K}^+)\)-ATPase activity in rat brain homogenates was \(0.184 \pm 0.18 \mu\text{moles} \text{ phosphate released per min per mg tissue protein}\). The effects of sodium, potassium, magnesium and calcium on activity were investigated and expressions equating activity with the concentrations of these ions were developed.

**MAGNESIUM DEFICIENCY-INDUCED HYPEREXCITABILITY**

That Mg deficiency is a cause of hyperexcitability among mammals, as manifested by seizure proneness and other symptoms, has been well known for some time (78). By today, the causal relationship has been soundly documented and occurs in several species including man (Part I, 39, 91, 121,
While most research has been intent upon identifying the relationship between Mg status and the symptomatology, little has been done to explain it. Why a lack of dietary Mg should induce hyperexcitability remains largely unknown, although a few theoretical postulates have been made and a few bits and pieces of what appears to be a gigantic puzzle have been found. Part of the complexity arises because Mg is involved in so many enzymatic processes. Nearly all reactions involving high energy phosphates require Mg as an enzyme cofactor or cosubstrate. Several enzymatic steps needed for the conversion of energy contained in raw sources—glucose, fatty acids, amino acids—into ATP, proceed in the presence of Mg. Mg is required to form an active substrate, Mg-ATP, before the energy can be effectively utilized to synthesize, for example, a specific protein or a neurotransmitter, or perform some other service.

Mg directly affects transmitter release at the synapse (66, 74) and at the neuromuscular juncture (18, 19, 64). Of these two, however, its action on the synapse appears to be the consequential one in the living mammal. Reduced peripheral Mg concentration alone does not induce hyperexcitability (Part I).

Just what central nervous system mechanism is responsible for Mg deficiency-induced hyperexcitability is unclear. It is apparent that the deficiency leads to
decreased ATP production, with the rate limiting step being an uncoupling of oxidative phosphorylation (21, 134, 135). Indeed, reduced P:O ratios have been observed in the brains of Mg deficient rats (145). Theoretically, the loss of ATP should cause cation pump failure, since Mg-ATP is the substrate. If the pump fails, intracellular sodium and water contents increase while potassium is lost, adequate electrical gradients are not maintained, cells discharge prematurely, and seizures and other symptoms ensue. This theory has been advanced (36) and is supported by research on several tissues in which electrolyte and fluid changes have occurred in the predicted manner during Mg depletion (40, 45, 90, 93, 151, 152). Unfortunately, the theory may not be entirely tenable when applied to brain. While we found a marginal increase in brain sodium during Mg deficiency in very young animals (Part II), Martindale and Heaton (93) observed a decrease in adult rats. Furthermore, dinitrophenol, a specific uncoupler of oxidative phosphorylation, causes hyperpolarization and decreased excitability of cortical neurons (52). It acts by decreasing intracellular potassium, increasing intracellular chloride and blocking the action of the excitatory transmitter, acetylcholine. This action responds to a complex chain of events in which ATP depletion increases intracellular calcium which is thought to induce the other effects (83, 84, 94). When administered to dogs and rodents, chronic sub-lethal doses of the drug have a
sedative effect, although acute lethal doses cause hyperexcitability (71, 137). Furthermore, it is well known that the behavioral effects of diabetic or hypoglycemic coma lie in a direction opposite that of hyperexcitability. Blood levels of glucose, a most important precursor of ATP production in brain, are positively related to ATP concentration (85). It is for these reasons that the relationship between decreased intracellular energy and hyperexcitability is, at best, mixed, and one needs to look further for a more satisfactory explanation of why Mg deficiency induces it.

Perhaps a most obvious direction to look is toward one or more of the neurotransmitter systems. Unfortunately, very little information is available on this score. Essman (42) found a marked decrease of serotonin in brains of weanling mice fed a low Mg diet 16 days. Itokawa (67, 68) reported ambiguous results using 80-100 g rats fed a low Mg diet 4 weeks. We (Part III) found an increase in brains of 150-200 g rats fed a low Mg diet 21 days. This trend of increased brain serotonin in Mg deficient rodents as they grow older parallels a decrease in nonspecific excitability level (NEL) (Parts III and IV). Serotonin may therefore modulate the excitability of these animals.

Although the older rats may display decreased NEL and have increased serotonin, they still exhibit some seizure activity (Part III). Furthermore, while decreasing seizure proneness and NEL, cerebral intraventricular Mg
injections may have some, although not a very pronounced effect on brain serotonin. This implies that at least one other mechanism operates in response to Mg deficiency and is more likely the dominant one leading from reduced brain Mg concentration to the symptomatology. A likely alternative is acetylcholine, a neurotransmitter, a release of which is known to respond to the Mg concentration of a bathing medium surrounding isolated nerve preparations (66, 74).

Of course other putative neurotransmitters may be involved, such as gamma amino butyric acid and histamine, or other substances, relative concentrations of which may affect the climate surrounding a nerve, which in turn may attenuate excitability.

In conclusion: A dietary Mg deficit is reflected in reduced serum Mg concentration within a few hours. Reduced cerebrospinal fluid Mg concentration follows in a few days with concomitant increased excitability as determined by NEL and audiogenic seizure susceptibility. Decreased brain Mg probably leads to reduced ATP levels; however, this may not cause hyperexcitability because of a complex compensatory mechanism which produces sedation, if anything. The solid link between reduced brain Mg and hyperexcitability remains hidden. However brain serotonin is implicated since concentrations may vary directly with acute brain Mg changes, and inversely if these changes are not drastic and time is allowed for compensation to occur. The effects of Mg
deficiency on acetylcholine and other putative neurotransmitters and substances, in whole brain and in discrete areas, have yet to be determined.

SUGGESTIONS FOR FUTURE RESEARCH

That Mg deficiency-induced hyperexcitability is mediated via some mechanisms or mechanism located in the central nervous system, as opposed to a peripheral mechanism, has been soundly established by the research presented in this dissertation (Parts I and III). Low serum Mg concentrations without a concomitant reduction in CSF Mg, neither increase NEL nor audiogenic seizure susceptibility. Very high serum Mg concentrations however reduce NEL in rats (Part I), produce death in some (Part IV), and cause drowsiness and coma in human beings (59). It is not known why this occurs, although a curare-like block of electrical transmission at the neuromuscular juncture seems the most promising possibility (18, 19, 64). That this is where and how the block occurs in the living mammal, when serum Mg concentrations are elevated, has yet to be researched.

Another interesting phenomenon is that audiogenic seizures can occur even when NEL is low due to elevated serum Mg (Part I). Does this imply that, under extreme conditions, the central nervous system may be hyperexcitable or close to seizure thresholds while outward pre-seizure behavior depicts a "calmness"? Might it be possible for an
individual to appear lethargic and yet feel "uptight?" The thought opens "Pandora's box" of possibilities. Also, are there effectors other than elevated serum Mg which could block efferent neural transmission under certain conditions? There is much that could be researched here.

A most promising aspect (Part I) is that NEL is a more sensitive indicator of Mg deficiency-induced hyper-excitability among young subjects than seizure susceptibility during the early stages of deficiency. This suggests that NEL determinations may also be useful for identifying pre-seizure activity induced by other means, such as convulsant drug injections, cobalt implantations and drug withdrawal (106) or kindling (49) or pyridoxine deficiency (61, 155).

Appropriate behavioral measures might be applied to other animals for the same purposes. A behavioral measure might be developed which could indicate pre-seizure activity in humans. The practical implications of this are enormous.

A sensitive behavioral measure that reflects central nervous system activity, such as the NEL determination originally developed by Lát and Gollová-Hémon (82) and refined by us, has obvious advantages over electronic devices, if it can be used. The expense and time saved are obvious. Also, in addition to its being more sensitive than the audiogenic seizure method for some applications, the NEL method has other advantages: first, it is far less traumatic for the subject; second, it responds to treatments which may
decrease excitability or produce sedation (Parts III and IV); finally, it can be applied to subjects which are not seizure prone (Part III). There are an unlimited number and variety of drug and nutrient studies for which this method might be utilized. An extensive computer analysis of the behaviors that comprise the NEL index is currently being undertaken to improve even more its discriminatory power.

While researching the Mg deficient rat as a model of epilepsy (Part IV), it was noted that older subjects did not display increased NEL if they were fed the low Mg diet for long periods. Indeed, if anything, NEL decreased. The present conjecture posits that it is the length of time rats are fed the low Mg diet, not age, that is the important cause. This needs to be confirmed. Apparently some adaptation is occurring. Research presented in Part III implicates serotonin as playing a role in this adaptive process. The serotonin involvement needs to be more firmly implicated and understood.

The exact mechanism by which Mg deficiency leads to hyperexcitability in the rat has yet to be fully understood. Our research has determined that it is not caused by decreased (Na$^+$ K$^+$)-ATPase (Part II), although a decrease in the supply of energy needed to run this cation pump is still a possibility. Investigations using intraventricular ATP injections should yield useful information, provided that extracellular administration is effectual.
The neurotransmitter, acetylcholine, is a prime suspect for involvement in the hyperexcitability phenomenon. Release of this excitatory transmitter has been shown to increase as Mg concentration decreases (66, 74). Unfortunately, acetylcholine data are not available on the Mg deficient mammal. Research in this area needs desperately to be done, first with whole brain preparations, then with preparations from discrete areas. Concentrations of other neurotransmitters in the brain of the Mg deficient rat need also to be determined in discrete areas. Turnover rates and binding studies would additionally clarify the situation.

Preliminary work only has been undertaken to examine the Mg-deficient rat as a model of epilepsy (Part IV). Length of time rats are fed a low Mg diet, age, sex, NEL and audiogenic seizure susceptibility relationships need to be delineated more precisely. The effects of other drugs on the model should also be considered before its total usefulness can be reported.

Finally, there is more that can be researched with respect to computer modelling of enzyme function. Although beginning steps have been taken (Part V, Appendix B), this area is wide open. More can be done fully to characterize (Na⁺ K⁺)-ATPase under a variety of ionic conditions. The activities of other enzyme systems, especially the more complex, may adapt themselves in a practical sense to mathematical modelling.


APPENDIX A

RESULTS OF AN ADDITIONAL EXPERIMENT COMPARING BRAIN (Na\(^+\) K\(^+\))-ATPase ACTIVITY OF MAGNESIUM DEFICIENT AND CONTROL RATS
During investigations of the relationships between Mg deficiency and hyperexcitability in the young rat, it was felt that non-developmental factors might affect (Na\(^+\) K\(^+\))-ATPase activity. In order to minimize the developmental factors, brain (Na\(^+\) K\(^+\))-ATPase activity was determined in more mature rats than used in the experiments reported in Part II, at a time when the brain is nearly fully developed.

Twenty-four weanling Sprague-Dawley male rats, weighing 50-60 g upon arrival in the laboratory, were fed a control diet containing 400 mg Mg per kg for eight days. At the end of this period they were assigned to two groups of twelve. One group was fed a low Mg test diet containing less than 40 mg Mg per kg. The other group was fed the control diet. Both were fed ad libitum. Fourteen days after initiating test feeding, one half of each group was terminated. Twenty-eight days after initiating feeding, the other half was terminated. Protein determinations and (Na\(^+\) K\(^+\))-ATPase assays were conducted on whole brain homogenates as described in Part II. Results are presented in the Table.

There were no significant (P < 0.05) dietary effects on brain protein or (Na\(^+\) K\(^+\))-ATPase activity in brains of older rats, although an effect was observed in the brains of younger animals (Part II). It does not appear that factors unassociated with the developing brain are instrumental in affecting (Na\(^+\) K\(^+\))-ATPase activity in the rat.
Table 1. Effect of Mg Deficiency on \((Na^+ K^+)\)-ATPase Activity in Brains of Older Rats Than Reported in Part II.

<table>
<thead>
<tr>
<th>Days Fed Test Diet</th>
<th>Diet</th>
<th>Body Wt (9)</th>
<th>Brain Wt (9)</th>
<th>Brain Protein (% wet wt)</th>
<th>((Na^+ K^+))-ATPase Activity (ug Phosphate released per min per mg tissue protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Low Mg</td>
<td>102±15</td>
<td>1.65±0.05</td>
<td>10.6±0.2</td>
<td>0.183±0.012</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>126±31</td>
<td>1.72±0.05</td>
<td>10.8±0.2</td>
<td>0.174±0.011</td>
</tr>
<tr>
<td>28</td>
<td>Low Mg</td>
<td>106±10</td>
<td>1.65±0.13</td>
<td>11.2±0.8</td>
<td>0.188±0.018</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>162±4</td>
<td>1.68±0.08</td>
<td>11.1±0.3</td>
<td>0.184±0.015</td>
</tr>
</tbody>
</table>
APPENDIX B

ADJUSTED CATION EFFECTS ON (Na\(^+\) K\(^+\))\(-ATPase

ACTIVITY IN RAT BRAIN
The data, from which equations expressing \((\text{Na}^+ \text{ K}^+)\)-ATPase activity responses to varying concentrations of \(\text{Na}^+\), \(\text{K}^+\), \(\text{Mg}^{++}\) and \(\text{Ca}^{++}\) derived in Part V, were corrected for day to day variation by multiple regression. Plots of the data are presented in the Figure. These plots show how enzyme activity changes as the concentration of one mineral at a time varies. It must be understood that the concentrations of all other minerals contained in the reaction media are fixed as specified (Part V, Table 1).

Plots of the unadjusted data are shown in Part V, Figures 1-4. The mean corrected \((\text{Na}^+ \text{ K}^+)\)-ATPase activity was 0.184 µmoles phosphate released per min. The average deviation of actual values from the regression was 0.018 µmoles per min, and the overall \(r^2\) was 0.90. Approximate literature values for cerebrospinal fluid (CSF) and whole brain (BR) concentrations are indicated by arrows.

These plots were placed in the Appendix because little additional information is provided which is not given in Part V, although there may be some advantage to viewing all plots concurrently.

A future study might consider the effects of varying each mineral at each concentration of all the other minerals. A general equation might then be developed which could predict \((\text{Na}^+ \text{ K}^+)\)-ATPase activity knowing the concentrations of the minerals in the reaction media, whatever they might be.
Figure 1. Plots of Corrected (Na⁺ K⁺) - ATPase Activity.
PUBLICATIONS AND MANUSCRIPTS IN PRESS


ASPIRATION:

A career in nutrition and related disciplines with teaching and research opportunities.

EDUCATION:

Ph.D. from Utah State University. 3.7 G.P.A. Courses have included biochemistry, physiology, neurophysiology, endocrinology, psychology, research methods, computer programming and advanced statistics, as well as requisite courses in human nutrition. Employed continually as a research assistant while engaged in the program.

M.S., Family Relationships (1974), from Brigham Young University.

B.A., History (1963), from Yale University including 24 semester hours of chemistry.

EXPERIENCE AND PREPARATION FOR TEACHING:

Appointed as Assistant Professor of Home Economics at the California State University, Chico. Responsibilities include teaching human nutrition, advising, and developing new courses and programs.

Taught a graduate level class on protein, carbohydrate, lipid and energy metabolism, and organized a nutrition laboratory techniques course at Utah State University. Substitute taught other nutrition classes. Taught three courses at Brigham Young University (Sept. 1969 - Jan. 1970) and worked as a driving instructor for Mortlock's Driving School (Vernon, Conn., 1968).

Taught and provided motivational training for college dropouts as part of Success Motivation Institute's Zoom program (Provo, Spring 1970). Taught as a missionary in Hong Kong and in the Republic of the Philippines (April 1965 - Nov. 1967).
Preparation for teaching has included teacher training courses and an educational tests and measurements course which stressed the importance of organization, setting objectives and effective evaluation.

PUBLICATIONS AND PROFESSIONAL PRESENTATIONS:

A list of publications and manuscripts in press, and papers presented at meetings precedes the resume.

LEADERSHIP AND HUMAN RELATIONSHIPS:

As president of the Bridgerland Toastmasters Club (1976), responsible for directing the club's public speaking and leadership training activities.

As Assistant to the Mission President of the Church of Jesus Christ of Latter-day Saints in Hong Kong (Oct. 1966 - Apr. 1967) responsible for motivating, teaching and coordinating the efforts of missionaries in the area. Supervised clerical activities of Church members from Taiwan to West Pakistan. Established a new branch in Bacolod City (Philippines, Summer 1967). Directed and expanded several programs resulting in improved quality of life for members.

Appointed Platoon Leader of U.S. Army basic training company (1964).

Captained the Yale Freshman Crew (1960).

AWARDS:

Received the Lea D. Widtsoe Scholarship Award presented to a graduate student at Utah State University (1975).

Selected by U.S. Army basic training unit as the company's "Outstanding Trainee" (1964).

Chosen from among all Yale Oarsmen in 1963 for "Outstanding Oarsmanship and Team Spirit".

Granted Raycroft Walsh Scholarship which paid all tuition and some expenses for 2½ years at Yale.

Received English Speaking Union Schoolboy Fellowship for one year at a British boarding school (1958 - 1959).

Awarded five varsity letters from high school and two varsity letters from Yale.
ACTIVITIES AND SPORTS:

At Logan and Utah State University: Bridgerland Toastmasters Club, Family Life Council, Phi Upsilon Omicron and Sigma Xi (Assoc. Mem.).

At Yale University: Varsity crew, Freshman Glee Club, Phi Gamma Delta Fraternity.

In high school: Varsity track, varsity crew, varsity rifle, football, rugby, band, choir, glee club and math club (Pres.).

AVOCATIONS AND INTERESTS:

Nutrition and behavior, energy conservation, carpentry, backpacking, systems and methods analysis.