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BEHAVIORAL AND BIOCHEMICAL METHODS

TO STUDY BRAIN RESPONSES TO ENVIRONMENT AND EXPERIENCE

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X.1 INTRODUCTION

Exposing laboratory rats to one or another laboratory environment was found in the early 1950's to affect their problem-solving ability (e.g. Forgays and Forgays, 1952; Hymovitch, 1952), and similar findings were later made with dogs (e.g., Melzack, 1962; Fuller, 1966), cats (Wilson et al., 1965), and monkeys (e.g., Gluck et al., 1973). Then, beginning in the late 1950's, experience in differential environments was discovered to affect numerous aspects of brain biochemistry and neuroanatomy of rodents (e.g., Krech et al., 1960; Rosenzweig et al., 1961; Rosenzweig et al., 1962; Bennett et al., 1964; Ferchmin et al., 1970; Greenough and Volkmar, 1973; Greenough, 1976; Walsh et al., 1969). Bodily growth (Rosenzweig et al., 1972b) and sleep-waking cycles (Tagney, 1973; McGinty, 1971; Lambert and Truong-Ngoc, 1976) were later found to be influenced by laboratory environments. Because not only these but undoubtedly many other aspects of behavior and physiology are modified by environmental experience, it is clear that--at the minimum-- investigators should describe the environments of their subjects in some detail in all research reports. Beyond this, selection of suitable environ- ment(s) for the particular investigation should be undertaken with care. Either the environment in which animals are housed or specific experiences or training given during experimental sessions can be employed as independent variables to modulate many aspects of brain, body, and behavior.

The present chapter will describe some of the environmental and experi- mental manipulations that have been tested, and it will note some of the results that have been found; some of the methods used to assay cerebral effects of experience will also be described and others will be cited. Specifically, this is how the content of the chapter will be apportioned: We will describe in some detail environmental and training situations and

compare their effectiveness in producing significant changes in cerebral measures. We will then mention rather briefly some behavioral tests that have been used to characterize effects of prior experience and describe one procedure in detail. Finally, we will present in considerable detail the methods used to dissect brain samples, to obtain brain weights, and the analytical procedures employed to measure content of RNA and DNA and activities of AChE and ChE. The stablest and most statistically significant effects of environment on the brain that have been reported to date are changes in tissue weights (especially the cortical/subcortical weight ratio) and the RNA/DNA ratio of cortical tissue.

X.2 ENVIRONMENTAL AND TRAINING TECHNIQUES

X.2.1. Differential Environments

X.2.1.1. Basic Description.

Beginning in the late 1950's our laboratory has done a large number of experiments in which we have compared the behavioral and cerebral consequences of assigning rodents for periods of time among three principal environments which we term "enriched condition" (EC), "standard colony" (SC), and "impoverished condition" (IC). The main characteristics of these environmental conditions can be seen in Fig. 1. Many variants of the enriched condition have been tested, as will be described below, but most appear to yield rather similar effects.

In the standard Berkeley EC, 10-12 same-sex animals are housed in a relatively large cage (76 x 76 x 45 cm) which is furnished with several varied stimulus objects. The exact size of the cage is not important nor does it matter whether the floor is a metal grid or is covered with shavings. Variety in stimulation from the objects is secured in either of two ways, and these seem to yield equivalent results: (a) About 6 different objects are placed in the cage each day from a pool of about 25 objects. (b) When N groups are being given EC experience at the same time, different arrangements

of objects are placed in N EC cages, and each group is moved from one cage
to another each day; every Nth day, all cages receive a new arrangement
of objects. The exact nature of the objects does not seem to be important.
They are chosen to induce exploratory behavior and to give the animals a
variety of surfaces, visual forms, tastes, and smells. Objects that we have
commonly used are illustrated in Fig. 2 (Rosenzweig and Bennett, 1969).

Standard Colony (SC) animals are housed in a group of 3 in a usual colony
cage. Our colony cages for rats measure 21 x 34 x 20 cm but any cage that meets
standards for animal care will be adequate.

For the impoverished condition (IC), animals are housed singly in standard
colony cages. In our early work, the IC subjects were housed in cages with
solid walls (as illustrated in Fig. 1), and these were placed in quiet and
dimly illuminated rooms. We later found that extracage stimulation is
of little importance, so in many experiments we have housed the IC animals
in SC cages and on the same cage racks as SC subjects.

All animals in all environmental conditions have food and water ad lib.
Standard laboratory chow is provided as food.

In our earlier experiments EC also included two additional features be-
sides those described above: (a) a few trials of formal training per day,
and (b) daily exploration in a 75 x 75 cm open field apparatus in which
a different pattern of maze barriers was placed each day. Because of the
inclusion of training (in the Krech Hypothesis Apparatus--a multiple unit
maze), we originally termed the enriched condition "environmental complexity
and training" (ECT). In the mid 1960's we tested whether the daily training
trials contributed to the cerebral effects and found that they did not,
so by 1968 (e.g., Rosenzweig et al., 1968) we dropped the training and referred
simply to the enriched condition (EC). We also eliminated the sessions in the

field with barriers in order to test whether the superiority of EC rats over IC rats in solutions of maze problems could be attributed to transfer of training from experience with barriers. The results showed that experience in the EC cage was sufficient to produce both superiority in maze solution and also the cerebral differences with respect to IC animals; in fact, the field experience did not contribute measurably to either the behavioral or the cerebral effects, so we have not included it in experiments done since 1970.

X.2.1.2. Dimensions of Differential Environments

From the foregoing description, and from other environmental conditions that various investigators have tried, it appears that the enriched environment included both social and inanimate stimuli and that the latter can be divided into intracage and extracage stimuli. Some evidence is available as to the effectiveness of each of these sorts of stimulation in producing changes in behavior and in brain, and conclusions will be stated briefly here. (This topic is taken up in some detail and citations are given in Rosenzweig and Bennett, 1977).

Effects of social grouping have been studied by comparing animals housed in SC versus IC environments. In some cases the social group has been enlarged by housing 10-12 animals in an EC cage but without providing any inanimate stimulus objects. (This has been called the Group Condition--GC.) Social grouping versus isolation aids subsequent learning and retention but not as much as does the EC treatment. Social grouping also produces some cerebral effects, but these are significantly smaller than those caused by experience in EC.

Inanimate stimulus objects within the EC cage aid subsequent learning, as can be seen by the superior performance of EC versus GC animals. Larger cerebral effects are also produced by EC than by GC. Even single rats placed in EC cages with stimulus objects can develop brain values similar to those

of EC rats and significantly different from those of IC rats, so social stimulation is not required. It should be noted, however, that single rats tend to be rather inactive and not to explore much, so production of EC cerebral effects in individual rats is facilitated if they are primed to interact with the stimulus objects, as described in the next section.

Extracage stimuli seem to be ineffective in producing either behavioral or cerebral effects in rats. We originally believed that ambient visual and auditory stimuli might be effective, so we placed the EC cages in a busy laboratory room and placed the IC cages in a quiet, dimly lighted room. Later we found that typical cerebral differences between EC and IC rats were produced even when the EC cage was kept in a quiet, dimly lighted room and the IC cages were put in the busy laboratory room. We also found that rats housed in small individual cages within EC cages developed cerebral and behavioral characteristics like those of IC littermates rather than like those of the EC rats (Ferchmin et al., 1975). Some investigators have referred to an environment as "enriched" when rats were housed singly and were provided only extracage stimulation (Yeterian and Wilson, 1976), but we believe that this appellation is misleading.

X.2.1.3. Enriched Experience for Individual Animals

X.2.1.3a. Individual Rats in EC.

In order to test effects of enriched experience that does not include the component of social stimulation, several conditions have been employed. The simplest has been just to place an individual in each EC cage. This was found not to work very well because individual laboratory rats tend to be rather inactive, remaining in a corner of the cage much of the time, resting or grooming. We found that the activity of each animal increases considerably when other animals are placed in the in the cage with it (Rosenzweig, 1971). We therefore undertook various methods

.to "prime" the activity of animals placed individually in EC. A method
found to be effective with young rats placed into the experimental condition
at weaning (about 25 days of age) or shortly thereafter was to give a small
injection of methamphetamine (2 mg/kg) shortly before placing the animal
into EC for a -hr period (Rosenzweig and Bennett, 1972). After an injection
of the drug the individual animal interacts vigorously with the varied
stimulus objects, and during a 2-week or 4-week experiment it develops brain
measures similar to those of the rat placed in EC in a group of 10 or 12.
The methamphetamine is even more effective if given to rats during the dark
phase of their daily cycle. This method cannot be used to prime the activity
of older rats, because even low doses of methamphetamine induce stereotyped
and abnormal behavior in adult rats.

X.2.1.3 b. Individual Maze Training. Individual trials in mazes is
another effective way of giving animals experience. Based on earlier experi-
ments, we were doubtful about finding clear cerebral effects of maze training
as compared with runway controls (Rosenzweig. 1971, pp. 335-336 but we later
obtained significant effects (Bennett, 1976, p. 284), and Greenough (1976)
has also reported effects of maze experience on brain measures.

Recently we have been studying central effects of self-paced maze trials
(Bennett et al., in press). In order to get rats to run self-paced trials,
we separated their sources of food and water so that the animal had to
traverse the maze to get from one to the other. The maze was a box made
of Plexiglas and measured 10 cm high x 74 cm wide x 74 cm deep; it was placed
within an EC cage on flanges that supported it 15 cm above the cage floor
(Fig. 3). Holes 7 cm in diameter were placed at the four corners of
the bottom and top of the plastic box, so that the rats could crawl in and out
of it, and any of these holes could be closed with a plastic door when desired.
Plastic barriers could be placed within the box to provide a variety of
maze patterns. Food pellets were made available, as usual, on the floor
of the cage, but the water bottle was placed above the plastic box so that

in order to get from food to water the rat had to climb into the plastic

box at an open corner in the bottom, traverse the box to an open corner

at the top, climb out of the box and stand on its top to reach the spout of

the water bottle. Rats that eat dry food pellets like to drink frequently,

so that they had to run up and down a number of times during each bout of

feeding; the rats were rarely able to carry a food pellet up into the maze and

never above it to the water station. In the experimental situation, the maze

pattern was changed daily for 30 days. In a control condition, no barriers were

placed in the plastic box, so that the animal had only to traverse the empty

box and did not have to learn maze patterns.

Results have shown that the "empty box" controls did not develop brain

values different from those of littermate IC rats, whereas the animals that

learned maze patterns differed significantly from IC and "empty box" litter-

mate controls. The maze-learning individuals developed brain values in

the direction of EC rats but somewhat below the EC level; the brain values

of the maze rats were essentially the same as those of littermates placed in the

Group Condition, that is, placed in a group of 12 in a large cage but with-

out access to varied stimulus objects. Thus the maze situation yields

significant cerebral effects without necessity of social stimulation and

in an inanimate situation that is considerable simpler and easier to describe

than that of the Enriched Condition.

X.2.1.4. Superenriched Environments

In the last few years some investigators have gone beyond the EC situation

in order to provide animals with even more enriched experience. The methods

used to enhance enrichment have included the following: (a) Placing rats

in succession in cages of a variety of sizes and shapes and placing naive

rats with experienced "guides" (Ferchmin et al., 1970). (b) Placing a large

number of rats in two or three interlinked cages (Kuenzle and Knüsel, 1974;

Davenport, 1976; Rosenzweig et al, in press). (c) Placing rats in a large
outdoor enclosure with a dirt floor (Rosenzweig et al., 1972a;Bennett, 1976;
Rosenzweig et al,in press). Each of these methods will be described in
some detail, and results will be noted.

X2.1.4a. Ferchmin EC. Ferchmin et al., (1970) working in Argentina, reported
that only four days in their enriched conditions sufficed to induce signi-
ficant increases in weight of the cerebrum and changes in cerebral RNA and DNA.
The environmental conditions were not spelled out in great detail in their
report, but we were fortunate to have Drs. Ferchmin and Eterović in our
laboratories during 1971-1974, and in discussion it became apparent that their
enriched conditions were considerably more complex than our EC. They rotated
rats twice a day among four cages of different sizes, two of the cages being
considerable larger than ours and offering more opportunities for climbing
and exploration. Also, since they had observed that the rats in their
laboratory in Argentina seemed rather timid and slow to venture into the
complex environment, they provided the naive rats with more experienced "guides"
who had already been in the environment for several days; in fact, the ex-
perimenters sometimes kept up a continuing production of enriched-experience
rats in which the originally naive rats then served as guides to a new
group before being removed for brain analyses.

We set up conditions similar to those of the Argentinian laboratory
and compared cerebral effects of Ferchmin EC (FEC) with those of our regular
EC and IC conditions, using subjects of the Berkeley S_1 strain of rats.
Results for weights of brain sections were reported by Bennett (1976,
Table 17.3) for several experiments ranging in duration from 4 to 15 days.
Both FEC and EC produced significant differences from IC in as little as
4 days; for occipital cortex, FEC vs IC caused a difference of 5.2%
(P < .001), EC vs IC, 6.6% (P < .001); for total cortex, FEC vs IC, 2.6%

(P <.001), EC vs IC, 2.2% (P <.001). The only apparent advantage of FEC over EC was seen in the rapidity of occurrence of differences in the ratio of weight of cortex to rest of brain. On this measure after 4 days, FEC vs IC yielded a difference of 1.7% (P <.001) whereas EC vs IC did not yet show a difference (0.2%, NS); after 8 days the effects reached 2.6% (P <.001) and 1.7% (P <.05), respectively, but by 12 days the two environments yielded comparable effects on the cortical/subcortical ratio--2.6% and 2.3% respectively, both significant at beyond the .01 level of confidence. Thus the more complex FEC environment was not more effective than the standard EC in altering most brain weight measures.

X.2.1.4b. Large Social Group in Interlinked Cages. Kuenzle and Knüsel (1974) designed a "superenriched environment" in which a group of 70 rats lived in two large interconnected cages and had to shuttle back and forth across a bridge with changing gates and signals in order to find food and water. Over the 29-day experimental period, the rats were given successively more complicated problems to solve, and they had to perform atheletic feats in order to survive. In two replications, groups were run simultaneously in the superenriched environment and in a reproduction of the Berkeley EC situation. The rats in the superenriched environment were reported to surpass the rats in EC in weight of occipital cortex, in length of cerebral hemispheres, in ChE/AChE in occipital cortex, but not in RNA/DNA in occipital cortex. While these results are encouraging in showing even larger brain effects than had been found heretofore, it is clear that this experiment confounds the factors of social and inanimate enrichment, since the superenriched environment contained more stimulation along both dimensions than does the EC condition. Also, the stress on agility in the superenriched environment renders questionable the assertion of Kuenzel and Knüsel that the superenriched condition "is superior to the original [EC] in that it

confronts the animal with true learning situations," whereas "the original
setup mostly improves the animal's motor performance but does not provide
for genuine learning situations."

Davenport (1976) first used the EC treatment and later a modified version
of the Kuenzle and Knüsel superenriched environment in experiments on environ-
mental therapy for neonatally hypothyroid rats. In an initial study in 1972
Davenport et al.,1976), the EC treatment was effective in helping to overcome
behavioral deficits in hypothyroid rats. A further study in 1973 did not, however,
yield these positive results, (Davenport, 1976).   Therefore in later work
Davenport used a version of the superenriched environment in which 45 rats were
housed in an apparatus constructed out of three large cages interconnected by
four tunnels with coded gates; the middle cage contained various objects to be
explored. Davenport found the superenriched environment to be highly effec-
tive in counteracting the deficiencies caused by experimental cretinism.
He did not, however, conduct a direct simultaneous comparison of the relative
effectiveness of the standard Berkeley EC vs the superenriched condition.

We have employed a version of the superenriched environment in our
own laboratory; it was similar to that devised by Davenport. For this purpose,
we placed three of our regular EC cages side by side on a table, spacing
them 12 cm apart; two tunnels made out of hardware cloth connected each
pair of cages. The tunnels were 8 cm square in cross-section; a swinging door
could be placed in the middle of each tunnel, and the door could be set to swing
in one direction only. On most days food was placed in one of the end cages and
water in the cage at the opposite end, so that the animals had to run back
and forth between the cages to obtain food and water. Varied stimulus objects
were placed in each of the cages. In our laboratory, the superenriched
condition did not produce larger cerebral effects than were found in an
EC group that was run simultaneously (Rosenzweig et al., in press).

X.2.1.4c. Seminatural Outdoor Environment. In a series of experiments we placed laboratory rats in a seminatural outdoor environment for 30 days and compared the cerebral effects with those found in littermates placed in EC or IC at the same time. The seminatural environment (SNE) was established in an outdoor "population pit" at the Field Station for Research in Animal Behavior above the Berkeley campus. The population pit is a 9 x 9 m concrete rectangle with a wire mesh roof; earth was placed on the floor to a depth of about 30 cm. Some stones, branches, and pieces of wood lay on the surface of the dirt, and weeds grew in it. Four stations for food and water were placed in the pit, and food and water was available at at least one of the stations each day. For each experiment, 12 animals were placed together in SNE.

We found that laboratory rats can thrive in an outdoor enclosure even during a wet winter when the temperature drops to the freezing point. When the ground was not too wet, the rats dug extensive burrows, something that their ancestors had no opportunity to do in the laboratory for over 100 generations. Even the enriched laboratory environment does not permit this kind of activity.

SNE was found to produce cerebral effects that were similar in their pattern of distribution over brain regions, but significantly larger in magnitude than those caused by EC. Some brain weight data from these experiments were reported by Bennett (1976, Table 17.4). More extensive brain weight data and also measures of RNA/DNA and AChE are given by Rosenzweig et al. (in press, Tables IV and V). In one set of three experiments with N = 35 per condition, SNE rats exceeded IC littermates by 11.4% (p <.001) in weight of occipital cortex and by 7.5% (p <.001) in the cortical/subcortical weight ratio, while the corresponding percentages for EC vs IC littermates were 8.5 (p <.001) and 5.2 (p <.001).

X.2.1.5. Assignment of Animals to Conditions

In order to be able to make critical comparisons between animals that have been assigned to the differential environmental conditions, it is necessary to follow certain procedures in the assignment. The groups assigned to the conditions should be rather closely equivalent, but in order not to bias the outcome and to be able to make statistical tests of differences, each animal should have an equal chance of being placed in any of the conditions. To reduce variability and also the possible loss of animals during the course of an experiment, all runts or ill animals are excluded at the start. Insofar as possible, we take litters with at least as many littermates of the desired sex as there are experimental conditions, so that we can make littermate comparisons. As a further restriction on variability, we take only litters within which the range of body weights does not exceed 15%. The littermates are then assigned randomly so that each one goes to a different experimental condition. In other cases when littermates are not available, animals are weight-matched, and a weight-matched group is then treated like a litter.

Various further measures can be taken to restrict variability among subjects to be assigned to differential envorinments. Here are a few methods that may be considered for this purpose: One is the use of highly inbred strains of rats. We have used the Fischer inbred strain for this reason, but we did not find them to be less variable in brain weights or brain chemistry than rats of our S_1 breeding stock. Culling litters down to a standard number of pups (e.g. 4) provides larger and more uniform pups at weaning than does allowing the mothers to suckle numbers that range from 4 to 12 or more. Since mothers differ in their ability to care for young, Herman Epstein of Brandeis University rotates mothers daily among four litters to produce pups that are as uniform as possible (personal communication). It is also possible to take

the differences between litters into account by appropriate statistical techniques.

Animals of only a single sex are used in a given experiment for three reasons. First, since there are sex differences among rats in brain size and weight and in some measures of problem solving behavior, the results must be analyzed separately for the two sexes. Secondly, including both sexes in group conditions can lead to territorial and aggressive behavior and, if the experiment lasts more than a few weeks, to the birth of young. These results of mixing the sexes may make conditions more "natural", but they greatly complicate the conditions. Also, pregnancy may alter brain values of rats (Diamond, et al., 1971; Hamilton, et al., 1977). Finally, female rats show smaller cerebral effects of environmental restriction than do males (Rosenzweig and Bennett, 1977), and female monkeys show less severe behavioral effects of isolation than do males (Sackett, 1972). Thus, while it has been worthwhile to investigate the effects of environment in each sex, it has been more convenient to perform parallel experiments with the sexes kept separate.

X.2.1.6. The Question of a Baseline Environment

As we have noted in a number of discussions (e.g., Rosenzweig, 1971), we use the terms "enriched environment" and "impoverished environment" only in relation to the baseline of the standard laboratory colony condition. This is a convenient baseline for many purposes, since it is the standard for much research in animal laboratories as well as in laboratories of biochemistry, pharmacology, nutrition, etc. It has been pointed out that even the "enriched condition" in the laboratory is undoubtedly impoverished compared to what rodents experience in a natural outdoor environment. As noted in section 2.1.4c, we have found that housing a group of 12 rodents in a 9 m x 9 m outdoor enclosure leads to somewhat greater development of brain values than does our indoor EC condition (Bennett, 1976; Rosenzweig, et al, in press).

But even this outdoor exposure is not a completely "normal" condition. A full comparison of the physiological and behavioral consequences of truly "normal" and laboratory conditions is difficult to undertake. Lessac and Solomon (1969) commented on this problem in the following way when describing their experiment on learning in "normally" reared and isolation-reared beagles:

> The definition of "normal" rearing is a difficult one. In a laboratory there is no "wild" environment, and so the ethologists stress how "abnormal" the laboratory is. But in the "wild" there are no psychologists teaching animals complex, abstract concepts! There seems to be no alternative, at the moment, to the purely arbitrary definition of normal rearing for each experiment...

Granted that environment is an important variable and that any choice of environment brings some benefits and some liabilities, how should environments be chosen to study many aspects of brain and of behavior? We suggest that for laboratory rodents an enriched environment of the sort described in section 2.1.1. and illustrated in Fig. 1 is suitable for many purposes. It is practical to set up and to maintain, and it assures more complete development of both brain and behavior than occur in restricted or standard colony environments. The trend toward automation of care in colonies that afford a minimum of stimulation runs counter to our recommendation and seems likely to produce inferior subjects for research. Whatever environment is employed in a given study, a full description of the environment and the handling should be a required part of each publication.

X.3 BEHAVIORAL TECHNIQUES TO TEST EFFECTS OF PRIOR EXPERIENCE

As we mentioned in the Introduction, it was found in the 1950s that exposing rats to an enriched environment led to a better subsequent learning of a variety of tasks than did experience in an impoverished environment. Davenport

(1976) has compiled reports of such experiments; he lists 32 papers giving
positive results and 14 findings of no significant enhancement of learning in
enriched-experience rats (see his Table 2, pp. 94-5). The positive results
came mainly from studies employing relatively complex maze tests (e.g. Hebb-
Williams maze, Lashley III maze), whereas lack of effect was mainly reported
from simpler maze tests (e.g. multiple T-maze, Y-maze) or from tests of sensory
discrimination, but this differentiation is not absolute. Even simpler situations,
such an observation of behavior of an animal in an "open field" (e.g. an
empty circular enclosure 1 m in diameter), have revealed differences between rats
or mice from differential environments (e.g. Woods, et al., 1960; Henderson,
1969). Since many investigators are now using operant or classical conditioning
procedures for refined analysis of behavior, it is regrettable that (with
the single exception of Ough et al., 1972) these techniques have not been employed
to characterize the effects of enriched or impoverished experience.

Description of procedures used in behavioral tests of prior experience would
go beyond the scope of this chapter. Even the procedures employed in the
apparently simple open field test turn out to incorporate many variables, and a
recent review of research with this instrument shows that seemingly inconsequential
details of procedure can alter the behavior significantly (Walsh and Cummins,
1976). Some useful sources on formal observation and experimental testing
of behavior of laboratory rodents are the following: Bureš et al., 1976; Munn,
1950.

Perhaps a few additional comments about behavioral procedures may not
be amiss for investigators from other disciplines who may decide to incorporate
behavioral tests in their programs of investigation:

1) As is true of other fields, published reports of behavioral research
are not exhaustive and they assume a background of knowledge and familiarity
on the part of the reader. As we will note later under biochemical analyses,

certain chemical procedures require exacting standards of cleaning glassware, while others do not, and we find analogies in the study of rodent behavior. In the case of a male rat placed in a maze for pretraining, the odor of a female rat that has traversed the area previously will be distracting--the male will try to locate the female. On the other hand, the presence of odor from other males of the same colony appears to be reassuring; a rat will begin to explore a maze more readily if it has been "ratted up" than if it is absolutely clean. Male mice, unlike male rats, are not distracted by the previous presence of females in the apparatus. Because of these characteristics of rats, we usually run experiments with only a single sex at a time.

2) Appropriate control of motivation of animal subjects during training and testing is of extreme importance. As Tolman demonstrated in the 1930s, a rat can know the shortest path through a maze but not run that path unless it is suitably motivated, e.g. by a food reward; this phenomenon is termed "latent learning"). Furthermore, there may be competing motivations, such as the exploratory tendency in rats, that complicate the outcome.

3) Behavioral aspects of research on brain and behavior are at least as complicated as the cerebral aspects, and a great deal of progress has been made in laboratory studies of animal learning since Thorndike initiated them in 1898. It would be a waste of time and effort if neuroanatomists, neurochemists and other biological scientists had to rediscover the same knowledge on their own and reinvent testing devices and techniques. For the present, the most promising approach seems to be interdisciplinary research in which investigators trained in behavioral sciences collaborate with investigators from the biological and chemical disciplines. Eventually there may be scientists who can encompass the essential disciplines within a single skull.

X.3.1. The Hebb-Williams Maze

The test that has most often been used to investigate behavioral differences as a consequence of previous enriched or impoverished experience is the Hebb-

Williams (H-W) maze. It has also been used to study effects of post-lesion enriched or impoverished experience on problem-solving behavior (e.g. Schwartz 1964; Will et al., 1976, 1977). We will describe here our own procedures with this test which differ somewhat from those given in the original description by Rabinovitch and Rosvold (1951). The following description is adapted from that prepared for technicians and students in our laboratory.

The Hebb-Williams maze test of problem-solving ability consists of a standard series of maze problems. These are set up in by means of wooden barriers in a field 76 cm square and 9.8 cm high with a start box extending from one corner and a goal box extending from an opposite corner (See Fig. 4A). The apparatus is covered with a Plexiglas lid. The rats run 8 trials per day for food reward; a different problem is presented on each day.

Phase 1: Pretraining

Since rats without prior experience may not run at all, or may behave erratically when placed in a maze, several days of pretraining preceed the series of actual test problems. During pretraining rats are:

(1) deprived of food (except for the time they spend in the goal box) and their weights brought down to 80% of predeprivation weight;

(2) introduced to eating a special kind of food (a mash made of ground-up rat chow mixed with water) in a strange situation (the goal box of the maze);

(3) trained to run down a straight runway to find food in the goal box (GB) at the end. (This also furnishes data on effects of prior experience on performance in a straightaway, a task that has been used frequently in other contexts);

(4) introduced to several simple maze problems.

At the end of pretraining most rats have reached a body weight at which they are optimally motivated and have learned to run through a maze to find food. They are now ready for the standard series of test problems.

Phase 2: Testing

Rats are tested on a new problem each day and are given eight successive trials for each problem: The rat is placed in the start box (SB), run through the maze to the GB, and allowed 30 seconds to eat, and the procedure is repeated until the rat has run through the maze eight times. The rat is then allowed extra time (about 5 minutes) to eat in a supplementary GB so it can get enough food to maintain its body weight over the next 24 hours of deprivation.

For each rat, body weight at the start and end of each day's testing is recorded. This way the rat's weight can be monitored to make sure it stays close to 80% of its predeprivation weight; too much above this, the rat may not be motivated to run; to much below, it may be in poor health and even die. In addition, on each of the eight trials, the experimenter:

(1) records how long it takes the rat to come out of SB after the door is opened (SB time);

(2) draws on the record sheet the path the rat takes through the maze and count any errors it makes,

(3) records how long it takes the rat to reach GB after leaving SB (running time).

The times and error scores are recorded first on a scoring sheet, then transferred to a face sheet which serves as a cumulative record of a single rat's performance throughout pretraining and testing.

On the score sheet for each problem eight identical maze diagrams like the one shown in Fig., 4B are reproduced . The wooden barriers that define the problem are indicated by heavy solid lines. The hatched lines mark the Error Zones. (These error zone lines are not present in the maze but are

only on our scoring sheets.) For each problem there is one most direct path from SB to GB. If the rat deviates significantly from this path it will cross one (or more) of the hatched lines. Each entry over such a line by the head and two front feet counts as an error.

The details of scoring start box time, errors, and running time are explained below. All other details of the procedure are best learned in practice.

How to Record Errors and Time in the Hebb-Williams Maze

This is simple in principle, but there are a few special features to note.

Initial errors. The first entry of a rat into an error zone on a given trial is an initial error. Therefore initial errors for a trial equal the number of different error zones that the rat entered. Repetitive errors. Any entry after the first into the same error zone on a given trial is a repetitive error. On most trials a rat will make few errors and one will have no difficulty in recording them. But occasionally a rat will make a large number of errors, and this can make recording difficult. From our experience, we have found that the following method works very well even when a rat makes so many errors that the tracing of its path is extremely complicated, so we recommend this procedure to you: As the rat runs through the maze, trace its path on the score test sheet and keep a running count of all errors that it makes, without distinguishing between initial and repetitive errors. When the rat reaches the goal box, that total is recorded. Now check to see how many error lines the rat crossed. This gives you the number of initial errors. Subtract initial errors from the total to obtain the number of repetitive errors.

Time measures.

Start Box (SB) time is the time the rat takes to emerge from the start box after the start box door has been opened. The stopwatch is started when the start box door is opened. As soon as the rat emerges into the apparatus

and the door behind it is closed, SB time should be recorded. Continue timing with the stopwatch until the rat enters the goal box, then subtract SB time from total time to obtain running time (R) and record R on the sheet.

Running Time (R) starts when the rat has left the start box and the door behind it can be closed. Running time ends when the rat has entered the goal box (GB) and the GB door can be closed.

Retracing. Sometimes a rat will enter the square just before the goal box and almost go into the GB, then stop, turn away and explore the maze further. When this happens, time taken to reach the GB initially is recorded as total time. The stopwatch is permitted to continue running to measure retracing time, that is, cumulative time minus the initial run.

Errors during retracing are tabulated separately. Typically rats don't make many errors while retracing, but it will be helpful to score all the errors a rat makes on the way to GB. Then, if the rat starts to retrace, jot down the total errors made to the initial contact with GB is recorded. Start a new series of initial and repetitive errors for the retracing.

Exploration. Sometimes on later trials after a rat has already learned a pattern well, it will slow down on a particular trial and start to explore. When this happens, write a big dot (0) beside both errors and total time. This is not done on early trials when most rats explore--exploration is recorded when a rat's performance on the problem has reached a high level and then the rat slows down and starts to nose around.

Detailed Schedule

Pretraining Procedures for Hebb-Williams Maze, using Pretraining Alley

Day 1. Weigh each rat and put it in a clean individual colony cage with water but no food.

Day 2. Place a dish of mash in GB. Weigh rat and put it by itself in GB with door closed for 15 min. Weigh it again after taking it out.

Starting this day and continuing through the experiment, weigh each rat just before putting into apparatus and just after removing it, and record these weights on the rat's record sheet.

Day 3. Same as Day 2.

Day 4. Same as Day 2 except only 10 min in GB.

Day 5. Place diagonal pretraining alley into the H-W apparatus. Open door to GB. Weigh rat and put it into SB. Open SB door and start stopwatch. When rat emerges from SB into pretraining alley, close SB door gently behind rat and record time to nearest second. This is SB time. When rat enters GB, close GB door gently and record time in alley. This is R (running) time. Give rat 30 sec in GB, then remove it, place it in SB, open door to GB and then to SB, and time both emergence from SB and alley running during each trial. Allow rat a maximum of 5 runs in 15 min total in the apparatus. The last 5 min of the 15 is to be allowed for eating; 30 sec of this can be in the GB of the apparatus, and then the rat can be moved to an extra GB for the remaining 4½ min. If the rat runs rapidly, it will not need all 15 minutes in the apparatus.

Some rats will not emerge readily from SB. It is best to give them a fair amount of time to emerge on their own, so follow this schedule: if rat has not left SB after 5 min, then guide it gently from SB into alley and close SB door behind it. If rat has not entered GB after 10 min in apparatus, guide it gently into GB and close GB door behind it. Record if the rat is guided.

Day 6. Same as Day 5.

Day 7. Remove diagonal alley from apparatus and set up barrier pattern D in maze. Errors are to be recorded from this day on. Detailed procedures are as follows.

1. Set up barrier pattern D in maze.

2. Open GB door; close SB door.

3. Weigh rat and record on face sheet.

4. Place rat in SB; open SB door when rat is facing door and start stopwatch.

5. After rat moves into field close SB door gently, record SB time, and keep watch running for R time.

6. Record path of run on maze form and mark a stroke for each error.

7. When rat reaches food cup in GB, close GB door gently and stop watch.

8. After 30 sec in GB remove rat to SB to begin next trial. Record path, errors, and times for each trial.

Allow rat as many trials (maximum 8) as it can run in a total of 10 min from time it is first placed in SB. At end of last trial allow rat 5 min in GB; thus total time in apparatus may reach 15 min. Maximum time in GB will be 9 min.

From this day on, the final period in GB can be adjusted to keep rat's weight between 80 and 85% of pretraining value. If weight at start of day is below 80%, increase final GB time by 1 min; if weight exceeds 85%, decrease final GB time by 1 min. Greater adjustments can be made but it is best not to change feeding time more than 1 min from preceding day.

Day 8. Same as day 7; give maximum of 8 trials with 5 min in GB after final trial. Thus maximum time in GB is 8.5 min. Use pattern E.

Day 9. Use pattern F.   Same as Day 7, maximum of 8 trials with only 5 min in GB after last trial.

Day 10. Use pattern G.   _   Give rat 8 trials with 30 sec in GB after trials 1-7 and 4 min after trial 8, total 8 min in GB.

Day 11. Use pattern H; otherwise same as Day 10. The rat has now completed pretraining and is ready to run the standard maze patterns.

Day 12. Pattern 1. Same as Day 10.

Days 13-21. Procedure the same, for problems 2-5, 7-11. (We have found that a series of 10 problems is sufficient to differentiate groups of rats, so we have shortened the series from 12 to 10 in interests of economy. In addition, it should be noted that problem 12 of the standard series does not correlate well with results of the other problems and does not reliably separate groups on the basis of previous experience.)

X.4 BIOCHEMICAL TECHNIQUES

X.4.1. General Considerations

One of the goals of the environmental manipulations and training that
have been described in the previous sections has been to determine if measure-
able differences in cerebral anatomy and biochemistry would result. At the
initiation of this research, it was assumed (and it has been subsequently
confirmed) that the differences produced by environmental manipulations would
be relatively small. Therefore, the analytical techniques that have been
chosen and developed are ones useful for processing relatively large number
of samples in a standardized fashion and to high degrees of accuracy and
reproducibility. Techniques for four biochemical measures are described.
These have been chosen from a large repertoire of measures that have been
used on the basis of their being reliable, sensitive, convenient, and relatively
easy indicators of prior environmental influences. These measures are (1) RNA
content, (2) DNA content, (3) acetylcholinesterase (AChE) activity, and (4)
cholinesterase (ChE) activity.

The brain weight measures are compatible with and indeed represent a
necessary first step in the measurement of either AChE and ChE activities
or RNA and DNA content. Anatomical measures that have been used to test effects
of differential experience on brain but that will not be described here include
cortical thickness (Diamond et al., / 1966,1967), counts of neurons and glia (Diamond
et al., 1964, 1966), dendritic spine counts (Globus et al, 1973), dendritic branching
(Greenough and Volkmar, 1973), and electron microscopic measurement of synapses
(West and Greenough, 1972; Diamond et al., 1975).

X.4.2. Weights of Brain Regions

It has been found that environmental manipulations produce responses
that differ among brain regions, and the most sensitive direct measures include
weight of occipital cortex and weight of total cortex. The ratio of cortex to sub-

cortex weight is an even more stable index and is the most reliable weight measure to use to detect the gross anatomical effects of environmental manipulations (Rosenzweig et al., 1972b). Total brain weight is relatively independent of overall body growth after weaning and has not been found to be a sensitive indicator of environmental manipulations, other than ones which would be classified as extreme (such as prenatal or infantile malnutrition).

We describe in detail below the procedure employed for the dissection of rat brain into the six basic sections that we use routinely. This procedure can be readily adapted for use with other small rodents; it has been employed with minor modifications in studies with gerbils and mice (Rosenzweig and Bennett, 1969).

X.4.2.1. Method of Dissection*

The unanesthetized rat is killed by decapitation, either by a Harvard guillotine (Harvard Apparatus Co., Dover, Mass.) or by inserting one blade of a pair of 8" double sharp scissors through the neck and rapidly severing the spinal cord; subsequently the head is completely removed. The calvarium is then removed, as described below, to expose the entire dorsal surface of the brain. (As an aid in visualizing the parts of the skull and brain, the reader may wish to refer to the excellent illustrations in Zeman and Innes, 1963). All dissection is carried out on brown waxed paper under a fluorescent light. The brown color provides good contrast with the tissue, and the waxed surface aids in removing bits of brain from the paper. A lamp with a magnifying glass attached (Luxo) is very useful for precise dissection.

After decapitation, the skin of the head is inverted and pushed forward from the neck toward the eyes so as to expose the muscles and the skull. At some points the muscles can be freed from the underlying bone by cutting with a sharp scissors. Be careful not to cut through the skin with the scissors

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*This description is taken from one distributed by our project starting in 1965, with subsequent revisions.

because this will probably get hairs into the dissection. To remove the calvarium, first cut bilaterally through the dorsal surface of the medial orbit with a small bone forceps. (We use a Liston bone cutting forceps, straight 5-1/2") This cut is at the anterior end of the olfactory bulbs; be careful not to damage the bulbs. With a scalpel, cut through the temporalis muscle 2-3 mm ventral to the attachment of the muscle. This provides an indication of the lateral extent to which the bone should be removed. Cut off the spinal column caudal to the supraoccipital bone. Clip away the calvarium, starting from the caudal extremity of the skull. Place the bone forceps in the foramen magnum and clip the sides of the foramen, enlarging it in an upward direction, again aiming toward the cut in the temporalis. Keep the point of the rongeurs turned outward toward the bone, in order not to damage the brain. Elevate the whole calvarium to the coronal suture. Continue cutting rostrally at the identical lateral level and then elevate the frontal bone to the first cut in the medial orbit.

After the calvarium has been removed, the dura mater is cut along the midline and reflected back laterally, removing it from the dorsal surface of both hemispheres. The plastic T-square is then positioned on the brain as shown in Fig. 5. The stem of the T is flat on one surface and wedge-shaped on the other. The wedge-shaped surface of the stem is placed downward so that it fits in between the two hemispheres along the midline.

There are three transverse marks near the end of the stem of the T (as shown at A in Fig. 5a) and three transverse marks on the cross-bar just beyond the point where the stem joins the cross-bar (at B). These transverse lines are used as follows in positioning the T-square along the longitudinal axis of the brain. In Fig. 5b the anterior extremity of the hemispheres is shown at A' (the frontal poles), and the posterior extremity at B' (the

occipital poles). The T-square is moved back and forth until these boundaries coincide with either the innermost, middle or outermost pair of transverse lines (for a small, medium, or large brain respectively). These placements are indicated for a small brain in Fig. 5c and for a large brain in Fig. 5d.

The length of the brain, as measured in this way, is used to determine which cross marks near the end of the cross-bar to employ in determining the lateral extent of the samples. Thus, with a small brain, the cortical samples will be bounded laterally by the innermost pair of guide lines on the cross-bar of the T, as in Fig. 5c. With a large brain, the boundaries go out to the side lines nearest to the end of the cross-bar of the T, as in Fig. 5d. For the occipital sample the lateral line should be an extension of the appropriate marking on the cross-bar. Beginning with the somesthetic sample, the lateral boundary should not be straight, but rather should follow the gentle curve of the lateral edge of the brain. See Figs. 5c and d.

Along the stem of the T three sets of samples are provided for, as shown in Fig. 6. The Occipital (O) sample has its anterior boundary indicated by the first crossline on the stem of the T anterior to the crossbar. There is then a small space (1.02 mm) between the occipital and somesthetic samples. The next pair of lines bounds the somesthetic sample. Another space of 1.02 mm separates the somesthetic sample from the sample of motor cortex.

To take each sample of cortical tissue, one circumscribes it with a scalpel, following the guide lines on the T-square. (We used a scalpel with a narrow blade, e.g., size 11 Bard-Parker.) The cuts made at the posterior end of the occipital sample and the cuts made adjacent to the midline between the cerebral hemispheres are to be made after the T-square has been removed from the brain. The medial boundaries of the sections are made 1 mm lateral to the midline; this is closer to the midline than the sides of the stem of the T would indicate, since the stem is 2.92 mm wide. The posterior border of the

occipital sample is a function of the size of the brain. For a small brain
(Fig. 5c) the posterior border of O should correspond to the anterior border
of the bar of the T, but for larger brains (Fig. 5d and 6), the posterior
limit of the occipital section is 0.5 to 0.8 mm behind the anterior border
of the crossbar of the T. In each case the posterior corners of the occipital
area reach practically to the boundaries of the brain, as shown in Fig. 6.

Before acquiring sufficient practice, a person tends to take occipital
samples that are too small. As a guide in the first dissections, one can refer
to Table I, and the weight values progressively should approach the tabulated
values as practice proceeds. Table I gives weight values at 4 ages, and later
tables will give RNA, DNA, AChE and ChE for the same ages and brain regions.
Since brains vary somewhat in size as a function of age, sex and strain (and even
among rats of the same age, sex and strain), it is especially the relations between
the different samples and the cortical/subcortical ratio that should tend
to approach the values indicated. Note that the cortical/subcortical ratio is higher
in young rats than in adults; it tends to stabilize at around 0.70 as the animals
reach the age of about 100 days. Not only should the means come to resemble the
tabulated values, but standard deviations should decrease for successive groups of
dissections and approach the tabulated values which are representative of
those obtained from groups of 12 rats.

The desired cortical samples are thus completely circumscribed and can
be peeled from the corpus callosum. To facilitate easy removal of the samples,
it is advisable that none of the cuts penetrate the corpus callosum. We
usually take the somesthetic sections from both hemispheres as one sample, and
subsequently take the occipital sections from both hemispheres as another
sample.

Following removal of the somesthetic and occipital areas (and motor
area if desired) the remaining dorsal cortex is taken. This sample includes
all the cerebral cortex remaining on the dorsal surface of the brain after

removal of the preceeding sections. We use the long, thin scalpel blade to dissect out the areas medial to the removed O and S samples, to proceed anteriorly to the frontal pole, then to clean off the left hemisphere anterior to the S area, and to continue laterally back to the occipital area and then medially to the original starting point. Take the dorsal cortex in segments in order to leave as much corpus callosum as possible. The same dissection is then performed on the right hemisphere. As one cuts laterally, the scalpel should rest on the inclined surface of the corpus callosum as it slopes downward and laterally, the point of the scalpel reaching the external surface of the cortex at the point where the brain shows its maximum width. The lateral boundary of this area is not clearly delineated, but with some practice it can be removed in a highly reliable way. By these procedures we remove all of the remaining dorsal cortex (as indicated by the dotted area in Fig. 7), thus leaving the cleaned white corpus callosum exposed.

At this point the brain is removed from the skull and turned upside down. The olfactory bulbs and the olfactory tubercles are cut off and put aside to be used later as part of the "subcortex" sample. The next section, called "ventral cortex", also includes such structures as the corpus callosum, hippocampus, and amygdaloid complex. Working on the ventral surface, we gently free the ventral cortex from the adjacent hypothalamus with a size 15 Bard-Parker scalpel blade. The brain is then turned right side up again and starting from the posterior aspect of the cortex, the choroid fissure is opened, exposing the internal capsule; this allows a clean separation of the ventral cortex from the underlying subcortex. Anteriorly, where the internal capsule becomes continuous with the corona radiata, the ventral cortical sample must be separated carefully from the underlying caudate nuclei, by cutting through the internal capsule and continuing around the frontal surface of the caudate nucleus. The presence of the lateral ventricle allows for a natural separation of the hippocampus from the underlying diencephalon.

The fifth section is called "rest of the brain". It includes the olfactory bulbs, the medulla and cerebellum, and all the core of the brain remaining after removal of the "ventral cortex". The medulla is separated from the spinal cord at the point where the medulla narrows to form the cord. When the medulla and cerebellum are desired as a separate sample, a cut is made at the cranial border of the pons while the brain is resting in an up-side-down position. The cut is aimed toward the cerebellum allowing for the natural separation between the cerebellum and caudal border of the inferior colliculus.

As each sample is removed, it is weighed and frozen. Rapid routine weighings accurate to 0.1 mg can be obtained with a semi-micro projection balance capable of weighing to approximately 0.02 mg. Even more convenient is an electronic analytical balance such as the Mettler HE10 or the Ainsworth D1000 Digmetric. These may be interfaced with data printing units. For purposes of weighing, we prepare in advance small squares of waxed paper on which we have written the number of the animal and the section designation. These pieces of paper are weighed first and are then weighed again as soon as the tissue samples are placed upon them. The order of removal and weighing are always the same, and the time of these operations is kept as uniform as possible in order to minimize errors due to drying of the tissues. When the five or six sections described above are removed, six or seven rats per hour can be sacrificed. The samples, on their pieces of paper, are placed on a block of dry ice and are stored in a deep freeze in petri dishes sealed with freezer tape until removal for chemical analysis.

The person performing the dissection and the person doing the weighing do not know the experimental condition to which any individual animal has been assigned, in order to guard against any possible bias in these procedures.

X.4.3. RNA and DNA Content

One of the most reliable biochemical indices of differentiated environ-
mental experience has been the content of RNA and the RNA/DNA ratio. Both
of these increase significantly in the cortex with enriched experience. In
over 600 paired comparisons of rats raised in enriched environments (EC) to
littermates raised in impoverished environments (IC), the RNA/DNA ratio of the
occipital cortex of the EC rats exceeded that of the IC rats by 7.6%; a difference
in this direction was found in 90% of the pairs. For total cortex, the correspond-
ing values were 3.7% increase and 89% of the comparisons.

The total RNA of the cortex of rats in EC exceeded that of rats in IC
by 4.9%. This value approximated the percentage difference in cortical weight
of the two groups (4.3%). We should note that, just as for behavioral procedures
and measurements of tissue weight, all chemical analyses are performed "blind"
with animals identified only by code number and members of all experimental
groups being interdigitated in an analytical series.

The following procedure for the determination of RNA and DNA in brain
employs cetyltrimethylammonium bromide as the initial precipitant and repre-
sents a modification of the method of Schmidt and Thannhauser (1945) which,
with various modifications, has frequently been employed for the determination
of nucleic acids in brain. The method is based upon the intrinsic UV absorption
of the separated and hydrolyzed nucleic acids, and no corrections for inter-
fering absorptions are required. Our method (Morimoto et al., 1974) avoids
problems of interference from contaminating materials that are particularly
abundant in brain and of low recovery that is found with the other modified
procedures.

Under routine conditions, 40 brain samples can be analyzed as a group
for RNA and DNA over a two-day period when the sample size permits duplicate

analyses, or 80 samples if only single determinations are desired. The re-
liability of the assay is high; the standard deviation of 14 RNA analyses of
one sample was found to be approximately 2%, and for DNA the standard deviation
was approximately 3.5%. The standard deviation of the RNA/DNA ratio of the
cortex in a sample of 12 rats from a given environmental condition is typically
in the range of 2 to 3%.

In general, duplicate assays result in good agreement. We typically find 75%
of our RNA assay values to agree within 3% and 60% of our DNA duplicate analyses
differ less than 4%. Occasionally when samples are combined or are large
(as in the case of the RS sample), triplicate analysis are performed. In
these cases, RNA values are discarded that are more than 5% from the mean
(6½% for DNA).

Values of the nucleic acids for 4 ages and each of our standard brain regions
are presented in 3 Tables: Table II, RNA/weight; Table III, DNA/weight; Table IV,
RNA/DNA. A comparable presentation of AChE and ChE activities will be found in
Tables VII VIII, and IX.

X.4.3.1. Reagents for RNA and DNA Analyses.

Ethylenediaminetetraacetic acid buffer (0.11M). Ethylenediaminetetra-
acetic acid, disodium salt, dihydrate (EDTA, Alrich Chemical Co.), 41.8 g/liter,
pH adjusted to 5.9 with KOH.

Cetyltrimethylammonium bromide (0.08M or 3%). An aqueous 30 g/liter
solution of hexadecyltrimethylammonium bromide (CTAB). Technical grade,
Eastman Kodak.

Deoxyribonucleic acid. Calf thymus DNA (A grade, Calbiochem) stock
solution 2 mg/ml in 0.01 M Tris, pH 8.6. Several drops of chloroform are
added to act as a preservative.

Scintillation solution. Forty milliliters Permafluor (Packard Instrument
Co., Inc., Illinois), 200 ml Bio-Solve (Beckman), and 33 ml butyric acid are
diluted to 1 liter with toluene.

<u>Ascorbic acid</u>. Ascorbic acid (200 mg/ml) (Calbiochem) in H_2O. With the chloroform preservative, the DNA standard is stable for 4 to 6 weeks at room temperature; the other reagents are stable for 3 to 4 months.

X.4.3.2. Instruments

The instruments required are those commonly found in any well-equipped chemistry or biochemistry laboratory and include the following items: (a) A centrifuge such as a Sorvall RC-3 capable of 7000 x g. An HG-4 head and 4 adaptors permitting the centrifugation of 40 samples is desirable. (b) Absorbance measurements are best made with a digital spectrophotometer equipped with a cell holder for 4 or 5 cuvettes. We have used a Beckman DU spectrophotometer updated with a Gilford Model 252-1 or a Gilford 220 unit. Other quality spectrophotometers such as a Cary 219 are also suitable. (c) A water bath shaker maintains temperature at 37°C for RNA hydrolysis, DNA hydrolysis is performed in an oil bath maintained at 70°C. (d) A Vortex mixer is used for mixing the samples.

X4.3.3. Analytical Procedures

<u>X.4.3.3a. Sample Preparation</u>. All operations of the sample preparation are carried out at 0-5°C using cold solutions. If the sample size exceeds 100 mg, the sample is homogenized using a Potter-Elvehjem homogenizer in approximately 4 ml of the EDTA (0.11 M) buffer. Additional buffer is used to rinse the homogenizer and to bring the sample to a final concentration of 25.0 mg/ml. In those cases when the sample size is less than 100 mg (e.g., samples of occipital or somesthetic cortex), the sample is initially homogenized in 2.0 ml of buffer, and two 1.0 ml rinses are used to transfer the entire sample to the culture tubes used for the analyses. Two ml of 3% CTAB is added to 4 ml of brain homogenate in 16 x 75 mm culture tubes, and the precipitate is allowed to form. After 1 hr, the precipitate is collected by centrifugation in a Sorvall RC-3 centrifuge at 7000 x g (5250 rpm) for 15 min. The super-

natant is discarded, and the pellet is washed twice with 1 ml H_2O and once with 0.1 N potassium acetate in absolute ethyl alcohol. The pellet is thoroughly dispersed using a Vortex test tube mixer and then centrifuged beween each washing.

X.4.3.3b. Analysis of RNA. The pellet is dispersed with 100 μl of H_2O and hydrolyzed in 1.1 ml of 0.3 N KOH at 37° for 1 hr in a shaker bath. During this one-hr hydrolysis, samples are given two additional vigorous mixings using the Vortex mixer. After cooling, the alkaline digest is made 0.2 N in acid by the addition of 500 μl of 1.3 N $HClO_4$, and allowed to stand for 15 min at 0°C. After centrifugation at 7000 x g for 15 min, the supernatant is recovered. The acid-insoluble fraction is washed twice with 50 μl of 0.2 N $HClO_4$. The three supernatants comprising the RNA are pooled, and the volume is adjusted to 5.0 ml (final concentration $HClO_4$ 0.1 N).

The RNA content is assayed by absorbance at 260 nm, and calculated on the assumption that an absorbance of 1.00 at 260 nm is equivalent to 32 μg RNA/ml. This value has been reported for rat liver (Munro and Fleck, 1966a,b). A value of 31.6 for rat brain RNA using the base composition data of Balazs and Cocks (1967) has been calculated, and values of 31.5 and 31.3 have been calculated for rat liver using data of Munroe and Fleck (1966), and Mahler and Cordes (1966), respectively.

X.4.3.3c. Analysis of DNA. The acid insoluble fraction is drained by inverting the tubes overnight over absorbant paper (e.g., Kimwipes). The pellet is thoroughly dispersed in the appropriate volume of 1 N $HClO_4$; 2.0 ml is used for cortical samples, 3.00 ml for rest of brain (RS) and medulla, and 6.00 ml for cerebellum. The DNA is heated with frequent mixing for 20 min at 70°C, cooled, and spun at 7000 x g for 15 min. The absorbance of the supernatant is determined at 266 nm. To control for minor variations in hydrolysis, for each group of samples, calf thymus DNA samples subjected to hydrolysis with

1 N HClO$_4$ are used as standards. An absorbance of 1.00 for 45 µg DNA/ml is typically found for calf thymus DNA; base analyses given for calf DNA and for rat tissues are very similar (Sorber, 1970).

X.4.3.3d. Determination of Radioactivity in the RNA Fraction. If desired, the radioactivity present in cerebral RNA of rats previously injected with [^{14}C] uridine or [^{3}H] uridine can be conveniently determined in the CTAB precipitate after hydrolysis with 0.3 N KOh. Aliquots are taken and further hydrolyzed overnight at 37°C; 500 µl of the hydroylsate is mixed in a glass scintillation vial with 18 ml of scintillation solution; 50 µl of 20% ascorbic acid is added to eliminate chemiluminescence. Samples are then counted in a scintillation counter (Packard Tricarb or Beckman LS-9000).

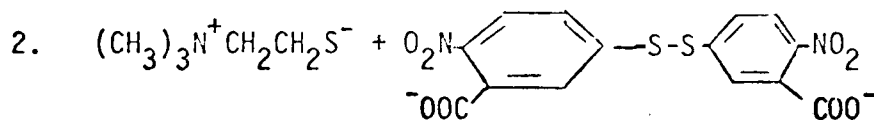X.4.4. AChE and ChE Activities.

As a class, cholinesterases constitute a group of esterases that hydrolyze choline esters at a higher rate than other esters when hydrolysis rates are compared at optimum conditions with respect to substrate concentration, ionic strength, pH, etc. Two classes of enzymes that hydrolyze choline esters exist in biological material--acetycholinesterase (AChE) and cholinesterase (ChE). Unfortunately, since 1932 when an enzyme was prepared from horse serum by Stedman and Easson (1932) and called "choline-esterase", considerable confusion has existed in the literature regarding the nomenclature of these two classes of enzymes. For example, AChE has been referred to as acetylcholinesterase, cholinesterase, e-type ChE, specific ChE, ChE I, and aceto ChE. ChE has been referred to as butyro ChE, propionic ChE, benzyl ChE, ChE, pseudo ChE, s-type ChE, non-specific ChE, ChE II, and X-ChE. To avoid further confusion the Commission on Enzymes of the International Union of Biochemistry (Florkin and Stotz, 1965) has recommended that acetylcholine acetyl-hydrolase (System No. 3.1.1.7) be the formal name and acetycholinesterase the trival name for the enzyme having the higher affinity for acetylcholine than for

any other choline ester. In like manner, acylcholine acyl-hydrolase (System No. 3.1.1.8) or cholinesterase (ChE) is the term to be used for the other enzyme(s) which hydrolyzes certain other esters of choline, i.e. butyrylcholine or propionylcholine, at a higher rate than acetylcholine. In spite of these recommendations, it is still common to find confusion in the literature with respect to AChE and ChE.

AChE is widely distributed in the brain and its principal function is to inactivate the neurotransmitter ACh after its release in the process of transmission of nerve impulses. The role of ChE in the mammalian CNS is still largely obscure. We have used ChE as an index of glial function since it appears to be mainly concentrated in glial cells and in white fiber tracts. The ratio of AChE activity to ChE activity varies widely from one part of an organism to another, and also varies among species. Numerous studies have shown that in brain, retina, and enthrocytes a high proportion of the total AChE-ChE activity is due to AChE. Unlike brain, intestine and blood serum are characterized by relatively high ratios of ChE to AChE. The properties of ChE vary more widely from species to species than do those of AChE. Isozymes of AChE have been demonstrated in numerous species.

Historically, acetylcholine, the natural substrate of AChE, or an analogue, acetyl-β-methylcholine (mechoyl) was the substrate of choice for AChE; butyrylcholine or benzoylcholine was the substrate of choice for ChE. Enzymatic activity was most frequently based upon the amount of acid liberated upon hydrolysis under standardized conditions of pH, substrate concentration, etc. An auto-titrator or "pH stat" was frequently employed to measure hydrolysis rates. While considerable precision could be obtained with this method under well-controlled and standardized conditions, it was relatively time consuming and required considerable attention to details of technique to achieve highly reproducible answers.

In 1961 Ellman _et_ _al_. described a colormetric method for the determination of AChE utilizing acetylthiocholine (AcSCh) for substrate and the Ellman reagent, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), as the indicator of the extent of hydrolysis. In addition, by substituting butyrylthiocholine (BuSCh) for AcSCh this method becomes a sensitive and convenient assay for ChE. The reactions involved are summarized below:

1a. $H_2O + (CH_3)_3N^+CH_2CH_2SCOCH_3 \xrightarrow[\text{or}]{\text{AChE (fast)}}$

$$\xrightarrow{\text{ChE (slow)}}$$

$(CH_3)_3N^+CH_2CH_2S^- + CH_3COO^- + 2H^+$

1b. $H_2O + (CH_3)_3N^+CH_2CH_2SCOCH_2CH_2CH_3 \xrightarrow[\text{ChE (fast)}]{\text{AChE (fast)}}$

$(CH_3)_3N^+CH_2CH_2S^- + CH_3CH_2CH_2COO^- + 2H^+$

2. $(CH_3)_3N^+CH_2CH_2S^- + O_2N$—⟨ ⟩—$S$-$S$—⟨ ⟩—$NO_2$
^-OOC COO^-

$\xrightarrow[\text{Non-enzymatic}]{\text{very fast}}$

$(CH_3)_3N^+CH_2CH_2$-S-S—⟨ ⟩—NO_2, COO^-

^-OOC—⟨ ⟩—S^-
O_2N
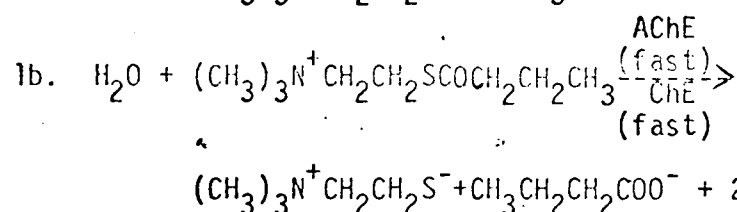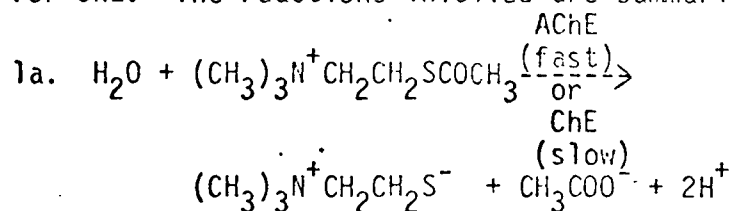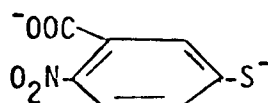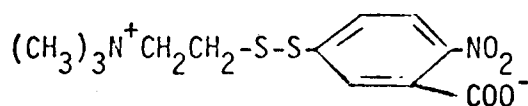
The product, 5-thio-2-nitrobenzoate, has an extinction coefficient of 13,600 at 412 nm, thus measurement of its rate of formation provides a sensitive and highly specific measure of the hydrolysis rate of thiocholine esters. With appropriate modern spectrophotometers, a skilled analyst can readily perform analyses of 50 samples daily for both AChE and ChE in duplicate with an average difference between duplicate samples of less than 3%. The details of the procedures for AChE and ChE were developed after extensive investigation and comparison of the characteristics of hydrolysis of AcCh, AcSCh, BuCh, and BuSCh as a function of pH, substrate concentration, source of

enzyme, and inhibitor type and concentration.

The hydrolysis rate of AcCh (pH stat assay) and AcSCh (spectrophotometric assay) increases as a function of pH, but the pH dependence is less at pH 8.0 than at pH 7.0 (Fig. 8). However, above pH 8.0, the non-enzymatic hydrolysis rate increases rapidly. (The apparent high activity of AcSCh by the pH stat method can be attributed to a shift in the numbers of acid equivalents titrated/mole hydrolyzed as a function of pH). The relative rates of hydrolysis of AcCh and BuCh (pH stat assay) and AcSCh and BuSCh (spectrophotometric assay) as a function of substrate concentration are compared in Fig. 9. The curves for the two acetyl substrates were very similar, with both substances having a maximum hydrolysis rate at about 10^{-3}M. Since the rate of hydrolysis of AcSCh is only slightly lower at 6 x 10^{-4} M, and the blank is reduced substantially, we recommend the lower concentration for routine assays. The best value---based upon more than 200 analyses---for the ratio of rate of hydrolysis of 6 x 10^{-4}M AcSCh to 7.7 x 10^{-4} AcCh by rat brain tissue at pH 7.95 is 0.94. A similar ratio should be obtained with other sources of AChE which contain less than 10% ChE.

Although the enzyme-substrate relationships are highly specific, when one enzyme in a tissue (e.g. AChE) is present in much larger amounts than another related enzyme (e.g. ChE) it is desirable to have good estimates of the relative specificities under the conditions of assay. The availability of separate inhibitors of AChE and ChE in combination with several sources of AChE and ChE has permitted such estimates to be obtained. At the time of development of these assays Bayless and Todrick (1956) had evaluated several selective inhibitors. These included 1:5-bis(4-trimethylammoniumphenyl)-pentan-3-one diiodide (BW62C47) and 1:5-bis-(4-allyldimethylammoniumphenyl)-pentan-3-one diiodide (BW284C51), effective as inhibitors of AChE, and 10-(2-diethyl-aminopropryl)-phenothiazine (Lysivane, ethopropazine), effective as an inhibitor of ChE. Bayless and Todrick had used a number of substrates for AChE and

ChE, but not the thiocholine esters. BW62C47 (5×10^{-6}M) and BW284C51

(1×10^{-7}M) each resulted in more than 95% inhibition of AcSCh hydrolysis

by brain homogenates and less than 20% inhibition of the small amount of BuSCh

hydrolysis. BW284C51 (5×10^{-7}M) reduced the hydrolysis of AcSCh by rat

intestine by 10% and had no effect on the hydrolysis of BuSCh. Since

BW284C51 is commercially available (Sigma Chemical Co.) it is the inhibitor

of choice to determine ChE in the presence of AChE.

We were unable to obtain Lysivane but were able to obtain a closely re-

lated compound 10-(2-dimethylaminopropyl) phenothiazine (Promethazine).

We found, using either rat brain or retina, that 2.5×10^{-5}M Promethazine

inhibited hydrolysis of AcSCh (AChE activity) less than 5%, while the hydrolysis

of BuSCh by intestine was inhibited more than 70%. This concentration is

recommended to essentially eliminate any possible interference of ChE in assays

for AChE in cerebral tissues of the rat.

Promethazine together with BW284C51 inhibited 99% of the hydrolysis of

AcSCh by rat brain. In addition, eserine inhibited 98% of the hydrolysis
                                          and
of AcCH, AcSCh,/BuSCh by brain or intestine, confirming that cholinesterases

are being measured by the methods described.

Since rat retina contains AChE with little ChE, it provides a convenient

source of tissue from the same species to further check the relative activities

of AChE against AcSCh and BuSCh and the specificity of inhibitors of choline-

sterases. Therefore, hydrolysis of AcSCh and BuSCh by homogenates of rat

retina was investigated and the effect of several concentrations of Promethazine

and of 5×10^{-7} M BW284C51 and 10^{-5}M eserine on these hydrolysis rates was

also studied. The results, summarized in Table V, provide the best evidence

that we have obtained to date of the high degree of specificity AChE has for

AcSCh as compared to BuSCh. Thus, even in the absence of inhibitor, the

hydrolysis of BuSCh by the retinal preparation is only 0.3   nM/min/g,

or 1.7% as rapid as the hydrolysis of AcSCh. When the effect of BW284C51 on the hydrolysis of BuSCh by the retinal preparation is considered, the hydrolysis of BuSCh by AChE is about 1% as rapid as the hydrolysis of AcSCh under our conditions of assay. The ChE inhibitor Promethazine inhibits less than 3% of the activity of the retinal homogenate against AcSCh at 4×10^{-5} M, a concentration that inhibits at least 80% of the activity of rat intestinal ChE against either BuSCh or AcSCh. These results confirm the selectivity of Promethazine as well as the high degree of "purity" of rat retina AChE. Over 98% of the activity against AcSCh is inhibited by BW284C51, and essentially all the activity is inhibited by 10^{-5} M eserine. Eserine inhibits the hydrolysis of BuSCh completely.

A limited number of studies with dog showed that while the ChE in cortex activity was approximately twice that of the rat, AChE activity was approximately one-third that of the rat (Table X). The fact that ChE represents an appreciable fraction of total cholinesterase activity in the dog but not in the rat emphasizes the desirability of determining the raltive activities of AChE and ChE by appropriate inhibitor studies when applying these methods to previously little studied sources of these enzymes.

X.4.4.1. Reagents for AChE and ChE Analyses.

5,5'-Dithiobis-(2-nitrobenzoic acid (0.01 M). DTNB reagent without inhibitor is prepared by dissolving 400 mg of DTNB in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0. This reagent is not used for routine assays, but is useful for studies of AChE and ChE activities in the absence of inhibitors. DTNB can be obtained from numerous suppliers including Pierce Chemical Co., Calbiochem Corp. and Sigma Chemical Co.

DTNB-Promethazine (DTNB-I ChE). The stock solution of DTNB containing promethazine, an inhibitor of ChE, is prepared by dissolving Promethazine hydrochloride (N,N, -trimethyl-10H-phenothiazine-10-ethanamine; 10-(2-

dimethylaminopropyl) phenothiazone 48.2 mg of Promethazine hydrochloride and 400 mg of DTNB in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0. This solution is 1.5×10^{-3} M in Promethazine and 0.01 M in DTNB. Promethazine has been obtained from Wyeth Laboratories and Purpac Pharmaceutical Co.

For some studies, promethazine may be omitted since the hydrolysis of AcSCh by the ChE in mouse and rat brain is less than 5% of the total hydrolysis rate. However, as already noted in this Section, relative ChE activity may be much higher in other tissues and/or species.

DTNB-BW284C51 (DTNB-I AChE). For routine assays of ChE activity, a DTNB reagent containing a specific inhibitor of AChE is used. The specificity and commercial availability 1,5-bis (N-allyl-N,N-dimethyl-4-ammoniumphenyl) pentan-3-one dibromide (BW284C51) (Sigma Chemical Co.) makes it the inhibitor of choice. A 3×10^{-3} M stock solution of BW284C51 is prepared by dissolving 20.1 mg of BW284C51 in 10 ml of distilled water; 1 ml of this/to is added 400 mg of DTNB dissolved in 100 ml of water.

THE EFFECTIVENESS OF BW284C51 AS AN INHIBITOR OF AChE CANNOT BE TOO STRONGLY EMPHASIZED: GLASSWARE USED FOR THE ANALYSIS OF ChE IN THE PRESENCE OF BW284C51 SHOULD BE CLEARLY MARKED AND SEPARATED FROM GLASSWARE USED FOR THE ANALYSES OF AChE. CELLS AND GLASSWARE THAT HAVE BEEN IN CONTACT WITH BW284C51 MUST NOT BE USED FOR THE ANALYSIS OF AChE UNLESS THE ARTICLES HAVE BEEN SCRUPULOUSLY CLEANED AND CHECKED AGAINST OTHER KNOWN CLEAN CELLS.

Acetylthiocholine (0.037 M). The AcSCh stock solution is prepared by dissolving 270 mg of acetylthiocholine iodide in 25 ml of distilled water. AcSChI can be obtained from numerous suppliers including Calbiochem, Sigma Chemical Co., Gallard-Schlesinger Chemical Mfg. Co., and Pfaltz and Bauer.

Butyrylthiocholine (0.063 M). The BuSCh stock solution is prepared by dissolving 500 mg of butyrylthiocholine iodide in 25 ml of distilled water. BuSChI can be obtained from the suppliers of AcSCh.

Unidentified impurities in AcSCh and BuSCh have resulted in markedly low values for AChE and ChE using brain homogenates. New lots of any reagents should be checked against samples of known purity.   .

We routinely prepare sufficient quantities of these stock reagents for assay of the tissues from one or more behavioral experiments. Aliquot quantities of these stock reagents sufficient for several days analyses are placed in individual small test tubes and stored frozen. Under these conditions, reagents are stable for at least one month.

X.4.4.2. Instruments

AChE and ChE activities are best determined with a recording spectrophotometer capable of determining the absorbance of 4 to 5 samples sequentially at frequent intervals. We have used a Beckman DU spectrophotometer equipped with a Gilford Model 220 absorbance detector, a Model 210 sample changer and a Model 208 position offset. More modern, more suitable units include the Gilford Model 2400-2 or the Cary Model 219 spectrometer. These units are equipped with a thermostated cell compartment. We have used an auxillary thermostated block to prewarm the samples to 37°C. This permits groups of samples to be run consecutively with no loss of time between groups.

To maximize the number of analyses per day per analyst, it is necessary to have two spectrometers, one system used for AChE, the other set at twice the recorder sensitivity to analyze ChE. These instruments can be used simultaneously by an analyst who puts samples into them alternately. For a limited number of samples the operator can change samples manually and read and record the absorbance at timed intervals over a 5 to 10 minute period. However, these results will be less precise.

X.4.4.3. Analytical Procedures

X.4.4.3a. Sample Preparations. Since AChE activities are stable in frozen cerebral tissue, it is generally most convenient to use brain sections that

have been dissected, weighed, frozen and stored as described above. On the day of analysis, a petri dish of samples is removed from the deep freeze and placed on a slab of dry ice in an insulated box. To homogenize the samples, a commercial glass homogenizer with a teflon pestle is used (A. H. Thomas No. 4288). The smaller size (A) is used for all samples weighing less than approximately 750 mg. The intermediate size (B) tissue grinder is used for the subcortical brain section when it includes the cerebellum and medulla. A Sunbeam Mixmaster motor, fitted with a Jacobs chuck into which the pestle can readily be locked, is recommended for homogenizing the samples. The motor is mounted on a heavy, stable supporting stand.

Frozen samples are readily transferred from the wax paper to the glass homogenizing tube and any small trace of material that remains on the wax paper is wiped off on the teflon pestle. A minimal size graduate (10, 25, 50, or 100 ml) is used for each sample. Samples are homogenized initially in a volume of the ice-cold 0.1 M sodium phosphate buffer, somewhat less than the final total volume that is desired, and then transferred to the chilled glass-stoppered graduate. The homogenizer and pestle are rinsed with several additional portions of buffer, and the grooves on the bottom of the pestle are inspected to make sure that no tissue is caught in them. These washings are added to the initial homogenate in the graduate. A plastic wash bottle containing chilled phosphate buffer is used for the rinses and to make the dilution to the desired final volume. The graduates containing the samples are stored in ice until the analyses are completed.

It is convenient to prepare a table with the desired final volumes before the samples are homogenized. The homogenate concentrations recommended for analyzing adult rat brain sections for AChE and ChE are summarized in Table VI with the approximate aliquot used in each 3 ml assay. These values are suitable for postweaning rats, and appropriate modifications of tissue homogenate

concentrations are made when greatly different activities are expected, as from tissues from very young rats or other species.

X.4.4.3b. Analysis of AChE. To determine AChE activity, 0.1 M sodium phosphate buffer, pH 8.0 and the desired sample aliquot to make a known final volume of about 3 ml are pipetted into standard spectrophotometer cuvettes (Beckman or similar). "Lamda" pipettes are used to deliver the samples. Repipettes (Lab-Line Instruments, Inc.) provide a convenient and accurate method of adding the required amount of buffer. Alternatively, glass volumetric pipettes may be modified and individually calibrated to deliver 2.6 ml. One hundred µl of DTNB reagent is added to each cell, and the contents are premixed by inverting several times. Small pieces of Parafilm provide convenient·and leakproof closures for the cells. The solutions are pre-warmed in incubator compartments (37°) for ten to twelve minutes, then removed and 50 µl of AcSCh reagent is added. Hamilton repeating syringes which dispense 50 µl/aliquot provide a convenient method for adding the DTNB and AcSCH rapidly and accurately. An alternative method is to prefill the required number of "Lambda" pipets with the appropriate reagent and place them horizontally on a grooved wooden or plastic pipet rack. The contents are again rapidly mixed and placed in the cell compartment (37°C) of the recording spectrophotometer. Optical density of 412 nm is recorded sequentially on each sample for 10 to 15 seconds over a ten to twelve minute period. Recorder sensitivity is set for full scale of the recorder to be the equivalent of a change of one absorbance unit. Duplicate analyses are routinely made, normally in separate incubations and runs. These duplicate analyses should check within 3%. If not, additional analyses are made.

A set of 4 reagent blanks is run at least twice daily by substituting phosphate buffer for the brain homogenate. The AcSCh reagent blank should be about .0063 absorbance units/min, or about 8% of the sample absorbance

change. Reaction rates are linear for at least 10 minutes under the conditions described. High blanks may indicate an accumulation of tissue in the cells and thorough cleaning is indicated.

X.4.4.3c. Analysis of ChE. The determination of ChE follows a procedure similar to that for AChE except that larger tissue aliquots and correspondingly less buffer diluent is required, 50 µl of DTNB-I AChE is used and BuSCh is substituted for AcSCh, and recorder sensitivity is set at 1/2 optical density unit per full scale deflection. The blank for BuSCh is approximately .0046 absorbance units/minute, about 20% of the rate of change observed with the brain samples. Duplicate ChE analyses should agree within 5% and the reaction rate should be linear.

Calculation of AChE or ChE activity is based upon the rate of change of absorbance (determined by fitting the best straight line to the recorder plot), the total assay volume, weight of sample, homogenate volume, and aliquot of sample. Answers are typically expressed in terms of nmoles of AcSCh or BuSCh hydrolyzed/min/mg wet weight tissue. As a guide in checking your procedure, typical values and standard deviations are given for the areas of brain that we usually analyze for rats of 4 different ages in Tables VII, VIII, IX. The 5-dinitrobenzoate anion has an extinction coefficient of 13,600 at 412 nm at pH = 8.0.

AChE and weights of 15 brain areas from 6 lines of rats have been compared by Bennett et al. (1966).

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.TABLE I

WEIGHTS OF STANDARD BRAIN AREAS FOR COLONY REARED S_1 MALE RATS

	.Age at time of sacrifice (days)			
	.30	60	1.00	250
	MEAN WEIGHT (mg) $\pm$ STANDARD DEVIATION			
CORTEX				
Occipital	63 $\pm$ 4	66 $\pm$ 5	65 $\pm$ 5	74 $\pm$ 5
Somesthetic	50 $\pm$ 3	57 $\pm$ 4	56 $\pm$ 5	58 $\pm$ 4
Remaining Dorsal	260 $\pm$ 14	299 $\pm$ 18	289 $\pm$ 17	286 $\pm$ 18
Ventral	210 $\pm$ 15	243 $\pm$ 15	273 $\pm$ 16	291 $\pm$ 19
Total	583 $\pm$ 30	665 $\pm$ 29	683 $\pm$ 29	709 $\pm$ 32
SUBCORTEX				
Cerebellum	179 $\pm$ 12	224 $\pm$ 11	239 $\pm$ 12	249 $\pm$ 16
Medulla	120 $\pm$ 9	162 $\pm$ 8	180 $\pm$ 9	205 $\pm$ 12
Remaining Subcortex	408 $\pm$ 20	477 $\pm$ 26	507 $\pm$ 32	550 $\pm$ 33
Total	708 $\pm$ 37	863 $\pm$ 40	925 $\pm$ 43	1004 $\pm$ 54
TOTAL BRAIN	1290 $\pm$ 65	1528 $\pm$ 65	1609 $\pm$ 63	1713 $\pm$ 83
TOTAL CORTEX/SUBCORTEX	.82 $\pm$ .02	.77 $\pm$ .02	.74 $\pm$ .03	.71 $\pm$ .02
BODY WEIGHT (g)	67 $\pm$ .12	200 $\pm$ 23	297 $\pm$ 29	410 $\pm$ 40

TABLE II   60

RNA OF STANDARD BRAIN AREAS FOR COLONY REARED S$_1$ MALE RATS

Age at time of sacrifice (days)

MEAN SPECIFIC RNA (μg/100 mg) ± STANDARD DEVIATION

	30	60	100	250
CORTEX				
Occipital	204 ± 7	171 ± 5	156 ± 5	152 ± 6
Somesthetic	199 ± 7	171 ± 6	146 ± 8	145 ± 6
Remaining Dorsal	196 ± 6	165 ± 5	146 ± .5	144 ± 5
Ventral	192 ± 6	160 ± 3	145 ± 5	138 ± 4
Total	196 ± 6	164 ± 3	147 ± 4	143 ± 3
SUBCORTEX				
Cerebellum	232 ± 8	187 ± 4	172 ± 3	167 ± 5
Medulla	195 ± 7	146 ± 3	129 ± 3	114 ± 4
Remaining Subcortex	186 ± 5	150 ± 3	138 ± 3	133 ± 4
Total	200 ± 6	159 ± 3	146 ± 3	138 ± 3
TOTAL BRAIN	198 ± 5	161 ± 2	146 ± 2	140 ± 2
TOTAL CORTEX/SUBCORTEX	.98 ± .03	1.03 ± .02	1.01 ± .03	1.04 ± .03

TABLE III
61

DNA OF STANDARD BRAIN AREAS FOR COLONY REARED S_1 MALE RATS

	Age at time of sacrifice (days)			
	30	60	100	250
	MEAN SPECIFIC DNA (μg/100 mg) $\pm$ STANDARD DEVIATION			
CORTEX				
Occipital	97 ± 6	92 ± 5	97 ± 5	98 ± 4
Somesthetic	92 ± 5	96 ± 4	96 ± 5	96 ± 4
Remaining Dorsal	93 ± 4	97 ± 5	98 ± 4	95 ± 5
Ventral	112 ± 5	104 ± 3	102 ± 6	95 ± 5
Total	100 ± 4	97 ± 3	99 ± 3	95 ± 3
SUBCORTEX				
Cerebellum	757 ± 34	672 ± 30	607 ± 26	584 ± 22
Medulla	127 ± 6	104 ± 4	91 ± 4	80 ± 4
Remaining Subcortex	148 ± 5	146 ± 9	143 ± 7	141 ± 9
Total	298 ± 12	275 ± 11	254 ± 11	239 ± 8
TOTAL BRAIN	208 ± 8	197 ± 7	188 ± 6	179 ± 6
TOTAL CORTEX/SUBCORTEX	.34 ± .02	.35 ± .04	.39 ± .02	.40 ± .02

TABLE IV

RNA/DNA OF STANDARD BRAIN AREAS FOR COLONY REARED S₁ MALE RATS

Age at time of sacrifice (days)

MEAN SPECIFIC RNA/DNA ± STANDARD DEVIATION

	30	60	100	250
CORTEX				
Occipital	2.10 ± .09	1.86 ± .06	1.63 ± .08	1.56 ± .06
Somesthetic	2.16 ± .08	1.78 ± .06	1.52 ± .08	1.51 ± .07
Remaining Dorsal	2.11 ± .07	1.70 ± .09	1.49 ± .07	1.52 ± .09
Ventral	1.73 ± .04	1.54 ± .04	1.44 ± .08	1.46 ± .06
Total	1.96 ± .04	1.70 ± .05	1.49 ± .05	1.51 ± .05
SUBCORTEX				
Cerebellum	.31 ± .02	.28 ± .01	.28 ± .01	.28 ± .01
Medulla	1.54 ± .03	1.40 ± .03	1.42 ± .04	1.42 ± .03
Remaining Subcortex	1.29 ± .03	1.03 ± .03	.97 ± .05	.94 ± .06
Total	.67 ± .02	.58 ± .02	.57 ± .03	.58 ± .02
TOTAL BRAIN	.95 ± .02	.82 ± .02	.78 ± .03	.78 ± .03
TOTAL CORTEX/SUBCORTEX	2.93 ± .07	2.99 ± .06	2.60 ± .13	2.62 ± .09

TABLE V

Hydrolysis of Thiocholine Esters by Rat Retina*

Substrate and Concentration (M)		Inhib-itor	and Concentration	Activity (nM/min/mg)	Relative Activity
AcSCh	7.7×10^{-4}	None		17.8	100
		Promethazine	4.6×10^{-6}	17.5	98
			4.6×10^{-5}	17.3	97
			9.2×10^{-5}	16.7	94
			2×10^{-4}	15.7	88
AcSCh	7.7×10^{-4}	284c51	5×10^{-7}	0.28	1.6
AcSCh	7.7×10^{-4}	Eserine	10^{-5}	0.28	0.5
BuSCh	1×10^{-3}	None		0.30	100
BuSCh	1×10^{-3}	284c51	5×10^{-7}	0.10	33
BuSCh	1×10^{-3}	Eserine	10^{-5}	0.00	0

*Rat retinas were homogenized in 0.1 M potassium phosphate buffer, pH 8.0; 1 mg of retina in a total volume of 1 ml was used when AcSCh in the absence of inhibitor was used as substrate, and 10 mg of tissue was used in all of the other assays.

TABLE VI

Recommended Tissue Concentrations

and Aliquots for Analyses of Rat Brain

AChE and ChE

	For AChE Assay			For ChE Assay	
	Tissue conc. (mg/ml)	Aliquot (μl)	pH 8.0 Phosphate Buffer (ml)	Aliquot (ml)	pH 8.0 Phosphate Buffer (ml)
Occipital Cortex	3.0	1000	2.0	3.0	---
Somesthetic	3.0	800	2.0	3.0	---
Remaining Dorsal Cortex	5.0	400	2.6	2.0	1.0
Ventral Cortex	5.0	300	2.6	3.0	---
Subcortical Brain	5.0	100	3.0	2.0	1.0
Cerebellum & Medulla	5.0	400	2.6	2.0	1.0
Cerebellum	5.0	800λ	2.0	2.0	1.0
Medulla	3.0	300λ	2.6	3.0	---

TABLE VII

ACETYLCHOLINESTERASE OF STANDARD BRAIN AREAS FOR COLONY REARED S_1 MALE RATS

	Age at time of sacrifice (days)			
	30	60	100	250
	AChE (nM AcSCh hyd./min/mg) $\pm$ STANDARD DEVIATION			
CORTEX				
Occipital	4.3 $\pm$ .5	5.8 $\pm$ .3	5.7 $\pm$ .4	5.5 $\pm$ .3
Somesthetic	5.5 $\pm$ .6	7.0 $\pm$ .4	7.0 $\pm$ .4	6.5 $\pm$ .4
Remaining Dorsal	5.9 $\pm$ .6	7.6 $\pm$ .4	7.4 $\pm$ .4	7.0 $\pm$ .4
Ventral	8.5 $\pm$ .9	11.7 $\pm$ .6	11.6 $\pm$ .7	10.6 $\pm$ .6
Total	6.9 $\pm$ .6	9.1 $\pm$ .4	9.1 $\pm$ .4	8.5 $\pm$ .4
SUBCORTEX				
Cerebellum & Medulla	10.9 $\pm$ .5	9.8 $\pm$ .4	8.9 $\pm$ .3	8.4 $\pm$ .3
Remaining Subcortex	24.0 $\pm$1.7	29.1 $\pm$1.4	28.0 $\pm$1.2	25.8 $\pm$1.1
Total	18.6 $\pm$1.3	20.5 $\pm$ .9	19.4 $\pm$ .9	18.0 $\pm$ .9
TOTAL BRAIN	13.3 $\pm$ .9	15.5 $\pm$ .6	15.1 $\pm$ .6	14.0 $\pm$ .6
TOTAL CORTEX/SUBCORTEX	.37 $\pm$ .03	.44 $\pm$ .02	.47 $\pm$ .02	.48 $\pm$ .02

TABLE VIII

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CHOLINESTERASE OF STANDARD BRAIN AREAS FOR COLONY REARED S_1 MALE RATS

Age at time of sacrifice (days)

ChE(nM AcSCh hyd./min/mg) $\pm$ STANDARD DEVIATION

	30	60	100	250
CORTEX				
Occipital	.32 $\pm$ .04	.34 $\pm$ .02	.34 $\pm$ .03	.35 $\pm$ .03
Somesthetic	.35 $\pm$ .04	.38 $\pm$ .02	.38 $\pm$ .03	.38 $\pm$ .02
Remaining Dorsal	.31 $\pm$ .04	.34 $\pm$ .02	.35 $\pm$ .03	.36 $\pm$ .02
Ventral	.33 $\pm$ .03	.33 $\pm$ .02	.31 $\pm$ .02	.31 $\pm$ .01
Total	.32 $\pm$ .03	.34 $\pm$ .02	.33 $\pm$ .02	.34 $\pm$ .01
SUBCORTEX				
Cerebellum & Medulla	.61 $\pm$ .05	.55 $\pm$ .03	.51 $\pm$ .02	.50 $\pm$ .03
Remaining Subcortex	.64 $\pm$ .05	.65 $\pm$ .03	.63 $\pm$ .02	.63 $\pm$ .03
Total	.64 $\pm$ .04	.59 $\pm$ .02	.58 $\pm$ .02	.57 $\pm$ .03
TOTAL BRAIN	.50 $\pm$ .03	.49 $\pm$ .02	.47 $\pm$ .02	.47 $\pm$ .02
TOTAL CORTEX/SUBCORTEX	.53 $\pm$ .05	.56 $\pm$ .03	.58 $\pm$ .03	.60 $\pm$ .03

TABLE IX

ChE/AChE OF STANDARD BRAIN AREAS FOR COLONY REARED S_1 MALE RATS

Age at time of sacrifice (days)

ChE/AChE X 100 ± STANDARD DEVIATION

	30	60	100	250
CORTEX				
Occipital	7.1 ± .7	6.1 ± .4	5.8 ± .6	6.3 ± .5
Somesthetic	6.0 ± .6	5.4 ± .4	5.4 ± .5	5.7 ± .4
Remaining Dorsal	5.6 ± .5	4.4 ± .3	4.6 ± .4	5.1 ± .4
Ventral	4.0 ± .4	2.8 ± .2	2.6 ± .3	2.8 ± .2
Total	4.9 ± .5	3.7 ± .2	3.7 ± .3	3.9 ± .3
SUBCORTEX				
Cerebellum & Medulla	5.8 ± .5	5.6 ± .3	5.6 ± .3	6.0 ± .3
Remaining Subcortex	2.7 ± .3	2.4 ± .2	2.3 ± .1	2.4 ± .2
Total	3.4 ± .4	2.9 ± .2	3.0 ± .2	3.2 ± .2
TOTAL BRAIN	3.7 ± .3	3.1 ± .2	3.2 ± .2	3.4 ± .2
TOTAL CORTEX/SUBCORTEX	.142 ± .012	.125 ± .008	.123 ± .009	.124 ± .008

TABLE X

COMPARISON OF RATES OF HYDROLYSIS OF CHOLINE ESTERS
BY RAT AND DOG BRAIN TISSUES

	Substrate and Concentration			
	$AcSCh[7 \times 10^{-4}\underline{M}]$		$BuSCh[10^{-3}\underline{M}]$	
Acetylcholinesterase Inhibitor	−	+	−	+
	Hydrolysis Rate (nM/min/mg)			
RAT				
Cortex	8.68	.53	.5	.32
Subcortex	18.4	1.1	.9	.57
DOG				
Cortex	2.91	.24	.70	.60
Subcortex	11.7	.99	2.20	1.88

XBL 7710-4720

LAHUE
Bennett et al

Fig. 1

70
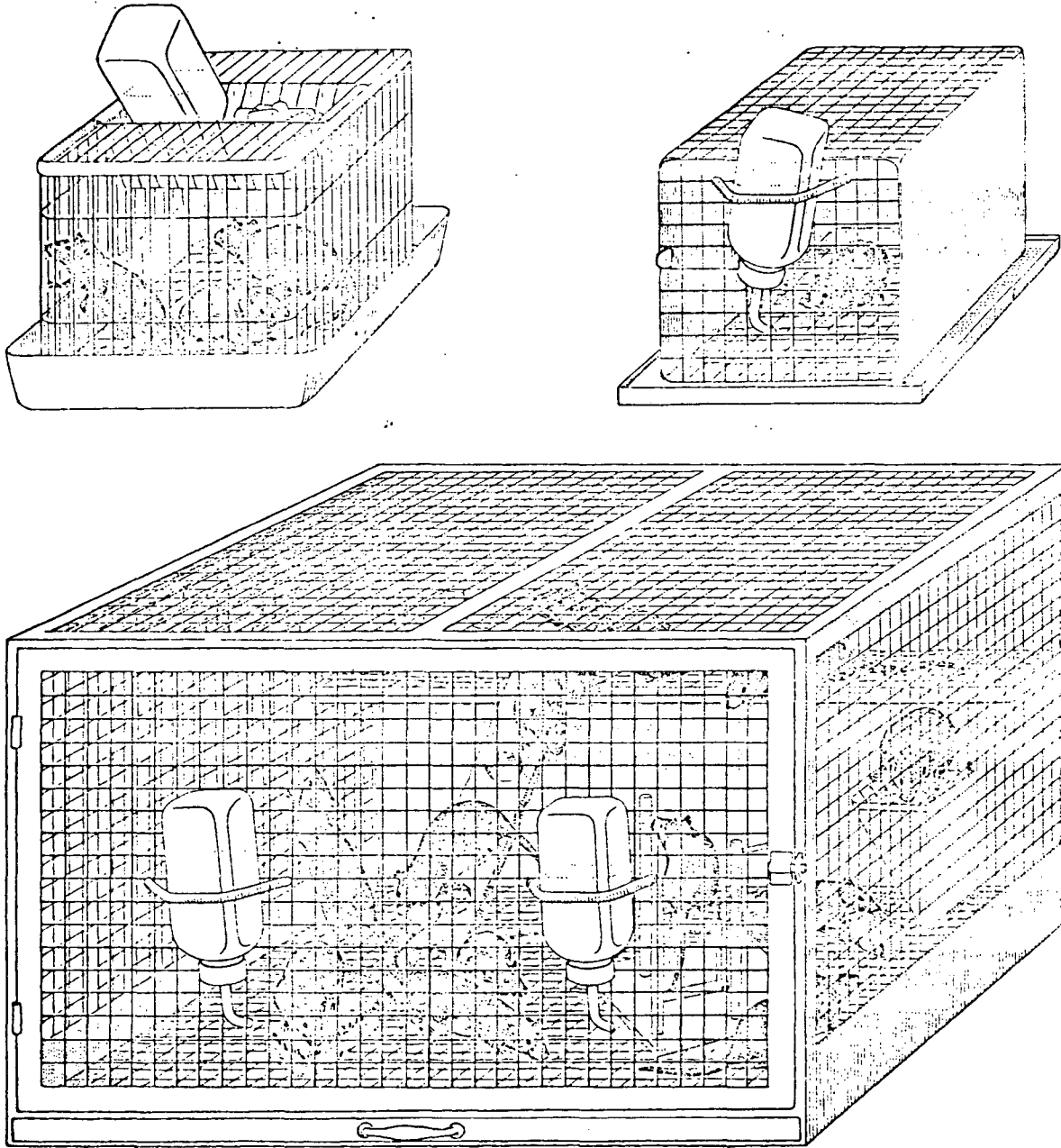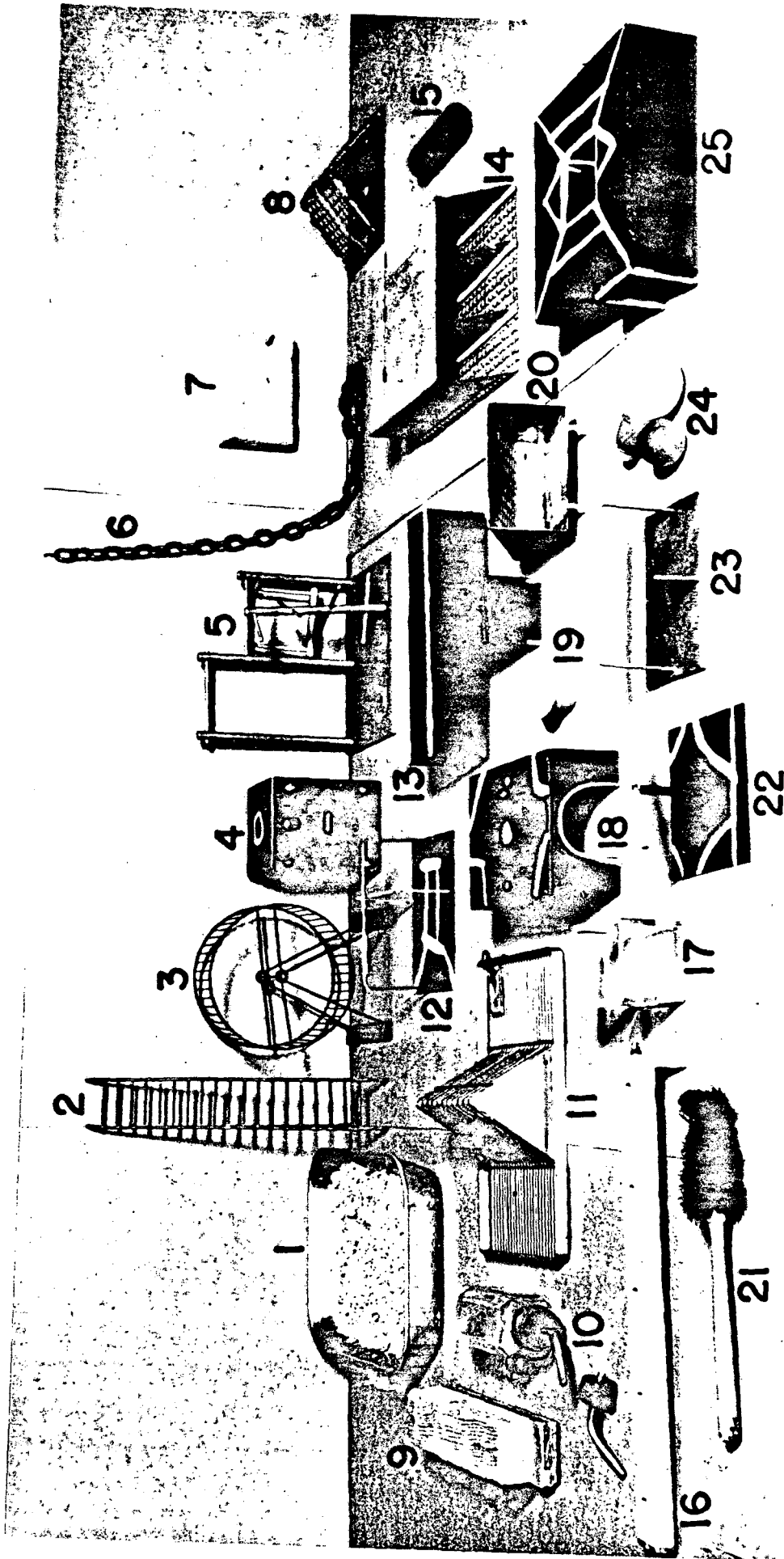
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Fig. 2
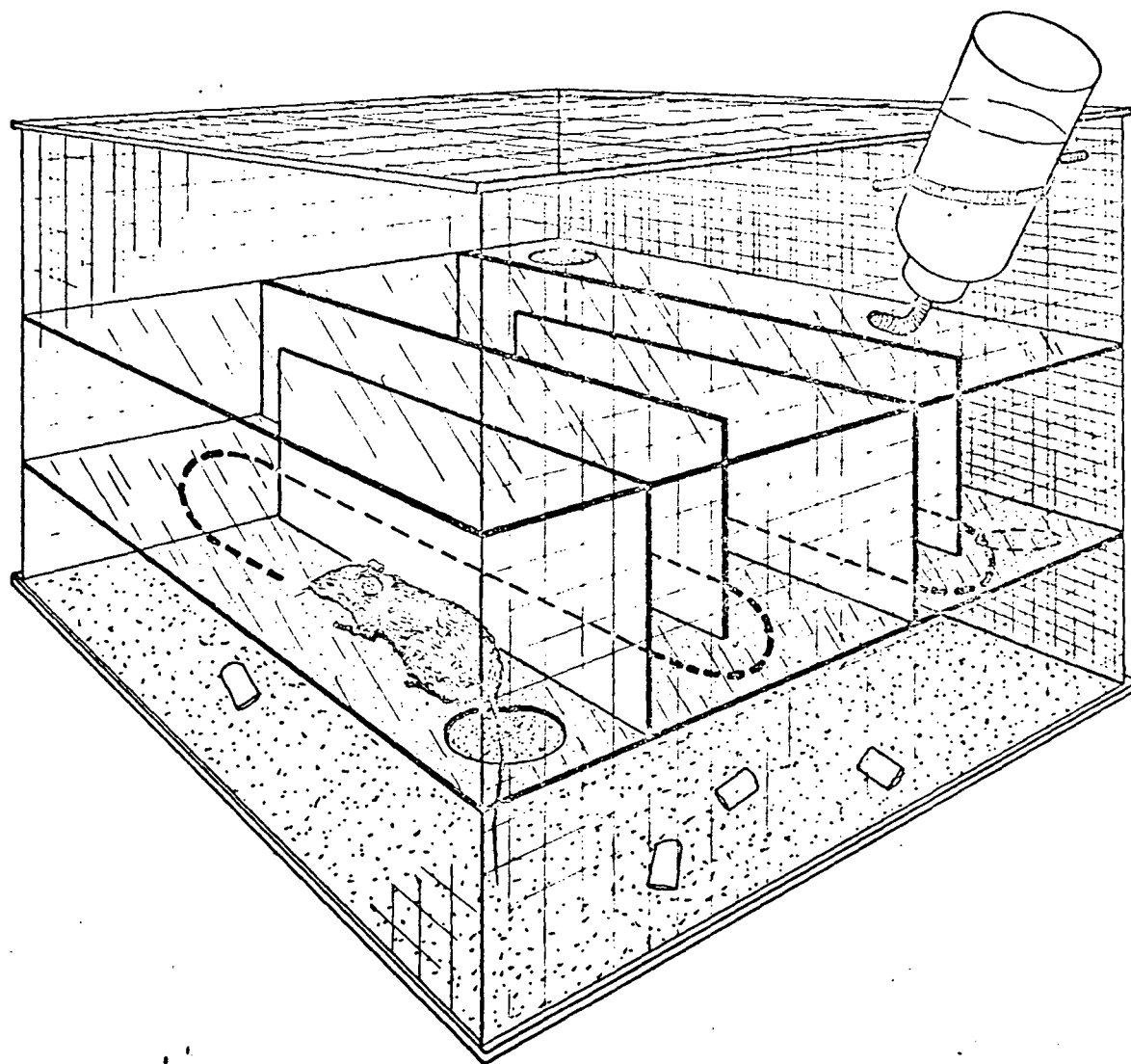
XBL 777 – 4519

Lahue
Bennett et al
Fig. 3

HEBB – WILLIAMS APPARATUS
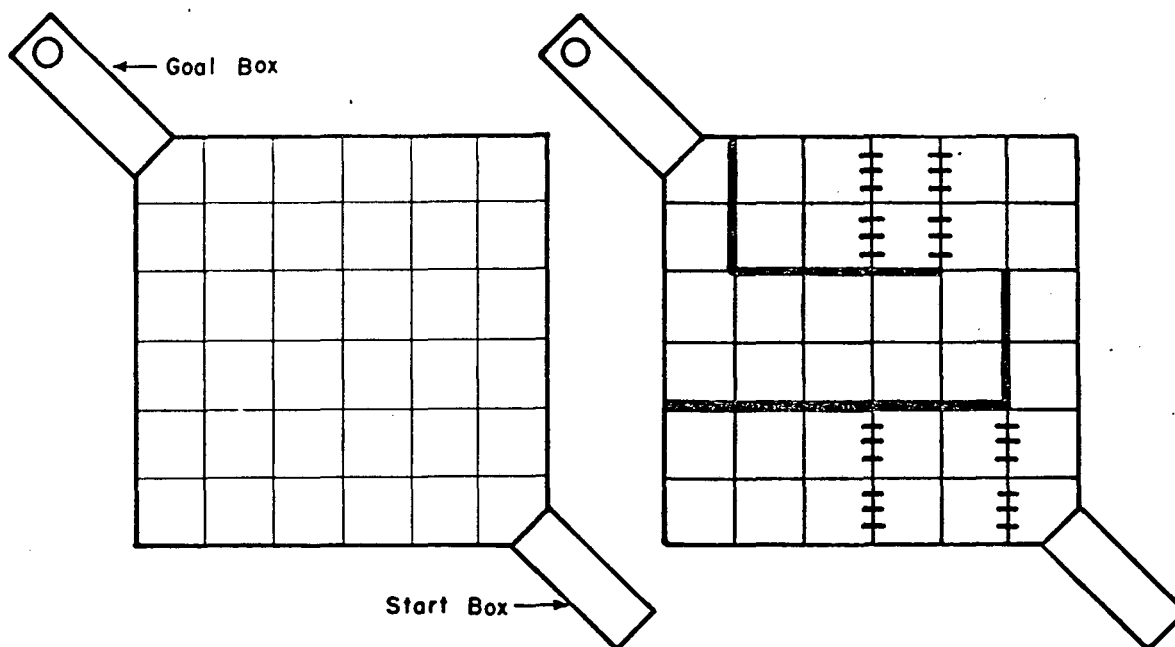
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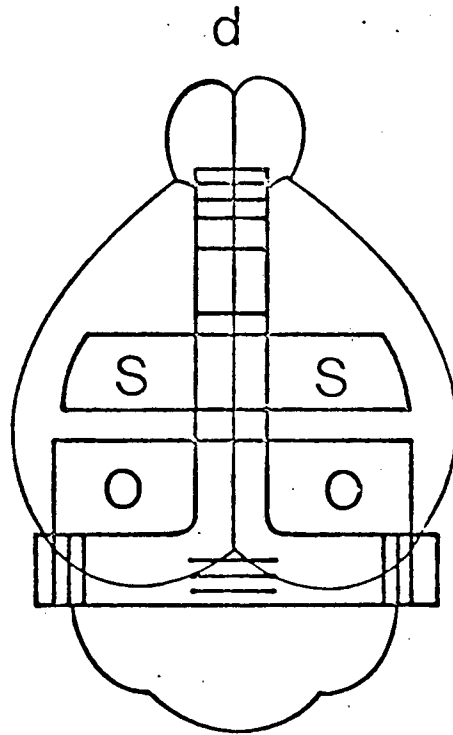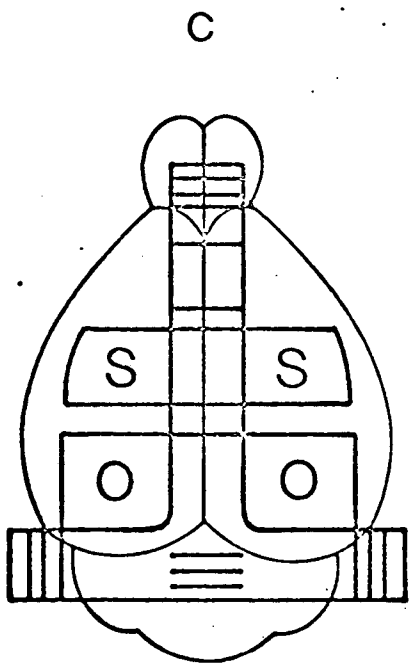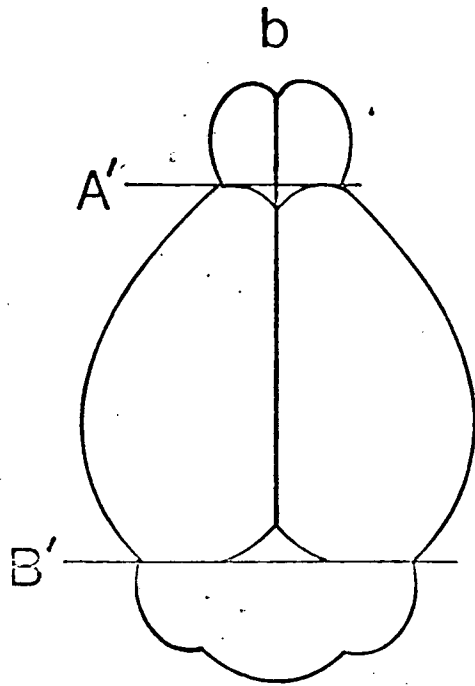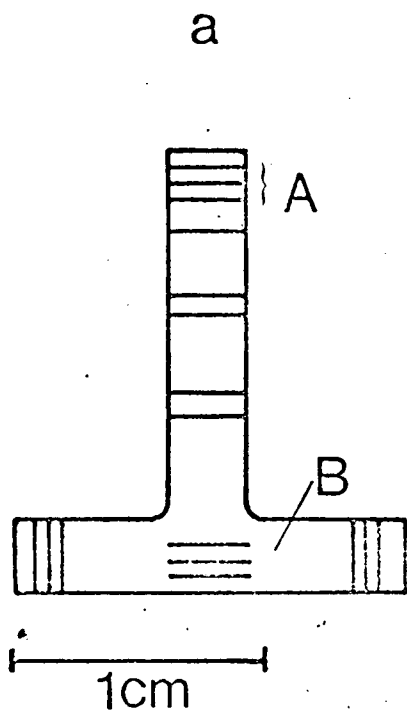
XBL 779-4621

RAT T-SQUARE —

Positioned on medium sized rat brain
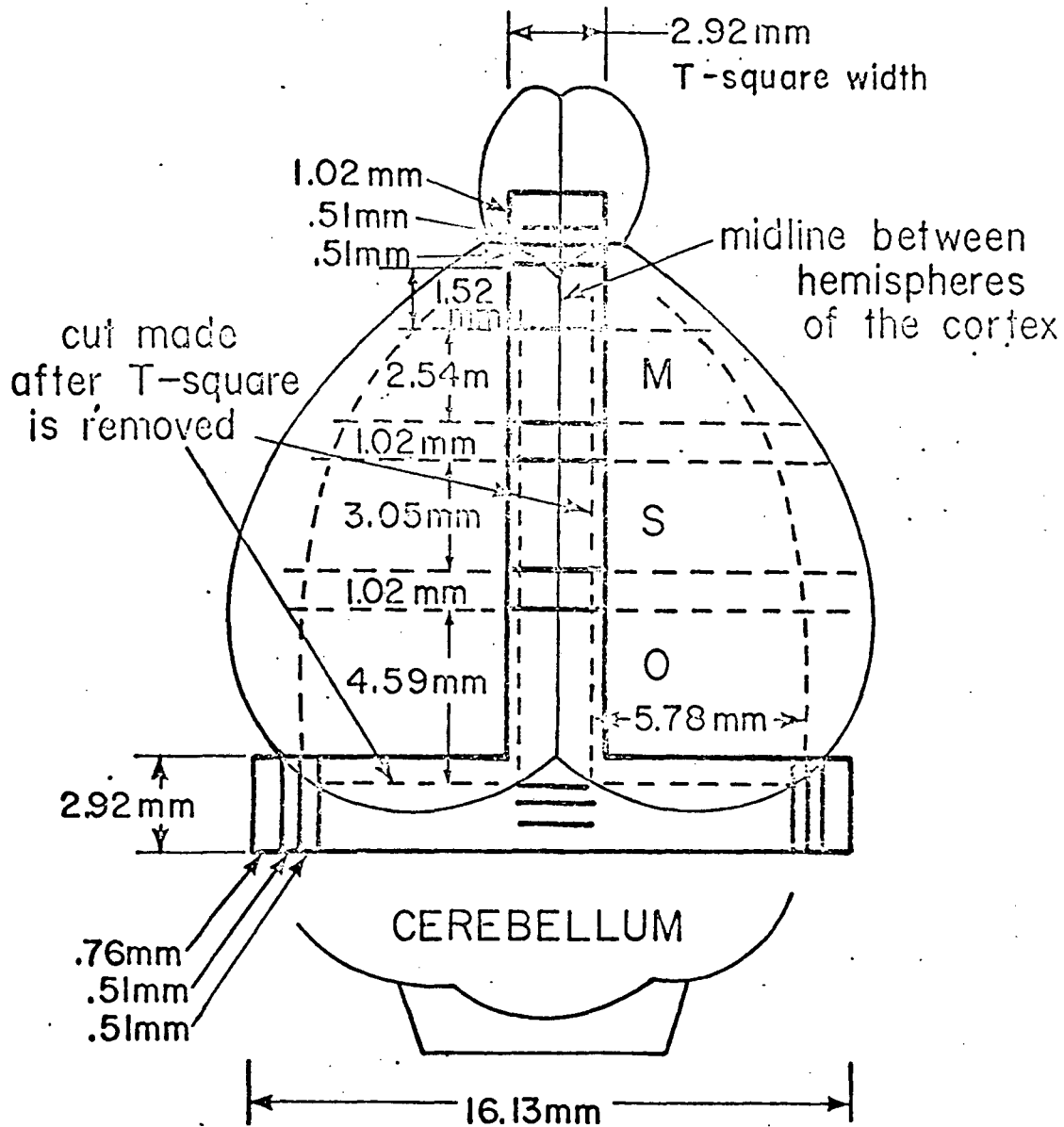
2.92 mm
T-square width

1.02 mm
.51 mm
.51 mm

midline between
hemispheres
of the cortex

1.52 mm

cut made
after T-square
is removed

2.54 m

M

1.02 mm

3.05 mm

S

1.02 mm

4.59 mm

O

←5.78 mm→

2.92 mm

.76 mm
.51 mm
.51 mm

CEREBELLUM

16.13 mm

XBL 779—4623

REMAINING DORSAL CORTEX

SUBCORTEX II
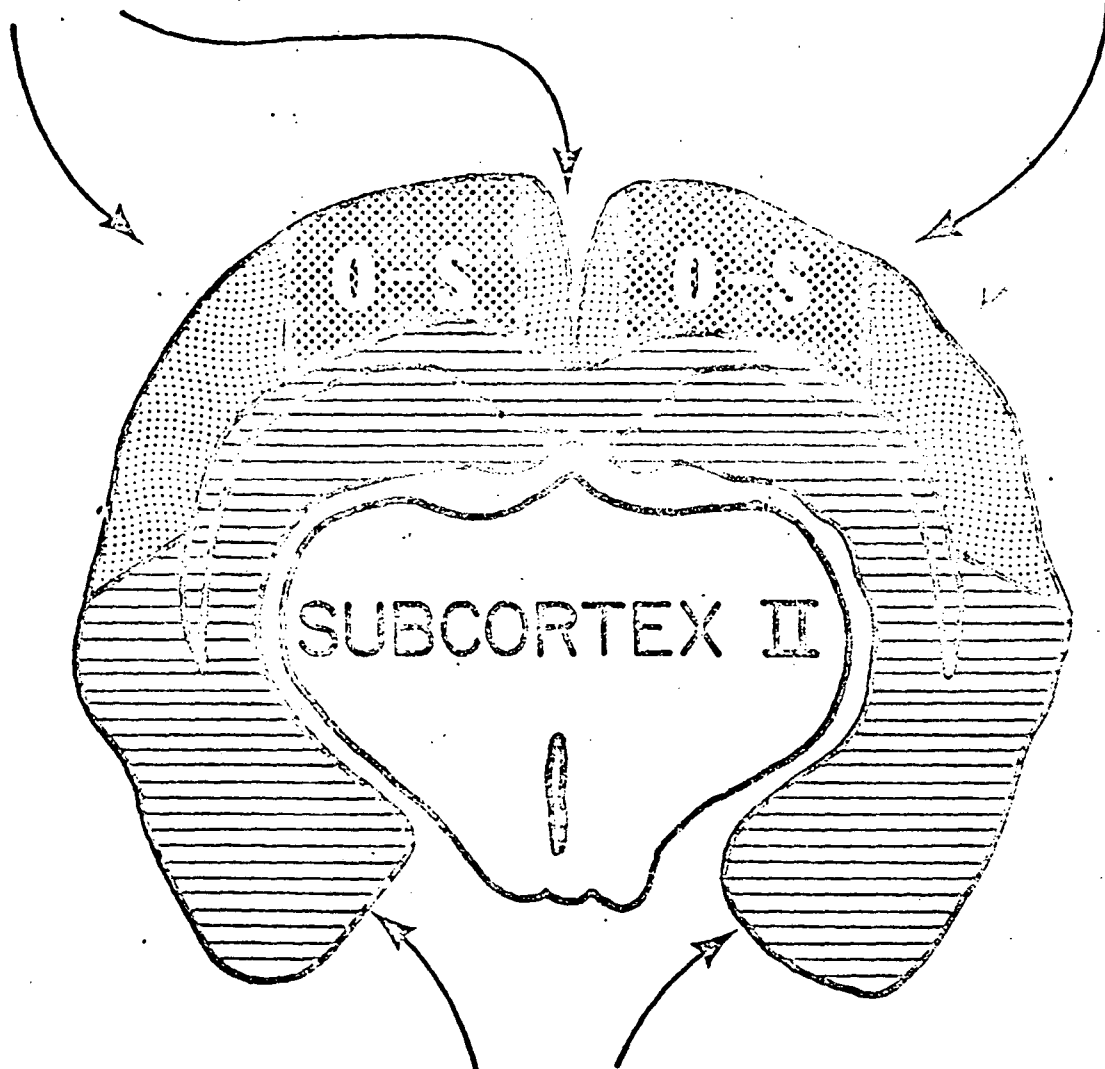
VENTRAL CORTEX

XBL 779-4622
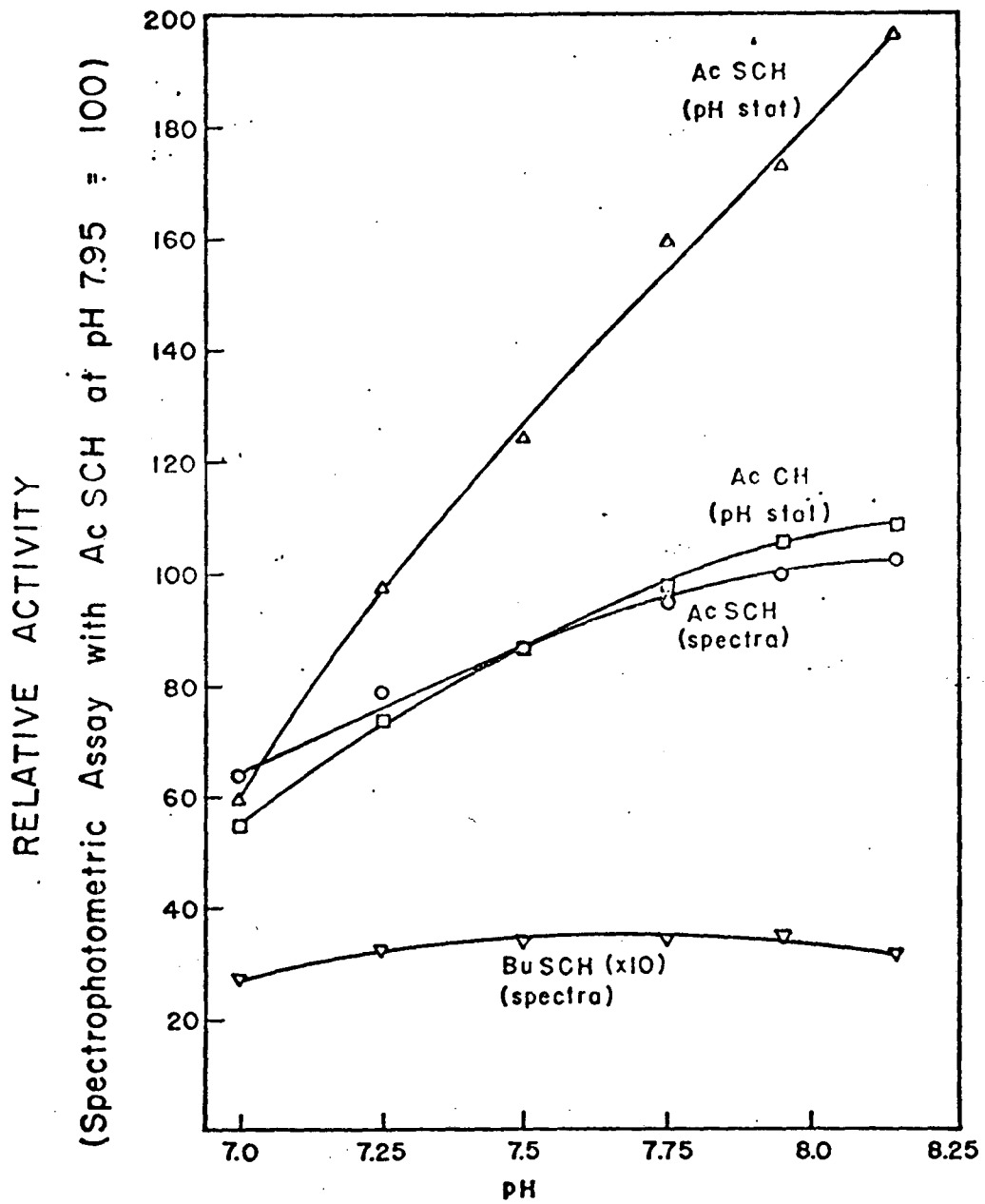
RELATIVE ACTIVITY
(Spectrophotometric Assay with Ac SCH at pH 7.95 = 100)

Ac SCH (pH stat)

Ac CH (pH stat)

Ac SCH (spectra)

Bu SCH (x10) (spectra)

pH
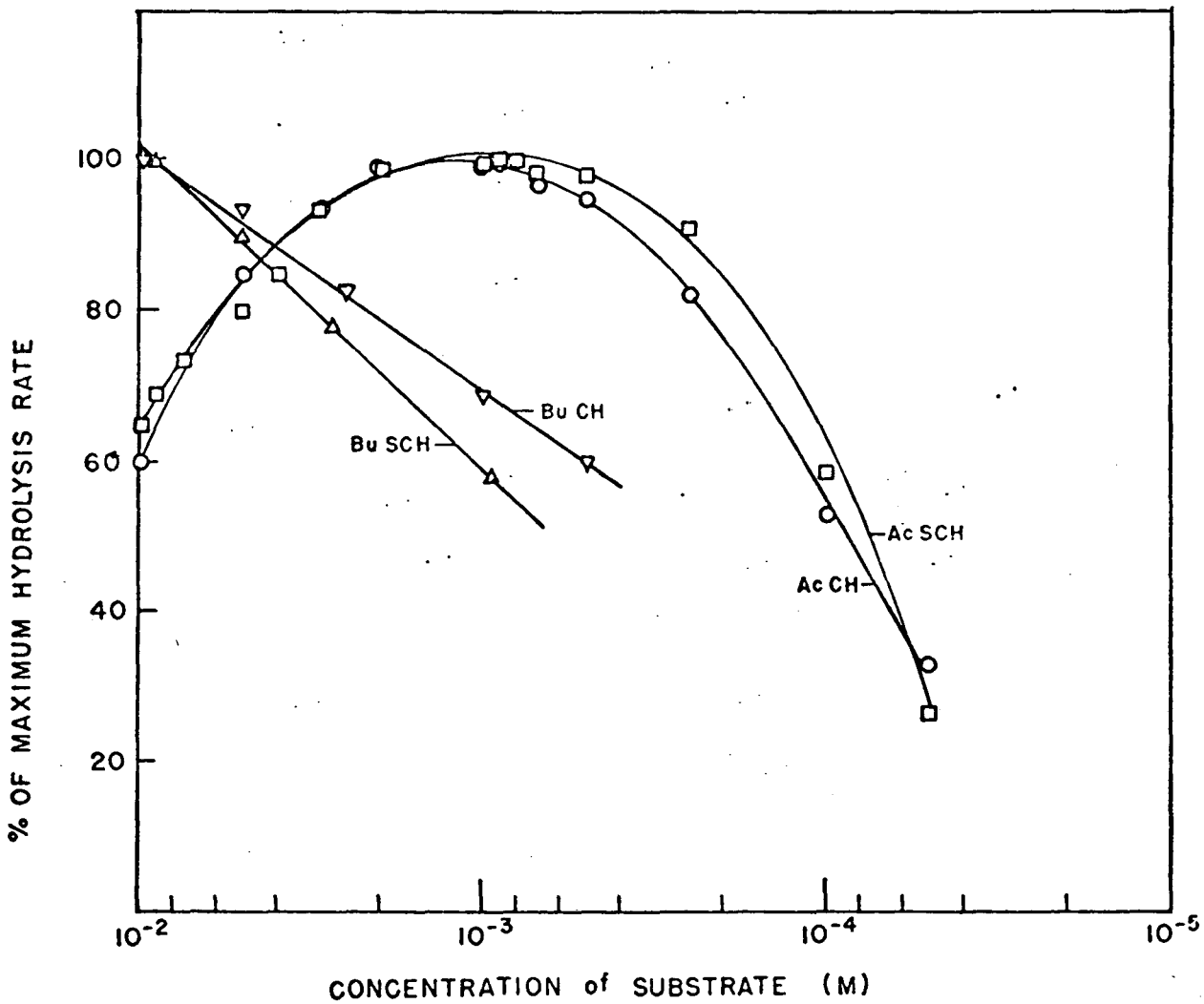
MUB-3305

Figure Captions

Fig. 1   Rats in three environments: Upper left, standard colony (SC);
         upper right, impoverished condition (IC); below, enriched condition
         (EC). (From Rosenzweig et al., Scientific American, 1972).
         Assigning rats to these environments for a period of days to
         weeks leads to significant changes in anatomy and chemistry of
         regions of the brain.

Fig. 2   Stimulus objects commonly used in the enriched condition. Typically
         about 6 objects from this pool are placed in an EC cage. (From
         Rosenzweig and Bennett, 1969).

Fig. 3   Plastic maze placed within a large cage. The rat runs self-paced
         trials to get from the food station below to the water station
         above. Training in this situation leads to significant changes in
         brain anatomy and brain chemistry, when comparison is made with
         an appropriate cortical condition.

Fig. 4   A. Hebb-Williams maze apparatus floorplan is shown to the left

         B. The barrier pattern and error zones for problem 3 are shown
             to the right. The light solid lines are painted on the floor
             of the apparatus. The heavy lines indicate the position of
             barriers. The hatched lines indicate error zones; these lines
             are not present in the maze and they appear on the score sheets
             that the experimenters use for recording performance in the
             maze.

Fig. 5   Delimitation of cortical samples by means of a calibrated
         plastic T-square. a. T-square with calibration. b. Dorsal surface
         of rat brain indicating anterior poles (A') and posterior poles
         (B') of the cerebral hemispheres. c and d. Small and large
         brains with locations of somesthetic (S) and occipital (O) samples
         demarcated by the T-square.

Fig. 6   T-square position on medium-sized adult rat brain. Detailed
         dimension of the T-square are shown.

Fig. 7   Schematic transverse section of rat brain showing the occipital
         or somesthetic samples (O-S), Remaining Dorsal Cortex, Ventral Cortex,
         and Subcortex.

Fig. 8   Effect of pH on hydrolysis rates of choline esters by rat brain
         homogenate determined by rate of acid liberation (pH stat) and by
         DTNB method (spectra). The apparent high rate of hydrolysis of
         AcSCH is measured by the pH stat is discussed in the text.

Fig. 9   Effect of substrate concentration on hydrolysis rates of choline
          esters by rat brain homogenate. The hydrolysis rates using the
          substrates for AChE are maximal at approximately 10^{-3}M; slightly
          lower concentrations are recommended to improve the ratio of net
          hydrolysis rate to blank hydrolysis rate. No concentration maximum
          is found with the substrates (BuCH, BuSCH) typically employed for
          ChE.