Otis Lloyd Byrd, Jr. SOME STUDIES ON SODIUM FLUX IN THE ISOLATED RETINA.

(Under the direction of Stanley Buckser and W. James Smith) Department

of Biology, August 1971.

Isolated retinas from Rana pipiens were excised in the dark and

equilibrated in a neural Ringer's solution containing small amounts of Na^{22}.

At the end of equilibration time the radioactive tissue was washed with a

nonradioactive Ringer's solution and the eluent tested for Na^{22} content.

Experiments were graphed as percent Na^{22} remaining in the retina as a func-

tion of time. Under control conditions the efflux showed a three slope

phenomena. The fast slope (A) goes from time 0-5 minutes, the intermediate

slope (B) goes from time 5-20 minutes and the slow slope (C) goes from

time 20 minutes until the termination of the experiment. Lowering the

temperature of the experiment 10° caused some increase in slope A which

could be explained by the Q_{10} rule and a marked change in Slope B indi-

cating a metabolically dependent function. Rats were treated with doses

of sodium-1-glutamate from days 2-18 in order to selectively degenerate

the bipolar and ganglion layers of the retina. Tests on these rats show

that the ERG is completely abolished. Na^{22} efflux profiles from the retinas

of glutamate treated rats show that slope A is slightly changed while

slopes B and C are completely abolished. Results from the three sets of

experiments show clearly that the major component of slope A is absorbed

Na^{22} associated with the fluid drop surrounding the retina and implies

that slope C is a combination of Na^{22} from Müller cells and Na^{22} which

follows a complex absorption-reabsorption route. By deduction, slope

B appears to be dominated by the Na^{22} efflux from the various neural ele-

ments. The importance of Na^{22} exchange in the rod outer segments cannot

be determined since it is not known if they actively transport sodium.

SOME STUDIES ON SODIUM FLUX IN THE ISOLATED RETINA

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts in Biology

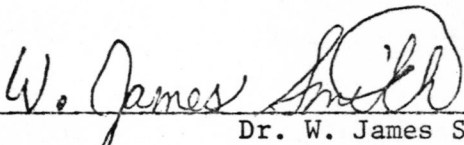by

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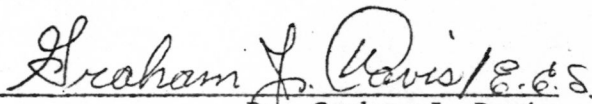SOME STUDIES ON SODIUM FLUX IN THE ISOLATED RETINA

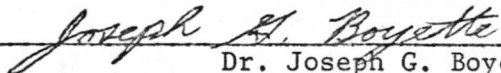by

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This thesis is lovingly dedicated to my wife, Patricia, who helped make a dream a reality and to my sons Jon and Stephen.

Table of Contents

LIST OF FIGURES

SOME STUDIES ON SODIUM EFFLUX IN THE ISOLATED RETINA

Historical. Ion flux studies began early in the eighteenth century

when differences between cell and bathing medium were noted. However,

it was 1899 when Overton made the first statement indicating membrane

transport. Working with both plant and animal tissues he discovered a

close simularity between the permeability rate and lipoid solubility.

For materials which were insoluble in lipids (amino acids, sugars, elec-

trolytes) he postulated "adenoid activity." Adenoid activity meant some

undefined type of active transport (1).

This earlier work paved the way for the first formal membrane

theory postulated by Pfeffer around the turn of the century. Essentially

his theory contained two main points: 1. There is a continuous barrier

around cells. 2. This barrier (not the cytoplasm itself) limits the

rate at which materials enter and leave the cell (2).

Structurally this idea of a cell barrier was elaborated upon by

Davson and Danielli in 1935. Coupling the knowledge of solubility rate

with the newfound knowledge of the surface behavior of organic molecules,

they proposed the first molecular model of the cell membrane. In essence,

the model was a bimolecular layer of lipid sandwiched between layers of

protein (3). Development of the electron microscope seems to support

this model and today it is universally accepted. This theory was expanded

by Robertson in 1959. He suggested that the membrane was a bimolecular

layer of phospholipid whose fatty acid chains are pointed inward (perpen-

dicular to the plane of the membrane) and whose polar portions are

attached to protein or carbohydrate. This membrane morphology is uniform for all organisms although there may be wide variations in membrane chemistry. He uses the term unit membrane to convey this idea of universal membrane simularity (4).

The text of this thesis deals with transport across nerve membrane and herein it is important to comment on the ultrastructure of the excitable membrane. With the electron microscope, the structure appears similar to other membranes. Biochemically, it has some unique features. Most noteable is the presence of gangliosides which are very specific for neurons (5). It seems likely that gangliosides play an important role in the function of the neuron. Studies indicate that in the absence of oxygen the gangliosides lose sialic acid and electrical excitability ceases. Electrical excitability is restored if gangliosides are added to the inactive tissue (6). Work by Tasaki has shown that the nature of the action potential depends not upon the specificity of the cell membrane but instead upon the presence of the divalent cation Ca^{++} on the inside of the membrane. It seems that almost any monovalent cation will substitute for Na^+ and K^+ (7). A proposed mechanism, wherein the ganglioside bound Ca^{++} is replaced by K^+ and a conformational change occurs in the membrane creating the action potential, has been presented. This two state concept for the neuronal membrane is widely accepted today by authorities and can be found outlined in great detail in a paper by Albert Lehninger "The Neuronal Membrane" (8).

Although the membrane structure has been reasonably well worked out, the transport phenomena remains very nebulous. Early investigators added to the membrane a pore concept. These pores were aqueous channels which allowed the smaller hydrated K^+ to easily pass through but barred the passage of the larger hydrated Na^+. The advent of tracer technology negated this theory when investigators such as Heppell [9] and Steinback [10] demonstrated that the Na penetrated the membrane with apparent ease.

The catchall solution to the problem was and is the hypothetical sodium pump theory which states that there is some mechanism within the membrane which maintains Na^+ concentration against its concentration and electrical gradients. Many investigators postulate an enzymatic carrier mechanism to explain active transport. Perhaps the foremost investigator in this area is Skou. He has found in nerve, muscle, red blood cells, and other tissue membrane fragments, an ATPase which is Na/K activated. Although many aspects are not yet worked out, at least three generalizations can be made about the system. It is located in the membrane; it is dependent on the relative concentrations of Na and K and it is inhibited by the cardiac glycoside, ouabain. Skou concedes that many aspects of the active transport phenomena cannot be explained by the energy dependent carrier mechanism [11].

Some of these unanswered questions seem to rule out the possibility of the carrier mechanism as a thorough solution. How can one postulate the mechanism for active movement of Ca, Mg, amino acids and sugars? Many investigators feel that the energy requirements for such a pump

are too stringent. Levi and Ussing (12), Harris and Burns (13), and
Keynes and Maisel (14), all calculated that the amount of energy avail-
able to the pump fell far short of the amount necessary to drive it
(generally less than 20%) even assuming 100% efficiency. Another inves-
tigator, Gilbert Ling, has calculated that the energy requirement would
be 1500-3000 times the amount actually available. Ling is a major pro-
ponent of the nonmembrane theory and explains distribution of materials
in terms of fixed charges in the cytoplasm. He describes the protoplasm
as a protein fixed charge system believing that in such a system both
solvent and solute behave differently. Ling and others believe that
flux can be explained by four types of interactions: 1. Substances may
compete for a site. 2. Due to a lower free energy, one material may
actually facilitate absorption of the other. 3. Indifference because
the absorption constant of one is so much greater than the other.
4. At first the two species show indifference but lowering the concen-
tration of one reveals competition (15).

Purpose of this study. The correct explanation for the mechanism
of active Na transport is then yet to be demonstrated and so currently
one must accept pore, carrier, fixed charge theories or any combination
of the three according to his own academic fancy. However, this thesis
is concerned primarily with the fact that the Na ion is actively trans-
ported out of the excitable cell and seeks to explain gross movements
of the ion in an isolated tissue, the retina. In an attempt to locate
the sites of active transport and explain the nature of the Na elution
curve the following experiments will be discussed:

I.   The nature of the efflux of Na^{22} from the frog and rat retina.

II. The effect of temperature on the Na^{22} elution.

III. Na^{22} efflux analysis from the retina of animals whose retinal cells have been selectively degenerated by daily injections of sodium glutamate.

Structure and function of the retina. The retina is frequently used as a model for gray matter since embryologically it develops as an out-pocketing of the forebrain. Although modified for photoreception, the tissue is very useful in "in vitro" studies for a number of reasons. It is a thin (100 u-200 u) tissue yet durable enough to remove and work with. Since it is thin, test materials reach all the cells quickly. There is no problem of a blood-brain barrier (16). There are only three types of neurons and the current flow is unidirectional. If one is not concerned with the visual process, the retina is still a good model since only about 15% of the excitable cells are specialized for photoreception (17). Its viability "in vitro" can be demonstrated since ERG's can be recorded from the isolated retina for very long periods (18).

The structure of the retina is well established and has been schematized in Fig. 1. Typically the retina is divided into 10 layers. They are (from brain toward vitreous):

1. Pigmented epithelium.
2. Photoreceptor-outer-segments.
3. Outer limiting membrane.
4. Outer nuclear layer.
5. Outer plexiform layer.
6. Inner nuclear layer-bipolar cell bodies.
7. Inner plexiform layer.
8. Ganglionic cells.
9. Layer of optic nerve.
10. Inner limiting membrane.

There are 2 synaptic areas in the retina. One is the outer plexiform layer wherein the axons of the photoreceptors synapse with the bipolar and horizontal cells; the other is the inner plexiform where the horizontal and bipolar cells synapse with the ganglionic cells. Other cells in the retina would be primarily the neuroglial cells of Muller. The nucleus of this cell is found in the inner nuclear layer and the cell body extends downward to form junctions below the nerve tract. This is seen in Fig. 1. as the inner limiting membrane. Other processes from these cells push throughout all parts of the retina and form sheathlike coverings around the various neural elements. At the region separating the receptor outer segments these Muller processes come together to form tight junctional complexes which due to their density form the outer limiting membrane. As with other neuroglial elements in other tissues, the function of these cells has been greatly underestimated. It was thought that these cells were simply supportative in nature but it has been recently discovered that they are metabolically important to the neural elements. It is important to understand that the outer and inner limiting membranes are not true membranes but are simply junctions between cells. In this light, they do not function as limiting membranes and probably offer no barrier to solute flow through the tissue.

The site for triggering the nerve impulse seems to be the lamellae of the rod outer segments. These outer segments contain the visual pigment rhodopsin. Rhodopsin is a lipoprotein reasonably high in phospholipids. The pigment is easily degraded by light to the protein
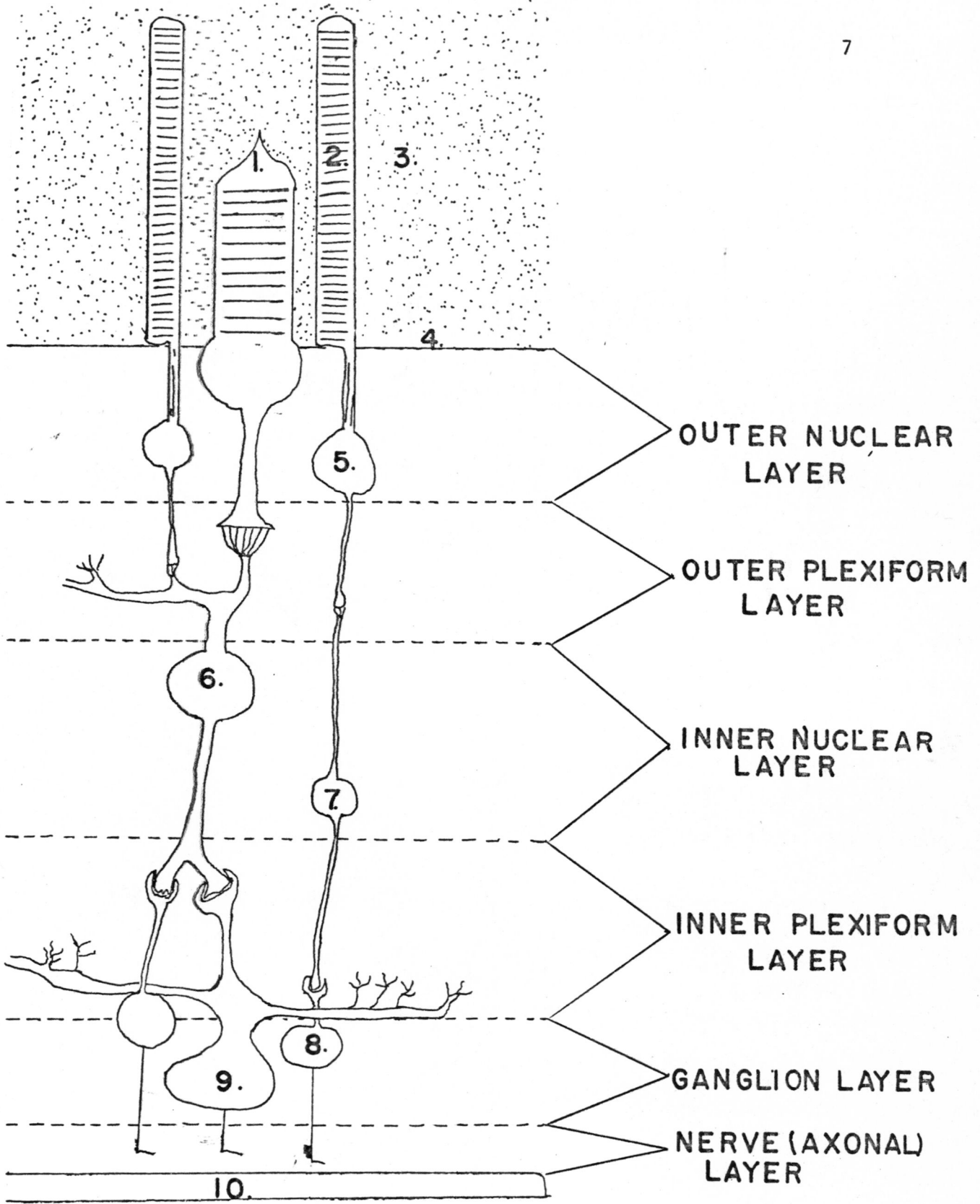
1. 2. 3.

4.

OUTER NUCLEAR LAYER

5.

OUTER PLEXIFORM LAYER

6.

INNER NUCLEAR LAYER

7.

INNER PLEXIFORM LAYER

8.

GANGLION LAYER

9.

NERVE (AXONAL) LAYER

10.

FIGURE I. Schematic illustrating the layers of the retina

opsin and vitamin A aldehyde. This degredation also involves a structural change. 11-cis retinene is transformed to all trans retinene (19). Beyond this point many theories are formed. It is not known how this reaction of rhodopsin actually causes an impulse to be registered. Yet ERGs from isolated retinas are routinely done. Wald feels that rhodopsin is a pro-enzyme which upon light stimulation bares its active sites (20). Bonting and Bangham have discovered high concentrations of a Na/K activated ATPase in the rod outer segments. This seems to indicate that the classical mechanism of the Na pump is operating in close association with the rhodopsin breakdown (21). These investigators believe that the structural change in rhodopsin causes a corresponding structural change in the disc membranes of the rod outer segments. The structural change would then be propagated to the brain.

METHODS AND MATERIALS

Control Studies. This thesis does not deal with vision, yet it was designed as some preliminary experiments whereafter the ionic events of photoreception could be better discussed. Toward this goal, test animals were dark adapted 24 hours. Retinas were excised under dim red light and experiments conducted in total or near total darkness. Under these conditions, the photoreceptors should still be functioning and thus should play an important role in the active Na transport. Because of this some summary comments follow on the triggering of nerve impulses by light.

Test animals were either rats or frogs. It has been demonstrated that the rat is an all rod animal and that the frog is also an all rod (or nearly all rod) animal. Hence, photochemistry of the cone can be omitted.

Frogs were used for all experiments except for the glutamate studies and here rats were used. The frogs (Rana pipiens) were purchased from Carolina Biological Supply in Burlington, N. C. and the rats were Holtzman strain. General procedure was the same for both frog and rat.

The dark adapted animal was sacrificed by: double pithing (frog); cutting the spinal cord with sharp scissors (rat). Immediately the eye lid and surrounding tissue was cut away and with 90 degree forceps the whole eyeball was gently lifted. The optic nerve was cut some distance from the eyeball. The eyeball was then placed in a small watchglass containing neural Ringer's solution.

Using very small iris scissors, an incision was made at the ora serrata and followed around the periphery of the cornea. The cornea and lens with most of the vitreous attached was then removed. Using 90 and 120 degree microforceps and working behind the retina next to the choroid, the connective tissue was gently severed (the plane of cleavage was between rod outer segment and the pigmented epithelium). This allowed the retina to float up much like a closed umbrella. It was then possible to reach under it and pinch the optic nerve. Once the optic nerve was cut, the retina could be floated out into the Ringer's solution. Both retinas were excised and the less damaged of the two was chosen for the experiment. The retina was then lifted in a folded position by an 18 gauge platinum hook. Initially it was felt that the metal hook might be depolarizing or otherwise damaging to the tissue, however, experiments using glass hooks seemed to show no difference. The retina was placed into a small flow chamber which held 12-15cc of neural Ringer's solution with approximately .5 micro curies of Na^{22} per cc. The entire operation was performed under dim red light (#2 Wrattan filter with 40 watt bulb) and took less than 5 minutes.

At least 2 factors are critical in the study of ion flux. One must approximate as close as possible the composition of extracellular fluid found in the living state. A neural Ringer's solution was made up in the following manner: NaCl 92.7mM, KCl 2.5mM, $CaCl_2$ 1.0mM, $MgSO_4 \cdot 3H_2O$ 1.2mM, $NaHCO_3$ 17.3mM, $NaH_2PO_4 \cdot H_2O$ 2.0Mm, $Na_2HPO_4 \cdot 12H_2O$ 1.2mM, Glucose 24.0 mM. This solution flowed past the retina at 10 cc/min.

The second important factor is having ample oxygen supplied to the tissue. Ames has done some studies which demonstrate the irreversable changes which take place during oxygen deprivation. He found the mitochondria become swollen. After 3 minutes the number of synaptic vessels at the rod inner segment were fewer. Those which remained were more irregularly shaped. Mitochondrial swelling was seen in the bipolar cells at the end of 3 minutes also. The same was true in the mylinated parts of the ganglionic axons. Similar changes were noted in glucose deprivation but they followed a slower time course and occurred to a lesser degree (22).

In order to assure oxygen saturation, pure oxygen was slowly bubbled into the Ringer's solution. In the experimental setup there were actually 2 reservoirs each containing 12-15 ml. Thus the total volume of radio-active solution available was approximately 28-30 ml. The second reser-voir was used occasionally to equilibrate 2 retinas simultaneously but its chief function proved to be as a site wherein oxygen and test materials could be added (see Fig. 2). Preliminary experiments indicate that the retina accumulates a maximum amount of labeled sodium at 1-1.5 hours so the equilibration time was set at 2 hours.

After equilibrating for 2 hours the retina was removed by hook and placed in a 2 cc aliquot of plain Ringer's solution for approximately 2 seconds. This 2-second dip helped to remove large quantities of the absorbed Na^{22} which might otherwise mask the true profile of the efflux studies. After dipping, the tissue was placed into another small flow chamber positioned above a Brinkman Linear 11 fraction collector and plain

Ringer's solution flowed through at 10 cc/min. The aliquots were contin-
uously collected with varying time intervals (smallest 2cc/12 seconds
and largest 20 cc/2 minutes). These aliquots were later counted for Na^{22}
in a Nuclear Chicago deep well scintillation detector. In experiments
of this type, it is best to express results in counts/gram of tissue.
This was impossible to do with the retina. At times a large drop of the
dip fluid would adhere to the tissue. Another problem was the vitreous
and aqueous humor. Varying amounts of these substances would adhere at
different times. Numerous attempts were made to correctly weigh the
retina (both wet and dry) however the results showed such wide variations
that all attempts were discontinued. The only alternative was the less
desirable method of expressing data in counts/time/whole retina. Of
course, some differences in retina weight should be expected from animal
to animal depending on age, size, etc. It is felt that this difference
would not significantly effect the efflux profile.

At the conclusion of each experiment, the retina was removed from
the flow chamber and the total Na^{22} in the tissue was counted. Gross
examination of the tissue was made and general appearance was recorded.
Frequently the tissue showed tears which were not apparent under the
dim red light. Small tears were disregarded since they should in no
way interfere with the function of the tissue.

Aliquots were taken for each sample and were counted for an arbi-
trary 2.5 minutes each. By adding to the counts remaining in the retina
the sum of the counts for the duration of the experiment, it is possible
to obtain the total amount of Na^{22} associated with the retina. It is

then possible to subtract from the total Na^{22} (A_t) the amount of Na^{22}

effluxed over a given time interval (A_{cum}) and obtain the amount of

Na^{22} remaining in the retina at a given time. This amount can be plotted

graphically as a function of time. The counts per whole retina were

plotted instead of per gram of tissue. It was not possible to obtain a

satisfactory weight for the whole organ because adhering vitreous and

saline proved to be uncontrolled variables.

If the Na^{22} efflux shows a simple diffusion coefficient when plotted

on semilog graph paper, it yields a straight line. If the diffusion is

not linear, then some type of complex process is operating. In order to

further standardize the curves, all data was plotted as per cent Na^{22}

remaining in the retina as a function of time ($A_t - A_{cum}/A_t$). This method

of plotting the data is equal to plotting relative specific activities.

It puts all graphs at 100 counts at 0 time and allows data to be super-

imposed for comparison. Although the data is in this manner forced into

a smoother pattern, it still shows a true profile of the raw data.

Thermodynamic study. The experiment was performed in water baths

using an external circulator and copper tubing. A Brinkman K2B Circu-

lator was used with a 1:1 water methanol mixture. The system consisted

of a series of open topped insulated aluminum cans. The cooling solu-

tion flowed through insulated hosing into the can where copper tubing

was used. One can contained the chamber with the radioactive Ringer's

solution, another can contained the plain Ringer's solution which was

used to wash the retina and a third can contained the flow chamber wherein

the retina was washed. The dip solution was placed into a refrigerator

before the experiment and was later transferred to one of the water

baths immediately prior to use.

The system allowed a wide range of temperatures in the individual cans for two reasons. The tops of the cans were open; and, secondly, wide temperature fluxuations occurred within the closed room containing the apparatus. The path of the cooling solution was from cooling unit to radioactive Ringer's solution to plain Ringer's solution to the wash chamber. The radioactive bath was always colder than either of the other two cans by several degrees. The difference could not be predicted from one experiment to the next. The minimum temperature to which the retina could be cooled while equilibrating was 5 C while the wash solution was 10 C. It should be pointed out that the temperature used in data analysis is the temperature of the flow bath and that the retina was equilibrated 2-4 C lower. Room temperature was generally 26-28 C.

Glutamate Study. Newborn rat pups were given daily injections of sodium -1- glutamate after the method of Potts. The pups were given daily I.P. injections from day two through day eighteen. The concentration of glutamate was increased daily starting with 2.2 mg/gm body weight and ending with 5.8 mg/gm body weight. Other pups were given placebo injections. The mortality rate of the placebo was around 25% while the treated animals was about 50%. Most of the pups were lost during the first 5 days of injections. The rats were not sacrificed until sometime after 3 months. After injections were discontinued, the pups exhibited normal behavorial patterns and in this manner could not be distinguished from their litter mates (control).

In order to determine the effectiveness of the treatment, ERG's were performed on the glutamate treated rats and the controls. The results are seen in Figure 5. The ERG is completely abolished except for some A wave and a slight rise that might be considered a B wave fragment. Microscopic examination was not done since the retinas were used to plot the washout curve. Excising the retina from the glutamate rat was a very tedious operation. The retina was in most cases so severely degenerated that it fragmented when touched. From a total of 18 eyes, only 4 remained whole enough to work with.

RESULTS

Control Curves. Data analysis of the efflux of Na^{22} in the manner
previously described seems to yield a three slope interpretation
($y=Ae^{-B}+Ce^{-Dt}+Ee^{-Ft}$). In Figure 2 a typical washout curve is shown dem-
onstrating the three compartments. The fast portion of the curve was
over at 4 or 5 minutes. The intermediate part occurred until 20-22.5
minutes. The third part-slow exponential exchange, occurred for the
duration of the experiment (total time 60 minutes or more). All curves
show this basic 3 slope design with some variations in slopes.

Reduced Temperature Curves. The results of the temperature studies
are seen in Figure 3. It exhibits a phenomena similar to that Ling (15)
finds in muscle. The three slopes disappear and the whole group become
more linear. Slope A is basically unchanged although it is slightly
slower (in all experiments) than at room temperature. This is contrary
to the findings of Mellon and Treherne (23). Slope C is practically un-
changed although slower exchange causes a higher percent of Na^{22} to
remain in the retina and thus extends its time course much beyond
that at room temperature. It is slope B that is radically changed. It
is practically lost as a separate function.

Glutamate Studies. The results of the glutamate studies can be
seen in Figures 4 and 6. Slope A remains about the same as control but
slopes B and C can no longer be determined. Slope A extends for about
10 minutes and then the remaining Na^{22} is quickly lost. The fast efflux
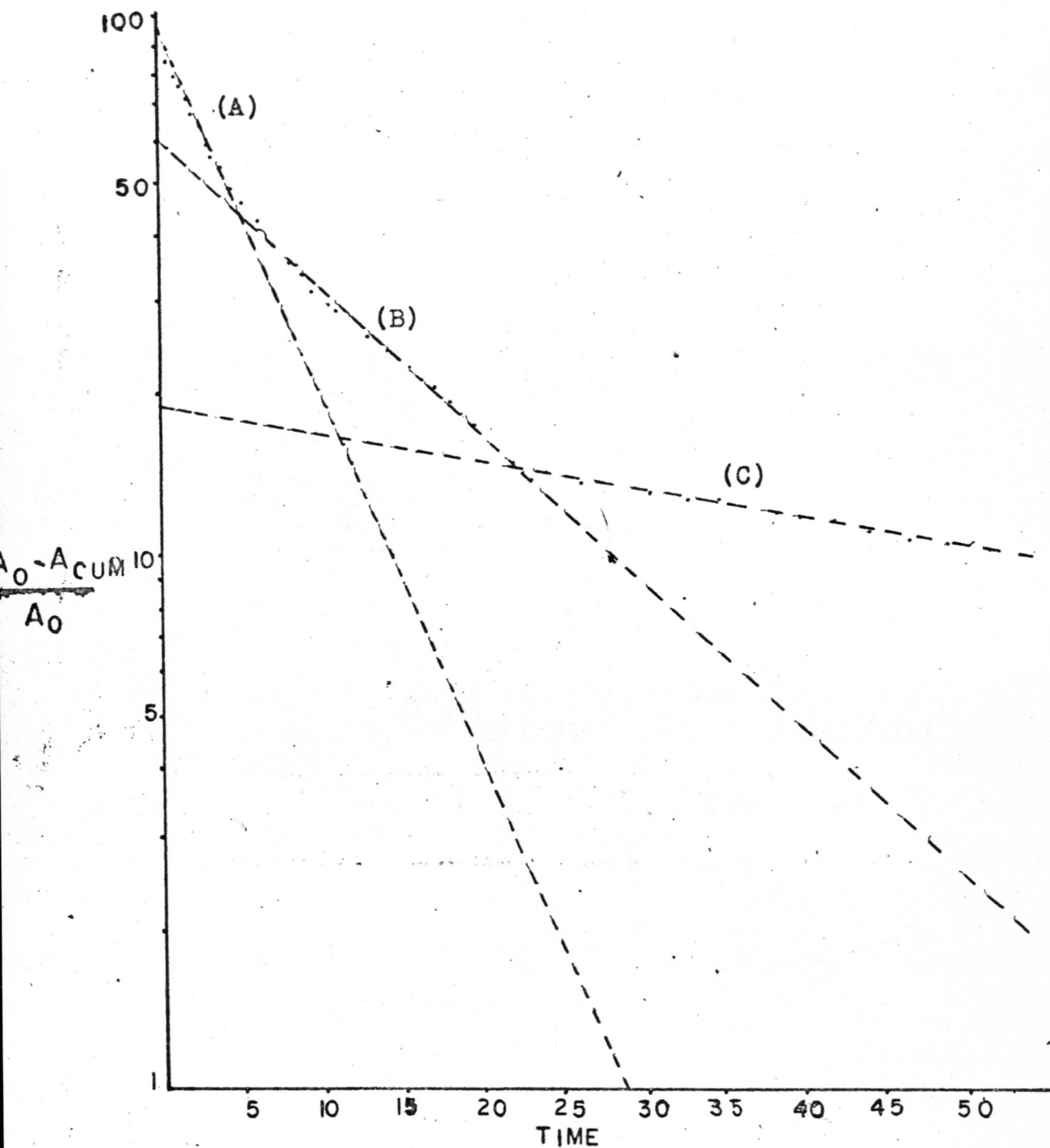showed no pattern of exchange but the time interval remained constant.

FIG. 2. Long washout Na^{22} curve of the isolated frog retina under control conditions. The A, B, and C portions have been manually extrapolated to 0.
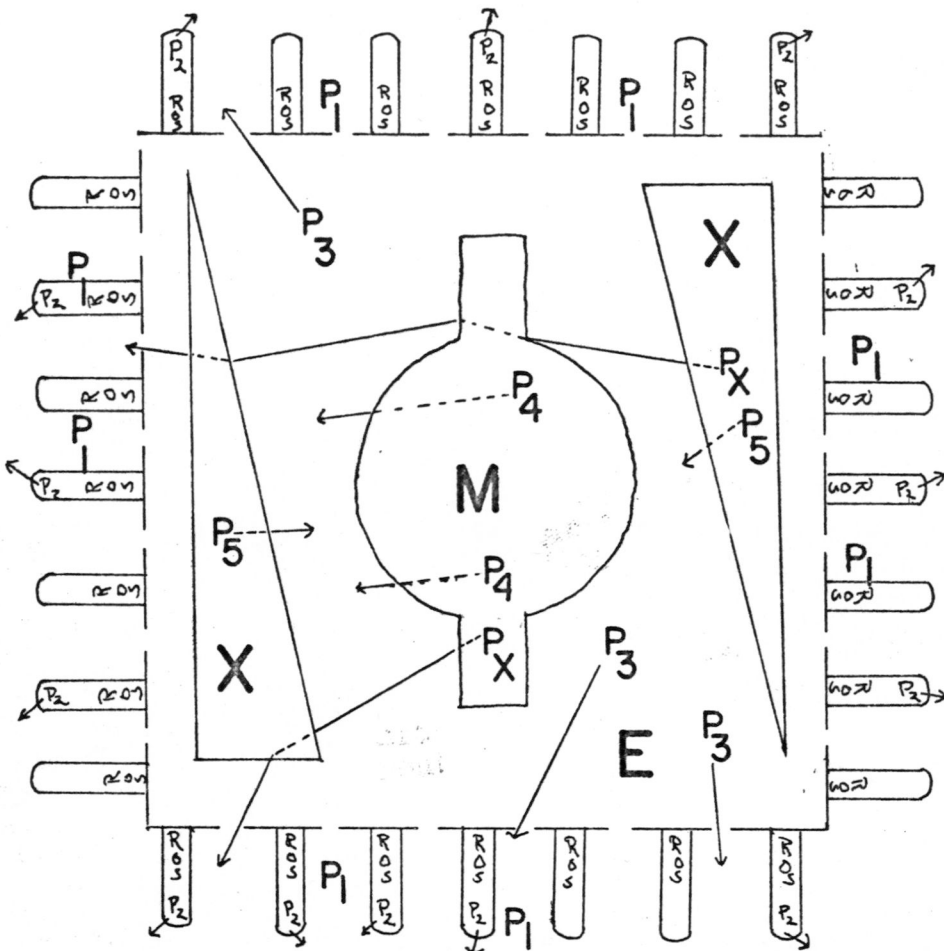
FIGURE 3. Model showing the exchange routes for
Na^{22} exiting the retina.

1. P_1-Adsorbed Na^{22}(all on the wet surfaces).

2. P_2-Na^{22} associated with the rod outer segments.

3. P_3-Extracellular Na^{22}.

4. P_4-Na^{22} associated with the various neural elements.

5. P_5-Na^{22} in intracellular sites primarily neuroglia.

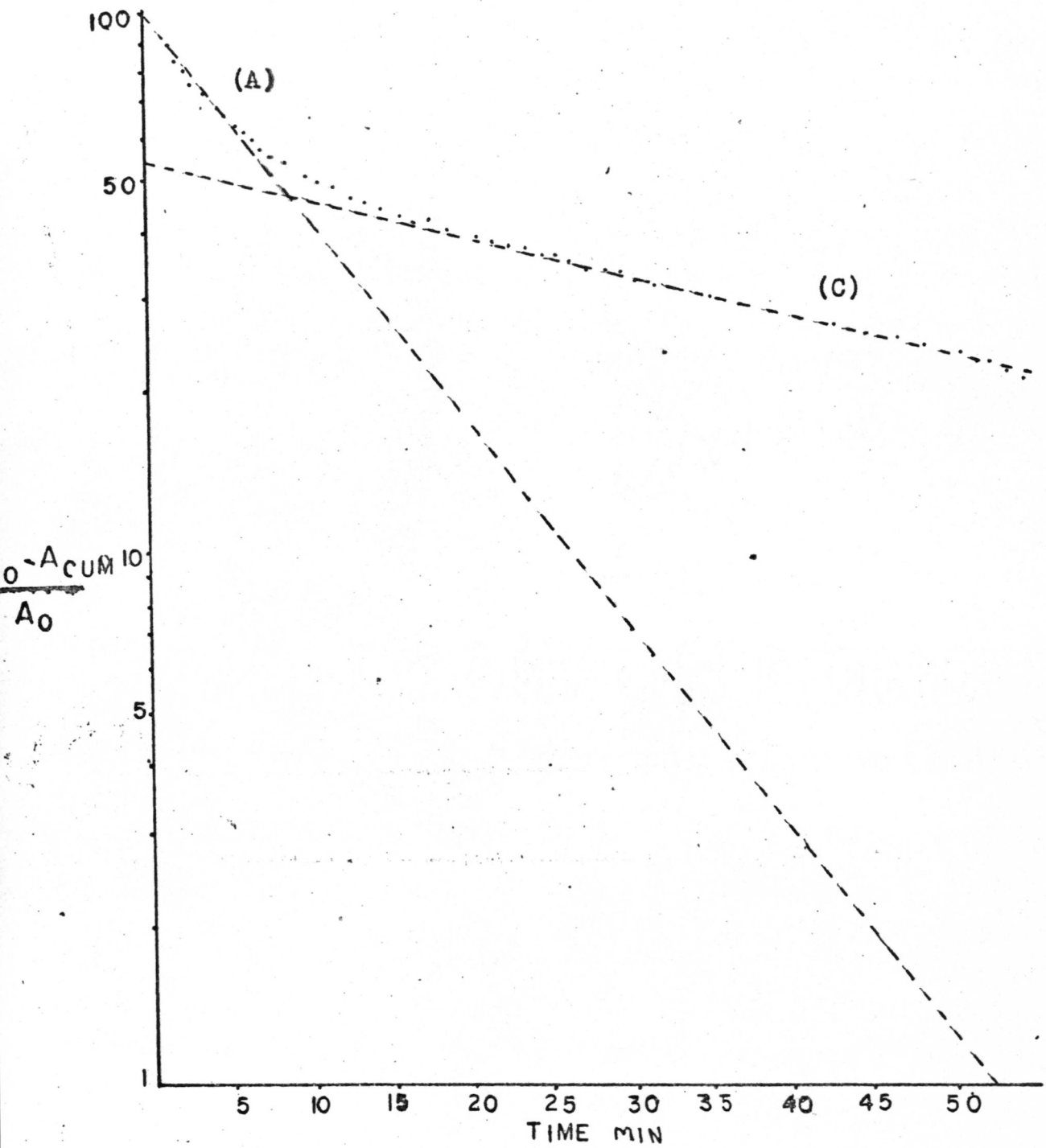6. P_x-Any Na^{22} which follows a complex absorption-reabsorption route.

FIG. 4. Long washout Na^{22} curve of the isolated frog retina with the temperature lowered to 14°C. The A and C portions of the curve have been manually extrapolated to 0.
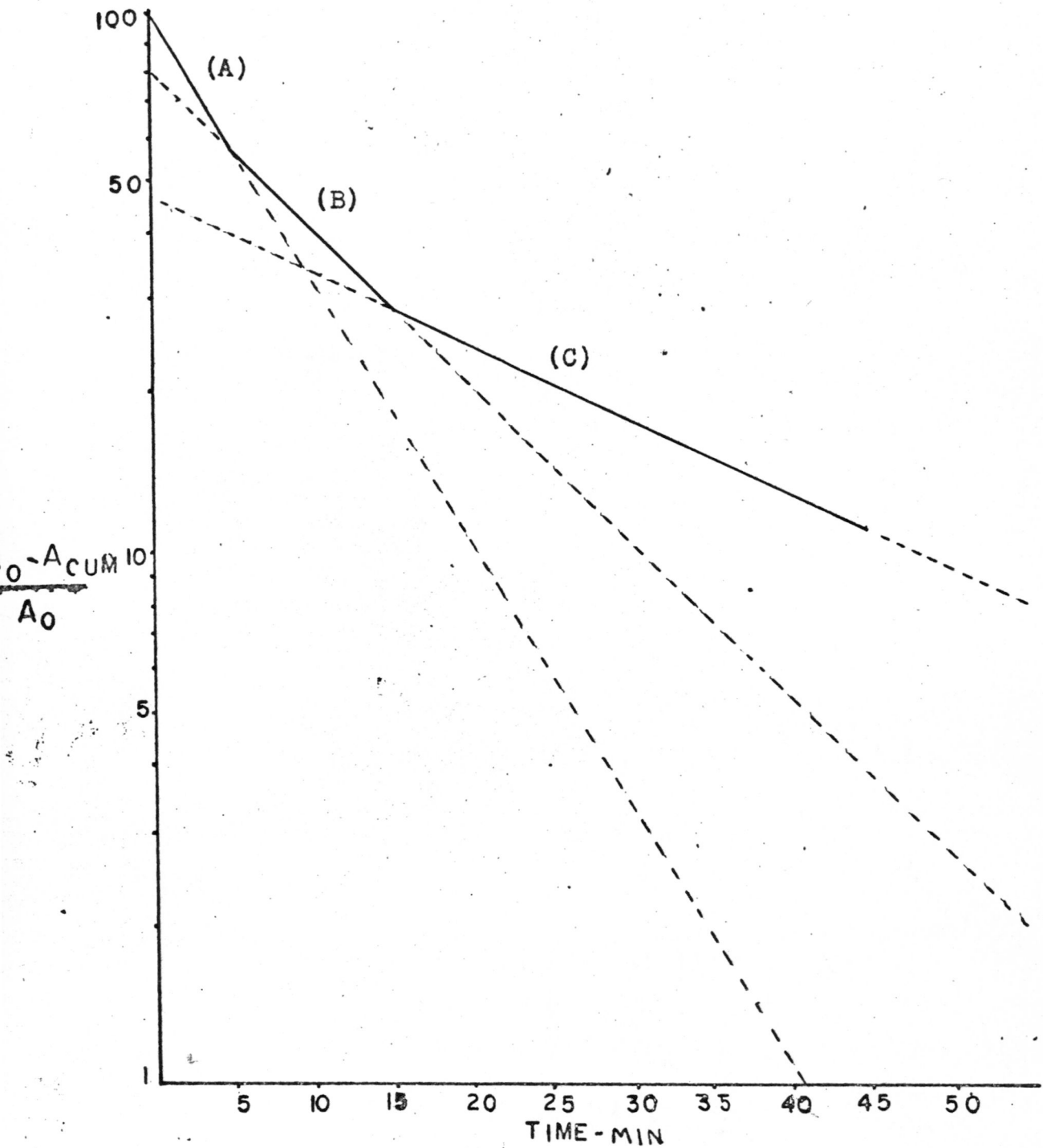
FIG. 5. Long washout Na^{22} curve of the isolated rat retina
under control conditions. The A, B, and C portions
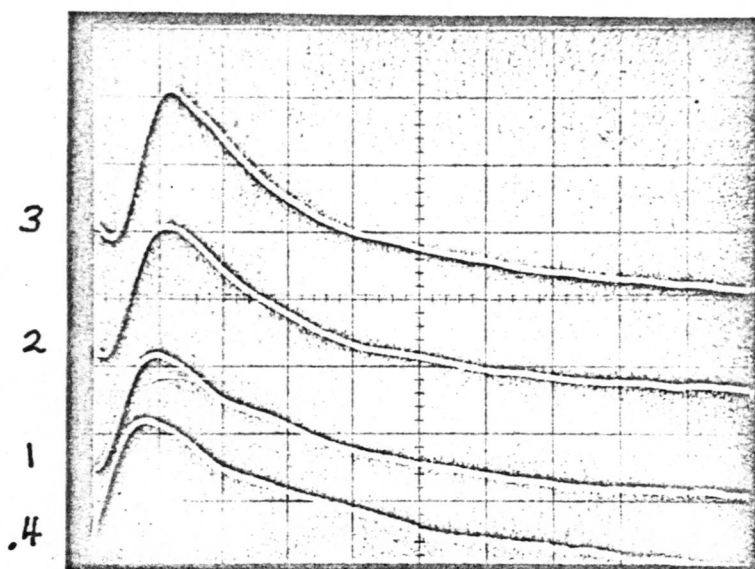of the curve have been manually extrapolated to 0.

Figure 6A. ERG from control rat. Sensitivity
6 x 10^3 mv/cm. Time 0.1 sec/cm.
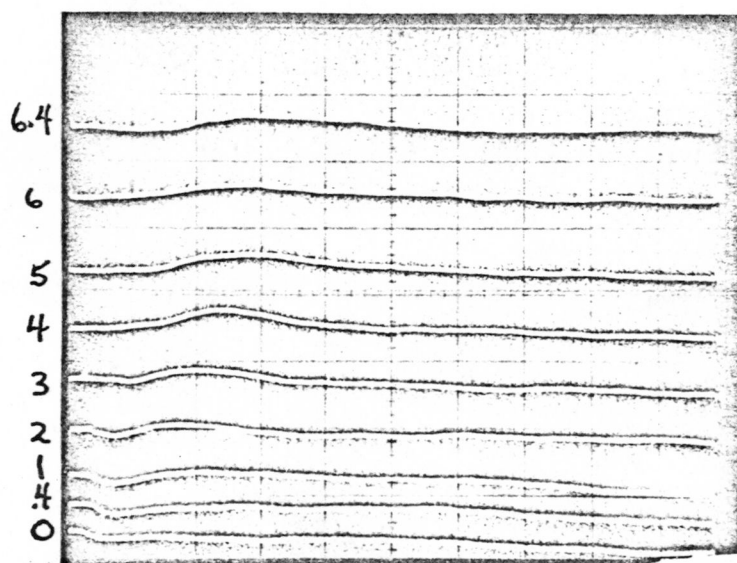Numbers on left margin indicate
neutral density filters.

Figure 6B. ERG glutamate treated rat. Sensi-
tivity 6 x 10^3 mv/cm. Time 0.05 sec/cm
Numbers on left margin are neutral
density filters.

DISCUSSION

General Comments. Profile analysis has been used extensively to
study ionic flux with radioactive tracers. Sodium has been studied
extensively in this manner using many different tissues. In muscle
and nerve the profiles are similar although there is considerable
difference in the interpretation of data. The major difference is
whether the efflux of Na^{22} follows a two slope curve. Keynes and Steinhardt
(24) and Frazier (25) using frog Sartorius muscle preparation have
described a two slope efflux. The fast phase is reasoned to come from
the sarcoplasm while the slow exponential decay is thought to be Na
exuding from the sarcoplasmic reticulum. The exact nature of this type
of efflux will be dealt with in more detail in the discussion of metabolic
inhibitors.

Gilbert Ling describes the efflux of Na^{22} from the sartorious
muscle as a three slope phenomena instead of a two slope one. The first
part is a very fast process which appears to be interstitial Na while
the last is a slow log diffusion--apparently Na which has become attached
to fixed sites within the cytoplasm. The second fraction occurs at a
rate intermediate to alpha or gamma. Ling believes that beta cannot be
dismissed as simply the change in rate between alpha and gamma and
demonstrates mathematically that it is not. If beta is simply the change
of rate between alpha and gamma, then by extrapolating the slope of gamma
and 0 and subtracting it from alpha and beta, a straight line should occur.
However, when this is done, a curvilinear relationship still persists
showing beta to be a real separate ellution process (26).

In the nervous system an analogous situation exists. Buckser has mentioned a three slope curve for the retina although not in any detail (27). Mellon and Treherne have discussed in detail a two slope analysis using axons in the central nervous system of Andonta cygenea (a freshwater bivalve). They state that the fast portion is initially complex but quickly becomes exponential with a halftime of 150 seconds. They postulate that the slow log diffusion is probably the escape of the intracellular ion fragment. The fast fraction is very hard to explain since reducing the temperature causes its rate to increase. This would indicate that the fast phase is a complex process not yet separated into its individual components (23).

Ames has done much ion transport work using the rabbit retina as a model. It must be mentioned at the onset that Ames works in the light, while the experiments in this thesis were done in total or near-total darkness. It was felt that maintaining the retina in the dark greatly enhances its excitability by keeping the photoreceptive (dendritic) endings operating. Under actual darkroom conditions, however, it is impossible to completely block all light and any pinpoint of light could cause a response in the photoreceptors and initiate firing in the bipolar, horizontal, amacrine and ganglionic cells. Ames describes in his work a two slope curve. The fast component of this curve occurs from 0-2 minutes while the slower phase persists for 24 minutes (termination of experiment). Using inulin to calculate the extracellular space, and gas phase separation, he has been able to analyze in detail the

component sites of the efflux. About one third of the fast component is due to surface wetting (absorbed) while the remaining two thirds is extracellular. The slow phase is then intracellular diffusion exchange. At the end of the initial 2 minutes, 95% of the labeled extracellular Na has exuded from the tissue while 8% of the intracellular Na has been lost. The intracellular Na^{22} constitutes 20% of the total Na^{22} (28).

Control Studies. The analysis was derived in the following manner. With a ruler the slow fraction (C) was extrapolated to 0. The same procedure was followed for the fast fraction (A). Between these two slopes a slope of best fit was drawn for the intermediate fraction (B). Time intervals were taken at points where slopes intersect. For a number of experiments slope C was subtracted from slopes A and B and the graph remained a curvilinear function. This demonstrates that fraction B does in fact exist. This was further illustrated by subtracting (B-C) from (A-C) and obtaining a straight line.

While time values for the slopes are close and reproduceable, values for percentile washout at a given time varied over a wide range. For this reason very little can be concluded from this particular set of data. Reasons for the wide variation come primarily from transfer techniques and/or adhering nonretinal tissue. Also, flow chamber dynamics may mask some process by dilution factors. Usually the retina floated off the transfer hook but on many occasions it adhered to the hook and had to be swished gently to free it. The result of this swishing, no matter how gently, is higher than normal counts in the first few aliquots. This changes both slope and percentile values. The same phenomena occurs when excessive amounts of vitreous or connective tissue remain attached to the retina, since the amount of extracellular Na^{22} is, in these cases, excessively high. In a few instances, fluid flow caused the retina to move and become caught in a vortex. This extra agitation also caused faster exchange. Since slopes were extrapolated from level parts of the curve, the time intervals for the exchange remain fairly constant.

Obviously the efflux is an example of complex absorption-reabsorption dillution processes acting simultaneously. To facilitate discussion, the major components will be broken down into the following processes:

1. P_1-Absorbed Na^{22} (all Na^{22} on the wet surface of the tissue).

2. P_2-Na^{22} associated with the rod outer segments.

3. P_3-Extracellular Na^{22}.

4. P_4-Na^{22} associated with the various neural elements.

5. P_5-Na^{22} in intracellular sites primarily in neuroglia cells.

6. P_x-Any Na^{22} ion which follows a complex absorption, reabsorption route.

There is some question as to whether the rod outer segments process exists. It has been shown that the rod outer segment contains a Na-K linked ATPase (21) but the actual exchange of Na across the lamellae has never been demonstrated. There is some question as to whether the depolarization occurs in the outer segment or further down the cell body. P_5 relates to the Na^{22} found on fixed cytoplasmic anionic sites primarily in Müller cells. Although the same sites are present in neural elements, the rate of ionic exchange in these cells is so great that they should be considered as a separate process. P_x occurs with some of the ions from P_3, P_4, and P_5. It is added to indicate that many ions are reabsorbed many times before leaving the tissue (i.e. it is possible for an ion which has been rapidly exchanged across a cell membrane to still require a long time constant to exit the tissue). Figure 7 shows a model with the mode of the various processes. With the skematic, slope A would be the result of $P_1+P_2+P_3+P_4+P_5+P_x$. The major component of this slope
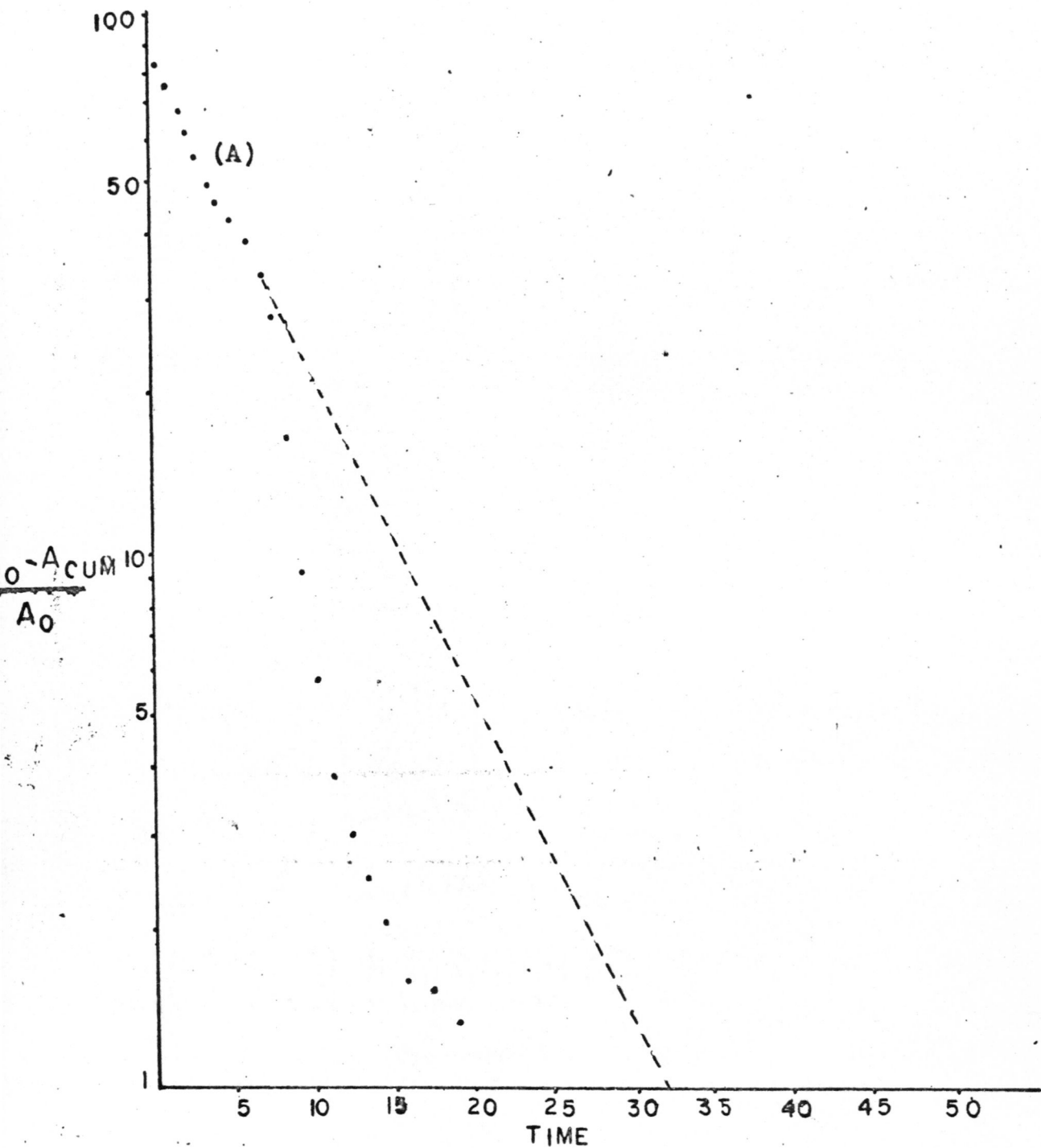
FIG. 7. Long washout Na^{22} curve of the isolated rat retina glutamate treated. The A slope has been manually extrapolated to 0.

would be P_1. This exchange would occur almost instantaneously with the unlabeled element. Next in magnitude would probably be P_3. This fraction of free Na^{22} would occur very rapidly also. Since the existence of P_2 is questionable, it is hard to weigh its relative contribution to slope A. The model shows the rod outer segment protruding above the plane of the tissue. This makes available an enormous surface area for ion exchange. Hence the ROS exchange should be very fast.

At the end of slope A, processes P_1 and P_2 should be exhausted, leaving P_3, P_4, P_5 and P_x. In contrast to these very fast processes the most logical choices for slow exponential decay would be P_5 and P_x. These ions bound inside nonexcitable membranes would offer very slow exchange while P_x would offer variable exchange rates directly proportional to the number of reabsorptions.

By deduction, slope B is a combination of $P_3+P_4+P_5$. P_3 would be the major component of the slope. The excitable membrane of the neural elements coupled with spontaneous firing of some of these elements would give a rapid exchange of Na^{22} but there would be some delay before it reached the outside of the tissue.

Although the profile data doesn't match closely the data of either Mellon and Treherne (23) or Ames (17), it is felt that it is more valid for several reasons. These investigators used both flame photometric analysis and radiotracer technique. In practically all cases with both techniques they used an isotonic sucrose solution while the data in this thesis was obtained using a neural Ringer's solution, comparable to the bathing medium in the living state. The fast portion of Mellon and

Treherne's graph after it has had the slow slope subtracted is curvilinear yet they simply call it a complex fast portion without trying to analyze their components. In Ames' work he does not always use darkroom conditions and, hence, turns off the photoreceptor mechanism. A big factor against Ames' data is the lack of a dip. This greatly alters the curve profile. In this thesis washouts were collected after a 2-second dip to remove absorbed Na^{22}. The dip aliquot frequently contained .5-.75 as many counts as the total counts of the retina. This is not considered in Ames' work and consequently accounts for the fact that 80% of the total sodium is lost in 2 minutes.

Already mentioned is the fact that isotonic sucrose solutions were used by both Ames and Treherne. It has been shown that a certain sodium concentration must be maintained in order to generate an action potential. The action potential of the frog muscle can be completely removed by a Na free medium (29). Hamasaki has found that in the frog retina, 95% Na replacement by choline or sucrose causes the ERG to be completely abolished (30).

Thermodynamic Study. Passive diffusion follows the Q_{10} rule closely.
That is, within a restricted range, a rise in temperature of 10 C
causes the efflux to proceed twice as fast and vice versa. Active trans-
port processes which are metabolically dependent would show a much
greater Q_{10} value (as great as 5). By reducing the temperature 10 C and
analyzing the profile of the Na^{22} efflux, some conclusions can be reached
which further define the compartments.

Results from other investigators are varied, Hertog and Ritchie
found in isolated c fibera a Q_{10} of 2.56 with a temperature change from
16 C to 26 C. In stimulation experiments they found that the net Na^{22}
influx was not affected by temperature change (31). Mellon and Treherne
found that when they lowered the temperature to 0 C, the slow efflux
was made even slower while the rate of escape in the initial fast phase
became even faster. This would serve to explain that the fast efflux
is extracellular but no explanation is offered for the rate increase
in the fast fraction. Ling found in frog muscle that at 0 C the complex
three phase curve tends to become straight due to the low rate of exchange
and states that the complex components are not apparent until 25 C (15).

Glutamate Study. The effects of sodium glutamate on the developing
retina was first discussed by Lucus and Newhouse in 1957. Using mice
for test animals, they microscopically demonstrated that daily injections
caused degeneration of the ganglion and inner nuclear layer. The degen-
erated retina consisted of epithelial cells, photoreceptors, neurologial
and resicual cells left from the degenerating cell layers. Microscop-
ically, the photoreceptors appeared in good condition (32).

Using the same technique of Lucus and Newhouse, Potts, et al studies the ERG of rats with degenerated retinas. They made some additional discoveries on the nature of the degenerative processes. The effects of glutamate are seen only with daily doseages given to suckling mice; adults were not affected. Injections from day two through day seven seem to give maximal results (ERG and microscopic analysis). The ERG was completely abolished except for the A wave which backs up existing information that the photoreceptors are the site of the A wave (33).

The most recent studies on the effects of glutamate were done by Hanson in 1969 using the scanning electron microscope. He found that the nerves of the inner nuclear layer and ganglion layer were lost causing the retina to be about .5 its original thickness. Nerve cells in general were greatly reduced in number and changed in structure. In most cases the cells were so changed that positive identification was impossible. Blood vessels were very few when compared to the control retina. The photoreceptors, epithelium and inner limiting membrane appeared to be normal. The rat retina was found to be much more sensitive to the glutamate treatment than the mouse retina (34).

The exact mechanism whereby the glutamate causes retinal degeneration is obscure. There is no simple reason for a normal metabolite causing such adverse affects. Potts et al theorized that an enzymatic reaction in which the end product is glutamate is repressed. Assuming that this reaction is necessary during the formation of the inner layers, the repression would subsequently cause degeneration of these layers.

Ames found that rabbit retinas when incubated "in vitro" with
varying concentrations of glutamate increased the total intracellular
water content. This was followed by a subsequent increase in intracell-
ular monovalent cation content. The amount of water and cation
increase is directly proportional to the concentration of the glutamate
(36).

CONCLUSION

The glutamate studies as well as the thermodynamic study seems to indicate that the initial fast portion of the Na^{22} efflux curve is predominantly extracellular and not energy dependent. It also demonstrates that slope B is a real function (not just the change in rate from A to C). Preliminary studies were done using metabolic inhibitors KCN and $NaIO_4$ as well as the cardiac glycoside Ouabain. The metabolic inhibors severely reduce metabolically dependent parts of the exchange, whereas Ouabain would block just the exchange of Na across the cell membrane. The results of these experiments are variable due to a very large experimental error. Yet they do seem to indicate that the slope A is unchanged throughout. They also show slope B to be most affected by the inhibitors and slope C to be most affected by Ouabain. These properties indicate that slope B is metabolically dependent and slope C is a diffusion. The data does not indicate which of the various tissue components are connected with the various parts of the slopes.

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