STUDY OF ION MOVEMENTS IN ISOLATED CHICKEN RETINAS DURING SPREADING DEPRESSION

Thesis by

Lee-Ming Kow

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1972

Abstract

Spreading depression (SD) is a phenomenon observed in several sections of vertebrate central nervous system. It can occur spontaneously or be evoked by a variety of stimuli, and consists of a wave of depression of the normal electrical activity of the nervous tissue which spreads slowly in all directions in the tissue. This wave of depression is accompanied by several concomitants including ion movements. All the concomitants of SD can be explained by an increase in the sodium permeability of the plasma membranes of cellular elements involved in this phenomenon.

In the chicken retina, SD is accompanied by a transparency change which can be detected with the naked eye. The isolated retina is a thin (0.1 mm) membrane in which the extracellular fluid quickly and completely equilibrates with the incubation solutions. This preparation was therefore used to study the ion movements during SD by measuring and comparing the ion contents and the extracellular space (ECS) of retinas incubated in various solutions of which some inhibited SD, whereas others allowed this phenomenon to occur.

The present study has shown that during SD there is a shift of extracellular sodium into the intracellular compartment of the retina, a release of intracellular K and a decrease in the magnitude of ECS.

These results are in agreement with previous postulates about SD, although the in vitro experimental condition makes the ion movements appear larger and the loss of ECS smaller than observed in the intact cortical tissue. The movements of Na and K, in opposite directions, are reversible. The development and magnitudes of SD is very little affected by deprivation of the oxygen supply.

It was established that the inward sodium shift is not a consequence of an arrest of the Na-pump. It can be prevented, together with SD by the membrane stabilizers, magnesium and procaine. Spreading depression and the ion movements are incompletely inhibited by tetrodotoxin, which blocks the sodium influx into nerve fibers during the action potential. The replacement of Na in the bathing solution by Li does not prevent SD, which is accompanied by Li accumulation in the intracellular compartment. From these experiments and others it was concluded that the mechanism underlying SD and the ion shifts is an increase in the sodium permeability of cell membranes.

Introduction

During an investigation of experimental epileptiform after-discharges, Leão (1944a) found that a spreading wave of depression of cortical activities could be triggered either by weak electrical or slight mechanical stimulation on any region of the exposed cerebral cortex of the rabbit, except the retrosplenial area. He called this phenomenon "spreading depression" (hereinafter designated as SD). Since then SD has been extensively investigated, and has become an established phenomenon of cortical physiology. The earlier investigations have been excellently reviewed by Marshall (1959) and Ochs (1962).

During SD the front of the depression wave spreads slowly (2 - 5 mm/min) from the triggered locus in all directions with little regard for the anatomical and functional features of the cortex. The time course of SD was the same, no matter how and where it was triggered. Later, Leão and Morison (1945) found that SD could also be elicited chemically with KCl. They found furthermore that this phenomenon was independent of subcortical connections, and was not affected by a brief (less than one minute) period of anemia, which arrests synaptic transmission. From the above observations and the slowness of the propagation they concluded that SD is not dependent on neuron-to-neuron transmission. Another electrical event, the slow potential change (SPC) was found by Leão (1947) to accompany SD. This SPC is a biphasic phenomenon. First the cortical surface becomes negative with respect to an indifferent electrode. After 2 - 3 min the polarity reverses and

the cortical surface becomes positive for 3 - 4 min. The magnitude of the negative (5 - 10 mV) phase is larger than that of the cortical positivity (1 - 2 mV). The characteristics of SD mentioned so far were confirmed by other investigators (Marshall, Essig and Dubroff, 1951; Van Harreveld and Stamm, 1951; and many others).

Since its first discovery by Leão, SD has been observed in the neocortex of all animals studied. Because of its ubiquitousness and the ease with which it can be evoked especially under unfavorable experimental conditions such as dehydration (Marshall, 1950) or lowered temperature (Marshall et al., 1951), SD must have confused or masked many observations on the electrophysiology of the cortex. For example, prior to the discovery of SD, Marshall, Woolsey and Bard (1937) observed the disappearance of cortical responses to peripheral tactile stimulation for short periods of time (1 to 5 minutes) for which no obvious explanation could be found. Many authors believed at that time that depression of the activity of certain cortical areas could be achieved by stimulating other regions, the so-called "suppressor areas." Presumably this "suppression" was transmitted through specific nervous tract. However, Dusser de Barenne and McCulloch (1941) found that "suppression" did not require the underlying fiber tracts, and could be evoked by stimulating unrelated cortical areas. They, therefore, concluded that suppression is independent of cortico-cortical connection, a conclusion conflicting with the original concept of this phenomenon. By making careful comparisons between suppression and SD,

Sloan and Jasper (1950) argued that most of the observations described as suppression were actually SD, and Marshall (1950), taking SD into consideration, was unable to find any "suppressor area" in the cat's cortex. Apparently, SD has confused many electrophysiological studies on the cerebral cortex, and has to be taken into consideration and avoided whenever cortical activity is to be observed. This caution not only applies to the cerebral cortex but to several other brain structures, for SD can also be evoked in the hippocampus (Leão and Martins-Ferreira, 1958; Monakhov, Fifkova and Bureš, 1962), the nucleus caudatus (Martins-Ferreira and Leão, 1958), the corpus striatum (Martins-Ferreira and Leão, 1958; Ookawa and Bureš, 1969), the retina (Gouras, 1958; Martins-Ferreira and de Oliveira Castro, 1966), the optic lobes of chickens (Ookawa and Bureš, 1969) and probably other structures. This is a troublesome side of SD for brain physiological research. On the positive side, its characteristic depressing effect has been used as "functional decortication" in the study of brain functions concerned with behavior (Bureš and Burešová, 1960a, b; Albert, 1966; Burešová and Bureš, 1969; etc.), and drug effects (Bohdanecký and Nečina, 1963). Spreading depression has furthermore been associated with migraine (Milner, 1958; Basser, 1969) and Jacksonian convulsions (Sloan and Jasper, 1950) because of the similarities in the manner and velocity of propagation.

After earlier descriptive stages, SD has drawn considerable attention which led to the discovery of other electrical, morphological

and chemical concomitants. An increase in cortical resistivity during SD was first discovered by Leão and Martins-Ferreira (1953) and confirmed later by many other investigators (Freygang and Landau,1955; Van Harreveld and Ochs, 1957; Ranck, 1964). These investigators found that SD was accompanied by a 15 to 20% increase in cortical resistivity. Even larger increases in tissue impedance were recorded during asphyxiation of the brain (Leão and Martins-Ferreira, 1953; Freygang and Landau, 1955; Van Harreveld and Ochs, 1956). After a latency of 1 - 3 min the resistance of the asphyxiated tissue may double in the ensuing 2 to 4 min. As in SD, this impedance increase was accompanied by a SPC. As will be described below, these two phenomenon, SD and the asphyxial changes, have so many concomitants in common that it can be concluded that they are caused by very similar mechanisms.

It was proposed by Van Harreveld and Ochs (1956) that the impedance increase in the brain would be due to a loss of extracellular electrolytes. This concept was based on the investigations of Fricke (1924) and Cole (1940) on the impedance of cell suspensions. Fricke used erythrocytes in serum and Cole sea urchin eggs in sea water for this purpose. The latter system has some advantages because of the simple spherical geometry of the marine eggs. Cole based his investigation on an equation derived by Maxwell in 1873 dealing with the specific resistance of a suspension (r) of a certain volume percentage (ρ) of spherical bodies of a resistance (r_2) in a medium of a resistance (r_1).

$$\frac{1 - r_1/r}{2 + r_1/r} = \rho \frac{1 - r_1/r_2}{2 + r_1/r_2} \qquad (1)$$

Cole showed that the volume percentage of the sea urchin eggs could be computed with Maxwell's equation (equation 1) assuming that the eggs had an infinite resistance. The equation can then be simplified as:

$$\frac{1 - r_1/r}{2 + r_1/r} = \frac{\rho}{2} \qquad (2)$$

making it possible to compute the volume percentage of cells from the specific resistance of the suspension and the suspending medium.

The assumption that marine eggs can be considered to have an infinite resistance needs some explanations. Cole (1928) modified another equation of Maxwell's for the specific resistance of a spherical body with a core resistance (r_3) and a radius A surrounded by a shell of a resistance (r_4) (expressed in ohm/cm^2). The specific resistance of the entire spherical body (R) can be computed with the following equation.

$$R = r_3 + \frac{r_4}{A} \qquad (3)$$

This equation shows that the resistance of such a particle is dependent not only on the resistance of the core and of the shell but also is inversely proportional to the radius of the particle. Since marine eggs are small (a radius of 36 μ, Shapiro, 1935) their resistance is high (27,900 ohm/cm, Van Harreveld, 1966) even though the resistance of the cell membrane (shell) is not particularly high (about 100 ohm/cm^2, Cole, 1942). Because the specific resistance of sea water is only

20 ohm/cm, the assumption of an infinite resistance of the eggs does not seem unreasonable.

Like cell suspensions, tissues including those of the central nervous system consist of extracellular fluid as suspension medium, and cells surrounded by cell membranes which impede ion movements and thus have a high resistance. The size of the cellular elements in the central nervous tissue are in general very small, giving them a high specific resistance, whereas the extracellular fluid probably does not have a specific resistance much higher than that of plasma or cerebrospinal fluid (CSF). The measuring current in a tissue is therefore mainly carried by extracellular electrolytes. Large losses in tissue resistance, as occur during SD and asphyxiation, can therefore be explained by a loss of extracellular electrolytes.

Central nervous tissue is abundantly vascularized, containing numerous vessels filled with blood which can be regarded as part of the extracellular compartment. Since blood has a greater conductivity than the tissue itself, a change in the filling of the vessels can be expected to change the impedance of the tissue. Therefore, in considering the mechanism of the impedance increase during SD the possibility that vascular changes are involved has to be considered. Changes in the vascular system during SD were discovered by Leão (1944b), and have been observed by several authors since. Most of the evidence shows that vasodilation occurs during SD (Leão, 1944b; Van Harreveld and Ochs, 1957; Burešová, 1957), although an initial vasoconstriction

for a brief period (Van Harreveld and Stamm, 1952) and a decrease in blood flow (Sonnenschein and Walker, 1956) have occasionally been observed. The vasoconstriction and the drop in blood flow will decrease the volume of the well conducting blood in the vessels and can therefore be expected to result in an increase in the tissue impedance. However, the drop in blood flow does not always occur during SD, and therefore cannot account for the very consistent impedance increase during this phenomenon. Furthermore, the duration of the initial vasoconstriction during SD is of too short to account for the impedance increase (Van Harreveld and Ochs, 1957). Finally, the blood in the cortical vessels contributes only about 5% to the conductivity of the cortex (Van Harreveld and Ochs, 1956), and its volume change is therefore too small to cause the 15 - 20% loss in conductivity observed during SD. The vasodilation, which was more often observed during SD, on the other hand would cause a decrease in tissue resistance, and is apparently masked by the impedance increase caused by another mechanism.

Since a change in the vascular system cannot account for large impedance increases, a loss of extracellular electrolytes seemed to be the most likely mechanism underlying the impedance changes observed during SD and asphyxiation. The predominant extracellular electrolyte is NaCl. In order to account for the pronounced and rather rapid impedance increase, a large part of the extracellular NaCl would have to be lost in a short period of time (few minutes). Such a loss could be caused by a transport of NaCl from the extracellular- into

the intracellular space (ICS) where the electrolytes are surrounded by cell membranes, or by removal by the circulatory system. Between the extracellular fluid and the blood in the cerebral circulatory system there is a blood-brain barrier, which impedes the movement of the electrolytes, as well as many other compounds, into and out of the brain. Because of the presence of this barrier it is unlikely that a large part of the extracellular NaCl can be removed by the circulatory system in a short period of time. Besides, such a removal of extracellular NaCl would have to be accompanied by a water movement to maintain osmotic equilibrium, and therefore by a loss of tissue volume. No evidence for such a volume decrease during SD or asphyxiation has been found. The possibility that the loss of tissue conductivity is not due to the loss of extracellular electrolytes but to the immobilization of ions has been considered. However, it is difficult to conceive of a mechanism which could account for a sudden immobilization of a large amount of extracellular NaCl (Van Harreveld and Ochs, 1956). As the most likely mechanism for the observed impedance increases during SD and asphyxiation there rests a movement of extracellular NaCl into the ICS.

A movement of extracellular Cl^- into the ICS during SD or asphyxiation has been demonstrated. Using a histochemical method to locate the chloride in the tissue, Van Harreveld and Schadé (1959) found that in the normal, oxygenated cerebral cortex Cl^- was rather evenly distributed, while in the asphyxiated or SD invaded cortex the

Cl^- was concentrated in apical dendrites. The retrosplenial area, which, as mentioned earlier, is not invaded by SD, did not show the Cl^- accumulation in apical dendrites. The uneven Cl^- distribution was more pronounced in the asphyxiated cortex than in that invaded by SD. Apparently, the movements of extracellular Cl^- into the ICS of the neuronal elements is a concomitant of SD, as well as of asphyxiation. Such a loss of extracellular electrolytes to the ICS is in agreement with the observed increase in tissue impedance during asphyxiation and SD, and must be accompanied by a movement of water into the tissue elements involved to maintain osmotic equilibrium.

Freeze substitution, which can preserve the water distribution of central nervous tissue better than chemical fixation methods, was used by Van Harreveld (1957, 1958) to detect changes in the size of apical dendrites in the cerebral cortex during asphyxiation and SD. The diameters of these dendrites were compared in cortices frozen while SD was in progress and in control cortices not invaded by SD. In the same way asphyxiated and normal, oxygenated cortices were compared. It was found (Van Harreveld, 1957) that the impedance increase caused by circulatory arrest was accompanied by an appreciable increase in the diameter of the apical dendrites. A similar increase was demonstrated during SD (Van Harreveld, 1958), although the increase is less than that caused by asphyxiation. The increase in diameter of apical dendrites was well correlated with the time course of SD. The increase was largest at the site where the maximum impedance

increase, as the index of SD, was recorded, and became smaller as the cortex was recovering from SD.

The diameter, and hence the volume increase of neuronal elements strongly supports the postulate that during SD and asphyxiation a shift of extracellular material into the ICS occurs. This was further supported by an electron micrographic investigation by Van Harreveld and Khattab (1967). Again using freeze substitution to preserve the electrolytes and water distribution in central nervous tissue they found in electron micrographs of cortices frozen during SD (as indicated by the presence of a SPC) less ECS than in those of normal control tissue. A swelling of cellular elements, mostly dendritic structures, in the cortex invaded by SD was also found. Furthermore, restoration of the original water distribution was demonstrated as the cortex recovered from SD. These observations provided further evidence that SD is accompanied by a transport of extracellular material into cellular elements resulting in an increase in the ICS at the expense of the ECS, and that the transport and the reciprocal volume changes are reversible. A similar loss of ECS and swelling of tissue elements was found to occur during asphyxiation (Van Harreveld, Crowell and Malhotra, 1965; Van Harreveld and Malhotra, 1967).

The demonstated uneven distribution of Cl^- and the reciprocal volume changes in the ICS and the ECS during SD and asphyxiation suggest that during these phenomena there is a shift of extracellular NaCl into the ICS. This can be explained by considering the membrane

properties of nervous tissue elements. In the resting state the cell membrane of nervous tissue is permeable to Cl^- and K^+. The latter ion is abundantly present in the ICS and hence is the dominant intracellular cation. Experiments with labelled sodium have shown that the resting membrane is also permeable for Na^+. The permeability is low, however, so that in the resting state only a small amount of extracellular Na^+ can diffuse into the ICS. These Na^+ are rapidly transported back into the ECS by an active ion transport system or ion pump. In this way the intracellular Na^+ concentration is kept low, even though the extracellular concentration is high and the membrane is actually permeable for Na^+. Therefore, functionally, the resting cell membrane is impermeable for Na^+. The cell membrane, both in the resting and the excited states, is considered impermeable for large intracellular organic anions, such as proteins.

The resting central nervous tissue can therefore be considered as consisting of two compartments, the ICS and the ECS, separated by a membrane permeable for K^+ and Cl^- but impermeable for the intracellular organic anions and Na^+, most of which is present in ECS. Under these conditions, the impermeable intracellular anions cannot exchange with the permeable extracellular anion, Cl^-, and the impermeable extracellular Na^+ cannot exchange with the permeable cation, K^+, in the ICS. Due to the concentration gradients, the K^+ tend to diffuse into the ECS, and Cl^- into the ICS. Since ions are electrically charged, the concentration gradients will create a

potential difference across the membrane which counteracts the tendency of the ions to move along the concentration gradients. Finally the two counteracting factors, the membrane potential and the diffusional forces, will reach an equilibrium at which there is no net ion movement. Because there are two species of impermeable ions, this situation has been called a "double Donnan" equilibrium. The potential difference across the membrane at this equilibrium, E, can be expressed as

$$E = \frac{RT}{F} \ln \frac{[K^+]_I}{[K^+]_E} = \frac{RT}{F} \ln \frac{[Cl^-]_E}{[Cl^-]_I} \qquad (4)$$

where R represents the universal gas constant; T the absolute temperature; F the Farady constant; $[K^+]_I$ and $[K^+]_E$ the intracellular and extracellular K^+ concentrations, respectively, and $[Cl^-]_I$ and $[Cl^-]_E$ the intracellular and extracellular Cl^- concentrations. This membrane potential, as mentioned before, counteracts the diffusion due to the concentration gradients of intracellular K^+ into the ECS and of extracellular Cl^- into the ICS and favors the movements of cations from the ECS into the ICS. However, in the central nervous tissue there is no true equilibrium, because the ion distribution depends on the ion pump to transport Na^+ that diffused into the ICS back to the ECS. Since the Na^+ concentration is higher in the ECS than in the ICS, and the resting membrane potential favors the inward movement of cations, the ion pump has to transport Na^+ against both the electrical and the chemical gradients, and hence needs energy.

The resting double Donnan equilibrium will be disturbed and ion

movements will occur when during SD or asphyxiation the membrane permeability for Na^+ is increased beyond the ability of the ion pump to return this cation back to the ECS or the sodium pump is arrested, for instance, by depriving it of the necessary energy. The increase in Na permeability creates a situation studied by Donnan in which two compartments containing different electrolytes are separated by a membrane which is impermeable for one of the ion species contained in one of the compartments. In the present case the membrane becomes permeable for K^+, Cl^- and Na^+ but would remain impermeable for the intracellular anions. Donnan showed that under such conditions an equilibrium is reached when the products of the concentrations of the anions and cations which can pass through the membrane are equal in the two compartments. Since one of the compartments contained originally only one permeating ion species this can be achieved only by the diffusion of an equivalent amount of cations and anions into this compartment. This results in an increase in osmotic pressure in this compartment which in Donnan's case was balanced by a hydrostatic pressure difference between the compartments. Since the development of a significant pressure gradient between the ECS and the ICS in a tissue is impossible, the expected inflow of NaCl into the intracellular compartment will have to be accompanied by a movement of water in the same direction.

There is considerable evidence for this ion and water movement during SD. The accumulation of Cl^- in intracellular structures was

demonstrated with a histochemical method for this ion. The loss of extracellular NaCl explains the impedance increase, and the shift of water into the ICS accounts for the swelling of certain cellular elements during SD. The increase in membrane permeability for Na^+ and the inward shift of this ion will depolarize the resting membrane potential. Depolarization of cellular elements has indeed been demonstrated in intracellular records made during SD (Morlock, Mori and Ward, 1964; Brožek, 1966; Collewijn and Van Harreveld, 1966; Karahasi and Goldring, 1966). The depolarization was always marked, sometimes almost complete. It developed quickly at the beginning of SD and the membrane potential returned to the resting level slowly during the recovery of the tissue. This depolarization may explain SPC observed during SD and asphyxiation.

The normal membrane potential prevents K ions from moving from the ICS into the ECS. Potassium will therefore tend to move outward as the depolarization develops and to exchange for extracellular Na^+. A release of intracellular K^+ during SD can therefore be expected.

By postulating an increase of the Na^+ permeability of plasma membranes, all the concomitants of the SD described above can be explained. As mentioned above the increase in Na^+ permeability may be a consequence of an arrest of the Na^+ pump. However, the resting membrane Na^+ permeability, which is counteracted by the pump, is low so that the Na shift caused by an arrest of the Na^+ pump will probably be too slow to account for SD. It has therefore been proposed that

SD is caused by a substantial increase in Na^+ permeability of the cell membrane leading to an inward Na shift.

The expected K^+ release during SD was first postulated by Grafstein (1956). She found that the invasion of the cortex by SD was preceded by a burst of neuronal activity. Such an intensive neuronal activity would cause a release of intracellular K^+ from the cellular elements involved in SD into the ECS. An accumulation of K^+ in the ECS would then cause the depolarization of adjacent cells and fibers resulting again in intensive neuronal activity and release of more K^+, which in turn would depolarize more distant elements, and so on. This chain-reaction of intensive neuronal activity and K^+ release was hypothesized by Grafstein (1956) as the mechanism underlying the propagation of SD.

According to Grafstein's hypothesis an amount of intracellular K^+ sufficient to depolarize tissue elements has to be released into the ECS during SD. Evidence for such a release has been found independently by two groups of investigators using very similar methods. Brinley, Kandel and Marshall (1960b) washed the surface of the cerebral cortex, which had previously been charged with radioactive ^{42}K, with Ringer's solution, and assayed the amount of ^{42}K in the washings before, during and after SD. The other group (Křivánek and Bureš, 1960) did not use labelled potassium for this purpose. They washed the cortical surface with a potassium-free solution and measured the amount of potassium washed out before and during SD. Both groups

found that intracellular K^+ was released from the depressed cortex in an amount that seemed sufficient to trigger SD. This evidence, which supported Grafstein's hypothesis, is also compatible with the Na^+ shift postulated above.

Grafstein's hypothesis about the propagation of SD was adequate before the shift of extracellular material into the ICS during SD was observed. As discussed above this shift can be explained by an increase in Na^+ permeability of the cell membrane. Grafstein's hypothesis does not account for such a permeability change.

There is, however, in the central nervous system a compound which has been shown to cause a marked increase in membrane permeability. This compound is the dicarboxylic amino acid, glutamate. Glutamate is present in considerable quantities in brain tissue (10 mM/kg w.t., Schwerin, Bessman and Waelsch, 1950; Berl and Waelsch, 1958) and has been shown to cause firing of nerve cells by depolarizing cell membranes (Curtis, Phillis and Watkins, 1960). This depolarization is due to an increase in ion permeability of the membrane. The release of this amino acid into the ECS could explain not only the firing of tissue elements which, as proposed by Grafstein, would be involved in the propagation of SD but also all the concomitants of SD mentioned above.

The postulate that propagation of SD is due to a release of glutamate from the ICS is supported by a number of observations. It has been found that glutamate, like potassium, can elicit cortical SD when applied topically to the rabbit's cerebral cortex (Van Harreveld, 1959). In this respect glutamate is a more potent stimulant

than potassium; the threshold concentration of the former is much lower (15 mM) than that of potassium (110 mM).

Glutamate added to the solution superfusing the isolated chicken retina elicits SD (Van Harreveld and Fifkova, 1970). The threshold concentration is quite low, 0.2 mM. In the isolated chicken retina charged with ^{14}C-glutamate, the label is released from the tissue during SD, elicited either electrically or chemically with KCl or with unlabelled glutamate (Van Harreveld and Fifkova, 1970). Since the threshold concentration of glutamate for SD is low, and it is present in considerable amounts in central nervous tissue, the amount of glutamate released from the SD invaded tissue could well be sufficient to affect adjacent tissue causing further depression and glutamate release, and so the propagation of SD.

Glutamate causes furthermore, an uptake of Na^+ by the isolated mammalian retina (Ames, 1956; Ames, Tsukada and Nesbett, 1967) and by brain slices (Bourke and Tower, 1966a, b; Harvey and McIlwain, 1968; Okamoto and Quastel, 1970a, b; Pull, McIlwain and Ramsay, 1970).

All these findings not only suggest that glutamate release is the mechanism underlying the propagation of SD, as proposed by Van Harreveld (1959), but also that glutamate does so by causing an increase in ion permeability of the cell membrane resulting in an inward shift of extracellular NaCl.

With the almost inescapable conclusion of a shift of Na^+ into the ICS during SD, it is surprising that no direct evidence for such

a shift has yet been provided, although an unsuccessful attempt to demonstrate a Na^+ shift during SD has been made by Křivánek and Bureš (1960). Since shifts of ions between intracortical compartments do not change the overall ion concentrations of the tissue, the Na^+ shift in the cortex can only be detected by determining the Na^+ distribution between the ICS and the ECS. For an easily diffusable ion, such as Na^+, such a distribution is difficult to demonstrate. To overcome this difficulty, one has to use a sample of central nervous tissue in which SD can be elicited and in which a change in Na^+ distribution between the ECS and the ICS can be more easily determined. Fortunately, such a nervous tissue, the retina, is available.

Spreading depression in the retina was, according to Marshall (1959), first noticed in the frog by Hartline. The first more extensive study of the retinal SD was made by Gouras (1958). He demonstrated in the excised toad retina a phenomenon resembling cortical SD in almost every respect. Retinal SD often occurred spontaneously, but could be triggered by the same stimuli that evoked cortical SD. The electroretinogram and the activity of the ganglion cells were both depressed, the wave of depression spread in all directions, and a typical SPC was present, although of smaller amplitude than in the cortex. In addition to all these events observed both in cortex and retina, there is a change in tissue appearance accompanying retinal SD. This change is due to an increase in transparency of the tissue, which can be detected without any elaborate devices, and is therefore very

useful for the study of SD. Similar phenomena, inlcuding the change in transparency were also observed in the isolated retina of chickens, pigeons and lizards (Martins-Ferreira and de Oliveira Castro, 1966).

The retina can be regarded as a sample of central nervous tissue, and the observations and analytical results obtained from it can be considered to represent those of gray matter. The retina has some special advantages for the electrolyte analysis of the tissue. First, it can be isolated quickly without mechanical damage, which might cause a profound alteration in ion distribution. The retina can be kept in almost normal physiological condition for at least one hour in suitable solutions (Ames and Nesbett, 1966). Secondly, it is very thin (about 0.1 mm) and is not separated from the bathing fluid by diffusion barriers; therefore its extracellular contents can readily exchange with the solutes in the bathing solution. Because of this easy exchange its ECS can be labelled with extracellular markers, or its extracellular fluid (ECF) can be replaced by ion-free solution. Furthermore since the ECS exchanges readily with the bathing solution, shift of electrolytes between the ECS and ICS can be expected to result in overall changes of the electrolyte content of the retinal tissue. Finally the retina is, unlike cortical slices, tough enough to be handled as an integrated tissue.

The present study attempts to provide direct evidence for a Na^+ transport during SD by determining the ion distribution in the isolated chicken retina with and without the occurrence of SD. Chicken retina

was chosen because its large mass (40 - 60 mg vs. about 10 mg of rat retina) is convenient for multiple analysis. Besides, the avian retina is believed to be void of blood vessels (Pumphrey, 1961) and hence offers less complications in the interpretation of the analytical results.

MATERIALS AND METHODS

Materials

Domestic chickens were narcotized with ether, the eyeballs were quickly excised and immersed in artificial chicken cerebrospinal fluid containing magnesium (Mg-CSF; see Solutions). The eyeballs were cut along the equator, and the anterior hemisphere together with most of the vitreous body were removed. The posterior part of the eyeball, which contains most of the retina was bathed in Mg-CSF. The remaining bits of vitreous body were carefully removed. In order to isolate the retina quickly at the end of the experiment the retina was completely and carefully separated from the underlying pigment epithelium by jets of the bathing fluid. The retina so prepared was still attached to the sclera through the pecten and the optic nerve, and lay upon the pigment epithelium, which served as a dark background making the detection of transparency changes possible. Gouras (1958) reported that in the toad once the retina was separated from the pigment epithelium no color change could be observed during SD. In the chicken retina, at least, this is not true (Martins-Ferreira and de Oliveira Castro, 1966; Van Harreveld and Fifkova, 1970; and the present study), although the transparency change may be less conspicuous when the retina is separated from the pigment epithelium. The retina, attached to the sclera which conveniently served as a handle, was used as the experimental material in the present study.

Solutions

Several solutions based on the composition of adult chicken CSF reported by Anderson and Hazelwood (1969) were used. They were designated as Mg-CSF, MA-CSF, Mg-Li-CSF, and MA-Li-CSF, and their compositions are listed in Table I. In the Mg-CSF 15 mM of NaCl were replaced by 10 mM of $MgCl_2$. The $MgCl_2$ was added to prevent SD since Mg salts are, according to Bureš (1960), the most effective in this respect. The concentration used (10 mM) was sufficient to suppress both spontaneous and evoked SD. In the MA-CSF the 10 mM $MgCl_2$ of the Mg-CSF was replaced by methylamine hydrochloride ($CH_3NH_2.HCl$) or sucrose. The concentration of these compounds was adjusted to keep the molarity and Na concentration of all solutions the same. Since MA-CSF hardly contains any magnesium, this solution will allow the development of SD. Both methylamine hydrochloride and sucrose are known to remain extracellularily, and hence not to compete with Na^+ in the ion movement during SD. No difference was observed by using sucrose or methylamine hydrochloride solutions. The lithium solutions, Mg-Li-CSF and MA-Li-CSF were made by substituting all the sodium in Mg-CSF and MA-CSF, respectively, with lithium.

During the experiment these incubation solutions were contained in 100 ml plastic weighing trays. Each tray contained at least 20 ml of solution, which is about 400 times the volume of one retina. These solutions were aerated with a mixture of oxygen (95%) and CO_2 (5%) before and throughout each experiment, except where otherwise stated.

Table I

Composition of the incubation and washing solutions.

The concentration of the constituents is given as mM/l.

Solutions	Mg-CSF	MA-CSF	Mg-Li-CSF	MA-Li-CSF	Ringer's Sucrose
Sucrose					318
KCl	4	4	3	3	4
$CaCl_2$	1	1	1	1	0.5
$MgSO_4$	0.5	0.5	0.5	0.5	10
Glucose	10	10	10	10	
NaCl	123	123			
$NaHCO_3$	20	20			
NaH_2PO_4	1	1			
$MgCl_2$	10		10		
CH_3NH_2-Hcl (or sucrose)		10			
Choline-Cl				10	
LiCl			111.5	111.5	
Li_2CO_3 (+HCl)			15.0	15.0	
Li OH			2.5	2.5	
KH_2PO_4			1.0	1.0	

The aeration stirred the solution gently and continuously, and hence also served as a means to facilitate the diffusion between the solution and the ECS of the incubated retina. The pH of the solutions was adjusted to 7.55 ± 0.1 with small amounts of isotonic NaH_2PO_4 or $NaHCO_3$ just before each experiment.

In most of the experiments labelled inulin (inulin-carboxyl-^{14}C, New England Nuclear) was used as an extracellular marker. A stock solution of inulin was prepared by dissolving 25 µC of inulin in 5 ml MA-CSF. A small amount of this stock solution (0.1 ml) was mixed with the bathing solution (20 ml) just prior to the experiment, imparting a radioactivity of about 2,000 cpm/5 µl to this solution.

An isotonic Ringer's sucrose solution and an isotonic Tris base solution were used to wash the retinas after incubation. The Ringer's sucrose solution contained the same concentration of KCl, $CaCl_2$, $MgSO_4$, and glucose as the other solutions. The lacking constituents were made up with sucrose (see Table I). This solution was used to remove NaCl from the ECS of the retinas in some experiments. It was used instead of a plain isotonic sucrose solution because it seems to preserve the ion distributions of nervous tissues better (Nicholls and Kuffler, 1965, and the present observation). The isotonic Tris base solution, which contains solely Tris base (340 mm) was used to remove the bathing solution adhering to the surface of the retina. Both isotonic solutions were cooled to 2 - 3°C with ice during the washing.

Methods

After having been prepared as described above, the retinas were washed with fresh Mg-CSF to remove the vitreous fluid and the blood that might still adhere to the preparation. Then they were transferred to another tray of Mg-CSF containing radioactive inulin and bathed in this solution for at least 25 minutes. This period of time is sufficient to allow the retina to recover from the traumatic effect of its preparation and from SD which might have been evoked in the process. This period also is long enough to reach an equilibrium between the inulin concentrations in the ECS and in the bathing fluid. This initial incubation period kept the retinas in a uniform resting state, and hence served as a common starting point in all the experiments.

At the end of the initial incubation period the retinas serving as controls were kept in the Mg-CSF. The experimental retinas were transferred to other solutions designed for the specific experimental purpose. In the experiments in which inulin was used the concentrations of this marker was made the same in all the solutions used for the control and experimental retinas. Routinely, the experimental and control experiments were paired, i.e., one retina of the chicken was used for the experiment while the other was used as control. The retinas were carefully observed throughout the incubation and experimental periods to detect changes in transparency, which served as an indication of SD. After the experiment had been performed, the sclera was cut away, and the retina, with a small piece of optic nerve or pecten still attached to serve as a handle, was taken out of the

incubation solution and washed.

In the earlier experiments two different processes of washing were employed. In one the retina was first washed with three changes of cold Ringer's sucrose solution for a total of 3 min. to remove the extracellular Na, and then rinsed briefly (about 3 sec.) in cold Tris base solution. The retina so washed was assumed to contain only intracellular sodium. This process will be referred to as the W-type washing. In the other type of washing the retina was not bathed in the sucrose solution but rinsed only with the Tris base solution (N-type washing). Such a retina would contain both the intracellular and extracellular sodium. The difference in Na^+ concentration of these retinas would be a measure of the extracellular sodium from which the extracellular Na space could be computed. Labelled inulin was used as an extracellular marker for an alternate determination of the ECS. These retinas were subjected to the N-type washing.

After the brief washing in the Tris base solution each retina was quickly blotted on a clean sheet of aluminum foil to remove the excess fluid, and at the same time the attached optic nerve or pecten was removed. The wet retina was then placed on a small piece of aluminum foil of known weight and immediately weighed. The dry weight of the retina was obtained by weighing the retina dried overnight in a 110°C oven. From these data the water content of the retina could be determined. For the measurements of the radioactivity and the ion contents, the dried retina was digested with 0.5 ml of concentrated HNO_3 in a 10 ml plastic (Nalgene) graduated cylinder at 110°C for 10

to 15 minutes.

Measurements of the radioactivity and of the ion contents

The volume of each digested retina was made up with distilled water to 1 ml. To measure the radioactivity of the retinas treated with labelled inulin, two 100-µl samples from the digest of such retinas were taken and deposited on two circles of glass fiber filter paper (Whatman, GF/A, 2.4 cm). To detect background activity, two samples were also taken from the digests of retinas not treated with labelled inulin. The radioactivities of the solutions bathing the control and the experimental retinas were also measured. Two 5-µl samples of each bathing solution were taken at the end of each experiment and deposited on two circles of the filter paper. Two blank samples of the inulin-free solution were similarly prepared. Each of the filter papers carrying the samples was dried completely (for more than six hours) in a 60°C oven and then immersed in 10 ml of sciltillation liquid (toluene plus PPO plus POPOP) contained in a glass scintillation counting vial. The radioactivity was counted with a Beckman LS-230 liquid scintillation counter for 10 minutes. The background activities for both blank retina and bathing solution samples were about the same (between 45 and 50 cpm/sample). The radioactivity of the bathing solutions containing labelled inulin, as mentioned before, was about 2,000 cpm/5 µl. That of the retinas treated with labelled inulin ranged from 280 to 600 cpm/100 µl digest, or 6 to 12 times the background activity.

To measure the ion contents, the retina digests were further diluted with distilled water. When lithium was not used, the digests were diluted 20 times. The concentrations of sodium and potassium in these diluted digests were measured with a flame spectrophotometer (Beckman Model DU) by comparison with a set of standard solutions. Since the retina digests contain both sodium and potassium which may interfere with each other in the measurement, the standard solutions were made up with both sodium and potassium salts at a ratio close to that in the retina digest.

When lithium was used, the concentration of lithium, in addition to sodium and potassium, was measured. The method described above was used with the following modifications. The retina digests were less diluted. Since the digests now contained lithium in addition to sodium and potassium, the standard solutions were made up with the chloride salts of all the three elements in the proper ratio. The ion contents of the bathing solutions were also measured. The concentration of potassium was measured at a wave length of 770 mμ, that of sodium at 589 mμ, and of lithium at 670.8 mμ.

Calculations and presentation of results

The results presented in the following section were calculated from the raw data obtained from the various measurements. In making these calculations it was assumed that the retina is a tissue made up of two compartments, the ECS and the ICS, and that the electrolytes and the water are distributed over these two components.

The water content of the retina was expressed as mg water per mg dry tissue (d.t.). Since the difference between the wet weight and the dry weight of a retina represents the amount of water contained in the tissue, the water content can be calculated as,

$$\text{Water Content (mg } H_2O\text{/Mg d.t.)} = \frac{\text{Wet weight of retina(mg)-Dry weight of retina(mg)}}{\text{Dry weight of retina(mg)}} \quad (5)$$

The contents of Na, K and Li were calculated by dividing the amount of each cation contained in the retina by the wet weight of the tissue, and were expressed as mM of each ion per kg of the wet tissue (w.t.). The raw data give the concentrations expressed as mM of the ion per liter of the retina digest. The ion contents can thus be computed with the following equation,

$$\text{Ion Content (mM/kg w.t.)} = \frac{\text{Ion mM/l} \times \text{Total volume of the retina digest (ml)} \times 10^{-3}}{\text{Wet weight of retina (mg)} \times 10^{-6}} \quad (6)$$

As mentioned earlier in this section, the Na contents determined in the retina after N-type washing, $(Na)_N$, and that after W-type washing, $(Na)_W$, may represent the total and the intracellular Na contents of the tissue, respectively. The extracellular Na content is therefore given by the difference between the $(Na)_N$ and the $(Na)_W$. Assuming that the Na concentrations in the ECF and the bathing solutions are the same, the extracellular Na space, which is expressed as the volume percentage of the wet weight of the retina, can be calculated

as follows,

$$\text{Extracellular Na space (\%)} = \frac{(Na)_N - (Na)_W}{[Na]_{B.S.}} \times 100 \qquad (7)$$

where $[Na]_{B.S.}$ represent the Na concentration in the bathing solution.

The inulin space is also used as a measure of the extracellular space. It was assumed that the radioactivities per unit volume (or the concentration of the labelled inulin) in the extracellular fluid and in the bathing solution are the same. With this assumption, the volume of the space in the retina occupied by inulin can be obtained by dividing the radioactivity of the retina (RR) by the radioactivity per unit volume of the bathing solution (RBS). The inulin space can therefore be expressed as,

$$\text{Inulin space (\%)} = \frac{RR}{RBS} \times 100 \qquad (8)$$

The RR is in the unit of cpm/kg w.t., and the RBS in cpm/l. Since the background activity (BA) has to be substracted, and each of the two retina samples used for the radioactivity measurement is one tenth the total retina digest, the RR can be expressed as,

$$\text{RR (cpm/kg w.t.)} = \frac{(\text{Mean radioactivity per retina sample} - \text{BA}) \times 10}{\text{Wet weight of retina (mg)} \times 10^{-6}} \qquad (9)$$

Similarly, the RBS, which is measured in 5 µl samples, can be expressed as,

$$\text{RBS (cpm/l)} = \frac{\text{Mean radioactivity per sample} - \text{BA}}{5\ (\mu l) \times 10^{-6}} \qquad (10)$$

Since it was assumed that the concentrations of each ion in the ECF and the bathing solution are the same, the extracellular ion contents of the retina treated with labelled inulin can be computed from the known ion concentrations in the bathing solution, $[Ion]_{BS}$, and the calculated inulin space (%) in the following way,

$$\text{Extracellular ion content (mM/kg w.t.)} = [Ion]_{BS} \times \frac{\text{Inulin space}}{100} \tag{11}$$

and therefore, in such retinas, the,

$$\text{Intracellular ion content (mM/kg w.t.)} = (Ion)_N - \text{Extracellular ion content} \tag{12}$$

where $(Ion)_N$ is the total content of the ion in the retina.

In the following section, the results (except that in Table III and IV) are presented as means with their standard deviations. The number of data used to calculate the mean is presented within parentheses. Whenever the comparison of the significance of the difference between two means (Dm) was necessary, the t test was used and the significance was expressed as the probability (P) of the result being a chance occurrence.

Results

Measurements of the extracellular space and of the ion contents in the resting retina

As described above, the retina, after its preparation, was immersed for a period of more than 25 minutes in Mg-CSF which prevents the development of SD. Since the immersion period was considered to be long enough to allow the tissue to recover from the adverse effect of the dissection and from SD that might have been elicited, retinas at the end of this period can be expected to be in the resting state. Data obtained from such retinas served as a basis for the changes that might occur during or after the SD. One of the two retinas of a chicken was, after the immersion period, treated with N- and the other with the W-type washing procedure. In this way the data obtained from 13 pairs of retinas were used to estimate both the extracellular sodium and the inulin spaces. Other retinas were analyzed following the N-type washing. The results of the measurements were listed in Table II.

Table II shows that the mean total K content measured in the retinas after N-type washing was significantly larger than that after W-type washing. This was true for every one of the thirteen pairs of retinas, in which each retina of the pair was washed differently. The difference between each pair of retinas was larger than 10 mM/kg w.t., in eleven of the thirteen pairs, and were 1.5 and 5.2 mM/kg w.t., in the two other pairs. Since the amount of the extracellular K in

Table II

Results of the measurements from resting retinas

Water Content	Total K Content		Total Na Content		Calculated Intracellular Na Content	Extracellular Na Space	Inulin Space
	N*	W*	N	W			
Mg H_2O/mg d.t.	mM/kg w.t.					%	%
4.563	64.1	51.2	37.0	12.3	17.5	15.2	13.4
±.140	±4.4	±3.4	±3.4	±2.5	±3.4	±2.2	±1.6
(18)	(20)	(13)	(20)	(13)	(15)	(13)	(15)
Dm	12.9		5.2			1.8	
p	<0.01		<0.01			<0.05	

*In this and the latter tables, N and W denote the data obtained from the retinas after N- or W-type washing, respectively.

The total Na content after W-type washing is believed to represent the intracellular Na. The extracellular Na content is the difference between the Na content after N- and W-type washing. From this value and the sodium concentration in the bathing fluid an extracellular Na space can be computed.

the retina (about 0.6 mM/kg w.t.) is smaller than any of these differences, the latter must be a result of the loss of the intracellular K from the tissue. Because the retinas of each pair were treated in exactly the same way, except that the one with lower K content was washed with the Ringer's sucrose solution (W-type washing), it is apparent that the loss of intracellular K takes place during the application of this solution. Thus, in spite of the addition of the Ringer's salts (except for Na) and glucose the washing with the Ringer's sucrose solution still causes the retina to lose intracellular K.

There is also a difference between the values of the inulin and the extracellular Na spaces. The latter was always larger than the former in each pair of retinas. As described above, the extracellular Na space was calculated from the difference between the Na contents measured in the retinas after N- and W-type washings. The Na content after the W-type washing would represent the intracellular sodium. The washing with Ringer's sucrose solution may cause a loss of intracellular Na as was observed for K. Such a loss may cause an erroneously high estimate of the amount of the extracellular Na, and hence of the extracellular Na space. The larger ECS estimated as the extracellular Na space as compared with the inulin space is therefore probably due to the loss of intracellular Na during the Ringer's sucrose washing. This was also found in the experiments involving SD that will be described below. In view of these findings, it seems that the inulin space is a better estimate for the ECS than the Na space.

Assuming that the inulin space represents the real ECS, then the

intracellular Na content of the retina can be calculated with equation (12). The intracellular Na content calculated in this way is significantly larger than that measured directly in the retina after W-type washing (see Table II). If the inulin space gives a correct estimate of the ECS, then the washing with Ringer's sucrose solution would cause a loss of about 5 mM/kg w.t., of intracellular Na from the retina.

Experiment on rabbit retinas

The ECS of the rabbit retina has been estimated by Ames and his coworkers (Ames and Hastings, 1956; Ames and Nesbett, 1966). They used methods very similar to those described in the present study for the chicken, but found that the ECS of the rabbit retina was about 30% of the total tissue space (see Table III). This figure is much larger than that of the chicken retina (about 13 - 15%) found in the present study. The large discrepancy may be due to a species difference or to differences in the experimental procedure, or both. In order to decide between these possibilities, experiments were carried out on two pairs of rabbit retinas using the methods described in the present study, except that artificial rabbit CSF, instead of chicken CSF was used, and the experiments were performed at the body temperature of rabbits (38°C) instead of room temperature (24°C). The results of these experiments on rabbit retinas, as shown in Table III, agree with those reported by Ames and his coworkers. Obviously the experimental procedure and the methods of the measurements do not account

Table III

Results of the experiments on rabbits retinas

Total K Content		Total Na Content		Calculated Intracellular Na Content	Extracellular Na Space	Inulin Space	Source of Data
N	W	N	W				
mM/kg w.t.					%	%	
74.9	79.0	63.2	16.3	21.2	32.1	29	Present Study
80.3	79.8	59.0	11.3	18.4	32.6	28	" "
72.4		62.3		21.3		29	Ames and Hastings (1956)
			14.1	15.7	33		Ames and Nesbett (1966)

For explanation see Table II.

Table IV

Results of Experiments Under Various Conditions

Conditions of Experiment			Total K Content		Na Content		Na Space	Inulin Space
Temperature	Osmolarity	Substance Added	N	W	N	W Extra-cellular		
°C	mOsm		mM/kg w.t.				%	%
24	340		64.1	51.2	37.0	12.3	15.2	13.4
(From Table II)			±4.4	±3.4	±3.4	±2.5	±2.2	±1.6
41	340		72.8	77.2	39.7	13.2	17.8	14.2
41	340		72.7	71.1	45.9	19.5	17.8	17.2
41	360	NaCl	76.5	70.4	46.2	16.0	18.9	
24	380	NaCl	68.0	68.2	54.2	19.4	20.5	
41	380	NaCl	70.3	65.1	57.8	15.8	24.7	
24	385	Sucrose	65.4	69.0	52.5	14.6	25.3	
41	400	NaCl	60.6	53.1	73.6	38.7	19.4	
41	400	Sucrose	69.4	58.2	51.9	26.0	17.2	

for the large discrepancy in the magnitudes of the ECS between the two species. To check the possible effect of the difference in temperature at which the retinas were kept, the experiment on chicken retina were repeated at its body temperature (41°C). The results of two such experiments were listed on the upper part of Table IV. For the convenience of comparison, the results from Table II were also listed at the top of Table IV. The comparison of these two sets of results shows that chicken retinas kept at body temperature have a higher K content than those at room temperature. Although the number of experiments is small, this comparison may indicate that under a physiologically more favorable condition (at body temperature) the chicken retina can better maintain its ion composition. Also, the ECS determined in these experiments was slightly larger than that in the experiment performed at 24°C. But, even under these conditions the magnitude of the ECS in the chicken retina, either estimated as inulin or extracellular Na space, is still far smaller than that in the rabbit retina.

From the results of the above experiments it is obvious that the large discrepancy between the ECSs in the retinas of the chicken and the rabbit is due to a species difference. This is further supported by the investigation of the effect of increasing the osmolarity of the bathing solution (Mg - CSF) on the ECS of the chicken retina. The increase in osmolarity was achieved by adding extra NaCl or sucrose to the bathing solution. The results of several such experiments are listed in the lower part of Table IV. They show that an increase

in the osmolarity of the bathing solution causes an enhancement of the extracellular Na space of the retina up to about 25%, which is still smaller than that of rabbit retina. It can therefore be concluded that the ECS of the chicken retina is actually smaller than that of the rabbit retina.

The species difference between rabbits and chickens is not unexpected, for there are some physiological, morphological, and may be metabolic differences known to exist between the retinas of the two species. For instance, in the avian retina there is a structure called pecten, which is not present in the mammalian retina, on the other hand, in the mammalian retina there are retinal blood vessels which are absent in the avian retina. This morphological difference suggests differences in the supply of oxygen and nutrients. In mammals the retina may receive its supply through the retinal blood vessels, while in the birds the pecten is believed to be involved in the supply to the retina.

The extracellular space and the ion contents of the retina after invasion by spreading depression

At the end of the initial incubation period the retinas were transferred from Mg-CSF to MA-CSF. After being transferred, SD developed spontaneously in about 30 seconds and was completed in about 2 minutes. The retina was then separated from the other tissues, which takes about 2 minutes. The retinas were therefore immersed in

the MA-CSF for a total of 4 minutes and 2 minutes after the completion of SD, before they were subjected to washing. Both types of washing were employed. The results of the experiments are given in Table V. A comparison of these results with the data of the resting retina (Table II) shows that after SD the total K content is decreased and that the intracellular Na content is increased.

The marked difference in the total K contents of the resting retinas due to the two procedures of washing is not observed in the retinas invaded by SD. Therefore, the decrease in K content caused by SD computed as the difference between the resting and the SD invaded retinas is larger after N-type washing (23.5 mM/kg w.t.) than after W-type washing (11.5 mM/kg w.t.). Both figures for the decrease in K due to SD are far larger than the extracellular K content (less than 1 mM/kg w.t.). Besides, in computing the decrease, the extracellular K contents are about cancelled. Therefore, it is obvious that the decrease is due to a loss of intracellular K. Hence, it can be concluded that during SD intracellular K is released.

The magnitude of the increase in the intracellular Na content due to SD is, again, different when computed from the data obtained from retinas after different procedures of washing. With data from the retinas after N-type washing the increase is 16.5 mM/kg w.t., while after W-type washing it is only 9.0 mM/kg w.t. This discrepancy, as will be discussed below, is due to a loss of intracellular Na from the SD invaded retinas during Ringer's sucrose washing. However,

Table V

Results from the retinas two minutes after spreading depression

	Total K Content		Total Na Content		Computed Intracellular Na Content	Extracellular Na Space	Inulin Space
	N	W	N	W			
	mM/kg w.t.					%	
	40.6	39.8	53.1	21.3	34.1	21.2	12.6
	±1.9	±4.1	±3.4	±1.3	±2.2	±3.0	±1.1
	(4)	(4)	(4)	(4)	(4)	(4)	(4)
Dm					12.8	8.6	
p					<0.01	<0.01	

both sets of data show that there is an increase in intracellular Na content after SD, indicating that during SD a significant amount of extracellular Na enters the intracellular compartment of the retina.

As shown in Table V, there is a large discrepancy between the magnitude of the extracellular Na and the inulin spaces in the SD invaded retinas. The former is almost twice as large as the latter. A comparison of these data with those in Table II shows that there is a contradiction between the effect of SD on the magnitude of these spaces. From the change in the extracellular Na space it would appear that SD causes an increase in the ECS from 15 to 21%, while the data of the inulin space indicates that SD causes a slight decrease. Obviously, at least one of these estimates is incorrect.

Inulin is a large molecule (M. wt. 5,000 - 5,500) as compared to water or the Ringer's electrolytes, and hence it will diffuse into and out of the narrow ECS in the isolated retina slower than these compounds because of steric hindrance. Therefore, when the magnitude of the ECS changes, that of the inulin space will lag behind, especially when there is a decrease in magnitude of the ECS. Only if enough time is allowed for diffusion, the magnitude of the inulin space may become equal to that of the ECS. Ames and Hastings (1956) found that when the retina was transferred from an inulin-free to an inulin-containing artificial CSF, the marker reached its final, maximal concentration in the tissue in about 3 minutes. In the present experiment the retina had been charged with inulin before it was transferred from

Mg-CSF to MA-CSF, both of which had the same concentration of inulin. Although the period of time that the retina was immersed in MA-CSF (4 minutes) is of the same order as that needed for the equilibration of the inulin between the ECS and the bathing solution, the ECS can be expected to change during this period. The change of the inulin space may therefore indicate the direction of a change in the ECS but cannot be expected to represent the magnitude of the ECS. Furthermore, SD observed in the present and all the subsequent experiments did not occur over the entire retina at once, but developed spontaneously at the margin of the retina and took about 1.5 to 2 min. to completion. Van Harreveld (1958) showed that the swelling of the cortical cellular elements is most conspicuous at the site where the maximal impedance change accompanying SD is recorded, and that the extent of the swelling decreases in the tissue that is recovering from this phenomenon. Thus, at 2 min. after the completion of SD (3.5 min. after SD first developed) some parts of the retina may be recovering, and the magnitude of the ECS in these parts may be on their way back to the resting level. Therefore, the inulin space determined does not represent the magnitude of the ECS of the entire retina at the maximum of SD. The decrease of the inulin space shown in Table V, although small, suggests therefore that the ECS in the retina invaded by SD is smaller than that in the resting state.

Since the ECS seems to decrease after SD, the large increase in the extracellular Na space in these experiments (from 15% to 21%)

must be due to other causes than a change in the ECS. This Na space was calculated from the Na contents measured in the retinas after the two different types of washing and the known Na concentration of the bathing solution. The Na concentration of the bathing solution is constant, and the Na content measured in the retina after N-type washing can be expected to supply a reliable value for the total Na content of the retina. Therefore the large Na space determined in the present experiment must be due to the loss of the intracellular Na from the retina during W-type washing. This is supported by the fact that the intracellular Na content measured in retinas after W-type washing is only about two thirds of that calculated from retinas after N-type washing (Table V). Apparently a significant amount of intracellular Na was lost from the SD invaded retinas during the Ringer's sucrose solution washing. Such a loss can be explained. Since the cell membrane of the retina is permeable to Na^+ even at the resting state, the washing of the retina, which contains a high concentration of intracellular Na^+ after SD, in the Na-free Ringer's sucrose solution will undoubtedly cause a loss of intracellular sodium. Besides, it was postulated that the Na^+ permeability of cell membranes is increased during SD and it is possible that such an increase will persist for a while after the termination of the transparency change accompanying SD. The washing with the Ringer's sucrose solution may therefore remove more intracellular sodium than in the resting state. Indeed, again

assuming that the inulin space represents the actual ECS, the loss of intracellular sodium in the present experiment involving SD is about 13 mM/kg w.t., which is much larger than that in the previous experiment (5 mM/kg w.t.) in which no SD was elicited.

From the above discussion it is apparent that the application of the Ringer's sucrose solution in the W-type washing procedure causes a serious loss of intracellular Na, and hence an increase in the extracellular Na space. Therefore, this washing procedure was not employed in the later experiments (except the lithium experiment).

Changes in the extracellular space and the ion contents of the retina during the course of spreading depression

From the relatively quick restoration of the electrical activity in the nervous tissue invaded by SD one would expect that the restoration of the resting ion distribution and the ECS occurs soon after the completion of SD. Accordingly, the actual changes in the ECS and the ion contents caused by SD must have been greater than those listed in Table V, for they were determined two minutes after the completion of SD and during these two minutes some restoration might have taken place. To investigate the maximal changes produced by SD and their restorations the following experiments were carried out. The retinas were prepared in such a way that they could be isolated completely almost instantly. The tissue was then taken out of the bathing solution at various designated times after the transferrance

from Mg-CSF to MA-CSF which initiates SD and subjected to the N-type washing and the measurements described above. The retinas of the control group were kept in the Mg-CSF solution all the time. The results from both groups of retinas are listed in Table VI and plotted in Figs. 1 through 5.

In the control retinas SD was never observed. The levels of the water content, the inulin space, and the Na and K contents of these control retinas remained fairly constant throughout an experimental period of 30 minutes (see Table VI and Figs. 1 through 5). This indicates that during the entire experimental period the isolated retinas are in a steady physiological state and hence that no serious deterioration occurs.

In the retinas invaded by SD there are various changes. The change in water content caused by SD is not statistically significant. However, there is a tendency for the water content to increase after the SD developed. The changes in inulin space, as shown in Fig. 1, are rather complicated. The average magnitude of the inulin space increases slightly immediately after the retina is transferred to MA-CSF, and starts to decline as the SD develops. The decline continues until two minutes after the completion of SD, then the inulin space increases rapidly and eventually reaches a plateau which is higher than the control value. The values of the inulin space in the retinas of the experimental group are not statistically different from those of the controls, except for those obtained 3 minutes after

Table VI

Results from the Control and the Experimental Retinas at Various Times After Initial Incubation Period

Time after Initial Incubation Period	Water Content		Inulin Space		Total Na Content		Total K Content	
	mg H_2O/mg d.t.		%		mM/kg w.t.		mM/kg w.t.	
Min.	Control	Exptl.	Control	Exptl.	Control	Exptl.	Control	Exptl.
0	4.604 ±.196 (15)		14.9±1.7 (12)		37.0±1.9 (14)		66.1±1.9 (11)	
0.5		4.603 ±.119 (8)		1.6±1.6 (5)		40.0±2.3 (6)		6.1±3.0 (8)
2	4.450 (5)	4.650 (14)	14.7±1.8 (5)	13.3±1.2 (10)	37.5±1.7 (5)	52.9±3.5 (12)	64.2±1.2 (4)	48.9±3.2 (14)
3		4.784 ±.157 (13)		12.3±1.1 (11)		51.2±2.7 (11)		45.8±2.4 (12)
4 (From Table V)		4.892 ±1.18 (4)		12.6±1.2 (4)		53.1±3.4 (4)		40.6±1.9 (4)
5	4.492 ±.110 (5)	4.680 ±.232 (6)	14.3±1.5 (5)	15.5±1.5 (4)	36.3±4.4 (5)	54.2±2.8 (7)	66.1±2.5 (3)	47.1±2.6 (5)

Table VI (continued)

Time after Initial Incubation Period	Water Content mg H_2O/mg d.t.		Inulin Space %		Total Na Content mM/kg w.t.		Total K Content mM/kg w.t.	
Min.	Control	Exptl.	Control	Exptl.	Control	Exptl.	Control	Exptl.
10	4.529 ±.33 (5)	4.845 ±.259 (7)	14.6±1.6 (5)	17.4±1.5 (3)	34.8±3.1 (5)	50.6±2.0 (6)	65.6±2.3 (3)	47.5±2.7 (7)
15		4.795 ±.234 (8)		18.2±1.4 (3)		49.4±4.1 (7)		50.3±3.9 (8)
20	4.632 ±.241 (4)		14.5±1.0 (4)		36.2±3.6 (4)		65.0±3.5 (4)	
30	4.459 ±.242 (5)	4.479 ±.139 (4)	14.4±1.4 (5)	18.8±2.6 (4)	34.2±2.0 (5)	41.9±4.4 (4)	68.4±1.7 (4)	54.1±2.5 (4)

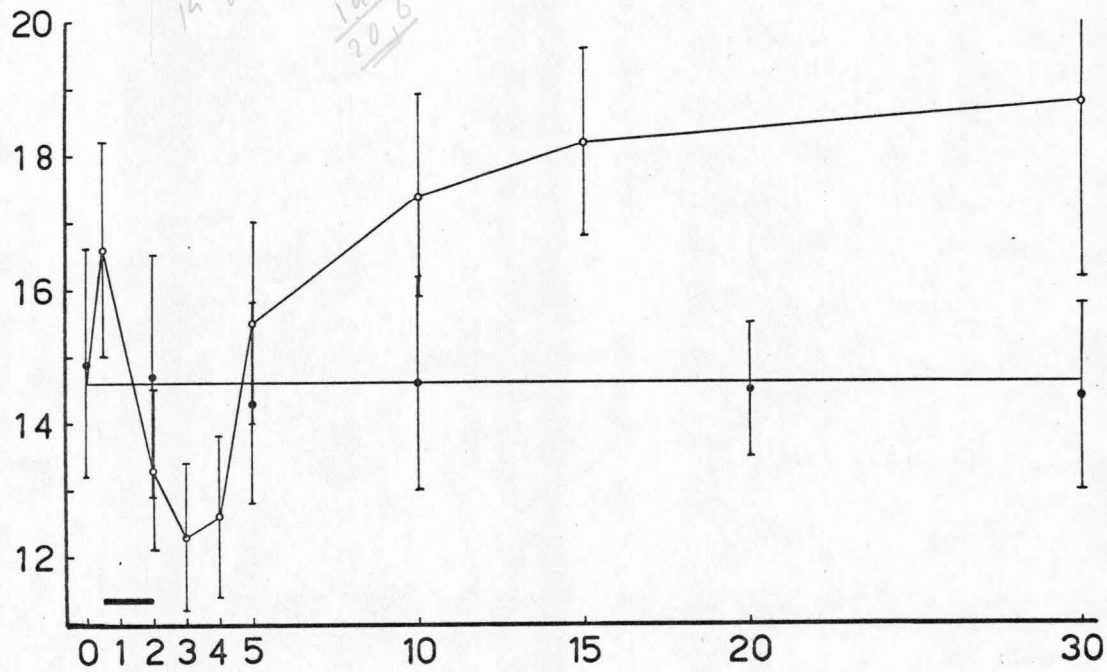

Fig. 1. The inulin spaces of the control and the experimental retinas at various times after initial incubation period under aerobic conditions. The inulin space in percentage is plotted on ordinate. In this and all the subsequent figures the filled circles represent the control values, the open circles the experimental values. The vertical lines through the data points represents the standard deviations of the means. The time in minutes after the initial incubation period is plotted on the abscissa in all the figures, and the horizontal bar above the abscissa represents the duration of the transparency change in retina accompanying SD.

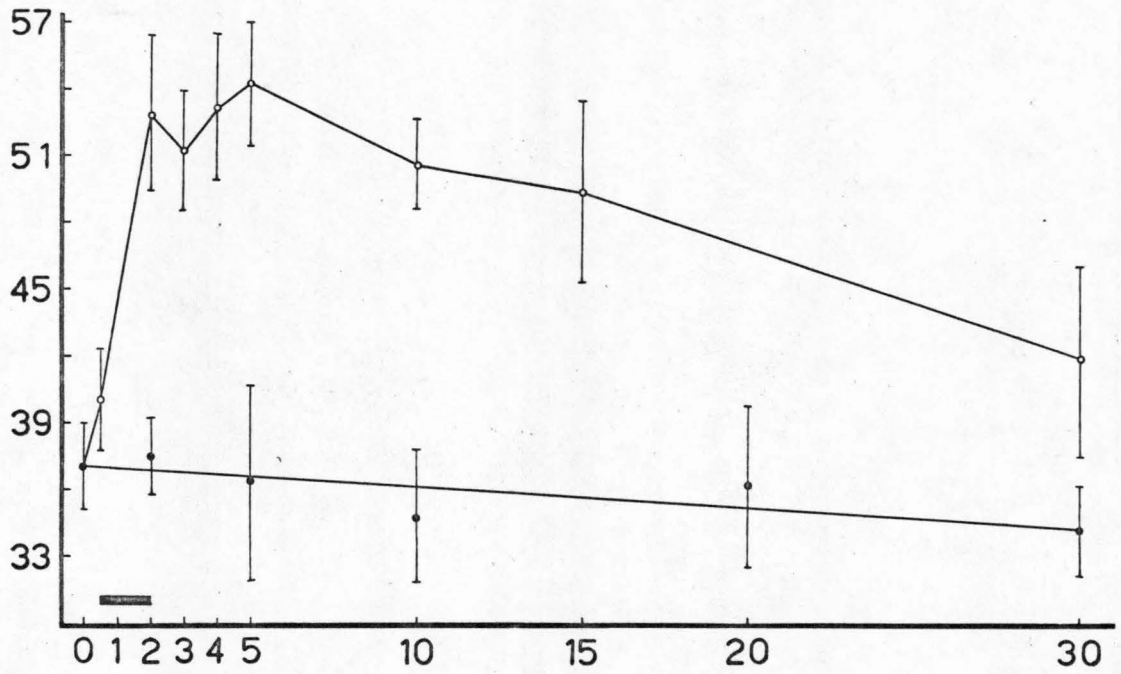

Fig. 2. Total Na contents of the control and the experimental retinas at various times after initial incubation under aerobic condition. On the ordinate the total Na content in mM/kg w.t. is plotted.

the completion of SD ($p < 0.01$). As discussed above the changes in the inulin space observed may lag behind those in the actual ECS. This may explain why the inulin space continues to decrease after SD was completed.

The changes in ion contents during SD are more pronounced than those in water content and inulin space. The data of the total Na content (shown in Table VI) are plotted in Fig. 2. This figure shows that during SD the total Na content rises rapidly and then stays at approximately the same level for 3 more minutes after the completion of SD, before it starts to decline. The decline in the total Na content is slow and does not reach the resting level 28 minutes after the completion of SD. Since, as indicated by the changes in the inulin space, SD is accompanied by a decrease in the ECS and hence the amount of extracellular Na, the pattern of the changes in the total Na content, which includes both intracellular and extracellular fractions, may not represent that of the intracellular Na content. The change in the intracellular Na content, which is more illustrative in showing the movement of the ion, was calculated with equation (12) on the assumption that the inulin space represents the ECS, and the results of the calculations were plotted on Fig. 3. Figure 3 shows that the intracellular Na content rises rapidly from the resting 15 mM/kg w.t. to 33 mM/kg w.t., at the end of SD, and stays at the high level for two more minutes before it declines first rapidly then

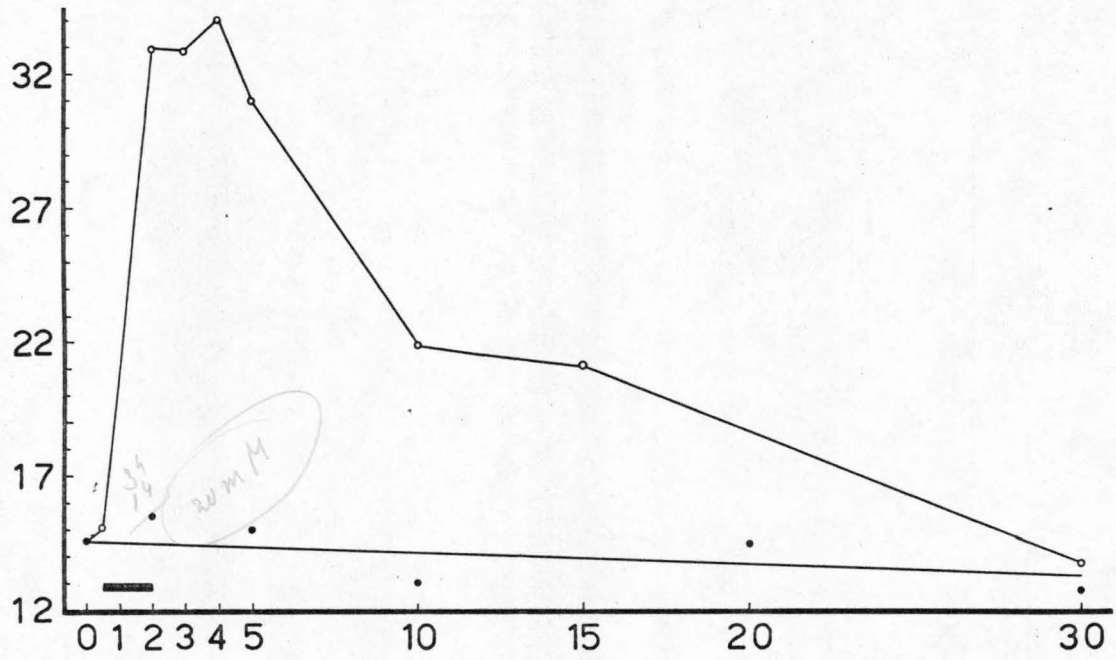

Fig. 3. The intracellular Na contents of the control and experimental retinas calculated from the values shown on Figs. 1 and 2. The intracellular Na content in mM/kg w.t. is plotted on ordinate.

more slowly to, and finally reaches, the resting level. The increase in the intracellular Na content during SD is the result of a movement of Na from the extracellular fluid and of the bathing solution into the intracellular compartment of the retinal cells. This inward Na movement, as mentioned in the Introduction, is probably due to an increase in the cell membrane permeability for Na although the arrest of the ion pump has to be considered. The mechanism causing the inward Na movement seems to persist beyond the completion of SD, as suggested by the delayed restoration. The rapid restoration indicates that the ion pump is functioning, and that the Na movement is not the result of the deterioration of the tissue but is a concomitant of the reversion of the SD changes.

The changes in total K content, plotted in Fig. 4, are similar, but opposite to those of the total Na content. The total K content starts to decrease rapidly even before the occurrence of SD and the decrease continues for 2 minutes after the completion of this phenomenon. Then the total K content increases toward the resting level, but does not reach this level at the end of the experimental period (28 minutes after the completion of SD). Since the K concentration in the extracellular fluid is very low (4 mM/kg w.t.), the value of intracellular K is not much different from that of total K content. Nevertheless, the intracellular K contents were also calculated and plotted in Fig. 5. It can be seen from this figure that unlike that of intracellular Na content, the restoration of the intracellular K content is slow and incomplete within the experimental period. This

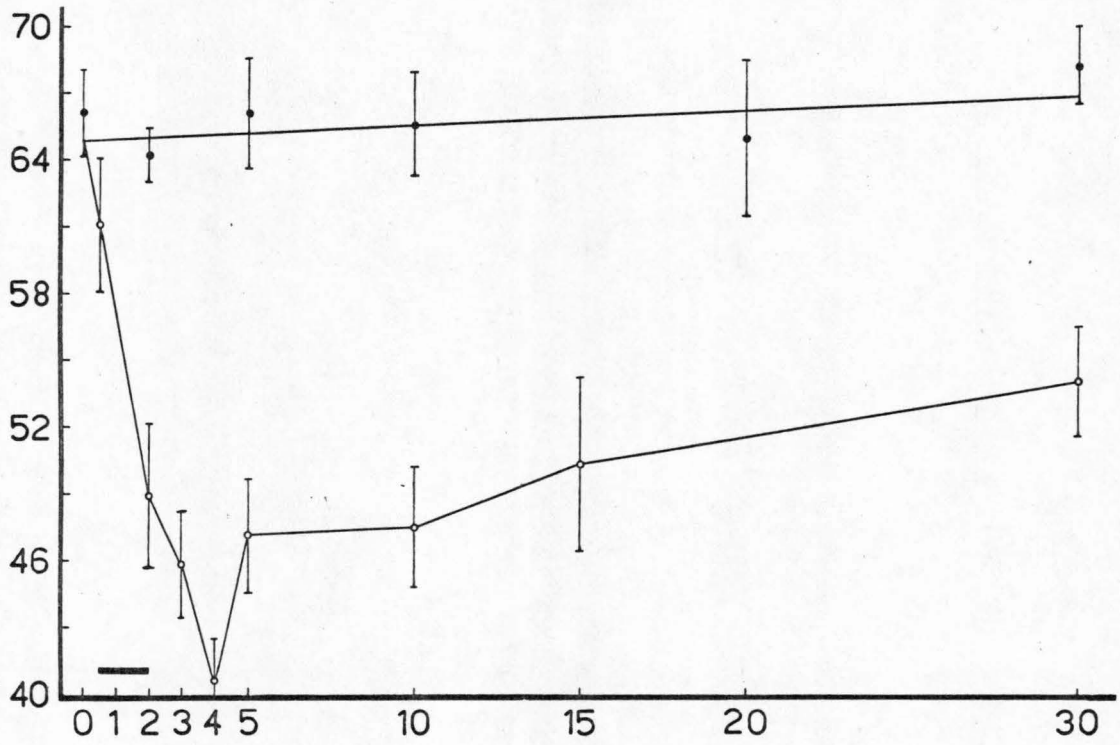

Fig. 4. The total K contents of the control and the experimental retinas at various times after the initial incubation under aerobic condition. The total K content in mM/kg w.t. is plotted on ordinate

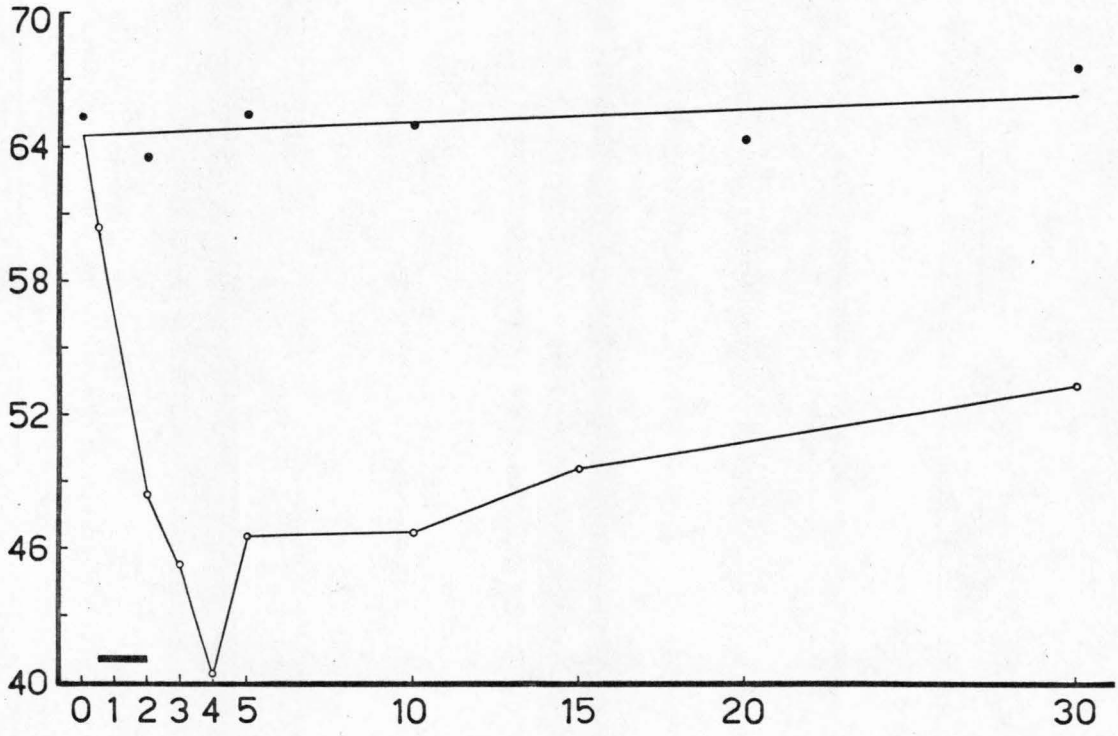

Fig. 5. The intracellular K contents of the control and the experimental retinas calculated from the values shown on Figs. 1 and 4. The intracellular K content in mM/kg w.t. is plotted on the ordinate.

phemonenon can be explained as follows. The volume of the bathing solution is so large (at least 400 times the volume of immersed retina) that the intracellular K released from the retina during SD and lost into the bathing solution will not cause any significant increase in the K concentration of the bathing solution. This makes the re-uptake of the extracellular K during the recovery phase more difficult in the present in vitro condition than in the in vivo condition, in which the released K is not lost but accumulates in the ECS. The difficulty in the re-uptake of K from the bathing solution with its low K content accounts for the slow restoration of the K content.

From the results obtained in the present experiment, it can be concluded calculated that during SD a significant amount of Na (19 mM/kg w.t., at maximum) shifts from bathing solution into the intracellular compartment of the retina, and a large amount of K (25 mM/kg w.t., at maximum) moves in the opposite direction. These ion movements are reversible although the restoration may take a long time.

Spreading depression at low temperature

It is not known whether the ion pump is inhibited during SD. If it is, then the inhibition of the pump alone, or in combination with an increase in the membrane permeability, may account for the inward Na shift during SD and the delay in restoration after SD. If it is not, then by inhibiting the ion pump a further increase in the total

Na content after SD can be expected. The present experiment was designed to investigate the significance of the ion pump on the development and the recovery of SD by inhibiting the ion pump. The ion pump can be inhibited either by pharmacological inhibitors or by cooling the tissue. In this experiment the latter method was chosen because the pharmacological agents in use may have other effects than those on the metabolism.

The retinas were prepared and incubated as usual, but after the initial incubation period the paired retinas were incubated in cold (2° - 3°C) Mg-CSF for 2 minutes. After that the experimental retina was transferred to cold MA-CSF while the control retina remained in the cold Mg-CSF. Spreading depression in cold MA-CSF occurred spontaneously, though it had a longer latency and duration, lasting for 4 - 6 minutes instead of 2 minutes at room temperature. Spreading depression was not observed in the retinas in cold Mg-CSF for up to 10 minutes, but beyond that time an increase in the transparency of the retina sometimes developed. Each pair of retinas were immersed in the cold solutions for the period of time necessary to complete SD in MA-CSF. Then the retinas were washed and subjected to the measurements described above. The data obtained from six pairs of retinas are shown in Table VII.

The results obtained at low temperature are qualitatively the same as those at room temperature. In the retinas invaded by SD at low temperature there also is a statistically insignificant increase in the water content, a small (statistically insigifnicant) decrease

Table VII

Results from the Low Temperature (2 - 3°C) Experiments

	Water Content		Inulin Space		Total Na Content		Total K Content	
	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
	mg H_2O/mg d.t.		%		mM/kg. w.t.			
	4.546 ±.168 (6)	4.975 ±.316 (6)	14.3 ±2.0 (6)	12.2 ±1.4 (6)	46.5 ±3.5 (6)	60.1 ±3.2 (6)	50.1 ±4.1 (6)	35.8 ±4.0 (6)
Dm	0.429		2.1		13.6		14.3	
p	<0.05		>0.05		<0.01		<0.01	
From Table VI	4.450 ±.249	4.650 ±.182	14.7 ±1.8	13.3 ±1.2	37.5 ±1.7	52.9 ±3.5	64.2 ±1.2	48.9 ±3.2
Dm					15.4		15.3	
p					<0.01		<0.01	
Dm†					9.0	7.2	14.1	13.1
p					<0.02	<0.05	<0.01	<0.01

†Differences between the results of the present and the previous experiments.

in the inulin space, a significant increase in the Na content, and a significant decrease in the K content. These results were compared with the corresponding ones of the experiment carried out at room temperature (Table VI, two minutes after the initial incubation).

The comparison shows that the lowering of temperature causes an increase in the total Na content and a decrease in the total K content in both the control and experimental groups, but does not affect the magnitude of the ECS in either group. In the control group, the lowering of temperature causes a 9 mM/kg w.t., increase in Na content and a 14 mM/kg w.t., decrease in K content. In the experimental group, the Na content increases by 7 mM/kg w.t., and the K content decreases by 13 mM/kg w.t. These changes, as shown at the bottom of Table VII, are statistically significant. Because the magnitude of the ECS does not change due to the lowering of temperature, the above significant changes in ion contents must be the results of an accumulation of Na in the ICS and a loss of intracellular potassium. In the retinas of the control groups, in which SD was never observed, these changes must be due to the arrest of the active ion transport system (ion pump) by the low temperature. Therefore, it can be concluded that at room temperature the ion pump in the resting isolated chicken retina is functioning.

In the cold the ion pumps are postulated to be arrested. The development of SD at 2 - 3°C thus indicates that SD is not caused by an arrest of the sodium pump. This observation supports the alternate

mechanism for SD, namely an increase of the sodium permeability of the plasma membrane.

If the functioning ion pump can remove the Na shifted into the retinal cells during SD, then the magnitude of the Na shift could be expected to be greater when the pump is arrested by low temperature. However, comparison shows that the magnitude of the Na shift occurring during SD at low temperature (13.6 mM/kg w.t.) is somewhat smaller than that at room temperature (15.4 mM/kg w.t.). This comparison therefore indicates that although the ion pump seems to be functioning during SD it is not able to remove a significant amount of the Na shifted inward during SD.

Spreading depression in lithium solutions

Lithium possesses the unique characteristic of being discriminated against by the Na-pump but not by the "Na-channels" in the cell membrane (Keynes and Swan, 1959). Or, in other words, lithium ions with few exceptions, behave as extracellular Na ions (Ozeki and Grundfest, 1967), but once inside the cell they will not be pumped out. This, as will be seen later, is also true for the retina. Thus, if there is an increase in the membrane permeability for Na during SD, then the retina will accumulate lithium by letting SD develop in a retina immersed in a solution, in which all the sodium is substituted by lithium. In this way, the effect of the active transport system will be circumvented, and the difference in the Li contents of the SD invaded and the control retinas will show the magnitude of the Na

shift during SD not masked by the transport system.

In the present experiment, the retinas were prepared and incubated as usual, but after the initial incubation period one of the paired retinas was transferred to Mg-Li-CSF to serve as control, the other to MA-Li-CSF to serve as experimental tissue. Spreading depression developed spontaneously in the retina bathed in MA-Li-CSF. The retina in the Mg-Li-CSF did not show SD for at least 10 minutes, after that a transparency change in the retina might take place. Each retina was immersed in the Li solutions for 7 minutes, which is enough tor SD to be completed and for the isolation of the retina from the other tissues. After the immersion in the Li solutions all the retinas were subjected to the W-type washing which removes extracellular Na and Li, therefore the Na and Li contents measured in these retinas were the intracellular fractions. The measured total K content, which is not much different from the intracellular K content, was also regarded as the intracellular fraction. The ECS of these retinas was not labelled with inulin. Since the N-type washing procedure was not employed, no reliable data for the ECS are available. The results of the present experiments are listed in Table VIII. The corresponding results obtained previously with Na solutions (Table II for the control and Table V for the SD group) are listed at the bottom of the table for the convenience of comparison.

In the present control group, the intracellular Li accumulated in the retina is about 30 mM/kg w.t., which is more than twice that of

Table VIII

Intracellular Cation Contents of the

Retinas Incubated in Lithium Solutions

	Na Content		K Content		Li Content	
	Control	Exptl.	Control	Exptl.	Control	Exptl.
	mM/kg. w.t.					
	6.6 ±1.4 (8)	4.2 ±0.8 (8)	28.2 ±2.9 (8)	21.5 ±2.8 (8)	30.2 ±2.5 (8)	44.1 ±215 (8)
Dm	2.4		6.7		13.9	
p	<0.01		<0.01		<0.01	
From Table II (w)	12.3 ±2.5		51.2 ±314			
From Table V (w)		21.3 ±1.3		39.8 ±4.1		

the resting intracellular Na content (12.3 mM/kg w.t., after W-type washing). The presence of such a large amount of intracellular Li in the resting retina indicates that Li ions, like Na ions, leak into the retinal cells at the resting state, but unlike Na ions, cannot be removed subsequently by the Na-pump. A comparison with the results obtained from the retinas treated with Na solutions (Table II) shows that the resting retina in the Li solution loses about 6 and 23 mM/kg w.t., of intracellular Na and K, respectively. Since the sum of these losses is about the same as the gain of intracellular Li, it is apparent that each intracellular Na ion and K ion lost are replaced by Li ions, and hence that the total intracellular cation content is not changed. This suggests that the size of the ICS, as well as ECS, is not changed by the leaking of Li ions into the cells.

The intracellular Li content of the retinas in the experimental (SD) group increases over that of the control group, while both the intracellular Na and K contents decrease significantly (see Table VIII). The statistically significant decrease in the intracellular Na content of the SD invaded retinas compared with that in the resting retinas is of interest, since in the normal physiological solution SD causes an inward shift of sodium. This will be discussed in the next section. The magnitude of the increase in intracellular Li content (14 mM/kg w.t.) is larger than the sum of the reductions in the Na and K contents (9 mM/kg w.t.). Spreading depression observed under the present conditions may therefore cause a net gain in intracellular cation content. Since no hydrostatic pressure can build up in the retinal

cells, the osmotic pressure created by the net gain in intracellular ion content has to be balanced by a diffusion of water into the cells. This will cause an increase of the ICS. In the in vivo, but not necessarily in the in vitro situation, such an increase in the ICS would be accompanied by a shrinkage of the ECS.

The results of the Li experiment suggest that SD causes an inward shift of the major extracellular cation, a release of intracellular K, and an increase of the ICS probably at the expense of the ECS. These results, though smaller in magnitude, agree with those of the previous experiments.

Spreading depression under anaerobic conditions

It has been mentioned in the Introduction that the retina in chickens may be metabolically different from that in mammalians. Indeed, there is evidence showing that the energy for the ion pump can be supplied by aerobic metabolism (Van Harreveld and Fifkova, 1972). It was therefore interesting to investigate the effect of deprivation of oxygen on SD and its concomitant ion movements.

In the present experiment the usual experimental procedures was employed, except that none of the incubation solutions used were oxygenated. In other words, the retina was deprived of oxygen supply from the beginning of the experiment. The results so obtaind are shown in Table IX and Figs. 6, 7 and 9. The intracellular ion contents calculated from the data in Table IX are plotted in Figs. 8 and 10. These results show that in the control group the ECS and the Na and

Table IX

Results of the Anaerobic Experiment.

Time after Initial Incubation Period	Inulin Space		Total Na Content		Total K Content	
Min.	%		mM/kg. w.t.		mM/kg. w.t.	
	Control	Exptl.	Control	Exptl.	Control	Exptl.
0	13.3±1.4 (4)		38.0±1.5 (4)		63.4±2.7 (4)	
2		12.4±1.4 (7)		50.4±2.4 (6)		49.9±0.3 (6)
3	13.6±0.3 (3)	11.1±1.9 (7)	38.0±1.6 (3)	48.8±3.4 (6)	64.6±1.9 (3)	47.4±2.2 (6)
4		11.3±2.4 (5)		50.7±2.9 (5)		46.6±2.0 (5)
5	13.1±1.1 (3)	13.1±2.3 (2)	37.6±1.8 (3)	49.6±3.1 (3)	62.5±2.3 (3)	45.3±1.4 (3)
15	14.3±1.4 (3)	17.3±1.4 (3)	37.0±1.0 (3)	47.8±1.4 (3)	64.9±2.1 (3)	49.7±1.3 (3)

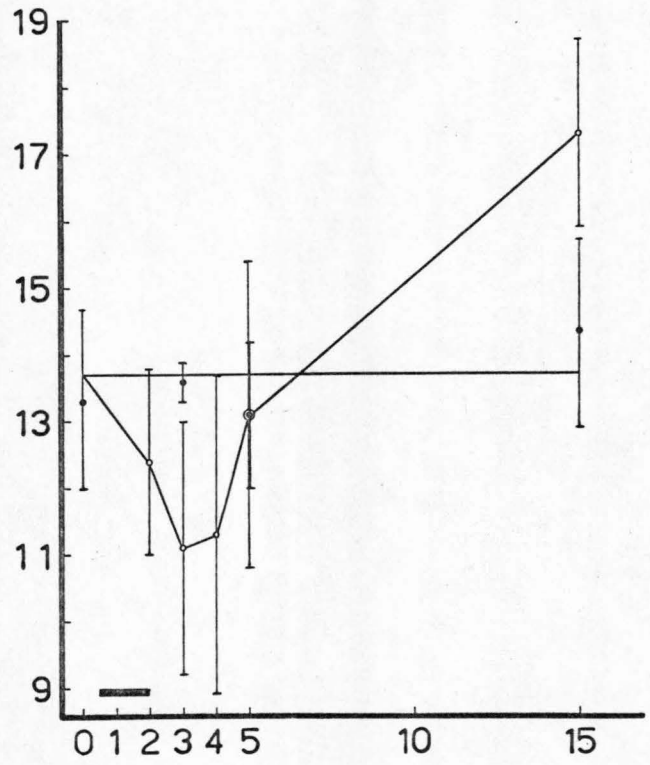

Fig. 6. The inulin spaces (in %, ordinate) of the control and the experimental retinas at various times after the transfer to MA-CSF (abscissa) under anerobic condition.

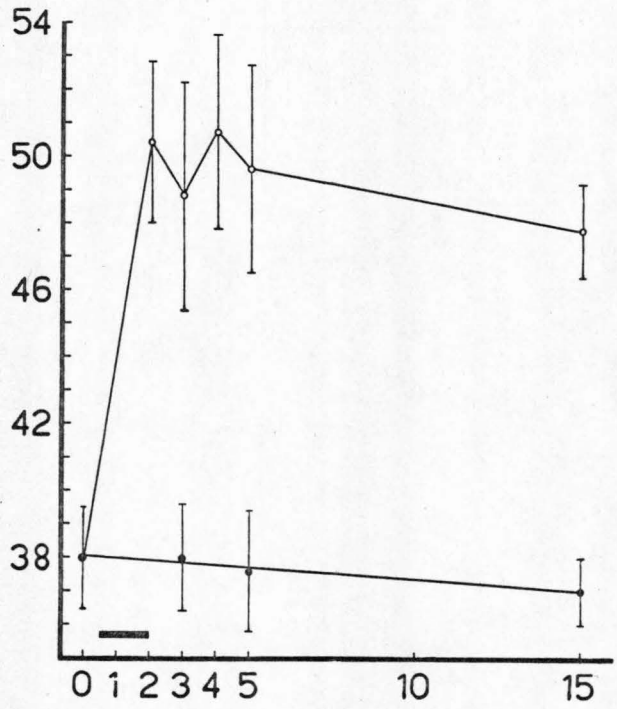

Fig. 7. The total Na contents (in mM/kg w.t., ordinate) of the control and experimental retinas at various times after the initial incubation (abscissa) under anaerobic conditions.

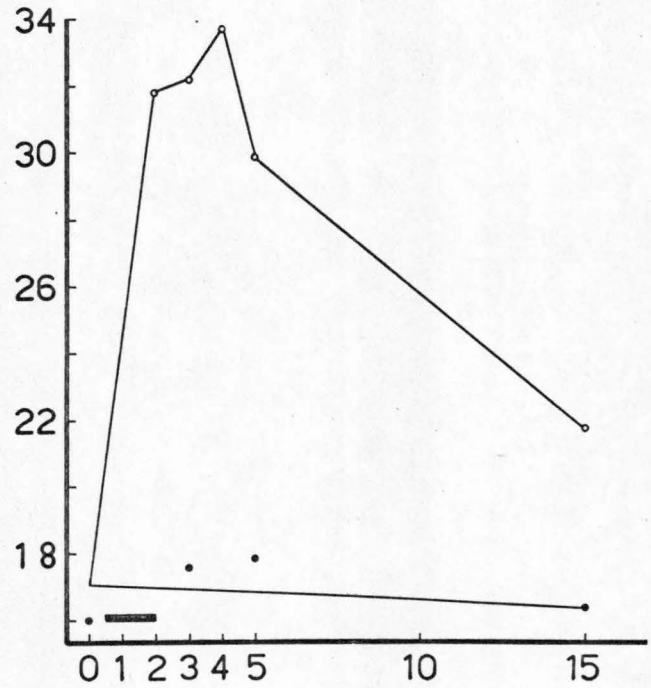

Fig. 8. The intracellular Na contents (in mM/kg w.t., ordinate) of the control and the experimental retinas calculated from the values shown on Figs. 6 and 7.

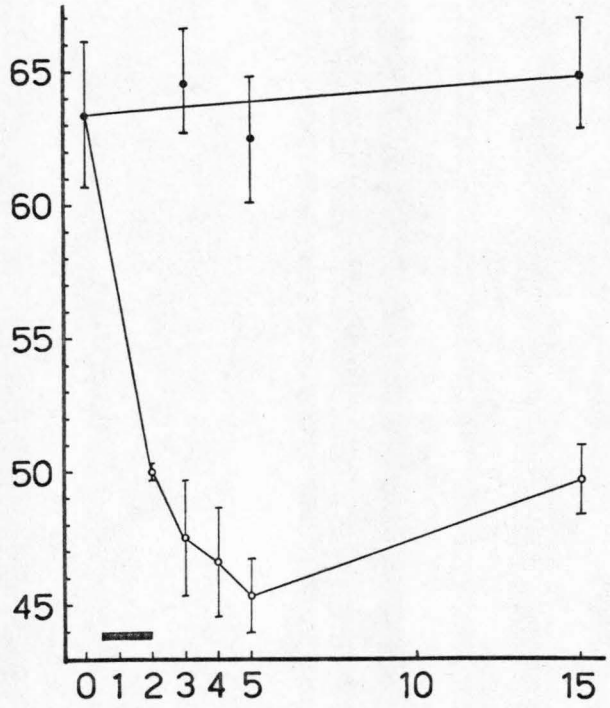

Fig. 9. The total K contents (in mM/kg w.t., ordinate) of the control and experimental retinas at various times after initial incubation (abscissa) under anaerobic conditions.

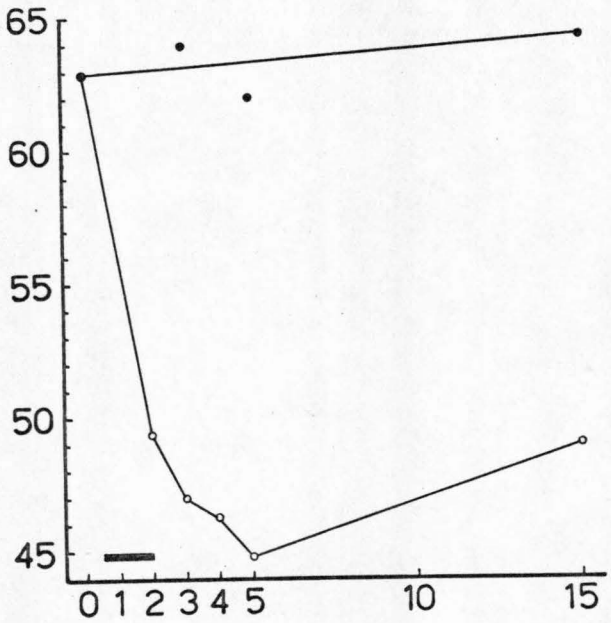

Fig. 10. The intracellular K contents (in mM/kg w.t., ordinate) of the control and experimental retinas calculated from the values shown on Figs. 6 and 9.

K content of the retinas remain quite constant throughout the entire experimental period (15 minutes). Furtheremore, these values are very close to the corresponding figures obtained in the experiments in which the retinas were bathed in oxygenated solutions (Tave VI and Figs. 1 through 5). It is therefore apparent that the deprivation of oxygen does not affect the maintenance of the steady resting state of the isolated chicken retina. The deprivation of oxygen also has very little effect on the changes which occur during SD. The patterns of the changes in the inulin space and of the total and intracellular ion contents observed in the anaerobic experimental group are very similar to that of the corresponding aerobic group, though the magnitude of the changes in the ion contents are smaller in the anaerobic experimental group. Thus, unlike the lowering of temperature which arrests the ion pump, the deprivation of oxygen does not cause an increase in the Na content in either the control or SD invaded retina, indicating that the ion pump in the chicken retina, at least under in vitro conditions, does not have to depend on aerobic metabolism. The energy supply to the ion pump may be derived from glycolysis, as suggested by Van Harreveld and Fifkova (1972). This is further supported by an experiment on two pairs of retinas in which the glucose-free (substituted by sucrose) bathing solutions were used. At the end of the initial incubation period (25 minutes) in the glucose-free solution, the Na content of the retinas was very high (71.5 mM/kg w.t., average of two) while the K content was very low (25 mM/kg w.t., average

of two). The transparency of the retina increased during the incubation period. There was no transparency change indicating the development of SD when the retinas were transferred from glucose-free Mg-CSF to glucose-free MA-CSF.

Effects of procaine on spreading depression and the concomitant ion movements

Leão (1944a) reported that cortical SD does not pass a narrow strip of cortex treated with cocaine. In his review, Marshall (1959) mentioned, although no reference was given, that the topical application of cocaine and procaine prevents SD and decreases the magnitude of K movement. The effects of procaine were studied in the present experiment on the retina.

Paired retinas, prepared as usual, were, after the initial incubation, pretreated with procaine for 5 minutes by transferring them to Mg-CSF containing 0.25% (9 mM/L) of the local anesthetic. This pretreatment allows the drug to diffuse into the ECS of the retina and act on the membrane of the retinal cells. At the end of the pre-treatment one of the paired retinas was transferred to MA-CSF containing the same concentration of procaine (experimental group), while the other retina remained in the original solution (control group). The retinas were immersed in these procaine solutions for periods ranging from 4 to 15 minutes. The ECS was labelled with radioactive inulin. The results of this experiment are presented in Table X.

Table X

Results of the procaine (0.25%) experiment

Water Content		Inulin Space		Total Na Content		Total K Content	
Control	Exptl.	Control	Exptl.	Control	Exptl.	Control	Exptl.
Mg H_2O/mg d.t.		%		mM/kg w.t.			
4.597	4.605	18.2	16.3	37.6	36.1	55.4	54.2
±0.257	±0.244	±1.0	±2.4	±1.4	±1.6	±2.0	±1.6
(8)	(10)	(8)	(10)	(8)	(10)	(8)	(10)
4.604		13.4		35.3		62.8	
4.531		12.8		38.1		61.3	

No SD was ever observed in the retinas of either group, even during 15 minutes immersion in MA-CSF. Neither were there differences in the Na and K contents between the experimental and the control groups. There is a slight but not significant decrease in the inulin space of the retinas in the experimental group. Evidently, SD and its concomitant ion movements are prevented by procaine.

In the control group of the procaine treated retinas the inulin space (18.2%) is higher, while the K content (55.4 mM/kg w.t.) is lower, than those determined in the absence of procaine (14% and 64 mM/kg w.t., respectively). To investigate the possibility that this could be an artifact, control experiments were carried out exactly as the procaine experiment but without using the drug. The results of such experiments on two retinas are listed at the bottom of Table X. The inulin space and K contents determined in the retinas in the absence of procaine are comparable with those listed in Table II. This suggests that the larger inulin space and lower K content observed in the procaine treated retinas are no artifacts. Procaine therefore seems to cause a loss of intracellular K and an increase in the ECS of the resting retina.

If the retina treated with procaine was washed with the usual (procaine-free) Mg-CSF thoroughly for a few minutes and then transferred to the MA-CSF, SD occurred as usual. The effect of procaine is thus reversible. When the retina bathed in the ordinary MA-CSF and partially invaded by SD was transferred to MA-CSF containing procaine, then the

progress of SD was soon arrested and the difference in transparency between the resting and the SD invaded parts of the retina remained unchanged for a long time (at least 20 minutes). It seems that procaine not only prevents the occurrence of SD, but also blocks its restoration.

Experiment with tetrodotoxin

The present study indicated that during SD Na moves from the bathing fluid into the ICS of the isolated retina. Evidence has also been provided that this Na movement is due to an increase in the membrane permeability for this ion during SD. Since tetrodotoxin (TTX) is known to block the Na influx into nerve fibers during the action potential (see Discussion), it was used in the present experiment in the hope of preventing SD by blocking the ion movement. A stock solution of TTX was freshly prepared on the day of the experiment by dissolving 1 mg of TTX in 1 ml of MA-CSF at pH 7. Of this stock solution 0.1 ml was added to 50 or 25 ml of the bathing solutions to make the TTX concentration 6 or 12 μM/L, respectively. These concentrations are more than 100 times that used to block the conduction of single axons, and are somewhat higher than the concentrations used by McIlwain et al. (1969) to antagonize ion movements in cortical tissue slice caused by glutamate. The procedure used in the procaine experiment was used and that the period of the pretreatment varied from 6 to 25 minutes.

No SD was observed in the retinas immersed in Mg-CSF containing TTX at either concentrations. In MA-CSF, TTX, either at 6 or 12 μM/L, unexpectedly did not prevent SD from occurring spontaneously, even when the pretreatment period was prolonged to 25 minutes. However, the transparency change accompanying SD in the TTX solution was less conspicuous, and SD had a longer latency (one minute vs the normal 30 seconds) and duration (3 minutes vs the normal 1.5 minutes). Since it took four minutes for the complete development of SD, all the retinas, either in Mg-CSF (control group) or in MA-CSF (experimental group), were taken out of the solutions for analysis four minutes after the pretreatment. The results of the measurements are listed in Table XI. At the bottom of this table the corresponding results from Table VI are shown for comparison.

In the control group, the inulin space and the Na content of the retinas are comparable to those listed in Table VI. The ECS and the Na content, and hence the Na distribution of the resting retinas did therefore not seem to be affected by the presence of TTX in the incubating solution. The K content is significantly lower in the TTX treated retinas (see Table XI). In the experimental group, SD was observed in every retina. A comparison of the control and the experimental groups shows that in the latter group both the inulin space and the K content are decreased and the Na content is increased. The changes in the ion contents are statistically significant. Evidently neither the occurrence of SD nor its concomitant ion movements are prevented

Table XI

Results of the Tetrodotoxin (6 or 12 μM/L) Experiment.

	Inulin Space		Total Na Content		Total K Content	
	Control	Exptl.	Control	Exptl.	Control	Exptl.
	%		mM/kg. w.t.			
	15.2 ±1.0 (6)	12.7 ±0.8 (6)	37.9 ±1.7 (7)	47.7 ±3.6 (7)	57.3 ±3.7 (7)	45.9 ±3.2 (7)
Dm			9.8		11.4	
p			<0.01		<0.01	
From Table VI	14.7 ±1.8	13.3 ±1.2	37.5 ±1.7	52.9 ±3.5	64.2 ±1.2	48.9 ±3.2
Dm			15.4		15.3	

by TTX. However, the magnitude of the Na accumulation during SD is lower in the presence of TTX (9.8 mM/kg w.t.) than in its absence (15.4 mM/kg w.t.). Also, the loss of K is smaller (11.4 mM/kg w.t., with TTX vs 15.3 mM/kg w.t., without TTX). It was found that the magnitudes of the changes in ion contents due to SD, like the occurrence of SD itself, were not affected by the difference in TTX concentration used, nor by a variation in the period of pretreatment.

It can be concluded that although TTX does not prevent the development of SD, it does partially block the ion movements occurring during SD. This may account for the prolonged latency and duration, as well as the less pronounced transparency change, of the SD observed in retinas treated with TTX.

Summary of Results

The results of the control and the experimental groups of all the experiments are summarized in Table XII. Of the lithium experiment only the ion fluxes are listed. For the purpose of comparison of all the experiments in which SD was observed, the data obtained at the end of the completion of this phenomenon are presented. The intracellular ion content was computed according to equations (11) and (12) by assuming that the inulin space determined represents the actual ECS. These computed values are shown underneath the corresponding total ion content in Table XII, and are used to calculate the magnitude of the ion movements occurring during SD.

Table XII

Summary of the Results of Various Experiments

Condition of Experiment	Inulin Space		Na Content		Na	K Content		K	Occurrence of SD
	Control	Exptl	Control	Exptl	Influx	Control	Exptl	Outflux	
	%		mM/kg. w.t.						
At Room Temperature (24°C)	14.7 ±1.8	13.3 ±1.2	37.5±1.7† 15.5	52.9±3.5 32.9	17.4	64.2±1.2 63.6	48.9±3.2 48.4	15.2	Yes
Anaerobic at 24°C	13.6 ±0.3	12.4 ±1.4	37.6±1.6 17.2	50.4±2.4 31.8	14.6	64.6±1.9 64.1	49.9±0.3 49.4	14.7	Yes
At Low Temperature (2-3°C)	14.3 ±2.0	12.2 ±1.4	46.5±3.5 25.0	60.1±3.2 41.9	16.9	50.1±4.1 49.5	35.8±4.0 35.3	14.2	Yes
With TTX at 24°C	15.2 ±1.0	12.7 ±0.8	37.9±1.7 15.1	47.7±3.6 28.6	13.5	57.3±3.7 56.7	45.9±3.2 45.4	11.3	Yes
Estimated from Li Experiment					14.0			9.0	Yes
With Procaine at 24°C	18.2 ±1.0	16.3 ±2.4	36.7±1.4 9.4	36.1±1.6 11.6	2.2	55.4±2.0 54.7	54.2±1.6 53.5	1.2	No

†The Upper figure represents total, while the lower figure represents intracellular ion content

Table XII shows that the inulin space of the experimental groups is always slightly, but usually not statistically significantly smaller than that of the control goup. The occurrence of SD is always accompanied by a significant increase in Na content and a significant decrease in K content. The calculation of the intracellular ion content shows that the changes in the ion contents during SD are not due to the changes in the ECS but are the results of the movement of Na from the bathing fluid into the ICS of the retina and a release of intracellular K into the bathing fluid. They occur only when SD is observed, indicating that the Na movement and the K release are consistent concomitants of SD.

The magnitudes of the inward Na movement and the K release at the end of SD are computed from the intracellular ion contents. The former is slightly larger than the latter in all the experiments, except those under anaerobic conditions, in which both are equal.

DISCUSSION

The evidence concerning SD reviewed in the Introduction has shown that during cortical SD there is an inward shift of extracellular water and chloride ions, a decrease in the magnitude of the ECS, an increase in tissue impedance, and a release of intracellular potassium. In interpretating the present observation one has to bear in mind that the previous results were obtained under *in vivo* conditions, whereas *in vitro* conditions were used in the present study. Under the *in vivo* condition tissue components only shift between the ECS and the ICS during SD. There is little or no net gain or loss of any material during this phenomenon. Accordingly, the intracellular K released during SD will accumulate in the ECS, causing an increase in the extracellular K concentration. Such an increase reduces the concentration gradient, which favors the outward movement of intracellular K, and therefore retards or prevents further release of K from the ICS, limiting the amount of intracellular K released during SD. Since there is no net gain of any material during SD, the amount of extracellular Na that can possibly shift into the ICS is also limited. The present study, on the other hand, was carried out under a quite different condition. The experiments were performed on the isolated chicken retina with its extracellular fluid equilibrated with a large volume of bathing solution. Under these conditions the amount of extracellular Na is practically unlimited. And, when Na is allowed to enter the ICS either due to an arrest of the ion pump or to an increase in membrane permeability, the

amount of extracellular Na that can potentially shift into the ICS is much larger than that under the in vivo condition. In fact, in some experiments the amount of Na shifted into the ICS exceeded the amount of extracellular Na in the resting retina. This obviously is not possible in the in vivo situation.

When positively charged Na ions are shifted into the ICS during SD, the resting membrane potential, which prevents the loss of intracellular K in the resting state, will be depolarized. Intracellular K then can diffuse into the ECS. In the isolated retina with its extracellular fluid equilibrated with the bathing solution, such a K release during SD, even if very large, will not cause any significant increase in the extracellular K concentration. The increase in extracellular K during SD under in vivo condition, does not develop in the in vitro situation employed in the present study. Therefore, the release of K will continue, leading to a serious K depletion of the retina. This is supported by the following quantitative comparison between the results of the present study and those of previous reports.

Křivaňek and Bureš (1960) estimated that in the intact cerebral cortex the amount of intracellular K released during 1 to 2 waves of SD would cause the extracellular K concentration to increase 3 to 5 times the resting value (about 4 mM). Brinley et al. (1960b) using a similar preparation estimated that the increase in the extracellular K concentration during one wave of SD was four-fold. These estimates were based on the increase in the amount of K washed out from the

cortical surface during SD. Since the K released by the cortical cells during SD has to pass certain barriers to reach the cortical surface, the latter investigators believed that the actual increase in the extracellular K concentration of the cortex might reach 7 to 8 times the resting concentration, (or about 30 mM.) In the present study the amount of intracellular K released during one wave of SD ranged from 11 to 15 mM/kg w.t. (see Table XII). If this K were not lost into the bathing solution but accumulated in the relatively small ECS (13.3%, Table XII), then the extracellular K concentration would rise to more than 80 mM. This increase is much higher than that estimated in the previous reports, and hence apparently a much greater loss of intracellular K has occurred during the SD in the isolated retina immersed in bathing solution.

Taking into consideration the special features of the in vitro experimental situations described above we can now consider the results of the present investigation. During SD a marked increase in the total Na content of retina was observed. Since a shift of Na between the compartments of the tissue does not change the overall Na content, the extra Na must have come from the bathing fluid. Two mechanisms can be involved in this Na uptake by the tissue during SD. The disturbance of the double Donnan equilibrium due to the increased Na permeability of cell membrane can cause an uptake of NaCl from the bathing fluid as discussed in the Introduction. With this mechanism,

the Na uptake must be accompanied by water to maintain osmotic equilibrium. The evidence for such an uptake of bathing fluid during SD has been provided by Van Harreveld and Khattab (1967). They fixed the cerebral cortex during SD by freeze substitution and studied this material with the electron microscope. They found that the dendritic spines in cortex invaded by SD exhibit a moderate swelling. However, if the cortex during SD was superfused with a physiological salt solution, the swelling of the spines was greatly enlarged. The enormous swelling of the spines in superfused cortex suggests that the tissue takes up water from the superfusing solution in addition to the extracellular fluid. This suggestion is supported by the observation that the greatly swollen dendrites are found only in the surface layer of the cortex.

The second mechanism which can lead to an increase in Na content of the retinal tissue is an exchange of intracellular K for Na from the bathing fluid. Such an exchange could take place without an uptake or loss of water, other than the difference in the hydration shells of these ions. If this ion exchange is the only mechanism causing the Na uptake, then the amounts of the K released and the Na taken up by the tissue must be equivalent. This is not the case in the present study, more K is released than Na is taken up. Potassium ions which have to be accompaneid by anions therefore seem to have diffused out of the retina. For osmotic reason, this diffusion of potassium salt

must be accompanied by water, resulting in a decrease of the water content of the tissue. The water in the tissue may thus be affected by two opposing mechanisms; an increase due to the uptake of NaCl from the bathing fluid, and a decrease due to the loss of intracellular K salts. This may explain the observation that the increase in the water content of the isolated retina during SD is small.

Under the in vivo condition, the inward shift of extracellular NaCl and water must result in a shrinkage of the ECS. In the present in vitro situation, in which the ECS of the retina communicates freely with the bathing solution, the loss of the extracellular material could be replenished by an inflow of this solution into the ECS if there exists a mechanism to cause such an inflow. The moderate decrease in the inulin spaces observed during SD which, as discussed in the Results, seems to be an indication of a more severe shrinking of the ECS, suggests that the ECS during SD is not completely replenished from the bathing solution. Furthermore, SD in the isolated retina is characterized by an increase in the tissue transparency, which has been explained by a shrinking of the ECS. This would bring cellular elements closer to each other, causing changes in the position of interphases formed by extracellular material, cell membranes and cytoplasm where passing light is dispersed (Van Harreveld and Fifkova, 1971). The marked increase in the transparency of the retina observed during SD in the in vitro condition would in this view indicate a significant decrease in the magnitude of the ECS. It would therefore

seem that no effective mechanism exists which could cause an inflow of the bathing fluid into the ECS during SD.

From the above discussion it follows that the electrolyte and water movements during SD in the retina under in vitro conditions is complex. It is not possible to estimate the importance of the various mechanisms which can cause shifts in Na, K and water in the retina. They all may contribute to the changes in electrolyte and water contents of the tissue actually observed. However, taking the in vitro conditions of the experiment into consideration the results of the present investigation are in agreement with the concept of SD discussed in the Introduction.

In the procaine experiments, the control retinas showed a significantly larger inulin space, a significantly lower K content and an unchanged Na content as compared with the values obtained from the resting retinas not treated with the drug. The differences are, as mentioned in the Results, not due to experimental artifact. And, since the control retinas had been immersed in the procaine solution for at least 9 min., the inulin space determined in these retinas may represent a reliable figure for the ECS. The water content of these retinas was not different from those not treated with the drug (Table II), suggesting that the local anesthetic does not significantly change the overall volume of the retina. It can be surmised that the increase in the magnitude of the ECS caused by procaine is the result of a shrinkage

of the ICS, as suggested by the loss of intracellular potassium. A potassium salt accompanied by water may have moved into the ECS and from there into the bathing fluid. The increase in inulin space may therefore not be an actual increase but only a relative one due to the shrinking of the ICS.

The inverse relationship between the magnitudes of the ECS and the ICS discussed in relation with the procaine treated retinas may explain the large increase of the inulin space observed during the later phase of the recovery from SD (see Fig. 1). During that phase the Na ions which invaded the ICS during SD are removed by the ion pump while the re-uptake of K may be retarded by the low extrcellular K concentration. It has to be assumed that the pump does not exchange Na for K during that phase of the recovery but removes Na ions accompanied by an anion. As a result of this, there would be a net loss of intracellular ions, causing a shrinkage of the ICS and hence a relative and an absolute increase in the magnitude of the ECS and the inulin space, respectively.

In the low temperature experiment the action of the Na pump was inhibited by the cooling, and the Na ions diffusing into the intracellular compartment of the retina could not be pumped out any more. Since the total Na content was increased in these retinas, as compared with those kept at room temperature, without a change in the inulin space, Na ions must have been taken up by the intracellular compartment from the bathing fluid. An equivalent amount of K was lost to the bathing fluid (Table VII). The changes in the water content, inulin

space and ion contents during SD in the retinas at low temperature were very similar to those observed in retinas at room temperature.

The function of the ion pump is also interfered with when the retina is bathed in a lithium solution, because the active transport mechanism for Na cannot handle lithium ions. Lithium diffusing into the intracellular compartment can therefore not be removed. Lithium ions thus replace K and Na ions in the ICS explaining the high Li content and the low Na and K contents of the control retinas bathed in the lithium solution. Spreading depression causes a marked increase in the Li content and a smaller loss of K in the retina bathed in the Li solution. The increase in the Li content even though marked is still smaller than the corresponding Na increase in the retinas bathed in the normal physiological solution. The smaller inward Li shift during SD can be explained by the fact that before the development of SD the retina in the Li solution has already had a relatively high intracellular Li content which reduces the concentration gradient favoring the inward shift of lithium ions.

The magnitude of the K outflux during SD estimated in the Li experiment is the lowest in all the experiments. The low K outflux seems to be due to the following reasons. The intracellular K content of the control retina is very low (28.2 mM/kg w.t.), suggesting that in the experimental retina too, before the development of SD, the amount of the intracellular K of the retina is very small. Accordingly, both the amount of the intracellular K available for release and the

concentration gradient favoring the outflux of K during SD are reduced. Furthermore, a significant amount of intracellular cations are bound to negatively charged impermeable organic ions, such as proteins or certain lipids, and hence are non-exchangeable. This further reduces the amount of K available for release during SD, and may explain why there is still a residual amount (4.2 mM/kg w.t.) of Na left in the ICS of retinas after they have been extensively washed in Na-free solutions and invaded by SD. In rat cerebral tissue, Katzman and Leiderman (1953) estimated that the amount of non-exchangeable K is about 20 percent of the total potassium content in the normal brain tissue. Since the retina is part of the central nervous system, it is reasonable to assume that the non-exchangeable K in the ICS of the chicken retina is of a similar magnitude. The total K content of the isolated chicken retina in optimal physiological condition (incubated at body temperature) is about 72 mM/kg w.t., which is practically the same as that measured from the retina immediately after its removal from the eye (Calculated from Table I, Van Harreveld and Fifkova, 1972). Therefore, there would be about 14.5 mM/kg w.t., of non-exchangeable K in the ICS of the chicken retina. Assuming that just before the occurrence of SD the retina in the Li solution contains the same amount (28.2 mM/kg w.t.) of intracellular K as the control retinas, then the amount of intracellular K available for release during SD would be only about 14 mM/kg w.t. From this 6.7 mM/kg w.t., is released during SD. The low resting intracellular K content thus

seems to be the main reason responsible for the low K release observed. The low resting intracellular K content, in turn, is due to the displacement of Li passively accumulated in the ICS of the retina. The accumulation of Li, therefore is indirectly responsible for the observed low K release.

It can be concluded from the above discussions that but for the accumulation of a large amount of Li in the ICS of the retina, the results of the Li experiment are in agreement with those of the experiments carried out in the normal salt solution, and hence support the conclusions derived from the latter experiments. Furthermore, as will be discussed below, the Li experiments supply evidence about the mechanism underlying the inward shift of extracellular Na during SD.

The present study has shown that during SD sodium shifts from the extracellular fluid and bathing solution into the ICS of retina. As mentioned in the Introduction, the Na shift can be a consequence of an arrest of the ion pump, or of an increase in Na permeability of cell membranes, or a combination of both mechanisms. The transparency change which may develop in retinas bathed for more than 10 min in the cold or in a Li solution in the presence of a high concentration of Mg ions may be caused by the arrest of or interference with the ion pump. The Mg ions, while capable of arresting the development of SD, cannot prevent the inward diffusion of Na (or Li) ions. This diffusion, although a slow process, will eventually result in an accumulation of NaCl or LiCl in the intracellular compartment, causing similar changes

in transparency as observed during SD. This slow uptake of extracellular material also occurs in non-nervous tissues maintained in vitro and subjected to conditions in which the ion pump can be expected to be inhibited. The tissues accumulate water and Na (Leaf, 1956), and show an increase in tissue impedance (Van Harreveld and Biber, 1962).

The results of the low temperature experiments show that SD can occur in a retina deprived of a functioning ion pump. The arrest of the ion pump can therefore not be the mechanism underlying the Na shift observed during SD. The alternate mechanism, the increase in Na permeability of the cell membrane, becomes therefore the mechanism of choice. This is further supported by the results of the Li experiments.

In the Li experiments, the accumulation of this ion in the ICS of the control retinas indicates that the resting cell membrane exhibits a moderate permeability for Li. This is not unexpected since the resting membrane shows a permeability for labelled Na ions, and Li behaves as extracellular Na and passes through cell membrane via "Na channels." During SD the ICS of the retina in Li solution accumulates more Li, indicating that during this phenomenon the membrane permeability for this ion, and hence for Na is increased, allowing Li to shift from the bathing fluid into the ICS. It is of interest to note that a loss of intracellular Na from the retinas bathed in Li solutions occurs during SD. With the retina immersed in Li solution, which contains no Na, the concentration gradient for Na favors its outflux.

The loss of intracellular Na observed during SD, therefore, also indicates that Na permeability of the cell membrane is increased during this phenomenon.

The increase in Na permeability of the cell membrane explains the depolarization of tissue elements observed during SD (Brožek, 1966; Collewijn and Van Harreveld, 1966; Karahashi and Goldring, 1966). This depolarization may cause the slow potential changes which accompany SD.

The concept that the Na shift observed during SD is due to an increase in Na permeability of cell membranes can also be consolidated in another approach. If the concept is correct one would be able to block the Na shift with membrane stabilizers which prevent the increase in membrane permeability. Procaine, a local anesthetic, is such a membrane stabilizer. It reduces both the influx and the outflux of K in the resting toad and squid nerve fibers without affecting the resting potential (Shanes, 1958). In the Purkinje fibers of calf and sheep hearts (Weidmann, 1955) and in the squid axon (Shanes, Frekgang, Grundfest and Amatnick, 1959) both the rates of rise and the maximum amplitude of the action potential are reduced by procaine. Using an iontophoretic technique to apply procaine to spinal neurons, Curtis and Phillis (1960) found that this local anesthetic abolished spike activity without significantly affecting the resting membrane potential. They interpreted their data in view of the membrane stabilizing effect of procaine, and concluded that the Na conductance

(permeability) was reduced by the drug to such an extent that a regenerative inward sodium current could not be elaborated. Brinley et al. (1960a) studied the effect of procaine on the ^{42}K outflux from the cerebral cortex and concluded that this local anesthetic acts on the mammalian cortex in a similar way (membrane stabilization) as on the peripheral nerve. From these observations, it is apparent that procaine stabilizes the nerve membrane in both the resting and active states. In the present study procaine was found to block not only the Na shift but SD itself as well (Table X). Such a blocking effect is also exhibited by another membrane stabilizer, magnesium.

Magnesium ion and another bivalent cation, calcium, are long known to be capable of decreasing the excitability of peripheral nerves (Hoeber and Strhe, 1929; Guttman, 1940; Frankenhäuser and Meves, 1958). In high concentration they increase the depolarizing threshold to achieve the same increase in Na conductance of the squid axon (Frankenhäuser and Hodgkin, 1957) and frog sciatic nerves (Frankenhäuser, 1957). Because of these pieces of evidence Mg and Ca ions are generally considered as the membrane stabilizer. Although this evidence comes from investigations on the peripheral nerve, it is not unreasonable to assume that the bivalent cations also have a membrane stabilizing effect on central nervous tissues, including the retina. In fact, this stabilizing effect was proposed by Bures (1960) to explain the observed effects of Mg and Ca ions in blocking cortical SD. In the present study, it was found that SD did not develop or

propagate in retinas immersed in solutions containing 10 mM/1 of Mg ions, even if stimulation (mechanical) was applied to the retina. Neither did the ion movements, which are the concomitant of SD, occur, as indicated by the constant levels of the inulin space, and the Na and K contents (control group, Table VI) throughout a period of 30 minutes. When the retina was transferred to a solution with a low Mg concentration, SD and its concomitant ion movements occurred spontaneously in 30 to 60 seconds. The fact that both membrane stabilizers block the development of SD as well as the Na shift not only supports the concept that the Na shift observed during SD is a consequence of the increase in Na permeability of the cell membrane but suggests that the increase in membrane permeability is the basic mechanism underlying SD.

In view of the above conclusion, the observation that TTX, which is known to block inward Na movement during action potential (see Kao, 1966; Dettbarn, 1971), did not completely block SD and its concomitant ion movements requires explanation. Like the membrane stabilizing effect of Mg ion and procaine, the blocking effect of TTX is also derived from the investigations of peripheral nerves. There is evidence, however, that TTX acts on the neurons in the central nervous system in the similar way. For example, TTX depresses the vasomotor and the respiratory centers (see Ogura, 1971). It was found that the electrical activity of the central nervous tissue was abolished by exposing the tissue to TTX (Colomo and Erulker, 1968; Blankenship,

1968). Frank and Pinsky (1966) found that in isolated slabs of cerebral cortex TTX inhibited the potentials that normally can be evoked by electrical stimulation. Furthermore, this toxin has been found (McIlwain et al., 1969; Pull et al., 1970; Okamoto and Quastel, 1970b) to depress the uptake of Na ion by cortical slices, caused by glutamate stimulation. These pieces of evidence are in agreement with the effect of TTX in producing the reductions of the extent of the transparency increase and the magnitude of the ion movements during SD, but do not explain why the blocking is incomplete. There are at least three possible explanations for this incomplete blocking. Firstly, it could be surmised that TTX acts only on the axonal membrane but not on the membranes of the cell bodies and dendrites. Thus, in the presence of TTX only the axons would be prevented from participating in SD. However, axons do not seem to change during SD (Van Harreveld, 1958). An exclusive effect on the axons can therefore not explain the effect of TTX on the retina. Secondly, one could postulate that TTX depresses Na movement in the plasma membrane of somas and dendrites, but less effectively than on the membrane of the peripheral nerves. This is not an unreasonable speculation since these parts of the neuron have, in addition to electrically excitable components, areas which are chemically excitable and hence may be insensitive to the toxin. The third explanation is that some of the cellular elements which participate in SD are not affected by TTX. These cellular elements

may be neurons or glial cells. The first possibility is suggested by the finding of Koketsu and Nishi (1969) that action potentials in the sympathetic ganglion of the frog are indeed resistant to TTX. Glial elements have been observed to show electrical responses similar to, but much slower than those exhibited by neuronal elements (Tasaki and Chang, 1958). Based on this observation and the slowness of SD, Galamos (1961) suggested that glial elements would be involved in SD. In the present study as much as 24 mM/kg w.t., of intracellular K were released during one wave of SD. This amount of K constitutes about half the exchangeable intracellular K (45 - 50 mM/kg w.t.) of the entire retina used for the observation, suggesting that some of the K ions released during SD are derived from the glial elements, or, in other words, that glial elements participate in SD. However, the possibility that glial elements participate in SD has been questioned. Van Harreveld and Stamm (1954) and Hull and Van Harreveld (1966) found that SD could not be evoked in, or propagated through an area of cerebral cortex damaged by asphyxia to such an extent that the spontaneous electrical activity was abolished; such cortices show an abundance of glia. Van Harreveld, Terres and Dernburg (1956) also showed that SD was not transmitted through a narrow region of cortex in which extensive neuronal degeneration with glial proliferation had been induced by a knife cut. Grafstein and Sastry (1957) found that in a chronically isolated slab of cerebral cortex, in which much more injury to neurons than to glia cells can be expected, much stronger electrical

stimuli were required to elicit SD. Furthermore, the accompanying SPC was of much lower amplitude, and the velocity of propagation of SD was slightly decreased. Finally, electron micrographs of the cerebral cortical tissue fixed by freeze substitution at the height of SD show swelling of dendritic elements but no clearcut evidence of a glial involvement (Van Harreveld and Khattab, 1967). These observations strongly suggest that neuronal elements are required for the development and the propagation of SD.

The results of the TTX experiments also shed light on the mechanism underlying SD. According to Grafstein's hypothesis (see Introduction), SD is initiated by an intensive neuronal activity which causes a release of intracellular potassium. Since the neuronal activity will certainly be abolished in the presence of TTX, the failure of this toxin to completely prevent SD indicates that neuronal burst is not necessary for SD. Furthermore, another basic requirement of Grafstein's hypothesis is that the amount of intracellular K released from the activated neurons is large enough to depolarize adjacent neural elements. In the present study, SD was observed in the isolated retina with its extracellular fluid in equilibrium with the bathing solution. The retina is very thin and the K ions can diffuse rapidly into the bathing solution. It is therefore doubtful that the intracellular K released during SD can accumulate in the ECS and build up a high enough concentration to markedly depolarize adjacent neurons. A mechanism other than that proposed by Grafstein is therefore required to account for the

development and propagation of SD.

As mentioned in the Introduction, a different mechanism underlying SD has been proposed by Van Harreveld (1959). The principal of this mechanism is similar to that of Grafstein's hypothesis, but glutamate, instead of K is believed to be the agent released by the cellular elements of nervous tissue. This amino acid would cause depolarization of cellular elements and thus be involved in the development and propagation of SD. It was found that a concentration of glutamate as low as 0.2 mM/l in the physiological solution bathing the isolated retina can elicit SD (Van Harreveld and Fifkova, 1970). It seems therefore possible that under the in vitro experimental condition employed in the present study the glutamate released from the cellular elements of the retina can elicit SD in adjacent tissues before the amino acid is lost into the bathing solution. Since TTX has been reported to antagonize the effect of glutamate in causing the uptake of extracellular material by the nervous tissues in vitro, one could argue that the failure of TTX to completely prevent SD and its concomitant ion movements is not in agreement with the proposed role of glutamate in the propagation of SD. However, TTX does not completely suppress, but only depresses the uptake of extracellular Na by cortex slices in response to glutamate (McIlwain et al., 1969; Pull et al., 1970; Okamoto and Quastel, 1970b). Also the transparency increases in isolated retinas stimulated with glutamate is not completely

abolished by TTX (Van Harreveld and Fifkova, 1972). These observations are in agreement with the incomplete blocking of SD and its concomitant ion movements by TTX observed in the present study.

CONCLUSION

The results of the present study on the isolated chicken retina are basically in agreement with, and hence support, the concepts of SD developed by previous investigators. Evidence was presented for the postulate that during SD an inward shift of extracellular Na occurs, and that this Na shift is not due to an arrest of the ion pump but is a consequence of an increase in Na permeability of cell membranes. It is furthermore suggested that this increase in membrane permeability is the basic mechanism underlying SD and is probably caused by a glutamate released from cellular elements of the nervous tissue.

REFERENCES

Albert, D. J. The effect of spreading depression on the consolidation of learning. Neuropsychologia 4:49-69. 1966.

Ames, A. Studies on water and electrolytes in nervous tissue. II. effect of glutamate and glutamine. J. Neurophysiol. 19:213-223. 1956.

Ames, A. and Hastings, A. B. Studies on water and electrolytes in nervous tissue. I. Rabbit retina: methods and interpretation of data. J. Neurophysiol. 19:201-212. 1956.

Ames, A. and Nesbett, F. B. Intracellular and extracellular compartments of mammalian central nervous tissue. J. Physiol. (London) 184:215-238. 1966.

Ames, A., Tsukada, Y. and Nesbett, F. B. Intracellular Cl^-, Na^+, K^+, Ca^{2+}, Mg^{2+}, and P in nervous tissue; response to glutamate and to changes in extracellular calcium. J. Neurochem. 14: 145-159. 1967.

Anderson, D. K. and Hazelwood, R. L. Chicken cerebrospinal fluid: normal composition and response to insulin administration. J. Physiol. (London) 202:83-95. 1969.

Basser, L. S. The relation of migraine and epilepsy. Brain 92:285-300. 1969.

Berl, S. and Waelsch, H. Determination of glutamic acid, glutamine, glutathione and γ-aminobutyric acid and their distribution in brain tissue. J. Neurochem. 3:161-169. 1958.

Blankenship, J. E. Action of tetrodotoxin on spinal motoneurons of the cat. J. Neurophysiol. 31:186-194. 1968.

Bohdanecký, Z. and Nečina, J. Course of some pharmacological tests in functionally decorticated animals. Physiol. bohemoslov. 12: 55-61. 1963.

Bourke, R. S. and Tower, D. B. Fluid compartmentation and electrolytes of cat cerebral cortex in vitro - I. swelling and solute distribution in mature cerebral cortex. J. Neurochem. 13:1071-1097. 1966a.

Bourke, R. S. and Tower, D. B. Fluid compartmentation and electrolyte of cat cerebral cortex in vitro - II. sodium, potassium and chloride of mature cerebral cortex. J. Neurochem. 13:1099-1117. 1966b.

Brinley, F. J., Jr., Kandell, E. R. and Marshall, W. H. Effect of gamma-amino butyric acid (GABA) on K^{42} outflux from rabbit cortex. J. Neurophysiol. 23:237-245. 1960a.

Brinley, F. J., Jr., Kandel, E. R. and Marshall, W. H. Potassium outflux from rabbit cortex during spreading depression. J. Neurophysiol. 23:246-255. 1960b.

Brožek, G. Changes in the membrane potential of cortical cells during spreading depression. Physiol. bohemoslov. 15:98-103. 1966.

Bureš, J. Block of Leão's spreading cortical depression by bivalent cations. Physiol. bohemoslov. 9:202-209. 1960.

Bureš, J. and Burešová, O. The use of Leao's spreading cortical depression on research on conditioned reflexes. EEG Clin. Neurophysiol. suppl. 13:359-376. 1960a.

Bureš, J. and Burešová, O. The use of Leao's spreading depression in the study of interhemispheric transfer of memory traces. J. Comp. Physiol. Psychol. 53:558-563. 1960b.

Burešová, O. Change in cerebral circulation in rats during spreading EEG depression. Physiol. bohemoslov. 6:1-11. 1957.

Burešova, O. and Bureš, J. The effect of prolonged cortical spreading depression on learning and memory in rats. J. Neurobiol. 1:135-146. 1969.

Colre, K. S. Electric impedance of suspensions of spheres. J. gen. Physiol. 12:29-36. 1928.

Colre, K. S. Permeability and impermeability of cell membranes for ions. Cold Spring Harbor Symp. 8:110-122. 1940.

Cole, K. S. Impedance of single cells. Tabul. biol., Hague 19 II, 24. 1942.

Collewijn, H. and Van Harreveld, A. Membrane potential of cerebral cortical cells during spreading depression and asphyxia. Exp. Neurol. 15:425-436. 1966.

Colomo, F. and Erulkar, S. D. Miniature synaptic potentials at frog spinal neurones in the presence of tetrodotoxin. J. Physiol. (London) 199:205-221. 1968.

Curtis, D. R. and Phillis, J. W. The action of procaine and atropine on spinal neurones. J. Physiol. (London) 153:17-34. 1960.

Curtis, D. R., Phillis, J. W. and Watkins, J. C. The chemical excitation of spinal neurones by certain acidic amino acids. J. Physiol. (London) 150:656-682. 1960.

Dettbarn, W.-D. Mechanism of action of tetrodotoxin (TTX) and saxitoxin (STX). In Neuropoison (L. L. Simpson, ed.) New York, London, Plenum Press. vol. 1, pp. 169-186. 1971.

Dusser de Barenne, J. G. and McCulloch, W. S. Suppression of motor response obtained from area 4 by stimulation of area 4_S. J. Neurophysiol. 4:311-323. 1941.

Frank, G. B. and Pinsky, C. Tetrodotoxine-induced central nervous system depression. Brit. J. Pharmacol. 26:435-443. 1966.

Frankenhäuser, B. The effect of calcium on myelinated frog fiber. J. Physiol. (London) 137:245-260. 1957.

Frankenhäuser, B. and Hodgkin, A. L. The action of calcium on the electrical properties of squid axons. J. Physiol. (London) 137: 218-244. 1957.

Frankenhäuser, B. and Meves, M. The effect of magnesium and calcium on the frog myelinated nerve fiber. J. Physiol. (London) 142: 360-365. 1958.

Freygang, W. H.,Jr. and Landau, W. M. Some relations between resistivity and electrical activity in cerebral cortex of cat. J. Cell. Comp. Physiol. 45:377-392. 1955.

Fricke, H. A mathematical treatment of the electric conductivity and capacity of disperse systems. I. the electric conductivity of a suspension of homogeneous spheroids. Phys. Rev. 24:575-587. 1924.

Galambos, R. A glia-neural theory of brain function. Proc. Natl. Acad. Sci. 47:129-136. 1961.

Gouras, P. Spreading depression of activity in amphibian retina. Am. J. Physiol. 195:28-32. 1958.

Grafstein, B. Mechanism of spreading cortical depression. J. Neurophysiol. 19:154-171. 1956.

Grafstein, B. and Sastry, P. B. Some preliminary electrophysiological studies on chronic neuronally isolated cerebral cortex. EEG Clin. Neurophysiol. 9:723-725. 1957.

Guttman, R. Stabilization of spider crab nerve membranes by alkaline earths as manifested in resting potential measurements. J. gen. Physiol. 23:343-364. 1940.

Harvey, J. A. and McIlwain, H. Excitatory acidic amino acids and the cation content and sodium ion flux of isolated tissues from the brain. Biochem. J. 108:269-274. 1968.

Hoeber, R. and Strohe, H. Über den Einfluss von Salzon auf die elektrotonischen Ströme, die Erregbarkeit und das Ruhepotential des Nerven. Pflüg. Arch. ges Physiol. 222:71-88. 1929.

Hull, C. D. and Van Harreveld, A. Absence of conduction of spreading depression through cortical region damaged by asphyxiation. Amer. J. Physiol. 207:921-924. 1964.

Kao, C. Y. Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. Pharmacol. Rev. 18:997-1049. 1966.

Karahashi, Y. and Goldring, S. Intracellular potentials from "idle" cells in cerebral cortex of cat. EEG Clin. Neurophysiol. 20: 600-607. 1966.

Katzman, R. and Leiderman, P. H. Brain potassium exchange in normal adult and immature rats. Am. J. Physiol. 175:263-270. 1953.

Keynec, R. D. and Swan, R. C. The permeability of frog muscle fibers to lithium ions. J. Physiol. (London) 147:626-638. 1959.

Koketsu, K. and Nishi, S. Calcium and action potentials of bullfrog sympathetic ganglion cells. J. gen. Physiol. 53:608-623. 1969.

Křivánek, J. and Bureš, J. Ion shifts during Leao's spreading cortical depression. Physiol. bohemoslov. 9:494-503. 1960.

Leaf, A. On the mechanism of fluid exchange of tissues in vitro. Biochem. J. 62:241-248. 1956.

Leão, A. A. P. Spreading depression of activity in the cerebral cortex. J. Neurophysiol. 7:359-390. 1944a.

Leão, A. A. P. Pial circulation and spreading depression of activity in the cerebral cortex. J. Neurophysiol. 7:391-396. 1944b.

Leão, A. A. P. Further observations on the spreading depression of activity in the cerebral cortex. J. Neurophysiol. 10:409-414. 1947.

Leão, A. A. P. and Martins-Ferreira, H. Alteration of electric impedance in the course of spreading depression of activity of the cerebral cortex. Ann. Acad. bras. Sci. 25:259-266. 1953.

Leão, A. A. P. and Martins-Ferreira, H. Nota sobre a ocorrencia da 'depressao alastrante' no hipocampo e no eropo estriado. Anais acad. brasil cienc. 30: No. 2:I. 1958.

Leão, A. A. P. and Morison, R. S. Propagation of spreading cortical depression. J. Neurophysiol. 8:33-45. 1945.

Marshall, W. H. The relation of dehydration of the brain to the spreading depression of Leao. EEG Clin. Neurophysiol. 2:177-185. 1950.

Marshall, W. H. Spreading cortical depression of Leao. Physiol. Rev. 39:239-279. 1959.

Marshall, W. H., Essig, C. F. and Dubroff, S. J. Relation of temperature of cerebral cortex to spreading depression of Leao. J. Neurophysiol. 14:153-166. 1951.

Marshall, W. H., Woolsey, C. N. and Bard, P. Cortical representation of tactile sensiblity as indicated by cortical potentials. Science 85:388-390. 1937.

Martins-Ferreira, H. and de Oliveira Castro, G. Light-scattering changes accompanying spreading depression in isolated retina. J. Neurophysiol. 29:715-726. 1966.

Martins-Ferreira, H. and Leão, A. A. P. Nota sobre o estudo da 'depressao alastrante' em repteis. Anais. acad. brasil cienc. 30: No. 2:I. 1958.

McIlwain, H., Harvey, J. A. and Rodriguez, G. Tetrodotoxin on the sodium and other ions of cerebral tissues, excited electrically and with glutamate. J. Neurochem. 16:363-370. 1969.

Milner, P. M. Note on a possible correspondence between the scotomas of migraine and spreading depression of Leao. EEG Clin. Neurophysiol. 10:705. 1958.

Monakhov, K. K., Fifkova, E. and Bures, J. Steady potential field of hippocampal spreading depression. J. Cell. Comp. Physiol. 59: 155-162. 1962.

Morlock, N. L., Mori, K. and Ward, A. A. A study of single cortical neurons during spreading depression. J. Neurophysiol. 27:1192-1198. 1964.

Nicholls, J. G. Kuffler, S. W. Na and K content of glial cells and neurons determined by flame photometry in the central nervous system of the leech. J. Neurophysiol. 28:519-525. 1965.

Ochs, S. The nature of spreading depression in neural networks. Int. Rev. Neurology 4:1-69. 1962.

Ogura, Y. Fugu (Puffer-Fish) poisoning and the pharmacology of crystalline tetrodotoxin in poisoning. In Neuropoisons (L. L. Simpson, ed.), New York, Plenum Press, vol. 1, pp. 139-158. 1971.

Okamoto, K. and Quastel, J. H. Water uptake and energy metabolism in brain slices from the rat. Biochem. J. 120:25-36. 1970a.

Okamoto, K. and Quastel, J. H. Tetrodotoxin-sensitive uptake of ions and water by slices of rat brain in vitro. Biochem. J. 120:37-47. 1970b.

Ookawa, T. and Bures, J. Spreading depression in the striatum and optic lobes of the chicken. Brain Res. 13:119-128. 1969.

Ozeki, M. and Grundfest, H. Crayfish muscle fiber: ionic requirements for depolarization synaptic electrogenesis. Science 155:478-481. 1967.

Pull, I., McIlwain, H. and Ramsay, R. L. Glutamate, calcium ion-chelating agents and the sodium and potassium ion contents of tissue from the brain. Biochem. J. 116:181-187. 1970.

Pumphrey, R. J. Sensory Organs: Vision. In Biology and Comparative Physiology of Birds (A. J. Marshall, ed.), Academic Press, vol. II, pp. 61. 1961.

Ranck, Jr., J. B. Specific impedance of cerebral cortex during spreading depression, and an analysis of neuronal, neuroglial, and interstitial contributions. Exp. Neurol. 9:1-16. 1964.

Schwerin, P., Bessman, S. P. and Waelsch, H. The uptake of glutamic acid and glutamine by brain and other tissues of the rat and mouse. J. Biol. Chem. 184:37-44. 1950.

Shanes, A. M. Electrochemical aspects of physiological and pharmacological action on excitable cells. II. the action potential and excitation. Pharmacol. Rev. 10:165-273. 1958.

Shanes, A. M., Frekgang, W. H., Grundfest, H. and Amatnick, E. Anesthetic and calcium action in the voltage calmped squid giant axon. J. gen. Physiol. 42:793-802. 1959.

Shapiro, H. The validity of the centrifuge methods for estimating aggregate cell volume in suspensions of the egg of the sea-urchin Arbacia punctulata. Biol. Bull. Marine Biol. Lab. (Woods Hole) 68:363-377. 1935.

Sloan, N. and Jasper, H. The identity of spreading depression and "suppression." EEG Clin. Neurophysiol. 2:59-78. 1950.

Sonnenschein, R. R. and Walker, R. M. Local cerebral blood flow in spreading cortical depression. Fed. Proc. 15:175. 1956.

Tasaki, I. and Chang, J. J. Electric response of glia cells in cat brain. Science 128:1209-1210. 1958.

Van Harreveld, A. Changes in volume of cortical neuronal elements during asphyxiation. Amer. J. Physiol. 191:233-242. 1957.

Van Harreveld, A. Changes in the diameter of apical dendrites during spreading depression. Amer. J. Physiol. 192:457-463. 1958.

Van Harreveld, A. Compounds in brain extracts causing spreading depression of cerebral cortical activity and contraction of crustacean muscle. J. Neurochem. 3:300-315. 1959.

Van Harreveld, A. Brain Tissue Electrolytes. Washington: Butterworths, Inc., p. 55. 1966.

Van Harreveld, A. and Biber, M. P. Conductivity changes in some organs after circulatory arrest. Amer. J. Physiol. 203:609-614. 1962.

Van Harreveld, A., Crowell, J. and Malhotra, S. K. A study of extracellular space in central nervous tissue by freeze-substitution. J. Cell Biol. 25:117-137. 1965.

Van Harreveld, A. and Fifkova, E. Glutamate release from the retina during spreading depression. J. Neurobiol. 2:13-29. 1970.

Van Harreveld, A., and Fifkova, E. Effects of glutamate and other amino acids on the retina. J. Neurochem. 18:2145-2154.

Van Harreveld, A. and Fifkova, E. Effects of metabolic inhibitors on glutamate release from the retina. (in press). 1972.

Van Harreveld, A. and Khattab, F. I. Changes in cortical extracellular space during spreading depression investigated with the electron microscope. J. Neurophysiol. 30:911-929. 1967.

Van Harreveld, A. and Malhotra, S. K. Extracellular space in the cerebral cortex of the mouse. J. Anat. 101:197-207. 1967.

Van Harreveld, A. and Ochs, S. Cerebral impedance changes after circulatory arrest. Amer. J. Physiol. 187:180-192. 1956.

Van Harreveld, A. and Ochs, S. Electrical and vascular concomitants of spreading depression. Amer. J. Physiol. 189:159-166. 1957.

Van Harreveld, A. and Schadé, J. P. Choride movements in cerebral cortex after circulatory arrest and during spreading depression. J. Cell. Comp. Physiol. 54:65-84. 1959.

Van Harreveld, A. and Stamm, J. S. On the conditions for the recording of Leao's spreading depression. EEG Clin. Neurophysiol. 3:323-328. 1951.

Van Harreveld, A. and Stamm, J. S. Vascular concomitants of spreading cortical depression. J. Neurophysiol. 15:487-496. 1952.

Van Harreveld, A. and Stamm, J. S. Relation between asphyxial damage to the cortex and the SD. Amer. J. Physiol. 178:117-122. 1954.

Van Harreveld, A., Terres, G. and Dernburg, E. A. Cortical discontinuity and propagation of spreading depression. Amer. J. Physiol. 184:233-238. 1956.

Weidmann, S. Effects of calcium ions and local anaesthetics on electrical properties of Purkinje fibers. J. Physiol. (London) 129:568-582. 1955.