On the R Membrane in the Frog's Eye: Its Localization, and Relation to the Retinal Action Potential

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It was reported by Brindley (1, 3, 5) that there is a layer of high electrical resistance and capacitance termed the R membrane in the retina and that the ERG develops across this membrane. He considers that the R membrane is the external limiting membrane. On the other hand, Brown and Wiesel (6) who worked more recently on the unopened cat's eye with microelectrodes concluded that the R membrane is not the external limiting membrane but Bruch's membrane lying just back of the pigment epithelium. It will be reported in this paper that (*a*) our results obtained from the excised opened eye of the frog support the conclusion of Brown and Wiesel, (*b*) the R membrane is an important physical factor that determines the configuration of the intraretinally recorded action potential, and (*c*) some of the conflicting evidence concerning the origin of the ERG obtained with microelectrodes can be resolved in terms of this factor.

METHOD

The localization of the R membrane was made by measuring the radial resistance of the frog's opened eye using a modification of Brindley's method. The arrangement used is illustrated in Fig. 1*A*. Constant current pulses 15 μa. in intensity and some 35 msec. in duration were passed across the eye from a wick-electrode in contact with the vitreous humor to a chlorided silver plate which also served as the indifferent lead. The eye was mounted in the concave upper face of a block of Ringer-soaked chalk placed on the silver plate. This arrangement served to produce a radial current of constant density across the eye. Potentials developed by the pulse currents across the tissue were led off by the internal and external pipettes of a pencil-type microelectrode (10, 11, 13). Silver wires inserted into each pipette were connected through cathode followers to a two channel amplifier as shown in Fig. 1*A*. By connecting the two pipettes differentially to one of the channels (channel I) of the amplifier,

the height of pulse potential recorded through this channel was directly proportional to the resistance of the tissue intervening between them. In addition, a minor polarization which might have occurred at the surface of the indifferent electrode as a result of the pulse current would be rejected by this differential connection.

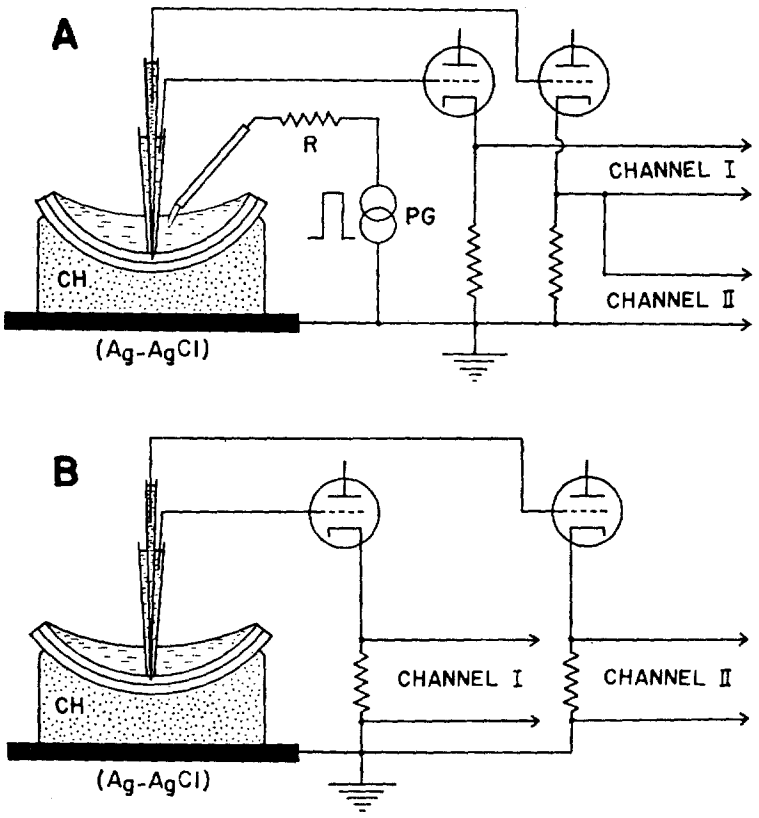

FIGURE 1. Arrangements for measurement of the radial resistance of the opened eye, *A*; and for recording of responses to light, *B*. *CH*, Ringer-soaked chalk; *PG*, square pulse generator; *R*, 1 MΩ resistance as a current limiter. See text.

The responses of the eye to light were usually recorded through both pipettes connected independently to the inputs of the two channel amplifier as shown in Fig. 1*B*. Simultaneous recording of the ERG (through channel I) and the intraretinal response (through channel II) thus obtained served for the comparison of their configuration.

The photostimulator used in the present experiment is shown diagrammatically in Fig. 2. It consists of a pair of nearly identical optical assemblies which produce beams of light that are combined by a half-mirror prism before

they are focussed on the retina. Control of areas and relative positions of the two light patches on the retina is made by adjusting the apertures and horizontal positions of the diaphragms, while their intensity is controlled by a

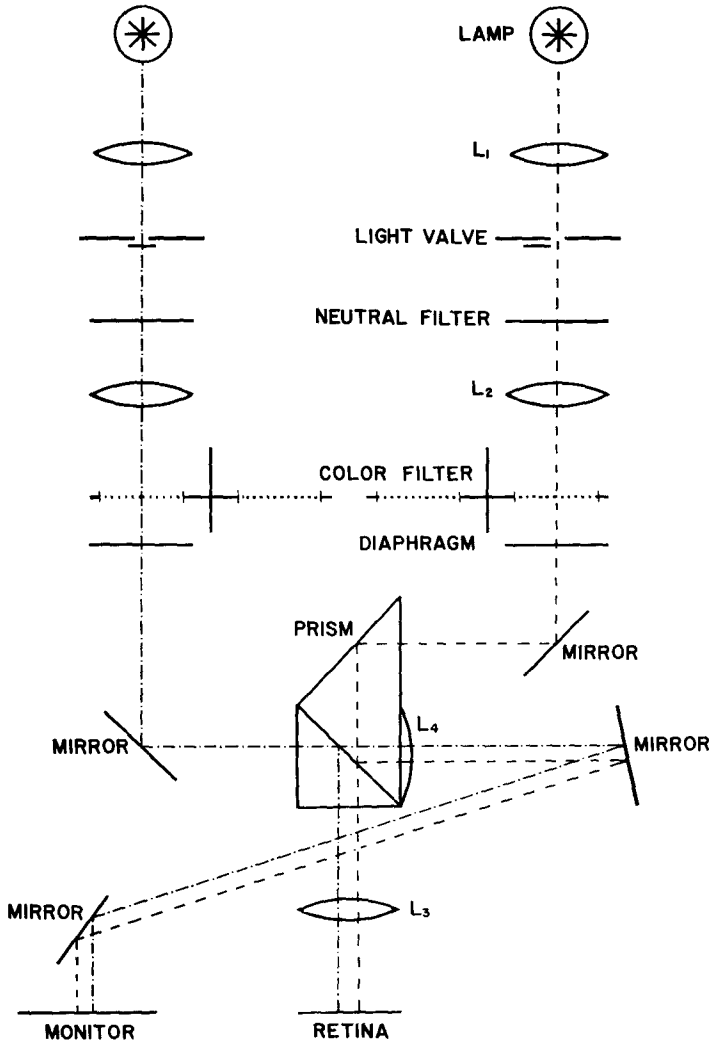

FIGURE 2. Schematic diagram of photostimulator. Power to each light source (single-coiled tungsten lamp) is supplied from A.C. line after it is stabilized, stepped-down properly, and rectified. Further explanation in text.

series of neutral density filters in each assembly. The light valves are operated automatically by a timer that synchronizes them with the sweep of the oscilloscope beam. Each assembly also contains a series of interference filters which cover the visible spectral range (400 to 750 mμ) in some 20 mμ steps and have

been adjusted to give colored light of equal energy. In the present experiment, however, only white light was used for stimulation.

RESULTS

The left column of Fig. 3 illustrates a series of recordings of pulse potentials resulting from pulse currents of 15 μa. across the eye. The recordings were

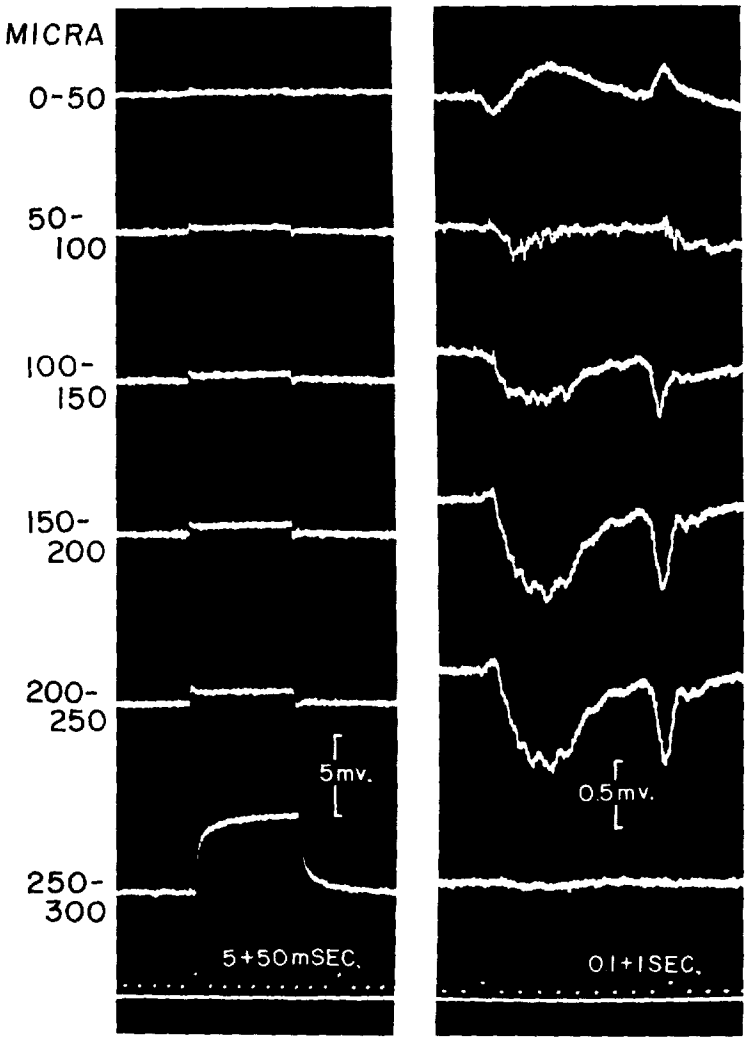

FIGURE 3. Depth recording of pulse potentials due to pulse currents of 15 μa. across the eye (left column), and of responses to diffuse illumination (right column). Channel I in Fig. 1*A* was used for recording of the pulse potentials whose amplitude at each depth was proportional to the resistance of the tissue layer intervening between the anterior retinal surface and the depth of recording. Responses to light were recorded through channel II in Fig. 1*A*.

made by a differentially connected pencil-type microelectrode whose external pipette was on the anterior retinal surface and the internal pipette at various depths in the tissue (Fig. 1*A*, channel I). The pulse potential which was proportional to the resistance of the tissue intervening between the tips of the pipettes was increased gradually as the depth of the internal pipette increased, but at a depth slightly over 250 μ a sudden marked increase occurred, indicating that the R membrane had been penetrated (bottom record). Recordings from opposite sides of this membrane could be repeated many times by moving the internal pipette up and down within a range of 20 μ. The right column of Fig. 3 shows the responses to diffuse illumination over the whole retina. Each record was obtained at the same depth as the pulse potential illustrated to the left of it. (Channel II of Fig. 1*A* was used to record these action potentials.) The intraretinal response to light dominated by a negative deflection at both "on" and "off" fell off abruptly when the pipette penetrated to the opposite side of the R membrane. It was noticed, however, that there was no reversal in polarity after the penetration. A trace of potential change similar to that before the penetration usually remained.

The pulse potential recording was then repeated on the eye cup after removal of the retina but with the pigment epithelium left intact. A resistance comparable to that of the R membrane in the intact opened eye was detected near the inner surface of this preparation. If the pigment epithelium happened to be removed with the retina, leaving the choroid and sclera, the above resistance was completely lost. This finding supported strongly the conclusion of Brown and Wiesel that the R membrane is the Bruch's membrane.

The resistance of the R membrane (R_{Rmemb}) and the resistance of the retina (R_{Retina}) were calculated by the following equations:

$$R_{\text{R memb}} = \frac{V_2 - V_1}{I} S,$$

$$R_{\text{Retina}} = \frac{V_1}{I} S,$$

in which V_1 and V_2 are the heights of pulse potentials just before and after penetration of the R membrane by the internal pipette, and S is the area of the retina across which the pulse currents of the intensity I (15 μa.) were passed. The resistance of the R membrane was 216 Ω cm.^{2} on the average (measurement in winter time at about 10°C.) and was several times greater than the resistance across the whole retinal tissue which was 43 Ω cm.^{2} on the average. Resistance across the choroid after removal of the pigment epithelium was negligibly small.

The existence of such a high resistance layer just back of the retina leads to the suspicion that at least some portion of the intraretinally recorded negative response might be caused by a reversal of the PII component of the ERG due

to the large potential drop across this resistance. The following circuit elements may be considered: (*a*) the source of the PII battery, (*b*) the vitreous humor, (*c*) the cut edge of the eye and some inactive regions in the retina if any exist (*cf.* Fig. 4), (*d*) the fluid conductor filling the space posterior to the R membrane plus the indifferent electrode, (*e*) the R membrane, (*f*) the sink of the PII battery. It is clear that the R membrane in this circuit acts to reduce the amplitude of the ERG recorded in the standard manner and to keep the distal portion of the retina more negative with respect to the indifferent electrode. This agrees, though only qualitatively, with our experience: In the surface recording, on one hand, the amplitude of the ERG is smaller in the retina of the opened eye than in the retina detached from the pigment

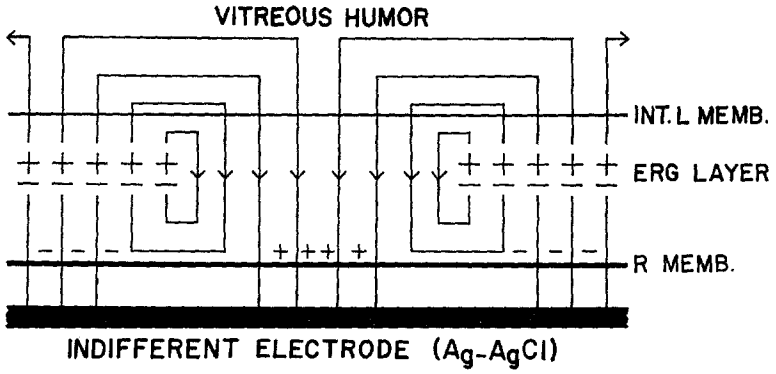

FIGURE 4. Schema of the flux of ERG current across an inactive region (central part of the diagram) and active regions of the retina. See text.

epithelium. Intraretinally, on the other hand, the size of the negative response is bigger in the retina of the opened eye than in the isolated retina.

The relation of the R membrane to the retina also makes the following inference a likely one. In the diagram shown in Fig. 4, one region of the retina is assumed to have been inactivated so that the layer which gives rise to the ERG does not generate any potential. The ERG originating in the neighboring active retinal region then gives rise to a current across this inactivated region which now acts simply as a passive external circuit for the ERG. As a result of such current, the R membrane under the inactivated region undergoes a polarization, the magnitude of which is

$$V_{\text{R memb}} = \frac{R_{\text{R memb}}}{R_{\text{Retina}} + R_{\text{R memb}}} V_{\text{ERG}},$$

in which V_{ERG} is the ERG potential between the vitreous humor and the in-

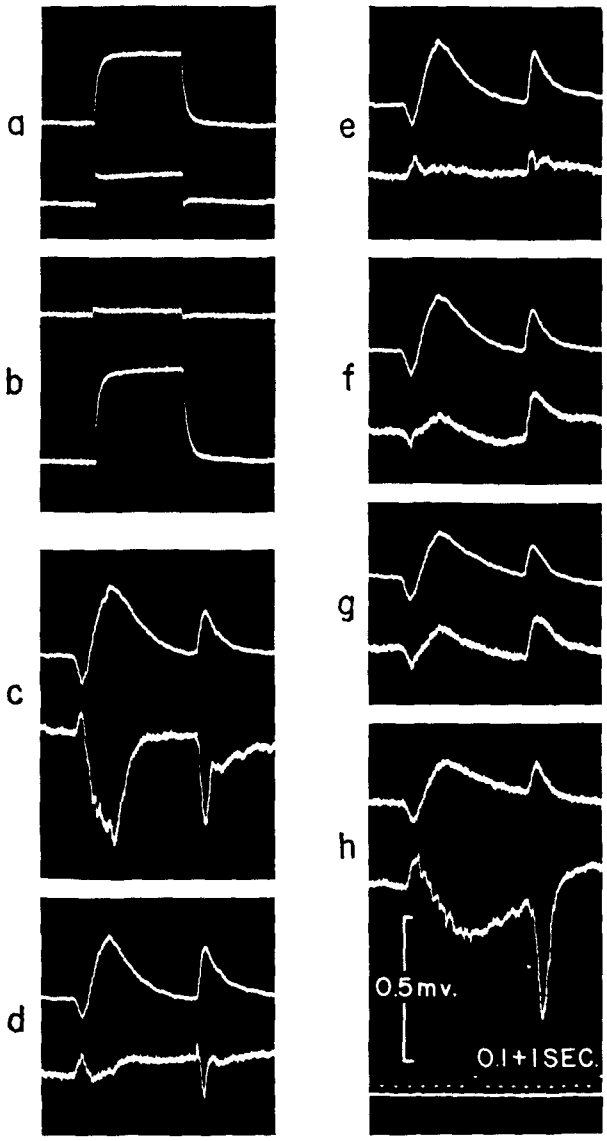

FIGURE 5. Effect of inactivation of the focal retinal region by cocaine upon the response to diffuse illumination. *a* and *b*, preliminary recordings of pulse potentials through channels I and II in Fig. 1*A* for locating the internal pipette of a pencil-type microelectrode to a depth some 30μ anterior to the R membrane (see text). *c*, control showing normal *ERG* (upper tracing) and intraretinal response (lower tracing) to diffuse illumination, obtained immediately after local application of 5 per cent cocaine. Times of subsequent recordings after cocainization: 30 sec. (*d*), 45 sec. (*e*), 2 min. (*f*), and 5 min. (*g*). *h*, recording obtained from a peripheral region of the same retina after recording of *g*. The arrangement in Fig. 1*B* was used for the recordings *c* to *h*.

different electrode. Substituting 43 Ω for R_{Retina} and 216 Ω for $R_{\text{R memb}}$, we obtain

$$V_{\text{R memb}} = 0.84\ V_{\text{ERG}}.$$

It thus becomes highly probable that an electrode located in an inactivated region of the retina records an apparently normal ERG at any depth above

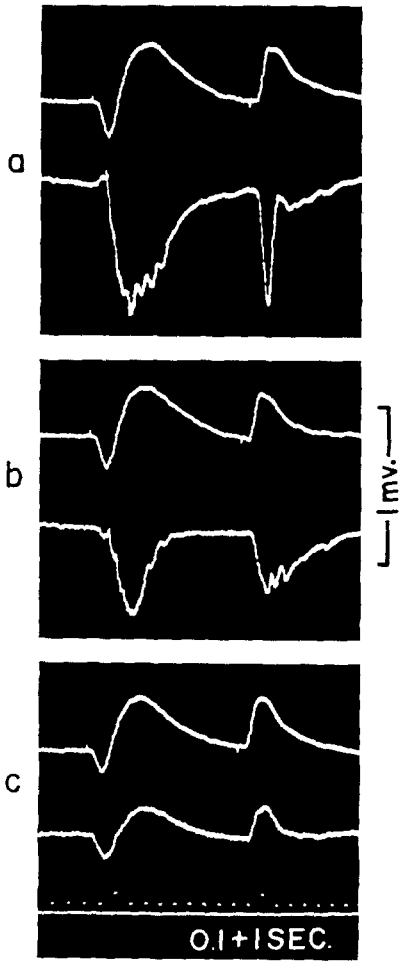

FIGURE 6. Responses obtained by different combinations of focal light (light spot of 2 mm. in diameter on the focal retinal region) and non-focal light (diffuse but with a shade of 4 mm. in diameter on the focal retinal region, 1 log unit less strong in intensity than the focal light). The arrangement in Fig. 1*B* was used. *a*, response to focal + non-focal illumination; *b*, response to non-focal illumination alone. *c*, response to non-focal illumination in the presence of focal light as a background.

the R membrane without much loss of the potential. In other words, the R membrane under the inactivated region behaves as if it were the origin of the ERG. This was proved in the following two experiments.

In one experiment, the retinal region around a recording electrode was inactivated by a small dose of 5 per cent cocaine applied locally through an injection needle placed close to the retinal surface near the electrode. In an example illustrated in Fig. 5, the first two records (*a* and *b*) show how the

internal pipette of a pencil-type microelectrode was located for subsequent recordings of the retinal response to illumination. The big pulse potential in the upper tracing of Fig. 5*a* was obtained through channel I of Fig. 1*A* im-

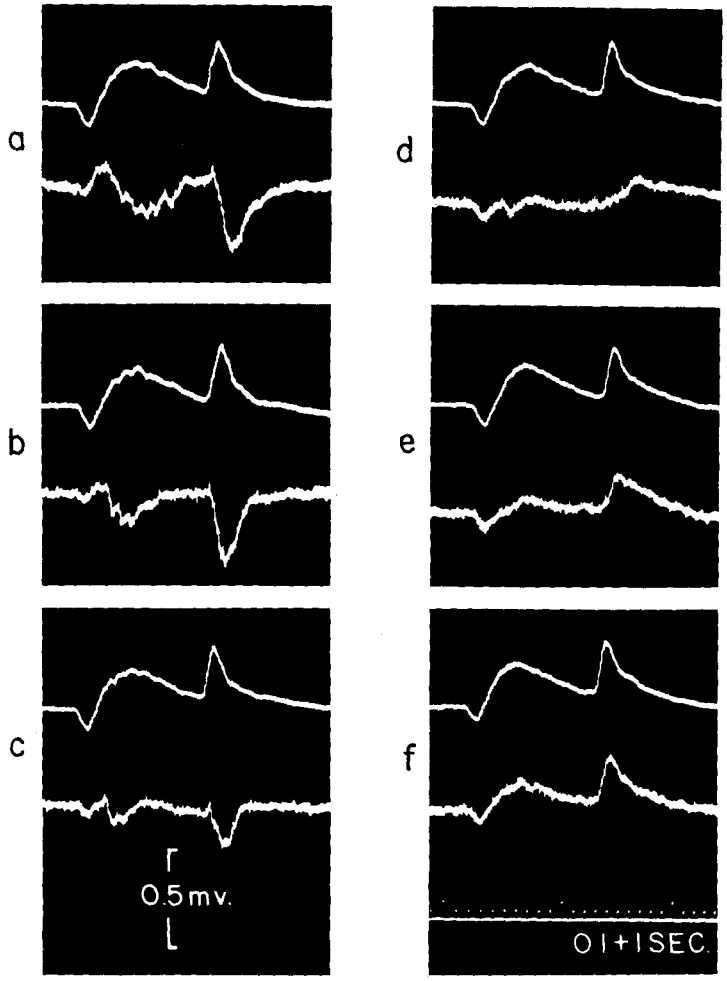

FIGURE 7. Responses to non-focal illumination in the presence of various intensities of focal light (2 mm. across) as a background. The arrangement in Fig. 1*B* was used. *a*, control, without focal light. Intensities in log unit of focal light in reference to non-focal light: −1.8 (*b*), −1.2 (*c*), −0.6 (*d*), 0 (*e*), and 1.0 (*f*).

mediately after the internal pipette has penetrated through the R membrane. The lower tracing in the same record was obtained simultaneously through channel II of Fig. 1*A*, showing the pulse potential between the internal pipette and the indifferent electrode. Fig. 5*b* was recorded after the internal pipette was withdrawn 40 μ. A marked decrease of the pulse potential in the upper tracing and a corresponding increase in the lower tracing indicate that the

internal pipette is now on the retinal side of the R membrane. With the internal pipette fixed at this depth, the connection was changed from *A* to *B* in Fig. 1. The region of the retina under the electrode was then cocainized and the change in configuration of the inraretinal response (lower tracings in all the subsequent records) was pursued together with the ERG (upper tracings). Fig. 5*c* is control, recorded immediately after local cocainization, both the ERG and the intraretinal response being normal. Later, the intraretinal response showed a rapid decrease in size (*d* and *e*), reversal in polarity (*f*), and finally took a configuration similar to the ERG in the surface recording (*g*). A response obtained at this stage from a neighboring retinal region was found to be normal (*h*).

In the other experiment, inactivation of the region under a recording electrode was achieved by exposure of this region to a strong light spot through one channel of the photostimulator. Illumination of the rest of the retina (non-focal illumination) by moderate intensity of light was made through the other channel. Fig. 6*a* is control during which the light valves of both channels were operated synchronously. Both the surface ERG (upper tracing) and the intraretinal response (lower tracing) were normal. For (*b*), the retina was illuminated only in the periphery while the light valve for focal illumination was kept closed. The intraretinal response though smaller in size was still dominated by negativity. There was no doubt in this case that the region under the electrode was stimulated effectively by the light scattered from the illuminated portion of the retina, because the same illumination applied in the presence of strong illumination on the region under the electrode produced only an ERG-like response (Fig. 6*c*). Fig. 7 illustrates a variety of configurations of the intraretinal response obtained by non-focal illumination in the presence of various intensities of focal light. The records show various degrees of inactivation of the response of the focal region to scattered light. The intraretinal response due to non-focal illumination is seen to change from the dominantly negative type to ERG-like type as the intensity of the background focal light is increased.

DISCUSSION

1. It is apparent from the above experiments that the intraretinally recorded action potential is a result of interaction of two potentials, the one the potential of the focal origin (the focal potential) and the other that of the non-focal origin (the non-focal potential). The former is featured by its predominant negativity, as was revealed by focal microillumination (12, 13). The latter is essentially the whole ERG potential which is distributed across the eye in proportion to the distribution of radial resistance. It was demonstrated in the present experiment that a major portion of the non-focal potential ap-

pears across the R membrane because of its high resistance, and that in the absence of the focal potential, therefore, an electrode located at any depth in the retina of the opened eye records a normal ERG potential. Thus, the intraretinally recorded response as a consequence of interaction of the focal and non-focal potentials can either be more of the predominantly negative or more of the ERG type according to the proportion of these two potentials (*cf.* Figs. 5 to 7). As a collorary, the depth at which the reversal in polarity of the intraretinally recorded response takes place changes according to the proportion of these two potentials.

2. It is important to note that the proportion of the focal and non-focal potentials in response to diffuse illumination of the whole retina cannot be constant if a functional non-uniformity exists in the retina. It should be admitted that all the previous attempts to localize the ERG by means of depth recording have been based on the implicit assumption that the retina is uniform everywhere forming a uniform double layer for each component of the ERG. There is evidence, however, that this assumption is not always appropriate. In the first place, Gouras (7) has described a phenomenon of spreading depression in the toad's retina which in many respects resembles the spreading cortical depression of Leão (9). The depression wave which is usually observed in aged preparations marches periodically across the surface of the retina and lasts for 2 to 3 minutes at any one point, during which even the most intense photic stimulation becomes unable to elicit a local response. Second, according to our experience, recordings at the same depth from various regions in the same retina do not always produce the same pattern of response, particularly when the eye has been removed from the animal for some time. A non-uniform development of deterioration is suggested. It should be emphasized in this respect that any interpretation of the intraretinal recording which neglects such a functional non-uniformity can lead to misleading conclusions with regard to the site of origin of the potentials. It is predicted that the use of the retina deprived of the R membrane provides more favorable conditions for this kind of experiment, since the potential field is less disturbed in the absence of the high resistance layer. However, the most important condition should be that the retina is functionally uniform, a condition that is considered to be better satisfied in the fresh retina.

3. In the above discussions, the focal potential has not been well defined. When the nature of this potential was discussed earlier (13), attention was focussed on two possibilities. The one was that the focal potential is entirely different in nature from the ERG, while the other was that the focal potential is the manifestation of individual cell activities that constitute the ERG when many are added together. At that time, the first possibility was thought more probable for the following reasons. First, the focal potential was highly localized in the illuminated retinal region while the ERG appeared to be a more

diffuse potential. Second, although the focal potential resembled the mirror image of the b-wave at "on" (and of the d-wave at "off"), its initiation was not always concurrent with that of the b-wave (and of the d-wave). Third, the focal potential was usually more susceptible to aging and chemicals than the ERG. In the light of new evidence available at this moment, however, none of the above reasons appears to be sufficiently well grounded. For instance, a highly localized nature of the ERG has been demonstrated by Brindley (2, 4) who established the fact that additivity holds in the ERG when the effect of scattered light is eliminated by background illumination. Our recent attempt to record responses from the retinal surface after draining off the vitreous humor also reveals that the response thus obtained is as highly localized as the intraretinally recorded focal potential. The reason why the ERG appeared to be a diffuse potential is now clear from the result of resistance measurement in the present experiment: calculation from the radial resistance of the retina (43 Ω cm.^{2}) and from the retina thickness (250 μ) gives a value of 1720 Ω cm. for the specific resistance of the retinal tissue. Compared with this value, the specific resistance of the vitreous humor, which is known to be very close to that of blood plasma (8) and may roughly be assumed to be 100 Ω cm. for the frog, is so small that a potential originating from any one region in the retina spreads readily throughout the vitreous humor. The facts that support the second and third reasons may be accounted for by assuming that the ERG is made up of many focal potentials whose latencies and susceptibilities to aging and chemicals have a certain statistical distribution. Under such circumstances, it is no longer necessary to stick to the view that the focal potential is different in nature from the ERG. Instead, we are tempted to the alternative and simpler view that the focal potential is a locally obtained ERG. In this sense, the focal and non-focal potentials are nothing but a kind of topographical subdivision of the retinal action potential with no strict border between them. An objection to this may be that the focal potential is usually far more complex in configuration than the ERG. However, the complexity of the focal potential is probably attributable to the proximity effect, the effect of recording with microelectrodes placed close to the origin of a potential that is changing in space as well as time. In view of the complex neural network in the retina, there is no doubt that even a microillumination gives rise to a number of sources and sinks of a complex spatial and temporal distribution. While such a complexity is reflected as the proximity effect in the configuration of the focal potential, its contribution to the ERG as the distance effect should be simpler, because a number of dipoles that are near to one another can be replaced by an equivalent single dipole when viewed a long way off.

4. The above discussions lead to the very simple conclusion that the

localization of the ERG in the retina of the opened eye is made by determining the depth at which reversal in polarity takes place in fresh materials in which the condition of the functional uniformity is better satisfied. Of course, the determination of this depth cannot be accurate owing to the proximity effect, but one can judge it from two other depths, the maximal depth for normal ERG and the minimal depth for inverted ERG. In Fig. 3, for instance, the maximal depth for normal ERG is 0 to 50 μ and the minimal depth for inverted ERG is 150 to 200 μ. From these values, the depth of reversal from the anterior retinal surface may be judged as to be about 100 μ. This depth is undoubtedly proximal to the receptors and is in good agreement with our earlier results of localization of the ERG (12, 13).

SUMMARY

1. The radial resistance of the frog's opened eye was measured using a method similar in principle to that of Brindley. The layer of high resistance (R membrane) was found to lie not in the retina but just back of it. The resistance of the R membrane was 216 Ω cm.^{2} on the average, being several times greater than the resistance across the whole retina (43 Ω cm.^{2}).

2. An intraretinally recorded response to diffuse illumination that resembles the mirror image of the ERG fell off abruptly when a recording microelectrode penetrated through the R membrane. No reversal in polarity of the response occurred after the penetration. If the retinal region under the recording electrode was inactivated locally by cocaine or by strong spot light kept shining on it, the response to diffuse illumination was always identical with a normal ERG recorded from the surface irrespective of the depth of the electrode in the retina. It fell off only after the electrode had passed through the R membrane. By means of partial inactivation of the focal region, any intermediate configuration of response could be obtained from just in front of the R membrane. Analysis of the potential field could justify all these observations.

3. The conventional view that the intraretinally recorded focal negative response is different in nature from the ERG was further discussed in the light of the new evidence. An alternative and simpler view is advanced that the above response is nothing but a locally obtained ERG.

4. It was stressed that the main origin of the ERG is proximal to the receptors.

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