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ISOLATION OF COMPONENTS OF ADMITTANCE CHANGE IN ROD OUTER SEGMENTS

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SUMMARY

1. Rods were separated by equilibration on a bovine serum albumin (BSA) density gradient into two major fractions, differing in their response to light.

2. In one fraction the response, measured as a change in the real part of admittance ΔG, appeared to consist exclusively of component I, while in the other, component II was prominent.

3. Evidence is presented that component I arose in damaged rods. This follows from observations on rods which have been deliberately damaged by freezing followed by thawing, or by fragmentation.

4. In such damaged rods, component II was absent while component I was increased in amplitude.

5. The frequency dependence of component I in isolation was characterized as a positive ΔG of constant amplitude from low frequencies up to the characteristic frequency f_Y for the major dispersion of admittance. Above this frequency, it declined to a variable extent.

6. The frequency dependence of component II observed in isolation was consistent with the previous analysis.

7. A negative-going ΔG is described which was linear with the amount of rhodopsin bleached and which was frequency independent up to the highest frequency of measurement (17 MHz).

8. The origins of component I and the negative component are discussed.

INTRODUCTION

Previous papers on admittance changes, evoked by illumination of suspensions of frog rod outer segments, have described how the response can be analysed to reveal two components (designated I and II) which depend on structural features of the rods (Falk & Fatt, 1968, 1973). The components are distinguishable by the time course and frequency

dependence of the measured quantity. Component I has a slow time course of development; its amplitude, measured as an increase in real part of admittance (ΔG), is independent of frequency from very low frequencies (presumably from zero frequency) up to the characteristic frequency of admittance (f_Y at about 2 MHz), but declines at higher frequencies. In contrast, component II has a fairly rapid time course; it is undetectable at very low frequencies, appears as a positive ΔG when a critical frequency ($f_{\mathrm{III}f}$, about 2 decades below f_Y) is exceeded, and then increases logarithmically with frequency up to the region of f_Y.

A problem which has been left untouched thus far is whether the population of rods yielding this composite response is homogeneous, in the sense that the contributions made by components I and II are in a constant ratio for all rods in a suspension. One is concerned here mainly with the possibility that, in the process of isolating rods from the retina, a proportion of them may have undergone a structural or chemical alteration which would affect their response. That a change of this kind may occur is suggested by the variability of the response observed in some sets of experiments in which rod suspensions were prepared under what were intended to be identical conditions.

The present paper describes differences between rods, separated from one another on the basis of their sedimentation behaviour, and the effect of treatments expected to cause mechanical damage. It is shown that differences in the response extend so far that some rods within a suspension give exclusively component I, while others give exclusively component II. Furthermore, evidence was obtained suggesting that the occurrence of component I, with the exclusion of component II, is a consequence of some kind of damage to the rods.

With the preparation of rod suspensions giving component I without component II, it becomes feasible to obtain more precise information on the frequency dependence of ΔG for this component in the region of f_Y and above. In addition, a component of response in the form of a frequency-independent negative ΔG of rapid time course is described and its probable mechanism of origin is considered.

METHODS

The methods were similar to those employed in the preceding paper (Falk & Fatt, 1973), except for a more refined control of the conditions of sedimentation, permitting rod suspensions to be fractionated into populations of rods having different response characteristics.

In a few experiments, the density of the suspending medium was adjusted with sucrose according to the method employed previously. In other experiments, which yielded most of the results reported in this paper, the density of the suspending medium was adjusted over wide limits without variation in osmotic pressure by the

addition of high concentrations of colloidal substances. Both BSA and Ficoll (a highly branched artificial polysaccharide of av. mol. wt. 400,000) were used for this purpose in different experiments. Apart from these density-controlling substances, the solutions had the composition of a modified Ringer solution: 60 mM-NaCl, 100 mM sucrose, 2 mM-EDTA, 3 mM phosphate, 0·01 g BSA/ml. (the last of these being added to the Ficoll-containing solutions) with 13 mM-Na^+ to give pH 7·0. In order to maintain a constant pH and osmotic pressure for solutions of differing BSA concentration, each 0·01 g BSA/ml. was accompanied by the addition of 1·0 mM-Na^+ together with the removal of 1·0 mM-Cl^-. When used in a concentration sufficient to cause rods to remain suspended in a gravitational field, Ficoll was found to have a serious disadvantage over BSA. The Ficoll solution was considerably more viscous than that of BSA and there was a greater tendency for particles to clump together in the Ficoll than in the BSA. Owing to these difficulties, Ficoll was not used in studies involving the equilibration of rods in a density gradient.

Density gradients were made up with BSA. These were discontinuous, but finely graded, consisting of twenty-five approximately equal steps of BSA concentration, extending from 0·20 to 0·30 g/ml. (each step corresponding to an increment in specific gravity of about 0·0015). The gradient was formed in the cylindrical stem (8 cm long × 0·2 cm inner diameter) of a glass centrifuge tube, similar to the type used for haematocrit measurements. About 1·8 ml. of a crude suspension of rods, obtained from the retinas of five to six frogs and filtered through nickel gauze, was placed in the bulb forming the upper part of the centrifuge tube. The solution used for suspending the rods was a modified Ringer solution having the composition given above. The tube was centrifuged at 7800 *g* for two or three periods of 25 min, until it was considered that the rods had reached positions of equilibrium within the density gradient. The distribution of rods was judged by the scattering of red light. The rods, which were distributed in bands along the gradient, were removed in a number of samples corresponding to regions of different densities. This was accomplished by means of a narrow-bore polyethylene tube with an expanded orifice which fitted closely to the inner wall of the cylindrical stem of the centrifuge tube. Lowering of the tube caused regions of the gradient to be forced upwards sequentially. The samples of rods, each in a small volume of concentrated BSA, were diluted with modified Ringer solution, which resulted in an increase in volume of about fiftyfold. The diluted samples, representing rods obtained from different regions of the density gradient, were placed in separate conductivity cells, and the rods were then packed by centrifugation to occupy the space between the electrodes. (The plan of the conductivity cell is shown in Fig. 1 of the preceding paper; Falk & Fatt, 1973.)

In other experiments to be described in the Results, rods were separated according to their rates of sedimentation.

Deliberate damage to rods. Some experiments included in the present study involved mechanical damage to rods suspended in modified Ringer solution. Damage was brought about either by freezing to −25° C and then thawing, or by forcing a suspension through several layers of filter paper under a pressure difference of several atmospheres.

RESULTS

Variation of response outside experimental control

A considerable degree of variability was noted in the response characteristics of different samples of packed rods prepared under similar conditions. An improvement in the reproducibility of results was achieved by

subjecting the suspension of rods in the conductivity cell to an initial, prolonged stage of low-speed centrifugation during which whole rods would sediment but not fine fragments of rods or other small particles (see Methods of Falk & Fatt, 1973). However, even with this improvement, some variability remained, especially in the amplitude of response recorded at very low frequencies, where for weak flashes component I would be expected to appear in isolation. Fig. 1 shows records of the response at

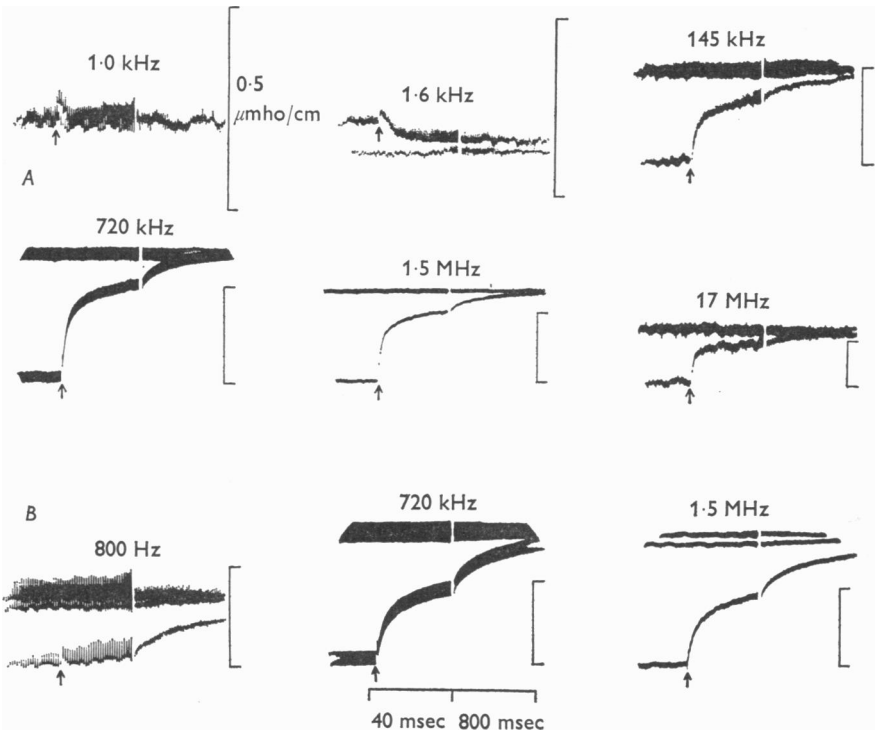

Fig. 1. Records of ΔG obtained in response to a flash bleaching 1% of the rhodopsin content of rods. Two experiments are represented: *A* by the upper 6 records, *B* by the lower 3 records. The rods were prepared similarly in both experiments. An isotonic suspending medium was employed throughout the preparation. Purification of the rods was accomplished by differential sedimentation during low-speed centrifugation of the suspension in the conductivity cell. The medium had the composition: 136 mM-Na^+, 73·3 mM-SO_4^{2-}, 12 mM Tris, 0·01 g methylcellulose/ml. (viscosity, 20 cP for 2% solution at 20° C), pH 7·2. As indicated in Methods of preceding paper (Falk & Fatt, 1973), this solution, although 0·8 times the osmolarity of Ringer solution, was isotonic for rods. Note the large variation in the sensitivity used for the display of ΔG at different frequencies, the vertical calibration bar having the same value for all records. In the records at low frequencies, the initial upward deflexion, coincident with the flash (arrow), is an artifact associated with operation of the flash lamp.

different frequencies for two samples of rods, obtained from different retinas, but prepared under identical conditions as far as these were under experimental control. The response illustrated in Fig. 1*A* represents one extreme in the range of variation, in that component I appears to be absent. No response could be detected at the lowest frequency of measurement, 1·0 kHz. (Note the high sensitivity of recording employed at the lower frequencies.) At a slightly higher frequency, 1·6 kHz, a small response is observed in the form of a negative ΔG. (In the record, this response follows immediately upon an upward deflexion which is an artifact associated with the operation of the flash lamp and is present also when the light is prevented from reaching the conductivity cell.) At higher frequencies, 3 kHz and above, the response reverses to appear as a positive ΔG and increases progressively in amplitude as the frequency is raised further, with a limiting value of ΔG being reached at about 1·5 MHz. It should be noted that the response at 1·6 kHz, although opposite in sign to that recorded at higher frequencies, is similar in its time course of development to the response at higher frequencies, provided correction is made for the flash artifact. In this experiment the time course of response was evidently independent of the frequency of measurement. These observations are consistent with the response consisting exclusively of component II. A limited region of frequencies in which ΔG due to component II takes on negative values has been described previously (Falk & Fatt, 1968), and it has been shown that this form of behaviour is expected for a network including an element of variable (increasing) conductance in series with a fixed capacitance, such as will give a sharp low-frequency cut-off of ΔG.

In the experiment of Fig. 1*B*, carried out under the same conditions as that of 1*A*, both components I and II are present as indicated by the positive ΔG at very low frequencies and the dependence of the recorded time course of response on the frequency of measurement. The difference in the responses obtained in these two experiments indicates the operation of some uncontrolled variable, possibly involving damage caused to the rods in the process of their detachment from the retinas.

Separation of rods by equilibration on a density gradient

Experiments were undertaken, making use of non-uniformities in the sedimentation behaviour of rods within a given suspension, to decide whether components I and II arise in the same or in different rods. A BSA density gradient was used to isolate different populations of rods from a suspension. The majority of rods initially suspended in an isotonic modified Ringer solution and equilibrated on a BSA gradient, as described in the Methods, comprised two distinct bands, one at a mean specific gravity of 1·082 and the other at 1·089. Samples from each of these bands were

examined in the light microscope. Most of the rods from the less dense band were refractile and had no obvious internal structure. Those from the more dense band, although refractile, had a cross-striated appearance. There were no obvious differences in the dimensions of the rods in the two bands (mean diameter 6 μm, 70% of the rods having a length between 40 and 60 μm).

The response of rods recovered from the low-density band is shown in Fig. 2*A*; the response of rods from the high-density band in Fig. 2*B*. The

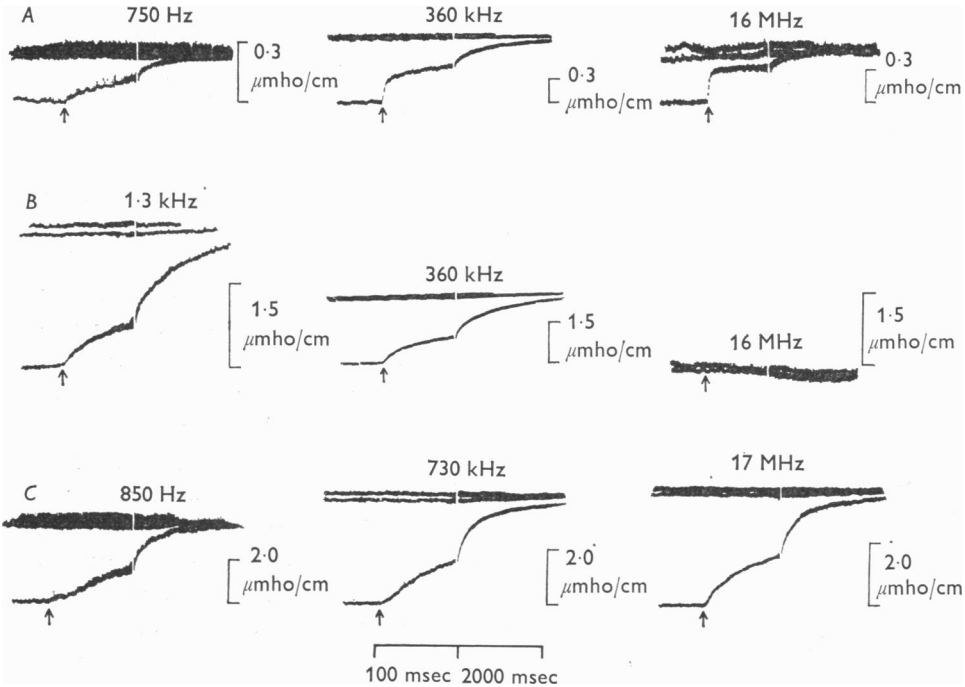

Fig. 2. Responses obtained from two fractions of a rod suspension separated by equilibration on a BSA density gradient. Rods were shaken off the retinas of six frogs in a solution consisting of 73 mM-Na^+, 2·5 mM-K^+, 62 mM-Cl^-, 2 mM-EDTA, 1·5 mM phosphate, 100 mM sucrose, 0·01 g BSA/ml. The suspension of rods was placed on top of a uniformly stepped gradient (see Methods) of BSA extending between 0·2 and 0·3 g/ml. Records of the response of a sample of rods recovered from the band extending between 0·248 and 0·266 g BSA/ml. (mean sp.gr. 1·082, the low density band) are shown in *A*; in *B* response of rods distributed in the band from 0·280 to 0·284 g BSA/ml. (mean sp.gr. 1·089, the high density band). An additional sample of rods from the low-density band was frozen and thawed and then sedimented in a third conductivity cell. The response of these freeze-thawed rods is shown in *C*. Note the different calibrations for ΔG in each set of records. Each flash was of an intensity which bleached 1% of the initial rhodopsin content.

response of the rods in the low-density band, for which ΔG is a function of frequency, is similar to what has been described as typical for rod suspensions with contributions from components I and II. In contrast, the response of the rods in the high-density band has a time course which is the same at all frequencies, and an amplitude which is constant between 1·3 and 730 kHz, but which declines at higher frequencies. The time course of this response is similar to that recorded at low frequencies for rods from the low-density band. It is furthermore seen that the response of rods from the high-density band has an amplitude about 10 times that of the response of rods from the low-density band recorded at low frequencies. A reasonable interpretation of these results is that component I and II arise in different rods; those rods giving component II are present only in the low-density band of the BSA gradient, which contains only a small proportion of rods giving component I.

In addition to the two bands which constituted the major portion of the rod material, there was a minor fraction, amounting to about 10% of the total material, which formed a diffuse layer extending from a specific gravity of 1·092 to 1·098. The microscopic appearance of these rods was similar to those which formed the sharp band at sp.gr. 1·089. The response to a flash was the same for the two fractions, except that, for those rods comprising the minor fraction, the amplitude of response was constant at all frequencies of measurements extending from 700 Hz up to 17 MHz.

A variation in the apparent density of rods within the gradient would occur if some rods were penetrated by BSA. Sidman (1957), using suspending media with graded concentrations of BSA to match the refractive index of isolated rods, found that some rods were penetrated rapidly by BSA while others excluded it for periods as long as 2 days. It seems probable that the separation of rods into different fractions by equilibration on a BSA gradient may be dependent upon such differences in penetrability. This view is supported by measurements of G_1 (the limiting low-frequency value of G) for packed suspensions of rods derived from the different regions of the gradient. In the experiment described above, the three fractions, in the order that the rods were derived from the least to the most dense regions, were found to have values for G_1 of 15, 19 and 31 μmho/cm. There is no reason to suppose there would be any variation in the fractional extracellular space, since the rods from each of the fractions were packed in conductivity cells from identical solutions containing only about 1·5% BSA. Therefore the observed differences in G_1 are attributable to differences in the conductance of the surface membrane, with the possibility that rods in the least dense fraction, giving rise to component II, may have had sufficiently low surface membrane conductance that this would not contribute appreciably to the value obtained

for G_1. The large rise in G_1 that distinguishes the most dense fraction from the others is of interest in relation to possible damage of the rods, since a similar rise in G_1, accompanied by a failure of ΔG to decline at high frequencies, occurs in rods that have been intentionally damaged by freezing or by being subjected to shear, as described later. A frequency dependence of G with a characteristic frequency, f_Y, in the region of 1·2 MHz, was found for all three rod fractions. The presence of such a dispersion may be taken as an indication that most rods present in each of the three fractions were bounded by membranes.

Response of damaged rods

Since there were indications that component I might arise in rods which were structurally altered, the response was examined in samples of rods which had been deliberately damaged.

Freezing-thawing. An experiment in which the damaging procedure consisted in freezing and then thawing is illustrated in Fig. 2*C*. In this particular case the initial sample was derived from the low-density band in the BSA gradient, the response before freezing being illustrated in Fig. 2*A*. As a result of freezing and thawing, the response recorded at low frequency increased in amplitude about fourteenfold, without alteration in time course. Furthermore, the response was then of similar time course and amplitude at all frequencies of measurement, from 850 Hz to 17 MHz. This contrasts with the behaviour of the same material before freezing and indicates that the effect of the treatment was to abolish component II, which contributed the early rapid rise of ΔG in the records of Fig. 2*A* obtained at high frequencies. The response of the freeze-thawed rods appeared to consist of component I alone.

In the case of the experiment illustrated, the rods were frozen while suspended in an isotonic solution containing EDTA. Similar results have been obtained after freezing and thawing rods suspended in solutions containing Ca^{2+} or in hypertonic sucrose solutions. On microscopic examination, rods, after being subjected to freezing, were still recognizable as such, but instead of appearing as circular cylinders, they were of irregular outline. They were also swollen and cross-striated.

Measurements of G_1 for the experiment illustrated in Fig. 2, as well as in others, gave strong indications that freeze-thawing caused the surface membrane to become leaky, as evidenced by a large increase in the low-frequency value of G. A dispersion was still present, though the characteristic frequency was usually decreased by about a factor of 0·8.

Fragmentation of rods. In an alternative procedure for causing damage, rods were disrupted by shear when a suspension of them was forced through several layers of filter paper under a pressure difference of a few

atmospheres. Rods were fragmented by this treatment into small particles of such dimensions that cleavage was clearly not restricted to the transverse plane. There were no recognizable rods.

The response of such rod fragments derived from rods in an isotonic solution is illustrated in Fig. 3*B*. The amplitude and time course were the same at all frequencies, indicating that the response consisted exclusively of component I. It is furthermore to be noted that the magnitude of this component was about 5 times greater for the rod fragments than for a

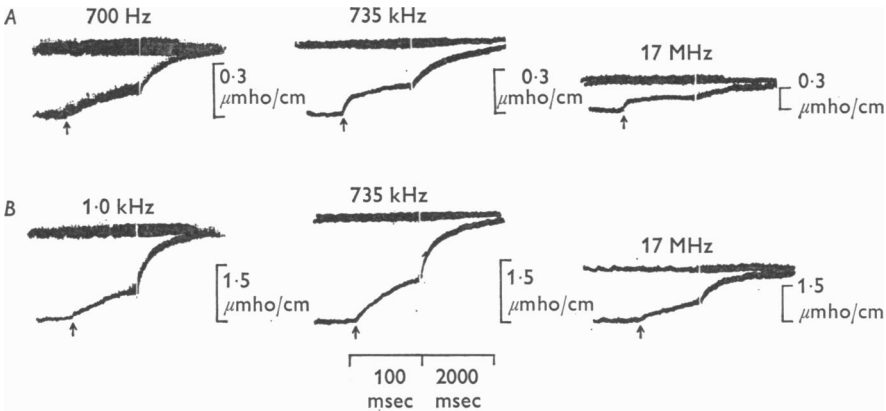

Fig. 3. Responses of fragmented rods to flashes bleaching 1 % of the initial rhodopsin content. *A*, control suspension of unfragmented rods. *B*, fragmented rods. Rods were detached from the retinas in an isotonic modified Ringer solution (as described in Methods). The suspension was layered on top of an isotonic, neutral solution containing 0·34 g BSA/ml. and centrifuged to separate the rods from other retinal material. Rods were collected from the interface and suspended in a relatively large volume of modified Ringer solution. This suspension was divided into aliquots, one of which, the control, was placed in a conductivity cell in which rods were packed by centrifugation. The other aliquot was forced under pressure through four layers of No. 42 filter paper (Whatman). The fragmented rod particles were then packed by centrifugation into a second conductivity cell.

sample of the same suspension not subjected to this treatment (records of Fig. 3*A*). In the latter case, components I and II were of about equal magnitude (estimated from a comparison of ΔG recorded at 1·5 MHz with that recorded at low frequencies). It will be seen that the time course of the response of the disrupted rods was the same as the low-frequency response of rods not so treated.

In contrast to rods which had been frozen and then thawed, fragmented rods showed virtually no dispersion in G over the frequency range between 1 kHz and 17 MHz. The ratio of the low-frequency value of G to the conductivity of the suspending medium was only 2·7, indicating that if the

fragments were membrane bound, that membrane was extremely leaky to ions.

Frequency dependence for component I in isolation

The frequency dependence of G and ΔG is shown in Fig. 4, for two experiments in which rod samples were obtained from a suspension in an isotonic Ficoll-containing solution. In both experiments the time course of response was invariant with frequency and was similar to what has been ascribed to component I. Also, in both experiments the amplitude of response was constant for all frequencies from below 1 kHz up to 700 kHz,

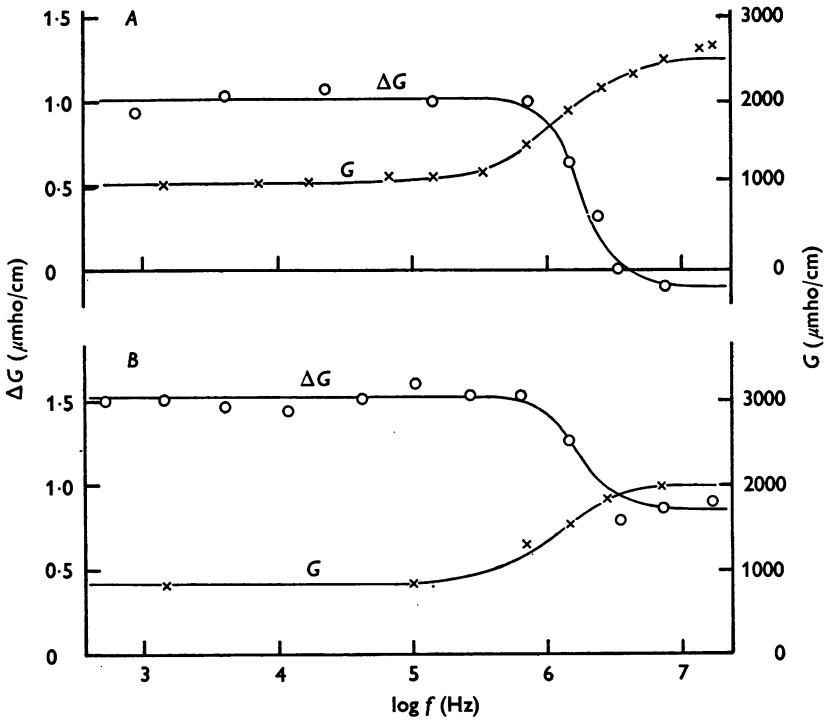

Fig. 4. Plots of ΔG and G against log frequency for two samples, A and B, of rod suspensions which gave responses consisting entirely of component I. Retinas were agitated in a solution similar to the standard isotonic solution (p. 223), except that the solution for A contained no EDTA. A concentrated Ficoll solution was added to the resulting suspension of rods together with other particulate matter so as to bring the specific gravity to 1·083 (at a Ficoll concentration of 0·17 g/g solution), while the other constituents of the solution were unchanged. Centrifugation at 7200 g for 8 min resulted in sedimentation of red blood corpuscles, melanin granules and some rods. The supernatant still containing rods was diluted with an equal volume of Ficoll-free solution and the mixture centrifuged in a conductivity cell in order to pack the rods between the electrodes.

but diminished as the frequency was increased further. In the experiment of Fig. 4*A*, ΔG had a small negative value at the highest frequency of measurement (8 MHz). The small negative ΔG at high frequency is attributable to the superposition of a different component of response described in a later section of this paper. A different behaviour was observed in the experiment of Fig. 4*B*. In this case ΔG remained positive at high frequencies; its amplitude was unchanged over the frequency range 1·5–17 MHz at about 55 % of its low-frequency value.

The experiments of Fig. 4 were selected for illustration because the measurements covered a wide range of frequencies. The basis for the isolation of component I in these experiments is not entirely clear. It is not known whether the separation of rods in the Ficoll solution of density 1·083 (see legend to the Figure) was dependent on differences in density among the rods or differences in rate of sedimentation. From other experiments in isotonic solutions containing Ficoll, the density of the rods was found to be greater than 1·08 but less than 1·09. When the responses of rods which had been floated in Ficoll solutions of density 1·095 (containing the entire rod population) were tested, these were found to consist both of components I and II. In the two experiments illustrated in Fig. 4, the rods which sedimented when the density was 1·083 were contaminated with other retinal particles and were discarded for this reason without being tested for their response to light. Microscopic examination of the rods giving the responses shown in Fig. 4 revealed them to be curved and distinctly cross-striated.

Frequency dependence for component II in isolation

Fig. 5 shows the frequency dependence of G and ΔG for a sample of rods constituting the rapidly sedimenting fraction of a rod suspension in a hypertonic sucrose solution. In this experiment there was no detectable ΔG at the lowest frequencies of measurement, indicating the absence of component I. The response, consisting of component II alone, had a low-frequency cut-off in the region of 3 kHz. It increased nearly linearly with log frequency from this region up to about 1·5 MHz where a limiting value for ΔG was reached. No further change was observed up to the highest frequency of measurement, which was 8 MHz in this experiment. This description of the frequency dependence of component II is in agreement with the results of the analysis of the compound response, consisting of components I and II, given in the preceding paper.

A component of response in the form of a negative ΔG

In rods which had been kept for a day or longer after their separation from the retina, components I and II were smaller than in freshly prepared

material. With the failure of the light flash to elicit components I and II, another component of response in the form of a negative change in G was observed. This component was usually barely discernible over base line noise when a flash bleaching 1 % of the rhodopsin was employed. The negative component was regularly observed in experiments on stored rods when the flash intensity was increased so that 4 % or more of the rhodopsin was bleached (calculated as a percentage of the initial rhodopsin content of the dark-adapted rods). The amplitude of the negative-going response was

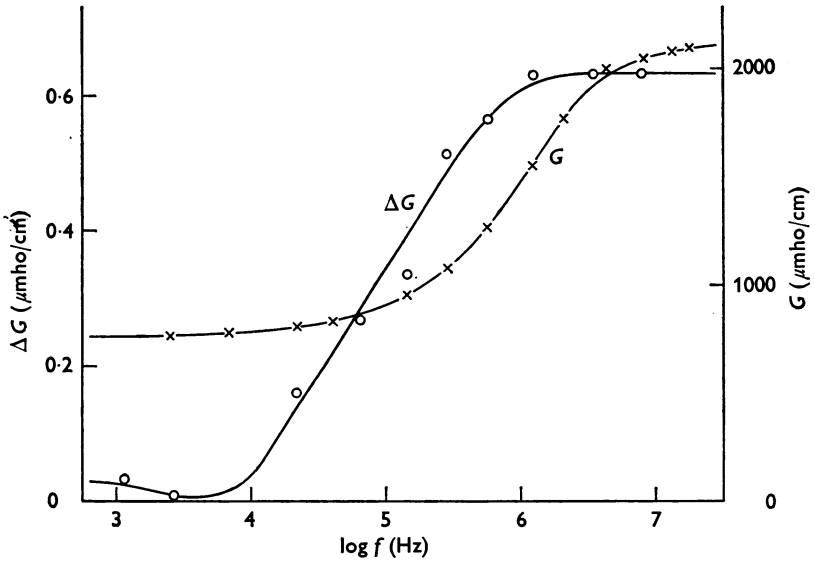

Fig. 5. Plot of G and ΔG against log frequency for a suspension of rods in which the more rapidly sedimenting rods were selectively packed between the electrodes. A suspension of purified rods was prepared in a solution containing 105 mM-NaCl, 6 mM phosphate, 10 mM-K^+, 0·01 g neutralized BSA/ml., 0·39 g sucrose/ml. After dilution of the sucrose to 0·25 g/ml., rods were packed between the electrodes of the conductivity cell by centrifugation at 150 g for 20 min followed by 5000 g for 3 min. Each flash used to obtain ΔG was of an intensity which bleached 1 % of the initial rhodopsin content of the rods.

proportional to the amount of rhodopsin bleached for all flash intensities that were employed (up to 20 % bleaching), thus differing from components I and II which saturated at a level of a few per cent bleaching.

Responses to fairly bright flashes, obtained from suspensions of stored rods which were incapable of yielding components I and II, are illustrated in Fig. 6, together with responses to flashes of infra-red radiation. The initial upward deflexion in the records of response to blue-green flashes corresponds to the early part of the heat component, arising largely from the dissipation of the radiant energy absorbed by rhodopsin.

The records shown in Fig. 6*A* were obtained on the same preparation at two widely separated frequencies (2·2 kHz and 17 MHz). The amplitude of the negative-going component of response was independent of the frequency of measurement covering the main dispersion of admittance.

The time course of the negative-going response cannot be reliably estimated from these records. The reason is that the heat component

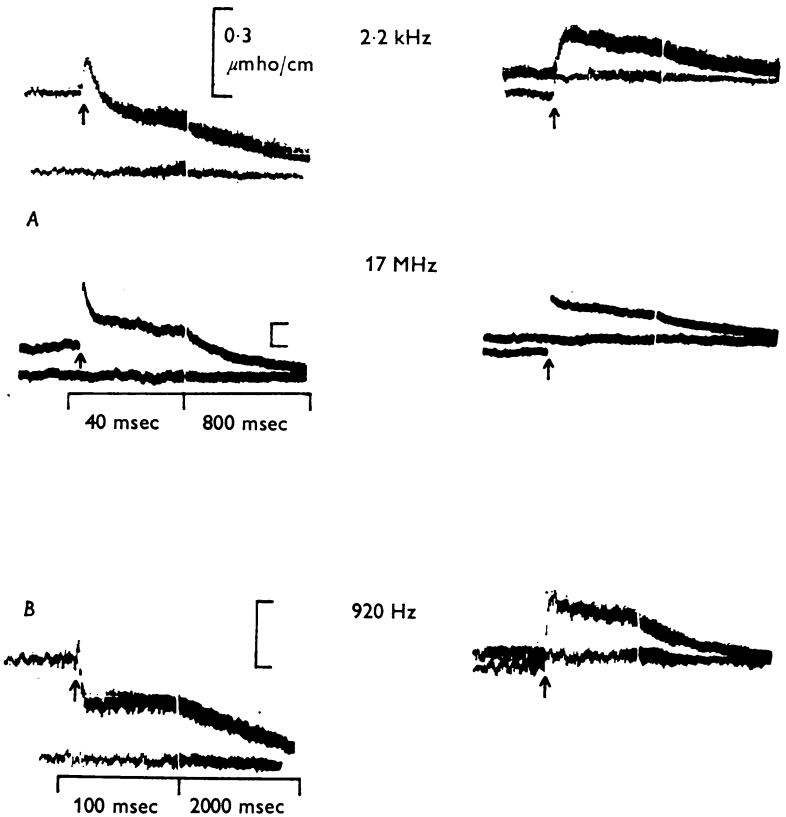

Fig. 6. Records showing the negative component observed in rod suspensions after storage. Records on the left show the response to a flash of blue-green light of an intensity which bleached 15% of the initial rhodopsin content. The records on the right show, for comparison, ΔG resulting from the application of an infra-red flash. The records in *A* were obtained 48 hr after the rod suspension was prepared. The frequency dependence of ΔG in response to a weak flash for this rod suspension, obtained shortly after isolation from the retina, is shown in Fig. 5. The records in *B* are from another experiment after 30 hr storage of the rod suspension. The suspending medium had a conductivity of13,400 μmho/cm. It contained 250 mM-NaCl, 0·8 M sucrose, 6 mM imidazole, 0·01 g BSA/ml., pH 7·0. Vertical calibration bars for the records at each frequency indicate a ΔG of 0·3 μmho/cm.

makes a significant contribution to the over-all response and the size of this contribution is frequency-dependent. The heat component may be expected to have the same frequency dependence as the response to infra-red radiation and a similar time course. On this basis, the contribution of the heat component would be about 3·5 times greater at 17 MHz than at 2 kHz in the records of Fig. 6*A* and probably accounts for the difference in the time course of the overall response recorded at the two frequencies. In the record on the left of Fig. 6*B*, the heat component is small so that the time course is largely determined by the negative component. It is inferred that the negative component has a rapid initial phase of development lasting 3–5 msec during which time G decreases to the extent of about one half of the final negative displacement recorded a few seconds later. The further decrease in G occurring during the second, slow half of the sweep in all the records of the response to a bleaching flash appears to be due, in part, to a slowly developing phase of negative ΔG. The extent of this phase of response is, however, difficult to estimate owing to the superposition of the heat component which exhibits a slow decay in the same range of time following a flash.

The question arises whether the frequency-independent negative component occurs only after the ageing of rods or is also present in fresh preparations, but is there masked by components I and II. There is evidence that the latter is the case. The presence of the negative component may have a considerable influence on the time course of ΔG recorded at low frequencies, which time course has previously been taken as indicative of the development of component I. This situation is illustrated in Fig. 7, which includes records of ΔG obtained at a number of different frequencies for a preparation in which component II was absent. In this experiment there was a large positive ΔG at frequencies below f_Y (the displacement to the steady level of G reached about 10 sec following the flash, seen in the records at 980 Hz and 280 kHz). When the frequency was raised past f_Y (estimated to lie at about 1·0 MHz), ΔG declined steeply to become negative at high frequencies, where it was again frequency independent (seen in the records at 4·3 and 16·5 MHz). The behaviour over the entire range of observation is consistent with the response consisting of the superposition of component I, having the frequency dependence previously described for it, on a frequency-independent negative component with a time course approximately that given by the records at high frequencies (together with the heat component giving the early rapid upward deflexion in most of the records). On the basis of such an analysis it would appear that the very slow initial development of ΔG recorded at

low frequencies is the result of a near cancellation of the early part of component I with a negative-going component, having a final amplitude about 30 % that of component I, but with a more rapid time course.

The amplitude of the negative component in this Figure was particularly large, being about 6 times what was usually observed. It may be relevant to note that the region between the electrodes of the conductivity cell was only partly filled with the rod suspension, the upper portion being filled with supernatant. A proposal for the origin of the negative component is given in the Discussion. It is there considered that the negative component results from an increase in volume of the rhodopsin molecule such that there is a small decrease in the amount of conducting material

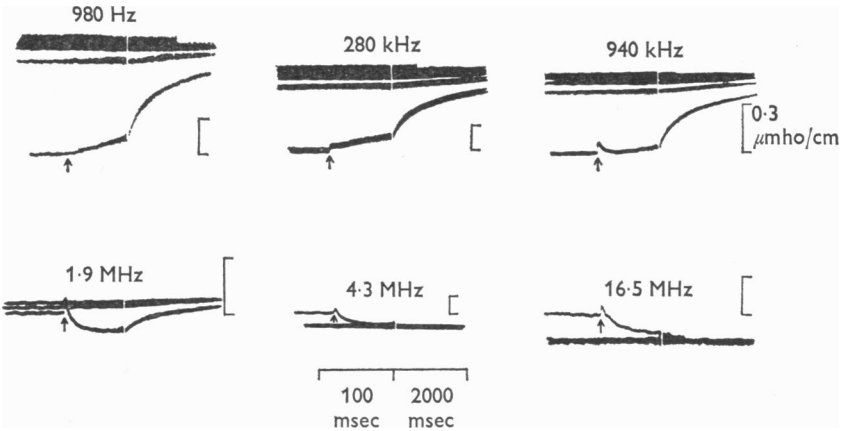

Fig. 7. Variation in time course of ΔG with different frequencies used in the measurement. In this experiment, the response was the result of superposition of component I and the negative component. Each flash bleached 1 % of the initial rhodopsin content of the rods. As noted in the text, the unusually large amplitude of the negative component may have been due to incomplete filling of the conductivity cell with the rod suspension. Account has been taken of the change in the cell constant. The preparation of the rod suspension and the composition of the solutions were nearly identical with the experiment illustrated in Fig. 4*B*.

contained between the electrodes. A displacement in the volume of supernatant would produce a very much greater change in the measured G than would a similar displacement in volume of the rod suspension. The greater amplitude of the negative component observed when the conductivity cell was incompletely filled with rods (account having already been taken of the change in cell constant) would therefore be consistent with the proposed mechanism.

The presence of the negative component in freshly prepared rods could be demonstrated by using paired, bright flashes separated by a short interval. If the flash was of sufficient intensity so as to saturate components I and II (a flash bleaching about 10 % of the rhodopsin), then the second flash following within a period of one or two minutes often evoked a

negative ΔG. This effect depends on the slow recovery of components I and II from saturation produced by a bright flash, while the negative component increases in proportion to the amount of rhodopsin bleached by the flash.

DISCUSSION

Component I

Component I has been identified as a slowly developing, positive ΔG which is independent of the frequency of measurement up to the region of the characteristic frequency of admittance, f_Y. Evidence is presented in this paper showing that component I arises in a different population of rods from that giving component II, and more particularly that it arises in damaged rods. In the absence of other kinds of observations providing more direct information on changes in structure and chemical composition, it is not possible to identify the essential nature of the damage which leads to the occurrence of component I. It may be noted, however, that the fractionation procedure involving suspension of the rods in media containing high concentrations of BSA indicates that the rods giving component I are penetrated by this protein. On the other hand, the similarity in the dispersion of admittance, which has been found between the two populations, makes it probable that rods giving component I have in general retained their surface membrane. (It is conceivable that the surface membrane might be lost and the rods still yield a normal dispersion if the disks were to swell so as to occlude the interdisk space, the frequency-dependent behaviour then arising from the capacitance of the disk membranes at the surface of the denuded disk stack. This kind of structural change is not supported by electronmicroscopic studies, in which the change of rods from their normal condition is generally seen to involve an increase in interdisk as well as intradisk spacing.)

Origin of component I. The dependence of component I on the frequency of measurement, especially in relation to the major dispersion of admittance, can be used to infer a probable mechanism of origin. The observation that at low frequency ΔG has a constant (i.e. frequency independent) positive value, but then declines to a variable extent when the frequency exceeds f_Y (the characteristic frequency for the suspension, corresponding to the frequency above which a major fraction of the current through the suspension penetrates the rods), is consistent with a decrease of rod volume.

A response arising from a decrease in rod volume could involve either a movement of electrolyte solution from the rod interior to the external space or a movement of water alone. The finding that component I develops over the course of about a second rather than over a minute or more, suggests that the latter mechanism applies. In this case, the response

would be the result of a redistribution of ions in the external space, rather than of an increase in the total number of ions in this space. In particular, an increase in the fractional external space would result in a reduction of the resistance in the path through the external space where current passes between closely spaced rods. Since the total number of ions does not change, this reduction in resistance of the narrow portions of the current

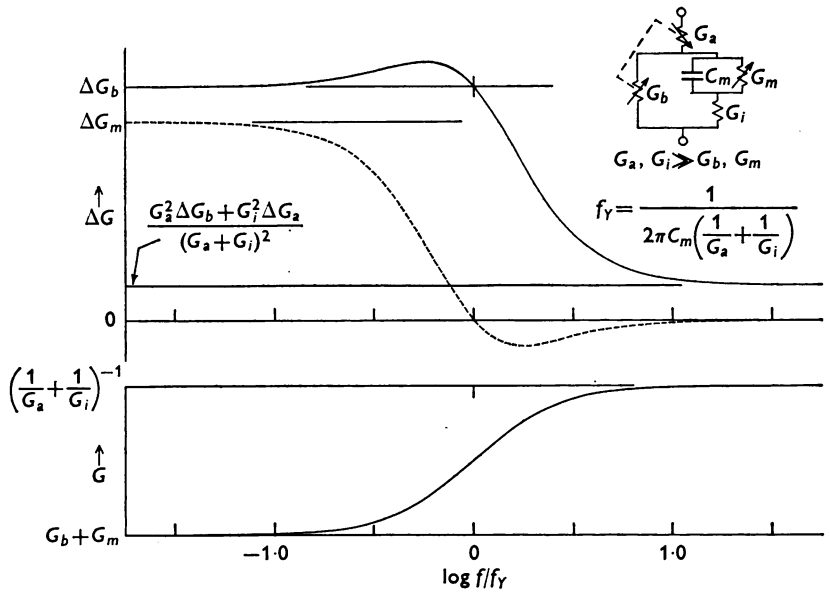

Fig. 8. Theoretical curves showing the frequency dependence of G and ΔG for a model containing a single capacitance and several conductances, some of them variable. C_m represents the surface membrane capacitance of the rods in a packed suspension, G_m the surface membrane conductance, G_i the internal conductance and G_a, G_b the conductance of the path external to the rods (divided into two parts distinguished according to whether the external path is in series with or by-passes the rods). The continuous curve for ΔG gives the effect of a decrease in rod volume involving an increase in G_b and a decrease in G_a. The dashed curve gives the effect of an increase in membrane conductance, G_m. The curves are derived according to the procedure described previously (Falk & Fatt, 1968).

path must be accompanied by an increase in resistance of the wide portions where the external space is effectively in series with the rod. This scheme is represented in the network model of Fig. 8 where G_a and G_b correspond to the conductance of the wide and narrow portions, respectively, of the external current path. The combined decrease in G_a and increase in G_b is used to account for the frequency dependence of ΔG in the neighbourhood of f_Y. In this scheme it is assumed that the internal conductance of the

rods, G_i, is unchanged. It is possible that G_i will change during the volume decrease, but the extent to which this will affect ΔG observed at high frequencies cannot be estimated.

The degree to which the negative variation in G_a balances the positive variation in G_b will depend on the detailed structure of the external and internal spaces which determines the values of G_a, G_b and G_i. Except for suspensions composed of rods which had been intentionally damaged, ΔG fell steeply as f_Y was exceeded. In the case of rods which had been subjected to freezing, ΔG continued constant up to the highest frequency of measurement, about a decade above f_Y. (A failure of ΔG to decline was also found in the case of rods which had been disrupted by shear. It is possible, however, that for this preparation f_Y was above the highest frequency of measurement.) It is conceivable that, in the case of the previously frozen rods, the failure of ΔG to decline significantly when the frequency exceeded f_Y was a consequence of the effective internal conductivity (G_i) having been decreased by disorganization of the structure of the disk stack.

The relation between a volume change and ΔG has been presented elsewhere (Falk & Fatt, 1972). For a preparation of rods, suspended in a solution of conductivity similar to Ringer solution and in which all of the rods are capable of yielding component I, ΔG, recorded at low frequencies, has been found to have a value of about 6 μmho/cm for a flash bleaching 1% of rhodopsin. This is calculated to correspond to a decrease in rod volume of 0·2%. Because of saturation of the response amplitude with flashes bleaching more rhodopsin, the maximum volume decrease obtainable in response to an intense flash amounts to about 0·6%.

For comparison, Fig. 8 also indicates the expected behaviour of ΔG for a response arising from a change in surface membrane conductance (dashed curve). A response, ΔG, arising from an increase in surface membrane conductance, would be expected to decline to zero over about a decade in frequency below f_Y.

Negative component

This component of response has not been previously recognized as a separate entity, though its presence seems likely to have influenced some previously reported observations. On the basis that it varied in amplitude in direct proportion to the amount of rhodopsin bleached, this component may be classified as similar to the heat and buffer components in depending directly on physicochemical changes in the rhodopsin molecule (or its photoproducts) and not involving interactions with other constituents of the rod which limit the magnitude of response, as occurs for components I and II at a few per cent rhodopsin bleached.

As regards its mechanism of origin, one may consider the possibility that the negative component, like component I, arises from a volume change – in this case an increase in volume of the rhodopsin molecule or of some portion of the disk membrane associated with it. Owing to the hydrophobic character of the structure undergoing this volume change, there may be a failure of water to penetrate this structure. The result would be an increase in rod volume which, without movement of water or of electrolyte across the surface of the rods, would lead to a small fraction of rod suspension being forced out of the region of the conductivity cell between the electrodes. Such a loss of conducting material from the region of measurement would give a frequency independent ΔG as observed.

Following a simple approach, one may suppose that the fractional decrease in G would be equal to the fractional increase in the volume of the rod suspension. In experiments such as that illustrated in Fig. 6, one has for a 15% bleach of the initial rhodopsin content $-\Delta G/G = 4 \times 10^{-4}$, approximately. In the suspension the initial rhodopsin content amounts to about 8% by weight or 6% by volume (assuming a partial molar volume for rhodopsin of 0·75). One may now calculate that, to produce a response of the observed magnitude, the water-excluding region of the rhodopsin molecule would have to increase fractionally on exposure to light to the extent of 0·04. An apparent fractional increase in volume of the rhodopsin molecule of 0·36 has been reported, based on the use of molecular sieve methods (Heller, 1969), which would not distinguish between changes in the water-penetrated and water-excluding regions of the molecule. It is concluded that a change in volume of the rhodopsin molecules is a possible mechanism of origin of the negative component.

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