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DISSECTION OF A GRADED VISUAL RESPONSE WITH TETRODOTOXIN*

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INTRODUCTION

THE *Limulus* eye has proved advantageous for intracellular studies because of the relatively large size of the visual cells (Miller, 1957) and because of the characteristic isolation of visual units in a compound eye. A further dividend has been provided by the relative ease with which both the graded generator potential and optic nerve activity can be monitored in a single preparation. The latter property has been especially useful for asking how the generator potential controls optic nerve activity, and this question has been approached with considerable experimental success (Hartline, Wagner and MacNichol, 1952; MacNichol, 1956; Fuortes, 1959; Behrens and Wulff, 1965; and others). A second question might be phrased as follows: How do the light reactions control the generator potential response? Experimental approaches to the latter problem have emphasized the properties of the generator potential itself, although these properties establish only one set of boundary conditions for the general problem.

A model of the generator response system is shown in Fig. 1 (Benolken, 1961). If the light reactions adjust R_2 "properly", the transient and steady-state components of the generator potential could be produced as shown. This model and other available models are probably an oversimplification. The data to be presented here suggested that the light reactions control at least two electrical parameters of the model, for example, R_1 and R_2. A further complication was also indicated by the data. It appears likely that the light reactions provide two independent inputs to the generator potential process as well as exerting control at two independent electrical points.

The transient ("initial") component of the response is affected differently than the steady-state ("final") component of the generator potential by calcium deprivation, by potassium concentration changes, and by probe withdrawal (Yeandle, 1957). Further chemical dissection of the generator potential response would be especially useful if this could be accomplished with agents of defined pharmacological specificity. Tetrodotoxin, a poison

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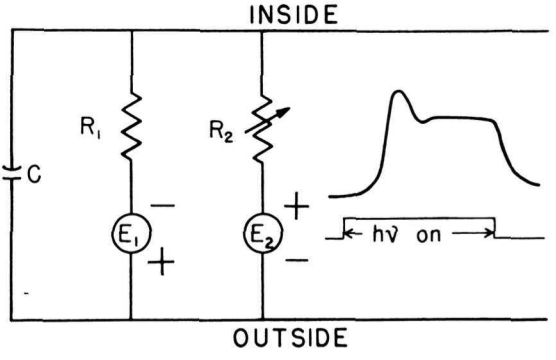

FIG. 1
Oversimplified electrical model of the generator potential response.

extracted from puffer fish, has been shown to block impulse activity in muscle and nerve (Furukawa, Sasaoka, and Hosoya, 1959; Narahashi, Moore and Scott, 1964; Nakamura, Nakajima and Grundfest, 1965; and others). In nerve, the drug appears to act specifically by inhibiting the ionic processes which are associated with the regenerative sodium conductance changes of a propagated impulse.

Loewenstein, Terzuolo and Washizu (1963) applied tetrodotoxin to two sensory systems, (a) the crustacean stretch receptor and (b) the mammalian pacinian corpuscle. In both cases these authors reported that the drug selectively blocked the propagated nerve impulses of the sense organs but that it had *no* effect on the graded generator potential. Results to be reported here indicate that such is not the case for the *Limulus* eye. Perhaps it should be pointed out that a different result for *Limulus* was not altogether unexpected. The graded generator potential of *Limulus* exhibits two properties which appear to be somewhat unusual in sensory systems: (1) the graded response can "reverse" the resting level of the visual cell (Benolken, 1961) and (2) a portion of the graded response domain may show regenerative transducing properties (Fuortes and Poggio, 1963; Adolph, 1964; Benolken, 1965a; and Benolken, 1965b).

METHODS

A lateral eye of *Limulus* was excised and cut transversely to expose the ommatidia. Solutions were exchanged in two ways: (1) when analyzing the effect of drug concentration, the whole eye was mounted in a clamp and solutions were exchanged with an infusion-withdrawal pump; (2) when multiple exchanges of drug and sea water were desired, thin sections of the eye were mounted in a small chamber as suggested by Adolph (1965), and tetrodotoxin was added to the chamber via a small capillary during continuous

(or intermittent) chase with sea water. Without exception crystalline tetrodotoxin was dissolved in sea water, and percentage concentration refers to weight in grams dissolved in 100 ml of sea water.

Micropipettes, filled with 2M KCl, were probed through an ommatidium until a light response could be recorded intracellularly. The micropipette provided a salt bridge between the preparation and an Ag–AgCl electrode connected to a negative capacitance preamp (MacNichol and Wagner, 1954). The circuit was completed through an Ag–AgCl electrode placed in the sea water surrounding the eye. The amplified output of the preamp was monitored by an oscilloscope and a high-speed ink writer. A Grass camera provided records of the scope traces.

Stimuli were provided by a tungsten source and an optical bench. Intensities were controlled by attenuating a constant source intensity with neutral density wedges. Scattered light was visually apparent at the air–solution interface of the preparation. If percentage scatter was reasonably constant for a given preparation, the experimental results should be independent of the scatter because of the experimental controls. Stimulus durations and stimulus cycles were programmed with an estimated reproducibility of ± 200 μsec, and flash waveforms were flat with rise times less than or equal to 500 μsec.

RESULTS

Physiological properties of a drug are not independent of dosage characteristics. However, it will be convenient to begin by considering tetrodotoxin as an "effective" dissecting tool and to defer a definition of effective dosage until later. The simplest way to analyze the generator potential response as a function of tetrodotoxin is to make all other parameters of the system as invariant as possible. A closely controlled stimulus program was set up to maintain constant stimulus conditions for the experiments unless otherwise noted. A stimulus of constant duration and intensity was presented to the eye every 20 sec for a period of some hours preceding the records of Fig. 2, and the same program was continued throughout the experimental period of the preparation. Before the first record the preparation had been presented with a continuous chase of sea water. After record 1 the chase was stopped and tetrodotoxin was injected into the system. Within 60 sec the transient component of the generator potential had been reduced as shown in record 4; then the sea water chase was started, and the effect of the drug was reversed in less than 60 sec as shown in records 7 and 8.

Prior to the records of Fig. 2 the preparation had been subjected to several injections of tetrodotoxin. Although the primary effects of the drug had been reversed by sea water, the responses appear to be "noisy" when compared to responses observed before exposure to the drug. A noisy response as shown in Fig. 2 was characteristic of preparations which had been subjected to tetrodotoxin.

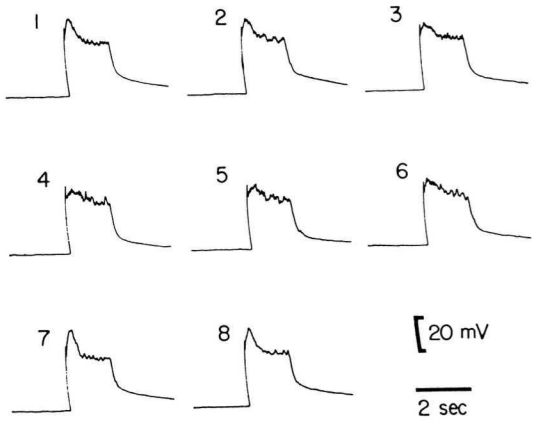

FIG. 2

Intracellular responses recorded when tetrodotoxin was added to the solution bathing the eye. Response 1 is a control recorded in sea water. Tetrodotoxin was added to the bathing solution after record 1. The drug was washed out by a sea water chase initiated after record 4. The "noisy" properties of the response are discussed in the text. The numbers above each record correspond to the numbered data points of Fig. 3. Stimuli were repeated every 20 sec. Each stimulus was of 2 sec duration and constant intensity. The recording baseline was established by the resting level of the cell. Positive polarity increases in an upward direction, and the curvilinear recording arc is defined for the voltage calibration.

Three response parameters were measured from the records of Fig. 2: (1) amplitude of "initial pulse", (2) maximum amplitude of the transient component, and (3) amplitude of the steady-state component at the end of the stimulus period. The initial pulse, or notch, occurs on all records of Fig. 2, but is especially obvious when the transient component is suppressed, e.g. on records 3 through 6. The initial pulse is not observed in all preparations, a point which will come up again later in the discussion of latency.

Figure 3 is a plot of the three response parameters vs. time. The numbered data points of the plot were derived from correspondingly numbered records of Fig. 2. A few general comments on the plot itself might be helpful. Although the points are not equidistantly spaced along the time axis, the preparation was controlled with a constant stimulus program, but only every third response was plotted when parameters were invariant in order to reduce congestion on the plot. The size of the symbols on the plot is a measure of the magnitude of uncertainty in reading a parameter from the records. The upward arrow indicates the end of a sea water chase and a short-term injection of tetrodotoxin, the downward arrow indicates when the sea water chase was started again, and the double arrow indicates that tetrodotoxin was injected for about 60 sec *while* a sea water chase was operating continuously. Thus the plot summarizes the response of one preparation to two injections

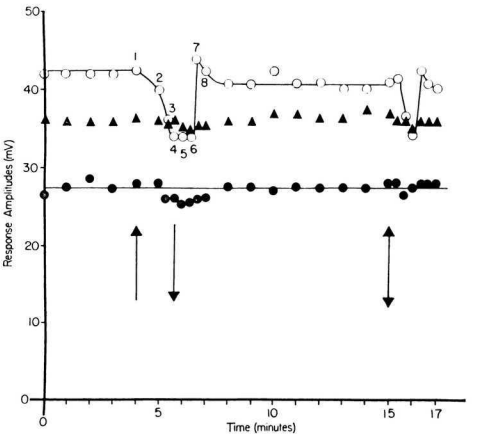

FIG. 3

The plot of response amplitude vs. time includes points derived from correspondingly numbered records of Fig. 2. The maximum amplitude of the transient component is represented by open circles, the peak of the initial pulse is represented by filled triangles, and the filled circles represent the amplitude of the steady-state component which was measured just before the light was switched off. All parameters were measured relative to the resting level of the cell before the stimulus was switched on. The uncertainty of reading amplitudes from the records is indicated by the magnitude of the symbols. The flow of sea water was stopped and tetrodotoxin was injected into the bathing solution at the upward arrow, sea water chase was resumed at the downward arrow, and the double arrow indicates a brief injection of tetrodotoxin *during* continuous sea water chase.

of tetrodotoxin, one injection with intermittent sea water chase and the other with continuous chase.

It is apparent from Fig. 3 that the transient component of the generator potential was inhibited selectively by tetrodotoxin, that the initial pulse and steady-state components were almost unaffected by the drug, and that the effect of the drug was reversed more rapidly under conditions of continuous rather than intermittent sea water chase. To anticipate the discussion on drug variability, it should be pointed out that the preparation of Figs. 2 and 3 was selected as an example of one extreme observed in the data. In the first place, the effect of the drug could be initiated and reversed rapidly. Under conditions of continuous chase the drug was delivered, the transient component reduced, and the effects were reversed in an interval of less than 80 sec. Secondly, the initial pulse and the steady-state component were almost invariant; the standard deviation of the mean for these two parameters was respectively less than or equal to the magnitude of uncertainty of measurement where calculations of standard deviation and mean value were based on the twenty-six responses shown in Fig. 3. Also the amplitude of the transient component was reduced markedly by tetrodotoxin, but it was not eliminated. Finally, the

amplitude of the transient component of the generator potential completely recovered in sea water.

The curve of open circles in Fig. 3 corresponds to the maximum amplitude of the transient component of the response. There was a suggestion of overshoot in this parameter when the effect of the drug was reversed. This is an interesting observation. Presumably the light reactions and the photopigment were controlled by a constant quantum flux density during the time that the drug inhibited the transient component, a fact indicated both by the stimulus conditions and by the constant amplitude of the steady-state component of the generator potential. To phrase the situation differently, the transient component of the response appears to dark adapt when blocked by tetrodotoxin even though flux density and the amplitude of the steady-state component of the response appear to be constant throughout the experiment. The overshoot is not spectacular in Fig. 3, probably (a) because of the extremely rapid reversal of the drug and correspondingly short time for selective adaptation, (b) because the relatively lengthy stimulus cycle of 20 sec permitted appreciable dark adaptation between stimuli even in the absence of the drug, and (c) the stimulus intensity was at a nearly saturating level for the response mechanisms. The question of selective adaptation will be examined again under more favourable conditions.

Stimuli of constant intensity and duration were presented to the preparation of Figs. 4 and 5. The stimuli were carefully programmed to repeat every 10 sec. The reduced amplitude of the transient component relative to the amplitude of the steady-state component in the control records of Fig. 4

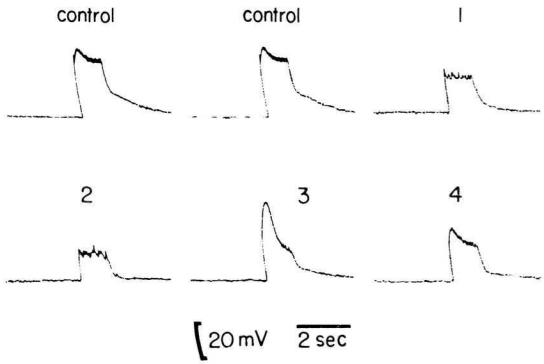

FIG. 4

Intracellular records. The two sea water controls were recorded immediately before tetrodotoxin was injected into the bathing solution. After about 5 min, response 1 was recorded. Sea water chase was started after record 1 in order to wash out the drug. The stimulus cycle was repeated every 10 sec, and the stimuli were of constant intensity and were of 1·3 sec duration. The numbers above each record correspond to numbered data points of Fig. 5. Other general recording features were similar to those of Fig. 2.

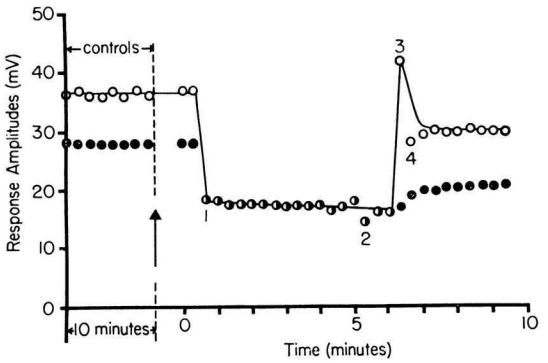

FIG. 5

Data points include amplitudes measured from correspondingly numbered records of Fig. 4. Open circles represent amplitudes of the transient component of the generator potential, and filled circles represent amplitudes of the steady-state component. The half-filled circles indicate responses for which there was no measurable transient component and only the steady-state component was observable on the records. The size of the symbols is a measure of reading precision. The flow of sea water was stopped and tetrodotoxin was injected into the bathing solution at the arrow. About 5 min elapsed from the arrow until time $t = 0$. No effect of the drug was observed until after $t = 0$. Sea water chase was started to wash out tetrodotoxin after the point numbered 1. Other general features of the plot were similar to Fig. 3.

indicates that the preparation was light adapted significantly by this shorter stimulus cycle. The general comments directed toward Fig. 3 also apply to the plot of Fig. 5. The numbered data points of Fig. 5 were derived from numbered records of Fig. 4, and the size of the symbols is a measure of precision. The arrow indicates that tetrodotoxin was presented to the preparation about 5 min before $t = 0$. At record 1, the sea water chase was resumed. The half-filled circles indicate that the transient component and the steady-state component were indistinguishable.

The effect of tetrodotoxin on the preparation of Fig. 5 should be compared to its effect on the earlier preparation of Fig. 3. For Fig. 5 the drug required considerable time to produce an effect and at least 5 min were required for its effect to be reversed in sea water. The amplitude of the steady-state component was reduced to about two-thirds of its control level after the preparation had been exposed to tetrodotoxin. The transient component was eliminated by the drug, that is, it was completely inhibited. Except for response record 3, the amplitudes of the transient and steady-state components never completely recovered their control value when returned to sea water. However, the *difference* in amplitude between the transient and steady-state component closely approached the control level. Thus it seems likely that the irreversible effect of the drug could be explained by an irreversible loss of amplitude of the steady-state component with almost complete

recovery of the transient component. Experience with the drug would suggest that this unusual loss of the steady-state component might be explained by some unknown clumsiness in exchanging solutions, but convenience is the only merit which can be claimed for this type of explanation.

The overshoot of response 3 in Fig. 5 is striking, especially since thereafter in sea water the amplitude of the generator potential never again achieved the control value. The amplitude of the response suggests that the transient processes had been dark adapting selectively while completely inhibited for a period of 5 min. An even more compelling argument is presented by record 3 in Fig. 4. The waveshape of this transient response is characteristic of a dark adapted eye which has been stimulated by relatively intense light. (But the eye had been exposed to a constant flux density and stimulus program throughout the run.) Typically after dark adaptation, intense stimuli produce a transient component which is prolonged in duration as well as of large amplitude. In other words dark adaptation is accompanied by waveshape changes as well as amplitude changes in the transient component of the generator potential.

Latency

Is the latency of the generator potential altered by tetrodotoxin? The data suggested that the answer to this question depends upon how it is asked. Unfortunately the light response of *Limulus* is complicated by at least two different types of intracellular recording situations, and this complication appeared to be crucial to the latency question.

The response of Fig. 6 exhibited a clearly defined "initial pulse". The record at 5:14 is a control. At 5:14 tetrodotoxin was presented to the prepara-

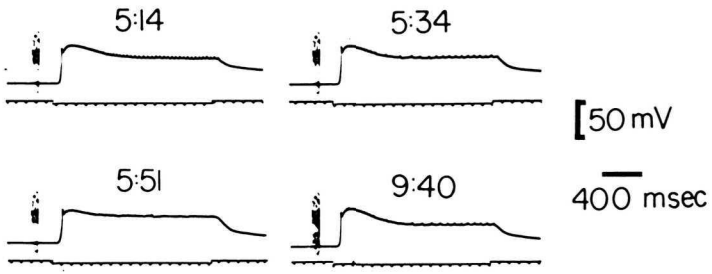

FIG. 6

The 5:14 record is a sea water control. Tetrodotoxin was injected immediately after 5:14, and the sea water chase was not resumed until after 5:51. A stimulus monitor deflected the lower scope trace of each record downward when the stimulus was on. A time base of 10 msec pulses repeated at 100 msec intervals was also recorded on the lower trace. A 1·6 sec stimulus of constant intensity was repeated on a 16 sec cycle. A ripple was observed especially on the upper trace of the 5:14 record and was almost absent from the 5:51 record. This was the result of nerve impulses which were poorly resolved at this writing speed.

tion and by 5:51 the drug had almost eliminated the transient component of the response. Sea water chase was started at 5:51 and by 9:40 the control amplitude of the transient response was almost recovered, although the nerve response did not completely reverse for this preparation. The preparation of Fig. 6 again demonstrated that the initial pulse of the generator potential was an almost invariant parameter in the presence of the drug. The latency of preparations showing an initial pulse probably was unaffected by tetrodotoxin.

Nerve impulses were not recorded from the preparation of Fig. 7, and the generator potential did not exhibit an initial pulse. (However, these two

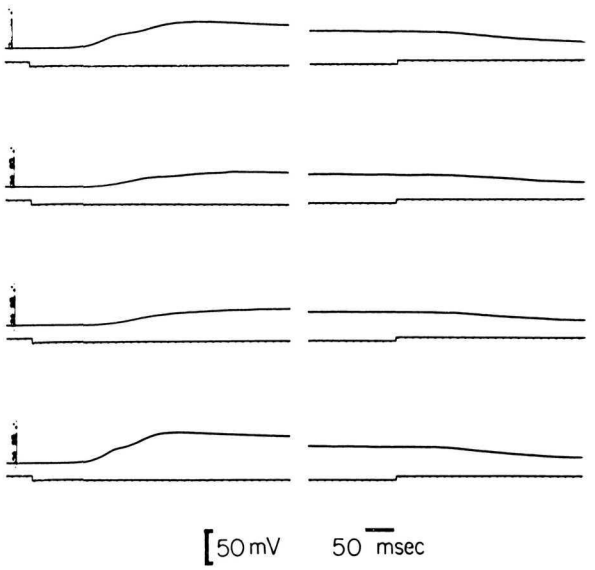

FIG. 7

Records of responses to 4 consecutive stimuli of a 16 sec stimulus cycle. The upper record is a sea water control. Tetrodotoxin was injected after the upper record. The sea water chase was resumed 32 sec later immediately after the third record. Again, a downward deflection of the lower scope trace in each record indicated when the light was on. The timing pulses on the lower trace were separated by an interval of 10 msec. Notice the absence of an initial pulse and optic nerve activity in the control and other records. The break in the records was convenient because of the expanded time scale.

conditions are not necessarily correlated.) The responses were recorded in consecutive 16 sec intervals. The upper record was a control. Tetrodotoxin was injected after the first response and exerted considerable effect on the second response 16 sec later. The transient component was eliminated by the third record, and sea water reversed the effect of the drug with some transient overshoot in the bottom record. The lower trace of the records in Fig. 7 indicates when a light flash was switched on, and the timing pulses on the

lower trace are separated by 10 msec. It would appear that the latency was increased by about 30 msec when the transient component was inhibited by tetrodotoxin. The latency change here is contrary to what was observed when the generator potential exhibited an initial pulse.

The initial pulse, when it occurred, was affected by tetrodotoxin in the same fashion as the steady-state component of the response. It is possible that there are three separable components of the generator potential, but for simplicity it will be assumed that there are only two until the data demand further complication. On these assumptions, the initial pulse seemed to be a part of the steady-state component.

While the latency problem was of interest for its own sake, the primary motivation for these experiments arose from a less obvious question. Perhaps the transient component of the graded response arises from activity near the distal process of the unique eccentric cell (see Miller, 1957, for an excellent description of the morphology of the *Limulus* ommatidium), and possibly the transient component of the response might exhibit a propagation velocity which would be a function of probe location in the ommatidium. The steady-state component was assumed to be a non-propagated process. One way to test the question of propagation was to follow latency changes as the probe proceeded from the transient electrogenic site toward the neural generator (recording sites 1 and 2 of Benolken, 1965a). The latency of the transient component would change as the probe position varied from one recording site to the other if the transient component exhibited a propagation velocity, while the steady-state component would provide a time reference. The steady-state reference could be precisely defined when tetrodotoxin eliminated the transient component. Then the latency of the transient component could be followed by subtracting the tetrodotoxin response from control sea water responses recorded before and after administration of the drug. In short, the time of appearance of a "difference" response should define the desired latency time. However, the "difference test" failed because of uncertainties in resolution of time and/or space. It quickly became obvious that the initial pulse, when present, always preceded the transient component. Therefore the transient component became bracketed in time-space by an initial pulse and the remainder of the steady-state component, and both bracketing parameters became noisy though relatively invariant in the presence of the drug (the noise problems associated with the drug were discussed in reference to Fig. 2). To the noise uncertainty must be added another uncertainty. Why do preparations presumably close to the transient electrogenic site exhibit or not exhibit an initial pulse? (Fig. 7). For that matter why do preparations presumably closer to the neural generator (Fig. 6) usually exhibit an initial pulse? It is the authors' opinion that the propagation question will not be resolved by data such as that of Figs. 6 and 7 until the spatial properties of the recording situation have been more precisely defined.

Optic Nerve Response

As expected, optic nerve impulses could be eliminated with tetrodotoxin, and the effect was reversible if the eye was again bathed in sea water. In general, tetrodotoxin eliminated the optic nerve response before and/or at lower concentrations than it eliminated the transient component of the graded generator potential. Presumably the drug acts on the receptor neuron, as it does on other nerves, by eliminating the initial regenerative component of current or the so-called sodium activation. However, stimulation of the receptor neuron here occurred by way of light reactions which initiate an electrical generator potential rather than by direct electrical stimulation as is the usual case for nerve experiments. Perhaps the drug acted on the eye by uncoupling the neural processes from the preceding light reactions, which include the generator potential, rather than by acting directly upon the neural process itself. This possibility suggests at least three alternatives: (1) the drug acts in the usual way upon the neural process itself, (2) the drug acts by uncoupling the neural process from the preceding light reactions, and (3) the drug acts both upon the neural process and upon the coupling process. The last alternative is the most conservative interpretation of the three, but an experimental test can be devised which should discriminate between the first two by providing a basis for rejecting one of them. Slightly spontaneous preparations proved to be useful for this test. Preparations were selected which exhibited a relatively low nerve-impulse frequency in darkness and which showed a marked increase of firing rate when the eye was stimulated with intense light.

Assume for argument that the drug acts by uncoupling the neural processes from preceding light reactions. On this assumption the drug should eliminate the increased firing rate observed during illumination of a spontaneous preparation but should have no effect on the spontaneous neural activity observed in darkness, since in darkness no generator potential is observed. That is, the coupling processes would be inhibited by the drug but the spontaneous neural processes would be unaffected. On the alternate hypothesis that the drug would act directly on the neural process, the drug should eliminate the spontaneous neural activity as well as the neural activity associated with illumination.

Selected records from a spontaneous preparation are shown in Fig. 8. The preparation was not stimulated at constant intensity as was the case for all previous records. In this case stimulus intensities were varied in steps over several log units, but the various steps were repeated in identical fashion from run to run. The stimulus program had been carefully controlled at least an hour before the first record and was maintained thereafter. Two responses are shown in each record; the response at the left was elicited by a stimulus ten times as intense as that for the response on the right. The record at 6:28 is a

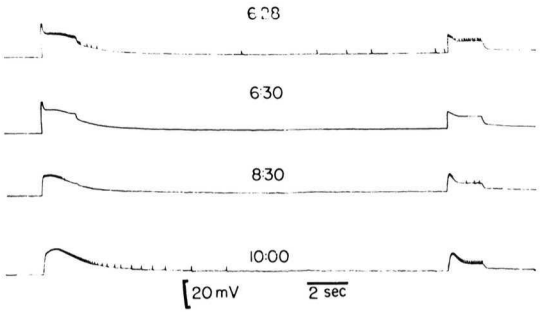

FIG. 8

Selected responses recorded from a spontaneous preparation. Light responses at the left were elicited by stimulus intensities which were 10 times greater than intensities at the right of each record. Tetrodotoxin was injected after the sea water control of 6:28. Sea water chase was resumed after the 6:30 record. During stimulation in some records impulses occurred at too high a frequency to be resolved with this time base; then the impulses appear only as a broadening of the generator potential trace.

control recorded in sea water. Spontaneous nerve impulses were observed on the record after the 2 sec stimulus had been switched off. Notice that the *spontaneous* neural activity occurs long after the generator potential had been recorded and the stimulus had been switched off. Tetrodotoxin was added to the bathing solution after the 6:28 record. The concentration of tetrodotoxin was sufficient to block nerve impulses but was too low to measurably affect the transient component of the generator potential. By 6:30 the drug had eliminated all nerve impulses, both those associated with illumination and the spontaneous impulses observed in darkness. From 6:30 to the end of the experiment the eye was subjected to a sea water chase. After 2 hr in sea water some neural activity was observed during periods of stimulation in the 8:30 record. Nerve impulses occurred both during stimulation and in darkness by 10:00.

If the 8:30 record of Fig. 8 is ignored, these data would seem to favor the hypothesis that the drug eliminates optic nerve impulses by acting on the neural process directly. The fact that tetrodotoxin eliminated spontaneous activity seems to demand at least, as a most conservative interpretation, that the drug acts both on the neural process itself *and* on the coupling processes.

Even the 8:30 record would support the argument that the drug acts directly on the neural process if the reasonable assumption is made that the drug acts in a quantitative rather than in an all-or-none fashion to inhibit sodium activation of the nerve. As the effect of the drug was slowly reversed during the sea water chase, the nerve threshold should be slowly decreased until the light reactions began to stimulate nerve activity although to a reduced extent relative to the control record. The data indicate that the threshold was

considerably higher than the control value, because even during stimulation the number of impulses was considerably less during the 8:30 record than during the corresponding controls of 6:28 and 10:00. It is reasonable to suppose that the higher neural threshold indicated at 8:30 was above the spontaneous threshold. This would account for the observation that spontaneous activity was not reversed when a reduced neural activity was recorded during illumination even though tetrodotoxin was acting directly on the neural process in both situations.

For our discussion a stable preparation is defined as one which exhibits a constant response under a given set of conditions for the period of an experiment. This must be true whether the experiment requires a few minutes or several hours. Obviously reversing the effect of a drug has no quantitative meaning unless a control response can be repeated before and after the experimental run. It is also obvious that the 10:00 run of Fig. 8 did not reproduce the original control of 6:28. The spontaneous activity occupies a different portion of the time domain in the stimulus cycle and the generator potential at 10:00 has a dark adapted characteristic. The preparation of Fig. 8 was not stable as defined above, and indeed it was not expected to be so. Spontaneous activity appears to be a pathological symptom for the *Limulus* ommatidium. Except when useful for a specific experimental purpose, spontaneous preparations were routinely discarded as a sign of damage probably inflicted by the probing micropipette. Although a simple experiment in principle, tetrodotoxin was applied to only two spontaneous preparations which could be maintained in a state of "bad health" for 5 or 6 hr. (By contrast, non-spontaneous preparations such as those of Figs. 2 and 3 were stable for many hours.) It should be pointed out that these data indicated only that tetrodotoxin inhibited spontaneous neural activity as well as the neural activity associated with illumination. While both types of neural activity could be recovered in the same cell with a sea water chase, the response of a spontaneous preparation was not reversible in the sense that it could be reproduced quantitatively over a period of several hours. However, qualitatively the results were clear for the two successful spontaneous preparations: tetrodotoxin eliminated spontaneous neural activity as well as the neural activity associated with illumination.

Pharmacological Parameters

"Effective" concentrations of tetrodotoxin were assumed for the preceding discussions. Whenever effective, the general physiological effects of the drug were reasonably consistent. The data shown earlier were selected to provide examples of extremes in physiological variability, while variability in the time course of drug action is indicated by a comparison of Figs. 2–8. Variations observed in dosage values (effective concentrations) require further comment.

Dosage variations could arise from physiological differences between preparations, differences in the flow patterns during solution exchanges or additions, and differences from sample to sample in the drug itself. Deciding which was the most important source of variation is a risky business at best, but the data would seem to support the conclusion that the principal source of dosage variation was the variation between drug batches. This appeared to be true even though all of the drug was prepared by Sankyo Corporation, No. 1 Ginza-Nichome, Chuo-ku, Tokyo, Japan. The major variations observed in dosage characteristics correlated perfectly with batch differences of the drug, while physiological variations and the time course of drug action did not appear to correlate with known batch differences.

The dosage conclusions suggested here were based on samples from three batches of tetrodotoxin and about twenty "successful" preparations. The word "successful" has the following meaning: a stable preparation was successful if it could not be discarded because of obvious blunders in exchanging solutions, and the drug could be shown to have some effect on the eye. The latter qualification might appear to bias a consideration of dosage characteristics except for the fact that the drug always produced a physiological effect upon otherwise successful preparations unless it could be demonstrated that the drug had decomposed with time in solution. The potency of samples of tetrodotoxin declined monotonically with time in solution; as a rough rule of thumb, the drug became useless for our purposes about 2 weeks after a sample of the crystalline material had been dissolved in sea water.

The dosage characteristics of the drug were most carefully examined for the batch of highest potency. For drug samples of highest potency the optic nerve response was eliminated at concentrations less than or equal to 10^{-7}% for freshly dissolved crystals. Concentrations of 10^{-7}% had no measurable effect upon either component of the graded generator potential. The transient component of the graded receptor response was eliminated by drug solutions of about 100 times the above concentration, and these experiments were performed on the same preparations as those in which the neural response had been eliminated at concentrations of 10^{-7}%. That is, the transient component of the graded response could be eliminated by tetrodotoxin at concentrations equal to or less than 10^{-5}%. The discrimination between the neural process and the graded response as a function of drug concentration was useful for experiments such as that of Fig. 8.

The batch of intermediate potency eliminated the transient component of the generator potential at concentrations less than or equal to 10^{-3}%. The third batch of drug samples eliminated the optic nerve response at concentrations of about 10–3%, but it did not affect the generator potential at maximum *practical* concentrations of about 5×10^{-3}%. Experiments with the last drug batch did not contribute to the number of successful preparations.

DISCUSSION

Clearly tetrodotoxin eliminated the transient component of the graded generator potential as well as the neural impulses of the visual response. If (1) tetrodotoxin acts by inhibiting regenerative sodium conductance changes and if (2) the drug acts by inhibiting sodium sites whenever it produces an effect regardless of membrane origin, the data support the hypothesis that the transient component of the generator potential is controlled by a regenerative sodium process. In the absence of conflicting evidence and with the support of other lines of evidence (Benolken, 1961 and 1965a), this hypothesis and the explicit conditions of this hypothesis will be assumed for simplicity of argument. Notice that the second condition above is not necessarily contrary to the observation that the neural response and the graded transient response could be separated as a function of drug concentration. Again it is sufficient to invoke quantitative action by the drug, where the drug would be specific for a regenerative sodium process in both cases and where the neural process would be 100 times more sensitive to the drug than would be the graded response.

Independent of the question of drug specificity, tetrodotoxin did provide a precise chemical tool for selective dissection of the visual response. A schematic result of the dissection is summarized in Fig. 9. The three separable

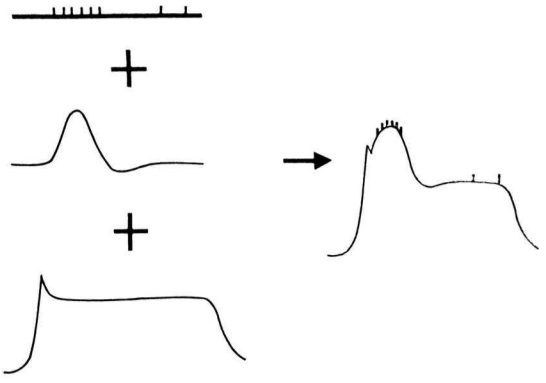

FIG. 9

The schematic light response at the right is represented as the sum of the three reversibly separable components at the left.

components are shown at the left, and the sum of the components—the intracellular light response of the *Limulus* eye—is shown at the right. The transient component of the total light response (right) shows characteristics of a slightly underdamped system. The response to a step function of light

often exhibits an initial overshoot of the steady-state level *and* a subsequent undershoot before the steady-state level is established. The data indicated that when measurable the undershoot, as well as the overshoot, was eliminated by tetrodotoxin. However, the initial pulse of the response was not eliminated by tetrodotoxin. This point was discussed when latencies were considered, where it was assumed that the initial pulse was either a part of the steady-state graded process or a separate component of the response. Since the data did not demand the further complication of a third component of the graded response, the principle of parsimony was applied and the initial pulse was assumed to be a part of the steady-state response. This complicates terminology. If the initial pulse is part of the steady-state component, these data indicate that the steady-state component *usually* precedes the transient component in time. The earlier literature often refers to the transient component of the response as the "initial" component and to the steady-state component as the "second" component. But in fact the time sequence of events usually appears to be the reverse of that implied by earlier terminology, and usage of the terms "transient" and "steady-state" might avoid possible ambiguity.

The authors were encouraged to use tetrodotoxin as a dissection tool for the graded response of the *Limulus* eye by Terzuolo (1962) because of the presumed specificity of the drug in nerve tissue. The drug proved to be an especially fine and selective tool for the intracellular response of *Limulus*, often eliminating the transient component altogether but having no measurable effect upon the steady-state component. However, it should be emphasized that Yeandle (1957) had shown that especially Ca and perhaps K had preferential effects upon the two components of the graded response of the *Limulus* eye. While apparently these two physiological ions were not as precise or as well defined a dissection tool as tetrodotoxin, it seems unfortunate that Yeandle's earlier work has not been published where it would be more accessible.

The transient and steady-state components of the response were represented by an electrical model in Fig. 1. The resistive arm R_2 of the model was selected as the single variable (graded) component in the model "until such time as the data indicate the necessity of further complication". Further complication is indicated by these data, and a single variable component does not appear to be adequate to describe the response.

Because of the polarity of generator E_2 and because only the transient component of the generator potential has been demonstrated to reverse the resting level of the cell, the resistive arm R_2 of the model was related to the transient component of the response. Consequently, tetrodotoxin should act on the model either to open-circuit resistive arm R_2 or to fix R_2 to a constant value. Either action of the drug would be sufficient to remove the one variable electrical parameter of the model, and such cannot be the case since the steady-state component of the generator potential continues to be a graded

function of light energy even in the presence of tetrodotoxin. With R_2 either fixed or eliminated (open-circuited) in the model, the response cannot be explained without a second variable electrical parameter. For example, the model would be inadequate unless, say, R_1 as well as R_2 were a variable parameter. It is possible to propose the not altogether trivial alternate hypothesis that the two variable components exist in the same arm of the model. Thus R_2 could be fixed at its maximum value by the drug, and a second variable resistor R_3, in the same arm E_2, could provide a graded steady-state function in the model. However, this alternative seems unlikely when considered from the perspective of the assumptions regarding drug action set forth in the beginning of this discussion section. The electrogenic source E_2 was presumably derived from membrane sites specific for a single ion, in this case sodium. If tetrodotoxin completely blocks these membrane sites, the entire arm E_2 would be open-circuited by the drug, and the variable component for the steady-state function could not occur in that same arm.

The transient processes appeared to dark adapt selectively when tetrodotoxin inhibited the transient component of the generator potential. The adaptation was selective for the transient response, since a controlled stimulus program was continued throughout the experiments and the steady-state component of the response was maintained at a constant state of adaptation. These observations provided the experimental basis for the earlier suggestion that the light reactions control the generator potential via two inputs to the electrical system. Notice that this statement is not equivalent to the proposition that the response mechanisms contain two variable electrical parameters; this hypothesis is stronger and demands two inputs into the electrical system as well as two variable parameters in the electrical system itself. By way of beginning consider another phenomenon which does not demand two independent processes. In the absence of drug the two components of the generator potential frequently light adapt at differing rates, the transient component being light adapted more rapidly than the steady-state component (see discussion of Fig. 4). This could be interpreted as indicating that a single light reaction step, which precedes the electrical event, inactivates the transient mechanism for a longer time than the steady-state processes. As an example suppose that a single chemical transmitter acts on both membrane sites, but after stimulation the transient receptor sites for the transmitter might be inactivated for a longer time than the steady-state sites. In the absence of evidence to the contrary, variations in the properties of membrane sites could account for the observed differences in the rate of light adaptation of the response components, and this interpretation is independent of whether or not the light reactions provide more than one kind of input into the electrical system. The same type of argument does not apply if selective adaptation occurs when one type of membrane site is inactivated by a light-independent agent.

A single input to the electrical process does not seem likely if the previous assumptions about drug action are correct, that is, if the drug simply acts on the receptor membrane to eliminate the specific permeability change of the transient process. When tetrodotoxin was applied, the stimulus program and the steady-state component remained constant while the transient process was inhibited. Apparently the transient electrical process was eliminated and the steady-state process continued without perturbation. Also the photochemical reactions were driven to the same state and at the same rate as they were before the drug was applied, and this must be the case if the photochemical reactions were entirely limited by light quanta. The simplest interpretation for selective adaptation of the transient component is that one product of the photochemical reactions accumulated when the transient process was eliminated by the drug. At the same time at least one other product of the light reactions proceeded at the usual rate with the steady-state process which remained invariant. True, this interpretation of the data is not conclusive (a common property of *all* positive scientific hypotheses). It is possible to explain the selective adaptation of the transient process on the basis of a "rebound" of the receptor membrane after removal of inhibiting drug. As a second of many possible alternatives it might be argued that there is an electrical–chemical transducer which senses the average electrical state of the receptor membrane and then acts on a feedback control to limit the photochemical reactions. However, until experimental evidence indicates otherwise the simplest hypothesis of two inputs to the electrical process will be assumed since it is consistent with these data.

An elegant analytical model has been proposed by Rushton (1965) which provides two points of electrical control for the visual process, one at the input of the electrical system and a second in a voltage feedback loop of an operational amplifier. The model is consistent with data which require two points of electrical control. The notion of two inputs to the electrical system was rejected by Rushton on the basis of human psychophysical data; the second control through the feedback loop was presented as an explicit function of the output voltage of the electrical network rather than as a function of a second input from the light reactions. *If* a common hypothesis is necessary here for psychophysical data and data on isolated receptor preparations, at least one of the two interpretations must be in error.

Spontaneous fluctuations or "quantum bumps" are observed when the *Limulus* eye dark adapts. What is the effect of tetrodotoxin on these fluctuations? Relatively unsystematic tests indicated that tetrodotoxin had no striking effect upon the fluctuations at concentrations which were physiologically significant for the light response. A more careful analysis would seem to be warranted, and the question should be examined further.

All of the physiological responses were measured intracellularly and hence, for completeness, a final remark should be directed toward the effect of

tetrodotoxin upon the resting membrane potential of the cell. The resting level might be of more than usual interest here because of the characteristic recovery processes which occur after illumination of the eye. However, the data indicated that tetrodotoxin did not exhibit a marked effect upon the resting level of the cell. The changes often observed were not consistent; apparently the cell might be hyperpolarized slightly or depolarized slightly by the drug in an unpredictable fashion. Either tetrodotoxin has no significant effect upon the resting level of the cell or present data are inconclusive on this point.

SUMMARY

The transient component of the generator potential of the *Limulus* eye is selectively and reversibly inhibited by tetrodotoxin, while the steady-state component of this intracellular light response is either unaffected or only slightly affected by the drug. These data seem to support the hypothesis that the transient component of this graded response involves a regenerative sodium process. When inhibited by the drug the transient component selectively dark adapts under conditions of constant quantum flux density suggesting that the light reactions control at least two inputs to the generator potential process. Latency studies indicate that an initial portion of the steady-state component *usually* precedes the transient component of the response. At low concentrations tetrodotoxin inhibits only optic nerve activity. This inhibition appears to be specific for the neural mechanisms, and the drug does not appear to act as an uncoupling agent between the generator potential and the neural process.

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