THE RESPIRATION OF THE ISOLATED ROD OUTER LIMB OF THE FROG RETINA

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I

INTRODUCTION

We have recently shown that cozymase (DPN) is required for the proper functioning of the rhodopsin cycle, thus establishing a link between the rhodopsin system and the more general metabolic activities of the visual cells (Wald and Hubbard, 1949, 1950; Hubbard and Wald, 1951). These cells, whose structure has been studied by many investigators for the past 100 years, consist of two discrete portions, the inner and outer limbs or segments (for a review of the literature, see Arey, 1932). The inner limb contains the cell nucleus, and has generally been considered the vegetative part of the cell. Rhodopsin is localized in the non-nucleated outer limb. The outer limb, which can easily be detached from the inner segment, has a well defined, almost crystal-like structure. According to Schmidt (1938, 1951), it consists of highly oriented molecules of lipid and protein, arranged in alternate layers. The rhodopsin is also oriented, and is responsible for the natural dichroism of the dark-adapted outer limb. This then is the anatomical site for photoreception. Here the absorption of light by rhodopsin is somehow converted into the electrical and chemical phenomena of a nerve impulse.

From the point of view of cellular economy, it seemed possible that the outer limb is specialized for photoreceptor functions, and depends for its nutrition on metabolic supplies from the nucleated inner limb or the adjoining pigment epithelium. We therefore decided to inquire whether the isolated frog rod outer limb has the enzymatic potential for respiration.

For this investigation, we chose the Cartesian diver method developed by Linderstrøm-Lang (1937), and Holter and Linderstrøm-Lang (1943). This enabled us to study the respiration of relatively small numbers of outer limbs, which could be isolated easily and quickly, and allowed us to check each cell sample under the microscope for the absence of inner limbs and nuclei.

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II

Methods

Retinas were dissected from freshly killed, dark-adapted frogs (*R. esculenta*), and the rod outer limbs were detached by gently scraping the posterior surface of the retina with a spatula (*viz.* Wald and Hubbard, 1949). This procedure was carried out in white light unless otherwise specified. Respiration was studied with samples containing between 10,000 and 100,000 outer limbs in a suitable suspension medium (frog-Ringer alone, or with supplements), in a total volume of about 0.8 μl. The suspension of outer limbs was introduced into the bulb of a conventional Cartesian diver having a volume of about 10 μl., and sodium hydroxide and oil (0.5 μl. each) were inserted as seals into the neck of the diver, to absorb carbon dioxide and minimize diffusion of gases. Manometer readings were usually begun within an hour after the frog had been killed. Experiments were carried out at 20°C., for periods of 2 to 8 hours. In experiments with cyanide, it was necessary to insert a loosely fitting stopper into the neck of the diver to minimize loss of cyanide by diffusion (*viz.* Linderstrøm-Lang and Holter, 1942). Parallel with each set of diver experiments, a measured sample of the suspension was diluted 10 to 50 times with Ringer solution, and the outer limbs were counted in a red blood cell counting chamber under the microscope, after checking for absence of inner limbs and nuclei.

III

RESULTS AND DISCUSSION

A summary of several representative experiments is shown in Table I. The table is self-explanatory, and we shall therefore only discuss some points that seem to us to be of particular significance. The experiments show unequivocally that isolated frog rod outer limbs are capable of respiring in Ringer solution, even in the absence of added organic substrates. They must therefore contain the requisite enzymes and coenzymes, along with a considerable stock of substrates. The rate of respiration was independent of the concentration of outer limbs over a range of roughly 20,000 to 80,000. In all experiments, the respiratory rate was steady for 1 to 2 hours, after which it began to decrease in some cases.

In Experiment 7 (Table I), we tried the effect of washing the outer limbs with Ringer solution. The washed outer limbs were then divided into four portions and suspended respectively in Ringer solution alone, and in Ringer containing 0.01 M fructose diphosphate, glucose, or succinate. Compared with other experiments, washing reduced the rate of respiration in Ringer solution, without, however, abolishing it. The rate was raised slightly by addition of fructose diphosphate or succinate.

The respiration was cyanide-sensitive (Experiments 10 and 11), and therefore probably mediated by the cytochromes.

The respiratory rate in Ringer solution was increased by fructose diphos-

TABLE I

Respiration of Isolated Frog Rod Outer Limbs in Ringer Solution, and in the Presence of Various Added Substrates

The outer limbs were suspended in the various media immediately after isolation, and respiration was measured in a total volume of 0.8 μl. Measurements were usually begun within an hour after the death of the animal. All measurements were made at 20°C., pH about 7.

Experiment	Suspension medium	No. of outer limbs in sample	Oxygen consumption (μl. oxygen per 10,000 outer limbs per hr.) $\times 10^{-3}$	Remarks
1	Ringer + fructose diphosphate (0.01 M)	14,400	3.90	
3 *a*	Same	22,000	3.24	Rate fell off after 2 ½ hrs.
5 *a*	Ringer	41,300	2.2	Rate steady for about 2 ½ hrs., then fell slowly to about 50 per cent after 4 ½ hrs.
5 *b*	Ringer + succinate (0.01 M)	38,600	3.66	After 2 ½ hrs. rate fell to 1.92
6 *a*	Ringer	76,000	2.01	After 1 hr. rate fell to 1.23
6 *b*	Ringer	42,700	2.55	After 1 hr. rate fell to 1.40
6 *c*	Ringer	22,700	2.60	After 1 hr. rate fell to 0.87
7 *a*	Ringer	19,800	1.73	Outer limbs washed three times with Ringer, then suspended in respective media, and measurements begun
7 *b*	Ringer + fructose diphosphate (0.01 M)	19,000	2.2	
7 *c*	Ringer + glucose (0.01 M)	18,300	1.84	
7 *d*	Ringer + succinate (0.01 M)	18,500	2.14	
10 *a*	Ringer	88,000	2.88	
10 *b*	Ringer + cyanide (0.008 M)	80,000	1.01	65 per cent inhibition
11 *a*	Ringer	78,400	2.10	
11 *b*	Ringer + cyanide (0.005 M)	73,500	1.02	51 per cent inhibition
14	Ringer + fructose diphosphate (0.01 M)	43,000	2.2	Measurements begun with dark-adapted outer limbs; then bleached about 2 ½ hrs. after animal killed. No change in respiratory rate on bleaching
15	Same	47,600	3.00	Same conditions, but bleached about 3 ½ hrs. after animal killed. No change in rate on bleaching (*viz.* Fig. 1)

phate or succinate (Tables I and II). Table II presents a summary of the rates in Ringer solution, in the presence and absence of fructose diphosphate. This substrate, though clearly not essential for respiration, increased the rate by about 55 per cent. It also increased the variability of the data. This is probably due to the fact that isolated rod outer limbs are depleted to a variable extent, also in soluble factors—coenzymes and substrates—other than fructose diphosphate. It should be remembered that anatomically, these tissues are only cell fragments, which present no barrier to the diffusion of water-soluble substances at their proximal ends. We therefore do not wish to imply

TABLE II

Comparison of Respiratory Rates of Rod Outer Limbs in Ringer Solution, and in Ringer Containing Fructose Diphosphate (0.01 M), at 20°C., about pH 7

Only the initial rates were used for this comparison. Addition of fructose diphosphate increases the rate by about 55 per cent.

Ringer solution		Ringer + fructose diphosphate	
Experiment	Oxygen consumption (μl. oxygen per 10,000 outer limbs per hr.) × 10^{-3}	Experiment	Oxygen consumption (μl. oxygen per 10,000 outer limbs per hr.) × 10^{-3}
5 *a*	2.2	1	3.90
6 *a*	2.01	3 *a*	3.24
6 *b*	2.55	4 *a*	5.61
6 *c*	2.60	4 *b*	4.29
9 *a*	2.23	4 *c*	3.50
9 *b*	2.26	13	3.2
10 *a*	2.88	14	2.2
11 *a*	2.10	15	3.00
Average.....	2.35 ± 0.10	Average.....	3.62 ± 0.36
Q_{O_2}.....	−0.7 μl./mg./hr.	Q_{O_2}.....	−1.0 μl./mg./hr.

that isolated rod outer limbs lack specifically fructose diphosphate. In fact, in another connection (Wald and Hubbard, 1949), we have compared the metabolic capacities of these tissues with those of "washed" muscle. The data, however, show clearly that the respiratory rate in Ringer solution can be enhanced by the addition of this substrate. Further experiments are needed to determine the complete metabolic requirements of isolated rod outer limbs, and the full extent of their respiratory capacities.

The average value for the respiratory rate in fructose diphosphate was 3.6×10^{-3} μl. oxygen per 10,000 outer limbs per hour (see Table II). For comparison with other tissues, this can be converted roughly into the more generally applicable unit of Q_{O_2}. Assuming that the volume of a frog rod outer limb is about 1.4×10^{-6} μl. (Schmidt, 1938, 1951), and its density about 1.0, and

since about 75 per cent of the wet weight is due to water (*viz.* Collins *et al.*, 1952), the dry weight of 10,000 outer limbs would be about 3.5×10^{-3} mg. The Q_{O_2} of isolated frog rod outer limbs is therefore about -1.0 μl. per mg dry weight per hour (the minus sign indicates oxygen uptake) at 20°C. This may be compared with the value -4.5 for whole frog retina at the same temperature (Kubowitz, 1929). The discrepancy between these two values is not surprising, since the retinal Q_{O_2} must include a large respiratory contribution from ganglion cells and other nervous tissue. There may also be a considerable diffusion of metabolites out of the outer limbs, rendering them less intact metabolically than is the isolated retina. It is, therefore, all the more remarkable that the respiratory rate of the outer limbs is entirely comparable with that of many other tissues (*cf.* Krebs and Johnson, 1948).

We have also examined the effect of light and dark adaptation on the respiration of isolated outer limbs. For many years, there have been conflicting reports concerning the effect of bleaching rhodopsin on the respiratory rate of isolated whole retinas. This literature has recently been reviewed by Hwang (1950). We shall therefore only mention in summary that most workers have not observed any effect; but some have. If these purported changes were associated directly with the bleaching of rhodopsin, they should be revealed most clearly by a comparison of the respiratory rates of isolated, dark-adapted outer limbs before and after bleaching. Observations on outer limbs would avoid the possibility, inherent in experiments on whole retinas, that small changes in the respiration of the visual cells are masked by large contributions from the non-photosensitive portions of the retina.

The effect of light on the respiratory rate of dark-adapted outer limbs was therefore studied in Experiments 14 and 15 (Table I). They show that bleaching rhodopsin does not change the rate of respiration. In these experiments, all preliminary operations such as dissecting and scraping the retinas, filling the divers, and inserting them in the diver vessels, were carried out in dim red light. As these preparations were more difficult in red light, measurements could not be begun until about 2 or 3 hours after the frogs were killed. The respiratory rate was first determined in red light, and then a white light, bright enough to bleach the rhodopsin in a few seconds, was turned on inside the thermostat bath containing the diver vessels, and measurements were continued in white light. Bleaching did not cause any change in the respiratory rate, as is shown in Fig. 1, which presents in detail the results of Experiment 15 (Table I).

These experiments show that the rod outer limb, though specialized for photoreception, has retained its general metabolic capacities, and that no special oxygen exchange is connected with the bleaching of rhodopsin.

It has recently been shown that the synthesis of rhodopsin from vitamin A requires DPN to oxidize the alcohol group of vitamin A to the aldehyde group

of retinene (Wald and Hubbard, 1950; Hubbard and Wald, 1951). This implies that a constant supply of DPN as well as vitamin A is needed for rhodopsin synthesis. The Q_{O_2} calculated above for the respiration of the rod outer

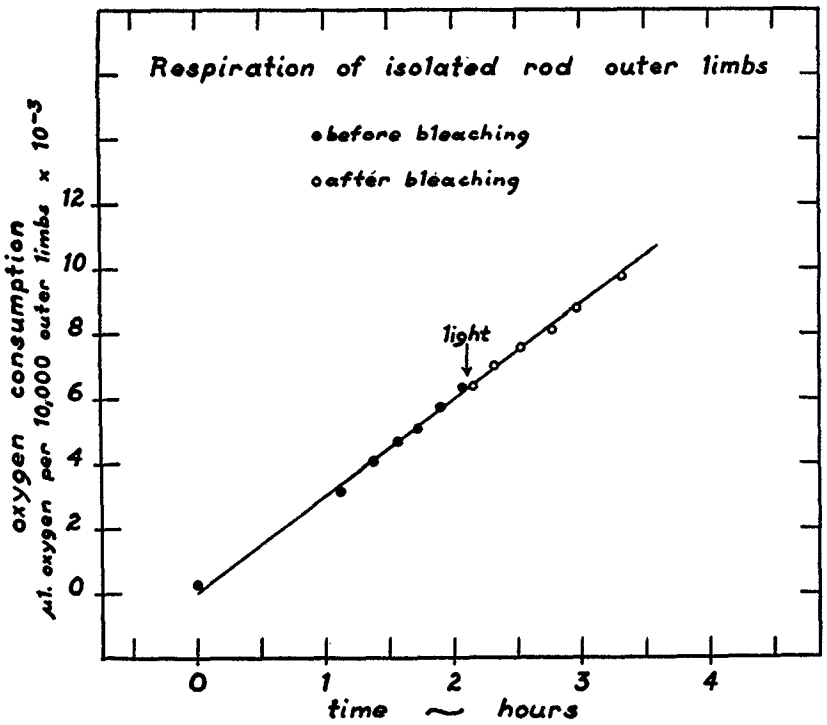

FIG. 1. Respiration of dark-adapted rod outer limbs before and after bleaching rhodopsin. Ordinate, oxygen consumption; abscissa, time from beginning of measurements. Data from Experiment 15 (Table I). The outer limbs were prepared from dark-adapted retinas, and measurements begun in red light. After about 2 hours, the outer limbs were exposed to a bright white light to bleach the rhodopsin. Measurements were then continued in white light. The rate of respiration was unchanged by the illumination and the data obtained before and after bleaching could best be fitted by a single straight line.

limb *in vitro*, makes it seem very likely that the outer limb *in vivo* contains sufficient DPN to maintain the concentration of rhodopsin required for vision.

IV

SUMMARY

The respiration of the isolated frog rod outer limb has been measured in the Cartesian diver. The outer limbs respire in Ringer solution without the addition of substrates, but the rate of respiration is increased by the addition

of fructose diphosphate or succinate. The respiration is cyanide-sensitive, and therefore presumably mediated by the cytochromes. The Q_{O_2} in 0.01 M fructose diphosphate is -1.0 µl. oxygen per mg. dry weight per hour at 20°C. This is lower than the Q_{O_2} of whole frog retina, but comparable with it and many other tissues. The respiratory rate is independent of the state of dark adaptation (rhodopsin content) of the outer limbs. The metabolism of the outer limb is probably adequate to provide the DPN required for the maintenance of the rhodopsin concentration necessary for vision.

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REFERENCES

Arey, L. B., Retina, Chorioid and Sclera, *in* Special Cytology, (E. V. Cowdry, editor), New York, Paul B. Hoeber, Inc., 2nd edition, 1932, **3,** 1211.

Collins, F. D., Love, R. M., and Morton, R. A., Studies in rhodopsin. 5. Chemical analysis of retinal material, *Biochem. J.*, 1952, **51,** 669.

Holter, H., and Linderstrøm-Lang, K., On the Cartesian diver, *Compt.-rend. trav. Lab. Carlsberg, Sér. chim.*, 1943, **24,** 333.

Hubbard, R., and Wald, G., The mechanism of rhodopsin synthesis, *Proc. Nat. Acad. Sc.*, 1951, **37,** 69.

Hwang, T. F., Respiration of retinal tissue, *Japan. J. Physiol.*, 1950, **1,** 169.

Krebs, H. A., and Johnson, W. A., Cell metabolism, *Tabulae Biologicae*, 1948, **19,** pt. 3, 100.

Kubowitz, F., Stoffwechsel der Froschnetzhaut bei verschiedenen Temperaturen und Bemerkung über den Meyerhofquotienten bei verschiedenen Temperaturen, *Biochem. Z.*, 1929, **204,** 475.

Linderstrøm-Lang, K., Principle of the Cartesian diver applied to gasometric technique, *Nature*, 1937, **140,** 108.

Linderstrøm-Lang, K., and Holter, H., Diffusion of gases through protective seals of oil and flotation medium in the Cartesian diver micro respirometer, *Compt.-rend. trav. Lab. Carlsberg, Sér. chim.*, 1942, **24,** 105.

Schmidt, W. J., Polarisationsoptische Analyse eines Eiweiss-Lipoid-Systems, erläutert am Aussenglied der Sehzellen, *Kolloid-Z.*, 1938, **85,** 137.

Schmidt, W. J., Polarisationsoptische Analyse der Verknüpfung von Protein—und Lipoidmolekeln, erläutert am Aussenglied der Sehzellen der Wirbeltiere, *Publ. stazione zool. Napoli*, 1951, **23,** suppl., 158.

Wald, G., and Hubbard, R., The reduction of retinene$_1$ to vitamin A_1 *in vitro*, *J. Gen. Physiol.*, 1949, **32,** 367.

Wald, G., and Hubbard, R., The synthesis of rhodopsin from vitamin A_1, *Proc. Nat. Acad. Sc.*, 1950, **36,** 92.