Biochimica et Biophysica Acta, 729 (1983) 90–96
Elsevier Biomedical Press

BBA 71553

ALKALINE PHOSPHATASE

A DOMINANT ENZYME OF MICROVILLUS STRUCTURE OF CEPHALOPOD PHOTORECEPTORS

OSCAR ROMERO-SARAVIA and KURT HAMDORF

Institut für Tierphysiologie, Ruhr Universität, Universitätsstrasse 150, D-4630 Bochum (F.R.G.)

(Received September 13th, 1982)

Key words: Alkaline phosphatase; Photoreceptor; Cephalopod; (Eledone aldrovandii)

The rhabdomeres of cephalopod photoreceptors, which are built up mainly of rhodopsin and phospholipid molecules, show a very high alkaline phosphatase activity. The enzyme has been partially characterized in purified rhodopsin vesicle fractions of the rhabdomeres by the following kinetic data: pH optimum 8.7; activation energy 9100 cal·m^{-1}; $V_{max} = 2.5$ μmol·min^{-1}·mg^{-1}; $K_m = 1.5 \cdot 10^{-4}$ M; its activity depends on Mg^{2+}. There is good evidence that the alkaline phosphatase is a membrane-bound enzyme with receptor sites presumably located on the inside of the membrane. This enzyme has not been purified but its high activity compared to that of other known alkaline phosphatases (see Table I) indicates that each mirovillus, the structural unit of the rhabdomere, contains 1–20 enzyme molecules. This finding supports the hypothesis that the alkaline phosphatase is involved in the biochemical amplification process of excitation, or adaptation.

Introduction

The details of the visual transduction process are still unknown, although physiological and psychophysiological experiments have shown that vision must depend on a relatively slow biochemical amplification process. This amplification process is very probably much more complicated than in other transduction processes, such as in mechanoreception, since light, causes the longest latency between energy uptake (quantum absorption by the rhodopsin molecule) and the receptor response: up to 100 ms. Thus phototransduction most probably involves enzyme reactions which are coupled to the photoreactions and which occur in the neighbourhood of the rhodopsin molecules associated with the light absorbing structures, i.e. the microvilli of invertebrate receptors and the disc membrane of vertebrate receptors. There is good evidence that in vertebrate photoreceptors the excited rhodopsin molecule controls the level of cGMP [1], but a corresponding system has not yet been clearly demonstrated in invertebrate photoreceptors. Weber [2] has suggested that alkaline phosphatase is involved in phototransduction in *Calliphora*. On the other hand, alkaline phosphatase associated with microvilli are important enzymes involved in cation transport, as in the brush border of the intestine [3]. In contrast to those of vertebrates, the visual pigments of invertebrates are thermostable and photoreversible and these properties can be used when studying the interactions between rhodopsin and the enzyme system. In the octopus *Eledone aldrovandii* used in this work, the rhodopsin P_{473} is maximally converted to metarhodopsin M_{530} (62%) by blue light of 430 nm–440 nm, and maximally reconverted to rhodopsin (100%) by red light (> 580 nm; [4]). Thus, by applying blue converting and red regenerating light alternately, the rhodopsin

0005-2736/83/0000–0000/$03.00 © 1983 Elsevier Science Publishers

content in the photoreceptor can be manipulated precisely.

Furthermore, the rhabdomeres of *Eledone* photoreceptors, which contain 0.3 mM rhodopsin, can be easily separated from other cell organelles and transformed into vesicles of similar diameter to the microvilli by sonification, without any detectable change in their molecular composition, as shown be freeze-fracture electron microscopy and biochemical analysis (Romero and Hamdorf, unpublished observations).

Materials and Methods

Specimens of the Mediterranian species *Eledone aldrovandii* [5] were supplied by the Instituto de Investigaciones Pesqueras, Barcelona. The animals were dark adapted for 12 h before dissection of the eyes. The material was frozen and stored at −30°C. The rhabdomeric membranes were purified by a method described earlier [6,7]. All the purification steps were carried out in dim red light. The rhabdomeres were separated by shaking the retina in a sodium acetate solution (pH 7.35; 5 mM) and then sedimented at 43 500 × *g* and 0°C. The sediment was homogenized at 50 rpm and the homogenate resuspended in a 1.5 M sucrose solution and again centrifuged at 23 300 × *g* with an overlay of an acetate solution. The rhabdomeric membranes obtained by this floatation method were then washed three times with an acetate solution and again sedimented. This purified material was used for preparing vesicles either immediately, or after storage at −30°C. To do this, the sediment was resuspended in an acetate solution and sonicated at 20 kHz 6 times for 30 s at 0°C under a nitrogen atmosphere. The suspension was then centrifuged 1 min at 12 100 × *g* and the supernatant pressure-filtered through Millipore filters of decreasing pore size (5.0, 1.2 and 0.8 μm diameter). By this stepwise filtration it was possible to remove multiple layered vesicles (about 60% of the sample) to give an almost homogeneous fraction of small vesicles between 200 and 2000 Å in diameter. The rhodopsin content of these vesicle fractions was determined from difference spectra obtained after photoequilibration at 430 nm and 570 nm [4,6,8] as well as by counting the rhodopsin particles in electron micrographs of freeze fractured vesicles.

In order to determine whether or not the specific activity of the phosphatase is dependent on the integrity of the membrane the vesicles were solubilized in 2% digitonin. As far as it is known, digitonin is the only detergent capable of stabilizing the rhodopsin-metarhodopsin system of invertebrates in a way similar to the way the lipids of the native membrane stabilize the system. The dephosphorylation activity of freshly prepared and of 2% digitonin-destroyed vesicles was measured by a method similar to that described by Schaffel and Hulett [9], where *p*-nitrophenylphosphate is used as a substrate, after being recrystallized in 87% ethanol. The enzyme activity results in *p*-nitrophenol and orthophosphate. The increase of *p*-nitrophenol was determined photometrically (at 400 nm) for a period of 30 min: an extinction coefficient of $1.88 \cdot 10^4\ l \cdot mol^{-1} \cdot cm^{-1}$ was used. For pre-incubation, 50 μl of the vesicle preparation (ca. 20–35 μg protein) were suspended in 2.97 ml of a Tris buffer (1.0 M Tris at 8.5 plus 0.5–1.0 mM $MgCl_2$) and adapted to 30°C for 5 min. Usually, 30 μl of 100 mM *p*-nitrophenyl phosphate substrate were then added. The consumption of the substrate caused by enzyme activity was measured against a blank containing the buffered substrate.

In order the determine the pH optimum of the dephosphorylizing enzyme, a barbital-sodium acetate buffer or a glycine buffer was used instead of the Tris buffer.

The temperature dependence of the enzyme reaction was determined in the range 5 to 65°C. In these experiments, 1.0 mM was used to saturate the enzyme, K_m and V_{max} were determined by using different substrate concentrations, from $0.1 \cdot 10^{-4}$ to $10 \cdot 10^{-4}$ M *p*-nitrophenyl phosphate, and a protein concentration of 21 μg per assay, which corresponds to a rhodopsin content of 17 μg per assay. The protein content was determined by a modified Lowry method [10]. The rhodopsin content (C_{Po}) was calculated from difference spectra obtained from rhodopsin (P) and its acid metarhodopsin (M) in a photoequilibrium created by monochromatic irradiation (suffixed 'x') at 434 nm [4,6,8].

The rhodopsin content is given by the equation:

$$C_{Po} = \Delta\epsilon \cdot (\alpha_{Px} + \alpha_{Mx}) / (\alpha_{Px} \cdot \alpha_M + \alpha_{Mx} \cdot \alpha_P)$$

where α_P and α_M are the molar extinction coefficients at the wavelength of the measuring beam, and α_{Px} and α_{Mx} are the extinction coefficients at the wavelength of irradiation. If a measuring wavelength is chosen where α_P is much smaller than α_M, as is the case at 570 nm, the equation simplifies to:

$$C_{Po}(\alpha_P \ll \alpha_M) = \Delta\epsilon \cdot (\alpha_{Px} + \alpha_{Mx})/(\alpha_{Px} \cdot \alpha_M)$$

Inserting the values for α_P and α_M at 570 nm and for α_{Px} and α_{Mx} at 434 nm, and given that α_{P470} is 40000 and α_{M530} is 60000, the C_{Po} concentration is given by $\Delta\epsilon \cdot 8.1 \cdot 10^{-5}$ M.

In order to find out whether or not the phosphatase is membrane-bound, the following experiments were carried out. (1) The enzyme activity of sonicated rhabdomeres before and after filtration was compared. (2) The activity of sedimented vesicles was compared with that of the supernatant (for the sedimentation of the vesicles the freeze-thawing technique was used).

Results

The dephosphorylating enzyme associated with the rhodopsin vesicles has the following characteristics. The pH optimum of the phosphatase is 8.7 at 30°C, as shown in Fig. 1. Under the same experimental conditions, *p*-nitrophenyl phosphate

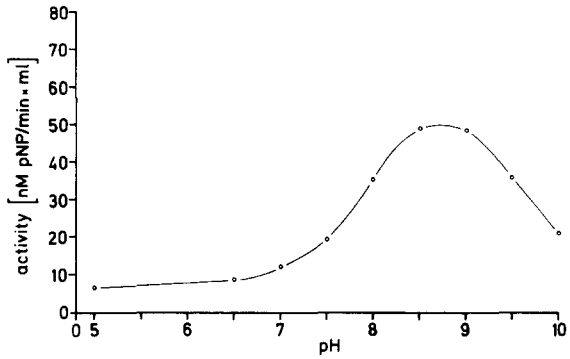

Fig. 1. The dependence of enzyme activity of rhodopsin vesicles upon pH at 30°C. Maximal activity is observed at pH 8.7. pNP, *p*-nitrophenol.

is 3-times more rapidly hydrolysed than arginine phosphate and 5.7-times more rapidly hydrolysed than ATP. These results show that the enzyme belongs to a group of well defined alkaline phosphatases (see Talbe I). The temperature optimum of the alkaline phosphatase is in the range (43°C; see Fig. 2b) as described for comparable enzymes of homeothermic animals (approx. 40°C). It is inactivated at temperatures above 60°C. The Arrhenius plot shown in Fig. 2b shows a linear relationship over the range $31.5 \cdot 10^{-4}$ $34.5 \cdot 10^{-4}$ K^{-1}, corresponding to the temperature range 44–17°C. The slope indicates an apparent activation energy of 9100 cal $\cdot$ mol^{-1}. Below 17°C the

TABLE I

ACTIVITY OF ALKALINE PHOSPHATASES FROM VARIOUS TISSUES

Source of material	M_r	Specific activity (μmol $\cdot$ min^{-1} $\cdot$ mg^{-1})		Turnover (mol/min)	Ref.
		starting material	purified enzyme		
Rhodopsin vesicles	–	2.5	–	125	–
1. Bovine kidney	170000	0.44	18550	314500	14
1a. Bovine kidney	–	0.20	6200	1054000	15
2. Bovine mammary tissue	150000	0.02	346	51900	16
3. Cardiac muscle	35000	0.021	73	2555	17
4. Egg yolk	150000	0.0005	21	3150	18
5. Liver	150000	0.024	25	3750	18
6. Blood plasm	150000	0.0043	34	5100	18
7. Intestine	120000	–	30	3600	18
8. Intestine	60000	–	–	236000?	19

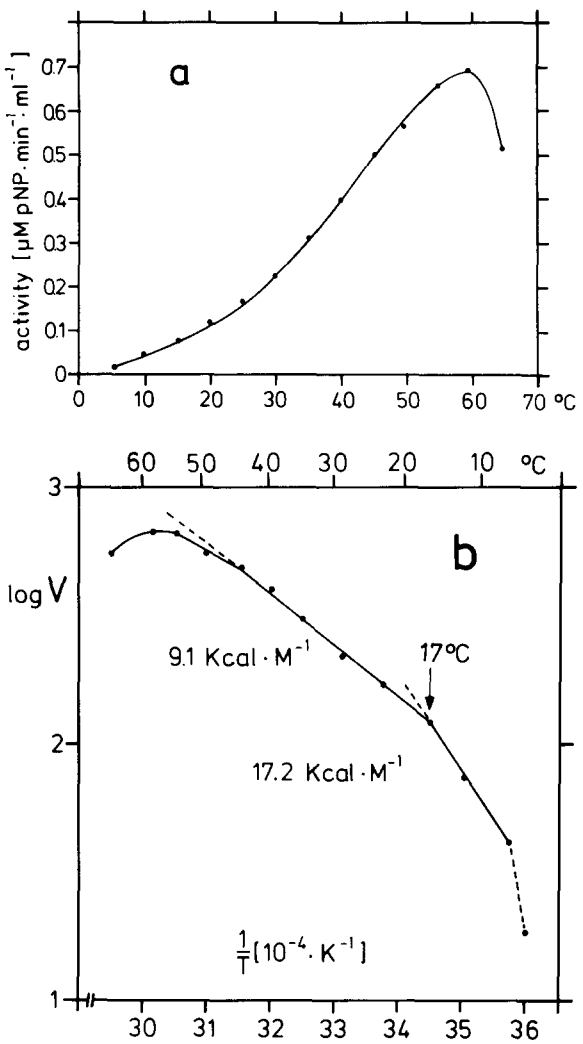

Fig. 2. The dependence of enzyme activity of rhodopsin vesicles on temperature. Maximal rate of alkaline phosphatase activity is reached at about 43°C. The Fig. 2b shows the Arrhenius plot of this relationship. In the physiological temperature range 15–40°C the slope indicates an apparent activation energy of 9100 cal·mol^{-1}. Below 17°C the calculated activation energy is 17,200 cal·mol^{-1}. Incubation medium: 1.0 M Tris at pH 8.5, 1.0 mM $MgCl_2$, 55 µg protein, 1.0 mM *p*-nitrophenyl phosphate. pNP, *p*-nitrophenol.

calculated energy is 17200 cal·mol^{-1}, which is similar to the value given for other integral membrane enzymes [11]. The temperature dependence of the enzyme activity is influenced strongly by the substrate concentration, i.e. at 10 mM *p*-nitrophenyl phosphate, which inhibits the enzyme activity by about 50% (see below), a lower activation energy of 7300 cal·mol^{-1} is calculated. The enzyme activity becomes saturated at a substrate concentration of $5 \cdot 10^{-4}$ M *p*-nitrophenyl phosphate, as shown in Fig. 3, and is inhibited at higher concentrations. The transformation of this relationship into a Lineweaver-Burk plot shows linearity over the range $2 \cdot 10^3$ to $10 \cdot 10^3$ M^{-1}, corresponding to the substrate concentration range $1 \cdot 10^{-4}$ M to $5 \cdot 10^{-4}$ M. (Data relating to very low substrate concentrations were excluded since the analysis of the enzyme kinetics based on a Gaussian distribution is no longer valid.) Extrapolation of the linear relationship gives crossover points at the *Y* and *X* axes at 0.4 and 6.4, respectively. These data indicate that $V_{max} = 2.5$ µmol·min^{-1}·mg^{-1} and $K_m = 1.5 \cdot 10^{-4}$ M.

The activity of the phosphatase depends on the presence of divalent cations, as shown in Fig. 4. Chelation of Ca^{2+} and Mg^{2+} by EGTA or EDTA abolishes enzyme activity, whereas increasing the Mg^{2+} concentration up to 0.2 mM markedly

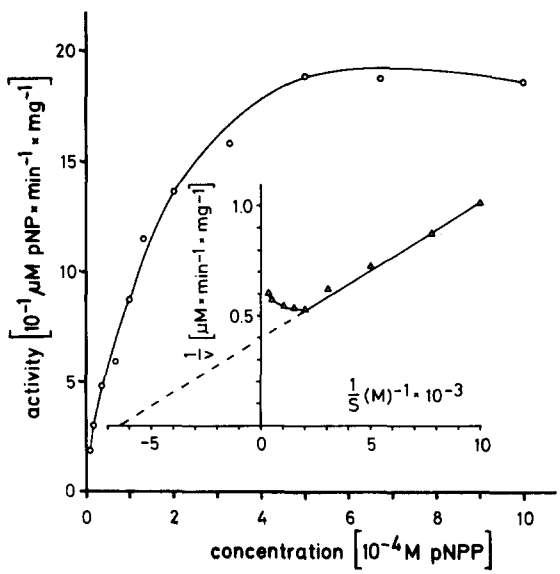

Fig. 3. The dependence of enzyme activity on substrate concentration (*p*-nitrophenol phosphate (pNPP)) at 30°C determined after 30 min incubation. Maximal rate of alkaline phosphatase activity is reached at 0.5 mM pNPP. The insert shows the Lineweaver-Burk plot giving an estimated V_{max} of 2.5 µM·min^{-1}·mg^{-1} and K_m of $1.5 \cdot 10^{-4}$ M. Incubation medium: 1.0 M Tris, pH 8.5, 0.5 mM $MgCl_2$, 21 µg protein (corresponding to 17.1 µg rhodopsin). pNP, *p*-nitrophenol.

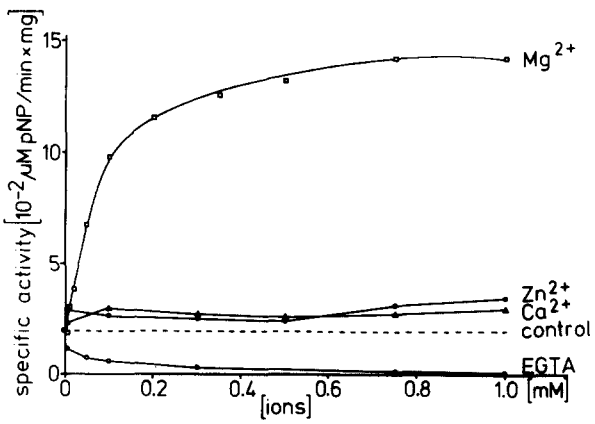

Fig. 4. The effect of divalent ions on alkaline phosphatase activity. Increasing Mg^{2+} concentration increases the enzyme activity about 7 times, whereas increasing the Ca^{2+} or Zn^{2+} concentration did not significantly influence the enzyme activity. Chelation of divalent cations by EGTA or EDTA (latter not shown) abolishes the enzyme activity. Incubation medium: 1.0 M Tris, pH 8.5, 34.8 µg protein. pNP, *p*-nitrophenol.

accelerates the reaction. On the other hand, higher concentrations of Ca^{2+} or Zn^{2+} do not seem to appreciably influence the velocity of the reaction. Other di- and trivalent cations were tested and these led to a reduction of enzyme activity of up to 45% ($Sr^{2+} > Fe^{3+} > Cd^{2+} > Cu^{+} > Ba^{2+} > Ni^{2+} > Co^{2+} > Al^{3+} > Cu^{2+} > Hg^{2+}$). The value for Hg^{2+} equaled that of the control.

The activity of the phosphatase markedly increased upon the addition of vitamin D-3 (1α,25-dihydroxycholecalciferol, $1.5 \cdot 10^{-4}$ M) by a factor 1.4.

The enzyme seems to be bound to the photoreceptor membrane, as indicated by the following experiments. (1) The enzyme activity of crude sonicated rhabdomeres (36 nmol · min^{-1} · mg^{-1} protein) and of filtered vesicles (37.3 nmol · min^{-1} · mg^{-1} protein) is almost identical. (2) After sedimentation of filtered vesicles, all the activity was found in the sediment which contained 93% of the total protein and none in the supernatant, which contained 7% protein. The specific activity of the sediment (51.1 nmol · min^{-1} · mg^{-1} protein) was about 40% higher than that of the starting material (37.3 nmol · min^{-1} · mg^{-1} protein). This increase can be only partially a result of which one expects by the fact that 7% of the protein (inactive) was washed out. Then the specific activity should increase only up to 40.1 nmol · min^{-1} · mg^{-1} protein. The method of freezing and subsequently thawing the vesicles increases the enzyme activity markedly, by a factor of 1.27 (51.1/40.1). The same increase in enzyme is observed after treatment with digitonin (45.2/35.7). The enzyme activity of vesicle suspensions was not influenced by monochromatic irradiation with a wavelength of light which maximally shifts rhodopsin to acid metarhodopsin or which produce a maximal photoregenerating effect. Thus, we conclude that the observed activity is not directly correlated to the number of P → M or M → P transitions.

Discussion

The substrate *p*-nitrophenyl phosphate is known to be highly specific for alkaline phosphatase [12,18]. The degradation of this substrate by an enzyme associated with rhodopsin vesicles prepared from purified rhabdomeres of cephalopod photoreceptors is several times faster (5.7- and 3.0-times, respectively) than the degradation of ATP or arginine phosphate. Furthermore, the pH optimum (8.7) and apparent K_m ($1.5 \cdot 10^{-4}$ M) of this enzyme are consistent with those of alkaline phosphatase from *Bacillus licheniformis* [9]. Similarly, the value for the apparent activation energy (9,100 cal M^{-1}) is close to 6,880 cal M^{-1}, as reported by Garen and Levinthal [13] for *Escherichia coli*. Lastly, the enzyme activity depends on the presence of magnesium ions. These features lead us to conclude that the observed *p*-nitrophenyl phosphate degradation was caused by an alkaline phosphatase associated with the microvilli, and as the total enzyme activity of the crude rhabdomeres was recovered in the sedimented 'filtered vesicles', the phosphatase must be an protein integral to or very strongly associated of the microvillus membrane.

This view is further supported by the fact that the observed break in the Arrhenius plot (see Fig. 2b) at 17°C coincides with the phase transition temperature of the microvillus membrane. The question, of whether the receptor sites of the enzyme are located inside or outside of the membrane, cannot be decided on the basis of the present experiments as the vesicle membrane is leaky to the substrate employed. However, the

slight increase of 1.27 in enzyme activity after vesicle sedimentation or digitonin treatment favours the view that the receptor sites are located on the inside of the vesicle membrane.

The observed alkaline phosphatase activity of the rhodopsin vesicles is about 10–100-times higher than that in other tissues with a high phosphatase activity which is primarily associated with a microvillar border (see Table I). This indicates that the alkaline phosphatase is a specific enzyme of the rhabdomer which, as with the phosphatase from the intestine [3], is similarly activated by vitamin D-3 like the phosphatase from the intestine. In view of this, it might be that the phosphatase activity resides in the rhodopsin molekule itself. Biochemical and spectrophotometric data show that about 80% of the total protein in the vesicle fraction is rhodopsin. Thus, the specific activity of the rhodopsin molecule ($M_r = 40000$) would be expected to be in the order of 125. This value is much smaller than the values for highly purified enzymes (see Table I) and therefore it seems unlikely that rhodopsin itself is the enzyme in question.

However, it is probable that, in vivo, rhodopsin serves as a substrate for the highly active alkaline phosphatase since rhodopsin, in the presence of Mg^{2+}, is phosphorylated after absorption of a quantum [7], as occurs also in vertebrate photoreceptors [21–25]. It should, however, be noted that a subsequent dephosphorylation of cephalopod rhodopsin has not yet been shown. In addition, because rhodopsin is membrane bound and has little freedom to diffuse laterally, each microvillus unit must contain sufficient (one or more) enzyme molecules to dephosphorylate the rhodopsin of that microvillus. This appears to be the case since, assuming the highest turnover rate of 10^6 mol/min (as for kidney alkaline phosphatase; Table I, enzyme 1a), the expected enzyme/rhodopsin ratio in the vesicles is 1/9000 whereas, assuming a lower activity (3000–5000 mol/min; Table I, enzymes 3–8), a ratio of 1/30 to 1/40 is obtained. Since each microvillus is about 2 μm long and contains about 1000 rhodopsin molecules, one expects a ratio of 0.1–24 alkaline phosphatase molecules per microvillus. Each microvillus could therefore act as a physiological unit with respect to phosphorylation, as it does with respect to excitation (as evidenced by electrophysiological data; [4,20] Razmjoo and Hamdorf in preparation). However, it may be that the alkaline phosphatase activity associated with the rhabdomeres of *Eledone* has nothing to do with the phosphorylation of rhodopsin but of some other substance(s). With regard to this possibility, it is noteworthy that the predominant enzyme of the proximal tubule of the kidney is a highly active alkaline phosphatase and this region of the tubule is responsible for sodium reabsorption. The high level of alkaline phosphatase activity associated with the rhabdomeres of *Eledone* suggest that this organelle may also be involved with an active cation exchange.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 114). We thank Ms. P. Jergolla for technical assistance, Dr. P. Arté Gratacós, Instituto de Investigaciones Pesqueras, Barcelona, for providing us with *Eledone* eyes, and Dr. A. Whittle for critical reading of the manuscript.

References

1 Kühn, H., Bennett, N., Michel-Villaz, M. and Chabre, M. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6873–6877
2 Weber, K.M. and Schorrath, G. (1971) Histochemie 28, 137–144
3 Norman, A.W. (1979) Vitamin D: The Calcium Homeostatic Steroid Hormone, pp. 341–343, Academic Press, New York, San Francisco, London
4 Hamdorf, K. (1979) in Handbook of Sensory Physiology (Autrum, H., ed.), Vol. VII/6A, pp. 146–224, Springer-Verlag, Berlin, Heidelberg, New York
5 Robinson, G.C. (1932) A Monograph of Recent Cephalopoda (British Museum, Natural History), Part II, pp. 258–270
6 Hamdorf, K., Schwemer, J. and Täuber, U. (1968) Z. Vergl. Physiol. 60, 375–415
7 Paulsen, R. and Hoppe, I. (1978) FEBS Lett. 96, 55–58
8 Schwemer, J. (1969) Z. Vergl. Physiol. 62, 121–152
9 Schaffel, S.D. and Hulett, F.M. (1978) Biochim. Biophys. Acta 526, 457–467
10 Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210
11 Kumamoto, J., Raison, J.K. and Lyons, J.M. (1971) J. Theor. Biol. 31, 47–51
12 Herz, F. and Nitowsky, H.M. (1962) Arch. Biochem. Biophys. 96, 506–515
13 Garen, A. and Levinthal, C. (1960) Biochim. Biophys. Acta 38, 470–483

14 Cathala, G. and Brunel, C. (1975) J. Biol. Chem. 250, 6040–6053
15 Mössner, E., Boll, M. and Pfleiderer, G. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 543–549
16 O'Keefe, R.B. and Kinsella, J.E. (1979) Int. J. Biochem. 10, 125–134
17 Li, H.-C., Hsiao, K.-J. and Sampathkumar, S. (1979) J. Biol. Chem. 254, 3368–3374
18 Debruyne, I. and Stockx, J. (1979) Int. J. Biochem. 10, 981–988
19 Morton, R.K. (1957) Biochem. J. 65, 674–682
20 Hamdorf, K. and Kirschfeld, K. (1980) Z. Naturforsch. 35c, 173–174
21 Kuhn, H. and Dreyer, W.J. (1972) FEBS Lett. 20, 1–6
22 Bownds, D., Dewes, J., Miller, J. and Stahlman, M. (1972) Nature New Biol. 237, 125–127
23 Frank, R.N., Cavenagh, H.D. and Kenyon, K.R. (1973) J. Biol. Chem. 248, 596–609
24 Miller, J.A. and Paulsen, R. (1975) J. Biol. Chem. 250, 4427–4432
25 Paulsen, R., Miller, J.A. Brodie, A.E. and Bownds, M.D. (1975) Vis. Res. 15, 1325–1332