# FURTHER DATA ON THE SPECIFICITY OF AEQUORIN LUMINESCENCE TO CALCIUM

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Received June 5,1973

SUMMARY. The possible triggering of luminescence of the photoprotein aequorin by 11 kinds of metal ions, in concentrations from $10^{-3}$ to $10^{-6}$ M, at pH 6.0 and at pH 8.0, were examined under conditions which minimized contamination with extraneous $Ca^{2+}$. $Y^{3+}$ and $La^{3+}$ were found to have activities nearly as great as that of $Ca^{2+}$ at pH 6.0, but gave evidence of quenching effects in the higher concentrations at pH 8.0. Cations indicating no activity, or only negligible activity at either pH 6.0 and 8.0 included $Be^{2+}$, $Ba^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Cu^{2+}$. Considerable activity was shown by $Pb^{2+}$ and $Cd^{2+}$ at pH 6.0, but very little at pH 8.0.

## INTRODUCTION

A light-emitting (bioluminescence) reaction of the photoprotein aequorin in aqueous solution at physiological pH's and temperatures is triggered by traces of $Ca^{2+}$, or to a less extent $Sr^{2+}$. Since we first described the extraction and purification of this protein from the luminous jellyfish Aequorea some ten years ago (1,2) it has seemed that the reaction is specific for these cations; no activity was found for 0.01 M solutions of $NH_4^+$, $K^+$, $Mg^{2+}$, $Ba^{2+}$, $Pb^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Cu^{2+}$, or $Zn^{2+}$. Because of the extreme sensitivity of the reaction to $Ca^{2+}$, together with the specificity indicated above, as well as other favorable properties, the use of aequorin for the microdetermination of $Ca^{2+}$ in biological reactions and physiological processes has become increasingly widespread (3-5).

According to a recent report by Izutsu et al. (6), numerous metal ions in addition to $Ca^{2+}$ and $Sr^{2+}$ can elicit the luminescence reaction of aequorin. Some of their results are in dire contrast to our experience with this system during the past decade. We have therefore re-investigated the problem with

[1]Aided by NSF Grant GB 30963X and ONR Contract N00014-67A-0151-0025.

reference to the questionable cations, and with due reference also to the influence of such factors as pH, quenching at relatively high concentrations of ions, and leeching or dissolving of $Ca^{2+}$ from walls of glass containers (cf. 7). In regard to the last mentioned factor, it is very easy to show that when deionized distilled water is added to a scrupulously cleaned Pyrex tube (especially when the tube is dry at the start) this water acquires a rapidly rising, luminescence-triggering activity for aequorin; in fact, this system might be used to assay the rate of leeching of $Ca^{2+}$ from glass or other materials. The ubiquitous sources of contamination with $Ca^{2+}$ together with the sensitivity of aequorin to the cation, make it necessary to add some suitable chelator to stabilize the aequorin in order to obtain quantitatively reproducible results in testing for the activity of different ions. Thus, the present reaction mixture contained a final concentration of $10^{-7}$ M EDTA; in our original study (1), the final concentration of EDTA was $10^{-4}$ M, but this was in conjunction with $10^{-2}$ M final concentration of the ion being tested. Present results also demonstrate the importance of controlling the pH of the reaction mixtures, a factor which apparently was not seriously taken into account by Izutsu *et al.* (6) as judged by their use of unbuffered solutions, in some instances at extremes of pH, such as pH 3 and pH 11, at which the activity of the photoprotein aequorin is quickly and greatly reduced at room temperature (1). We have omitted from the present study those cations which have been repeatedly and consistently found inactive in eliciting a light-emitting reaction of aequorin, *viz*, $K^+$, $Mg^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, and $Zn^{2+}$ (1,6).

## MATERIALS AND METHODS

Purified aequorin, showing a single band in polyacrylamide disc-electrophoresis, was prepared as previously reported (8,9) and was desalted by a small column of Sephadex G-25 (fine) equilibrated with 0.1 mM EDTA, pH 7.5, and kept frozen until ready for use. Chemicals of "Ultrapure" grade, generally containing less than 1 ppm of $Ca^{2+}$ ($CH_3COONa$, $Pb(CH_3COO)_2$, CoO, CuO, $Cd(HCOO)_2$ and $LaCl_3$) and 99.999% $Y_2O_3$ were obtained from Alpha Inorganics.

Glycylglycine was obtained from Sigma Chemical Co. $BeCl_2$, $SrCl_2$, $BaCl_2$, $NiCl_2$ and all other chemicals were reagent grade, with care taken to choose those containing a minimum amount of Ca. Hydrochloric acid was redistilled in a quartz still before use. Deionized distilled water having a resistance of more than 10 megaohms was used throughout. Because of the virtual impossibility of a prior leeching out of all $Ca^{2+}$ from glass (7), all solutions were prepared and kept in polypropylene (Nalgen) containers from which they were dispensed with plastic pipettes and they were not allowed contact with glass throughout the experiments, except for dissolving the metal oxides with HCl in Vycor test tubes. The metal oxides and carbonate were first dissolved in a small amount of diluted HCl, then neutralized with $NaHCO_3$, and finally diluted with buffer; other metal salts were dissolved directly in buffer.

The luminescence reaction was initiated by rapid addition of 4 ml of the metal salt solution to 5 µl of aequorin solution which had just been delivered into a polycarbonate tube from an Eppendorf pipette. Light was measured by a photomultiplier-amplifier-recorder assembly with a pen response time of approximately 20 msec for the full scale.

## RESULTS AND DISCUSSION

Table 1 summarizes the activity of 11 metal ions towards eliciting a bioluminescence reaction of aequorin at two different pH's, i.e., 6.0 and 8.0. These differences in pH obviously are important factors in the results observed. Qualitative confirmation of Izutsu *et al.*'s results with $Y^{3+}$ and with $La^{3+}$ (6) is indicated in Table 1 showing that these rare earth cations are almost as active as $Ca^{2+}$, especially at pH 6.0. Thus, the previously made suggestion for the use of lanthanides as replacements for $Ca^{2+}$ to study the binding sites of $Ca^{2+}$ in biological systems in general (10,11,12) appears applicable to the aequorin system.

Izutsu *et al.* (6) reported that, at a concentration of $10^{-5}$ M, the activities of $Sr^{2+}$, $Pb^{2+}$, $Co^{2+}$, and $Cu^{2+}$ were almost equivalent to that of $Ca^{2+}$, and that the activities of $Ba^{2+}$, $Ni^{2+}$, and $Cd^{2+}$ were somewhat less.

Table 1. Maximum Intensities of the Luminescence Response of Aequorin to Various Metal Ions.[a]

| Metal ion Added | In 0.01 M sodium acetate buffer, pH 6.0 | | | | In 0.01 M glycylglycine-NaOH buffer, pH 8.0 | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^{-3}$ M | $10^{-4}$ M | $10^{-5}$ M | $10^{-6}$ M | $10^{-3}$ M | $10^{-4}$ M | $10^{-5}$ M | $10^{-6}$ M |
| None | * | * | * | * | * | * | * | * |
| $Be^{2+}$ | * | * | * | * | * | * | * | * |
| $Ca^{2+}$ | 35 | 29 | 17 | 2.1 | 36 | 27 | 19 | 5.6 |
| $Sr^{2+}$ | 9 | 3 | 0.3 | * | 16 | 7.2 | 0.9 | 0.2 |
| $Ba^{2+}$ | * | * | * | * | * | * | * | * |
| $Pb^{2+}$ | 9 ($\phi$=23%) | 11 | 10 | 6 | b | 0.2[c] ($\phi$=20%) | 0.3[c] ($\phi$=32%) | 0.4 |
| $Co^{2+}$ | * | * | * | * | 0.5 | 0.7 | 0.3 | * |
| $Ni^{2+}$ | * | * | * | * | * | * | * | * |
| $Cu^{2+}$ | * | * | * | * | 0.7 ($\phi$=6.5%) | 0.4 | * | * |
| $Cd^{2+}$ | 0.9[c] ($\phi$=35%) | 10 | 6.6 | 2.5 | [c] ($\phi$=4.7%) | [c] ($\phi$=4.7%) | * | * |
| $Y^{3+}$ | 31 | 27 | 22 | 5 | 11 ($\phi$=27%) | 6.3 ($\phi$=52%) | 4.4 | 2.4 |
| $La^{3+}$ | 22 | 19 | 17 | 2.4 | 16 ($\phi$=32%) | 14 ($\phi$=64%) | 7.2 | 2.4 |

[a] The data are expressed in terms of unit intensity = $10^{12}$ quanta/sec. An aliquot of 8 $\mu$g of aequorin was reacted in 4 ml of solution at 24 - 25° C. The final concentration of EDTA was $1.25 \times 10^{-7}$ M. An asterisk (*) indicates that the response was less than $0.1 \times 10^{12}$ quanta/sec. The total quanta emitted in each run was $3.2 - 3.5 \times 10^{13}$, except where the quantum yield, $\phi$, was found to differ so as to result in the amount indicated in parenthesis, taking $3.4 \times 10^{13}$ quanta as $\phi$ = 100%.

[b] Data were not obtained due to precipitation.

[c] A momentary flash, amounting to $1.2 - 2.5 \times 10^{12}$ quanta in 0.1 - 0.2 sec, was observed at the beginning of the reaction.

The present study has shown that, at the corresponding concentration of $10^{-5}$ M and at pH 6.0, $Ba^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Cu^{2+}$, are almost completely inactive, and that $Sr^{2+}$ is very slightly active. Only the activities of $Pb^{2+}$ and $Cd^{2+}$ at pH 6.0 were qualitatively consistent with the data of Izutsu et al., and even these were only slightly active at pH 8.0. At pH 8.0, $Co^{2+}$ and $Cu^{2+}$ were very slightly active at relatively high concentrations. The previously unreported effects of $Be^{2+}$ were found to be negative.

Although the present paper demonstrates a different interpretation of many of the observations described by Izutsu et al. (6), their experiments have nevertheless indirectly aided the reliability of the aequorin test for $Ca^{2+}$ by showing the ease with which misleading results may be obtained under certain conditions. Present data make it convincingly clear that the luminescence reaction of aequorin is highly specific for $Ca^{2+}$ at pH 8 in the absence of rare earth metal ions and in the absence of a relatively high concentration of $Sr^{2+}$. Thus, in using the aequorin test for $Ca^{2+}$, it is desirable to regulate the pH to between 7.5 and 8.0 or somewhat higher, in order to achieve a high degree of specificity to this cation, as well as to avoid the spontaneous aggregation of aequorin which occurs at lower pH (13).

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