immunofluorescence using anti-pig zona rabbit serum, normal rabbit serum and human serum samples. The same antibody titer (1:512) could be detected with the standard antiserum by these 2 methods and no reaction was found with normal rabbit serum (table 1). Of 103 sera from men and women tested using latex reagent, 32 (31%) gave positive reactions regardless of the sources of sera, showing a maximum titer of 1:4 (table 2). All the positive sera in latex agglutination showed a positive reaction on the outer surface of the porcine zonae as determined by indirect immunofluorescence, whereas negative sera did not. No serum sample gave a contradictory reaction with these 2 methods. The reactivity of the latex reagent did not change for more than a month when stored at 4 °C.

These results indicated that the slide latex test investigated in this study is comparable in sensitivity and specificity to the method using indirect immunofluorescence. Moreover, considering the simplicity of the procedure, as well as the short time of the reaction, this new method should be valuable as a simple screening method for the detection of anti-zona pellucida activity in human sera instead of indirect immunofluorescence.

It has been reported that a common antigen is shared by human and porcine zonae[11], but differences in antigenic composition of human and pig zona have also been suggested[12]. Considering these reported differences, as well as the possible presence of hetero-agglutinin in human sera, the detection of anti-pig zona activity in this study does not necessarily indicate the presence of anti-zona antibody. Further characterization of anti-zona activity in human sera is now being done in our laboratory using the slide latex test.

1 Ownby, C.L., and Shivers, C.A., Biol. Reprod. *6* (1972) 310.
2 Sacco, A.G., and Shivers, C.A., J. Reprod. Fert. *32* (1973) 415.
3 Yanagimachi, R., Winkelhake, J., and Nicolson, G.L., Proc. natl Acad. Sci. USA *73* (1976) 2405.
4 Shivers, C.A., in: Immunological approaches to fertility control, p.223. Ed. E.D. Deczfalusy. Karolinska Institute, Stockholm 1974.
5 Shivers, C.A., and Dunbar, B.S., Science *197* (1977) 1082.
6 Mori, T., Nishimoto, T., Kohda, H., Takai, I., Nishimura, T., and Oikawa, T., Fert. Steril. *32* (1979) 67.
7 Nayudu, P.L., Freemann, L.E., and Trounson, A.O., J. Reprod. Fert. *65* (1982) 77.
8 Dietl, J., Knop, G., and Mettler, L., J. Reprod. Immun. *4* (1982) 123.
9 Noda, Y., Mori, T., Takai, I., Kohda, H., and Nishimura, T., J. Reprod. Immun. *3* (1981) 147.
10 Noda, Y., Kohda, H., Takai, I., Hayashi, S., Shimada, H., Mori, T., and Tojo, S., J. Reprod. Immun. *5* (1983) 161.
11 Sacco, A.G., Biol. Reprod. *16* (1977) 164.
12 Takai, I., Mori, T., Noda, Y., and Nishimura, T., J. Reprod. Fert. *61* (1981) 19.

0014-4754/83/080926-03$1.50 + 0.20/0


## Bioluminescence assay of calcium with a liquid scintillation counter

C. Oveby, R. Kant and S. Batra[1]

*Leo Research Laboratories, AB Leo, Box 941, S-251 09 Helsingborg (Sweden), August 23, 1982*

*Summary.* The luminescence produced by Ca aequorin reaction in dilute solutions decays exponentially over a relatively prolonged period of time. The concentration of total Ca and of free Ca in Ca-EGTA buffers could be determined by measuring the decay of luminescence in a liquid scintillation counter. The method is also suitable for determining total Ca concentration in small tissue samples.

A number of reports in the past have drawn attention to the possibilities offered by use of scintillation counters for the measurement of bioluminescence produced by firefly extract in the presence of ATP[2-4]. With the commercial availability of aequorin, a calcium-sensitive luminescent protein, we decided to explore the possibility of measuring calcium concentration using a liquid scintillation counter. Conditions are described for the measurement of total calcium and, under certain conditions, of free calcium in calcium-EGTA system using aequorin. The method is also suitable for determining total calcium concentration in small tissue samples.

*Materials and methods.* Calcium aequorin reaction was carried out in a buffer solution containing 2 mM each NaCl, KCl, $MgSO_4$ and 10 mM of Tris, pH 7.0. This solution which was made from ultra pure chemicals was passed through a Chelex column to remove contaminating calcium. Preliminary experiments showed that without Chelex treatment the blank values were about 10 times higher than those obtained with Chelex-treated solutions[5]. 10 ml of ice-cold buffer solution were pipetted into polyethylene bottles (Packard) kept on ice. 50 or 100 µl of aequorin solution (1 mg aequorin, Type 3, dissolved in 1 ml $H_2O$) were added and the contents shaken. 100 µl of $CaCl_2$ standards (0–40 nmoles) were then added to different bottles and vortexed for 10 sec. After 15 or 30 sec of waiting, samples were counted repeatedly for 10 0.1-min intervals in a Packard Tri-Carb liquid scintillation counter in a tritium channel. Total tissue calcium concentration in the uterus and aorta was determined after removing the adhering fat and connective tissues. Tissue pieces weighing about 100 mg were taken, weighed and thereafter dried for 16–24 h at 100 °C. After determining the dry weight, tissues were digested by heating at 200 °C with 5 drops of concentrated $HClO_4$ and $HNO_3$ (1:1). The tissues were completely ashed after 24 h. The ash was dissolved in 2 ml of water and 100 µl of the sample as that of the standard was used for Ca assay.

Total calcium in rabbit uterus and aorta

| Tissue | Total Ca (mmoles/kg) Wet weight | Dry weight |
|---|---|---|
| Uterus | 1.35 ± 0.14 | 10.16 ± 1.17 |
| Aorta | 3.32 ± 0.36 | 12.87 ± 1.66 |

Values are means ± SE of 4 determinations.

*Results.* The concentrations of Ca and all other agents shown in the figures represent final values in the reaction mixture. Figure 1 shows some typical decay-curves of the Ca-aequorin reaction with different Ca concentrations. Above a certain Ca concentration the decay curve shows a rapid fall which probably represents the declining phase following a maximum[5,6]. This behavior is due apparently to an excess Ca over aequorin as can be seen from decay curves shown in figure 2. Here, after the 1st set of decay curves, the measurements were repeated following a 2nd addition of aequorin after 15 min. At low Ca concentrations, additional aequorin had no effect whereas at high Ca concentration (3.94 µM), Ca was still available for reaction with aequorin. With appropriately diluted Ca standards, decay curves were parallel over the period used for counting (fig. 3,A). A standard curve using the values measured in the last period (fig. 3,A) was constructed as shown in figure 3,B. The standard curve was linear at Ca concentrations between 0.5 and 2 µM. Almost identical curves were obtained on different occasions as long as the same batch of aequorin was used. Using this kind of standard curve (fig. 3,B) Ca concentration in the rabbit uterus and aorta were measured and the values, expressed on the basis of both wet and dry weight, are shown in the table. The values are in agreement with those reported by others[7,8].

Decay curves obtained with 1 µM Ca concentration in the presence and absence of ATP and EGTA are shown in figure 4. Whereas ATP reduced Ca response by about 20%, EGTA reduced it considerably. With an EGTA concentration which was slightly higher than that of Ca, the aequorin response as expected was practically abolished, the affinity constant of Ca-EGTA being at least an order of magnitude higher than that of Ca-aequorin[6]. The free Ca ion concentration in 5 different Ca-EGTA buffer solutions was measured and the data used for the determination of the apparent association constant of Ca-EGTA (log

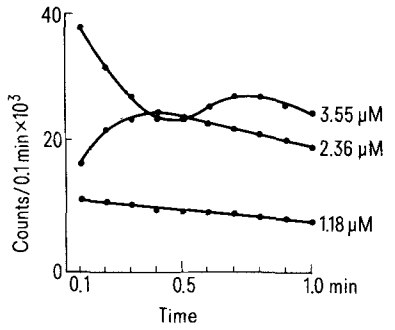


Figure 1. Luminescence curves with different concentrations of Ca. First measurement after 15 sec.

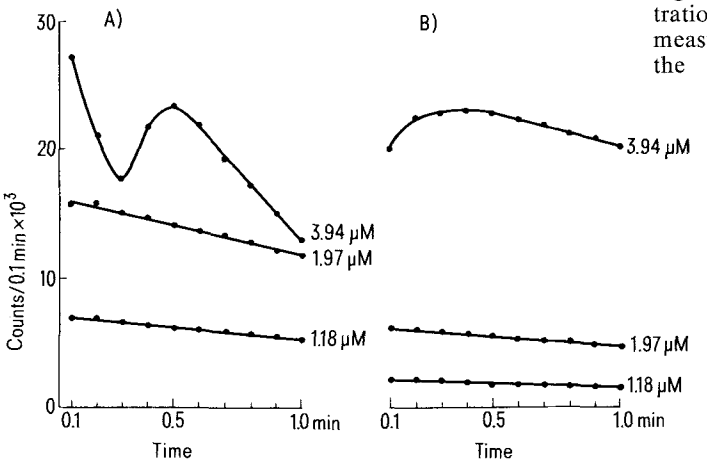


Figure 2. Luminescence decay curves with different Ca concentrations. *A* First period after 15 sec and *B* after 15 min with a further addition of 50 µl aequorin.

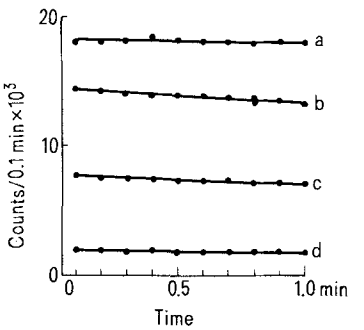


Figure 4. Decay curves with 1 µM Ca in the presence of ATP or EGTA. A, No additions; B, 0.62 or 1.23 µM ATP; C, 0.62 µM EGTA; D, 1.23 µM EGTA.

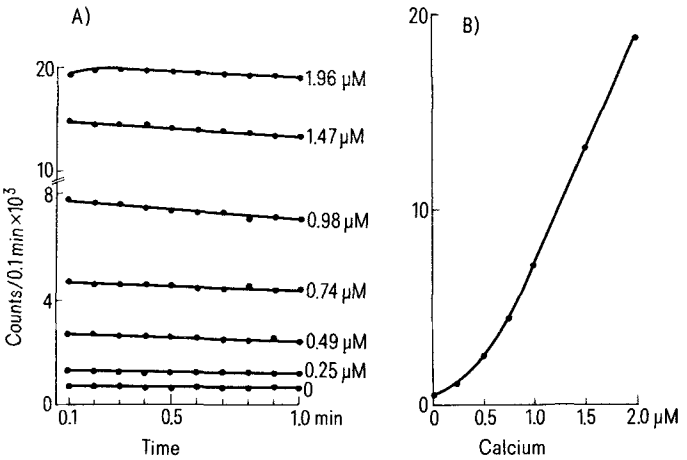


Figure 3. *A* Decay curves with different Ca concentrations. First period after 30 sec. *B* Standard curve constructed from the last point of measurements in (A).

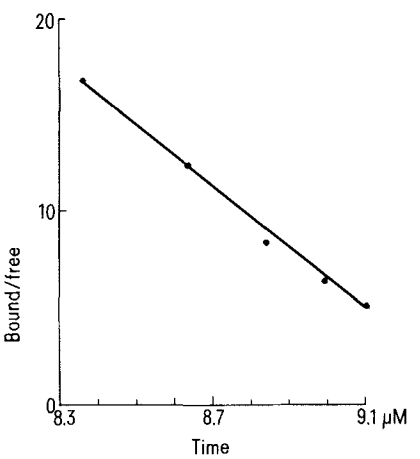


Figure 5. Scatchard plot of the data obtained using Ca-EGTA buffers. Ionic strength = 0.024, pH = 7.0. Log $K'_{EGTA}$ = 7.23.

$K'_{CaEGTA}$) by a Scatchard plot (fig. 5). A value of 7.2 for log $K'_{CaEGTA}$ was obtained. This value is slightly higher than that reported by others at pH 7[9,10]. This may be explained by the lower ionic strength used in the present determinations. Since the sensitivity of the reaction, between Ca and aequorin, decreased as the ionic strength was increased[5], comparative measurements of free Ca using EGTA could not be made in solutions of higher ionic strength. The present data also show that in the presence of Ca complexes of lower affinity than that of Ca and aequorin, free Ca concentrations cannot be measured; this is in agreement with the observations of Izutsu et al.[11].

*Discussion.* The proportionality between Ca concentration and luminescence, observed at the maximum of the curve, is the basis of various methods for the assay of Ca concentration[4,5]. Because of the time spent in introducing the vial into the counter, it is not possible to record the starting portion of the curve with the present instrument. However, if the reaction is slowed down by the dilution of the reactants, not only is the initial maximum less pronounced but the luminescence decays exponentially over a relatively prolonged period of time. Along the exponential portion of the curve, the light intensity happens to be proportional to the Ca concentration. The rate of decay appears to be related to the intensity of the initial peak. This phenomenon may provide an explanation for the anomalous behavior of the luminescence signal at high Ca concentration reported by Johns[7], although no information by the author was given about the time at which the samples were counted. The time elapsed between mixing the sample and counting, as shown by the present data, is very critical. Since liquid scintillation counters are available in most biochemical laboratories, and aequorin can be obtained commercially, the present method may find widespread application.

1 To whom reprint requests should be addressed.
2 Addanki, S., Sotos, J.F., and Rearick, P.D., Analyt. Biochem. *14* (1966) 261.
3 Cole, H.A., Dimpenny, J.W.T., and Hughes, D.E., Biochim. biophys. Acta *143* (1967) 445.
4 Cheer, S., Gentile, G.S., and Hegre, C.S., Analyt. Biochem. *60* (1974) 102.
5 Blinks, J.R., Mattingly, P.H., Jewell, B.R., van Leeuwen, M., Harrer, G.C., and Allen, D.G., Meth. Enzym. *57* (1978) 292.
6 Shimomura, O., and Johnson, F.H., in: Calcium in biological systems, p. 41. Cambridge University Press, Cambridge 1976.
7 Johns, A., J. pharmac. Meth. *6* (1981) 45.
8 Carrier, O., Murphy, J.C., and Tenner, T.E., Eur. J. Pharmac. *24* (1973) 225.
9 Bjerrum, J., Schwarzenbach, G., and Sillén, L.G., in: Stability constants part 1: Organic ligands, p. 76. The Chemical Society, London 1957.
10 Ohnishi, T., Masoro, E.J., Bertrand, H.A., and Pal Yu, B., Biophys. J. *12* (1972) 1251.
11 Izutsu, K.T., Felton, S.P., Siegel, I.A., Nicholls, J.I., Crawford, J., McCough, J., and Yoda, W.T., Analyt. Biochem. *58* (1974) 479.

0014-4754/83/080928-03$1.50+0.20/0


## In Memoriam

It is with profound sorrow that we announce the tragic and untimely death of Hugo Aebi. We have lost a dear friend and respected colleague. Professor Aebi joined the Experientia Editorial Board in 1974. As editor, he skillfully sought a balanced representation of disciplines covered by the journal and was an energetic proponent of Experientia's interdisciplinary goals. A tribute expressing our thanks and indebtedness to Professor Aebi will appear in a forthcoming issue of Experientia.

Hans Mislin
Werner P. Koella