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CONTROL OF THE Ca^{2+}-TRIGGERED BIOLUMINESCENCE OF *VERETILLUM CYNOMORIUM* LUMISOMES

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Summary

Calcium ions can trigger an emission of light from *Veretillum cynomorium* lumisomes (bioluminescent vesicles) under conditions where they are not lysed. This process does not require a metabolically-linked source of energy, but is dependent upon the nature of the ions present inside and outside the vesicles. The Ca^{2+}-triggered bioluminescence is stimulated by an asymmetrical distribution of cations or anions. Either high internal sodium or high external chloride is required for the maximal effect. When sodium is present outside the structure and potassium inside, the slow inward diffusion of calcium is decreased. Unbalanced diffusion of internal cations also stimulates the bioluminescence, suggesting control of the calcium influx by an electrochemical gradient. It is assumed that rapid outward diffusion of sodium or inward diffusion of chloride generates an electrical potential difference (inside negative) which drives the Ca^{2+}-influx. With purified lumisomes it has been shown that Ca^{2+}-triggered bioluminescence and calcium uptake (presumably net uptake) were correlated. In two instances uptake of the lipophilic cation dibenzyldimethylammonium has given direct evidence for the existence of a potential difference. With NaCl-loaded vesicles, it has not been possible to demonstrate an uptake of lipophilic cations but experiments with ^{22}Na and ^{42}K indicated a higher rate of sodium efflux, in accord with the proposed hypothesis.

Introduction

The bioluminescent *Anthozoan Coelenterates* emit flashes of light, which indicates a precise control of the light-emitting reaction. A considerable progress in the comprehension of the mechanism of control has been made recently [1—6]. Specialized organelles isolated in vitro and termed lumisomes give rise to a rapid emission of light in presence of calcium ions [2,7].

The mechanism of the flashing has been elucidated by Cormier et al. [1,3]. The luciferin is normally bound to a protein and thus is not available for the luciferase; when Ca^{2+} is added, these ions bind to the protein and the luciferase can then oxidize the luciferin. The excited molecules produced during the oxidation process transfer their energy efficiently to a green fluorescent protein which determines the color of the emission [1,7—9].

Lumisomes are closed vesicles and for the initiation of the flash, an increase of the internal Ca^{2+} concentration is required [1]. This allows another possibility of control since various treatments of the vesicles have been described which have very different effects on Ca^{2+} influx [4—6]. If the calcium ions are added to the preparation in isotonic NaCl solution, they trigger only a low-level light emission, indicating limited Ca^{2+} influx, whereas addition in KCl solution induces a strong flash, suggesting rapid influx. This observation might be physiologically relevant since in vivo the suspension of the animal in KCl induces a strong light emission [10]. Anderson and Cormier have recently given an interpretation of this phenomenon [5]. They showed that sodium must be present inside the vesicles for the external potassium to be active. They therefore suggested that Ca^{2+} entered the vesicles by a Na^{+}-Ca^{2+} exchange mechanism [11]. More recently they have defined that property as a sodium gradient-dependent calcium transport [6]. We present an alternate possibility to the Na^{+}-Ca^{2+} exchange mechanism according to which calcium influx is driven by a transmembrane potential difference, due to the asymmetrical distribution of the monovalent cations and to the selective permeability of the membrane to these ions.

Materials and Methods

Preparation of crude NaCl lumisomes. Veretillum cynomorium were obtained from the Arago Laboratory (Banyuls/mer, France). They were kept at 13° C for short periods of time in artificial sea water.

Crude NaCl lumisomes were prepared by a modification of previously described procedures [2,4,5]. Three animals were kept for 30 min at the aquarium temperature in 2 l of 0.01 M Tris · HCl buffer (pH 7.5) containing 0.55 M NaCl and 0.05 M $MgCl_2$. This treatment induced the contraction of swollen animals, either during the incubation period or when they were brought to the bench in broad daylight. They were homogenized in a Waring blendor for 60 s, then in a Polytron grinder (Kinematica GmbH; Lucerne) for 30 s, in 200 ml of 0.01 M Tris · HCl/0.025 M EGTA/0.6 M NaCl buffer (pH 7.5) cooled to 4° C. The homogenate was centrifuged at 650 × *g* for 10 min and the supernatant saved. The pellet was repetitively extracted with decreasing volumes of the same buffer by homogenization for 15 s with the Polytron grinder and centrifugation, until solubilization of most of the activity. The pooled active 650 × *g* supernatants, amounting to more than 50% of the homogenate activity were centrifuged at 20 000 × *g* for 20 min. The pellet was resuspended by manual operation with a Teflon tissue homogenizer (Thomas, Philadelphia, Pa.) in 50 ml homogenization buffer in which EGTA concentration was reduced to 0.01 M (buffer A). This fraction, after being washed twice

with buffer A, was used as crude lumisomes. It usually contained 40% of the homogenate activity.

Preparation of other crude lumisomes. The same technique has been used to prepare lumisomes in other media. The animals were pretreated in vivo in the buffer previously described then homogenized and purified in the desired buffer. The following media were used: 0.6 M KCl, 0.01 M EGTA, 0.01 M Tris · HCl; 0.6 M sodium and potassium methylsulfate, 0.01 M EGTA, 0.01 M Tris · HCl; 0.3 and 0.6 M potassium acetate, 0.01 M EGTA, 0.01 M Tris · HCl; 0.15 and 0.25 M potassium phosphate, 0.01 M EGTA, all adjusted to pH 7.5. In several instances (sodium and potassium methylsulfate), the EGTA concentration of the homogenization buffer was 0.025 M and in the case of KCl lumisomes, it contained 0.05 M $MgCl_2$.

The osmotic pressure of these preparations was determined by checking their activity in hypo- and hypertonic media. The protein concentrations were measured by the Folin technique, using bovine serum albumin as a standard [12].

Purification of lumisomes. Crude NaCl lumisomes were purified by centrifugation on discontinuous Ficoll gradients. The extract from three animals was layered onto a 18% Ficoll solution in buffer A. The Ficoll solution had a refractive index of 1.3685 at 20°C. Each centrifugation tube contained 30 ml Ficoll and 24 ml crude extract. They were centrifuged at 24 000 rev./min for 2 h in a SW 25.2 rotor of a Spinco ultracentrifuge. At the end of the run the interface appeared to consist of a white fluffy layer and of a pink precipitate. The other major component was a thick brown pellet. The tube content was separated in five fractions by suction with pipettes. Each fraction was washed twice by dilution in buffer A and centrifugation at 20 000 × *g* for 20 min. The relative activities of the fractions of a typical experiment are given in Table III.

Crude potassium phosphate lumisomes were purified by the same technique, using 0.25 M potassium phosphate (pH 7.5)/0.01 M EGTA instead of buffer A.

Assay of the lumisomes. Lumisomes were routinely assayed by measuring the maximal intensity (I_{max}) of the flash of light obtained by addition of hypotonic Ca^{2+} [4]. The standard assay mixture contained 0.35 ml 0.01 M Tris · HCl (pH 7.5), 0.25 mM EGTA, 0.6 M NaCl and 0.05 ml lumisomes in a scintillation vial, to which 1.6 ml 0.01 M Tris · HCl (pH 7.5) containing 3 mM $CaCl_2$ was added with a syringe. Light emissions were measured with a photomultiplier [13]. The same apparatus could also be used to measure total light emitted (L).

Electron microscopy of the preparations. Preparations were centrifuged at 27 000 × *g* for 15 min. The pellets were gently washed with 0.25 M sucrose, 0.1 M sodium phosphate (pH 7.4), fixed in 2.5% glutaraldehyde (1 h at 4°C), washed in the same buffer, post-fixed with 0.5% OsO_4 (1 h at 4°C) and embedded in Araldite. For negative staining, phosphotungstic acid (2%) was used. Micrographs were obtained with a Philips E 300 apparatus.

Uptake experiments. Concentrated vesicle suspensions (approx. 40 mg/ml protein) were diluted (usually 20-fold) in the radioactive media listed in the legends of the various figures. Unless otherwise stated, experiments were conducted at 25°C. Aliquots (0.2 ml) of the suspension were directly filtered

through Millipore filters (HAWP 025), washed with 10—15 ml of the indicated media then dried. Preliminary experiments indicated that the filters retained more than 80% of the lumisome proteins. The isotopes (^{3}H and ^{45}Ca) were counted in an Intertechnique scintillation counter with 0.5% PPO and 0.03% POPOP in toluene (5 ml). In each experiment, control filtrations (without lumisomes) were included and the background due to non-specific binding was subtracted from the total count for each sample. A small aliquot (2 μl) layered onto a dry filter was used to determine the total activity of the incubation.

Apparent internal concentrations of the substrates taken up by the vesicles were calculated using a value of 2 μl of intravesicular fluid/mg membrane protein. This value was derived from ^{14}C-labelled serine uptake experiments with overnight incubation at 0° C.

Synthesis of [^{3}H]dibenzyldimethyl ammonium chloride. [^{3}H]Methyl iodide (250 μmol, 100 Ci/mol, Radiochemical Centre, Amersham, U.K.) was added to *N*-methylbenzylamine (0.09 ml) in acetone (0.3 ml). The reaction mixture was incubated for 24 h at room temperature in a conical glass-stoppered tube. The solvent was evaporated and 0.5 ml of ether was added to the residue. The excess of amine was dissolved and after removal of the solvent, large crystals were obtained which were dissolved in water. This solution was passed over a 1.5-cm column of Dowex-1 (chloride form) in a Pasteur pipette, and the column was eluted with water. The eluate was lyophilized and the residue dissolved in 0.6 ml ethanol. The yield of the reaction was 70% estimated either radiochemically of spectrophotometrically, assuming an extinction coefficient of 830 $M^{-1} \cdot cm^{-1}$ at 262 nm [14].The purity of the product was checked by thin-layer chromatography in chloroform/methanol/0.1 M aqueous acetic acid (50 : 70 : 36, v/v/v).

Synthesis of triphenylmethylphosphonium bromide. [^{3}H]Methyl iodide (250 μmol, 100 Ci/mol) and triphenylphosphine (100 mg) were mixed in ether (1 ml) and incubated overnight. The products were then processed as described for dibenzyldimethylammonium chloride, using a Dowex column in the bromide form.

The yield of the reaction was 35%. The purity was checked by thin-layer chromatography in the solvent previously described and by ultraviolet absorption. An extinction coefficient of 3100 $M^{-1} \cdot cm^{-1}$ at 267 nm was assumed [14].

Other radioactive chemicals. [U-^{14}C]Sucrose (556 Ci/mol) was obtained from the Radiochemical Centre, Amersham, U.K. [U-^{14}C]Serine (120—150 mCi/mol), $^{45}CaCl_2$ (22.5 mCi/mg), ^{42}KCl (0.08—0.8 mCi/mg) and $^{22}NaCl$ (30 mCi/mg) were from C.I.S.

Results

Factors required for the bioluminescent flash of lumisomes

Lumisome preparations are characterized by a Ca^{2+}-triggered emission of light which has a maximal intensity weaker in isotonic NaCl (I_{Na}) than in isotonic KCl (I_K), the latter being comparable to that obtained in hypotonic solutions (I_0) [4,5]. This property is not under metabolic control as indicated by the lack of effect of ATP (0.8 mM) and of cyanide ions (1 mM) on the

intensities of the isotonic flashes (I_{Na} and I_K). Ouabain (0.1 mM), an inhibitor of ($Na^+ + K^+$)-ATPase was also without effect. An ATPase activity has been detected in purified lumisomes (data not shown) but this activity is not directly related to the Ca^{2+}-induced flash.

In contrast, the capacity for the preparation to discriminate between sodium and potassium ions ($I_{Na} < I_K$) is dependent on the integrity of the membrane and the nature of the cations present on both sides of it. An osmotic shock resulted in a 46% decrease of activity and a nearly complete loss of its discriminating capacity (Table I). This effect was reversible since washing and resuspension in a 0.6 M NaCl-containing medium restored its discriminating property to the lysed preparation. If after lysis the lumisomes were suspended in KCl in place of NaCl the preparation did not recover its discriminating property, I_K being equivalent to I_{Na}. This experiment indicates that lumisomes are vesicles which can be reversibly opened and that sodium but not potassium ions must be present inside the structure to observe the above behaviour.

The internal ion can also be exchanged without a lysis step, by resuspending a preparation in a KCl-containing medium (Table I) as described by Anderson and Cormier [5], but in this case the exchange reaction is time-dependent. When sodium lumisomes (vesicles isolated in Na^+ buffers) were preincubated in 0.6 M KCl for various lengths of time, the flash of light obtained by addition of Ca^{2+} in the same medium decreased with time (Table II). This effect was not seen when the vesicles were kept in 0.6 M NaCl before injecting Ca^{2+} in the presence of Na^+; in this case the flash height increased slowly with time. The discriminating capacity ($1 - I_{Na}/I_K$) of the preparation decreased in a biphasic

TABLE I

EXCHANGE OF INTERNAL CATIONS

Conditions **	I_0 * (arbitrary units)	I_{Na}/I_0 *	I_K/I_0 *
Experiment I: NaCl lumisomes ***			
Initial preparation	35.6	0.15	0.62
After two washes			
in 0.6 M NaCl	17.7	0.19	1.16
in hypotonic buffer	9.5	0.39	0.52
Suspension of shocked lumisones			
in 0.6 M NaCl	11.6	0.42	0.94
in 0.6 M KCl	9.9	0.41	0.57
Suspension of non-shocked lumisomes			
in 0.6 M NaCl	20.5	0.19	0.80
in 0.6 M KCl:			
after 3 min	19.3	0.23	0.61
after 30 min	14.2	0.32	0.46
Experiment 2: KCl lumisomes ***			
Initial preparation	18.1	0.28	0.34
After suspension (30 min) in 0.6 M NaCl	19.4	0.32	0.62

* Activities were measured by dilution of the preparation in 0.35 ml of the same buffer and addition of 1.6 ml $CaCl_2$ (3 mM) in 0.1 M Tris · HCl(I_0), in Tris/0.6 M NaCl(I_{Na}) or in Tris/0.6 M KCl (I_K)

** All buffer changes were done by centrifugation at 20 000 × *g* for 20 min and homogenization of the pellet with a Teflon homogenizer. The buffers were 0.01 M Tris · HCl (pH 7.5), 0.02 M EGTA and, when indicated, 0.6 M NaCl or KCl

*** Crude lumisones were isolated either in NaCl or KCl media as described.

TABLE II

EFFECT OF PREINCUBATION IN 0.6 M KCl OR NaCl OF NaCl-LOADED LUMISOMES BEFORE CALCIUM ADDITION

Lumisomes (50 μl) were incubated for various lengths of time in 1.45 ml 0.6 M KCl or NaCl, 0.25 mM EGTA, 0.01 M Tris · HCl (pH 7.5). They were assayed by addition of 0.5 ml $CaCl_2$ (9.6 mM) in 0.01 M Tris · HCl (pH 7.5) containing either 0.6 M NaCl (I_{Na}) or KCl (I_K).

Time (min)	I_{Na} (arbitrary units)	I_K (arbitrary units)	$1 - \frac{I_{Na}}{I_K}$
0.12	4.45	16.75	0.735
0.20	4.5	16.1	0.720
0.33	5.1	17.0	0.700
0.50	5.45	17.5	0.689
0.75	5.7	18.35	0.689
1	6.05	17.4	0.659
2	6.6	16.2	0.593
5	6.6	15.55	0.582
10	6.8	13.7	0.504
15	6.5	11.8	0.449
20	6.5	11.2	0.420
30	6.4	10.45	0.388

way, the first part of the curve being characterized by a half lifetime of 1—6 min, quite short compared to the second one (45 min). Since sodium ions must be present inside the vesicle to obtain a flash of light when Ca^{2+} is added in isotonic KCl [5], the observed kinetics should reflect the exchange of internal sodium ions.

Preparation of lumisomes with different internal salts

When potassium was substituted for sodium in all the buffers used for preparing crude lumisomes the preparation obtained had the characteristics of the K^+ lumisomes described above. Addition of Ca^{2+} (3 mM) in presence of either isotonic Na^+ or isotonic K^+ did not give rise to an intense flash of light ($I_K/I_0 = 0.29$; $I_{Na}/I_0 = 0.18$) and resuspension of the preparation in 0.6 M NaCl increased I_K but not I_{Na}. This experiment shows that by changing the homogenization and purification buffers the internal salt can be modified.

All the experiments hitherto reported were in chloride buffers. Since Cl^- might be a permeant anion [15], we decided to replace it by methylsulfate which should be an impermeant monovalent ion [15,16]. For this purpose, crude lumisomes were isolated in buffers containing sodium methylsulfate (0.6 M) in place of NaCl. The preparation thus obtained had an activity comparable to that of NaCl lumisomes when assayed by injection of hypotonic calcium (2.4 mM). Increasing the concentrations of sodium methylsulfate in the calcium solution resulted in a sharp drop in the light emission at concentrations higher than 0.6 M showing that the structure was not normally permeable to Ca^{2+}. If potassium methylsulfate was added with Ca^{2+} a similar drop in the light intensity was observed at high salt concentrations, but the effect was less marked, indicating that under these conditions the rate of entry was somehow faster. Under isotonic conditions (0.6 M salts), figures of 0.22 and 0.45 were obtained for I_{Na}/I_0 and I_K/I_0, respectively, showing that chloride

and methylsulfate vesicles have the same basic property. The intensity of the flash obtained in isotonic potassium methylsulfate is weaker than that of hypotonic Ca^{2+} solutions, thus indicating that in the presence of methylsulfate the passage of Ca^{2+} through the membrane is slower than in the presence of chloride. Nevertheless, the potassium methylsulfate-stimulated Ca^{2+} entry is an efficient process since after 1 min the total light emitted under these conditions is 117% of that emitted under hypotonic conditions and is 2.8 times that emitted in isotonic sodium methylsulfate.

Vesicles isolated in other sodium salts such as acetate (0.3 M) or phosphate (0.15 M) were also more permeable to Ca^{2+} in presence of external potassium salts.

Reversed sodium-potassium effect

Since, when sodium ions are internal and potassium external, entry of Ca^{2+} is facilitated it was of interest to examine the effect on Ca^{2+} uptake of the reverse situation (internal potassium and external sodium) by comparing it with a symmetrical situation (for instance internal and external potassium). The experiment was done using crude potassium methylsulfate lumisomes. This preparation gave rise to a weak emission of light after addition of Ca^{2+} in isotonic potassium methylsulfate (Fig. 1), which was interpreted as a result of the diffusion of these ions through the vesicle membrane [4,5]. The intensity of the emission increased continuously with Ca^{2+} concentration, up to 20 mM, whereas that of the flash obtained under hypotonic conditions levelled

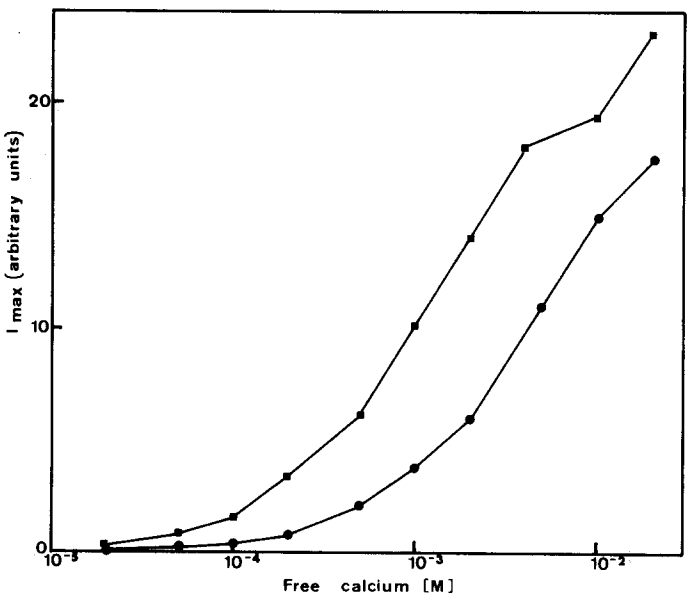

Fig. 1. Effect of Ca^{2+} concentration on the luminescence of potassium methylsulfate-loaded lumisomes. Lumisomes (50 µl) from a preparation twice washed with 0.01 M Tris · HCl (pH 7.5), 0.6 M potassium methylsulfate, 10 µM EGTA were suspended in 0.35 ml of either the same buffer (■) or the corresponding one with sodium in place of potassium (●). Calcium was then added in 1.6 ml 0.01 M Tris · HCl (pH 7.5) containing 0.6 M potassium (■) or sodium (●) methylsulfate. The concentrations given in the figure are free calcium after addition.

off at about 1 mM. Fig. 1 shows that when sodium methylsulfate is the external salt a similar glow is obtained but that its intensity is weaker for all calcium concentrations. Thus in the reverse situation (internal potassium and external sodium) Ca^{2+} did not rapidly enter the vesicles and the diffusion rate was slower than in a symmetrical situation.

Effect of anions on the bioluminescent flash

Since the observations outlined above indicated that an asymmetry between external and internal cations is required to stimulate the Ca^{2+}-induced bioluminescence, it was of interest to check if asymmetrical distributions of anions might be found which would have the same effect. It has been found that this is the case for crude phosphate lumisomes (isolated in 0.25 M potassium phosphate) suspended in KCl. The flash of light elicited by addition of Ca^{2+} (1 mM) was more intense when these ions were added in isotonic or hypertonic KCl than in potassium phosphate. When the effects of isotonic calcium in chloride medium and hypotonic calcium additions were compared a figure of 0.65 was obtained for I_{Cl}/I_0. In contrast, when the same experiment was done with phosphate as the external anion the ratio was 0.14. The low values obtained in presence of phosphate were not a result of a simple precipitation of Ca^{2+} since when the concentration of the latter ion (1 mM) was decreased by a factor of 2, light intensities were proportionally diminished. It has also been shown that acetate and propionate ions were intermediate between chloride and phosphate, being characterized by figures of 0.47 and 0.40 for $I_{acetate}/I_0$ and $I_{propionate}/I_0$, respectively.

Using lumisomes isolated in 0.6 M potassium acetate, it has also been possible to demonstrate a chloride effect. This ion, when present outside the vesicle, increased Ca^{2+}-triggered luminescence. In an experiment similar to that described for phosphate vesicles, values of 0.79 and 0.26 were obtained for I_{Cl}/I_0 and $I_{acetate}/I_0$, respectively. In Fig. 2 the experiment was done in a different way, the effect of increasing concentrations of calcium being studied in hypotonic and isotomic chloride or isotonic acetate buffers. It can be seen that light emissions observed in isotonic media were weaker than those in hypotonic conditions, but also that the Ca^{2+} addition was more efficient in chloride than in acetate.

An anionic effects was also noted when Ca^{2+} in isotonic KCl was added to particles isolated in 0.6 M potassium methylsulfate (methylsulfate lumisomes) (data not shown). These experiments and others previously described thus indicate that distribution of the anions can stimulate the bioluminescent flash and presumably the calcium uptake of the lumisomes. Moreover, they also suggest that chloride plays a special role which is reminiscent of that of Na^+ in the cation effect.

Effect of diffusion potentials on the bioluminescent flash

A possible interpretation of the data is that the asymmetrical distribution of the anions and cations and the permeability of the membrane to these ions might result in the appearance of an electrical potential difference which would, in turn, act on the Ca^{2+} flux. In order to check such a hypothesis, conditions which should produce such a potential difference were examined.

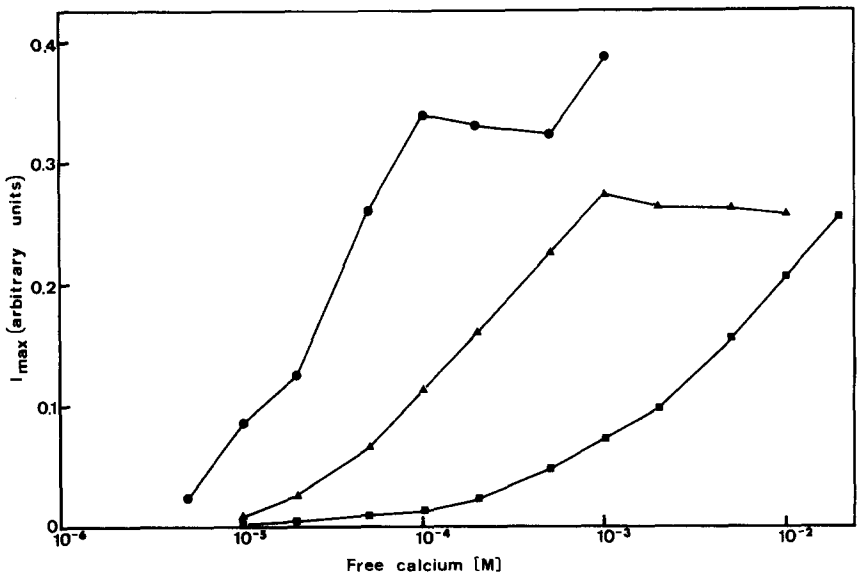

Fig. 2. Effect of Ca^{2+} concentration on the luminescence of potassium acetate-loaded lumisomes. Same procedure as in Fig. 1. Washing was done with 10 μM EGTA, 0.6 M potassium acetate, 0.01 M Tris · HCl. Calcium was injected in: (●) 0.01 M Tris · HCl (pH 7.5); (■) 0.6 M potassium acetate, 0.01 M Tris · HCl (pH 7.5); (▲) 0.6 M KCl, 0.01 M Tris · HCl (pH 7.5).

From the study of chloroplasts [17] and of closed vesicles derived from bacterial membranes [18,19], it appears that association of a permeant cation and an impermeant anion inside a structure will generate an electrical potential across the membrane (inside negative) when the cation can diffuse outwards. Accordingly, crude lumisomes were prepared in 0.15 M potassium phosphate (pH 7.5) and assayed by measuring the light intensity after 100-fold dilution in buffers containing 1 mM Ca^{2+} and variable amounts of potassium (as phosphate). Sucrose was added to the diluting solution to maintain a constant osmotic pressure slightly higher than that of the vesicles. The results are presented in Fig. 3 where light intensities are plotted as a function of the log of the ratio of internal and external cation concentrations. It can be seen that when the internal potassium concentration is 100 times that of the external, addition of Ca^{2+} induced an emission of light 15 times more intense. This light emission was also faster than the one obtained under hypotonic conditions.

If the observed phenomenon is indeed associated with a diffusion potential, it suggests that the lumisomes are permeable to K^+. It was repeated with sodium and cholinium cations (Fig. 3). The latter gave only a very slight effect, as might have been predicted knowing the small permeability of lipidic membranes for this ion. The former produced an effect stronger than the one obtained with potassium, a result which, according to the assumptions, should reflect a higher permeability to Na^+. Addition of valinomycin (10 μM), a well known K^+ ionophore, did not increase the potassium effect. The differences observed with the three cations tested indicated that the decrease of the flash height observed at high phosphate concentration was not due to calcium

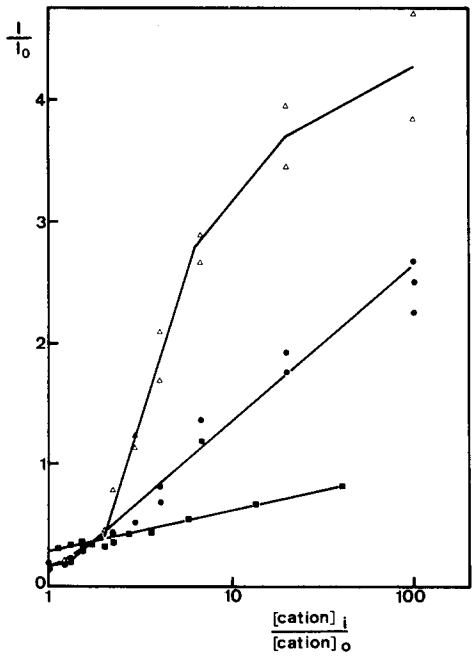

Fig. 3. Effect of the concentration of the external salt at constant osmotic pressure on the luminescence of phosphate lumisomes. Lumisomes (20 µl, 0.8 mg protein) were pipetted in a scintillation vial and $CaCl_2$ (1 mM) was added in 2-ml mixtures of sucrose and sodium (△), potassium (●) or choline (■) phosphate (pH 7.5). The lumisomes had been isolated in 0.01 M EGTA/0.15 M potassium phosphate (pH 7.5) and dialyzed against that buffer (●) or the sodium salt (△) before the experiment or were isolated in 0.01 M EGTA/0.15 M choline phosphate (■). The osmolarity of the calcium solutions was kept constant and equal to 600 mosM/l by addition of sucrose; the osmolarity of 0.2 M phosphate (pH 7.5) was assumed to be 600 mosM/l. The data are presented as a semi log plot of the ratio of internal and external cation concentrations.

precipitation. In addition, acetate (0.3 M) can be substituted for phosphate in this type of experiment. A 40-fold decrease of the external sodium concentration induced a 3-fold increase in the bioluminescent flash.

A feature of diffusion potentials is their short lifetime. To investigate this point, sodium and potassium acetate lumisomes were diluted 40-fold in buffered isotonic sucrose, incubated for various lengths of time and then assayed by recording their light emission after addition of a concentrated Ca^{2+} solution (Fig. 4). A semi log plot of the results shows that the kinetics are biphasic. The sodium and potassium curves decay with similar rates initially (half lifetimes of 9 and 8 s, respectively) but shift to slower rates which are quite different for the two cations, K^+ showing a slower rate (half lifetimes 160 s for K^+ and 26 s for Na^+).

Evidence for an electrochemical control of calcium influx

Up to now only bioluminescence experiments have been reported and it has been postulated that triggering of the precharged bioluminescent system by calcium ions reflects influx of these ions inside of the vesicles. Experiments will now be presented which confirm the hypothesis of an electrochemical control of calcium influx and which are based on independent techniques.

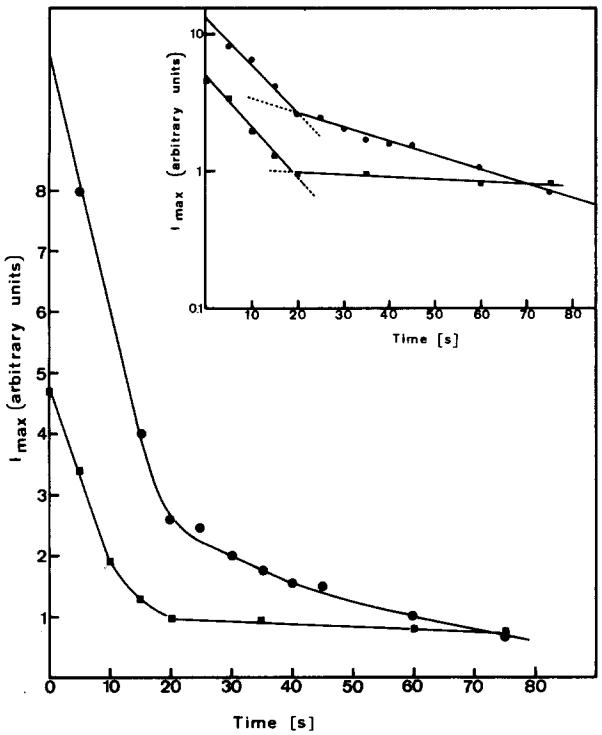

Fig. 4. Effect of preincubation in sucrose of acetate lumisomes. Lumisomes were isolated in 0.3 M sodium acetate/0.01 M EGTA/0.01 M Tris · HCl (pH 7.5) and dialysed against that buffer (•) or potassium acetate (■). Aliquots (50 μl) were incubated for various lengths of time in 2 ml 0.6 M sucrose/0.01 M Tris · HCl (pH 7.5) at room temperature and then assayed by addition of 0.2 ml $CaCl_2$ (33 mM) in the same buffer.

Purification was a prerequisite to such experiments. A purification procedure was devised, in which lumisomes where centrifuged on a layer of Ficoll (18%) in NaCl buffer. The density of the Ficoll was such that only light lumisomes were blocked at the interface. Under these conditions, from 25 to 60% of the activity of crude lumisomes was accounted for and the pink interface fraction contained 75% of the activity of the gradient (Table III). This fraction was designated as purified lumisomes. It had the discriminating property shown by the crude lumisomes when their Ca^{2+}-triggered biolumiescence was assayed in isotonic KCl or NaCl media [4,5]. It consisted mainly of vesicles as judged by electron microscopy of negatively stained or of glutaraldehyde-fixed preparations (Fig. 5). The vesicles were fairly uniform in size and most of them appeared to be empty. In contrast, the brown pellet of the Ficoll gradient contained few vesicles and an amorphous unidentified material.

If purified lumisomes are incubated with [^{14}C]serine or [^{14}C]sucrose, a slow uptake of these compounds occurs which is detected after filtration on Millipore filters or after centrifugation of the preparation to wash external components. Experiments done with several different preparations gave an average of 2 and 0.84 μl/mg protein for the internal volume of the vesicles determined with [^{14}C]serine and [^{14}C]sucrose, respectively. Control experiments per-

TABLE III

CENTRIFUGATION OF CRUDE LUMISOMES ON DISCONTINUOUS FICOLL GRADIENTS

Crude lumisomes from three animals (880 mg protein) were centrifuged as described in the experimental part. The lower interface fraction (purified lumisomes) contained 120 mg protein.

	Volume (ml)	Activity (I_{max}) (arbitrary units)	Total activity ($v \cdot I_{max}$)	Relative activity (%)
Upper layer	51	0.15	7.65	0.37
Upper interface	19	0.9	17.1	0.82
Lower interface	29	54.0	1566.0	75.0
Lower layer	68	1.4	95.2	4.56
Pellet	35	11.5	402.5	19.27

formed under hypotonic conditions showed a large decrease of the internal volume, indicating that in isotonic media the tracers were indeed trapped inside the vesicles.

Calcium uptake of purified lumisomes

It has already been noted [2,4] that addition of Ca^{2+} in 0.6 M KCl to purified lumisomes gave an emission of light which was much brighter than when Ca^{2+} was added in 0.6 M NaCl and this fact has been ascribed to an increased calcium influx through the membrane. To check this point, lumisomes were incubated with ^{45}Ca in either KCl or NaCl buffers, filtered on Millipore filters and washed. In order to obtain measurable rates of calcium entry, the

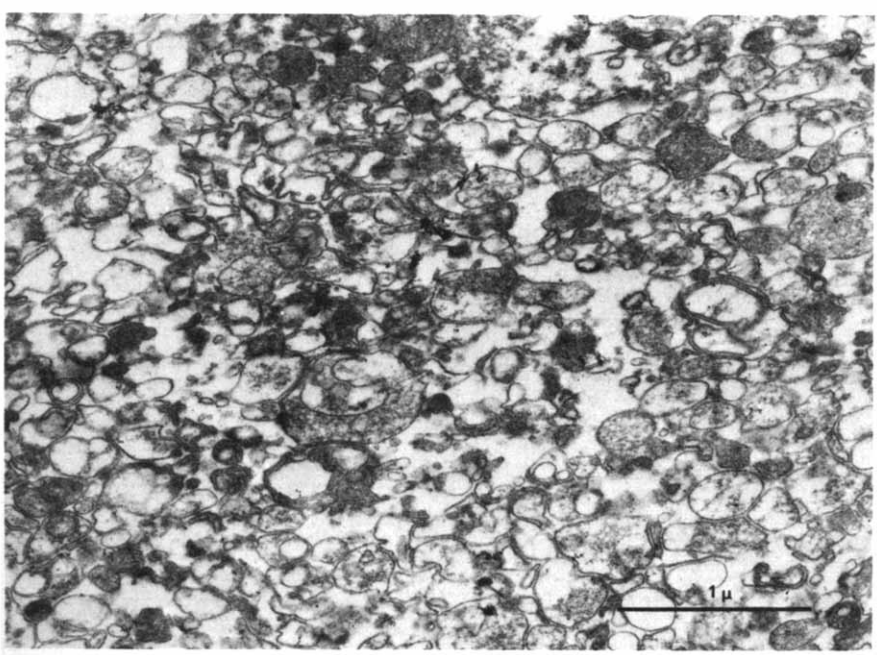

Fig. 5. Electron micrograph of purified lumisomes. The preparation was obtained by centrifugation on discontinuous Ficoll gradients.

calcium concentration was lower than in the bioluminescence experiments (0.17 mM) and Mg^{2+} (5 mM) was added. Fig. 6 shows that in presence of external K^+ there is a 4-fold increase in ^{45}Ca uptake. This phenomenon is rapid since it is complete in 2 min. When the same lumisome preparation was assayed for bioluminescence activity, the flash obtained in KCl was 3 times more intense than in NaCl. The concentrations of internal ^{45}Ca were calculated assuming a figure of 2 μl/mg protein for the internal volume of the vesicles and found to be 0.89 and 3.35 mM in presence of external NaCl and KCl, respectively. It has been shown that this phenomenon is due to a net uptake of calcium and not only an exchange reaction (Table IV). The experiment was done by incubating crude lumisomes with Ca^{2+} (0.1 mM) in NaCl or KCl buffers, centrifuging and assaying the calcium content of the supernatant by atomic absorption.

As previously reported with crude lumisomes an asymmetrical distribution of anions stimulated the Ca^{2+}-triggered bioluminescence and when phosphate was present inside the vesicles addition of calcium in chloride gave rise to a stronger emission of light than calcium in phosphate. Purified phosphate lumisomes were prepared by substituting potassium phosphate (0.25 M) for NaCl in all buffers. The bioluminescence (I_{max}) of such a preparation was stimulated 2.3-fold in presence of KCl (0.5 M). Calcium uptake was measured using ^{45}Ca as previously described (Fig. 7A). Here again the phenomenon was

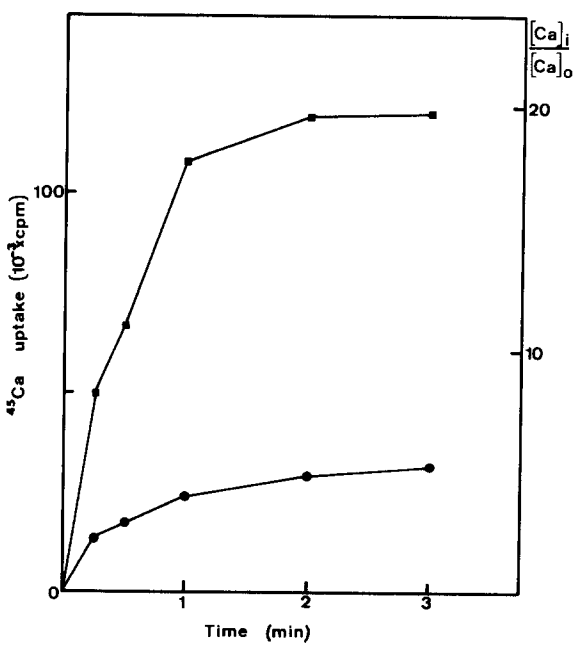

Fig. 6. Effect of KCl on the uptake of ^{45}Ca by purified NaCl lumisomes. The sample was washed twice with 0.6 M NaCl/5 mM $MgSO_4$/0.01 M Tris · HCl (pH 7.5) prior to incubation. The incubation mixture (1.2 ml) contained 3.5 mg protein, 0.6 M NaCl (•) or KCl (▪), 5 mM $MgSO_4$, 0.01 M Tris · HCl (pH 7.5) and 0.17 mM $^{45}CaCl_2$ ($3.1 \cdot 10^7$ cpm/μmol). At intervals 0.2-ml aliquots of the suspension were filtered and washed 3 times with 5 ml Ca-free buffer. A background of 180 cpm due to non-specific binding was subtracted.

TABLE IV

NET UPTAKE OF CALCIUM BY CRUDE LUMISOMES

Crude lumisomes (13.9 mg protein) were incubated in 1 ml 0.01 M Tris · HCl (pH 7.5) containing either 0.6 M NaCl or 0.6 M KCl with or without $CaCl_2$ (100 nmol), for 1 min at 22°C. The incubations were stopped by addition of 0.1 ml 20 mM EGTA in Na^+ or K^+ buffer. The samples were centrifuged at 27 000 × *g* for 15 min and the calcium content of the supernatants was analyzed by atomic absorption, after diluting 4 times in distilled water. Only plastic tubes were used. The buffers alone gave a figure of 10 nmol, which was subtracted from all measurements.

Sample	Calcium content (nmol)	
	NaCl medium	KCl medium
Supernatant of lumisomes incubated without Ca	21.9	21.9
Supernatant of lumisomes incubated with Ca	99.4	81.9
Ca uptake	22.5	40.0

rapid and after 5 min, the internal ^{45}Ca concentrations were 0.7 and 1.24 mM in presence of external phosphate or chloride, respectively. These figures were obtained for an external Ca^{2+} concentration of 0.16 mM and using the same internal volume of the vesicles.

In the first part of this communication, it has also been noted that suspension in sucrose buffer of crude lumisomes containing potassium phosphate as the internal salt gave rise to strong Ca^{2+}-triggered bioluminescence. The same experiment could be done with lumisomes purified in 0.25 M potassium

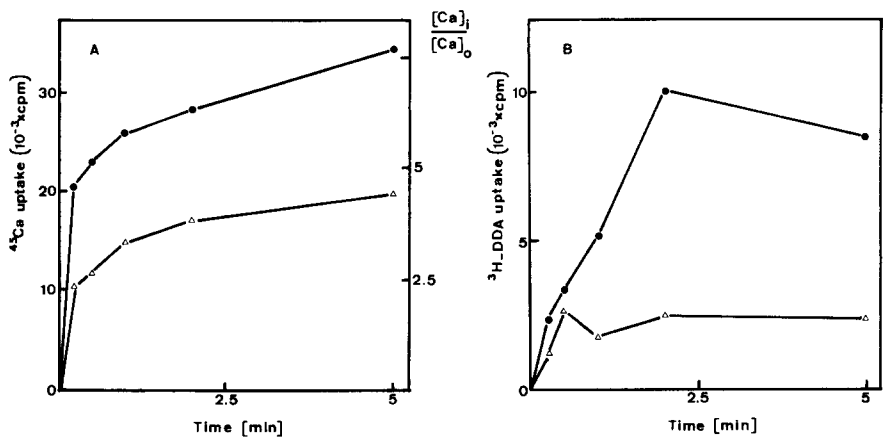

Fig. 7. Effect of KCl on potassium phosphate-loaded lumisomes. (A) Effect on ^{45}Ca uptake. The procedure was that described in Fig. 6. The lumisomes (2 mg protein) were washed in 0.25 M potassium phosphate (pH 7.5), 5 mM $MgSO_4$ and incubated in 0.25 M potassium phosphate (△) or in 0.6 M KCl/ 0.01 M Tris · HCl (●) in both cases containing 5 mM $MgSO_4$ and 0.16 mM $^{45}CaCl_2$ ($4.0 \cdot 10^7$ cpm/μmol). The background level was 1100 cpm. (B) Effect on [^{3}H]dibenzyldimethyl ammonium uptake. The lumisomes (2 mg protein, 60 μl) were prepared as for ^{45}Ca uptake, but prior to incubation pretreated with 5 μl of 1 mM sodium tetraphenylborate for 5 min at 25°C. The incubation contained the same components as for ^{45}Ca uptake, with 0.36 mM [^{3}H]dibenzyldimethyl ammonium ($3.1 \cdot 10^7$ cpm/μmol) in place of ^{45}Ca. The figure shows incubations in phosphate (△) and in chloride (●) buffers. The filters were washed with the same buffers containing 6.1 mM dibenzyldimethyl ammonium. The background level was 1200 cpm.

phosphate. A 40-fold dilution of such a preparation in 0.75 M sucrose buffer containing 1 mM $CaCl_2$ and 5 mM $MgSO_4$ resulted in light emission which was 6.5 times more intense than that obtained after diluting in 0.25 M phosphate buffer. The calcium movements in presence of either sucrose or phosphate have been investigated by the ^{45}Ca technique (Fig. 8A). The uptake stopped after two minutes. The final internal ^{45}Ca was 0.47 mM in presence of external phosphate and 1.96 mM in presence of external sucrose. Fig. 8A also shows that preincubation of lumisomes with the lipophilic anion sodium tetraphenylborate (80 μM) does not alter the results.

Rate of sodium and potassium ion-exchange across the lumisome membrane

Our hypothesis on the cause of Ca^{2+}-stimulated bioluminescence has been investigated as well by measuring the rates of exchange of these ions by a tracer technique (Fig. 9). Lumisomes were loaded with either ^{22}Na or ^{42}K and then diluted in non-radioactive buffers. The radioactivity remaining in the particulate fraction after filtration and washing on Millipore filters was studied as a function of time. A semi log plot of the results gave straight lines, suggesting that on the time-scale used the experiment reflected the rate of exit of the cations. It can be seen that the sodium efflux is significantly faster than that of potassium, being characterized by half-lifetimes of 16.5 and 48.25 min, respectively. The average values ($P = 0.1$, $n = 4$) obtained for half-lifetimes of ^{22}Na and ^{42}K efflux were 20.3 ± 3.8 and 45.6 ± 24.4, respectively. It should be noted that the internal concentrations of the radioactive salts extrapolated to zero time ranged from 20 to 40% of the external one. A likely explanation of those low values is a rapid efflux of the cations under the conditions of filtration used.

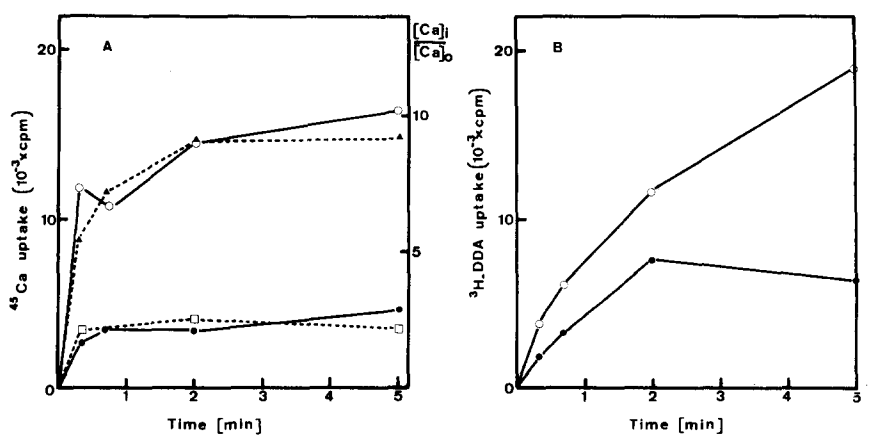

Fig. 8. Effect of sucrose on potassium phosphate-loaded lumisomes. (A) Effect on ^{45}Ca uptake. The procedure was that described in Fig. 7A. In two experiments (- - - - -) the lumisomes (2.55 mg protein, 50 μl) were preincubated with 4 μl 1 mM tetraphenylborate. The incubation contained 0.25 M potassium phosphate (● and □) or 0.75 M sucrose, 0.01 M Tris · HCl (▲ and ○) and 5 mM $MgSO_4$, 0.2 mM $^{45}CaCl_2$ ($4.0 \cdot 10^7$ cpm/μmol). The background level was 2500 cpm. (B) Effect on [^{3}H]dibenzyldimethyl ammonium uptake. The procedure was the same as for ^{45} Ca uptake (●, potassium phosphate; ○, sucrose). The lumisomes (2.55 mg protein) were pretreated with tetraphenylborate. In place of Ca^{2+}, 0.44 mM [^{3}H]dibenzyldimethyl ammonium ($2.4 \cdot 10^7$ cpm/μmol) was used. The background level was 2500 cpm.

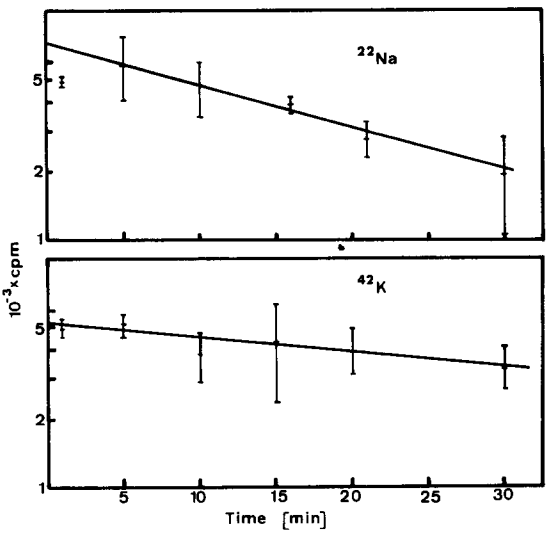

Fig. 9. Rates of ^{22}Na and ^{42}K exchanges through lumisome membrane. Purified NaCl-lumisomes (8.0 mg protein, 0.3 ml) and KCl-lumisomes (11.1 mg protein, 0.3 ml) were loaded with ^{22}Na and ^{42}K by addition of 15 and 16.9 μCi, respectively, of these isotopes to the preparations in their isolation buffers and incubated at 0°C for 3 h. The suspensions were then warmed and diluted with 30 ml of 0.6 M NaCl or KCl/5 mM $MgSO_4$/0.01 M Tris · HCl (pH 7.5). Aliquots (1.65 ml) were withdrawn at intervals, filtered and washed 3 times with 5 ml dilution buffer. The background level was 150 cpm. Each point is the result of three measurements; standard errors were calculated by Student's *t*-test ($P = 0.1$).

The effect of osmotic pressure on ^{42}K uptake of lumisomes showed that this ion was stored in an osmotically sensitive compartment.

Uptake of lipophilic cations by purified lumisomes

Asymmetrical diffusion of ions through a membrane should produce a potential difference across that membrane. Since the size of the lumisomes prevents direct measurements of the postulated potential difference, the indirect technique originally devised by Skulachev and Liberman [14,20] which has been applied to the closed vesicles derived from bacterial membranes [18,19] has been used. According to this technique, uptake of artificial lipophilic cations inside a structure is taken as evidence of the existence of a potential difference, inside negative. If the lipophilic cation is labelled, its uptake may be followed by filtration of the particulate fraction. By this technique, it was possible to demonstrate that diffusion of internal potassium ions produces a potential difference (Fig. 8B). The lipophilic cation was [^{3}H]-dibenzyldimethyl ammonium and the conditions of the experiment were those used for demonstration of ^{45}Ca uptake (Fig. 8A). When the lumisomes containing potassium phosphate as the internal salt were diluted in sucrose buffer, an uptake of [^{3}H]dibenzyldimethyl ammonium was observed which was 1.7 times that obtained when the lumisomes were diluted in potassium phosphate. This effect was small but it was increased to a 4.3-fold stimulation when the vesicle preparation was preincubated for 5 min with sodium tetraphenylborate (80 μM), a lipophilic anion. Catalysis of the uptake of dibenzyldimethyl ammonium by this salt has already been described [14,18]. The stimulation

of [^{3}H]dibenzyldimethyl ammonium uptake by diffusion of internal K^+ was compared with that of ^{45}Ca uptake and of calcium-triggered bioluminescence obtained with the same preparation (Fig. 8).

Other conditions which resulted in an increase of ^{45}Ca uptake were investigated by the method outlined above. The suspension of NaCl lumisomes in KCl or choline chloride buffers gave results which were not conclusive in spite of numerous attempts. More than fifty experiments have been performed to set up conditions of incubation and of filtration and most of the time the data were scattered and non-reproducible. The various trials included the use of [^{3}H]triphenylmethylphosphonium in place of [^{3}H]dibenzyldimethyl ammonium as the lipophilic cation and substitution of chloride by methylsulfate as the counterion. As the background levels were high, other types of filters have been tried, particularly cellulose acetate which has been reported to give better results [19]. Only six experiments indicated a stimulation of the tritiated cation uptake when the sodium lumisomes were suspended in potassium or choline buffers. The stimulation ranged from factors of 1.5 to 11.

In contrast, good results were obtained when the technique was applied to the anion effect. The uptake of [^{3}H]dibenzyldimethyl ammonium by phosphate containing vesicles was higher when they were suspended in chloride than in phosphate buffers (Fig. 7B). The time course of the reaction was short since it stopped after 2 min. At that point the uptake in presence of chloride was 3.8 times higher than in phosphate. If the lumisomes were not pretreated with tetraphenylborate, the enhancement figure dropped to 1.3. The uptake of [^{3}H]dibenzyldimethyl ammonium can be compared with that of ^{45}Ca and the calcium-triggered bioluminescence, which have all been studied under similar conditions and with the same preparation (Fig. 7).

Discussion

The stimulation of Ca^{2+}-triggered bioluminescence by K^+ is reminiscent of the K^+ depolarization of brain synaptosomes, a phenomenon which is also accompanied by uptake of calcium ions [21,22]. Nevertheless the two preparations are different in that the latter is dependent on a metabolic source of energy which is not required in the former. The lack of effect of cyanide and the fact that lumisomes can be kept for days without loosing their discriminating capacity indicate that this property is not metabolically linked. Although some ATPase activity has been detected in lumisome preparations, ATP does not seem to be involved in the bioluminescence flash. Another difference between synaptosomes and lumisomes is that the bioluminescent particles can recover their discriminating property after an osmotic shock, if they are resuspended in 0.6 M NaCl. This suggests that the property is independent of the vesicle content. In fact the cellular origin of the lumisomes is not known in the case of *Veretillum* and their vesicular shape might be the result of the isolation procedure.

It has been shown that KCl can replace internal NaCl in three different ways: (i) by incubating NaCl-containing lumisomes in KCl, (ii) by resealing in KCl media vesicles previously osmotically shocked or (iii) by using KCl buffers during the preparation. The first process is the only one to be time-dependent.

These observations led us to study the properties of vesicles isolated in media of various compositions and various osmolarities assuming that the matrix of the vesicles had the composition of the homogenization buffer.

A striking result was the stimulation of the Ca^{2+}-triggered bioluminescence which was observed when lumisomes isolated in potassium phosphate were diluted in sucrose buffers since it provided evidence that internal sodium is not an absolute requirement for light emission. This stimulation is not an artifact due to calcium precipitation by external phosphate in the control experiment as shown by similar experiments with potassium acetate and by the lack of stimulation observed when the non-permeant choline cation was substituted for potassium phosphate. Neither is the stimulation a result of a lysis induced by the sucrose medium since the nature of the internal cation affects the stimulation of phosphate lumisomes and the decay rate of acetate lumisomes preincubated in sucrose before Ca^{2+} addition (Fig. 4). Stimulation of ^{45}Ca uptake under the same conditions is another evidence against lysis in sucrose. We thus propose that this stimulation is a result of unbalanced internal potassium diffusion. Such a diffusion generates a potential difference (inside negative). The experiments with the lipophilic cation dibenzyldimethyl ammonium provide direct evidence for such potential difference. The uptake of [^{3}H]-dibenzyldimethyl ammonium by K^+ loaded lumisomes diluted in sucrose is to be compared with that observed when K^+ containing membrane vesicles of *Escherichia coli* are suspended in sucrose [18]. Both were greatly enhanced by the lipophilic anion tetraphenylborate, but only the latter required incubation of the vesicles with valinomycin indicating a larger permeability of the lumisomes to K^+. In principle, the [^{3}H]dibenzyldimethyl ammonium concentration gradient is related to potential difference by the Nernst formula. In fact, application of this simple equation could be misleading since this is not an equilibrium situation and some exchange might have occurred during filtration, complicating the measurements of the internal salt concentration. The final concentration gradient for [^{3}H]dibenzyldimethyl ammonium in *E. coli* vesicles was twice that of lumisomes.

We finally propose that the potential difference across the lumisomes membrane is driving the calcium ion influx and thus the bioluminescent flash. The comparable values obtained for ^{45}Ca and [^{3}H]dibenzyldimethyl ammonium uptakes are consistent with this idea. More evidence for this hypothesis comes from the linear increase of light intensity as a function of the log of the ratio of internal and external cations.

Diffusion experiments have given information on the permeability properties of the membrane. Thus phosphate, acetate and cholinium ions appear to be impermeant whereas sodium and potassium are permeant. Chloride is also permeant since addition of Ca^{2+} in sucrose to NaCl lumisomes does not induce a flash of light because of the simultaneous diffusion of the two internal ions. The low permeability of lipidic membranes to large charged anions such as phosphate and methylsulfate or cations such as choline is generally accepted [15,16]. On the contrary, acetate is often a permeant anion [23]. Although some experiments indicate a small permeability of the lumisome membrane to this ion, it seems that the organelle is better characterized by a high permeability to Cl^-. The same property is also shared by postsynaptic vesicles [15] and

sarcoplasmic reticulum fragments [16]. Interestingly, the results also suggest that the membrane is more permeable to Na^+ than to K^+ (Fig. 3).

Once the properties of the membrane are known, it is possible to utilize the same hypothesis to interpret the effect of cations and anions on the Ca^{2+}-triggered bioluminescence. If the vesicles contain a slowly diffusing anion such as phosphate, methylsulfate or acetate and if they are suspended in chloride buffer, the inward diffusion of the latter ion will generate a potential difference across the membrane. It will have the same polarity (inside negative) as that produced by the outward diffusion of a cation. We postulate that this electrical phenomenon is responsible for the Ca^{2+} influx, these ions moving into the vesicles down an electrochemical gradient. Inward chloride diffusion induces a potential difference and a calcium influx as shown by ^{45}Ca and [^{3}H]dibenzyldimethyl ammonium uptake experiments. The two final concentration gradients here again are comparable.

In the anionic effect as in the diffusion potential effect, no sodium gradient was required to stimulate Ca^{2+} influx. The hypothesis proposed to account for these two effects can also explain the cationic effect. The intense light emission which is observed when calcium in isotonic KCl is added to NaCl-containing lumisomes is a result of the large permeability of the membrane to Na^+, in accord with the formulation of a sodium gradient-dependent calcium transport. The outward diffusion of sodium is faster than the inward one of external K^+, thus building an electrical potential difference which drives the Ca^{2+} flux. According to this interpretation the decay of the discriminating capacity of the lumisomes which is observed when they are preincubated in KCl buffer before addition of calcium (Table II) should reflect the decay of the potential difference. As for the diffusion experiments (see Fig. 4) a biphasic time course is observed, the meaining of which is unclear. Another consequence of the hypothesis is that changing the relative positions of the cations (Na^+ being outside and K^+ inside) reverses the polarity of the potential and therefore slows the inward diffusion of Ca^{2+} which now are moving against an electrochemical gradient (Fig. 1).

Surprisingly, the lipophilic cation technique failed to demonstrate a potential difference in this case. It has nevertheless to be pointed out that the results were unconclusive rather than negative and they might reflect an unknown technical difficulty originating from the chosen experimental conditions. In addition the existence of such a potential difference is likely knowing the rates of diffusion of Na^+ and K^+ through the lumisome membrane. Experimental values of 20 and 46 min have been found for the half lifetimes of efflux of Na^+ and K^+, respectively. In these experiments, the same cation was present on the two sides of the membrane and thus exchange rates were measured. The lumisome membrane is different from excitable ones, which are characterized by a greater permeability to potassium than to sodium. For instance, the excitable microsacs of *Electrophorus electricus* have times for half equilibration of 55 and 22 min for Na^+ and K^+, respectively [15].

To our knowledge, the calcium uptake of lumisomes appears to be unique. Mitochondria accumulate Ca^{2+} very efficiently [24] and it has been shown that the ion flux is driven by an electrical potential but in this case the origin of the potential is a respiratory-linked proton gradient. Sarcoplasmic reticulum

vesicles are also able to accumulate calcium against a large concentration gradient but at the expense of ATP. Some anionic effects observed in absence of ATP have also been reported [16] but they resulted in simultaneous increases of the influx and efflux rates, suggesting an electrically controlled change of permeability. Synaptosomes (pinched-off nerve endings) are characterized by a rapid, potassium-dependent calcium influx, but potassium induces depolarization, and not polarization, of the system [22]. Sodium-calcium exchange is another mechanism of calcium uptake which has been shown in synaptosomes [11], but there is some evidence against such a type of mechanism in lumisomes. First, as already pointed out, lumisomes can accumulate calcium in absence of Na^+. Secondly, in the Na-Ca exchange process Ca^{2+} can move against an electrochemical gradient and against a concentration gradient if Na^+ is going downhill. This shows that in this mechanism calcium movements are not necessarily driven by a potential difference as suggested for lumisomes.

Acknowledgements

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