A fluorescence assay for monitoring and analyzing fusion of biological membrane vesicles in vitro

Andres Stutzin

Laboratory of Cell Biology and Genetics, NIADDK, NIH, Building 4, Room 312, Bethesda, MD 20892, USA

Received 30 December 1985; revised version received 8 January 1986

A new technique has been developed to study fusion of biological membrane vesicles. Bovine chromaffin granule ghosts (CGG) were loaded with fluorescein isothiocyanate-dextran (FITC-dextran) at self-quenching concentrations. Loaded ghosts were then made to fuse with empty CGG. Fusion was induced by synexin, a protein previously proposed to be involved in exocytosis. The fusion process was monitored by measuring the dequenching of the fluorescence. Dequenching occurred as FITC-dextran was diluted into the increased volume due to fusion with empty ghosts. Spurious signals from leakage or breakage of vesicles were removed by including a specific anti-fluorescein antibody in the reaction medium. This new technique may prove to be of more general use for studying membrane fusion processes in other systems.

Membrane fusion *Chromaffin granule* *Fluorescein* *Exocytosis* *Synexin*

1. INTRODUCTION

Membrane fusion is a basic property of biological systems and the mechanism(s) of the process has been the subject of intense theoretical [1] and experimental [2] interest. One of the fundamental limitations on fusion studies has been the lack of reliable in vitro assays which might be used both in artificial and biological membranes. One popular approach has been to measure the mixing of labelled phospholipids or lipid-soluble probes which occurs during membrane fusion [3]. However, the interpretation of such experiments might not be straightforward due to the possible exchangeability of the probes [4]. More compelling, in principle, are assays which attempt to monitor the mixing of membrane-enclosed spaces of fusing particles [5,6]. However, until now, such assays have been fraught with technical problems involving leakage to the medium and have been limited mainly to artificial membrane vesicles [7,8].

Abbreviations: PMSF, phenylmethylsulfonylfluoride; DTT, dithiothreitol

As reported here, many of the problems hitherto encountered with such volume mixing experiments have been solved. This was achieved by loading one of the membrane-enclosed compartments with a self-quenching concentration of fluorescein, chemically linked to high-M_r dextran. Upon fusion of the dye-loaded vesicle with an empty vesicle, dilution and dequenching of the probe occur. A specific anti-fluorescein antibody [9] present in the reaction medium has been used to suppress the signals due to leakage. Using this technique it is now possible to follow synexin [10]-induced fusion of chromaffin granule membrane ghosts. This process seems to be highly specific and potentially quite general.

2. EXPERIMENTAL

2.1. *Preparation of chromaffin granules*

Bovine chromaffin granules were prepared by differential centrifugation and purified over a 1.6 M sucrose-density gradient as reported in [11]. The isolation medium was 300 mM sucrose, 40 mM Hepes-K, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, pH 7.2. The final granule suspen-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

sion was stored at −80°C or used immediately for preparing chromaffin granule ghosts (CGG). Protein content was measured as in [12], with bovine serum albumin as a standard.

2.2. *Chromaffin granule ghost preparation*

The chromaffin granules were lysed in a 25-fold volume of either distilled water or 5 mM Tris-maleate, pH 7.2, for 10 min at 4°C, followed by a 30 min incubation at 37°C. The resulting CGG were then washed twice and resuspended in the experimental medium containing 140 mM KCl, 20 mM Hepes-K and 0.1 mM EGTA, pH 7.2.

2.3. *Incorporation of fluorescein isothiocyanate-dextran (FITC-dextran) into CGG*

An aliquot of the CGG was resuspended in 0.5 ml of the experimental buffer containing 0.1 mM FITC-dextran, M_r 20000 (Sigma). Another CGG aliquot was resuspended in the same buffer but without FITC-dextran. Both samples were submerged in liquid nitrogen for 1 min and subsequently allowed to thaw at room temperature. This cycle was repeated 3 times to increase entrapping of the probe by the vesicles [13]. The FITC-dextran-loaded CGG were then washed twice by centrifugation in buffer and subsequently passed through a Sephacryl S-300 superfine column (Pharmacia) to remove the excess dye. The non-loaded vesicles were treated similarly. The protein contents of both samples were adjusted to 0.3 mg/ml.

2.4. *Incorporation of octadecylrhodamine B into CGG*

Octadecylrhodamine B (R_{18}) (Molecular Probes, Junction City, OR) was dissolved at 10 mg/ml in ethanol; the probe was incorporated into the membranes at a 5% molar lipid ratio [4]. The membranes were incubated for 30 min at 37°C and subsequently passed through a Sephadex G-25M column (Pharmacia) to remove the excess R_{18}.

2.5. *Measurement of apparent trapped volume*

A sample of CGG was loaded with a non-quenched concentration of FITC-dextran. The vesicles were separated by centrifugation and the fluorescence of the supernatant and of the pellet was measured. Corrections were done for protein content and for dilution volumes. Under these conditions, the apparent trapped volume was 9.4 μl/mg protein.

2.6. *Anti-fluorescein antibodies*

Anti-fluorescein antibodies were prepared as in [9]. The conjugation protein was keyhole limpet hemocyanin, KLH (Calbiochem, CA), and the molar ratio FITC/KLH was 40:1.

2.7. *Fluorescence measurements*

Continuous monitoring of the fluorescence was carried out in a Spex Fluorolog 2 spectrophotofluorimeter (Spex Industries, Metuchen, NJ), equipped with a digital plotter and a microprocessor (DM1B). For experiments with FITC-dextran, the excitation and emission wavelengths were 465 and 520 nm, respectively. In the R_{18} experiments the respective wavelengths were 540 and 580 nm. A cutoff filter (498 nm for FITC-dextran and 565 nm for R_{18}) was regularly used. The sample chamber was equipped with a magnetic stirrer and the temperature was controlled with a thermostatted circulating water bath.

2.8. *Conditions for fusion experiments*

The medium used was 140 mM KCl/20 mM Hepes-K, pH 6.03, and the experiments were run at 37°C. Free calcium (p*Ca*) and pH were calculated as described [14]. Synexin was prepared as described in [15]. The calibration experiments were run as follows: the vesicles were mixed in a 1:5 ratio (loaded/empty, respectively) in the absence of anti-fluorescein antibodies. The total amount of CGG protein per experiment was 9 μg. When the signal was stabilized, synexin was added. Nonidet P-40 (NP-40, British Drug House; 0.1% final concentration) was then added and the fluorescence at infinite dilution was obtained. The disruption of the vesicles was followed by the addition of small aliquots of the anti-fluorescein antibodies, to quench the fluorescence to background levels. In each fusion experiment, the vesicles were mixed at the above mentioned ratio simultaneously with an excess amount of anti-fluorescein antibodies, as calculated from the calibration experiments. Once the system was stabilized, synexin was added. NP-40 was added at the end of the experiment to confirm that the concentration of anti-fluorescein antibodies was adequate to correct for any leakage.

3. RESULTS

3.1. *Loading the chromaffin granule ghosts with FITC-dextran*

The optimal concentration of FITC-dextran incorporated into the CGG for measuring dilution-dependent dequenching of fluorescein fluorescence was approx. 100 μM (fig.1). CGG loaded at such a concentration showed the maximum degree of fluorescence dequenching induced by the addition of the detergent. The fluorescence dequenching was linear between 10 mM and 10 μM.

3.2. *Detecting fusion of FITC-dextran-loaded ghosts with empty ghosts*

FITC-dextran-loaded ghosts were mixed with a 5-fold mass excess of unloaded ghosts. As shown in fig.2, no changes in fluorescence of the system

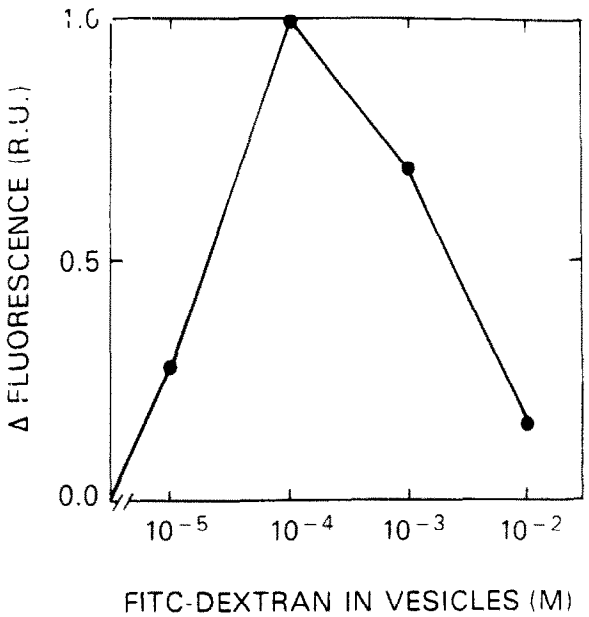

Fig.1. Self-quenching property of FITC-dextran inside vesicles. The ordinate represents the change in fluorescence as a function of the FITC-dextran concentration entrapped in the vesicles. Each point is obtained by measuring the fluorescence output of a sample of FITC-dextran-loaded CGG after and before the addition of NP-40 (infinite dilution) and plotting the difference. The actual fluorescence increase is about 2-fold. The optimal concentration is ~100 μM. As inferred from this curve, the fluorescence yield per molecule gives a monotonically increasing curve.

(zero-time) were recorded. Pure synexin was then added to this membrane mixture and a biphasic increase in fluorescence (~2-fold) was detected thereafter (fig.2, upper trace). The system reached equilibrium in ~2 min and remained stable for up to 15 min. Further addition of NP-40 (detergent) disrupted the membranes, diluted the entrapped probe, and raised the fluorescence to the maximum unquenched level. Under these experimental conditions the estimated extent of fusion was >70%. The possibility that the recorded increase in fluorescence was due to light scattering was ruled out by independently measuring the change in the absorbance of the sample throughout the experiment. After the addition of synexin, the change measured at the excitation wavelength was 0.01 absorbance units, a change of only 5%. The synexin-induced fusion was highly specific, since no fusion was detected using bovine serum albumin or non-specific IgG (not shown).

A prime concern in experiments such as these is the contribution of leakage to the signal. This was tested by inclusion of anti-fluorescein antibodies in the reaction medium, since such antibodies are reported to quench entirely the fluorescence when binding to the ligand [9]. This was also the case in this system as shown in the inset in fig.2. In the case of synexin-induced fusion the anti-fluorescein antibodies decreased the extent of the fluorescence dequenching induced by synexin by only ~10%. By contrast, it rapidly quenched ~95% of the fluorescence of the system when detergent was subsequently added. It thus appeared that the majority of the signal induced by synexin indeed indicated fusion, while only a small portion of the dequenching signal seemed due to simple leakage. However, it was also clear that inclusion of anti-fluorescein antibodies in the reaction mixture seemed to eliminate entirely even the small contribution of leakage to the signal.

Further analysis of the leakage showed that this event had a fast initial rise, which paralleled the initial event of fusion, and subsequently almost remained constant. The time constant for the leak was less than 5 s, compared to that for the slow component of the fusion event which averaged ~100 s. Also, no kinetic differences were detected with or without the anti-fluorescein antibodies, suggesting that the antibody did not affect the reaction per se and that leakage was expressed as

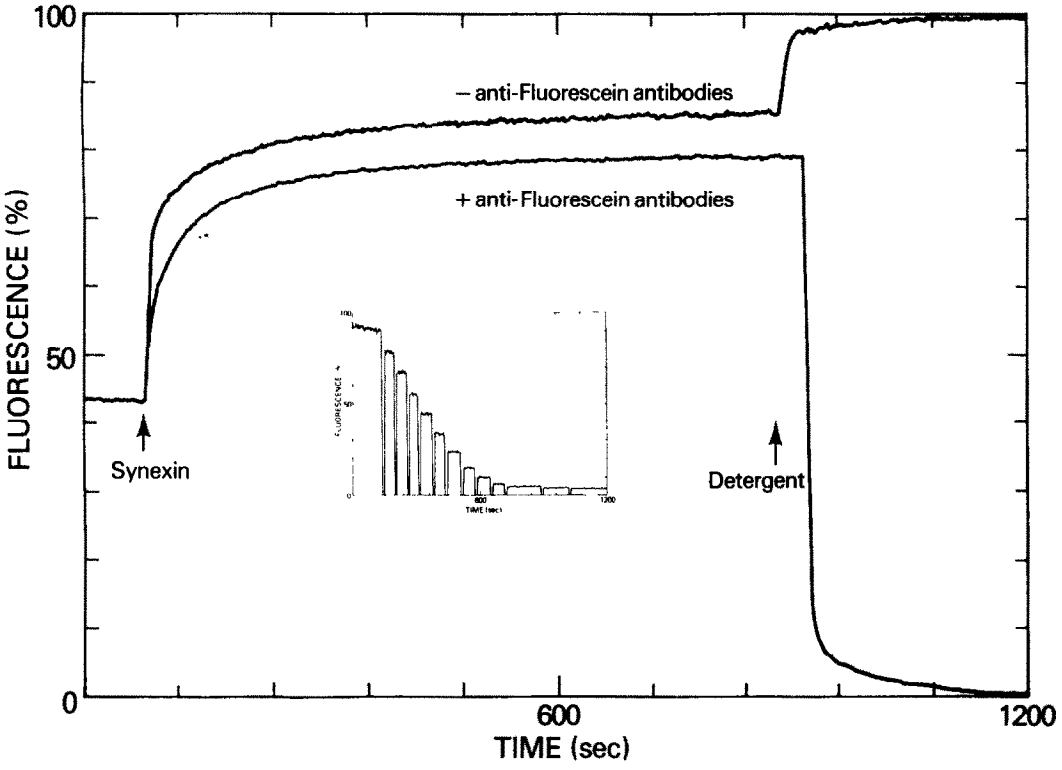

Fig.2. Synexin induces fusion of CGG. The increase of fluorescence due to the addition of synexin (34.08 μg) (first arrow) is shown in the absence and presence of the anti-fluorescein antibody. In this experiment the medium is 140 mM KCl and 20 mM Hepes-K (2 ml total volume), pH 6.03, p*Ca* 7, at 37°C. The absorbance of the sample was adjusted to 0.2 *A* units at the beginning of the experiment. After the addition of NP-40 (second arrow) the fluorescence either increased (calibration experiment, upper trace) or decreased (fusion experiment, lower trace). The anti-fluorescein antibody induced quenching is a fast event, at least for ~95% of the original fluorescence. The acquisition time was 1 s. Calibration experiments (n = 2) and fusion experiments (n = 3) were averaged point-by-point. The experiments considered for analysis showed a variability less than 5%. Correction for leakage was done by subtracting both averaged signals. The leakage component is ~10%. Inset: determination of the antibody activity. Small aliquots (1.2 μl) of the anti-fluorescein antibodies were added to a 65 μM FITC-dextran solution. Each addition is shown by a decrease in the fluorescence and the final dilution of the antibody is 1/1666. The inhibition obtained is greater than 95%.

an increase in the final extent of fluorescence. Furthermore, substitution of the anti-fluorescein antibodies by a non-specific rabbit IgG had no effect on the fusion reaction. Thus, inclusion of the anti-fluorescein antibodies in the reaction medium provides an adequate correction for the leakage.

3.3. *Detecting fusion by an alternate membrane-probe transfer assay*

In the past, fusion of biological membranes has often been followed by studying the transfer of lipid-soluble probes from one membrane surface to another [3]. For example, high concentrations of R_{18} can be used to label a membrane such that the fluorescence of R_{18} is self-quenched [4]. Upon mixing with native non-labelled membranes R_{18} diffuses into the native membranes thereby being diluted and thus unquenched in terms of fluorescence. To test if the mixing of contents was correlated with the mixing of membranes, independent experiments were run with R_{18}-labelled CGG mixed with non-labelled CGG. The experimental conditions were exactly as described for the previous experiments. No spontaneous change in fluorescence was detected, and neither bovine serum albumin nor non-specific IgG was able to induce any fusion. However, upon addition of synexin (fig.3, trace b) a prompt increase in

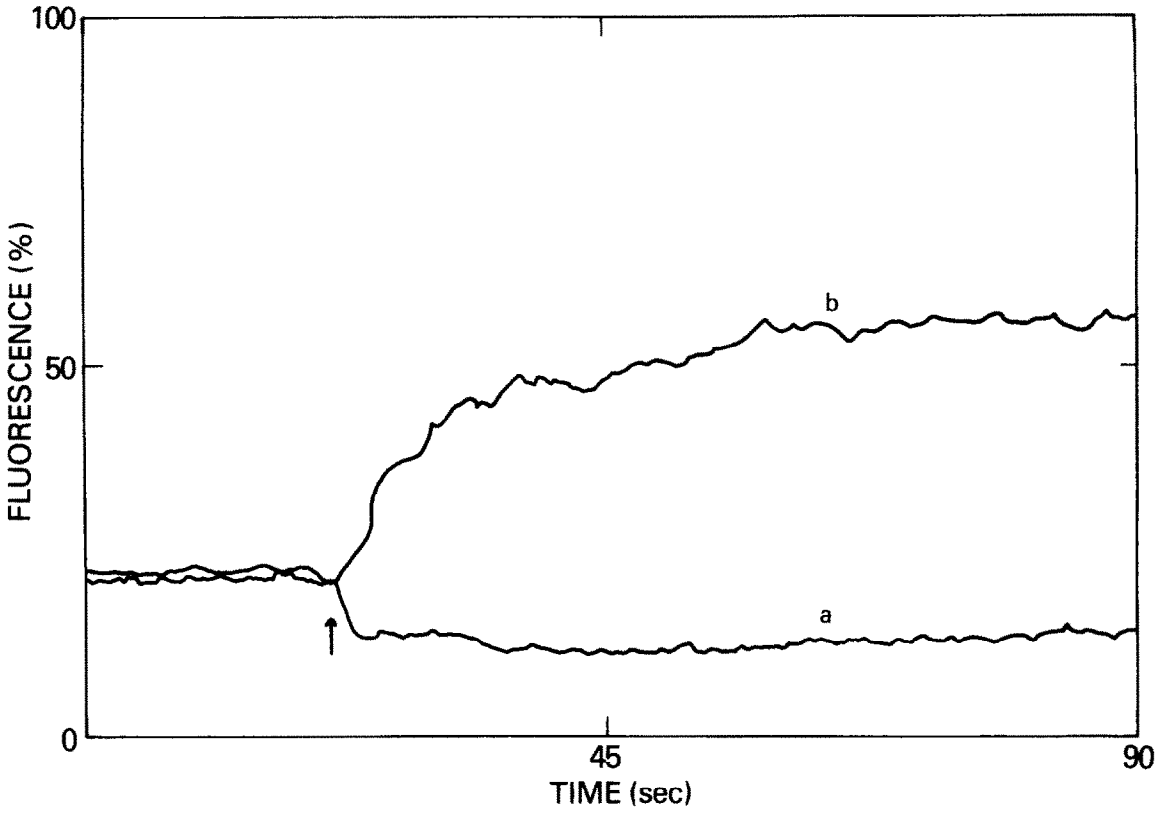

Fig.3. Synexin induces fusion of CGG as determined by the membrane-lipid (R_{18}) transfer experiments. R_{18} molecules were incorporated into the CGG membranes at a 5% molar lipid ratio. The buffer and the temperature were the same as described for the FITC-dextran experiments, as well as the number of experiments and the variability criterion. Trace (a) shows the effect of synexin (34.08 μg) (arrow) when added to labelled vesicles. The fluorescence decreases slightly. Trace (b) depicts the effect of synexin when added to a mixture (1:5) of labelled and blank CGG vesicles. Synexin in this case enhances the fluorescence.

fluorescence was observed, being essentially complete in 1 min. By comparison, the same experiment performed in the absence of non-labelled CGG (fig.3, trace a) resulted in a modest, immediate and sustained reduction of fluorescence. Analysis of the data showed that >80% of the vesicles fused, which indicates a high proportion of active CGG in terms of their fusing capabilities. The data also suggest that in synexin-induced membrane fusion, membrane mixing may occur slightly faster than mixing of internal volumes.

4. DISCUSSION

Fusion of chromaffin granule membrane vesicles can now be directly studied by detecting the mixing of vesicle volumes. The basic principle for this technique rests on the fact that FITC-dextran can be incorporated into vesicles at a self-quenched concentration, and that fusion of loaded vesicles with empty vesicles results in dilution and dequenching of fluorescence. This general approach has several advantages for measuring biological membrane fusion. First, spontaneous leakage before, during and after the fusion event, or leakage across juxtaposed, but not fused, membranes is minimized by the large size and charge of the FITC-dextran molecule. Second, the availability of anti-fluorescein antibodies for inclusion in the reaction mixture allows precise control of the contribution of leaked contents to the fusion signal. Finally, the intactness of the system for small molecules can be evaluated by taking advantage of the pH dependence of fluorescein

fluorescence. For example, adding Mg-ATP to the reaction medium, hence activating the inwardly directed H^+ pump, leads to further quenching of the dye (not shown).

The data reported here also may yield information about separate events occurring during fusion. Fusion of membranes is a complex process and in biological systems usually not spontaneous [16]. One example of fusion of native membranes has been demonstrated with red blood cell ghosts [17]; however, under normal circumstances they do not undergo fusion. In contrast, chromaffin granule membranes do naturally fuse with plasma membranes and with each other during exocytosis from chromaffin cells, and synexin has been considered one of the substances involved in membrane contact and fusion in this system [18].

Therefore, it may not be surprising that synexin supports fusion of chromaffin granule membrane ghosts. However, the simple experiments described here have demonstrated at least 3 separate kinetic events occurring during fusion in this system. The most rapid event is a leak of granule contents, corresponding to ~10% of the total signal, over in less than 5 s. Leakage could be directly induced by the fusion reaction, i.e. a transient spillover of the vesicle contents during the fusion event. Another possibility, however, is that synexin may induce some degree of leakage and/or lysis of vesicles which do not actually fuse. The latter possibility is indirectly supported by the observation that synexin is indeed able to induce leakage of CGG loaded with carboxyfluorescein (Stutzin, A. and Lelkes, P.I., unpublished).

A less rapid event during fusion seems to be the mixing of membrane lipid components, using R_{18} as a detector, which occurs in ~10 s. Finally, the volume mixing event seems to be slower, and shows a complex behaviour, reaching steady-state in ~100 s. While the membrane mixing event is measured by a separate chemical system, the leak and the volume mixing are both detected using FITC-dextran. Thus, the apparent kinetic differences between the latter two systems are probably related to the process, not to the detectors.

These differences do make mechanistic sense and serve to validate further the fusion system reported here. Leakage as an intrinsic component of fusion is not unknown as a process in biological membrane fusion. Arachidonic acid-induced fusion of synexin-aggregated CGG is accompanied by a leak of 10% protein and 30% catecholamines [19]. In human platelets loaded with mepacrine, stimulation of secretion results in secretion of mepacrine. However, intracellular leakage from secreting platelet dense bodies has been also observed microscopically (Pollard, H.B. and Allen, R.D., joint observation, Dartmouth College, 1978).

The technique presented here may prove to be of more general use for studying membrane fusion processes in other systems which could operate in vivo and also may yield further information regarding this specific synexin-dependent fusion process.

ACKNOWLEDGEMENTS

The author is indebted to Dr Harvey B. Pollard and Dr E. Rojas for conceptual and experimental suggestions and for their criticism, to Dr Ioav Cabantchik for his critical comments and help in preparing the anti-fluorescein antibodies, and to Dr Peter I. Lelkes for his help and advice throughout the experimental work, mainly with the R_{18} experiments. I also wish to thank Mrs Louise Hickman for the preparation of the chromaffin granules and Mrs Diane Seaton for the preparation of synexin.

REFERENCES

[1] Nir, S., Bentz, J., Wilschut, J. and Duzgunes, N. (1983) Prog. Surf. Sci. 13, 1–124.
[2] Blumenthal, R. (1985) Curr. Top. Membranes Transp. 27, in press.
[3] Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099.
[4] Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) Biochemistry 23, 5675–5681.
[5] Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692.
[6] Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489.
[7] Wilschut, J., Duzgunes, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021.
[8] Papahadjopoulos, D., Poste, G. and Vail, W.J. (1979) Methods Membrane Biol. 10, 1–121.

[9] Darmon, A., Bar-Noy, S., Ginsburg, H. and Cabantchik, Z.I. (1985) Biochim. Biophys. Acta 817, 238–248.
[10] Creutz, C.E., Pazoles, C.J. and Pollard, H.B. (1978) J. Biol. Chem. 253, 2858–2866.
[11] Smith, A.D. and Winkler, H. (1967) Biochem. J. 103, 480–482.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[13] Straus, G. (1984) in: Liposome Technology (Gregoriadis, G. ed.) vol.I, pp.197–219, CRC Press, Boca Raton, FL.
[14] Portzehl, H., Caldwell, P.C. and Ruegg, J.C. (1964) Biochim. Biophys. Acta 79, 581–591.
[15] Scott, J.H., Kelner, K.L. and Pollard, H.B. (1985) Anal. Biochem. 149, 163–165.
[16] Wilschut, J. and Hoekstra, D. (1984) Trends Biochem. Sci. 9, 479–483.
[17] Hoekstra, D., Wilschut, J. and Scherphof, G. (1983) Biochim. Biophys. Acta 732, 327–331.
[18] Creutz, C.E., Pazoles, C.J. and Pollard, H.B. (1979) J. Biol. Chem. 254, 553–558.
[19] Creutz, C.E., Scott, J.H., Pazoles, C.J. and Pollard, H.B. (1982) J. Cell Biochem. 18, 87–97.