Interaction of the Fluorescent Probe 1,6-Diphenyl-1, 3, 5-Hexatriene with Biomembranes

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Abstract. The relationship between the conditions of membrane labelling by the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and its fluorescence parameters was investigated. In the labelling solutions prepared by the usual method, the presence of DPH microcrystals was revealed which led to the lower resultant fluorescence anisotropy values. Lower labelling efficiency was observed with DPH solutions in tetrahydrofuran when compared with solutions in acetone. Modifications of the labelling procedure are proposed which give better reproducibility of the results. There modified method involves the preparation of a 2×10^{-4} mol . l^{-1} DPH stock solution in acetone, a 100-fold dilution in an appropriate buffer, subsequent bubbling through with nitrogen for 30 min and mixing the resulting solution with cell/membrane suspension in a 1 : 1 (v/v) ratio. Changes in intensity, anisotropy and spectra of DPH fluorescence in the course of membrane labelling were studied. A two-stage model of the incorporation of DPH into membranes was proposed, according to which DPH molecules first quickly adhere to the membrane surface and then are slowly translocated to the apolar regions of the membrane.

Key words: Fluorescence polarization — Diphenylhexatriene — Erythrocyte ghost — Membrane fluidity — Spectrofluorometry

Introduction

Since Shinitzky and Barneholz introduced 1,6-diphenyl-1,3,5-hexatriene (DPH) on a wide scientific forum, it has become the favourite fluorescent probe for the structural and dynamic studies of hydrophobic regions in biological membranes. The suitable spectroscopic properties of the probe, the relatively simple experimental equipment needed and illustrative interpretation of results prompted a plethora of papers on steady-state fluorescence anisotropy of DPH in connection with the concept of the so called membrane fluidity (for a review, see

Shinitzky and Barenholz 1978; Shinitzky and Henkart 1979; Shinitzky and Yuli 1982; Shinitzky 1984).

For any fluorescent probe used in dynamic or structural studies it generaly holds that the more we learn about the probe, about its chemical and spectral properties and about its interaction with the object of interest, the more information on the dynamics and architecture of the object studied can be obtained.

This is also the case of DPH. Nowadays several dozens of theoretical papers are available dealing with the interpretation of fluorescence anisotropy values, and proposing several hundred applications of the DPH fluorescence polarization method. Nevertheless, we still do not know the exact localization of DPH either in the membrane or in the cell. Moreover, the interaction of DPH with the membrane during sample labelling and the process of its incorporation into the membrane have not been clarified as yet (Shinitzky and Barenholz 1974; Inbar et al. 1974; Shinitzky and Inbar 1976).

According to the original articles by Shinitzky and coworkers (Shinitzky and Barenholz 1974; Inbar et al. 1974), DPH enters the membrane with a simple saturable kinetics and, in the steady-state, it is localized in the hydrophobic core of the membrane. Since then several other papers have appeared that have called in question the original idea of the exclusive localization of DPH molecules in the centre of the lipid bilayer (Mély-Goubert and Freedman 1980) and, what is even more serious, also the idea of the localization in the plasma membrane only (Bouchy et al. 1981; Van Hoeven et al. 1979). These studies suggested that DPH not only resides in the plasma membrane but that it enters the cell and is incorporated into the membranes of the cellular organelles and into the intracellular lipid droplets. DPH fluorescence from the intracellular membranes and lipidis is less polarized. Owing to this, the decrease of fluorescence anisotropy during the first 10—15 min of fluorescent labelling, as observed by Bouchy et al. (1981) and Van Hoeven et al. (1979), has been explained as resulting from the incorporation of DPH into the intracellular membranes and lipids. Furthermore, Van Hoeven et al. (1979) observed that some growth in DPH fluorescence intensity in transformed murine cells may persist even after the removal of the free probe from the suspending medium.

As far as the preparation of samples for routine assessment of membrane fluidity is concerned, the situation is similar. The majority of authors adhere to the membrane labelling procedure proposed by Inbar et al. (1974). The usual labelling technique consists of a 1000-fold dilution of a 2×10^{-3} mol . l^{-1} stock solution in tetrahydrofuran (THF) in an appropriate buffer and subsequent mixing of 1 volume of cell/membrane suspension with 1 volume of DPH dispersion.

Obviously, the results of membrane labelling may be influenced by the

condition under which the hydrophobic probe, DPH is introduced into the water medium. However, the most suitable labelling procedure has not been discussed in more detail as yet, except in a paper by Lakowicz and Sheppard (1981).

The aim of the present paper is to investigate in detail the changes in intensity, anisotropy and spectra of DPH fluorescence in the course of membrane labelling and to study the relationship between the technique of preparation of the solution for membrane labelling (further labelling solution) and the resulting DPH fluorescence parameters.

Materials and Methods

Chemicals

DPH from three different sources (Koch-Light, Fluka and ICN Pharmaceuticals) was used. Since no difference in fluorescence properties were found, only DPH manufactured by ICN Pharmaceuticals (scint.grade) was used. Tetrahydrofuran (THF) and acetone were from Lachema Czechoslovakia in further experiment. THF (analytical grade) was freshly distilled before use. Acetone for UV spectroscopy was used without purification.

Membrane preparations

Erythrocyte ghosts were obtained from Dr. Mařík (Institute of Hematology and Blood Transfusion, Prague). The ghosts were prepared according to Dodge et al. (1963). The isolated membranes were stored in PBS at 4 °C.

Saccharomyces cerevisiae T 158 C yeast strain was obtained from Dr. Vondrejs (Faculty of Science, Charles University, Prague). The cells were cultured for 2 days at 20 °C in a medium containing 0,5 % yeast extract and 1 % glucose. The medium was buffered at pH 4.7 with 5×10^{-3} mol. l^{-1} citric acid and K_2HPO_4, (C-P buffer). All samples were washed with C-P buffer before the measurements.

Human peripheral lymphocytes were obtained from Dr. Haškovec (Institute of Hygiene and Epidemiology, Prague). After separation on a discontinuous Ficoll 400 (Pharmacia) — Verografin (Spofa) density gradient, the lymphocytes were maintained in Eagle's minimal essential medium with 1×10^{-3} mol. l^{-1} Hepes, pH 7.2 — 7.3 (ÚSOL). Prior to the measurements, the cells were washed and resuspended in PBS.

DPH solutions

Concentrated stock solutions of DPH in THF and acetone (2×10^{-3} and 2×10^{-4} mol. l^{-1}) were prepared. Labelling solutions of DPH in PBS were prepared by a 1000-fold dilution of a 2×10^{-3} mol. l^{-1} or by a 100-fold dilution of a 2×10^{-4} mol. l^{-1} stock solution. For spectroscopic studies of the fluorescence of DPH dissolved in water, a standard solution was obtained by dissolving DPH in distilled water for a long time. The dissolution of some DPH in water was indicated by an increase in UV absorption and fluorescence intensity. The concentration of DPH in this saturated DPH water- solution was not determined.

Fluorescent labelling of the cells/membranes

The erythrocyte ghost suspensions were labelled as follows: (I) 1 μl of 2×10^{-3} mol. l^{-1} stock solution of DPH was added directly into 2 ml of diluted ghost suspension (40 μg protein per ml). The

final concentration of the probe in the sample was 1×10^{-6} mol . l^{-1}. (II) Ghost suspension (80 μg of protein per ml) was mixed 1 : 1 with 2×10^{-6} mol . l^{-1} DPH labelling solution prepared by a 1000-fold dilution of 2×10^{-3} mol . l^{-1} stock solution. (III) Ghost suspension (80 μg of protein per ml) was mixed 1 : 1 whith 2×10^{-6} mol . l^{-1} DPH labelling solution prepared by a 100-fold dilution of 2×10^{-4} mol . l^{-1} stock solution. Yeast cells and lymphocytes were labelled by procedure III. With stock solutions of low concentrations, a high amount of organic solvent is added to the sample (up to 0.5 % of the total volume). To avoid the disturbing effects of organic solvents on membranes, the solvent should be removed from the labelling DPH solution before mixing it with the cell suspension. In case of acetone, this can easily be achieved by heating it to 60 °C or by bubbling the labelling solution with nitrogen.

Fluorescence measurements

Fluorescence measurements were performed with two fluorescence spectrometers. The weak fluorescence spectra of DPH in water were measured by an EG & G OMA 2 optical multichannel analyzer equipped with SIT 1254 detector and a Jobin Yvon HR 320 S polychromator (the spectral resolution was 0.5 nm per channel). The luminiscence of a sample placed at the thermostatted holder was excited at $\lambda = 365$ nm by a Narva XB0 500 xenon lamp using a Carl Zeiss Jena SPM-1 monochromator. The optical multichannel analyzer enabled us to separate precisely the spectra of the very weak DPH fluorescence in water media from the stray light background.

Changes in intensity and fluorescence polarization during the process of membrane labelling were studied with a spectrofluorometer consisting of a Narva HB0 200 high pressure mercury lamp, two Carl Zeiss Jena SPM-2 monochromators with added emission wavelenght scanning, a thermostatted sample holder, an EMI 9789QB photomultiplier and a Keithley 610 C electrometer. Carl Zeiss Jena dichroic filters were used as the excitation polarizer and emission analyzer. DPH fluorescence was excited at $\lambda = 365$ nm, changes in both fluorescence intensity and anisotropy were monitored at $\lambda = 430$ nm. The spectral bandwidth of the emission monochromator was 2 nm and 6 nm for the measurement of emission spectra and intensity changes, respectively. The measured fluorescence signal was either plotted on an X-Y plotter or collected on-line by a Commodore CBM 4032 minicomputer which performed both computation of fluorescence anisotropy and correction of emission spectra. In addition to the measurement of the average fluorescence intensity in macroscopic samples, the differences in fluorescence properties of individual membranes were visually estimated with a Carl Zeiss Jena Fluoval II fluorescence microscope.

Results

Emission spectra of DPH solutions

The spectrum of very weak fluorescence of saturated DPH solution in water consists of a single broad band with maximum at (448 $\pm$ 3) nm (Fig. 1*B*). It differs substantially from the well-known spectra of DPH fluorescence in membranes and organic solvents of low polarity (see e. g. Cehelnik et al. 1975; Nikitina et al. 1977). For comparison, the fluorescence spectra of 1×10^{-6} mol . l^{-1} DPH solutions in THF and hexane are shown in Fig. 1*A*. These spectra exhibit a distinct vibronic structure, the wavenumber of the vibrations observed being (1350 $\pm$ 70) cm^{-1}.

In Fig. 1*C* the spectra of DPH fluorescence in the different labelling solutions are shown. The spectrum of 2×10^{-6} mol . l^{-1} DPH in water, measured for samples prepared by a 100-fold dilution of 2×10^{-4} mol . l^{-1} DPH in acetone

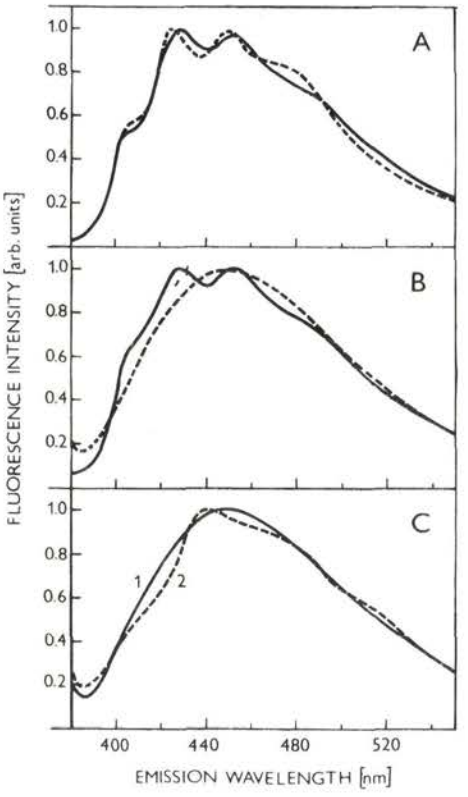

Fig. 1. Corrected emission spectra of DPH fluorescence in solutions. *A*) 2×10^{-6} mol . l^{-1} DPH in hexane (– – –), 2×10^{-6} mol . l^{-1} DPH in tetrahydrofuran (——). *B*) Saturated water solution, concentration undertermined (– – – –); suspension of DPH aggregates prepared by mixing 0.1 ml of 2×10^{-3} mol . l^{-1} DPH stock solution in acetone, with 0.9 ml water (———). *C*) 2×10^{-6} mol . l^{-1} DPH in PBS; 100-fold dilution of the 2×10^{-4} mol . l^{-1} DPH stock solution in acetone (——); 1000-fold dilution of the 2×10^{-3} mol . l^{-1} DPH stock in acetone (– – –).

stock solution, resembles closely the fluorescence of the standard solution of DPH in water (see Methods: DPH solutions). In 2×10^{-6} mol . l^{-1} aqueous solution of DPH prepared by diluting the concentrated stock solution of DPH (2×10^{-3} mol . l^{-1} DPH in acetone), the fluorescence spectrum exhibits, to a certain degree, a fine structure which is not characteristic of DPH fluorescence in the standard aqueous DPH solution.

When DPH concentration in water medium exceeds significantly the saturation level, the sample becomes turbid due to the appearance of DPH aggregates. The fluorescence spectrum of DPH aggregates is shown in Fig. 1*B*. It was measured in a highly turbid sample, prepared by adding 0.1 ml of 2×10^{-3} mol . l^{-1} solution of DPH in acetone to 0.9 ml of distilled water. The sharp vibronic structure is typical of the fluorescence spectra of DPH aggregates.

Obviously, the weak vibronic structure of the fluorescence spectra of DPH labelling solutions may be due to a contribution of DPH aggregates. To estimate this contribution, fluorescence spectra $F(\lambda)$ of various DPH labelling solutions were analyzed in terms of a superposition of normalized fluorescence spectra of

the standard DPH water solution $F_w(\lambda)$ and of that of DPH aggregates $F_a(\lambda)$. By the leasts-squares fitting we got $F(\lambda) = 0.02\,F_a(\lambda) + 0.98\,F_w(\lambda)$ for curve (1) in Fig. 1*C*, and $F(\lambda) = 0.17\,F_a(\lambda) + 0.83\,F_w(\lambda)$ for curve (2) in Fig. 1*C*. This means that the relative contribution of DPH aggregates to spectra (1) and (2) is different. An increased concentration of DPH aggregates must be considered for spectrum (2).

Effect of the labelling procedure on the intensity and anisotropy of DPH fluorescence in membranes

Suspensions of erythrocyte ghosts were labelled with DPH stock solutions in acetone or THF. Since there was no difference between the samples labelled with DPH stock solutions in acetone or freshly distilled THF, only the results obtained with DPH stock solutions in acetone are presented here.

The erythrocyte ghosts were labelled at 25 °C or at 37 °C using three different procedures as described in Methods.

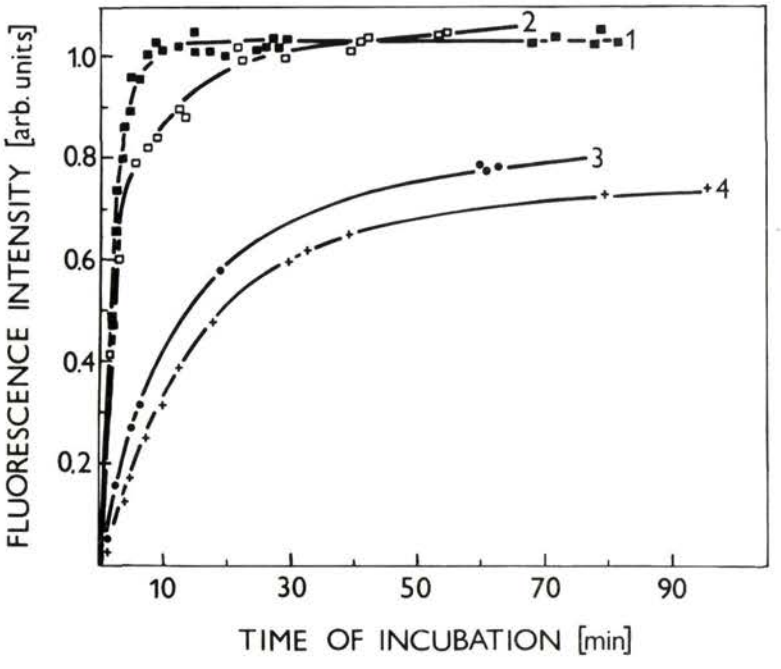

Fig. 2. Increase in DPH fluorescence intensity during labelling of erythrocyte ghosts. Curves 1 and 2: ghosts labelled according to procedure I at 37 °C and 25 °C, respectively; curve 3: ghosts labelled according to procedure II at 37 °C; curve 4: ghosts labelled according to procedure III at 37 °C.

The effect of the labelling procedure on the probe uptake was judged from the increase in DPH fluorescence in the course of labelling and from the anisotropy of equilibrium fluorescence. It was found that the choice of the labelling protocol may influence both the intensity and anisotropy of DPH fluorescence (Fig. 2).

The steepest increase in the fluorescence intensity of the probe was observed with the concentrated DPH stock solution diluted in the membrane suspension (procedure I). This labelling procedure also yielded the highest intensity of equilibrated fluorescence. Membrane labelling at 25 °C or 37 °C gave similar results (curves 1 and 2). Moreover, there was a small but important difference in fluorescence anisotropies in membranes labelled by the three different procedures. In particular, at 37 °C, the anisotropy of equilibrated fluorescence in membranes labelled by procedure III was $r = 0.217 \pm 0.005$. Procedures I and II gave lower values of equilibrated fluorescence anisotropy, $r = 0.205 \pm 0.005$ and $r = 0.207 \pm 0.005$, respectively.

The measurements of mean intensity and anisotropy of DPH fluorescence of membrane suspensions were supplemented by microscopic observations of fluorescence of labelled erythrocyte ghosts. No apparent differences in the intensity of fluorescence from individual membranes were observed with samples labelled by procedure III. A quite different picture was typical of samples labelled by procedure I. Fluorescence from most membranes was considerably weaker than that typical of membranes labelled by procedure III. Among these weakly fluorescing membranes, a few bright objects could be observed. They made up a low proportion of the total number of membranes. The fluorescence intensity of the weakly labelled membranes was insufficient to be photographically recorded.

Changes in DPH fluorescence parameters during membrane labelling

Fluorescence intensity. The course of membrane labelling by DPH was studied with erythrocyte ghosts, *Saccharomyces cerevisiae* yeasts and human lymphocytes. In addition to the usual increase in probe fluorescence during approximately 90 min of incubation of membranes with DPH, an extraordinary increase in DPH fluorescence was found after the removal of the free probe from the labelling solution.

This effect was observed with samples treated as follows: The membrane suspension was incubated with 1×10^{-6} mol . l^{-1} DPH at 21 °C for 4 min. Subsequently, the cell suspension was centrifuged and the supernatant removed to eliminate most of the free DPH. After resuspending the cells in a pure buffer, the concentration of the free probe remaining in resuspended samples was less than 10^{-8} mol . l^{-1}.

In erythrocyte ghosts (20 μg of protein in 1 ml of membrane suspension) and yeast cells (10^6 cells/ml) the fluorescence intensity in resuspended samples grew further to reach about 60 % of that measured in samples continuously incubated with 1×10^{-6} mol . l^{-1} DPH (Fig. 3). A similar effect, though less pronounced, was observed with lymphocytes.

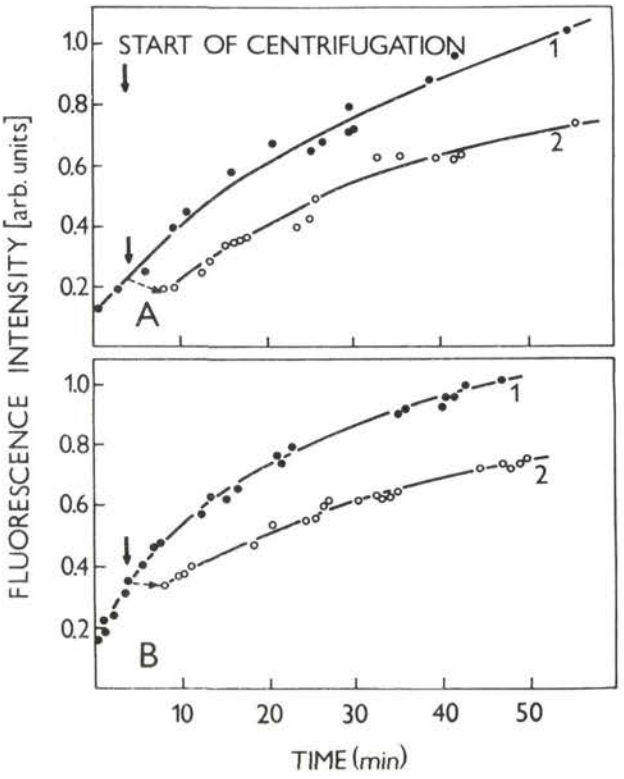

Fig. 3. Rates of DPH labelling of yeast cells (*A*) and erythrocyte ghosts (*B*) upon continuous incubation of membranes with DPH (●●●) and after the removal of free DPH from the cell suspension (○○○).

Fluorescence anisotropy. Evolution of DPH fluorescence anisotropy during membrane labelling was studied in lymphocytes (10^6 cells/ml) and erythrocyte ghosts (20 μg protein in 1 ml suspension). In both cases, a distinct decrease of the probe fluorescence anisotropy was observed in the early stages of membrane labelling (Fig. 4). The erythrocyte ghosts at 25 °C exhibited an initial fluorescence anisotropy $r = 0.27$ after 1 min of incubation. After 15 min of incubation, the DPH fluorescence anisotropy decreased to a value close to the

final plateau of $r = 0.21$. A similar effect was observed with lymphocytes at 37 °C, the fluorescence anisotropy being $r = 0.20$ after 3 min and $r = 0.14$ after 40 min of incubation. At 21 °C, there was only a slight decrease of DPH fluorescence anisotropy in lymphocytes. For the cell concentrations in our sample, the fluorescence depolarization due to both excitation and emission light scattering was negligible.

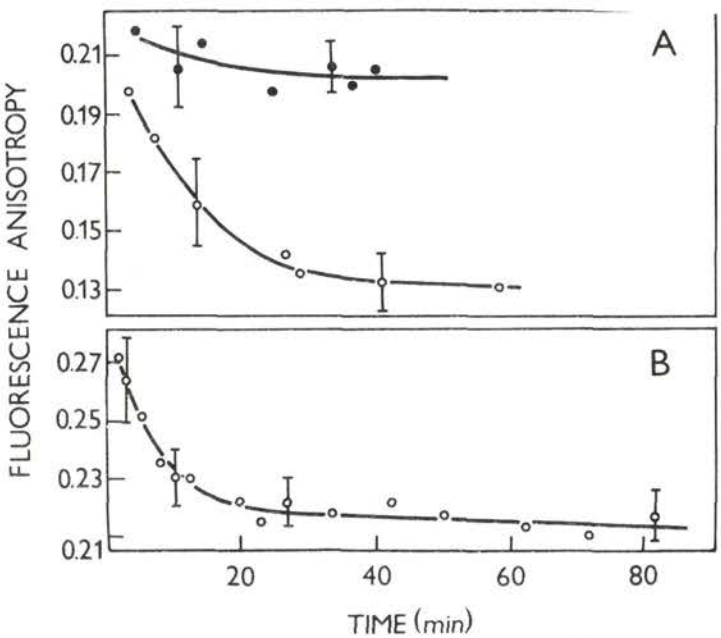

Fig. 4. Time course of DPH fluorescence anisotropy during membrane labelling: (*A*), human peripheral lymphocytes at 21 °C (●●●) and 37 °C (○○○), (*B*) human erythrocyte ghosts at 25 °C

Emission spectra. The suspension of erythrocyte ghosts (100 μg of protein in 1 ml suspension) was incubated in a 1×10^{-6} mol . l^{-1} DPH solution PBS at 20 °C. Using OMA 2, an optical multichannel analyzer, the fluorescence spectra were recorded at different times after mixing the membranes with the probe. A gradual blurring of the vibrational structure of the DPH fluorescence spectrum was observed during the incubation. This effect is illustrated in Fig. 5 by the spectra measured after 1 and 35 min of incubation. The spectra presented were corrected for blank fluorescence and normalized.

Discussion

In the fluorescence spectra of aqueous labelling solutions of DPH prepared from concentrated ($\sim 10^{-3}$ mol . l^{-1}) DPH stock solutions a contribution of

structured fluorescence of DPH aggregates was observed. This indicated the presence of higher amount of DPH aggregates in these samples. On the other hand, the fluorescence spectroscopy indicated only negligible amounts of such aggregates in the labelling solutions prepared from 2×10^{-4} mol . l^{-1} DPH stock solutions.

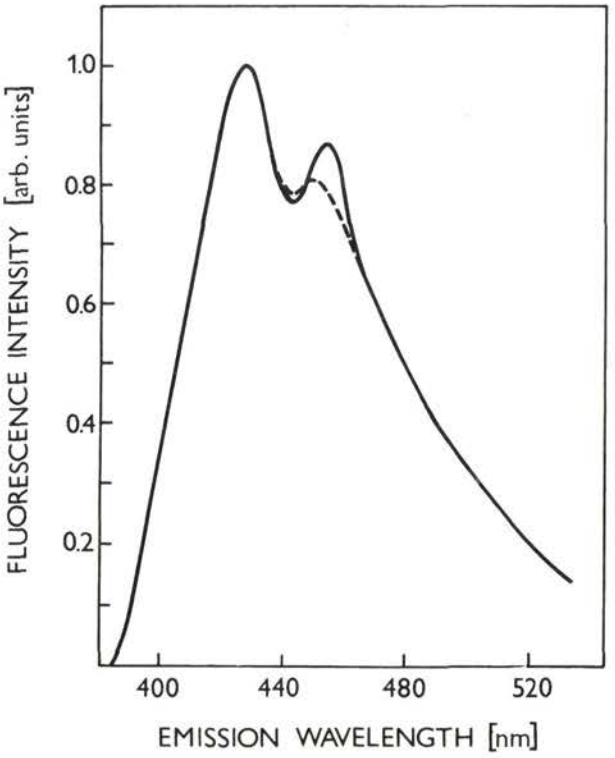

Fig. 5. Emission spectra of DPH fluorescence in human erythrocyte ghosts after 1 min (——) and 35 min of incubation (– – –).

We tried to estimate to what extent the formation of probe aggregates would influence the resulting fluorescence parameters of labelled membranes. In comparing the fluorescence parameters of erythrocyte ghosts labelled by procedures I, II (higher amounts of aggregates in the labelling solution) and III (low amounts of aggregates in the labelling solution), lower fluorescence anisotropy is measured in samples labelled by both procedure I and II. Moreover, in these samples the fluorescence microscopy revealed very intense fluorescence in a small number of membranes which can be explained as being a results of the interaction of some membranes with whole aggregates. Thus, the low value of

DPH fluorescence anisotropy in the aggregate containing samples should be attributed to the contribution of extremely strong fluorescence observed in some erythrocyte ghosts labelled by DPH aggregates. In the membranes containing an excessive amount of DPH molecules the decreased fluorescence is a result of fluorescence depolarization by intermolecular energy transfer (so called concentration depolarization, see e. g. Parker 1986).

This effect should always be taken into account when assessing membrane fluidity by means of DPH. Owing to this we prefer to label cells/membranes with an labelling solution containing low amounts of aggregates, i. e. with a solution prepared by procedure III.

As to the choice of the solvent for the preparation of the stock solution, we prefer acetone to tetrahydrofuran. The main advantage of acetone is its stability. In contrast to acetone, stored THF tends to form oligomers and other products (Haugland 1985) which decrease its miscibility with water. According to our experience, the use of aged THF results in a low efficiency of cell labelling and a low intensity of the probe fluorescence.

The growth in fluorescence intensity in washed samples may be explained by a two-stage interaction of DPH with the membrane. According to this model, the probe molecules first adhere to the cell surface and only then are translocated to the hydrophobic sites of the membrane, i. e. to the lipid bilayer interior and probably also to the hydrophobic sites of integral membrane proteins. We suppose that the first phase of this process must be relatively fast since a 4 min incubation before washing is sufficient to increase fluorescence up to intensities comparable with those reached after 60 min of continuous incubation. The DPH quantum yield is low on the membrane surface due to the high extent of DPH-water interactions. As shown by Thulborn et al. (1979), the fluorescence quantum yield increases when a fluorophore is moved to the center of the bilayer. Thus, the gradual translocation of the probe molecules from the cell surface to apolar regions of the membrane may produce the observed continuous growth in DPH fluorescence intensity after the removal of the free probe.

This idea can be supported further by an examination of energetics of the incorporation of extrinsic probes into model vesicles. In case of a series of *n*-(9-anthroyloxy) fatty acids ($n = 2$, 12 and 16) it was found by Rockley and Najjar (1981) that the insertion energy increased with the movement of the anthracene moiety down the acyl chain, i. e. when the fluorophore approached the centre of the lipid bilayer. For DPH, the insertion energy was even higher (96 ± 8 kJ mol^{-1} in dipalmitoylphosphatidylcholine vesicles) which indicates that DPH translocation from the surface into the membrane can be slow.

Furthemore, it was repeatedly shown that the order of lipid chains decreases towards the center of the bilayer, (e. g. Seelig and Seelig 1974). It was also found

for a set of *n*-(9-anthroyloxy) fatty acids that there is a decrease of fluorescence anisotropy as the fluorescent anthracene moiety is moved towards the acyl chain (Thulborn and Sawyer 1978; Thulborn et al. 1989). The mobility of DPH molecules on the membrane surface is relatively low and thus the fluorescence anisotropy is higher than in the less ordered membrane interior. Hence, the decrease of DPH fluorescence anisotropy may be again explained as a result of a gradual translocation of DPH to the core of the lipid bilayer.

A similar decrease of DPH fluorescence anisotropy during labelling of murine ascites tumour cells was described earlier (Bouchy et al. 1981; Van Hoeven et al. 1979). In these papers, the decrease of fluorescence anisotropy was interpreted as being a result of DPH passage through the plasma membrane into the cell interior and labelling of organelle membranes and intracellular lipid droplets. Since the fluidity of these intracellular lipids is higher than that of the plasma membrane the resulting steady-state anisotropy decreases with the DPH passage into the cell interior. This may be the case with kinds of cells used previously; however, we found the same decrease of fluorescence anisotropy also with erythrocyte ghosts which lack endomembranes and intracellular lipid droplets. We thus believe that, similarly to the increase in fluorescence intensity, also the decrease of fluorescence anisotropy may be due to the proposed two--stage kinetics of the probe-membrane interaction.

Changes in the vibronic structure of molecular fluorescence spectra generally reflect some changes in the fluorophore-solvent interaction. Thus, the differences observed in DPH fluorescence spectra during the labelling should be attributed to the changes in the probe enviroment which support the idea of a gradual DPH translocation into the membrane interior.

Finally, we expect that the two-stage interaction of DPH with biomembranes is a phenomenon which is not limited to this fluorescent probe. Presumably, it may also occur with various other lipophilic molecules.

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Homokaryons from Animal and Plant Cells Generated by Electrofusion

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Abstract. A new apparatus was constructed which enables the use of the electrofusion method to obtain polynuclear cells of various mammalian cell lines, erythrocytes and plant protoplasts. This technique was applied to both suspensions and monolayers. Electrical and other physical parameters were monitored to find optimal conditions for mutual contact of cells (dielectrophoresis) and subsequent fusion. In the suspension technique, dielectrophoresis of mouse erythrocytes occurred at a field frequency of 20 kHz and a strength of 500 $V.cm^{-1}$, whereas cultured mammalian cells and plant protoplasts required a frequency of 1 — 1.4 MHz and a strength of 250—800 $V.cm^{-1}$. Fusion of cells was induced after the application of 1 to 10 high-voltage pulses of 1—5 $kV.cm^{-1}$, 10—36 μs duration. After these high-voltage pulses were applied to the monolayer of mouse L cells, about 12 % viable homokaryons were obtained.

Key words: Electrofusion — Permanent cell lines — Nicotiana protoplasts — Homokaryons

Introduction

Recently cell hybridization has become a method frequently used in various biological laboratories. The technique of fusion of complete cells or cell fragments (e. g. minicells, cytoplasts) permits production of hybrid cells, especially hybridomas, cybrids and reconstituted cells, which are of great importance not only for the understanding of fundamental questions of gene expression in cells, but also for practical applications in medicine and agriculture (see Ringertz and Savage 1976; Köhler and Milstein 1975; Zimmermann et al. 1984). The most frequently used fusion inducers are chemical agents (polyethylene glycol, lysolecithin) or virus particles. Over the last years a new fusion technique based upon the application of very brief electrical pulses to cells in contact was developed

(Zimmermann et al. 1985). This procedure has the following advantages: easy microscopic control of the fusion process, exactly defined fusion conditions, and fusion efficiency exceeding that of chemical or viral induction.

Electrofusion in suspension consists of two steps: (i) dielectrophoresis, when in an alternating field chains of cells in intimate contact are formed ("pearl chains"); (ii) controlled electrical breakdown of the adjacent membranes as a result of high-voltage direct current pulse (Zimmermann 1982; Zimmermann et al. 1985).

It is also possible to induce electrical breakdown of the membranes in monolayer cultured cells without prior dielectrophoresis (Teissié et al. 1982; Finaz et al. 1984; Orgambide et al. 1985). Some authors enhance the fusion efficiency using proteolytic enzymes (pronase or dispase), which are presumed to protect cells against field pulses of high intensity (Zimmermann 1982; Zimmermann et al. 1982a).

In the present paper we describe electrofusion experiments performed with the aid of an apparatus constructed in our institute. The simple power supply proved suitable for dielectrophoresis and fusion of suspensions of both mammalian and plant cells. The viability of fusion products was not influenced. In the monolayer technique about 12 % of polykaryons were scored after 24 h cultivation.

Materials and Methods

Cell types. Mouse erythrocytes were obtained from peripheral blood of C 57 Bl strain mice, washed with phosphate balanced saline solution (PBS) and sodium citrate (0.01 mol . l^{-1}). Mouse lymphosarcoma cells LS/BL were propagated in vivo in the peritoneal cavity of females of host mice. After removal, the cells were washed in PBS. Permanent cell lines of mouse L libroblasts, Chinese hamster V 79 cells and human HeLa cells were cultivated in vitro in minimal Eagle's medium (MEM) supplemented with 10 % fetal calf serum (FCS). Cells were trypsinized and washed in PBS. Protoplasts of *Nicotiana plumbaginifolia* were isolated from mesophyl leaf tissue by enzymatic digestion and resuspended in 0.4 mol . l^{-1} mannitol (Saunders and Gillespie 1984).

In the case of fusion in monolayer the L cells were plated in plastic culture dishes containing MEM supplemented with 10 % FCS. The 24-hour-culture was washed with PBS before use.

Fusion protocol. Fusion of cells in suspensions. The suspensions of animal cells were resuspended in 1 mmol . l^{-1} phosphate buffer (pH 7.4) containing saccharose (250 mmol . l^{-1}) and 1 mmol . l^{-1} $MgCl_2$ (slightly modified from Finaz et al. 1984). In some experiments Pronase P (Serva, 1 mg . ml^{-1}) was added and the cells were preincubated for 20—40 minutes. Immediately before fusion the cells were transferred to saccharose without pronase (250 mmol . l^{-1}), adjusted to a density of 10^4 cells per ml, and pipetted in a plastic fusion chamber with Pt electrodes (wires 200 μm in diameter, gap width 150 μm). *Nicotiana* protoplasts were transferred from mannitol solution to saccharose (300 mmol . l^{-1}) and placed in a fusion chamber. The alignment of cells by

dielectrophoresis was induced after application of non-uniform alternating (AC) field. The process of fusion was immediately induced using one or more high voltage current (DC) pulses. The field strength and pulse duration were monitored on a Philips PM 3266 model memory scope.

Fusion in monolayers. Similarly to the method of fusion in suspensions, cells in dishes were washed in phosphate-saccharose-$MgCl_2$ buffer (pH 7.4) and then overlaied by 250 mmol.l^{-1} saccharose (0.5 ml per dish 60 mm in diameter). Stainless steel flat electrodes (2.4 mm apart) were applied to a cell monolayer and fusion was initiated by 3 to 10 high voltage pulses. MEM with 10 % FCS was added to the dishes and the cell monolayer was fixed using a mixture of glacial acetic acid and methanol (1:3), and stained with methyl-green pyronine after 24 hours.

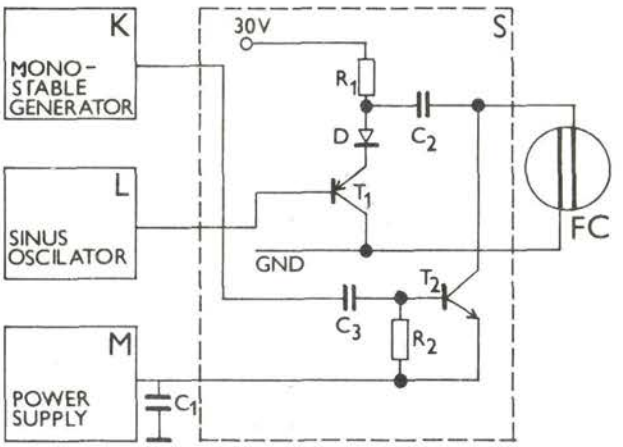

Fig. 1. Electrofusion circuit. The apparatus contains four basic electronic blocks: (K) pulse generator with monostable circuit (switched on only at the moment the pulse is generated), (L) Wien bridge oscilator with amplitude stabilization, (M) power supply and switching converter. To obtain strong energy of fusing pulse the output is connected to large capacitor C_1. (S) electronic switch allows immediate attachment of the DC pulse to the sine wave AC field. C_1, 3 × 10 μF/450 V; 390 pF/400 V; C_3, 15 nF/400 V; R_1, 470 ohms; R_2, 12 kohms; T_1, transistor type PNP; T_2, BF 459; D, KY 199. (FC) fusion chamber, (GND) ground.

Electrofusion apparatus: An AC — DC power supply of original design was developed (Fig. 1). The supply is tunable up to a frequency of 2 MHz and the output is variable over a range of 0—9 V, and it generates DC square pulses 0—240 V of 10—50 μs duration.

Results

The technique of fusing cells in suspensions makes direct investigation of dielectrophoresis and fusion itself under an optical microscope and thus determina-

tion of optimal AC and DC parameters, possible. To obtain sulfficiently high field values fusion experiments had to be carried out in isotonic media of a relatively low conductance (Zimmermann and Vienken 1982). Owing to this the experimets were performed in 250 mmol . l^{-1} saccharose for animal cells, and in 300 mmol . l^{-1} saccharose with plant protoplasts. In Table 1 optimal voltage values and frequencies of AC field for particular cell types are shown at which formation of "pearl chains" exhibiting intimate cell-to-cell contact occurred. Mouse erythrocytes formed contact even in a field with a frequency of 20 kHz and strength 500 V . cm^{-1}, while optimal conditions for mammalian permanent cell lines and plant protoplasts were 1 — 1.4 MHz and 250—800 V . cm^{-1}. In the case of dielectrophoresis of two cell types with various requirements for frequency and field strength, the higher ones were also optimal for the mixture of cells (Fig. 2).

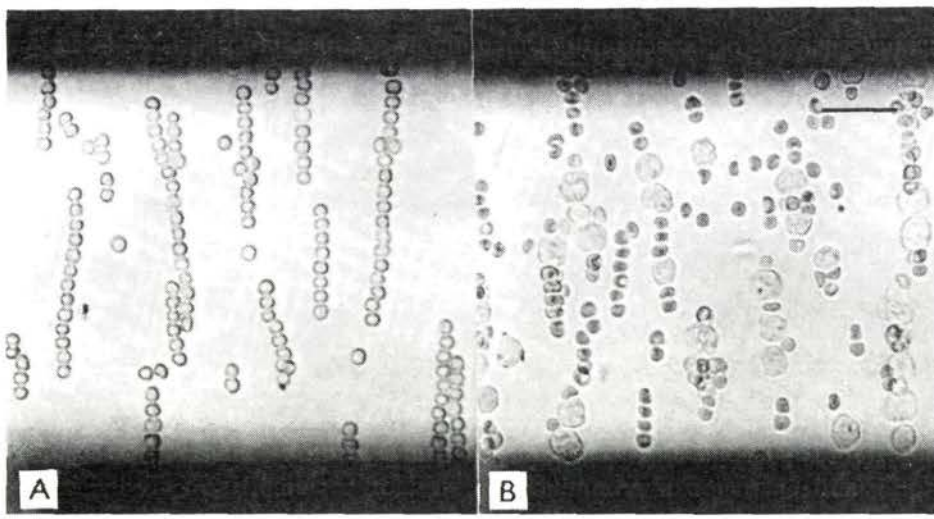

Fig. 2. Dielectrophoresis of cells in AC field. Mouse erythrocytes (*A*) formed "pearl chains" by 0.5 kV . cm^{-1} field strength and 20 kHz frequency. In a mixture with lymphosarcoma LS/BL cells (*B*) dielectrophoresis was achieved by the application of AC field 0.8 kV . cm^{-1}, 1 MHz. Cells were resuspended in 250 mmol . l^{-1} saccharose; bar: 20 μm.

Cell fusion was induced immediately after dielectrophoresis occurred using one or more pulses (1—5 kV . cm^{-1}, 10—36 μs duration). Effective values for various cell types are also presented in Table 1. Higher field strength or longer pulse duration resulted in considerable cell lysis. Repeated pulsation (up to 10,

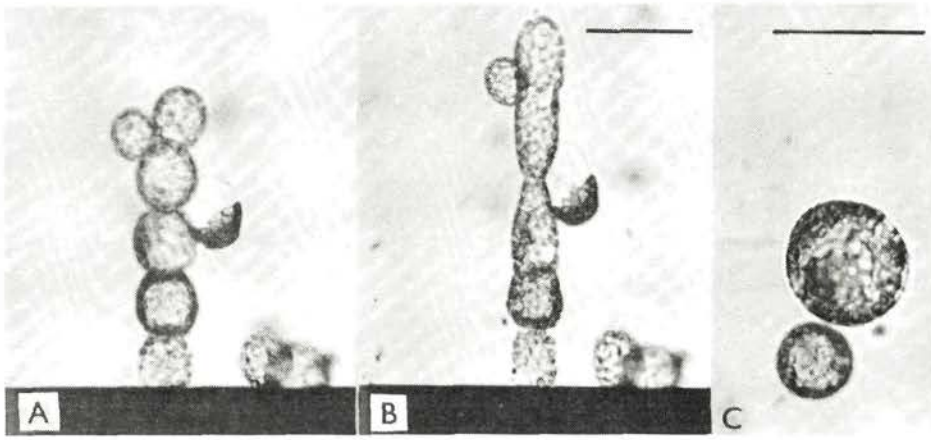

Fig. 3. Fusion of *Nicotiana* protoplasts. (*A*) Dielectrophoresis conditions: field strength 0.2 kV . cm^{-1}, frequency 1 MHz. (*B*) Cell fusion immediately after breakdown pulse application (3 pulses in 1 s intervals, intensity 1 kV . cm^{-1} and 10 μs duration). (*C*) Fused protoplast 5 min after pulse application. Bar: 50 μm.

separated by one-second intervals) resulted in a partially higher yield of multinuclear cells, but a further increase also induced cell lysis. Cell fusion was observed immediately after the application of breakdown pulses and was usually completed within few minutes (Fig. 3). Binuclear cells were observed most

Table 1. Electrofusion parameters optimal for various cell types

Cells	Dielectrophoresis		Electrical pulse	
	Field strength (V/cm)	Frequency (MHz)	Field strength (kV/cm)	Duration (μs)
Mouse erythrocytes	500	0.02	5	20—36
Mouse fibroblasts L	500	1—1.4	1	10
Mouse lymphosarcoma LS/BL cells	500—800	1	2	36
Chinese hamster V 79 cells	800	1	1	20
Human HeLa cells	250—500	1	1—2	20
N. plumbaginifolia protoplasts	250	1	1	10

Fusions were performed in suspensions in the medium described in Materials an Methods. The distance between electrodes was 150 μm. One pulse was applied to cells immediately after dielectrophoresis.

frequently, but multinuclear homokaryons also occurred (Fig. 4). After fusion of mouse erythrocytes large cells were often obtained, arising from several cells aligned not only in parallel to the field lines (Fig. 5). In this case, fusion was not completed until several minutes later.

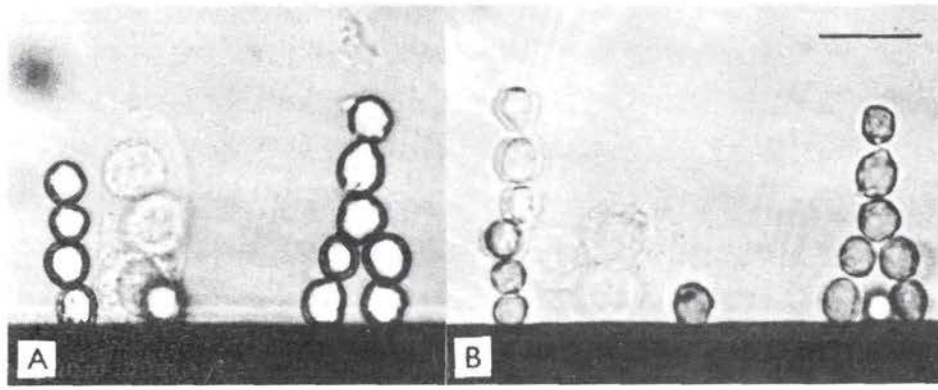

Fig. 4. Electrofusion of Chinese hamster V 79 cells. Dielectrophoresis: 0.8 kV . cm^{-1}, 1 MHz. Fusion: one pulse 1 kV . cm^{-1}, 20 μs duration. Enlargement of the diameter of fusing cells occurred immediately after the pulse was applied. (*A*) homokaryon 3 s after pulse application, (*B*) rounding up after 15 s. Bar: 30 μm.

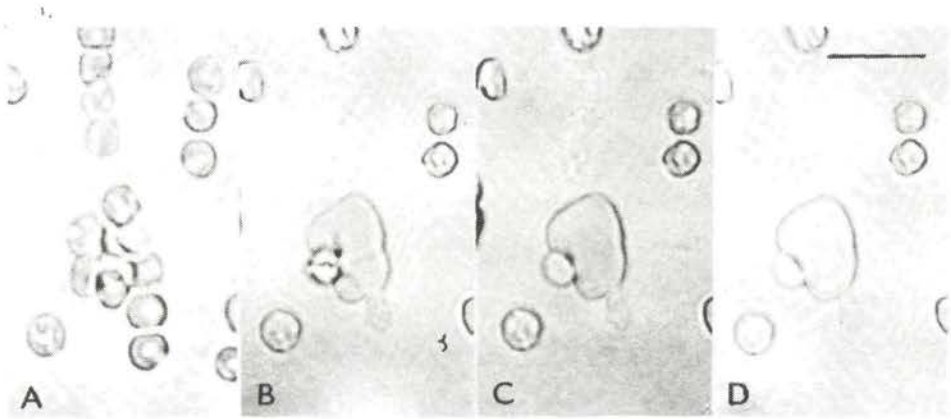

Fig. 5. Electrofusion of mouse erythrocytes. (*A*) Dielectrophoresis 0.5 kV . cm^{-1}, 20 kHz. (*B*—*D*) Fusion after breakdown pulse of 5 kV . cm^{-1}, 20 μs duration. Photographs were taken 20 s (*B*), 40 s (*C*) and 60 s (*D*) after pulse application. Bar: 10 μm.

To induce fusion of plant protoplasts after dielectrophoresis DC pulses of 1 kV . cm^{-1}, 10 μs duration were used. Contrary to the mammalian cells, protoplasts elongate at the moment of the pulse application (Fig. 3*b*). This elongation

is oriented parallel to the field lines and disappears immediately after the AC field is switched off; rounding up of the protoplasts is completed within few minutes (Fig. 3*c*).

The stimulation effect of pronase treatment on the fusion of mammalian cells was studied in the presence of Pronase P in fusion mixture or after 20—40 minutes of pretreatment and subsequent enzyme removal. The presence of pronase during the fusion process decreased "pearl chain" formation as well as the efficiency of fusion; on the other hand twenty minutes' pretreatment slightly increased the yield of fusion compared to control experiments without pronase. Pretreatment prolongation caused cell lysis.

Table 2. Fusion indices after fusion of mouse L fibroblasts in monolayers

	Number of polykaryons					
	2 nuclei	3 nuclei	4 nuclei	5 or more nuclei	Total	%
Control	10	1	1	0	12	0.6
3 pulses	195	36	9	3	243	12.5
10 pulses	187	25	0	4	216	10.8

Pulses (1 kV . cm^{-1}, 36 μs duration) were repeated at 1 s intervals. As controls, non-pulsed cells were scored. In each case 2000 cells were evaluated.

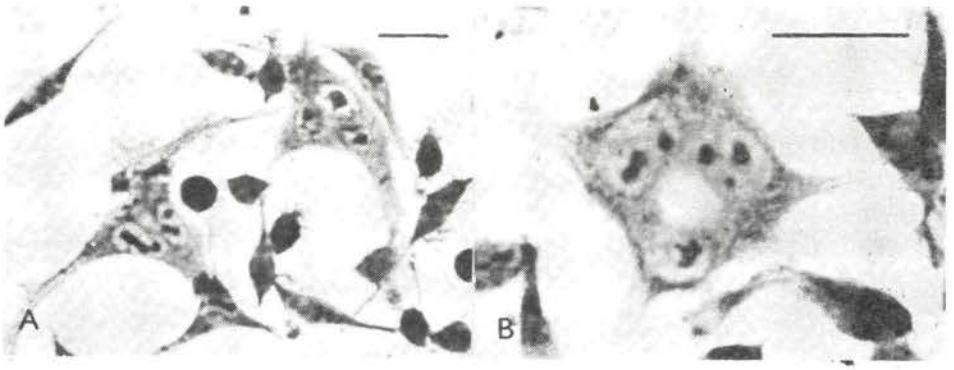

Fig. 6. Electrofusion of mouse L fibroblasts in monolayer culture. Cells were fused by 3 pulses of 0.9 kV . cm^{-1}, 36 μs duration, repeated at 1 s intervals. Multinuclear cells were fixed 24 h after fusion. Bar:20 μm.

The fusion of cells in monolayers was performed by setting stainless steel electrodes to the cells attached at the bottom of Petri dishes. After 3—10 pulses (1 kV . cm^{-1}, 1-s intervals), no visible morphological changes and/or damage to cells were noted in light microscope. The extent of fusion of mouse L cells was

determined after 24 hour-cultivation in fixed preparations. The frequency (in %) of multinuclear cells is presented in Table 2. As compared to control (non-pulsed) cells an increase in bi- and trinuclear homokaryons was noted (Fig. 6). Moreover, cells having five or more nuclei were scored; similar cells were not observed in controls. Data in Table 2 show that increasing the number of pulses from 3 to 10 did not improve fusion frequency; on the other hand, no adverse effects on cell morphology were seen after repeated pulsation.

Discussion

Cells in suspensions subjected to non-uniform alternating electric field behave like neutral or charged particles and move toward regions of highest field intensities. If the frequency of the field ranges between kHz and MHz and if field strengths of the order of $100\,V.cm^{-1}$ are applied, the cells come into intimate contact, forming "pearl chains" (Zimmermann et al. 1985). Frequency values for optimal dielectrophoresis depend on cell type and cell diameter, e. g. mouse erythrocytes formed "pearl chains" at lower frequencies (20 kHz), whereas for permanent cell lines a frequency of 1 to 1.4 MHz was required. It is important to determine optimal conditions for dielectrophoresis, because at lower frequencies the cells rotate, which prevents their fusion. Another important condition for dielectrophoresis to occur is AC field strength. The optimum for mammalian cell lines lies between 250—$800\,V.cm^{-1}$, whereas at field strengths exceeding $250\,V.cm^{-1}$ plant protoplasts lysed in our experiments. If AC field voltage reaches breakdown voltage or exceeds it, it causes cell deformation or even lysis (Zimmermann and Vienken 1982; Saunders et al. 1986). During the application of pulses inducing fusion, some deformation of cells also occurs. One example of such deformation is protoplast elongation (Fig. 3*b*) also observed by Bates et al. (1983) in Petunia and Zea protoplasts. This kind of deformation has no adverse effect on cell fusion.

After cell contact had been established by dielectrophoresis, fusion was induced by 1—10 pulses (10—36 μs duration, 1—$5\,kV.cm^{-1}$). A lower voltage, about $1\,kV.cm^{-1}$, induced fusion of two or three cells, oriented in parallel to the field lines, but at $5\,kV.cm^{-1}$ erythrocytes also fused at various angles (Fig. 5). The formation of giant cells arising from thousands of human erythrocytes was also decribed by Zimmermann et al. (1982b) and Zimmermann and Vienken (1982) who applied $6\,kV.cm^{-1}$ and stimulated fusion with pronase.

High fusion efficiency of erythrocytes has been also described by other authors working with human erythrocytes (Scheurich and Zimmermann 1981; Zimmermann 1982). Fusion efficiency of erythrocytes can be increased due to long-lived fusogenic state of the membranes as shown by Sowers (1986) in erythrocyte ghosts after stimulation by electrical pulses. It is also known that

differences in the fusion capacity exist between malignant and non-malignant cells when the fusion is stimulated by viral or chemical inducers. A no less important role is played by cell origin, culture conditions, phase of the cell cycle etc. (Poste 1972; Ringertz and Savage 1976). In our experiments no differences in fusion capacity were found after electrical stimulation between cells derived from tumours (HeLa, LS/BL) and permanent cell lines of non-malignant origin (L, V 79).

The yield of fusion products has been reported to be enhanced by pronase in some works (Zimmermann et al. 1982a; Zimmermann and Vienken 1982; Berg et al. 1983). This phenomenon is explained by a protective effect of pronase against the higher pulse voltages, which cause cell disruption. Such a stimulating effect of pronase was not observed in our experiments either for mouse erythrocytes, or for fusion of permanent cell lines. The influence of pronase is probably not the same for all cell types; moreover, it has been postulated that enzymes have to be present in the medium at the moment of fusion (Zimmermann et al. 1982a). It was not possible to arrange experiments in the same way, because pronase partially enhanced medium conductance and consequently decreased pulse voltage and fusion efficiency. The presence of pronase seems to be important only for some cell types and experimental conditions because high fusion efficiency for LM mouse line ID clones without pronase treatment has been reported by Finaz et al. (1984).

Employing a modified method of pulsing the cells in monolayer in close contact omitting dielectrophoresis, we obtained multinuclear mouse L cells ($1\,kV \cdot cm^{-1}$, 3—10 pulses, $36\,\mu s$ duration). Their viability was confirmed after 24-h cultivation. The fusion frequency was higher than 10%, which is in accordance with the results of Teissié et al. (1982), who fused mouse 3T3 cells under similar conditions. We did not reach 90 % fusion efficiency, as did Finaz et al. (1984) for LM mouse line, but the frequency was higher than those observed after PEG (3—10%) or Sendai virus (1—4%) induced fusion in similar cell systems. In particular, the number of multinuclear cells was higher than after treatment with PEG or Sendai. Moreover, a varying fusion efficiency was obtained after these inducers since the fusion conditions are not exactly reproducible. On the other hand, electrical parameters for electrofusion can be well determined and adjusted. In addition, fusogenic compounds and viruses can also affect the viability of fusion products because of uncontrollable interactions with the membrane surface (Zimmermann and Vienken 1982).

Contrary to the report by Teissié et al. (1982), the fusion frequency was not influenced by multiplication of pulses to ten. Increased pulse numbers (over 10) also led to massive cell destruction or detachment from the substrate. Preliminary results obtained after fusion of mixed cell populations give good prospects for isolation of hybrid cells.

It can be assumed that the method of electrofusion, especially in monolayers, is very successful, and acceptable for many laboratories. The equipment is now commercially available, but it presents no problems to construct not too complicated apparatus suitable for electrofusion. It should be mentioned that at a lower output only non-conductive media have to be used and the electrode distance should permit a pulse strength of at least 1 to 6 kV . cm^{-1}. Owing to the advantages mentioned above, electrofusion is becoming an attractive technique in studying the transport of genetic material and its expression.

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