ELSEVIER

Biochimica et Biophysica Acta 1324 (1997) 320–332

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

5-Iodonaphthyl-1-azide labeling of plasma membrane proteins adjacent to specific sites via energy transfer

Bruce I. Meiklejohn [1], Noorulhuda A. Rahman [2], Deborah A. Roess, B. George Barisas *

Departments of Chemistry and Physiology, Colorado State University, Ft. Collins, CO 80523, USA

Received 24 June 1996; revised 12 November 1996; accepted 18 November 1996

Abstract

We have examined conditions optimal for 5-iodonaphthyl-1-azide (INA[4]) labeling of membrane proteins proximal to known membrane sites. Membrane-bound INA can be indirectly activated by energy transfer from visible chromophores. We demonstrate that the efficiency of this sensitized activation is enhanced by use of triplet-forming chromophores such as eosin and by deoxygenation. Variation of sensitized activation efficiency with INA concentration indicates that the critical distance for eosin-INA energy transfer in solution is 8–14 Å. We suggest that photosensitization occurs through triplet exchange and present an improved labeling protocol based on these findings. This protocol was used to examine whether different accessory proteins are associated with isolated and crosslinked Type I Fc_ϵ receptors on 2H3 rat basophilic leukemia cells. 2H3 cells were incubated with eosin-conjugated IgE and irradiated at 514 nm yielding [^{125}I]INA derivatized peptides at 53, 38, 34, and 29 kDa. Crosslinking IgE with mouse anti-rat IgE prior to irradiation labeled three additional proteins at 60, 54, and 43 kDa. These results demonstrate the utility of sensitized INA labeling in characterizing protein–protein interactions in membranes of intact cells and indicate the importance of considering photophysical factors when selecting sensitizers and reaction conditions. We discuss estimation of the size of the membrane region surrounding a sensitizing chromophore within which INA labeling of membrane proteins occurs.

Keywords: Membrane protein labeling; Photosensitization; 5-Iodonaphthyl-1-azide

1. Introduction

Interactions between membrane proteins are central to the function of many membrane receptor systems. These include receptor aggregation such as exhibited by receptors for insulin [1], epidermal growth hormone [2], and gonadotropin releasing hormone [3]. They also involve associations between receptors and accessory membrane proteins such as are known for the B- and T-cell receptors [4,5]. However, it is difficult to identify unknown proteins near known sites on the plasma membrane. Immunoprecipitation of protein complexes has been of

Abbreviations: EITC, eosin isothiocyanate; INA, 5-iodonaphthyl-1-azide; INS, *N*-(5-iodo-1-naphthyl)-succinamic acid; PBS-Tween, phosphate-buffered saline with 5% polyoxyethylene sorbitan; BSS, balanced salt solution; HRP, horseradish peroxidase; Fc_ϵRI, Type I (high-affinity) Fc_ϵ receptor

* Corresponding author. Fax: +1 (970) 4911801.

[1] Present address: Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, USA.

[2] Present address: PerSeptive Biosystems, Inc., Framingham, MA 01701, USA.

PII S0005-2736(96)00237-4

tremendous value, for example in the study of the B cell receptor, but questions invariably arise as to whether important interactions survive detergent extraction or whether apparent interactions are in fact generated by crosslinking in the immunoprecipitation protocol.

There is thus a real need for techniques which covalently label in situ unknown membrane proteins physically proximal to a known membrane site, thus facilitating the identification and isolation of these new proteins. The most promising such method involves the hydrophobic photochemical reagent 5-iodonaphthyl-1-azide (INA), originally synthesized by Bercovici and Gitler [6]. This material readily inserts into cell membranes [7] where it can be directly activated by light near 310 nm to form a nitrene or activated azide [8,9], either mechanism resulting in covalent attachment of INA to adjacent proteins and lipids [10]. In 1987 Raviv et al. [11] discovered that INA was capable of sensitized activation by fluorescent chromophores including fluorescein, NBD and others. When both INA and hydrophobic sensitizers were introduced into cell membranes, irradiation at wavelengths absorbed by the sensitizer, but above those absorbed by INA, caused activation of the azide. This in turn produced covalent INA labeling of membrane proteins. The method has found subsequent application in identifying proteins in cytoplasmic environments to which particular lipid analogs are transported or processed [12,13]. Moreover, sensitizing chromophores could also be attached to extracellular domains of membrane proteins. When membranes of such cells were treated with INA and irradiated at sensitizing wavelengths, transmembrane domains of the fluorophore-bearing proteins were covalently derivatized with INA [11]. This showed that the energy transfer mechanism of sensitized activation could operate over a substantial distance. Later studies showed that chromophore-tagged lectins bound to rod outer segment and lymphocyte plasma membranes could induce the labeling of a very specific subset of membrane proteins [14]. Nonetheless, the degree of protein labeling obtained in such studies has been small and the technique has thus far not proven to be of general utility in identifying new membrane proteins associated with known membrane receptors.

Our goal was to improve protein labeling efficiency and, if possible, to better understand the photochemistry and distance relationships of sensitized INA activation. In this paper we combine chemical and biophysical studies of the sensitized INA labeling of membrane proteins. These studies provide insight into the mechanism of this labeling and permit improvements in labeling protocols to be suggested. This has resulted in substantially better labeling and identification of membrane proteins proximal to known membrane sites. We illustrate these improved methods with labeling of proteins adjacent to the high-affinity Fc_{ϵ} receptor.

2. Materials and methods

2.1. Preparation of 5-iodonaphthyl-1-azide (INA) and N-(5-iodo-1-naphthyl)-succinamic acid sulfo-NHS ester (INS ester)

5-Iodonaphthyl-1-azide (INA) was synthesized by complete diazotation of 1,5-dinitronaphthalene and sequential treatments with one mole sodium azide and one mole sodium iodide as described by Gitler and coworkers [6]. Product was dissolved in ethanol and stored at 4°C. [^{125}I]INA was prepared by the same route using [^{125}I]NaI (ICN Biomedicals, Costa Mesa, CA). The preparation of the reactive derivative INS ester has been described previously [15]. In summary, hydrazine reduction of 1,5-dinitronaphthalene afforded 5-amino-1-nitronapthalene. Diazotation and treatment with sodium iodide provided 5-iodo-1-nitronaphthalene, which upon tin reduction, yielded 5-iodo-1-naphthylamine. This amine was succinylated to obtain *N*-(5-iodo-1-naphthyl)-succinamic acid. The product was homogeneous according to TLC and possessed a molar absorptivity at 296 nm of 10 586 $cm^{-1}\,M^{-1}$ and a melting point of 213°C. The overall yield from 1,5-dinitronaphthalene was 35%. The active sulfo-NHS ester [16,17] was prepared by treatment with *N*-hydroxysulfosuccinimide and dicyclohexylcarbodiimide in dry DMF. Removal of insoluble urea and precipitation of product with ethyl acetate allowed collection of INS ester which was stored desiccated under nitrogen.

2.2. Preparation of protein conjugates

N-(5-iodo-1-naphthyl)-succinamic acid (INS) conjugates of rabbit IgG and bovine serum albumin were

prepared in 0.1 M phosphate buffer pH 7.8 at 1 mg/ml. Fifteen equivalents of INS ester were added to these protein solutions which were mixed for 30 min with gentle stirring and then quenched with ten equivalents of 1.0 M Tris. Protein–INS conjugates were separated from free reactive ester by size exclusion chromatography. Extinction coefficients used to calculate INS-to-protein ratios were as follows: ϵ_{296}(INS), 10 586 $M^{-1}\,cm^{-1}$; ϵ_{278} (IgG), 1.47 ml/mg; and ϵ_{278} (BSA), 0.69 ml/mg. Typical INS-rIgG and INS-BSA preparations possessed 8 and 2.6 moles INS per mole protein, respectively.

2.3. Protein derivatization with eosin isothiocyanate

Ten mg of eosin isothiocyanate (EITC) was dissolved in 100 μl of DMSO, diluted to 1 ml with 0.05 M borate buffer (pH 9.5) and mixed for 10 min in the dark. Two mg of antibody or bovine serum albumin were dissolved in 1 ml of 0.05 M borate buffer (pH 9.5). Eosin solution was added to the protein and mixed gently overnight. The reaction was stopped by adjusting the pH to 6.0 with saturated Tris-HCl. To remove unbound dye, a Sephadex G 25-150 column was packed with eight times the reaction volume and equilibrated with 0.05 M phosphate (pH 7.2) before adding the protein–dye mixture. The column was run at 0.5 ml per min and monitored at 280 nm. The first peak containing the EITC–protein conjugate was collected and then dialyzed against 0.05 M phosphate buffer (pH 7.2) for 24 h. The protein-to-eosin ratios were determined using an ϵ_{533} of 83 000 $M^{-1}\,cm^{-1}$. The reaction conditions were adjusted to obtain a ratio of five eosin molecules per protein for BSA used in triplet lifetime measurements and for goat anti-mouse IgM used in murine B lymphocyte experiments. In experiments examining IgE molecules on 2H3 cells, the ratio of eosin to protein was 3:1. The protein solutions containing 0.02% sodium azide were stored in the dark at 4°C.

2.4. Preparation of anti-INA antibodies

Three 5-week-old New Zealand white rabbits were immunized by intramuscular injection with 0.5 ml of 300 μg/ml INS-rIgG in Freund's complete adjuvant followed 2 weeks later by 0.5 ml of INS-rIgG (300 μg/ml) in Freund's incomplete adjuvant. Animals were boosted after 2 months with 0.5 ml of INA-IgG (300 g/ml) in Freund's incomplete adjuvant. Antisera were collected 10 days post-boost and tested for anti-INS and anti-INA antibody activity by a ELISA procedure [15]. Affinity purification was performed by passing antiserum over a column of Reacti-Gel carbonyldiimidazole-activated support (Pierce Chemical Co., Rockford, IL) conjugated with INS-BSA. Unbound proteins (i.e. albumin) were washed from the column and antibody was eluted from the column using pH 2.2 phosphate buffer. ELISA showed that the affinity-purified antibodies recognized the both INS- and INA-determinants but neither the succinyl linker arm nor the rabbit IgG carrier protein.

2.5. Quantitation of sensitized INA activation in solution

In the presence of eosin Y or other sensitizer in alcohol solution, INA photolysis yields primarily 1-iodo-5-aminonaphthalene [15]. Unreacted INA can easily be extracted from acid-soluble products and its concentration determined spectrophotometrically. This permits the extent of sensitized activation to be quantitated. Experiments were performed in 70% ethanol in water to increase INA solubility and to approach more closely the hydrophobic conditions in membranes. In a representative experiment, 1 ml of $6.6 \cdot 10^{-6}$ M eosin Y stock solution in 70% ethanol in water was placed in a jacketed cuvette and various amounts of INA in a total volume of 200 μl in ethanol were added in the dark. Ice-cold 0.1 M potassium ferricyanide was circulated through the cuvette jacket to absorb stray UV light. The cuvette was then typically irradiated for 5 min with 50 mW of 514 nm radiation from a Coherent Radiation 100 argon laser expanded, for cell samples, to about 1 cm diameter using an $8\times$ Galilean telescope. The sample was then acidified and extracted with 1 ml of hexane. The extract was diluted 1:5 with fresh hexane and the absorbance from 260 nm to 360 nm recorded using a Beckman DU70 spectrophotometer. The amount of unreacted INA remaining in the sample was calculated using a molar absorptivity at 310 nm of 21 500 $M^{-1}\,cm^{-1}$. Photon absorption was calculated from solution absorbance at the laser wavelength. Eosin triplet lifetimes in 70% ethanol solution and in water were determined from time-resolved

phosphorescence emission using a time-domain triplet spectrometer [18].

2.6. SDS-PAGE electrophoresis and autoradiography

Gel electrophoresis was performed using the method described by Laemmli [19]. Cells were sonicated for 1 h in 300 μl of 1% Triton X-100 in the dark. To complete solubilization of membrane proteins, 300 μl of Laemmli SDS sample buffer was added to the solution. Both reducing and non-reducing conditions were employed. Samples were applied to the gel and run at 80 V for 5 h in the dark [20]. The gel was fixed for 1 h in 10% glutaraldehyde, washed in four changes of distilled water over 24 h and silver stained using the method described by Oakeley et al. [21]. The gel in a plastic bag, Dupont Cronex Lighting Plus intensifying screens and Kodak X-OMAT AR film were placed in an tray exposure cassette and placed in a -70°C freezer. The film was developed 2 weeks later. The film was scanned at 300 dot-per-inch resolution on an Hewlett Packard Scan Jet Plus white light scanner interfaced to a Microscan 2000 video image analyzer (Technology Resources, Nashville, TN). This system provides displays of protein band absorbances after removal of the underlying, slowly varying background absorbance of the autoradiograph.

2.7. ELISA detection of INA-labeled and other proteins

In some experiments, proteins were transferred from SDS gels to nitrocellulose. An LKB transfer unit (LKB, 2117 Multiphor II, Bromma, Sweden) was employed to transfer the proteins as described in LKB product literature using 0.8 mA per cm^2 for 1 h. The gel was then fixed in 10% glutaraldehyde for 1 h and the nitrocellulose paper blocked with 2% (w/v) dry milk for 20 min with gentle agitation. Nitrocellulose paper was removed from the milk solution, washed three times in PBS-Tween, and then incubated for 2 h in a solution of $1 \cdot 10^{-6}$ M rabbit anti-INA or other antibody. The paper was washed in PBS-Tween to remove unbound proteins and then incubated in the secondary antibody solution, 0.5 mg/ml horseradish peroxidase-conjugated goat anti-rabbit antibody (American Qualex, LaMirada, CA) diluted 1:2000, for 1 h, washed with PBS-Tween and once with distilled water. Paper was developed for 20 min in 3 mg/ml 4-chloro-1-naphthol in methanol diluted 1:5 with 0.1 M Tris (pH 7.2) containing 0.9% NaCl and 0.01% H_2O_2. Assay sheets were photographed and the amount of purple color developed estimated visually.

2.8. Sensitized INA labeling of red blood cell membrane proteins

Human red blood cells obtained from healthy volunteers were suspended in PBS at a concentration of 10^5 cells/ml. One-ml samples were treated with 200 μl 10^{-4} M [^{125}I]INA in ethanol or 200 μl of ethanol alone and some samples contained 10^{-6} M eosin Y. Where preserving the arrangement of cell membrane proteins is critical, lower ethanol concentrations should generally be used (see below). All samples were mixed for 1 h, irradiated at 100 mW for 1 h as described above and extracted with 9 volumes of cold toluene. One ml of the aqueous layer was removed and placed in a Beckman LS1701 scintillation counter to detect proteins derivatized with [^{125}I]INA. Samples were also examined by ELISA with anti-INA antibody.

2.9. Specific labeling of B cell mIgM μ-chains by sensitized INA activation

Splenic lymphocytes from 6–8-week-old female BALB/c mice were isolated and depleted of T cells by treatment with Dynabeads bearing polyclonal anti-T cell antibodies (Dynal, Fort Lee, NJ) as per manufacturer's instructions. T cells bound to the beads were removed with a magnet. The remaining T cell-depleted cell population was resuspended in 6 ml BSS. 600 μl of mM [^{125}I]INA was added to the cell suspension and incubated for 1 h in the dark with gentle mixing. Cells were centrifuged at $300 \times g$ for 10 min and suspended in 6 ml of BSS. Three 1-ml samples were prepared. Sample 1 was incubated with underivatized goat anti-mouse IgM antibody (American Qualex, LaMirada, CA), deoxygenated with 0.1 mg/ml glucose oxidase, 0.1 mg/ml catalase and 25 mM glucose for 60 min and then exposed to 40 mW 514 nm light for 30 min as described above. Two 1-ml samples were labeled with 40 μl of 0.5

mg/ml EITC-goat anti-mouse μ-chain antibody for 1 h at 21°C in the dark, washed and resuspended. After deoxygenation, Sample 2 received no further treatment while Sample 3 was exposed to 40 mW of 514 nm laser light for 30 min.

2.10. Identification of plasma membrane proteins near IgE on 2H3 cells

2H3 cells were grown as described previously [18], harvested by centrifugation and resuspended in 10 ml of BSS. 10 μl of 26 mM [^{125}I]INA in ethanol was added to the cell suspension in the dark which was incubated for 2 h with gentle mixing. The ethanol concentration did not exceed 1%. The cell suspension was centrifuged for 10 min at $300 \times g$ and the cell pellet resuspended in 500 μl BSS. INA-Labeled cells were incubated with 250 μl of $1.4 \cdot 10^{-7}$ M EITC-rat IgE in 0.1 M phosphate buffer (pH 7.2) (Chemicon International, Temecula, CA). The EITC-IgE had a 3:1 dye to protein ratio. After 1 h cells were spun down, washed twice with BSS and divided into two 500-μl samples. One sample received no further treatment. The second sample was treated with 50 μl of a 25 mg/ml stock solution containing mouse anti-rat IgE antibody (Bioproducts for Science, Indianapolis, IN). Both samples were then incubated for 2 h in the dark, washed three times with BSS, and finally deoxygenated in 1 ml of solution containing 0.1 mg/ml glucose oxidase, 0.1 mg/ml of catalase and 25 mM glucose in 0.1 M phosphate buffer (pH 7.2) for 1 h. Samples were irradiated for 2 h with 40 mW of 514 nm radiation. The cell samples were collected and membrane preparations were subjected to SDS–gel electrophoresis and autoradiography.

3. Results

3.1. Sensitized photolysis of INA in solution is enhanced by triplet state-forming donors and by deoxygenation

We compared the efficiencies of $2 \cdot 10^{-4}$ M concentrations of eosin Y and sodium fluorescein, the non-halogenated parent molecule of eosin, in sensitizing the photolysis of INA. Representative spectra of INA extracted from control solutions (marked 'A'), irradiated solutions (marked 'B') and deoxygenated and irradiated solutions (marked 'C') are shown in Fig. 1. The sensitized photolysis of INA by eosin, particularly in deoxygenated solutions, is apparent. An appropriate measure of the efficiency of a sensitizer is the apparent quantum yield for the sensitized reaction, here, the ratio of the number of molecules of INA photolyzed to photons absorbed at constant

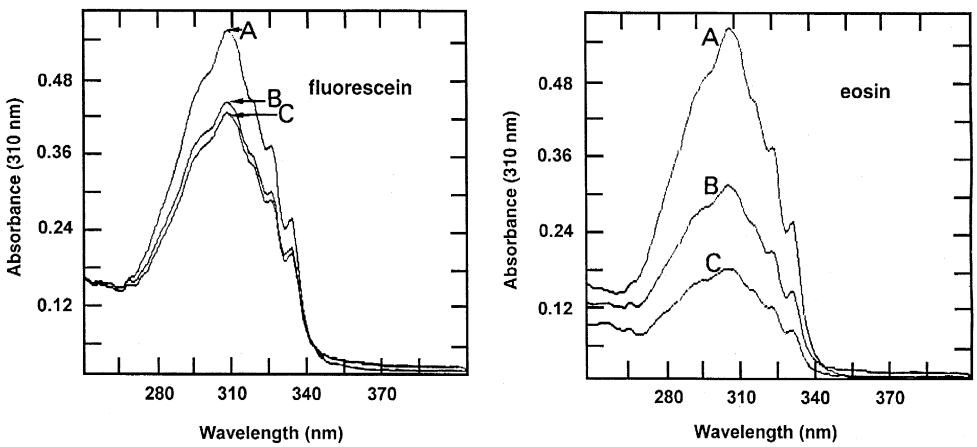

Fig. 1. Comparison of $2 \cdot 10^{-4}$ M fluorescein and eosin Y as sensitizers of INA photolysis. The efficiency of fluorescein (left panel) and eosin Y (right panel) in sensitizing INA photolysis was examined under various conditions. Curve A: no irradiation. Curve B: irradiated 15 min at 488 nm and 93 mW (fluorescein) or 514 nm and 73 mW (eosin). Curve C: deoxygenated 15 min with N_2 purging then irradiated as above. Initial INA concentration was $4.2 \cdot 10^{-5}$ M. The higher efficiency of eosin, particularly in deoxygenated solutions, is apparent.

INA concentration. Under these conditions the apparent quantum yield for eosin- and fluorescein-sensitized INA photolysis had values of $4.39 \cdot 10^{-4}$ and $1.61 \cdot 10^{-4}$, respectively [15]. Deoxygenation increased the quantum yield for eosin to $6.72 \cdot 10^{-4}$, but had little effect on fluorescein where the yield remained at $1.87 \cdot 10^{-4}$. Given this result we focused further attention on eosin as a sensitizer.

3.2. Sensitized photolysis of INA is proportional to time and power of illumination and to the sensitizer concentration

In general, the amount of INA remaining in solution after irradiation decreased more or less linearly as irradiation time or power or sensitizer concentration was increased [15]. For example, after 5 min irradiation under specified conditions, 13% of INA was converted while 38% was converted after 15 min. When irradiation power was varied at 15 min duration, a 9 mW irradiation reduced the INA concentration by 16% while 18 mW reduced it 25%. The concentration of donor, eosin, affected the efficiency of energy transfer. A 1.3 μM eosin solution produced 9% INA photolysis while a 3.3 μM solution produced 15%. At lower power levels and eosin concentrations, the ratio of molecules INA photolyzed to photons absorbed was quite consistent at a value of about $8 \cdot 10^{-4}$ [15]. This matter is discussed further below.

3.3. Sensitized photolysis efficiency plateaus at 10^{-4} M acceptor concentrations

The original studies of Förster [22] demonstrate the use of varying acceptor concentrations to estimate the maximum distance over which energy is transferred in a given system. We irradiated various INA concentrations in the presence of eosin sensitizer under constant conditions. As described previously, the amount of INA photolyzed was determined and compared with the number of photons absorbed by the solution to determine the apparent quantum yield (Fig. 2). It is difficult to employ higher INA concentrations and therefore difficult to decide precisely where the conversion plateaus. Nonetheless, one can estimate that half-maximal efficiency is reached at an INA concentration of perhaps $8 \cdot 10^{-5}$ M. Under these conditions, approximately one INA molecule was converted per 1000 photons absorbed.

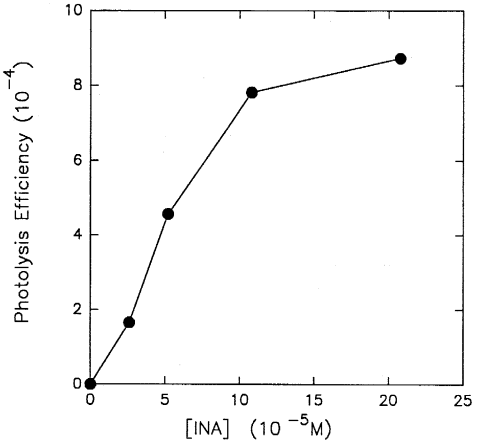

Fig. 2. Apparent quantum efficiency of sensitized photolysis of INA measured as a function of initial INA concentration. Various concentrations of INA in 70% ethanol were irradiated 15 min with 45 mW of 514 nm light. The eosin concentration was $6.6 \cdot 10^{-6}$ M.

3.4. A simplified model predicts acceptor concentration effects on efficiency of triplet exchange

The relation between the concentration of a solution phase acceptor and the efficiency of energy transfer permits estimation of the effective distance over which sensitized activation can occur. The general formalism for such situations where diffusion of acceptor occurs during the donor excited state lifetime was presented by Steinberg and Katchalski [23]. The difficulty of the resulting calculations depends upon what form is assumed for the dependence of the probability of energy transfer upon donor-acceptor separation. For example, the r^{-6} dependence of singlet transfer leads to a differential equation requiring numerical solution. However, the application at hand permits some simplification. For the expected triplet transfer mechanism, the probability of transfer from donor to acceptor is a very steep inverse function of separation [24]. This suggests that approximating the transfer process as diffusion into a spherical sink should provide useful semi-quantitative results.

If some chemical process is activated by energy transfer, the overall quantum yield for this reaction is the product of the quantum yields Φ_e for excitation of the donor, Φ_t for donor decay through energy

transfer and Φ_r for the desired reaction following energy transfer.

$$\Phi = \Phi_e \Phi_t \Phi_r \quad (1)$$

Steinberg and Katchalsky [23] give the transfer quantum yield (their 1-η) as

$$\Phi_t = 1 - \tau^{-1} \int_0 \exp(-\epsilon n - t/\tau)\, dt \quad (2)$$

where τ is the excited state lifetime. $\mathrm{Exp}(-\epsilon n - t/\tau)$ represents the probability that a donor molecule excited at time 0 has not decayed through energy transfer by time t and is defined by

$$\epsilon n = aC_A \int_{r=a} erfc \frac{r-a}{(\pi Dt)^{1/2}} 4\pi r dr \quad (3)$$

where a is the radius of the region within which energy transfer has unit probability, D is the relative diffusion coefficient of donor and acceptor and C_A is the acceptor concentration. Evaluation of the integral in Eq. (2) gives

$$\Phi_t = 1 - e^{-4\pi C_A^3/3} \left[\frac{1}{1 + 4\pi C_A D\tau} \right] \times \left[1 - \sqrt{\pi}\, b e^{b^2} erfc(b) \right] \quad (4)$$

where

$$b^2 = \frac{16\pi C_A^2 a^4 D\tau}{1 + 4\pi C_A a D\tau} \quad (5)$$

The results of this calculation are presented in Fig. 3, where a is the same as the Förster distance R_0 used by Elkana et al. [25] and where the excited state lifetimes are the same. Thus, direct comparison with their Figs. 3 and 4 is possible. This comparison shows that, if a and R_0 are equated, the 'spherical sink' approximation predicts somewhat higher diffusion enhancement of energy transfer for a given D than does the r^{-6} model appropriate to singlet–singlet transfer.

3.5. INA concentration effects on sensitized activation efficiency suggest a triplet exchange mechanism with a critical distance of 8–14 Å

A central question in the application of sensitized activation of INA to labeling membrane proteins is

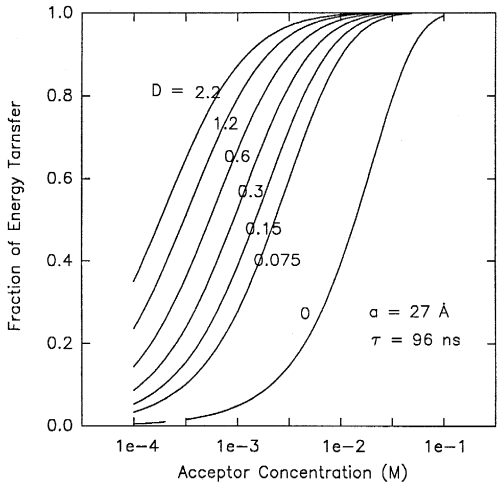

Fig. 3. Energy transfer efficiency as a function of acceptor concentration for various relative diffusion coefficients as calculated from Eq. (4). This calculation models energy transfer as diffusion into a spherical sink. R_0 is 27 Å and the excited state lifetime is 96 ns. Diffusion coefficients are given in units of 10^{-5} $\mathrm{cm^2\,s^{-1}}$. These values are those used by Elkana et al. [25] and can be compared directly with those authors' Figs. 3 and 4 which present diffusion effects on energy transfer efficiency calculated for different models.

the distance over which energy can be transferred from some sensitizer to membrane INA. This distance can be estimated from the dependence of sensitization efficiency on acceptor concentration. If one applies the original Förster treatment, which ignores diffusion, to the above result, an impossibly large critical distance of about 150 Å is inferred. It is thus immediately clear that diffusion during the excited state lifetime has to be considered. We previously outlined Steinberg and Katchalsky's [23] treatment of diffusion effects in singlet–singlet transfer and presented an approximate model for diffusion effects on triplet exchange. We measured the triplet lifetime of eosin in 70% ethanol water to be 2.0 μs and estimated the relative diffusion for the INA–eosin pair to be $1.2 \cdot 10^{-5}$ $\mathrm{cm^2\,s^{-1}}$ [25]. When the data of Fig. 2 are analyzed by Eq. (1) using these parameters, the spherical sink radius must be taken as 8 Å to satisfactorily fit the observed data. If one adjusts the results of Elkana et al. [25] for our lifetime value, a Förster distance R_0 of 14 Å satisfactorily predicts our acceptor concentration results. Either of these values is realistic for triplet–triplet exchange being the mechanism by which eosin excitation energy is transferred

Table 1
INA labeling of RBC membrane proteins via energy transfer from solution-phase eosin sensitizer [a]

Treatment	Irradiation	^{125}I-incorporation (cpm)
None	None	142
[^{125}I]INA	310 nm	18411
[^{125}I]INA + eosin	None	131
[^{125}I]INA + eosin	514 nm	255
[^{125}I]INA + eosin, $-O_2$	514 nm	904

[a] Red blood cells were prepared as described in Section 2. Following exposure to light, membranes were isolated and ^{125}I-incorporation evaluated.

to INA with the ultimate result of nitrene formation or other activation.

3.6. Irradiation of eosin in solution achieves sensitized activation of INA in RBC membranes

The above various results suggest that choice of eosin as an energy donor and use of deoxygenating conditions should produce improved labeling efficiency for membrane proteins. To assess this, red blood cells were treated with [^{125}I]INA, suspended in buffer containing eosin Y and irritated under various conditions (Table 1). Total membrane proteins were then extracted and the degree of ^{125}I-incorporation determined by counting. When RBCs were exposed to 310 nm ultraviolet light, maximum [^{125}I]INA derivatization of RBC membrane components occurred. Some labeling was observed in oxygenated eosin Y-containing solutions irradiated at 514 nm, but the degree of INA incorporation increased 3-fold when comparable samples were deoxygenated prior to irradiation. This labeling required both 514 nm irradiation and the presence of eosin. When red blood cells were irradiated with 514 nm light in the absence of eosin there was no detectable ^{125}I-incorporation (data not shown). Similarly, in the absence of light, there was no detectable [^{125}I]INA bound to membrane components. Protein from the various samples was transferred to nitrocellulose and subjected to an ELISA using polyclonal anti-INA antibody to detect the presence of INA-derivatized proteins (data not shown). Detectable levels of INA were present in the samples illuminated with 310 nm light and in both regular and deoxygenated samples illuminated with 514 nm light in the presence of eosin.

3.7. Activation of [^{125}I]INA via EITC-anti-μ Fab labels B cell μ chains

An important application for eosin-sensitized INA activation is the labeling of transmembrane domains of specific plasma membrane components. Primary cultured murine B lymphocytes were incubated with 2.6 mM [^{125}I]INA, washed and labeled with 0.5 mg/ml EITC-anti-μ Fab. After exposure to 514 nm light, B cell proteins were separated using gel electrophoresis and silver stained or transferred to nitrocellulose. Western blots using horseradish peroxidase-conjugated goat anti-μ chain antibody demonstrated that both the IgM standard and the membrane protein extracts were stained at a location corresponding to a molecular mass of 66 kDa. The autoradiograph is shown in Fig. 4. Examination of this figure

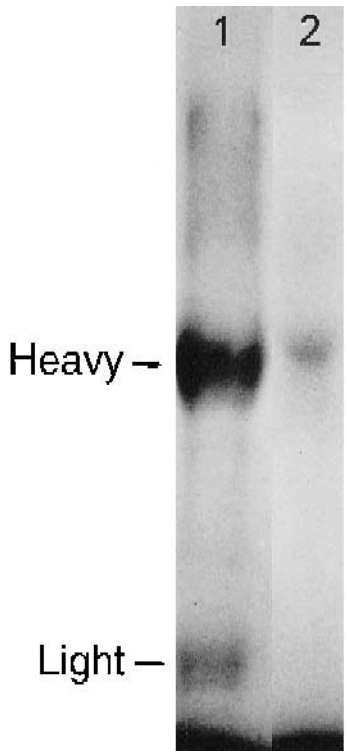

Fig. 4. Transmembrane domains of B cell μ chains labeled with [^{125}I]INA by energy transfer from cell bound EITC-anti-μ chain antibody. Lane 1: ^{125}I-labeled IgM. Both the 66 kDa heavy chain and the 23 kDa light chain are visible. Lane 2: membrane proteins from cells containing INA, binding EITC-anti-μ antibody and irradiated at 514 nm. Only the mIgM μ chains are labeled and only when EITC-antibody is bound and cells irradiated.

demonstrates the remarkable specificity that is achieved by this labeling method. On reducing gels an [^{125}I]IgM standard appeared as two bands with molecular masses of 66 kDa and 23 kDa corresponding to immunoglobulin heavy and light chains (lane 1). No ^{125}I-incorporation was observed on non-irradiated cells (not shown). However, following 1 h exposure of a deoxygenated sample to 514 nm light, a single 66 kDa band appeared on the autoradiograph indicating selective derivatization of the IgM heavy chain (lane 2). In contrast silver stained gels show an almost continuous distribution of proteins at all molecular weights. For radiation in the absence of eosin sensitizer, no INA-derivatized proteins were seen suggesting that INA was not activated by thermal decomposition or other means (not shown).

3.8. Three additional peptides are recruited into association with the 2H3 cell Type I Fc$_\epsilon$ receptor upon receptor crosslinking

Sensitized INA activation by donor chromophores attached to specific membrane sites allows chemical labeling of other membrane proteins proximal to these sites. There are few if any other ways to accomplish such labeling of unperturbed membrane complexes. The 2H3 rat basophilic leukemia cell line was used to evaluate this potential since crosslinking

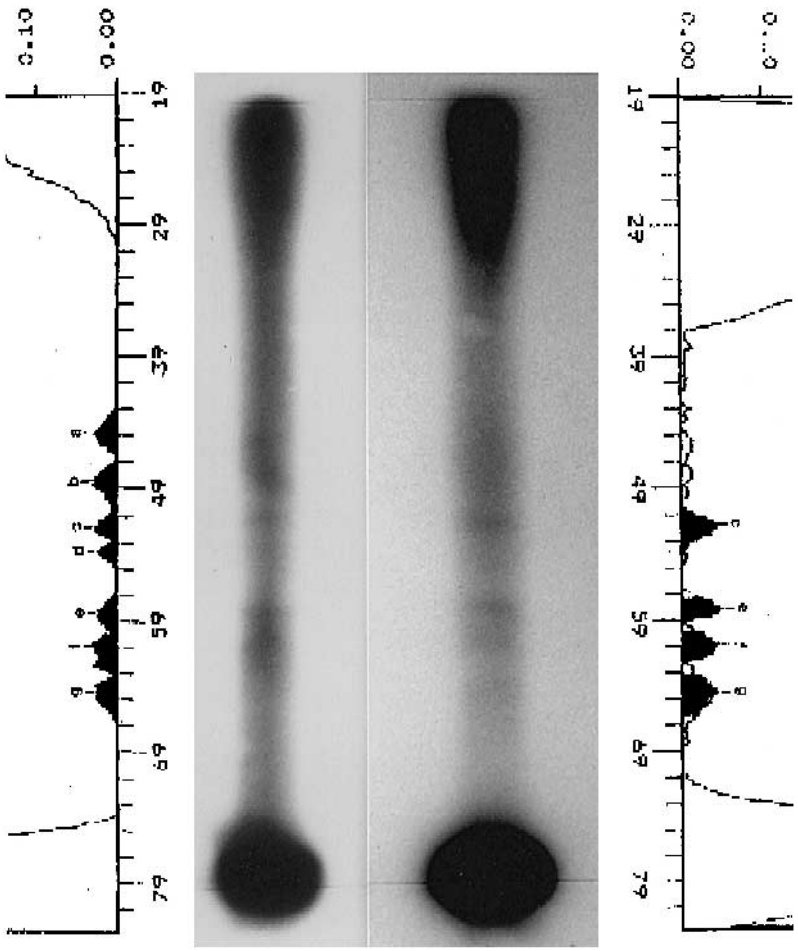

Fig. 5. Autoradiograph of SDS-gel showing membrane proteins isolated from 2H3 cells subjected to [^{125}I]INA labeling sensitized by eosin isothiocyanate-conjugated IgE. The optical density scan, corrected for low spacial frequency background absorbance, is shown adjacent to each autoradiograph. When Type I Fc$_\epsilon$ receptors remained uncrosslinked, four peptides of 29 kDa (g), 34 kDa (f), 38 kDa (e) and 49 kDa (c) were labeled (right lane). When receptors were crosslinked by anti-IgE antibody before labeling, three additional peptides of 43 kDa (d), 54 kDa (b) and 60 kDa (a) were detected (left lane).

of the Type I Fc_ϵ receptor is believed to recruit additional proteins into association with the receptor. 2H3 cells were incubated with either EITC-rat IgE, which leaves the receptor as a monomer, or with EITC-rat IgE followed by an anti-rat IgE antibody which crosslinks receptors into large molecular mass structures [26]. In each experiment, antibody binding to 2H3 cells was confirmed by fluorescence microscopy. Following labeling with [^{125}I]INA and irradiation with 514 nm light, proteins were separated using gel electrophoresis. The gel autoradiograph is shown in Fig. 5 and demonstrates that INA-labeling is confined to the vicinity of the membrane protein to which the sensitizer-conjugated ligand binds extracellularly. The samples shown in the right lane are from cells where the $Fc_\epsilon RI$ are not extrinsically crosslinked. The autoradiograph for these non-crosslinked samples shows ^{125}I-incorporation in four peptides of approximate molecular mass 49, 38, 34 and 29 kDa. The receptor is composed of one 55 kDa α, one 34 kDa β and two 9 kDa γ chains [27,28]. The first three peptides may thus correspond to the receptor α chain, β chain and a proteolytic fragment of the α chain [29]. The γ chain, even if labeled, would not been recognized on the gels used. The nature of the 29 kDa peptide is unknown but it might well represent the so-called Mast cell Function-Associated molecule or MAFA [30]. This 63 kDa dimeric protein appears as two 29 kDa protein bands on reducing gels such as these. When IgE was crosslinked by antibody (left lane), three additional proteins of 43, 55, and 60 kDa were identified. The nature of these peptides is unknown. All INA peaks appearing on the autoradiographs, four for the isolated receptors and seven for the crosslinked receptors, also gave positive ELISAs with anti-INA antibody.

4. Discussion

The question of the mechanism of sensitized INA activation is a difficult one. Studies of aryl azide photolysis suggest that both triplet and singlet mechanism may contribute to sensitization in any given system. It has been observed that azides can be photodecomposed using aromatic hydrocarbons or aromatic ketones as singlet or triplet sensitizers, respectively [31]. In the case of INA, the fact that substantial sensitized photolysis occurs at 10^{-5} M acceptor concentrations effectively rules out singlet–singlet energy transfer mechanisms. Either diffusion coefficients or R_0 values would have to be impossibly large for singlet sensitization mechanisms to be involved. Thus charge transfer mechanisms would appear to be the most likely mechanisms favoring singlet donors [31].

Our initial interest in this system arose from our hypothesis that triplet sensitization was the major mode of INA activation. Consideration of Eq. (1) and Eq. (4) suggests that two parameters of a chromophore are important for its participation in triplet sensitization, namely quantum yield triplet formation and triplet lifetime. Eosin was selected as the donor for these experiments partially because of its high triplet yield, approximately 0.70 [32] as compared to only about 0.05 for fluorescein. The triplet lifetime of eosin is also important in determining the probability of acceptor activation [33]. The triplet lifetime of protein-bound eosin increases from approximately 10 μs in aqueous solution at atmospheric oxygen tensions to 1 ms after aggressive deoxygenation. This excited state lifetime enhancement upon deoxygenation probably represents a major contributor to the high efficiency of cellular labeling achieved by our protocols. Our solution studies were performed in 70% ethanol, itself a good triplet quencher, and this fact probably accounts for only the modest increase in INA photolysis efficiency achieved upon deoxygenation of the sample solutions.

What are the implications of these studies for optimizing sensitized INA labeling of membrane proteins? Clearly, selection of triplet-forming chromophores as sensitizers improves the efficiency of INA activation. The sensitizers used to date for this purpose typically have poor quantum yields for intersystem crossing to the triplet state. Moreover, maximum excited state lifetimes are necessary to realize the benefits of any sensitizer. Triplet sensitizers thus need a deoxygenated environment for optimal function and deoxygenated reaction conditions have not previously been employed. Also, the difference in the triplet energies of the sensitizer and INA must govern the quantum yield Φ_r for sensitized reaction. It seems likely that the triplet energy of INA is close to that of eosin, namely about 41 kcal/mol [34]. This suggests that sensitizers with slightly higher triplet energies

might increase the overall efficiency of sensitized INA activation markedly and we are currently examining some alternatives in this area. Finally, the distance between donor and acceptor over which excitation energy can be transferred is apparently quite small, as would be expected for triplet–triplet exchange. Such exchange mechanisms all have comparable critical distances of 11–15 Å [33], so improvements in this area are unlikely to be obtained. On the other hand, INA in cell membranes *is* activated by extracellular chromophores with surprising efficiency, as for example, the Fc_ϵ receptor experiments demonstrate. Perhaps this is to be understood in terms of fluctuations of proteins and of the cell surface generally. As Cooper showed [35], although the *time-average* thermodynamic fluctuation of any large structure about its equilibrium position is small, in any given time period the *maximum* fluctuation will typically be enormously larger. Perhaps now and again the various motions of chromophore, antibody, receptor and adjacent membrane come together to bring the chromophore into suitable proximity with the membrane surface and the INA inside. If this happens even once during the 1 ms lifetime of an eosin triplet, that is sufficient for efficient triplet exchange yielding sensitized INA activation in the vicinity of the receptor. Such a mechanism is supported by results of experiments examining INA derivatization of EITC-conjugated anti-IgM on murine B cells. EITC-anti-IgM binds to the extracellular portion of the IgM molecule. It is unlikely that eosin is able to insert into the membrane in this configuration. Therefore, the transfer of energy necessary for activation apparently occurs at *equilibrium* distances greater than those explained by either triplet or charge transfer (10–15 Å) [33].

A fundamental question is what is the spacial reach of the technique, namely the effective distance from the sensitizing chromophore site within which membrane proteins are actually labeled. Experimentally, in 2H3 cells where the type I Fc_ϵ receptor binds eosin-conjugated IgE, we apparently observe labeling of transmembrane segments of the receptor α-and β-chains plus perhaps four accessory peptides. The equivalent hydrodynamic diameter of this receptor is about 60 Å [36] and one might expect the IgE-bound eosin to lie across the receptor's volume from its transmembrane segments. This would suggest that labeling might occur at distances as great as 60 Å. On the other hand, the true figure may be somewhat smaller. Labeling within a 60 Å radius would include nearly 10% of the cell surface, but autoradiographs seem to contain substantially fewer bands than 10% of those apparent on silver-stained total membrane protein gels.

Another factor possibly contributing to the spacial reach of the technique is diffusion of the activated INA species during its reactive lifetime. Photochemical activation occurs when an excited chromophore comes within some critical distance of an INA molecule, but the activated INA species then diffuses until it labels some membrane molecule or is nonproductively quenched. The distance of this diffusion might be substantially greater than the distance over which initial activation occurs. To determine how far the activated INA species diffuses during its lifetime requires determining experimentally both the diffusion coefficient and the lifetime in situ of the active species. We are currently working on both these questions. If the nitrene or azide active species *did* have a relatively long lifetime [37], within this time, an active molecule *might* encounter other proteins not associated with the target complex. However, the extreme simplicity of the autoradiographs obtained for the B cell receptor (Fig. 4), the Type I Fc_ϵ receptor (Fig. 5) and the luteinizing hormone receptor (in progress) all suggest highly specific labeling of receptors and accessory proteins is already being achieved. Should non-specific labeling be encountered in the future, use of more reactive compounds such as carbenes might help to lower any such random labeling [38,39].

In an early study involving direct INA labeling, INA-loaded 2H3 cells were irradiated at 350 nm, detergent extracted and peptides isolated through chromatography on immobilized IgE. Label was observed only on a 30 kDa peptide identified with the Type I Fc_ϵ receptor chain [40]. Applying our protocol for sensitized INA labeling to non-crosslinked receptors yields four labeled peptides, three of which might be of receptor origin. Comparison of these results suggests the improved sensitivity of the sensitized activation method and this is all the more striking in that no fractionation or chromatography was performed on our cell lysates. All the specificity to receptor and receptor-associated proteins was con-

ferred by the localization of donor chromophores on receptor-bound IgE. Labeling of crosslinked receptors identified three additional proteins of molecular weights 43, 54, and 60 kDa which are recruited into association with the receptor upon its crosslinking. Whatever the actual identities of these peptides, this appears to be the first instance where new proteins physically proximal to an individual, known membrane protein have been labeled through sensitized INA methods, since the additional bands appearing upon receptor crosslinking necessarily signify accessory protein recruitment into the proximity of the receptor. In the case of the B cell receptor, we note that no peptide corresponding to either CD79a or b (Ig α, Ig β) is visible in the autoradiograph of Fig. 4. Since the association of CD79a,b with IgM is typically studied only in immunoprecipitated samples, it is not clear whether these proteins ought to be strongly associated with the receptor as examined here. Moreover, the autoradiograph was not heavily exposed and it is possible that the B cell receptor accessory proteins would be visible following prolonged exposure.

Although photoaffinity labeling techniques are capable of identifying primary and associated proteins in intact cells under physiological conditions [11,13,14], certain limitations still remain. To achieve satisfactory protein labeling, long irradiation times of up to 1 h are currently needed. With adequate solution cooling, higher laser intensities should be usable to shorten these times. Heretofore, use of [^{125}I]INA has been the only practical means of detecting labeled products, given the relatively low levels of protein labeling achieved. However, such isotopes are inconvenient and the radiochemicals themselves have short shelf lives. Enzyme-linked immunoassays for the INA group such as we have described here, possibly modified to employ chemiluminescence detection, promise a future strategy to avoid such isotope use.

Acknowledgements

The Authors are grateful to Dr. Yossef Raviv for helpful discussions. This project was supported in part by NIH Grants AI21873 and AI36306 to B.G.B. and HD23236 to D.A.R. The manuscript was prepared during the tenure of B.G.B. as Max Varon Visiting Professor of Chemical Immunology at the Weizmann Institute of Science, Rehovot 86100, Israel.

References

[1] Kahn, C., Baird, K., Jarrett, D. and Flier, J. (1978) Proc. Natl. Acad. Sci. USA 75, 4209–4213.
[2] Schechter, Y., Hernaez, L., Schlessinger, J. and Cuatrecasas, P. (1979) Nature 278, 835–838.
[3] Conn, P.M., Rogers, D.C. and McNeil, R. (1982) Endocrinology 111, 335–337.
[4] Reth, M., Hombach, J., Wienands, J., Campbell, K.S., Chien, N., Justement, L.B. and Cambier, J.C. (1991) Immunol. Today 12, 196–201.
[5] Janeway Jr., C.A. (1992) Annu. Rev. Immunol. 10, 645–674.
[6] Bercovici, T. and Gitler, C. (1978) Biochemistry 17, 1484–1489.
[7] Gitler, C. and Bercovici, T. (1980) Ann. NY Acad. Sci. 346, 199–211.
[8] Lewis, F. and Saunders, W., Jr. (1968) J. Am. Chem. Soc. 90, 7033–7038.
[9] Lwowski, W. (ed.) (1970) Nitrenes. Wiley and Sons (Interscience), New York.
[10] Sidman, C.L., Bercovici, T. and Gitler, C. (1980) Mol. Immunol. 17, 1575–1583.
[11] Raviv, Y., Salomon, Y., Gitler, C. and Bercovici, T. (1987) Proc. Natl. Acad. Sci. USA 84, 6103–6107.
[12] Rosenwald, A.G., Pagano, R.E. and Raviv, Y. (1991) J. Biol. Chem. 266, 9814–9821.
[13] Raviv, Y., Pollard, H., Bruggemann, E., Pastan, I. and Gottesman, M. (1990) J. Biol. Chem. 265, 3975–3980.
[14] Raviv, Y., Bercovici, T., Gitler, C. and Salomon, Y. (1989) Biochemistry 28, 1313–1319.
[15] Meiklejohn, B.I. (1991) 5-Iodonaphthyl azide labeling of specific membrane proteins via energy transfer from donor chromophores. Ph.D. thesis, Colorado State University (Ft. Collins).
[16] Anderson, G., Callahan, F. and Zimmerman, J. (1967) J. Am. Chem. Soc. 89, 178.
[17] Yip, C., Yeung, C. and Moule, M. (1978) J. Biol. Chem. 253, 1743–1745.
[18] Rahman, N.A., Pecht, I., Roess, D.A. and Barisas, B.G. (1992) Biophys. J. 161, 334–361.
[19] Laemmli, U.-K. (1970) Nature 222, 680–685.
[20] Staros, J., Bayley, H., Standring, D. and Knowles, J. (1978) Biochem. Biophys. Res. Commun. 80, 568–572.
[21] Oakeley (1980) Anal. Biochem. 105, 361–363.
[22] Forster, T. (1949) Zeitschr. Naturforsch. 4a, 321–327.
[23] Steinberg, I. and Katchalski, E. (1968) J. Chem. Phys. 48, 2404–2410.
[24] Dexter, D. (1953) J. Chem. Phys. 21, 836–850.
[25] Elkana, Y., Feitelson, J. and Katchalski, E. (1968) J. Chem. Phys. 48, 2399–2404.

[26] Segal, D.M., Taurog, J.D. and Metzger, H. (1977) Proc. Natl. Acad. Sci. USA 74, 2993–2997.
[27] Orloff, D., Ra, C., Frank, S., Klausner, R. and Kinet, J.-P. (1990) Nature 347, 189–191..
[28] Alcaraz, G., Kinet, J.-P., Liu, T.-Y. and Metzger, H. (1987) Biochemistry 26, 2569–2575.
[29] Goetze, A., Kanellopoulos, J., Rice, D. and Metzger, H. (1981) Biochemistry 20, 6341–6349.
[30] Ortega, E., Schneider, H. and Pecht, I. (1990) Int. Immunol. 3, 333–342.
[31] Leyshon, J.L. and Reiser, A. (1972) J. Am. Chem. Soc. Faraday Trans. 268, 1918–1927.
[32] Johnson, P. and Garland, P. (1982) Biochem. J. 203, 313–321.
[33] Calvert, J.G. and Pitts, J.N.J. (1966) Photochemistry, pp. 240–352, John Wiley and Sons, New York,.
[34] Parker, C.A. (1968) Photoluminescence of Solutions, pp. 544 Elsevier,.
[35] Cooper, A. (1976) Proc. Natl. Acad. Sci. USA 73, 2740–2741.
[36] Metzger, H., Alcaraz, G., Hohman, R., Kinet, J.-P., Pribluda, V. and Quarto, R. (1986) Annu. Rev. Immunol. 4, 419–470..
[37] Reiser, A. and Leyshon, L. (1970) J. Am. Chem. Soc. 92, 7487.
[38] Bayley, H. and Knowles, J. (1978) Biochemistry 17, 2420–2423.
[39] Shih, L. and Bayley, H. (1985) Anal. Biochem. 144, 132–141.
[40] Holowka, D., Gitler, C., Bercovici, C. and Metzger, H. (1981) Nature 289, 806–808.