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IMMUNOFLUORESCENCE STUDIES WITH 4-ACETAMIDO-4'-ISOTHIOCYANATO STILBENE-2,2'-DISULPHONIC ACID (SITS)

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The application of the fluorochrome 4-acetamido-4'isothiocyanato stilbene-2,2'-disulphonic acid (SITS) in immunofluorescence was studied. The optimal excitation wave length is 350 nm, and optimal fluorescence is obtained at 420 nm.

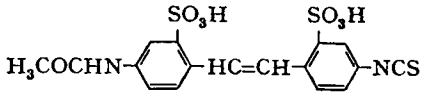
After purification of the commercial compound, conjugation is performed in a strong buffer at pH 9.0—9.5. SITS conjugates were very satisfactory for immunofluorescence studies of the cytoplasmic antigens of cell preparations, but their blue emission was difficult to distinguish from autofluorescence in sections of human tissues.

Good results with immunofluorescence on membrane bound antigens were obtained by using an ultra-violet laser beam as light source. SITS can be used simultaneously with FITC and TRITC conjugates thus making it possible to show three antigens in one preparation.

INTRODUCTION

Important technical developments in the past 10 years have had a considerable impact on progress in immunofluorescence microscopy. The introduction of the epi-illuminator (Ploem, 1969) increased the possibilities and the sensitivity of immunofluorescence, and the development of techniques for automated immunofluorescence and especially the construction of cytofluorographs and fluorescent cell sorters have considerably enlarged the field of application of immunofluorescence (Loken and Herzenberg, 1975).

In connection with these developments the need for other fluorochromes to be used in simultaneous examination of substrates with fluorescein-isothiocyanate (FITC) and tetramethyl rhodamine-isothiocyanate (TRITC) became obvious (Hijmans, 1971). For this reason we have studied the potential value of the fluorochrome 4-acetamido-4'-isothiocyanato stilbene-2,2'-disulphonic acid (SITS — fig. 1) in immunofluorescence. Synthesis of SITS and its application in staining of biological membranes was first described by Maddy (1964). Its use was further studied for biological or haematological



4-Acetamido,4′-isothiocyanostilbene-2,2′-disulphonic acid.
SITS

Fig. 1.

purposes by Marinetti and Gray (1967), Benjaminson and Katz (1970) and Rothbarth et al. (1976). In a preliminary paper Rothbarth et al. (1975) gave a short description of a procedure for conjugation of SITS to antibodies. This paper deals with the application of SITS as fluorochrome in immunofluorescence, particularly the purification of the dye, the experimental conditions for conjugation and the various aspects of fluorescence microscopy using this new fluorochrome.

MATERIALS AND METHODS

Analysis and purification of SITS

For our studies several different batches of SITS were obtained from British Drug Houses (BDH; Poole, U.K.) and two different batches from Nutritional Biochemical Corporation (NBC; Cleveland, OH, U.S.A.). Qualitative analysis of SITS was carried out by thin-layer chromatography on silica gel plates (Kieselgel G DC Fertigplatten, 0.25 mm, Merck, Darmstadt, West Germany), using the solvent system ethyl acetate/ethylmethylketone/formic acid/distilled water (5 : 3 : 1 : 1 by vol.), as described by Stahl and Schorn (1962). Compounds were detected under UV light; for preparative purposes the compounds were recovered from the silica (PSC Kieselgel 60 plates, Merck) by extraction with phosphate buffered saline (PBS), pH 7.4, followed by lyophilization. To determine the *cis/trans* ratio thin-layer chromatography was carried out with the solvent system isopropanol/water/25% ammonia (6 : 3 : 2 v/v/v), as described by Marinetti and Gray (1967).

Quantitative analysis was carried out spectrophotometrically at 330 nm, using the value ϵ SITS = 42.300 cm^{-1} (Marinetti and Gray, 1967). Fluorescence at 420 nm was measured in arbitrary units on a Perkin-Elmer fluorescence spectrophotometer with excitation at 350 nm. IR and NMR spectroscopic analysis of SITS and the subfractions isolated by thin-layer chromatography was carried out in the Laboratory of Organic Chemistry of the State University of Utrecht, and elemental analysis (C, H, S) was performed at the Organic Chemical Institute TNO in Utrecht. For the NMR analysis SITS was dissolved in D_2O.

Preparation of conjugates

Antisera used in this study were goat anti-human IgA and goat anti-human light chains. The antigens used, the immunization protocol and the specific-

ity criteria applied after absorption have been previously described (Mul et al., 1975). Purified IgG fractions were prepared from these antisera by ammonium sulphate precipitation (at 40% saturation, pH 7.2), followed by chromatography on DEAE/Sephadex A-50 and elution with PBS, pH 7.4. Conjugation of purified IgG was effected in principle as described by Hijmans et al. (1969) except that the reaction was allowed to proceed at room temperature for 1 h. All conjugates were prepared using SITS fraction A, obtained by preparative thin-layer chromatography, as described below. In each case 2 mg of purified SITS were added per 100 mg of IgG isolated from the antisera. Free SITS was removed by filtration through Sephadex G-75 with PBS, pH 7.4.

The conjugates were examined by immunoelectrophoresis using rabbit anti-goat IgG. The dye/protein (F/P) ratio was determined by measurement of the extinction at 330 nm and 280 nm respectively. This ratio was 2.0 for the anti-IgA conjugate and 3.0 for the anti-light chain preparations.

Staining with the conjugates

Performance tests were carried out on human bone-marrow cell preparations as described by Hijmans et al. (1969). In these experiments the ability of the SITS conjugates to stain intracellular immunoglobulins was studied. In addition, skin biopsies from a patient with parapemphigus and kidney tissue from a patient with immune complex nephritis were used. The experimental conditions for the demonstration of membrane-bound immunoglobulins on normal human peripheral blood lymphocytes have been described earlier (Gmelig Meyling et al., 1974). Quantitative aspects were studied using the defined antigen substrate spheres (DASS) system as described by Capel (1975), Knapp and Ploem (1974) and Haaijman (1977). Tests of specificity were performed on Sepharose beads coupled with human immunoglobulin light chain or with bovine serum albumin. The following working dilutions of SITS conjugates were employed: 1 : 10 for staining bone-marrow cells, skin and kidney; 1 : 4 for peripheral lymphocytes and 1 : 2 for tests on Sepharose beads.

Fluorescence microscopy

SITS-immunofluorescence was examined with a Leitz Dialux fluorescence microscope with a Ploemopak type 2,3 epi-illuminator (Koch, 1973) and equipped with a HBO 100 mercury lamp.

The heat-filter and the BG 38 filters were omitted to obtain maximum output of ultra-violet light. For excitation two 1 mm UG 1 filters (Schott und Gen.) were used with TK 400 mirror, and LP 430 was employed as barrier filter.

Quantitative immunofluorescence was performed with a Leitz MPV microfluorimeter. In some experiments a more intense light source was necessary

and for this purpose an Argon laser, type 165-03 was used (Spectra-Physics, Mountain View, CA, U.S.A.). The filter combinations in the fluorescence microscope were not changed, but a 10 D quartz lens was installed at the light exit of the laser to focus the light.

RESULTS

Analysis of SITS

Thin-layer chromatography revealed two fractions, one (fraction A) had a R_f value of 0.60, the other (fraction B) had an R_f value of 0.15 and was eluted with the polar front of the eluent. Re-chromatography of the isolated fractions resulted in only one band in each with a R_f value identical to that found in the first run. Semi-quantitative estimations based on spectrophotometry revealed that in the batches of SITS obtained from BDH the A : B ratio was about 1 : 2. Chromatography carried out in the light or in the dark did not show any difference. The absorption, excitation and emission spectra did not show any qualitative differences between the fractions or between fractions and the original material. However, the fluorescence intensities, measured quantitatively on a weight basis of fractions A and B revealed considerable differences (table 1).

From the absorption, excitation and emission spectra it could be concluded that both fractions contained the stilbene core, and therefore that different secondary groups at the main core are the cause of their different mobilities. To determine the precise differences infra-red and NMR-analysis were carried out on the original compound, since the phosphate content of the freeze-dried fractions interfered with accurate analysis. On IR and NMR analysis, a very low acetyl signal was found, indicating that the commercial SITS is incompletely acetylated. This would explain the differences in polarity between fraction A (*N*-acetylated) and fraction B (non-*N*-acetylated). The data obtained by analysis of carbon, hydrogen and sulphur content finally confirmed that the SITS preparation had a lower carbon content than would be expected from its molecular formula (analysis: C, 32.91; H, 3.89; S, 17.41; $C_{17}H_{14}N_2O_7S_3$ calc.: C, 37.0; H, 3.3; S, 17.4%). The A/B ratio

TABLE 1

Characteristics of SITS fractions obtained by preparative thin layer chromatography.

	R_f	420 nm fluorescence at 350 nm excitation (arbitrary units)
Fraction A	0.60	10
Fraction B	0.15	2
Unfractionated SITS		5

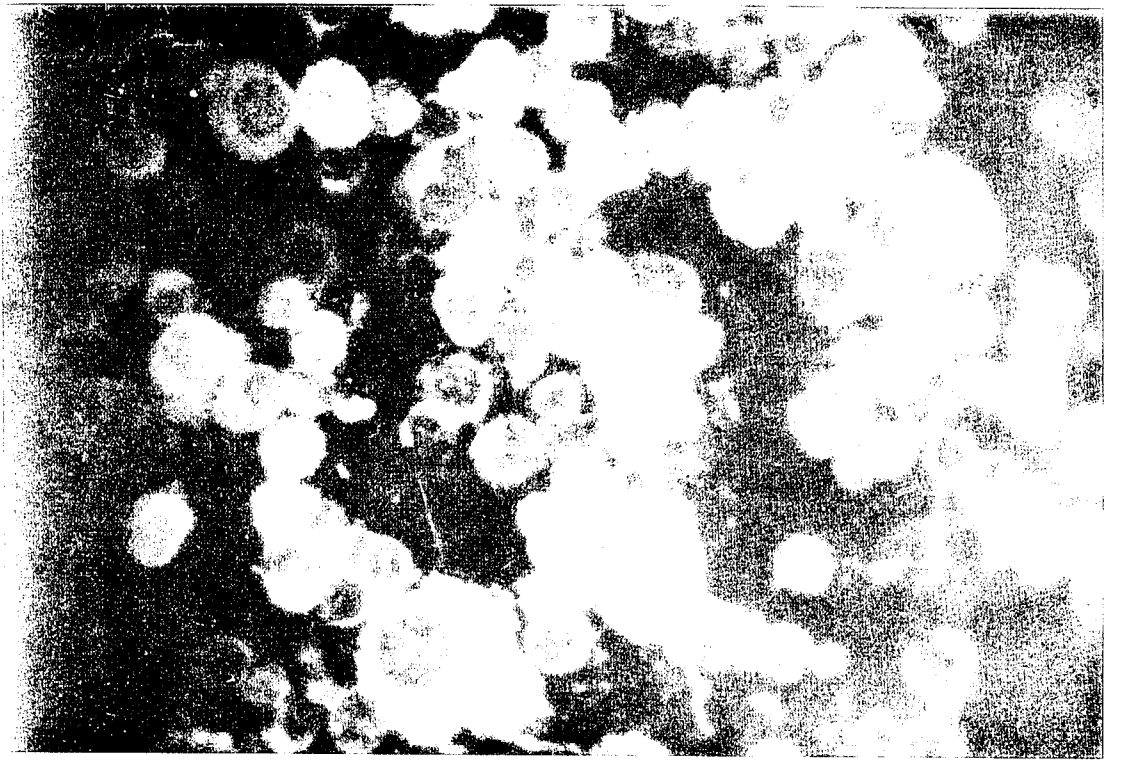


Fig. 2. IgA myeloma bone-marrow plasma cells. anti IgA SITS. ×280.

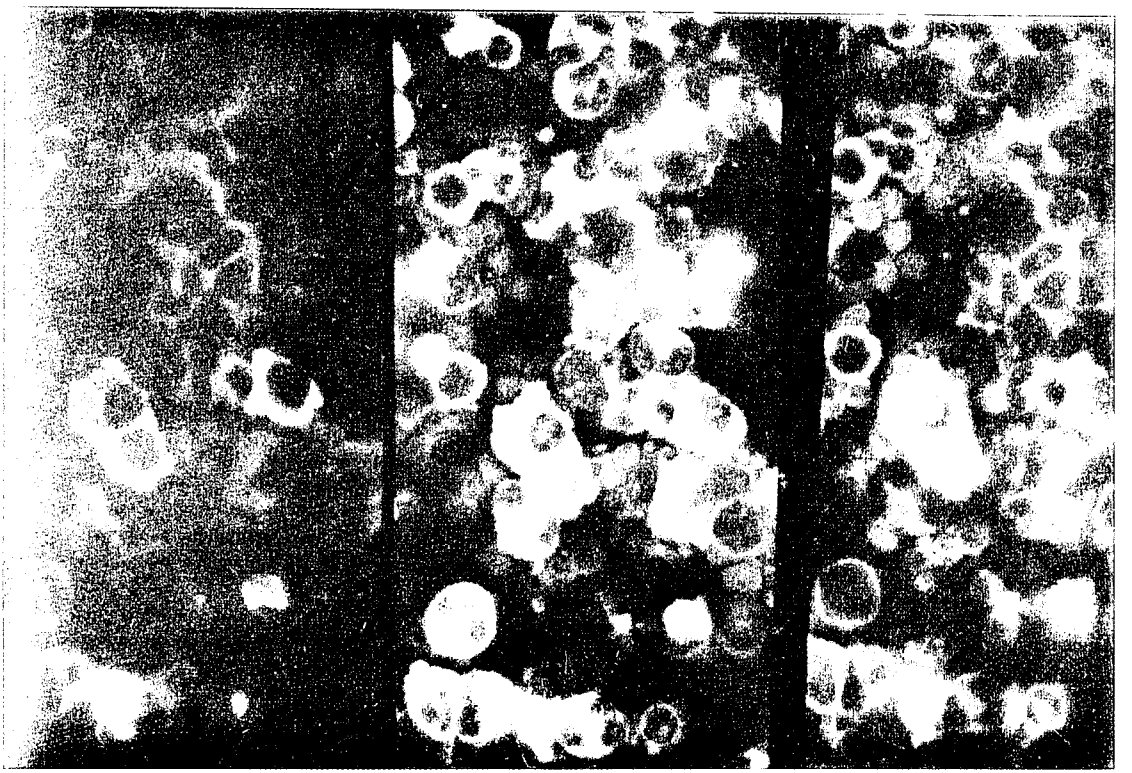


Fig. 3. Triple staining of IgA myeloma bone-marrow plasma cells. anti IgA SITS, anti kappa FITC, anti lambda TRITC. ×250.

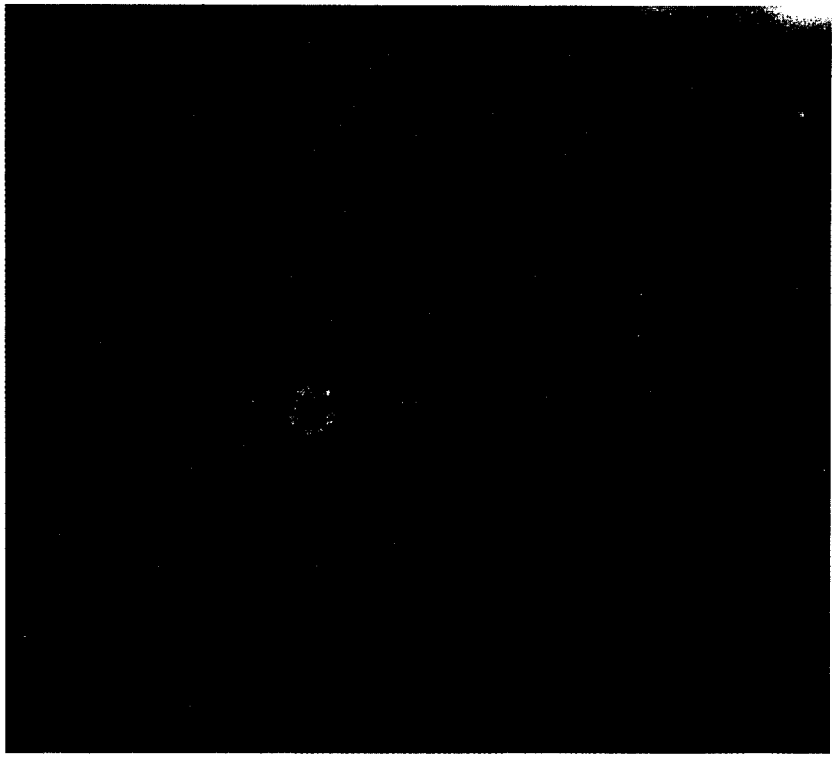


Fig. 4. Membrane anti-light chain staining on peripheral lymphocytes with anti-light chain SITS. Excitation with Argon laser. ×250.

was the same in different batches of SITS tested. For further preparative and analytical experiments SITS (BDH) was used. Thin-layer chromatography with isopropanol/water/ammonia revealed a negligible quantity of *cis*-isomers of SITS (BDH).

Cytoplasmic fluorescence

Specific cytoplasmic immunofluorescence was obtained on bone-marrow cells from IgA myeloma patients stained with SITS-anti IgA conjugate (fig. 2); myeloma cells from patients with IgG and IgM monoclonal gammopathy were unstained. Fig. 3 shows triple staining of a bone-marrow preparation with SITS, FITC and TRITC conjugates. In bleaching experiments SITS did not show any fading within 60 sec.

Immunofluorescence on tissue sections

Immunofluorescence studies with SITS-conjugates on sections were unsuccessful because the tissues (skin and kidney) showed autofluorescence on

excitation at 360 nm, making it impossible to distinguish specific immunofluorescence.

Membrane immunofluorescence

Poor results were initially obtained in attempts to demonstrate surface bound immunoglobulins on normal human lymphocytes stained with anti-light chain-SITS conjugate. A very weak ring fluorescence was observed. By using a UV Argon laser however, distinct membrane fluorescence could be obtained (fig. 4). This fluorescence was fainter than we obtained with FITC and TRITC conjugates under conventional conditions. Excitation of SITS conjugates by the laser beam was followed by marked fading comparable with the fading that occurs when FITC is exposed to UV-light. This phenomenon was not noted after excitation with a HBO 100 mercury lamp as light source (see Cytoplasmic fluorescence).

Quantitative studies with laser light

Quantitative immunofluorescence studies using Sepharose beads coated with light chains showed an increase of fluorescence intensity by a factor of 3.3 with the laser light. Background fluorescence, however, was considerable due to autofluorescence on excitation at 353 nm of the objectives, immersion oil and Sepharose beads. The laser beam was therefore filtered through two 1 mm UG 1 filters. This eliminates blue light, which otherwise produces a blue background and poor contrast.

DISCUSSION

SITS is synthesized from 4-amino 4′-nitrostilbene 2,2′-disulphonic acid (Maddy, 1964; BDH, information upon request). The first step involves the synthesis of the acetamido group followed by the introduction of the isothiocyanato group on which the substrate is heated to 80°C at pH 3—4. During this last step the acetyl part of the acetamido group can be hydrolysed, yielding the amino analogue. This last compound is more polar than SITS, which explains the results obtained in thin-layer chromatography. Maddy (1964) and Marinetti and Gray (1967) described the existence of *cis*- and *trans*-isomers of SITS. Only the *trans*-configuration has fluorescent properties. Exposure to light induces conversion of the *trans* configuration to the *cis*-form.

The presence of 2 components on thin-layer chromatography of SITS cannot be explained by *cis-trans*-isomery on the basis of the following arguments: (1) The *cis*-form has negligible fluorescence, whereas the relative fluorescence intensity of fraction B was considerable (20% of that of fraction A). (2) Most chromatography was performed in daylight; it might be expected that re-chromatography of the purified fraction A would result in

the reappearance of fraction B. In fact this was not found. Chromatography in a dark environment gave the same results. Furthermore, the SITS used contained a negligible quantity of *cis*-form. (3) *Cis-trans* differences cannot explain the large difference in polarity of the two fractions. (4) The infra-red and NMR spectrometry showed loss of *N*-acetyl groups in fraction G. (5) The congruent absorption spectra of the fractions A and B is in contradiction with the difference of the absorption spectra between *cis*- and *trans*-stilbene (Lewis et al., 1940).

Fully carboxylated SITS, obtained by preparative thin-layer chromatography, can be conjugated to antibodies with good results. It must be emphasized, however, that SITS is a very polar compound and therefore the conjugation has to be carried out in a buffer with good buffering capacity at pH 9.0—9.5. The pH must be checked and if necessary corrected. In our hands the SITS conjugates were most useful in cytoplasmic immunofluorescence of plasma cells. In this field simultaneous immunofluorescence with three fluorochromes is now possible. Unfortunately, application of different SITS conjugates to study tissue sections was hampered by autofluorescence of the tissue. This fluorescence is not due to the reported non-specific binding of SITS to intracellular structures or to plasma membranes, because this occurs only at considerably higher concentrations of SITS (Maddy, 1964; Marinetti and Gray, 1967; Benjaminson and Katz, 1970).

Although membrane fluorescence with SITS conjugates using the conventional fluorescence microscope is faint, it does not exclude SITS conjugates for use in membrane immunofluorescence studies. In our opinion SITS conjugates can be of value in studies using fluorescent cell sorters as described by Loken and Herzenberg (1975), Shapiro et al. (1976) and Curbelo et al. (1976). These cell sorters employ laser beams as light source and can be equipped with an extra fluorescence canal. In conclusion it can be stated that SITS might be added as a third fluorochrome in immunofluorescence studies of cell preparations and in studies in which fluorescent cell sorters are used.

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REFERENCES

Benjaminson, M.A. and I.J. Katz, 1970, Stain Technol. 45, 57.
Capel, P.J.A., 1975, Ann. N.Y. Acad. Sci. 254, 108.

Curbelo, R., E.R. Schildkraut, T. Hirschfeld, R.H. Webb, M.J. Block and H.M. Shapiro, 1976, J. Histochem. Cytochem. 24, 388.
Gmelig Meyling, F., L. Kooy-Blok and R.E. Ballieux, 1974, Eur. J. Immunol. 4, 332.
Haaijman, J.J., 1977, Ph.D.-thesis, University of Leiden.
Hijmans, W., 1971, Ann. N.Y. Acad. Sci. 177, 405.
Hijmans, W., H.R.E. Schuit and F. Klein, 1969, Clin. Exp. Immunol. 4, 457.
Koch, K.F., 1973, Leitz Mitt. Wiss. Tech. 6, 49.
Knapp, W. and J.S. Ploem, 1974, J. Immunol. Methods 5, 259.
Loken, M.R. and L.A. Herzenberg, 1975, Ann. N.Y. Acad. Sci. 254, 163.
Lewis, G.N., T.T. Magel and D. Lipkin, 1940, J. Am. Chem. Soc. 62, 2973.
Maddy, A.H., 1964, Biochim. Biophys. Acta 88, 390.
Marinetty, G.V. and G.M. Gray, 1967, Biochem. Biophys. Acta 135, 580.
Mul, N.A.J., L.E.M. de Boer, J.A. Taminiau and R.E. Ballieux, 1975, Eur. J. Clin. Invest. 5, 63.
Ploem, J.S., 1969, Z. Wiss. Mikrosk. 68, 129.
Rothbarth, P.H., I. Hendriks-Sturkenboom and J.S. Ploem, 1976, Blood 48, 139.
Rothbarth, P.H., J.G. Olthof and N.A.J. Mul, 1975, Ann. N.Y. Acad. Sci. 254, 65.
Shapiro, H.M., E.R. Schildkraut, R. Curbelo, C.W. Laird, R.B. Turner and T. Hirschfeld, 1976, J. Histochem. Cytochem. 24, 396.
Stahl, E. and P.J. Schorn, 1962, in: Dünnschichtchromatographie, ed. E. Stahl (Springer Verlag Berlin, Göttingen, Heidelberg) p. 388.