COMPARISON OF DIIODOSULPHOPHENYLISOTHIOCYANATE WITH OTHER REAGENTS AS SURFACE LABELS FOR LYMPHOCYTES

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1. Introduction

The determination of the orientation and arrangement of proteins within the plasma membrane is crucial to our understanding of the structural basis of membrane functions. Methods for selective radiolabelling of the components exposed on the cell surface play a vital role in these structural studies and a number of reagents, which modify proteins or carbohydrates, have been proposed as suitable candidates for vectorial labelling [1–9]. The ideal cell surface reagent should interact uniformly with all exposed polypeptide or carbohydrate groups (i.e., be non-specific) and not penetrate into the cell to react with internal components. Several reagents have been claimed to fulfill the latter requirement, usually by virtue of their size or lipophobicity [3–10]. Limitations in the reactivities of the reagents have, however, not been studied widely [3,11]. There is currently a need for information on the latter aspect as well as for the designation of new non-specific vectorial reagents.

Here, the preparation and use as a cell surface reagent of a novel derivative of sulphanilic acid, namely II [^{125}I]diiodosulphophenylisothiocyanate (DISPITC), is described. This reagent is more stable and generally reactive than its diazotised analogue [10] and less expensive than the ^{35}S-labelled non-iodinated reagent [11]. It does not appear to penetrate the cell surface membrane and reacts with amine groups on the cell surface under conditions which do not significantly reduce cell viability. Several published methods for cell surface labelling were compared with DISPITC in order to assess the specificity of these reagents. A human lymphoblastoid B cell line, BRI-8, was chosen as the model target system because of the availability of cell suspensions of high viability and its relatively well-characterised and uniform surface structure [12]. The results obtained showed that all of the reagents tested were restricted in their reactivity but to varying degrees, lactoperoxidase and DISPITC being the most general.

Abbreviations: HBS, 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid), 0.15 M NaCl (pH 8.5); TBS, 15 mM Tris–HCl, 0.15 M NaCl (pH 7.3); DISPITC, diiodosulphophenylisothiocyanate; DDISA, diazodiiodosulphanilic acid; SDS, sodium dodecyl sulphate

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2. Experimental

2.1. *Materials*

Sulphanilic acid, pyridoxal phosphate and sodium periodate (meta) were purchased from BDH Chem. Ltd., Poole. Thiophosgene was obtained from Koch-Light Lab. Ltd., Colnbrook and stored in a double-sealed container in a fume hood. Na^{125}I(carrier free), NaB^{3}H$_4$(7.2 Ci/mmol) and formyl-methionine [^{35}S]sulphone were purchased from The Radiochemical Centre, Amersham. NaB^{3}H$_4$ was dissolved in 10 mM NaOH to 1 mCi/μl and stored in 10 μl aliquots at −70°C. Neuraminidase was obtained from Behringwerke AG, Marburg-Lahn and galactose oxidase from Kabi, Stockholm; both enzymes were described as being free from proteolytic activity. Lactoperoxidase and glucose oxidase were obtained from Sigma Chem. Co., Poole.

2.2. *Cells and membranes*

BRI-8 cells were obtained from Searle Diagn., High Wycombe. The viability of cells used in all labelling experiments was > 95%, as judged by exclusion of eosin. Microsome and plasma membrane fractions were prepared after mechanical disruption of the cells as in [13].

2.3. *Preparation and use of [^{125}I]diiodisulphophenylisothiocyanate*

DISPITC was prepared from sulphanilic acid by combination and modification of the methods for the preparation of [^{125}I]diazodiiodosulphanilic acid [2] and [^{35}S]sulphophenylisothiocyanate [11]. Diiodosulphanilic acid was synthesised from sulphanilic acid [2] and stored in the dark at 4°C. It was radioiodinated (100 nmol in 10 μl of 0.05 M $NaHCO_3$) by exchange with Na^{125}I (1.5 mCi) in the presence of ICl (5.5 nmol) [2]. The exchange was terminated after 15 min at 20°C by the addition of 10 nmol sodium metabisulphite. Thiophosgene (1 μl containing 13 μmol) was then added and the mixture stirred for 1 h at 20°C. The products were diluted by the addition of 100 μl water and excess reagents removed by two extractions with 100 μl diethyl ether. DISPITC was isolated from the aqueous phase by 3 extractions with 100 μl butan-1-ol, dried in vacuo over $CaCl_2$ and stored dry at −70°C.

In some experiments the reagent was further purified by thin-layer chromatography on silica gel in methanol:diethyl ether (1:4, v/v). It was detected by autoradiography (R_F 0.81), eluted with butan-1-ol and dried in vacuo over $CaCl_2$. The reagent was redissolved in 10% aqueous pyridine immediately prior to use.

Cells (10^8) were suspended in 200 μl HBS and 20 μl DISPITC solution (7–14 nmol; 50–100 μCi) was added. After 15 min at 20°C, the cells were washed by sedimentation (500 g_{av}, 10 min) using the following sequence: twice with TBS; once through a 2 ml cushion of 5% bovine serum albumin in TBS; twice more with TBS.

Plasma membrane (100 μg protein) was suspended in 100 μl HBS and 5 μl DISPITC solution (20 μCi) was added. After 15 min at 20°C the membranes were washed twice with TBS by sedimentation at 100 000 g_{av} for 30 min.

2.4. *Radiolabelling of cells with other reagents*

Radioiodination by the lactoperoxidase/glucose oxidase method was carried out using conditions recommended for vectorial labelling [8]. [^{125}I]-DDISA was synthesised [2] and reacted with BRI-8 cells for 20 min at 37°C as recommended for vectorial labelling [10]. The methyl phosphate anhydride of formylmethionine [^{35}S]sulphone was prepared [4] and added to BRI-8 cells in 0.1 M $NaHCO_3$, 0.06 M NaCl (pH 8.5) (20 min at 20°C). Cells were also radiolabelled using pyridoxal phosphate/NaB^3H_4 [3], sodium periodate/NaB^3H_4 [6] and neuraminidase/galactose oxidase/NaB^3H_4 [9].

2.5. *Analytical methods*

Polyacrylamide gel electrophoresis was performed in sodium dodecyl sulphate (SDS) on 10% (w/v) slab gels [14] after reduction of the samples in 2% (w/v) SDS, 0.1 M dithiothreitol, 10% (w/v) glycerol, 80 mM Tris–HCl (pH 6.8) at 100°C for 1 min. Radiolabelled proteins were detected after drying the gel, by direct autoradiography for ^{125}I and fluorography for ^{3}H [15]. Incorporation of radioactivity into protein was measured by counting an aliquot dried on a Whatman GF/A filter disc before and after sequential washing in 7% trichloroacetic acid, ethanol and diethyl ether.

3. Results and discussion

The butanol extract of DISPITC routinely contained ~ 50% of the radioactivity originally added as Na^{125}I. This figure compares well with the recovery of ^{125}I in crystals of DDISA prepared using a similar procedure (~ 60%). Thin-layer chromatography of the butanol extract separated two radioactive constituents: a major one in the position of DISPITC (R_F0.81); a minor one in the position of diiodosulphanilic acid (R_F0.59). By this method a reagent of specific activity 7.5–15 mCi/μmol was routinely prepared. No deterioration in its reactivity was noted upon dry storage at −70°C for up to 2 months. Various derivatives of phenylisothiocyanate have been used as both general protein and membrane protein reagents [11,16–18].

The ultraviolet spectrum of non-radioactive DISPITC showed an A_{302} max and this absorbance decreased upon reaction with glycine ($t_{½}$ < 1 min at

20°C with 0.03 mM reagent and 0.25 mM glycine). On mixing [^{125}I]DISPITC (3 nmol) with cytochrome *c* (50 nmol) in 0.5 ml HBS at 20°C, the incorporation of radioactivity into protein was maximal after 30 min and 80% of maximal after 15 min. Using similar conditions to these 2 trial experiments (see section 2.3), 2–5% of the added radioactivity was routinely incorporated into the protein of whole cells; the corresponding value for the lactoperoxidase labelling procedure was 0.5–2%. The recovery of cells was 60–70% and their viability decreased by < 10% during the procedure.

The labelling pattern of BRI-8 plasma membrane prepared from whole cells which had been reacted with DISPITC was identical to that of the original cells, but differed considerably from that obtained when the isolated membrane was exposed to the reagent (fig.1). This observation suggests that DISPITC is prevented from reacting with many plasma membrane proteins in intact cells presumably because it does not penetrate the membrane. Two reagents with similar structures to DISPITC have also been reported to be excluded by intact cells [10,11]. The labelling pattern of whole cells contained one major band (mol. wt 55 000) and ~ 15 less intense bands. The major band was apparently also strongly labelled by pyridoxal phosphate (fig.2) and may be related to the anion transport protein of erythrocytes which preferentially binds similar anionic reagents [3,11].

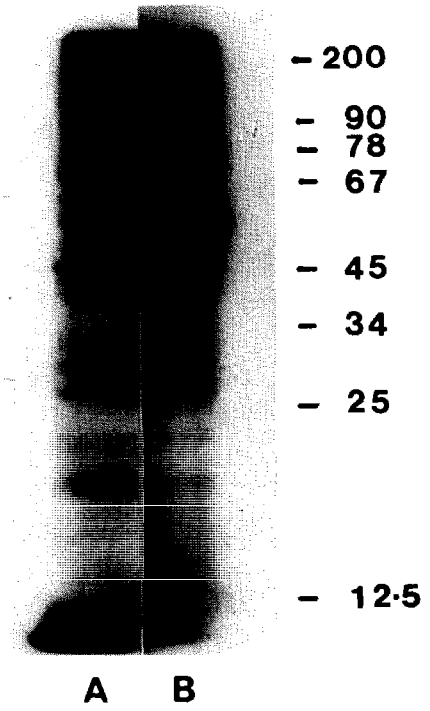

Fig.1. Autoradiograph of SDS–polyacrylamide gel electrophoretograms of BRI-8 plasma membrane. (A) Membrane reacted with DISPITC; (B) membrane prepared from whole cells reacted with DISPITC. The positions of standard proteins of known molecular weights ($\times 10^{-3}$) are indicated on the right.

The capacities of DISPITC, and other putative cell-surface labelling reagents [2–6,8,9], to interact uniformly with cell surface proteins was assessed by comparing the labelling patterns obtained using BRI-8 cells as a model. Each reagent was used under conditions reported to give no penetration of the membrane. Of the reagents tested (see section 2.4) only the formyl methionine sulphone methyl phosphate failed to label the cells to any extent (< 0.01% incorporation of added radioactivity). This failure is probably attributable to the lower pH necessary to keep lymphocytes viable (pH 8.5) compared with the conditions used for the reaction of erythrocytes with this reagent (pH 9.6) [4]. The labelling pattern obtained with each reagent was unique, although some proteins were clearly labelled by several of the reagents (fig.2). The patterns obtained with the two reagents which are specific for carbohydrate (periodate and galactose oxidase) differed only in the labelling of one band (mol. wt 90 000 with periodate and 110 000 with galactose oxidase). A similar difference was observed in erythrocytes [6]. Surprisingly, these glycoproteins, which are assumed to be major cell-surface proteins, were not labelled by all of the other reagents. DDISA and pyridoxal phosphate were the most limited in their reactions. None of the reagents tested labelled all of the proteins which were capable of being labelled by the sum of the reagents and so none of them can be classified as a general cell-surface labelling reagent. This observation advises caution in interpretation of data obtained by use of such reagents, particularly in respect of conclusions derived from a lack of labelling. DISPITC was one of the more general reagents. It labelled more proteins than its diazotised analogue, and should prove a useful addition to the complement of cell-surface and pro-

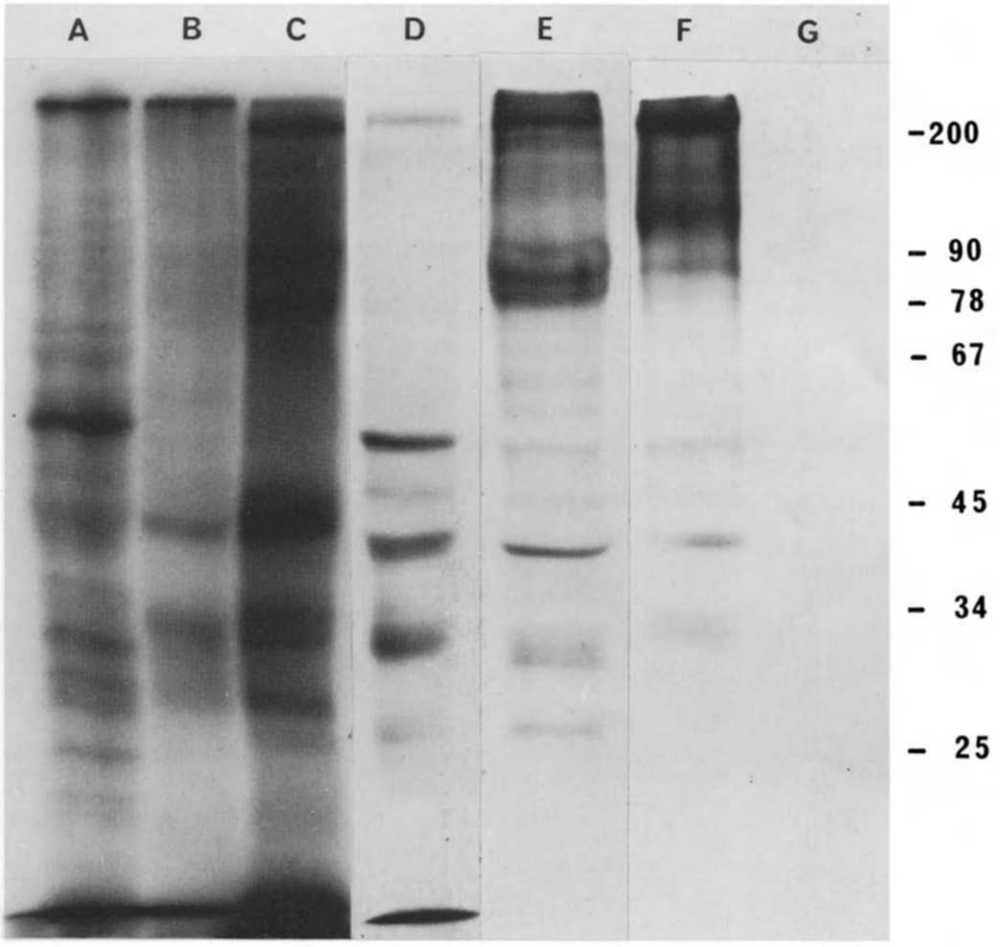

Fig.2. Autoradiograph (A–C) and fluorograph (D–G) of SDS–polyacrylamide gel electrophoretograms of BRI-8 cells labelled with :(A) DISPITC; (B) DDISA; (C) lactoperoxidase/glucose oxidase; (D) pyridoxal phosphate/NaB^3H_4; (E) sodium periodate/NaB^3H_4; (F) neuraminidase/galactose oxidase/NaB^3H_4; (G) NaB^3H_4. The alignment between tracks is not exact because of the different extent of swelling associated with the different radioactive detection procedures. The positions of standard proteins of known molecular weights ($\times 10^{-3}$) are indicated on the right.

tein-labelling reagents especially because of its stability and inexpensiveness.

References

[1] Carraway, K. L. (1975) Biochim. Biophys. Acta 415, 379–410.
[2] Helmkamp, R. W. and Sears, D. A. (1970) Int. J. Appl. Radiat. Isotopes 21, 683–685.
[3] Cabantchik, I. Z., Balshin, M., Breuer, W. and Rothstein, A. (1975) J. Biol. Chem. 250, 5130–5136.
[4] Bretscher, M. S. (1971) J. Mol. Biol. 58, 775–781.
[5] Whiteley, N. M. and Berg, H. C. (1974) J. Mol. Biol. 87, 541–561.
[6] Gahmberg, C. G. and Andersson, L. C. (1977) J. Biol. Chem. 252, 5888–5894.
[7] Gabel, C. A. and Shapiro, B. M. (1978) Anal. Biochem. 86, 396–406.
[8] Hubbard, A. L. and Cohn, Z. A. (1976) in: Biochemical Analysis of Membranes (Maddy, A. H. ed) pp. 427–501, Chapman and Hall, London.
[9] Gahmberg, C. G. and Hakomori, S. -I. (1973) J. Biol. Chem. 248, 4311–4317.

[10] Edwards, R. M., Kempson, S. A., Carlson, G. L. and Dousa, T. P. (1979) Biochim. Biophys. Acta 553, 54–65.
[11] Ho, M. K. and Guidotti, G. (1975) J. Biol. Chem. 250, 675–683.
[12] Snary, D., Barnstaple, C. J., Bodmer, W. F., Goodfellow, P. N. and Crumpton, M. J. (1977) Scand. J. Immunol. 6, 439–452.
[13] Snary, D., Goodfellow, P., Hayman M. J., Bodmer, W. F. and Crumpton, M. J. Nature (1974) 247, 457–461.
[14] Laemmli, U. K. (1970) Nature 227, 680–685.
[15] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.
[16] Sigrist, H. and Zahler, P. (1978) FEBS Lett. 95, 116–120.
[17] Edman, P. (1956) Acta. Chem. Scand. 10, 761–770.
[18] Braunitzer, G., Schrank, B. and Ruhfus, A. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 1589–1590.