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Enzymic Iodination

A PROBE FOR ACCESSIBLE SURFACE PROTEINS OF NORMAL AND NEOPLASTIC LYMPHOCYTES

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1. Radioactive iodide was covalently bound to living cells from normal mouse spleen and a variety of lymphoid tumours by a system consisting of lactoperoxidase, hydrogen peroxide and iodide. 2. About 3×10^5–6×10^5 molecules of [^{125}I]iodide/cell could be incorporated without affecting cell viability. 3. Electron-micrographic radioautography showed that the radioactive label was associated with the outer surfaces of the cells. 4. Radioiodinated proteins were solubilized in 9M-urea–0.2M-mercaptoethanol and analysed by gel-filtration and disc electrophoresis. 5. Comparison of distinct tumour lines by disc electrophoresis showed qualitative and quantitative differences in protein distribution patterns.

Proteins associated with the surfaces of cells play dominant roles in the determination of the antigenic configuration of the particular cell (Aoki, Hämmerling, de Harven, Boyse & Old, 1969; Reisfeld & Kahan, 1970*a*,*b*; Nathenson & Davies, 1966). Further, these molecules serve as receptors for specific ligands such as hormones (Lefkowitz, Roth & Pastan, 1970) and antigenic determinants (Ehrlich, 1900; Byrt & Ada, 1969). Although knowledge of the nature of the cell surface proteins involved in immunological and endocrinological recognition and differentiation is necessary for elucidation of the mechanisms of these processes, it is difficult to obtain detailed information about these molecules because only small quantities of material can be isolated in relatively pure form (Reisfeld & Kahan, 1970*a*,*b*). Moreover, highly sensitive methods for the analysis of these cell proteins are needed because the cell membrane constitutes only 2–5% of the cell mass (Dowben, 1969) and a given receptor would probably comprise less than 5% of the membrane protein or approx. 10^{-13}g/cell.

The aim of the present study was to devise a method of increasing the sensitivity of analysis of cell surface proteins. In addition, we sought an approach that could be applied to actively metabolizing cells without causing loss of viability, so that the events of activation and differentiation could be followed. Radioiodination of proteins has been a useful technique in various analytical studies (Greenwood, Hunter & Glover, 1963; Ada, Nossal & Pye, 1964; McConahey & Dixon, 1966) including the characterization of minute quantities of immunoglobulins produced by single cells (Marchalonis & Nossal, 1968). We previously reported the use of the enzyme lactoperoxidase to catalyse the radioiodination of tyrosine residues present in serum proteins under conditions where no physicochemical evidence of denaturation was observed (Marchalonis, 1969).

In this paper we describe an adaptation of this enzymic method that allows the covalent labelling of proteins present on the outer surfaces of normal and neoplastic lymphoid cells. We also provide results on the resolution of these accessible proteins by gel filtration and disc electrophoresis under dissociating conditions.

MATERIALS

Buffers. Reagents were dissolved in phosphate-buffered saline, pH 7.3, containing 0.05M-sodium phosphate–0.15M-NaCl. Cells were prepared and washed in Eisen's balanced salt solution (Eisen, Kern, Newton & Helmreich, 1959), and resuspended in phosphate-buffered saline before iodination.

Ficoll. Ficoll, mol.wt. 400000, was obtained from Pharmacia, Uppsala, Sweden.

Foetal calf serum. This was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia.

Lactoperoxidase. This enzyme was prepared from the whey of fresh skim milk by the procedure of Hogg & Jago (1970). The purified lactoperoxidase had an E_{412}/E_{280} ratio of 0.70. Enzyme concentrations were estimated by measuring E_{412} [$\epsilon_{412} = 11.1\times10^4\,\text{M}^{-1}\cdot\text{cm}^{-1}$ (Morrison, Hamilton & Stotz, 1957)].

Radioactive iodide. Carrier-free [^{125}I]iodide was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., at a concentration of 90–160mCi/ml. Carrier-free

[^{131}I]iodide was supplied by the Australian Atomic Energy Commission, Lucas Heights, N.S.W. The specific radioactivity of this preparation was 500mCi/ml.

Hydrogen peroxide. A stock solution of 10M-H_2O_2 (analytical grade) was obtained from Ajax Chemicals Ltd., Melbourne, Australia. Dilutions of the stock solution were made with phosphate-buffered saline. Opened bottles of 10M-H_2O_2 were discarded after three weeks.

Animals. Male and female CBA mice, weighing approx. 25g, were used as a source of normal lymphoid cells.

Tumour cell lines. Cells of a murine lymphoma (SIAT. 4), myeloma (MOPC 460), thymoma (WEHI 22) and mastocytoma (P815) maintained *in vitro* were supplied by Dr A. W. Harris, Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia.

METHODS

Preparation of cell suspensions. Single-cell suspensions were obtained from the spleens of CBA mice that were older than 100 days. Mice were killed by cervical dislocation and the spleens were removed and teased on a stainless-steel mesh into chilled Eisen's balanced salt solution. Cell debris and clumps were removed by allowing the cell suspension to settle through 1ml of foetal calf serum for 5min at 4°C. The cell suspension was then layered over 34% Ficoll (w/v) in Eisen's balanced salt solution and centrifuged at 500*g* for 20–25min to remove erythrocytes and dead lymphoid cells (Perper, Zee & Mickelson, 1968). The band of cells above the Ficoll–buffer interface was removed with a Pasteur pipette and washed twice with Eisen's balanced salt solution. The cells were resuspended (4×10^7 cells/ml) in phosphate-buffered saline before iodination. Cells prepared in this fashion were 92–98% viable as judged by Eosin dye exclusion.

Tumour cells were washed three times with chilled Eisen's balanced salt solution and resuspended in phosphate-buffered saline before iodination. The cells were 98–99% viable as judged by exclusion of Eosin dye.

Lactoperoxidase-catalysed iodination. This procedure was similar to that described by Marchalonis (1969) except that the reaction was carried out at 30°C. Iodination of cells was performed in 12ml polystyrene centrifuge tubes. The reaction mixture usually contained 0.25ml of cells at a concentration of 5×10^6–10×10^6 cells per ml, 10μl of lactoperoxidase (0.3mg/ml in phosphate-buffered saline), and 5μl of iodide solution. For studies of incorporation where a high specific radioactivity was not critical, a solution consisting of [^{125}I]-iodide diluted in K^{127}I carrier to give a specific radioactivity of 1mCi/ml was used. Carrier-free [^{125}I]- or [^{131}I]-iodide was used in experiments involving fractionation of iodinated proteins. The iodination reaction was initiated by the addition of 10μl of 10μM-H_2O_2. The cell suspension was vigorously mixed after the addition of all reagents. When desired the reaction was stopped by dilution with 10ml of chilled Eisen's balanced salt solution containing 5% (v/v) foetal calf serum. The cells were washed two times with Eisen's balanced salt solution containing foetal calf serum and resuspended to 1ml. Before radioautography or lysis, the cells were washed twice on discontinuous foetal calf serum gradients (100%, 50% and 25% in phosphate-buffered saline). All washes were performed at 4°C. The amount of radioactive iodide was assessed by counting the radioactivity of the cells in a Packard Autogamma scintillation spectrometer equipped with a deep-well sodium iodide crystal detector.

Disc electrophoresis on polyacrylamide gel. This procedure was carried out under dissociating conditions by procedures described in detail by Parish & Marchalonis (1970). The gels were sliced into 35–40 fractions by using a Canalco slicer. The radioactivity of slices was counted in plastic tubes by using the Packard Autogamma Spectrometer. Mobilities are expressed as distance migrated relative to that of a Methyl Green dye marker (R_F).

Gel filtration. Cell proteins that were soluble in 9M-urea were fractionated by gel filtration on a column (95cm × 1.5cm) of Sephadex G-100 (Pharmacia) equilibrated with 6M-urea–1M-propionic acid. The flow rate was 15ml/h. The procedure has been described by Marchalonis & Edelman (1965, 1968). Polypeptide chains of human immunoglobulins were used as molecular-weight calibration standards in the gel-filtration and disc-electrophoresis analyses. These were human light chain (mol.wt. 22000), γ-chain (mol.wt. 53000) and μ-chain (mol.wt. 70000); the mol.wt. values are taken from Edelman & Gall (1969). These polypeptide chains were prepared by cleaving interchain disulphide bonds with 2-mercaptoethanol under the conditions described by Edelman & Marchalonis (1967).

Radioautography. (*a*) Light microscopy. Radioautography of smears of cells iodinated with [^{125}I]- or [^{131}I]-iodide was performed on the scale of light microscopy by the method of Byrt & Ada (1969).

(*b*) Electron microscopy. Cells were resuspended in foetal calf serum and spun down gently in small cellulose nitrate ultracentrifuge tubes and then fixed in three changes of 2.5% glutaraldehyde in sodium cacodylate buffer, pH 7.4. After a total of 1h of fixation the end of the tube containing the cell pellet was cut off and left in cacodylate buffer overnight. The next day the samples were post-fixed in 2% osmic acid for 2–4h, dehydrated in graded acetone solutions and infiltrated and embedded in Araldite (CIBA Basel, Switzerland).

Ultrathin sections were cut with an LKB Ultratome III and processed for radioautography by the method of Salpeter & Bachmann (1964), as slightly modified by Mitchell & Abbot (1965). The sections were exposed for 1–3 weeks, developed with Dektol (Kodak) and mounted on 100-mesh copper grids or 1mm × 2mm slotted discs. The specimens were examined unstained in a Phillips EM300 electron microscope by using a 20μm ultrathin objective aperture and an accelerating voltage of 60kV.

RESULTS

Iodination of cells

We tried a variety of methods including modifications of the chloramine-T method of Hunter & Greenwood (1962) and the lactoperoxidase method (Marchalonis, 1969) that we had developed for the iodination of serum proteins. In the present studies, we found that 10^7 lymphoid cells was the most con-

Table 1. *Effect of reagent concentration on the incorporation of* [^{125}I]*iodide by spleen cells*

All samples contained 10^7 cells.

Experiment	Lactoperoxidase (μg in reaction mixture)	H_2O_2 (μmol in reaction mixture)	[^{125}I]Iodide incorporated (% of added material)
A	0.35	0.1	1.8
	0.75	0.1	3.7
	1.50	0.1	6.6
	3.00	0.1	6.5
	4.00	0.1	7.9
	6.00	0.1	9.7
B	3.00	0.025	2.8
	3.00	0.05	4.2
	3.00	0.10	5.7
	3.00	0.20	9.0
	3.00	1.00	12.4
	3.00	10.00	3.4
C	3.00	0.00	0.04
	0.00	0.1	0.03

venient number to use in the standard assay. This quantity of normal and tumour cells was readily available and could incorporate radioactivity sufficient for a variety of analytical procedures, under conditions where cell viability was not adversely affected. Table 1 presents results from experiments designed to assess the effects of variation of lactoperoxidase and hydrogen peroxide concentration on the labelling of spleen cells. Incubation with enzyme, [^{125}I]iodide and hydrogen peroxide was carried out at 30°C for 5min in these experiments. Fig. 1 shows that this incubation time was sufficiently long to allow maximum iodination to be achieved.

As Table 1 shows, the addition of 1.5–6.0μg of lactoperoxidase to 10^7 cells in the presence of 0.1μmol of hydrogen peroxide allows the incorporation of 7–10% of the available iodine. This value corresponds to 3×10^5–6×10^5 molecules of iodine/cell. Part B of Table 1 illustrates the dependence of the iodination reaction on the concentration of peroxide. The inhibitory effect of a high concentration of peroxide, as well as the fact that concentrated peroxide solutions deteriorate on standing, are critical factors in the application of this experimental method. Section C of Table 1 shows that peroxide and enzyme must both be present for significant incorporation of radioactive iodide to occur. We concluded that iodide incorporated under these conditions was covalently bound to protein because it was not removed by exhaustive washing, was precipitated by fixatives used as a routine to render proteins insoluble, and remained with proteins when these were analysed under rigorous dissociating conditions. Cells labelled with [^{125}I]-iodide by this method exhibited no loss of viability as judged by the exclusion of Eosin. The initial viability of these cells in these experiments was at least 95%. To exclude the possibility that radioiodinated lactoperoxidase or some adventitiously iodinated component of the washing media was cytotropic, the following experiment was performed. To 0.25ml of phosphate-buffered saline were added the standard amounts of [^{125}I]iodide, lactoperoxidase and hydrogen peroxide. After 5min at 30°C, 10^7 spleen cells in 5ml of chilled Eisen's balanced salt solution that contained 5% (w/v) of foetal calf serum were added. After this, standard washing procedures were carried out at 4°C. Under these conditions, less than 0.1% of the [^{125}I]iodide was incorporated into cells. This value corresponded closely to those obtained in Table 1 when peroxide and enzyme were excluded from the reaction mixture.

It was possible to incorporate more iodide into the cells by using a gentle chloramine-T method. The number of cells used and the [^{125}I]iodide solutions were the same as those described above, but 20μg of chloramine-T was added to oxidize the iodide to the reactive I^+ form. The reaction was allowed to proceed for 5min at 4°C, and then the washes were carried out as described above. Under these circumstances, 10–20% of the iodide was taken up by the cells. However, the viability of the lymphoid cells was often decreased, and electron-micrographic radioautography showed that the label had penetrated the interior of the cells. Although cell membranes were usually intact when examined with the electron microscope, most of the chloramine-T-labelled cells showed some evidence of deterioration. We therefore concluded that the lactoperoxidase method gave optimum results. Although the initial standardization experiments were performed by using heterogeneous

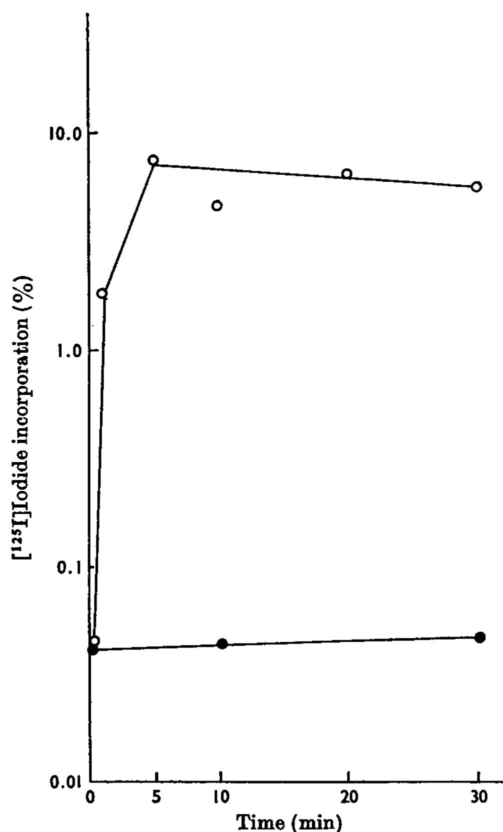


Fig. 1. Kinetics of incorporation of [^{125}I]iodide into lymphocytes obtained from mouse spleen. Incubation was performed at 30°C under the conditions described in the text. ○, Complete reaction system; ●, the reaction system contained cells, H_2O_2 and [^{125}I]iodide solution, but no lactoperoxidase. The scale of the ordinate is logarithmic.

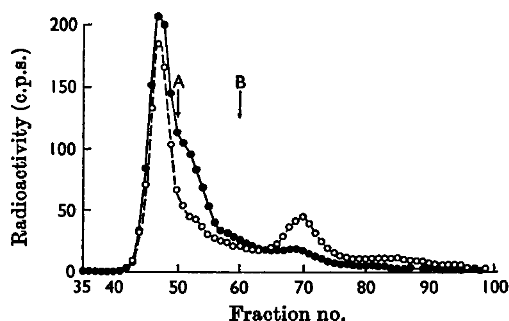


Fig. 2. Fractionation by gel filtration on Sephadex G-100 in 1M-propionic acid–6M-urea of urea-soluble cell proteins radioiodinated by the lactoperoxidase method. ○, Radioactivity of [^{125}I]iodide-labelled protein from spleen cells; ●, radioactivity of [^{131}I]iodide-labelled proteins from lymphoma SIAT-4 cells; A, B, elution volumes of proteins included as reference standards: A, immunoglobulin γ-chain (mol.wt. 53000); B, immunoglobulin light chain (mol.wt. 22000).

suspensions of cells from spleens and thymuses, the tumour-cell populations that had been grown *in vitro* provided much better material for precise analytical work because these were homogeneous populations of cells and the initial cell viability in the experiments was often as high as 99%.

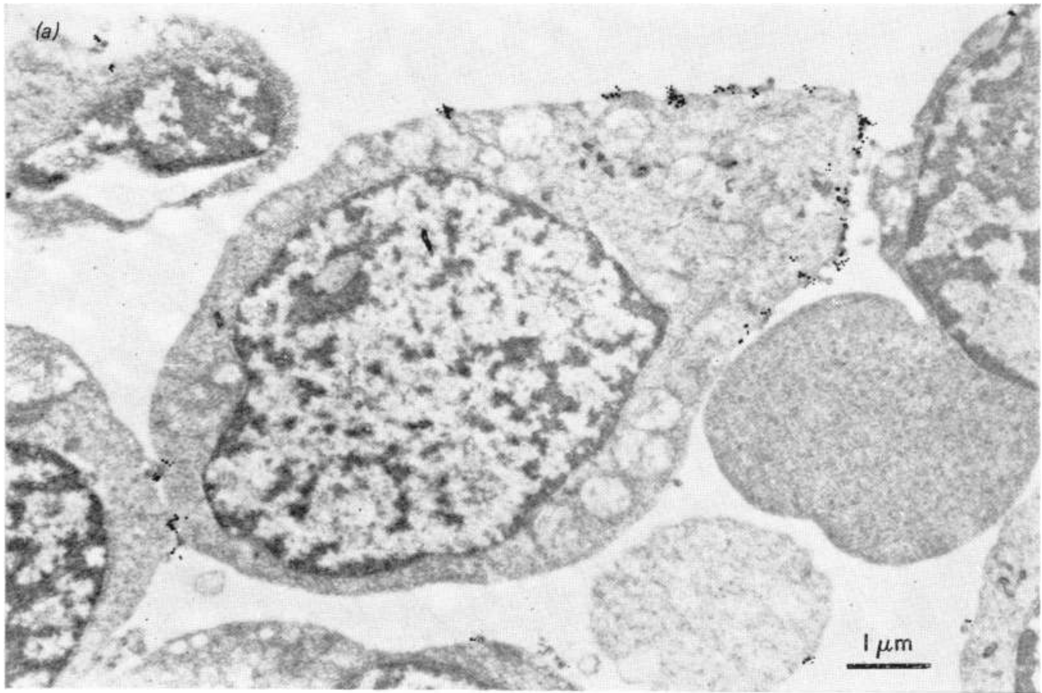
Radioautographic analyses of radioiodinated cells

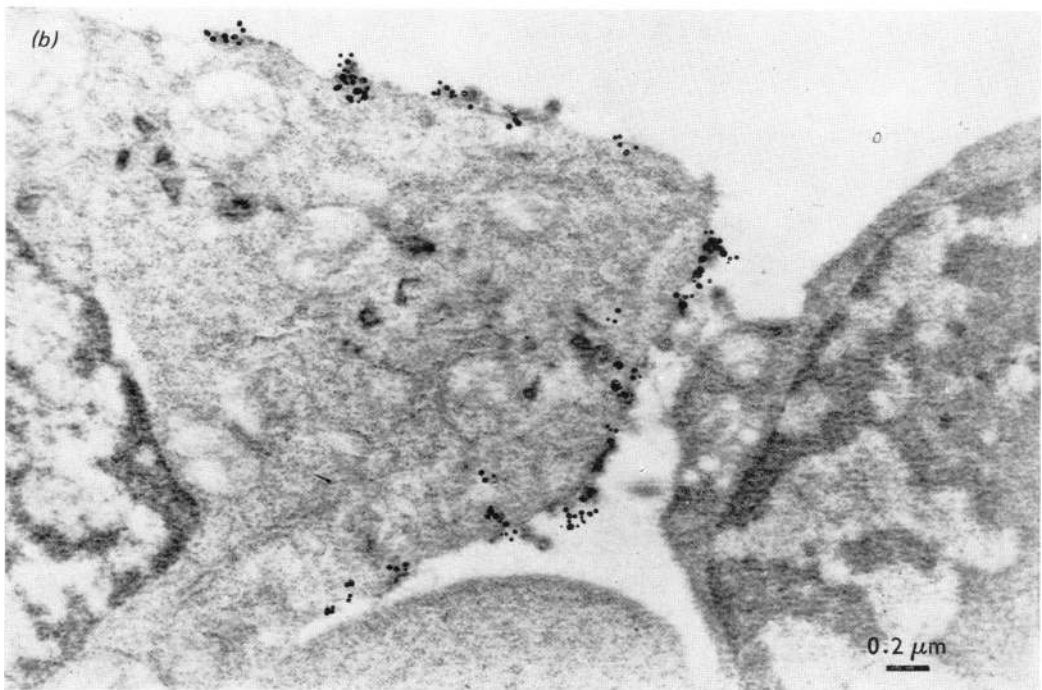
Radioautography of [^{125}I]- or [^{131}I]-iodide-labelled cell preparations was carried out on the scale of light microscopy by the procedure of Byrt & Ada (1969). With spleen-cell suspensions, lymphocytes were labelled consistently better than were monocytes. Erythrocytes were labelled rather poorly under the conditions used. This observation was confirmed by studies of lactoperoxidase-catalysed iodination of pure erythrocyte suspensions, which showed that these cells were labelled only 10% as well as lymphoid cells. In general, more than 95% of the lymphoid cells in each preparation of normal or tumour-cell suspensions were iodinated. The degree of labelling was moderate, with each cell possessing 20–50 grains.

A more detailed analysis was performed by subjecting cells iodinated with [^{125}I]iodide to electron-micrographic radioautography. This procedure allowed the radioiodinated protein to be located with a high degree of precision. Plate 1 shows electron micrographs of radioiodinated lymphoma SIAT.4 cells. All of the cells were labelled and the grains were associated only with the outer surfaces of the intact cell.

Fractionation of radioiodinated cell-surface proteins

The general approach followed in the fractionation of radioiodinated proteins from cell surfaces was to solubilize labelled protein in 9M-urea and to perform gel filtration or disc electrophoresis in buffers containing this dissociating solvent. Fig. 2 illustrates the results of gel filtration of the radioiodinated accessible proteins of spleen cells and SIAT.4 tumour cells. After iodination and washing, the cells were dissolved in 9M-urea containing 0.2M-mercaptoethanol. Addition of this thiol was found to double the amount of material with mol.wt. between 10000 and 100000. The preparations were incubated at 37°C for 2h and refrigerated overnight





EXPLANATION OF PLATE I

Electron-microscopic radioautography of SIAT-4 lymphoma cells radioiodinated by the lactoperoxidase method. The sections are unstained to maximize contrast between the grains and the cells. Radioautographs were exposed for 2 weeks.

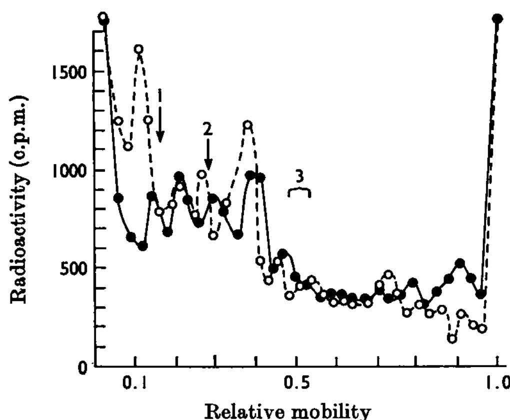


Fig. 3. Comparison by disc electrophoresis in 9M-urea–acetic acid of urea-soluble surface proteins of cells from lymphoma SIAT.4 (○) and myeloma tumour MOPC 460 (●). The abscissa shows the mobility relative to that of Methyl Green dye marker. Numbers 1, 2 and 3 refer to the R_F values of standard proteins subjected to electrophoresis under these conditions: 1, immunoglobulin μ-chain; 2, immunoglobulin γ-chain; 3, immunoglobulin light chain.

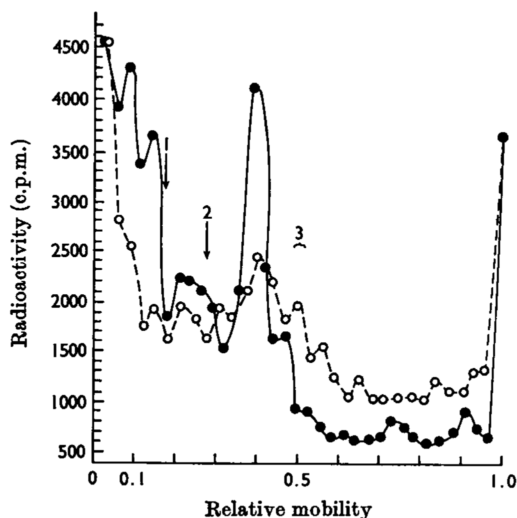


Fig. 4. Comparison by disc electrophoresis in 9M-urea–acetic acid of urea-soluble surface proteins of thymoma WEHI 22 (●) and mastocytoma P815 (○). The abscissa and meanings of numbers 1, 2 and 3 are the same as those in Fig. 3.

before fractionation. Nucleic acids and other insoluble cell materials tended to aggregate under these conditions and were removed by centrifugation. Gel filtration was carried out in Sephadex G-100 equilibrated with 6M-urea–1M-propionic acid (Marchalonis & Edelman, 1965). Spleen and SIAT.4 cells both possessed radioactive proteins characterized by molecular weights larger than that corresponding to the exclusion volume of the gel. These proteins constituted 43% and 31% of the urea-soluble proteins of the spleen and lymphoma cells respectively. In addition, both cell types possessed urea-soluble proteins characterized by elution volumes consistent with molecular weights between 10000 and 70000. Further, although the protein-elution profiles were generally similar, reproducible differences between these two cell types were noticed. The lymphoma possessed relatively more material of mol.wt. approx. 50000 and less material of mol.wt. 10000 than did the spleen cells.

It was possible to compare the fraction of cell protein labelled under enzymic conditions with total cell protein by two approaches: (a) direct staining of bulk proteins and (b) by first dissolving the cells in 9M-urea, then radioiodinating with the same amount of [^{125}I]iodide but using the chloramine-T oxidation method. Pronounced differences were observed between proteins localized by these methods and proteins iodinated by lactoperoxidase. The major component of the bulk protein was characterized by an R_F value of 0.21–0.23, whereas this mobility region did not contain the predominant amount of radioactivity in the lactoperoxidase-treated cells. As Figs. 3 and 4 illustrate, a protein with an R_F value of 0.35–0.40 usually constituted the major protein fraction. Further, although the protein patterns obtained in the enzymic case contained multiple bands, they were less complex than that of total cell protein.

Accessible proteins obtained from four distinct murine tumour lines are compared by disc electrophoresis in Figs. 3 and 4. The arrows designate the R_F values of proteins that were included as reference standards, namely human immunoglobulin light chain (mol.wt. 22000), γ-heavy chain (mol.wt. 53000), and μ-heavy chain (mol.wt. 70000). The electrophoresis conditions used here were those established by Parish & Marchalonis (1970) to resolve immunoglobulin chains and fragments in the mol.wt. range 10000–100000. Although the present results provide a much more detailed analysis than that obtained by gel filtration, they are consistent with those in Fig. 2. The distinct cell lines shared some components but were different quantitatively and qualitatively in others. All the cells possessed a relatively large component in the R_F range 0.35–0.40 but differed markedly in the R_F range 0.15–0.35. Further, the protein pattern of a mastocytoma, P815, was quite distinct from those of the three lymphoid tumour cells.

DISCUSSION

The object of this study was to devise a means of radioiodinating proteins associated with the surfaces of living lymphoid cells. We found that a system consisting of purified lactoperoxidase and hydrogen peroxide would catalyse the incorporation of as many as 6×10^5 molecules of [^{125}I]iodide/lymphoid cell without decreasing the cell viability. We also tried modifications of the chloramine-T oxidation method (Hunter & Greenwood, 1962; Greenwood *et al.* 1963) as a way of radioactively labelling cell surfaces. This method proved unsatisfactory for our purposes because electron-micrographic radioautography indicated that radioiodinated protein was found within cells and these cells often showed evidence of deterioration. By contrast, the radioiodinated protein of cells labelled by the lactoperoxidase method was associated only with the cell surfaces.

The amount of radioactivity incorporated into cell protein by the enzymic method was sufficient to enable fractionation of these molecules by gel filtration and disc electrophoresis. Analysis by gel filtration on Sephadex G-100 of iodinated cell-surface proteins that were soluble in urea indicated that 58–69% of this material possessed molecular weights within the range of resolution of the gel. Disc-electrophoresis conditions were designed to provide optimum resolution of components that had molecular weights from 10000 to 100000 (Parish & Marchalonis, 1970). Under these conditions, 12–18 bands could be detected. Comparisons with total cell protein, either by use of Amido Black stain or by iodination of proteins from lysed cells, suggested that certain components were preferentially labelled by the enzymic method. All of the cell types studied possessed a highly radioactive component characterized by a mobility intermediate between those of the comparison standards human immunoglobulin light chain and γ-heavy chain. Molecular-weight estimates of the highly radioactive protein by the method of Parish & Marchalonis (1970) give values of approx. 40000. The major components detected by staining or iodination of total cell protein were located in the R_F range 0.15–0.30, which was markedly distinct from that of the above protein.

Comparisons by disc electrophoresis of radioiodinated proteins from the different cell lines established that each cell type possessed a characteristic protein-distribution pattern. Although certain components were present in all four tumour-cell lines, the amount of each relative to the other labelled proteins often varied between cell types. Further, components specific to a particular cell line were occasionally present. These observations are consistent with a number of reports that various lymphocyte populations may differ in surface properties reflected in their capacity to bind antigen (Byrt & Ada, 1969) and presence or lack of distinctive antigens such as TL, θ and H2 (Aoki *et al.* 1969).

The identification of the accessible cell-surface proteins resolved in the present study is of direct relevance to the elucidation of physiological events mediated through cell surface proteins. A cell could conceivably possess a variety of surface components that are involved in recognition and activation. Among these are the following: receptors for antigen (Byrt & Ada, 1969), receptors for hormones (Lefkowitz *et al.* 1970) and cell-specific antigens (Aoki *et al.* 1969). We have found that murine cells obtained from normal spleen and cultured tumours possessed a surface protein with mol.wt. approx. 40000. It may prove significant that this value is comparable with that observed by Reisfeld & Kahan (1970*a*,*b*) for HL-A antigen derived from human lymphocytes. The exact identification of the proteins detected in the present work requires the application of immunological techniques, the use of affinity labelling reagents (Singer & Doolittle, 1966) and, conceivably, the utilization of peptide 'mapping' techniques.

Berg (1969) used a conceptually similar approach to study proteins associated with the outside of erythrocyte membranes. The diazonium salt of [^{35}S]sulphanilic acid did not penetrate the cell membrane. It bound primarily to a protein with an estimated mol.wt. of 140000. The effects of reagents such as these on actively metabolizing cells should be investigated.

The advantages of the enzymic method of labelling cell surface proteins are its gentleness and sensitivity. Sufficient radioactive iodide can be incorporated into all surface proteins from 10^7 lymphocytes to facilitate a variety of analytical studies. Further, physiological studies of the living cells, such as the turnover of membrane components, can be followed. We hope that this procedure may prove useful in biochemical and immunlogical studies.

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