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AN EVALUATION OF TECHNIQUES FOR LABELLING THE SURFACE PROTEINS OF CULTURED MAMMALIAN CELLS

R. L. JULIANO[a, b] and M. BEHAR-BANNELIER[a]

[a]*The Research Institute, Hospital for Sick Children and* [b]*The Department of Medical Biophysics, University of Toronto, Toronto (Canada)*

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SUMMARY

We have evaluated four techniques for labelling the surface proteins of cultured mammalian cells. The techniques are: (a) the lactoperoxidase system; (b) the pyridoxal phosphate–[^{3}H]borohydride system; (c) the [^{3}H] 4,4′–diisothiocyano-2,2′-dihydrostilbene disulfonate system and (d) the galactose oxidase–[^{3}H]borohydride system. The subcellular distribution of radiolabel produced by these techniques has been evaluated by autoradiography at the light microscope level and by cellular fractionation. We find that while all four systems label the surface membranes in the majority of the cell population, they also heavily label internal sites in a small subpopulation of nonviable cells. The contribution of the internally labelled cells to further biochemical analysis may represent a severe problem in investigations which rely solely on surface labels for the study of plasma membrane organization.

INTRODUCTION

At the present time a good deal is known about the organization of the proteins of the erythrocyte membrane. Much of this information has resulted from studies employing surface labels (for a review see ref. 1). By this we mean reagents which react with proteins, but whose size or solubility properties render them unable to enter cells, thus restricting their sites of reaction to the cell periphery. Several techniques for surface labelling have been developed, including enzymatic radioiodination, using the lactoperoxidase system [2] ^{3}H-labelling, using pyridoxal phosphate and [^{3}H] borohydride [3], reaction with radioactive compounds such as ^{35}S-labelled formylmethionylsulfone methylphosphate (FMMP), and ^{3}H-labelled 4,4′-diisothiocyano-2,2′- dihydrostilbene disulfonate (DIDS) [4, 5] and the galactose oxidase [^{3}H]borohydride method for labelling glycoprotein and glycolipids [6].

Surface label techniques usually have considerable chemical, as well as topological specificity; for example DIDS binds to protein non-covalently, but can also

Abbreviations: FMMP, formylmethionylsulfone methylphosphate; DIDS, 4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonate.

form covalent bonds with protein (or lipid) amino groups [1,5]. Pyridoxal phosphate forms a Schiff base with amino groups which may then be reduced by borohydride to form a stable link [3], while lactoperoxidase primarily iodinates tyrosine and possibly histidine residues in proteins [1, 2]. Galactose oxidase specifically oxidizes the 6-position of terminal galactose moieties [6].

It would be interesting to be able to study the arrangement of proteins in the membranes of cultured mammalian cells utilizing the surface label approach. Before this can be done in a judicious manner, however, one must evaluate the merits and pitfalls of this approach, as applied to the cell type in question. The lactoperoxidase system has been studied in terms of the subcellular distribution of label in red cells [2] and in lymphoid cells [7,8]. This technique has also been applied to other cell types such as platelets [9], fat cells [10] and tissue culture cells [11 12], although in the latter two cases little effort was made to ascertain whether the enzymatic iodination was confined to the cell surface. Other labelling techniques have been evaluated in red cells [4,5] and in the influenza virion [3].

In this study we have examined four of the surface label techniques namely, the DIDS system, the lactoperoxidase system, the pyridoxal phosphate–borohydride system and the galactose oxidase system as applied to Chinese hamster ovary cells and to mouse neuroblastoma cells. We have examined the kinetics and other aspects of the labelling procedures, we have evaluated the immediate and long-term effects of the labelling procedures on cell viability, and we have studied the cellular distribution of label through autoradiography and subcellular fractionation.

Our results suggest that a small subpopulation of non-viable cells, which is inevitably present in all cultures, may become heavily labelled at internal sites. This subpopulation may represent an important artifact in further biochemical analysis of whole cell populations treated with surface labels. However, the contribution of internally labelled cells may be minimized by engaging in cell fractionation prior to further analysis.

METHODS

Cells

Chinese hamster ovary cells (CHO cells) were obtained from the Ontario Cancer Research Institute and were maintained as monolayer cultures in medium α plus 10 % fetal calf serum, or as suspension cultures in a 1/1 mixture of medium α and RPMI 1640 medium plus 10 % fetal calf serum. Mouse neuroblastoma cells (N2A cells) were obtained from the American Type Culture Collection and were maintained as monolayers in medium α plus 10 % fetal calf serum. Cells for experiments were harvested during exponential growth.

Labelling techniques

(*a*) *DIDS.* Cells were washed twice in ice-cold Dulbecco phosphate-buffered saline (an isotonic buffer), resuspended in this buffer and incubated on ice with [^{3}H] DIDS for the times and at the concentrations indicated in the legends. The cells were then washed twice in buffer plus 1 % bovine albumin and twice more in cold buffer. No further radioactivity was removed from the cells after the third wash in this case, or in the other labelling procedures.

(*b*) *Lactoperoxidase.* The procedure used was essentially that of Marchalonis et al. [7], except that the cells were washed twice after iodination with buffer plus 1 % albumin plus 10 mM NaI and twice more with buffer; other reaction parameters are given in the legends.

(*c*) *Pyridoxal phosphate–borohydride.* Cells were washed twice with cold buffer and incubated with pyridoxal phosphate in buffer at a pH of 7.5–8.0 and usually at 37°C. The cells were then washed twice with ice-cold buffer and then treated with [^{3}H] borohydride in buffer at ice temperature. The ^{3}H labelling was terminated by the addition of 10 vol. of buffer plus 1 % albumin and the cells were washed twice in this solution and twice more in cold buffer. The[^{3}H] borohydride was dissolved in ice cold 0.01 M NaOH just prior to use.

(*d*) *Galactose oxidase.* Washed cells were treated with 50 units of *Vibrio cholerae* neuramidase for 5 min at 37 °C and then washed. Cells were subsequently treated with 15 units of galactose oxidase for 5 min at 37°C and then with [^{3}H] borohydride for 5 min at 0°C. Labelled cells were washed twice in buffer–albumin and twice in buffer prior to further analysis.

Cell Number and Viability Determinations

Cells were counted by hemocytometry or with the Celloscope particle counter. Viability determinations were made by trypan blue exclusion for the short term [13], or by measurement of relative plating efficiency for the long term [14].

Cell Fractionation

Subcellular fractions were isolated using the methods of Brunette and Till [15] and of Vaughn et al. [16]. Washed cells were swollen in cold 1 mM Zn Cl_2 and homogenized in a Dounce homogenizer with a tight pestle (Johns Glass Co.) until more than 90 % of the cells were broken. The nuclei and membrane fragments were sedimented with a centrifugation of $2 \cdot 10^4 g \cdot$min.The supernatant was recovered and centrifuged at $2.5 \cdot 10^6$ $g \cdot$min; this second supernatant was considered to be the cytoplasmic or soluble fraction. The pellet of the first centrifugation was respun under the same conditions as before and then resuspended in 12 ml of an aqueous two-phase polymer system which was identical to that used in ref. 15, except that $MgCl_2$ was substituted for $ZnCl_2$. The two-phase system was centrifuged at $2 \cdot 10^5$ $g \cdot$min; the "supernatant", including the plasma membranes, was decanted, remixed and recentrifuged under the same conditions. The interface of the second two-phase centrifugal separation, which contained mainly plasma membranes, was recovered and washed twice in cold 2 mM $MgCl_2$. The pellet of the first two-phase centrifugal separation which contained mainly nuclei, but also some whole cells and membrane fragments, was extracted with 1.5 % deoxycholate in 15 mM NaCl plus 2 mM $MgCl_2$ [16] so as to remove membranous material. The resulting pellet contained almost entirely nuclei, free of whole cells and membranes. Thus the homogenate was separated into soluble, plasma membrane and nuclear fractions.

Autoradiography

Autoradiography was generally conducted according to the methods of Prescott [17]. Labelled coverslips or Petri-dish cultures were washed, fixed in 1 % gluteraldehyde in buffer and extracted several times with cold 5 % trichloroacetic acid

and with water. Smears of labelled, washed, suspension cells were fixed in Carnoys solution for 20 min and extracted with ethanol and with water. Slides holding smears or mounted coverslips were dipped in Kodak NTB-2 emulsion and exposed to radiation for 1 week in light-tight boxes at refrigerator temperature. The slides were developed with Kodak Dektol and Rapidfix and were sometimes counterstained with Giemsa. Slides were observed and photographed in a Zeiss universal microscope.

Other methods

Cells and subcellular fractions were frequently precipitated by mixing 1 vol. of sample with 1 vol. of 0.1 % albumin and then adding 2 vol. of cold 5 % trichloroacetic acid. The samples were allowed to precipitate at ice temperature and the precipitates were collected in Millipore filters and washed with cold 5 % trichloroacetic acid. Some samples were also precipitated with cold 90 % ethanol. Lipid extractions were done by mixing precipitated samples with 10 vol. of ethanol-diethyl ether (1:1, v/v) for 15 min at room temperature and separating the precipitate and supernatant by centrifugation. ^{3}H was detected in a Packard Tri Carb liquid scintillation counter, using toluene plus 4.2 % Liquifluor and 10 % Protosol as a solvent, or using Brays solution. ^{125}I was detected using a Nuclear Chicago γ- counter. Proteins were determined by the method of Lowry et al. [18]. $(Na^+ + K^+)$-ATPase activity was measured by the methods of Kimelberg and Papahadjopoulos [19]while DNA was measured by the method of Schneider [20]. Gel electrophoresis was conducted according to Fairbanks et al. [21].

MATERIALS

Medium RPMI 1640, fetal calf serum and sterile buffered salt solutions were purchased from Grand Island Biological Co. Medium α was obtained from the Ontario Cancer Research Institute. [^{3}H] DIDS was prepared according to the methods of Cabantchik and Rothstein [5] from a precursor (their Compound I) catalytically ^{3}H–labelled by New England Nuclear (spec. act. $3.3 \cdot 10^{15}$ dpm/mole. Unlabelled DIDS was prepared as described previously [5]. ^{125}I (80–140 Ci/l) and sodium [^{3}H] borohydride (5000–20 000 Ci/M) were obtained from Amersham, Searle. Lactoperoxidase and neuraminidase were purchased from Calbiochem, while pyridoxal phosphate, bovine albumin and galactose oxidase were obtained from Sigma. Scintillation solvents were purchased from New England Nuclear or from Eastman Organics. Other chemicals were of reagent grade and were purchased from Fisher Scientific Ltd. Photographic supplies were obtained from Kodak Canada Ltd.

RESULTS

Aspects of Surface Labelling Techniques

(*a*) *DIDS.* [^{3}H] DIDS binds rapidly to cells at ice temperature and the reaction goes to completion in less than 2 min, even at very low chemical concentrations of DIDS (Fig. 1). The degree of labelling is approximately the same, whether the treated cells are washed with buffer or with buffer plus albumin, indicating that the binding of DIDS is not readily reversible [5]. Essentially, all of the bound DIDS is precipitable by cold trichloroacetic acid and only about 25 % is extracted when the cells are

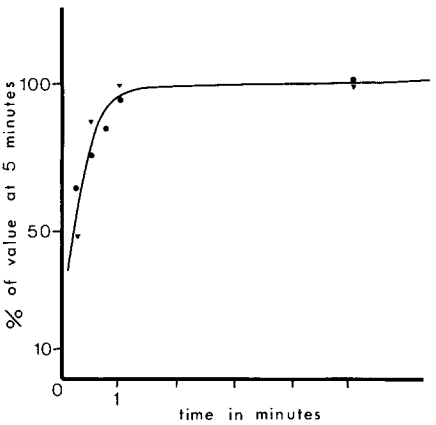


Fig. 1. Kinetics of DIDS binding. CHO suspension cells ($1.5 \cdot 10^6$ /sample) were treated with [^{3}H]-DIDS at a final concentration of 10^{-4} M (▼) or 10^{-7} M (●) in buffer at 0 °C. The reaction was terminated by adding DL-lysine to a final concentration of 10^{-3} M and the cells were washed four times in cold buffer, solubilized in scintillation fluid and counted.

treated with lipid solvents such as ethanol-diethyl ether. Thus most of the bound DIDS is probably covalently, or at least tightly, bound to cellular proteins. Only a small proportion of the applied label was bound to the cells under the conditions used ($< 1\,\%$ at 10^{-7}M and $< 10\,\%$ at 10^{-9}M); however the reaction was more efficient in terms of the ratio of incorporated label to applied label than the others used.

Binding studies with [^{3}H] DIDS indicate the existence of at least two types of binding sites (Fig. 2). These are high affinity (possibly covalent) sites which are present

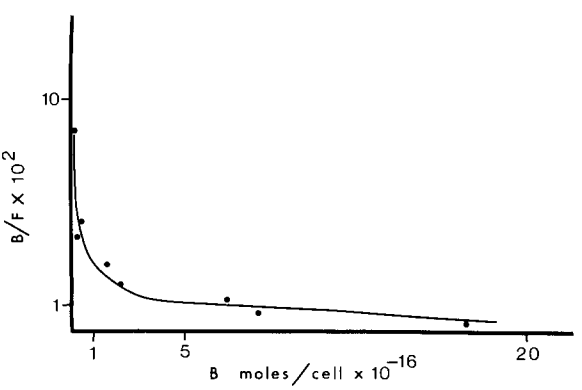


Fig. 2. Scatchard Plot of DIDS binding. CHO suspension cells ($1 \cdot 10^7$/sample) were treated with 1 ml of [^{3}H]DIDS in buffer at final concentrations of 10^{-9} M/ml to $5 \cdot 10^{-6}$ M/ml at 0 °C. After 5 min the cells were washed twice with cold buffer plus albumin and twice with cold buffer and the cell pellets were dissolved in scintillation fluid and counted. The amount of bound DIDS was calculated from the known specific activity. The results are plotted according to the Scatchard equation [22] where B is the amount of agent bound to the cells, F is the amount of free agent, B_0 is the maximum number of binding sites and K is the dissociation constant.

$$B/F = Bo/K - B/K$$

at a level of less than 10^{-16} moles per cell, and a much larger number of lower affinity sites which may be of more than one type [22]. Binding curves of this type have been seen in cases where the reagent binds first to cell surface sites and then slowly penetrates to reach additional sites in the cell interior [23]. However, because of the rapid binding kinetics seen in this system, it seems likely that high-affinity and low-affinity sites are accessible to the reagent from the start of the reaction.

(*b*) *Pyridoxal phosphate.* [^{3}H] Borohydride itself has the capability of labelling cellular lipids or other chemical moieties [3]; however, the amount of acid-precipitable label incorporated into [^{3}H] borohydride-treated cells can be increased 10-fold by pre-treating the cells with pyridoxal phosphate. The reaction with pyridoxal phosphate occurs rather rapidly at 37°C (half-time of 0.8 min at 10 mM), while the reaction at 0°C occurs more slowly, but apparently also may reach equilibrium. Only about 10% of the label retained by thoroughly washed cells is precipitated by cold trichorloacetic acid, while the remainder seems to be trapped or bound borohydride or its breakdown products. Only about 0.1–0.3% of applied radioactivity is recovered as acid-precipitable counts in this system. Dilution of [^{3}H] borohydride with unlabelled borohydride, or the presence of excess pyridoxal phosphate during the ^{3}H -labelling, reduce the efficiency of the reaction still further.

(*c*) *Lactoperoxidase.* In other cell types it has been reported that very little iodination of cellular protein occurred in the absence of exogenous lactoperoxidase [7,11]. In the CHO cell, however, the addition of up to 40 μg/ml of lactoperoxidase produced only a 4–5-fold enhancement above the basal level of iodine incorporation. We suspect that CHO cells might contain endogenous peroxidases.

In our hands, lactoperoxidase-catalysed labelling proceeded rapidly at 37°C, but not at ice temperature. The reaction continues for about 5 min after the addition of H_2O_2, and the degree of labelling is the same whether the same amount of peroxide is added initially as one large dose, or is applied sequentially in several small doses. In the present study, 0.5–1.0% of the applied label was recoverable as acid-precipitable counts. A very large proportion of the radio-iodine which remained associated with the cell after exhaustive washing was not precipitable by trichloroacetic acid; the proportion of acid-precipitable material varied from 20% in the absence of added peroxidase to 50% in the presence of 80 μg/ml of the enzyme.

(*d*) *Galactose oxidase.* Although previous workers [6] have utilized incubation of several hours with galactose oxidase, we find maximal stimulation of ^{3}H incorporation occurs after only 5 min of enzyme treatment under the conditions indicated in Methods. As we shall see later short reaction times are very advantageous in surface label studies. Incorporation of ^{3}H in the absence of enzyme pre-treatment amounts to less than 10% of that in the presence of enzyme. Some of the same considerations apply to the use of ^{3}H borohydride in this system as in the case of the pyridoxal phosphate system. Steck and Dawson [24] have suggested that galactose oxidase treatment may cause proteolysis, however when intact cells were treated under the conditions specified in Methods, no proteolysis was detected in sodium dodecylsulfate gel electrophoretic examination of membranes from treated versus control cells.

Effects on Cell Viability

All of the surface label reagents used in this study have little effect on the viability of cultured cells. We have previously shown that DIDS at concentrations

TABLE I

PLATING EFFICIENCY OF DIDS TREATED CELLS

CHO cells (10^6/sample) were washed, treated with the indicated concentration of DIDS and resuspended in complete medium at a density of approximately 100 cells/ml. Three 2-ml aliquots were then placed in Falcon dishes and the cells were maintained in a tissue culture incubator for one week. Thereafter the dishes were stained with giemsa and the number of colonies counted.

DIDS (M)	No. colonies formed ($N = 3$)
0	123.8± 3.3
10^{-9}	91.3± 5.6
10^{-8}	104.0± 9.7
10^{-7}	100.0± 5.9
10^{-6}	111.6±12.0
10^{-5}	92.3± 5.9
10^{-4}	99.0± 2.6
10^{-3}	83.0± 9.6

of up to 10^{-3}M causes no impairment of the ability of CHO and N_2A cells to exclude trypan blue [25], which is a measure of the integrity of a cell's permeability barrier [13]. Neither does DIDS treatment have any substantial effect on the plating efficiency of CHO cells, which is a manifestation of the cell's ability to engage in replication (Table I). Treatment with pyridoxal phosphate in the range necessary for efficient labelling and with borohydride, even at levels far in excess of those used in labelling studies has no effect on the short- or long-term viability of cells as measured by maintenance of cell number, trypan blue exclusion and plating efficiency (Table II).

TABLE II

EFFECTS OF PYRIDOXAL PHOSPHATE AND BOROHYDRIDE ON CELL VIABILITY

Borohydride mM	Pyridoxal phosphate (mM)	Cells/plate	% viable	Colonies/plate*
0	0	119	98.9	92.7±6.5
2.8	0	101	—	—
56	0	118	95.8	—
56	2	—	88.6	—
56	4	—	—	90.0±5.0

* Separate experiment.

In a like manner, the reagents used in the lactoperoxidase reaction have little effect on CHO cell viability per se. Thus the viability of a sample of cells iodinated in the usual manner was 94 % that of a control sample which was handled in the same fashion, but not exposed to the labelling reagents. However, one must keep in mind that although the reagents used in the labelling procedures do not in themselves cause damage to the cell, the extensive washing used in all the procedures and especially the need to maintain cells in salt solution at 37°C*, as required by the lactoper-

* The lactoperoxidase reaction may be carried out at room temperature, but not at ice temperature. The same considerations apply, however, since even at room temperature, cells are unstable in protein-free buffers.

oxidase and borohydride procedures, can definitely impair cell viability. Thus the viability of a cell population declined from 98 % when freshly harvested from culture to a value of 53 % immediately after iodination by lactoperoxidase. Since non-viable cells are leaky to small molecules such as pyridoxal phosphate and DIDS, and since they avidly bind many types of macromolecules [13], the presence of even a small proportion of non-viable cells during a surface labelling reaction may constitute a serious potential artifact. In our hands, monolayer cells seemed more resistant to washing and labelling procedures than were suspension cells.

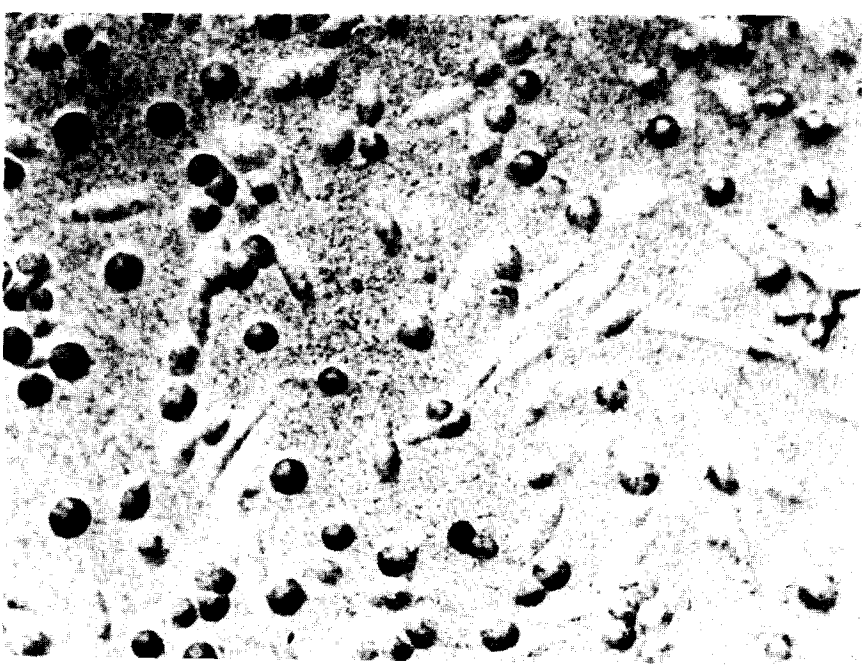
Autoradiography

Autoradiograms of samples treated by any of the surface label techniques employed here clearly show two populations in terms of grain density. Most of the cells have a relatively small number of grains associated with them, while a small

TABLE III

AUTORADIOGRAPHY

Cell type	Surface labelling technique	Heavily labelled/lightly labelled cells (per field)
CHO suspension	$1 \cdot 10^8$ dpm/ml [^{3}H]DIDS	1/51, 2/39, 0/32, 0/41
CHO suspension	$1 \cdot 10^7$ dpm/ml [^{3}H]DIDS	2/28, 0/26, 2/38
CHO monolayer	$1 \cdot 10^7$ dpm/ml [^{3}H]DIDS	0/163, 1/212
CHO suspension	^{125}I 20 μCi	2/44, 3/71, 3/79
CHO monolayer	^{125}I 20 μCi	0/56, 0/39, 1/78
CHO monolayer	PDP-borohydride 500 μCi	2/77, 2/41, 2/79



(a)

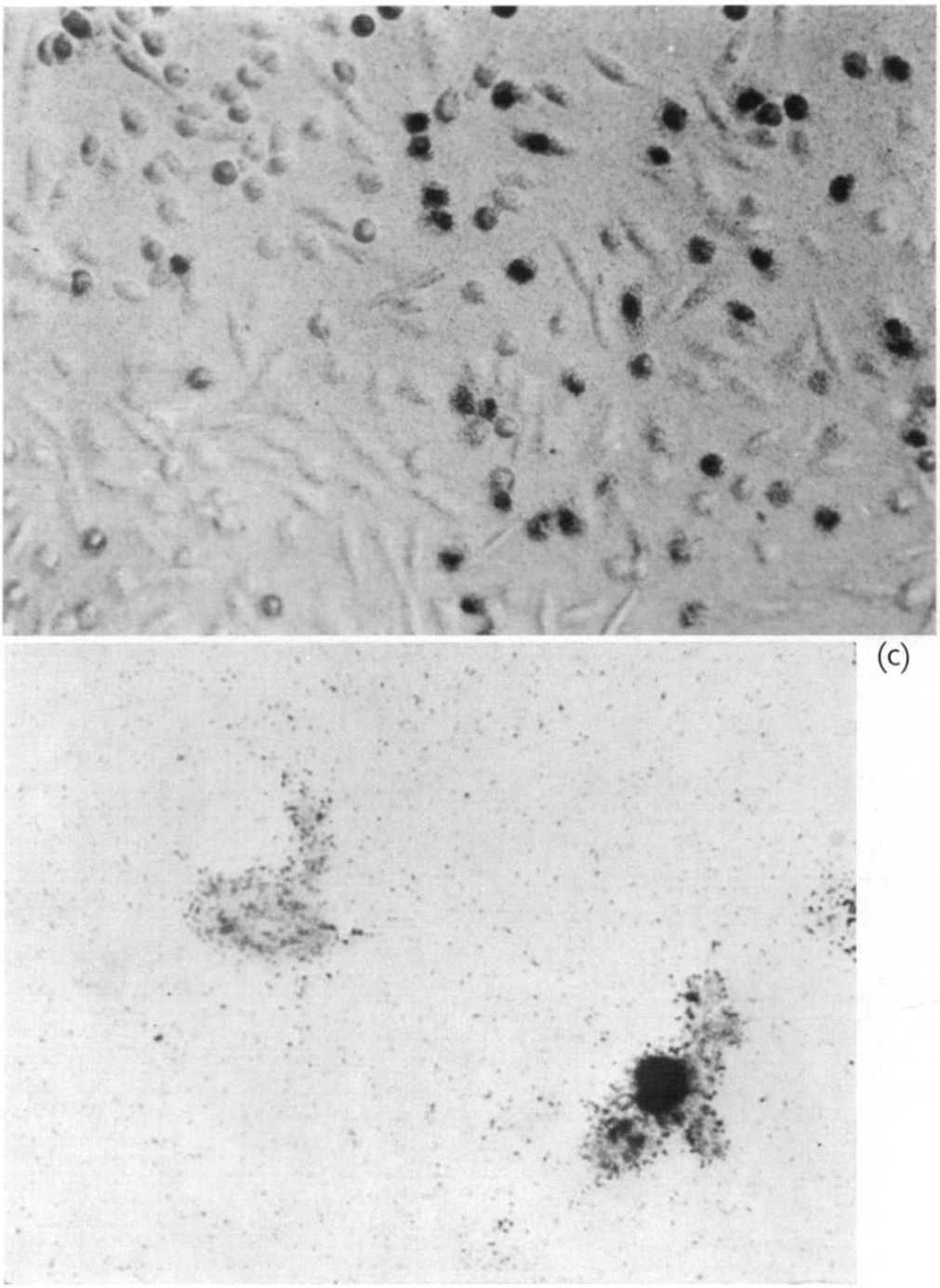
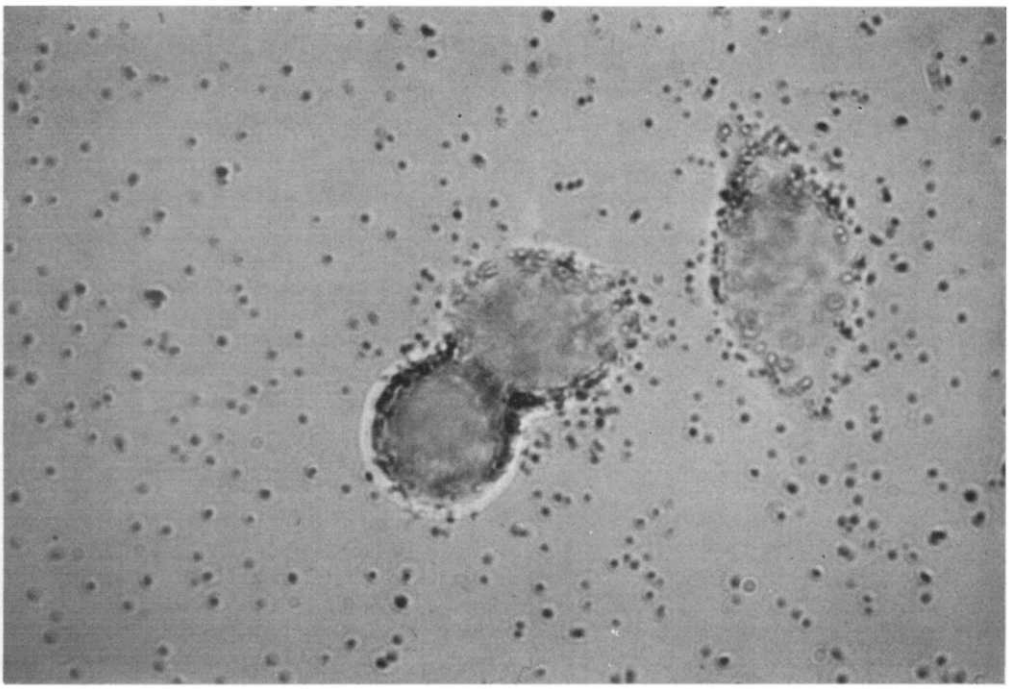


Fig. 3. Autoradiograms. (a) CHO monolayer labelled with $1 \cdot 10^7$ dpm of [^{3}H]DIDS. Note the single heavily labelled cell. × 80. (b) Cells and labelling as above. Note the cluster of heavily labelled cells, some showing a concentration of label in the nuclear region. This field is atypical in that an unusually high percentage of cells are heavily labelled. × 64. (c) Nucleii of cells labelled with [^{3}H]-DIDS. Note intensely labelled nucleus in cluster of lightly labelled ones. × 64.

(a)



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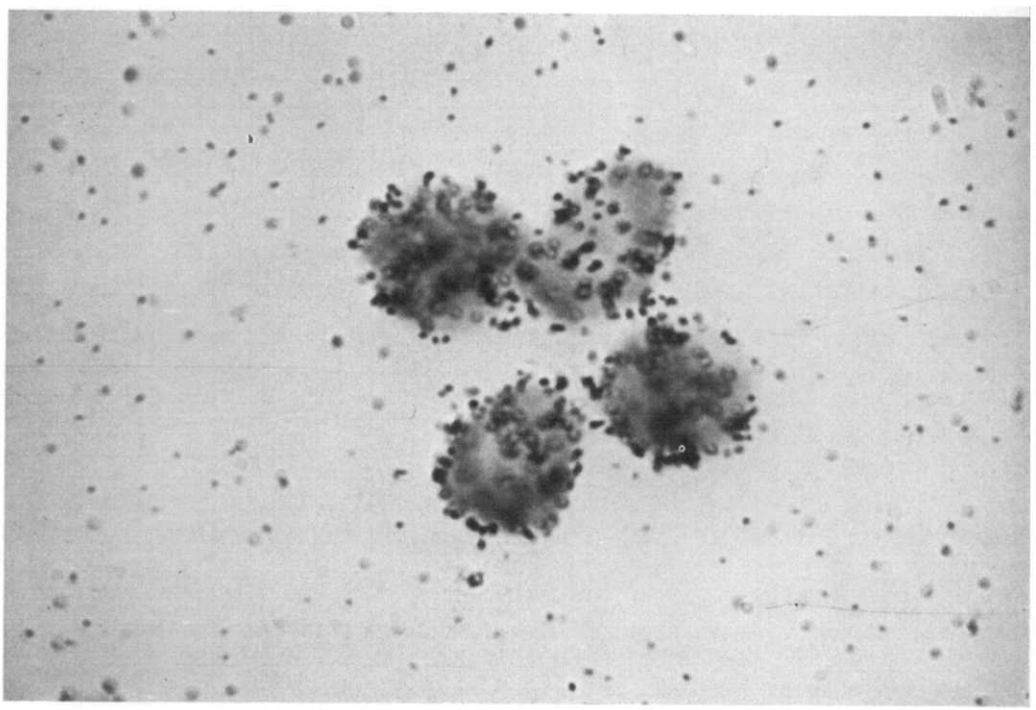

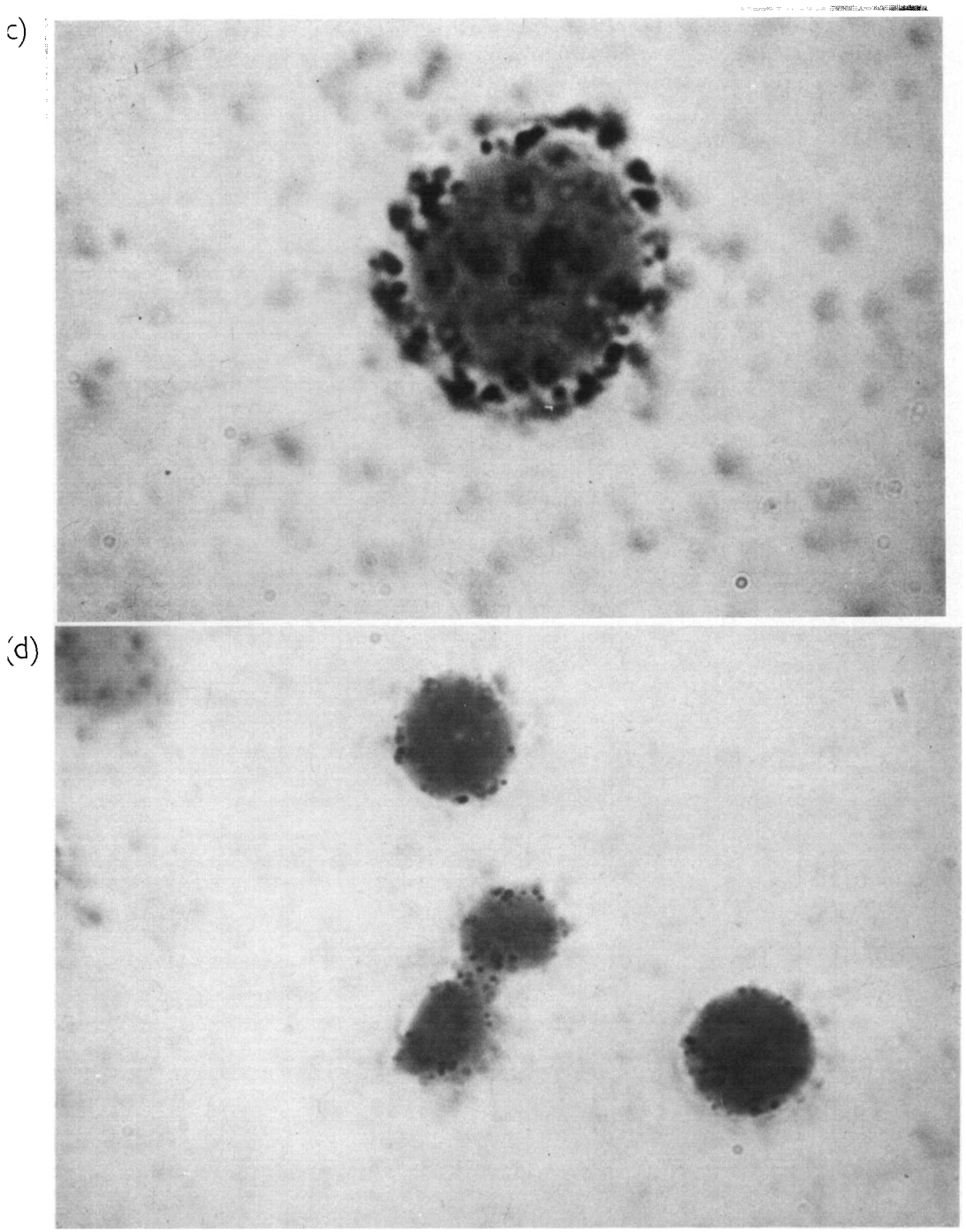

Fig. 4. Autoradiograms. (a) N_2A monolayer cells, plated in serum free medium and labelled with pyridoxal phosphate and borotritide $\times 256$, (d) CHO suspension cells labelled with pyridoxal phosphate and borotritide (500 μCi) $\times 400$, (c) CHO suspension cells labelled with [^{3}H]DIDS ($5 \cdot 10^8$ dpm) $\times 800$, (d) CHO suspension cells labelled by the lactoperoxidase method with ^{125}I (30 μCi) $\times 400$.

proportion of the cells have a much more dense concentration of grains. The ratio of heavily labelled to lightly labelled cells for several experimental conditions is given in Table III. In the case of suspension cells most of the label is associated with the cells, while in the case of monolayers the proteinaceous layer between the cells [26] is labelled to approximately the same degree as the majority of cells (See Fig. 3). When monolayer and suspension cells are labelled under the same conditions, the monolayers contain a lower proportion of heavily labelled cells than do the suspensions (Table III). When autoradiograms of the isolated nuclei of DIDS or pyridoxal phosphate-treated cells are examined one finds that most of the nuclei are very lightly labelled but a few are heavily labelled (see Fig. 3). This indicates that only in a small subpopulation of cells is the nucleus accessible to labelling.

While it is not possible to make a definitive statement as to the cellular location of radiolabel by light microscopic autoradiography, our observations suggest the following: (a) the label on the lightly stained cells is associated with the cell periphery (Fig. 4);(b) the label on the few densely stained cells penetrates the cell interior, and in the case of DIDS and pyridoxal phosphate is mainly associated with the cell nucleus (Fig. 3b). A parallel situation exists in the case of DIDS binding to cells as studied by fluorescence microscopy [25]. Most of the cells exhibit a halo of DIDS fluorescence at the cell periphery while a few cells are brightly fluorescent throughout their volume and especially in the nuclear region.

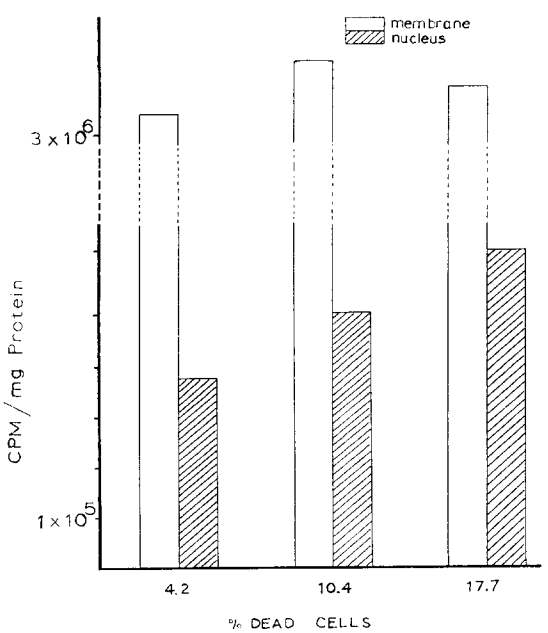

Fig. 5. Nuclear vs membrane labelling. Cells were maintained in buffer at 37 °C for varying periods, labelled with [^{3}H]DIDS and then fractioned. The specific activity of the membrane and nuclear pellet fractions are shown as a function of cell viability.

Cellular Fractionation

Subcellular fractionation of suspension or monolayer cells yielded the following: (a) a cytoplasmic fraction poor in particulates: (b) a plasma membrane fraction which was free of nuclei and (c) a nuclear fraction with little plasma membrane. This is illustrated in Table IV which shows that the putative plasma membrane fraction (Fraction E) is enriched in about 3-fold in the marker enzyme, alkali cation-activated ATPase, while it is essentially depleted of cytochrome *c* reductase and of DNA, which are markers for the endoplasmic reticulum and nucleus, respectively. This fraction is also observed, by phase contrast microscopy, to contain identifiable cell envelopes or "ghosts". The nuclear fractions (Fractions F and G) have a specific activity of ATPase which is substantially lower than the homogenate, but they are enriched in DNA.

The pattern of distribution of the surface label [^{3}H] DIDS shows that the reagent is most enriched in the plasma membrane fraction (E Fraction), and that the degree of enrichment is comparable to that of ATPase. The recovery of DIDS in Fraction E (5.9%) is also comparable to the recovery of ATPase (4.1 %). However a substantial amount of DIDS (80 %) is recovered in the crude nuclear fraction (Fraction F). This fraction does contain ATPase activity and thus presumably is contaminated with plasma membranes, but the level of activity is one-third that of the homogenate and only 10 % of total cellular activity is recovered in this fraction as opposed to 32 % of total cell protein. This suggests that not all of the[^{3}H] DIDS present in the crude nuclear fraction is due to contaminating plasma membrane material. As another approach to this question one might consider that if all of the DIDS binding sites were on the plasma membrane then the ratio of [^{3}H] DIDS/mg to ATPase/mg should be a constant: varying amounts of non-membrane protein would cause both values to change in parallel. However one observes that this ratio is 382 (arbitrary units) in the plasma membrane fraction (Fraction E) and is 1850 in the crude nuclear fraction (Fraction F), once again indicating that the DIDS in Fraction F is associated with other than membrane material.

An anomaly observed in our experiments of this type is the presence of a large proportion of the cellular ATPase in the "cytosol" fraction Fraction B). The source and nature of this activity is unknown at present.

We have measured the amount of trichloroacetic acid precipitable radioactivity per mg of protein incorporated into each of the above fractions after the cells had been treated with surface label reagents. Our results indicate that relative to the homogenate the soluble fraction is depleted of radioactivity while the membrane and nuclear pellet fractions are enriched in most cases (see Table V). As the nuclear pellet fraction was treated with detergent so as to disaggregate plasma membranes and then pelleted by a low-speed centrifugation to avoid sedimentation of small membrane residues, we must conclude that the surface label techniques employed here directly label at least some of the nuclei in the cell population. Further experiments (Fig. 5) show that the degree of nuclear labelling is correlated with the percentage of dead cells in the culture, while the labelling of the membrane is insensitive to such variation in cell viability. This again suggests that the nuclear labelling is contributed by a subpopulation of non-viable cells while the plasma membrane is the site of labelling in the majority of viable cells. The subcellular distribution of radiolabel in fractionated N_2A or CHO monolayer cells was similar to that seen in CHO suspension cells,

TABLE IV

SUB CELLULAR FRACTIONATION

In this experiment $5 \cdot 10^7$ CHO cells were prelabelled with [^{3}H]DIDS, the labelled cells were mixed with $5 \cdot 10^8$ unlabelled cells and the entire sample was subjected to sub cellular fractionation as described in the Methods section. Fractions: (A) whole cell homogenate, (B) $2.5 \cdot 10^6 \cdot g \cdot$ min supernatant ("cytosol"), (C) $2.5 \cdot 10^6 \cdot g \cdot$ min pellet, (D) supernatant of second $2 \cdot 10^4 \cdot g \cdot$ min centrifugation, (E) interphase of two phase separation ("membranes"), (F) pellet of two phase separation ("nuclear pellet"), (G) pellet of detergent extract of Fraction F.

$$R = \frac{\text{activity in fraction}}{\text{activity in homogenate}}, \quad \% = \frac{\text{activity in fraction} \cdot \text{mg protein}}{\text{activity in homogenate} \cdot \text{mg protein}} \cdot 100 \quad \text{(i.e. recovery in fraction)}$$

Fraction	Protein			[^{3}H]DIDS			$(Na^+ - K^+)$-ATPase			Cytochrome *c* reductase			DNA
	mg/ml	Total mg	%	cpm/mg	%	*R*	nM/mg/h	%	R	nM/mg/h	%	*R*	(μg/mg)
A	3.06	114.0	100.0	6 700	100.0	1.0	26.0	100.0	1.0	49.0	100.0	1.0	48.0
B	0.56	28.0	24.5	2 000	7.3	0.3	36.0	34.0	1.4	3.5	1.8	0.07	nd
C	0.60	12.0	10.5	1 750	2.8	0.3	nd**			nd			73.0
D	0.12	4.8	4.2	3 100	2.0	0.5	nd			nd			nd
E	0.88	1.8	1.6	25 700	5.9	3.8	67.0	4.1	2.7	2.8	1.0	0.06	nd
F	3.66	36.6	32.0	16 800	80.4	2.5	9.0	11.0	0.3	167.0	110.0	3.4	60.0
G	0.88	3.5		13 500		2.0	18.0		0.7	nd			100.0
B + C + D + E + F (recovery)			73.0*		98.4			49.1			112.0		

* Some material is lost in washing Fractions E and F; this has not been included in the recovery.

** nd, not detectable.

TABLE V

CELL FRACTIONATION

	Surface labelling technique	Fraction	cpm/mg	cpm/mg / cpm/mg homogenate
(a)	Pyridoxal phosphate 0.01 M at 37 °C for 5 min + tritiated borohydride at 0 °C for 5 min CHO suspension	Homogenate	19 218	1.0
		Soluble	8 319	0.4
		Membrane	61 646	3.2
		Nuclear pellet	138 880	7.2
(b)	Lactoperoxidase (40 μg) + ^{125}I 20 μCi + 5 μM H_2O_2 at 37 °C for 5 min CHO suspension	Homogenate	8 569	1.0
		Soluble	7 673	0.9
		Membrane	17 561	2.0
		Nuclear pellet	24 563	2.9
(c)	[^{3}H]DIDS at 0 °C $3.2 \cdot 10^{-8}$ M/ml CHO suspension	Homogenate	14 730	1.0
		Soluble	6 774	0.5
		Membrane	85 937	5.8
		Nuclear pellet	68 596	4.7
(d)	[^{3}H]DIDS $4.4 \cdot 10^{-9}$ moles/ml	Membrane	—	8.1
	$2.2 \cdot 10^{-8}$ moles/ml	Membrane	—	5.8
	$1.8 \cdot 10^{-6}$ moles/ml	Membrane	—	2.7
(e)	Lactoperoxidase (20 μg) + 30 μCi ^{125}I + 5 μM H_2O_2 at 37 °C for 5 min CHO monolayer	Homogenate	9 908	1.0
		Membrane	29 827	2.1
		Nuclear pellet	18 712	1.9
(f)	Lactoperoxidase (20 μg) + 30 μCi ^{125}I + 5 μmoles H_2O_2 at 37 °C for 5 min N_2A monolayer	Homogenate	6 188	1.0
		Membrane	13 584	2.2
		Nuclear pellet	8 400	1.4
(g)	Galactose oxidase CHO suspension	Homogenate	19 824	1.0
		Soluble	21 628	1.1
		Membrane	111 491	5.6
		Nuclear pellet	5 934	0.3

except that the nuclear pellet fraction was not labelled to quite as high as a specific activity. Galactose oxidase labelling shows little incorporation into the nucleus, but some enrichment of label in the soluble fraction. This is presumably due to a paucity of glycoproteins in the nucleus and the presence of glycoproteins or glycolipids, possibly as very small microsomes, in the soluble fraction. The degree of enrichment of most surface label reagents in the membrane fractions in the experiments shown in Table V (from 2.0- to 5.8-fold, mean 3.3-fold) is similar to the degree of enrichment (2.7-fold) of alkali cation ATPase in the membrane fraction of the experiment shown in Table IV.

Since the binding curve for [^{3}H] DIDS suggested the existence of high affinity sites on the CHO cells, we wish to ascertain if these sites might be associated with the plasma membrane. As a test of this idea, we labelled cells with different concentrations of DIDS, fractionated the cells and measured the ratio of incorporated radioactivity in the membrane to that in the homogenate. If the high affinity sites were located on the membrane, then one would expect to increase the specificity of labelling

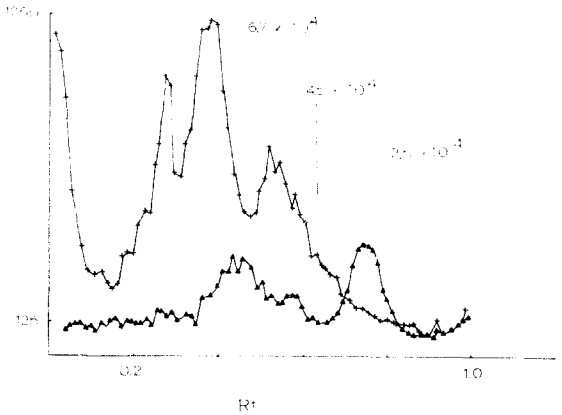

Fig. 6. Sodium dodecylsulfate gel analysis of labelled polypeptides. Intact CHO cells were labelled with the galactose oxidase ^{3}H borohydride system as described in Methods. The whole cell homogenate and plasma membrane fractions were run on 5.6 % sodium dodecylsulfate gels and the gels sliced and counted for tritium. An equal number of counts was layered on each gel. A peak (R_f 1.0) which runs ahead of the dye marker and which is presumably lipid is not shown. The ordinate is cpm/slice. Molecular weight markers were run in parallel and migrated to the positions shown. Homogenate, ▲ ▲, membrane, [illegible].

(i.e. the membrane /homogenate ratio) at a low concentration of [^{3}H] DIDS. This was found to be the case (Table Vd) suggesting that the high-affinity sites might indeed be associated with a plasma membrane component.

Gel Electrophoresis

A sodium dodecylsulfate gel analysis of whole cell homogenate and plasma membrane fractions derived from intact cells labelled with the galactose oxidase methods is seen in Fig. 6. The homogenate shows two broad peaks of apparent molecular weights of 80 000 and 33 000. The membrane fraction on the other hand shows three components of apparent molecular weights 140 000, 102 000 and 66 000 which do not correspond to the major peaks seen in the homogenate.

CONCLUSIONS

Our observations indicate that mammalian cells may be treated with surface label reagents and suffer only minor impairment of cell viability. Autoradiograms and cell fractionation studies support the concept that the plasma membrane is preferentially labelled in most members of the cell population. However, it is inevitable that a small number of non-viable cells will be exposed to the surface label reagents and these cells may be labelled internally, as well as on the surface. Autoradiographic and fluorescence [25] studies indicate that much more label is bound on the interior of a non-viable cell than on the surface of a viable cell. This also agrees with previous studies, indicating that a relatively few membrane sites are available for labelling [27]. Surface label reagents, most especially pyridoxal phosphate and DIDS, intensively label the nucleus when they have access to this organelle. This result should not be surprising as DIDS and pyridoxal phosphate are amino-reactive anions

and the nucleus contains many positively charged, lysine-rich histones which would be expected to both avidly bind and react with such reagents.

Since most biochemical manipulations do not discriminate between dead and live cells, a minor proportion of non-viable, but heavily labelled cells may present a substantial artifact in the biochemical analysis of cell membrane proteins using the surface label approach. For example in Table Ve, with lactoperoxidase labelling, the membrane fraction and nuclear pellet fractions have spec. act. of $3.0 \cdot 10^4$ cpm/mg and $1.9 \cdot 10^4$ cpm/mg, respectively. In our experience the membrane comprises less than 5 % of total cell protein while the nucleus comprises about 30 %. Therefore, in 100 mg of whole cell homogenate a total of 30 times $1.9 \cdot 10^4$ cpm equals $5.7 \cdot 10^5$ cpm would be contributed by the nucleus, while only five times $3.0 \cdot 10^4$ cpm $= 1.5 \cdot 10^5$ cpm would be contributed by the plasma membrane. Thus, during further analysis with gels or columns the contribution of labelled nuclear proteins might well mask the contribution of plasma membrane proteins. The situation can be remedied by subcellular fractionation; one may readily remove more than 95 % of the nuclei from the membrane fraction thus reducing the nuclear contribution to $2.5 \cdot 10^4$ cpm, a relatively insignificant level. This illustrates the need for careful cell fractionation in conjuction with surface label studies. A further reinforcement of this point is the result seen by sodium dodecylsulfate gel analysis of cells labelled with the galactose oxidase system. The labelled components of the plasma membrane must constitute only small percentage of the total labelled components, as the membrane peaks are not readily visualized in a gel of this whole cell homogenate. In fact the predominate labelled components of the homogenate are absent from the plasma membrane fraction and thus are likely to stem from other cellular loci. In view of these results supposed cell surface differences between galactose oxidase treated normal and virally transformed fibroblasts should be interpreted cautiously [28].

We wish to point out that the problem of internal labelling in non-viable cells is not peculiar to the Chinese hamster ovary line, which indeed is a very hardy cell. With few exceptions cultured mammalian cells are unstable in the protein-free buffers required for surface labelling and thus dead cells will rapidly accumulate during labelling procedures. This emphasizes the need for rapid labelling techniques and suggests that some surface label approaches utilizing incubations of 30 min to several hours at 37°C require modification before they can be applied to nucleated mammalian cells [3, 6].

At first glance our lactoperoxidase labelling results seem to disagree with those of previous investigators [7] who demonstrated by electron microscopic autoradiography that lactoperoxidase-catalyzed iodination occurred at the cell membrane and that cytosol enzymes were not labelled by this system. We agree that lactoperoxidase iodinates mainly the plasma membrane of most cells. However, autoradiography cannot evaluate the contribution of a small percentage of atypical internally labelled cells to a biochemical analysis. Our cell fractionation studies suggest that the quantitative contribution of internally labelled cells may be very substantial. In addition, we find that the extent of lactoperoxidase catalyzed iodination of cytosol protein is not a valid measure of the extent of penetration of this enzyme to the cell interior. The enzyme lactoperoxidase seems to have an affinity for cell particulates and labels particulate proteins far more effectively than soluble ones. Thus, in one experiment, isolated cytosol ($2.5 \cdot 10^6 g \cdot$ min supernatant) was labelled

at the level of $2 \cdot 10^5$ cpm/mg, while whole cell homogenate treated in an identical fashion was labelled at the level of $2 \cdot 10^6$ cpm/mg. This affinity for particulate protein was not observed in the case of [^{3}H] DIDS which labelled cytosol and homogenate proteins equally well. Thus, rather than measuring the degree of labelling of cytosol enzymes, a better measure of lactoperoxidase penetration into the cell interior might be a measure radioiodination of internal cell particulates such as mitochondria [10].

Another frequently used criterion of the specificity of lactoperoxidase for labelling cell surfaces is that the enzyme labels many more proteins in hypotonically disrupted or homogenized cells than in intact cells [11]. This criterion is not really valid since either of these perturbations not only introduces the enzyme into the cell interior, but also causes drastic rearrangement of cellular structures and may expose many new sites for labelling. It is well known that even in intact cells, a relatively minor osmotic stress may expose new sites to the actions of enzymes [29].

In our hands somewhat less undesirable labelling of internal components occurs when monolayer cells are used, rather than suspension cells. Against this advantage must be balanced the fact that treatment of monolayers with surface labels also results in radiolabelling of the proteinaceous layer between cells and an unknown and variable amount of this material may remain adherent to the cell when it is removed from the substrate.

In this report we have tried to evaluate some of the problems and limitations of the surface label approach as applied to cultured mammalian cells. The main problem seems to be that of discriminating between the contribution of the majority of the cell population which have been labelled on their surfaces, and the contribution of the few cells which have been more heavily labelled at internal sites. The latter contribution may be reduced by the use of cellular fractionation [10,12], or by means of immunological recognition of specific membrane components [7,8]. Studies which rely solely on the specificity of surface label techniques to distinguish between peripheral and internal proteins in whole cells are likely to be plagued with artifacts

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REFERENCES

1 Juliano, R. L. (1973) Biochim. Biophys. Acta 300, 341–378
2 Phillips, D. R. and Morrison, M. (1971) Biochemistry 10, 1766–1771
3 Rifkin, D. B., Compans, R. W. and Reich, E. (1972) J. Biol. Chem. 247, 6432–6437
4 Bretscher, M. S. (1971) J. Mol. Biol., 58, 775–781
5 Cabantchik, Z. I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311–330
6 Gahmberg, C. G. and Hakamori, S. I. (1973) J. Biol. Chem. 248, 4311–4317
7 Marchalonis, J. J., Cone, R. E. and Santer, V. (1972) Biochem. J. 124, 921–927
8 Baur, S., Schenkein, I. and Uhr, J. (1972) J. Immunol. 108, 748–754
9 Phillips, D. R. (1972) Biochemistry 11, 4582–4588
10 Czech, M. P. and Lynn, W. S. (1973) Biochemistry 12, 3597–3601
11 Hynes, R. O. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3170–3174
12 Poduslo, J. F., Greenberg, C. S. and Glick, M. C. (1972) Biochemistry 11, 2616–2621

13 Juliano, R. L. and Mayhew, E. (1972) Exp. Cell Res. 73, 3–12
14 Thompson, L. H. and Baker, R. M. (1973) Methods in Cell Biology, Vol. VI, pp. 209–281, Academic Press, New York
15 Brunette, D. M. and Till, J. E. (1971) J. Membrane Biol. 5, 215–224
16 Vaughan, M. H., Warren, J. R. and Darnell, J. E. (1967) J. Mol. Biol. 25, 235–251
17 Prescott, D. M. (1967) Methods in Cell Physiology, Vol. 1. p. 365
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19 Kimelberg, H. K. and Papahadjopoulos (1972) Biochim. Biophys. Acta 282, 277–292
20 Schneider, W. C. (1957) Methods in Enzymology (Colowick, S. P. and Kaplan, N., eds), Vol. III, Academic Press, New York
21 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
22 Tanford, C. (1968) Physical Chemistry of Macromolecules, p. 543, Academic Press, New York
23 Van Steveninck, J., Weed, R. I. and Rothstein, A. (1965) J. Gen. Physiol. 48, 617–632
24 Steck, T. L. and Dawson, G. (1974) J. Biol. Chem. 249, 2135–2142
25 Juliano, R. L. (1974) Exp. Cell Res., 86, 181–184
26 Weiss, L. (1967) The Cell Periphery Metastasis and Other Contact Phenomena, p. 199, North-Holland, Amsterdam
27 Cabantchik, Z. I. and Rothstein, A. (1974) J. Membrane Biol., 15, 227–248
28 Gahmberg, C. G. and Hakamori, S. I. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3324–3333
29 Woodward, C. B. and Zwaal, R. F. A. (1972) Biochim. Biophys. Acta 274, 272–278