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IODINATION OF CELL MEMBRANES
I. OPTIMAL CONDITIONS FOR THE IODINATION OF EXPOSED MEMBRANE COMPONENTS

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SUMMARY

Iodination of red blood cells under optimal conditions by the Phillips–Morrison method leads to the iodination of two surface proteins. Modification of these conditions leads to the labeling of additional membrane proteins; labeling of hemoglobin can also occur. These results lead to the conclusion that, depending on the conditions of iodination, proteins located at various depths of the membrane can be labeled. This information was used in establishing an assay for the optimal iodination conditions of HeLa cells. Such iodinated HeLa cells grow at the same rate as control HeLa cells; most of these iodinated surface proteins can be removed by subsequent treatment with pronase.

INTRODUCTION

A long range objective of this work is to characterize some of the membrane components unique to leukemic cells. Since no reliable or generally applicable methods exist for the isolation of membranes of leukemic cells we decided to develop the method from well-established observations made on HeLa cells.

We proposed to use the Phillips and Morrison method [1] for the iodination of red blood cell membranes (in the presence of lactoperoxidase, H_2O_2 and ^{125}I), as a means of identifying the membrane proteins of eukaryotic cells. Such a method would permit us to do two things: (1) to provide an assay method for the identification of the membranes as a unique fraction, and (2) to identify the proteins within this fraction, which became labeled with ^{125}I, as distinct from those membrane protein components which are not so labeled.

This method depends on the following series of reactions. Lactoperoxidase reacts with H_2O_2 to form a lactoperoxidase–O complex; this in turn reacts with $^{125}I^-$ to form a lactoperoxidase–^{125}I complex; this can iodinate proteins predominantly in exposed tyrosine groups. Since the lactoperoxidase–^{125}I complex has a mol. wt of about 78000, it is presumed to be impermeable to cells; consequently, it should only iodinate proteins containing exposed tyrosine groups on the cell

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surface. (In our own experiments with red blood cells and lactoperoxidase we in fact find no assayable amount of lactoperoxidase within the red blood cells after long-term incubation of red blood cells with lactoperoxidase.) Although in their original investigations, Phillips and Morrison [1] reported that only one protein could be detected to be radioactive in red blood cells using 5% acrylamide–sodium dodecylsulfate gels, their investigations subsequent to the work of Bretscher [2], using 10% acrylamide–sodium dodecylsulfate gels revealed two labeled proteins [3].

The iodination of surface membrane proteins by the lactoperoxidase method has been used to iodinate mouse lymphocytes [4], L cells [5] and human platelets [6,7]. More recently this method has been modified so that the H_2O_2 necessary for the reaction is not added manually but generated by glucose–glucose oxidase [8,9] in the presence of lactoperoxidase and iodide. Before we used the Phillips–Morrison method we had to establish a working baseline, especially since we were planning to apply the method to a cell type which is much more complex than the red blood cell in terms of internal architecture. (a) How much can the conditions of labeling of red blood cells be changed and still provide only labeling of two membrane proteins? (b) Do conditions exist under which labeling of internal red blood cell proteins (i.e. hemoglobin) is possible?

A knowledge of these parameters would permit us to establish the flexibility of the method and to provide us with a degree of confidence in our results. Because, if under no conditions does internal labeling occur and if under all conditions only two membrane proteins are labeled then the method can be used with a reasonable degree of confidence. However, should some conditions permit internal labeling or labeling of more than two membrane protein components then these conditions would have to be avoided when eukaryotic cells were being labeled.

In this communication we present our results which indicate that unless one adheres to conditions very close to those defined by Phillips and Morrison additional membrane components of red blood cells can become iodinated. The further these conditions deviate from the optimal conditions, increased labeling of internal cytoplasmic protein components can also occur. An indication of this internal labeling is the isolation of ^{125}I-labeled crystalline hemoglobin. We have proceeded to adapt these optimal conditions to the labeling of external HeLa cell membrane proteins.

MATERIALS AND METHODS

Radioactive ^{125}I were purchased from New England Nuclear. Lactoperoxidase was purified by the method of Morrison and Hultquist [10] through the CM-cellulose step of their purification procedure. Joklik modified minimum essential medium containing streptomycin and penicillin and fetal calf serum were obtained from Grand Island Biological Company. Pronase (B grade) from Calbiochem. and phenylmethylsulfonylfluoride from Sigma Co. Sodium metrizoate solution was obtained from Nyegaard and Co., Norway. All other chemicals were of reagent grade. We wish to thank Dr Lon Hodge of the Department of Microbiology, Yale University School of Medicine, for providing us with HeLa S_3 cells.

Preparation of red blood cells

The red blood cells were obtained from a single individual, type O, RH^+.

10 ml of blood were diluted with 90 ml 0.106 M sodium phosphate, pH 7.4 containing 3% dextran. The red blood cells were allowed to settle for 30 min in a 100-ml graduated cylinder at room temperature. The supernatant was siphoned off and this procedure repeated two more times. In this way the possibility of contamination with white blood cells which settle more slowly was minimized [11]. The red blood cells were then suspended in 0.106 M sodium phosphate, pH 7.4 (phosphate buffer) and washed several times in this buffer. The washed red blood cells were used the same day or the following day.

Preparation of HeLa cells

HeLa S_3 cells were grown in suspension culture in minimum essential medium supplemented with 10% fetal calf serum and 2 mM glutamine. Cells were harvested at the density of $5 \cdot 10$–$6 \cdot 10^5$ cells/ml; they were centrifuged at $300 \times g$ for 10 min and washed two times with minimum essential medium alone.

Iodination procedure

I. Red blood cells. The iodination technique used was a modification of the Phillips–Morrison method [1]. 3 ml of red blood cells were iodinated at room temperature in the presence of 0.106 M sodium phosphate buffer, pH 7.4, $5 \cdot 10^{-7}$ M lactoperoxidase and 10^{-6} M $K^{125}I$ (10^{-5} M, 2 Ci/l) from a reservoir, by a Radiometer autotitrator which was connected to a Radiometer silver billet electrode and a reference K_2SO_4 electrode; the autotitrator would readjust the I^- concentration to the initial concentration. The reaction was initiated and continued by the manual addition of H_2O_2 to which the automatic titrator responded by the addition of $K^{125}I$. 1 mM H_2O_2 in phosphate buffer was added with a Hamilton syringe in 5–10 μl portions (the maximal amount of H_2O_2 never exceeded 3 μM). One aliquot of H_2O_2 was consumed before another aliquot was added to reinitiate the reaction. The sequential additions were employed to avoid the accumulation of H_2O_2. After iodination, red blood cells were collected by centrifugation, they were washed two times in a large excess of phosphate buffer and two more times in the same buffer containing 5 mg KI/ml.

II. HeLa cells. The minimum essential medium-washed cells were washed once with about 40 ml cold phosphate buffer/10^8 cells just before iodination. HeLa cells at $2 \cdot 10^7$–$4 \cdot 10^7$ cells/ml were iodinated as described above. The iodination of HeLa cells was completed in 5–7 min. Radioactive HeLa cells were diluted with minimum essential medium and centrifuged. The cells were washed four more times with minimum essential medium.

Isolation of plasma membranes

I. Red blood cell membranes and cytoplasmic fraction. Red blood cells were lysed by freeze–thawing three times. These were then centrifuged at $30000 \times g$ for 30 min to yield the membrane-free cytoplasm. The membrane ghosts were isolated and dialyzed [1].

II. Isolation of HeLa plasma membranes. HeLa cell membranes were isolated acccording to the procedure of Atkinson and Summer [12]. Membrane preparations were purified through two cycles of discontinuous sucrose gradients.

Separation of red blood cells from a mixture of HeLa cells and red blood cells

The mixture of HeLa cells and red blood cells was iodinated in 3 ml of 0.106 M phosphate buffer, pH 7.4. After iodination the mixture was diluted to 10 ml with minimum essential medium and centrifuged at $250 \times g$ for 10 s to remove most of the HeLa cells; this pellet was discarded. The supernatant fraction was recentrifuged at $300 \times g$ for 10 min; the resultant cell pellet was resuspended in about 2 ml of minimum essential medium. Of this, 0.5-ml portions were layered on top of a ficoll–metrizoate mixture (3.6 ml of 9% Ficoll in water + 1.5 ml sodium metrizoate) and centrifuged at $450 \times g$ for 5 min at room temperature [13]. The labeled red blood cells at the bottom of the tubes were collected free of HeLa cells by microscopic examination and $2 \cdot 10^8$ non-radioactive red blood cells were added as carrier before the washing of radioactive red blood cells was initiated as described above.

Gel electrophoresis

Radioactive red blood cells membrane preparations were dialyzed against 6.8 mM phosphate, pH 7.4 containing 5 mM EDTA and 5 mM 2-mercaptoethanol overnight at 4 °C. The dialyzed samples were centrifuged at $34000 \times g$ for 20 min and the pellets were resuspended in a small volume of 6.8 mM phosphate buffer, pH 7.4. 10 mM Tris–10 mM sodium azide–5 mM EDTA–5 mM 2-mercaptoethanol, pH 8.0, was used as the dialyzing buffer for ^{125}I-labeled HeLa membranes. The dialyzed HeLa membranes were centrifuged at $20000 \times g$ for 15 min and resuspended in a small volume of 10 mM Tris, pH 8.0. The membranes were dissolved in 2–3% sodium dodecylsulfate by heating the membranes at 60 °C for 5 min; occasionally the HeLa cell membranes were heated at 100 °C for 3 min. 10% acrylamide gel (6 mm × 10 mm) was used for analysis of red blood cell membranes and 7.5% gel for HeLa membranes by the method of Lenard [14] modified so that the final bisacrylamide was 1.5% of the monoacrylamide concentration. The gels were sliced to 1.5-mm sections by hand or to 1.0-mm sections by the Gilson gel fractionator. The gel fractions in counting vials were suspended in 10 ml toluene–POPOP–Triton X-100 with 3% Protosol and shaken overnight at 37 °C. The radioactivity was counted in a Packard liquid scintillation counter.

Pronase digestion of HeLa cells

HeLa cells suspended in minimum essential medium were added to an equal volume of a pronase solution to give final concentrations of $6 \cdot 10^5$ cells/ml and 25 μg/ml of pronase. The cell suspension and the pronase solutions were prewarmed separately to 37 °C before mixing and the mixtures were kept at 37 °C without any shaking whatsoever. Adherence to these conditions both in terms of cell number and avoidance of any shaking were found to be essential to avoid clumping of the cells. After incubation they were cooled in ice–water and 10% fetal calf serum was added. The pronase-treated cells were centrifuged and washed two times with a volume of minimum essential medium equal to the original volume, containing 10% fetal calf serum; they were washed two more times with the same volume of minimum essential medium alone. This was done in order to avoid any further degradation of the membranes by the proteases.

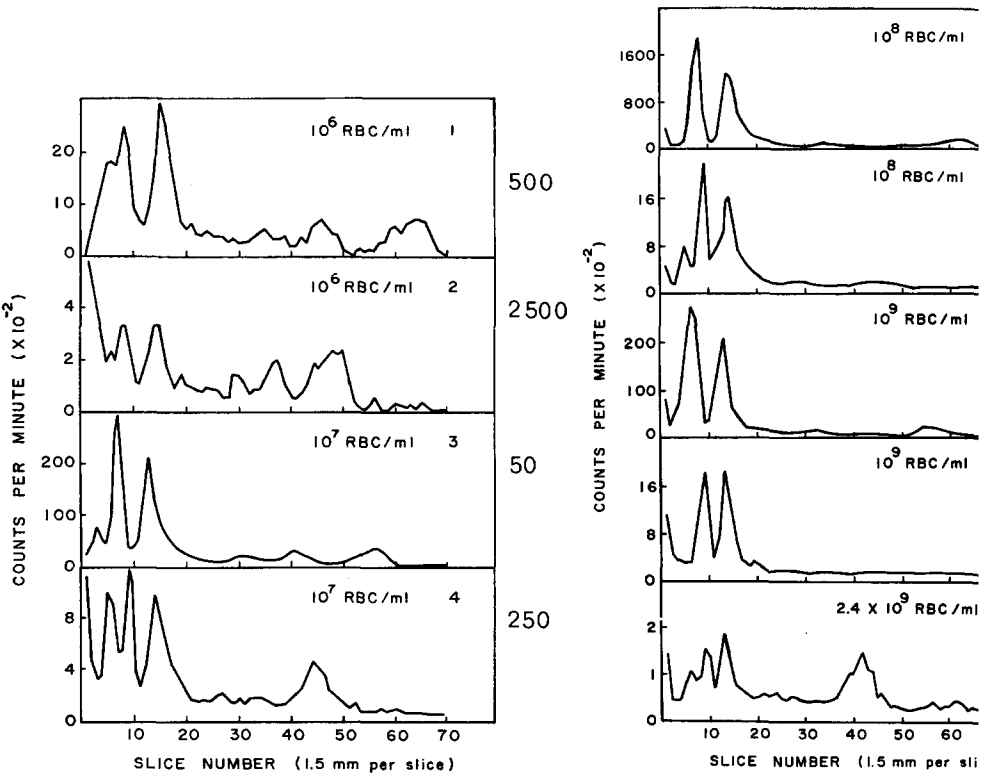
Other procedures

The cell number was determined with a Coulter counter. The concentrat of H_2O_2 was determined from the absorbance at 230 nm using a molar extinc coefficient of 72.4 [1].

RESULTS

1. The labeling of red blood cell membranes

We established that the Phillips–Morrison [1] method when applied to blood cells under the conditions described by the authors yields two labeled m brane proteins. The question then became, what flexibility exists in the meth We decided to concern ourselves with two variables only. Since we were intere in applying the method to other cell types one obvious difference would be the size; this would determine the maximum number of cells that could be contai per ml. Another variable would be the degree of iodination.



Figs 1 and 2. ^{125}I profile of red blood cell membrane proteins obtained on acrylamide–so dodecylsulfate gels; variables include red blood cell/ml and degree of iodination. Washe blood cells were iodinated by the Phillips–Morrison procedure as described in the text. ^{125}I-labeled red blood cells were washed repeatedly in 0.106 M phosphate buffer (sodium pH 7.4, lysed and the membranes were further purified [12]. The isolated membranes wer sociated with sodium dodecylsulfate, and 25–50 μg were electrophoresed on 10% sodium doc sulfate–acrylamide gels [17]. The numbers in each panel refer to the number of red blood (RBC)/ml iodinated. The consecutive numbers 1–9 refer to the experiment number prese Table I which includes iodination conditions, etc.

TABLE I

Sample numbers 1, 3, 5 and 7 contained starting concn of $K^{125}I$ of $2 \cdot 10^{-7}$ M and were titrated to this level. The remaining samples contained and were titrated to 10^{-6} M $K^{125}I$. Radioactivity % refers to macromolecular-bound ^{125}I. Cytoplasm refers to ^{125}I bound to the cell sap which elutes within the void volume of a G-25 Sephadex column (3 cm × 35 cm). Membrane refers to that bound ^{125}I which is associated with the washed membrane fraction. Normalized values correspond to the number of nmoles of titrant added per 1×10^9 cells. Our calculations show that for experiments 1, 3, 5 and 7 the number of I atoms attached per cell are $1.12 \cdot 10^6$, $5.12 \cdot 10^5$, $7.1 \cdot 10^4$ and $3.5 \cdot 10^3$, respectively.

Expt	Initial red blood cell concn (cells/ml)	Titrant $K^{125}I$ (mole $\times 10^{10}$ added)	Radioactivity %		Polyacrylamide gel (number of radioactive bands)	Normalized value (nmoles titrant added/10^9 cells)
			Cytoplasm	Membrane		
1	10^6	5	13	87	> 3	500
2	10^6	25	12	88	> 3	2500
3	10^7	5	4	96	> 3	50
4	10^7	25	10	90	> 3	250
5	10^8	5	1	99	2	5
6	$2 \cdot 10^8$	25	1	99	3	12.5
7	10^9	5	2	98	2	0.5
8	10^9	25	3	97	2	2.5
9	$2.4 \cdot 10^9$	1700	10	90	> 3	71

Figs 1 and 2 and Table I present the results obtained when both the concentration of red blood cells per ml as well as the degree of iodination is changed. When the conditions described by Phillips and Morrison are strictly followed (Fig. 2 and Table I, Expt 7) only two membrane proteins are labeled.

For the same amount of added I^-, additional membrane proteins become labeled if the red blood cell concentration is decreased. In addition, a more complex pattern of membrane labeling is obtained when the red blood cell concentration is kept constant and additional I^- is added. Note also the additional cytoplasmic labeling that occurs as these conditions are varied. From these results it becomes apparent that iodination conditions used for Samples 5 and 7 are optimal. Consequently, we could conclude that the unique labeling of two membrane proteins occurs only under well defined conditions and that more extensive labeling can occur if these conditions are not strictly adhered to; it should be emphasized that in these experiments proteins are being extracted from well-washed red blood cell ghosts.

In addition, it should be noted that it is not only the concentration of red blood cell that is important but also the degree of iodination. Because as can be seen in Fig. 2, Expt 9, excessive iodination in the presence of an optimal amount of red blood cells can also lead to the labeling of more than two membrane proteins. With these experiments we established two of the parameters involved in this reaction; the remaining parameters, amount of lactoperoxidase, and salt concentration did not need to be defined since, for our purpose, they could be kept constant.

2. The labeling of intracellular red blood cell components

The determination of the ^{125}I associated with the cytoplasmic fraction (Table I), indicated that iodination could occur within the cell. However, it could also be argued

that, during the lysis of red blood cell to separate the membrane from the cell sap, loosely associated ^{125}I-labeled membrane proteins were solubilized and that in fact no internal labeling occurred. In order to examine this possibility we isolated hemoglobin which can be easily purified by repeated crystallization [15]. We used iodination conditions similar to those of Fig. 1, Expt 4, and were able to show that repeated recrystallization of hemoglobin leads to the isolation of samples of radioactive hemoglobin of constant specific activity.

TABLE II

THE CRYSTALLIZATION OF HEMOGLOBIN (Hb) FROM THE CELL LYSATE OF RED BLOOD CELLS

A total of $5 \cdot 10^7$ red blood cells were iodinated according to the conditions described in Fig. 1 and Table I, Expt 4, and washed. The membranes were separated by centrifugation and the macromolecular components of the cell sap were isolated in the void volume of a Sephadex G-25 column. To this radioactive fraction was added the membrane-free lysate derived from 50 ml of packed non-radioactive red blood cells in order to provide enough hemoglobin for repeated recrystallization. The specific activity was determined (cell lysate); the hemoglobin (Hb) was then crystallized from the complete mixture four times [15] and the specific activity determined after each time.

Fraction	Specific activity (cpm/unit of $A_{410\,nm}$)
Cell lysate	12 500
Hemoglobin	
Hb after 1st crystallization	3 520
Hemoglobin	
Hb after 2nd crystallization	3 660
Hemoglobin	
Hb after 3rd crystallization	3 540
Hemoglobin	
Hb after 4th crystallization	3 620

Table II shows that approx. 25% of the radioactivity of the cell sap associated with the fraction which elutes in the void volume of Sephadex G-25 recrystallizes with hemoglobin. This experiment establishes that iodination of an intracellular component can occur under certain conditions. However, when labeling of red blood cells was performed under optimal conditions, Fig. 2, Expt 7, where only two membrane proteins are labeled, the hemoglobin was not found to be radioactive, at least to a level less than 1% of the total membrane labeling.

3. Definition of conditions for the optimal labeling of HeLa cells

The situation which we faced was that we are dealing with nucleated cells of much larger dimensions than the red blood cells. We could not possibly reproduce the optimal red blood cells cell number/ml because the packed cell volume of HeLa cells is less than 10^9 cells/ml. (Packed cell volume for HeLa cells is approx. $2 \cdot 10^8$ while for red blood cells it is approx. $6 \cdot 10^9$.) Yet, we knew from the above that unless we picked correct iodination conditions, extensive labeling of membrane proteins as well as intracellular labeling could occur.

The problem was resolved as follows. We knew (Fig. 1, Expt 4) that iodination of 10^7 red blood cells/ml under our standard conditions results in the labeling of many red blood cell membrane proteins. Upon adding HeLa cells to such a suspension of red blood cells we found that we could decrease the extent of labeling of the additional red blood cell membrane proteins until, when $2 \cdot 10^7$ HeLa cells/ml were added only the original two red blood cell membrane proteins were labeled. After the iodination the red blood cells were separated from the HeLa cells [13] (see Materials and Methods), the red blood cell membranes isolated and the sodium dodecylsulfate–acrylamide gel pattern determined as usual (Fig. 3C).

The implication of this result is that now there is enough additional membrane surface to yield only the two radioactive red blood cell membrane proteins. By inference, we concluded that an optimal labeling of exposed surface protein on HeLa cells occurred. Using these conditions we could then further increase the HeLa cell number and see if any further change occurs in the labeled HeLa membrane protein profile.

When this series of experiments was performed, the results summarized in Fig. 3 were obtained. Addition of $2 \cdot 10^6$ HeLa cells to 10^7 red blood cells resulted

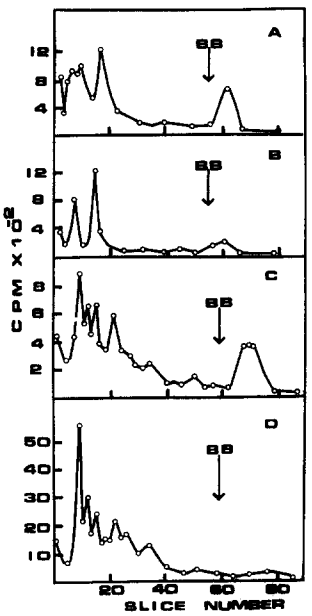


Fig. 3. (A and B) Iodination of red blood cells in the presence of Hela cells. ^{125}I profile of sodium dodecylsulfate-acrylamide gel pattern of red blood cell membranes obtained when 10^7 red blood cells (RBC)/ml are iodinated in the presence of $2 \cdot 10^6$ HeLa cells/ml (A) and in the presence of $2 \cdot 10^7$ HeLa cells/ml (B). The ^{125}I-labeled red blood cells were isolated [13] free of HeLa cells, washed and electrophoresed using the conditions in the legends to Fig. 1 and Table I, Expt 4. BB refers to the bromophenol blue marker. Protein added 50 μg.

Fig. 3 (C and D). Iodination of HeLa cells. ^{125}I profile of acrylamide–sodium dodecylsulfate gel pattern of HeLa cell membranes. $2 \cdot 10^7$ HeLa cells/ml (C) and $3.4 \cdot 10^7$ HeLa cells/ml (D) were iodinated using the above conditions, the membranes were isolated [12] and electrophoresed on 7.5% gel. Protein added, 50–100 μg.

in a decrease of the number of radioactive red blood cell membrane proteins but not in a conversion to the two membrane protein labeling patterns (Fig. 3, Expt A, versus Fig. 1, Expt 4). However, addition of $2 \cdot 10^7$ HeLa cells to the red blood cell suspension converted the red blood cell membrane pattern to a two-component pattern (Fig. 3, Expt B versus Fig. 1, Expt 4). The radioactive pattern of the Hela membrane under these conditions is shown in Fig. 3, Expt C. Iodination of HeLa cells at approximately two times this concentration does not change the HeLa cell membrane labeling pattern (Fig. 3, Expt D). The low molecular weight peak which appears after the bromophenol blue marker is discussed in the accompanying article [16].

4. Is the HeLa cell labeling limited to the cell surface?

We have done a variety of experiments to establish this fact, however, only one experiment gives reasonably conclusive evidence. Although radioautography shows extensive labeling of the cell membrane, it does not conclusively eliminate the possibility that labeling of the cytoplasm occurs because we have found that ^{125}I labeling of red blood cells or HeLa cells followed by extensive washing of the cells in the presence of non-radioactive I^- still leaves an appreciable amount of acid-soluble intracellular radioactive I^-. In addition, it leaves open the possibility of pinocytosis of labeled membrane proteins. A different approach, the separation of cell homogenates on sucrose gradients always yields cross contamination with membrane fractions of various sizes.

We decided to label HeLa cells, treat them with pronase and measure the residual radioactivity of the whole cells; the assumption being that pronase would only degrade proteins on the cell surface. The treatment was done under conditions which left the cells intact and impermeable to trypan blue. Fig. 4 shows an initial rapid decline followed by a slower progressive decrease of radioactivity associated with the whole cell. Such a pattern of decrease would be in agreement with a pro-

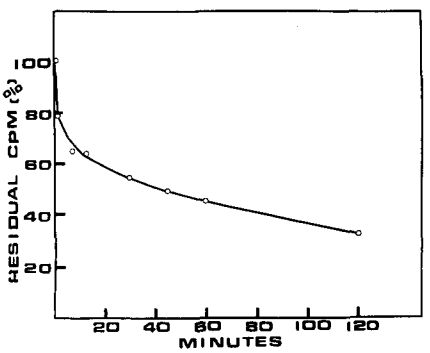


Fig. 4. Loss of radioactivity from ^{125}I-labeled HeLa cells upon treatment with pronase. HeLa cells were iodinated as in legend Figure 3D. Two sets of tubes which contained 2 ml of HeLa cells in minimum essential medium at $6 \cdot 10^5$ cells/ml were incubated at 37 °C. One set contained pronase at 25 μg/ml; the control set did not contain pronase. At different periods of time control and pronase tubes were removed and washed as described in the text. The cell number was redetermined after washing and the radioactivity per cell was determined. The percent of radioactivity remaining after pronase treatment, as compared to the control, was then calculated.

gressive decrease in accessibility of the cell surface proteins to the pronase. That such a situation may exist is indicated by the fact that the cells become extremely sticky in the first few minutes, they cannot be pipetted without clumping and very special conditions have to be maintained in order to make this experiment possible. (See Materials and Methods.)

Fig. 4 shows that when HeLa cells were incubated with pronase (25 μg/ml) for 10, 30 and 120 min and compared to controls incubated for the same time in the absence of pronase that 36, 46 and 66%, respectively, of the radioactivity was removed. In separate experiments, incubation of HeLa cells for 30 min in the presence of 25 μg, 100 μg and 250 μg/ml of pronase resulted in the loss of 25, 50 and 71% of the radioactivity. This cleavage of exposed membrane proteins by proteolytic enzymes is best understood within the context of the experiments of Hubbard and Cohn [8]. These authors reported that trypsin digestion of iodinated red blood cells removed 40% of the radioactivity while pronase treatment removed 45% of the radioactivity. Both enzymes completely removed one of the two labeled proteins. However, whereas trypsin did not cleave the other labeled protein (mol. wt 110000), pronase cleaved it to a smaller protein (mol. wt 72000) which remained on the membrane and contained all the original radioactivity. In addition, it should be mentioned that trypsin digestion of iodinated human platelets removed only 10% of the total radioactivity [6]. Consequently, it may be concluded that availability of a protein to iodination does not carry as a consequence its availability to complete digestion by pronase. Our results emphasize that well over 50% of the membrane radioactivity can be digested by a variety of proteolytic enzymes; the particular protein which is digested depends on the nature of the proteolytic enzyme used. This topic is further emphasized in the adjoining paper [16].

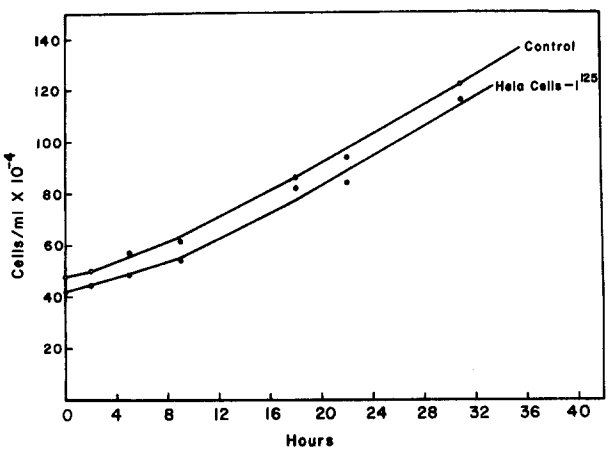


Fig. 5. The growth curve of ^{125}I-labeled HeLa cells. HeLa cells were iodinated, washed and resus ended in growth medium and their growth rate compared to that of control non-iodinated cells. After 18 h, HeLa cell culture was diluted with an equal volume of the medium but cell number was doubled and presented in the Figs. In order to maintain optimal growth rates, at 18 h the medium was diluted to maintain the cells in the logarithmic phase of growth. The curves presented have been corrected for this dilution.

5. The effect of iodination on the growth of HeLa cells

If the iodination procedure leads to labeling of surface proteins, the question that can be raised is whether one is modifying the cell structure in such a way that the cell becomes non-viable. If this does occur, is the membrane surface altered sufficiently to distort the cell surface? If such a distortion were to occur, the radioactive acrylamide gel protein pattern could then be a reflection of this altered state of the membrane rather than of its normal state.

Fig. 5 shows that, under our conditions of iodination, the ^{125}I-labeled HeLa cells grow at the same rate as the controls and that no obvious gross or irreversible damage has occurred as measured by this criterion.

DISCUSSION

In these experiments we have made every effort to ensure a pure population of red blood cells. We have first used the methods normally used to isolate white cells, platelets, etc. [13] and discarded this fraction. Only after three such fractionations did we proceed to isolate red blood cells.

The Phillips–Morrison method [1] appears to be an excellent method for labeling exposed membrane proteins of cells and probably of other cell particles. Like all methods, it has an optimal functional range. If this range is exceeded additional labeling can occur. Our results indicate that two variables, the cell concentration and the degree of iodination, are very critical and unless strictly adjusted the method can yield variable results.

The advantage that red blood cells have as a test system is that the membranes can be easily purified free of adhering cytoplasmic components, since no internal architecture is evident in these cells. Although optimal conditions of labeling will yield only two radioactive membrane components, we have shown that changes in the cell number or the degree of iodination will label additional membrane components. If the conditions are varied, further excessive labeling of hemoglobin, a cytoplasmic component, can also occur.

This is interesting because of its implications. It implies that the lactoperoxidase–I^- complex will not necessarily travel always as one component. We have performed experiments in which lactoperoxidase is kept separate from albumin by a dialysis sac and have been able to demonstrate the iodination of the albumin in this discrete compartment, separate from the lactoperoxidase by a membrane many times the thickness of a cell membrane. Under some conditions, iodide radicals can be formed which will penetrate deeper into the membrane and also reach the cytoplasm.

We offer a tentative explanation of this phenomenon. The lactoperoxidase–I^- complex has a finite life time. If during this interval it meets an iodinatable site, the site will be iodinated. If it does not meet an iodinatable site, during this time interval, the lactoperoxidase–I^- complex dissociates into lactoperoxidase and an iodide radical. This iodide radical can now penetrate structures otherwise impermeable to the lactoperoxidase–I^- complex and iodinate them.

The implication, therefore, is that unless one uses the correct number of cells to decrease the mean free path between the cell surface and the lactoperoxidase–I^- complex, one can also iodinate structures other than those which are most exposed. This observation has its positive side, that by judicious arrangement of the concen-

tration of cells one can define a pattern of membrane proteins arranged according to their distance from the exterior surface of the membrane. Of course, this can be further modulated by proper pretreatment with proteolytic enzymes, neuraminidase, etc.

These results also suggest that attempts to label, by this method, cells attached to solid surfaces or large structures like nerves, etc., are bound to end up by having labeled much more than highly exposed surface proteins. The mean distance between the surrounding fluid and the object to be iodinated will necessarily be such as to permit iodination by an iodide radical free of lactoperoxidase.

We decided to label HeLa cells as a model system of an eukaryotic cell with complex internal architecture because a reliable method existed for isolation of its membranes. However, we were faced with the difficulty of choosing the correct iodination conditions for this cell type. The size of the HeLa cell excludes using cell concentrations equal to those of red blood cells. Consequently, we decided to use an internal control, a cell whose labeling characteristics were known to us, the red blood cells. We added red blood cells at a concentration which in itself would ensure excessive surface labeling. By gradual addition of HeLa cells we were able to establish conditions which converted the multiple protein labeling pattern of the red blood cells to a two protein labeling pattern. Of course, we do not have an independent check that this is the optimal condition for labeling of HeLa cells, except for the fact that a further increase in HeLa cell concentration does not result in more discrete labeling as in the case of the red blood cells. The logic of this experiment was that if one uses a suboptimal number of red blood cells one can acquire optimal labeling by adding more red blood cells; we did the same thing by adding more HeLa cells. We believe that this internal control provides a critical method for the delineation of optimal iodination conditions of various cell types.

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