MACROMOLECULAR GLUCOSAMINE-CONTAINING COMPONENT OF THE SURFACE OF CULTIVATED MOUSE CELLS

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SUMMARY
It has been demonstrated that a glucosamine-containing macromolecular component of the cell surface of 3T3 mouse cells, and SV40-transformed cells, is released from cells by treatment with trypsin under conditions in which the plasma membrane remains functionally intact. This was shown by the fact that the treated cells could be cloned with high plating efficiency and remained impermeable to the vital stain, erythrocin. A method for specifically marking this surface component has been devised based on the finding that in 3T3 cells growing synchronously after subculture by trypsin maximum incorporation of glucosamine into this material occurs 12-13 h thereafter. Of the total radioactive glucosamine incorporated into macromolecular cell constituents, over 80% was recovered in surface component.

Studies on the biosynthesis of surface component revealed that this was periodic during a cycle of cell duplication, with an increased rate of formation immediately after cell division. It was found that the surface component of 3T3 cells differed from that of SV40-transformed cells.

INTRODUCTION
There is now considerable work with bacterial (Ganesan & Lederberg, 1965; Ryter, 1968; Marvin, 1968) and animal cells (Abercrombie & Ambrose, 1962; Mayhew, 1966; Weiss, 1967), which indicates that the cell surface plays a key role in the regulation of cell and DNA duplication. The direct or indirect participation of the cell surface in these processes is emphasized by the demonstration that in cells transformed by viruses (Vogt & Dulbecco, 1962; Stanners, Till & Siminovitch, 1963), or other carcinogens (Borek & Sachs, 1966; Huberman & Sachs, 1966), in which the pattern of cell multiplication is grossly altered, changes have been brought about in the surface components (compare Abercrombie & Ambrose, 1962; Habel, 1963; Burger, 1968; Irlin, 1967).

Very little is at present known about the mechanism(s) of regulation effected by the cell surface. Before undertaking a study of this problem it is first necessary to characterize the chemical composition of this organelle.

With this goal in mind, investigations were begun to isolate and chemically characterize the plasma membrane of mouse 3T3 cells (Todaro & Green, 1963) and 3T3 cells transformed by SV40 virus (Oxman et al. 1967). In the course of these studies, procedures were developed for the specific labelling and isolation of a surface component.
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of \(3T_3\) mouse cells and SV40-transformed \(3T_3\) cells. These procedures are described herein, as are some of the physiological properties of the surface component. A preliminary report of this work has already been made (Onodera & Sheinin, 1968).

MATERIALS AND METHODS

Cells

\(3T_3\) cells, which were adapted from fresh mouse embryo cells to grow continuously in vitro (Todaro & Green, 1963), were used. Although preliminary studies were carried out with cells obtained from Dr Howard Green, most of the experiments described herein were done with a clone of the original \(3T_3\) cell line, isolated by Dr Marguerite Vogt. A clone of SV40-transformed \(3T_3\) cells (designated clone 479) was derived from Dr Vogt's line of \(3T_3\) cells by Dr Michael Oxman (Oxman et al. 1967). The cultures of both cell types have been checked periodically for the presence of Mycoplasma by Miss Patricia A. Quinn of the Hospital for Sick Children, Toronto. The results have always proved negative.

Cultivation of cells in vitro

For maintenance of the \(3T_3\) and SV40-transformed \(3T_3\) cells, cell sheets were grown to confluence and were then subcultured at a concentration of no greater than \(10^6\) cells/cm\(^2\) in 8-oz plastic bottles. For experimental purposes cultures were prepared in 32-oz Brockway bottles. Under these conditions of cultivation the cells showed the growth properties originally described by Todaro & Green (1963).

To subculture \(3T_3\) or SV40-transformed \(3T_3\) cells, the medium from cell sheets which had just grown to confluence was decanted and the cells were processed as follows. A solution of 0.1 % crude trypsin (w/v) in citrate saline (compare Sheinin, 1961) (2 ml/8-oz plastic bottle and 10 ml/32-oz bottle) was added to the cells and these were incubated for 1-2 min at room temperature. The culture bottles were inverted to remove excess trypsin solution from the cells, which were further incubated for up to 5 min. The trypsin solution was then decanted and the cells were suspended in growth medium. Approximately 15-ml and 60-ml volumes of the cell suspension were dispensed into 8-oz and 32-oz bottles respectively. The cells were cultivated in medium 1066 (compare Parker, Castor & McCulloch, 1957) supplemented with 10 % (v/v) foetal calf serum at 37 °C in an incubator flushed with a gas mixture of 95 % air and 5 % CO\(_2\).

Synchronized growth of \(3T_3\) cells

Synchronized cell populations, used in all experiments, were obtained using a method based on the observation of Nilausen & Green (1965). Cultures of \(3T_3\) cells were grown to confluence and then subcultured as described above. If plated in small (10 x 35 mm) or large (15 x 60 mm) plastic Petri dishes or in 32-oz Brockway bottles at \(10^6\) cells/ml (2, 5 and 60 ml, respectively) the cells underwent synchronized cell duplication as shown in Fig. 1. The first cycle of DNA replication was initiated at approximately 10-12 h after the cells were subcultured, reached maximal cell involvement at about 24 h and was complete by 48 h. Mitoses were first detected at about 30-32 h after subculture and the first cycle of cell division was complete at approximately 48 h. Two cycles of synchronized cell duplication were obtained when cells were plated at \(5 \times 10^4\) cells/ml.

SV40-transformed cells, if subcultured according to the above regimen could be only partially synchronized, since cell division continued to occur in cultures in which the cell sheets had become confluent. However, gentle shaking of confluent cultures of SV40-transformed cells resulted in the removal of the majority of dividing cells and permitted the preparation of synchronously growing cultures by the procedure just described.
Fig. 1. Pattern of DNA synthesis and mitosis in synchronized populations of \(3T_3\) cells. Confluent cultures of \(3T_3\) cells were subcultured and plated at \(10^5\) cells per plastic Petri dish (10 × 35 mm) each containing a 2-cm square coverslip. At various intervals the cultures were exposed for 1 h to medium containing TdR-methyl-\(^3\)H, and they were processed for the counting of mitotic cells and for cells which had synthesized DNA, by the technique of radioautography (cf. Sheinin & Quinn, 1965). The mitotic index (○—○) indicates the number of dividing cells/100 cells and the DNA synthetic capacity of the culture is reported in terms of the percentage of cells with radioactive nuclei (●—●).

Specific labelling of surface component of mouse cells grown in vitro

The procedure used for the specific radioactive-labelling of surface component of \(3T_3\) and SV40-transformed \(3T_3\) cells was developed on the basis of experiments to be discussed in the Results section. Confluent cultures of \(3T_3\) cells or SV40-transformed \(3T_3\) cells were subcultured as described above to obtain populations of synchronously growing cells. After 12 h the cells were pulse-labelled for 1 h with medium containing radioactive d-glucosamine hydrochloride (at a concentration and specific activity noted for each experiment). As will be discussed later, this regimen resulted in specific labelling of material at the surface of \(3T_3\) mouse cells.

Chromatography on columns of DEAE-cellulose

Columns (approximately 1.2 cm × 10 cm) of DEAE-cellulose were prepared in 0.01 M potassium phosphate, pH 7.0. The material (approximately 5–10 ml) to be chromatographed was first dialysed overnight in the cold, against 6 l. of water, and was then applied to the columns. The columns were developed with NaCl of increasing concentration, in potassium phosphate buffer. Fractions (9 ml) were collected. The NaCl concentration of each fraction was measured using a calibration curve relating salt concentration to conductivity (measured using a Philips Conductivity meter). The distribution of radioactively labelled material was analysed as noted in the following section.

Measurement of incorporation of radioactive substances into macromolecules

For analysis of the radioactivity of any macromolecular fraction, the material was chilled in ice and to it was added cold trichloroacetic acid (TCA) to give a final concentration of 5% (w/v). The well mixed suspension was left at least 1 h in ice and the precipitated macromolecules were
then collected on Millipore filters (0.45 μm pore size; type HA) and washed with two 5-ml volumes of cold TCA solution (in 95% ethanol). The filters were transferred to scintillation vials, dried, and 10 ml of non-aqueous scintillation fluid (compare Arnold, 1963) were added to each vial. The radioactivity of the precipitated material was counted in a liquid scintillation counter.

Preparation of plasma membrane of 3T3 mouse cells

Confluent cultures of 3T3 mouse cells (in 32-oz Brockway bottles) were employed for the isolation of plasma membrane. The cells, washed with phosphate-buffered saline of Dulbecco & Vogt (1954) (PBS) were harvested by scraping and were combined in PBS. The resulting cell suspension was processed, using an adaptation of methods developed by Warren, Glick & Nass (1966), for the preparation of cell membrane. To the cell suspension (in 17.5 ml PBS) was added 0.5 ml 0.001 M ZnCl₂. This was incubated at room temperature for 10 min and then 1 ml 0.01 M ZnCl₂ and 1 ml 1% (v/v) Tween 20 were added. After 5 min the cells were disrupted (as judged by phase-contrast microscopy) using a Dounce homogenizer. The homogenate was passed through a column of glass beads (Superbrite 150) and the cell membrane fraction was recovered by elution with water. The eluent was centrifuged at 4000 g for 20 min. The sedimented membrane-containing material was resuspended in 1 ml water and layered over a 45–60% (w/v) sucrose gradient. This was centrifuged for 1 h at 23000 rev/min (Spinco Rotor SW 25.1). A white layer of membrane was present at an approximate density of 1.26. It has been established in studies described elsewhere (K. Onodera & R. Sheinin, in preparation) that the material so isolated is indeed plasma membrane.

Chemicals and reagents

Unless otherwise noted all chemicals were AnalR grade, obtained from British Drug Houses. Crude trypsin in salt solution was obtained as a 2.5% suspension from Grand Island Biological Co. Worthington Chemical Corporation was the source of 3 x crystallized trypsin. The radioactive chemicals were purchased from New England Nuclear Corporation and Volk Chemicals; the 1,4-bis-2-(5-phenyloxazolyl)benzene and 2,5-diphenyloxazole for the scintillation fluid from Packard, the soybean trypsin inhibitor from Nutritional Biochemicals, and the DEAE-cellulose from the Whatman Company.

RESULTS

Experimental conditions for the specific labelling of glucosamine-containing material at the surface of 3T3 mouse cells and SV40-transformed 3T3 cells

The extensive use of trypsin in the continuous in vitro cultivation of animal cells attests to the fact that surface materials can be removed from cells without necessarily destroying, or even impairing, those functions of the plasma membrane essential to the maintenance of viability. In the present study this enzyme has been used to examine the surface localization of a glucosamine-labelled moiety of 3T3 cells.

Cultures of normal or SV40-transformed 3T3 cells, synchronized as described in Materials and Methods, were treated with radioactive glucosamine for 1 h, 12 h after subculture. The washed cells were scraped from the growth surface and were then treated with trypsin. Viability of the cells was assessed and the trypsin-digested material was analysed to determine the distribution of glucosamine-containing material.

As is seen in Table 1, brief treatment of 3T3 cells with either crude or crystalline trypsin under the particular conditions described, yielded a cell suspension in which a majority of cells remained viable as shown by the fact that they were able to form
Mouse cell surface

Macroscopically visible colonies after 10 days of culture in vitro. Although a small proportion of the enzyme-treated cells did not exhibit this high degree of resistance to trypsin treatment, nevertheless they did survive the incubation period to the extent that they retained their impermeability with respect to the non-vital stain, erythrocin. In addition, microscopic examination of the culture dishes on the day of staining showed that about 10% of the original cell population had given rise to abortive clones.

In Fig. 2 are presented data illustrating the effect of trypsin on the release of radioactive, acid-insoluble material from cells incubated as above with [3H]glucosamine. Release of such material began immediately upon addition of enzyme, and approached a maximum after about 5 min. Little or no further recovery from cells of glucosamine in acid-insoluble material occurred even after 10 min. The viability data shown in

Table 1. Viability of mouse cells treated with trypsin

<table>
<thead>
<tr>
<th>Treatment of cells</th>
<th>3T3 cells</th>
<th>SV40-transformed 3T3 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min, 0.1% crystalline trypsin</td>
<td>98</td>
<td>90</td>
</tr>
<tr>
<td>5 min, 0.1% crystalline trypsin</td>
<td>84</td>
<td>102</td>
</tr>
<tr>
<td>10 min, 0.1% crystalline trypsin</td>
<td>67</td>
<td>85</td>
</tr>
<tr>
<td>1 min, 0.1% crude trypsin</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>5 min, 0.1% crude trypsin</td>
<td>87</td>
<td>90</td>
</tr>
<tr>
<td>10 min, 0.1% crude trypsin</td>
<td>70</td>
<td>81</td>
</tr>
</tbody>
</table>

Cultures of 3T3 or SV40-transformed 3T3 cells were treated at 37 °C with crystalline or crude trypsin for the periods noted, under conditions described for cultivation of cells in Materials and Methods. The cells were suspended in PBS, counted in the presence of erythrocin (0.01% w/v) and then diluted in medium and plated in 5-ml volumes in Petri dishes (15 x 60 mm) to give 20, 50, 100 and 200 cells/dish. Triplicate samples were plated at each concentration. The cells were incubated for 10-14 days, the colonies formed were stained with methylene blue (Stanners et al. 1963) and counted. The plating efficiency is reported in terms of the number of colonies obtained as a percentage of the number of cells plated per dish.

Table 1, together with the finding that release of glucosamine-containing macromolecules from 3T3 cells approached a plateau at about 5 min, suggested that the particular trypsin treatment of 3T3 mouse cells used resulted in the removal from the cell of glucosamine-containing material, primarily on the outer surface of the plasma membrane.

Support for this conclusion was derived from studies on the effect of similar enzyme treatment on the protein of cells pulse-labelled with radioactive amino acids under the conditions used to label with glucosamine. 3T3 cells were subcultured as noted above. Twelve hours later they were incubated for 1 h with [14C]valine or [14C]leucine. The labelled cells were treated with trypsin for 10 min as described in Fig. 2. It was found that of the total acid-insoluble, cell-associated radioactive amino acid, approximately 7% was released by trypsin.

In Table 2 are presented data showing the distribution of radioactive glucosamine among fractions of cells labelled with [3H]glucosamine as described above, and then
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treated with trypsin. They show that the major portion (about 80%) of radioactive glucosamine, which had been incorporated by 3T3 cells into acid-insoluble linkage was recovered in the supernatant fraction of trypsinized cells. Thus under conditions of trypsin treatment in which only 7% of cell protein is lost from 3T3 cells, 80% of glucosamine-containing macromolecules are released.

Fig. 2. Kinetics of release of acid-insoluble material from 3T3 cells pulse-labelled with [3H]glucosamine and treated with trypsin. 3T3 mouse cells (plated at 5 x 10^5 cells/2-oz Brockway bottle) were labelled with D-[6-3H]glucosamine.HCl (0.54 μM; 3.7 Ci/mmole), as described in Materials and Methods. The medium was decanted and the cells were washed 3 times with PBS. To control and test cultures, respectively, was added 0.5 ml prewarmed PBS or 0.5 ml 0.1% (w/v) crystalline trypsin. The cultures were incubated at 37 °C. At the times noted, triplicate control or test cultures were processed as follows: The cultures were chilled in ice and the cells remaining attached to the growth surface were scraped therefrom. The cell suspension was quantitatively transferred into a centrifuge tube with two 5-ml volumes of cold PBS. One ml of the resulting suspension of about 11 ml was removed and precipitated with 5 ml cold 5% TCA (w/v) to determine the total acid-insoluble radioactive material. To the remaining cell suspension was added foetal calf serum to a final concentration of 1% (v/v). The cells were harvested by centrifugation (for 8 min at 800g at 2 °C), washed twice with 5 ml cold PBS containing 1% foetal calf serum, and suspended in 5 ml of cold 5% TCA. To the original supernatant was added 1 ml of 50% cold TCA. The radioactivity of the precipitated macromolecules was measured as described in Materials and Methods. ○—○, Control cultures; ●—●, trypsin-treated cultures.

These observations have been used to formulate an operational definition of a glucosamine-containing surface component of 3T3 cells. This definition depends upon 2 operations. Thus the particular surface component under study is that material specifically marked when synchronously growing 3T3 cells are pulse-labelled for 1 h with radioactive glucosamine, 12 h after subculture, using trypsin to dissociate cells
previously grown to confluence. This glucosamine-containing surface component is released from cells by trypsin treatment under conditions which leave the cells viable and impermeable to non-vital stains.

Table 2. Analysis of the distribution of [14C]glucosamine incorporated into acid-insoluble material of mouse cells pulse-labelled at 12 h after subculture

<table>
<thead>
<tr>
<th>Cell type tested</th>
<th>Total cpm</th>
<th>% of total</th>
<th>Trypsin-treated cpm</th>
<th>% of total</th>
<th>Trypsin digest (supernatant) cpm</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 cells</td>
<td>3732</td>
<td>100</td>
<td>867</td>
<td>23.4</td>
<td>3006</td>
<td>83.5</td>
</tr>
<tr>
<td>SV40-transformed 3T3 cells</td>
<td>6204</td>
<td>100</td>
<td>1065</td>
<td>17.1</td>
<td>4940</td>
<td>79.8</td>
</tr>
</tbody>
</table>

Synchronously growing cultures of 3T3 cells (plated at 5 x 10^4 cells/ml) in Petri dishes (60 x 15 mm) were incubated with 14C-labelled glucosamine (0.49 mM, 8.9 mCi/m mole) as described in Materials and Methods under 'the specific labelling of surface component'. The cells were washed and treated with 0.1% (w/v) crude trypsin for 10 min as described in Table 1. The cells were harvested by centrifugation for 10 min at 8000 g. The amount of acid-insoluble glucosamine present in the cells and the trypsin-digested supernatant fraction was determined.

Table 3. Stability of the surface component of 3T3 mouse cells

<table>
<thead>
<tr>
<th>Period of incubation after surface component was labelled, h</th>
<th>Total culture cpm</th>
<th>Cell-associated cpm</th>
<th>Released by trypsin cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1409</td>
<td>1185</td>
<td>807</td>
</tr>
<tr>
<td>4.3</td>
<td>2269</td>
<td>1896</td>
<td>1020</td>
</tr>
<tr>
<td>24</td>
<td>2114</td>
<td>1805</td>
<td>1049</td>
</tr>
<tr>
<td>48</td>
<td>2138</td>
<td>1573</td>
<td>1121</td>
</tr>
</tbody>
</table>

Synchronously growing cultures of 3T3 mouse cells (in 32-oz Brockway bottles) were prepared as described in Materials and Methods. The cells were plated at a concentration of 5 x 10^4 cells/ml. Twelve hours after subculture the surface component of the cells was pulse-labelled for 1 h with [3H]glucosamine (0.88 mM; 1.25 Ci/m mole) as described in Materials and Methods under 'the specific labelling of surface component'. The radioactive medium was decanted. The cells, washed 3 times with non-radioactive medium, were incubated with similar medium. At the intervals noted, triplicate cultures were harvested and analysed (as indicated in Table 2) for the presence of radioactive acid-insoluble material in the fractions listed.

Experiments were carried out to determine if the surface component detected in the present study was a stable cell constituent once formed, or whether it was secreted as such or in degraded form into the medium. Confluent cultures of 3T3 cells were subcultured with trypsin as noted in Table 3. After 12 h the cells were pulse-labelled for 1 h with [3H]glucosamine to label surface component. The radioactive medium was replaced with non-radioactive medium and the cells were further incubated. At various times thereafter the cultures were analysed for the distribution of radioactive glucosamine among various cell and culture fractions.
The data obtained (Table 3) revealed that maximum total labelling of macromolecules in the culture was obtained within 4 h after the period of incubation with radioactive glucosamine. Of this total radioactive material over 80% was found to be cell-associated. The major fraction of the cell-bound material was resident at the cell surface as indicated by its sensitivity to release by trypsin treatment. Little change was seen to occur in the glucosamine-labelled macromolecules of the whole culture during the next 48 h, during which time the cells divided twice and reached the stationary phase of their growth. The labelled material remained cell-associated and did not appear in the medium. Trypsin treatment of cells revealed that at any time a major portion (about 84%) of the glucosamine-containing macromolecules of 3T3 cells was released by this enzyme under conditions leaving cells viable. These observations were interpreted as indicating that the major portion of the incorporated glucosamine was present in surface component. In addition they suggested that the surface component once formed remained stable in the sense that it was not degraded or released into the environment, and that it remained at the cell surface.

Because studies were to be made using SV40-transformed cells, experiments like those just described for the parental 3T3 cells were carried out to determine whether these cells too formed a surface component. It was found that trypsin treatment yielded a majority of fully viable cells (Table 1) and that incubation of synchronously growing cells with radioactive glucosamine under defined conditions (Table 2) did result in specific labelling of surface material as defined above. Indeed, in experiments analogous to all of those described above for 3T3 cells, essentially similar results were obtained with SV40-transformed cells. Using the afore-noted procedures it has therefore been possible to identify surface component on the external surface of the plasma membrane of both cell types.

Isolation of a cell fraction containing a specifically marked surface component of mouse cells grown in vitro

In order to carry out biochemical and physiological studies on the surface component specifically marked as described in the foregoing section, it was desirable that it be isolated in intact form without the use of trypsin. A fraction of 3T3 cells containing this component was isolated in the following way: The surface component was labelled with radioactive glucosamine as previously described. The labelled cells were washed, scraped from the glass and suspended in PBS at a concentration of no greater than 10^7 cells/ml. The cell suspension was chilled in ice and treated with sonic vibrations. The suspension of disrupted cells was subjected to centrifugation as is indicated in Fig. 3, in which the results of an analysis of the distribution of labelled material amongst the various cell fractions obtained, are also presented.

Ninety-four per cent of the labelled material was recovered in the supernatant 1 fraction obtained when the sonicated cell suspension was spun at 27,000 g, and 67% appeared in the supernatant 2 fraction obtained after subsequent centrifugation of the S1 fraction at 104,000 g. Because the work presented in the previous section showed that approximately 80% of the glucosamine-containing macromolecules of labelled cells was present as surface component, it was therefore concluded that the majority
of this material was released by sonic treatment of \textit{3T3} cells, as a soluble cell fraction.

Partial purification of the labelled material obtained from sonicated \textit{3T3} cells was achieved by chromatography of the supernatant 1 fraction (obtained as described above—Fig. 3) on DEAE-cellulose columns. In Fig. 4 are presented data obtained with a supernatant 1 fraction obtained from cells in which the surface component was

![Diagram](attachment:image.png)

\textbf{Fig. 3.} Distribution of [\textsuperscript{14}C]glucosamine in macromolecules of fractions of \textit{3T3} cells with labelled surface component. Surface component of \textit{3T3} cells was labelled (as described in Materials and Methods) with D-[\textsuperscript{1-14}C]glucosamine.HCl (0.48 \mu M; 39.5 mCi/mmole). The cells were then harvested and fractionated as noted in text and in figure. During the manipulations the temperature of the materials varied from 0-8 °C. (*The largest temperature variations occurred in the course of sonication.)*

A portion of each cell fraction was treated with TCA (5%, w/v) to precipitate the macromolecules. The total radioactivity of each cell fraction (measured as described under Materials and Methods) is given in brackets in terms of absolute counts and as a percentage of the total radioactive material present in the original cell suspension.

labelled with \textsuperscript{[3H]}glucosamine. The majority of the incorporated glucosamine was eluted from DEAE-cellulose with salt of concentration greater than about 0.22 M as 2 major components. The bulk of the cell protein (as measured by O.D. 280 nm) clearly separated from the trailing major radioactive components was recovered primarily in two main fractions. One was washed right through the column and the other was eluted with salt of concentration lower than about 0.22 M. Thus, considerable purification of the glucosamine-containing material from other cellular material was achieved in the chromatography on DEAE-cellulose columns.

Because the cell fraction carrying the surface component specifically labelled with radioactive glucosamine was isolated by sonic disruption, it was possible that some portion of the material obtained by the above-described chromatographic procedure might originate from another cell moiety. To determine which of the radioactively labelled materials eluted from DEAE-cellulose were derived from surface component, a comparison was made between surface component extracted from cells which were harvested by being scraped from the growth surface, and from cells which were harvested by trypsinization and which should therefore have been denuded of this substance.
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Two populations of synchronized $3T_3$ cells were prepared. In one the surface component was labelled with $[^3H]$glucosamine and the cells were removed from the growth surface by scraping; in the other the surface component was labelled with $[^{14}C]$glucosamine and the cells were removed from the growth surface by trypsin treatment. The differentially labelled and harvested cell lots were combined, the supernatant $S_1$ fraction was obtained and chromatographed on DEAE-cellulose columns.

![Figure 4](image-url)

**Fig. 4.** Chromatography on DEAE-cellulose columns of a soluble component of $3T_3$ cells containing $[^3H]$glucosamine-labelled surface component. The surface component of a population of synchronized $3T_3$ cells was labelled as described under Materials and Methods with $D-[6-^{3H}]$glucosamine·HCl ($0.88 \mu M; 1.15 \text{ Ci/mmole}$). The supernatant $S_1$ fraction of the cells was obtained as noted in Fig. 3. It was dialysed against distilled water and chromatographed on columns of DEAE-cellulose. The acid-insoluble radioactivity ($\bullet$) and O.D. 280 nm ($\times$) of each fraction were measured. The NaCl concentration is given by the unmarked line. Of the total macromolecular radioactivity and O.D. 280 nm units of material put on to the column approximately 90.4% and 68.4% respectively, were recovered in the fractions collected.

The data obtained with the extracts of these two lots of cells are shown in Fig. 5. (It will be noted that the overall profile of Fig. 5 differs somewhat from that shown in Fig. 4. This is due to differences in chromatographic procedures employed. The data of Fig. 5 were derived early in the course of this study at which time chromatography was carried out with a fairly sharp NaCl gradient. More recent purification, an example of which is presented in Fig. 4, has employed a very shallow NaCl gradient for development of DEAE-cellulose columns.) It is clear from Fig. 5 that the material derived
from cells labelled with $[^3H]$glucosamine, and scraped from the growth surface yielded one major fraction, eluted with NaCl at concentration of about 0.20–0.38 M and 3 minor glucosamine-containing fractions eluted with NaCl of higher concentration. A very different pattern of chromatography on DEAE-cellulose was obtained with the extract of cells in which the surface component had been labelled with $[^14C]$glucosamine, and which were removed from the growth surface with trypsin under conditions shown previously (Table 2) to result in the release of the labelled surface component from cells. Little $[^14C]$-labelled material was present in the position of the major fraction recovered from cells labelled with $[^3H]$glucosamine and harvested by scraping. In addition, the amount of $[^14C]$glucosamine-labelled material eluted in the position of 2 of the 3 $[^3H]$glucosamine-labelled minor fractions appeared to be reduced.

It should be noted that analogous results were obtained if the procedure of harvesting of cells was reversed. Thus, if cells labelled in the surface component with $[^3H]$glucosamine were harvested by trypsin treatment and then combined with cells labelled with $[^14C]$glucosamine and harvested by scraping, then the typical pattern of
chromatography on DEAE-cellulose was obtained with the $^{14}$C-labelled material. Little tritium-labelled material was recovered from the column.

On the basis of these observations it was concluded that the chromatographic procedure just described does result in the partial purification of a specifically labelled surface component of $3T_3$ cells as defined operationally in the first section of Results. This material was recovered in fractions eluted from DEAE-cellulose columns with 0.22–0.38 M NaCl.

**Possible association of surface component with other cell constituents**

Radioactive glucosamine has been used in these studies to label specifically a macromolecular component of the cell at the external surface of the plasma membrane. However, it was not clear whether the isolated radioactively-labelled surface component of cells contained other non-labelled surface constituents. As a particular approach to this problem an attempt was made to detect the presence of constituents of cell or of plasma membrane in preparations of surface component in the following way. $3T_3$ cells or SV40-transformed cells were grown through several generations to confluence in a medium containing one of $^{14}$C-amino acid, $[^{14}$C]glucosamine, or $[^{14}$C]choline to label uniformly plasma membrane (as well as other cell organelles) in peptide.

### Table 4. Distribution of radioactive macromolecules in fractions of cells prelabelled with precursors of lipid, protein and carbohydrate

<table>
<thead>
<tr>
<th>Cells*</th>
<th>Prelabelled with</th>
<th>Total cell suspension, cpm</th>
<th>Plasma membrane fraction, cpm</th>
<th>Surface component,† with 0.19–0.22 M NaCl, cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>$3T_3$-SV</td>
<td>$[^{14}$C]choline</td>
<td>$2.51 \times 10^4$</td>
<td>$1.100 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>$3T_3$-SV</td>
<td>$[^{3}$H]choline</td>
<td>$6.19 \times 10^5$</td>
<td>$2.430 \times 10^6$</td>
<td>0</td>
</tr>
<tr>
<td>$3T_3$</td>
<td>$[^{14}$C]choline</td>
<td>$3.65 \times 10^4$</td>
<td>$1.372 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>$3T_3$</td>
<td>$[^{3}$H]choline</td>
<td>$-$</td>
<td>$3.690 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>$3T_3$-SV</td>
<td>$[^{14}$C]leucine</td>
<td>$6.07 \times 10^6$</td>
<td>$1.720 \times 10^6$</td>
<td>$4.720 \times 10^4$</td>
</tr>
<tr>
<td>$3T_3$-SV</td>
<td>$[^{14}$C]glucosamine</td>
<td>$7.18 \times 10^6$</td>
<td>$5.520 \times 10^6$</td>
<td>$3.100 \times 10^3$</td>
</tr>
<tr>
<td>$3T_3$</td>
<td>$[^{14}$C]glucosamine</td>
<td>$2.31 \times 10^6$</td>
<td>$3.290 \times 10^6$</td>
<td>$7.800 \times 10^2$</td>
</tr>
</tbody>
</table>

* Approximately $5 \times 10^6$–$5 \times 10^7$ cells were grown for 4–5 generations to confluence in medium containing tracer amounts of L-$[^{1}$H]leucine ($0.21 \text{ mM}$; 263 $\mu$Ci/mmole), D-$[^{1}$H]glucosamine. HCl ($1.12 \text{ M}$; 13.4 mCi/m mole) or $[^{3}$H]methylcholine. HCl ($0.02 \text{ M}$; 72.7 mCi/m mole). One-half of the cultures were processed for isolation of plasma membrane and the remainder were used to prepare specifically labelled surface component. Cells prelabelled with $^{14}$C-molecules were subsequently pulse-labelled with $[^{3}$H]glucosamine (see Fig. 2), those prelabelled with $^{3}$H-molecules were pulse-labelled with $[^{14}$C]glucosamine, in each case to mark the surface component to facilitate isolation.

† Glucosamine-labelled surface component (eluted with 0.19–0.22 M NaCl) and material eluted with 0.19–0.22 M NaCl were obtained from DEAE-cellulose in the course of partial purification of surface component (see Fig. 4).

- indicates not measured; 0 indicates none detectable.
carbohydrate or lipid moieties, respectively. Portions of the labelled cultures were used for the preparation of plasma membrane and the remaining $^{14}\text{C}$-labelled cells were subcultured and incubated with $[^3\text{H}]$glucosamine to label surface component specifically. Cell extracts were then processed for the isolation of surface component (see Fig. 4). The material eluted with 0.19–0.22 M NaCl from DEAE-cellulose columns, as well as glucosamine-labelled surface component (eluted with 0.22–0.38 M NaCl), was analysed to determine the distribution of radioactive macromolecules. At the same time the purified plasma membrane preparations were also so analysed. In some experiments cells were prelabelled with $[^3\text{H}]$choline and the surface component was pulse-labelled with $[^{14}\text{C}]$glucosamine.

The data obtained are summarized in Table 4. They show that no choline of the macromolecules of prelabelled cells or plasma membrane was detected in the labelled surface component preparations. In contrast both glucosamine and amino acid-containing macromolecules of prelabelled cells were recovered in the surface component.

Some prelabelled macromolecules of the cell containing amino acid and glucosamine were also detected in the material eluted from DEAE-cellulose columns with low concentrations of NaCl. It is at present not clear what this material is. But these studies, as well as others using the velocity sedimentation analyses, suggest that it differs considerably from surface component.

**Formation of glucosamine-containing surface component in cultures of 3T3 cells synchronized by trypsin subculture**

The foregoing studies have provided tools for the study of a component at the outer surface of 3T3 cells. These have been used to examine the following: (a) regeneration of surface component previously removed by trypsin digestion; and (b) the formation of this material during synchronous growth.

Confluent cultures of 3T3 cells were subcultured, and at various intervals thereafter the newly seeded cultures were examined with respect to DNA synthesis and the capacity to synthesize the specifically labelled surface component discussed above. The data presented in Fig. 6 revealed 2 features of the synthesis of this surface component. The first was that its resynthesis began within 2 h after trypsinized cells were plated. The rate of such resynthesis increased throughout the prolonged $G_1$ period to reach a maximum at about 12 h after which it declined as the cultures passed into and through the $S$ phase of growth. The second observation of interest was that the rate of synthesis of the surface component increased temporarily after the cultures passed through mitosis, and again entered a $G_1$ period of growth.

**Indications of dissimilarity between surface components of 3T3 cells and SV40-transformed 3T3 cells**

As discussed above, it has proved possible to identify and isolate in normal 3T3 cells, and in SV40-transformed cells, surface component resident in vivo at the external limit of the plasma membrane. Studies were therefore carried out to determine whether the surface changes associated with virus transformation might be reflected in the surface component of these 2 cell types.
This study was made using trypsin as a tool; for it has been found (R. Sheinin & K. Onodera, in preparation) that the surface component contains a peptide moiety. In order to compare directly the surface component of 3T3 cells with that of SV40-transformed 3T3 cells the following experiment was performed: Confluent cultures of 3T3 cells and of transformed cells were subcultured and their surface components were labelled with [14C]glucosamine and [3H]glucosamine, respectively. Equal numbers of both lots of cells were scraped from the growth surface and were combined in PBS. The differentially labelled cell suspension was treated with crystalline trypsin and the digested material was chromatographed on DEAE-cellulose yielding the elution profiles shown in Fig. 7.

Fig. 6. Synthesis of surface component in populations of 3T3 cells synchronized by trypsin subculture. Confluent cultures of 3T3 cells were subcultured with trypsin and plated at a concentration of 5 x 10^4 cells/ml in Petri dishes (15 x 60 mm). At the intervals noted duplicate sets of 3 cultures were incubated for 1 h at 37 °C in medium containing TdR-methyl-3H (1.3 nM; 0.77 Ci/mMole) or D-[6-3H(N)]glucosamine.HCl (0.54 nM; 3.7 Ci/mMole). For measurement of DNA synthesis, medium from triplicate cultures incubated with 3H-TdR was decanted, the cultures were washed 3 times and the cells were removed from the growth surface by trypsin treatment. The cells were collected by centrifugation of the resulting suspensions for 10 min at 800 g. Cell number was determined and the incorporation of 3H-TdR into acid-insoluble material (O—O) was measured. The synthesis of surface component was assessed, using triplicate cultures incubated with 3H-glucosamine, in terms of the amount of acid-insoluble radioactive tracer present in macromolecules released by trypsin) (●—●). The cultures were washed 3 times with non-radioactive medium and the cells were treated with trypsin for 10 min as noted in Fig. 2. The supernatant fractions were harvested, chilled in ice and precipitated with cold TCA (final concentration 5%, w/v). The precipitated material was collected on Millipore filters and its radioactivity measured. The bar graph at the top of the figure indicates the period during which cell division (measured in triplicate cultures) occurred.
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On the basis of an arbitrary classification shown on the figure, the peptide fragments derived from the $^{14}$C-labelled surface component obtained from normal $3T_3$ cells were ordered as fractions I–V. The digest derived from the SV40-transformed cells, labelled with $[^3H]$glucosamine, yielded fractions III–V only. Analogous results were obtained if the labelling procedure was reversed. Thus if normal $3T_3$ cells were labelled with $[^3H]$glucosamine and SV40-transformed cells with $[^{14}C]$glucosamine,

![Chromatography on DEAE-cellulose of an incomplete digest of surface component of 3T3 cells and SV40-transformed 3T3 cells.](image)

Fig. 7. Chromatography on DEAE-cellulose of an incomplete digest of surface component of $3T_3$ cells and SV40-transformed $3T_3$ cells. The surface components of equal numbers ($5 \times 10^5$) of $3T_3$ cells and of cells transformed by SV40 virus were labelled with $[^{14}C]$glucosamine. HCl ($1.12$ mM; $13.4$ mCi/mmole) and $[^6H]$glucosamine. HCl ($0.54$ mM; $3.7$ Ci/mmole), respectively. The 2 cell lots were removed from their growth surface by scraping and were combined. The differentially labelled cells were treated with 0.1% (w/v) crystalline trypsin for 10 min at 37°C. Trypsin soybean inhibitor was added to a final concentration of 4 mg/ml. The cell suspension was spun at 800 g for 10 min. The supernatant containing the partially digested surface component was dialysed and chromatographed on DEAE-cellulose columns (pH 7.0). The acid-insoluble radioactivity of each fraction was determined: ○, Material derived from $^{14}$C-labelled surface component of $3T_3$ cells (recovery = 85.2%); ●—●, material derived from $^3H$-labelled surface component of $3T_3$ cells transformed by SV40 virus (recovery = 78.8%); ×—×, NaCl concentration.
5 peptide fractions were detected in the \(^3\)H-labelled material and only 3 were observed in the \(^14\)C-labelled material. On the basis of these observations it was concluded that the surface structure of SV\(^{40}\)-transformed 3T3 cells differs, either quantitatively or qualitatively, from that of the parental cell type.

**DISCUSSION**

The investigations discussed above have provided us with tools for the study of surface component of 3T3 cells and SV\(^{40}\)-transformed 3T3 cells. This surface component can be specifically labelled with radioactive glucosamine and is released from cells by trypsin treatment under conditions which leave the cells viable and the cell membrane presumably intact. Release of surface molecules from the plasma membrane of various cells has been achieved using analogous methods (Cook & Eylar, 1965; Rambourg & Leblond, 1967; Winzler et al., 1967; Borle, 1968).

Procedures are described for the isolation of surface component from 3T3 cells and SV\(^{40}\)-transformed cells. Studies of the isolated materials (Onodera & Sheinin, 1968; R. Sheinin & K. Onodera, in preparation) suggest that the surface component is comprised of carbohydrate and peptide moieties. These findings are in agreement with results reported by others (compare Fitton-Jackson, 1964; Dukes & Goldwasser, 1965; Eylar, 1966; Rambourg & Leblond, 1967; Winzler et al. 1967; Cook, 1968), who have obtained evidence by methods of specific staining, direct chemical analysis and studies on the incorporation of radioactive carbohydrate precursors, that carbohydrate-protein complexes exist on the outside of a variety of cell types.

The precise physiological role of the surface component of 3T3 cells was not delineated by the experiments described herein. These studies did however show that, under the conditions of *in vitro* cultivation employed, 3T3 cells appear to have a requirement for the presence of the surface component. Removal of this material by trypsin treatment was followed very rapidly by its reformation when cells were plated in the absence of the enzyme. The resynthesis *in vitro* of a sialic-acid containing component of a number of animal cell types after trypsin treatment, has been reported by Kraemer (1967). In addition, Gasic & Gasic (1962) have described regeneration of a coating of tumour cells and Borle (1968) noted the reformation of a calcium-containing surface material in trypsin-treated HeLa cells.

That the surface component under study is indeed an integral part of the cell surface is indicated by the finding that once synthesized it is stable. It remains cell-associated and at the cell surface, at least while the cells are undergoing growth. These results are in accord with those of Warren & Glick (1968), who found that the plasma membrane of exponentially growing L-cells did not turn over.

The work described herein revealed that not only is the surface component resynthesized after cells are subcultured by trypsin treatment, but its synthesis is significantly enhanced after mitosis when cells enter a second G\(_1\) period. If one examines 3T3 cells microscopically at various stages after subculture, it is clear that immediately after subcultures the cells are rounded and raised from the growth surface. At about 4 h the cells are still rounded but appear to be firmly attached to the substratum. At about 8 h
the cells begin to spread on this surface and begin to assume the appearance of epithelioid cells, and by 12–14 h the cells acquire the spindle-shape of fibroblasts in culture. It is therefore possible that the surface component of mouse fibroblasts participates in their adhesion to the growth surface. This conclusion is given support by the finding that a second burst of synthesis of surface component occurs in synchronized cell populations immediately after cell division. It has been observed (Axelrad & McCulloch, 1958; Terasima & Tolmach, 1963) that cells in mitosis detach from the growth substrate and subsequently re-attach and progress through a second cycle of duplication. The role of the surface component in a single mitotic cycle and in the cell-to-cell interactions of a growing population remains to be established.

Comparative studies of the properties of the surface component of 3T3 cells and 3T3 cells transformed by SV40 virus have revealed interesting differences. Analysis of surface component released by trypsin digestion from 3T3 cells revealed the presence of at least 5 peptide fractions. Similar treatment of surface component of SV40-transformed 3T3 cells revealed the presence of only 3 major fractions. These findings have been confirmed in studies using purified surface components (R. Sheinin & K. Onodera, in preparation). In addition, preliminary experiments with other virus-transformed 3T3 cell lines suggest that the surface components of these cells also differ from that of the normal parent cell. Differences in carbohydrates of membrane components of 3T3 mouse cells and SV40-transformed cells have recently been reported by others (Meezan, Wu, Black & Robbins, 1969; Wu, Meezan, Black & Robbins, 1969).

The structural basis of the difference between the surface component of 3T3 and SV40-transformed 3T3 cells is not defined by the studies reported here. Since the operational definition of surface component depends upon the use of trypsin for its release from the plasma membrane, the observed differences in the surface component, as isolated, could be due to a change either in surface component per se, or to an alteration in the plasma membrane, with which it is associated in vivo. The relationship of surface component to plasma membrane will be clarified in studies on the precise chemical nature of this material and of purified cell membrane. The role played by either or both of these surface structures in the cycle of cell duplication and in the altered cell-cell interactions leading to neoplastic transformation remains to be elucidated.

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