We have reported the isolation and preliminary biochemical characterization of two mutants of mouse fibroblasts selected for a decrease in cell-to-substrate adhesion (Pouysségur, J., and Pastan, I. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 544-548). We attributed the adhesive defect of these mutants (AD6 and AD8) to the absence of iodinatable cell surface proteins.

This study demonstrates that a defect in glycoprotein synthesis is the biochemical basis for the reduction in proteins exposed at the outer surface of the mutant cells. When D-glucosamine or L-fucose was used as radioactive precursors, analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a decrease in the labeling of all the glycoproteins of both clones. This decrease in glycoprotein labeling was not due to a defect in D-glucosamine uptake, since this precursor was taken up at a higher rate in the mutants than in the wild type. In spite of this high uptake, the rate of D-glucosamine incorporation into macromolecules was decreased by 60% and the carbohydrate content of membranes (mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid) from clone AD6 was diminished by 40 to 60%.

When the various cell lines were labeled for 1 to 3 h with glucosamine and the acid-soluble pool analyzed, the wild type cells were found to accumulate UDP-N-acetylhexosamine as the major component. In contrast, clones AD6 and AD8 accumulated glucosamine 6-phosphate as the major component. This last finding suggests that there is a block in the N-acetylation of glucosamine 6-phosphate in both mutants. This suggestion is supported by the finding that feeding the mutant cells 10 mM N-acetylglucosamine reverts these mutants (AD6 and AD8) to the absence of iodinatable outer membrane proteins responsible for the defect in adhesion and the altered morphology of the mutant cells.

To establish the biochemical basis of these alterations, we have investigated the synthesis of glycoproteins in the mutant and parent cells. We report here that the mutant clones have an overall decrease in glycoprotein synthesis due to a limitation in the rate of formation of N-acetylglucosamine phosphate. A preliminary report of this work has been presented previously (4).

**EXPERIMENTAL PROCEDURES**

*Materials—* D-[6-3H]Glucosamine, D-[1-14C]Fucose, D-[U-14C]Glucose, UDP-N-acetyl[1-14C]glucosamine, 2-deoxy[6-3H]glucose, and carrier-free Na1, were purchased from New England Nuclear Corp. The reagents for polyacrylamide electrophoresis (sodium dodecyl sulfate, acrylamide, methylenebisacrylamide, Tris, glycine, and Coomasie brilliant blue) were from Bio-Rad.

**Cell Culture and Cell Lines**—BALB/c 3T3 mouse fibroblasts were obtained from Dr. S. Aaronson of the National Institutes of Health. The clones AD6 and AD8 were derived from mutagenized BALB/c 3T3 cells after a specific selection for nonadherent cells described previously (1). Approximately every 2 months a new vial of mutant cells was defrosted from stocks kept in liquid N2. Routinely, the cells were grown in Dulbecco-Vogt's modified Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.), penicillin (50 units/ml), streptomycin (50 g/ml), and glutamine (2 g/l). Wild type and mutant cells grown in monolayer (Falcon tissue culture flasks) were passaged using a solution containing 0.25% trypsin and 0.1% sodium phosphate, pH 7.2, as described previously (1). The cell lines were free of mycoplasma by electron microscopy and thymidine incorporation (5).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—
Cell monolayers were homogenized in a solution containing 2% sodium dodecyl sulfate, 10 mM sodium phosphate (pH 7.0), and 1 mM phenylmethylsulfonyl fluoride; the homogenates were centrifuged to remove denatured protein. The supernatants were mixed with 20% dodecylmaltoside and 8.0 M guanidine hydrochloride, incubated at 37°C for 1 h, and the protein precipitated by addition of 2.5 M sodium acetate. The precipitated protein was collected by centrifugation and dissolved in 1 M sodium hydroxide. The solubilized proteins were applied to a SE-Sephacel column and eluted with a linear gradient of NaCl. The fractions containing glycoproteins were pooled and precipitated with acetone.

The glycoproteins were subjected to electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate and stained with Coomassie brilliant blue. The gels were scanned for radioactivity using a bioimage analyzer. The radioactive bands were cut out and counted for radioactivity. The glycoproteins were further purified by high-performance liquid chromatography on a Sephadex G-75 column.

RESULTS

Cell Surface Glycoproteins of Mouse 3T3 Fibroblasts - To investigate the glycoproteins present in 3T3 cells, the cells were incubated with [3H]glucosamine and the labeled proteins separated by electrophoresis. The proteins labeled with [3H]glucosamine did not correspond to the proteins observed by Coomassie blue staining. For example, myosin, a major cell protein, was not labeled. Thus [3H]glucosamine is not significantly utilized as a glycolytic substrate in 3T3 cells and as reported by others (14) is a reliable precursor for glycoprotein biosynthesis. Fig. 1A shows a typical glycoprotein pattern (○-○) of whole cell extract. The main characteristic is the presence of two large peaks (A and B) of molecular weights over 100,000 and 45,000 to 60,000. These peaks correspond to the two classes, A and B, of glycoproteins reported by Hunt and Brown (15) in 3T3 mouse fibroblasts and also found in many other fibroblast cell lines. The major peak (A) is the main component detected by the periodic acid-Schiff reaction (16, 17) but does not stain as a discrete band with Coomassie blue even in a crude membrane fraction where this glycoprotein is enriched. Therefore, Peak A might be composed of a protein with a high carbohydrate content similar to periodic acid-Schiff 1 or glycoporphin of human erythrocytes (18). Besides these two main groups of glycoproteins, five other glycoproteins with molecular weights over 90,000 were detected (Fig. 1B, ○-○). These glycopeptides are membrane-associated since the radioactive pattern does not change when crude plasma membranes were analyzed (Fig. 2).

To establish which of the membrane-associated glycoproteins are exposed on the outer surface of the cell, duplicate dishes were iodinated with the lactoperoxidase-catalyzed reaction (3). Fig. 1A, ○-○ and B shows the iodinated pattern resolved in the same polyacrylamide slab gel electrophoresis as the [3H]glucosamine homogenate. It is noteworthy that the high molecular weight glycoproteins, with the exception of Peak A, correspond to the iodinated bands 1 to 6 (see Table I for molecular weight). Iodinated band 6 had been resolved from Peak A as a separate [3H]glucosamine protein by increasing the time of electrophoresis. In other experiments band 6 has also been found to bind concanavalin A.1

These data suggest that most of the membrane glycoproteins of 3T3 cells are exposed on the cell surface. This finding is in agreement with the results of Gahmberg (19) who studied the external labeling of human erythrocyte glycoproteins.

Glycoprotein Analysis of Wild Type and Mutant Cell Homogenate - Previously we reported that the major cell surface alterations of clones AD6 and AD8 were loss of iodinated bands 4 and 6 and a decrease in the intensity of band 5; these three polypeptides have now been identified as glycoproteins (Table I). To investigate whether or not the mutants synthesize these polypeptides, mutant and wild type cells were grown at different cell densities in the presence of [3H]glucosamine for 48 h. Whole cell homogenates were solubilized in sodium dodecyl sulfate, electrophoresed, and the radioactive glycoproteins analyzed. Fig. 3 shows the autoradiograms of the polyacrylamide

1 K. Yamada, personal communication.
2 J. Pouyssegur, and I. Pastan, manuscript in preparation.
both mutants; clone AD6 was decreased more than AD8. Cell presented in Table II and Fig. 5. In sparse and confluent cells, growing cells. in the three phases of growth but was more marked in non-

top of the appropriate gels in Fig. 4). This defect was observed incorporation was strongly reduced. The reduction involved all of the glycoproteins as well as the complex polysaccharides not

profile of Experiment B. In both mutants the [*Clglucosamine had been confluent for 2 days. Fig. 4 shows the radioactivity

incorporation into macromolecules was reduced in both mutants. This glycoprotein synthesis and [*C]glucosamine for comparison, is

saccharides at confluency could account for a difference in the

gels corresponding to sparse (A) and subconfluent (B) exponentially growing cells and also to nongrowing cells (C) that had been confluent for 2 days. Fig. 4 shows the radioactivity

uptake decreases (40-fold decrease in the mutants) to reach about the same rate in the three cell lines. It is thought that n-glucosamine is taken up through the n-glucose carrier because glucose and glucosamine compete with each other, both uptake systems are competitively inhibited by cytochalasin B (20), and both are decreased at high cell densities.4 We, therefore, measured the 2-deoxyglucose uptake. No significant difference was found in the rate of uptake of the mutants and parental cells.

Despite the high rate of glucosamine uptake in AD6 and AD8, incorporation of glucosamine into macromolecules was decreased by 80% in both mutants (Fig. 6). We also performed this experiment in cells growing in medium containing glucose and serum. (The conditions were similar to those in Fig. 4.) Although the total uptake of glucosamine by AD6 was slightly lower (30%) than in the wild type under these conditions, the rate of incorporation into macromolecules measured for 6 h was still markedly decreased (70%) (Fig. 7). Furthermore, this rate was not limited by the amount of n-glucosamine taken up by the cells. Indeed, the amount of

4 J. Pouysségur, unpublished data.
FIG. 2. Autoradiograms of sodium dodecyl sulfate gel electrophoresis of plasma membrane (M) and whole cells (WH) labeled with [14C]glucosamine. 3T3 cells were planted at 10^6 cells/100-mm dish and grown for 2 days; then the medium was changed and supplemented with [14C]glucosamine (51 mCi/mM) at 1.25 &mu;Ci/ml. Two days later cells were harvested and crude membrane was prepared as follows. Cells were resuspended in hypotonic buffer (10 mM Tris/HCl, pH 7.5; 10 mM NaCl; and 5 mM MgCl2) and homogenized with a Dounce homogenizer. The nuclei were discarded by low speed centrifugation (800 x g, 10 min) and the supernatant centrifuged for 60 min at 100,000 x g. The 100,000 x g membrane pellet (M) and the whole cell homogenate (WH) were solubilized in sodium dodecyl sulfate and electrophoresed (40 &mu;g of protein) in a 5% polyacrylamide gel. The dried gel was exposed for 5 days to x-ray film.

TABLE I

Molecular weights of main glycoproteins and cell surface iodinated proteins of 3T3 BALB/c

| Peak number referred on Fig. 1 | Molecular weight x 10^3 | Glycopeptide | Iodinated peptide |
|-------------------------------|------------------------|--------------|-------------------|
| 1                             | 290,000                | 290,000      |                   |
| 2                             | 230,000                | 230,000      |                   |
| 2.1                           | 210,000                | 210,000      |                   |
| 3                             | 175,000                | 175,000      |                   |
| 4                             | 140,000                | 140,000      |                   |
| 5                             | 110,000–125,000        | 110,000–125,000 |               |
| A                             | 100,000–110,000        |              |                   |
| 6                             | 92,000                 | 92,000       |                   |
| B                             | 46,000–65,000          |              |                   |

Fig. 3. Autoradiograms showing glycoprotein pattern of AD6, AD8, and wild type cells grown at different cell densities in the presence of [14C]glucosamine. The three cell lines were planted at three cell densities: 5 x 10^4 cells/cm^2 (A), 2.6 x 10^5 cells/cm^2 (B), and 5.2 x 10^5 cells/cm^2 (C). Twenty-four hours later, fresh medium was supplemented with 10 &mu;M [14C]glucosamine (228 mCi/mMol). Glucose concentration was lowered to 1 g/liter. Two days later, the cells were harvested, solubilized in sodium dodecyl sulfate, and electrophoresed in a 5% polyacrylamide gel. To each lane 25 to 30 &mu;g of protein were applied: 6 (clone AD6), 8 (clone AD8), WT (wild type cells). The gels were dried and exposed to an x-ray film for 6 days. The three autoradiograms correspond to sparse cells (A), nearly confluent cells in log phase (B), to non-growing confluent cells (C).

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole cells labeled with [14C]glucosamine. Cells were planted at 2.6 x 10^5/60-mm dish. One day later, the medium was replaced with fresh medium and the glucose concentrations were lowered to 1 g/liter and [14C]glucosamine (228 mCi/mMol) added at 10 &mu;g. Cells were harvested 2 days after addition of [14C]glucosamine, solubilized, and electrophoresed in 5% polyacrylamide slab gels. The same amount of protein was applied in each lane (7 &mu;g). Strips corresponding to wild type cells (WT), AD6 mutant cells (AD6), and AD8 mutant cells (AD8) were cut, frozen, sliced (width 1 mm), and counted.
TABLE II

| Cells | n-Glucosamine | L-Fucose | n-Glucosamine | L-Fucose |
|-------|---------------|---------|---------------|---------|
| Protein (mg/dish) | AD6 | AD8 | WT | AD6 | AD8 | WT | AD6 | AD8 | WT | AD6 | AD8 | WT |
| Acid-insoluble fraction, cpm/mg protein (× 10^4) | 215 | 354 | 385 | 60 | 72 | 91 | 102 | 175 | 306 | 116 | 133 | 146 |
| Mutant/parent (%) | 54 | 89 | 100 | 66 | 79 | 100 | 33 | 57 | 100 | 79 | 91 | 100 |

Cells were planted at 2 × 10^4 and 2 × 10^5 cells/60-mm dishes. One day after planting, fresh medium was supplemented with 2 µCi/ml of either L-[4C]fucose (48 nCi/mm) or n-[4C]glucosamine (51 nCi/mm) for 62 h. Cells were washed three times in NaCl/P and scraped off the dish. For acid-insoluble determinations one aliquot was precipitated with 7% trichloroacetic acid in the presence of 100 µg of bovine serum albumin as a carrier. At the time of harvesting the cells planted at low density were still sparse and those planted at higher density were confluent.

Fig. 5. Autoradiograms showing glycoproteins of AD6, AD8, and wild type cells labeled with either glucosamine or fucose. Exponentially growing cells were labeled with o-[4C]glucosamine for 48 h, then solubilized in sodium dodecyl sulfate, and electrophoresed in a 5% polyacrylamide slab gel. The autoradiograms shown here correspond to cells harvested at confluency; the three left lanes represent o-[4C]glucosamine labeling, the three right lanes o-[4C]fucose labeling.

Analysis of Acid-soluble Pool after n-Glucosamine Pulse

The metabolic fate of exogenous glucosamine in eukaryotic cells is outlined in Fig. 8. Exogenous glucosamine is phosphorylated, N-acetylated, and converted after several steps into three nucleotide sugars: UDP-N-acetylglucosamine, UDP-N-acetyl galactosamine, and CMP-sialic acid. These constitute the immediate precursors of heteropolysaccharides, glycolipids, and glycoproteins (21). The hexosamine pathway in mutant and wild type cells was investigated analyzing the radioactive soluble intermediates derived from glucosamine labeling. Fig. 9 shows the identification of the major radioactive peaks after separation by paper chromatography of the acid-soluble extracts. In the three extracts free n-glucosamine represents less than 5% of the total counts. In wild type extracts, the main peak (75% of the radioactive pool) migrates slower than the major component of mutant extracts. The major component in wild type cells was identified as UDP-N-acetylglucosamine because it had the same Rf as the standard compound and was resistant to hydrolysis by alkaline phosphatase (Fig. 9). Identical experiments with a line of SV40 transformed 3T3 cells also gave rise to a single peak of UDP-N-acetylglucosamine. These findings are in agreement with those of Kornfeld and Ginsburg (22) for HeLa cells. In contrast to the normal and SV40 transformed cells, AD6 and AD8 almost exclusively accumulated glucosamine 6-phosphate; it constituted 90% of the acid-soluble pool. The identification of this compound is based on the fact that treatment of soluble extracts with alkaline phosphatase converts the labeled product to glucosamine (Fig. 9). The other intermediates of the glucosamine pathway, N-acetylglucosamine 6-phosphate and N-acetylglucosamine 1-phosphate, would be converted to N-acetylglucosamine by alkaline phosphatase which has a higher mobility than glucosamine (Fig. 9). Small amounts of N-acetylglucosamine were found when wild type extracts were treated with alkaline phosphatase, indicating that small amounts of N-acetylglucosamine phosphate accumulated in the parent cells.

The acid-soluble pool was also analyzed after 1 h and 3 h in cells growing in the presence of 5 mM glucose and 10% serum. The acid-soluble pool of AD6 was still composed of only glucosamine 6-phosphate, whereas wild type cells accumulated UDP-N-acetylhexosamine and N-acetylglucosamine phosphate (data not shown).

These results suggest strongly that the hexosamine pathway of the mutant cells is impaired by a block in the acetylation of glucosamine 6-phosphate (Step 2 of the scheme, Fig. 8).
FIG. 6. Rates of \( n \)-glucosamine uptake and incorporation into acid-insoluble material of wild type (WT) and mutant cells (AD6, AD8). Uptake in glucose and serum-free medium. Cells were planted at 10<sup>5</sup> cells/60-mm dish, medium changed 48 h later, and the experiment performed 1 day later; in Experiment A, cells were sparse; in Experiment B, duplicate dishes were assayed 2 days later when the cells were confluent but still in log phase. The uptake studies were performed with cells in monolayer, incubated at 37° in a CO<sub>2</sub> incubator. The cells were washed three times with medium without glucose and serum and the reaction was started by addition of \( n \)-[\(^{3}H\)glucosamine (0.5 μM, 2.9 μCi/ml) in 1 ml of medium without glucose and serum. The reaction was stopped by removing the medium and washing four times with glucose and serum-free medium (the four washings took less than 30 s). The cells were then scraped off the dish and counted to determine total uptake (left panel) and uptake into the acid-insoluble fraction (right panel).

Carbohydrate Analysis of AD6 and Wild Type Cell—The carbohydrate composition of AD6 and the parent cells is shown in Table III and Fig. 10. Compared to the wild type, there is a general decrease in all of the carbohydrates analyzed: a 56% decrease in mannose, a 66% decrease in galactose, a 58% decrease in N-acetylglucosamine, a 59% decrease in N-acetylglucosamine, and a 40% decrease in sialic acid. Fucose is also reduced (Fig. 10) but the radioactivity was too low to calculate an exact percentage. Only the glucose content of the mutant cells was increased, but this could have resulted from a higher glycogen content in AD6.

FIG. 7. Rates of \( n \)-glucosamine uptake and incorporation into acid-insoluble fraction. Uptake was done in medium containing glucose and serum. AD6 and wild type (WT) cells were planted at 2 × 10<sup>5</sup> cells/60-mm dish, medium was changed 2 days later, and assays were performed 24 h after medium change. At zero time, \( n \)-[\(^{14}C\)glucosamine (228 mCi/mmol) at 10 μM was added to glucose (5 mM) and serum (10%) containing medium. Total associated counts (---) and acid-insoluble counts (-----) were determined as described under "Experimental Procedures."

DISCUSSION

We have previously shown that many of the iodinatable proteins in the plasma membranes of the adhesion-deficient clones (AD6 and AD8) are altered; some are decreased and others are undetectable. This reduction could have been due to decreased synthesis of membrane proteins or to a conformational change in the cell surface resulting in decreased accessibility of these membrane proteins to lactoperoxidase.

It is generally believed that many of the proteins exposed on the outer surface of cells and detected by covalent labeling with various nonpenetrating probes (23, 24) are glycosylated. This concept has been strengthened by recent studies by Gahmberg (19) in human erythrocytes and Hunt and Brown (15) in mouse L cells. Our data with 3T3 cells showing that the same six polypeptides are labeled by lactoperoxidase iodination and \([\(^{14}C\)glucosamine incorporation are in keeping with this concept and furthermore suggested that one possible ex-
Fig. 9. Chromatograms of acid-soluble pools of wild type (WT), AD8, and AD6 cells after 1-h glucosamine pulse. Cells were pulsed for 1 h with 1-[^3H]glucosamine (10 μCi/ml, 1 μM) in glucose and serum-free medium 48 h after planting (6 × 10⁵/100-mm dish) and 24 h after medium change. Cell homogenates were divided in two parts. One part was extracted with HCl/ethanol, concentrated, and directly chromatographed to analyze the soluble pool (- - -). The second part was incubated with 6 units/ml of alkaline phosphatase (Sigma) in 50 mM Tris/HCl, pH 8.0, for 1 h at room temperature. The hydrolyzed homogenate was then HCl/ethanol extracted and chromatographed (- - -).

The percentages of mannose and galactose were obtained from the area of the radioactive peaks resolved by paper chromatography (see Fig. 10) and derived from analysis of whole cell extracts. The amino sugars were analyzed from crude membrane preparation (see "Experimental Procedures") and represent the average of duplicate experiments.

Fig. 10. Radioactive scans of chromatograms of neutral sugars obtained from [14C]-labeled wild type and AD6 cells. Acid-insoluble extracts of [14C]-glucose-labeled cells (see "Experimental Procedures") were hydrolyzed and neutral sugars isolated according to Ref. 9. The hydrolysates were separated on descending paper chromatography and radioactivity scanned. The same amount of hydrolyzed proteins was chromatographed for both cell lines. (Note that ribose peak derived from total cellular RNA is identical for both extracts.) Sugars were identified with authentic standards run in parallel.

TABLE III
Carbohydrate composition of AD6 and wild type 3T3 cells

| Carbohydrate                  | AD6 mutant | 3T3 wild type | AD6/3T3 |
|-------------------------------|------------|---------------|---------|
| Mannose                       | 14.0       | 35.9          | 44      |
| Galactose                     | 1.6        | 3.7           | 43      |
| N-acetylglucosamine           | 4.6        | 7.6           | 60      |
| N-acetylgalactosamine         |            |               |         |
| Sialic acid                   |            |               |         |
| DMN                           |            |               |         |

The percentages of mannose and galactose were obtained from the area of the radioactive peaks resolved by paper chromatography (see Fig. 10) and derived from analysis of whole cell extracts. The amino sugars were analyzed from crude membrane preparation (see "Experimental Procedures") and represent the average of duplicate experiments.

plation for a decrease in the number of iodinatable surface proteins might be a defect in glycoprotein synthesis.

The data presented in this paper demonstrate that both mutants do have an alteration in glycoprotein synthesis. All of the mutant glycoproteins labeled with [3H]glucosamine and resolved by sodium dodecyl sulfate-polyacrylamide electrophoresis are decreased. This reduction in the labeling of glycoproteins is not due to a selective impairment in the ability to utilize glucosamine since in both clones (a) the incorporation of L-fucose, a precursor which is utilized by a different pathway of glycoprotein synthesis (25), is also decreased and (b) the uptake of glucosamine is not rate-limiting. Indeed, instead of being diminished the uptake of glucosamine is 3- to 4-fold higher in the mutants incubated at a low glucosamine concentration and in glucose-free medium. The reason for this increase is not known but it is not associated with an enhancement of glucose uptake. Instead of this increase in uptake, AD6 and AD8 incorporate considerably less glucosamine into macromolecules (30 to 40% of the control rate). These findings are best explained by an overall decrease in glycoprotein synthesis. However, the evaluation of glycoprotein synthesis using an exogenous precursor can be complicated by an aberrant compartmentation of the carbohydrate precursor pools in the mutants. Such a possible artifact was ruled out by direct carbohydrate analysis of mutant and wild type membranes. In clone AD6 all of the sugars measured (sialic acid, galactose, mannose, N-acetylglucosamine, and N-acetylgalactosamine) were reduced by 40 to 60%.

What is the molecular basis of this defect? At least four molecular levels could be considered for such an alteration: (a) a block in carbohydrate metabolism preventing the synthesis of sugar nucleotides, (b) a block in the transfer of these precursors to the polypeptide backbone, (c) an alteration in the synthesis of the polypeptide chain, or (d) a block in the transport of the glycoprotein to the plasma membrane. The analysis of the low molecular weight carbohydrate pool clearly indicates that both mutants are blocked in an early step of the amino sugar pathway. Instead of accumulating UDP-N-acetylgalactosamines after a [14C]glucose pulse as do wild type cells, the mutants accumulate glucosamine 6-phosphate. This compound is the second intermediate of the hexosamine ana-bolic pathway (21). The fact that we did not detect any acetylated hexosamine intermediates in AD6 and AD8 strongly suggests that the acetylation reaction is the limiting step.

From the outline of the amino sugar pathway (Fig. 8), inhibition of glycoprotein synthesis due to a block of Step 2 should be overcome using N-acetylglucosamine as glycoprotein precursor. When AD6 cells were grown in the presence of...
10 mM N-acetylglucosamine, the morphology, the cell-to-substratum adhesion, and the cell surface protein iodination pattern were restored to normal (2, 4). In addition, after the biochemical reversion, the rate of incorporation of $\alpha$-fucose was restored to normal, whereas the incorporation of $\beta$-glucosamine was still impaired (2).

This finding which shows that the block can be bypassed using N-acetylglucosamine supports our suggestion that the acetylation of glucosamine 6-phosphate is limited in both mutants. Apparently such a defect is sufficient to account for all of the abnormal properties of the mutant cells.

A number of important questions remain unanswered. One is whether the defect in acetylation leads to a reduction in the number of glycoprotein molecules synthesized and inserted into the membrane or to the formation of incompletely glycosylated membrane proteins. Another is related to the role of carbohydrates in membrane structure. One of the major iodinated glycoproteins of the surface of 3T3 cells, band 6 of $M_s = 92,000$, is not detected in the mutants. However, a noniodinated protein with a $M_s$ of 90,000 accumulates in the plasma membranes of AD6 cells which could be precursor of the 92,000 protein. This preliminary observation suggests that incompletely glycosylated or nonglycosylated proteins are still attached to the membrane of AD6 and that the reduction of the number or size of the oligosaccharide chain reduces the exposure of the protein to the aqueous environment of the cell surface or changes the nature of its insertion into the membrane.

The nature of the mutation leading to a limitation in the acetylation of glucosamine 6-phosphate is not yet established. The enzyme glucosamine 6-phosphate N-acetylase is a likely candidate since the formation of acetylated amino sugars is an obligatory step for their interconversion. This enzyme has been described in yeast, bacteria, and different animal tissues (26–28).

Preliminary attempts to assay this activity in fibroblasts using the colorimetric method of Reissig et al. (29) were unsuccessful because of its low sensitivity. We are currently developing a more sensitive assay using a radioactive substrate. It will be of interest to know whether the regulation or the physical characteristics of this enzyme are modified in AD6. It is clear that the mutants are “leaky” in the acetylation reaction since the glycosylation process is not totally abolished. A further reduction in glycosilation might be lethal for normal cells which require some attachment to substratum to grow. AD6 which displays a more marked alteration than AD8 is vacuolated and grows poorly in low serum or if the medium is not changed regularly.

We have emphasized in our study the alteration of glycoproteins in the mutant cells. Glycoproteins constitute one of the major carbohydrate containing components of the cell surface. However, the early position of the block in the mutant cells should affect the carbohydrate composition and the synthesis of glycolipids and mucopolysaccharides. An investigation of these specific carbohydrate-containing components is needed to assess to what extent their biosynthesis is altered.

Other mutants with defective cell surface carbohydrates have already been reported. Morphological mutants of Neurospora crassa were found to have an altered carbohydrate composition in their cell wall (30). Further characterization of these mutants demonstrated the existence of mutations in the glycolytic pathway leading to the abnormal surface carbohydrate composition (31, 32). More recently, mammalian cell mutants have been isolated based on their resistance to the toxicity of some plant lectins. Some of these have an altered glycoprotein pattern due to a defect in UDP-$N$-acetylglucosamine—glycoprotein-$N$-acetylglucosaminyltransferase (33, 34). These mutants also appear to have an altered morphology and a decreased adhesiveness to substratum. Clones AD6 and AD8 provide another example of cell surface carbohydrate mutants of eukaryotic cells. Such cell lines should be very useful for the studies of membrane structure and to evaluate the role of cell surface proteins and carbohydrates in biological processes.

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