Effect of Growth on the Glycoproteins from the Surface of Control and Rous Sarcoma Virus Transformed Hamster Cells*

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ABSTRACT: Recent studies have indicated that cell surface components may vary with the state of growth of the cell. Because of this, the effect of cell growth on the surface glycoproteins of control and Rous sarcoma virus transformed BHK_{21}/C_{13} cells was investigated. The glycopeptides from the surface of cells grown under various conditions in the presence of radioactive L-fucose were compared by gel filtration. It was found that the more rapidly migrating glycopeptides constituted a larger proportion of the glycopeptides from the surface of rapidly growing cells than from the surface of slowly growing or plateau cells. This was the case for both control and Rous sarcoma virus transformed cells.

Previously, we have shown by gel filtration that glycopeptides from BHK_{21}/C_{13} cells transformed with Rous sarcoma virus (RSV)[1] were enriched in a population of more rapidly migrating glycopeptides when compared to those of control BHK_{21}/C_{13} cells (Buck *et al.*, 1970). Small amounts of this material were detected, however, in all of the control cells which were examined.

Several observations suggested that the glycoproteins of a cell may change with the state of growth of the cell. Meezan *et al.* (1969) noted differences in the elution profile of glycopeptides from growing and confluent 3T3 cells. Changes have been noted also in the sialic acid content of L cells relating to the growth of the cells in culture (Glick *et al.*, 1970) and in the individual monosaccharides of KB cells during the mitotic cycle (Glick *et al.*, 1971). The apparent discrepancy between the results of Hakomori and Murokami (1968) who reported a decrease in hematosides in transformed cells and Mora *et al.* (1969) who failed to note this decrease but found a decrease in the more complex glycolipids could also be explained by differences in the state of growth of the cells.

For these reasons, we have compared the glycopeptides from control BHK_{21}/C_{13} cells and from these same cells transformed with the Bryan strain of RSV (C_{13}/B_4) exposed to radioactive L-fucose during various stages of growth. In both cases, quantitative changes in the glycopeptides from the surface of the cells were seen when material from rapidly growing (approximating a logarithmic phase of growth) and slowly growing (plateau) cells were compared.

Materials and Methods

Cell Culture. Cell lines and the details of the methods for culturing have been described previously (Buck *et al.*, 1970). The cultures were periodically examined for *Mycoplasma* and were negative. All cells were grown in disposable roller bottles having a growth area of 654.51 cm^2 (Bellco Glass Co., Vineland, N. J.) which were rotated on a Bellco roller apparatus. Each bottle was inoculated with 2×10^7 cells. Growth medium consisted of Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% tryptose phosphate broth (Difco), 5% fetal calf serum (Microbiological Associates, Rockville, Md.), penicillin (50 units/ml), streptomycin (100 μg/ml), and anti-PPLO agent (Grand Island Biological Co.). Fresh medium (50 ml) was added to each bottle daily until the total volume of medium reached 200 ml/bottle. At this time all old medium was discarded and 50 ml of fresh medium was added. Daily addition of 50 ml of medium was then continued. Cells were grown in the presence of L-fucose-1-^{14}C (0.5 mCi/mmole, Calbiochem, Los Angeles, Calif.) or L-fucose-*t* (generally labeled, 4 Ci/mmole, New England Nuclear Corp., Boston, Mass.) as described previously (Buck *et al.*, 1970).

Trypsinization. Monolayers of cells were washed with TBS and digested with three-times crystallized trypsin (Worthington Biochemicals, Freehold, N. J.) for 15 min at 37°. Soybean trypsin inhibitor (Worthington Biochemicals) was then added. The supernatant solution remaining after removal of cells by centrifugation will be referred to as the "trypsinate." The details and further processing of the trypsinate have been described (Buck *et al.*, 1970).

Preparation of the Surface Membranes. Cells were washed as described previously (Buck *et al.*, 1970) and surface membranes were isolated by the zinc ion procedure (Warren and Glick, 1969).

Pronase Digestion. All samples to be compared were pooled prior to digestion with pronase (Calbiochem, Los Angeles, Calif.). Digestion was carried out for 5 days at 37° in the presence of 1 mg/ml of pronase. Fresh pronase was added daily (Buck *et al.*, 1970).

Gel Filtration. All fractionations were performed on a Sephadex G-50 column.

Preparation of Samples for Scintillation Counting. Fractions were dissolved directly in Aquasol (New England Nuclear Corp.) and counted in a Packard TriCarb liquid scintillation counter.

Results

Growth of BHK_{21}/C_{13} and C_{13}/B_4 Cells in Roller Bottles. A

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[1] Abbreviations used are: RSV, Rous sarcoma virus; SV40, Simian virus 40; log, cells in a state of rapid or logarithmic growth; plat, slow-growing cells that have reached a plateau phase of growth; SDS, sodium dodecyl sulfate; TBS, buffer solution containing 0.15 M NaCl–0.02 M Tris-HCl (pH 7.5).

TABLE I: Number of Cells per Culture at Time of Harvesting.[a]

Cell Line	Phase of Growth	Cells/Culture	Cells/cm² of Culture Surface
BHK_{21}/C_{13}	Logarithmic	9.6×10^7	1.5×10^5
BHK_{21}/C_{13}	Plateau	2.6×10^8	3.9×10^5
C_{13}/B_4	Logarithmic	9.0×10^7	1.4×10^5
C_{13}/B_4	Plateau	2.5×10^8	3.8×10^5

[a] Cells were harvested as described (Materials and Methods and Figure 2). Cells were counted using a Coulter counter.

typical growth curve of cells grown in roller bottles is shown in Figure 1. The cells grew most rapidly during the first 3 days in culture and less rapidly, if at all, from days 4 to 7. For subsequent experiments, material from rapidly growing cells (log) was obtained after exposing the cells to radioactive L-fucose during the first 72-hr growth. Material from slowly growing cells or cells which have apparently reached a plateau phase of growth was obtained after exposing the cells to radioactive L-fucose from day 4 to day 7 in culture.

The number of cells per culture in bottles used for the experiments described below is shown in Table I. The cells from both C_{13}/B_4 and BHK_{21}/C_{13} cultures were harvested at similar cell densities.

Cochromatography on Sephadex G-50 of Pronase Digests of Trypsinates from Rapidly Growing and Plateau Cells. Cells were grown in the presence of either L-fucose-^{14}C or L-fucose-*t* for the first 3-days culture (rapidly growing cells) or from day 4 to 7 (slowly growing cells). The cells were washed and digested with trypsin as described in Materials and Methods. Samples of the appropriate trypsinates were pooled and digested with pronase and subsequently chromatographed on Sephadex G-50. The results of cochromatographing the trypsinates from rapidly growing (log) and slowly growing (plat) BHK_{21}/C_{13} cells are shown in Figure 2a. The material was eluted from the column in three general regions. The first region, eluting just after the Blue Dextran marker, consisted of material eluting in a rather broad area between fractions 20 and 35. The second region consisted of that material eluting as a more well-defined peak between fractions 36 and 50. Finally, the third region, apparently lower molecular weight material, eluted between fractions 50 and 85. Of these three general areas, trypsinates from the more rapidly growing cells appeared to be enriched in material migrating in the first area relative to that in the second when compared to material from the plateau cells. It is difficult to attach any significance to the differences seen in the third area of the eluate as the differences noted were not readily reproducible.

Figure 2b shows the results of a similar experiment in which pronase digests of trypsinates from rapidly growing and plateau C_{13}/B_4 cells were compared. Again the material from the rapidly growing cultures was enriched in the glycopeptides eluted earlier from the column. Unlike the profiles shown in Figure 2a, however, glycopeptides from C_{13}/B_4 cells were eluted as distinct peaks in both the first and second region of the profile. There was consistently more material seen in the third region of the profile (fractions 50–80) of plateau C_{13}/B_4 cells than in that of rapidly growing cells.

Cochromatography on Sephadex G-50 of Pronase Digests of

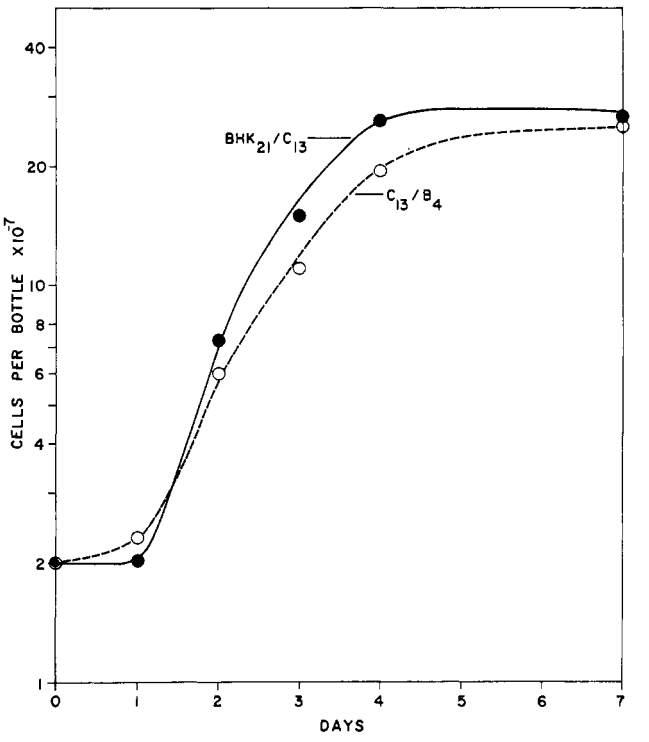

FIGURE 1: Growth of BHK_{21}/C_{13} and C_{13}/B_4 cells in roller bottles. 2.0×10^7 cells in 50 ml of medium were added to each bottle: every 24 hr, 50 ml of fresh medium was added to each bottle as described in Materials and Methods. At the designated times, monolayers were washed with TBS and 10 ml of TBS containing 1 mg/ml of three-times-crystallized trypsin was added to each bottle. After 15 min at 37°, the cells were dispersed and counted in a Coulter counter. BHK_{21}/C_{13} (●); C_{13}/B_4 (○).

Surface Membranes from Rapidly Growing and Plateau Cells. In order to determine if the profiles observed for the trypsinates were representative of the complete cell surface, the purified surface membranes from the cells described in Figure 2 were digested with pronase and cochromatographed. The profile obtained for the surface membranes from rapidly growing and plateau C_{13}/B_4 cells is shown in Figure 3a. The membranes from the rapidly growing cells (log) contained a mixture of glycopeptides which could be resolved into two general areas corresponding to the first two areas seen in the profiles of pronase-digested trypsinates (Figure 2b). The membranes from plateau cells (plat) contained considerably less material eluting in the first region of the profile.

Figure 3b shows the results of cochromatographing pronase digests of surface membranes from rapidly growing C_{13}/B_4 and BHK_{21}/C_{13} cells. Again, the two distinct regions were seen in the profile of glycopeptides from the C_{13}/B_4 cells. The material from the surface membranes of BHK_{21}/C_{13} cells contained some glycopeptides which migrated in the first region of the column, but, as was seen with the trypsinates from the same cells (Figure 2a), the material in this region did not resolve into a distinct peak.

When surface membranes from plateau C_{13}/B_4 and BHK_{21}/C_{13} cells were digested with pronase and cochromatographed, the results shown in Figure 3c were obtained. The major portion of the glycopeptides from the surface membranes of both cell lines migrated in an area corresponding to the second region of the profile seen for the surface membranes of rapidly growing C_{13}/B_4 cells (Figure 3a). There was little material migrating in the first region of the column when compared to

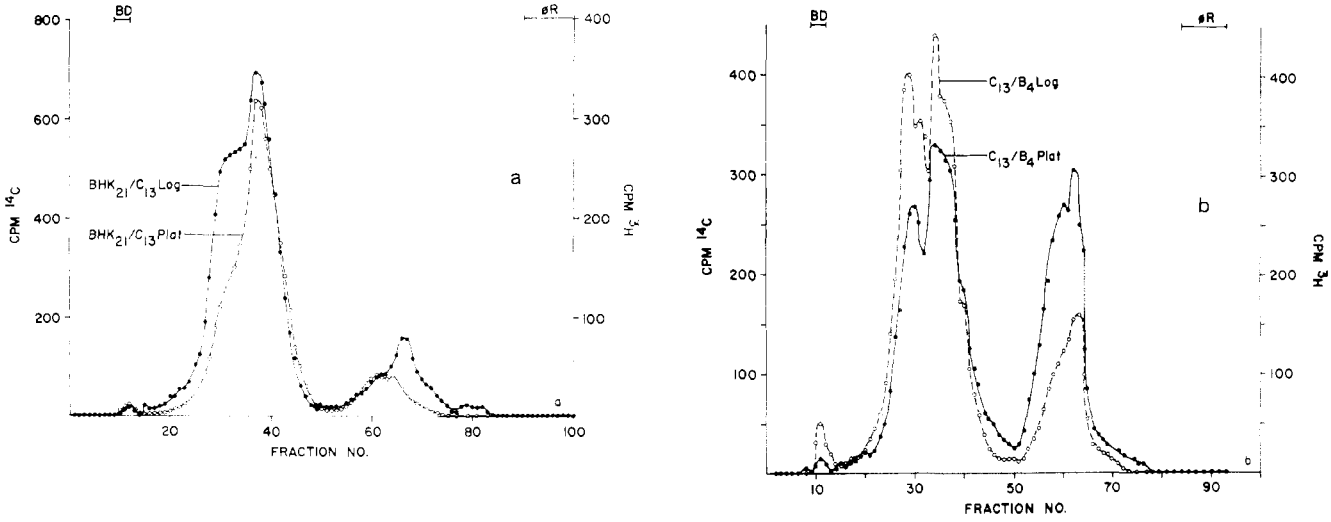

FIGURE 2: Cochromatography on Sephadex G-50 of pronase-digested trypsinates from cells in logarithmic and plateau phases of growth. Cells were grown as described in Materials and Methods. Cells were grown in the presence of 100 μCi of L-fucose-*t* or 10 μCi of L-fucose-^{14}C. The log cells were exposed to radioactive L-fucose during the first 3 days in culture, and the plat cells were exposed to radioactive L-fucose during the last 3 days in culture. Cells were harvested and trypsinates were mixed and digested with pronase (1 mg/ml) for 5 days prior to chromatographing on a column of Sephadex G-50 (0.8 $\times$ 100 cm). The column was eluted with 0.1 M Tris-acetate buffer (pH 9.0), 0.1% SDS, 0.01% EDTA, and 0.1% mercaptoethanol. Blue Dextran and phenol red were present as high and low molecular weight markers, respectively. Samples of 0.7 ml were collected at a flow rate of 13 ml/hr. (a) Glycopeptides from BHK_{21}/C_{13} cells in log phase of growth in the presence of L-fucose-*t* (●); glycopeptides from BHK_{21}/C_{13} cells in plat phase of growth in the presence of L-fucose-^{14}C (○). (b) Glycopeptides from C_{13}/B_4 cells in log phase of growth in the presence of L-fucose-^{14}C (○); glycopeptides from C_{13}/B_4 cells in plat phase of growth in the presence of L-fucose-*t* (●). BD, fractions in which Blue Dextran was eluted; ϕR, fractions in which phenol red was eluted.

the profiles of Figure 3a,b. In every case, when glycopeptides from surface membranes were chromatographed, very little of the low molecular weight material migrating just ahead of the phenol red marker (fractions 50–80) was seen.

Discussion

Several investigators have studied the effects of cell-to-cell contact on various aspects of cell movement (Abercrombie, 1970) and metabolism (Todaro *et al.*, 1967). Cunningham and Pardee (1969) found that the addition of serum to confluent monolayers of 3T3 cells resulted in increased transport of uridine-^{14}C and increased phospholipid synthesis within minutes after the addition of serum. The increased uridine uptake and phospholipid synthesis were not seen when serum was added to growing 3T3 cells or Polyoma virus transformed 3T3 cells. From these observations they suggested that membrane changes were involved in contact inhibition. This suggestion is consistent with the observations of Meezan *et al.* (1969) in which differences were seen in the elution profile of glycopeptides from growing and confluent 3T3 cells maintained in the presence of radioactive D-glucosamine. Although BHK_{21}/C_{13} cells are not as sensitive to density-dependent inhibition of growth (Rubin and Stoker, 1967) as 3T3 cells (Stoker and Abel, 1962) the results reported here add further to the idea that biochemical changes on the surface of cells are associated with cell contact or growth.

We have previously reported that the surface membranes of hamster cells transformed with RNA-containing tumor viruses are enriched in a group of apparently high molecular weight glycopeptides when compared to the surface membranes of control cells (Buck *et al.*, 1970). We also noted, however, that glycopeptides from control BHK_{21}/C_{13} cells contained some of the high molecular weight glycopeptides characteristic of the transformed cells. In order to see if this might be a growth related phenomenon, the glycopeptides of cells in logarithmic and plateau phases of growth were compared after growth in the presence of radioactive L-fucose. The glycopeptides obtained from the trypsinates of the rapidly growing BHK_{21}/C_{13} cells were enriched in the higher molecular weight glycopeptides when compared to the glycopeptides from plateau BHK_{21}/C_{13} cells. Since fresh medium was added daily to the cultures, this change could not be due to medium deficiencies. When the glycopeptides from the surface membranes of these same cells were compared following digestion with pronase, it could be seen that the membranes from the plateau cells contained only small amounts of the early-eluting glycopeptides, showing that the deficiency of these glycopeptides in the trypsinate accurately reflected the surface membrane. This same result was obtained whether the cells were grown in the presence of L-fucose-^{14}C or L-fucose-*t*. The amount of the apparently higher molecular weight glycopeptides found on the surface of the rapidly growing normal cells was not increased by harvesting the cells at lower cell densities (0.5×10^5 cells/cm^2) than used for the experiments reported here (Table I).

The relative amount of the early-eluting glycopeptides was also decreased as RSV-transformed cells entered the plateau phase of growth. This decrease was reflected in both the trypsinate and the surface membrane preparations from these cells. Regardless of the state of growth, however, trypsinates from transformed cells were always more enriched in the higher molecular weight glycopeptides than trypsinates from the control cells harvested at similar cell densities. It should be pointed out that this relative enrichment could reflect increased synthesis of the early-eluting glycopeptides or a decreased synthesis, or increased degradation, of the material migrating just after these more rapidly migrating glycopeptides. Any one or all of these processes could result in the relative changes in glycopeptide elution profiles reported here and earlier (Buck *et al.*, 1970). Further comparisons of the apparently higher molecular weight glycopeptides from

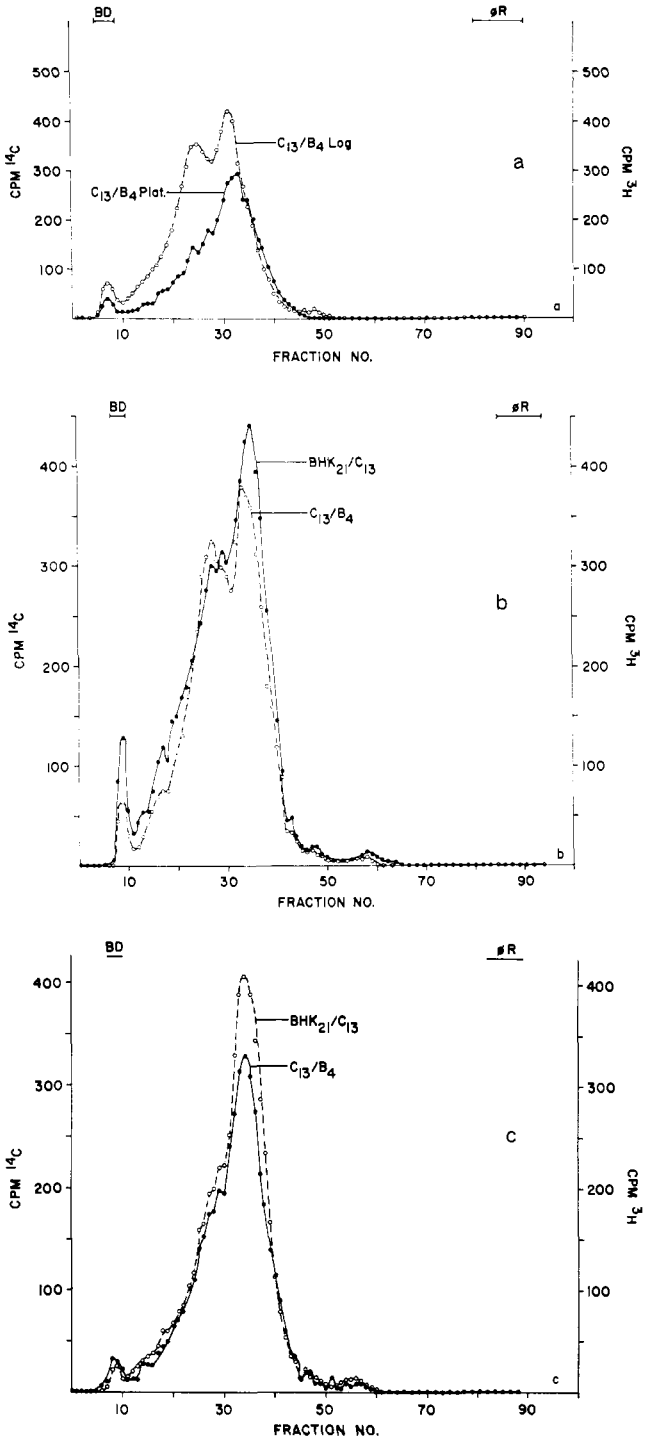

FIGURE 3: Cochromatography on Sephadex G-50 of pronase-digested surface membranes from cells in logarithmic or plateau phase of growth. Cells were grown in the presence of radioactive L-fucose and surface membranes isolated and digested with pronase as described in Materials and Methods and Figure 2. Glycopeptides were chromatographed as stated in Figure 2. (a) Glycopeptides from surface membranes of C_{13}/B_4 cells in log phase of growth in the presence of L-fucose-^{14}C (○); glycopeptides from surface membranes of C_{13}/B_4 cells in plat phase of growth in the presence of L-fucose-*t* (●). (b) Glycopeptides from surface membranes of C_{13}/B_4 cells in log phase of growth in the presence of L-fucose-^{14}C (○); glycopeptides from surface membranes of BHK_{21}/C_{13} cells in log phase of growth in the presence of L-fucose-*t* (●). (c) Glycopeptides from the surface membranes of C_{13}/B_4 cells in plat phase of growth in the presence of L-fucose-*t* (●); glycopeptides from the surface membranes of BHK_{21}/C_{13} cells in plat phase of growth in the presence of L-fucose-^{14}C (○). BD, fractions in which Blue Dextran was eluted; φR, fractions in which phenol red was eluted.

control and transformed cells will have to await more detailed chemical characterization of this material.

The material eluting in the later fractions, just prior to the phenol red marker, may prove to be of interest, but the fact that short peptides remaining after pronase digestion were eluted in this region (Buck *et al.*, 1970) may account for some of the heterogeniety seen in this area of the profile.

The following summary of previous observations using these same cell lines suggest that the results reported here reflect alterations in glycoproteins of the cell surface during growth, and not procedural artifacts (Buck *et al.*, 1970). Trypsin digestion resulted in the removal of about 23% of the total sialic acid from both BHK_{21}/C_{13} and C_{13}/B_4 cells. Following the growth of cells in radioactive L-fucose, 90% of the radioactivity was recovered as fucose. Similar chromatographic results were obtained whether the cells were grown in the presence of radioactive L-fucose or D-glucosamine. Digestion of trypsinates or surface membranes with pronase was uniform as determined by the absence of radioactive amino acids from the first 60 fractions of the elution profile from Sephadex G-50. Finally, the specific activity of radioactive L-fucose was similar in both cell lines, and any fluctuation in specific activity did not alter the chromatographic results.

Previously, it has been reported that treatment of nontransformed cells with proteolytic enzymes would render them as susceptible to agglutination by wheat-germ agglutinin (Burger, 1969) or concanavalin A (Inbar and Sachs, 1969) as virus-transformed cells. Ben-Bassat *et al.* (1970) have reported that the exposure of concanavalin A reactive sites on cells following SV40 infection requires at least one cell division. Recently Häyry and Defendi (1970) have found that surface antigens similar to those found following SV40 transformation could be exposed on the surface of control cells by trypsin digestion. These observations and those reported here indicate that under certain conditions normal cells may transiently acquire some of the characteristics of transformed cells. Because of this, it is imperative that comparisons be made only of membranes from control and transformed cells in nearly identical phase of growth.

Two points should be stressed. First, the glycoproteins of the surface membranes from transformed and control BHK_{21}/C_{13} cells change with the state of growth of the cell. Second, regardless of the state of growth of the cells, the surface membranes from RSV-transformed BHK_{21}/C_{13} cells are enriched in the more rapidly migrating glycopeptides when compared to control BHK_{21}/C_{13} cells.

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Stereospecific Decarboxylation of Specifically Labeled Carboxyl-^{14}C Aminomalonic Acids by L-Aspartate β-Decarboxylase*

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ABSTRACT: Studies with specifically labeled carboxyl-^{14}C aminomalonic acids obtained by oxidation of L-serine-*1*-^{14}C and L-serine-*3*-^{14}C showed that L-aspartate β-decarboxylase catalyzes the removal of the carboxyl group of aminomalonic acid that is in the position analogous to the CH_2COOH group of L-aspartate rather than the α-carboxyl group. The findings constitute experimental proof of Ogston's hypothesis concerning aminomalonic acid.

We have previously reported that L-aspartate β-decarboxylase decarboxylates animomalonic acid to glycine and that this reaction is stereospecific (Palekar *et al.*, 1970). Thus, we found that enzymatic decarboxylation of aminomalonic acid in the presence of H_2O-*t* gave (*S*)-Gly-*t*; in the corresponding nonenzymatic reaction (*SR*)-Gly-*t* was formed. (*S*)-Gly-*t* is also formed in the reaction catalyzed by serine hydroxymethylase (Wellner, 1970). Thus, in the enzyme-catalyzed decarboxylation of aminomalonic acid, the carboxyl group that is lost may be considered to be in a position on the enzyme that is analogous to the CH_2OH group of L-serine and to the CH_2COOH group of L-aspartate. However, one cannot exclude the possibility that the carboxyl group of aminomalonic acid that is lost is in the position analogous to to that of the α-carboxyl group of aspartate and therefore that addition of a proton is accompanied by inversion of configuration. In the present studies, which were carried out in order to answer this question, we have used specifically labeled carboxyl-^{14}C aminomalonic acids prepared by oxidation of L-serine-*1*-^{14}C and L-serine-*3*-^{14}C. In this paper the carboxyl-labeled aminomalonic acid obtained by oxidation of L-serine-*1*-^{14}C will be referred to as aminomalonate-*1*-^{14}C; the product obtained by oxidation of L-serine-*3*-^{14}C will be designated as aminomalonate-*3*-^{14}C.

Materials

L-Aspartate β-decarboxylase was isolated from *Alcaligenes faecalis*, strain N (ATCC 25094), as described by Tate and Meister (1968). Hog kidney D-amino acid oxidase was kindly supplied by Dr. Daniel Wellner. L-Serine-*3*-^{14}C was obtained from Amersham Searle, and DL-serine-*1*-^{14}C was obtained from the New England Nuclear Corp. L-Serine-*1*-^{14}C was obtained from the racemate by selective destruction of the D isomer by treatment with D-amino acid oxidase followed by adsorption of the L-serine on Dowex 50 (H^+) followed by elution with 2 M ammonium hydroxide.

Methods

The reactions were carried out in stoppered tubes in which a polyethylene well containing 0.1 ml of 1 N potassium hydroxide was suspended. The reactions were stopped by injecting 1 ml of ethanol into the tubes, which were then placed on a shaker for 90 min. The potassium hydroxide was mixed with 10 ml of liquid scintillation medium (Bray, 1960) for the determination of $^{14}CO_2$. The denatured protein was removed by centrifugation and the supernatant solution was evaporated to dryness *in vacuo*. The residue was dissolved in 0.2 ml of water and the pH was adjusted to 6 by addition of sodium hydroxide. This solution was applied to the top of a column of Dowex 1 (acetate form); the glycine was eluted with 1 ml of water and the eluate was evaporated to dryness. The residue was dissolved in 0.4 ml of water. Aliquots of this solution were taken for the determination of glycine-^{14}C by scintilla-

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