Release of Macromolecules from BALB/c Mouse Cell Lines Treated with Chelating Agents†

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ABSTRACT: Balb/c 3T3 mouse cells and SV40-transformed 3T3 cells (SVT2) were treated with Mg^{2+}- and/or Ca^{2+}-specific chelating agents to determine what types of macromolecules were extracted from various cell compartments. Ca^{2+}-dependent functions were found to be important in establishing cell-to-dish attachments, while Mg^{2+}-dependent functions were found to be more important in maintaining the impermeability of the surface membrane. Neither cation was critically important in cell-to-cell adhesion in confluent cultures. Protein and RNA which were extracted from SVT2 cells by ethylenediaminetetraacetic acid (EDTA) or ethylenebis-(oxyethylenenitrilo)tetraacetic acid (EGTA) treatment apparently leaked from the cell cytoplasm. The ethylenediaminetetraacetic acid extracted protein coelectrophoresed in polyacrylamide gels (containing sodium dodecyl sulfate) with soluble cytoplasmic proteins. The ethylenediaminetetraacetic acid extracted RNA was characterized as tRNA by its sedimentation properties and methylated base composition. Leakage probably occurred from a large number of cells, and not from the small proportion of nonviable cells which were stained with the vital dye Trypan Blue. Considerably more RNA and protein were extracted from 3T3 than from SVT2 cells. In contrast, "glycoproteins" were extracted slowly as a function of time from cellular membranes from both 3T3 and SVT2 cells. During short treatment periods with ethylenebis(oxyethylenenitrilo)tetraacetic acid, which were sufficient to detach cells, membrane "glycoproteins" were conserved with the intact cells. Three classes of "glycoproteins" were identified in cultures of normal and transformed cells; these included "glycoproteins" which were associated with the cell, those that remained attached to the dish after removal with chelating agents, and those that were secreted into the medium. The relative amounts of these "glycoproteins" were determined as a function of cell growth in 3T3, SVT2, and concanavalin A revertant (Culp, L. A., and Black, P. H. (1972), *J. Virology* (in press)) variant cells isolated from populations of SVT2 cells. 3T3 and revertant cells, both of which are contact inhibited, deposited much more "glycoprotein(s)" onto the dish and secreted more into the medium than SVT2 cells. Secretion of "glycoprotein(s)" into the medium continued while deposition of "glycoprotein(s)" onto the dish terminated, after growth inhibition of contact-inhibited cell lines.

Evidence suggests that viral transformation of mammalian cells results in loss of contact inhibition of growth and acquisition of neoplastic potential; these changes may result from altered chemical and/or architectural properties of the surfaces of transformed cells (Culp *et al.*, 1971; McNutt *et al.*, 1971; Culp and Black, 1972; Black *et al.*, 1971). Studies of the properties of revertant cells (contact-inhibited variants of virus-transformed cells) further support the hypothesis that surface membrane components are responsible for mediating contact inhibition of growth (Culp *et al.*, 1971; Culp and Black, 1972).

The isolation and identification of surface components, particularly glycoproteins, have been based primarily on the use of trypsin to release cells from dishes and to remove surface glycopeptides, but little attention has been paid to (1) the destruction of the protein backbones of glycoproteins during the isolation procedure and (2) the leakage of macromolecules from the cytoplasm as a result of membrane damage which may occur during treatment (Snow and Allen, 1970). The use of trypsin may be efficacious if the investigator is only interested in the polysaccharide portion of glycoproteins, provided measures are taken to prevent leakage from the intracellular matrix.

Our studies attempted to find methods whereby mammalian cells in culture can be removed from culture dishes with minimum surface membrane damage and minimum leakage of macromolecules from the cytoplasmic space. We have used as criteria of damage: (1) leakage of RNA, which is present in very high concentrations in the cytoplasm of the cell; and (2) the ability of cells to concentrate the vital dye Trypan Blue (Paul, 1965). We have placed special emphasis on agents which chelate the divalent cations Mg^{2+} and/or Ca^{2+} and have avoided glycolytic and proteolytic agents which hydrolyze the surface membrane components which are of particular interest. The distribution of three classes of macromolecules—ribonucleic acid, protein, and "glycoproteins"—were examined in some detail to determine whether particular classes of macromolecules were "stripped" from cell surfaces or were "leaked" from the cytoplasm. Some information has been reported by Snow and Allen (1970) and Codington *et al.* (1970) on release of macromolecules from cells subsequent to EDTA or trypsin treatments.

Materials and Methods

Cells. BALB/c 3T3 cells (clone A31), SV40-transformed 3T3 cells (SVT2 cells), and the concanavalin A selected revertant[1] of SVT2 cells (revertant clone 84) have been described (Culp and Black, 1972). These cell lines were only

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[1] Revertant denotes a contact-inhibited variant of SVT2 cells selected by concanavalin A treatment (Culp and Black, 1972).

used between their 5th and 15th passages in our laboratory. The term "subconfluent" will indicate that cells are in their exponential phase of growth and that approximately 40–60% of the surface of the dish is covered with cells. The term "confluent" indicates that 100% of the surface of the dish is covered with cells. Confluent 3T3 and revertant cells, which are contact inhibited, no longer divide; SVT2 cells, however, which are not contact inhibited, continue to divide after confluence has been achieved and produce multilayered colonies.

Contamination Controls. These cells were routinely assayed for and found to be free of *Mycoplasma* contamination as determined by the culture assay of Madoff (1960), and a radiolabel assay performed as follows. Sparsely seeded cell cultures in 60-mm Petri dishes were radiolabeled with [^{3}H]-thymidine (18 Ci/millimole, 5 μCi/ml) for a 4-hr period. The medium was then decanted and the dishes were washed twice with phosphate-buffered saline (pH 7.4) (PBS),[2] fixed twice with cold 5% trichloroacetic acid (w/v), and washed five times with cold 95% ethanol. Autoradiograms of the fixed cells were prepared by layering the dishes with Kodak AR10 film and setting at room temperature for 3 days before the film was developed and fixed. *Mycoplasma*-infected cells yielded patterns of silver grains over the cytoplasm of cells with a paucity of silver grains over the nucleus (probably due to the preferential use of thymidine by *Mycoplasma* in the cytoplasm where they grow (Nardone *et al.*, 1965)). *Mycoplasma*-free cultures displayed heavy silver grain patterns only over the nuclei of cells.

BALB/c 3T3 and SVT2 cells were found to be free of infectious mouse leukemia virus as assayed by the Bassin procedure (Bassin *et al.*, 1971).

Growth and Radiolabeling of Cells. Cells were grown in Eagle's minimal essential medium supplemented with a four-fold concentration of vitamins and amino acids, 10% fetal calf serum, penicillin, and streptomycin (MEM×4). They were maintained at 37° in a humidified atmosphere of 5% CO_2 in air. In experiments to be described, cells were dispersed with trypsin and seeded into 29-oz glass bottles; 24 hr later when the cultures were approximately 5–10% confluent, the medium was changed to remove residual trypsin. Forty milliliters of fresh medium was added containing a radiolabeled precursor: 25 μCi of [5-^{3}H]uridine (specific activity 25 Ci/mmole) to radiolabel RNA, 100 μCi of [5-^{3}H]-thymidine (specific activity 17 Ci/mmole) to radiolabel DNA, or 100 μCi of [1-^{3}H]glucosamine hydrochloride (specific activity 2.6 Ci/mmole) to radiolabel "glycoproteins."[3] In order to radiolabel proteins, cells were exposed to 40 ml of leucine-deficient MEM×4, supplemented with 5.0 mg/l. of leucine and 25 μCi of [4,5-^{3}H]leucine (specific activity 35–45 Ci/mmole). Cells were incubated in the presence of the radiolabel for 48 hr until they were 40–60% confluent in order to study macromolecule synthesis and release in subconfluent cultures. To study synthesis and release of macromolecules from cells of confluent cultures, medium containing isotope was added approximately 24 hr before the cells became completely confluent; incubation in radioactive medium was carried out for 48 hr. Thus, synthesis of molecules was being measured both during the period when cell-to-cell contacts were being established and for a period of time after confluence of the cells had been achieved.

To determine the methylated base compositions in RNAs, SVT2 cells were grown for 48 hr, in methionine-deficient MEM×4, supplemented with 5.0 mg/l. of [*methyl*-^{3}H]-methylmethionine (specific activity 250 μCi/μmole) and 2 mg/ml of sodium formate (Culp and Black, 1971).

Assay for Released Materials. To quantitate the release of cells from glass surfaces to which they are attached and to assay the release of macromolecules from whole cells, the following procedure was used. After growth in the presence of the radioactive precursor, medium was decanted from the bottle, and the cell layer was washed twice with PBS containing 100 mg/l. of $CaCl_2$ and 100 mg/l. of $MgSO_4 \cdot 7H_2O$. Fifty milliliters of chelating agent in PBS (sodium pyrophosphate which chelates Mg^{2+} and Ca^{2+}, EDTA which chelates Mg^{2+} and Ca^{2+}; or EGTA which specifically chelates Ca^{2+} and not Mg^{2+} (Blaustein and Wiesmann, 1970), all at a final concentration of 0.5 mM) was added to the culture, and the culture was shaken gently in a reciprocal shaker at 37°. Aliquots were removed periodically, a portion of which was precipitated by 10% trichloroacetic acid after the addition of 100 μg of carrier bovine serum albumin to determine the total amount of radioactive macromolecule per volume of extract at a particular time period; the remainder of the extract was filtered through a 0.45 μ Millipore[4] membrane to filter out whole cells and an equal-volume aliquot of the filtrate was trichloroacetic acid precipitated (in the presence of 100 μg of carrier bovine serum albumin) to determine the fraction of macromolecule which is released from cells. The percentage of released macromolecules was determined by dividing the amount of trichloroacetic acid precipitable radioactivity in 0.1 ml of filtrate by the amount[5] of trichloroacetic acid precipitable radioactivity in 0.1 ml of whole extract and multiplying by 100. The standard deviation in measuring the radioactive content of duplicate aliquots from one culture in these experiments was ±4%. The standard deviation in measuring the percentages of filterable radioactivity between different culture bottles grown and treated under the same conditions was approximately ±10%.

An additional method for the assay of released macromolecules was attempted. Cell suspensions were centrifuged at 600*g* for 10 min to pellet whole cells; aliquots of the supernatant fluids were then precipitated with trichloroacetic acid. There was generally 10–20% more protein, RNA, and glycoprotein in supernatant fluids after centrifugation than in filtrates after Millipore filtration. At least two reasons may explain this discrepancy: (1) centrifugation may not pellet intact, dead cells which are less dense than viable cells, whereas these would be trapped by the 0.45 μ pores of membranes; (2) chelation treatment may cause budding of vesicles from the surface membrane which are light in density and cannot be pelleted at 600*g*, but which are sufficiently large to be trapped by membrane pores. We have found no evidence for

[2] Abbreviations used are: PBS, phosphate-buffered saline; EGTA ethylene glycol diaminetetraacetic acid (or ethylenebis(oxyethyleneni trilo)tetraacetic acid); TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

[3] The term "glycoprotein" in this study is used to denote glucosamine-radiolabeled, trichloroacetic acid precipitable macromolecules; these include simple glycoproteins and some mucopolysaccharides as well. All of the latter macromolecules are rich in derivatives of glucosamine and some are trichloroacetic acid precipitable (Kraemer, 1971). Paper chromatography of glucosamine indicated that it was 98% pure.

[4] Suspensions of cells are filtered through a Millipore membrane with 0.45 μ pores. This should filter out whole cells and large vesicles such as nuclei, mitochondria, lysosomes, and plasma membrane vesicles, but not free macromolecules which will appear in the filtrate. Millipore membranes were washed several times with PBS before use.

[5] Determined when 100% of the cells had been removed from the surface.

nonspecific adsorption of protein, RNA, or glycoprotein to Millipore membranes. Filtration through Millipore membranes was used to routinely assay for released macromolecules.

Assay for Dish-Associated Materials. In order to determine whether there is macromolecular material which remains attached to the dish after treatment with a chelating agent, and detachment of 100% of the cells, cultures (29-oz bottles) were treated as described above and shaken for 30 min with EGTA in PBS. This extract was decanted, and the dish was rinsed several times with distilled water. Then 10 ml of 1% sodium dodecyl sulfate in H_2O or 0.1 N NaOH was added to the bottles which were shaken for 30 min at 37°. The dodecyl sulfate or NaOH extract was assayed for trichloroacetic acid precipitable, radioactive macromolecules.

Isolation of rRNA and tRNA. SVT2 cells which had just become confluent were dispersed with trypsin, pelleted by centrifugation at 600*g* for 10 min, and washed once with PBS. Approximately 10^8 cells were suspended in 1 ml of 2.5×10^{-3} M $MgCl_2$ and homogenized with 30 up-down strokes in a Potter-Elvejhmn homogenizer rotating at approximately 400 rpm. Four milliliters of a solution containing 0.25 M sucrose, 0.025 M KCl, 0.004 M $MgCl_2$, and 0.05 M Tris (pH 7.4) were added, and nuclei were pelleted by centrifugation at 600*g* for 10 min. DNase (RNase free), at a final concentration of 10 μg/ml, was added to the supernatant fluid and incubated at 4° for 30 min; sodium deoxycholate (0.5% final concentration) was then added and an additional 30-min incubation at 4° was carried out. Cell debris was pelleted by centrifugation at 10,000*g* in the Servall centrifuge for 20 min. The supernatant was then centrifuged at 110,000*g* for 3 hr in an International B-60 ultracentrifuge. The pellet (microsomes) was resuspended in PBS to be used as sources for ribosomes and rRNA. The supernatant was used as the source for tRNA.

RNAs were purified by adding sodium dodecyl sulfate (2% final concentration) and shaking vigorously at room temperature for 5 min. The extracts were shaken with an equal volume of distilled phenol at room temperature for 15 min. The aqueous phase was separated from the phenol layer by centrifugation at 10,000*g* in the Servall centrifuge, extracted two more times with one-half volume of phenol, and finally extracted with three volumes of ether.

Analysis of Methylated Bases in RNA. To determine the methylated base compositions of RNAs (Iwanami and Brown, 1968a,b), cells were grown in the presence of [*methyl*-^{3}H]-methylmethionine as described above. The appropriate RNA types were purified, taken to dryness, and hydrolyzed in 0.5 ml of 88% formic acid at 175° for 1.5 hr to liberate purine and pyrimidine bases. The methylated bases were separated by paper chromatography in 1-butanol–acetic acid–water (4:1:1, v/v) and analyzed as described previously (Culp and Black, 1971).

Materials. Materials were purchased from the following sources: [4,5-^{3}H]leucine, [5-^{3}H]uridine, [5-^{3}H]thymidine, and Aquasol scintillation fluid from New England Nuclear Corp.; [*methyl*-^{3}H]methylmethionine from Tracerlab; [1-^{3}H]glucosamine from The Radiochemical Centre, Amersham, England; 29-oz glass culture bottles from Eastern Drug Co., Dedham, Mass.; 60-mm plastic Petri dishes from Falcon Plastics; three-times-recrystallized trypsin from Nutritional Biochemicals; DNase (free of RNase) and TPCK-trypsin (free of chymotryptic activity) from Worthington Biochemical Co.; EDTA and EGTA from Eastman Organic Chemicals; sodium pyrophosphate and sodium deoxycholate from Fisher Chemicals; Millipore membranes (25-mm HAWP 0.45 μ pore size) from Millipore Corp.; sodium dodecyl sulfate from Matheson Coleman & Bell; 5-methylcytosine, adenine, guanine, and thymine from Sigma Chemical Co.; Trypan Blue from Grand Island Biologicals Co.

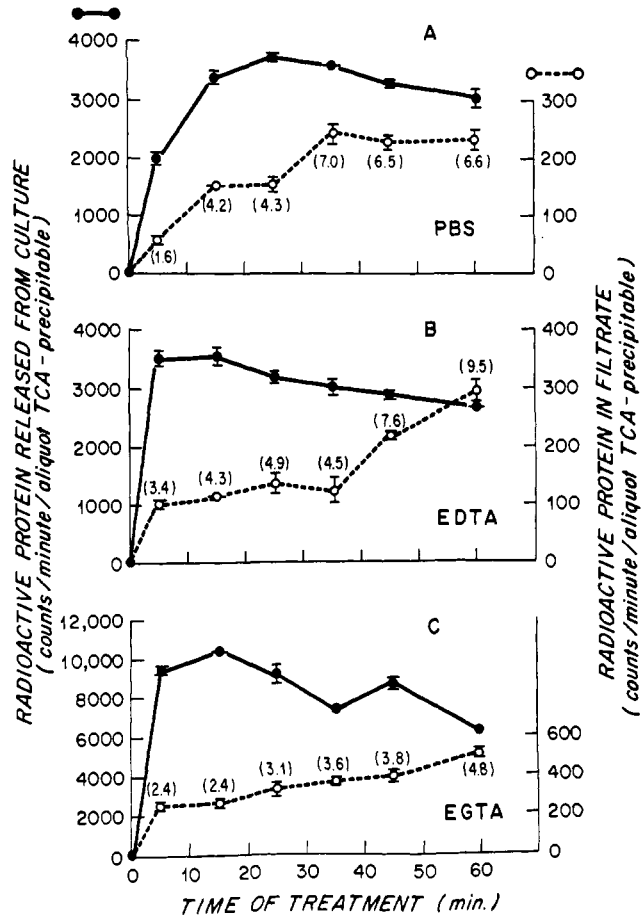

FIGURE 1: Protein release from subconfluent SVT2 cells. Cells were radiolabeled with [^{3}H]leucine and treated as described in the Materials and Methods section. At the indicated times of incubation, an aliquot was withdrawn, part of which was filtered through a Millipore membrane with 0.45 μ pores. Equal aliquots of the unfiltered extract and the filtrate were trichloroacetic acid precipitated. The number in parentheses adjacent to a datum point for the filtrate is the percentage of material which is present in the filtrate as compared to the maximum amount of material in the unfiltered aliquot after all the cells have been detached (*e.g.*, at 5-min treatment in part B).

Results

Release of Protein from SVT2 Cells. Figure 1A illustrates the effect of treating subconfluent SVT2 cells with PBS. Cells were released from the dish slowly; after 25-min treatment, however, no more radioactive protein could be liberated from the dish, but visual observation of the cultures revealed that approximately 95% of the cells had been released from the surface and were present as a suspension of single cells. There was always a small fraction of cells (approximately 5%) which could not be released from the dish even after treatment periods up to 2 hr. An increasing proportion of the protein became filterable up to a treatment period of 35 min; this percentage did not increase thereafter.

3T3 cells, on the other hand, were highly resistant to detachment from the dish with PBS. Thus, PBS treatment was not uniformly effective in removing cells from surfaces.

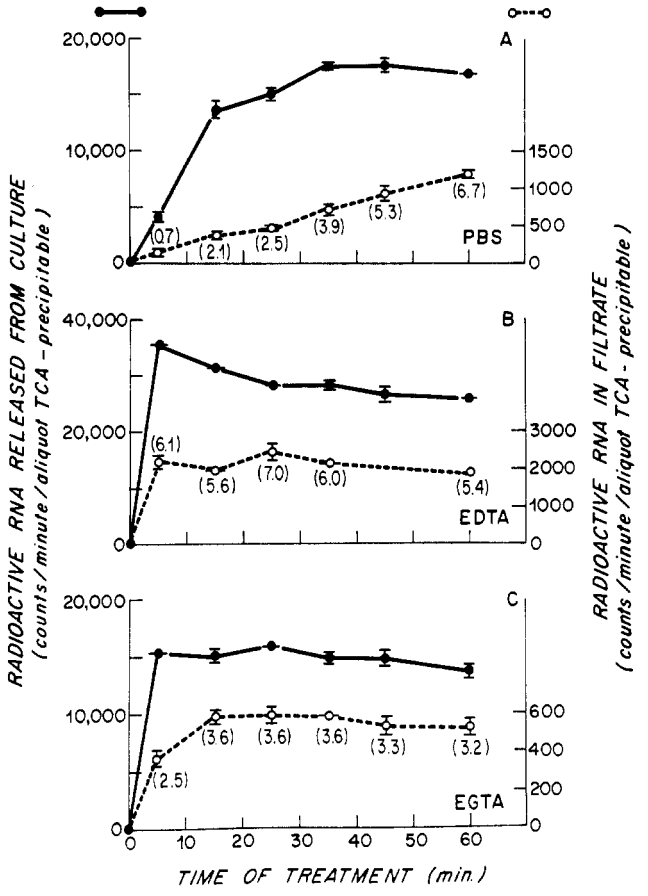

FIGURE 2: RNA release from subconfluent SVT2 cells. Cells were radiolabeled with [^{3}H]uridine and treated as described in Figure 1.

EDTA treatment (Figure 1B) released cells from the glass surface within the first 2- to 3-min treatment. After 20 min, the amount of material in the cell extract began to decrease because of extensive agglutination of cells and the adherence of these clumps to the side of the glass bottle despite continuous shaking; this problem was partially overcome by vigorously shaking the bottle to resuspend these clumps before taking samples.

EDTA treatment resulted in the rapid release of 3.5–4.5% of the protein from the cells as measured in the filtrate between 5- and 30-min treatment (Figure 1B). This fraction of cellular protein increased to as much as 9.5% after 60-min treatment. Profiles identical with that of Figure 1B were obtained by treatment of cells with sodium pyrophosphate, which also chelates both Mg^{2+} and Ca^{2+} ions.

EGTA, an agent which chelates Ca^{2+} specifically, produced markedly less protein release from the cell, while effectively releasing 100% of the cells from the dish within the first 5-min treatment (Figure 1C). Only 2.4% of cell protein was released within the first 5 min; an increase to 4.8% was noted after 60-min treatment. Thus, EGTA was just as effective as EDTA and pyrophosphate at removing cells from glass surfaces; however, appreciably less trauma to the cell resulted as indicated by the reduced amounts of filterable protein.

Varying the concentration of EDTA or EGTA in PBS between 0.05 and 2.5 mM did not affect the ability of these agents to remove cells from the surfaces or to alter the percentage of released protein or RNA.

When the temperature of incubation was lowered to 4°, cells were released much more slowly; 45- to 60-min treatment were required to suspend 100% of the cells. Thus, the release of cells from glass surfaces by Mg^{2+} and/or Ca^{2+} chelating agents is temperature dependent, perhaps because of metabolic reactions at the cell surface or temperature-dependent conformational rearrangements of "binding-factor" protein(s) which makes Ca^{2+} ions available to the chelating agent.

TABLE I: Trypan Blue Stainability.

Time of Treatment (min)[a]	Chelating Agent[b]	Percentage Trypan Positive (%)[c] SVT2[d]	3T3[d]
60	PP	13.5	
2	EDTA	4.8	
10	EDTA	3.5	
30	EDTA	5.3	
60	EDTA	9.3	
15	EDTA		20
30	EDTA		26
60	EDTA		28
60	EGTA	7.1	
60	EGTA	7.0	
15	EGTA		15.7
15	EGTA		16.4

[a] Cells were treated for the indicated periods of time as described in the Materials and Methods section. Duplicate aliquots of cells were centrifuged at 600*g* for 10 min. The pellet of cells was resuspended in 0.5 ml of PBS (containing chelating agent) or 0.5 ml of PBS which contained 100 mg/l. of $MgSO_4 \cdot 7H_2O$ and 100 mg/l. of $CaCl_2$ by gentle pipetting; to this was added 0.5 ml of Trypan Blue solution. At least 2–300 cells were counted in a hemocytometer. [b] PP is sodium pyrophosphate; EDTA is ethylenediaminetetraacetic acid; EGTA is ethylenebis(oxyethylenenitrilo)tetraacetic acid. [c] Determined by dividing the number of cells which were stained with Trypan Blue by the total number of cells and multiplying by 100. [d] Cultures in which the cells were 50–60% confluent were used for these determinations. Duplicate aliquots were averaged to give the indicated numbers.

Table I summarizes data on the loss of viability of cells as a result of surface membrane damage. Nonviable cells concentrate the vital dye Trypan Blue, whereas viable cells are not stained with this dye. During the first 30-min treatment of SVT2 cells with EDTA, the percentage of stained cells remained quite low—between 3 and 5%. By 60-min treatment, this percentage increased to 9.3. Other data have indicated that the percentage of Trypan-stainable cells increases dramatically after 60-min treatment.

EGTA treatment was somewhat milder in that 60-min treatment produced 7% stainable cells. Sodium pyrophosphate appeared to be much more deleterious to SVT2 cells—13.5% of the cells was stained after 60-min exposure. It is notable that the percentage of Trypan-stainable cells was the same whether they were resuspended in buffer containing chelating agent or buffer containing Mg^{2+} and Ca^{2+}, an indication that membrane damage cannot be easily repaired by restoring divalent cations.

Release of RNA and DNA from SVT2 Cells. The release of macromolecular RNA from cells with PBS treatment is depicted in Figure 2A. Cells were released from the surface

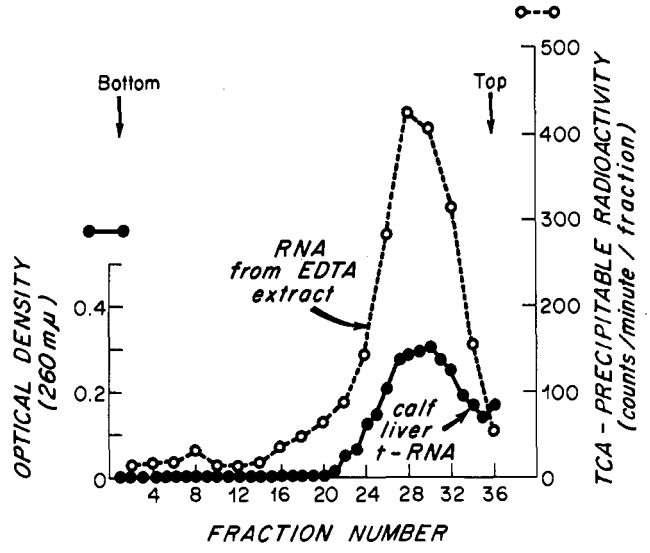

FIGURE 3: Sedimentation velocity analysis of EDTA-released RNA. Subconfluent SVT2 cells, which had been grown for 48 hr in the presence of [^{3}H]uridine, were treated for 10 min with EDTA as described in the Materials and Methods section. An aliquot of cell suspension was filtered, and the filtrate was extracted as described in the Materials and Method section to purify radiolabled RNA. An aliquot of radioactive, EDTA-extracted RNA was mixed with 200 μg of carrier calf liver tRNA and centrifuged in a 5-ml 5–20% sucrose gradient (in 0.5% sodium dodecyl sulfate, 0.01 M Tris (pH 7.4), 0.001 M EDTA, and 0.1 M NaCl) at 20° for 18 hr in the SB405 rotor of the International B-60 ultracentrifuge. Fractions (0.15 ml) were collected, combined with 100 μg of carrier bovine serum albumin, precipitated with trichloroacetic acid, and analyzed for radioactivity.

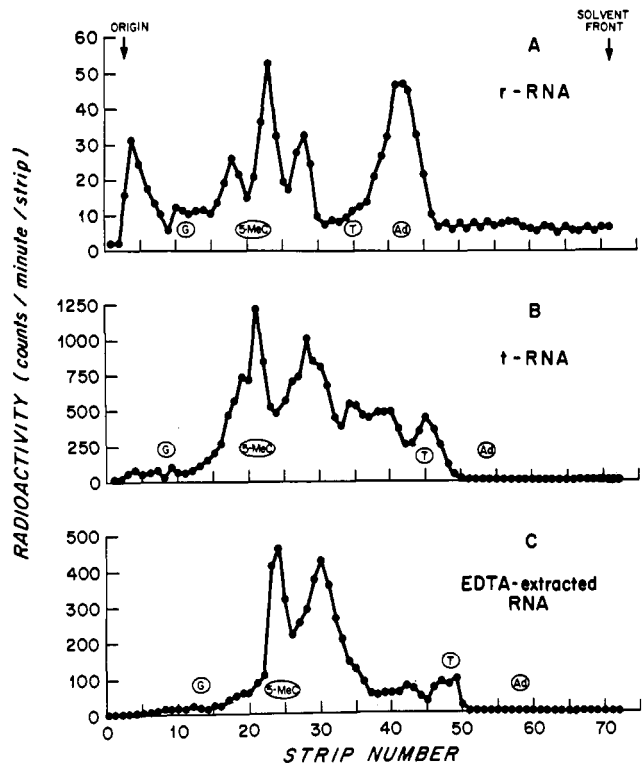

FIGURE 4: Methylated components of cytoplasmic and EDTA-extracted RNA. The purification of the various types of RNA are described in the Materials and Methods section. The hydrolysis and paper chromatography of RNAs have been described previously (Culp and Black, 1971). Ten micrograms of carrier guanine (G), 5-methylcytosine (5-MeC), thymine (T), and adenine (Ad) were cochromatographed with the radioactive hydrolysate. Marker purine and pyrimidine spots were identified under an ultraviolet lamp. The chromatogram was cut into 4 × 50 mm strips which were analyzed for the presence of radioactivity as described (Culp and Black, 1971).

of the dish over a period of 30 min, while the fraction of cellular RNA which was released from the cell and was therefore filterable increased with time to 6.7% after 60-min treatment. EDTA treatment (Figure 2B) caused the rapid release of approximately 6% of cellular RNA during the first 5 min (when 100% of the cells were released from the surface of the dish); the proportion of this material did not increase between 5- and 60-min treatment. This suggests that there is a particular class of RNA which can be rapidly and efficiently released from the cell, while the remaining 93% of cellular RNA is quite refractile to extraction with EDTA.

EGTA treatment (Figure 2C) resulted in relatively less release of RNA (3% of RNA compared to 6% with EDTA treatment) and slower release; plateauing of release of RNA did not occur until 15-min treatment (compared to 5 min for EDTA treatment).

In order to identify the origin of this RNA, sedimentation and methylation studies were performed. The sedimentation properties of the RNA which was released from cells by EDTA treatment are depicted in Figure 3. It cosedimented with carrier calf liver tRNA; very little high molecular weight RNA was present in the EDTA extract.

tRNA and rRNA, the major RNAs found in the cytoplasm of the cell, have distinctive methylated base patterns by which these RNAs are characterized (Iwanami and Brown, 1968a,b). The methylated components of rRNA are depicted in Figure 4A after hydrolysis and paper chromatography. The major peak at the origin was methylribose which was present in large quantity in rRNA and was polymerized during formic acid hydrolysis. A second major class of methylated bases which was only found in rRNA was 6-methyladenine and 6-dimethyladenine which cochromatographed with carrier adenine between fractions 38 and 45. No 5-methylcytosine (5-MeC) is present in rRNA and thus no major radioactive peak which cochromatographed with carrier 5-MeC was observed.

Figure 4B is the distribution of methylated components in tRNA. There was a large radioactive peak which migrated with carrier 5-MeC and a smaller radioactive peak which migrated with thymine, both of which were only found in tRNA. There was no methylribose and no methylated adenine (the N^6 isomers) in tRNA (Iwanami and Brown, 1968a).

The methylated components of EDTA-extracted RNA are depicted in Figure 4C; this profile resembles that of tRNA and not rRNA. There was a major peak of radioactive 5-MeC and a minor peak of thymine; there was no methylribose and no methylated adenine. Thus, the methylated components of EDTA-extracted RNA were more characteristic of tRNA than of rRNA as determined by analyses of both sedimentation properties and methylated components.

The question remains as to whether RNA in ribosomes can be broken down to low molecular weight RNA under these conditions of treatment with EDTA. Ribosomes, radioactively labeled by incorporation of [^{3}H]uridine into RNA, were prepared as described in the Materials and Methods section. Two different types of incubations were then performed: (A) ribosomes were added to 0.5 mM EDTA in PBS alone to determine whether EDTA treatment alone is deleterious to

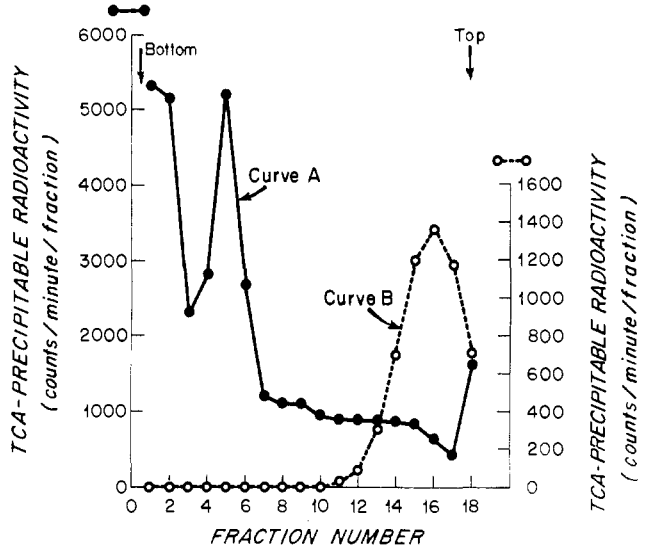

FIGURE 5: Breakdown of RNA in ribosomes. [^{3}H]Uridine-labeled ribosomes were prepared as described in the Materials and Methods section. Incubation A: 0.1 ml of radioactive ribosomes was mixed with 0.9 ml of 0.5 mM EDTA in PBS and incubated at 37° for 15 min; RNA was then extracted from the incubation as described in the Materials and Methods section and analyzed on a 5–20% sucrose gradient as described for Figure 3 (●). Incubation B: 1.0 ml of radioactive ribosomes was mixed with 25 ml of 0.5 mM EDTA in PBS, added to a washed layer of 50% subconfluent SVT2 cells in a 29-oz glass bottle, and incubated with gentle shaking at 37° for 15 min. An aliquot of the suspension was filtered through a 0.45 μ pore Millipore membrane, RNA was extracted from the filtrate as described in the Materials and Methods section, and analyzed on a 5–20% sucrose gradient as described in Figure 3 (○). Fractions (0.2 ml) of gradients were collected, combined with 100 μg of carrier bovine serum albumin, precipitated with trichloroacetic acid, and analyzed for radioactivity.

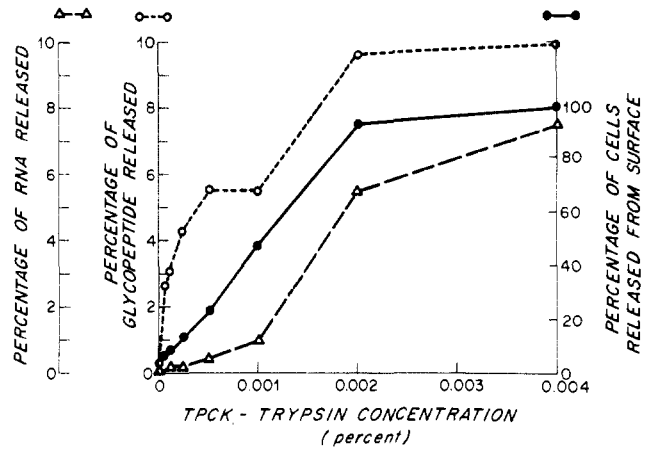

FIGURE 6: RNA and glycopeptide release after TPCK-trypsin treatment of SVT2 cells. Cells were grown in the presence of [^{3}H]uridine to radiolabel RNA or [^{3}H]glucosamine to radiolabel "glycoproteins" as described in the Materials and Methods section. Medium was decanted from cultures (in 4-oz glass prescription bottles) and the cell layers (60% subconfluent) were washed with PBS (100 mg/l. of $CaCl_2$ and $MgSO_4 \cdot 7H_2O$). Then 5 ml of the indicated concentration of TPCK-trypsin in PBS (plus Mg^{2+} and Ca^{2+}) was added to each bottle, which were shaken gently at 37° for 10 min. Aliquots were then withdrawn and treated to determine the fraction of released RNA or glycopeptide (see Materials and Methods section and Figure 1). The percentage of cells released from the surface of the dish was determined by dividing the amount of radioactive RNA in the trypsin suspension by the total amount of RNA in the culture and multiplying by 100.

the RNA; and (B) ribosomes were added to subconfluent SVT2 cells that were being treated with 0.5 mM EDTA in PBS. Sedimentation velocity analyses of the RNAs from these two incubations are displayed in Figure 5. RNA from ribosomes treated with EDTA alone in the absence of SVT2 cells maintained its high molecular weight properties (curve A). RNA from ribosomes incubated with EDTA-treated SVT2 cells was broken down to low molecular weight RNA (curve B) characteristic of that analyzed in Figure 3. It is therefore possible that some low molecular weight RNA that is EDTA extractable is rRNA that has been hydrolyzed to low molecular weight fragments, although the absence of rRNA-characteristic methylated components suggests that there may be very little, if any, of this RNA present.

The question arises whether RNA which is extracted from the cell with EDTA or EGTA "leaks" from the cytoplasmic space and is a measure of the breakdown of cell membrane integrity or is "stripped" from the surface of the cell because it is a surface-associated RNA. To answer this question, a comparison was made between RNA and glycopeptide release from cells. Cell membranes are very rich in glycoproteins (see data below). TPCK-trypsin was used to detach cells; the loss of glycopeptide, which was "stripped" from the surface of the cell by proteolytic action (Codington *et al.*, 1970), was then compared to the loss of RNA. If RNA is a structural component of the cell surface, the release of RNA as a function of TPCK-trypsin concentration should parallel the loss of glycopeptide.

Figure 6 demonstrates that this is not the case. Between TPCK-trypsin concentrations of 0.0001 to 0.001%, an appreciable fraction of cellular glycopeptide was liberated (3–6%) while less than 0.5% of cellular RNA was released. Above a concentration of 0.001%, somewhat comparable yields of RNA and glycopeptide were released. At 0.004% TPCK-trypsin concentration, 9.8% of the glycopeptide had been released; likewise, 7.5% of cellular RNA was released. After 30-min treatment with 0.004% TPCK-trypsin, the cells were completely destroyed, and there were large clumps of aggregated chromatin in the culture.[6]

DNA release from SVT2 cells during EDTA treatment is illustrated in Figure 7. There was much less DNA release (0.2%) than RNA release (6%) from subconfluent SVT2 cells (Figure 7A). When cells became confluent, however, the fraction of DNA (Figure 7B) which was released from cells increased to 0.5%, probably as a result of increased cell death and lysis when multilayering of the cells occurred. When confluent layers were treated with EDTA or EGTA, large sheets of cells were detached from the dish which never became single-cell suspensions. This indicates that cell-to-cell associations are of a different nature than cell-to-dish associations and may not be dependent upon divalent cation interactions.

Release of "Glycoprotein" from SVT2 Cells. Studies have shown that most glycoproteins of the cell are associated with the membranes of the cell (Spear and Roizman, 1970) which are principally at the cell surface and in the cytoplasm. The data of Table II confirm this and indicate that approximately 75% of cellular "glycoprotein" was membrane associated in cell types that possessed quite different morphologies, growth properties, and cytogenetic compositions (Culp and Black,

[6] 3T3 cells, on the other hand, were quite stable in this concentration of TPCK-trypsin for periods in excess of one hour.

TABLE II: "Glycoprotein" Distribution in 3T3, SVT2, and Revertant[a] Cells.

Cell[b]	Fraction[c]	"Glycoprotein," cpm (Percentage of Total)[d]	Protein, μg (Percentage of Total)[e]
3T3	Soluble	13,500 ± 600 (26.8)	2570 (61.7)
	Membrane	36,700 ± 1200 (73.2)	1590 (38.3)
SVT2	Soluble	26,800 ± 900 (24.0)	4100 (62.0)
	Membrane	84,300 ± 2300 (76.0)	2520 (38.0)
Revertant clone 84	Soluble	11,200 ± 300 (22.7)	3600 (59.7)
	Membrane	38,000 ± 1700 (78.3)	2440 (40.3)

[a] Revertant refers to the contact-inhibited variant of SVT2 cells which was selected by concanavalin A treatment (Culp and Black, 1972). [b] Cells were grown in 100-mm plastic Petri dishes for 48 hr (from 10% confluence to 50–60% confluence) in the presence of 50 μCi of [1-^{3}H]glucosamine hydrochloride (2.6 Ci/mmole). [c] Cells were washed twice with PBS (containing 100 mg/l. of $MgSO_4 \cdot 7H_2O$ and $CaCl_2$) and scraped with a rubber policeman into 5 ml of this buffer. The cell suspensions were sonicated at 5° for 5 min to lyse cells and nuclei, incubated with RNase (10 μg/ml) and DNase (10 μg/ml) for 30 min at 37°, and centrifuged at 110,000*g* for 3 hr in the A-321 rotor of the International B-60 ultracentrifuge. The supernatant is called "soluble" material. The pellet was homogenized in 2.5 ml of PBS (containing Mg^{2+}, Ca^{2+}, and 0.5% sodium dodecyl sulfate) with a ground-glass homogenizer and is called "membrane" fraction. [d] Aliquots of 0.3 ml were precipitated with trichloroacetic acid in the presence of 100 μg of carrier bovine serum albumin onto glass fiber disks, whose radioactivity was determined in a scintillation counter. The total amount of radioactivity in each fraction is given with the percentage of material. [e] The protein content of each fraction was determined by the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as a standard.

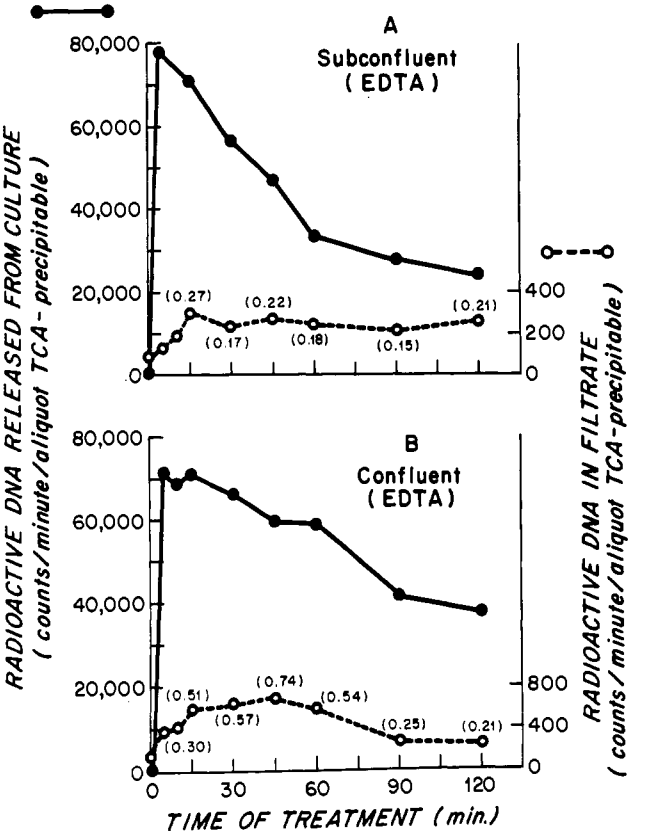

FIGURE 7: DNA release from subconfluent SVT2 cells. Cells were radiolabeled with [^{3}H]thymidine and treated as described in Figure 1 and the Materials and Methods section.

1972). Approximately 60% of whole cell protein was found in the soluble fraction.

Time courses of the release of "glycoproteins" from subconfluent SVT2 cells are illustrated in Figure 8. In contrast to protein and RNA release which plateaued after only 5- to 10-min treatment with EDTA or EGTA, "glycoprotein" release increased almost linearly with time. After 10-min treatment with EDTA or EGTA, only 2% of the cell's complement of "glycoproteins" was released. After 90-min treatment with EDTA (Figure 8A), 12% of cellular "glycoprotein" was released, while EGTA treatment (Figure 8B) resulted in release of 8.2%. Perhaps after short periods of treatment, release of "glycoproteins" resulted from an increase in cellular permeability and leakage of the soluble "glycoprotein" pool, while longer treatment may have resulted in slow extraction of "glycoproteins" from the surface of cells.

Release of RNA, Protein, and "Glycoprotein" from 3T3 Cells. The time courses of release of subconfluent 3T3 cells from glass surfaces and RNA from cells after EGTA treatment are depicted in Figure 9A. Although 3T3 cells are spread on glass surfaces to a greater extent than SVT2 cells, they were detached within the first few minutes of treatment with the Ca^{2+}-chelating agent. The percentage of RNA that leaks from 3T3 cells (as much as 20%) was much greater than that leaked from SVT2 cells (3.5%). The loss of filterable RNA as a function of treatment time was probably the result of RNA breakdown, presumably due to liberation of lysosomal enzymes subsequent to cell death; 3T3 cells were far more sensitive to treatment with chelating agents than SVT2 cells (see Table I).

Protein release (Figure 9B) plateaued after 20-min EGTA treatment at a level (16–18%) which is much higher than that observed for SVT2 cells (2.5%).

"Glycoprotein" release from 3T3 cells (Figure 9C) was similar to the pattern of release from SVT2 cells in that a plateau was never achieved and the fraction released increased as a function of time. However, the proportion of "glycoprotein" released from 3T3 cells at any particular time of treatment was approximately twice that observed with SVT2 cells.

Thus, there were much higher percentages of RNA, protein, and "glycoprotein" released from 3T3 cells when compared to SVT2 cells. The surface membrane of 3T3 cells may be uniformly more labile than that of SVT2 cells. This was verified by Trypan Blue stainability data for 3T3 cells in Table I. Four or five times as many 3T3 cells as SVT2 cells concentrated Trypan Blue after EDTA treatment. 3T3 cells were somewhat less fragile after EGTA treatment than after EDTA treatment.

Dish-Bound Glycoproteins in 3T3, SVT2, and Revertant Cells. The data above dealt with two classes of "glycoproteins" that could be removed from the cell culture with EDTA or EGTA treatment; one class remained cell associated and was retained by the Millipore membranes, while a second class of "glyco-

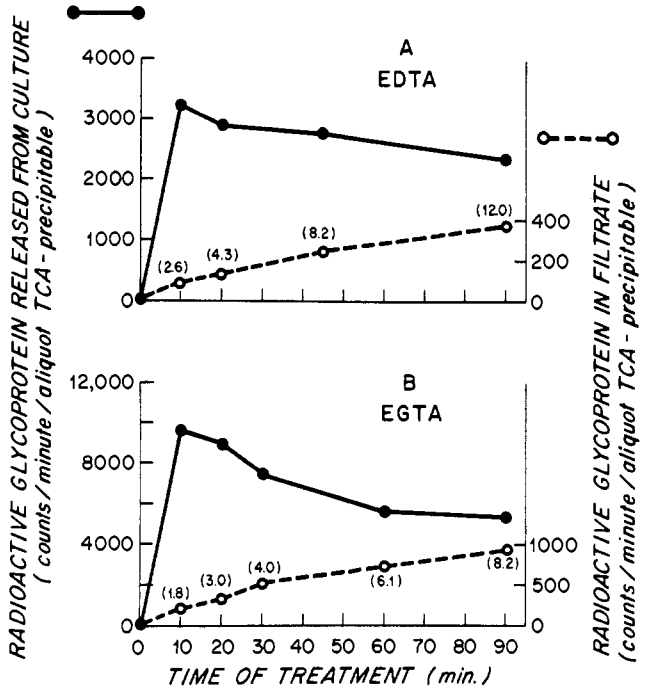

FIGURE 8: "Glycoprotein" release from subconfluent SVT2 cells. Cells were radiolabeled with [1-^{3}H]glucosamine and treated as described in Figure 1.

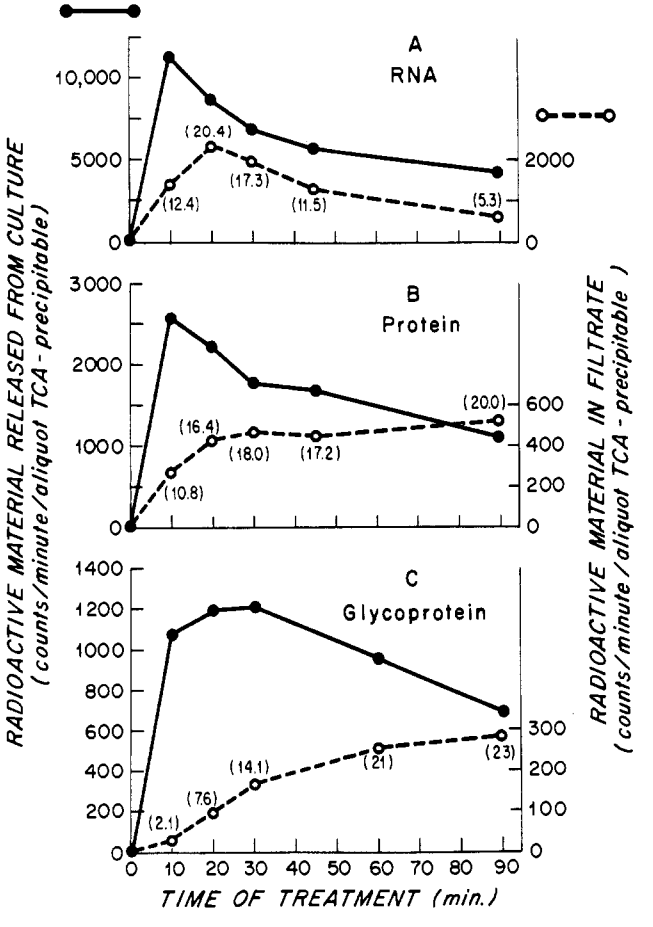

FIGURE 9: RNA, protein, and "glycoprotein" release from subconfluent 3T3 cells. Cells were radiolabeled with [^{3}H]uridine, [^{3}H]-leucine, or [^{3}H]glucosamine as described in the Materials and Methods section. Cell layers were treated with 0.5 mM EGTA in PBS and data were determined as described in Figure 1.

TABLE III: Dish-Bound Macromolecules in 3T3 and SVT2 Cultures.

Type of Macromolecule[a]	Amt in Fraction (cpm)[b]	Dish-Bound (%)[c]
	Subconfluent 3T3	
RNA	EGTA extract (1,830,000)	
	Dish bound (10,600)	0.58
Protein	EGTA extract (426,000)	
	Dish bound (17,200)	4.0
"Glycoprotein"	EGTA extract (147,000)	
	Dish bound (13,400)	9.1
	Subconfluent SVT2	
"Glycoprotein"	EGTA extract (240,000)	
	Dish bound (6000)	2.5
	Confluent SVT2	
RNA	EGTA extract (70,000,000)	
	Dish bound (78,000)	0.11
Protein	EGTA extract (1,850,000)	
	Dish bound (23,000)	1.25
"Glycoprotein"	EGTA extract (7,400,000)	
	Dish bound (92,800)	1.25

[a] For each class of macromolecules, cells were grown in media containing the radioactive precursor as described in the Materials and Methods section. [b] Medium was decanted and the cell layer was washed once with PBS (plus Mg^{2+} and Ca^{2+}); 25 ml of 0.5 mM EGTA in PBS was added. The culture was shaken gently at 37° for 30 min. An aliquot of the EGTA extract which contained 100 μg of carrier bovine serum albumin was precipitated with trichloroacetic acid to determine the total amount of radioactive macromolecule in 25 ml of the EGTA extract. The glass surface was rinsed three times with distilled water; 10 ml of 0.1 N NaOH was added, and the culture was shaken gently at 37° for 30 min. An aliquot of this extract was trichloroacetic acid precipitated in the presence of 100 μg of carrier bovine serum albumin to give the total amount of radioactive macromolecule in the NaOH extract. [c] Determined by dividing the amount of radioactivity in the dish-bound fraction by the amount of radioactivity in the EGTA extract and multiplying by 100.

proteins" was released from the cell and passed through the 0.45 μ pore.

Two more classes of "glycoproteins" were analyzed. One class appeared in the medium of growing cells and has been investigated by others (Halpern and Rubin, 1970). A second class of "glycoproteins" remained attached to the glass after all cells had been detached with EDTA or EGTA. We shall refer to this second class as dish-bound "glycoproteins."

When SVT2 or 3T3 cells had been detached from glass surfaces by EGTA treatment, there were no morphologically identifiable materials which remained on the surface of the culture as determined by examination of replicas of the glass surface in the electron microscope.[7] However, if the surface of the glass was treated with 0.1 N NaOH or 1% sodium dodecyl sulfate in H_2O, a considerable amount of material was found

[7] N. S. McNutt, L. A. Culp, and P. H. Black, manuscript in preparation.

TABLE IV: Time Course of Dish-Bound "Glycoprotein" Release.[a]

Time of Treatment (min)	Amt of Cl_3CCOOH Precipitable (cpm)[b]
0	13
5	280
10	478
15	563
30	571
60	678
90	475

[a] A culture which had been grown in [^{3}H]glucosamine for 48 hr was treated as described in Table III. Fifteen milliliters of 1% sodium dodecyl sulfate was added at $t = 0$. [b] Duplicate 0.25-ml fractions were withdrawn at the indicated times and trichloroacetic acid precipitated in the presence of 100 μg of carrier bovine serum albumin.

TABLE V: "Glycoprotein" Distribution in 3T3, SVT2, and Revertant Cell Cultures.

Cell Line[a]	Culture Fraction (cpm)[b]	Percentage Relative to Cell-Associated Material (%)[c]
3T3	Cell associated (330,000)	
	Medium (756,000)	227
	Dish bound[d] (63,900)	19.2
SVT2	Cell associated (598,000)	
	Medium (868,000)	145
	Dish bound[d] (21,000)	2.7
Revertant clone 84	Cell associated (325,000)	
	Medium (660,000)	199
	Dish bound[d] (42,700)	13.2

[a] Cells were grown for 48 hr (from 10% confluent to 60% confluent) in the presence of 100 μCi of [^{3}H]glucosamine (2.6 Ci/mmole) and 40 ml of MEM×4 as described in the Materials and Methods section. [b] Medium was decanted, 50 μl of which was precipitated with trichloroacetic acid to determine the amount of radioactive "glycoprotein" in the 40 ml of medium; there were generally 5–10,000 floating cells in 40 ml of medium as compared to 2–10 × 10^6 cells which were attached to the glass. The cell layer was washed with PBS (plus Mg^{2+} and Ca^{2+}); 25 ml of 0.5 mM EGTA in PBS was added, and the culture was shaken gently for 15 min. An aliquot of unfiltered EGTA extract was precipitated with trichloroacetic acid in the presence of 100 μg of carrier bovine serum albumin to determine the "cell-associated" glycoprotein. The glass surfaces were then treated as described in Table III to determine "dish-bound" glycoproteins. [c] Determined by dividing the amount of radioactive "dish-bound" glycoprotein or "medium" glycoprotein by the "cell-associated" glycoprotein and multiplying by 100. [d] Extractable with 1% sodium dodecyl sulfate in H_2O.

in association with the surface. This material is surface membrane material and/or intercellular matrix from which the cell is dissociated by a Ca^{2+}-chelating agent and which may be important in cell-to-dish attachments.

The data of Table III present the amounts of RNA, protein, and "glycoprotein" left on the dish after growth and detachment of 3T3 or SVT2 cells (either subconfluent or confluent). There was very little RNA bound to the glass in cultures of both normal and transformed cells but considerable proportions of protein and "glycoprotein." 3T3 cells deposited a larger fraction of protein and especially "glycoprotein" than SVT2 cells deposited. This dish-bound material was not the result of deposition of protein, RNA, or "glycoprotein" which had first been secreted into the medium of growing cells.

The dish-bound material analyzed in Table III was extracted with 0.1 N NaOH. Similar amounts of material could be extracted with 1% sodium dodecyl sulfate in H_2O; a somewhat higher yield of "glycoprotein" was obtained with sodium dodecyl sulfate extraction, perhaps because some polysaccharide–protein linkages in glycoproteins are labile to NaOH (Bertolini and Pigman, 1970).

The time course of release of "glycoproteins" from the glass surface during sodium dodecyl sulfate treatment is presented in Table IV. The amount of extracted "glycoprotein" increased and reached a maximum after 15-min treatment. Treatment for as long as 90 min liberated no additional material.

The proportions of "glycoproteins" secreted into the medium of growing cells and deposited on the glass surface of 3T3, SVT2, and revertant cell cultures was determined and are listed in Table V. Revertant cells selected by the concanavalin A procedure (Culp and Black, 1972) are flat and contact inhibited, very similar in morphology and growth properties to 3T3 cells. Interestingly, both these cell lines secrete on a percentage basis much more "glycoprotein" into the medium when compared to SVT2 cells. Also, both cell lines deposited much larger amounts of "glycoprotein" on the surface of the dish.

The kinetics of incorporation of glucosamine into medium, cell-associated, and dish-bound "glycoproteins" of 3T3 cells after long-term exposure to the radioactive precursor are shown in Figure 10. Cell-associated "glycoproteins" which appeared in the EGTA extract increased until the cell layer had been confluent for 24 hr; the amount of this material did not increase during the next 48-hr growth. Production of dish-bound "glycoprotein" occurred with a similar pattern. The fact that cells continued to make cell-associated and dish-bound "glycoproteins" for 24 hr after confluence had been achieved, and when the cell number did not increase, suggests that cell membrane and dish-bound material, both rich in "glycoprotein," continue to be synthesized without further cell division and that this synthesis for some unknown reason is eventually inhibited.

"Glycoproteins" continued to be secreted into the medium (Figure 10) at a linear rate for at least 3 days after the attainment of confluence and growth inhibition of 3T3 cells. Thus, there appears to be an absence of control over "glycoproteins" which are secreted into the medium.

Discussion

In order to study the properties of macromolecules on the surface of mammalian cells and their possible relationship to growth control (Culp *et al.*, 1971; Culp and Black, 1972), it is

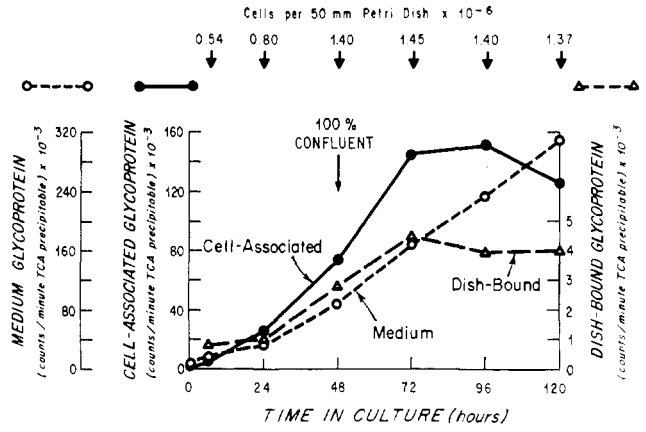

FIGURE 10: Production of different classes of "glycoproteins" in 3T3 cells. 3T3 cells were inoculated into 60-mm plastic Petri dishes containing 5 ml of MEM×4. After 24 hr, when cells were approximately 30% confluent, the medium was changed to MEM×4 containing 2.5 μCi/ml of [1-^{3}H]glucosamine hydrochloride (2.6 Ci/mmole) at t = 0 hr. After the indicated periods of incubation, duplicate plates were trypsinized to determine the number of cells/60-mm dish. An aliquot of medium was trichloroacetic acid precipitated to determine the amount of radioactive glycoprotein in 5 ml of medium. Duplicate dishes were also treated as described in the Materials and Methods section with 2 ml of 0.5 mM EGTA in PBS for 15 min, an aliquot of which was precipitated with trichloroacetic acid to determine the amount of cell-associated "glycoprotein" in 2 ml of EGTA-suspended cells. The dishes were rinsed with distilled water and extracted with 1.0 ml of 1% sodium dodecyl sulfate in H_2O for 15 min at 37°; an aliquot of this was precipitated with trichloroacetic acid to determine the amount of dish-bound "glycoprotein."

necessary to find methods for efficiently detaching cells from culture dishes and for separating surface components from the large quantities of intracellular macromolecules which may be present. The use of trypsin and other proteolytic agents should be avoided, since they partially destroy the surface proteins and glycoproteins which may be of major interest. For these reasons, we initiated experiments to determine the ability of Ca^{2+} and/or Mg^{2+} chelating agents to remove cells from culture dishes and to release various classes of macromolecules from the cell.

Both subconfluent 3T3 and SV40-transformed 3T3 (SVT2) cells were completely detached from glass surfaces within the first 2- to 3-min treatment with either EDTA (a Ca^{2+}- and Mg^{2+}-chelating agent) or EGTA (a Ca^{2+}-chelating agent) to form single cell suspensions. This suggests that Ca^{2+} is important in maintaining cell-to-dish attachments. During treatment for periods longer than 30 min, cells began to aggregate into clumps which tended to attach to glass; similar phenomena have been investigated in detail (Edwards and Campbell, 1971). Confluent 3T3 or SVT2 cells were detached much more slowly (5–15 min) and were released as sheets of cells; this indicates that cell-to-cell adhesion may be less dependent on Ca^{2+}- and Mg^{2+}-dependent ionic interactions than cell-to-dish attachment.

Two approaches were used to determine the extent of the breakdown of the integrity of the cell membrane with chelating agents: (1) Trypan Blue stainability (Paul, 1965) and (2) measurements of RNA and protein release from within the cell (Snow and Allen, 1970). A small fraction of SVT2 cells were stained with Trypan Blue after EDTA treatment (Table I), while a much larger fraction of 3T3 cells were stained. This was true with EGTA treatment as well, although EGTA was less deleterious to both SVT2 and 3T3 cells.

Studies of cellular protein released from SVT2 cells revealed that a higher proportion was released after EDTA treatment (4%) than after EGTA treatment (2.5%) (Figure 1). The available evidence indicates that the protein was released from the cytoplasm, presumably due to an increase in the permeability of the plasma membrane. Protein was released into the filtrate very early during the treatment and the proportion of this material did not increase to a major extent during 60- to 90-min further treatment; this indicates that these may be a unique class of proteins which were released from the intracellular matrix.

The EDTA-released protein ([^{3}H]leucine labeled) was coelectrophoresed in 7.5% polyacrylamide gels (containing 0.5% sodium dodecyl sulfate) with soluble cytoplasmic proteins ([^{14}C]leucine labeled).[8] Qualitatively, the profiles were very similar, an indication that EDTA-released proteins may be soluble cytoplasmic proteins. Quantitatively, there was a higher proportion of lower molecular weight proteins in the EDTA-released extract than in the soluble cytoplasmic extract. This suggests that there is a tendency for leakage of low molecular weight protein from the cell. It is therefore more likely that EDTA-released protein results from breakdown of the integrity of the cell membrane and leakage of soluble cytoplasmic material, rather than from dissociation of membrane-bound proteins.

Analysis of RNA released after exposure to chelating agents revealed many similarities with protein release. The kinetics of release were quite comparable; in addition, the proportion released was greater with EDTA (6%) than with EGTA (3%) treatment (Figure 2). Several lines of evidence suggest that EDTA-released RNA was leaked from within the cell and was not dissociated from the cell surface (Weiss, 1969). (1) EDTA-released RNA has sedimentation (Figure 3) and methylated-base properties (Figure 4) identical with tRNA, a component of the soluble matrix of the cytoplasm of the cell (Bernhardt and Darnell, 1969). (2) Release of this RNA occurs rapidly, and the amount of released material does not increase as a function of time. In contrast, "glycoprotein," which is a major component of surface membranes, is released slowly as a function of time (see below). (3) Comparison of glycopeptide release with RNA release as a function of the concentration of TPCK-trypsin, which is known to dissociate surface membrane components (Snow and Allen, 1970), reveals that at concentrations where RNA release is minimal (less than 0.5%) glycopeptide release (Figure 6) is appreciable (3–6%); at higher TPCK-trypsin concentrations, under conditions where the cells are being increasingly damaged, RNA and glycopeptide release are comparable. Thus, it is quite likely that RNA is leaked from the cytoplasm; we have found no evidence for an RNA species which is a structural component of the cell surface, as has been suggested (Weiss, 1969).

Is the small amount of leakage of RNA (6%) and protein (4%) from SVT2 cells occurring in all the cells of the population or only a small fraction of the cells, such as the Trypan stainable cells? Since the only RNA which appears to be leaked is tRNA, which comprises only 10–12% of the whole-cell RNA (Watson, 1970), a small fraction of cells would have an insufficient amount of this RNA to yield the 6% release. There did not appear to be any rRNA in the released fraction; rRNA comprises 80–85% of the cell's complement of RNA, most of which is in membrane-bound ribosomes. Thus, for SVT2 cells, leakage probably occurs from a large fraction of the cells.

[8] L. A. Culp and P. H. Black, unpublished data.

Although subconfluent 3T3 cells were detached from surfaces within 2- to 3-min of treatment with EDTA or EGTA, there were higher proportions (Figure 9) of protein and RNA released from these cells than from SVT2 cells. This was also reflected in the higher percentage of these cells which were stained with Trypan Blue (Table I). Perhaps the surface membrane of 3T3 cells is intrinsically more permeable, allowing for the higher proportions of released RNA and protein. As discussed above, it is likely that leakage occurs from the majority of 3T3 cells. It is also possible that small vesicles bud and detach from 3T3 surface membranes after EDTA or EGTA treatment (Weiss, 1967). Different experimental approaches must be taken to resolve these possibilities.

The kinetics of "glycoprotein" release from SVT2 cells (Figure 8) or 3T3 cells (Figure 9C), which were treated with EDTA or EGTA, were much different than the kinetics of protein or RNA release. During the first 5-min treatment, very little material was solubilized (generally less than 1%). As time of treatment increased, the proportions of released "glycoprotein" increased in a linear fashion. Since only a small proportion of whole-cell "glycoproteins" was found in the cell's soluble matrix (Table II), the "glycoproteins" were probably being extracted slowly from the surface, and perhaps cytoplasmic, membranes. If cells are treated with EGTA for very short periods of time, it may be possible to conserve surface "glycoproteins" with the intact cell. It will be necessary to use more sophisticated criteria to determine whether "glycoproteins," which are solubilized by long-term treatment with EDTA or EGTA, are derived from the surface or intracytoplasmic membranes.

Similarly, Codington *et al.* (1970) found that very little polysaccharide material was released from mouse ascites cells during EDTA treatment, as compared to treatment with TPCK-trypsin, although little information was available on leakage of material from the cell. On the other hand, Snow and Allen, (1970) studying baby hamster kidney cells grown on glass surfaces found approximately the same amount of RNA and DNA release after EDTA treatment as we did utilizing SVT2 cells. Unfortunately, the latter study used precipitation of glucosamine-radiolabeled material with 1% $CaCl_2$ in EtOH to quantitate glycoprotein release; we have found[8] that as much as 90% of the radioactive material which was precipitable with 1% $CaCl_2$ in EtOH cochromatographed with a leucine marker during gel filtration and probably represented unincorporated glucosamine.

Replicas of cells in culture were prepared and examined in the electron microscope.[7] They revealed long fibers which rested on the upper surface of cells and on some areas of the culture dish. When cells were treated with EGTA, cells and fibers were completely removed from the dish and there were no morphologically identifiable materials remaining on the dish. These fibers were also dissociated from the surfaces of the cell with EGTA and were found in the supernatant of low-speed centrifugations of cell suspensions. They were digestible with highly purified collagenase, an indication that they may be collagen fibrils. They may be some of the released protein and/or "glycoprotein" which is described above in filtrates. Thus, there is at least one type of surface-associated material which can be dissociated from cells with these chelating agents.

A measurable fraction of protein and "glycoprotein" remained on the dish after removal of the cells with EGTA. This material was extracted with NaOH or sodium dodecyl sulfate treatment (Table III). The slow release of these materials during dodecyl sulfate treatment (Table IV) is further evidence of the tenacity by which they are attached to the glass. 3T3 cells and revertant cells (contact-inhibited variants selected from SV40 transformed cells (Culp *et al.*, 1971; Culp and Black, 1972)), perhaps because of their highly flattened morphology, deposited much more of these materials than the spindle-shaped SVT2 cells (Table V). Little is known whether this dish-bound material is related to the morphology of these cells.

It is possible that these materials are cell-to-dish attachment factors that appear on the bottom surface of cells. It would be interesting to determine whether these materials are found over the entire cell surface or are only localized on the underside. In addition, 3T3 and revertant cells elaborated more "glycoprotein" material into the medium (Table V) than SVT2 cells. Very little RNA was deposited on the dish, further evidence that whole cells or vesicles were not being left on the dish.

The kinetics of the production of secreted, cell-associated, and dish-bound "glycoproteins"[9] during long-term cultivation of contact-inhibited 3T3 cells in radioactive glucosamine revealed the following (Figure 10). Dish-bound and cellular "glycoproteins" continued to be produced for a short time after the cultures had achieved confluence; thereafter, their level remained stationary. In contrast, "glycoproteins" continued to be secreted into the medium for at least 3 days. Thus growth control in 3T3 cells seems to be reflected in the inhibition of the accumulation of cellular and dish-bound "glycoproteins" but not medium-secreted "glycoproteins." More study must be performed to determine whether the "glycoprotein(s)" in the medium results from turnover of membrane components or secretion of differentiated products of the cell.

The nature and turnover of dish-bound, cellular, and medium-secreted "glycoproteins" will be further examined in an attempt to determine which surface components may be important in growth control of mammalian cells. Some of the "glycoproteins" described above may be mucopolysaccharides, many of which are precipitated by trichloroacetic acid (Kraemer, 1971); very little is known about the production of these materials with respect to the cell's state of growth (Kraemer, 1971; Bischoff, 1971) and in relation to the production of another differentiated product of fibroblastic cells, collagen (Culp *et al.*, 1971; Culp and Black, 1972). The topology of various "glycoprotein" classes around the surface of the cells also needs further exploration (Spiro, 1969).

By several criteria, EGTA treatment appears to be a milder method than EDTA treatment for protecting the cell's permeability barriers and minimizing surface "glycoprotein" release, while effectively detaching cells from culture dishes. Evidence that Mg^{2+} is an important cation in maintaining the compactness of cell membranes was attained utilizing artificial fetuin–phospholipid monolayer films (Tiffany and Blough, 1970). It also appears that (1) Ca^{2+}-dependent interactions are most important for cell-to-dish attachment, (2) Mg^{2+}-dependent interactions are most important in preserving the impermeability of the cell membrane, and (3) cell-to-cell associations are dependent on stronger forces than simple divalent cation interactions between complementary surface molecules.

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[9] Medium-secreted "glycoproteins" are not the result of sloughing of whole cells into the medium, since less than 1% of the entire population of cells is ever found in the medium.

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Stoichiometry of the Pyrimidine Deoxyribonucleoside 2′-Hydroxylase Reaction and of the Conversions of 5-Hydroxymethyluracil to 5-Formyluracil and of the Latter to Uracil-5-carboxylic Acid†

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ABSTRACT: The oxidative demethylation of thymidine by *Neurospora* is thought to involve the following α-ketoglutarate- and O_2-dependent conversions: thymidine to thymine ribonucleoside (2′-hydroxylase reaction), thymine to 5-hydroxymethyluracil (7-hydroxylase reaction), 5-hydroxymethyluracil to 5-formyluracil, and 5-formyluracil to uracil-5-carboxylic acid. Subjection of extracts of *Neurospora* to a purification scheme, which includes calcium phosphate gel, ammonium sulfate, Sephadex G-150, and DEAE-cellulose fractionation procedures, yielded several enzyme fractions. One contained the 2′-hydroxylase and none of the other enzymes involved in the demethylation of thymidine and another fraction contained the 7-hydroxylase as well as the capacity to oxidize 5-hydroxymethyluracil and 5-formyluracil but no detectable 2′-hydroxylase activity. Using these enzyme fractions the oxidation of thymidine, 5-hydroxymethyluracil, and 5-formyluracil was shown to be coupled to the decarboxylation of α-ketoglutarate so that the oxidized product, succinate, and CO_2 are produced in a 1:1:1 molar ratio. In addition, the 2′-hydroxylase reaction was shown to be stimulated by inclusion of catalase in the incubation mixture.

The conversion of thymidine to the pyrimidines of RNA (Fink and Fink, 1962) is thought to be effected by the following enzymatic reactions: thymidine to thymine ribonucleoside, pyrimidine deoxyribonucleoside 2′-hydroxylase reaction (Shaffer *et al.*, 1968); thymine ribonucleoside to thymine plus ribose (Shaffer *et al.*, 1972); thymine to 5-hydroxymethyluracil, thymine 7-hydroxylase reaction (Abbott *et al.*, 1967); 5-hydroxymethyluracil to 5-formyluracil (Abbott *et al.*, 1968); 5-formyluracil to uracil-5-carboxylic acid (Watanabe *et al.*, 1970); and uracil-5-carboxylic acid to uracil and CO_2, uracil-5-carboxylic acid decarboxylase reaction (Palmatier *et al.*, 1970). The 2′-hydroxylase, which catalyzes the first step in this pathway, also converts deoxyuridine to uridine and requires α-ketoglutarate, Fe^{2+}, ascorbate (Shaffer *et al.*, 1968), and O_2 for activity (Shaffer *et al.*, 1972). Although the role of α-ketoglutarate had not been studied in this reaction, it has been shown that α-ketoglutarate is decarboxylated in the thymine 7-hydroxylase reaction (Holme *et al.*, 1970; Mc-

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