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Studies on the Effects of Simple Sugars on Mammalian Cells in Culture and Characterization of the Inhibition of 3T3 Fibroblasts by L-Fucose[1]

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SUMMARY

Studies concerning the effects of naturally occurring simple sugars on the morphology and metabolism of various mammalian cell lines further illustrate the selective effects of different individual sugars on specific cell lines. Attempts to characterize the selective effects of L-fucose on 3T3 fibroblasts showed that the marked changes in morphology and inhibition of incorporation of radioactive precursors were reversible. Also, the plating efficiency of 3T3 cells grown in medium containing L-fucose for 24 hours was not diminished. Studies of the sequence of inhibition of incorporation of radioactive precursors in fucose-containing cultures showed that uridine incorporation was decreased before leucine and thymidine. In contrast to the striking effects of L-fucose on rapidly growing cultures of 3T3 cells, the addition of this sugar to confluent cultures caused no apparent effects. The mechanisms by which L-fucose causes these changes in 3T3 cells are unknown. However, it does not appear to be caused directly by interference with the uptake or utilization of glucose. Furthermore, the selective inhibition by L-fucose appears to require very little of this sugar to be taken up by the cells. Parallelisms between the characteristics of inhibition by L-fucose and those of cell contact inhibition of mitosis raise the possibility that similar mechanisms operate in both phenomena. Regardless of the mechanisms responsible for the selective effects of L-fucose, the results suggest that naturally occurring sugar constituents of cells may play a unique role in influencing cell growth and metabolism.

INTRODUCTION

In previous studies it was found that the pattern of growth and metabolism of several mammalian cell lines were altered by the addition of certain simple sugars to culture medium (4). These effects were selective in that (*a*) some, but not all, cell lines were altered by at least one of the sugars tested; (*b*) of the cell lines which were affected by sugars, not all lines were most strikingly altered by the same sugars; and (*c*) each cell line which was affected by a specific sugar was not affected by several closely related sugars.

In the present study, the effects of simple sugars is further delineated by comparing responses of a wider variety of cell lines. In addition, the selective effect of L-fucose on one cell line (3T3 fibroblasts) (19) is further characterized. The results demonstrate that certain naturally occurring saccharide constituents of mammalian glycoproteins and cell structures, when added to cells in culture, cause drastic alterations in the morphology and metabolism of certain cell lines. The mechanisms by which these sugars cause these profound effects is still unclear, but the characteristics of these effects raise the possibility that the saccharide constituents of macromolecules may play a unique role in affecting cell growth and metabolism.

MATERIALS AND METHODS

Cell Lines. Identification and characteristics of the cell lines used in the study are described in Table 1.

Media. Waymouth's medium containing 10% calf serum and antibiotics (penicillin 50 units/ml, streptomycin 50 μg/ml, and kanamycin 30 μg/ml) were used in all experiments (23). Waymouth's medium contains 5 mg/ml of glucose. Each sugar to be tested for its effect was added to complete Waymouth's medium so as to provide 12.5 mg/ml of *added* sugar. The sugars used include: D-glucose, D-galactose, and D-mannose (Fisher Scientific Co.); L-mannose and *N*-acetyl-D-mannosamine (Cal. Biochem. Co.); *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine (Sigma Chem. Co.); D-fucose (Mann Research Lab., Inc.); and L-fucose (Mann Research Lab., Inc. and Sigma Chem. Co.).

Methods of Culture. The methods used for the preparation of cell suspensions and for monolayer cultures were the same as those employed in previous studies (4, 5). In each experiment the general procedure was to inoculate an equal number of cells either into 30-ml tissue culture Falcon plastic flasks (for morphologic studies) or 2-, 4-, or 6-oz Flint glass bottles (for studies of cell growth and metabolism). After 24 hours, the medium was decanted and replaced with Waymouth's medium containing additional substances appropriate for the individual experiment. In most experiments where incorporation of leucine-1-^{14}C, uridine-2-^{14}C, and thymidine-2-^{14}C were studied,

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the isotope was added to the cultures 18 hours before harvesting. The isotopes were added to cell cultures so as to provide a final concentration of 200 mμc/ml of leucine, 20 mμc/ml of uridine, and 70 mμc/ml of thymidine in the culture medium. Variations in the details of the procedures are described where used (see table legends).

Assay for Protein and Radioactivity. Total cell protein was determined by the method of Lowry *et al.* (13) on cells lysed with deoxycholate. This measurement was used as an evaluation of cell growth. The incorporation of radioactive isotopes into trichloracetic acid (TCA) insoluble residues of cells was determined by scintillation counting as previously described (11).

Cytology. Replicate cultures were examined periodically with an inverted microscope during growth. When an experiment was terminated, the medium was decanted from the culture bottle, and the adherent monolayer was washed with saline and fixed for 24 hours with Kahle's solution. The monolayer was washed twice with deionized water and stained by Giemsa's method.

RESULTS

Selectivity of Effects of Simple Sugars on the Cell Morphology and Pattern of Growth of Different Cell Lines. A variety of cell lines were cultured in the presence of several simple sugars in order to determine if any of the sugars would cause gross changes in the morphology or pattern of growth of the individual lines. Table 1 depicts the usual morphologic characteristics of each of these cell lines and the degree to which changes occurred in cultures to which either L-fucose or D-mannose was added. The effects of these two sugars on the different cell lines are the only ones listed because the other sugars tested, *viz.*, D-glucose, D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-mannosamine caused no apparent gross morphologic alterations in any of the cell lines screened. The effects of L-fucose and D-mannose varied depending on the cell line tested. The morphology of some cell lines was markedly altered when grown in medium containing L-fucose and minimally and inconsistently altered in medium containing D-mannose (e.g., 3T3 and BHK_{21}). In other cell lines, the reverse was observed. Dramatic alterations in morphologic appearance occurred with D-mannose and little if any effect was seen in L-fucose-containing cultures (e.g., BSC-1 and HeLa Ch). In some cell lines (e.g., HeLa S_3 spinner cells adapted to monolayer culture and in the mouse L-cell line—NCTC clone 929L), neither L-fucose or D-mannose appeared to cause any morphologic change.

Of the cell lines which appeared to be susceptible to alteration with L-fucose, all were not affected to the same degree. 3T3 was the cell line which was the most dramatically and consistently altered by this sugar (Fig. 1). Cells of this line grown in the presence of L-fucose appeared more spindle-shaped, and few dividing cells were seen. Individual cells in such cultures tended to remain isolated from one another and did not achieve the confluent pattern found in cultures grown in the presence of each of the other seven sugars studied. Other closely related cell lines such as 3T6, a fibroblast line derived from the same species as 3T3, showed considerably less change with L-fucose, and 3T3 cells transformed by oncogenic viruses such as SV40 and polyoma virus also were substantially less altered by L-fucose than the ordinary 3T3 cells. In these lines the degree of susceptibility to morphologic alteration by L-fucose paralleled the degree of inhibition of mitosis ordinarily exhibited by these lines in confluent cultures.

Several cell lines derived from monkey kidney and from certain human tissues were selectively altered by D-mannose. For example, the established African green monkey kidney cell line BSC-1 was consistently altered by this sugar. BSC-1 cells grown in D-mannose were more spindle-shaped, and the individual cells appeared larger than those grown in medium containing each of the other sugars tested (Fig. 2). Furthermore, there was a diminution in the extent of cell overlapping, and the monolayer appeared less confluent. HeLa Ch cells were also selectively altered by D-mannose (Fig. 3). The cells of this line ordinarily grew in tightly packed clusters (Fig. 3*a*). However, when cultured in media containing D-mannose, the HeLa Ch cells were more loosely associated (Fig. 3*b*).

It should be emphasized that the consistency of response to a sugar depended on the cell line used. Some cell lines showed very little variation in response from experiment to experiment —e.g., in every experiment the morphology and pattern of growth of 3T3 fibroblasts were altered by L-fucose and was never altered by D-mannose. Also, BSC-1 cultures were consistently altered by D-mannose and not by L-fucose. On the other hand, some cell lines were more variable in their responses. For example, in most experiments the morphology of human skin fibroblasts were clearly altered by D-mannose and minimally altered by L-fucose. However, in a few experiments L-fucose seemed to cause substantial changes in cell morphology, and occasionally the effects of D-mannose were not impressive. The established cell lines capable of continuous cultivation *in vitro* generally were most consistent in their degree of response to individual sugars, whereas the responses of primary cell lines were generally more variable.

Selectivity of Effects of Simple Sugars on the Metabolism of Different Cell Lines. In addition to selectively altering the morphologic appearances of different cells, individual sugars also selectively affected the growth of certain cell lines (Table 1). As previously reported, 3T3 mouse fibroblasts showed marked inhibition of growth and incorporation of labeled leucine and uridine per mg of cell protein when cultured in medium with L-fucose (5). Equal amounts of other sugars added to the medium, including D-fucose, did not cause similar effects. It is of interest that D-mannose, which caused no morphologic changes in the cells nor any significant decrease in incorporation of labeled leucine or uridine, did occasionally cause a slight decrease in average cell protein (5). This one inconsistent effect of D-mannose was never found to be as great as that consistently observed with L-fucose.

In contrast to 3T3 mouse fibroblasts, the incorporation of radioactive leucine and uridine into BSC-1 cells was not selectively inhibited by growth in medium containing L-fucose (Table 2). Instead, in this cell line, D-mannose was the sugar which appeared to be most effective in inhibiting growth and incorporation of leucine and uridine. The decrease in average cell

Table 1

	Cell characteristics		L-Fucose[b]		D-Mannose[b]		Reference on cell characteristics and origin of lines[e]
	Morphology	Contact[a] inhibition	Growth[c]	Morphology[d]	Growth[c]	Morphology[d]	
3T3	Thick bipolar fibroblasts	++++	++++	Increase spindle shaped; increase in size; few mitoses	± to +	No change	19
Polyoma virus transformed 3T3	Stellate-shaped fibroblasts	+	++	No change	0	No change	20
SV40 transformed 3T3	Spindle-shaped fibroblasts	±	+	No change	0	No change	20
3T6	Spindle-shaped fibroblasts	+	+ to ++	No change	0	No change	19
C3H mouse cell line NCTC clone 929-L	Thick bipolar fibroblasts	±	0	No change	0	No change	16
Baby hamster kidney BHK_{21}	Spindle-shaped fibroblasts grow in parallel array	++	++ to +++	Bipolar thick fibroblasts; cells rounder	+	No change	17
Established green monkey kidney BSC-1	Thick bipolar fibroblasts	++	± to +	No change	++ to +++	Cells more spindle shaped and remain separate; increase in cell size	12
HeLa Ch	Cuboidal cells grow as closely packed cell clusters	+ to ++	0	No change	± to ++	Cells less closely associated in clusters	11
HeLa S_3 spinner	In monolayers grow as round cells in clumps	± to 0	0	No change	0	No change	15
Human skin fibroblasts	Spindle-shaped fibroblasts grow in parallel array	++	0 to ++	Variable increase in spindle shape	+ to ++	Cells generally more spindle shaped	6

Characteristics of cell cultures and comparison of the effects of L-fucose and D-mannose on growth and morphology of several mammalian cell lines.

[a] Contact inhibition is used here as described in Footnote 4. ++++, a very high degree of contact inhibition with little cell overlapping and few mitotic figures are observed in confluent cultures, maximum number of cells achieved in confluent cultures is 5 to 7 $\times 10^4$ cells/sq cm; ++, a moderate degree of contact inhibition, although cells continue to divide in confluent cultures and pack together or form several layers of cells, the maximum number of cells achieved in confluent cultures is about 5×10^5 cells/sq cm; ±, a low degree of contact inhibition, cells continue to divide in confluent cultures forming multilayered piles of cells, the maximum number of cells achieved in confluent cultures is often in excess of 1×10^6 cells/sq cm.

[b] Each of the cell lines was grown in medium to which 12.5 mg/ml of a sugar was added. In each cell line the effects of adding D-glucose, D-galactose, D-mannose, L-fucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, or *N*-acetyl-D-mannosamine were tested. Addition of each of the sugars caused a slight reduction (10 to 20%) in cell growth. However, only L-fucose or D-mannose caused marked changes in certain cell lines. The degree of effects noted in L-fucose or D-mannose cultures is based on a comparison between replicate cultures grown in medium with equal amounts of D-glucose added.

[c] The effects of L-fucose and D-mannose on cell growth was determined by measuring amounts of cell protein per bottle at 3 days after adding medium containing sugar and comparing to the average cell protein of replicate cultures grown in medium containing equal amounts of D-glucose. ++++, 50 to 70% decrease of cell growth; +++, 30 to 50% decrease of cell growth; ++, 20 to 30% decrease of cell growth; +, 10 to 20% decrease of cell growth; ± Variable but slight effect; 0, No measurable effect.

[d] The morphologic appearance and pattern of growth of the mammalian cell cultures was evaluated by viewing replicate cultures daily during culture and after fixation and staining. Morphologic effects were generally maximal at 2 to 3 days after adding medium containing sugar.

[e] The established mouse embryo lines were obtained from Drs. Howard Green and George Todaro at New York University Medical Center. NCTC clone 929-L and the BHK_{21} lines were purchased from Microbiological Associates, Bethesda, Maryland. The BSC-1 line was obtained from Dr. Michael Balsalmo of New York University Medical Center. The HeLa S_3 Spinner line was furnished by Dr. M. Scharff of the Albert Einstein School of Medicine and was adapted to monolayer culture in our laboratory. The HeLa Ch cell line is a clonal derivative of a HeLa cell population maintained in our laboratory for six years. The human skin fibroblasts cell strains were isolated in this laboratory by trypsinizing foreskins from newborn infants.

Table 2

Sugar added	Average cell protein (mg/bottle)[a]	Specific activity (leucine-1-^{14}C incorporation)[b]	Specific activity (uridine-2-^{14}C incorporation)[b]
None	0.470 ± 0.025	42.6 ± 2.5	7.2 ± 0.4
D-Glucose	0.410 ± 0.041	40.8 ± 3.8	6.6 ± 0.4
D-Galactose	0.497 ± 0.028	41.8 ± 1.6	6.8 ± 0.3
D-Mannose	0.310 ± 0.034	34.5 ± 4.6	5.5 ± 0.6
L-Mannose	0.413 ± 0.018	43.6 ± 3.9	6.9 ± 0.3
L-Fucose	0.385 ± 0.015	42.8 ± 5.6	7.2 ± 0.6
N-Acetyl-D-glucosamine	0.405 ± 0.046	41.2 ± 2.7	7.1 ± 0.4
N-Acetyl-D-galactosamine	0.410 ± 0.038	42.4 ± 1.8	7.0 ± 0.5
N-Acetyl-D-mannosamine	0.403 ± 0.042	39.8 ± 2.1	7.0 ± 0.4

Effect of simple sugars on cell growth and on leucine-1-^{14}C and uridine-2-^{14}C incorporation in BSC-1 monkey kidney cell cultures. Cultures were grown for 3 days in Waymouth's medium containing the sugar indicated. Average cell protein was 0.067 mg/bottle at the time medium containing the sugar to be tested was added.

[a] Average of 4 replicate 2-oz bottles.

[b] Specific radioactivity is expressed as cpm per mg of protein × 10^{-3}. Each value is the average of two replicate cultures.

protein seen in L-fucose-containing cultures of BSC-1 cells was minimal and variable from one experiment to another; it never was as great as that observed with D-mannose in this cell line.

HeLa Ch cells whose morphology was markedly altered when grown in medium containing D-mannose (Fig. 3) showed minimal and inconsistent alterations in average cell protein, and incorporation of leucine-1-^{14}C or uridine-2-^{14}C when grown in medium containing any of the sugars including D-mannose. With HeLa S_3 spinner cells adapted to monolayer culture, whose morphology was not altered by any of the sugars, no clear inhibition of growth or incorporation of the isotopes was observed with any of the sugars tested. Similarly, none of the sugars tested appeared to alter growth and incorporation of leucine and uridine in L-cells (NCTC clone 929-L).

General Characteristics of Effects of L-Fucose on 3T3 Fibroblasts

The selective effect of a simple sugar in altering the morphology and metabolism of a particular cell line was consistently and dramatically illustrated by the effects of L-fucose on 3T3 mouse embryo fibroblasts. This cell line was therefore chosen for further studies.

Sequence of Inhibition by L-Fucose of Incorporation of Leucine-1-^{14}C, Uridine-2-^{14}C and Thymidine-2-^{14}C into 3T3 Fibroblasts. A typical experiment, shown in Table 3, indicates the sequence in which incorporation of leucine-1-^{14}C, uridine-2-^{14}C, and thymidine-2-^{14}C is inhibited in 3T3 cells cultured in medium containing added L-fucose. A decrease in incorporation of uridine was detected within a few hours after culturing cells in L-fucose-containing medium, while inhibition of radioactive leucine and thymidine incorporation were not apparent until later.

Comparison of Effects of L-Fucose on Growing and Confluent Cultures of 3T3 Fibroblasts. In contrast to the striking effects of L-fucose on growing cultures of 3T3 cells, the addition of this sugar to confluent 3T3 cultures caused no significant effects on incorporation of radioactive leucine, uridine, or thymidine (Table 4).

Reversibility of Effects of L-Fucose on 3T3 Fibroblasts. When 3T3 cells are grown in L-fucose-containing medium for 24 to 48 hours, the cells assume a different morphologic appearance than cells cultured in medium with other sugars added. The cells appear somewhat larger, have longer stellate processes,

Table 3

Time after adding sugar (hr)	Specific activity[a]					
	Uridine-2-^{14}C		Leucine-1-^{14}C		Thymidine-2-^{14}C	
	D-Glucose	L-Fucose	D-Glucose	L-Fucose	D-Glucose	L-Fucose
2	3.5 ± 0.6	2.8 ± 0.3	3.4 ± 0.2	4.2 ± 0.4	1.1 ± 0.3	1.4 ± 0.1
4	3.9 ± 0.3	2.2 ± 0.5	2.5 ± 0.4	2.8 ± 0.6	1.2 ± 0.1	1.2 ± 0.1
6	4.2 ± 0.4	1.6 ± 0.2	2.8 ± 0.3	2.9 ± 0.6	1.6 ± 0.2	1.6 ± 0.3
18	4.6 ± 0.6	2.8 ± 0.4	3.8 ± 0.2	1.4 ± 0.1	1.8 ± 0.2	0.4 ± 0.1

Sequence of effects of L-fucose on the incorporation of leucine-1-^{14}C, uridine-2-^{14}C, and thymidine-2-^{14}C into 3T3 fibroblast cultures. Cultures were grown in Waymouth's medium containing 12.5 mg of added D-glucose or L-fucose for the time indicated. One hour before harvesting the cultures, 1000 mμc of radioactive isotope were added.

[a] Specific radioactivity is expressed as cpm per mg of protein × 10^{-3}. Each value is the average of 3 cultures.

Table 4

	Average cell protein[a] (mg/bottle)		Specific activity[b]					
			Leucine-1-^{14}C		Uridine-2-^{14}C		Thymidine-2^{14}C	
	D-Glucose	L-Fucose	D-Glucose	L-Fucose	D-Glucose	L-Fucose	D-Glucose	L-Fucose
Growing (non-confluent)[c]	0.135	0.084	48.6	23.8	9.4	4.7	15.4	2.8
Stationary (confluent)[c]	0.518	0.511	15.7	15.1	1.6	1.3	1.2	1.4

Comparison of effect of L-fucose on growing and on confluent 3T3 fibroblast cultures.

[a] Average of 9 replicate 2 oz bottles. Before adding the sugars, the average cell protein was 0.072 mg/bottle in the rapidly growing culture and 0.512 mg/bottle in confluent cultures.

[b] Specific radioactivity is expressed as cpm per mg of protein $\times 10^{-3}$. Each value is the average of 3 cultures.

[c] The sugars were added to the growing culture by decanting the medium and adding fresh Waymouth's medium containing 12.5 mg/ml of added sugar. Since it is known that fresh medium will permit limited cell division in confluent 3T3 cultures (21), the medium in which the confluent cells were maintained was decanted, appropriate sugars were added (12.5 mg/ml), and the medium returned immediately to its original bottle. Radioisotopes were added to the cultures as soon as the cultures were in the medium containing added sugar. All samples were harvested 18 hours later.

and generally appear more "fibroblastic". When the fucose-containing medium is replaced with Waymouth's medium without added sugar, most of the cells resume their usual appearance within a few hours.

Just as the selective effect of L-fucose on the morphologic appearance of 3T3 cells is reversible, so too are the selective effects on the growth and incorporation of leucine-1-^{14}C and uridine-2-^{14}C (Table 5).

Plating Efficiency of 3T3 Cells Grown in Medium with Added L-Fucose. Plating efficiency of cells is generally taken as a critical test for cell viability. After 24 hours of growth in L-fucose-containing medium (12.5 mg/ml), the plating efficiency of 3T3 cells was as great as that found in cells grown in medium to which D-glucose (12.5 mg/ml) was added (Table 6). The plating efficiency of cells grown in medium containing either added D-glucose or added L-fucose was less than that found in cells which were cultured in Waymouth's medium with no added sugar. This slight, apparently "nonspecific" decrease in plating efficiency was also observed when other sugars were added to the medium in a concentration of 12.5 mg/ml. Thus, after 24 hours of growth in medium with added L-fucose, there was no apparent *selective* decrease in the efficiency of plating of 3T3 cells.

After 48 hours of growth in medium containing 12.5 mg/ml L-fucose, there was a modest decrease in the ability of 3T3 cells to establish colonies as compared to cells grown for 48 hours in medium with added glucose (Table 6). In two experiments, after 72 hours of growth in L-fucose-containing medium, the plating efficiency was reduced to about one-half of that observed in replicate cultures grown in D-glucose.

Table 5

Sugar added	Average cell protein (mg/bottle)[a]	Specific activity[b] (leucine-1-^{14}C incorporation)[c]	Specific activity[b] (uridine-2-^{14}C incorporation)[d]
D-Glucose 1 day	0.260 ± 0.035	14.6 ± 1.1	3.6 ± 0.6
L-Fucose 1 day	0.166 ± 0.020	7.8 ± 0.8	1.9 ± 0.4
D-Glucose 2 days	0.495 ± 0.056	13.5 ± 0.5	3.4 ± 0.8
L-Fucose 2 days	0.240 ± 0.027	6.8 ± 0.4	1.4 ± 0.6
D-Glucose 1 day, no added sugar 1 day	0.530 ± 0.042	12.6 ± 1.8	2.8 ± 0.1
L-Fucose 1 day, no added sugar 1 day	0.430 ±0.048	11.4 ± 1.2	3.1 ± 0.5

Reversal of the effects of L-fucose on 3T3 fibroblast cultures. Each of the cultures was fed daily with the medium indicated. The concentration of added sugar was 12.5 mg/ml. Average cell protein was 0.120 mg/bottle at the time medium containing the sugar to be tested was first added.

[a] Average of 4 replicate 4 oz bottles.

[b] Specific radioactivity is expressed as cpm per mg of protein $\times 10^{-3}$. Each value is the average of 2 cultures.

[c] 200 mμc of L-leucine-1-^{14}C were added 6 hours before the cultures were harvested.

[d] 20 mμc of uridine-2-^{14}C were added 6 hours before the cultures were harvested.

Table 6

Medium in which cultures were grown before cloning	Percent plating efficiency 24 hr	48 hr
Waymouth's medium	39.8	37.6
Waymouth's medium + D-glucose (12.5 mg/ml)	28.2	30.5
Waymouth's medium + L-fucose (12.5 mg/ml)	30.4	24.2

Plating efficiency of 3T3 fibroblasts after growing in D-glucose and L-fucose. Replicate cultures were grown in Waymouth's medium or Waymouth's medium with 12.5 mg of added sugar. Following 24 and 48 hours of growth, the medium was decanted and replaced with Waymouth's medium without added sugar. Four hours later the cells were harvested and counted, and 500 to 800 cells were inoculated into flat-bottomed bottles (surface area 55 sq cm) containing 10 ml of Waymouth's medium with 20% calf serum and 10% fetal calf serum. After incubating these cells for 10 days, the medium was decanted, the cell colonies were washed twice with buffer, and the colonies were fixed, stained with Giemsa, and counted. The percent plating efficiency = $\frac{\text{No. of colonies}}{\text{No. of cells inoculated}} \times 100$. Each value shown is the average of 4 replicate cultures.

Studies on the Mechanism of L-Fucose Effects

The mechanism(s) by which L-fucose selectively inhibits the metabolism of 3T3 cells is unknown. The purpose of the following experiments was to investigate the means by which L-fucose might be inducing its effects.

Effect of Additional Glucose or Pyruvate on the Inhibition of Metabolism of 3T3 Cells by L-Fucose. One means by which L-fucose could be altering the metabolism of 3T3 cells is by interfering with the uptake or utilization of glucose. It was therefore of interest to determine whether the addition of more glucose to cultures of 3T3 cells would alter the effects of L-fucose. As shown in Table 7, additional glucose did not appear to interfere with the effects of L-fucose. In this experiment the concentration of L-fucose was reduced to 6 mg/ml, and the total glucose content of Waymouth's medium supplemented with glucose was 11 mg/ml, so as to avoid greater nonspecific effects at higher total sugar concentrations. Still the added glucose did not diminish the inhibitory effect of L-fucose.

Since it has been found that pyruvate enhances growth in medium containing suboptimal amounts of glucose (3), it was also of interest to determine whether adding pyruvate to the medium might reverse the effects of L-fucose. However, pyruvate, at a concentration of 2 mg/ml, was also ineffective in altering the effects of L-fucose.

Effect of L-Fucose on the Uptake and Utilization of Glucose-U-^{14}C by 3T3 Fibroblasts. The evidence that adding pyruvate or additional glucose to Waymouth's medium was ineffective in reversing the effects of L-fucose does not necessarily mean that glucose is taken up and utilized in a normal manner by 3T3 cells. In order to investigate this problem further, labeled glucose was added to 3T3 cell cultures grown in medium containing L-fucose, and the amounts of label present in TCA-soluble and -insoluble fractions were determined (Table 8). The total uptake of radioactivity derived from glucose-U-^{14}C in cultures of 3T3 cells grown in medium without sugar added or with *N*-acetyl-D-glucosamine added was greater than in replicate cultures grown in medium containing L-fucose. This might be expected, since cell multiplication is markedly inhibited in L-fucose-containing cultures and is only slightly decreased in cultures grown with *N*-acetyl-D-glucosamine or other sugars added. When uptake and incorporation of the label is calculated on the basis of radioactivity per mg cell protein in these cultures, there is no clear difference in L-fucose-containing cultures and the control cultures (Table 8); *N*-acetyl-D-glucosamine was used here for comparison, since it is one of the sugars which does not appear to cause selective alterations in 3T3 fibroblasts (5) and would be unlikely to compete with glucose as an energy source.

Uptake and Utilization of L-Fucose-1-^{14}C by 3T3 Fibroblasts. L-fucose-1-^{14}C (0.1 mg/ml) was added to Waymouth's medium, and the radioactivity of TCA-soluble and -insoluble cell residues and radioactivity remaining in the medium was assayed at various time intervals. In this experiment no unlabeled fucose was added to the cultures. As shown in Table 9, the amount of radioactivity associated with TCA-insoluble cellular material was extremely small, and only a minimal decrease in the radioactivity of the supernatant medium could be de-

Table 7

Sugar added	Average cell protein (mg/bottle)[a]	Specific activity (leucine-1-^{14}C incorporation)[b]	Specific activity (uridine-2-^{14}C incorporation)[b]
None	0.250 ± 0.05	37.1 ± 7.0	8.2 ± 0.5
D-Glucose 12 mg/ml	0.225 ± 0.01	38.8 ± 3.2	7.8 ± 0.3
D-Glucose 6 mg/ml	0.280 ± 0.02	40.4 ± 1.5	9.1 ± 0.4
L-Fucose 6 mg/ml	0.142 ± 0.01	18.8 ± 1.2	5.1 ± 0.8
L-Fucose 6 mg/ml + D-glucose 6 mg/ml	0.150 ± 0.03	15.8 ± 2.0	4.9 ± 0.6

Failure of additional glucose to reverse the effects of L-fucose on 3T3 fibroblast cultures. Cultures were grown for 2 days in Waymouth's medium containing the sugar indicated. Average cell protein was 0.089 mg/bottle at the time medium containing the sugar to be tested was added.

[a] Average of 4 replicate 2 oz bottles.

[b] Specific radioactivity is expressed as cpm per mg of protein × 10^{-3}. Each value is the average of 2 replicate bottles.

Table 8

Added sugar	1 hr TCA Insoluble	1 hr TCA Soluble	3 hr TCA Insoluble	3 hr TCA Soluble	6 hr TCA Insoluble	6 hr TCA Soluble	18 hr TCA Insoluble	18 hr TCA Soluble
None	0.51[a]	1.37	0.85	1.45	1.09	1.80	Not done	Not done
N-Acetyl-D-glucosamine	0.59	1.40	0.99	1.60	1.31	1.46	2.75	1.30
L-Fucose	0.56	1.33	1.07	1.60	1.18	1.84	2.05	1.37

Effect of L-fucose and *N*-acetyl-D-glucosamine on the uptake and incorporation of glucose-U-^{14}C by 3T3 fibroblast cultures. Replicate cultures were grown in Waymouth's medium alone or with 12.5 mg/ml *N*-acetyl-D-glucosamine or L-fucose and 500 mμc of glucose-U-^{14}C for the times indicated. Prior to harvesting, the culture bottles were cooled at 0°C for 10 minutes and then washed three times with ice cold buffer containing 0.5% glucose and 12.5 mg/ml of the appropriate sugar. The washed cultures were then air dried and harvested by lysis in 0.5% deoxycholate in 0.9% NaCl.

[a] Specific radioactivity, expressed as cpm per mg of protein [trichloracetic acid (TCA)-insoluble or -soluble fractions] × 10^{-3}. Each value is the average of 2 replicate cultures.

Table 9

Time after adding L-fucose-1-^{14}C	Specific activity of cells[a] TCA insoluble	TCA soluble	Medium (cpm/ml/ × 10^{-4})	Calculated maximum incorporation (pg L-fucose/mg cell protein)
1 minute	190	21	35.6	
48 hr	1450	1018	34.9	28
72 hr	1721	1383	34.4	34

Uptake and incorporation of L-fucose-1-^{14}C by 3T3 fibroblast cultures. Replicate cultures were grown in Waymouth's medium containing 0.1 mg/ml of L-fucose-1-^{14}C (250 mμc/ml). TCA, trichloracetic acid.

[a] Cell values are the average of 2 cultures. Specific radioactivity is expressed as cpm/mg protein.

tected during the course of the experiment. When the supernatant was analyzed by ascending paper chromatography (Whatman #1) utilizing pyridine:*n*-butyl alcohol:water solvent (4:6:3) and developed in a silver nitrate dip (1, 22), all of the radioactivity coincided with L-fucose. When ^{14}C-labeled L-fucose was added to cultures containing 5 or 12.5 mg of unlabeled fucose per ml, the amount of radioactivity associated with TCA-insoluble cellular material was only slightly above background.

Effect of Guanosine on the Inhibition of Uridine Incorporation in 3T3 Fibroblasts by L-Fucose. L-fucose might produce its effect by tying up the available pool of guanosine triphosphate (GTP) as guanosine diphosphofucose (10), thereby depleting the availability of GTP for the synthesis of macromolecules such as the nucleic acids. It was therefore of interest to determine if the addition of guanosine to culture medium (200 μg/ml final concentration) would delay the effect of L-fucose on the inhibition of uridine incorporation.[3] Maximum uptake and utilization of guanosine occurs in mouse fibroblasts at this concentration (14). However, addition of guanosine to the medium did not delay or reduce the inhibition of uridine incorporation by L-fucose.

DISCUSSION

The present studies illustrate the selective effects of naturally occurring simple sugars on specific cell lines. Attempts to further characterize the selective effect of L-fucose on 3T3 mouse fibroblasts revealed that: (*a*) decrease in incorporation of uridine (presumably RNA synthesis) was detectable before inhibition of leucine or thymidine incorporation (presumably protein and DNA synthesis); (*b*) in contrast to the striking effects of L-fucose in rapidly growing cultures, there was no further measurable reduction in metabolism of confluent cultures of 3T3 cells by L-fucose; (*c*) the marked changes in growth and metabolism induced within 24 hours of growth in L-fucose-containing medium were reversible; and (*d*) the plating efficiency of 3T3 cells grown in L-fucose-containing medium for 24 hours was not selectively reduced. These findings seem of interest not only because they describe some of the characteristics of the effects of L-fucose on 3T3 fibroblasts, but also because of some apparent relationships between them and the characteristics of inhibition of mitosis in confluent cultures of this cell line.[4] For example, the effects of L-fucose on the sequence of inhibition of incorporation of radioactive labels into macromolecules is similar to that observed in confluent cultures of 3T3 cells (G. Todaro, personal communication; 21). Also

[3] This experiment was suggested by Dr. Victor Ginsberg of the National Institutes of Health.

[4] Contact inhibition is used here in the sense of restriction of cell growth observed in confluent cultures of 3T3 fibroblasts (18, 21). This usage is especially appropriate for 3T3 cells since, at relatively low population densities (0.5 × 10^5 cells/sq cm), mitosis is completely inhibited (19, 21). Moreover, studies of DNA synthesis in clones of 3T3 cells indicates that, when a cell is surrounded by other 3T3 cells on all sides, DNA synthesis is completely inhibited (9).

the alterations produced by growth in L-fucose for 24 hours, like those occurring in cell contact-inhibited cultures are reversible. Cells inhibited by cell contact for 24 hours or longer resume rapid multiplication when subcultured. 3T3 cells inhibited for 24 hours by L-fucose also have a high plating efficiency. However, after 48 hours in L-fucose, plating efficiency is decreased, while 3T3 cells inhibited by cell contact for a corresponding period of time have a relatively unimpaired cloning efficiency. The finding that L-fucose causes little or no reduction in the metabolism of confluent cultures of 3T3 cells may simply mean that inhibition of metabolism by cell contact is so great in this cell line that further reductions by any means short of extensive cell damage would be difficult to detect. However, the fact that L-fucose does not further reduce the low levels of isotope incorporation in cell contact-inhibited cultures raises the possibility that common mechanisms of inhibition may be operative in both situations.

The similarities between the characteristics of L-fucose inhibition and the properties of contact inhibition[4] in 3T3 fibroblasts described in this report may of course be coincidental, but there are additional relationships between the two phenomena which are of interest. There was a parallelism between the susceptibility to alteration by L-fucose and the degree of contact inhibition ordinarily exhibited by a number of mouse cell lines. For example, 3T3 which was the mouse line found to be most susceptible to alteration by L-fucose, was, correspondingly, the mouse cell line which exhibits the highest degree of contact inhibition of mitosis. Other established mouse cell lines which were less susceptible to alteration by L-fucose, such as 3T6 and L cells, correspondingly exhibit a lesser degree of contact inhibition. Furthermore, when 3T3 cells are transformed by oncogenic viruses, the cells not only lose their property of contact inhibition, but they also become less susceptible to inhibition by L-fucose (5).

These relationships raise the possibility that the mechanisms by which L-fucose inhibits 3T3 cells might be similar to the mechanisms which account for cell contact inhibition. However, this hypothesis is not easily tested, since the mechanisms accounting for cell contact inhibition as well as those operating in L-fucose inhibition are unknown. Several attempts were made to elucidate the mechanism by which L-fucose produces its effects. The results suggest that L-fucose does not inhibit the metabolism of 3T3 cells primarily by interfering with glucose uptake and utilization. Nor does it appear that L-fucose inhibits synthesis of nucleic acids by depleting the available pool of guanosine. Furthermore, it is clear that increased acid production, which has been shown to decrease cell growth in cultures containing high concentrations of certain rapidly metabolized sugars (8), is not responsible for the inhibition induced by L-fucose in this cell line, since the pH of medium does not fall as much in L-fucose-containing cultures of 3T3 cells as in cultures with other sugars added (4). Thus, some of the more apparent means by which L-fucose could have been inhibiting 3T3 cells do not appear to explain the effect. Whatever the mechanism is, it would seem to require very little utilization of L-fucose by the cells, since exceedingly small amounts of radioactivity are found in cellular fractions of 3T3 cultures grown in medium containing labeled L-fucose.

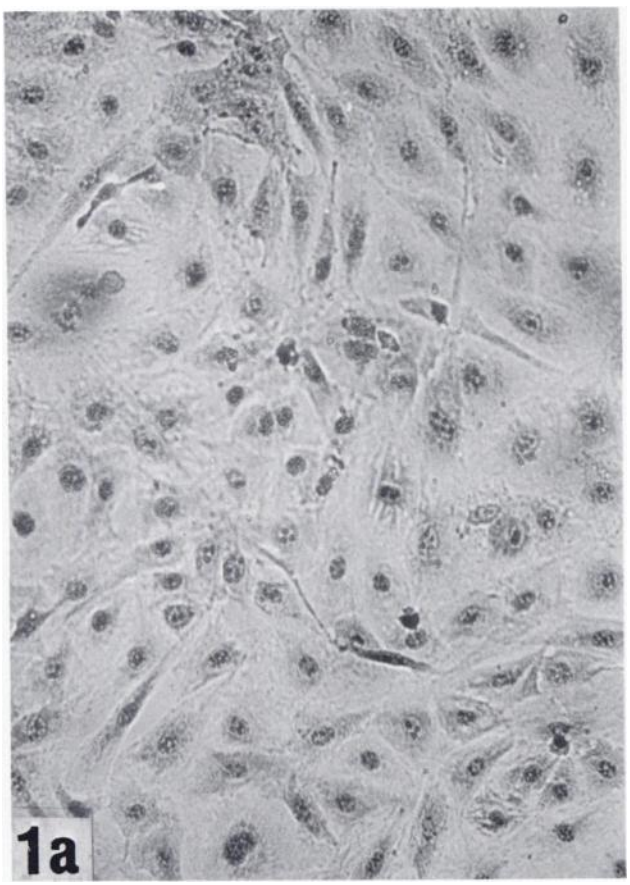
If the mechanisms whereby L-fucose inhibit 3T3 cells and those which occur in cell contact inhibition are related, one possible explanation for the parallelisms between the phenomena might be that L-fucose produces its effects by substituting for a fucose-containing natural constituent of 3T3 cells, which, when combining with complementary structures at the surface or within susceptible cells, induces the cells to undergo the changes characteristic of cell contact inhibition. Such a fucose-containing constituent might occur at the surface of normal cells and act by combining with complementary sites at the surfaces of susceptible cells, or it might occur as a macromolecular constituent of normal cells which gains entrance into susceptible cells and acts intracellularly. If contact inhibition is due to an increase in fucose-containing macromolecules, then L-fucose might produce its effects not only by substituting for these macromolecules, but also by causing an increase in the synthesis of fucose-containing macromolecules through an increase in the production of guanosine diphosphate L-fucose. Since, in some cells, guanosine diphosphate D-mannose can be converted to GDP-fucose, such a mechanism might account for the slight effect of D-mannose on some cells primarily susceptible to L-fucose. It has been suggested by several investigators that saccharide-containing structures play a role in cell contact inhibition (2, 7).

While all of the evidence concerning the effects of L-fucose on 3T3 cells and related lines is consistent with the preceding speculation, it should be emphasized that the mechanisms accounting for the effects of L-fucose are unknown, and that parallelisms with cell contact inhibition may be coincidental. Furthermore, it should be noted that the mechanism which accounts for the selective effects of D-mannose on other cell lines are also unknown, and whether both L-fucose and D-mannose produce their effects by the same mechanisms are unclear. Nevertheless, the findings demonstrate that naturally occurring saccharide constituents can profoundly alter the metabolism of selected mammalian cells, and this raises the possibility that the saccharide components of mammalian glycoproteins and cell structures might play a unique role in the regulation of cell growth and metabolism.

REFERENCES

1. Anet, E. F. L. J., and Reynolds, T. M. Isolation of Mucic Acid from Fruits. Nature, *174:* 930, 1954.
2. Bürk, R. R. Growth Inhibitor of Hamster Fibroblast Cells. Nature, *212:* 1261–1262, 1966.
3. Chang, R. S., and Geyer, R. P. Propagation of Conjunctival and HeLa Cells in Various Carbohydrate Media. Proc. Soc. Exptl. Biol. Med., *96:* 336–340, 1957.
4. Cox, R. P., and Gesner, B. M. Effect of Simple Sugars on the Morphology and Growth Pattern of Mammalian Cell Cultures. Proc. Natl. Acad. Sci. U. S., *54:* 1571–1579, 1965.
5. Cox, R. P., and Gesner, B. M. Comparison of Effects of Simple Sugars on 3T3 Cells Transformed by Infection with Oncogenic Viruses. Cancer Res., *27:* 974–979, 1967.
6. Cox, R. P., and MacLeod, C. M. Regulation of Alkaline Phosphatase in Human Cell Culture. Cold Spring Harbor Symp. Quant. Biol., *29:* 233–251, 1964.

7. Defendi, V., and Gasic, G. Surface Mucopolysaccharides of Polyoma Virus Transformed Cells. J. Cellular Comp. Physiol., *62:* 23–31, 1963.
8. Fare, G., Sammons, D. C. H., Seabourne, F. A., and Woodhouse, D. L. Lethal Action of Sugars on Ascites Tumor Cells *In Vitro*. Nature, *213:* 308–309, 1967.
9. Fisher, H. W., and Yeh, J. Contact Inhibition in Colony Formation. Science, *155:* 581, 1967.
10. Ginsberg, V. Studies on the Biosynthesis of Guanosine Diphosphate L-Fucose. J. Biol. Chem., *236:* 2389–2393, 1961.
11. Griffin, M. J., and Cox, R. P. Studies on the Mechanism of Hormone Induction of Alkaline Phosphatase in Human Cell Cultures. II. Rate of Enzyme Synthesis and Properties of Base Level and Induced Enzymes. Proc. Natl. Acad. Sci. U. S., *56:* 946–953, 1966.
12. Hopps, H. E., Bernheim, R. C., Nesalak, A., Tjio, J. H., and Smadel, J. E. Biological Characteristics of a Continuous Kidney Cell Line Derived from the African Green Monkey. J. Immunol., *91:* 416–424, 1963.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurements with Folin Phenol Reagent. J. Biol. Chem., *193:* 265–275, 1951.
14. McFall, E., and Magasanik, B. The Control of Purine Biosynthesis in Cultured Mammalian Cells. J. Biol. Chem., *235:* 2103–2108, 1960.
15. Puck, T. T., Marcus, P. I., and Cieciura, S. J. Clonal Growth of Mammalian Cells In Vitro: Growth Characteristics of Colonies from Single HeLa Cells with and without a "Feeder" Layer. J. Exptl. Med., *103:* 273–284, 1956.
16. Sanford, K. K., Earle, W. R., and Likely, G. D. The Growth In Vitro of Single Isolated Tissue Cells. J. Natl. Cancer Inst., *9:* 229–246, 1948.
17. Stoker, M. G. P., and MacPherson, I. Syrian Hamster Fibroblast Cell Line BHK_{21} and Its Derivatives. Nature, *203:* 1355–1357, 1964.
18. Stoker, M. G. P., and Rubin H. Density Dependent Inhibition of Cell Growth in Culture. Nature, *215:* 171–172, 1967.
19. Todaro, G. J., and Green, H. Quantitative Studies on the Growth of Mouse Embryo Cells in Culture and Their Development into Established Cells. J. Cell Biol., *17:* 299–313, 1963.
20. Todaro, G. J., Green, H., and Goldberg, B. D. Transformation of Properties on an Established Cell Line by SV_{40} and Polyoma Virus. Proc. Natl. Acad. Sci. U. S., *51:* 66–73, 1964.
21. Todaro, G. J., Lazar, G. K., and Green, H. The Initiation of Cell Division in a Contact-Inhibited Mammalian Cell Line. J. Cellular Comp. Physiol., *66:* 325–334, 1965.
22. Trevelyan, W. E., Procter, D. P., and Harrison, J. S. Detection of Sugars on Paper Chromatograms. Nature, *166:* 444–445, 1950.
23. Waymouth, C. Rapid Proliferation of Sublines of N.C.T.C. Clone 929 (Strain L) Mouse Cells in a Simple Chemically Defined Medium (M B 7521/1). J. Natl. Cancer Inst., *22:* 1003–1015, 1959.



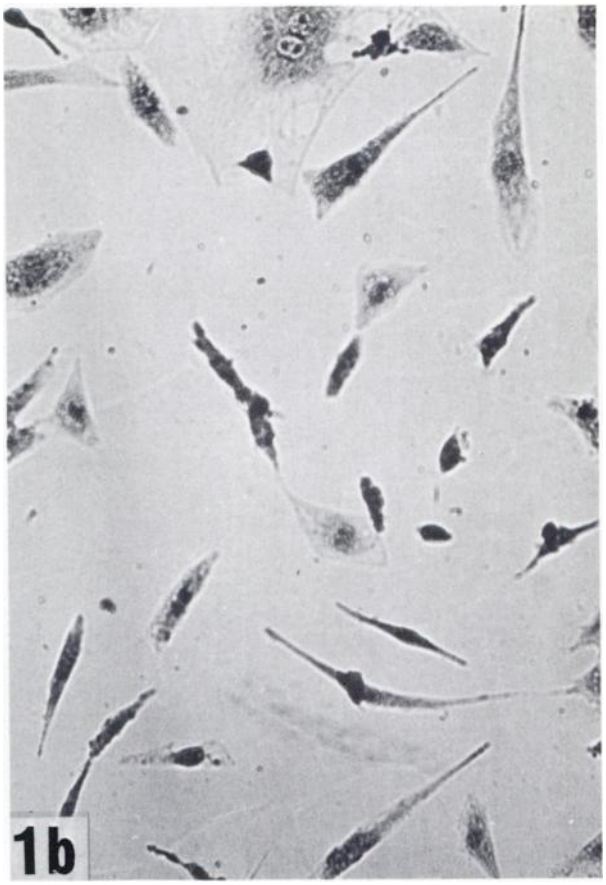


Fig. 1*a*. A representative area of 3T3 mouse fibroblast culture grown for 72 hours in Waymouth's medium containing 12.5 mg/ml D-glucose. Similar patterns of growth were observed with all of the other sugars tested except L-fucose. × 150.

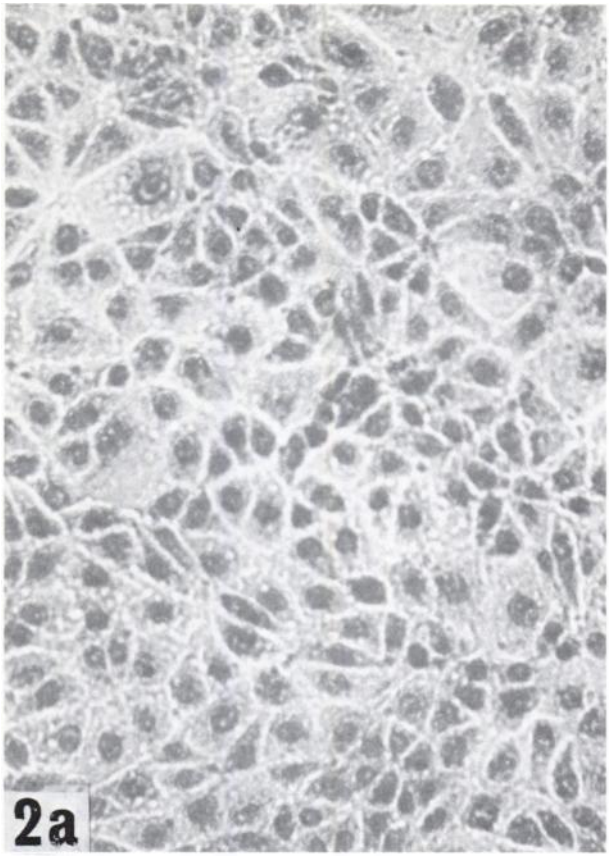
Fig. 1*b*. A representative area of 3T3 mouse fibroblast culture grown for 72 hours in Waymouth's medium containing 12.5 mg/ml L-fucose. Giemsa stain, × 150.

Fig. 2*a*. A representative area of BSC-1 African green monkey kidney culture grown for 72 hours in Waymouth's medium containing 12.5 mg/ml D-glucose. Similar patterns of growth were observed with all of the sugars tested except D-mannose. × 150.

Fig. 2*b*. A representative area of BSC-1 African green monkey kidney culture grown for 72 hours in Waymouth's medium containing 12.5 mg/ml D-mannose. Giemsa stain, × 150.

Fig. 3*a*. A representative area of HeLa Ch cell culture grown for 72 hours in Waymouth's medium containing 12.5 mg/ml D-glucose. Similar patterns of growth were observed with all of the other sugars tested except D-mannose. × 150.

Fig. 3*b*. A representative area of HeLa Ch cell culture grown for 72 hours in Waymouth's medium containing 12.5 mg/ml D-mannose. Giemsa stain, × 150.



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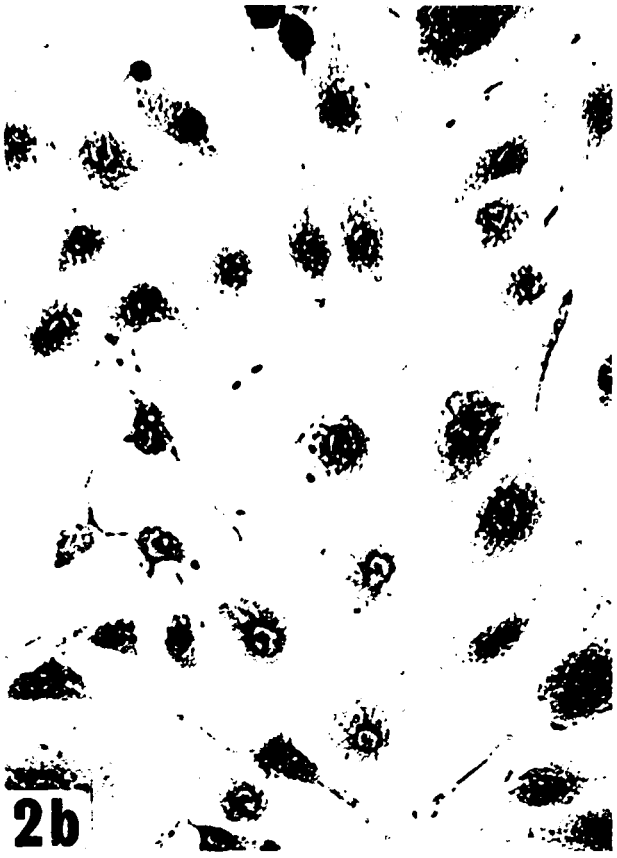

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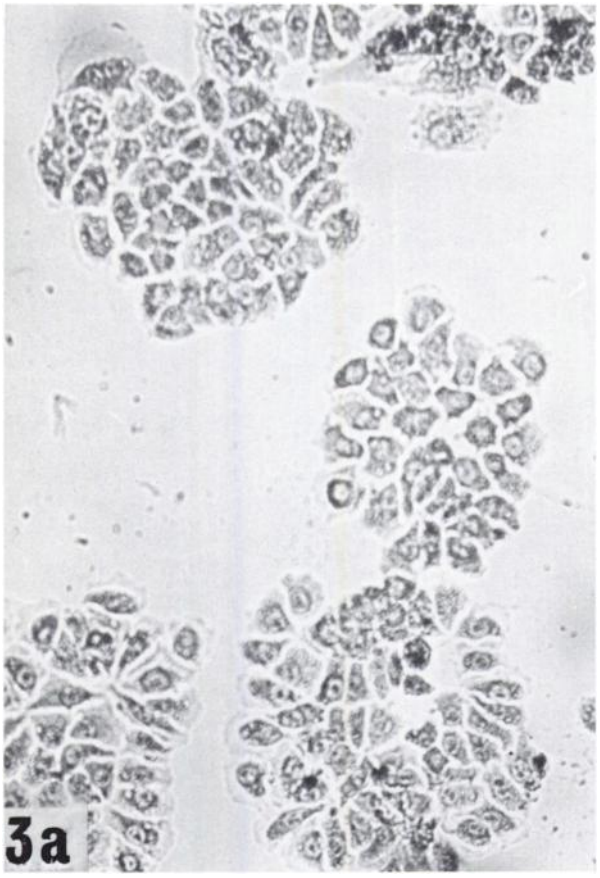

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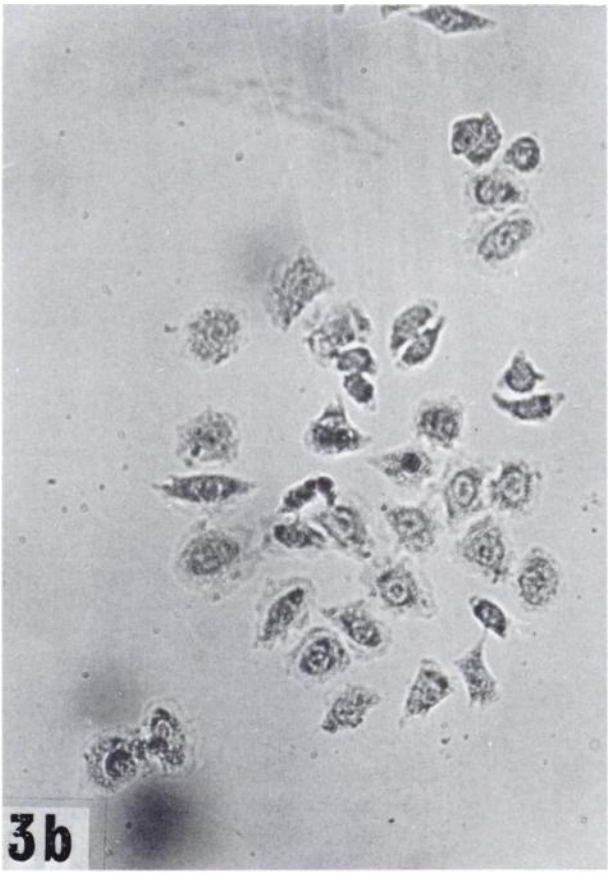

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