GENETIC STUDY OF HUMAN CELLS IN VITRO

CARBOHYDRATE VARIANTS FROM CULTURES OF HELA AND CONJUNCTIVAL CELLS*

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Established laboratory strains of human cells manifest some attributes of individual cells undergoing rapid and continuous multiplication in artificial biochemical environment. As other populations of single cells, variants with properties sufficiently different from those of the parent cultures may be readily isolated through the use of suitable selecting environments. Indeed, several investigators have reported on the successful isolations of variants from cultures of human and animal cells.

Examples are the adaptation of L strain of mouse fibroblast in a chemically defined medium (1), the isolation of variant cultures from human fibroblasts which did not require inositol or choline for growth (2), the isolation of clonal lines of HeLa cell with quantitative differences in their requirement of serum (3), the adaptation of human skin cell to chemically defined media (4), the isolation of variants from the HeLa and conjunctival cells capable of prolonged continuous growth with ribose, xylose, or lactate as sole source of added carbohydrate (5), those HeLa cells with increased resistance to poliomyelitis virus (6), the asparagine independent cultures from Jensen sarcoma cells (7), the increase in resistance to 8-azaguanine of variants from human bone marrow cell (8), and the isolation of fibroblast with increase resistance to puromycin and azaguanine (9). Some of these reports however, contained insufficient experimental data for critical evaluation.

In a previous communication, the propagation of the HeLa and conjunctival cells in various carbohydrate media was described (10). A subsequent paper reported the successful isolation of nutritional variants from these cells which differed from their parent cultures in their ability to multiply continually with ribose, xylose, or lactate as the sole carbohydrate constituent of the nutrient medium (5). Since these variants may provide useful tools in the genetic study of human cells *in vitro*, of the role of carbohydrate metabolism in cell differentiation (11) and of the possible relationship between abnormal carbohydrate metabolism and neoplastic growth (11), this observation was further investigated.

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This report describes the isolation of carbohydrate variants; factors influencing the frequency of appearance of variants; the morphology, growth rate, cloning efficiency and subzero preservation of some established stable variants; the effect of glucose, glucose analogue, and glucose oxidase on their propagation; the incorporation of C^{14}-labelled sugars; the synthesis and composition of polysaccharides, and their biosynthetic mechanisms as indicated by the syntheses of several viruses. The results of heterologous transplantation in hamster cheekpouch will be described in a separate report.

Materials and Methods

Nutrient Media.—Basal carbohydrate-free medium consisted of 10 per cent dialyzed serum in Eagle's basal medium (12) with the following modifications: (*a*) the omission of biotin and glucose; (*b*) the addition of mesoinositol to final concentration of 10 to 20 micromolar; and (*c*) the substitution of Earle's balanced salt solution by Hanks. The carbohydrate under study was added to a final concentration of 5 mM. To facilitate further description, a nutrient medium was named according to the carbohydrate it contained. For example, a ribose medium consisted of the basal carbohydrate-free medium containing 5 mM of ribose per liter.

Dialyzed Serum.—Sera were collected sterilely and stored at below −40°C. within 5 days after bleeding. Dialyzed serum was prepared by dialysis in cellulose tubing (Visking) with thickness and diameter of 0.0008 and 0.8 inches respectively against 10 to 15 changes of 10 to 40 times its volume of 0.85 per cent NaCl at about 0–10°C. for 4 days with constant stirring. Dialyzed serum was sterilized by filtration through Seitz SI sterilizing pads. To minimize possible loss of unidentified growth factor through absorption, at least 500 ml. of serum was passed through each pad of 2 inch diameter. Serum so dialyzed was invariably negative for glucose by the glucose oxidase test. This extent of dialysis has also been found adequate in removing C^{14} activity of 4 serums to which 0.1 μc. of glucose-1-C^{14} was added for each milliliter of serum prior to dialysis. The C^{14} glucose used had been previously dialyzed and the dialyzable portion was used.

Glucose Oxidase Test.—For the detection of the possible presence of glucose, the enzyme paper stick method was used (13). Final reading was made exactly 15 minutes after application of test solution. The minimum concentration of glucose which gave a postive reaction was about 0.05 mM.

Compounds.—Commercially available compounds of highest purity were used. All hexoses and pentoses used in this study were found chromatographically pure at 10 per cent concentration; and, with the exception of D-xylose, D-mannose, and D-galactose, no glucose could be detected at concentration of 2 M by the glucose oxidase test. Xylose, mannose and galactose were treated with glucose oxidase before use as described previously (10). The specific activities of C^{14}-labelled sugars were 2.93, 2.2, 2.45, 0.18, and 4.18 mc. per mM of glucose-C^{14}, ribose-C^{14}, ribose-1-C^{14}, xylose-1-C^{14}, and lactate-2,3-C^{14} respectively.

Human Cell Cultures.—The conjunctival (14) and the HeLa (15) cells were used. Parent cultures used in this study were propagated from single lots of cells preserved at below −70°C. by the method of Scherer (16). The glucose medium was used exclusively. Whenever recognizable degeneration or bacterial contamination appeared in the parent cultures, they were discarded and fresh cultures reinitiated from the frozen materials. It was hoped that alteration in the properties of the parent cultures through unintentional selection may thus be minimized. All cultures were checked at intervals of 1 to 4 weeks and at each critical experiment for possible contamination by pleuropneumonia-like organisms by Edward's method (17); no such contamination has been detected since 1957. Experimental results obtained prior to 1957 were

not included because several cultures were contaminated by the PPLO (18). Evaluation of rate of cell multiplication has already been described (10).

Isolation of Variants.—Parent cultures were propagated in a 200 ml. serum dilution bottle containing 10 ml. of glucose medium at 36°C. The medium was renewed every 2nd day until a confluent sheet of polyhedral cells was formed; such cultures had been found to contain about 2 to 5 million cells. After three successive washings with the basal carbohydrate-free medium, the cells were nourished in a carbohydrate medium under study. About 0.9 of the medium was renewed every 2 to 3 days until the appearance of advanced degeneration and then every 4 to 7 days. Visible colonies were counted at weekly intervals beginning on the 3rd week. If colonies failed to develop in 60 days, the experiment was terminated.

Relative Incorporation of C^{14}-labelled Sugars.—Parent and variant cultures were grown in 200 ml. serum dilution bottles containing 10 ml. of respective carbohydrate media at 36°C. When the total number of cells reached 1 to 3 million per culture, the culture was washed once with 10 ml. of carbohydrate-free medium, and then 10 ml. of carbohydrate-free medium containing 1 μc. of C^{14}-labelled sugar was added. After 24 hours at 36°C., carbon dioxide was trapped by a device described previously (19). The culture was rinsed thoroughly 3 times with 20 ml. each of 0.85 per cent NaCl, and the cells were then dissolved in exactly 10 ml. of water containing sodium lauryl sulfate at 0.025 to 0.05 per cent. Small aliquots of this solution of cell constituents were saved for deoxyribonucleic acid (DNA) and protein determinations, using the diphenylamine (20) or the indole method (21) and Folin's reagent (20). The remainder was fractionated by Schneider's method (22) into acid-soluble, alcohol- and ether-alcohol–soluble (lipids), hot trichloracetic acid–soluble (nucleic acids), and hot trichloracetic acid–insoluble fractions (proteins). In many experiments, the cell number per culture was also determined from replicate culture. In certain other specified experiments, more than 1 μc. of C^{14}-labelled sugar were used.

Composition and Synthesis of Cellular Mucopolysaccharides.—Solutions of cell constituents were prepared as described in the preceding paragraph. Trichloracetic acid was added to a final concentration of about 10 per cent and the mixture was shaken vigorously for 2 hours at about 4°C. and then kept at 0–2°C. for another 48 hours. After centrifugation at 2000 R.P.M. for 20 minutes, the supernatant fluid was dialyzed against 5 changes of about 100 times its volume of distilled water for 24 hours; this dialysis caused the formation of varing amount of white precipitate which was discarded. The water-soluble material was pervaporated to a convenient volume and then mixed with 9 volumes of absolute ethanol containing 1 ml. of 2.5 M sodium acetate. After 7 days at 0–2°C., the precipitate was collected and hydrolyzed in 2 N HCl at 100°C. for 4 hours. The HCl was removed under vacuum and the residue dissolved in several milliliters of water, which was the final product used in various analyses. Preliminary studies showed that the amount of Molisch-positive materials in the trichloracetic acid–insoluble fraction can be readily accounted for by the ribonucleic acid, the trichloracetic acid–soluble but water-insoluble materials contained proteins (Folin reaction) and the carbohydrate (Molisch reaction) at ratio of more than 10 to 1, and the trichloracetic acid– and water-soluble but alcohol-insoluble materials gave a strongly positive Molisch test and a weakly positive Folin test for protein. It was concluded that most of the cellular mucopolysaccharides were present in the last fraction (soluble in 10 per cent trichloracetic acid and in water, but insoluble in 90 per cent alcohol). Approximately 10^8 cells had been found satisfactory as starting material.

Total carbohydrate content was estimated by the standard Molisch test (20) using glucose as standard. Glucose was determined by the quantitative glucose oxidase test and inositol by the standard microbiological assay using *Saccharomyces cerivisiae* as test organism. Hexosamines, hexuronic acids, and pentoses were estimated by the methods of Elson-Morgan cited in reference 20, Dische (23), and Tracy (24) respectively. Since many other sugars are known

to give weakly positive readings in the last 3 tests, their results gave higher values than actually present, especially when the relative concentrations of hexosamines, hexuronic acids, and pentoses were low in comparison to other sugars.

To demonstrate the conversion of glucose and ribose into other hexoses and hexuronic acid, cells were grown in medium containing uniformly labelled C^{14} glucose or ribose as described. The mucopolysaccharides were extracted as described but omitting the step of alcohol precipitation due to the exceedingly small amount of C^{14}-labelled cellular materials available for such a study. After hydrolysis and the removal of amino acids with Dowex-50 column (this procedure also removes hexosamines), the C^{14} activities were analyzed chromatographically. Preliminary experiments showed that mixtures of glucuronate, galactose, glucose, L-sorbose, L-fucose, L-rhamnose, and ribose may be readily resolved into individual components on paper using ethyl acetate:pyridine:water as solvent. Therefore, the C^{14} activities were analyzed using these 7 sugars as carriers. Activities recovered from each spot were taken as evidence that glucose or ribose have been converted into certain other sugars. The well known limitations of paper chromatographical study that a spot may contain other unknown substance with similar R_f value should be taken into consideration in the interpretation of such results.

Virus Synthesis.—Viruses used in this study were polio type 1, Coxsackie group B type 1, adeno type 3, and herpes simplex; the former 2 were presumably RNA while the latter two, DNA viruses. Virus pools were harvested from infected HeLa or conjunctival cells and were dialyzed for 3 hours against 3 changes of about 1000 times their volume of 0.85 per cent NaCl to remove most of the glucose. Cultures infected with 30 ID_{50} doses of virus were observed daily for the appearance and progression of characteristic cytopathogenic changes. Infectivity of culture showing advanced cytopathogenic changes were assayed by the standard tube dilution method.

RESULTS

1. The Isolation of Variants

Two to five days following the replacement of glucose medium by xylose, ribose, arabinose, lactate, or pyruvate medium, evidences of degeneration were invariably present in cultures of HeLa and conjunctival cells. At this time, mitoses were conspicuously absent. In most experiments, the degeneration progressed rapidly to completion by the 2nd or 3rd week, and such experiments were terminated on about the 8th week. In some experiments the degeneration progressed at a slower pace and by the 3rd week many plaques of cells remained. Microscopically, the plaque consisted of healthy polyhedral, rounded granular degenerating and mitotic cells in varying proportions. Many isolated individual cells were also present at the same time. Subsequent observation revealed the frequent disappearance of existing colonies and the appearance of colonies at new sites. Some of the colonies or plaques would consist only of degenerating cells, while others mostly of polyhedral and mitotic cells. By the 8th week serial subcultivation was attempted, some successfully while others, ending in complete degeneration. In still other experiments, the degeneration progressed rapidly; and, by the 3rd week, only occasional cells, usually thin vacuolated and with long processes, were left. By the 6th to 8th week, one to several colonies made their appearance. Like other colonies, some degenerated while others enlarged rapidly detaching partially to form colonies at other sites.

Although variants were considered established after three successful subcultivations, many were lost subsequently through bacterial contamination, technical errors and unidentifiable causes. It should be emphasized that the results of variant isolation were extremely variable, expecially when quantitation of variant frequencies in parent cultures were attempted. To exclude the possibility that complete degeneration following the initial appearance of variants may be due to exposure to trypsin during subcultivation, variant isolations were also attempted without subcultivation for at least 3 months after variants first appeared; in many experiments these variants also degenerated completely. The results of 146 unselected and uncontrolled variant

TABLE I

*Results of 146 Unselected and Uncontrolled Variant Isolations**

Culture	Xylose‡	Ribose	Arabinose	Lactate	Pyruvate	None
Conjunctival (wild)	4/6	0/7	0/4	1/18	0/4	0/14
Conjunctival (low glucose)§	0/2	2/4	0/3	0/4	0/3	0/9
HeLa (wild)	1/7	1/6	0/6	1/11	0/2	0/14
HeLa (low thiamine)‖	2/2	2/2	2/2	4/4	2/2	0/7

* Results expressed as the number of successful isolation/total number of trials.

‡ Basal carbohydrate media to which various carbohydrates were added to final concentration of 5 mM.

§ Culture propagaged in a 0.1 mM glucose medium for over 6 months.

‖ Culture propagated in a glucose medium without thiamine for over 1 year.

isolations attempted during 1957 and 1958 are summarized in Table I. These results suggest that the outcome of variant isolations may be affected by the parent culture (see HeLa, low thiamine) and by the carbohydrate used (xylose and ribose in contrast to arabinose). Of interest is the complete failure in isolating variants capable of multiplying in the basal carbohydrate-free medium.

2. Some Factors Affecting the Outcome of Variant Isolation

The great variability of results of variant isolation prompted us to investigate certain factors, inherent in the cell culture system, that may affect such results. It has been possible to demonstrate through controlled experiments that the parent culture, source of dialyzed serum and the carbohydrate medium may contribute to such variabilities. Table II shows the results of one such experiment in which 4 parent cultures, 3 different dialyzed horse sera, and 2 carbohydrates were investigated. It should be emphasized that all other factors were controlled in so far as was possible; 6 cultures from each cell were replicate culture, the same 6 media (3 sera and 2 carbohydrates) were used for

the corresponding culture of each cell and the cultures were treated in a similar way at all times. By comparing the number of colonies appearing in cultures 4, 5, and 6 with those in 16, 17 and 18, the effect of parent culture was quite apparent. Similarly, those of cultures 3 and 5 demonstrated the effect of serum lots; and, Nos. 3 and 4, the carbohydrates. Of interest was the considerable

TABLE II

Effect of Parent Culture, Dialyzed Serum, and Carbohydrate on the Appearance of Variant Colonies

Culture No.	Parent culture	Serum	Media	No. of colonies on following wks.			
				3rd	4th	6th	8th
1	HeLa (wild)	A	Lactate	174	117	51	55
2	" "	A	Ribose	2	2	0	0
3	" "	B	Lactate	130	74	41	40
4	" "	B	Ribose	0	0	0	0
5	" "	C	Lactate	0	0	0	0
6	" "	C	Ribose	0	0	0	0
7	HeLa (low thiamine)	A	Lactate	141	98	14	12
8	" " "	A	Ribose	20	8	17	12
9	" " "	B	Lactate	160	65	6	28
10	" " "	B	Ribose	60	15	16	14
11	" " "	C	Lactate	181	143	100	116
12	" " "	C	Ribose	4	2	2	2
13	Conjunctival (wild)	A	Lactate	29	6	2	9
14	" "	A	Ribose	0	2	9	15
15	" "	B	Lactate	4	12	33	137
16	" "	B	Ribose	0	0	7	53
17	" "	C	Lactate	0	18	95	165
18	" "	C	Ribose	8	2	8	13
19	Conjunctival (low thiamine)	A	Lactate	64	28	19	42
20	" " "	A	Ribose	3	0	0	0
21	" " "	B	Ribose	0	0	0	0

fluctuation in the number of visible colonies from the 3rd to 8th week: in some instances, there was a decrease (culture 7); in others, increase (Nos. 16 and 17); in still others, a decrease followed by an increase (Nos. 9 and 19). This illustrated the unreliability of the number of variant colonies in the quantitation of variant frequency.

In view of the importance of thiamine in carbohydrate metabolism, experiments were performed to see if thiamine depletion would detectably affect variant frequencies. Eight experiments with both the conjunctival and HeLa

cells using 2 different lots of dialyzed sera were performed. Thiamine was omitted from Eagle's basal media as modified in our laboratory and the cultures were observed for at least 2 months. In no instances was there evidence of gross cellular degeneration. These cultures were subcultivated at 1 to 2 week intervals with net increase in cell number of 4- to 25-fold per week. Carbohydrate variants were isolated regularly from HeLa cell depleted of thiamine for more than 1 year (see HeLa "low thiamine" of Tables I and II); this was not so, however, with the conjunctival cell depleted of thiamine for 3 months. This observation of cell multiplication in the glucose medium without thiamine prompted us to assay for thiamine in the amino acid and vitamin solutions and in two dialyzed sera: 5 and 12 millimicrograms were found in each milliliter

TABLE III

Effect of Dialyzed Serum on Multiplication of a Xylose Variant and Its Parent Culture

Dialyzed serum	Parent culture		Xylose variant	
	0 day	7th day	0 day	7th day
Horse 1	19*	214, 226‡	34	24, 26
" 2	19	84, 106	34	60, 74
Human 1	19	262, 196	34	66, 142
" 2	19	164, 186	34	150, 188

* No. cell $\times 10^3$ per culture.

‡ Results of replicate cultures.

of dialyzed sera but none (<2 mμg.) in the amino acid and vitamin solutions from which thiamine had been omitted.

3. Properties of Established Stable Variants

An established stable variant is defined as one which has been propagated continuously in its carbohydrate medium for at least 6 months, subcultivated successfully at regular intervals and reestablished after subzero storage. A strain each of xylose, ribose, and lactate variant isolated from the conjunctival, and, xylose and ribose from the HeLa cells were included in this study.

Growth Rate.—Growth rate of the variants (as determined by net increase in cell number per week) has been observed to vary from 0- to 6-fold in contrast to 0- to 50-fold found with the parent cultures. There was marked cyclic fluctuation of growth rate at irregular intervals; periods of satisfactory multiplication with 2- to 6-fold weekly increase in cell number were frequently followed by intervals of partial degeneration. Cause for this fluctuation is not known. Similar fluctuation of lesser degree has also been observed with the parent culture. On account of this fluctuation, experiments were performed

during those intervals when satisfactory multiplication occurs (2- to 6-fold weekly increase for variants, and 6- to 50-fold for parent culture). One factor which significantly affects the multiplication of the variants has been identified as the source of dialyzed serum as illustrated in Table III: horse serum 1 which supported satisfactory cell multiplication of the parent HeLa culture in the glucose medium failed to maintain multiplication of the xylose variant in the xylose medium. Similar effect has been observed for other variants.

Cloning Efficiency.—Cloning or plating efficiencies were tested according to method of Puck (25) with minor modifications. Approximately 1000 cells were inoculated into 2 serum dilution bottles, one containing 10 ml. of respective carbohydrate medium and the other with the addition of glucose to 5 mM. The number of visible colonies were counted after 10 to 14 days. The results were dependent on the lot of dialyzed serum used. At least 3 dialyzed equine serums had been found incapable of supporting clonal growth of parent and variant cultures. In most other serums, the cloning efficiencies of the parent cultures varied from 2 to 40 per cent, while those of the variants varied from 0 to 5 per cent. For a particular dialyzed serum, the cloning efficiencies of the variants were very much lower than the parent culture (*e.g.* with a pool of dialyzed human serum, the cloning efficiencies were 22, 5, 0.1, and 0 per cent for the conjunctival parent, ribose$^+$, xylose$^+$, and lactate$^+$ cultures, respectively). The addition of glucose invariably increased the cloning efficiencies of these variants by at least 2- to 10-fold. Also of interest is that trypsinization procedure for the preparation cell suspension of parent culture gave rise to cell suspensions with numerous large cell clumps when applied to the ribose variants (*e.g.* in a representative experiment, two clumps of 2 and 3 cells among 112 single cells were noted for a suspension of parent culture in contrast to 9 large clumps of over 10 cells and 4 smaller clumps of about 5 cells among 150 individual cells for the ribose variant).

Subzero Storage.—The method described by Scherer (16) has been found satisfactory for both parent cultures. Cultures have been regularly reestablished from parent cells stored at below −70°C. for over 2 years in the glucose medium containing 10 per cent glycerine (approximately 500,000 cells per ml.). When the same procedure was applied to the variants using their respective carbohydrate media, the results were invariably negative with an occasional exception for the ribose variant from the conjunctival cell. However, by allowing the variants to propagate in the glucose medium plus 10 per cent whole serum for several days before freezing and several weeks following thawing, it has been possible to store many more variants at below −70°C. Even with this modification, the interval required for variant to reestablish (that is, to reach a population of over 100,000 cells in a roller tube culture) is about 2 to 4 weeks in contrast to about 1 week for the parent culture. It was clearly evident that a much smaller percentage of the variant cells were morphologically intact

after the freeze-thawing procedure. There is suggestive evidence that prolonged cultivation in their respective carbohydrate media increases the chance of survival following subzero preservation.

Morphology.—Both ribose variants have consistently shown morphological features differing from the parent cultures. Figs. 1 and 2 demonstrate the circular colonies formed by the ribose variant and the solid cell sheets of the

TABLE IV

Effect of D-2-Deoxyglucose and Glucose Oxidase on the Propagation of Parent and Variant Cultures

Culture*	Anti-metabolite or enzyme‡ added	No. cell × 10^3 per culture on 0 and 7th day			
		Conjunctival		HeLa	
		0	7	0	7
Parent (glucose)	D-2-Deoxyglucose	20	202, 176	22	162, 218
	Glucose oxidase	20	0, 0	22	0, 0
	None	20	99, 125	22	178, 182
Ribose variants	D-2-Deoxyglucose	34	3, 17	25	9, 17
	Glucose oxidase	34	55, 80	25	93, 55
	None	34	92, 116	25	61, 70
Xylose variants	D-2-Deoxyglucose	47	0, 0	20	3, 2
	Glucose oxidase	47	42, 28	20	20, 19
	None	47	83, 61	20	57, 29
Lactate variant	D-2-Deoxyglucose	60	0, 0		
	Glucose oxidase	60	76, 71		
	None	60	75, 84		

* Propagated in their respective carbohydrate media.

‡ Final concentrations were 0.1 mM for D-2-deoxyglucose and 1 μg. per ml. medium for glucose oxidase.

parent culture. When the glucose was added at concentration of 5 mM, most of the variant colonies assumed the morphological characteristics of its parent culture. Stained preparations are shown in Fig. 3; there are more multinucleated cells in the ribose culture and the individual cells are more closely apposed. The morphological peculiarities of the xylose and lactate variants have not been sufficiently consistent at this time to warrant description.

Effect of Glucose, D-2-Deoxyglucose, and Glucose Oxidase.—Upon the addition of glucose to concentration of 5 mM, growth rates of all variants tested so far increased greatly. Following withdrawal of glucose, their growth rates decreased. Their abilities to propagate in their respective carbohydrate media,

however, were unaffected by continuous multiplication in the glucose medium for 2 months.

D-2-deoxyglucose had been shown to competitively inhibit glucose metabolism (26). With both parent cultures, complete cell degeneration occurred within 7 days when the concentrations of glucose and D-2-deoxyglucose (D2G) were both 5 mM. By decreasing the concentration of D2G to 0.1 mM, cell multiplication was not detectably affected. Attempts to overcome the effect of D2G (5 mM concentration) have been unsuccessful with the following metabolites used also at concentrations of 5 mM:—glucose-1-phosphate, glucose-6-phosphate, hexose-1,6-diphosphate, mannose, fructose, galactose, and

TABLE V

Relative Incorporation of Glucose-C^{14} by Parent and Variant Cultures

Cell	Fraction	C^{14} activities, expressed as C.P.M. per 10^6 cells			
		Parent	Ribose	Xylose	Lactate
Conjunctival	CO_2	817	2313	1072	1840
	Acid-soluble	3290	4700	4840	5950
	Lipids	2504	749	248	605
	Nucleic acids	3400	1140	344	1070
	Proteins	9450	4210	441	2900
HeLa	CO_2	840	1720	1470	
	Acid-soluble	6304	3070	4500	
	Lipids	0	146	33	
	Nucleic acids	1256	1130	1530	
	Proteins	845	1760	207	

fructose plus glucose. Of interest is an observation that the cytotoxic effect (complete cell degeneration in 7 days) of D2G at 1 mM concentration was completely overcome by mannose but not by glucose both at 5 mM concentrations. With all the variants tested thus far, advanced cellular degeneration appeared within 7 days after the addition of D2G to 0.1 mM (see Table IV). Xylose and lactate variants appeared to be more sensitive to the effect of D2G than ribose variants.

Purified glucose oxidase (Nutritional Biochemical Corp., Cleveland) at concentration of 1 μg. per ml. medium regularly destroyed both parent cultures within 7 days in the glucose medium. The effect was specifically related to glucose since with the substitution of glucose by mannose or galactose, cell multiplication was unaffected. The addition of this enzyme at similar concentration did not significantly affect the multiplication of the variants in their respective carbohydrate media. With some experiments, suppression of the growth rates of the xylose and lactate variants had been observed.

Relative Incorporation of C^{14}-Labelled Sugars.—The results of the incorporation of glucose-C^{14} are presented in Table V. An experiment with each variant and parent culture was presented to illustrate that, under the described conditions, significant incorporation of glucose occurred in all fractions. No attempt has been made to quantitate differences between the parent and variant cultures since preliminary results indicated large variations between experiments and between replicate cultures of the same experiment.

TABLE VI

Relative Incorporation of Xylose-1-C^{14} by Variant and Parent Culture

A. (Amount of xylose-1-C^{14} = 1 μc. per culture)

Cell	Fraction	C^{14} activities expressed as c.p.m./10^6 cells			
		Xylose variant		Parent culture	
		Range	Mean	Range	Mean
Conjunctival	CO_2	112–132	120	15–80	36
	Acid-soluble	92–960	484	0–242	106
	Lipids	0–1600	440	0–8	2
	Nucleic acids	24–260	138	0–28	15
	Proteins	0–128	42	0–10	4
HeLa	CO_2	107–488	263	0–44	16
	Acid-soluble	270–860	528	0–27	9
	Lipids	0–30	14	0	0
	Nucleic acids	123–195	158	0	0
	Proteins	0–103	59	0–18	6

B. (Amount of xylose-1-C^{14} = 6 μc. per culture)

Fraction	C^{14} activities expressed as c.p.m./10^6 cells			
	Conjunctival xylose	Conjunctival parent	HeLa xylose	HeLa parent
CO_2	328	198	1230	47
Acid soluble, total	946	744	3225	231
" " non-dialyzable	270	31	684	6
Lipids	86	18	200	9
Nucleic acids	490	40	655	123
Proteins	540	14	646	167

The results of 8 experiments comparing the incorporation of xylose-1-C^{14} by the xylose variant and parent culture are presented in Table VI. Table VI A shows that the xylose variants incorporate significantly greater amounts of xylose than the parent culture. These differences are magnified when the

quantity of xylose-1-C^{14} per culture was increased by 6-fold (see Table VI B). The differences are especially striking between the corresponding macromolecules such as proteins, nucleic acids, and non-dialyzable acid solubles (presumably mucopolysaccharides).

The incorporation of ribose-1-C^{14} is presented in Table VII. When 1 μc. (38 μg.) of ribose-1-C^{14} per culture of 2 to 3 million cells were used, no significant

TABLE VII

Relative Incorporation of Ribose-1-C^{14} by Variant and Parent Cultures

Cell	Fraction	C^{14} activities, C.P.M. per 10^6 cells			
		Ribose variant		Parent culture	
		A*	B‡	A*	B‡
Conjuncti-val	CO_2	102	4596	98	885
	Acid-solubles				
	Total	1579	4653	1347	755
	Non-dialyzable		1690		320
	Lipids	25	1650	22	149
	Nucleic acids	856	6925	723	1237
	Proteins	292	5305	459	916
HeLa	CO_2	324	1365	168	197
	Acid-solubles				
	Total	1124	3256	1528	1276
	Non-dialyzable		1930		192
	Lipids	7	443	38	61
	Nucleic acids	855	1493	1204	117
	Proteins	474	1720	862	107

* Averages of 3 to 5 experiments; 1 μc. (38 μg.) of ribose-1-C^{14} used for each culture of 2 to 3 $\times$ 10^6 cells.

‡ Ten μc. (380 μg.) of ribose-1-C^{14} and 3.5 mg. of unlabelled Ribose used per culture of 2 to 3 $\times$ 10^6 cells. Ribose-C^{14} used in HeLa cells experiments.

differences could be detected. By increasing the amount of ribose to about 2.5 mM (10 μc. of ribose-1-C^{14} and 3.8 mg. of unlabelled ribose per culture of 2 to 3 million cells), the ribose variants metabolized 5 to 17 times more ribose than the parent cultures.

Lactate-2,3-C^{14} incorporation by the lactate variant and parent culture are presented in Table VIII; the results are in general similar to those of the ribose experiments.

Synthesis and Composition of Mucopolysaccharides.—Several of the differences of the stable ribose variants and the parent cultures described earlier in this manuscript suggest the possibility of some alterations in the properties of

TABLE VIII

Relative Incorporation of Lactate-2,3-C^{14} by Variant and Parent Conjunctival Cells

Fractions	C^{14} activities, C.P.M. per 10^6 cells			
	Lactate variant		Parent culture	
	A*	B‡	A*	B‡
CO_2	490	4608	452	1024
Acid-solubles				
Total	749	7260	575	1613
Non-dialyzable		6480		1840
Lipids	21	1995	386	886
Nucleic acids	33	1420	103	416
Protein	363	6968	4666	2218

* Averages of 3 to 5 experiments; 1 μc. (30 μg.) of lactate-2,3-C^{14} used for each culture of about 2×10^6 cells.

‡ Averages of 2 experiments; 10 μc. (300 μg.) of lactate-2,3-C^{14} and 2.3 mg. of sodium lactate used per culture of about 2 million cells.

TABLE IX

Conversion of Glucose-C^{14} and Ribose-C^{14} into Other Component Sugars of Mucopolysaccharides Extracted from Ribose Variant and Parent Culture

Experiment*	Cell	C^{14} substrate	Total activities‡ in the following carriers§						
			Glu. a.	Gal.	Glu.	Sor.	Fuc.	Rham.	Rib.
1	Parent	Glucose, 5 μc.	476	1181	748	350	98	332	101
2	Parent	Glucose, 5 μc.	101	422	46	154	29	263	13
	"	Ribose, "	5	20	3	6	0	126	0
3	Parent	Ribose, 10 μc.	107	54	16	26	11	171	8
	Ribose	" "	832	137	48	62	50	686	8
4	Parent	Ribose, 5 μc.	137	19	17	26	32	99	9
	Ribose	" "	243	88	43	43	57	261	7
5	Parent	Ribose, 5 μc.	Not resolved; total activity ½ that of variant						
	Ribose	" "	77	79	22	22	30	51	6
6	Parent	Ribose, 10 μc.	135	74	22	38	9	59	?‖
	Ribose	" "	514	194	49	51	37	340	10

* Experiments 1 to 4 were performed with the conjunctival cell; 5 and 6, HeLa cell.

‡ Total C.P.M. in each component sugar of mucopolysaccharides extracted from each culture of 2 to 3 million cells.

§ Glu. a. = glucuronic acid; Gal. = galactose; Glu. = glucose; Sor. = sorbose; Fuc. = fucose; Rham. = rhamnose; Rib. = ribose.

‖ Ribose spot ran off paper.

cell membranes. Since mucopolysaccharides are known to be important constituents of cell membranes of many microorganisms, studies were made to determine whether the ribose variants and the parent cultures differed in their syntheses and composition of mucopolysaccharides. Table IX shows the results of 6 representative experiments on the conversion of glucose and ribose into other component sugars and derivatives of mucopolysaccharides extracted. It may be concluded that glucose and ribose have been converted into other hexoses and derivatives, and that more ribose is incorporated into the muco-

TABLE X

*Analysis of Mucopolysaccharides from Ribose and Parent Cultures**

	Conjunctival cell			HeLa cell		
	Parent		Ribose	Parent		Ribose
	1	2		1	2	
"Molisch value"‡	124	201	20	157	115	8
Glucose	92	57	9	75	23	10
Inositol§	6	4	12	19	5	19
Hexosamines‖	<3	<4	<2	<2	<4	<2
Hexuronic acids‖	<14	<30	<5	<21	<13	<12
Pentoses‖	<27	<16	<30	<44	<12	<36
Protein§	257		170			268

* All values are expressed as micrograms per milligram of cell DNA.

‡ Results of quantitative Molisch test using glucose as standard; it is well known that such sugars as rhamnose, fucose, and galactose give much lower readings in this test than equal amount of glucose.

§ These readings are close to the lower limits of the test, and therefore may not be accurate quantitatively.

‖ The low values obtained in these 3 tests may have been due to other interfering sugars; the results are therefore expressed as "less than a certain amount."

polysaccharides by the ribose variants than by the parent cultures. Of interest is the high C^{14} activity associated with rhamnose. When another solvent (butanol:acetic acid:water) was used, similar high activity was again demonstrated in spots formed by rhamnose. Other results presented in this table should be interpreted with realization of the limitation of the method. For instance, it is well known that glucuronic and galacturonic acids are inseparable, and that, other sugars such as sorbose and mannose, or glucose and galactose, are frequently incompletely resolved. Furthermore, considerable C^{14} activities were regularly demonstrated in the intervals between the point of origin and glucuronic acid as well as between glucuronic acid and galactose. Therefore, the presence of other substances should be considered. The "tailing" of some substances, such as ascorbic acid, in this solvent may also complicate the

results. The contradictory finding of high C^{14} activity associated with glucuronic acid and low values of hexuronic acids in actual analysis (see Table X) suggests the presence of other substances with similar R_f value as glucuronic acid.

The results of several analyses on mucopolysaccharides extracted from the ribose variants and the parent cultures are presented in Table X. Of interest are the presence of inositol and proteins or polypeptides. The low "Molisch value" of both ribose variants in comparison to those of the parent cultures may be due to an actual decrease in the amount of mucopolysaccharides per cell, or differences in relative concentrations of their component sugars, or both. The low values for hexosamines and hexuronic acids are interesting but should be considered as preliminary since it is not known whether the present method of extraction is adequate for all species of mucopolysaccharides. Further study is indicated.

Virus Synthesis.—No significant difference has been observed in the onset and progression of cytopathogenic changes in all variant and parent cultures following infections with the polio, Coxsackie, adeno, or herpes simplex viruses. The production of poliovirus was also similar.

DISCUSSION

The experimental findings presented in this report lead one to inquire into the mechanism through which a variant culture appears, the fundamental biochemical changes which enable the variant to propagate in the selecting environment, and, the possible relationship between these biochemical, physiological, and morphological changes occurring in the variants. The initial degeneration of the vast majority of parent cells in the selecting environment and the subsequent ability of the established variants to continue propagation in their respective carbohydrate media after prolonged cultivation in the glucose medium strongly favor the genetic selection hypothesis over that of adaptive enzyme induction. The genetic selection hypothesis by itself, however, does not explain adequately the slow resumption of multiplication of those cells surviving the selecting environment and the prolonged continuous degeneration of their progenies. One would also have to consider simultaneously the concept of cellular interdependence. After exposure to a selecting environment (ribose medium for example), only those cells in the parent culture genetically endowed with the capacity to utilize sufficient ribose survive. For the resumption of cell multiplication, however, some other unidentified substances presumably synthesized and excreted by the cells are also essential. The few surviving cells would therefore have to accumulate slowly these essential substances in order to reinitiate cell multiplication. Once cell multiplication is resumed, progenies with varying degree of susceptibility to deficiency in these essential substances are produced. Similar to the concept of

unbalanced growth (27), the more actively metabolizing progenies would degenerate rapidly as a result of this deficiency. Thus, the processes of multiplication and degeneration would go on simultaneously until the entire culture degenerates or until a sufficiently high cell population is attained. It is not difficult to visualize how some environmental conditions, such as the source of dialyzed serum, may influence the outcome of this delicate balance. The concept of cellular interdependence is well known among the developmental biologists (28), tissue culture investigators (29), and bacteriologists (30).

The results of the C^{14} sugar incorporation studies clearly demonstrate that the variants are capable of utilizing significantly more of their respective sugars than the parent cultures. Thus, the parent cultures have degenerated in the xylose, ribose, or lactate medium owing to their inabilities to utilize sufficient xylose or ribose, or to convert sufficient lactate to hexose-monophosphate to meet their energy and biosynthetic requirements. One may postulate that there is a significant increase in the intracellular concentrations of xylose-kinase and isomerase which increases the rate of xylose utilization through the pentose phosphate pathway by the xylose variants. A similar increase in ribose-kinase may be postulated for the ribose variants. For the lactate variant, the simplest hypothesis would be the development of new mechanisms which overcome the two almost irreversible reactions in the glycolytic pathway; sufficient hexose-monophosphate could then be synthesized from lactate to meet the requirements of nucleic acid and mucopolysaccharide syntheses. Various mechanisms involved in overcoming these virtually irreversible reactions have been adequately discussed by Horecker and Hiatt (11).

Undoubtedly, many other important alterations in the over-all carbohydrate metabolism must have occurred as a consequence of these primary changes. For instance, the greater sensitivity of the variants to the cytotoxic effect of D-2-deoxyglucose, which blocks carbohydrate metabolism at the phosphoglucoisomerase level (31), may be taken as suggestive evidence that the rate of conversion of fructose-6-phosphate to glucose-6-phosphate has been reduced. This reduction in turn leads to a slower synthesis of other component sugars of cellular mucopolysaccharides through the uronic acid pathway. The reduction and possible alteration in the synthesis of mucopolysaccharides may be reflected in such observed morphological and physiological changes as giant cell formation, closer apposition of cells, decreased resistance to subzero storage, lower cloning efficiency, and the inadequacy of the standard trypsinization procedure for preparing single cell suspension. It is tempting to postulate that the following sequence of interrelated events has occurred: genetic selection, alteration in carbohydrate metabolism, changes in the cellular mucopolysaccharides, alteration in the surface properties of the cells, and evidence of primitive morphogenesis. Of interest in this connection is the electromicro-

scopic study of Birbeck and Mercer (32) suggesting a possible relation between properties of cell membrane and morphogenesis.

The striking difference in the results of C^{14} ribose and lactate incorporation study when small and large amounts of substrates were used (see A and B of Tables VII and VIII) deserves some emphasis. Using small quantities of C^{14}-labelled substrates, Hiatt was unable to demonstrte quantitative difference between ribose and glucose in percentage conversion of C^{14} to liver glycogen *in vivo* (33). However, by increasing the amount of substrate injected, glucose was much more extensively incorporated than pentose (34). A similar situation exists *in vitro*. When 1/200 of the usual amount of ribose was used, the parent culture and the ribose variant incorporated approximately the same amount of ribose. When the amount of ribose is increased a 100-fold the ribose variant incorporated 5 to 17 times more ribose than the parent culture. Thus, only at the higher concentration is the difference in ribose-metabolizing apparatus between the ribose variant and parent culture demonstrated.

The demonstration of inositol in the hydrolysate of mucopolysaccharides is of some interest. Inositol is an essential nutrilite for most established strains of human cells and is required at concentration much higher than most other vitamins. Depletion of inositol results in rapidly progressing cellular degeneration similar to the depletion of glucose (35, 36). Thus, one important function of inositol may possibly be in the synthesis of certain important cellular structure consisting of mucopolysaccharides.

With the current interest in applying the technics and principles developed in bacterial genetics to human cell culture, it is desirable to emphasize that the results of quantitation of variant frequencies by counting cell colonies emerged in the new environment are subjected to large variation. Factors inherent with the cell culture system itself, such as the unpredictable evolution of parent culture and the source of dialyzed serum, have been shown to affect the outcome of variant isolation (see Table II). It is not unreasonable to suspect that the interaction of these two factors and others not yet identified may further introduce variability. It is also obvious that each colony may have originated from more than one variant cell and that some colonies are formed through partial detachment of existing colonies. The demonstration of prolonged continuous multiplication of both the conjunctival and HeLa cells in the so called minimal growth medium (12) with the omission of thiamine focuses once again on the inadequacy of this minimal medium in certain types of metabolic and genetic studies. Since many vitamins, including thiamine, have been found in the dialyzed serum (18), other evidences are mandatory before one is justified in concluding that a thiamine-independent variant has appeared.

The results of experiments on susceptibility to several RNA and DNA viruses suggest that the synthesis of viral nucleic acids and proteins is not detectably altered in these variants.

Finally, it should be emphasized that the results presented in this report should not be generalized. For instance, whether all ribose variants would have properties similar to the one under study remains to be demonstrated. These data should also be interpreted with full realization of the various important limitations of the methods used.

SUMMARY

The isolation of carbohydrate variants from cultures of HeLa and conjunctival cells was described. Factors inherent in the cell culture system, such as parent populations and dialyzed serums, have been shown to influence the outcome of variant isolations. Established stable variants incorporated significantly more pentoses or lactate into various cell fractions than the parent cultures. Besides their abilities to propagate continuously in the selecting environments, the variants multiplied slower, were more susceptible to subzero preservation and the cytotoxic effect of D-2-deoxyglucose, showed lower cloning efficiencies and were less susceptible to the deleterious effect of glucose oxidase.

The ribose variants also differed from the parent cultures in morphological appearance such as formation of multinucleated cells and ring-shaped colonies. They converted more ribose into other component sugars of mucopolysaccharides than the parent cultures. Preliminary analyses of the mucopolysaccharides extracted from the ribose variants and parent cultures showed large difference in their carbohydrate (Molisch-positive materials) and DNA ratios. Evidence suggests that a sequence of interrelated events from genetic selection to primitive morphogenesis has been established.

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PLATE 17

FIG. 1. Colonies of the conjunctival cell (A) and its ribose variant (B).

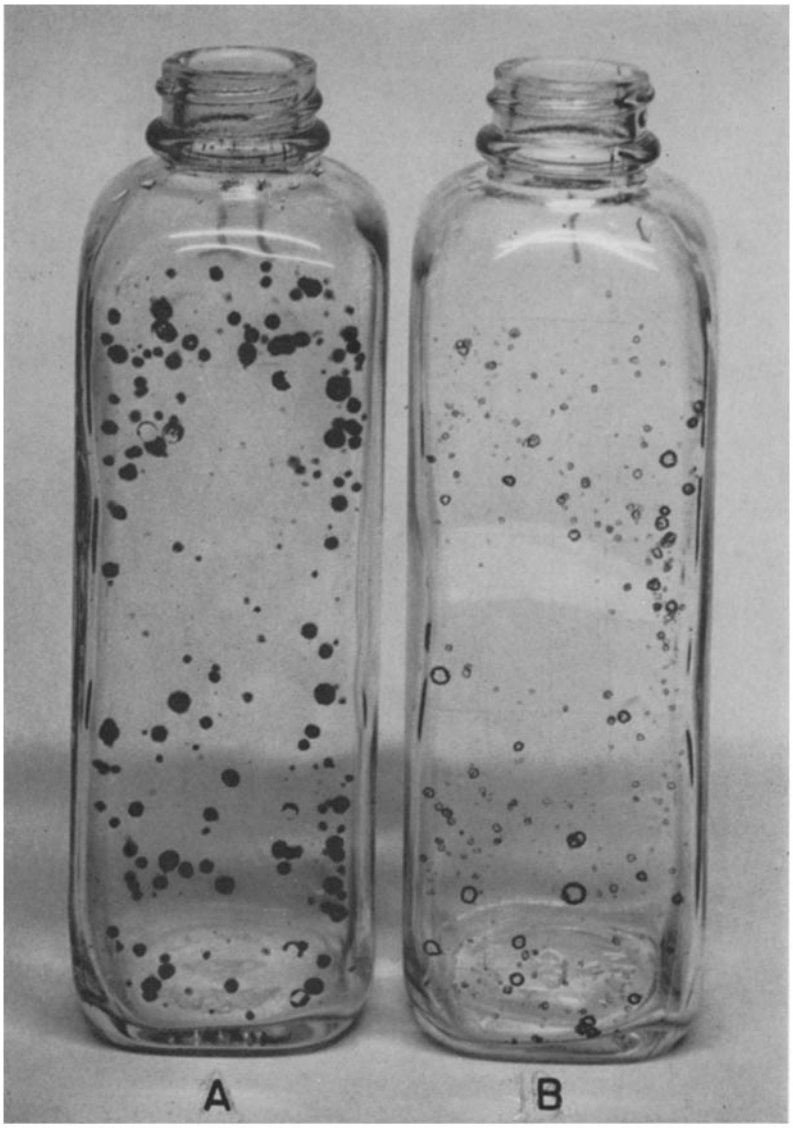
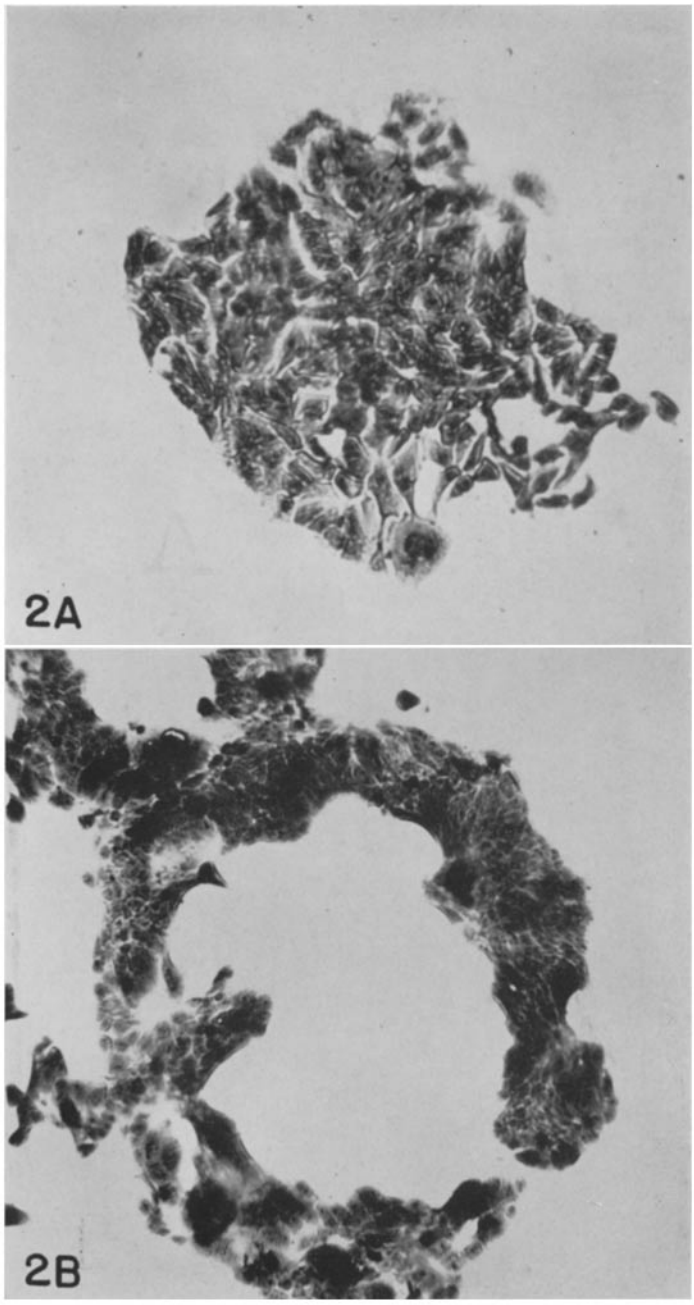


FIG. 1

(Chang: Genetic study of human cells *in vitro*)

PLATE 18

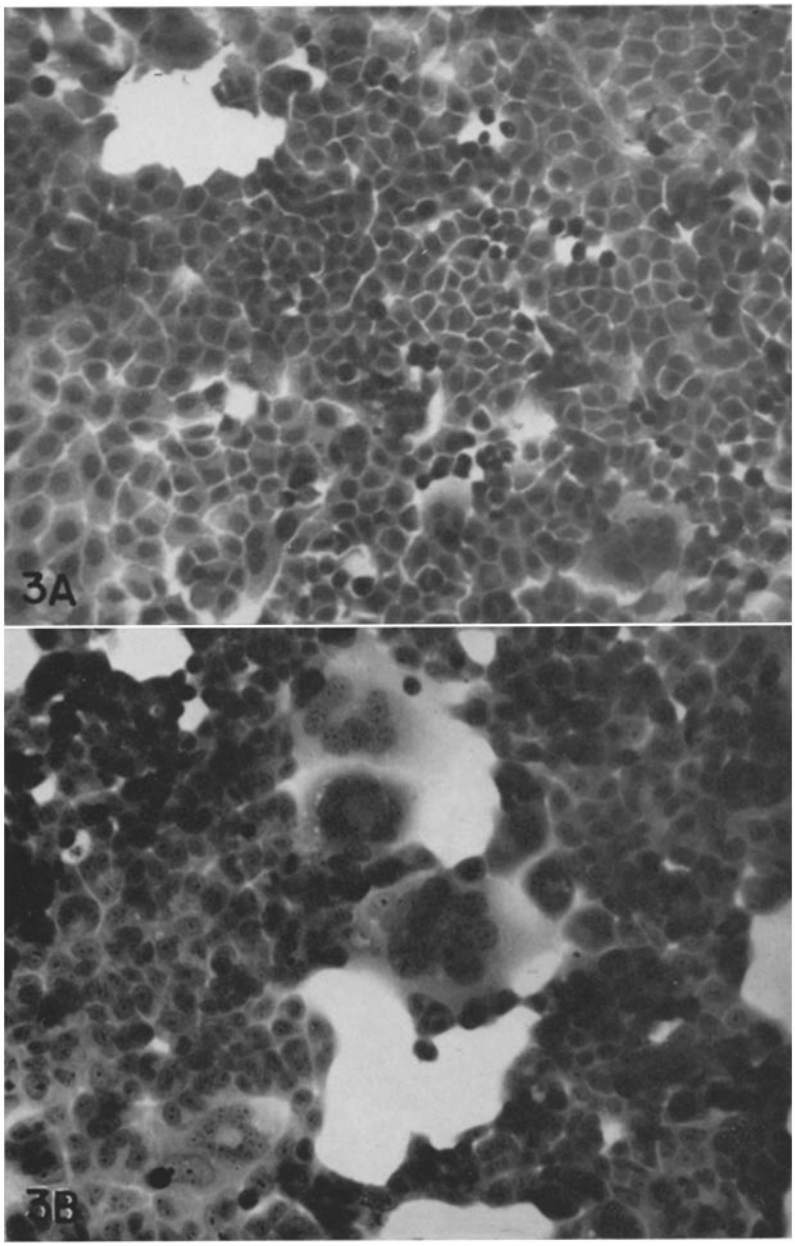
FIG. 2. Colonies of the conjunctival cell (A) and its ribose variant (B); stained with Giemsa. × 20.



(Chang: Genetic study of human cells *in vitro*)

PLATE 19

FIG. 3. Sheets of the HeLa cell (A) and its ribose variant (B); fixed with Bouin's fluid and stained with hematoxylin-eosin. × 100.



(Chang: Genetic study of human cells *in vitro*)