Propagation of Conjunctival and HeLa Cells in Various Carbohydrate Media.* (23471)

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Establishment of epithelial-like cells in continuous culture from human tissues has made available a more effective system for the study of metabolism of human cells *in vitro* than the previously used tissue explant method. Under controlled experimental conditions, using media of relatively well defined composition, these established cells may be maintained in a state of active multiplication and the rate of multiplication can be measured quite accurately. This report deals with propagation of these human cells in the presence of certain individual carbohydrates.

Materials and method. Human cells. Conjunctival(1) and HeLa cells(2) were used as representative cell lines, the former derived from normal and the latter from malignant human tissues. Stock cultures were propagated in 20% inactivated human serum diluted in Eagle's basal medium(3). Cultures were checked at regular intervals, and also during each critical experiment, to assure freedom from contamination by pleuro-pneumonia-like organism(4) and bacteria. *Media.* Basal carbohydrate-free medium consisted of 10% dialyzed serum in a modified Eagle's basal medium; the modifications consisted of elimination of glucose, addition of meso-inositol to final concentration of 10 to 20 micromolar, and the substitution of Earle's balanced salt solution by Hanks'. Carbohydrates being tested were added to this medium to final concentrations of 5 mM. *Sera* were collected and stored at −60°C as described (5). Dialyzed serum was prepared by dialysis of about 100 ml serum containing about 100,000 units of penicillin and 10 mg of streptomycin against 10 changes of 0.85% NaCl of 1 liter each at about 2°C for 48 hours. Sera so dialyzed were negative for glucose by the glucose oxidase test. *Glucose oxidase test.* The enzyme paper stick method(6) was used, and the final reading was made exactly 1 minute after application of test solution. The minimum concentration of glucose which gives a positive reaction is about 0.05 mM (0.5 to 1.0 mg per 100 ml). *Carbohydrates.*† Included in the present study were soluble starch, glycogen, maltose, sucrose, lactose, d-fructose, d(+)galactose, l(+)rhamnose, l(−)fucose, l(−)sorbose, d-ribose, d(+)xylose, l(−)xylose, d(−)lyxose, l(+)arabinose, d(−)arabinose, d-2-deoxyribose, i-erythritol, dihydroxyacetone, sodium pyruvate and sodium lactate. When tested for presence of glucose as impurity by the glucose oxidase test, the following results expressed in terms of highest concentration giving a negative test were obtained: Starch‡ 0.02 M, glycogen‡ 0.02 M, maltose 0.02 M, lactose 0.3 M, d(+) galactose 0.01 M (this preparation was specified as "glucose free"), d-mannose 0.03 M, d(+) xylose 0.5 M and all others negative at saturation (more than 1 M). Since, as will be shown, glucose is effective in supporting cell propagation at 0.05 to 0.1 mM, it is essential that the glucose present in starch, glycogen, maltose, d(+) galactose and d-mannose be removed. The amount of glucose in lactose and d(+)xylose was too small to be of consequence in the present studies. Tenth molar starch and glycogen solutions were dialyzed against running water for about 24 hours at 0-2°C; these dialyzed polysaccharide solutions, after passage through bacterial filters, no longer gave positive glucose oxidase tests. Maltose, d(+)galactose and d-mannose were treated with the enzyme, glucose oxidase

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† Starch and sodium lactate were purchased from Mallinckrodt Co.; lactose, maltose and sucrose, from Merck; d(+)galactose and d(+)xylose, from Pfanstiehl; d-fructose and d-mannose, from Eastman Kodak; and, all the others from Nutritional Biochem. Corp.

‡ Concentrations of all polysaccharides were based on glucose.

(Glucostat, Worthington Biochemical Corp.); about 150 mg of enzyme were used for each 10 millimoles of carbohydrate. After aeration for 3 hours, the enzyme-carbohydrate mixture was dialyzed against a small volume of sterile water for 16-20 hours at 2°C. The amount of carbohydrate present in the dialysing fluid was determined by dry weight analysis; such determinations were performed after filtration through an ultrafine glass filter. Maltose so treated gave a negative glucose oxidase test at 0.8 M concentration, galactose at 0.15 M, and d-mannose at 0.05 M. This failure to reduce substantially the reaction of d-mannose and d(+) galactose with glucose oxidase may possibly be explained by the oxidation of these 2 carbohydrates in the presence of this enzyme(7).

Method of evaluation. About 20,000 cells suspended in 0.2 ml basal medium were inoculated into a series of 6 to 12 new culture tubes, 18 x 150 mm, containing 0.8 ml basal medium plus the carbohydrate to be tested. After slanting at 36°C for about 20 hours, the tubes were placed on a roller drum also at 36°C. Media were renewed on the 3rd and then on every other day. The number of cells/tube was counted, generally on 4th and 8th day; 2-4 tubes were used at each counting. To facilitate description, this will be referred to subsequently as the *direct inoculation method.* To eliminate possible unknown factors introduced through tryptic digestion and mechanical agitation (both are essential steps in the preparation of homogeneous cell inoculum), all carbohydrates incapable of sustaining cell growth by the direct inoculation method were further tested by a *medium replacement method.* One-half ml of basal medium containing 0.2 mM glucose and about 30,000 cells was inoculated into each of a large series of culture tubes. On the 3rd day, when sheets of polyhedral cells were formed, the average number of cells/tube was calculated from cell counts in any 4 tubes picked at random. After 3 successive washings with basal carbohydrate-free medium, the nutrient medium in remaining tubes was replaced by the basal medium plus a carbohydrate to be tested; this was then considered as the initial day of the test period. Six tubes were generally used for each carbohydrate. Media were renewed every second day. The number of cells/tube was determined when definite degeneration appeared in the carbohydrate-free controls, and, again when degeneration was completed. Technical details of a similar quantitative study have already been described(5). In this particular study, reagents such as trypsin and antibiotics were dissolved in 0.85% NaCl rather than the balanced salt solution which contained glucose; the cell inoculum was washed twice in 10 ml carbohydrate-free medium; and, 2 controls, the basal carbohydrate-free and the basal plus 5 mM glucose, were included in each experiment. No attempt was made to evaluate minor differences in growth rate. Adaptive changes will be considered in another communication.

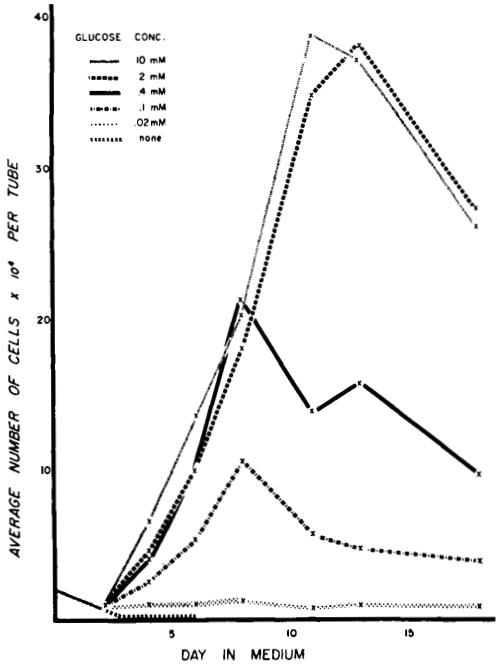

FIG. 1. Propagation of conjunctival cells in various concentrations of glucose.

Results. Cell growth in various concentrations of glucose. To provide basic data for subsequent comparison, multiplication patterns of the conjunctival cell in medium containing glucose at final concentrations of 10, 2, 0.4, 0.1, 0.02, 0.004, and 0.0 mM were used. Results are presented in Fig. 1. Similar growth curves were obtained in media containing glucose at concentrations of 10 and 2

TABLE I. Propagation of Conjunctival Cells in the Presence of Individual Carbohydrates.*

Carbohydrate	Cells × 10^3/tube on the following days: 0	4th	8th
None	29	3	0
Glucose	29	104	158
Starch	29	95	143
Sucrose	29	19	43
d-ribose	29	9	0

* See text for pertinent data.

mM; both showed a lag phase of about 2 days, followed by logarithmic growth period of about 10 days, which terminated in partial cell degeneration after a maximum cell population of about 300,000/tube was reached. At 0.4 mM, partial cell degeneration appeared several days earlier when the maximum population of about 200,000 was attained. At 0.1 mM, the slope of the logarithmic growth phase was less steep and when the cell population was about 100,000/tube, partial degeneration became apparent. At 0.02 and 0.004 mM glucose, evidence of cell multiplication and degeneration were present after about the 3rd day, no logarithmic growth phase was observed, and cell populations were maintained at approximately 5,000 to 10,000/tube. In the basal carbohydrate-free medium, degeneration was completed by the 4th to 7th day. Minor variations were observed depending on the physiological state of cell inoculum, sera used and cell lines tested. These variations were in length of the lag phase, the maximum population attainable before onset of partial degeneration, and the number of days in the carbohydrate-free medium for completion of degeneration. It is therefore essential to compare only results obtained within a single experiment.

Both conjunctival and HeLa cells have been maintained and subcultivated in the basal medium plus glucose at a concentration of 0.1 mM for 2-3 months. At this low concentration of glucose, the net increase in number of cells/week is significantly lower than that in medium containing glucose at concentration of 10 mM. No evidence of partial degeneration could be observed when the cell population per 1 ml medium did not exceed 50,000. It seemed that the actual rate of multiplication of both cells may have been reduced appreciably at this low concentration of glucose.

Cell propagation in carbohydrate other than glucose. Results for the conjunctival cells by the direct inoculation method are presented in Table I. Starch, glycogen, maltose, d(+)galactose, d-fructose and d-mannose supported cell multiplication satisfactorily; the net increase in the number of cells in 8 days in the presence of these carbohydrates was similar to that with glucose at a concentration of 5 mM. Results obtained with sucrose were dependent on the dialyzed serum used. When tested with 2 dialyzed horse sera, results such as those presented in Table I were obtained. However, when 3 individual dialyzed human sera were used, sucrose failed completely to sustain cell growth. Lactose, l(+)rhamnose, l(−)fucose, l-sorbose, l(−)xylose, d(+)xylose, d(−)lyxose, d(−)arabinose, l(+)arabinose, d-2-deoxyribose; i-erythritol, dihydroxyacetone, sodium pyruvate and sodium lactate were unable to support cell propagation; the results were similar to those obtained with d-ribose, which are shown in Table I. Essentially similar results were obtained with the HeLa cells. Except in the case of sucrose, no appreciable difference in the results was obtained whether a dialyzed horse or a dialyzed human serum was used. Those carbohydrates incapable of sustaining cell growth were further tested by the medium replacement method. Results are shown in Table II. Sodium pyruvate, sodium lactate, d-ribose and d(+)xylose delayed the degeneration of both kinds of cells. L(+)rhamnose, l(−)fucose and l(−)sorbose appeared to retard the degeneration of the HeLa cells more effectively than the conjunctival cells. Lactose, l(−)xylose, d(−)lyxose, d(−)arabinose, l(+) arabinose, d-2-deoxyribose, i-erythritol and dihydroxyacetone failed completely to produce any beneficial effect.

Carbohydrases in dialyzed serum. The satisfactory cell propagation in such carbohydrates as starch, glycogen, maltose and sucrose may be explained by the existence of amylase, maltase and, perhaps, even sucrase in the serum. These enzymes would hydrolyze the poly- and di-saccharides into glucose

TABLE II. Degeneration of Conjunctival and HeLa Cells in Certain Selected Carbohydrates.

	Cell × 10^3/tube on the following days:							
	0		4th		8th		20th	
Carbohydrate	Conj.	HeLa	Conj.	HeLa	Conj.	HeLa	Conj.	HeLa
None	33	26	2	22	0	0		
Glucose	33	26	132	112	217	397		
Pyruvate	33	26	9	14	7	11	5	7
l(+)rhamnose	33	26	2	20	3	7	0	6
d-2-deoxyribose	33	26	3	5	0	0		

and fructose, both of which are readily utilized by the cells. In several actual determinations, we were able to demonstrate positive glucose oxidase reaction with dialyzed serum containing starch, glycogen, and maltose after incubation of these serum-carbohydrate mixtures at 37°C for 16-20 hours; 2 individual dialyzed horse sera and 3 dialyzed human sera tested showed the ability of hydrolyzing these 3 carbohydrates. With sucrose, a positive glucose reaction was obtained when incubated with the dialyzed horse but not with the dialyzed human sera. No glucose was detected after incubation of lactose with any of the 5 dialyzed sera tested. This finding corresponded closely with the results of cell propagation studies using these dialyzed sera and carbohydrates. Satisfactory propagation occurred in starch, glycogen and maltose with any of the sera, sucrose would support cell growth only when dialyzed horse sera were used and lactose failed completely to support growth with all of the sera.

Glucose sparing effect of alanine and pyruvate. In view of the finding that sodium pyruvate was able to delay cell degeneration, the addition of this intermediate to medium containing a low concentration of glucose may spare some of the glucose from being oxidized as a source of energy, thereby increasing the amount of glucose available for synthesis of essential cell components. Results of experiments presented in Table III appeared to support this hypothesis. The addition of pyruvate and dl-alanine to media containing 0.1 mM glucose prevented the partial cell degeneration normally occurring between the 10th and 18th days. Cell multiplication occurred in media containing 0.01 mM glucose when pyruvate and alanine were added, while at 0.001 mM glucose, the addition of pyruvate and alanine delayed the rate of cell degeneration.

In an experiment with the conjunctival cells, when glucose was substituted by d-mannose or d(+)galactose, both at concentrations of 0.01 mM, satisfactory cell multiplication was observed in the presence of alanine and pyruvate; d-fructose was an effective substitute only at higher concentrations. This glucose sparing effect was shown to be due largely to pyruvate; alanine without pyruvate was completely ineffective; and, pyruvate without alanine was not as effective as pyruvate with alanine.

Discussion. Harris and Kutsky(8) reported that glycogen, maltose, d-mannose, d-fructose supported vigorous growth of chick heart fibroblast, that d-galactose was inhibitory and that sucrose, d-ribose and d-xylose were inert. Our results with human cells showed that d (+)galactose supported cell growth as well as glucose and that d-ribose and d-xylose retarded cell degeneration. Differences in the cell system used, the method of quantitation and the assay medium may account for some of these disagreements.

The presence of carbohydrases in serum

TABLE III. Glucose Sparing Effect of Pyruvate and dl-alanine.*

Medium†			Cells × 10^3/tube on following days:			
Glucose	Pyruvate	Alanine	0	6th	10th	18th
.1	0	0	14	67	158	38
.1	5	5	14	64	197	246
.01	0	0	14	3	4	0
.01	5	5	14	9	47	58
.001	0	0	14	0	0	
.001	5	5	14	3	1	0

* Results obtained with conjunctival cell. Tests with HeLa cells gave similar results.

† Basal carbohydrate-free medium with addition of glucose, pyruvate and dl-alanine concentrations indicated (expressed in mM).

may be an explanation for the satisfactory cell propagation in starch, glycogen, maltose and sucrose, as suggested by Harris(8). The ability of the 2 dialyzed equine sera and the inability of the 3 human sera to hydrolyze sucrose sufficiently to support cell growth tends to support this view.

The difference in the sucrase activities of the various individual sera is of interest in studies of the nutritional requirement of human cells. It illustrates how the results of certain studies may be influenced by the source of serum incorporated into the assay medium. Other differences of sera have already been described(9).

Summary. 1) Under the described experimental conditions, both conjunctival and HeLa cells propagated satisfactorily in the following individual carbohydrates: starch, glycogen, maltose, d-glucose, d-fructose, d-mannose and d(+)galactose. Cell propagation in sucrose depended largely on source of dialyzed serum. Sodium pyruvate, sodium lactate, d-ribose and d(+)xylose were unable to support cell multiplication, though cell degeneration in the presence of these carbohydrates and intermediates was definitely retarded. L(+)rhamnose, l(−)fucose and l-sorbose appeared to delay the degeneration of the HeLa cells more effectively than that of the conjunctival cells. Lactose, l(−)xylose, d(−)arabinose, l(+)arabinose, d-2-deoxyribose, i-erythritol and dihydroxyacetone neither supported growth nor retarded degeneration. 2) Glucose at low concentration of 0.1 mM is capable of supporting cell growth for at least several months. Net increase in number of cells at a concentration of 0.1 mM is significantly lower than that in 10 mM glucose. When pyruvate and alanine were added to the medium, the concentration of glucose could be further reduced to 0.01 mM without resulting in cell degeneration. D-mannose and d(+)galactose both at 0.01 mM concentrations, and d-fructose at somewhat higher concentration, are effective substitutes for glucose.

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1. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

2. Gey, G. O., Coffman, W. D., and Kubicek, M. T., *Cancer Research,* 1952, v12, 2164.

3. Eagle, H., *J. Exp. Med.,* 1955, v102, 37.

4. Robinson, L. B., and Wichelhausen, R. H., *Science,* 1956, v124, 1147.

5. Chang, R. S., and Geyer, R. P., *J. Immunol.,*

6. Adams, E. C., Burkhart, C. E., and Free, A. H., *Science,* 1957, v125, 1082.

7. Keilin, D., and Hartree, E. F., *Biochem. J.,* 1948, v42, 221.

8. Harris, M., and Kutsky, P. B., *J. Cell. Comp. Physiol.,* 1953, v42, 449.

9. Chang, R. S., *Conf. on Virus, Nucleic Acids and Cellular Biol.,* N. Y. Acad. Sciences.

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Effect of Beta-Aminopropionitrile on Incorporation of Glycine by Croton Oil Pouches in Rats.* (23472)

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Although several investigators have studied the influence of beta-aminopropionitrile (BAPN) on mesodermal tissues, the mechanism of the BAPN effect is still not understood. Dasler(1) pointed out that it is tissues which are high in collagen that appear to be primarily affected by the lathyrus factor, and he has suggested that lathyrism involves a disturbance in the metabolism of connective tissue or of a connective tissue component. Ponseti and Shepard(2) also concluded that mesenchymal tissues are influenced by the lathyrus

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