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Isolation of Nutritional Variants from Conjunctival and HeLa Cells.* (23618)

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Properties acquired by human cells upon prolonged cultivation have been a subject of considerable interest. Attention, however, has been directed chiefly toward the problem of acquired malignancy as evidenced by the numerous publications on this subject(1). While changes in the nutritional requirements of various laboratory cell strains have long been suspected, a report on the appearance of a variant capable of utilizing a chemical compound in lieu of an essential nutrient to support growth has been conspicuously lacking. Those of Haff and Swim(2), Puck(3) and Chang(4) referred to quantitative differences in the requirement of, or tolerance to, certain biologicals of unknown complex composition. This publication describes the successful isolation of nutritional variants from our cultures of human conjunctival(5) and HeLa cells(6). These variants are capable of utilizing certain chemical compounds in lieu of glucose to support continuous cell growth.

Material and methods. The maintenance of stock cell cultures; collection, storage and dialysis of serum; composition of the basal carbohydrate-free medium; glucose oxidase test; enumeration of cell number, and, assessment of cell multiplication rate have already been described(7). *Compounds.*† D(+)xylose, d-ribose, d-arabinose, sodium lactate, sodium pyruvate and alanine were used. These compounds were tested for possible trace contamination with glucose by the glucose oxidase test, and for their ability to prevent progressive degeneration of the stock conjunctival cells at concentrations of 125, 25 and 5 mM in the basal media. With the exception of d(+)xylose, these compounds gave negative glucose oxidase tests at concentrations of 2M, and were unable to prevent progressive cell degeneration. The three preparations of d(+)xylose tested gave positive glucose oxidase reactions at concentration of 0.1 to 0.2 M, and 2 of them were able to prevent progressive cell degeneration at concentrations of 25 and 125 mM. Although treatment of these xylose preparations with glucose oxidase, as described(7), failed to reduce appreciably their reactivities in the glucose test,‡ their ability to prevent cell degeneration was abolished. Thus, d(+)xylose which has been pretreated with glucose oxidase was used exclusively in this study. *Isolation of variants.* About one million cells

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† D(+)xylose, C. P., was purchased from Pfanstiehl, Fisher and Nutritional Biochemicals; d-ribose, d-arabinose and sodium pyruvate, from Nutritional Biochemicals; sodium lactate, from Mallinckrodt; and dl alanine, from Eastman Kodak.

‡ Slow oxidation of d(+)xylose in the presence of glucose oxidase has been described(8).

from stock cultures were inoculated into a 200 ml capacity serum dilution bottle containing 10 ml of 20% inactivated human serum in Eagle's basal medium(9). The bottle was incubated at 36°C with nutrient renewal every second day until a confluent sheet of polyhedral cells was formed; such bottles had been found to contain about 4 or 5 million cells. After 3 successive washings with the basal medium, the cells were then nourished in the basal carbohydrate-free medium plus a test compound at 5 mM concentration. This medium was renewed every 2-4 days until the appearance of advanced degenerative changes, and then, every 7 to 14 days. Serum collected from one single horse and dialyzed extensively was used exclusively in preparing the basal medium. If variants failed to develop in 60 days, the experiment was terminated. This method is based on the principles developed in the isolation of bacterial mutants(10).

Results. When the conjunctival or HeLa cells were nourished with the basal medium plus d(+)xylose at 5 mM concentration, progressive degeneration appeared on the 4th to 7th day. In certain experiments, several small colonies of cells were observed on about the 20th to 30th day. These colonies consisted of several polyhedral, an occasional mitotic and many rounded granular cells. Gradual enlargement of these colonies with more polyhedral, more mitotic and less rounded granular cells was observed on further examination at regular intervals. Eventually, stable strains of variants capable of continuous propagation with d(+)xylose as the sole source of added carbohydrate were obtained. All the 3 xylose preparations, with or without pretreatment with glucose oxidase, were capable of supporting the propagation of these variants.

This pattern of apparent degeneration followed by regeneration was also studied quantitatively by repeating similar experiments with HeLa cells grown in a series of culture tubes. When advanced degeneration appeared in all tubes, the average number of cells per tube was calculated from results of cell counts of 2 tubes picked at random. The remaining tubes were observed at regular intervals for the appearance of variants. In a successful experiment, areas of polyhedral, mitotic and degenerating cells were observed on about the 42nd day in 1 of the remaining 4 tubes. After about 7 more days, when sufficient polyhedral cells for actual cell count were observed, the cells were trypsinized, enumerated and reinoculated into fresh xylose medium. This procedure was repeated at intervals of 1 to 2 weeks. Results are shown in Table I.

TABLE I. Growth Pattern of HeLa Cells in Xylose Medium.

Tube	No. of cells × 10^3/tube on the following days: 0*	5th†	50th	57th	70th
A	20	9	0		
B	20	9	0		
C	20	9	0		
D	20	9	34	23	138

* Based on cell number in inoculum.

† Based on avg of 2 tubes picked at random during advanced degeneration; such tubes were discarded.

The development of d-ribose variants and sodium lactate variants is in general similar to that of d(+)xylose. As of this date, we have isolated one variant capable of continuous propagation in d-ribose and one in sodium lactate from the HeLa but none from the conjunctival cells. There is suggestive evidence that d-ribose and sodium lactate variants appeared less frequently than d(+)xylose variant from our cultures of cell at this time under the described experimental conditions.

We have been unsuccessful, to date, in isolating variants from either cells capable of continuous propagation in the carbohydrate-free medium, in d-arabinose or in sodium pyruvate plus dl alanine.

Discussion. A previous communication(7) described *in vitro* propagation of these cells in various carbohydrate media. The minimal concentration of glucose capable of supporting growth, under those experimental conditions, was about 0.1 mM. With the addition of pyruvate and alanine, this concentration could be further reduced to 0.01 mM. Among the monosaccharides and intermediates tested, d-mannose, d(+)galactose and d-fructose were the only effective substitutes. It was then postulated that at least 2 μg of glucose per ml must be supplied under those conditions for the synthesis of certain essential cell components. One is tempted to hypothesize that,

while the cells from stock cultures were unable, the variants were capable of converting sufficient ribose, xylose or lactate into glucose to meet the requirements of cell growth.

It should be pointed out that the basal carbohydrate-free medium is of unknown chemical composition, on account of the presence of dialyzed serum. However, since serum from one single horse or, in critical experiments, single batch of dialyzed serum from one single bleeding was used, this variability of nutrient media composition has been reduced.

I would like to emphasize that the described experiments were not designed to study quantitative differences. The apparent frequent appearance of d(+)xylose variants may or may not occur under other experimental conditions. The successful isolation, as of this date, of d-ribose and sodium lactate variants from HeLa and not conjunctival cells may or may not be due to difference between the two cell lines. Finally, the failure to isolate d-arabinose or sodium pyruvate variants does not imply that such variants cannot be eventually isolated.

Summary. A method used successfully in isolation of nutritional variants from the human conjunctival and HeLa cells has been described. Variants capable of continuous propagation, under prescribed experimental conditions, with d(+)xylose, d-ribose or sodium lactate as the sole source of added carbohydrate or intermediate have been isolated.

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Selective Accumulation of Cd^{115} by Cortex of Rat Kidney.* (23619)

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Up to the present time the only elements with sufficiently specific localization in an organ to warrant use of their radioactive isotopes as physiological research tools have been iodine, which is concentrated in the thyroid, and zinc, which is concentrated in the rat dorsolateral prostate(1). This study using radioisotopes reports another element, cadmium, which selectively accumulates in the cortex of rat kidney.

Procedure. For the study of Cd^{115} distribution in the tissues of the rat, 40 male and female Wistar rats, 12-16 weeks of age, were used. Cd^{115} (half-life 43 days) was administered by intracardiac injection to etherized rats in doses of 8 μc/kg.† The animals were sacrificed by chloroform and exsanguination at various intervals from 1 hour to 8 months after injection. The tissues to be studied were removed from the animal, dried, weighed, and

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† Cd-115-P processed, high specific activity, was purchased from Union Carbide Nuclear Co. as $Cd(NO_3)_2$ in HNO_3 solution. Dilutions were made with physiological saline.