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Growth and Glycolysis in Two Cell Lines Derived from Embryos of Salmonid Fish* (32767)

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A number of studies of glucose utilization and lactic acid production in cultures of cell lines derived from homoiothermic animals have been reported (1–6). Similar data on cell lines from poikilothermic vertebrates however is meager. Two lines of cells derived from embryos of coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Salmo gairdneri*) have been established in this laboratory (7). Various aspects of the metabolism of these cells *in vitro* are being studied, and the experiments reported here are concerned with a comparison of glycolysis in these salmonid cell cultures and in cultures of human cells of comparable embryonic derivation growing in the same nutrient medium.

Materials and Methods. Cell lines CSE 119 and STE 137 are heteroploid, fibroblast-like, and were derived from embryos of coho salmon and steelhead trout, respectively (7). The CSE 119 had been carried through 65 culture passages and STE 137 through 46 such passages when these experiments were begun. The human embryonic cells were diploid, fibroblast-like, and derived from 3 to 12 week old human embryos. They were obtained from Flow Laboratories, Rockville, Maryland.

Eagle's minimum essential medium (8) supplemented with 20% dialyzed newborn agamma calf serum[1] was used for all cell cultures. The serum was dialyzed in the cold against 10 volumes of Earle's balanced salt solution without glucose for 24 hours, with one change of salt solution at 12 hours. This removed most of the lactic acid and other low molecular weight metabolites. Penicillin at 100 units/ml and streptomycin at 100 μg/ ml were included in the nutrient medium.

For each experiment 20 or more 150-ml Pyrex milk dilution bottles were inoculated with an 8-ml volume of cell suspension con-

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taining from 300,000 to 500,000 cells/ml in the case of the fish cell lines. The inoculum for the human cell strain contained 200,000–300,000 cells/ml of medium. Fish cell cultures were incubated at 23°C. After 2-days incubation of CSE 119 cultures, 4 bottles were removed from the incubator and the culture medium pooled. The pH was measured immediately and the pooled fluid was frozen until chemical analysis. The cell monolayer was removed from each bottle by exposure to a known volume of 0.2% trypsin in phosphate buffered saline, pH 7.4, for 30 min and gentle scraping with a rubber policeman. The cells in the 4 bottles were dispersed by repeated trituration through a pipette. The suspensions plus rinsings, were pooled and adjusted to a known volume. The cells were then enumerated by the nuclei counting method of Sanford and Earle (9). Four separate counts were made on each suspension, involving a total of at least 400 cells, and from the mean value obtained the total cell population in the 4 bottles was calculated. Groups of 4 bottles were removed from the incubator after 4, 6, and 10 days incubation, and the above process was repeated at each interval. After 6 days, the culture fluid was removed and replaced with fresh medium. In the case of cell line STE 137, which had a slower growth rate than CSE 119, groups of culture bottles were removed for analysis after incubation periods of 2, 6, 10, 14, and 18 days. The nutrient medium was changed at the 10-day interval. The human embryonic cells, which had the most rapid growth rate, were incubated at 35°C. Groups of cultures were removed for analysis after incubation periods of 2.5, 4.0, 5.5, and 7.0 days, and the medium was changed after each 1.5-day interval.

The fish cell lines were tested for mycoplasma contamination by both cultural and chemical methods, but none was detected.

The culture fluid was freed from protein and analyzed for glucose by the glucostat (glucose oxidase) method of Worthington Laboratories, Freehold, N. J. Lactic acid was determined on the protein free extract by the Hullin and Noble modification of the Barker Summerson method (10).

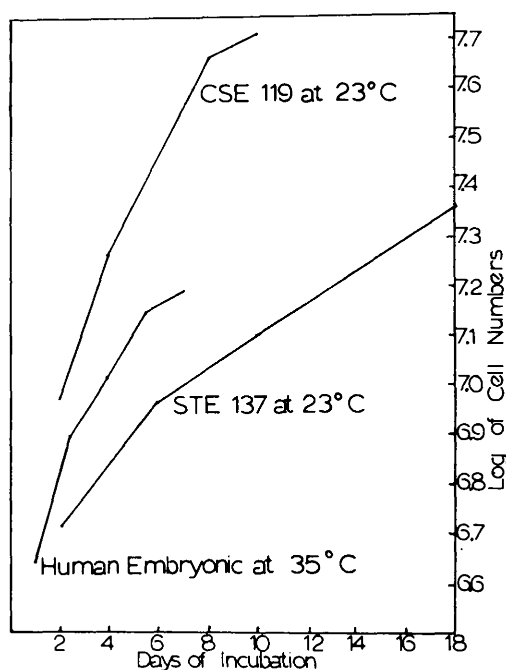

FIG. 1. Growth rates of 3 cell lines in Eagle's MEM with 20% dialyzed agamma calf serum.

The dry weights of cell lines were determined by first washing monolayer cultures with phosphate buffered saline and suspending the cells in some of the same diluent. The suspension was dispersed by repeated trituration by means of a pipette and a nuclei count was made in a hemocytometer. A 5.0-ml volume was transferred to a weighing bottle and dried to constant weight in a vacuum desiccator over P_2O_5.

Results. The growth rates of the 3 cell cultures studied were measured during the course of experiments on glycolysis. The mean rates obtained from 3 experiments with each cell line are shown in Fig. 1. The cell generation times measured during periods of most rapid growth were 122 hours for STE 137, 50 hours for CSE 119, and 45 hours for the human embryonic cells.

The rates of glucose utilization and lactic acid production measured in cultures of CSE 119 during four 2-day intervals in a 10-day growth period are shown in Fig. 2. The curves in Figs. 2, 3, and 4 represent the mean values obtained from 3 replicate experiments. The cell numbers indicated are

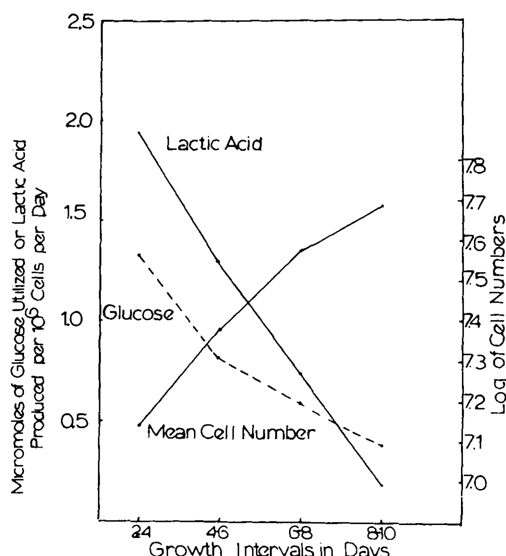

FIG. 2. Glycolysis in cultures of coho salmon cell line CSE 119.

the means of the cell counts at the beginning and end of each measurement interval. It is apparent that the rates in Fig. 2, in terms of μmoles per 10^6 cells per day were highest in the first 2-day interval, when the growth rate was maximal (Fig. 1). Thereafter they decreased steadily through the remainder of the experimental period. This

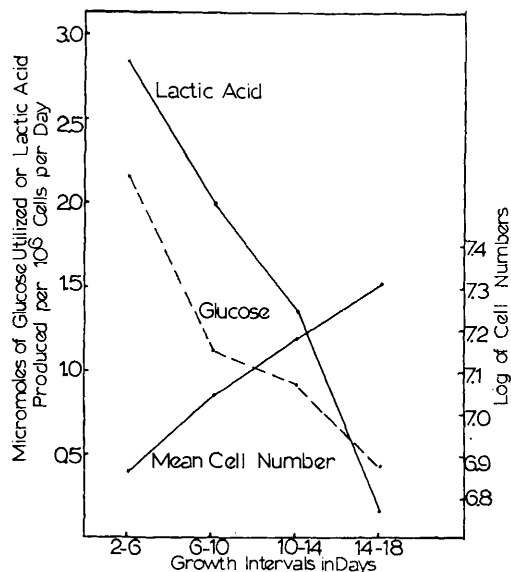

FIG. 3. Glycolysis in cultures of steelhead trout cell line STE 137.

puzzling phenomenon has been observed by others in both monolayer and suspension cultures, and appears to be characteristic of animal cell cultures growing *in vitro* (2,3,12). The molar ratio of lactic acid produced to glucose utilized indicates that the proportion of glucose appearing as lactic acid was about 74%, 80%, and 66%, respectively, during the first 3 measurement intervals. This proportion dropped to about 26% during the 8- to 10-day period. The pH of animal cell cultures has been found to influence the rate of glucose consumption and lactic acid production (11). In the experiments reported here no attempt was made to maintain a constant pH, but the range of pH variation was limited by changing the culture medium

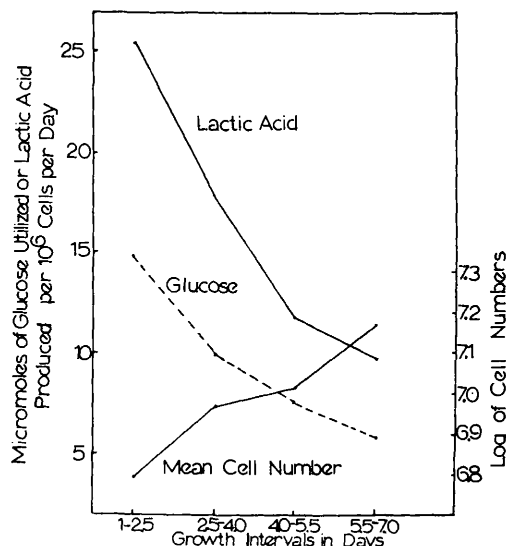

FIG. 4. Glycolysis in cultures of human embryonic cells.

at appropriate intervals. In experiments with CSE 119, the greatest pH variation occurred during the first 6 days, when the initial value of 7.5 or 7.6 dropped to 7.1 or 7.2.

Figure 3 shows comparable data obtained with STE 137. In this case glycolysis was measured during each of four 4-day intervals in an 18-day growth period because of the slower growth rate of this cell line. The same steady decrease in the rates of glucose consumption and lactic acid formation observed in Fig. 2 is also evident in Fig. 3. Both rates are seen to be somewhat higher during the

TABLE I. Comparison of Glycolysis in 2 Salmonid Fish Cell Lines and Human Embryonic Cells.

Cell line	Glucose utilized[a] (μmoles/10^6 cells/day)	Lactic acid produced[a] (μmoles/10^6 cells/day)	$Q^{O_2}_{CO_2}$
CSE 119	1.33	1.96	
STE 137	2.14	2.83	
Human embryonic	14.74	25.45	
	(μmoles/mg of dry wt./day)	(μmoles/mg of dry wt./day)	
CSE 119	5.81	8.56	7.97
STE 137	3.65	4.82	4.50
Human embryonic	8.18	14.13	13.19

[a] All values refer to mean rates from 3 experiments, during the first portion of the experimental growth period (1.5–4.0 days).

first measurement interval than those found with CSE 119. The proportion of glucose utilized appearing as lactic acid varied from about 66% in the first 4-day interval to about 90% and 74%, respectively, in the second and third intervals. It fell to less than 20% in the 14- to 18-day period. The greatest pH variation in these cell cultures occurred during the first 6 days of incubation, ranging from the initial value of 7.6 or 7.5 to 7.2 or 7.1.

In order to compare the data obtained with the 2 fish cell lines growing at 23°C with a mammalian cell culture of comparable origin growing at 35°C glycolysis was studied in cultures of diploid, fibroblast-like cells derived from 3- to 12-week human embryos. Because of their more rapid rate of growth, an experimental growth period of 7 days with 4 measurement intervals of 1.5 days was employed. The data obtained with these cells is shown in Fig. 4, where it may be seen that the general course of the glucose and lactic acid rate curves is similar to those found with the 2 fish cell lines. However the actual numbers of μmoles of glucose consumed and of lactic acid formed per 10^6 cells per day were 7- to 10-fold higher than for the fish cells. The proportion of glucose used appearing as lactic acid was about 86%, 89%, 78%, and 84%, respectively, for the first, second, third, and fourth measurement intervals. These values were somewhat higher than most of those observed with the fish cell lines, and the sharp fall noted with the latter toward the end of the experimental growth period did not occur. The pH range in the human cell cultures varied from the initial value of 7.6 or 7.7 down to about 6.8 at the end of the 7-day incubation period, despite changes of culture medium at each 1.5-day interval.

Comparisons of metabolic rates on the basis of cell numbers are commonly employed, but because of the variation in cell volume and weight between different cell cultures, it is desirable to determine such rates in terms of another frame of reference. The dry weight of cells has been most frequently used as a basis for comparing the data of different investigators. For this reason the dry weights of the 3 cells employed in this study were determined. The mean values from at least 4 separate measurements in each case were (i) 229 μg/10^6 cells from cultures incubated 10–12 days for CSE 119 (ii) 587 μg/10^6 cells from cultures incubated 15–20 days for STE 137 and 1803 μg/10^6 cells from cultures incubated 10–12 days for the human embryonic cells. From these values the rates of glucose consumption and lactic acid formation during the first measurement interval, when they were highest, were calculated for all 3 cells in terms of μmoles/mg of dry weight/day. They are shown in the lower half of Table I, in comparison with the parallel values in terms of cell numbers in the upper half. It is evident that when compared on a dry weight basis, the metabolic activity of CSE 119 appears greater than that of STE 137, whereas the reverse is true on the basis of cell numbers. Furthermore the differences between the fish cells and the human embryonic cells are much smaller on the

weight basis, but the activity of the human cells is still considerably greater.

From the values for μmoles of lactic acid produced per mg of dry weight per day in Table I, *Q* values for aerobic glycolysis (μl of CO_2 per mg of dry wt. per hour) can be estimated. These values for the 3 cell lines are also indicated in Table I. They are similar in magnitude to values reported for other lines of cells in culture (2,5,12), and the figure for the human cells is again significantly higher than those for the fish cell lines.

Discussion. The purpose of the present study was to compare the rates of glycolysis of the 2 lines of salmonid fish cells growing at 23°C with that of a mammalian cell line of comparable embryonic derivation growing at 35°C. When compared on the basis of cell numbers, the fish cells utilized only 9% and 15% as much glucose as the human cells, but on the dry weight basis they consumed 71% and 45% as much, respectively (Table I). On this basis their glucose consumption appears to fall about midway in the range of values reported for other established cell lines, i.e., from about 2.7 to 10.9 μmoles/mg of dry wt./day (2). However, these values may not be directly comparable to those reported here because the culture media and conditions were not the same.

The *Q* values for aerobic glycolysis for the fish cell lines were distinctly below that of the human embryonic cells. They were also below the value of 11.2 reported by Cristofalo and Kritchevsky for growing cultures of human cell strain WI-38 (5) and 9.5 reported by Graff and McCarty for growing strain L fibroblasts (12). These *Q* values indicate a lower level of aerobic glycolysis in the fish cell lines than in the human cells. It was also noted that the proportion of glucose utilized which appeared as lactic acid was in most cases somewhat lower in the fish cell lines than in the human cells. This suggests that in the fish cells a greater proportion of the glucose was being metabolized by one of the oxidative pathways than was true in cultures of the human cells.

The steadily decreasing rates of glucose utilization observed with all 3 cell lines may perhaps be accounted for in part by decreasing pH values and O_2 concentration (11). However, replacement of the culture fluid with fresh medium did not restore these rates to their initial values. Another possibility is suggested by the work of Munyon and Merchant (3) who found that in suspension cultures of L cells, the mean cell volume as well as the rate of glucose consumption, decreased progressively with the age of the cultures. They proposed the hypothesis that the rate of carbohydrate metabolism is directly related to the mean cell volume.

The gradual fall in pH which occurs in these fish cell cultures is in contrast to the rapid fall characteristic of most mammalian cell lines. This difference is apparently due in part at least to the smaller amounts of lactic acid produced by the salmonid cells.

Summary. Measurements of growth and glycolysis in 2 established heteroploid fibroblast-like cell lines derived from embryos of coho salmon and steelhead trout, growing at 23°C, have been compared with similar measurements in cultures of a strain of diploid cells derived from human embryos, growing at 35°C. The growth rate of the coho salmon cells was nearly as great as that of the human cell strain. The rates of glucose utilization and lactic acid production by the salmonid cell lines were lower than those of the human cells. The proportion of glucose used which appeared as lactic acid in the culture medium was frequently lower in the fish cell cultures. The data obtained seemed to indicate that the process of glycolysis in these lines of salmonid fish cells proceeds in a manner very similar to that in mammalian cell cultures and that differences which exist are chiefly quantitative in nature.

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A Quantitative Difference in the Immune Response between Male and Female Mice* (32768)

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In studies using Swiss Albino (Hale-Stoner) mice, a difference in the immune response of male and female mice to bovine serum albumin (BSA) has been frequently noticed. In order to confirm this observation a systematic investigation of the difference was undertaken. The quantities of antibody produced were compared as was the minimum dose required to elicit a primary response.

Materials and Methods. Mice used in these experiments were raised in a closed colony in this laboratory and were from 4 to 8 weeks of age when used. To minimize thyroidal uptake of radioactive iodine in mice injected with ^{125}I labeled antigen, NaI (0.1%) was added to the drinking water (1). In designated experiments, 0.5 mg Thephorin (phenindamine) was injected subcutaneously 15–20 min prior to the test dose of ^{125}I labeled antigen in order to reduce the incidence of fatal anaphylaxis (2).

Antigen. Crystalline bovine serum albumin (BSA) was obtained from Armour Laboratories, Chicago, Illinois. The BSA was labeled with ^{125}I using monoiodochloride (ICl) as described elsewhere (3). In all experiments, the primer and the booster were administered subcutaneously and the challenge was administered intravenously 7 days following the booster. The BSA dilutions were made with a solution containing 1% normal mouse serum.

Antibody. An hyperimmune mouse anti-BSA antiserum was prepared by injecting mice with 16 mg of fluid BSA given in four equal doses followed by 10 mg of BSA in complete Freund's adjuvant. The injections were given on alternate days and the animals were bled 18 days after the final injection.

Immune degradation. The degradation of antigen was followed by whole body measurement of retained radioactivity. The amount of antigen degraded at an accelerated rate (ADAR), which is linearly related to log circulating antibody concentration, was calculated as follows (4):

$$\mathrm{ADAR} = \left(1-\frac{S}{N}\right)_t 100.$$

S = percent of antigen retained in experimental mice at time (t); N = percent of antigen retained in control mice at time (t); t = time of determination. Mice which were fatally shocked when tested were assigned an ADAR of 85.

Farr Technique. The primary interaction of iodine labeled antigen with antibody was measured by ammonium sulfate precipitation as previously described (5). The antibody concentrations are expressed as the amount of antigen that was 10% precipitated by 0.1 ml of the antiserum. This quantity has been found to be linearly related to the concentration of antibody (6) such that 0.66 log ratio of the amounts of BSA that are 10% precipitated equals log ratio of the antibody

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