COMPARATIVE STUDIES OF THE GROWTH REQUIREMENTS, CHROMOSOME NUMBER, AND ISOENZYMES OF TWO HUMAN CELL LINES (HELA & HEP-2)

by

Diane Krill

Submitted in Partial Fulfillment of the Requirements
for the Degree of
Master of Science
in the
Biological Sciences
Program

Paul C Peterson 8/12/80
Advisor Date

Dean of the Graduate School 8/18/80
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YOUNGSTOWN STATE UNIVERSITY

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ABSTRACT

COMPARATIVE STUDIES OF THE GROWTH REQUIREMENTS, CHROMOSOME NUMBER AND ISOENZYMES OF TWO HUMAN CELL LINES (HELA & HEP-2)

Diane Krill

Master of Science

Youngstown State University, 1980

A characterization of the Hep-2 epidermoid carcinoma cell line was conducted to determine the functional differences between Hep-2 cells and a suspected contaminant, HeLa cervical carcinoma. Parameters utilized to measure differences between the two cell lines were based on chromosome number, serum and pH requirements, rate of growth, and isoenzyme analysis.

Results were consistent with earlier reports indicating that Hep-2 and HeLa cells had identically migrating bands on cellulose acetate for phosphoglucose isomerase (PGI) and glucose-6-phosphate dehydrogenase (G6PD). Significant differences were noted between the cell lines in regard to growth requirements, generation time, and chromosome number.

ACKNOWLEDGEMENTS

I would like to extend my appreciation to Dr. Paul Peterson for his continued assistance, facilities, and advice in the pursuit of this project. I also wish to thank Dr. Monika Rudzik for the initiation of this project and for her assistance with the cellulose acetate electrophoresis system.

Additionally, I would like to express my appreciation to Dr. Barry Wessels for providing the Hep-2 cell line used in this study and for the opportunity of learning tissue culture technique under his direction.

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INTRODUCTION

Since the first established human cell line, HeLa, was initiated into tissue culture (Gey, et al., 1952), the usefulness of human tumor cells in vitro has been demonstrated in the clinical laboratory to support viruses (Syverton and McLaren, 1957), in the establishment of new anti-cancer drugs (Durkin, et al., 1979), and in determining the effect of ionizing radiation on the single cell (Hall, 1976). Recently chemotherapeutic treatment therapy has attempted to associate drug sensitivity to specific tumor locations through the use of cultured cell lines (Salmon, et al., 1978). For this purpose, and particularly for those studies addressing induced cellular transformations or immunological cross-reactivity of cultured cells and infecting animal viruses, the correct identification of the cell lines is of critical importance.

Recent survey studies performed on a large number of existing cell lines have brought forth the possibility that a considerable proportion of human lines may be contaminated with the HeLa cervical carcinoma line (Nelson-Rees, et al., 1974). The basis for the proposed contamination depends largely on karyological evidence (Lavappa, et al., 1976) and electrophoretic typing (Nelson-Rees and Flandermeyer, 1976). Electrophoretic typing is a recent innovation designed to recognize the multiple molecular forms of an enzyme within the same species, designated as isoenzymes. The identification

of differences in the isoenzyme patterns has made possible the distinction between human cell lines at the population level. This is due to the observation that certain allozymes occur with greater frequency in one population than another within the same species. One of the most commonly employed allozymes with respect to cell culture is glucose-6-phosphate dehydrogenase (G6PD), which is a sex-linked enzyme. Most Caucasians are type B G6PD, while in some African descendants type A is found with a frequency of up to 30% (Thompson and Thompson, 1973). The unexpected appearance of the type A G6PD in cells of Caucasian origin in a survey of cell lines (Nelson-Rees and Flandermeyer, 1976) may suggest the following possibilities: (i) the previous generation had been heterozygous genotypically, (ii) the transformation of a tumor into cell culture involves the acquisition of the type A G6PD, or (iii) the cell lines have been contaminated by HeLa. Relevant to the second possibility, Linder (Linder and Gartler, 1965) reported the selection for G6PD type A from a G6PD heterozygote in which the ratio of A to B changed from 40:60 to 80:20 over only twenty-five passages in culture.

Also under recent investigation is the chromosome complement of the suspect cell lines as a species-specific characteristic. Human cell lines cultured in vitro appear to have retained their original properties as cancer cells, particularly the capacity to vary in chromosome number and structure (Lin and Goldstein, 1974). Cultured cells typically exhibit a wide range of variant karyotypes around a modal number that

often differs greatly from the mode of the species from which the culture was taken. Despite the aneuploidy, each cell line has its own unique cytogenetic features (Ford, 1974) -- a recurring modal number of chromosomes, a limited range of chromosome number, and usually one or more marker chromosomes. An alternative to the contamination theory has been suggested (Fogh and Loveless, 1977) which maintains that the appearance of several marker chromosomes common to many cell lines may be an indication of selective factors favoring certain chromosomal rearrangements as a result of continued passage of the cell line, rather than the contamination of one cell line with another.

In view of the above considerations, a characterization study of a suspected HeLa contaminant was undertaken to analyze other cell-specific characteristics such as the length of cell cycle, plating efficiency, and pH and serum requirements as compared to HeLa characteristics. Additionally the chromosome complement and electrophoretic type of G6PD and PGI were determined to verify earlier reports in regard to this particular cell line.

The Hep-2 cell line was chosen for this study for a number of reasons. First a voluminous amount of research has been conducted on this cell line reaching into many other areas. Hep-2 cells have been used for experimental studies of tumor production in rats (Toolan, 1957), hamsters (Patterson, et al., 1957), and embryonated eggs (Toolan, 1955). Dose response relationships have been determined employing Hep-2 cells for the

newly developed anti-cancer drugs, methotrexate (Bishun, et al., 1976) and 5-fluorouracil (Laskin, et al., 1979). In order to substantiate these results, accurate identification of the cell line is requisite. Secondly Hep-2 cells have been identified by numerous studies as G6PD type A and thus termed a HeLa contaminant. Lastly, Hep-2 cells were isolated and cultured by a similar method and at approximately the same time as the HeLa cells. Gey and coworkers (1952) employed a plasma clot, roller drum technique to implant the tumor from Henrietta Lack (HeLa) into tissue culture. After two generations in rats, the Hep-2 epidermoid carcinoma was implanted by this same method and was subsequently characterized (Moore, et al., 1955). In this study comparisons are made experimentally between the Hep-2 strain isolated by Moore and the currently available HeLa strain (ATCC #2). Further comparisons are made between the original Hep-2 culture described by Moore and the Hep-2 strain in its present form.

This study statistically describes the significant differences between HeLa cervical carcinoma and Hep-2 epidermoid carcinoma of the larynx by examination of the generation time, chromosome number, isoenzymes, and pH and serum requirements. This information may then provide more insight into whether Hep-2 is a HeLa contaminant, a unique cell line, or a Hep-2/HeLa hybrid expressing properties of both cells.

MATERIALS AND METHODS

The Hep-2 strain used in this study was supplied by Dr. B. Wessels, Department of Radiation Therapy, Youngstown Hospital Association, Youngstown, Ohio. The HeLa cell strain was obtained from the American Type Culture Collection, Rockville, Maryland.

The methodology for the determination of the cell cycle length was primarily a coupling of cell synchronization technique followed by microscopic observation of mitosis. Cell synchronization was achieved by the mitotic harvest technique (Terasima and Tolmach, 1963), selected due to the large degree of synchronization achieved with a minimum of physiological disturbance. Between 1 and 2 X 10^5 Hep-2 cells in 10 ml of complete medium (Basal Media Eagles' 15% newborn calf serum and .25% antibiotic) were cultured in a 25 cm^2 Falcon flask in a 37° C incubator as the initiation culture. The medium was discarded after 24 hours and replaced with fresh, prewarmed medium of the same composition to remove any dead cells that are detached from the surface. After six hours the medium was again removed and discarded, and 5 ml of prewarmed medium was forcibly ejected from a 10 ml pipette to wash off the loosely attached dividing cells. This cell suspension was pipetted into a sterile flask and incubated at 37° after the 24-hour reattachment period. Microscopic observations of the newly-plated synchronized cells were made at one hour intervals.

The same procedure was repeated simultaneously for HeLa cells.

Standard growth curves were prepared from equally confluent cultures of HeLa and Hep-2 cells. Eight flasks, four of each cell type were innoculated with approximately 20,000 cells per flask and incubated at 37^{o} in 5% CO_2 humidified atmosphere. At periodic intervals, one flask of each cell type was trypsinized and the number of cells counted by hemocytometer.

Comparisons of growth requirements and plating efficiency were accomplished with a multifactor analysis of variance in which three types of serum, three levels of pH, and the two types of cells were tested simultaneously. The day preceding the experiment, three separate flasks of media were prepared consisting of 1) Basal Media Eagle's, 15% newborn calf serum, and .25% antibiotic 2) Basal Media Eagle's 15% horse serum, and .25% antibiotic, and 3) Basal Media Eagle's, 15% calf serum, and .25% antibiotic. Trypsin (Shipman, 1973) was then added to two stock cultures, one of Hep-2 and one of HeLa, which were selected on the basis of equal confluency and age. The trypsinized cell suspension was then divided into three parts of .5 ml each and one of three sera in medium was added to each cell suspension in 10 ml quantities. Hemocytometer counts determined the cell concentration, and then approximate dilutions were made to arrive at a final concentration of 1,000 cells per flask. Prewarmed medium of each type was added to each set of four replicate flasks and three pH levels were maintained at 7.15,

7.45, and 7.75 by the addition of $NaHCO_3$ to the media. The 72 cultures were placed in the incubator in alternating rows and positions so as to reduce any effect of temperature variance by location. The cultures were allowed to grow over a period of thirteen days, then were stained with Wright's stain and analyzed for colony growth.

The chromosome number per cell of Hep-2 cells was determined with the incorporation of a metaphase-arresting agent, colchicine (Grand Island Biological Company) added to a culture ninety minutes before harvest at a final concentration of .1 g/ml. The cells were detached with .25% trypsin, centrifuged and resuspended in warm hypotonic (.75 M KCl) solution and incubated for 30 minutes at 37^0 C. These cells were spun down again at 1,000 rpm for 8 minutes and fixed in 75% methanol-25% acetic acid. Slides were prepared with a staining solution of 10 mM phosphate at pH 6.8 containing 1% concentrated Giemsa, followed by two rinses in distilled water. Chromosome counts of fifty metaphases were made using a camera lucida, available on a Wild Heebrugg microscope. Representative chromosome spreads were photographed with a microscope equipped with a 35 mm Nikon camera.

Cellulose acetate electrophoresis was carried out to verify the G6PD type of Hep-2 cells, following a methodology developed for enzyme variants in Leishmania (Kreutzer and Christensen, 1980). The cell buffer system for the G6PD analysis was prepared as follows: 28.4 g Na_2HPO_4 (.2M) and 24 g NaH_2PO_4 (.2M) were dissolved in 1000 ml distilled water.

The pH of the solution was adjusted by adding NaH_2PO_4 to the Na_2HPO_4 to reach a pH of 7.0. The membrane buffer was 1 part cell buffer and 14 parts distilled water. The reaction buffer was prepared by adding 3.64 g of hydroxymethyl aminomethane (Tris) in 400 ml of distilled water, adjusting the pH to 8.0 with 50% HCl and bringing the final volume to 500 ml.

Homogenates of the cell cultures were extracted using the dry ice freezing-thawing technique (Peterson, et al., 1979). The monolayer cultures were suspended with trypsin, centrifuged, and washed three times with .9% NaCl to remove all traces of media. The cell suspension was frozen in 50% methanol-50% dry ice solution and then allowed to thaw at room temperature. This procedure was repeated three times for each culture. The suspension was then centrifuged at 3,000 rpm for 10 minutes, and the supernatant liquid was transferred to a new tube and stored at -20° C.

The cellulose acetate strip was presoaked (20 minutes) in the membrane buffer. Care was taken to slowly and steadily immerse the strip in the buffer to insure complete diffusion of the buffer and to prevent the formation of air bubbles. Next 100 ml of cell buffer was poured into the outer sections of the Helena electrophoretic chamber and filter paper wicks were placed over the cell/membrane contact areas. Samples of Hep-2 cells, HeLa cells, and control (hydra) were thawed and .5 µl of each was transferred to the sample well plate. The samples were applied to the

cellulose acetate strip with a Helena slot applicator (double applications), and the strip was then quickly blotted and placed in the chamber.

The samples were run for 15 minutes at 170 volts. After electrophoresis, the plate was again blotted and placed in a petri dish containing the reaction components in agar for G6PD was prepared as follows: .075 g D-glucose-6-phosphate, .015 g MTT tetrazolium (Mtt), .015 g triphosphopyridine nucleotide (TPN), .010 g phenazine methosulfate (PMS), were dissolved in 30 ml reaction buffer, then mixed with 30 ml 2% Noble Agar in the reaction buffer. The zymograms were placed in 5% acetic acid, washed and air dried. They were then photographed and stored for later study.

The same basic procedure was followed for a PGI analysis. The cell buffer consisted of 12.11 g Tris, 11.63 g Maleic acid, 2.92 g disodium ethylenediamine tetraacetic acid (EDTA) and 2.03 g $MgCl_2$; the membrane buffer was a 1:14 dilution of cell buffer in distilled water; and the reaction buffer consisted of 3.64 g Tris in 400 ml distilled water, pH adjusted to 8.0 with 50% HCl and final volume brought to 500 ml.

For a control in the PGI enzyme determination, 75 μl of human blood cells were separated from plasma by allowing to stand several minutes. To the blood cells 200 μl of distilled water were added. The tubes were capped and frozen in 50% methanol - 50% dry ice solution, then allowed to thaw at room temperature. This procedure was repeated three times

then the homolysate was centrifuged and stored at -20° C.

PGI reaction plates were prepared by adding 25 units G-6-PD, .006 g Nicotinamide Adenine Dinucleotide Phosphate (NADP), .012 g Phenazine Methosulfate (PMS), .012 g MTT Tetrazolium (MTT), .120 g Magnesium Chloride ($MgCl_2$) to 30 ml reaction buffer, combining this mixture with 2% Noble Agar and pouring into Petri dishes. Electrophoresis for PGI was was 25 minutes at 200 volts.

RESULTS

The first category of differences between cell types were assessed on the basis of cell generation time. Synchronized cells of both cell types were observed microscopically for the generation time as an indicator of overall metabolic efficiency of the cell population. An increase in the cell number was measured by repeated counting of the number of cells in a specified number of microscopic fields, delineated by marks fixed to the bottom of the culture flask. Approximately one thousand cells were innoculated into the flask which resulted in only one or two cells appearing in each field. The flask was carefully monitored and changes were scored in total cell number due to the division of individual cells. Grossly abnormal cells were disregarded and not scored.

From the graphs in Figure 1, it can be observed that the Hep-2 cells underwent mitosis 37 hours after the last consecutive mitotic period. An increase in the number of cells began to appear after 28 hours, with the most rapid increase occurring from 36 to 37 hours. An instantaneous decrease is then seen due to the cells greatly reduced attachment to the flask at this point in the cell cycle. Comparatively, HeLa cells required 42 hours to double in cell number. The initial increase began at 35 hours and had been completed by eight hours later. These results are in agreement with the findings of Terasima, et. al., (1963) for the HeLa cell cycle. A sig-

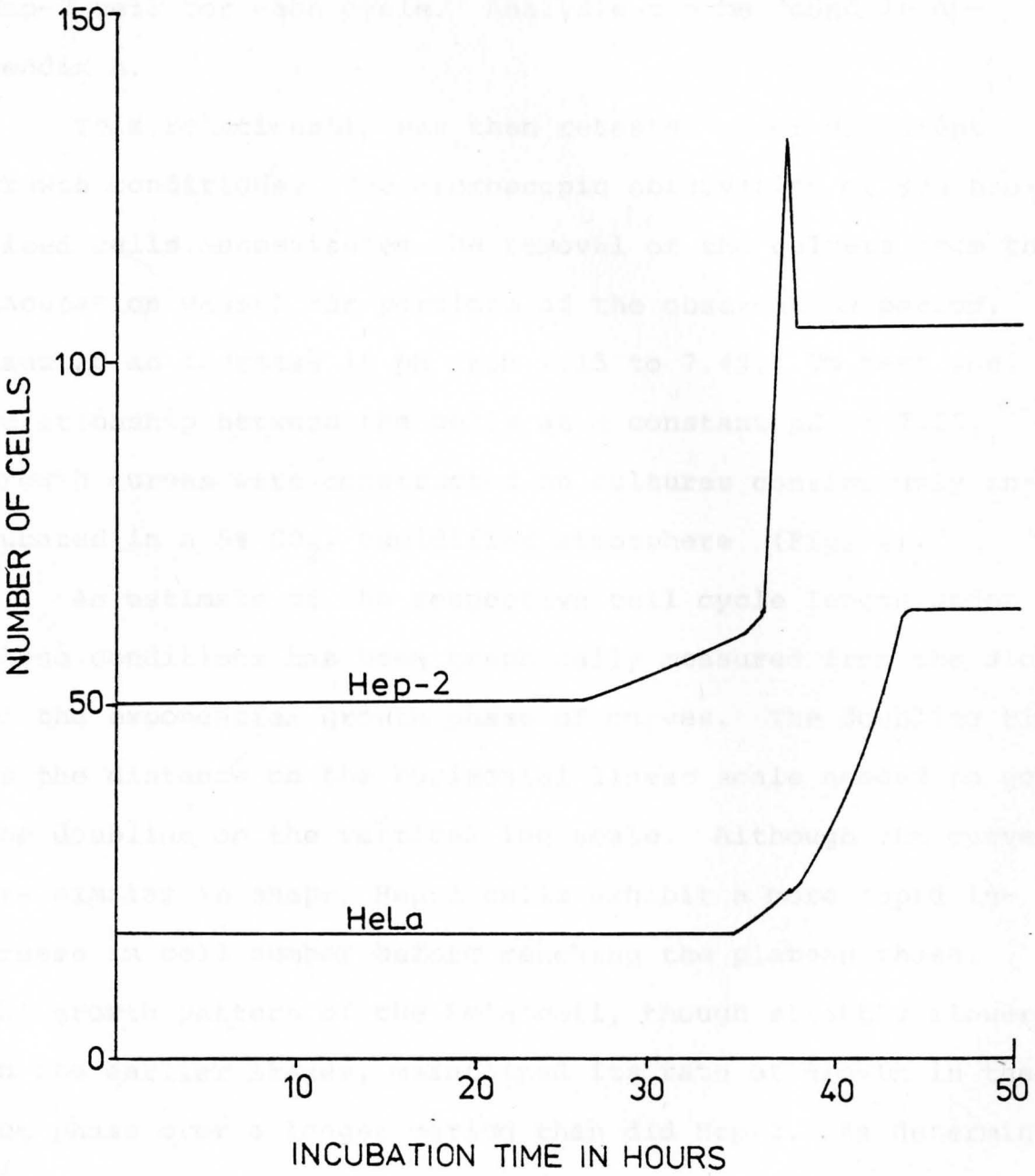


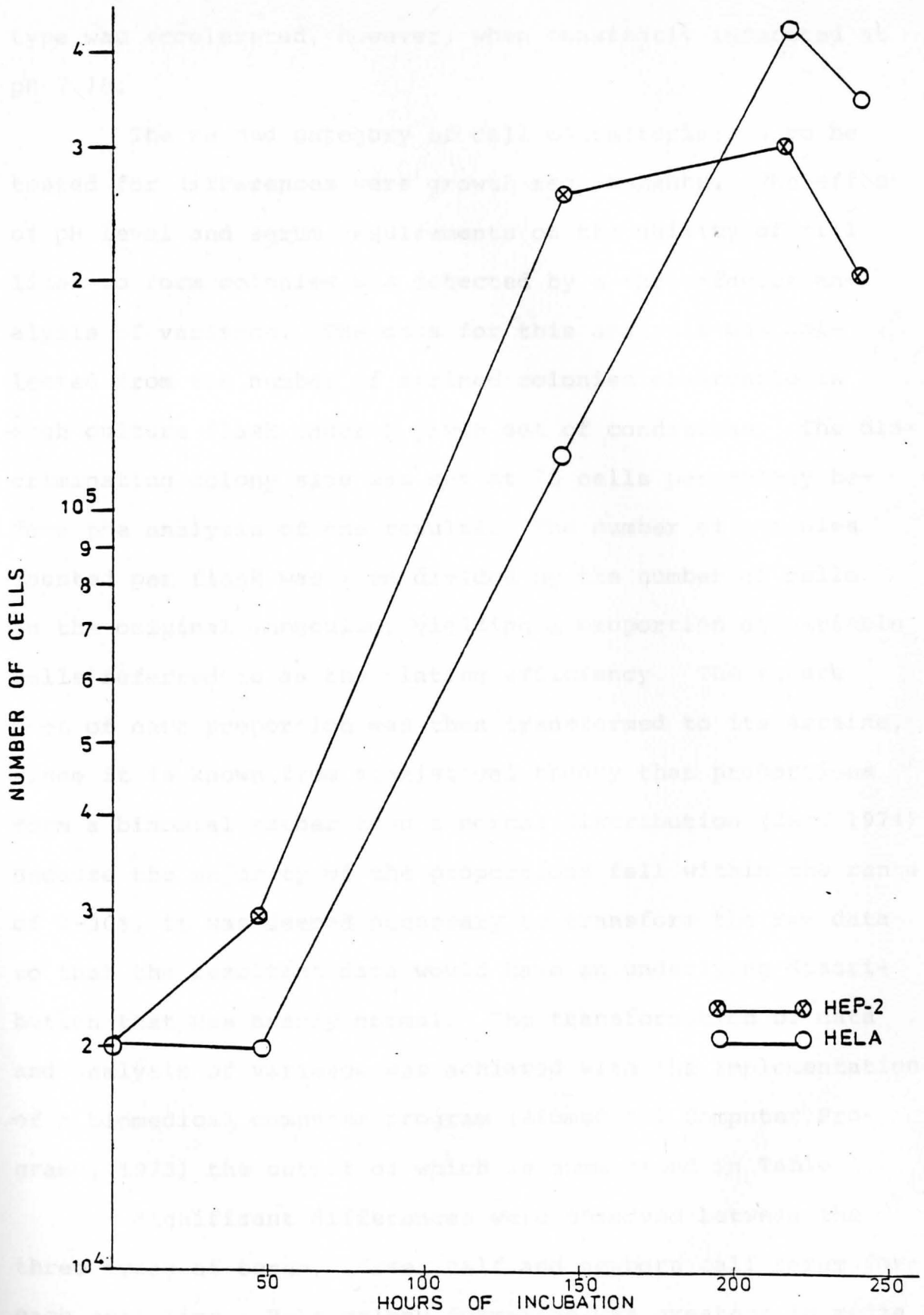
Fig. 1. Comparative Doubling Time Graphs for HeLa and Hep-2 Cell Lines Utilizing Synchronized Cells.

nificant difference between doubling times for the cell lines is 5 hours; HeLa requiring 5 hours longer than the Hep-2 cell for each cycle. Analysis can be found in Appendix A.

This relationship was then retested under different growth conditions. The microscopic observation of synchronized cells necessitates the removal of the culture from the incubation vessel for portions of the observation period, causing an increase in pH from 7.15 to 7.45. To test the relationship between the cells at a constant pH of 7.15, growth curves were constructed on cultures continuously incubated in a 5% CO_2, humidified atmosphere (Fig. 2).

An estimate of the respective cell cycle length under these conditions has been graphically measured from the slope of the exponential growth phase of curves. The doubling time is the distance on the horizontal linear scale needed to go one doubling on the vertical log scale. Although the curves are similar in shape, Hep-2 cells exhibit a more rapid increase in cell number before reaching the plateau phase. The growth pattern of the HeLa cell, though slightly slower in the earlier stages, maintained its rate of growth in the log phase over a longer period than did Hep-2. As determined from the graphs, the amount of time required for a HeLa cell to undergo one division cycle is 37 hours; for Hep-2, 32 hours. In both situations described, when maintained under identical conditions, the HeLa cell required 5 hours longer to double its cell number than the Hep-2 cell. The growth of both cell

Fig. 2. Comparative Growth Curves of HeLa and Hep-2 Cells Incubated in 5% CO_2 at 37° C; Data Points Reflect the Means of Three Experiments.


NUMBER OF CELLS
4
3
2
10^5
9
8
7
6
5
4
3
2
10^4
50
100
150
200
250
HOURS OF INCUBATION
HEP-2
HELA

type was accelerated, however, when constantly incubated at pH 7.15.

The second category of cell characteristics to be tested for differences were growth requirements. The effect of pH level and serum requirements on the ability of cell lines to form colonies was detected by a three-factor analysis of variance. The data for this analysis was collected from the number of stained colonies observable in each culture flask under a given set of conditions. The discriminating colony size was set at 75 cells per colony before the analysis of the results. The number of colonies counted per flask was then divided by the number of cells in the original innoculum, yielding a proportion of variable cells referred to as the plating efficiency. The square root of each proportion was then transformed to its arcsine, since it is known from statistical theory that proportions form a binomial rather than a normal distribution (Zar, 1974). Because the majority of the proportions fell within the range of 0-30%, it was deemed necessary to transform the raw data so that the resultant data would have an underlying distribution that was nearly normal. The transformation of data and analysis of variance was achieved with the implementation of a biomedical computer program (Biomedical Computer Programs, 1973) the output of which is summarized in Table 1.

Significant differences were observed between the three types of serum, horse, calf and newborn calf serum for each cell line. HeLa colony formation was greatest in media con-

TABLE 1

ANALYSIS OF VARIANCE SUMMARY TABLE FOR COMPARATIVE COLONY GROWTH OF HELA AND HEP-2 CELLS VARYING SERUM AND PH LEVELS.

Source of Variation	Degrees of Freedom	Mean Squares	F	Critical F	P
Serum	2	.10602	35.3	$F_{0.05(1)2,54=3.18}$	p<<.0005**
pH	2	.12725	42.3	$F_{0.05(1)2,54=3.18}$	p<<.0005**
Cell Type	1	.29831	99.3	$F_{0.05(1)1,54=4.03}$	p<<.0005**
Serum x pH	4	.00531	1.67	$F_{0.05(1)4,54=2.56}$	p> .10
Serum x Cell	2	.01292	4.33	$F_{0.05(1)2,54=3.18}$	.01<p<.025*
pH x Cell	2	.01194	4.00	$F_{0.05(1)2,54=3.18}$	.01<p<.025*
Serum x pH x Cell	4	.00146	.49	$F_{0.05(1)4,54=2.56}$	p>.25
Within Replicates	54	.00314			
Total	71				

taining newborn calf serum, following by horse serum, and least in calf serum. Hep-2 colony formation was highest in horse serum-supplemented media, followed by newborn calf serum, and least also, in calf serum. These results are summarized in Table 2.

Marginal means showed colonial survival, irrespective of cell type, decreased with an increase in pH over the range of 7.15 to 7.75. Hep-2 cells, however, decreased less than the HeLa cells at the higher extreme of the pH scale (Table 3). This interaction of cell type with pH level was found to be significant at the $p<.025$ level.

Overall, the Hep-2 colony formation significantly surpassed HeLa in the number of colonies scored for each category. The marginal mean recorded for the cell line variable was 2.19 for HeLa compared to 7.67 for Hep-2, a statistically significant difference at the $p<.0005$ level. HeLa colonies, though fewer in number, tended to be larger in size. Hep-2 colonies tended to be evenly dispersed, small colonies of approximately 50 to 100 cells per colony (Fig. 3).

In another experiment employing the growth of Hep-2 and HeLa at a pH range 7.3 ± .5, the media was removed from the flasks after a period of 24 hours and the pH determined again. In the flask containing the HeLa cells, a change had taken place in the direction of neutrality, to a pH of 7.1. In the Hep-2 flask, a change had taken place in the opposite direction, and the pH had readjusted at 7.45. No further significant change was noted thereafter during the 8 day observation period. This buffering capacity to the requisite

TABLE 2

SUMMARY OF SERUM EFFECTS BY CELL TYPE

CELL TYPE	SERUM		
	NEWBORN CALF	HORSE	CALF
HeLa	Best Growth	Fair Growth	Poor Growth
Hep-2	Fair Growth	Best Growth	Poor Growth

TABLE 3

TABLE OF CELL MEANS OF COLONY FORMATION FOR HELA AND HEP-2 CELLS VARYING PH LEVELS AND SERUM TYPE

SERUM	NEWBORN CALF			HORSE			CALF		
pH	7.15	7.45	7.75	7.15	7.45	7.75	7.15	7.45	7.75
CELL									
HeLa	7.69	4.08	.04	6.21	3.58	.96	2.91	1.20	.02
Hep-2	13.96	11.44	6.06	13.28	10.97	10.04	5.61	3.11	1.17

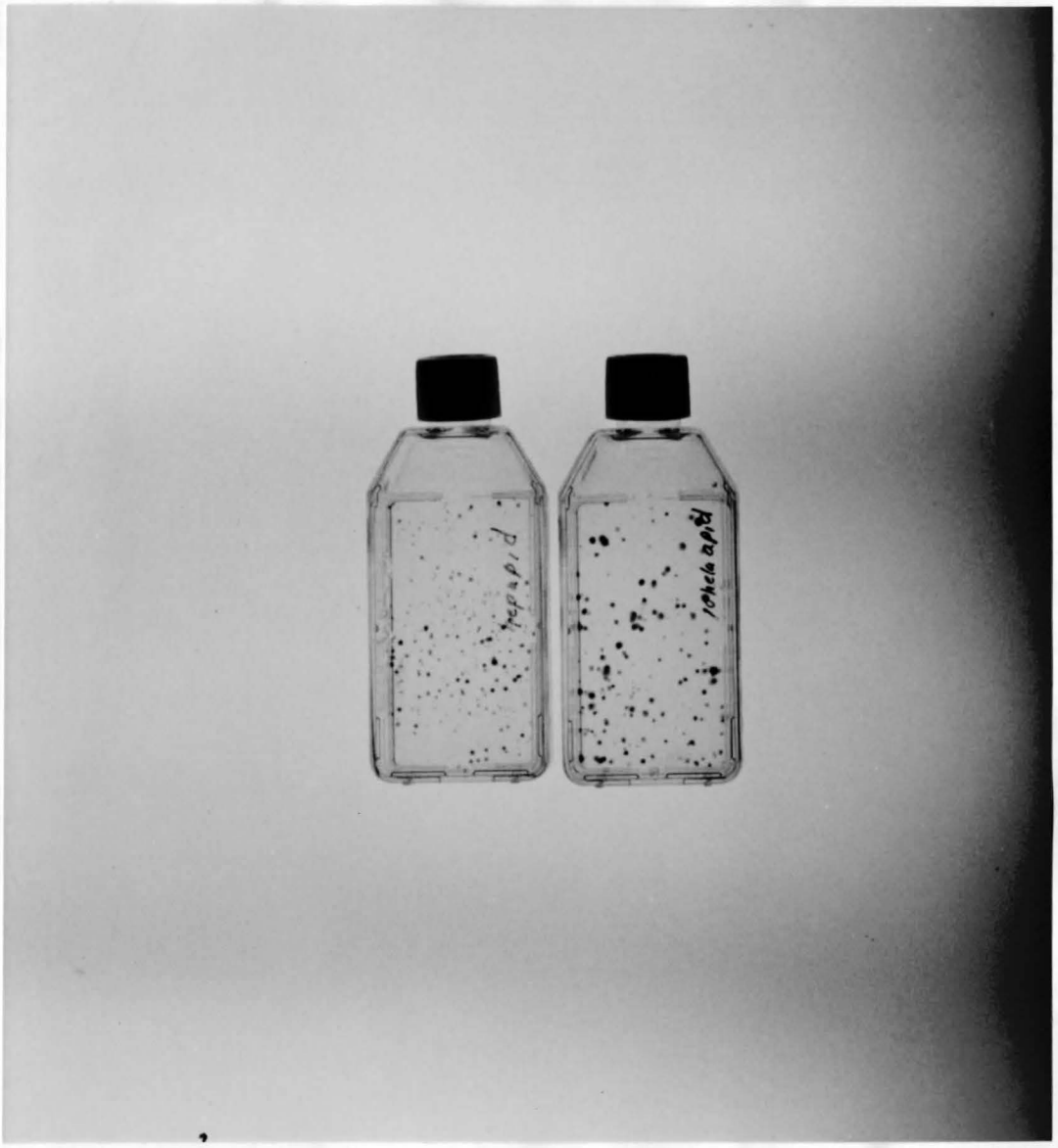


Fig. 3. Culture Flasks Containing Equal Concentrations of Hep-2 (LEFT) and HeLa (RIGHT) Cells Reflect Different Growth Patterns When Incubated Under Identical Conditions.

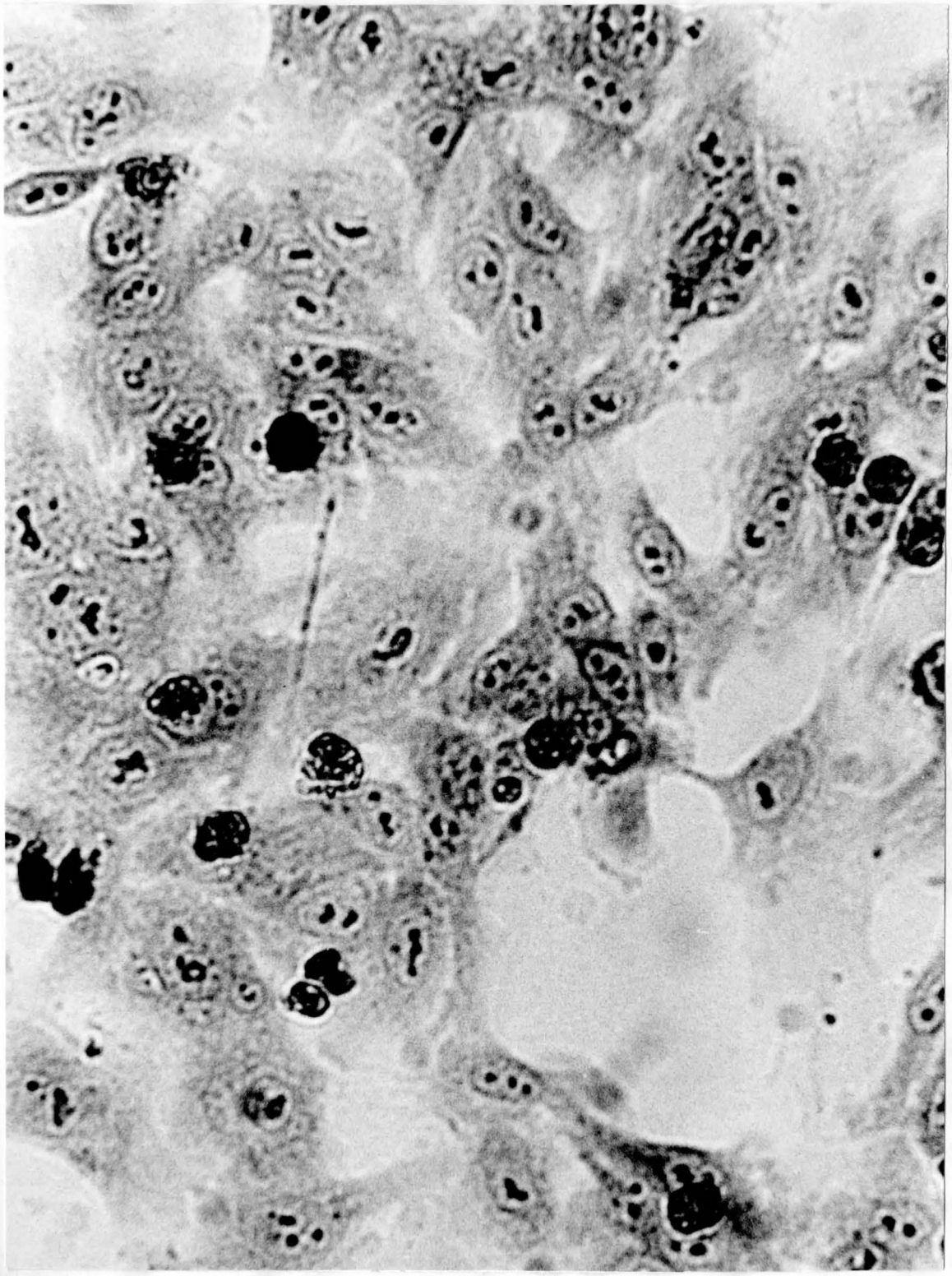


Fig. 4. Photograph of a Hep-2 Culture at Typical Saturation Density.

pH for the cell line appears to be exerted very rapidly.

The third parameter analyzed in this study was the number of chromosomes represented in the nucleus of the particular cell line. Chromosome analysis of fifty Hep-2 cells stained with Giemsa at the metaphase stage showed a range of 35-149 chromosomes per cell. In Fig. 5, a representative chromosome spread of the heteroploid human carcinoma line of Hep-2 is shown. In Fig. 7, a histogram expressing the frequency of appearance of chromosome number (Fig. 6) per Hep-2 cell, displays a bimodal distribution pattern. A mean value of 75 chromsomes per cell was determined for this Hep-2 cell line with a median value occurring at 69 chromsomes per cell. Other measures of central tendency can be found in Table 4. This experimental data obtained for the current Hep-2 cell line was then compared to Hep-2 and HeLa literature values (American Type Culture Collection, 1979). A statistically significant difference was determined ($p<.05$) between Hep-2 experimental data and the HeLa standard. No difference was detected between the Hep-2 experimental value and the Hep-2 literature value, indicating that the procedure itself had not caused an abnormal fluctuation in results. Calculations and tests of significance are provided in Appendix B.

Lastly, as a final comparison, extracts from HeLa and Hep-2 cells were prepared and the enzymes electrophoretically separated on cellulose acetate. Shown in Fig. 8 is a photograph of the results of G6PD analysis for HeLa and Hep-2 cells. Both cell lines exhibited the type A banding pattern. The band for HeLa cells is slightly lighter due to a decreased num-

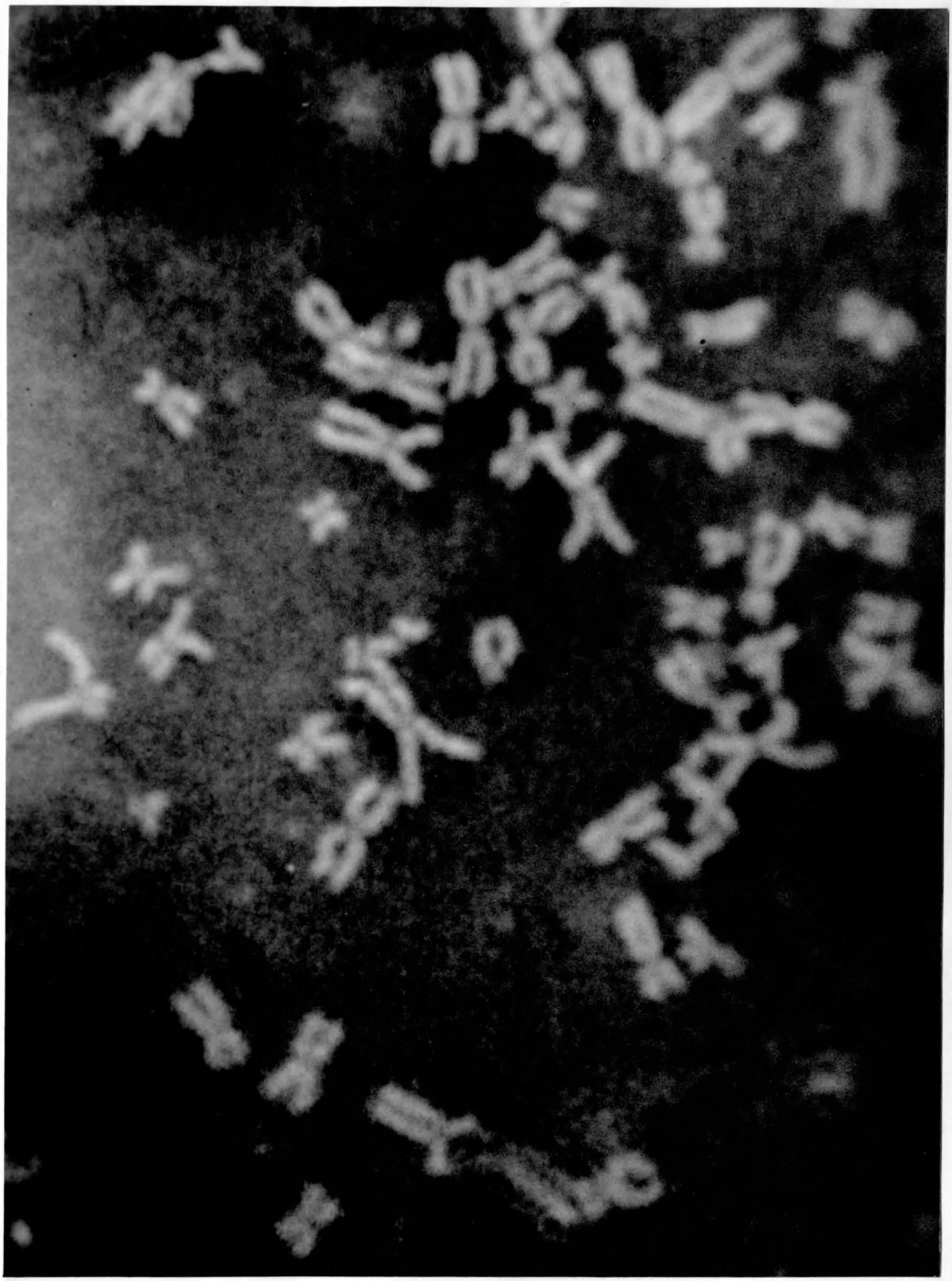


Fig. 5. A Representative Chromosome Spread of the Heteroploid Human Carcinoma Line, Hep-2.

Fig. 6. Chromosome Frequency Distribution of Fifty Hep-2 Cells.

Fig. 7. Frequency Histogram of Chromosomes of Hep-2 Cells.

FIG 6

CELLS:	1	1	1	1	1	1	2	3	2	3	1	4	1	2	2	4	3	1	1	2	2	1	1	1	1	2	1	2	1
CHROMOSOMES:	35	42	48	51	53	54	57	59	60	61	62	65	66	67	69	70	71	72	74	75	77	78	80	90	99	104	118	127	148	149

FIG 7

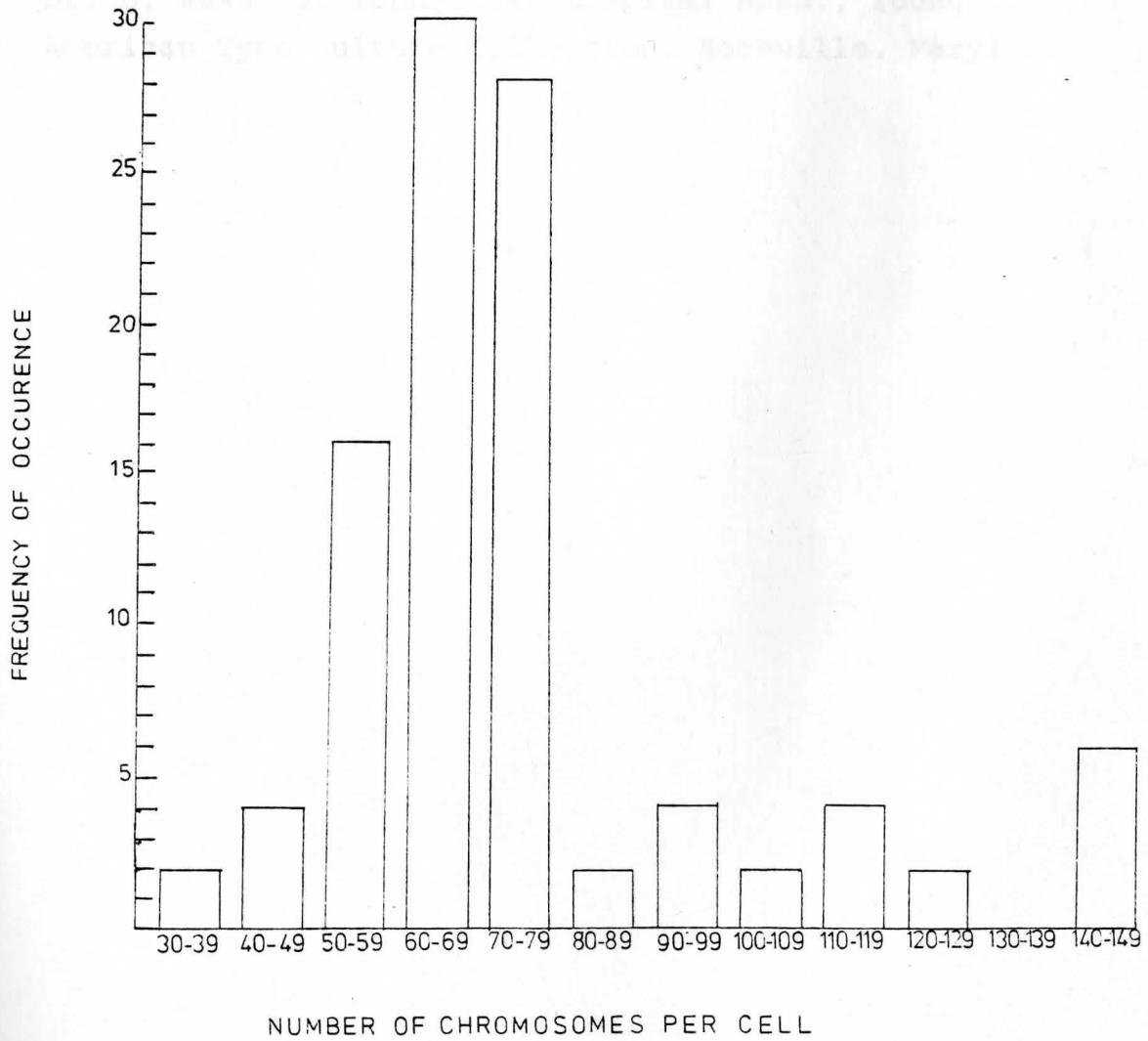


TABLE 4

COMPARATIVE EXPERIMENTAL AND LITERATURE VALUES FOR HEP-2 AND HELA CHROMOSOMES

Cell line	N	Median	Mode	Range	Sources	Sign. Level
1. Hep-2	50	69	65, 70	35-149	Exp. +	1 vs 2* p 05
2. HeLa	50	82	82	70-164	Lit. ++	2 vs 3
3. Hep-2	47	76	76	59-195	Lit. ++	1 vs 3

\+ Dr. B. Wessels, Youngstown Hospital Assn., Youngstown,OH

++ American Type Culture Collection, Rockville, Maryland

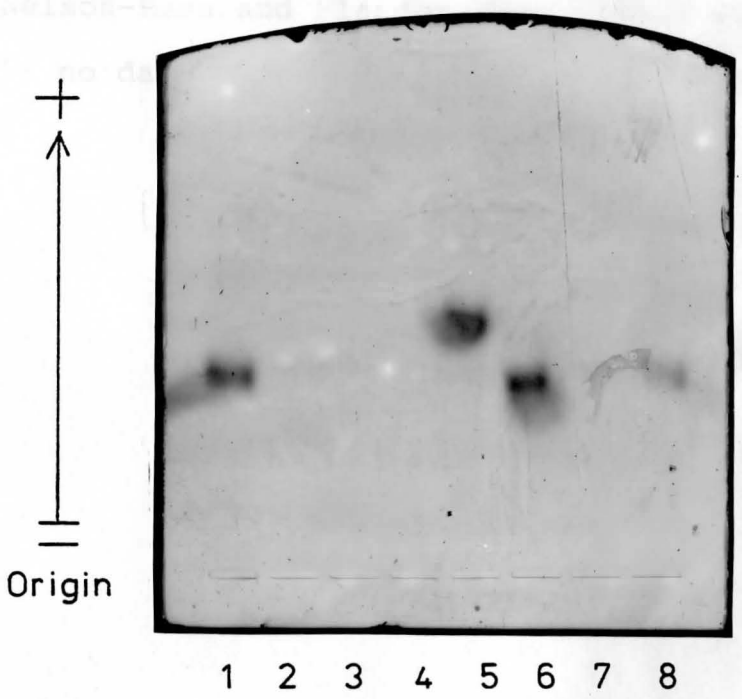


Fig. 8. Electrophoretic Patterns of Glucose-6-Phosphate Dehydrogenase for HeLa and Hep-2 Cell Lines. Slots as follows: 1, Hep-2; 5, control; 6, Hep-2; 8, HeLa.

ber of cells available for extraction rather than decreased activity of the enzyme. In Fig. 9 and 10 are schematic representations of the zymograms produced when the isoenzymes of G6PD and PGI were separated. No difference was detected between the banding pattern of the cell lines for either of these enzymes. The G6PD result is in agreement with other studies (Nelson-Rees and Flandermeyer, 1976 and O'Brien, et. al., 1977); no data is available on PGI studies.

FIG 9

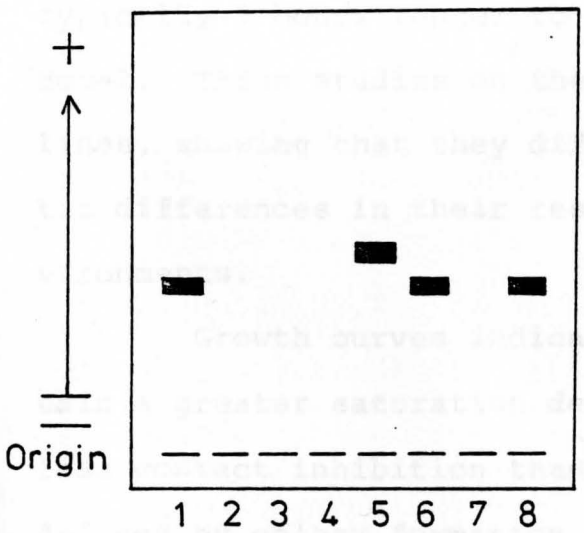


FIG 10

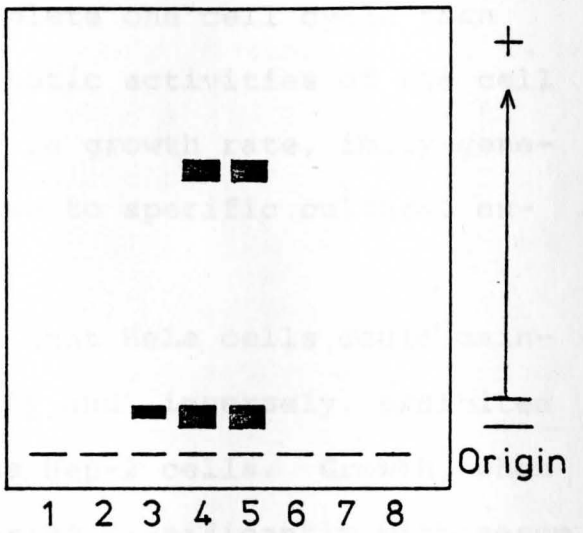


Fig. 9. Schematic Representation of Electrophoretic Patterns of Glucose-6-Phosphate Dehydrogenase for Hep-2 and HeLa cells. Slots as follows: 1, Hep-2; 5, control; 6, Hep-2; 8, HeLa.

Fig. 10. Schematic Representation of Electrophoretic Patterns of Phosphoglucose Isomerase for Hep-2 and HeLa cells. Slots as follows: 3, control; 4, Hep-2; 5, HeLa.

DISCUSSION

The results of these comparisons between the human epidermoid carcinoma of the larynx (Hep-2) and the cervical carcinoma (HeLa) show that measurable differences exist among several of the cell-specific characteristics. Analysis of the doubling time of each cell showed that HeLa took typically 5 hours longer to complete one cell cycle than Hep-2. These studies on the mitotic activities of the cell lines, showing that they differ in growth rate, imply genetic differences in their response to specific cultural environments.

Growth curves indicated that HeLa cells could maintain a greater saturation density and, inversely, exhibited less contact inhibition than the Hep-2 cells. Growth, when defined by colony formation, varied significantly with serum type and pH level between cell types. It was found that Hep-2 had a better survival rate than HeLa at high (7.75) pH levels by a statistically significant margin. This capacity to adapt to alkaline conditions was noted as an original characteristic of the Hep-2 strain described by Moore (1955) when she compared them with HeLa cells shortly after the initiation of both cultures. After having been passed in tissue culture for almost thirty years, the same relationship in regard to pH exists between the two cell lines. Thus growth characteristics can be useful tools in recognizing cell

characteristics and appear to be as reliable as many other methods employed.

Chromosome number was found to be lower in the Hep-2 cell than HeLa; each had a typically wide range of individual counts within the cells. This variation of chromosome number from one cell to another within the same population may suggest evidence of continuous selection. If it is assumed that the genomes represented by the varying karyotypes of a cell line differ from one another in their content of active genetic loci, the cells of such a population then would be expected to express different biochemical properties ties. These properties may be expressed collectively in an altered capacity for growth, doubling time, or saturation density. The resulting variation may be either advantageous or disadvantageous to the cell population. If all the genomes were equally advantageous, there would be an increase in the total chromosome variability, as none would be selected against. As reflected by the data collected, this does not occur. Instead both the modal number and range have been conserved, and the mean chromosome number for the currently available Hep-2 cell line is not significantly different from the original Hep-2 cell strain. The conclusion that can be drawn from this information is that stabilizing selection is most likely continuously occurring in the cell cultures. This tendency to maintain the status quo indicates that the extreme variants are not advantageous; that they are at a selective disadvantage and continuously eliminated.

Further cell characterizations were performed by electrophoretically separating G6PD and PGI isozymes. This system proved to be very useful for cell culture enzyme monitoring. The sample size was small (5 µl) and easy to obtain from a standard stock culture containing 1 to 2 x 10^6 cells. This is approximately seven times fewer cells than are needed for other electrophoretic methods, such as agar-gel (Peterson, 1968).

Identical banding patterns were obtained for both cell lines when tested for G6PD and PGI variation. This result of G6PD, type A for Hep-2 and HeLa cultures is in agreement with previous studies utilizing G6PD (Nelson-Rees and Flandermeyer, 1976 and O'Brien, et al., 1977); no other sources have been reported utilizing PGI in cell cultures. The probability of Hep-2 and HeLa having the same type G6PD is considered the strongest evidence indicating contamination among the cell lines. The improved methods of electrophoresis, allowing the resolution of isoenzymes, however, have only recently been applied to cell culture. The original type of G6PD in the tumor cells before being implanted in tissue culture in the 1950's can only be surmised at this point by the sex and race of the tumor donor. Glucose-6-phosphate dehydrogenase is now known to be an X-linked locus (Parr, 1974); as a result only females can express the heterozygous genotype, which appears electrophoretically as two bands. The family pedigree of Henrietta Lack, the black female patient from whom the HeLa cell originated, showed she

was heterozygous, since she had a son who was found to be type B (Hsu, et al., 1976). Either due to a unicellular origin of the tumor, X-inactivation, or selection, the resulting tumor cells were found to express G6PD, type A when tested by electrophoresis after several years passage. Linder (Linder and Gartler, 1965) obtained similar results experimentally with G6PD heterozygote in which the ratio A to B changed from 40:60 to 80:20 over a relatively short period.

Since the incidence of type A G6PD is essentially zero to Caucasians, it was assumed that the white male from which Hep-2 cells originated were G6PD, type B. Type B is considered the normal form of the enzyme as it occurs most frequently in all populations (Brock and Mayo, 1978). About one hundred million people in the world, however, exhibit a variant form of G6PD which results in slightly reduced activity of the enzyme. In order for a deleterious gene to reach such a high population frequency, a selective advantage has been postulated (Thompson and Thompson, 1973). It is believed that many G6PD variants, like sickle cell hemoglobin, may confer some protection against malaria (Brock and Mayo, 1978).

At a molecular level, the same process may be occurring in cell culture. The rarer form of G6PD may afford the cell some type of protection or improved growth condition. However, in order for a cell line to express G6PD type A, it must be either in the donor tissue or be produced by mutation. The difference between type A and B is the replacement of a

single asparagine residue in the B type enzyme by an aspartate residue in the A type molecule (Parr, 1974). Auersperg's report (Auersperg and Gartler, 1970) that G6PD type B was stable over 30 months in culture is a relatively short period compared to the thirty years that the Hep-2 line has been in culture.

Morphologically and culturally, these representative Hep-2 and HeLa cells exhibit different patterns of growth and behavior. They function as two distinct cell lines, distinguishable on the basis of three out of four parameters tested in this characterization. The test for G6PD yielded inconclusive results, since no comparable data is available on the Hep-2 cell at the time of the origin of the cell line.

Chemical and X-ray studies have shown additional differences. Survival curves show that the HeLa cell (Bono, et al., 1975) is more sensitive to the effects of radiation than the Hep-2 cell (Wessels, et al., 1980). HeLa and Hep-2 cells have also been found to be different on the basis of sensitivity to 5-fluorouracil, a growth inhibitor (Laskin, et al., 1979). These differences evident at present and indicating the different biochemical properties presumably under control of the nucleus of each cell type, are the result of either (1) a very early contamination with HeLa cells and years of separate subculturing have produced two unique cell lines or (2) the two separate cell lines have both been subject to the same selection pressure with respect to glucose-6-phosphate dehydrogenase. Further tests are necessary as to

the stability of G6PD over long periods of time, in order to disclude the second possibility.

APPENDIX A

Tests For Significance Between HeLa and Hep-2 Doubling Time

A two-tailed variance ratio test was conducted with Hep-2 and HeLa doubling time data from five experiments to test the following hypotheses:

$$H_O: \quad \sigma_2^2 = \sigma_2^2$$

$$H_A: \quad \sigma_1^2 = \sigma\ ^2_2$$

$$\sigma_1^2 = \text{Hela}$$

Where:

$$\sigma_2^2 = \text{Hep-2}$$

The data are the number of hours required for each type of cell to complete one cell cycle. This test was conducted prior to a t-test for difference between two means, because one must be able to assume $\sigma_1^2 = \sigma_2^2$ to insure the validity of that test.

HeLa	Hep-2
$n_1 = 2$	$n_2 = 3$
$v_1 = 1$	$v_1 = 2$
$SS_1 = .03$	$SS_2 = 1.04$
$s_1^2 = .03$	$s_2^2 = .52$

$$F = \frac{s_1^2}{s_2^2} = .06$$

$$F0.05\ (2)\ 1,\ 2 = 5.42$$

Therefore, do not reject H_O. Since the two populations have equal variances, then a t-value for testing a difference between two means can be computed.

A t-test was then implemented to test for signif-cance between the means of experimental doubling time for HeLa and Hep-2.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S\bar{x}_1 - \bar{x}_2}$$

and $$S\bar{x}_1 - \bar{x}_2 = \sqrt{\frac{s^2p}{n_1} + \frac{s^2p}{n_2}}$$

so $$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s^2p}{n_2} + \frac{s^2p}{n_2}}}$$

where HeLa = $\bar{x}_1 = 42.13$

Hep-2 = $\bar{x}_2 = 37.42$

$$t = \frac{42.13 - 37.42}{.55} = 8.56$$

$$t0.05\ (2)\ (v_1+v_2) = t0.05\ (2)\ (2 + 3) = 2.57$$

Therefore, reject H_0.

The result indicates the means are significantly different at the $p < .05$ level.

APPENDIX B

Tests of Significance Between HeLa and Hep-2 Chromosome Number

HeLa and Hep-2 cell lines were tested for a difference between their average number of chromosomes using the t-test. The sample sized analyzed were 50 chromsomes spreads for each cell type.

Case 1:

Where $\bar{x}_1$ = HeLa mean chromosome complement = 84

$\bar{x}_2$ = Hep-2 mean chromosome complement = 75

The following hypotheses were tested:

H_O: There is no difference between Hep-2 and HeLa average chromosome count.

H_A: There is a difference between Hep-2 and HeLa average chromosome count.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s^2_p}{n_1} + \frac{s^2_p}{n_2}}} = \frac{84 - 75}{4.2} = 2.14$$

$t_{O.05(2)(98)} = 1.984$

Therefore, reject H_O.

Case 2:

Where $\bar{x}_1$ = Hep-2 mean literature value = 80.9

$\bar{x}_2$ = Hep-2 experimental value = 74.8

The following hypotheses were tested:

H_O: There is no difference between Hep-2 experimental and literature value.

H_A: There is a difference between Hep-2 experimental and literature value.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s^2_p}{n_1} + \frac{s^2_p}{n_2}}} = \frac{80.9 - 74.8}{4.9} = 1.2$$

$$t = 0.05\ (2)\ (98) = 1.98$$

Therefore, do not reject H_O.

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