THE EFFECT OF pH ON GROWTH, PROTEIN SYNTHESIS, AND LIPID-RICH PARTICLES OF CULTURED MAMMALIAN CELLS

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ABSTRACT

A simple procedure has been established for controlling and measuring the pH of media in which the bicarbonate-carbonic acid system is the predominant buffer. The HCO^-_3 concentration was maintained at 22.5 mM and the H_2CO_3 concentration was varied by equilibrating the media with 0.5 to 40 per cent CO_2 in air. The curve relating extracellular pH to 3 day cell growth was similar for glass-attached HeLa and Chang liver cells. Maximum growth occurred over a pH range of 7.38 to 7.87. Cell growth declined precipitously on the alkaline side and more gradually on the acid side of the optimal pH range. Comparable pH growth curves were also obtained with newly isolated cells from rat liver and skeletal muscle. It was shown that the effect of pH on growth was independent of the CO_2 concentration and that the essential nutrients in the medium were stable over the pH range studied. Although alkalosis depressed the 3 day cell population, cells exposed to a pH of 8.0 to 8.2 grew at the maximal rate for the first 12 to 24 hours. Growth then ceased abruptly and the cells entered a steady state with respect to net protein synthesis. This was followed by cytoplasmic retraction and cell death. Increasing the concentrations of calcium or magnesium in the medium failed to prevent the effects of alkalosis. Moreover, the increase in CO^-_3 concentration of the media and the concomitant decrease in Ca^{++} ion concentration that occur at high pH were eliminated as determining factors in the growth failure and death. While acidosis had a less pronounced effect on the 3 day cell population, its effect on the growth rate was immediate. The increase in cell generation time was proportional to the H^+ ion concentration. In each of the cell lines studied, acidosis was accompanied by a striking increase in the number of cytoplasmic perinuclear granules. These granules which stain supravitally with Janus green are extracted from fixed cells with lipid solvents. They maintain their identity in cell homogenates and may be isolated from the other subcellular structures by differential centrifugation; at 100,000 *g* they form a distinct layer at the top of the supernatant fraction. On the basis of their physical and chemical properties, these granules have been called lipid-rich particles. The accumulation of lipid-rich particles in acidosis was independent of the growth rate and the CO_2 concentration.

The H^+ ion concentration is one of the most important parameters which determines the rates and steady state concentrations in chemical and biochemical reactions. However, in animals, it is often difficult to establish whether the profound chemical changes which accompany alterations in the blood pH are directly due to the altered H^+ concentration, or whether they are caused by the concomitant and subsequent shifts in the concentrations of other plasma electrolytes. Furthermore, it is not known whether the biochemical and physiological responses of the whole animal to

acidosis and alkalosis are caused by effects on all cells or whether they are mediated primarily through specific organs, tissues, or cell types. Indeed, because of compensatory reactions, it is practically impossible to alter and maintain the plasma pH at a new steady state for extended periods of time. It seemed desirable, therefore, to begin a study of the effects of the extracellular pH on mammalian cells grown in tissue culture. In contrast to the whole animal, the ionic composition of the medium can be varied under controlled conditions and pH measurements can be made directly on the extracellular fluid. Moreover, conditions can be chosen which exclude significant changes in the medium as the result of metabolic reactions.

It also seemed important to determine the effect of pH on the growth of cultured cells for purely practical reasons. The growth response is the primary criterion used for testing essential nutrients and, in general, cultured animal cells produce lactic acid at an appreciable rate. Consequently, the faster the growth, the more rapidly the medium becomes acid. It is therefore essential to know at which H^+ concentration growth is inhibited and to what degree. Indeed, without a detailed knowledge of this variable, many growth and metabolism experiments are difficult, if not impossible, to interpret.

In order to determine the relation between cell growth and pH, certain simple but nonetheless important criteria must be met. First, the buffer system employed should be a physiological one. Second, the pH of the medium should remain constant during the course of an experiment. Third, growth should be measured over a wide range of pH values in each experiment. Fourth, the pH levels used in successive experiments should overlap in order to detect differences that might result from changes in the stock cell cultures or in the composition of the medium.

The carbonic acid–bicarbonate buffer was selected for use in our experiments because it is the predominant buffer of interstitial fluids and plasma, and also for most free-living cells in the oceans and other natural waters of the world (2). Furthermore, the bicarbonate system is unique in that the acid component may be held at any desired steady state by maintaining a constant pressure of CO_2 in the atmosphere above the culture medium. Consequently, a decrease in the basic HCO_3^- ion, as a result of the metabolic production of acid, will not change the steady state concentration of the H_2CO_3 component. We therefore employed a medium based on Earle's salt solution (3), the organic constituents as defined by Eagle (4), and 10 per cent of horse serum. The approximate concentrations of buffers in this medium were 22.5 mM bicarbonate, 3 mM amino acids, 1 mM phosphate, and 0.6 gm. of serum proteins per 100 ml.

Equilibration of Medium with CO_2

Before studying the effect of pH on cell growth it was first necessary to determine whether or not the diffusion of CO_2 into the medium, contained in covered petri dishes, proceeded at a sufficient rate for the establishment of equilibrium pH values in a reasonable period of time. For this purpose the assembly shown in Fig. 1 was gassed with air containing different concentrations of CO_2 at 5 liters per minute for 10 minutes and thereafter at 1 liter per minute. A typical curve showing the rate of equilibration of the medium at pH 7.9 and 37.5°C with 5 per cent CO_2 is given in Fig. 2. Similar curves were determined for CO_2 concentrations ranging from 1.5 to 15 per cent. In all of the experiments, the equilibrium pH remained

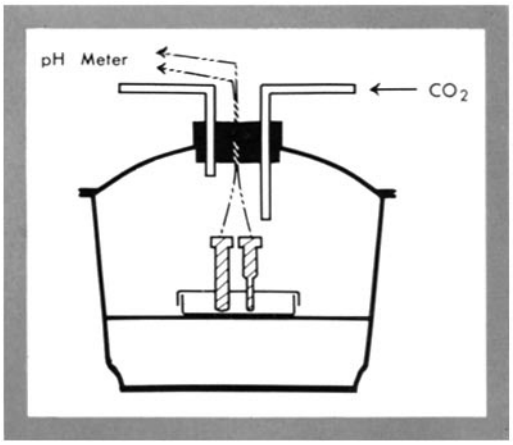


FIGURE 1

Assembly for measuring the pH of the medium in covered petri dishes exposed to various partial pressures of CO_2. The electrode lead wires entered the desiccator through an incision in the rubber stopper and the electrodes entered the petri dish through rubber dam gaskets in the cover. The petri dish rested on a small stand attached to the lead wires, so that the dish and the electrodes were introduced and removed from the desiccator as a unit attached to the lid.

constant for 24 hours with gas flows ranging from 0.5 to 2 liters per minute. In the growth experiments, the initial pH of the medium was adjusted to 7.4 to 7.5 with HCl before gassing with CO_2.

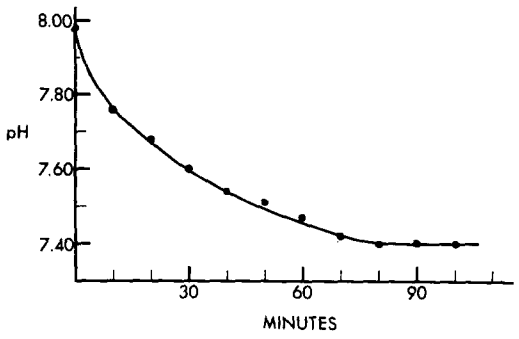


FIGURE 2

Equilibration of medium with 5 per cent CO_2 in air at 37.5°C; 5 ml. of medium were placed in a covered petri dish (6 cm. diameter) and the pH was measured in the assembly illustrated in Fig. 1.

TABLE I

pH of Bicarbonate Solutions Equilibrated with CO_2 by Diffusion and by Bubbling

5 ml. of bicarbonate solution were used in all experiments. The gas phase consisted of 5.21 per cent CO_2 in air at 630 mm.Hg and 26°C. In the diffusion experiments, the solutions were gassed in a covered Petri dish (6 cm. diameter) placed in the apparatus shown in Fig. 1. In the bubbling experiments, the gas was introduced through a capillary tube inserted at the bottom of a 10 ml. beaker. All pH measurements were made with indwelling electrodes. The calculated pH was obtained from the Henderson-Hasselbalch equation

$$pH = pK_1 + \log \frac{[HCO_3^-]}{pCO_2 \times \alpha \times 0.0591}$$

where the concentration of HCO_3^- is in millimoles per liter.

Solution	Diffusion	Bubbling	Calculated
26 mM $NaHCO_3$	7.59	7.57	7.60*
26 mM $NaHCO_3$ + 129 mM NaCl	7.49	7.48	7.49‡

* $pK_1 = 6.34$; $\alpha = 0.736$.
‡ $pK_1 = 6.22$; $\alpha = 0.721$.

Measurement of pH of Medium

Next, the pH obtained by the diffusion of CO_2 into bicarbonate solutions in covered petri dishes (Fig. 1) was compared with the pH obtained by bubbling the CO_2 directly into the same solutions. As shown in Table I, the steady state pH values obtained in these two procedures were in good agreement. Furthermore, they agreed with the pH values calculated from the Henderson-Hasselbalch equation when the pK and α at 26°C and at the ionic strengths of the solutions employed were calculated from the data of Hastings and Sendroy (5), Kendall (6), and Van Slyke and co-workers (7). These results indicate that the pH measurements made on the medium in covered petri dishes were correct.

It was not feasible to measure the pH of the medium with indwelling electrodes in growth experiments involving large numbers of desiccators and petri dishes. We therefore determined the effect on the pH of equilibrated medium of opening the desiccator, removing the dish, and rapidly pouring the medium into a 10 ml beaker. Over the range of CO_2 concentrations used, transfer of the medium was found to increase the equilibrium pH values by 0.06 ± 0.02 unit. This procedure was used in the growth experiments and the pH readings were corrected accordingly. The pH values obtained on the first 3 dishes to be removed from a desiccator agreed to within 0.02 pH unit.

Maintenance of Constant pH During Cell Growth

Next, a preliminary experiment was carried out to determine the effect of cell growth on the pH of the medium when the medium was changed daily. The HeLa cell, originally isolated from a human carcinoma of the cervix by Scherer, Syverton, and Gey (8), was used for this purpose. As shown in Fig. 3, an initial population of 26,000 cells grew at a logarithmic rate for 3 days without reducing significantly the pH of the medium. By the end of the fourth day, when the initial cell protein had increased 16 times, the pH had fallen by 0.08 of a unit. In view of these results, a 3-day growth period was selected for studying the effect of pH on cell growth and protein synthesis.

A subsequent experiment showed that the number of cells also increased at a logarithmic rate over a 3 day period and that the average protein per cell was approximately 8.1×10^{-4} μg. at

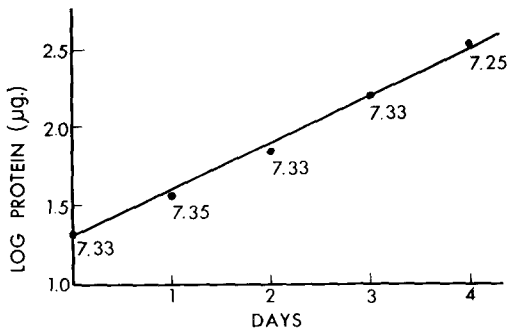


FIGURE 3

The effect of HeLa cell growth on the pH of 5 ml. of medium equilibrated with 5 per cent CO_2 in air. The initial pH of the medium and the pH at the end of each 24 hour period is given below the growth curve. The medium was changed every 24 hours.

the beginning and end of the experiment. Furthermore, the 260 mμ absorbing material, obtained by dissolving cells in 0.5 N NaOH, bore a constant relation to cell protein. It was therefore concluded that the increase in total cell protein was a satisfactory measure of cell division and growth under our experimental conditions.

Effect of Extracellular pH on the Growth of HeLa Cells

Using the foregoing procedures, the effect of the extracellular H^+ ion concentration on the growth of HeLa cells was studied. In each experiment, 20 to 30 petri dishes were plated with equal aliquots of a suspension of single cells and the medium (pH 7.4) was equilibrated with 5 per cent CO_2 at 37.5°C. Eighteen hours later, when the cells had attached to glass and entered the log phase of growth, the cell protein was determined in 4 dishes. The average value was taken as the "zero time" cell protein. In different experiments this value corresponded to zero time cell populations ranging from 7,000 to 30,000 cells. The remaining petri dishes were distributed in groups of 4 or 5 in desiccators which were gassed with different CO_2 concentrations ranging from 0.5 to 40 per cent. The medium was changed daily and total cell protein was measured at the end of 3 days. The increase in cell protein was used to calculate the number of cell generations from the formula

$$\text{Generations} = \frac{\log F - \log I}{\log 2}$$

where I is the initial cell protein and F is the final cell protein.[1]

In each of 8 experiments with HeLa cells, the greatest growth always occurred between pH 7.38 and 7.87. Furthermore, the greatest divergence in growth within this pH range in a single experiment was 2.9 and 3.2 generations. Consequently, the results of the individual experiments were averaged to obtain the composite curve (Fig. 4) relating pH to the number of cell generations.

The horizontal plateau in the curve extending from pH 7.4 to 7.9 may mean that in this region the cell's over-all synthetic, replicating, or transport mechanisms are approaching a maximum rate; or, alternatively, that the concentration of some essential nutrient in the medium is limiting growth. In either case, it is of interest that at pH 7.4, a value employed frequently in tissue culture experiments, the ability of the medium to support optimal growth in the face of metabolic acid production is approaching a minimum.

Effect of pH on Growth of Chang Liver Cells

Experiments similar to those described for the HeLa cell were performed on the cell isolated by Chang (9) from normal human liver, but of unknown cellular origin within that organ. As shown in Fig. 5, the relation between pH and growth of the Chang cell was similar to that observed with the

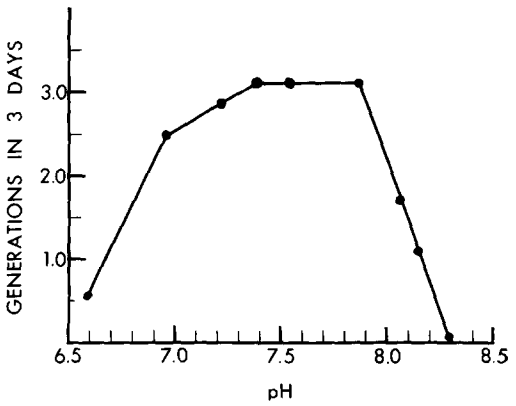


FIGURE 4

The effect of extracellular pH on the growth of HeLa cells.

[1] The factor by which the initial cell protein was increased at any pH can be obtained from the expression, $F = 2^x$, where x is equal to the number of cell generations.

HeLa cell. Since both of these cell lines had been in continuous culture for years, it seemed desirable to extend our studies to include newly isolated mammalian cells.

Effect of pH on Growth of Newly Isolated Rat Liver and Muscle Cells

A cell which resembles the Chang liver cell was isolated from rat liver (Fig. 6), and a cell with quite a different morphology was isolated from rat skeletal muscle. The latter cell, when attached to glass, covers a larger area and its cytoplasm contains numerous parallel "folds" (Fig. 7). Nevertheless, its volume in the detached, spherical form is approximately the same as that of the other cell lines studied. Although the precise origin of the two rat cells from within their respective tissues is not known, they have maintained their differences in appearance for over 6 months.

The effect of extracellular pH on the growth of these two lines of rat cells during their first month in tissue culture is shown in Table II. Similar results were obtained in later experiments, and they are in agreement with the results obtained with HeLa and Chang liver cells. It appears, therefore, that the relation between extracellular pH and the size of the 3 day cell population, illustrated in Fig. 4, holds for a variety of mammalian cells.

Effect of CO_2 Concentration on Cell Growth

In the preceding experiments, the pH varied not only with $-\log [H_2CO_3]$ but also with $-\log$ per cent CO_2. It was necessary, therefore, to determine the effect on cell growth of the CO_2 concentrations used in our experiments. This was particularly important at the 0.5 per cent CO_2 concentration (pH 8), in view of the recent reports of Geyer and Chang (10) and Swim and Parker (11) that animal cells require CO_2 for growth. The minimum effective concentration has not yet been determined.

In an experiment with HeLa cells, the pH of the medium equilibrated with 1.5 per cent CO_2 was raised from 7.8 to 8.0 by increasing the HCO_3^- ion concentration from 22.5 to 34 mM at the expense of Cl^- ions. As shown in Table III, the growth depression at pH 8 under these conditions was just as great as that obtained with 0.5 per cent CO_2 and the regular medium. Since 1.5 per cent CO_2 supported good growth at pH 7.8, it may be concluded that the growth failure observed routinely at pH 8.0 was not due to a deficiency of H_2CO_3 or dissolved CO_2.

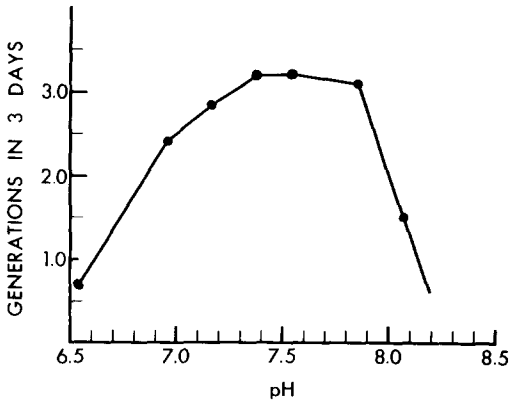


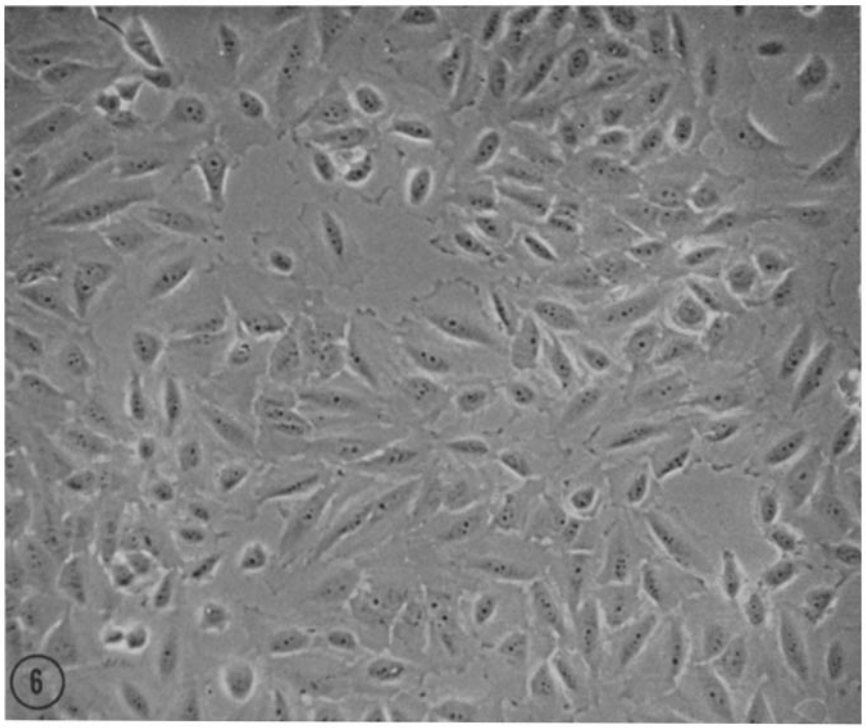
FIGURE 5

The effect of extracellular pH on the growth of Chang liver cells.

In a similar experiment with a slightly larger initial cell population, the effect on cell growth of increasing the acidity, either by reducing the HCO_3^- ion concentration, or by increasing the CO_2 concentration, was compared. As shown in the lower half of Table III, both operations reduced cell growth to the same extent. Thus, growth inhibition at pH 6.9 cannot be ascribed to CO_2 toxicity or O_2 deficiency. These two experiments indicate that the effect of pH on cell growth in the bicarbonate-carbonic acid buffer system is a function of the H^+ and (or) OH^- ion concentrations.

Effect of pH on the Organic Constituents of the Medium

Experiments were next conducted to determine whether the depression of growth at either end of the pH curve was due to the destruction of one or more essential nutrients in the medium. For this purpose, HeLa cells were grown at pH 7.4 in a medium that had been incubated during the preceding 24 hours at either pH 6.9 or pH 8.1 in petri dishes at 37.5°C. As shown in Table IV, this treatment did not affect the 3 day cell growth. It appears probable, therefore, that the reduction in growth at both ends of the optimal pH range is due to an effect of H^+ or OH^- ions on cell metabolism or on the cell membrane, or on both.



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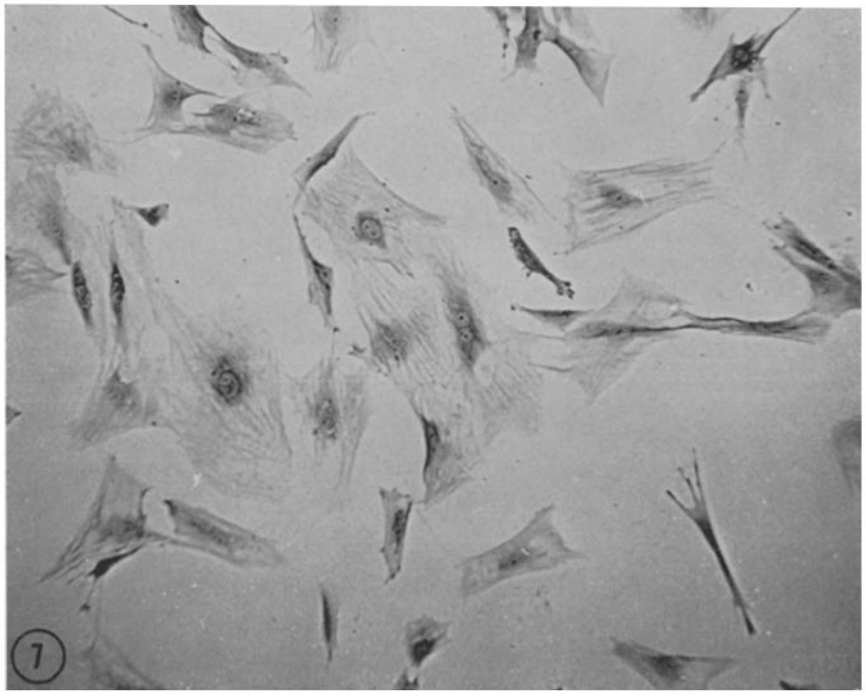

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TABLE II

Effect of Extracellular pH on the Growth of Cells Isolated from Rat Liver and Rat Muscle

The cells were attached to glass and had entered the log phase of growth at the start of the pH-growth experiment. The number of generations was calculated from total cell protein values determined at the start of the experiment and at the end of 3 days.

CO_2 *per cent*	pH	Generations in 3 days	
		Liver	Muscle
0.5	8.1	0.3	0
1.5	7.8	3.1	3.1
3.0	7.5	3.0	3.0
5.0	7.4	3.0	3.1
15.0	7.0	2.7	2.6

TABLE III

Exclusion of CO_2 Concentration as a Determining Factor in Growth Inhibition at pH 8.0 and 6.9

HeLa cells were grown for 3 days at the indicated CO_2 and HCO_3^- concentrations. Cell protein values are the averages of 4 determinations made on replicate dishes. In the alkalosis experiment (lines 1 to 3), the initial cell population contained 25 μgm. of protein. In the acidosis experiment (lines 4 to 6), the initial cell population contained 38 μgm. of protein.

CO_2	HCO_3^-	pH	Cell protein
Per cent	*mM*		*μg.*
0.5	23	8.03	76
1.5	34	8.02	71
1.5	23	7.80	209
8.0	23	7.20	277
8.0	10	6.88	202
15.0	23	6.92	209

Mechanism of Growth Failure in Mammalian Cells at pH 8

So far as we know, the foregoing experiments are the only ones to be reported thus far which describe the relation between pH and growth and protein synthesis in glass-attached mammalian cells. Studies on pH and the growth of chick fibroblast explants were reported by several workers in the 1920s and 1930s. However, because of the limited methods then available for culturing cells and measuring growth and pH, the results are difficult to evaluate. For example, the organic components of the media were added in the forms of body fluids or tissue extracts, the pH was not maintained at a constant level, the final pH was not always measured, and cell growth was estimated by the increase in the area of the explants employed. It is not surprising, then, that different investigators obtained different results. For example, with chick fibroblast explants Fischer (12) observed a growth maximum at pH 7.4, Lewis and Felton (13) at at pH 6.8 to 7.0, and Sankaran (14) at 7.4 to 7.6. Examination of the individual growth curves reported by Fischer shows that a precipitous drop in chick fibroblast growth always occurred at pH 8.0.[2]

In 1950, Dubin and Yen (15) reported the effect of pH on the growth of chick macrophages explanted in fragments of chick embryo spleen which had been infected with *Plasmodium gallinaceum*. The pH was measured with glass electrodes, and maintained at constant levels by continuous gassing with appropriate mixtures of CO_2. Macrophage growth, as estimated by the area of the

[2] With respect to the growth of 48 hour cultures, Fischer wrote, "The curves reached a maximum at pH 7.4 and fell very rapidly on both sides of this point, appearing always to be steeper on the alkaline side. . . ." Speaking of subdivided and transferred cultures he said, "The optimum growth occurred between pH 7 and 7.8." However, in his *Conclusions* he wrote, "The optimum growth of fibroblasts occurs at pH 7.4 to 7.8."

FIGURE 6

Rat liver cells grown in medium at pH 7.4. Phase contrast. × 25.

FIGURE 7

Rat muscle cells grown in medium at pH 7.4; fixed with Bouin's solution and stained with Janus green B to delineate the cytoplasm for photography. Phase contrast. × 50.

TABLE IV

Growth Response of Cells in Media Preincubated at pH 8.1 and 6.9

HeLa cells were grown for 3 days in ordinary medium or in medium preincubated for 24 hours at high and low pH levels at 37.5°C.

Preincubation pH	Growth pH	Generations
8.1	7.4	3.0
No preincubation	8.1	1.1
7.4	7.4	3.0
No preincubation	7.4	3.1
6.9	7.4	3.2
No preincubation	6.9	2.5

TABLE V

Effect of Added Ca and Mg on HeLa Cell Growth under Conditions of Alkalosis

The cells were grown for 3 days in each experiment. The added Ca and Mg doubled the quantities present in the original medium.

Addition to medium	pH	Generations
None	7.83	3.02
None	8.20	0.13
1.8 mM $CaCl_2$	8.17	0.85
0.8 mM $MgCl_2$	8.20	0
None	7.95	1.15
1.8 mM Ca vers. + 0.8 mM Mg vers.*	7.95	0.38

* The chelates of Ca and Mg with disodium ethylenediamine tetraacetic acid.

culture, was optimal between pH 6.8 and 8.2, and declined rapidly on both sides of this range.

Of particular interest are the studies of Lodge and Hinshelwood (16) on the relation between pH and the total cell population obtained with *Bacterium lactis aerogenes* in glucose-phosphate and lactose-tartrate media. In both cases, there was a precipitous fall in growth when the initial pH of the media was 8.0. It appears, therefore, that pH 8 is toxic for a number of cells which differ widely in their origin and genetic composition. While exceptions may occur, this relation suggests that an alkalinity of pH 8 inhibits growth by operating on some fundamental biochemical system.

The conditions under which our cells were grown resemble those that exist in the whole animal in an uncompensated respiratory alkalosis.[3] In this syndrome, a tetany sometimes develops which is generally ascribed to a reduction in the concentration of ionizable plasma Ca. Consequently, we examined the effect on cell growth at pH 8.2 of doubling the Ca, and also the Mg, content of the medium. As shown in Table V, neither addition affected cell growth significantly. Ca and Mg were also added to the medium at pH 7.95 as the chelates of disodium ethylenediamine tetraacetate. These compounds also failed to promote growth (Table V).

Inspection of the solubility products of Ca^{++} and Mg^{++} with OH^-, HCO_3^-, $CO_3^{=}$, $HPO_4^{=}$ and $PO_3^{=}$ ions showed that only the solubility products of $Ca_3(PO_4)_2$ (17, 18) and $CaCO_3$ (19) are exceeded in our medium. Although $Ca_3(PO_4)_2$ is the least soluble of the two compounds, with a solubility product of $10^{-26.6}$ (18), the phosphate concentration in the medium is not sufficient to react with all of the Ca^{++} ions even when the reaction goes to completion:

$$1.8 \text{ mM } Ca^{++} + 0.9 \text{ mM } PO_4^{=} \rightarrow 0.45 \text{ mM } Ca_3(PO_4)_2 + 0.45 \text{ mM } Ca^{++}$$

However, such is not the case with respect to $CO_3^{=}$, because the medium contains, for all practical purposes, an unlimited supply of $CO_3^{=}$ in the form of HCO_3^- ions. According to Hastings, Murray and Sendroy (19), the solubility product of $CaCO_3$ is $10^{-7.3}$ in the presence of serum salts diluted with 10 per cent whole serum, which is essentially the composition of our medium. Consequently, in the presence of 0.45 mM Ca^{++}, the solubility product will be exceeded when the concentration of $CO_3^{=}$ reaches 0.111 mM. As calculated from the pK_2 of H_2CO_3 = 9.79 (19), this concentration is attained in the medium at pH 7.5. Therefore, at this or at any higher pH, the addition of Ca^{++} ions will result in the following stoichiometric reaction,

$$Ca^{++} + CO_3^{=} + 2HCO_3^- \rightarrow CaCO_3 + CO_3^{=} + H_2O + CO_2$$

unless, of course, supersaturation occurs.

[3] The terms "alkalosis" and "acidosis" will be used in accordance with their classical usages in biochemistry and medicine, *i.e.*, deviations of the pH from a "normal" range which in higher animals is located on the alkaline side of neutrality.

TABLE VI

Exclusion of Carbonate Ion Concentration as Cause of Growth Inhibition in Alkalosis

HeLa cells were grown for 3 days in media containing CO_2 and HCO_3^- at the indicated concentrations. The $CO_3^=$ concentration was calculated from the pK_2 of H_2CO_3 = 9.79 at 38°C. and 0.16 ionic strength. The Ca^{++} concentration was calculated from the solubility product of $CaCO_3 = 10^{-7.3}$ at 38°C. in serum salts containing 10 per cent serum proteins.

pH	CO_2	HCO_3^-	$CO_3^=$	Ca^{++}	Generations
	Per cent	*mM*	*mM*	*mM*	
8.12	0.5	23	0.48	0.11	0
7.67	8.0	65	0.49	0.10	3.1
7.70	1.5	23	0.19	0.27	3.4

In view of these considerations, it was necessary to determine whether or not the growth failure observed at pH 8 was due to the increased concentration of $CO_3^=$ ions. For this purpose, the HCO_3^- ion concentration in the medium was raised to 65 mM at the expense of Cl^- ions, and the pH was kept at 7.67 by gassing with 8 per cent CO_2 (Table VI). Under these conditions, the $CO_3^=$ ion concentration, as calculated from the Henderson-Hasselbalch equation, exceeded the concentratioe present in the ordinary medium at pH 8.1 Nevertheless, the growth of cells at the lower pH far exceeded that observed at pH 8.0 and was in good agreement with the growth obtained at pH 7.7 in the usual medium. From these results it appears that growth failure under conditions of simulated alkalosis is not due to the increase in $CO_3^=$ ion concentration or to the concomitant decrease in Ca^{++} ion concentration.

The microscopic appearance of HeLa cells grown at high pH for 3 days is shown in Fig. 8. The area of cell attachment to glass is greatly reduced in many cells and as a result they have assumed an ellipsoidal or spherical shape. The cells are also granular in appearance; this is due, in part at least, to the appearance of small particles on the cell membrane. These particles are probably

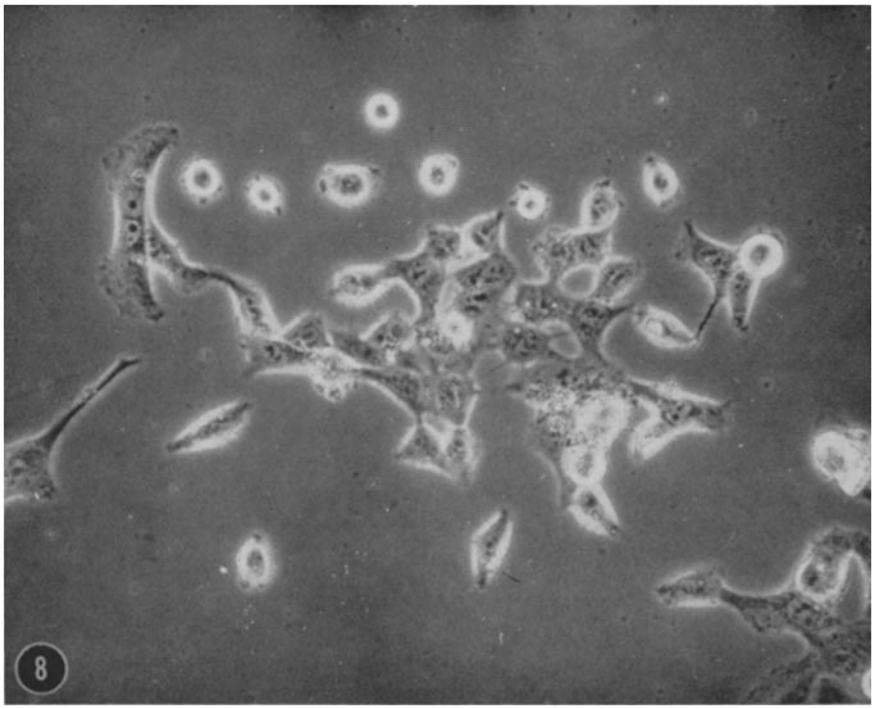


FIGURE 8

HeLa cells grown at pH 8.1 for 3 days. Although the cytoplasm was retracted, all of the cells pictured were still attached to glass. Phase contrast. × 50.

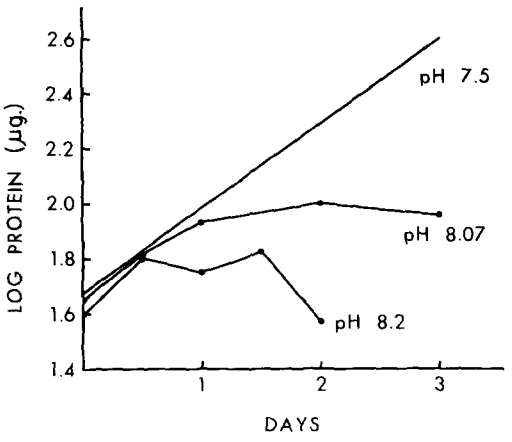


FIGURE 9

The effect of alkalosis on the growth of HeLa cells as measured by total cell protein.

liberated from disrupted cells, for they are also present in the medium in a free form but are not present in medium incubated at the same pH in the absence of cells.[4] The fact that these structural changes were not prominent until cells had been exposed to a high pH for 2 or 3 days suggested that growth inhibition might also be a delayed reaction.

In experiments designed to test this possibility, it was found that the initial rate of protein synthesis was maximal at both pH 8.05 and 8.2 (Fig. 9). However, after 24 and 12 hours, respectively, the net synthesis of protein ceased and the cells entered a steady state or stationary phase. After 36 hours at pH 8.2, there was a loss of cell protein, presumably as the result of cell death and disintegration. The results of these experiments are in agreement with our observations on the structural changes caused by alkalosis. They are also reminiscent of Lodge and Hinshelwood's (16) observation that at pH 8.0 *Bact. lactis aerogenes* multiply at a normal rate for several generations and then suddenly stop growing.

In view of the rapidity of reactions involving H^+ and OH^- ions, the delay in growth inhibition at high pH probably represents the time required for some direct reaction to exert its effect on net protein synthesis. Such a situation may be depicted most simply as the inhibition at pH 8 of an enzymatic reaction, A $\rightarrow$ B, where A is an inhibitor that must reach a critical concentration, or B is an essential metabolite normally produced at a nonlimiting rate. The enzyme system could be located inside the cell or in the membrane.

Effect of Acidosis on Cell Growth and Lipid-Rich Particles

The effect of acidosis on the growth rate of cells was quite different from the effect of alkalosis. For example, when the pH of the medium was reduced from 7.5 to 6.9, HeLa cells continued to grow at a logarithmic rate, but the generation time was increased from 23 hours to 29 hours. In other words, the increase in the extracellular concentration of H^+ ions had a prompt inhibitory effect on growth and net protein synthesis. Similar results were obtained with HeLa cells grown at pH 6.74.

Acidification of the medium also caused a striking increase in the number of cytoplasmic granules present in all the cell lines studied (Figs. 10 and 11). In HeLa cells grown at pH 6.9 for 2 days, the average number of granules per cell was 60, as compared with 0 to 15 in cells grown at pH 7.4. At pH 6.6, the average number per cell exceeded 100. Although these spherical particles were not normally present in Chang liver, rat liver, and rat muscle cells grown at pH 7.4 to 7.8, they appeared when the cells were grown at acid pH. For example, rat liver cells grown for 2 days at pH 6.6 contained an average of 40 granules per cell. These particles were concentrated around the nucleus, and had an average diameter of approximately 1 μ. They appeared as black bodies under

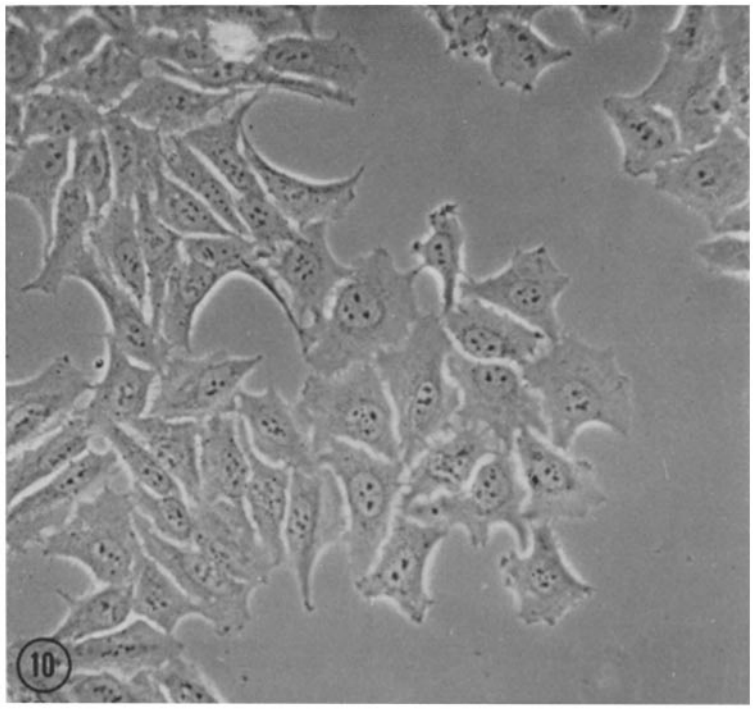
[4] Medium stored in a frozen state often develops a fine precipitate and should not be used in such experiments without being refiltered.

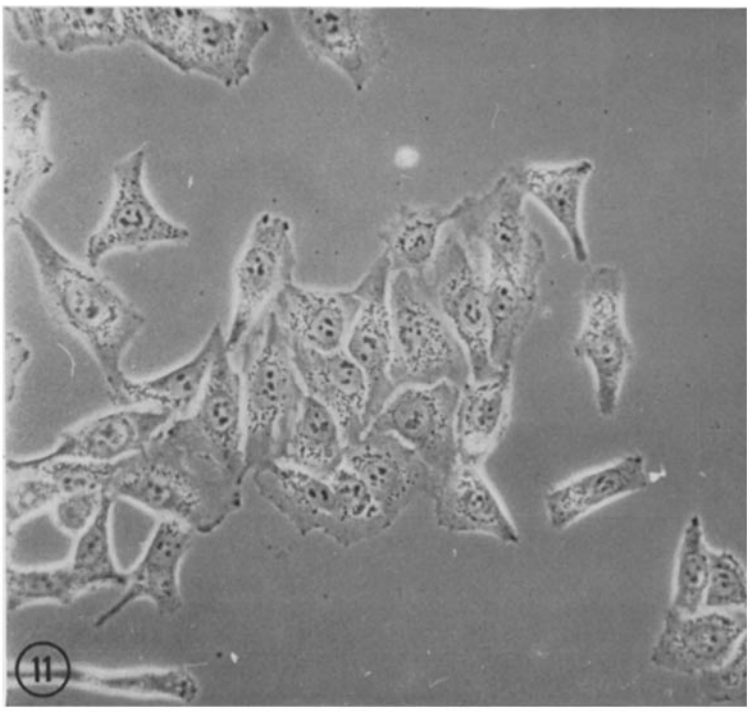
FIGURE 10

HeLa cells grown at pH 7.4 for 3 days. Phase contrast. × 50.

FIGURE 11

HeLa cells grown at pH 6.9 for 3 days. The dark granules concentrated around the nuclei are the lipid-rich particles with an average diameter of 1 μ. Phase contrast. × 50.





the phase contrast microscope and as pale yellow particles under the ordinary microscope. When cells grown at acid pH were fixed either in Bouin's solution or in osmium tetroxide vapor, the particles were stained selectively with oil red O dissolved in isopropanol. Prior extraction of fixed cells with xylol, toluene or dioxane eliminated the staining reaction completely.

These particles resemble in their size, microscopic appearance, and staining properties the lipid granules described recently by Crawford (20) in cultured snail amebocyte cells. Somewhat similar particles were observed sporadically in newly cultured chick embryo cells by the Lewises (21) in 1915, in their classical study on the shape and movement of mitochondria *in vivo*. They stated that the appearance of these particles in particular cultures was due to an unknown cause. It appears now that one of the determining factors may have been the pH of the medium.

The development of a procedure for the fractionation of cultured cells in sucrose enabled us to segregate the spherical particles from the other subcellular structures.[5] When glass-attached HeLa cells, which had been grown at pH 6.8, were disrupted by scraping in 0.25 M sucrose and passed through a No. 27 gauge hypodermic needle, the spherical particles were present in the cell-free suspension together with the nuclei and mitochondria. They were not sedimented in appreciable numbers with the nuclei, mitochondria, or microsomes, and at a centrifugal force of 100,000 *g* for 30 minutes rose to the top of the sucrose solution to form a clearly distinguishable band. The isolated particles did not agglutinate or coalesce, and their average diameter corresponded to the diameter of the perinuclear particles in the living cells.

The low specific gravity of these particles is further evidence that they are rich in lipids. However, they probably contain other materials and we have found that they are colored when the cultured cells are stained supravitally with Janus green B by the procedures of Conn (22) or Cowdry (23). Although they take the dye more slowly than some of the mitochondria, they are usually colored a deep blue or purple in 10 to 15 minutes and are clearly discernible when the cells are viewed under the conventional microscope (Figs. 12 and 13). Many of the particles swell after being stained with Janus green. In the swollen particles, the reduced dye remains concentrated at the periphery, whereas the center loses much of its color. In view of the properties described above we have called these bodies the *lipid-rich particles.*[6]

The increase in the number of lipid-rich particles in mammalian cells grown at acid pH was not due simply to the decreased growth rate, for HeLa cells whose growth at pH 7.4 was restricted by reducing the glucose content of the medium to 10 mg. per 100 ml., or omitting it completely, did not show this response. Furthermore, the increase in the number of particles in acidosis was observed irrespective of whether the pH was lowered by increasing the CO_2 concentration or reducing the HCO_3^- ion concentration. It appears, therefore, that one of the factors which causes an accumulation of lipid-rich particles is an increase in the H^+ ion concentration of the medium.

When the medium of HeLa cells that had entered the logarithmic phase of growth was acidified, the increase in lipid-rich particles was discernible in 1 day, and after 2 or 3 days it had approached a maximum. When such a culture

[5] The experiments on the fractionation of tissue culture cells were carried out in collaboration with Dr. Oscar K. Reiss.

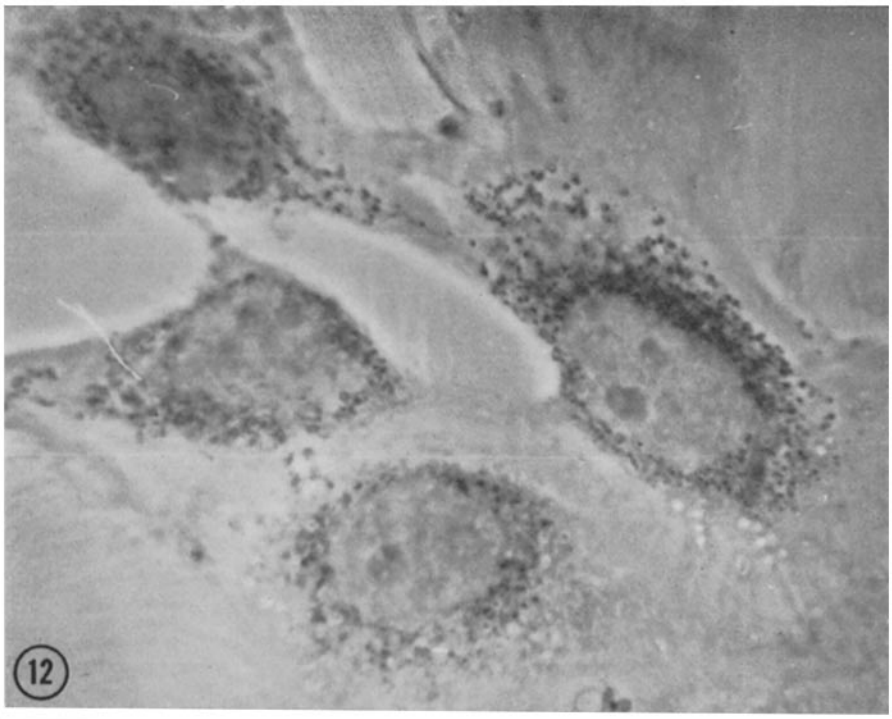
[6] Preliminary electron microscopic observations at × 40,500 made by Dr. James F. Reger of the Department of Anatomy showed these particles to be electron-dense bodies surrounded by a membrane. Many of the particles were close to, or in contact with, mitochondria.

FIGURE 12

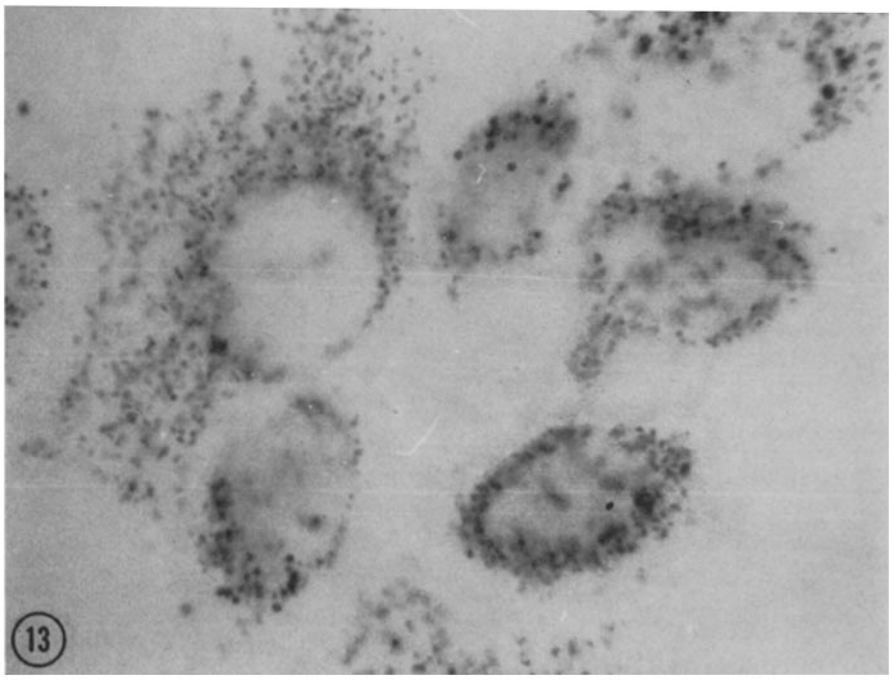
Rat liver cells grown at pH 7.0 for 3 days and stained supravitally with Janus green B. Phase contrast. × 240.

FIGURE 13

Rat liver cells grown at pH 7.0 for 3 days and stained supravitally with Janus green B. Conventional light microscope. × 240.



(12)



(13)

TABLE VII

Composition of Medium

In addition to the constituents listed, the medium contained 10 per cent horse serum,* 1 per cent beef embryo ultrafiltrate, and 50,000 units per liter of penicillin and streptomycin. All amino acids were of the L configuration.

Constituent	Quantity	Constituent	Quantity
	mM		*mM*
Na^+	133.0	Cl^-	117.0
K^+	4.8	HCO_3^-	22.5
Ca^{++}	1.8	$HPO_4^=$	0.9
Mg^{++}	0.8	$SO_4^=$	0.7
Glucose	7.0	Glutamine	2.0
Threonine	0.2	Valine	0.2
Leucine	0.2	Isoleucine	0.2
Cystine	0.05	Methionine	0.05
Phenylalanine	0.1	Tyrosine	0.1
Lysine HCl	0.2	Arginine	0.16
Histidine	0.05	Tryptophane	0.02
	μM		*μM*
Thiamine HCl	3.0	Riboflavin	0.3
Pyridoxal HCl	6.0	Ca pantothenate	4.5
Nicotinamide	8.0	Folic acid	2.0
Biotin	4.0	Choline chloride	8.0
m-Inositol	5.0	Phenol red	1.4

* Inorganic components and glucose of 10 per cent horse serum have been included in the concentrations given in the table (24).

of cells was transferred every third day to fresh medium at pH 6.9, the high cytoplasmic concentration of lipid-rich particles was still present at the end of 2 weeks.

It is probable that the lipid-rich particles play an important role in the metabolism of normal and "abnormal" cells. Their perinuclear distribution places them in apposition to the mitochondria which are concentrated in the same region. The possibility that the particles also possess enzymes and that they furnish substrates for the mitochondria is an attractive one that is being investigated in isolated preparations.

EXPERIMENTAL

Medium: With minor modifications, the medium used for both stock and experimental cells was made up of 830 ml. of Earle's solution (3), 100 ml. of horse serum, 10 ml. of beef embryo ultrafiltrate (Microbiological Associates), the organic constituents as defined by Eagle (4), and water to make up 1 liter. The composition of the medium, including the inorganic constituents and glucose present in horse serum (24), is given in Table VII. Before use, the medium was filtered through an O3 porosity Selas candle and the pH was adjusted to 7.4 with 0.5 N HCl.

Cells: Cultures of HeLa and Chang liver cells were obtained from Microbiological Associates. The rat liver cell was isolated from a primary explant of liver obtained from a 150 gm. male rat. The rat muscle cell was isolated from a primary explant of muscle obtained from a 17 day old rat embryo. Clones were prepared from all of the cell lines by the procedure of Puck, Marcus, and Cieciura (25).

Stock cultures of the cells were grown in milk dilution bottles and transferred once a week. To prepare cell suspensions for use in the growth experiments, cultures which had been transferred 3 days earlier and fed daily were washed 3 times with 10 ml. portions of a solution of the following composition; 2.7 mM KCl, 137 mM NaCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. The pH was adjusted to 7.4 with HCl. The cell monolayer was then scraped into 8 ml. of the same solution with a triangular rubber policeman and the cells were dispersed by pipetting gently for 1.5 minutes with a 10 ml. pipette. An aliquot of the suspension was diluted tenfold with medium and the cells were counted in a hemocytometer under the phase contrast microscope. The original suspension was then diluted with the above electrolyte solution

to obtain the desired cell concentration and 0.5 ml. aliquots were transferred to 6 cm. diameter petri dishes containing 5 ml. of medium.

Gassing of Cultures with CO_2: Dishes containing cells were placed in groups of 5 or more in sterilized 9 liter pyrex desiccators in a constant temperature room at 37.5 C. ± 0.1°. The bottoms of the desiccators were covered with water and the tops were sealed with glycerol. CO_2-air mixtures (National Cylinder Gas Co.) contained in large cylinders warmed to 37.5° were bubbled through water and delivered to the desiccators in sterilized pure gum rubber tubing. Gas was passed through the desiccators at 5 liters per minute for 10 minutes and thereafter at 1 liter per minute. The desiccators were opened and the petri dishes were removed for about 10 minutes every 24 hours in order to replace the medium. The desiccators were then regassed as described above.

Before use, the CO_2 content of all the cylinders used was checked by bubbling the gas through a standard $NaHCO_3$ solution and measuring the equilibrium pH. A cylinder containing 5.21 per cent CO_2, as determined by Haldane analysis, was used as a control.

Measurement of pH: All pH determinations were made with Beckman Model H-2 pH meters. The instruments were standardized against Sorensen's buffers over the pH range of 6.0 to 8.5 and with the Beckman buffer at 7.0. Readings were reproducible to ± 0.01 pH unit.

Determination of Cell Protein: The medium was removed from the petri dishes by aspiration and the dish and attached cells were washed 3 times with 5 ml. portions of the electrolyte solution described above. Additional washings did not remove significant amounts of protein. Six ml. of alkaline copper tartrate (C reagent) were added to each dish, the cells were disrupted by scraping thoroughly with a glass stirring rod, and the solution was allowed to stand at room temperature for 30 minutes. The protein content of suitable aliquots of the solution was determined by Oyama and Eagle's (26) modification of the procedure of Lowry and co-workers (27). In our analyses, aliquots of the cell solution were diluted to 6 ml. with C reagent and water was not added to the final mixture. 0.1 ml. of the stock solution of bovine albumin standard, 10 mg. per 1 ml. of H_2O, was diluted to 10 ml. with C reagent and appropriate aliquots of this solution were brought to a final volume of 6 ml. with additional C reagent.

Cell Counts: The cells were washed 3 times with 5 ml. portions of the electrolyte solution described above. To each dish was then added 2 ml. of a 0.1 per cent solution of trypsin 1-300 (Nutritional Biochemicals, Inc.) dissolved in the same solution with the phosphate reduced to 1 mM. The cells were incubated at 37.5°C. for 8 minutes, by which time all of the cells were detached from the glass. The cell suspension was then passed through a No. 30 hypodermic needle and 3 ml. of medium was added. The cells were counted at once in a hemocytometer under a phase contrast microscope. Whenever suspensions so prepared were too concentrated for accurate counting, they were diluted further with a suitable volume of medium. Ten samples were counted from each dish. In the growth experiments, the cells in 4 dishes were counted daily to obtain the average cell population.

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