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REDUCTION OF LACTIC ACID SECRETED FROM SV3T3 CELLS BY A SERUM FACTOR AND BIOTIN AND ITS RELATIONSHIP TO CELL GROWTH

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SUMMARY: Supplementation of media containing a low concentration (0.15-0.30% v/v) of calf serum with biotin or a low molecular weight serum growth factor (Peak III) reduces the amount of lactic acid secreted by simian virus 40-transformed 3T3 cells. While biotin and Peak III (which has been tentatively identified as biotin) can stimulate "stationary phase" cells to resume viable cell division, this growth promotion is not due to an alleviation of lactic acid toxicity per se. This conclusion is based on the finding that, although higher concentrations of lactic acid are cytotoxic, lactic acid added at concentrations found during "stationary phase" to cells plated in fresh medium is not growth inhibitory. These results suggest, instead, a possible major role for biotin and Peak III in energy production.

INTRODUCTION:

The excessive production of lactic acid by tumor tissue and by transformed and other cells in tissue culture is a well known experimental observation (1). The biochemical cause of this metabolic imbalance is still unclear despite various attempts at an explanation (2). Like other transformed cells, simian virus 40-transformed 3T3 mouse fibroblasts (SV3T3)[2] also secrete high concentrations of lactic acid into their medium.

Over the past several years, a low molecular weight ($<$ 500 daltons) component from calf serum, which enhances the viability or "growth" of these cells in low serum (3,4) has been partially purified in our laboratory. This agent, termed Peak III or Serum Factor III, is apparently not growth limiting for untransformed 3T3 cells in low serum medium (3,4). Preliminary evidence has suggested that the active agent in Peak III may be the vitamin biotin (manuscript in preparation).

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[2]Abbreviations: SV3T3, simian virus 40-transformed 3T3 cells; DME, Dulbecco's Modified Eagle's medium.

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This communication reports the finding that Peak III and pure biotin are both capable of greatly reducing the amount of lactic acid secreted by SV3T3 cells. Since addition to fresh medium of lactic acid at the secreted concentrations does not hinder growth appreciably, the significance of the lactic acid reduction does not appear to lie in an alleviation of a toxic effect but in an unexpected role for this serum agent (and biotin) in the generation of cellular energy.

MATERIALS AND METHODS:

Cells: The Swiss SV3T3 cells (obtained from Dr. Robert Holley) were maintained in Dulbecco's Modified Eagle's medium (DME) containing 4500 mg glucose per liter and supplemented with 10% ($^{v}/_{v}$) calf serum. The cells were kept in a 5% CO_2 incubator, transferred twice weekly with 1:250 trypsin as previously described (5), and examined for mycoplasma by exposure to ^{3}H-thymidine and autoradiography.

Chemicals: All routine tissue culture supplies, including powdered DME, 1:250 trypsin, calf serum, etc., were supplied by Grand Island Biological Co. (Gibco). Sephadex G-100 and G-25, lactic acid, and lactic acid dehydrogenase kit #826B used for the lactic acid assay were purchased from Sigma Chemical Co. Biotin was a product of Calbiochem.

Preparation of Peak III: Peak III was obtained from fresh calf serum from a local slaughterhouse in Cambridge, MA using a modification of an earlier process. In the original process, which is described in detail elsewhere (3), calf serum was acidified to pH 2, passed first through pH 2 Sephadex G-100, and then, following detection of the Peak III fractions, through a pH 2 Sephadex G-25 column. In the modified version, the Sephadex G-100 step was replaced by ultrafiltration. Calf serum was acidified to pH 4.5 and placed in a boiling water bath for 30 min. The supernatant was then ultrafiltered through Millipore Pellicon membranes (molecular weight cutoff of 10,000 daltons). Following neutralization and concentration by lyophilization, the material was then re-acidified to pH 2 and passed through a pH 2 Sephadex G-25 column (180 x 2.5 cm) using 0.01 M HCl as the eluting solution. The active fractions were then neutralized, lyophilized, and used for all experiments in this report.

Growth Assay: All growth experiments were performed as previously described (3). Briefly, stock SV3T3 cells were plated at 4×10^{4} cells per 3.20 cm Lux dish in 2.0 ml of DME containing 0.15-0.30% ($^{v}/_{v}$) calf serum. After 2-4 hr test samples (biotin, Peak III, lactic acid) were added in volumes not greater than 0.2 ml. Lactic acid solutions were first neutralized with NaOH prior to addition. At the indicated times, the viable, substratum-attached cells were removed by trypsinization and counted in a Coulter Counter. Each point was the average of at least 3-4 dishes.

Lactic Acid Determination: Cells were plated following the Growth Assay procedure described above with the exception that 6.0 cm Lux dishes were used and the cell number and medium volume were accordingly scaled up (1×10^{5} cells in 5.0 ml of 0.2% $^{v}/_{v}$ calf serum DME). At the times indicated 2 ml of culture medium were added to 4 ml of cold 8% ($^{v}/_{v}$) perchloric

acid. Following centrifugation of the precipitate, 0.2 ml of the clear supernatant was enzymatically analyzed for lactic acid using Sigma Chemical Co. kit #826B and following their instructions (Sigma Chemical Co. Technical Bulletin #826-UV). The assay measured the amount of NADH produced (monitored at 340 nm) from the oxidation of lactic acid by lactic acid dehydrogenase.

RESULTS:

When SV3T3 cells are plated in DME medium containing a low concentration of serum (0.15-0.3% $^{v}/_{v}$), growth ensues for a few days after which the number of viable, substratum-attached cells levels off and may start to decrease. Although cells in this "stationary phase" continue to make DNA and divide, half or more of the dividing population die and detach from the surface of the dish. If Peak III (3) or biotin (6) is added to "stationary phase" cultures, the number of viable cells increases again and fewer dead cells are released into the medium. This indicates that SV3T3 cells deplete Peak III (or biotin) in low serum media and that Peak III or biotin re-supplementation permits the resumption of healthy, viable cell division.

The growth active component in Peak III appears to be biotin. A substance in the Peak III fraction can be stained with the biotin-specific dye, p-dimethylaminocinnamaldehyde, and migrates on thin layer chromatography with the same R_f as biotin, while the growth activity in Peak III binds to avidin-Sepharose but not Sepharose alone (manuscript in preparation). Since biotin supplementation appears to retard the gradual acidification of SV3T3 cultures (as noted by visual observations of color changes in the phenol red pH indicator), it was thought that Peak III and biotin may enhance cell viability by lowering the amount of lactic acid present in the medium. The results of Table I show that Peak III and biotin do reduce the amount of lactic acid secreted by these cells. While the actual concentrations of this acid are higher in biotin and Peak III supplemented cultures than in the control, the supplemented cultures also contain much greater numbers of cells and when the amount of lactic acid is normalized with respect to the cell number, this ratio is significantly lower for cells growing in biotin or Peak III.

Table 1. Reduction of Lactic Acid Production by Peak III and Biotin

Time (Hr)	Control			Peak III			Biotin		
	Lactic Acid (mM)	Cells/ Dish ($\times 10^{-5}$)	Lactic Acid (μmoles) / 10^5 cells	Lactic Acid (mM)	Cells/ Dish ($\times 10^{-5}$)	Lactic Acid (μmoles) / 10^5 cells	Lactic Acid (mM)	Cells/ Dish ($\times 10^{-5}$)	Lactic Acid (μmoles) / 10^5 cells
70	2.69	3.59	3.75	2.64	5.65	2.35	2.79	5.19	2.70
167	8.08	3.85	10.50	15.47	18.8	4.10	14.85	16.6	4.45
190	11.36	3.85	14.75	21.45	19.0	5.65	20.22	17.1	5.90
212	12.29	3.44	17.85	28.92	24.9	5.80	21.14	19.0	5.55

The experimental protocol is described under Lactic Acid Determination in MATERIALS AND METHODS. The lactic acid values and the cell counts were the averages from three, separate, 6.0 cm dishes. This experiment was performed many times with results similar to the above.

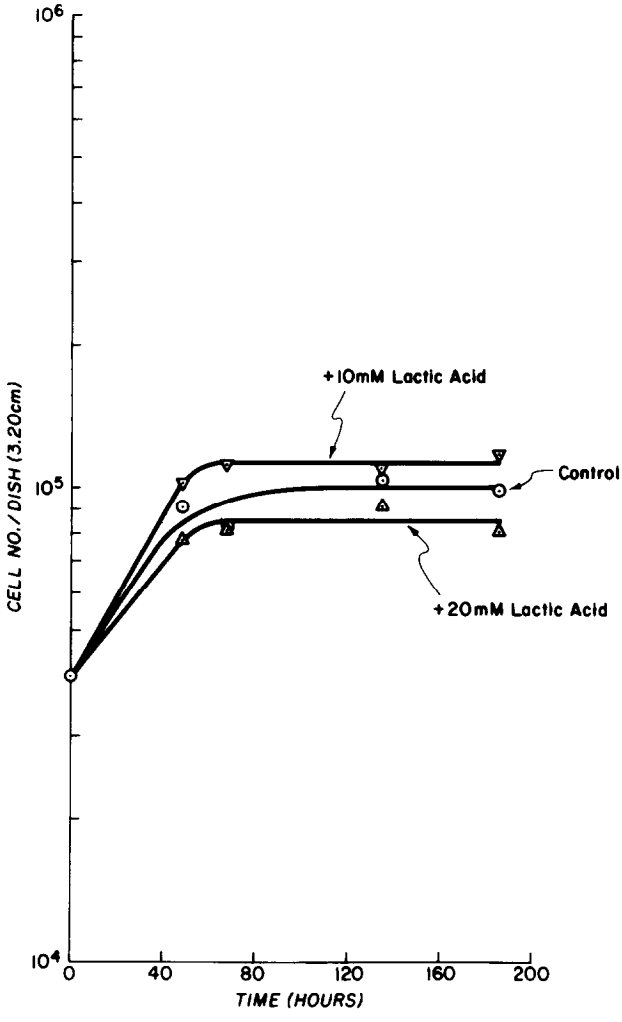

Figure 1: Growth of SV3T3 cells in low serum medium and in the absence and presence of 10 and 20 mM lactic acid. The experimental protocol followed the Growth Assay procedure outlined in MATERIALS AND METHODS. o-o, Control; ▽-▽, plus 10 mM lactic acid; △-△, plus 20 mM lactic acid.

If lactic acid is responsible for the growth suppression that is seen at "stationary phase", then its addition to cells newly plated in fresh medium should inhibit cell growth nearly immediately. As seen in Figure 1, lactic acid at concentrations of 10 and 20 mM do not immediately prevent cell growth and, although 10 mM is almost four times the concentration (2.69 mM) that prevails in the control culture at the onset of "stationary phase" (Table 1), growth in 10 mM lactic acid is as good as the untreated control.

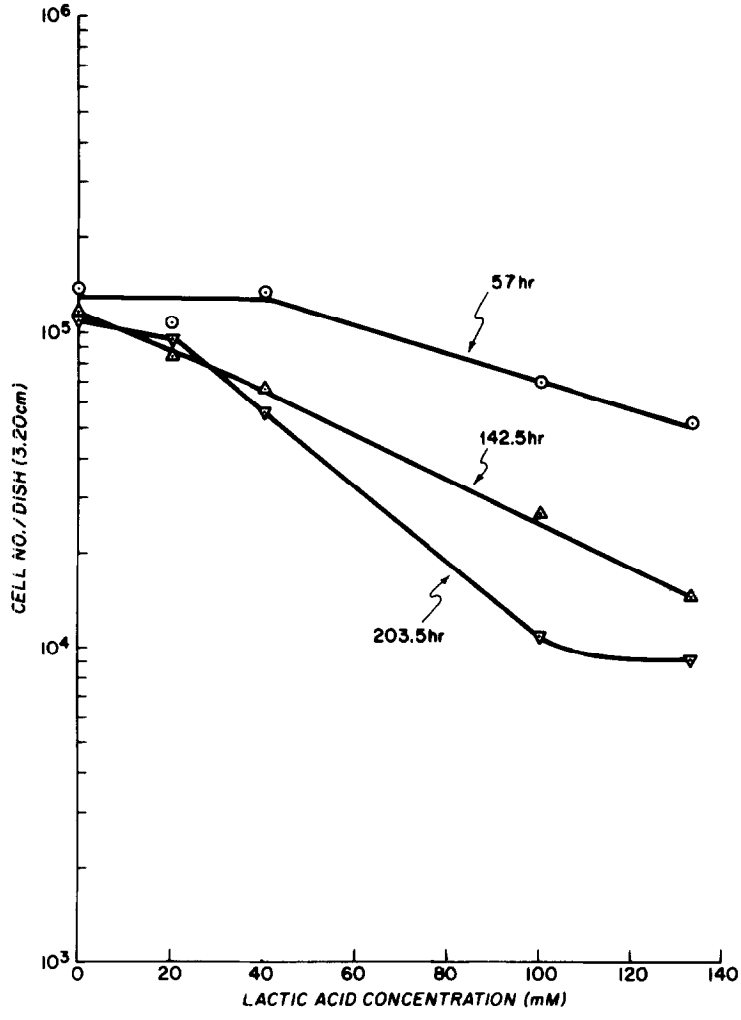

Figure 2: Toxicity of higher concentrations of lactic acid. The experimental procedure was as described in Figure 1. Cell counts at o-o, 57 hr; Δ-Δ, 142.5 hr.; ▽-▽, 203.5 hr.

Indeed, a careful examination of Table 1 reveals the fact that biotin and Peak III cultures attain the lactic acid concentration of 2.69 mM early but continue to grow rapidly while accumulating up to ten times this concentration. However, lactic acid can be cytotoxic, as observed in the extensive cell dying which occurs at higher concentrations (Fig. 2).

DISCUSSION:

The production of high concentrations of lactic acid in tumor tissue was first observed by Warburg who proposed that glycolysis played a greater

role in energy production than respiration in neoplastic cells (1,2). This phenomenon has also been observed in tissue culture not only for transformed cells but also for untransformed cells, too. Holley (7) has demonstrated that lactic acid is one of several compounds released by BSC-1 cells that inhibits at a concentration of 11 mM BSC-1 cellular growth, although the inhibition seen by Holley was small (~15%). On the other hand, lactic acid does not appear to be the primary cause for the commencement of "stationary phase" in SV3T3 cells.

The accumulation of lactic acid suggests that a blockage exists in the conversion of pyruvic acid to Krebs Cycle intermediates, which is apparently circumvented by the addition of biotin or Peak III. This statement is supported by the experimental observation that iron supplementation of SV3T3 cultures does not promote faster growth unless biotin (8) or Peak III (unpublished results) is simultaneously added. If the iron is needed for respiration, then it would be useless unless the prior blockage in the Krebs Cycle is first circumvented. The nature of the circumvention by biotin or Peak III is unclear, although pyruvic acid carboxylase, which forms oxaloacetic acid from pyruvic acid, is a biotin-requiring enzyme.

Additional evidence for this hypothesis can be found in those experiments which have shown that supplementation of culture media with α ketoacids and some Krebs Cycle intermediates can enhance cellular growth (9). Preliminary data from our laboratory also reveal that some Krebs Cycle intermediates are growth stimulatory for SV3T3 cells. Recently, a striking finding has been reported (10) which indicates that glutamine is the major energy source for HeLa cells, presumably by conversion to α ketoglutaric acid. The significance of all these results on energy production in neoplastic tissue and as a possible explanation for the original Warburg observation awaits the biochemical elucidation of the putative Krebs Cycle defect.

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REFERENCES:

1. Warburg, O., Posener, K., and Negelein, E. (1924) Biochem.Z. 152, 309-344.
2. Bustamante, E., and Pedersen, P.L. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3735-3739.
3. Young, D.V., Nakano, E.T., Wiggins, D.S., and McElaney, M.A. (1978) J. Cell. Physiol. 96, 147-154.
4. Paul, D., Lipton, A., and Klinger, I. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 645-648.
5. Young, D.V. (1976) J. Cell. Physiol. 89, 133-142.
6. Messmer, T.O., and Young, D.V. (1977) J. Cell. Physiol. 90, 265-270.
7. Holley, R.W., Armour, R., and Baldwin, J.H. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1864-1866.
8. Young, D.V., Cox III, F.W., Chipman, S., and Hartman, S.C. (1979) Exp. Cell Res. 118, 410-414.
9. McKeehan, W.L., and McKeehan, K.A. (1979) J. Cell. Physiol. 101, 9-16.
10. Reitzer, L.J., Wice, B.M., and Kennell, D. (1979) J. Biol. Chem. 254, 2669-2676.