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TECHNICAL MANUSCRIPT 584

# RELATIONSHIP OF OXIDATION-REDUCTION POTENTIAL TO GROWTH OF TISSUE CULTURE MEDIA POISED BEFORE INCUBATION


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TECHNICAL MANUSCRIPT 584

# RELATIONSHIP OF OXIDATION-REDUCTION POTENTIAL TO GROWTH OF TISSUE CULTURE MEDIA POISED BEFORE INCUBATION

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Project 1B563603DE32 February 1970

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## ABSTRACT

Earlier observations revealed that incubation of media and the attendant changes in oxidation-reduction potential (ORP) were related to improved cell production. This is a report of work done to show that the higher levels and increased rates of growth of cells grown in incubated medium are associated with the ORP level of the medium before its inoculation with cells. Work was done using 250-ml centrifuge spinner bottles as the culture vessels. Further work is needed to establish the desirability of deliberate poising of media prior to use for studies in small vessels and flasks.

# I. INTRODUCTION*

In a previous study, the authors** investigated growth and associated oxidation-reduction potential (ORP) patterns of Earle's L cells in centrifuge spinner bottles. That study was conducted with 100-ml portions of medium that was either stored frozen or incubated at 37 C for 3 days prior to inoculation. Routinely, samples of the medium were incubated to determine sterility.

In analyzing these data, we noted differences in cell counts between cultures grown in incubated medium and those grown in non-incubated medium. These observations led us to a separate investigation to measure the significance of these differences. Attempts were also made to determine whether changes in the medium induced by incubation might be reflected in change of ORP.

# II. EQUIPMENT AND PROCEDURES

## A. EXPERIMENTAL EQUIPMENT

### 1. Culture Vessels

Culture vessels were adapted from 250-ml centrifuge bottles. The adaption for routine cell propagation provided for agitation of medium by the spinner device shown in Figure 1. For uses involving measurement of ORP, the silver - silver chloride and platinum electrodes (Fig. 1) were used. The electrodes were sealed into holes in the bottles with Dow Corning Silastic silicone rubber adhesive RTV 731. To eliminate oxygen uptake by the medium during incubation, the spinner was replaced by a tightly fitting rubber stopper.

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* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

** Daniels, W.F.; Garcia, L.H.; Rosensteel, J.F. September 1969. Oxidation-reduction potential and concomitant growth patterns of cultures of Earle's L cells in centrifuge spinner bottles, (Technical Manuscript 550). Process Development Division, Fort Detrick, Frederick, Maryland.

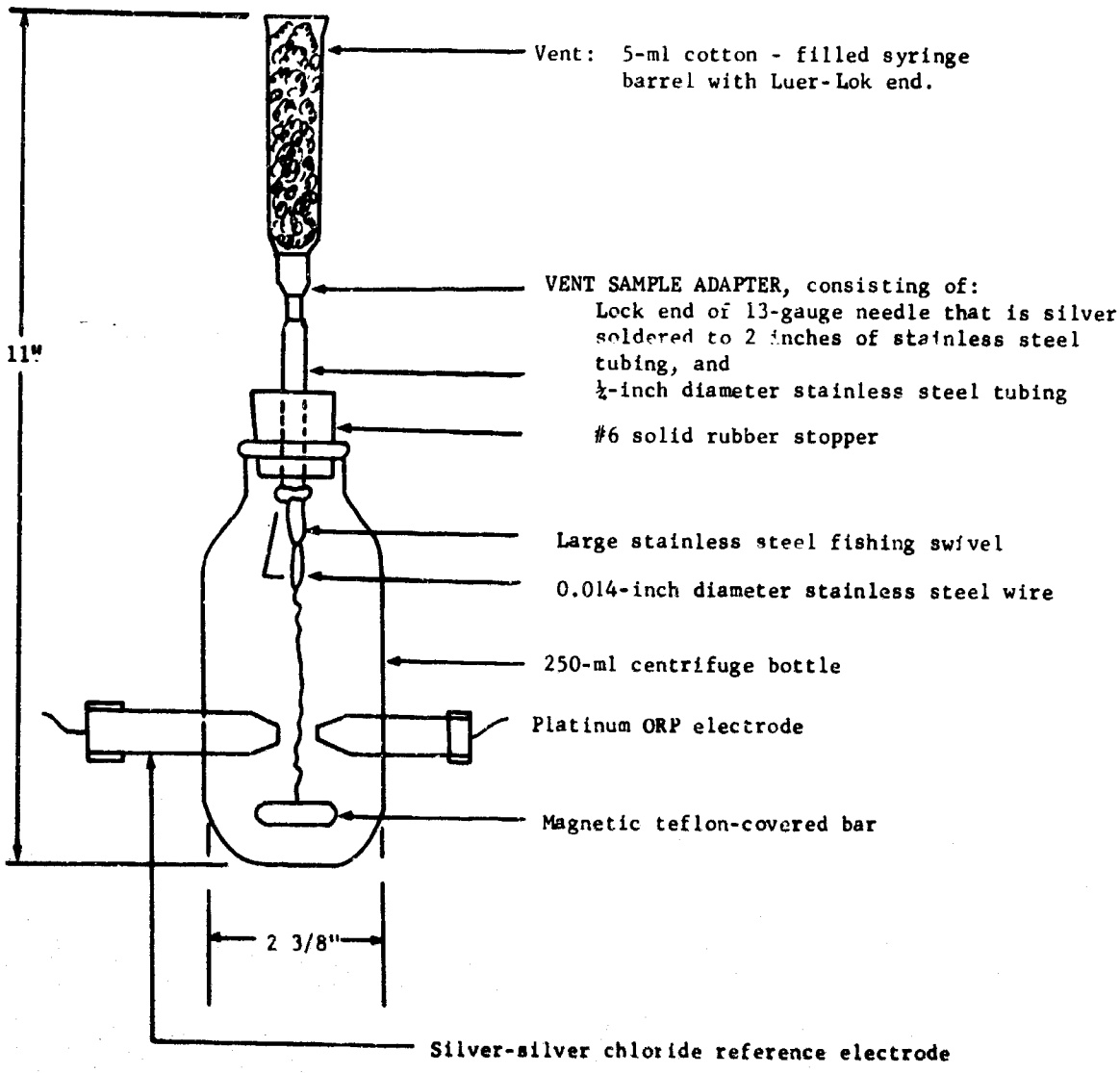


**FIGURE 1. Centrifuge Spinner Bottle.**

2. Recorders

The spinner bottles were connected with electrode wires to instrument panels similar to those described earlier for a 1-liter tissue culture fermentor by Daniels et al.* ORP values were continuously recorded during 3-day incubation periods prior to seeding with cells.

B. PROCEDURES

1. Medium and Inoculum

The medium previously described by Rosensteel et al.** consisted of Eagle's minimum essential medium (double strength) with the nonessential amino acids as a substitute for the essential amino acids, plus modified Earle's balanced salt solution. To this was added: lactalbumin hydrolysate, 0.5%; cysteine, 260 μg/ml; ascorbic acid, 50 μg/ml; glucose, 1%; and bovine serum, 10%. Each bottle contained 100 ml of medium. The pH at time of use was 7.2 to 7.4.

2. Samples

Each day a 2-ml sample was withdrawn aseptically from the bottle, and pH, cell count, viability, and cell size were determined.

The experiments were repeated eight times to insure reasonable validity of conclusions drawn. A few runs were lost because of malfunctioning spinner bars. The data from 43 vessels with electrodes were thus recorded. Of these, 22 contained medium that was not incubated, but was frozen, thawed at time of use, and inoculated with cells, and 21 contained medium that was incubated and then inoculated.

3. Apparatus and Operations

Three centrifuge spinner bottles were fitted with electrodes, one without electrodes was used for the control. The fitted bottles were placed in an incubator at 37 C and connected to instrument panels that continuously recorded ORP.

After three days' incubation these three bottles, plus the unincubated control, were inoculated with Earle's L cells at a level of $1 \times 10^5$/ml of final medium from an actively growing culture in the log phase, employing vessels without electrodes containing the same medium. The level of inoculation was intended each time to be $1 \times 10^5$ cells/ml and for the most part was obtained. All spinner bottles were returned to the 37 C incubator for growth.

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* Daniels, W.F.; Parker, D.A.; Johnson, R.W.; Schneider, L.E. 1965. Controlled pH and oxidation-reduction potential with a new glass tissue-culture fermentor. Biotechnol. Bioeng. 7:529-553.

** Rosensteel, J.F.; Jordan, W.C.; Daniels, W.F. 1969. Growth potential of a variation of Eagle's minimum essential medium for spinner cultures. Biotechnol. Bioeng. 11:263-266.

The described procedure was applied also to testing frozen medium, with the exception, of course, that the incubation period prior to inoculation does not apply.

## III. DISCUSSION AND RESULTS

Table 1 gives average peak growth levels and growth rates with the 95% confidence limits for the two types of media. The media poised by incubation had significantly higher (at 0.01 level) average peak growth than the non-incubated media, with a calculated $t = 4.21$. The variability of the incubated peak growth levels was also significantly higher (at the 0.01 level) with a calculated $F = 9.90$. This increase in average peak growth agrees with results of earlier experience that prompted this investigation of ORP changes during incubation.

TABLE 1. DIFFERENCES IN L CELL GROWTH IN INCUBATED MEDIUM COMPARED WITH L CELL GROWTH IN NON-INCUBATED MEDIUM

| Medium | Observations | Avg. Peak Growth, $10^6$ viable cells/ml | 95% Conf. Limits | Avg. Growth Rate, $10^5$ viable cells/ml per day | 95% Conf. Limits |
|---|---|---|---|---|---|
| Incubated | 21 | 2.87 | 2.45 - 3.29 | 2.8 | 2.29 - 3.35 |
| Frozen | 22 | 2.28 | 2.15 - 2.41 | 2.9 | 2.54 - 3.29 |

Growth rates and their variability were not significantly different at the 0.05 level with calculated $t = 0.32$ and $F = 1.91$ respectively.

Figure 2 illustrates typical behavior of the ORP changes during incubation of the medium without access to air other than that within the centrifuge bottle itself. Thus, for the three flasks represented here, the ORP of the medium was -100 mv at the outset. In spite of oxygen from the air within the vessels at inoculation, ORP levels dropped to about -230 mv, varying considerably one from the other in the time required to achieve this level. After 36 hours, variation became minimal, and the ORP of the three samples began to rise, leveling off at the end of the 3rd day. The three ORP values show remarkable agreement during this period, being within ±20 mv of each other by the end of this 3-day period. Figure 2 shows the ORP pattern of only one of the eight groups of three vessels incubated prior to inoculation with cells. The others were similar, differing only in initial ORP at the beginning of the experiments and that shown at the end.

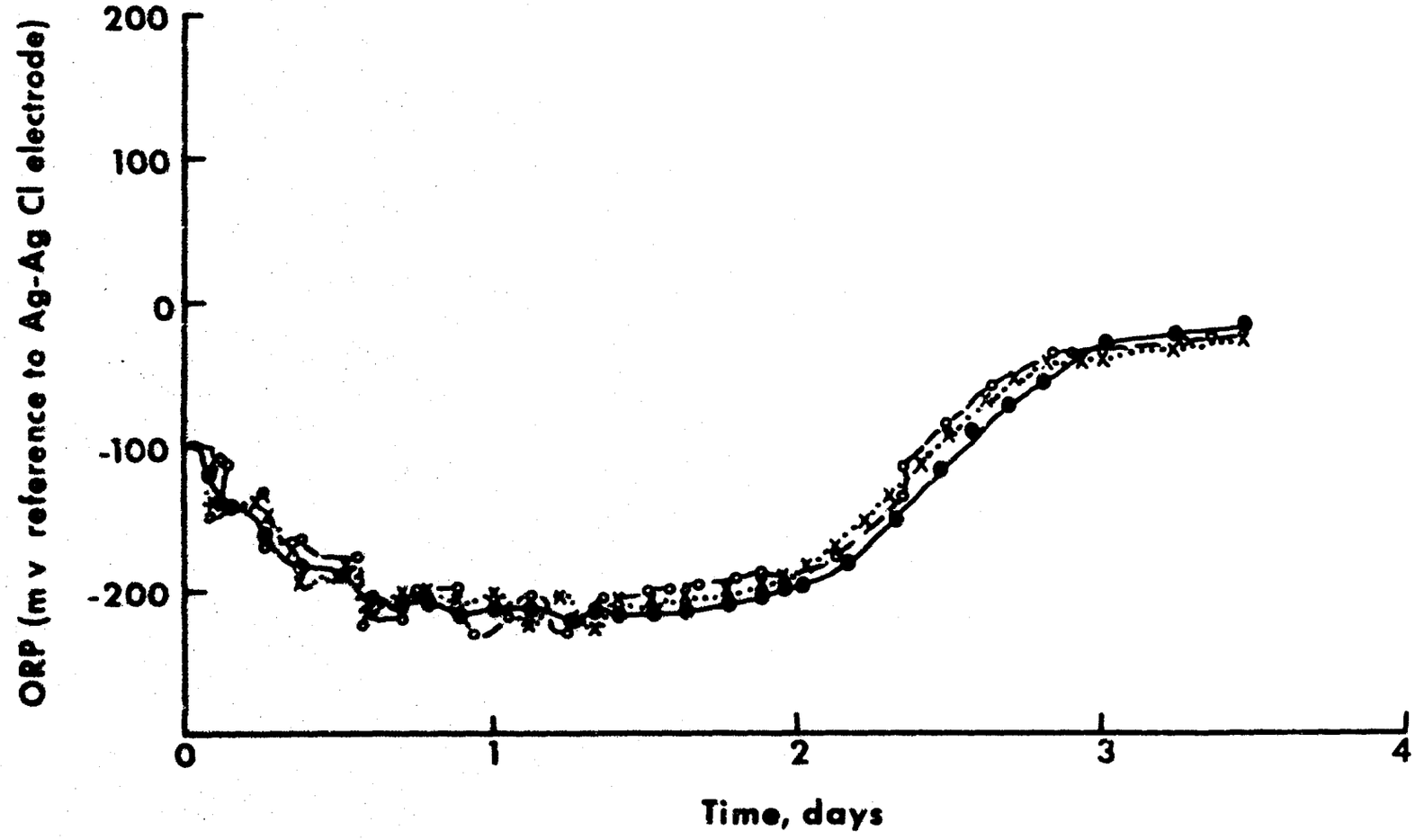


FIGURE 2. ORP Patterns Generated Without Aeration During Incubation of Medium in Three Centrifuge Spinner Bottles.

Further analysis of the data revealed several interesting facts. In Tables 2 and 3, all available data are compiled into four groups according to ORP level. Table 2 shows that groups 3 and 4 (with high initial ORP) had a significantly lower average growth rate and a longer generation time than groups 1 and 2. Group 4 also had a significantly lower average growth rate than group 3, as shown by a calculated $t = 6.85$.

The confidence limits in Table 3 show that group 4, incubated medium with high initial ORP, had an average peak growth higher than the other three groups and that the variance of the peak growth for group 4 was also significantly higher than the variance of the other three groups.

Figures 3 through 6 show the composite growth curves for groups 1, 2, 3, and 4. These data represent averages for the different groups listed in Tables 2 and 3. Interestingly, group 1 presents a marked lag phase (Fig. 3), but the fastest growth - 17.3 hours' generation time. An explanation for the differences in growth patterns of group 1 (characterized by short generation time, intermediate peak cell population, with decline at the 7th day) and of group 4 (much longer generation time, highest cell population, and decline on the 11th day) is not apparent at this time. Glucose levels at the end of the runs were still about 0.5% down from the initial 1.0%. Aeration was the same in all vessels, so we find it hard to believe that growth in the first three groups was restricted by lactic acid formation and accumulation, but not in group 4, which reached $5 \times 10^6$ cells/ml or double that in the first three groups. That the reason for the anomaly is ORP-associated seems probable at this time.

Tables 2 and 3 point out also that there are groups of incubated and nonincubated media with fast and slow growth rates, and incubated medium with no better peak cell growth than that shown by the nonincubated group. On the other hand, maximum peak growth was exhibited by a group in which the medium was incubated. Puzzling, also, is the fact that peak growth in this group was achieved only after 11 days.

TABLE 2. COMPARISON OF GROWTH RATES AND GENERATION TIMES OF L CELLS IN INCUBATED AND NON-INCUBATED MEDIA

| Group | Initial ORP Range, mv | Medium Treatment[a] | Number of Observations | Generation Time, hr | Avg. Growth Rate, $10^5$ viable cells/ml per day | 95% Conf. Limits |
|---|---|---|---|---|---|---|
| 1 | -100 to -60 | NI | 13 | 19.5 | 3.6 | 3.43 - 3.74 |
| 2 | -60 to 0 | I | 12 | 17.3 | 3.8 | 3.71 - 3.89 |
| 3 | -50 to +100 | NI | 9 | 25.8 | 1.95 | 1.84 - 2.06 |
| 4 | +140 to +170 | I | 9 | 38.3 | 1.5 | 1.41 - 1.62 |

a. I denotes incubated; NI, non-incubated.

TABLE 3. COMPARISON OF PEAK GROWTH OF L CELLS IN INCUBATED AND NON-INCUBATED MEDIA

| Group | Initial ORP Range, mv | Medium Treatment[a] | Number of Observations | Peak Growth | | |
|---|---|---|---|---|---|---|
| | | | | Days | $10^6$ Viable Cells/ml | 95% Conf. Limits |
| 1 | -100 to -60 | NI | 13 | 6 | 2.2 | 2.05 - 2.38 |
| 2 | -60 to 0 | I | 12 | 6 | 2.4 | 2.16 - 2.63 |
| 3 | -50 to +100 | NI | 9 | 9 | 2.4 | 2.13 - 2.61 |
| 4 | +140 to +170 | I | 9 | 11 | 3.5[b] | 2.68 - 4.32 |

a. I denotes incubated; NI, non-incubated.
b. Three of nine observations were $5.07 \times 10^6$, $4.7 \times 10^6$, and $4.9 \times 10^6$.

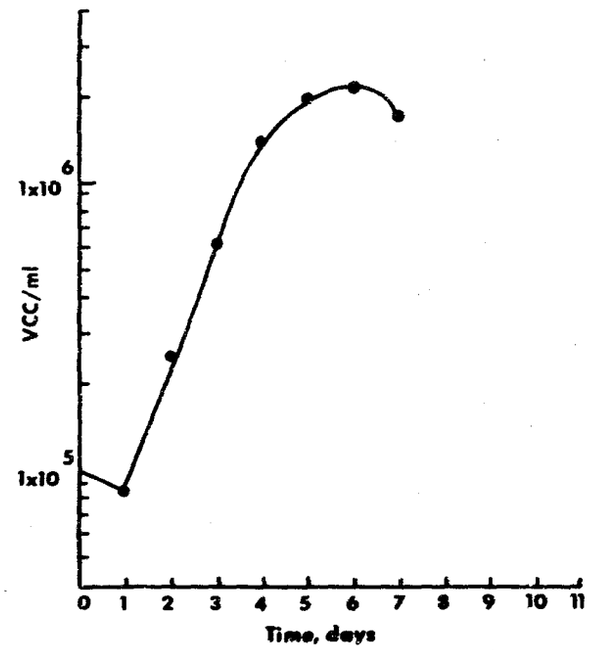


FIGURE 3. Average Growth Curve for Media Grouped with Initial Oxidation-Reduction Potentials in the Range -60 to -100 Millivolts (Ag-Ag Cl reference electrode).

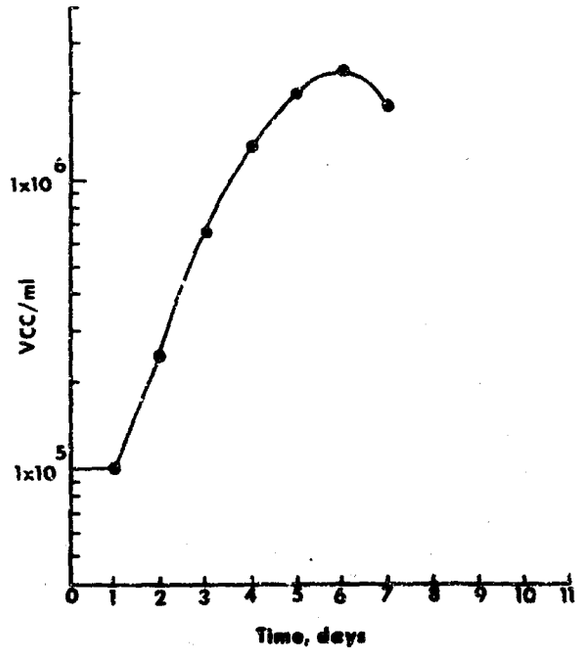


FIGURE 4. Average Growth Curve for Media Grouped with Initial Oxidation-Reduction Potentials in the Range -60 to 0 Millivolts (Ag-Ag Cl reference electrode).

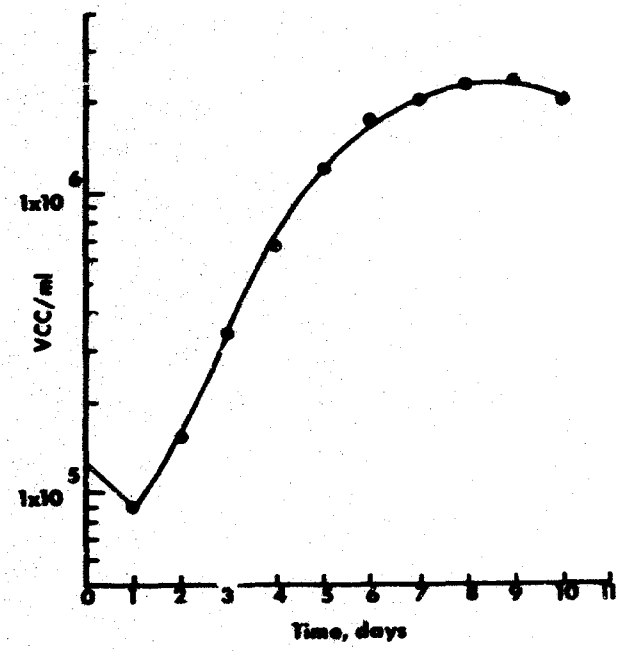


FIGURE 5. Average Growth Curve for Media Grouped with Initial Oxidation-Reduction Potentials in the Range -50 to +110 Millivolts (Ag-Ag Cl reference electrode).

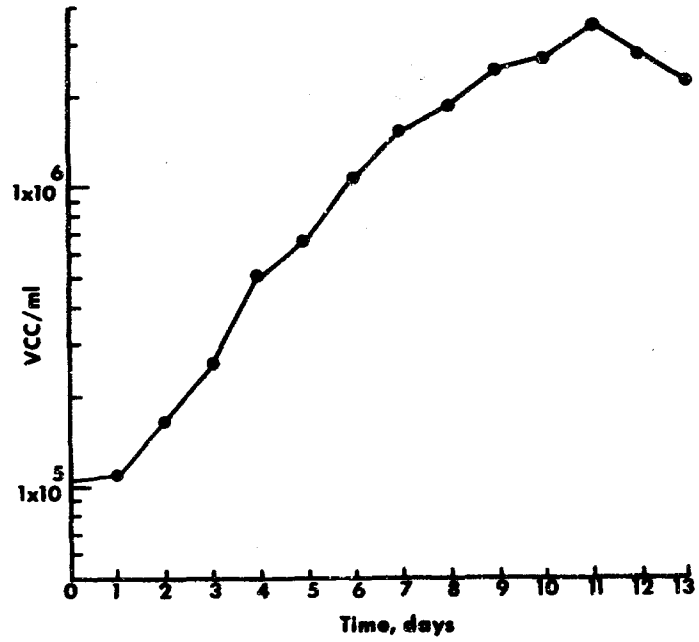


FIGURE 6. Average Growth Curve for Media Grouped with Initial Oxidation-Reduction Potentials in the Range +140 to +170 Millivolts (Ag-Ag Cl reference electrode).

## IV. CONCLUSIONS

Certain conclusions can be drawn from the data presented. First, the levels of growth achieved by the cells seem ORP-related. Second, the rates of growth are likewise associated with ORP. Third, the course of growth for the systems was in a great measure thus governed before inoculation ever occurred.

The implications for research using uncontrolled ORP levels appear obvious. Thus, one may question the value of studies made without due consideration of the effect of ORP on the system.

Whether or not the establishment of given levels of ORP when the medium is being processed does indeed alter the medium for greater potential over medium produced in the usual manner is highly pertinent.

Finally, contrary to the implication of Table 1 (that incubation is a desirable treatment for this medium prior to initiation of growth of cells) the change associated with the ORP levels of the medium during the preinoculation period probably accounts for the superior performance of the incubated medium with respect to total growth, and for inferior growth rates.

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13. ABSTRACT

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