

# The Influence of Progressive Growth on the Specific Catalase Activity of Human Diploid Cell Strains

## III. EXPERIMENTS BEARING ON THE MECHANISM RESPONSIBLE FOR CHANGE IN SPECIFIC ACTIVITY

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**ABSTRACT** It was shown previously that the specific catalase activity of human diploid cell strains falls immediately after subculture and then progressively rises in an exponential fashion. In this paper evidence is presented suggesting that the rise in catalase activity cannot be due to an accumulation within the cell of a small molecule which enhances enzyme activity in cell-free extracts. It is also shown that activity per cell, as well as per unit cell protein, rises as the culture grows.

The rate of fall of specific catalase activity immediately after subculture is greater if the cells are at a low population density than if they are at a high one. The rate of fall can be made more sharp by increasing the frequency with which the cultures are fed.

It is shown that used medium, which has previously been incubated with cultured cells of the same strain, does not significantly change either the rate of fall of specific catalase activity following subculture, or the rate of its subsequent rise. It is postulated, as one possibility, that the cells liberate into the medium an enhancer of cell catalase activity which is highly labile. The steady state concentration of this enhancer in the medium might be expected to increase as the culture grew, but to decrease when the cells are subcultured into fresh medium or when the frequency of feedings is increased.

In the two preceding papers (Pan and Krooth, '67; Pan, Krooth and Takahara, '68) we have shown that human diploid cell strains increase their specific catalase activity as they grow. The increase was observed in two cell strains carrying Mendelian mutations which affect the level of catalase activity, as well as in normal strains.

The kinetics of development of specific catalase activity during growth reveal four distinguishable phases (figs. 3, 4 in Pan and Krooth, '68).

1. There is a sharp fall in specific catalase within the first 24 hours following subculture.

2. Over the next several days, specific activity either does not change, or more usually, continues to fall though at a slower rate than initially.

3. Thereafter specific activity increases and the increase is exponential with time.

4. The rise in specific activity stops or slows at about the time that measureable net protein synthesis by the culture ceases.

The magnitude of the change in specific activity during growth is not great; the lowest level of catalase activity in the life cycle of the culture is rarely less than one-half the highest one. Nonetheless, the kinetics of change are highly repeatable.

In this paper we shall describe experiments bearing on the mechanism of this effect and particularly on the nature of the stimulus which causes the cell to develop augmented levels of activity.

### MATERIALS AND METHODS

The agar overlay was applied as follows: A single bottle of cells was subcultured in complete medium into several replicate plastic petri dishes (Falcon Plastics, Los Angeles) which were 15 cm in diameter; 72 hours later the original medium was removed and the monolayers were overlaid with freshly mixed medium containing agar. The final concentration of agar ("Bacto-Agar," Difco Co., Detroit) was 1.5 gm percent. At this time the monolayers

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in control dishes, which were to be free of agar, were overlaid with an identical medium except that distilled water in place of agar was added. The agar medium solidified within a few minutes. At the time of harvest, the agar overlay was loosened from the sides of the petri dish with the blade of a metal spatula. Then the plastic dish was deformed manually and inverted, so that the agar fell or slid off the monolayer of cells — usually in one piece. About half the cells were removed (inadvertently) with the agar. These cells were discarded. The cells which remained on the floor of the dish were harvested in the same way as the cells in the control dishes and flasks containing liquid medium (Pan and Krooth, '68).

"Used media" were obtained by incubating fresh media in replicate bottles, each of which contained a monolayer of growing diploid cells of the RU strain (CC). Following the period of incubation (specified for each experiment), the media were collected, pooled, and frozen. (Media were stored for no longer than two weeks and at  $-10^{\circ}\text{C}$ ). The media were then thawed and passed through a Millipore GS filter (Millipore Corp., Bedford, Mass.). The mean pore diameter of this grade of filters is  $0.2\ \mu$ , and is sufficient to exclude cultured cells.

Cells employed to generate used media are referred to as "donor cells;" those receiving the used media are described as "recipient cells."

Cells were grown as monolayers beneath medium in stoppered glass bottles with a surface area of  $187\ \text{cm}^2$ . The atmosphere, above the medium, contained 5% carbon dioxide and 95% air. The cells were removed from the glass, during subculture, by incubation in a dilute solution of trypsin. They were then sedimented by centrifugation, resuspended in fresh growth medium, and a fraction of the suspension was inoculated into new bottles.

## RESULTS

A. Does the augmented level of catalase activity observed in cultures which have grown to a high population density reflect increased enzyme within the cell?

We have previously shown (Pan and Krooth, 67) that when an extract of normal cells harvested at high population density is dialyzed in darkness for 72 hours, specific catalase activity falls. However, the rate of fall is very slow, and is probably not due to simple dilution of an unbound, freely-diffusible molecule. In addition, if undialyzed extracts from cells harvested, respectively, at high and low population densities are mixed, the catalase activity of the mixture is equal (approximately) to the arithmetic sum of the activities of the two extracts in the mixture (table 1).

We have also examined the possibility that the rise in specific activity which occurs as the culture grows is due not to increased enzyme within the cell, but to

TABLE 1  
*Effect of mixing cell extracts from cultures at high and low population density on the specific catalase activity of cells of the RU strain (CC)*

Cell population density at time of harvest <sup>1</sup>	Specific activity <sup>2</sup>	Protein concentration <sup>3</sup>	Predicted specific activity <sup>4</sup>
0.03042 (Low)	462	0.913	—
0.5250 (High)	949	1.312	—
1:1 Mixture of Sonic Extracts from High and Low Cultures	775	1.113	749

<sup>1</sup> Milligrams cell protein per  $18.7\ \text{cm}^2$  of surface area available for growth.

<sup>2</sup> Micromoles hydrogen peroxide decomposed per milligram of cell protein per hour of incubation.

<sup>3</sup> Milligrams per milliliter of extract.

<sup>4</sup> Specific activity predicted on the assumption that the activity of the mixture is the arithmetic sum of the activities of the two extracts mixed.

contraction of the pool containing most of the *other* cell proteins. In other words, if the total amount of protein per cell were diminishing as the culture grew, but the amount of catalase per cell were constant, one might see a "spurious" rise in specific activity. To test this idea, replicate cultures were inoculated at low population density, and harvested during growth at, respectively, low and high densities. The catalase activity per milligram cell protein *and per cell* was measured at the time of each of the two harvests. The results are shown in table 2. As can be seen, activity per cell as well as activity per milligram cell protein rose with cell population density. The total protein content of the cells also rose somewhat.

While these experiments are consistent with the view that the elevated levels of activity reflect increased enzyme within the cell, they do not rigorously prove this possibility to be the correct one. Even, however, if it is given that there is increased enzyme within the cell, we cannot at present distinguish between three general mechanisms which might account for the increase: 1. Accelerated *de novo* enzyme synthesis, 2. Decelerated enzyme catabolism or inactivation, or even, 3. Diminished excretion of enzyme by the cell.

What does seem clear, however, is that the amount of enzyme activity per cell increases as the culture grows, and the simplest explanation, at present, to account for the increase is that at high population density there is more enzyme inside the cell.

B. Nature of the stimulus which causes the culture to develop increasing levels of catalase activity as it grows.

1. *Introduction.* We have inquired experimentally into two possible mechanisms which might cause specific catalase activity to rise as the culture grows:

1. The medium might contain a diffusible compound which somehow reduces the cell catalase activity of the culture and which is also consumed or inactivated by the cells. Thus as the number of cells increases, the medium would be progressively depleted of the inhibiting molecule and specific catalase activity might rise.

2. The cells of the culture might liberate into the medium a diffusible compound which somehow augments cell catalase activity so that as the culture grows, the concentration of this compound rises, and specific catalase activity increases.

There are of course other possible mechanisms which could account for the increase in specific catalase activity with growth. For example, the cells of the culture, for an unknown reason, might develop higher levels of catalase activity in response to increasing cell-to-cell contact or proximity. Alternatively, the rise in catalase activity might reflect an autonomous response of each cell. For example, if the rise in specific activity reflected merely the "recovery" of the cell from some operation performed in the subculturing process, the "recovery" might proceed autonomously within each cell and be independent of the number of other cells in the vicinity or in the same container. The exponential character of the increase in specific activity (once it begins) tends slightly to favor a mechanism which allows the activity of an individual cell to be responsive to the number of other cells in the flask.

TABLE 2  
Effect of progressive growth on the catalase activity per cell: RU strain (CC)

Days after subculture	Cell population density <sup>1</sup>	Specific catalase activity <sup>2</sup>	Number of cells per milligram cell protein	Catalase activity per cell <sup>2</sup>
1	0.022 (0.347 × 10 <sup>5</sup> )	457	1.6 × 10 <sup>6</sup>	0.284 × 10 <sup>-3</sup>
12	0.472 (5.32 × 10 <sup>5</sup> )	763	1.1 × 10 <sup>6</sup>	0.675 × 10 <sup>-3</sup>

<sup>1</sup> Cell population density is expressed as milligrams of cell protein or, in parenthesis, number of cells, per 18.7 cm<sup>2</sup> available for growth.

<sup>2</sup> Catalase activity is expressed as micromoles of hydrogen peroxide decomposed per hour. Specific activity is activity per milligram cell protein.

2. *Effect of daily subculture on specific catalase activity in cultures maintained at two different population densities.* In these experiments parallel cultures were inoculated at two different initial population densities. They were then subcultured every 24 hours. The details of the experimental design are as follows:

A single bottle of cells was subcultured into a number of replicate bottles. When a confluent monolayer formed in the replicate bottles, the cells were harvested and pooled. A measured fraction of the cells was removed for the determination of cell protein and specific catalase activity. The remaining cells were then subcultured into two sets of bottles. One set was inoculated at very high population density; the other set was inoculated at a low population density. All bottles received the same lot of medium. Both sets of bottles were subcultured every day for four days. Each new recipient bottle received all the cells from the previous bottle. At the time of each

subculture one or more bottles was removed for the estimation of cell population density and specific catalase activity.

The results are shown in figure 1. Note that there is little change in cell population density, in both low and high inoculum cultures, during the course of the experiment. Hence it is unlikely that daily trypticinization caused the loss of a large fraction of the cells. Figure 1 reveals that during the experiment the specific catalase activity in both sets of bottles fell. However, the rate of fall was much steeper in the case of the cells maintained at low population density. Similar results have been obtained in other experiments. Thus the decline in catalase activity immediately following subculture is influenced by the population density of the cells.

3. *Effect of frequency of feeding on the specific catalase activity of replicate cultures.* To test the idea that the cells might be liberating or consuming a compound in the medium which somehow influences catalase activity, the medium was replaced in replicate flasks according to several different schedules. As can be seen from figure 2, the rate of fall of specific catalase activity increased with the frequency of

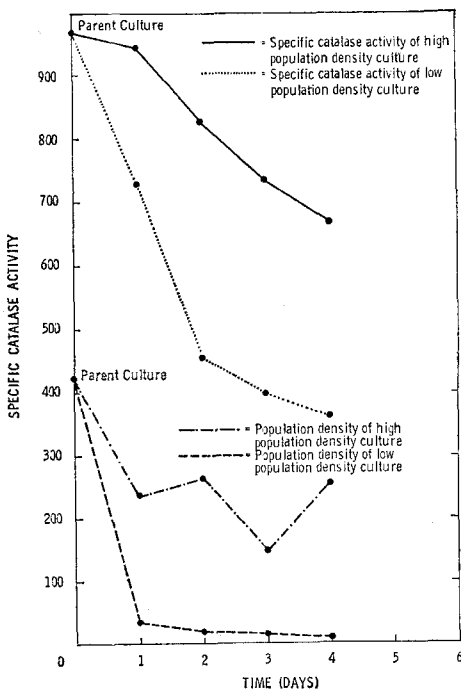


Fig. 1 Effect of daily subculture on the specific catalase activity of cells maintained at high and low population density, RU strain (CC). The upper pair of curves describes specific catalase activity (left ordinate). The lower pair of curves describes cell population density (right ordinate).

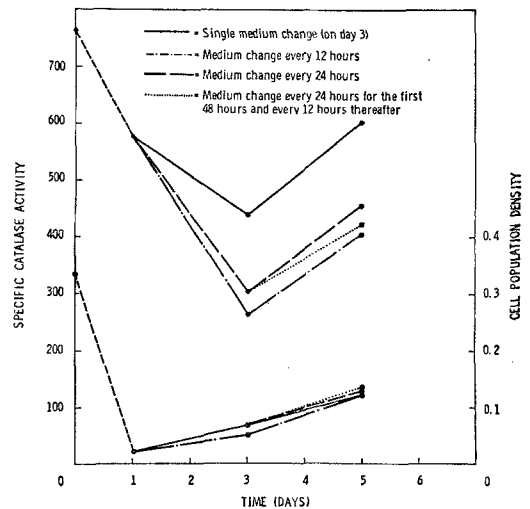


Fig. 2 Effect of repeated medium change on cellular specific catalase activity. RU strain (CC). Replicate bottles were fed with identical media but according to different regimens. The upper family of curves described specific catalase activity (left ordinate). The lower family of curves describes cell population density (right ordinate).

medium changes. Moreover once specific catalase activity began to rise, the rate of increase was slightly less in the cultures fed more frequently. Note that in these experiments the frequency of feeding had no significant effect on the rate of net protein synthesis. Results like these have now been observed in three independent experiments.

4. *Effect of used medium on the specific catalase activity of replicate cultures.* The effect of used medium on replicate cultures is shown in figure 3. The specific catalase activity of the parent culture was driven down by daily subcultures, for three days. On the third day, the bottle was subcultured into a number of replicate flasks. One set of flasks received fresh medium. The other set received medium which had been incubated for 48 hours in the presence of cells (of the same strain) which were growing at a high population density and had a high specific catalase activity.

As can be seen from figure 3, the recipient cultures which received used medium did not develop higher levels of specific catalase activity than the control cultures which received fresh medium. For the first 48 hours the two sets of cultures had about the same specific activity, and by 72 hours the cultures which received used medium had a specific activity significantly lower than the control cultures. By 72 hours also, it was clear that the cultures fed with used medium were not growing as rapidly as the control cultures. In experiments in which 1:1 and still higher dilutions of used medium in fresh medium were employed, this difference in growth rate was not observed. However, in these experiments also, the

cultures which received used medium did not have higher levels of specific activity than the control cultures.

The legend to figure 3 describes the specific catalase activity of the donor cultures — the replicate flasks that contributed the used medium. The donor cultures were at high population density at the time they were placed in the medium which they were to donate for the experiment. During the 48 hours they were incubated with this

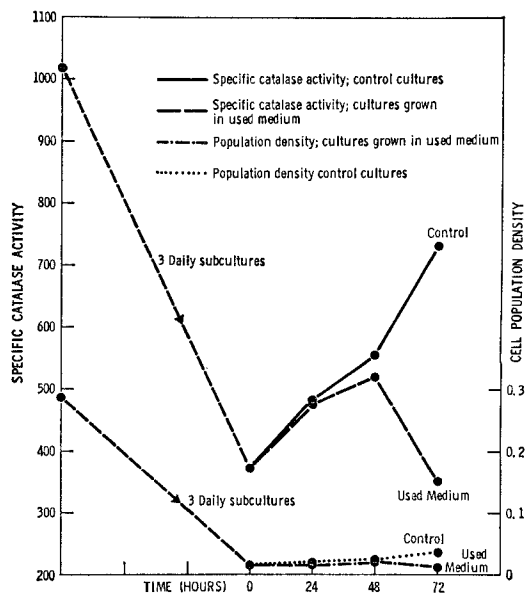


Fig. 3 The effect of growth in used medium on cellular specific catalase activity. RU strain (CC). The used medium was obtained by incubating "donor cultures" of the same strain (RU) with fresh medium. The details of the procedure by which the used medium was obtained are as follows:

Day	Operation	Specific catalase activity of donor cultures <sup>1</sup>	Cell population density <sup>2</sup> of donor cultures
0	Donor cultures made by subculturing a pool of cells from replicate flasks into new replicate flasks	—	—
3	Donor cultures receive fresh medium	920	0.300
5	Used medium harvested from donor cultures	974	0.396

<sup>1</sup> Micromoles of hydrogen peroxide decomposed per hour per milligram cell protein.

<sup>2</sup> Milligrams cell protein per 18.7 cm<sup>2</sup> of surface area available for growth.

medium, they increased their cell protein by about 33%, and their specific catalase activity, which was very high at the beginning of the incubation, by only about 5%. It can be argued that although the medium employed in figure 3 had been incubated with cells having a high specific catalase activity, the donor cells did not notably increase their specific activity while they were incubated with the medium. In other words, although the donor cells may have liberated into their medium a molecule which enhances cell catalase activity, the synthesis or liberation of this molecule need not be an unremitting process. Once either enzyme activity or cell population density reached a certain level, the release of this compound, for some reason, might cease. On this formulation, the used medium employed in the experiment described in figure 3 would have been obtained from a culture that no longer liberated the theoretical compound for which we are assaying. To rule this possibility out, experiments were performed in which the donor cultures markedly increased their specific catalase activity while they grew in the experimental medium. Here too there was no evidence that the used medium caused an augmentation of the specific catalase activity of the recipient culture.

Experiments measuring the effect of used medium on the rate of fall of specific catalase activity during the first 24 to 72 hours after subculture have been performed. The rate of fall, like the subsequent rise (fig. 3), is not influenced by used medium. In some of these experiments, medium was incubated in cell-free flasks during the period that the used medium was incubated in flasks containing the donor cells. The recipient cultures receiving fresh, incubated, and used medium all had about the same specific catalase activity. So did recipient cultures receiving a 1:1 dilution of used medium in fresh medium.

5. *Specific catalase activity of cells grown under an agar overlay.* In order to test further the possibility that the cells liberate during growth a labile and diffusible compound which augments specific catalase activity, cultures were grown under an agar overlay. If the theoretical compound were a macromolecule, then the agar

might retard its diffusion away from the cell, and thereby cause an increase in cell catalase activity. Table 3 describes one such experiment. As can be seen, the cells grown under agar had a slightly lower specific catalase activity than the control cells grown under a liquid medium. In a few such experiments, the cells under agar had a higher specific activity than the control cells, but in these experiments also the difference was very small.

#### DISCUSSION

We have shown that the rise in specific catalase activity during growth is not due to the accumulation within the cell of a small molecule which will augment catalase activity *in vitro* (i.e., in cell-free extracts). We have also shown that the rise can be detected as an increased amount of catalytic activity per cell as well as per unit cell protein.

The data presented on mechanism indicate that the sharpness of the initial fall in specific catalase activity following subculture is dependent on the population density of the cells; it is more pronounced when the cells are at a low population density than when they are at a high one. It is likely that at least one of the operations of subculture which causes specific catalase activity to fall is the introduction of the cells into fresh medium, for the

TABLE 3  
*Effect of growth under an agar overlay on the specific catalase activity of cells of the RU strain (CC)*

Medium	Cumulative duration of growth <sup>1</sup> (Days)	Milligrams cell protein per dish	Specific catalase activity <sup>2</sup>
Complete	3	0.77	798
Agar Medium	8	0.90	827
Control Medium	8	2.43	903

<sup>1</sup> The cells were left in complete medium for the first three days following subculture, at which point two replicate dishes were removed for determination of specific catalase activity and cell protein. The remaining dishes received either the agar overlay or a liquid medium identical with the agar overlay except that it contained distilled water instead of agar. These dishes were then incubated for five further days, and were harvested on the eighth day following subculture.

<sup>2</sup> Micromoles hydrogen peroxide decomposed per hour per milligram cell protein.

specific catalase activity of the cells can be reduced by frequent feedings.

The kinetics of change in cell catalase activity are probably not due to the fact that fresh medium contains an inhibitor or repressor of enzyme activity; for "used medium" which has been previously incubated with cells neither diminishes the initial fall in specific activity after subculture nor does it accelerate the subsequent rise. This latter result also means that either the cells are not liberating an enhancer or "inducer" of catalase activity into the medium, or, if they are, the active molecule is highly labile.

We slightly favor the idea that the cell is liberating into the medium a highly labile "inducer" or an enhancer of catalase activity. For one thing, the effects of cell population density and frequency of feeding are predicted by such a model. Second, on this theory we might expect the exponential kinetics of development of catalase activity which we have observed, since the cells themselves are increasing exponentially. Third, the changes in the specific activities of mammalian cells during growth seem likely in several other cases to result from an alteration in the composition of the medium — an alteration which is effected by the metabolic activity of the cells themselves. Cox and MacLeod ('62) have shown that the presence of cystine or cysteine in the medium tends to reduce the specific alkaline phosphatase activity in cultures of human diploid cells. Their evidence strongly suggests that as the culture grows, the medium is progressively depleted of cystine (or cysteine) and specific alkaline phosphatase activity rises as a consequence. DeMars ('64) has shown that the specific beta-glucuronidase activity of cultured human diploid cells rises progressively during growth. He reports that cultures receiving used medium had a five to six-fold increase in specific activity compared to control cultures growing in fresh medium. Littlefield ('65) has noted that in cultured mouse heteroploid cells ("L"-cells), the change in specific thymidine kinase activity during growth can be abolished if thymidine is present.

Recently DeLuca ('66) has studied the kinetics of development of specific cata-

lase activity in a heteroploid hamster line (RPMI-2402) and a heteroploid mouse line (L-cells; clone 929). Both lines were grown in suspension culture (whereas in our experiments and the ones quoted above the cells were grown in monolayer). The heteroploid hamster cells increased their specific catalase activity during the first 24 hours after subculture and then specific activity progressively fell. On the other hand the heteroploid mouse cells behaved more nearly like the human diploid cell strains we have worked with. It may be significant that the mouse cells, like the human diploid cell strains, are "fibroblastic" in morphology, while the heteroploid hamster cells DeLuca studied appear to have been mainly epitheloid.

DeLuca found that if the cultures of hamster cells were aereated, they developed progressively higher levels of catalase activity as they grew. In other words they then showed a kinetics of development of specific catalase activity similar to the one that both our cells and the heteroploid mouse cells display spontaneously. Aeration of the heteroploid mouse cell culture had no effect on the kinetics of development of catalase activity.

We plan in further experiments with cultured human diploid cells to develop a perfusion system in which monolayer cultures at high and low population density can be bathed in the same medium. If a labile "inducer" or enhancer of specific catalase activity is being liberated by the cells into the medium, then both the high and low population density cultures should tend to have the same level of activity. If, on the other hand, cell-to-cell contact or proximity (or some other factor) is required for cell catalase activity to rise, the cultures will behave independently.

On the basis of the literature we have summarized elsewhere, (Pan and Krooth, '67) we think it is fair to conclude that:

1. The specific activity of cultured mammalian cells with respect to a number of enzymes changes as the culture grows.
2. The kinetics of change of specific activity for a single enzyme is frequently different in different cell lines, and, within a single line, can be quite different for different enzymes.

In most instances, the mechanism responsible for the change in specific enzyme activity with growth is not known, but it seems reasonable at present to suspect that the underlying mechanism, as well as the nature of the change, depends upon the particular cell line and enzyme system studied (DeLuca and Nitowsky, '64). In this connection we should like to emphasize that both alkaline phosphatase and betaglucuronidase show a kinetics of development of specific activity in human diploid cell strains very similar to that of catalase. However, used medium previously incubated with live cells will cause a rise in specific activity for both these enzymes (Cox and MacLeod, '62, '65; DeMars, '64), whereas it does not for catalase.

These phenomena are therefore more interesting than they would be if the changes were stereotyped and reflected some quite general property of growing mammalian cells. Whenever cells in a specific way alter their catalytic activity in response to the presence of the other cells around them, the process has at least a superficial resemblance to cellular differentiation, and partly for that reason is of interest. Moreover, if the cells affect temporal changes in their catalytic activity by altering, through their own metabolism, the composition of the medium in which they live, the same process may occur *in vivo*. In the mammalian body, large numbers of cells live at population densities several logarithms higher than any obtainable in

cell culture, and in compartments of the body fluid which in many cases do not quickly equilibrate with the systemically circulating blood.

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