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

I. EFFECT OF CELLULAR GENOTYPE: HOMOZYGOUS STRAINS

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ABSTRACT The specific catalase activity of human diploid cell strains increases with progressive growth of the culture, and falls again following subculture. Although the increase is small, it is readily demonstrable, and is exponential with time.

The response of catalase activity to progressive growth of the culture was studied in three abnormal human cell lines. A diploid cell strain, developed from a patient homozygous for the gene causing acatalasia I, had no detectable catalase activity throughout the life cycle of the culture. Another diploid cell strain, developed from a patient homozygous for the gene causing acatalasia II, had about 5% normal catalase activity, but the *proportionate* increase in specific activity as the culture grew was the same as for normal cells. Thus the mutation causing acatalasia II does not change the responsiveness of the cell in terms of catalase activity to progressive growth of the culture. The behavior of a heteroploid line was similar to that of the normal diploid strains, but when the growth of the heteroploid cultures reached a plateau, their population densities were four times higher than those of the diploid strains and they had about twice the specific catalase activity.

In 1960 Weisman, Smellie, and Paul ('60) reported that the specific thymidine kinase activity of L-cells, a heteroploid mouse line, changed as the culture grew. Since then a number of other cases have been reported (table 1) where specific activity — total enzyme activity in the culture divided by total cell protein — was found to change during growth.

We have examined the enzyme catalase from this standpoint in human diploid cell strains. The purposes of the present paper are:

1. To describe the change in specific catalase activity as cultures of diploid cells grow.
2. To contrast the change in catalase activity with growth in normal cells and in cells carrying mutant Mendelian genes which affect catalase activity.
3. To contrast the change in catalase activity in human diploid cell strains with a human heteroploid line during growth.

The mutant Mendelian genes studied are the ones causing acatalasia I and acatalasia II. Acatalasia I is a recessive abnormality observed in a small number of Japanese and Korean families (Takahara, '52; Wyngaarden and Howell, '66). Diploid cell strains from affected persons have a deficiency of catalase activity (Krooth, Howell and Hamilton, '62; Kitamura, Ogata and Takahara, '62; Takahara, Sadamoto and Ogata, '66). Acatalasia II has been observed in several Swiss families; the erythrocytes and diploid cell strains of homozygous persons are reported to have about 15% normal catalase activity. (Aebi, Baggiolini, Dewald, Lauber, Suter, Micheli, Frei, '64).

MATERIALS

A. Medium. All cultures were grown in "nucleomedium" which contains 12% whole human sera. Most of the supplementary small molecules are present at the concentrations specified by Eagle ('59). The exact composition is given elsewhere (Krooth, '64).

B. Biochemicals. Hydrogen peroxide was obtained from Fisher Scientific Company (Fair Lawn, N. J.) and sodium azide from K. and K. Laboratories, Inc. (Plainview, N. Y.). Purified beef liver catalase was obtained from Calbiochem (Los Angeles), and had a specific activity approximately ten thousand times that of crude (normal) human cultured cell extracts.

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TABLE 1

Specific enzyme activities of cultured mammalian cells which change as the culture grows

Responsive activity	Cell type in which response was reported	References
Malic acid dehydrogenase	Human homoploid and heteroploid; rat heteroploid	Miedema and Kruse, '65
Thymidine kinase	Mouse heteroploid	Weissman et al., '60
Alkaline phosphatase	Human homoploid and heteroploid	Cox and Pontecorvo, '61; Nitowsky and Herz, '61
Glucose-6-phosphate dehydrogenase	Human homoploid and heteroploid; mouse heteroploid; hamster heteroploid(?); rat heteroploid	DeMars, '64a,b; DeLuca, and Nitowsky, '64; DeLuca, '66a; Miedema and Kruse, '65
β-glucuronidase	Human homoploid	DeMars, '64a; DeMars and Krooth, '64b
Lactic acid dehydrogenase	Human heteroploid and homoploid; mouse heteroploid; hamster heteroploid(?); rat heteroploid	DeLuca and Nitowsky, '64; Miedema and Kruse, '65; DeLuca, '66b
Uridine kinase	Mouse heteroploid	Kit et al., '64
Orotidine-5′-monophosphate decarboxylase	Human homoploid	Krooth, '64
Catalase	Human homoploid and heteroploid; hamster heteroploid	Krooth, '65; DeLuca, '66a,b; Krooth and Pan, '66; Krooth, '66
Glutamine oxalacetic transaminase	Mouse heteroploid	DeLuca, '66b
Galactose-1-phosphate uridyl transferase	Human homoploid	Russel and DeMars, '66, '67
UDP-galactose-4-epimerase	Human homoploid	Krone and Brunschede, '66
Thymidine-5′-monophosphate phosphatase	Human heteroploid	Eker, '65
Thymidine-5′-diphosphate phosphatase	Human homoploid	Eker, '65

C. Cell strains. The RU and the OFR cell strains, which have normal levels of catalase activity, have been previously described (Krooth, '64). The BU strain was developed from a biopsy on an adult Swiss male who is homozygous for acatalasia II. The biopsy was kindly sent to us by Professor Hugo Aebi of the University of Zurich. The CA strain was developed from a biopsy on an adult Japanese male (who is homozygous for acatalasia I), and was kindly sent to us by Dr. Howard B. Hamilton of the United States Atomic Bomb Casualty Commission, Hiroshima. The donor of the CA strain is not the same individual as the donor of the ACA strain, on which we have previously reported (Krooth et al., '62). In experiments on a human heteroploid line, the S-3 (Tjio and Puck, ('58) subline of HeLa was employed.

In the genetic notation, "C" will denote the normal allele for acatalasia. "c_I^*" will denote the allele for acatalasia I, and "c_{II}^*" for acatalasia II. This notation has obvious disadvantages, the most serious being that it implies that the genes for acatalasia I and II are different from one-another and are at the same locus. Neither of these implications is as yet known to be true. Unfortunately the customary notation for Mendelian genes, which we have used, assumes that information of this kind is available.

METHODS

A. Cell culture methods. The general methods employed in culturing and harves-

ting the cells have been described elsewhere (Krooth et al., '62; Krooth and Weinberg, '61). Cells were grown as monolayers in glass flasks having 187 cm^2 of surface area available for growth ("Blake bottles"). Cultures were fed every 48 to 72 hours with 100 ml of fresh medium. Replicate bottle experiments were performed by inoculating a large number of flasks with the same size inoculum from a single parent culture. At the time of inoculation an aliquot of cells from the parent culture was harvested for determination of specific catalase activity and total cell protein. Thereafter replicate flasks were removed from the experiment at 24 hour intervals for determination of cell protein and catalase activity. Both determinations were performed on crude sonic extracts of the cells, as previously described (Krooth et al., '62).

The unit of cell population density employed in this paper is defined as 1.00 mg of cell protein per 18.7 cm^2 of surface area available for growth. Cell protein was measured by the method (Lowry, Rosebrough, Farr and Randall, '51) used previously (Krooth et al., '62).

B. Assay for catalase activity. As in our earlier experiments, (Krooth et al., '62) catalase activity was measured spectrophotometrically (Beers and Sizer, '52; Lieberman and Ove, '58). The cell extract, buffer, and hydrogen peroxide are mixed at 25°C, and the kinetics of fall of optical density at 240 mμ is recorded. Specific catalase activity is expressed as micromoles hydrogen peroxide decomposed per hour of incubation per milligram protein.

C. Conditions of Dialysis. Dialysis was carried out at 4°C. A solution of cell extract, or purified enzyme, was poured into a bag made of cellulose casing (The Visking Co., Chicago). The bag was placed in a large bottle containing 0.01 molar (or in some experiments 0.02 molar) potassium phosphate buffer, pH 7.0. Turbulence was produced in the bath by a teflon coated magnetic stirrer. The ratio of volume of enzyme solution to volume of bath was 1:400. The bath contained 0.02 M potassium phosphate buffer (pH 7.0) and 0.86% sodium chloride. In some experiments 0.86% sodium chloride or 75 μg per milliliter of bovine serum albumin (Nutritional Biochemicals Corp., Cleveland) were added to the bath.

The catalase activity of human diploid cell strains, as measured by this assay, falls during prolonged dialysis of the cell extract, and the rate of fall is accelerated if the dialysis is performed in fluorescent light. The same phenomena are encountered with purified beef liver catalase. When the dialysis is carried out in light-shielded containers, about one-half the catalytic activity is lost over a period of 72 hours, in the case of both preparations. If dialyzed and undialyzed extracts of normal human cells are mixed, the catalase activity of the mixture is equal to the arithmetic mean of the activities of the unmixed extracts.

Photoinactivation of mammalian catalase has been described by Mitchell and Anderson ('65). We have confirmed their finding that purified beef liver catalase loses activity, even in the absence of dialysis, when exposed to visible light. We have not, however, detected photoinactivation, in the absence of dialysis, with crude extracts of diploid cell strains (perhaps because of the opacity of the extracts). Mitchell and Anderson ('65) postulated that visible light inactivates the enzyme by its effect on the heme groups, and Aronoff ('65) has suggested that photooxidation of a heme group is responsible for the loss of activity, since the inactivation requires an aerobic atmosphere. If these ideas are correct, it may be that the newly oxidized heme group does not in itself deprive the molecule of catalytic activity, but simply has less affinity for the apoenzyme and is lost. Hence one might expect enzyme activity to decay more rapidly during dialysis in the presence than in the absence of visible light. In any case, to minimize the loss of authentic enzyme activity, the duration of dialysis has been confined to 12 hours and both enzyme and dialyzing bath have been shielded from light. Less than 20% of the activity of both normal cell extract and purified beef liver catalase are lost under these conditions.

RESULTS

A. Effect of progressive growth on specific catalase activity. Figure 1 shows the change in specific catalase activity during

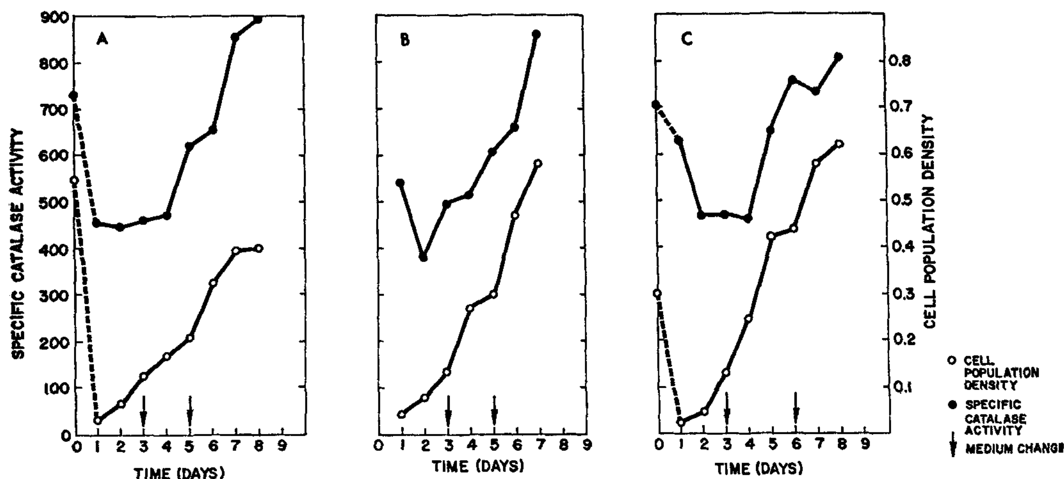

Fig. 1 Effect of progressive growth on the specific catalase activity of two homozygous (CC) diploid cell strains (RU and OFR) which have normal catalase levels. A and B are experiments 1 and 2 on the RU strain. C is an experiment on the OFR strain. The values of specific catalase activity and cell population density in the parent culture are plotted above day zero, except for one experiment (RU experiment 2) where they were not determined. On day zero the parent culture was subcultured into a large number of replicate bottles. On day 1, and each day thereafter, a fraction of these bottles was harvested, and cell population density and specific catalase activity were measured.

the growth of two normal human diploid cell strains. Note that specific activity falls following subculture and then gradually rises. The magnitude of the rise is not great, for there is only about a two-fold difference between the lowest and highest values. However, the increase in activity is exponential with time (as will be seen) and is repeatedly observed. Figure 2 contrasts, on the same scale, the rise in specific catalase activity and cell protein, in a normal human diploid cell strain and in a human heteroploid line — the S-3 subline of HeLa. At the time the growth of the diploid cells had reached a plateau, the HeLa cultures had a cell population density 4 to 5 times that of the diploid cells. At this point in growth, the HeLa cells had about twice the specific catalase activity of the diploid cells.

Figure 3 shows the kinetics of development of specific catalase activity in a diploid cell strain developed from a patient with acatalasia I. By the spectrophotometric assay used in these experiments there was no detectable activity throughout the growth cycle. Figure 4 presents a similar plot for the acatalasia II cell strain. As can be seen this cell strain does have appreciable enzyme activity. The acatalasia II cells, like the normal cells, developed progressively higher specific catalase activity as the culture grew, but throughout growth they had much lower specific activities than the normal cells.

Figures 5 and 6 summarize data from experiments on two cell strains that had normal catalase activity, and from two experiments on the acatalasia II cell strain. In these experiments all of the cells were growing at about the same rate (with a doubling time for cell protein around 33 hours) and began growth from approximately the same initial population density (0.012 to 0.040 milligrams cell protein per 18.7 cm^2 available for growth) as shown in figure 5. Figure 6 plots specific catalase activity, on a logarithmic scale, versus time on an arithmetic one. Note that with a semilogarithmic plot, the increase in specific catalase activity is linear for cells of both genotypes. Figure 6 also shows that the specific activity of the acatalasia II cells failed to increase after about day 10. However, by this time the cells were in the plateau phase of growth (fig. 5). Normal cell strains which have been followed this long also sometimes show a leveling off

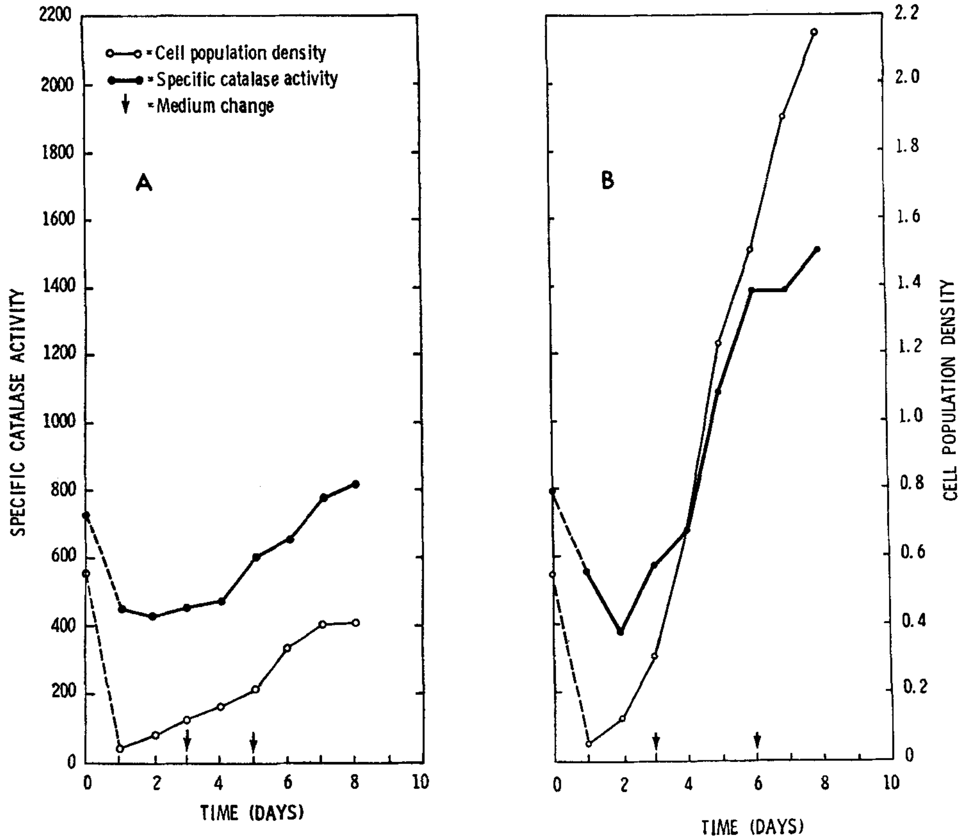

Fig. 2 Comparison of effect of progressive growth on the specific catalase activity of both a normal homozygous (CC) diploid cell strain (RU), shown in A, and a heteroploid line — the S-3 subline of HeLa, shown in B. Both graphs are drawn to the same scale. See legend of figure 2 for details.

of specific activity when net protein synthesis ceases or slows. Figure 7 shows that, during the period when specific activity is increasing, the lines corresponding to cells of each of the two genotypes have about the same slope. In other words. the *proportionate* increase in specific catalase activity with growth is about the same for acatalasia II cells as for normal cells. Although homozygosity for the acatalasia II gene reduces specific catalase activity by about 25-fold, it does not seem to alter the responsiveness of the cells, in terms of catalase activity to the effect of progressive growth.

B. The residual activity of the acatalasia II cells. Table 2 shows that the catalase activity of the acatalasia II strain is sodium azide sensitive (Cohen and Hochstein,

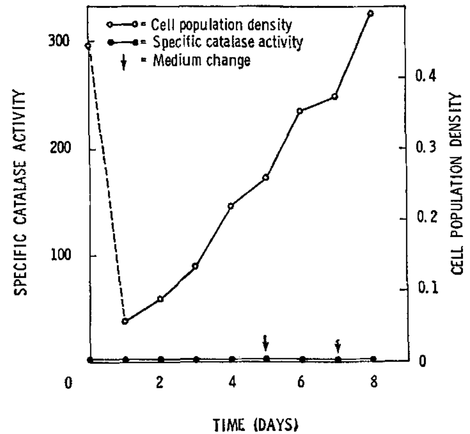

Fig. 3 Effect of progressive growth on the specific catalase activity of a diploid cell strain (CA) homozygous ($c_I{}^*c_I{}^*$) for acatalasia I. See legend to figure 2 for details.

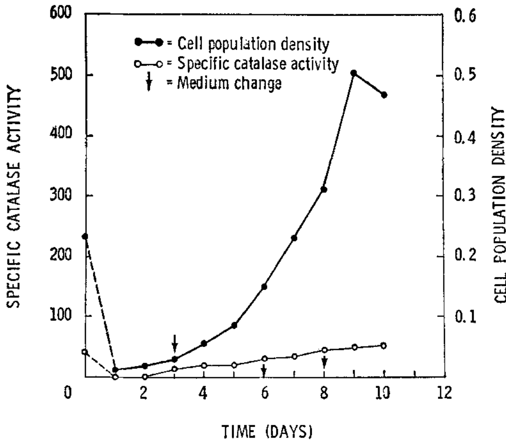

Fig. 4 Effect of progressive growth on a diploid cell strain (BU) homozygous ($c_{II}{*}c_{II}{*}$) for acatalasia II. See legend to figure 2 for details.

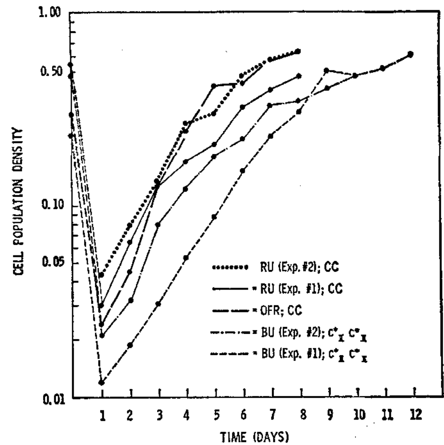

Fig. 5 Growth of diploid cell strains during the experiments summarized in figure 7.

'63) and thermolabile. When a sonic extract of acatalasia II cells was dialyzed in the dark for 12 hours, very little loss in activity was observed (table 3). These observations are consistent with the view that activity is due to a heme protein.

Aebi et al. ('64) have shown that patients who are mutant homozygous for acatalasia II have significant residual catalase activity in both their erythrocytes and cultured diploid cells. They have also shown that in the case of erythrocytes the activity is almost certainly due to a catalase molecule with an altered structure (Matusubara, Suter and Aebi, '67). Our experiments with cultured cells confirm their original finding, and suggest that in these cells also the residual activity is likely to be due to molecules of catalase.

DISCUSSION

It has been shown that cultures of human diploid cells increase their specific catalase activity as the culture grows. Although the rise is not large, it is exponential with time and repeatable. Following subculture, specific catalase activity falls again. Similar results were found with a human heteroploid line, suggesting that the heteroploidy had not produced a qualitative change in whatever mechanism accounts for this phenomenon. Cells from a donor who was homozygous for acatalasia I had no detectable activity by the spectrophotometric assay we have employed,

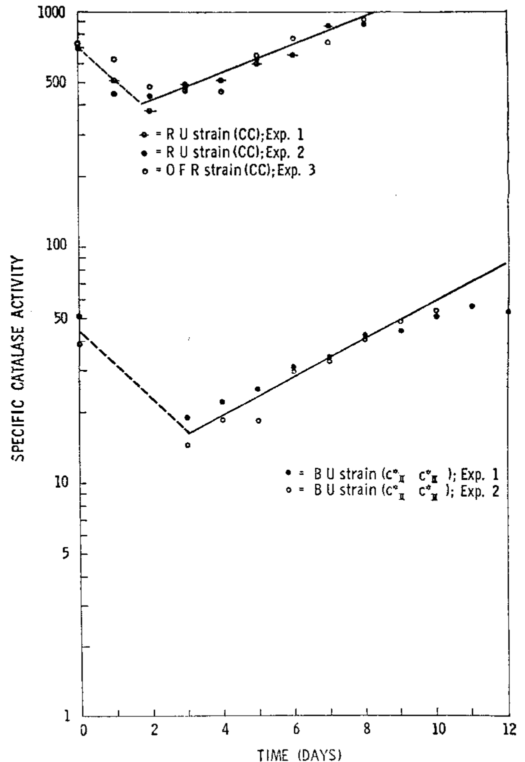

Fig. 6 Progressive increase of specific catalase activity during growth of two cell strains homozygous normal (CC) for the catalase phenotype and in a cell strain homozygous ($c_{II}{*}c_{II}{*}$) for acatalasia II. Semilogarithmic plot.

TABLE 2a

Effect of sodium azide on the catalase activity of sonic extracts of cells of the BU strain (c_{II} c_{II}*) and the RU strain (CC)* [1]

Cell strain and genotype	Concentration of sodium azide in reaction flask (Moles per liter)	Catalase activity: μM H_2O_2 decomposed per hour per 2.0 ml	Cell protein: Concentration per 2.0 ml	Specific catalase activity
BU (c_{III}* c_{III}*)	0	15.7	0.360	43.6
BU (c_{III}* c_{III}*)	1.54×10^{-5}	0	0.360	0
RU (CC)	0	174	0.194	896
RU (CC)	1.54×10^{-5}	0	0.194	0

[1] The extract was assayed within ten minutes of the addition of sodium azide.

TABLE 2b

Effect of boiling for ten minutes on the catalase activity of diploid cell strains

Cell strain and genotype	Treatment	Catalase activity: μM H_2O_2 decomposed per hour per 2.0 ml	Cell protein: Concentration per 2.0 ml	Specific catalase activity
RU (cc)	Control	330	0.406	812
	Boiled	0	0.406	0
BU (c_{II}* c_{II}*)	Control	31.9	0.611	52
	Boiled	0	0.611	0

and could not be further investigated from this standpoint. However, Takahara et al. ('66) have reported that by the manometric and perborate assays residual catalase activity can be detected in both hemolysates and cultured cells from acatalasia I homozygotes. Hence, by these methods, it may prove possible to explore the change with growth in the catalase activity of acatalasia I cells. Another approach would be to study a cell strain from an acatalasia I heterozygote. A mutant gene which qualitatively changed the kinetics of development of specific catalase activity might have interesting effects even when present in a cell that also contained the normal allele. The gene mutation might appear dominant in the heterozygote when the whole growth cycle of the culture is studied.

The acatalasia II cells had detectable catalase activity by the spectrophotometric assay. It is of interest that, by the perborate assay, Aebi et al. ('64) found that the ratio of specific activities of a normal to a homozygous acatalasia II diploid cell strain was about 7:1. In our experiments, using spectrophotometric assay, the ratio was about 25:1. The acatalasia II cells

TABLE 3

Effect of dialysis in darkness on sonic extracts of cells of the BU strain (c_{III} c_{III}*)* [1]

Duration of dialysis or control period in hours	Dialyzed extract	Control extract
0	47.3 (59.4)	47.3 (59.4)
12	33.3 (41.8)	47.2 (59.3)

[1] The concentration of cell protein was 0.398 mg per ml. Activity per 2.0 ml and specific activity, in parenthesis, are given in each cell of the table. The bath was not changed during dialysis. The undialyzed control extract was suspended in a cellulose casing bag which was not immersed in the bath.

developed catalase activity with time at the same proportionate rate as normal cells. The Mendelian mutation (which is probably one affecting the primary structure of the enzyme (Matusubara et al., '67) would therefore appear to have left the mechanism responsible for this effect intact. In contrast, the other Mendelian mutations which affect the catalytic activity of human diploid cells, and which have been studied in this way, appear to change the curve relating specific enzyme activity to time. These mutations are the ones causing the Negro variant of glucose-6-phosphate dehydrogenase deficiency (De

Mars, '64a), galactosemia (Russel and DeMars, '66, '67) and perhaps orotic aciduria (Krooth, '64).

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