PROPERTIES OF ACATALASIC CELLS GROWING IN VITRO

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Acatalasia is a rare disease characterized by congenital absence of the enzyme catalase (1–5). Over 50 cases are at present known. All of these cases have occurred in Japan, in Japanese persons and in one Korean family; about half of the patients are symptom-free, and the remainder develop recurrent infections of the gums and related oral tissues. Infection is chiefly with anaerobic organisms and can often be successfully treated with antibiotics. Extraction of the teeth usually prevents subsequent infection.

Acatalasic individuals lack the enzyme in all tissues which have thus far been examined; in contrast to its ubiquitous distribution in normal individuals, it is lacking in liver, red cells, muscle, oral mucosa, etc. in acatalasic persons.

Acatalasia is due to homozygosity for a recessive gene. Heterozygotes, called hypocatalasics, have red cell catalase activities, which average about one-half normal. The distribution of red cell catalase activities of the heterozygotes does not overlap the distribution for normal individuals (5).

In this report, we should like to describe certain properties of cell lines developed from skin biopsies from a hypocatalasic individual and an acatalasic patient. The interest of developing cell lines from the tissues of patients with inborn metabolic errors has recently been emphasized by several writers (6–10). Human euploid cell lines each biochemically marked by the action of a Mendelian gene may eventually prove useful in the study of genetic changes, such as somatic mutation, transformation, and recombination. The tissues of patients with certain inborn metabolic errors appear at present to be the only source of such mutant lines. Studies analogous to the ones reported here have also been performed on cell lines developed from the tissues of patients with galactosemia (8, 11).

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Materials and Methods

A. Clinical Data.—

Cell lines were developed from skin biopsies from an acatalasic, a hypocatalasic, and eight other persons who were either normal or had an unrelated disease. Clinical data on these patients is given in Table I. The ITA line (a normal control line) was developed from a biopsy on an American donor of Japanese origin. Some of the lines have been used in other experiments in this laboratory, and the appropriate references are given in Table I.

TABLE I

Clinical Data

Line	Donor's age	Donor's sex	Diagnosis	Previous published data on line (Ref. No.)
	yrs.			
ACA	60	M	Acatalasia	
TAC	28	M	Hypocatalasia	15
BA	30	F	Myositis ossificans	
ITA	40	M	Normal control	23
DUF	39	M	Chronic Tophaceous Gout	
TDU	11	F	Galactosemia	8, 11
MAD	38	F	Galactosemia heterozygote	8, 11
BE	21	F	Primary amyloidosis	8, 11, 15
ZOL	12	M	Wilson's Disease	
LBR	11	M	Galactosemia	

In Text-fig. 1 the pedigrees of the hypocatalasic (TAC) and the acatalasic (ACA) patients are given. Both patients were free of symptoms. Catalase assays on the red cells of these patients, performed by a method previously described (5), are as follows:

Patient	K_{cat} (velocity constant of red cell catalase)
ACA	0
TAC	2.17
259 Japanese Controls	5.38 ± 0.73 (Takahara *et al.* 5)

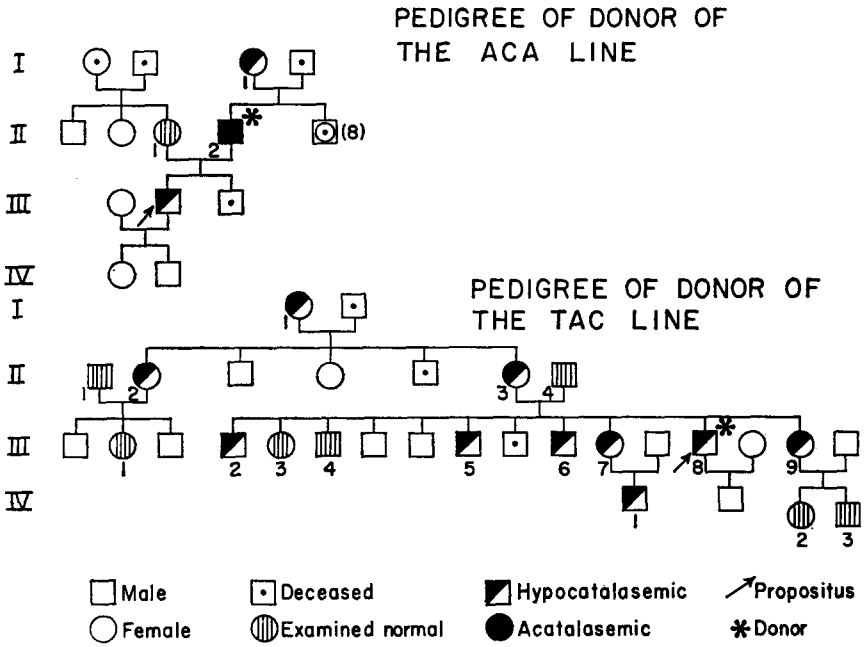
B. Techniques of Cell Culture.—

1. General techniques: All cell lines were developed from skin biopsies. In most instances a 4 mm punch biopsy was removed from the skin area. The biopsies on the acatalasic and hypocatalasic patients were performed in Hiroshima. Directly after excision the tissue was cut sterilely into pieces several millimeters in diameter and was placed in glass test tubes containing a medium made up of 40 per cent N 15 (12), 59 per cent saline G (12), and 1 per cent of Puck's antibiotic solution (12). The tissue was shipped by air (at cabin temperature) to the National Institutes of Health in Bethesda, and was in both cases processed about 80 hours after the biopsy.

The general method employed to develop the cell lines is based on principles originally set out by Puck and his associates in 1958. The details of the technique used in our laboratory

have been published elsewhere (8). The cell type which grows out is the "fibroblast"—a fusiform transparent cell (Fig. 1). In the cases of biopsies of normal skin on persons of normal karyotype, these cells will be euploid, and the euploidy will persist as a stable feature of the line (12–15). The cells are grown as monolayers on one durface of a glass bottle or dish.

2. Selection experiments: A large bottle of cells ("Blake bottle") was subcultured into 50 to 100 smaller ones ("T_{15} 's"). Twenty-four hours after subculturing the cells were washed twice



TEXT-FIG. 1. Pedigrees of the hypocatalasic (TAC) and the acatalasic (ACA) patients.

with 3 ml of the following balanced salt solution (in distilled water):

Compound	*gm/liter*
NaCl	6.80
KCl	0.40
$NaH_2PO_4 \cdot 2H_2O$	0.15
$NaHCO_3$	2.20

The cells were then overlayed with 2 ml of the above solution now containing varying concentrations of hydrogen peroxide. The overlay was incubated with the cells for 20 minutes (at 37°C), and was then poured off. The cells were washed once with 3 ml liters of growth medium; then the cells were incubated with fresh growth medium and fed every 72 hours.

The concentrations of hydrogen peroxide used were:

0.8×10^{-5} molar
0.8×10^{-4} molar
0.8×10^{-3} molar

After the second wash with the balanced salt solution, and before exposure to hydrogen peroxide, 6 bottles were frozen for subsequent determination of cell protein. Thereafter, three of the bottles exposed to each concentration of hydrogen peroxide were frozen every 72 hours. Cell proteins in the selection experiments were determined by the method of Oyama and Eagle (16). The unit of cell protein in graphing is defined as an amount of protein giving the same optical density as 0.1 mg of bovine albumin. Acatalasic cells and normal cells were handled and grown simultaneously in these experiments so that they might be washed and treated with the same solutions and fed with same media.

C. Chemical Determinations.—

After the first two or three subcultures, the cells were transferred to large bottles. When a confluent monolayer formed, the cells were subcultured. Periodically a bottle was removed for assay of cell catalase. The cells were harvested when the monolayer was confluent, as in subculturing, and a single bottle usually yielded between 5 and 10 mg of cell protein.

The cells were digested off the glass with a 0.05 per cent trypsin solution (8), spun at 1500 RPM for 5 minutes, the trypsin was removed, and the cells were then washed three times in 0.86 per cent sodium chloride. After the final wash the cells were suspended in 5 to 15 ml of 0.05 molar potassium phosphate buffer (pH7) and transferred to a plastic non-conical centrifuge tube (Spinco). After placing the tube in a beaker of ice, the cells were sonicated on a Mullard probe sonicator, the sonic vibration being delivered in 4 pulses of 30 seconds' duration each. Between each pulse the suspension was allowed at least 30 seconds to cool, and at no time did the temperature within the tube exceed 10°C. When a large number of tubes were run the probe was chilled with ice after every eight pulses. In most instances control cells were run simultaneously with acatalasic or hypocatalasic ones.

The sonicates were spun at 200 RPM for 10 minutes at 4°C. Two ml aliquots of the supernatant were removed and transferred to a Beckman cuvette for a spectrophotometric assay of the catalase activity of the sonicate. The method employed was that of Beers and Sizer (17, 18) and has been used previously on cultured cells (19).

Thirty percent H_2O_2 (Fisher Scientific Company) was diluted in 0.01 M KH_2PO_4 buffer (pH ≈ 7) to 0.03 molar. One ml of this dilution was then added to the 2 ml aliquot of the solution of cellular sonicate in the cuvette. Following addition of the hydrogen peroxide, optical density was taken at 10 second intervals at 2400 A in a model DU Beckman spectrophotometer. The first reading was obtained 10 seconds after the introduction of peroxide into the cuvette. The net change in optical density for the first minute was computed as

$$\Delta(\mathrm{OD}) = \frac{(\mathrm{OD})_{10\ \mathrm{sec}} - (\mathrm{OD})_{60\ \mathrm{sec}}}{5/6}$$

where the two terms in the numerator refer to optical density at 10 and 60 seconds, respectively. The individual readings were also plotted, to determine whether the initial reaction rate was constant over this time interval in each assay and could therefore be used to quantitate enzyme activity. Assays were run in triplicate. A millimolar extinction coefficient of 0.040 was used for hydrogen peroxide.

A 0.2 ml aliquot of the sonicated solution was taken for determination of the cell protein by Lowry's method (20).

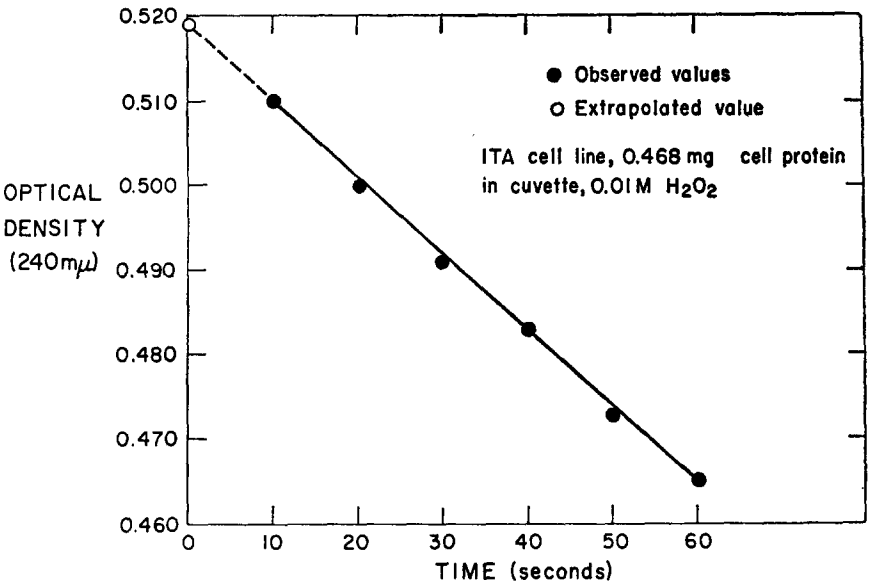
Using the computed change in optical density over 1 minute, the millimolar extinction coefficient of hydrogen peroxide and the concentration of cell protein in the cuvette, the specific activity of catalase in the cellular sonicate was obtained. This latter figure was then multiplied by 60 to give a virtual quantity: micromoles of hydrogen peroxide decomposed per hour per milligram cell protein. Except where otherwise specified, this unit of cell catalase activity will be employed in the graphs and tables to follow and has also been used by Lieberman and Ove (19).

Virtually no spontaneous decomposition of hydrogen peroxide is noted, over 1 minute, when the cellular sonicate is not added to the cuvette. The spectrophotometer readings are of course corrected for a protein blank.

RESULTS

A. Properties of the Catalase Assay System.—

1. General properties of the assay: In Text-fig. 2, the 10 second optical density readings from a typical assay are plotted against time. It will be noted that the curve is linear. In Text-fig. 3 catalase activity is plotted against the quantity



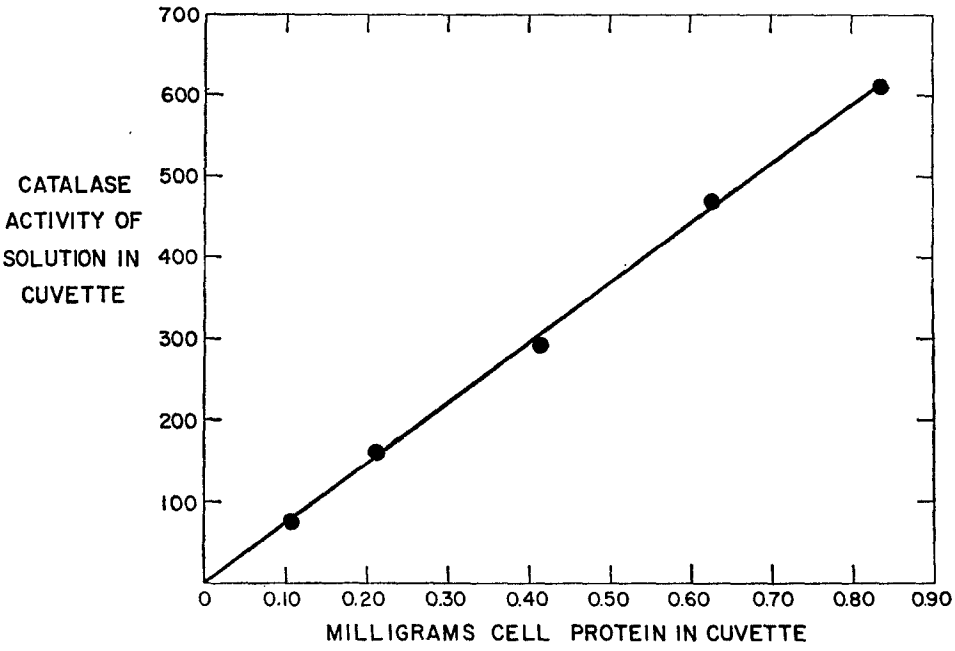
TEXT-FIG. 2. Kinetics of hydrogen peroxide disappearance in a solution of cellular sonicate.

of cell protein in the cuvette (using the ITA line). The relationship of catalase activity to cell protein is linear and the line passes through the origin.

In order to inquire further whether the catalase activity measured could be assumed to be proportional to the quantity of catalase present in the sonicate, another experiment was done.

Crystalline beef liver catalase (Biochemica Boehringer) was dissolved in the buffer described above and diluted to approximately 2×10^{-5} mg per ml. A solution of sonicate from cells of the ITA line was also made up with a protein concentration of 0.417 mg per ml. These two solutions were combined, in a cuvette, in varying proportions—to give a total volume of 2 ml in each case. One ml of 0.03 molar hydrogen peroxide was then added.

The catalase activity observed was compared with theoretical values computed from the values for the solutions before they were mixed. The data are set out in Table II.



TEXT-FIG. 3. Relationship of catalase activity to concentration of cell protein (ITA line). The quantities of protein present in the usual assay generally fell near the mid portion of this curve.

TABLE II

Catalase Activities of Mixtures of Cell Sonicate and Crystalline Catalase

Volume of crystalline catalase solution added to cuvette	Volume of ITA (cell sonicate) solution added to cuvette	Volume of 0.03 M H_2O_2 solution added to cuvette	Observed catalase activity of solution in cuvette*	Theoretical activity
ml	*ml*	*ml*		
2	0	1.0	428	428
1.5	0.5	1.0	491	497
1.0	1.0	1.0	558	564
0.5	1.5	1.0	624	636
0	2.0	1.0	702	702

* μMoles per hour per 3 ml of solution (the total volume in the cuvette).

It will be noted that good agreement between the theoretical and observed values is obtained, suggesting that the concentration of crystalline catalase can be accurately measured in the presence of the cellular sonicate.

2. Role of extraneous variables in determining the catalase activity of a cell line: A number of variables have been examined to determine whether they influence

the catalase activity of the lines. The chief variables studied have been the following:

(*a*) The age of the line (the time between the date of the biopsy and the date of the cells).

(*b*) The number of subcultures.

(*c*) The estimated number of times the cells have increased in culture (See Krooth and Weinberg, 8, for method of estimation).

(*d*) The interval between enzyme assay and last subculture; *i.e.*, the number of days the cells have been in the bottle.

TABLE III

Catalase Activities of the Several Cell Lines

Presumed genotype	Line	No. of determinations	Range in ages	Range in subculture Nos.	Mean catalase activity	Standard error of mean
			days			
c*c*	ACA	7	33–93	4–11	0	(all values zero)
Cc*	TAC	15	78–182	3–13	211.2	±14.0
CC	BA	11	61–126	3–12	756.4	±56.3
CC	ITA	9	29–104	3–14	675.9	±42.2
CC	DUF	3	73–78	3	669.0	±46.1
CC	JDU	4	286–307	21–24	844.2	±50.3
CC	MAD	2	284–306	19–22	883.0	±113.0
CC	BE	2	303–308	34–35	1109.0	±109.0
CC	ZOL	3	29–40	3–5	546.0	±33.2
CC	LBR	4	36–53	4–6	612.5	±55.7
—	Hela (LP-8)	7	—	—	712.0	±42.1

Subculture number is defined as the number of times the cells have been subcultured at the time they are harvested for assay. Catalase activity is expressed as micromoles of hydrogen peroxide decomposed per hour per milligram cell protein.

* The presumed abnormal gene.

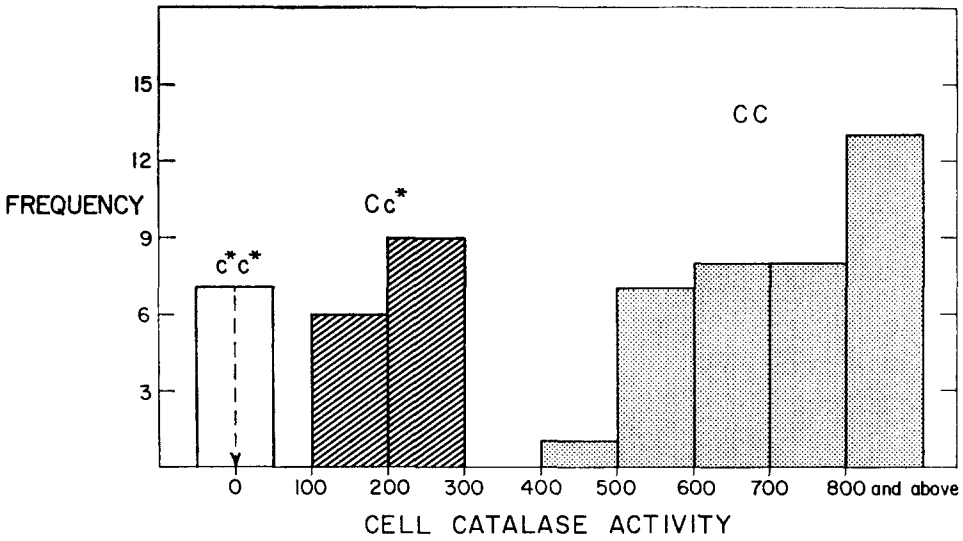
No significant effect of any of these variables on the catalase activity of the lines was found. The data on the effect of age of the line are typical and will subsequently be set out in more detail.

Assays of catalase activity done in triplicate during each determination gave almost no variation in results. Assays of bottles of the same cell line on different days did not, however, give precisely the same values. The coefficient of variation (standard deviation times 100 divided by the mean) lies between 10 and 18 per cent, the value depending on the line. The acatalasic line is an exception. No variation in the results from this line could be detected by our method.

B. Catalase Values of the Several Lines.—

In Table III, the mean catalase activities of the lines employed are set out.

Activity, computed as described previously, is expressed as micromoles of hydrogen peroxide decomposed per hour per milligram cell protein. Note how the control lines differ in their range of ages and in their range of subculture numbers. Some of the lines are older, while others are younger, than the hypo-



TEXT-FIG. 4. Frequence with which specified values of cell catalase were observed in 38 determinations on the 8 normal lines (CC), of 15 determinations on the hypocatalasic line (Cc*) and of 8 determinations on the acatalasic line (c*c*). The asterisk denotes the presumed abnormal gene.

TABLE IV

Summary of Data on Catalase Activity

Presumed genotype	No. of lines	No. of determinations	Mean catalase value	Standard error of mean
c*c*	1	7	0	(all values zero)
Cc*	1	15	211.2	±14.0
CC	8	38	762.0	±21.0

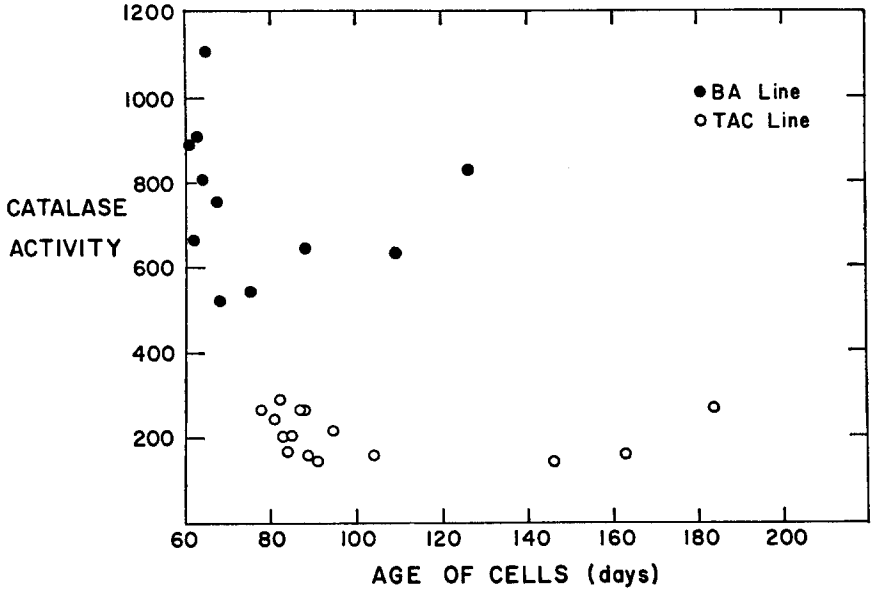
$\frac{Cc^*}{CC} = 0.28$

*The presumed abnormal gene.

catalasic and acatalasic lines, and corresponding variation is seen in the subculture numbers. These two parameters, however, appear to have no discernible effect on the catalase activity. In Text-fig. 4, the distribution of catalase activities for the 8 normal lines (38 determinations), the hypocatalasic line (15

determinations), and the acatalasic line (8 determinations) are graphed. Note that the three presumptive genotypes do not overlap. In Table IV the data are summarized. It will be seen that all the activities for the acatalasic line were zero. The ratio of the mean activity of the heterozygous line to the mean activity of the normal lines was 0.28.

In Text-fig. 5, the catalase activities of the hypocatalasic cells and of the BA cells are plotted against the age of the line. There is a suggestion from the graph that the catalase activity may fall as the line ages. However, the effect



TEXT-FIG. 5. Effect of "age' on the catalase activity of a normal cell line (BA) and the hypocatalasic cell line (TAC).

is small, and does not impair discrimination between the lines. Moreover, the effect is not significant. The product-moment correlation coefficients for age and catalase activity are:

Line	r
TAC	−0.165
BA	−0.137

The probability, using the "z" transformation (21) of getting these data (or a worse fit), on the hypothesis that the population correlation equals zero, is greater than 0.5 in the case of both lines.

Several experiments were done in which catalase and lactic acid dehydrogenase were measured on aliquots from the same cellular sonicate. The data are set out in Table V. Note that one can apparently discriminate between the three genotypes by the ratio of catalase activity to lactic acid dehydrogenase activity. The mean catalase activity of the normal lines is about 750 micromoles of peroxide decomposed per hour per milligram cell protein. This figure represents the catalase activity of around 7×10^{-5} mg of crystalline beef heart catalase. Assuming human cultured cell catalase and beef heart catalase have nearly the same molecular weight and turnover number, one can say that the human cell catalase is close to 0.01 per cent of the cell protein. The fact that cultured cells have less catalase activity than many intact tissues has been noted by other authors (19, 22).

TABLE V

Comparison of Lactic Acid Dehydrogenase and Catalase Activities in Certain Cell Lines

Presumed genotype	Line	Subculture No.	Age	LDH activity of cell protein (1)	Catalase activity (2)	Ratio (2)/(1)
			days			
CC	ITA	6	49	146.2	834	5.7
CC	BA	13	143	162.0	834	5.1
CC	DUF	4	75	73.5	761	10.3
Cc*	TAC	12	115	103.0	142	1.4
Cc*	TAC	14	132	96.0	160	1.7
c*c*	ACA	6	37	160.0	0	0

* The presumed abnormal gene.

It is of interest also that the medium in which the cells were grown contained 12 per cent pooled human serum, and had considerable catalase activity. One ml of fresh medium (before incubation with cells) could decompose roughly 7 micromoles of hydrogen peroxide per hour. The cells assayed were grown in 75 to 100 ml of medium (which was changed every 72 hours). It is notable that there was about as much catalase activity in the medium as in a milligram of cell protein. However, after several months of growing in such media, no catalase activity could be found in the cell protein of acatalasic cells.

C. Mixing Experiments.—

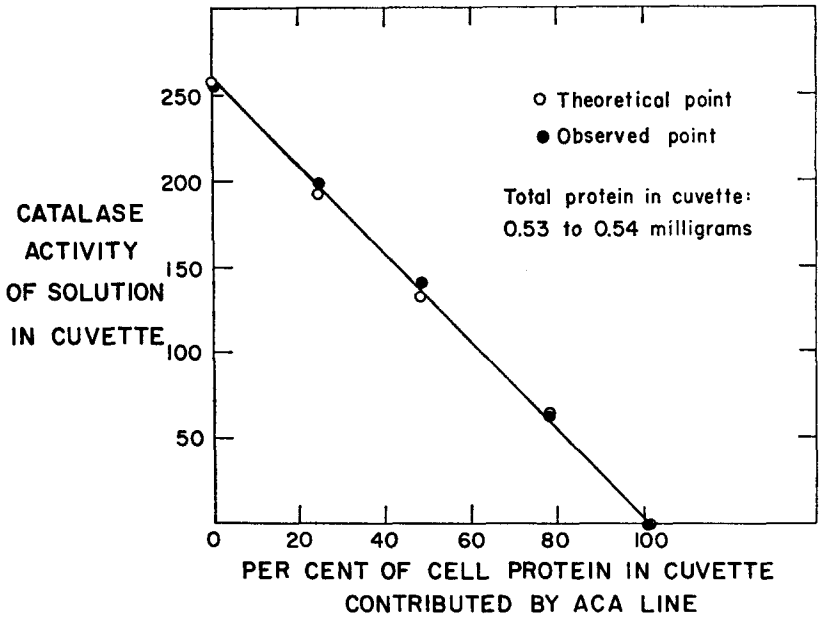
To inquire whether acatalasic cells might contain a demonstrable inhibitor of catalase activity, mixing experiments were performed. A sonicate of acatalasic cells was mixed in varying proportions with a sonicate of cells of the ITA line. Catalase activities were determined on the mixtures and on the two sonicates separately. Theoretical values (assuming no inhibitor) were computed from the unmixed sonicates. The data are set out in Table VI, and are plotted in

Text-fig. 6. The observed and theoretical values agree well, and the catalase activity of the normal cell sonicate was not reduced by the presence of the acatalasic sonicate. Nor did the presence of the acatalasic sonicate in the mix-

TABLE VI

Mixing of Sonicates of the ITA and ACA Cell Line

Protein present in cuvette		Total protein in cuvette	Per cent of total protein which is ACA protein	Catalase activity of solution in cuvette	Theoretical catalase activity
ITA line	ACA line				
mg	*mg*	*mg*			
0.529	0	0.529	0	256	256
0.397	0.135	0.532	24	198	193
0.265	0.270	0.535	50	140	128
0.133	0.405	0.538	77	63	65
0	0.540	0.540	100	0	0



TEXT-FIG. 6. Catalase activities of mixtures of cell sonicates of a normal cell line (ITA) and the acatalasic cell line (ACA).

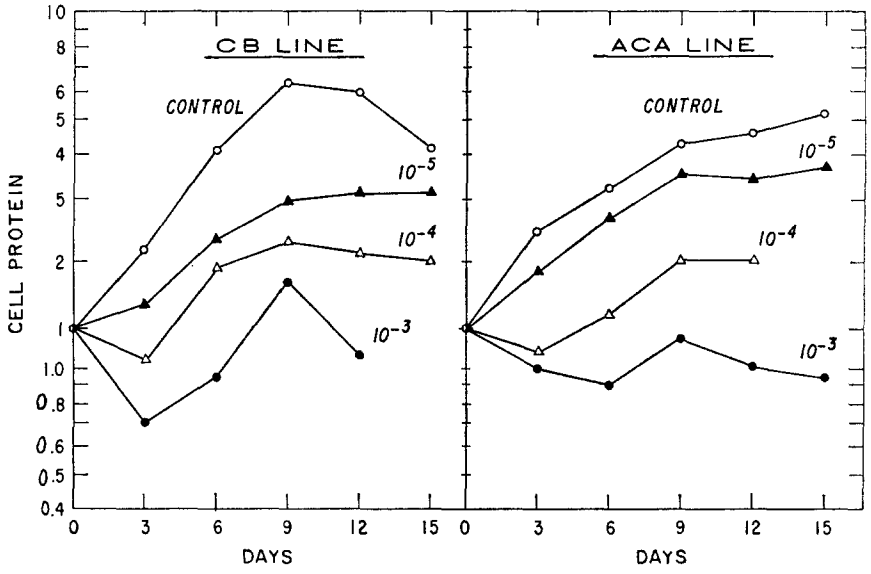
ture increase catalase activity over the theoretical value—which might occur if the ITA sonicate "released" the enzyme in the acatalasic sonicate. Note that from Table VI one can compute the catalase activity of the "synthetic heterozygote," namely, of the sonicate that contains half normal and half

acatalasic protein. The values are:

Sonicate	Catalase activity
"Synthetic heterozygote"	
Observed value	266
Theoretical value	244
Actual heterozygote	
Mean of 15 determinations on the TAC line	211

These values have been compared merely to show the absence of any gross difference between them.

Analogous experiments have been done on the hypocatalasic cell line—sonicates of the BA and TAC line were mixed. The same result as that described above was obtained.



TEXT-FIG. 7. Growth following 20 minute exposure of normal cells (CB) and acatalasic cells (ACA) to varying concentrations of hydrogen peroxide.

D. Selection Experiments.—

The value of a mutant cell line, in genetic experiments, is augmented if one can select against the mutant cells under circumstances where normal cells will survive and grow. We have therefore inquired whether acute exposure to various concentrations of hydrogen peroxide affects the growth of acatalasic and normal cells differently.

In Text-fig. 7, the results of one selection experiment are set out. The CB line, which is the control line in this experiment, was developed from a skin

biopsy on a 38 year old man with alcaptonuria. The cells were 56 days old at the beginning of the experiment and had a catalase activity of 1122. The acatalasic cells were 105 days old. The initial cell protein was the same for both lines.[1] The cells were treated, as described, with hydrogen peroxide at zero time. The increase in cell protein after exposure to the experimental concentrations of hydrogen peroxide was then followed.

Note that the general shapes of the growth curves of the two lines are fairly similar for each concentration of hydrogen peroxide. No gross difference between the lines is discernible. The CB line grew slightly more rapidly than the acatalasic one. Note that at a concentration of hydrogen peroxide of 0.8×10^{-4} molar, the cells in both cases were able to double the initial protein but could not grow further.

These experiments and others tend to suggest that acute exposure to hydrogen peroxide will not preferentially select against acatalasic cells, at least not to a readily measurable extent. Chronic exposure to low concentrations is at present rendered difficult, owing to the high quantity of protein (and other reducing substances) in the medium which promptly tend to decompose the peroxide.

DISCUSSION

Acatalasia appears to persist as a recognizable defect in cells growing in culture. It may therefore be used as a biochemical marker in genetic experiments. Acatalasic human cells have no counterpart in microbial genetics thus far and might eventually be of help in elucidating the physiologic function (if any) of catalase at the cellular level. Since ionizing radiation is sometimes thought to affect cells through the creation of hydrogen peroxide, the radiation sensitivity of these mutant lines may also be of interest.

Our inability at present to develop a system for selecting against these cells limits their usefulness as tools in genetics. When the function of an enzyme is not clearly known, it is difficult to challenge cells lacking the enzyme in an appropriate manner. On the other hand, galactosemic cells can be selected against in culture under conditions where normal cells will grow (8). The simultaneous use of both these markers should permit us to perform more elaborate genetic experiments than would be possible with either one singly.

SUMMARY

Acatalasia, a disease due to homozygosity for a Mendelian gene, is characterized by the absence of the enzyme catalase from the tissues of the human body. Red cells from heterozygotes have enzyme activities about one-half

[1] The value for the cell protein includes an increment (about 0.5 units) contributed not by the cells but by the protein in the medium. However, what is important for this experiment is that the true initial cell protein can be assumed to be the same for both lines and for all the different concentrations of hydrogen peroxide.

normal. In this paper, the development of cell lines from skin biopsies on an affected homozygote, a heterozygote, and eight control patients is described. The cell type is the euploid "fibroblast."

It was found that acatalasic cells lacked the enzyme, even after growing for many months in a medium rich in catalase. The control lines all had mean catalase activities double or more that of the heterozygous line. Selection experiments, in which the growth of cells exposed for 20 minutes to varying concentrations of hydrogen peroxide was measured, did not provide a system for preferentially eliminating acatalasic cells.

Certain other experiments bearing on the enzymatic defect in this disease were performed.

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EXPLANATION OF PLATE 34

FIG. 1. Photomicrograph of a culture of the acatalasic (ACA) cell line showing mitosis. The cells which grow out are fusiform and transparent. × 150.

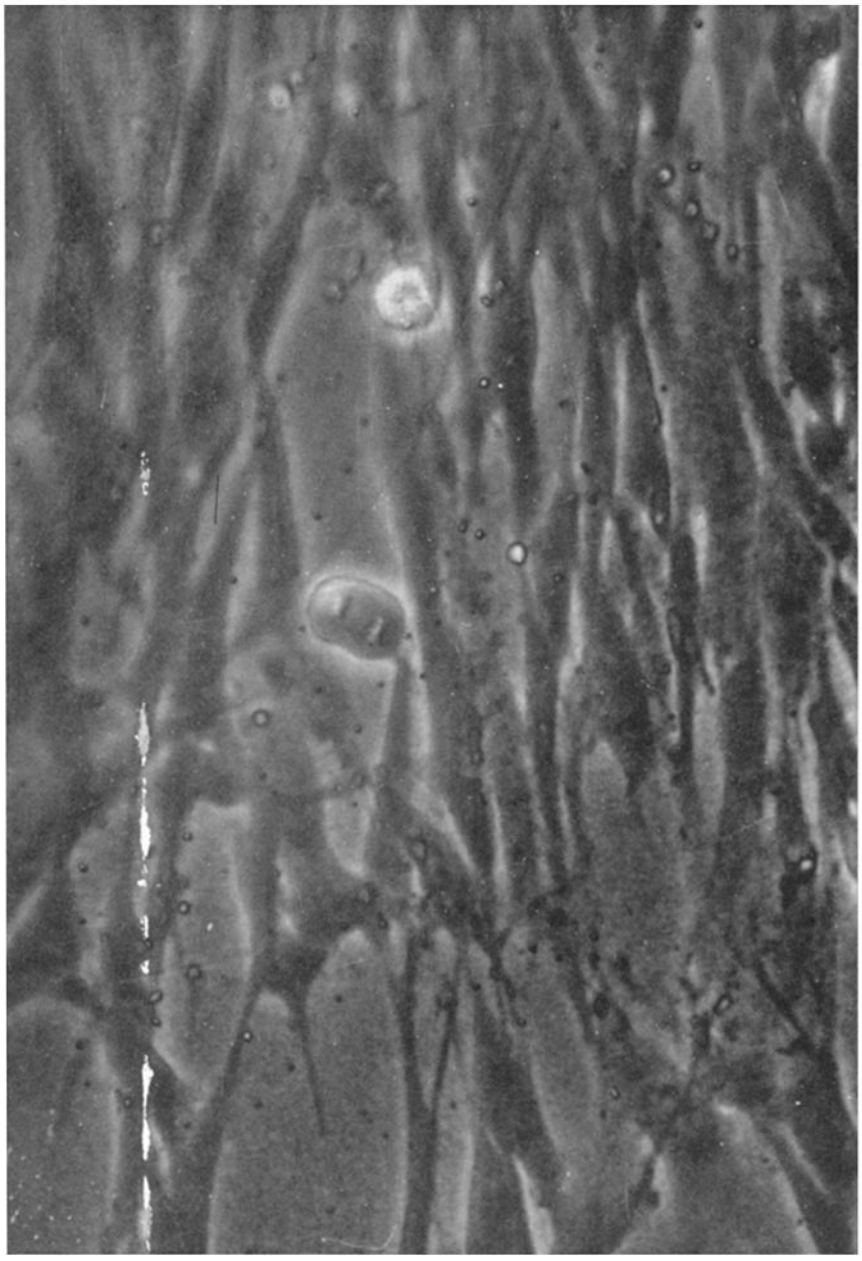

FIG. 1

(Krooth *et al.*: Acatalasic cells growing *in vitro*)