CATALASE REQUIREMENT FOR MAMMALIAN CELLS IN CULTURE*

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The studies of Puck and his coworkers (1) have made possible the growth of single mammalian cells by a procedure comparable to the plating technique of microbiology. Knowledge of the growth requirements peculiar to single cells (2) or small numbers of cells, however, is essentially lacking. Although the mutually advantageous interaction of cells growing close together was indicated by Sanford *et al.* (3), and was later ingeniously exploited with "feeder" layers (1), the exact nature of the cellular interplay has not been determined.

During growth studies with mammalian cells in various serum-free media (4), the unexplained rapid death of the inoculum was often encountered, particularly when small numbers of cells were used. While serum was protective, it was effective only in relatively huge concentrations. Examination of various biologic materials revealed that liver extracts, on a protein basis, were several hundredfold more potent than serum. The active material in liver required for the survival of small numbers of cells, but not for "mass" inocula, has been identified as catalase.

Thus, it would appear that prevention of peroxide formation or assistance in its removal is one of the important benefits of proximal growth and one of the functions of the "feeder" layer. With added catalase, growth of relatively small numbers of cells is now consistently obtained in serum-free medium.

The purpose of this report is to describe the protective effect of catalase preparations and to present the evidence for the identity of the protective agent with the enzyme catalase.

Materials.—The cell cultures used were as follows:—[1]

Culture	Clone	Origin	Morphology	Reference
"Altered" Kidney	2-2	Normal monkey kidney	Fibroblast-like	(5)
Appendix	A1	Normal human appendix	Epithelioid	(6)
HeLa	—	Human cervical carcinoma	Epithelioid	(7)

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[1] "Altered" kidney was kindly supplied by Dr. L. Siminovitch and Dr. R. C. Parker; Appendix was obtained from the Carver Foundation, Tuskegee Institute, Alabama; and HeLa was generously provided by Dr. J. E. Salk.

The basal medium contained the amino acids, inorganic salts, glucose, and antibiotics suggested by Healy, Fisher, and Parker (8), and the vitamin mixture of Eagle (9). The growth medium contained in addition 15 per cent beef serum. For the growth of small numbers of cells, a mixture of human and horse serum (1) was substituted for the bovine serum.

Crystalline beef liver catalase was obtained from the Worthington Biochemical Corporation, partially purified horseradish peroxidase, crystalline bovine hemoglobin, and crystalline bovine hemin, from the Sigma Chemical Company. Fetuin (10) was purified from fetal calf serum by chromatography on a column of DEAE[2]-cellulose (11).

Culture Methods.—The Kidney cells were grown in suspension in 50 ml. test tubes fitted with silicone stoppers. To prevent the cells from settling out and adhering to the glass, the cultures were incubated horizontally in a drum revolving at 40 R.P.M. (12). The Appendix and HeLa cultures were grown on glass surfaces.

Determinations.—Cell counts were made with Levy counting chambers. Catalase activity was measured spectrophotometrically by the decrease in optical density at 240 mμ according to the procedure of Beers and Sizer (13). Protein was estimated by the method of Lowry *et al.* (14).

Assay.—The protective activity of a test compound was determined by counting "healthy" cells after incubation with it. Cells which were round, shrunken, and highly granular, whether floating in the medium or attached to the glass surface, were considered to be dead and were not counted (their non-viability was occasionally established by the addition of a medium capable of supporting the growth of single cells with a plating efficiency of about 70 per cent). Such cells could be readily distinguished from "healthy" cells which were large, non-granular, bipolar (Kidney), or epithelioid (Appendix, HeLa) and could be propagated upon the addition of growth medium.

"Altered" Kidney.—Cell suspensions were sedimented by centrifugation for 5 minutes at 1500 *g* and the supernatant growth medium was discarded. The sedimented cells were resuspended to yield about 15,000 cells per ml. in basal medium supplemented with human serum (0.001 ml. per ml.). Two ml. aliquots of the suspension were then distributed to T-flasks (approximate floor area 15 cm.^{2}) which had previously received various amounts of the test solutions (generally up to 0.1 ml.). Control flasks containing only the cell suspension were also prepared.

The assay flasks, sealed with tight fitting white ("non-toxic") rubber stoppers, were incubated at 37°, and were examined as routine after 14 to 16 hours, occasionally after a shorter period.[3] Surviving ("healthy") cells were estimated by counting with a chemist's microscope several fields (radius of field = 1 mm.) along a line equidistant from the sides of the flask. Fourteen cells per field was taken to indicate 10,000 cells per flask.

Appendix and HeLa.—Assay with these cells was carried out in the same manner except that cultures were harvested by scraping with a rubber spatula, a smaller number of cells was used (20,000 per flask), and the concentration of serum in the assay medium was increased (0.002 ml. per ml.).

RESULTS

Effect of Catalase Concentration.—The effect of different concentrations of catalase was studied with three cell cultures, "altered" Kidney, Appendix, and HeLa. As shown in Table I, with the inoculum of the standard assay (about

[2] DEAE, diethylaminoethyl.

[3] In the absence of a protective agent, cell death first appeared after 2 to 4 hours of incubation. Essentially all the cells were dead after 6 to 10 hours.

15,000 cells per ml.), essentially complete survival of the Kidney cells occurred with approximately 0.02 μg. of catalase per ml. Similar results were obtained with the Appendix culture, but with HeLa, larger concentrations of the enzyme were required to protect the cells (about 0.15 μg. per ml.).

Also shown in the table are the results obtained with inocula of 100 Kidney cells. With no added catalase, after 6 hours in the standard assay medium, no cells capable of colony formation remained. On the other hand, with a sufficient

TABLE I

Effect of Catalase Concentration

In the experiment designated "mass inoculum," the "altered" kidney cells were treated and enumerated according to the standard assay procedure. The experiment designated "single clone" was carried out in Petri dishes (60 mm.). Two ml. aliquots of assay medium supplemented with the indicated amounts of crystalline catalase were inoculated with 100 Kidney cells. After 6 hours, (37°, atmosphere = 5 per cent CO_2-air), 2 ml. of basal medium containing 30 per cent human serum and 10 per cent horse serum was added. After 8 days clones were enumerated following fixation and staining (5).

Catalase	Survivors	
	"Mass inoculum," No. of "healthy" cells	Single clone, No. of clones
μg. protein per ml.		
0	0	0
0.007	1,400	—
0.011	11,000	—
0.014	19,000	37
0.018	25,000	—
0.021	30,000	32
0.028	33,000	47
0.035	32,000	61
0.055	—	59

concentration of the enzyme, the number of viable cells did not decrease appreciably during incubation in the assay medium.

Effect of Cell Numbers.—Survival in the unfavorable environment of the standard assay medium was studied as a function of cell numbers with the Kidney cells. With less than 100,000 cells per flask, few or no cells survived (Table II) while with added catalase, complete survival occurred even with the smallest number of cells studied (20,000 per flask).

Heat Lability of Catalase.—The minute quantity of crystalline catalase required (see Table I) suggested that the enzyme itself and not a contaminant is responsible for its protective action. Further support for this contention was obtained by comparing the residual enzymatic and cell-protective activities of heated catalase preparations (Table III). As shown in the table, comparable recoveries were obtained with both methods of assay.

Peroxidase, Reducing Agents, and Other Compounds.—Additional evidence for the identity of catalase and the protective factor was obtained with horseradish peroxidase and reducing agents. With "altered" Kidney cells approximately 0.3 μg. of peroxidase per ml. offered complete protection. Hemoglobin

TABLE II

Survival as a Function of Cell Numbers

The "altered" Kidney cells were treated and enumerated as described for the standard assay procedure. Catalase (0.05 μg. protein) was added to the T-flasks as indicated immediately before the addition of the cell suspension.

Initial No. of Cells	Catalase	No. of Survivors
20,000	−	0
20,000	+	19,000
40,000	−	0
40,000	+	33,000
60,000	−	0
60,000	+	70,000
80,000	−	10,000
100,000	−	62,000
150,000	−	167,000
200,000	−	189,000

TABLE III

Comparison of Enzymatic and Protective Activities of Heated Catalase Preparations

The catalase solution was heated for 10 minutes in a waterbath maintained at the temperatures indicated. Enzymatic activity was estimated by the spectrophotometric procedure of Beers and Sizer (13); cell protective activity, with "altered" Kidney cells under the conditions of the standard assay.

Temperature	Recovery of activity	
	Enzymatic assay	Cell culture assay
°C.	*per cent*	*per cent*
61	25.7	30
63	8.8	10
65	1.1	5
70	0.0	0

in larger concentrations (50 μg. per ml.) also prevented cell death but hemin and cytochrome C (each 50 μg. per ml.) were ineffective.

Several reducing agents were capable of substituting for catalase. Thus, sodium ascorbate, sodium thioglycollate, and sodium thiosulfate, tested with the Kidney and Appendix cultures,[4] completely protected cells at levels of 2, 2, and 5×10^{-4} M, respectively.

[4] With HeLa, ascorbate showed little or no activity, thioglycollate was fully active, and thiosulfate was not tested. No investigation was made into the cause of the inactivity of ascorbate.

Inactive, however, when tested with the same cultures were L-cysteine, reduced glutathione, sodium nitrite (each 2×10^{-3} M), and reduced diphosphopyridine nucleotide (2×10^{-4} M). Also without effect were sodium formate (0.02 M), ethanol (0.05 M), L-ethionine (0.001 M), vitamin B_{12} (1 μg. per ml.), crystalline soybean trypsin inhibitor (5 μg. per ml.), crystalline lactic and alcohol dehydrogenase, collagenase, and fetuin (each 50 μg. per ml.). None of the compounds tested inhibited the protective action of catalase.

Effect of O_2 Tension.—The survival of the "altered" Kidney cells in the absence of added catalase or effective reducing agent was studied as a function of

TABLE IV

Effect of O_2 Tension on Cell Survival

The "altered" Kidney cells were inoculated into T-flasks as for the standard assay. The flasks were immediately flushed (2 minutes) with a vigorous stream of gas containing the indicated concentration of O_2, 5 per cent CO_2, and N_2. Catalase (0.05 μg. of protein) was added as indicated before the addition of the cell suspension. Incubation and enumeration of surviving cells were carried out as for the standard assay.

O_2	Catalase	No. of survivors
per cent of total gas		
0	−	34,000
1	−	29,000
2	−	25,000
4	−	5,700
	+	32,000
8	−	0
	+	31,000
20*	−	0
	+	35,000

* Air atmosphere, not flushed.

O_2 tension (Table IV). Under anaerobic conditions, complete survival occurred; under O_2 tensions of about 8 and 16 mm. of Hg (1 and 2 per cent O_2, respectively), almost all the cells were protected; but with higher O_2 tensions, marked or complete cell death occurred.

Serum.—The ability of human serum to protect cells under the conditions of the standard assay was tested with the "altered" Kidney and Appendix cultures. No protective action was noted with 1 per cent serum, and for complete protection, approximately 5 per cent serum was required with both cultures. No diminution of activity was detected when the serum was heated (55°, 30 minutes).

DISCUSSION

The observation that mammalian cells kept in an atmosphere of air require added catalase for survival indicates an imbalance under these conditions be-

tween H_2O_2 formation and destruction. Their inability to cope with peroxide is somewhat surprising in view of the catalase levels found in extracts of these cells (15). On the other hand, cell damage may be preventable only by instantaneous removal of peroxide, requiring high levels of catalase activity. The protective role of catalase, minimized in favor of its peroxidatic activity (16), appears to warrant reemphasis.

The site of action of the added catalase is not known. Perhaps it functions in or on the cell membrane rather than in the internal milieu of the cell.

Cell survival in an O_2-poor atmosphere and with reducing agents was not surprising. In the O_2-poor atmosphere, the production of peroxide by the cell's flavin enzymes is probably depressed or abolished. Not so clear, however, is the mechanism of action of the effective reducing agents (ascorbate, thioglycollate, thiosulfate). Like catalase, they may remove H_2O_2, or more likely, may prevent its formation. The inactivity of cysteine and glutathione, despite their low redox potentials (17), is not understood.

The striking importance of the density of the cell population once again emphasizes the considerable interdependence of cultured mammalian cells. The results suggest that these cells are leaky and that adequate levels of catalase or of a vital reducing agent can be maintained inside the cell or attained in the surrounding medium only with sufficiently dense populations.

It is of interest to note that the protective effect of catalase on cells was first observed with a bacterium, the pneumococcus (18), which forms H_2O_2 but is not enzymatically equipped to dispose of it.

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