ENZYME FORMATION IN NON-VIABLE CELLS*

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INTRODUCTION

One of the crucial problems in the early history of "enzymatic adaptation" in microorganisms centered around the attempt to decide which of the following two mechanisms was involved in the phenomenon: (*a*) induction by substrate of enzyme synthesis; (*b*) selection by the substrate of existent mutants capable of forming the homologous enzyme. This is now a question of only historical interest since it has been well established with a variety of microorganisms and enzyme systems that both mechanisms exist and function. Particular attention has been focused in recent years on systems exhibiting induced synthesis in non-dividing cells and the results have been reviewed recently (1–3).

For the purpose of the present paper it is, however, of interest to note one particular approach used by the earlier workers in their efforts to decide the issue. It is quite evident that the decision as to the nature of the biological mechanism involved is greatly simplified in instances in which the enzyme activity can appear in populations in the absence of significant cell division. Here there can be no question of the selection of mutant types and one must in such cases conclude that substrate-induced synthesis of enzyme is occurring. The first apparently successful attempt to meet this criterion was that of Dienert (4) in the case of adaptation of yeast to galactose. The technique he employed depended primarily upon the use of populations suspended at extremely high densities in non-nutrient media under the assumption that cell division would not occur under such conditions. This method is obviously not completely free of possible complications. Dienert (4) was led to conclude from his findings that non-dividing yeast cells are able to "adapt" to the fermentation of galactose.

Subsequent authors used a somewhat different approach involving the use of lethal agents and conditions. Kluyver's (5) paper initiated a series of experimental attacks on the problem using this methodology. He attempted to obtain adaptation at temperatures high enough to prevent cell divisions with

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completely negative results. Euler and Nilsson (6) and subsequently Euler and Jansson (7) tried, also without success, to obtain adaptation in the presence of concentrations of phenol which inhibited cell division. Abderhalden (8) reported that he had observed adaptation with dried dead yeast cells. His experiments, however, involved extended incubations and his failure to check viability and the possibility of growth of a few surviving cells throws doubt on the validity of his conclusion. Stephenson and Yudkin (9) offered the first clear experimental evidence suggesting that non-viable cells were capable of enzyme synthesis. They subjected yeast cells to ultraviolet radiation and compared viability and capacity to form galactozymase. At doses yielding 30 per cent survivors they still retained over 70 per cent of the ability to form enzyme.

Despite the relative paucity of encouraging results in the literature it appeared desirable to extend the search for conditions which would yield a clear cut separation between viability and adaptability. It seemed not too unlikely that the information so obtained might be useful in the possible eventual realization of enzyme formation in an *in vitro* cell-free preparation. Accordingly a survey was conducted of the effect of various lethal agents at differing survival dosages on the capacity of yeast cells to synthesize maltozymase and galactozymase.

It is the purpose of the present paper to summarize the results and present the details of some representative experiments. In the course of this survey it was found that complete dissociation between viability and enzyme-synthesizing capacity could be obtained with x-rays. Enzyme formation was found to be unimpaired in cells subjected to 380,000 r which results in a 99.996 per cent kill. The findings with x-rays were briefly described in a preliminary communication by Spiegelman, Baron, and Quastler (10). Simultaneously, Brandt, Freeman, and Swenson (11) reported analogous results with yeast adapting to galactose. These authors achieved a 90 per cent kill with no observable effect on enzyme synthesis. While highly suggestive, such results do not constitute conclusive evidence that enzyme formation was observed in cells incapable of division. In the first place, a tenfold stimulation of the 10 per cent surviving cells by products released from the non-survivors could explain the observation. More important the dose employed was extremely low, being at a level of 4,580 r. Holweck and Lacassagne (12) have shown that below 30,000 r, death of yeast cells exposed to x-rays is not an immediate but a postdivisional event. In view of these findings it is not impossible that Brandt *et. al.* (11) were examining the enzyme-forming capacity of their irradiated suspensions during a period in which the vast majority of the cells were still capable of one or more divisions.

Methods and Materials

Strain and Conditions of Growth.—A diploid representative of *Saccharomyces cerevisiae* (strain K) was used in these experiments. It can be induced to form malto-

zymase and galactozymase when suspended in a phosphate buffer solution of substrate. The cells were grown in a medium made by adding the following to 1 liter of water: glucose, 40 gm.; difco-bactopeptone, 5.0 gm.; difco-yeast extract, 2.4 gm.; $(NH_4)_2SO_4$, 2.0 gm.; $CaCl_2$, 0.25 gm.; $MgSO_4$, 0.25 gm.; 60 per cent sodium lactate, 6 ml. Solid medium was made by adding 20 gm. of bacto-agar to the above. Cultures were developed in 125 ml. Erlenmeyer flasks containing 50 ml. of the above medium unagitated at 30°C. Unless otherwise specified 24 hour cultures were employed. Cells were harvested by centrifugation immediately prior to use and washed twice with cold buffer. They were then resuspended in cold M/15 KH_2PO_4–succinate buffer (pH 4.5) and adjusted to the desired cell density by the use of a Klett-Summerson photoelectric colorimeter previously calibrated for this purpose.

Measurement of Enzyme Formation.—Enzyme synthesis was followed manometrically at 30°C. under aerobic conditions in the usual Warburg apparatus. The two cup method (13) was used which permits the simultaneous and continuous measurement of Q_{O_2} and $Q_{CO_2}^{O_2}$. Enzyme activities are expressed as $Q_{CO_2}^{O_2}$ observed in the presence of the corresponding substrate and corrected for endogenous respiration. Final volume in all vessels was 2.0 ml. and substrate concentration was 2 per cent.

In control inductions with untreated cells each vessel contained in the neighborhood of 4 mg. dry weight of cell material. When, however, experimental treatments resulted in severe loss in viability and enzyme-forming capacity, the amount used was increased accordingly.

Merck's pure maltose and galactose were each further purified by two recrystallizations from 60 per cent alcohol.

Viability Measurement.—Determinations of viable cell counts were performed on the contents of the Warburg vessel subsequent to the measurement of enzyme-forming capacity. Suitable aliquots of appropriate dilutions were spread by means of bent sterile glass rods on agar plates containing the medium described above. These plates were incubated at 30°C. and counted at the end of four days.

In certain crucial experiments to be detailed, viability in terms of the capacity to go through one cell division was determined by direct microscopic examination of cells plated on the agar medium.

RESULTS

Lethal Agents or Treatments Which Lead to Physical Destruction.—There are a number of methods of varying degrees of effectiveness available which lead to the disruption of the yeast cell. Presumably a population exposed to anyone of those procedures would contain in various proportions individuals injured to differing degrees extending from unharmed viable cells to the completely disrupted. The procedures used may be listed and briefly described as follows:—

Freezing and Thawing.—A paste of freshly prepared cells was mixed with powdered solid CO_2 and ground in an automatic grinder for 15 minutes, allowed to thaw, and the process repeated.

Supersonic Vibration.—A suspension of cells was placed in the vessel of a Raytheon and subjected to an exposure of 30 or more minutes at 10 kilocycles. The temperature was kept low by circulating cold water.

Grinding with Alumina.—The procedure followed stemmed from the description of McIlwain (14). An amount of alumina (Alcoa No. 301) equal in weight to the packed cells was added. The mixture is ground in the cold for 5 minutes. Cell disruption is easily recognized by the onset of liquefaction.

Grinding with Powdered Glass.—Corning powdered glass (150 mesh) was used. The procedure is identical to that employed in the case of alumina except that more extensive (15 to 30 minutes) periods of grinding are required.

Cytolysis with Toluene.—0.1 ml. of toluene was added per 10 ml. of a thick suspension of cells in phosphate-succinate buffer. This was then shaken at 30°C for 30 minutes.

TABLE I

Comparison of Viability and Residual Capacity to Form Maltozymase

Viable cells were determined by the usual spread plate technique on aliquots removed from the Warburg vessels at the end of the determination of enzyme-forming capacity. The latter was measured by the usual two cup method in the presence of 2 per cent substrate. The enzyme activities achieved by the treated preparations are reported as the percentage of that attained in the control cells.

Method of cell destruction	Survivors	Enzyme activity achieved as per cent of controls
	per cent	
1. Freezing and thawing	20	24
2. Supersonic vibration	10	18
3. Grinding with alumina	2	<1
4. Grinding with powdered glass	8	12
5. Cytolysis with toluene	0.1	<1
6. Cytolysis with solid lactose	66	63
7. Vacuum drying	10	20

Cytolysis with Solid lactose.—Solid lactose was added to and mixed with a freshly prepared cell paste until cytolysis, as evidenced by liquefaction, is observed.

Dried Cell Preparations.—A freshly prepared cell paste was spread on the inside walls of a beaker which was then placed in a desiccator over $CaCl_2$. Continuous vacuum was applied for a 5 hour period.

Following each of the treatments an aliquot of the mixture was removed and a suitable dilution tested for enzyme-forming capacity by the manometric method. In each case untreated controls from the same cells were run in parallel. Table I summarizes the data obtained in some representative experiments in which viability and the capacity to synthesize maltozymase were examined. Similar results were obtained in the case of galactozymase formation. It is evident that the data do not suggest that any of the procedures employed effects a significant separation of viability and enzyme-synthesizing capacity. Attention was therefore turned to lethal agents such as ultraviolet light, nitrogen mustard, and x-rays which leave cell structure more or less intact.

Ultraviolet and Nitrogen Mustard.—

The effect of ultraviolet irradiation was studied with the aid of a 15 watt germicidal bulb of the low pressure type (General Electric Co.). The beam emitted contains radiations mainly in the region of 2537 A. The irradiation was carried out on 4 ml. aliquots contained in open petri dishes 7.1 cm. in diameter which were placed at a distance of 15 cm. from the lamp. Stirring of the cell suspension during the irradiation was accomplished with a magnetic stirrer. After suitable exposures the cells were removed, washed, and resuspended in buffer to the desired density and their enzyme-forming capacity measured manometrically. Aliquots were removed from the Warburg vessels at the end of the run for viability determinations.

TABLE II

Comparison of Viability and Capacity to Form Maltozymase in Cells Exposed to Ultraviolet Irradiation and Nitrogen Mustard

Viable cells were determined by the usual spread plate technique on aliquots removed from the Warburg vessels at the end of the determination of enzyme-forming capacity. The latter was measured by the usual two cup method in the presence of 2 per cent of substrate. The enzyme activities achieved by the treated preparations are reported as the percentage of that attained in the control cells.

Agent	Dose	Survivors	Enzyme activity achieved as per cent of controls
		per cent	
Ultraviolet	0.5 min.	32	76
	1.0 min.	4	<1
	2.0 min.	0.01	<1
Nitrogen mustard	0.05 per cent	13	62
	0.2 per cent	0.01	<1

In examining the effect of nitrogen-mustard, cell suspensions in phosphate buffer were exposed to varying concentrations of this agent for 10 minutes. Subsequent to this treatment the cells were washed and resuspended in buffer. Maltozymase-forming capacity and viability were determined as described above.

The results with both ultraviolet and nitrogen-mustard are summarized in Table II. It will be noted that in agreement with the results of Stephenson and Yudkin (9) some separation of viability from adaptability is suggested by the fact that 76 per cent of enzyme-forming capacity is observed in suspensions containing only 32 per cent surviving cells. Swenson and Giese (15) also provide evidence of separation of viability and adaptability in yeast cells exposed to ultraviolet. They observed that the inhibition of enzyme-forming capacity can be more easily reversed by photoreactivation.

It is of interest to note from Table II that essentially similar results are obtained with nitrogen-mustard, a substance which mimics ultraviolet in many

of the latter's mutagenic and other biological effects. In the case of the nitrogen-mustard experiments, suspensions are obtained containing only 13 per cent survivors which nevertheless exhibit 62 per cent of the control enzyme-forming capacity.

As was noted in the introduction, it is unfortunately not possible to conclude with certainty from such results that we have here an instance of enzyme formation by non-viable cells. The possibility remains that these or the analogous observations cited earlier can be explained by stimulation of the surviving cells by substances released from the killed cells and that the latter do not in fact participate in the process of enzyme formation.

TABLE III

Effects of X-Rays on Rate and Amount of Maltozymase Synthesized

Proportion of viable cells was determined by the usual spread plate technique on aliquots removed from the Warburg vessels subsequent to the determination of enzyme-forming capacity. Rates of enzyme synthesis were determined from the slopes in the linear portions obtained in semilog plots of $Q^{O_2}_{CO_2}$ on maltose *versus* time. Final enzyme activity attained is recorded in terms of $Q^{O_2}_{CO_2}$.

Dose	Survival	Rate of enzyme formation	Per cent of control	Final activity achieved ($Q^{O_2}_{CO_2}$)	Per cent of control
r	*per cent*				
20,000	60	0.042	110	200	130
73,000	20	0.043	105	235	120
97,000	6.1	0.041	102	240	138
240,000	1.0	0.042	111	250	158
365,000	0.01	0.042	112	315	160
380,000	0.006	0.043	106	300	159

X-Rays.—

Irradiations with x-rays were carried out with a machine set at 150 kv. at 8 ma. with no filter and capable of delivering 2500 roentgens per minute at the platform setting used. Cell suspensions were irradiated in shallow vessels prepared from 50 ml. beakers. Stirring during the irradiation was accomplished with a magnetic stirrer.

Subsequent to the x-irradiation the cells were treated as noted in the description of the ultraviolet experiments of the previous sections.

Table III summarizes the results of these experiments. Rates of enzyme formation were determined by the slopes of the linear portions of the semilog plots of the log $Q^{O_2}_{CO_2}$ *versus* time. The final levels of enzyme achieved in terms of $Q^{O_2}_{CO_2}$ are also included. It is evident that x-rays appear to effect a complete separation of viability from adaptability. Dosages which depress the survivors

to 0.006 per cent of the initial viable count leave the enzyme-forming capacity of the population unimpaired. In point of fact, there is a consistent and sig-

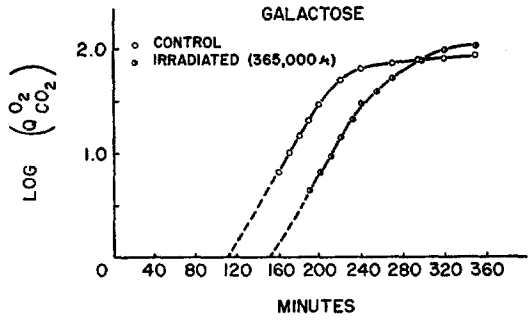

FIG. 1. Effect of x-irradiation on the formation of galactozymase. Substrate was tipped in at zero time. Enzyme was measured continuously by the two cup method and expressed as $Q^{O_2}_{CO_2}$. The rates of enzyme synthesis are proportional to the slopes of the linear portion of the semilog plots indicated. The length of the lag period is obtained from the distance to the y-axis of the points of intersection of the extrapolations to the zero time enzyme levels. The latter are virtually zero in the case of galactozymase.

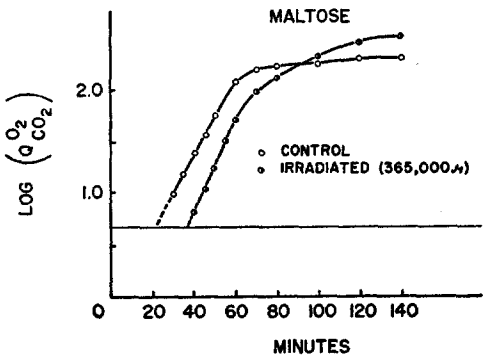

FIG. 2. Effect of x-irradiation on the formation of maltozymase. The conditions and methods of measurement are identical to those indicated in Fig. 1. Maltose at a level of 2 per cent was tipped in at zero time. The horizontal line to which the curves are extrapolated represents the zero time or "basal" enzyme content of unadapted cells.

nificant increase in both the rate and amount of enzyme formed as compared with the untreated controls. Figs. 1 and 2 detail the results of an experiment at a dosage of 365,000 r which yielded 0.01 per cent survivors. As may be seen from a comparison between treated and control cells of Figs. 1 and 2, the galactozymase- and maltozymase-forming systems responded in an identical

manner to the irradiation. In both cases the only inhibitory effect seen is a slight increase in the lag period which preceded the onset of enzyme formation. Once begun, the rate of enzyme formation in the irradiated cells is virtually identical to that observed in the controls in the case of galactose, and slightly higher in the case of maltose. It will be noted that in neither system does the irradiation affect the nature of the kinetics, being exponential in both treated and control cells.

The rather sharp dissociation between viability and adaptability apparently achieved with x-rays required a more detailed analysis. In the experiments thus far described, viability was investigated in terms of the production of visible clones on agar plates, a process which requires many divisions and a considerable amount of time. On the other hand measurement of enzyme-forming capacity is a procedure which is completed in a matter of hours. It was conceivable that many of the x-irradiated cells examined were capable of undergoing a limited number of divisions, a possibility which would not be detected by the usual viability test. Its existence would, however, clearly vitiate the conclusion that enzyme formation was being induced in cells incapable of division. Enzyme synthesis might well have occurred during the period in which one or more cell divisions are still possible. That this is indeed a not unlikely possibility follows from the work of Holweck and Lacassagne (12) which has already been cited as well as from the similar results obtained by Wyckoff and Luyet (16).

The x-ray dosages included in the present investigation were much higher than were indicated as minimally necessary for "immediate" killing by the investigations of Holweck and Lacassagne (12). However, to make certain, experiments were performed using their procedure for determining the proportion of "immediate" *versus* "postdivisional killing" at dosages of 365,000 r. This involves direct microscopic observation on agar plates for the capacity of irradiated cells to produce one or more buds after suitable periods of incubation which extended over 18 hours. The results indicate that less than 0.1 per cent of the treated cells was capable of 1 division at this dosage. Hence the vast majority of the irradiated cells were non-viable by this direct examination. As a further test, a comparison was made of the effect of incubation subsequent to x-irradiations at a level of 380,000 r on the observed enzyme-forming capacity. No differences were observed even with incubations of 18 hours. One may therefore be relatively certain that enzyme formation was being observed in cells completely incapable of undergoing cell division.

In all the experiments thus far described, the examination for enzyme-forming capacity was done on cells suspended in buffer solution of substrate. Spiegelman and Dunn (17) had demonstrated that enzyme formation under such conditions could be greatly stimulated by the addition of small amounts of a nitrogen source. It was of interest to see whether x-irradiated cells responded in the same manner. To test this, cells prepared in the usual manner

were irradiated with a dose of 380,000 r yielding a survival of 0.005 per cent. The ability of such cells to form maltozymase in the presence and absence of an added external nitrogen source in the form of NH_4Cl was examined. The results are described in Fig. 3 which includes for comparison the data obtained with the unirradiated controls. It is evident that the enzyme-forming capacity of irradiated cells responds just as dramatically to the addition of an external nitrogen source as does the capacity of the untreated controls. It must be concluded that though unable to divide, the vast majority of cells treated with 380,000 r are still capable of assimilating nitrogen and employing the resulting amino acids formed for the synthesis of enzyme.

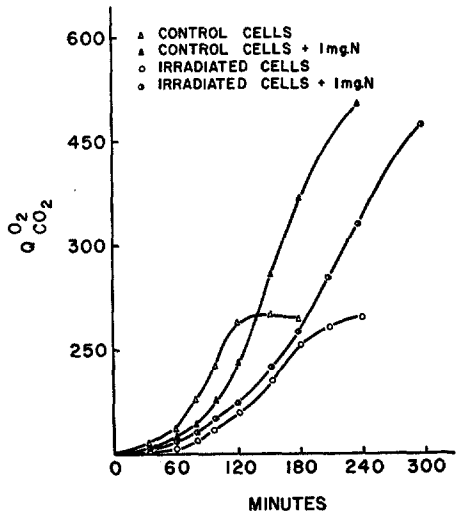

FIG. 3. The effect of an external nitrogen source on the enzyme-forming capacity of irradiated and non-irradiated cells.

DISCUSSION

The data obtained in the present investigation are consistent with the earlier reports in the literature which indicated that the application of most lethal agents or conditions results in a parallel loss of viability and ability to form enzyme. The findings of Stephenson and Yudkin (9) which suggested a partial separation of these two properties by means of ultraviolet irradiation have been confirmed. The fact that an almost identical situation can be obtained with nitrogen mustard adds another instance of similarity between these two mutagenic agents. These results recall the quite analogous finding of Herriot and Price (18) who demonstrated that mustard gas could inhibit cell division without interfering with virus synthesis.

It is of interest to note that for both nitrogen-mustard and ultraviolet the indication of a dissociation between cell division and enzyme-synthesizing

capacities holds in a relatively narrow region. As soon as dosages are reached with either agent which effect a kill of 99 per cent or over, enzyme-forming ability drops to the vanishing point. It is of course this feature which makes it difficult to be certain that a real separation has been achieved with these agents, even at the lower doses. Actually, ultraviolet represents a markedly effective agent for stopping enzyme formation and this property has been employed by Swenson (19) to analyze the nature of the enzyme-forming system. The action spectrum obtained suggests that a nucleic acid or nucleoprotein is involved.

The behavior of x-rays is in sharp contrast to all other lethal agents tested. A clear cut dissociation is provided at survival levels which preclude ascribing the residual enzyme-forming capacity solely to the stimulation of the small proportion of surviving cells. A stimulation of the order of 1×10^5 would be required to explain the observed results.

In the course of the present investigation no level of x-ray dosage achievable exerted other than a stimulatory effect on the rate and amount of enzyme formation in yeast. It is to be presumed, however, that dosages could be attained which would inhibit enzyme synthesis. With bacteria at any rate such levels have been reached. Billen and Lichstein (20) found that doses exceeding 60,000 r virtually eliminated the capacity of *Escherichia coli* to form hydrogenlyase. It is to be noted, however, that with a dose of 15,000 r which yielded 1.5 per cent survivors, 59 per cent of enzyme-forming capacity was retained. Again, at 30,000 r which yielded 0.03 per cent survival, approximately 28 per cent of the enzyme-synthesizing ability of the untreated controls was observed. Though not stressed by the authors, it would appear that they achieved a significant dissociation in this system.

Recent investigations with yeast by Halvorson and Spiegelman (21, 22) into the nature of the precursor in the induced synthesis of enzyme revealed that the free amino acids constitute the primary nitrogen source in the fabrication of new enzyme molecules. Any agent or condition which makes the free amino acid pool unavailable exerts a parallel and proportionate inhibition of enzyme synthesis. The results obtained indicate that any cell capable of synthesizing enzyme is capable of incorporating free amino acids into protein. The data reported in the present paper are consistent with this view since they indicate that cells exposed to 380,000 r retain along with their enzyme-forming capacity the ability to incorporate and utilize for enzyme synthesis externally supplied inorganic nitrogen. A more direct analysis of this aspect of the problem seems desirable.

SUMMARY

An attempt has been made to find conditions which would completely dissociate viability from capacity to synthesize enzymes in yeast cells. The only

lethal agent or condition found which yielded complete dissociation was x-irradiation. Dosages leading to 99.9 per cent lethality and greater exhibited no inhibitory action on the capacity of the treated suspensions to synthesize maltozymase and galactozymase. Such suspensions also retained their ability to assimilate externally supplied nitrogen and employ it in the synthesis of enzyme.

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