THE FORMATION AND STABILIZATION OF AN ADAPTIVE ENZYME IN THE ABSENCE OF ITS SUBSTRATE*

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I

INTRODUCTION

One of the most crucial aspects of enzymatic adaptation is the rôle of substrate in the induction and maintenance of enzyme activity. The experimental facts available are apparently unambiguous. The presence of substrate seems to be necessary for the appearance of the corresponding enzyme, and the removal of substrate leads to the disappearance of the enzyme it induced.

Most attempts to explain both the inducing and stabilizing effects of substrate on the so called "adaptive enzymes" have assumed that these enzymes form a class characterized by an unique *instability* in the absence of substrate. However, if such explanations are not to remain mere restatements of the experimental findings, a more precise concept of enzymatic stability must ultimately be provided. The need for such clarification is pointedly emphasized by the results of recent investigations in bacteria (1) and the yeasts (2), which have indicated the existence of competitive interactions among the enzyme-forming systems of the cell. The induction of a new enzyme in a cell can result in the disappearance of an existent enzyme and the suppression of others. In yeasts (2) the severity of this type of interaction can be reduced greatly by furnishing an exogenous nitrogen source.

Since the formation of a new enzyme can modify the activity levels of existent enzymes, enzymatic stability may be a more complex phenomenon than the more or less purely chemical concept which has thus far been considered. The existence of competitive interactions makes it pertinent to inquire whether the observed instability of the adaptive enzymes necessarily reflects their *chemical stability* as organic molecules.

It is evident that the maintenance of a particular enzyme in the cytoplasm can be determined by either one or both of the following factors: (1) The chemical stability of the enzyme as a protein molecule. (2) The competitive effectiveness of the synthesizing mechanism involved in its formation.

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The effect of substrate in the induction and maintenance of a specific enzymatic activity could be mediated by either of these two factors.

It is the object of this paper to present experiments designed to assess the relative importance of these factors in the mechanism whereby substrates influence cellular enzymatic constitution.

The experimental procedures adopted depend primarily on arranging conditions which would tend to minimize or completely to abolish competitive interaction among the enzyme-forming systems of the cell. It was reasoned that, if the latter is a controlling element in the modification and maintenance of enzymatic constitution, then the reduction of such interactions should markedly increase the stability of the unstable adaptive enzymes even in the absence of their substrate. It might also be expected that the ability of substrate both to induce and maintain its enzyme would be augmented under such conditions.

Two general methods were employed to reduce the severity of competitive interactions among the enzyme-forming systems of the cell. One was to supply sufficient nitrogenous material and optimum conditions for their use, so that the requirements of all nitrogen-synthesizing systems could be satisfied. The second was to inhibit all enzyme formation so that no competitive interaction could occur. As will be seen, both of these methods resulted in marked stabilizations of enzyme content independent of the presence or absence of substrate.

All the experiments reported here were done with adaptation to galactose fermentation in yeast. This system was chosen because it represents one of the most carefully and fully investigated cases of enzymatic adaptation.

II

Methods and Materials

Two representatives of *S. cerevisiae* and one of *S. carlsbergensis* were used in the experiments reported. The strains employed, A1, K1, and C1, are the same as those used in previous investigations.

The details relating to the media employed, the methods for handling stock cultures, and the preparation of standard suspensions may be found in preceding (3) papers.

All enzyme activity measurements were performed at 30.2°C. with the standard Warburg apparatus. Anaerobiosis was established by replacing the air with nitrogen. Enzyme activities are expressed in terms of $Q_{CO_2}^{N_2}$ (cubic millimeters of CO_2 released anaerobically per milligram of dry weight per hour). In certain instances which are detailed in the text, it was more convenient to express activities in numbers proportional to $Q_{CO_2}^{N_2}$. Inaccuracies due to retention of CO_2 were reduced by using M/15 KH_2PO_4 as the suspending medium for the cells. Unless otherwise specified, measurement of enzymatic activity with respect to a given substrate was made with the latter at 3 per cent.

With the exception of modifications noted in succeeding sections, all adaptations were carried out with washed cells suspended in M/15 KH_2PO_4 in the presence of 4

per cent of the adapting substrate. These suspensions were shaken in flasks in a water bath held at 30.2°C. Adaptations under anaerobic conditions were carried out in large Warburg flasks, filled with nitrogen.

The density of the experimental suspensions was determined with the aid of a colorimeter (Klett-Summerson photoelectric colorimeter, filter 42), which was calibrated in terms of dry weight (milligrams per cubic centimeter of suspension).

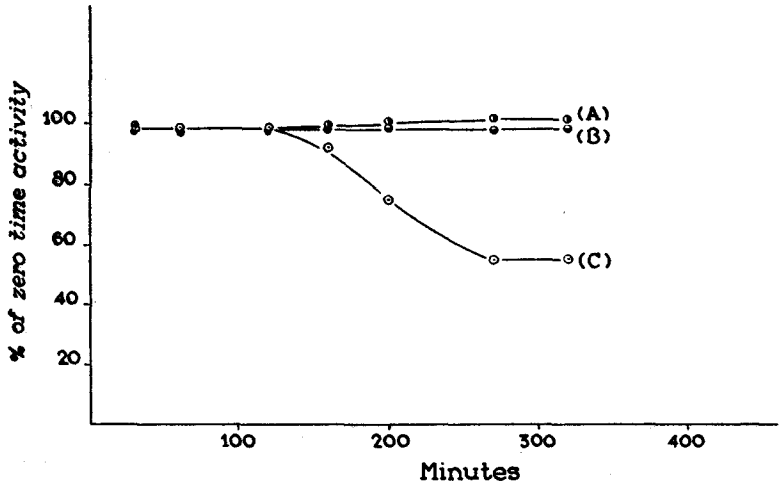

FIG. 1. The loss of adaptive galactozymase activity during the anaerobic fermentation of galactose (*C*). Illustrating the protective effect of exogenous nitrogen on the enzyme activity (*A*). Curve (*B*) shows the relatively greater stability of glucozymase even in the absence of nitrogen.

III

EXPERIMENTAL RESULTS

(*a*) *Factors Influencing the Capacity of Galactose to Stabilize Galactozymase*

There is no doubt that the removal of substrate often leads to the rapid disappearance of the corresponding enzyme. Nevertheless, the stabilization capacity of substrate is not an absolute one, and its presence does not guarantee the indefinite maintenance of activity. This can be seen clearly in some of the experiments reported by Stephenson and Yudkin (4), who showed that, even in the presence of galactose, the introduction of glucose can result in a marked diminution of galactozymase activity. However, the introduction of another substrate is not necessary to observe such losses. All strains of yeast examined, when fully adapted to galactose and allowed to ferment this hexose in phosphate buffer, will begin to lose activity after varying periods of time.

A case of this kind is exhibited in curve (*C*) of Fig. 1. This curve was ob-

tained by starting with a fully adapted culture of strain A, which was suspended in phosphate buffer containing 4 per cent galactose and allowed to ferment under anaerobic conditions. At intervals aliquots were removed, and the galactozymase and glucozymase activities determined. Even though this culture had been in continual contact with substrate, the galactozymase activity fell to 55 per cent of its initial activity. During the same period the glucozymase activity, which is given by curve (*B*), remained constant.

The loss of galactozymase during the metabolism of galactose is connected with the conversion of nitrogenous compounds. This is clear from curve (*A*) of Fig. 1, which describes the galactozymase activities of a suspension identical in origin with that employed for curve (*C*). In the case of curve (*A*), however, the fermentation of the galactose occurred in the presence of an exogenous source of nitrogen. The amount of nitrogen added (as $(NH_4)_2SO_4$) was equivalent to only 50 per cent of the nitrogen content of the cells. This amount of nitrogen is not sufficient to yield more than 5 per cent new cells under the conditions of the experiment. Nevertheless, complete stabilization of enzyme activity is observed. In the absence of substrate this amount of nitrogen is completely incapable of maintaining the galactozymase.

That relatively small amounts of nitrogen greatly augment the capacity of substrate to influence enzymatic constitution has already been seen (2) in the curves of enzyme *appearance*. Here, we see that the same amount of nitrogen can greatly augment the capacity of substrate to prevent the *disappearance of* the homologous enzyme. It seems likely, therefore, that we are dealing with the same phenomenon in these two instances; *i. e.*, the addition of an extra nitrogen source makes it easier for the galactozymase-forming system to make more enzyme.

These experiments do not permit a decision as to the primary mechanism underlying the disappearance of the adaptive enzyme in the presence of the substrate. They serve, however, to emphasize that conditions and factors other than substrate availability are of importance in determining enzyme activity levels. They further make unlikely any hypothesis of substrate function which depends solely on the capacity of substrate to stabilize the molecular structure of the enzyme. The marked ability of glucose to depress the stabilizing capacity of galactose and of ammonia to increase it are also not readily interpretable in such terms.

On the other hand, if competitive interactions are quantitatively important in determining enzymatic constitution, these results are not unexpected. The ability of substrate to stabilize its enzyme would be interpreted in terms of such factors as availability of nitrogen and the nature of other enzymes being used and formed by the cell.

(b) Galactozymase in Resting Suspensions Metabolizing Various Substrates

The competitive interaction interpretation of the inability of galactose completely to prevent galactozymase disappearance implies that enzyme turnover is taking place in a metabolizing cell. Under such conditions a slow loss of enzyme protein by the galactozymase-forming system to more active synthetic components could occur.

This viewpoint would predict that the capacity of a cell to retain galactozymase activity in the absence of exogenous nitrogen and galactose would depend on the level of metabolic activity and on the type of substrate being

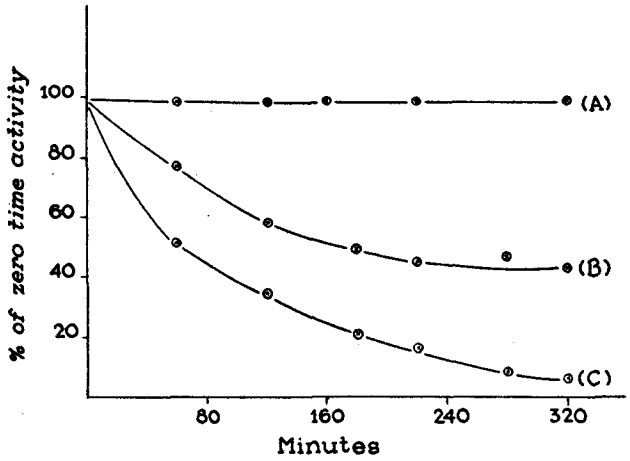

FIG. 2. The effect of endogenous respiration (*B*) and glucose utilization (*C*) on the stability of the adaptive galactozymase under aerobic conditions. The control curve (*A*), representing the results of aerobic incubation with galactose, illustrates the greater stability of the enzyme in this strain (K) as compared with the strain used in the experiments of Fig. 1.

metabolized. Presumably, the utilization of certain substrates could result in more severe interactions with the galactozymase system than others. It was therefore of some interest to see whether this was the case.

In order to magnify any differences in interaction effects, it was necessary to carry out these experiments with suspensions in phosphate buffer without any exogenous nitrogen. To provide a better basis of comparison, strains were employed here, which possessed a relatively better capacity to retain galactozymase activity than that exhibited by strain A in Fig. 1. Cultures fully adapted to galactose were washed free of medium and suspended in M/15 KH_2PO_4, to which was added the substrate to be metabolized. These suspensions were then incubated aerobically while shaking in a 30°C. bath. Aliquots

were taken at intervals, washed by centrifugation in the cold, resuspended in their original volumes of M/15 KH_2PO_4, and galactozymase activities determined.

The behavior of such suspensions is clearly illustrated in Fig. 2 which sets forth the galactozymase activities of strain K at various intervals of time while metabolizing galactose (curve (*A*)), endogenous reserves (curve (*B*)), and glucose (curve (*C*)). The relative stability of the galactozymase system in this strain metabolizing galactose in a nitrogen-free medium is illustrated by curve (*A*). It must, however, be noted that a fall in activity is observed if the incubation is carried out much longer. It is clear that the metabolism of the endogenous reserves leads to a considerable loss in galactozymase activity. The rate of decrease diminishes after about 120 minutes, and this may be connected with the fact that the rate of the endogenous respiration also begins to

TABLE I

Effect of the Metabolism of Various Substrates on Stability of Galactozymase

Activities are reported as microliters of CO_2 produced anaerobically from galactose per 30 minutes. The per cent entry gives the decrease in activity as per cent of zero time control. Respiration rate is given in terms of c. mm. of O_2 taken up per 30 minute period in the presence of the corresponding substrate at 0.01 M concentration.

Sample	Zero time	Galactose	Buffer	Glucose	Fructose	Alcohol	Pyruvate
Activity	474	429	316	149	147	261	265
Decrease, *per cent*	—	9	33	69	69	45	42
Respiration rate	—	191	87	200	179	256	131

drop sharply at this point. If the incubation is carried out for extensive periods of time (20 hours or more) the activity will, under these conditions, finally drop to about 5 per cent of its initial value. In the case where glucose is metabolized, a very rapid disappearance of the adaptive enzyme is observed. The reason for the more rapid decline of galactozymase activity in the suspension metabolizing glucose cannot be arrived at from these experiments. There exists here a difference in substrate as well as in metabolic rate, and either or both of these factors may be responsible. Nevertheless, it is evident that retention of the adaptive enzyme in the absence of substrate is dependent upon the metabolic activity of the cells.

Similar experiments in which these as well as other substrates were tested were carried out with strain C. Some typical results are recorded in Table I. For other than the zero time value, the numbers in the activity row in this table are those attained after 280 minutes of aerobic incubation at 30°C. in the presence of the corresponding substrate. The general pattern of behavior of the galactozymase with respect to the metabolism of galactose, endogenous reserves,

and glucose is the same as that observed with strain K. The metabolism of fructose appears to have a quantitative effect similar to that obtained with glucose. The consumption of both alcohol and pyruvate, however, results in a significantly smaller deterioration of galactozymase than that of the two hexoses. A comparison of the respiratory rates shows that this difference cannot be attributed to correspondingly lower metabolic rates for the alcohol and pyruvate. The former is actually metabolized at a higher rate than either hexose. Further, although alcohol is oxidized twice as fast as pyruvate, their efficiency in depressing galactozymase activity is virtually identical.

We have here a relatively clear cut case in which the type of substrate metabolized influences the stability of an adaptive enzyme in the absence of its substrate. This result is consistent with the hypothesis that competitive interactions are fundamental in the maintenance and modification of enzyme constitution. Aside from the general finding that different substrates vary in their effectiveness in suppressing the galactozymase activity, it is of some interest to note the order. Those which are most similar to galactose, *i. e.* glucose and fructose, are the most effective. This is the kind of order which would be expected if the intensity of competitive interaction is most pronounced between enzyme-forming systems controlling activity levels of enzymes handling the most closely related substrates.

(*c*) *Stabilization of Galactozymase in the Absence of Substrate*

The experiments described thus far show that the presence of substrate alone is not *sufficient* for the complete maintenance of the corresponding enzyme. It is the purpose of the present section to show that the presence of substrate is not *necessary* for enzyme maintenance.

In discussing the nature of adaptive enzyme formation and the rôle of substrate, it was pointed out that an assessment of the relative importance of competitive interactions could be attained by comparing enzyme stability under conditions where such interactions exist with those in which they have been suppressed.

Since effective competitive interaction presumably would occur only between actively synthesizing systems, suppression of interaction would be attained with the aid of any agent or condition which depressed enzyme formation to a considerable extent.

There are adequate experimental data available which demonstrate that the existence of an active metabolism is necessary for enzyme formation. It has been shown by various investigators (5, 6) that yeasts suspended under anaerobic conditions in the absence of an exogenous fermentable substrate metabolize at a negligible rate. Under similar conditions adaptive enzyme formation either does not occur at all or does so at a very low rate (7). These facts suggest that competitive interaction among enzyme-forming systems would

be sharply reduced in cells suspended in a substrate-free medium under anaerobic conditions. If such interactions do determine enzymatic stability to any considerable extent, an adaptive enzyme in the absence of substrate should be much more stable under anaerobic than under aerobic conditions, provided no other exogenous substrate is present.

A comparison of this kind between the aerobic and anaerobic stability of galactozymase is shown in Fig. 3. A galactose-adapted culture was washed and resuspended in M/15 KH_2PO_4. A portion of this suspension was shaken in air (curve (*A*)) at 30°C. and another shaken in nitrogen (curve (*N*)) at the same temperature. Samples were removed at intervals and galactozymase

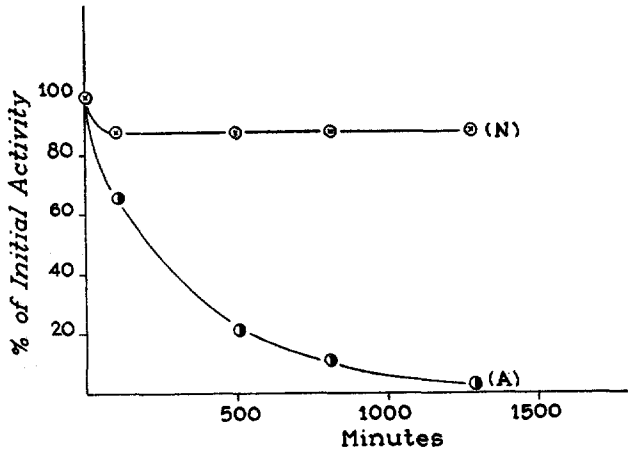

FIG. 3. The stabilization of adaptive galactozymase by anaerobiosis in the absence of substrate. The rapid disappearance of activity under aerobic conditions (curve (*A*)) contrasts with the marked stability appearing during incubation under N_2 (curve (*N*)).

activity levels determined. It is clear from a comparison of curves (*N*) and (*A*) that in the absence of active metabolism the presence of substrate is not necessary for the maintenance of the adaptive enzyme.

Another and perhaps more significant method of accomplishing a similar situation is provided by some recent experiments with inhibition of enzyme formation. It was shown (8) that the presence of NaN_3 in concentration of 2×10^{-3} M prevented the cell from utilizing the energy generated by anaerobic carbohydrate metabolism for adaptive enzyme formation. It should be recalled that this concentration of azide does not interfere with anaerobic fermentation of carbohydrate. In every instance the addition of the azide stopped any further enzyme formation, and the suspension maintained the activity attained at the time of the azide addition. If azide effectively blocks enzyme synthesis

in general, it can be expected, in view of the findings with anaerobiosis, that azide would also prevent the loss of any existent enzyme activity, no matter what substrate is being metabolized by the cell. Fig. 4 records the results of some experiments devised to test this expectation.

A fully adapted culture was washed and a suspension in M/15 KH_2PO_4 prepared in the usual manner. Galactozymase activity level was determined on an aliquot immediately. The remainder was dispensed in equal amounts into four flasks, into each of which was introduced an amount of glucose sufficient

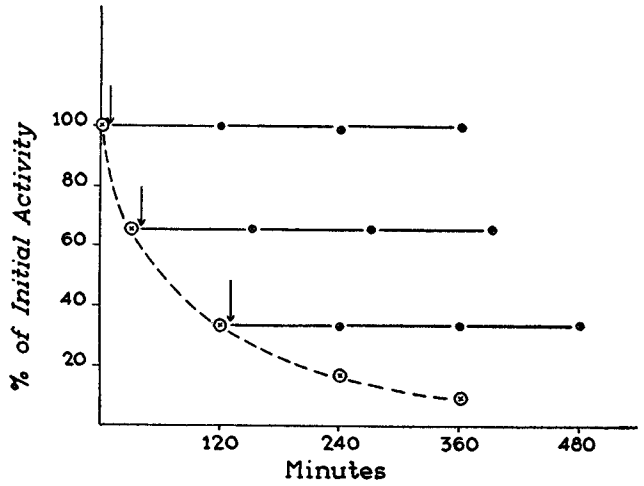

FIG. 4. The stabilization of adaptive galactozymase by NaN_3 during the anaerobic fermentation of glucose. The arrows indicate points where NaN_3 was added. The small circles (solid lines) show that NaN_3 prevented enzyme breakdown no matter at what level of enzyme activity the inhibitor was added. The large circles (dotted curve) show the loss of enzyme which occurs in the control suspension not treated with NaN_3.

to make a 4 per cent solution after anaerobiosis was established. In one flask azide (2.5×10^{-3} M final concentration) was put in immediately with the glucose. In a second, the azide was introduced after 30 minutes of glucose fermentation, by which time the galactozymase activity had fallen 34 per cent. The third flask received azide after 2 hours' anaerobic incubation with glucose. The fourth flask acted as an azide-free control. In each case, immediately before the introduction of the azide a sample was removed, washed in chilled buffer in the cold to remove the glucose, and galactozymase activity determination made. The large circles in Fig. 4 represent the galactozymase activity values attained by suspensions fermenting glucose in the absence of azide. The smaller circles show the behavior of galactozymase in cells fermenting

glucose subsequent to the addition of the azide. It is evident that in every instance the addition of azide stops any further enzyme disappearance, and the suspension maintains the activity attained at the time of the azide addition. A comparison of these results with those obtained with azide on anaerobic enzyme formation (8) reveals that the effect of azide on enzyme appearance is exactly similar to its effect on enzyme breakdown. In both cases azide can "freeze" the enzymatic constitution of the cell at that composition obtaining at the moment of its addition.

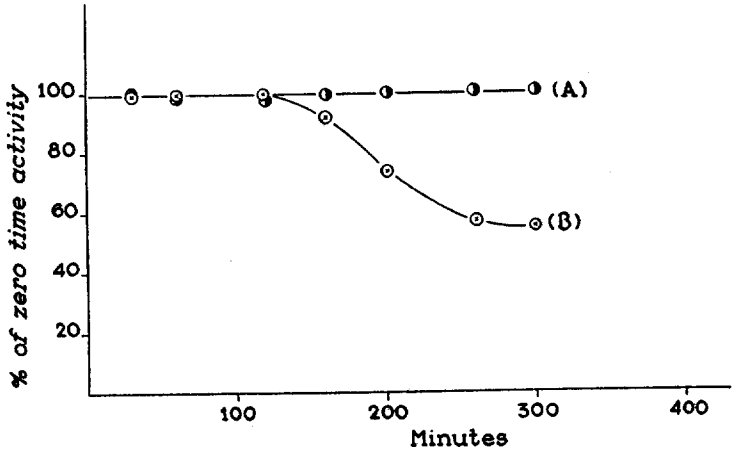

FIG. 5. The stabilization of adaptive galactozymase by NaN_3 during the anaerobic fermentation of galactose. The same conditions as those of Figs. 1 and 4. Curve (*B*) is a control, curve (*A*) shows the behavior in the presence of NaN_3. This brings out the essential similarity between the instability in the presence of the substrate and the instability in the presence of a competitive substrate.

A similar experiment performed in the presence of galactose instead of glucose (Fig. 5) shows that the loss of activity which occurs even in the presence of the adaptive substrate (see Fig. 1) can be prevented by azide just as can the loss in glucose, and that azide is able to replace exogenous nitrogen (Fig.1, curve (*C*)) in this respect. These results support the view that a common process of competitive interaction underlies the loss of enzyme activity under various conditions, and that the loss can be prevented equally well by stopping all competition or by removing the limiting factors which lead to competition.

(*d*) *Formation and Maintenance of Galactozymase in the Absence of Galactose*

As was suggested in the introduction, the intensity of competitive interaction among enzyme-forming systems would in principle be decreased by

providing sufficient material to satisfy all their needs. Theoretically, one would suppose that this state of affairs would best be obtained under conditions in which growth is occurring. The very fact that growth is taking place implies that all or at least the essential protein-forming components are functioning effectively. If this be the case, it might be expected that the capacity of growing cells to maintain enzymes, unstabilized by substrate, would be observably greater than that of resting cells.

Experiments bearing on this point were performed with galactose-adapted cultures of strains K and C. 48 hour galactose-grown cultures were centrifuged, washed, and resuspended in glucose and galactose medium. In order to compare the enzymatic content of fast growing with that of slow growing suspensions, two different densities were made in the resuspensions. In one, called "heavy inoculation," the cells were resuspended in their original volume of medium. In the other, called "light inoculation," the density was adjusted to one-sixth that attained in the 48 hour culture. For purposes of comparison with results in glucose medium, a heavy type of inoculation was made into galactose medium. These suspensions were allowed to incubate, and samples were removed for galactozymase, glucozymase, and density determinations. As usual, enzymatic activity was assayed in terms of rate of anaerobic evolution of CO_2 in the presence of the corresponding substrate.

There were two measurements of primary interest. One was the total enzymatic activity, and the other was the enzymatic activity per cell. An estimate of the former could be obtained by measuring the activity per unit volume of the incubating suspension, and the latter could then be estimated from this measurement by correction for changes in cell density. In order to attain greater accuracy in the determination of enzyme activity, it was necessary to take large aliquots for measurement from the lightly inoculated suspensions, particularly in the case of the early samples. In all instances these samples were washed free of glucose and medium by centrifugation in the cold, and resuspended to adequate density in M/15 KH_2PO_4.

The results of these experiments are recorded in Table II. The density columns list milligrams dry weight of yeast cells per cubic centimeter. The total activity columns (4, 8) record the cubic millimeters of CO_2 evolved per hour per cubic centimeter. Division of these numbers by the corresponding density figures yields the values recorded in columns 2 and 6. These are therefore $Q_{CO_2}^{N_2}$ values since they represent cubic millimeters of CO_2 evolved per hour per milligram and in turn are proportional to activities per cell.

An examination of the density column shows that increases in the number of cells occurred in all cases. However, with both strains the lightly inoculated suspension showed over an eightfold increase as compared with slightly more than twofold for the heavy inoculation. The behavior of galactozymase activity during the course of this experiment can most readily be seen from Fig. 6,

TABLE II

Formation of Galactozymase in the Absence of Galactose during Growth

Strain	Treatment	Time	Density	Glucozymase (1) Ratio to zero time	Glucozymase (2) Activity/cell	Glucozymase (3) Ratio to zero time	Glucozymase (4) Activity/cc.	Glucozymase (5) Ratio to zero time	Galactozymase (6) Activity/cell	Galactozymase (7) Ratio to zero time	Galactozymase (8) Activity/cc.	Galactozymase (9) Ratio to zero time
		hrs.										
KG	Heavy inoculation into glucose medium	0	1.8	1.0	319	1.0	574	1.0	231	1.0	416	1.00
		5	2.6	1.4	808	2.5	2100	3.7	127	0.55	430	1.03
		21	4.2	2.3	584	1.8	2455	4.3	103	0.45	435	1.04
	Light inoculation into glucose medium	0	0.3	1.0	319	1.0	96	1.0	231	1.00	69	1.00
		5	1.0	3.3	660	2.1	660	6.9	79	0.34	79	1.15
		21	2.6	8.6	629	1.9	1630	17.0	51	0.22	132	1.92
	Heavy inoculation into galactose medium	0	1.8	1.0	319	1.0	574	1.0	231	1.00	416	1.00
		5	2.4	1.3	637	2.0	1530	2.7	477	2.07	1145	2.75
		21	4.5	2.5	422	1.3	1900	3.3	304	1.31	1369	3.30
CG	Heavy inoculation into glucose medium	0	1.7	1.0	418	1.0	711	1.0	185	1.00	315	1.00
		6	3.7	2.2	654	1.6	2420	3.4	85	0.46	315	1.00
		26	3.8	2.2	453	1.1	1700	2.4	93	0.50	349	1.11
	Light inoculation into glucose medium	0	0.28	1.0	418	1.0	118	1.0	185	1.00	53	1.00
		6	0.90	3.8	689	1.6	620	5.2	90	0.49	82	1.55
		26	2.30	8.2	616	1.5	1400	11.9	35	0.19	98	1.85
	Heavy inoculation into galactose medium	0	1.7	1.0	418	1.0	711	1.0	185	1.00	315	1.00
		6	3.2	1.9	450	1.1	1440	2.0	376	4.93	1205	3.83
		26	4.3	2.5	380	0.9	1615	2.3	138	0.75	586	1.86

The data were obtained by measuring anaerobic CO_2 production in the presence of glucose or galactose by aliquots of cultures, treated as described in the first column. Columns (3) and (8) are the activities of equal aliquots of the experimental cultures; they show the course of the total enzyme activity of each culture during its growth. Columns (2) and (6) were obtained from columns (3) and (8) by correcting the values for the progressive change in the quantity of yeast resulting from growth. This is given by the density column, which is expressed as milligrams dry weight of yeast per cubic centimeter of culture. The values in (2) and (6) are therefore proportional to the $Q_{CO_2}^{N_2}$ and to the activity per cell. Each of the ratio columns (1), (3), (5), (7), and (9) gives the ratios of the values in the preceding column to those at zero time. They permit a comparison on a relative basis of the changes occurring with time under various conditions in the two enzyme systems examined.

in which are plotted the per cent of initial activity, both total and per cell, for both types of suspensions in the case of strain K. Included also is a curve obtained for the same strain fermenting glucose in the complete absence of growth.

It is seen that, for both the heavy and lightly inoculated suspensions, the enzyme activity per cell drops markedly. The decrease is more drastic in the case of the more rapidly dividing culture. However, the total activity remained absolutely constant in the case of the heavily inoculated suspension, and increased almost twofold in the more rapidly dividing suspension. This

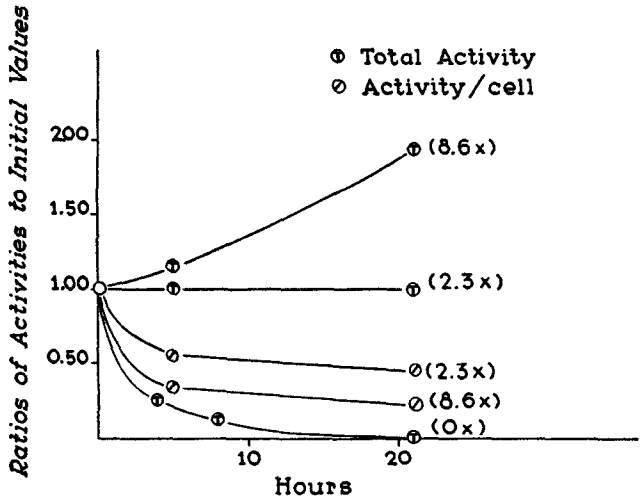

FIG. 6. The formation and maintenance of adaptive galactozymase by growing cells in the absence of galactose. Glucose was the carbohydrate source, and growth occurred in a complete medium (yeast extract-peptone broth). The designations (2.3 ×) and (8.6 ×) give the factor by which the initial number of cells increased during the experiment. The lowest curve shows the fall of enzyme activity in the absence of growth (medium: buffer plus glucose). The next two curves show the loss of activity per cell during increase of cell number; the more rapidly growing culture showed the most loss. The two uppermost curves show the total activity of the culture. They demonstrate the ability to maintain (slowly growing) and even to increase (rapidly growing) the initial enzyme activity in the absence of substrate under favorable conditions.

is to be compared with the total activity curve of the non-dividing suspension. In this latter case, rapid disappearance of the galactozymase occurs.

We have here a rather remarkable instance of adaptive enzyme formation in the absence of its substrate. The fact that an increase in galactozymase activity occurred in the more rapidly dividing suspension, whereas it was only maintained in the more slowly growing culture, is not unexpected. Presumably, the closer the approximation to optimal conditions of growth, the less severe are the competitive restrictions, resulting therefore in a more favorable condition for the synthesis of unstabilized enzymes.

Examination of Table II shows that an exactly similar situation obtained in the case of strain C. Here again, the rapidly dividing suspension exhibited the capacity to form galactozymase in the absence of galactose, and the more slowly growing suspension maintained its activity. As in the case of strain K, the activity per cell decreased in both suspensions, and again it is seen that the more rapidly growing culture exhibits a greater drop in activity per cell. This is probably due to the fact that the rate of enzyme formation is not rapid enough to keep up with the rate of production of new cells. Thus, as time went on, the new cells received less and less enzyme.

As an example of the possibility of rather extensive variations in the content of a so called constitutive enzyme, it is of some interest to examine column 2 of Table II. This records the glucozymase activity obtained during the course of the incubation. It will be noted that the glucozymase activity per cell rose to a peak at the 5 hour period and then fell. The same situation is observed for the galactozymase activity of the heavily inoculated suspension in the galactose medium (column 6).

DISCUSSION

The data presented permit a discussion of some of the fundamental aspects of enzymatic adaptation and a comparison of the adequacy of various hypotheses which have been offered in an attempt to explain this phenomenon. We need not concern ourselves here with those explanations which are based on a supposed "activation" of a preexisting enzyme system. These have already been discussed fully in a previous paper (3). It may merely be pointed out here that such theories are made quite untenable by the finding (9) that enzymatic adaptation in the case of galactose involves a modification of the apoenzymatic or protein moiety of the enzyme system. In addition, they either ignore or fail to explain the rôle of the substrate in the adaptive processes.

In the main, there have been proposed three types of theories which provide a reasonable mechanism whereby a cell can modify its enzymatic constitution in response to substrate. We may summarize these in the following way:

1. The Mass Action Theory.—Yudkin (10) assumed that the presence of substrate stimulates the production of its homologous enzyme by shifting an equilibrium between the enzyme and its precursor in favor of more enzyme formation. The underlying assumption is that the combination of enzyme and substrate, by removing the free enzyme from the reaction system, leads to further enzyme production.

2. Pre-Enzyme Theory.—The pre-enzyme theory of Monod (11) assumes the existence of a pool of common precursor from which a number of different enzymes can be formed. This precursor or pre-enzyme is supposed to have a small but finite affinity for any of the substrates. The combination of one of

these substrates with the pre-enzyme changes it, presumably by conferring a definite specificity upon it, in such a way as to form the particular enzyme.

3. The Plasmagene Theory.—This hypothesis asserts (12) that each enzyme is produced from non-enzymatic material by a specific enzyme-forming system, the plasmagene, which is self-duplicating. It is assumed that substrate, by combining with enzyme, increases the stability of the plasmagene-enzyme complex. Since the unit stabilized is autosynthetic, an increased net rate of enzyme formation would necessarily result.

(*a*) *The Mass Action Hypothesis.*—The adequacy of the mass action theory, as formulated by Yudkin, has previously been criticized on kinetic grounds (12–14). The mass action theory predicts that the adaptive curve should always be concave to the time axis. The observed rate of increase in enzyme activity should be maximal at the onset and decrease continuously until full activity is reached. A careful analysis of the kinetics of adaptation in all instances in which adequate data are available reveals that, on the contrary, the early portion of the adaptive curve is exponentially increasing in character.

Any theory of enzymatic adaptation which proposes to explain the rôle of substrate in inducing the appearance of enzymatic activity must also provide an explanation of why the enzyme disappears on the removal of the substrate. The mass action hypothesis provides a perfectly clear cut and testable mechanism for the stabilizing capacity of substrate. It implies that the reaction between precursor and enzyme has its equilibrium point far over to the side of the precursor. The mechanism suggested to explain the disappearance of enzyme on removal of substrate is therefore the essential instability of the enzyme molecule when it is uncombined with its substrate.

Such a hypothesis would obviously fail to explain some of the facts reported in the present paper. It would be difficult to predict from this hypothesis, for example, that the utilization of some other substrate in the presence of the adaptive one should lead to a decrease in activity of the adaptive enzyme.

It would also be difficult to predict the possibility of stabilizing enzymes in the absence of their substrates by the very conditions which inhibit or prevent enzyme formation. The fact that both anaerobiosis and the presence of sodium azide lead to maintenance of galactozymase activity, even in the absence of galactose, almost directly contradicts one of the primary postulates of the mass action hypothesis: for it shows that the adaptive enzyme is *not an inherently unstable unit in the absence of the substrate.*

Finally, the rôle assigned to substrate in this theory is one that would not permit measurable amounts of enzyme to be formed in the absence of substrate. Yet, as we have seen, under appropriate conditions precisely this result can be obtained.

(*b*) *The Pre-Enzyme Hypothesis.*—Monod formulated the pre-enzyme hypothesis largely in order to account for a discrepancy between the mass

action theory of Yudkin and Monod's own results (11) on the effect of substrate concentration on rate of adaptation. According to the mass action theory, substrate concentrations greater than that required to saturate the number of enzyme molecules present should have no effect upon the precursor-enzyme equilibrium, and therefore should have no effect upon the rate of enzyme formation. Monod, studying adaptive maltozymase formation in *E. coli*, observed a marked effect of substrate concentration above the concentration which he finds to be the saturation value for fully adapted cells. He argues from this that substrate must have another rôle than that of combining with and stabilizing the enzyme. Accordingly, he assigns substrate the function of combining with the precursor or pre-enzyme and converting it to enzyme. Since the quantity of hypothetical pre-enzyme may be very large, and its combining capacity low, it is possible to understand how more substrate would be required to saturate it than is required to saturate the maximal amount of enzyme formed.

Monod's theory was thus primarily devised to explain the effect of substrate concentration on enzyme formation. Accordingly, it does not specifically discuss the reasons why an enzyme, once formed from pre-enzyme by substrate, disappears when the substrate is removed from the medium. By implication it imputes a greater degree of instability to the enzyme in the absence of substrate.

The pre-enzyme theory does imply that competition occurs during enzyme formation—competition of substrate for the pre-enzyme. However, it does not provide for competitive interactions which are not directly mediated by substrate. It is therefore difficult to explain in terms of this hypothesis some of the experiments reported here on enzyme disappearance in the presence of substrate.

A crucial test of the applicability of Monod's theory must of necessity center around the rôle assigned to substrate in enzyme formation. One must conclude from the primary postulate of his proposed mechanism that the presence of substrate is *necessary* for enzyme formation. It is clear that this conclusion, and therefore the postulate from which it is derived, is untenable. The data presented above on rapidly growing cells demonstrate that adaptive enzyme can be formed in the absence of the corresponding substrate. One must conclude from these experiments that the presence of substrate is a *sufficient* rather than a *necessary* condition for enzyme formation.

It must be emphasized that none of the experiments described here rule out the existence of a pre-enzyme in the sense of an immediate enzyme precursor already possessing some specificity towards substrate. Neither do these results disprove the possibility that substrate can aid in the conversion of pre-enzyme to enzyme. They do prove that this conversion can occur without the intervention of substrate.

(*c*) *The Plasmagene Theory.*—The plasmagene theory differs from the other

theories of enzyme formation in that it does not assign the primary part in determining enzyme formation to substrate. The assumption that independent enzyme-forming units exist and that they are self-duplicating permits the utilization of what is already known concerning competitive relations between systems of self-duplicating units. Within this framework substrate provides *one* of the factors which can determine the successful competition of one particular system of self-duplicating units. Other factors known to operate are availability of energy, availability of nitrogenous building materials, and the general metabolic rate of the cell. The evidence for competitive interaction in enzyme formation, together with the effects of these factors, has already been discussed elsewhere (2).

The plasmagene theory does not assume any inherent instability of the molecules of active enzymes. It is clear that if raw materials or energy, or both, are limiting factors in enzyme formation, one would expect that some enzymes would be built up at the expense of others—the more able competitors at the expense of the less able. If combination of plasmagene and enzyme with substrate increases the stability of the complex, it would be expected that the presence of substrate would promote the appearance of measurable quantities of enzyme under conditions where otherwise no such quantities would survive the competitive process.

This point of view implies that the presence of substrate by itself does not guarantee the stability of enzyme once it has been formed. If cells are placed in a medium containing no external nitrogen source, and various plasmagene systems are striving to build enzyme, one would anticipate a loss of adaptive enzyme even in the presence of substrate. This is precisely what we have found to be the case in the experiments reported in the first section of this paper. Moreover, one would predict that the addition of a supply of nitrogen sufficient for synthetic purposes should ameliorate the competition, and possibly abolish the interaction altogether. This also was found to be the case.

It obviously also follows from the theory that the competitors of a particular enzyme system should be more effective in wiping it out when placed in the presence of their own specific substrates. The experiments of the second section fulfill this prediction. They also indicate a certain order of enzyme-forming systems with respect to the intensity of competition between them. Thus, several substrates increase the breakdown of galactozymase above the rate found in buffer. But also, the hexoses glucose and fructose appeared to compete more keenly with the galactose system than alcohol or pyruvate. There is a suggestion in this that enzymes which act on chemically similar compounds (so that one would expect the enzymes to be structurally similar) are more easily interconvertible. This would be expected to lead to stronger competition between such systems than between systems dealing with chemically rather different substrates.

On the view proposed here, when an enzyme disappears it is essentially

because some other enzyme is actively being constructed, not because it spontaneously disintegrates. Therefore, anything which prevents enzyme formation should prevent enzyme breakdown equally well. The experiments of our third section show that this is the case. Enzyme-forming processes can be blocked by instituting anaerobic conditions in the absence of any fermentable material. They can also be blocked by the use of sodium azide. These conditions presumably stop synthesis by interfering with the production or the transfer of the energy required for synthesis. That they do prevent enzyme synthesis has already been amply demonstrated. In the experiments reported above, it is seen that they also effectively prevent the disappearance of an adaptive enzyme in the absence of its substrate.

According to the plasmagene theory, enzyme formation is not a simple response to the stimulus of added substrate. Plasmagenes are units which form their corresponding enzymes whenever favorable conditions for synthesis and self-duplication are provided. This would lead us to expect that, beginning with an adequate number of plasmagenes, enzyme could be formed in the complete absence of substrate, provided the supply of energy, nitrogen, and all other requirements for synthetic activity were favorable. The experiments with rapidly growing cultures in the fourth section of this paper provide this kind of situation. There is a low population density, an ample supply of energy, of nitrogen in various forms, and of growth factors. Accordingly, we find that in the presence of glucose and the absence of galactose—two conditions which would be expected to lead to a rapid decrease of galactozymase—the initial amount of galactozymase in the culture was actually doubled. Even a more slowly growing culture under these conditions was able to maintain its initial amount of adaptive enzyme intact, whereas the same culture, under conditions less favorable to synthesis, lost it very rapidly.

The experiments reported and discussed in this paper constitute a critical test for any theory of enzyme formation. If we assume that enzymatic constitution is determined by competitive interactions between self-duplicating enzyme-forming systems, we arrive at the simplest interpretation so far suggested which is consistent with all the facts known about enzymatic adaptation. The effects of various agents and conditions on the ability of cells to form new enzymes and maintain existent ones can then be understood in terms of their influence on the outcome of this competitive interaction.

SUMMARY

The effect of various conditions on the stability of adaptive enzymes in the absence of their substrates has been studied. In particular, it is shown that even the presence of substrate is unable to maintain adaptive enzyme indefinitely under conditions unfavorable to synthesis. The stability of the enzyme can, however, be insured by providing an exogenous nitrogen source.

It is shown that various substrates accelerate the disappearance of an adaptive enzyme when its own substrate has been removed from the medium. The order of effectiveness of such substrates appears to be connected with their chemical similarity to the adaptive substrate.

It is shown that two conditions which are able to inhibit the formation of adaptive enzymes—anaerobiosis and the presence of sodium azide—are equally able to prevent the disappearance of an adaptive enzyme after the removal of its substrate. Finally, it is shown that rapidly growing cultures, under optimal conditions for synthetic activity, are able to maintain and even appreciably to increase their initial content of an adaptive enzyme, in the absence of its specific substrate and in the presence of a normally competitive substrate.

In the light of these results, the three major theories of enzyme formation hitherto proposed are evaluated.

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