THE COMPONENTS OF MALTOZYMASE IN YEAST, AND THEIR BEHAVIOR DURING DEADAPTATION[1]

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The term maltozymase designates the enzyme system involved in the production of carbon dioxide from maltose in yeast. The discovery of a hydrolytic α-glucosidase led Fisher (1895) to propose that the following reactions represent the action of maltozymase:

$$\text{maltose} \xrightarrow{k_1} \text{glucose} \xrightarrow{k_2} CO_2, \text{etc.} \quad (1)$$

Recent studies on the induced synthesis of α-glucosidase (Halvorson and Spiegelman, 1957) support Fisher's hypothesis. During the early stages of induction, the rate of the α-glucosidase reaction (k_1) limits the over-all activity of maltozymase. However, the observation that maltose is not metabolized, while glucose is, under conditions in which α-glucosidase would be expected to operate *in vivo* (Willstätter and Bamann, 1926; Leibowitz and Hestrin, 1945) suggests that maltozymase may contain other enzyme reactions.

Since the reactions produced by maltozymase include all those involved in glucose metabolism, it is clear that the appropriate method for searching for other components of maltozymase is to establish conditions in which its activity can be varied independently from glucose metabolism. Such conditions are provided when yeast cells grown in maltose are incubated aerobically with glucose in nitrogen-free medium (Spiegelman and Reiner, 1947). The resulting loss of maltozymase activity has been termed deadaptation. Although the phenomenon of deadaptation has led to speculation about the existence of competitive reactions between different enzyme-forming systems (Spiegelman and Dunn, 1947), the basis of the loss of maltozymase activity is not understood. Dinitrophenol and azide prevent deadaptation (Spiegelman and Reiner, 1947), suggesting that the process requires energy. Deadaptation is prevented also by nitrogen or by the inducing agent.

This paper reports the results of a study undertaken to determine the various components of the maltozymase system. The identity of these and their behavior during deadaptation are described.

METHODS AND MATERIALS

A diploid representative of *Saccharomyces cerevisiae* strain LK2G12, was used. Cells were grown in stationary flasks at 30 C in a medium prepared by adding the following to 1 L of water: peptone (Difco), 5 g; yeast extract (Difco), 2.5 g; $(NH_4)_2SO_4$, 2 g; $CaCl_2$, 0.25 g; $MgSO_4$, 0.25 g; KH_2PO_4, 2 g; and glucose (or maltose), 40 g.

Cells in the log phase (12 hr) were harvested by centrifugation, washed twice in cold water, and resuspended, either in water or in a buffer free of nitrogen and carbohydrate (Halvorson and Spiegelman, 1952), to a density of 2.84 mg dry weight of yeast per ml.

To induce the enzyme synthesis, cells were suspended in buffer supplemented with 3 per cent maltose. In the deadaptation experiments, glucose was substituted for maltose as the energy source. The suspensions were incubated aerobically at 30 C, either in a standard Warburg apparatus or in Erlenmeyer flasks, on a rotary platform shaker. Maltozymase was followed in the washed intact cells by the two-cup method, after stabilization by ultraviolet light (Halvorson and Spiegelman, 1954).

Three methods were employed to rupture the cells: (1) Fast-dried preparations were obtained by lyophilizing samples which had previously been shell-frozen at −30 C. After evacuation for 4 hr, the samples were stored overnight over

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anhydrous $CaCl_2$ in the cold. The enzyme content remained constant over a period of several months; the viability of the preparations was almost completely abolished. (2) Cells treated with toluene were prepared by shaking a washed suspension with 20 per cent toluene (reagent grade) at room temperature for 30 min. (3) Two cycles of freezing and thawing were also found to be optimal for extraction of the enzyme. Cell suspensions were alternately shell-frozen at −40 C and thawed at room temperature.

α-Glucosidase is measured by the rate of α-phenylglucoside hydrolysis in disrupted cells. The assay is based on a colorimetric determination of free phenol. This rate was previously found to be proportional to the maltose-splitting capacity of the enzyme during deadaptation and fractionation (Robertson, 1955). A 0.1-ml aliquot of the disrupted cells was added to 1.8 ml of .067 M phosphate buffer, at pH 6.8, containing 0.15 ml of cysteine per ml. After thermal equilibration at 30 C, 0.1 ml of an aqueous solution of 0.8 per cent α-phenylglucoside was added. At intervals, 0.1-ml samples of the reaction mixture were removed and added to 5.0 ml of borate buffer at pH 9.8 to stop the reaction. Then 0.2 ml of a 0.2 per cent solution of 2,6-dibromoquinone in ethyl alcohol were added, and the mixture was stirred and incubated at room temperature for 45 min. The contents were diluted to 10 ml with water and stirred, and the optical density was determined at 660 mm. The activities of α-glucosidase were calculated from a standard phenol curve and expressed as the μmoles of phenol liberated per hr per mg of dry cells.

α-Methyl-glucoside was synthesized from uniformly labelled C^{14} glucose (Helferich and Schafer, 1932). The product, twice recrystallized from absolute methyl alcohol, was nonreducing, had an mp of 163 to 164 C (uncorrected) and a specific activity of 0.354 mc per mmole. The mp was unchanged when the substance was mixed with a known recrystallized sample. Radioautographs prepared from chromatograms in butyl alcohol-acetic acid:water (4,1:1) indicated only the presence of α-methylglucoside (Rf 0.34) and not of glucose (Rf 0.16).

The total radioactivity of intact cells was measured on samples which were filtered and washed on membrane filters. A 5-ml suspension of cells was filtered through Co 5 membrane filters and washed rapidly with 15 ml of cold water; a few drops of 10 per cent glycerol were then added, to facilitate the adherence of the cells to the membrane during subsequent drying under an infrared lamp. The membranes were counted directly with an end window counter.

Crystalline ribonuclease (RNAase) and desoxyribonuclease (DNAase) were obtained from Nutritional Biochemicals Co. Uniformly C^{14}-labelled glucose was furnished by the Laboratoire des Isotopes de l'Institut Pasteur. A culture of *Saccharomyces italicus* strain Y1225 was obtained from Dr. S. Spiegelman (University of Illinois) and cultures of *Saccharomyces marcianus* Hansen and *Saccharomyces fragilis* Jöng from Dr. P. Slonimski (Institut de Biologie Physicochimique, Paris).

RESULTS

Kinetics of deadaptation. When maltose-grown cells are shaken aerobically in the presence of glucose and in the absence of exogenous nitrogen, they lose their maltozymase activity. This loss is known as deadaptation. In order to provide a basis for understanding this loss, the changes in maltozymase, α-glucosidase, and glucose fermentations were followed during deadaptation. The results obtained (figure 1) indicate that the phenomenon is complex. The deadaptation of

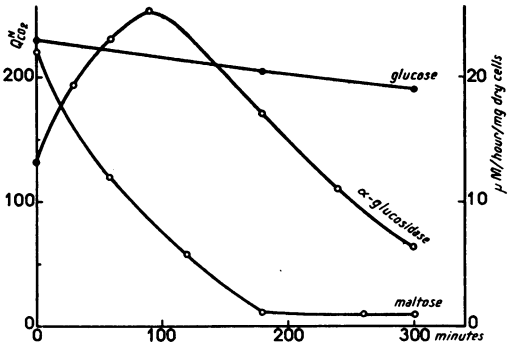

Figure 1. Kinetics of deadaptation of *Saccharomyces cerevisiae* strain LK2G12. Maltose-grown cells were incubated aerobically in nitrogen-free medium containing 3 per cent glucose. At intervals aliquots were withdrawn, washed, and their fermentative capacity (Q^NCO_2: μL CO_2/hr × mg dry weight cells) of maltose or glucose measured in the Warburg apparatus. Parallel samples were disrupted by lyophilization, resuspended in buffer pH 6.8, and measured for α-glucosidase by the α-phenyl glucoside assay.

TABLE 1

Comparison of methods of treatment of cell suspensions on the measurement of α-glucosidase behavior during deadaptation

A suspension containing 2.84 mg dry wt cells/ml of maltose-grown cells was aerated at 30 C/in N-free buffer containing 3 per cent glucose. At intervals 3 samples were withdrawn and washed by centrifugation. These cell suspensions were disrupted by lyophilization, or subjected to two cycles of freezing and thawing, or shaken with 10 per cent toluene for 30 min (see Methods for details). After resuspension to original volumes, α-glucosidase was measured with α-phenyl glucoside as substrate.

Minutes of Deadaptation	μmoles M Phenol/Mg Dry Cells × Hr		Toluenized
	Lyophilized	Frozen-thawed	
0	15.0	15.0	12.1
30	18.0	18.0	15.3
60	22.5	22.8	18.0
90	24.0	23.7	18.3
180	15.0	15.0	12.0

maltozymase began immediately after the addition of glucose and was complete within 180 min. During this period, the constitutive ability to ferment glucose, a presumed intermediate, did not change appreciably. On the other hand, the α-glucosidase increased 70 per cent during the first 90 min of deadaptation. After this period, the α-glucosidase was inactivated at approximately the same rate as the initial inactivation of maltozymase. This behavior of α-glucosidase during deadaptation was also evident in experiments in which cells were disrupted by lyophilization, repeated freezing and thawing, or treatment with toluene (table 1). In these experiments and in others carried out later, slightly lower α-glucosidase activities were consistently observed in toluenized preparations. In our experience the α-phenylglucoside-splitting capacity of extracts was always proportional to the hydrolytic activity on maltose.

During deadaptation, the delay in the inactivation of α-glucosidase results in a situation in which the intact cell becomes cryptic to maltose. The degree of crypticity (crypticity factor) is measured by the ratio of the maltose-splitting capacity in cell extracts/intact cells (α-glucosidase/maltozymase).

As for the behavior of the α-glucosidase, it is

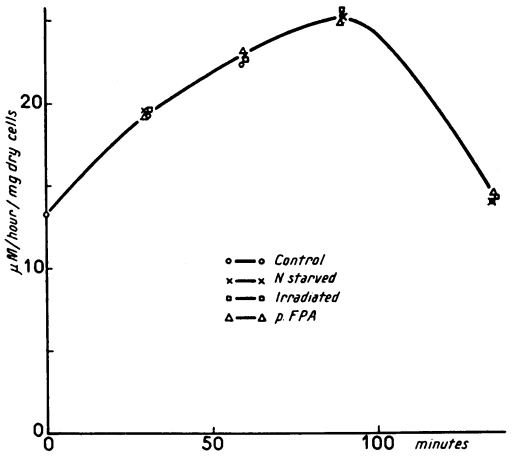

Figure 2. The effect of the limitation of protein synthesis on the *in vivo* rise of α-glucosidase during deadaptation. A suspension of washed maltose-grown cells in N-free medium was divided into 4 aliquots: (1) control, (2) irradiated for 180 sec, (3) suspended in 10^{-2} M *p*-fluorophenylalanine and (4) nitrogen-starved for 10 hr by maltose fermentation, washed and resuspended in fresh N-free medium. Glucose at a level of 3 per cent was added to each flask, and at intervals samples were removed, washed, and lyophilized. After resuspension in buffer, α-glucosidase was determined.

TABLE 2

Release of bound enzyme by sonic disruption

Maltose grown cells were deadapted as previously described (see table 1). At intervals duplicate lyophilized samples of cells (16 mg dry weight) were prepared. Both were suspended in 20 ml of buffer containing 0.25 M sucrose and M/15 phosphate pH 6.8. The first was assayed immediately. To the second 5 g of Superbrite beads were added and the suspension was placed in the receptacle of a Raytheon 10-kc sonic oscillator maintained at 0 C. After a 30-min treatment, the suspension was warmed to 30 C and assayed immediately for α-glucosidase.

Minutes of Deadaptation	μmoles/Mg Dry Cells/Hr	
	Lyophilized	Lyophilized, sonic disrupted
0	14.7	25.0
60	23.1	24.9
90	24.9	25.0
180	14.9	14.8

clear that maltozymase deadaptation involves at least three phenomena: (1) increase in α-glucosidase in the absence of an exogenous inducer, (2) eventual metabolic inactivation of α-glucosidase, and (3) increase in the crypticity factor.

These apparently independent phenomena all have a direct bearing on the problem of the components of maltozymase and on its deadaptation. We will first discuss the increase in α-glucosidase and then the problem of the increase in the crypticity factor. The inactivation of α-glucosidase during deadaptation will be the subject of a later communication.

Nature of the rise in α-glucosidase activity. Yeasts contain a free amino acid pool which is capable of supporting *de novo* enzyme synthesis in the absence of exogenous nitrogen (Halvorson and Spiegelman, 1952). It therefore seemed possible that the observed rise in α-glucosidase during the early stages of deadaptation might represent actual enzyme synthesis. This possibility was ruled out, however, by the following observations: (1) The inhibition of protein synthesis by ultraviolet light (Halvorson and Spiegelman, 1954), amino acid analogues, or nitrogen starvation (Spiegelman and Halvorson, 1953) did not prevent the rise in activity during deadaptation (figure 2). (2) A sonic disintegration of induced cells resulted in an immediate expression of the maximum α-glucosidase activity (table 2).

When it was evident that the enzyme which appeared *in vivo* during deadaptation already existed in induced cells, attempts were undertaken to reproduce its activation *in vitro*. Increases in α-glucosidase identical to that observed previously *in vivo* were observed when suspensions of disrupted cells were incubated at 4 C or 30 C (table 3). At the lower temperature a 3-hr delay in α-glucosidase expression was observed (figure 3), and during this period experiments were carried out to locate the origin of the increased activity. Maximal activity was attained after 6 hr and remained constant thereafter.

TABLE 3

The effect of temperature on the release of bound enzyme in disrupted cells

A series of tubes containing 4-ml samples of lyophilized maltose-adapted cells (14.2 mg) were incubated without agitation at 4 C and at 30 C. At intervals, aliquots were removed and assayed for α-glucosidase by the α-phenyl glucoside method.

Disrupted Cell Suspension at	μmoles Phenol/Hr × mg Dry Cells				Total Δ E
	0	½ hr	2 hr	24 hr	
C					
4	19.2	19.2	19.2	33.2	14
30	19.2	20.4	32.6		13.4

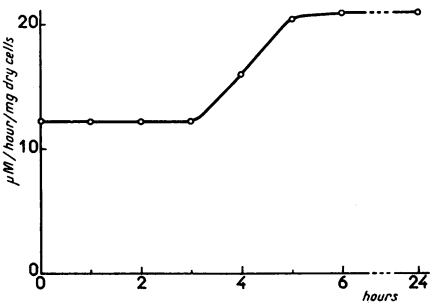

Figure 3. Kinetics of release of bound enzyme from lyophilized cells at 4 C. See table 3 for details.

By means of the *in vitro* assay, a fractionation of disrupted cells was undertaken during the period of delay. Results showed that the increase in α-glucosidase arose from the pellet fraction and could not be removed by extensive washing (table 4). The unimpaired ability of the washed pellets to release α-glucosidase upon aging seems to rule out the participation of soluble, cellular enzymes in this process. The rise in activity involved a solubilization. The supernatant α-glucosidase, as well as that released from the washed pellets either by aging or physical disruption of the pellet by sonic disintegration, were not sedimented at 20,000 × G (table 4) or at 140,000 × G. The viable count of the preparation was less than 0.001 per cent of the original preparation. The release of the bound enzyme from washed particles, which were devoid of free amino acids (table 5), further eliminated the possibility that this enzyme arose from residual intact cells. If this had been the case, one should have found 0.26 μmoles of amino nitrogen per ml based on the free amino acid content per intact cell.

The enzyme which is released in the *in vitro* experiments is clearly the same as that released *in vivo* during deadaptation: (1) In parallel experiments, the same percentage increase is obtained *in vivo* (figure 1) and *in vitro* (figure 3). (2) The properties of the soluble and released α-glu-

TABLE 4

Demonstration of the release of enzyme from the pellet fraction

A 300-mg sample of dried maltose-grown cells was added to 5 g of Superbrite beads and mixed with sufficient M/15 phosphate buffer to make a thick paste. After grinding for 30 min at 4 C in an automatic mortar and pestle, the suspension was diluted with buffer to 100 ml and centrifuged for 2 min to remove the beads. A second centrifugation at 21,000 × G for 10 min gave a supernatant (S_1) and a pellet (P_1) of which an aliquot was saved. The pellet was resuspended to original volume with buffer and recentrifuged (S_2 and P_2). This procedure was repeated two more times giving finally a 3-times-washed pellet (P_4). All fractions were assayed immediately and after aging for 24 hr to release the bound enzyme. The α-glucosidase activities are calculated on the basis of 1 mg of original dry cells.

Fraction	μmoles Phenol/Hr		ΔE
	Immediate	Aged	
Disrupted cell suspension	21	34.4	13.4
S_1	11.5	11.0	−0.5
P_1	9.0	22.3	13.3
S_2	2.5	2.5	0
S_3	1.2	1.0	−0.2
S_4	0	0	0
P_2	7.0		
P_3	5.1	18.5	13.4
P_4	5.1	18.7	13.6

cosidase are identical (Robertson, 1955). (3) The α-glucosidase is released *in vitro* under conditions which inhibit protein synthesis (table 6).

The localization of the bound enzyme with subcellular particles raised a number of interesting problems concerning the nature of this binding and the specificity of the site of attachment. The observation that catalase in yeast is attached to interfaces (Kaplan, 1954) may represent a similar phenomenon. In the present study, however, treatment of the subcellular particles, either with toluene (which releases catalase) or with surface active agents, as polymyxin, failed to liberate the bound enzyme. The degrading of over 90 per cent of the RNA or DNA in this sediment by RNAase or DNAase treatment neither released the bound enzyme immediately nor accelerated its release. However it is still possible that the bound enzyme is associated with a small fraction of the nucleic acid and is therefore resistant to depolymerization, or that it is only secondarily associated with nucleic acids. The activity per mg of protein of the released enzyme is only slightly higher than that of the soluble enzyme in the original extract, indicating that the release of protein was largely nonspecific (table 5). The small amounts of free amino acids released in parallel with the enzyme suggest that the linkage of this protein may occur through peptide links. A further analysis of the site and nature of the binding must obviously await a more extensive fractionation of these particles.

Conclusion: Approximately 30 to 50 per cent of the total α-glucosidase in induced cells is firmly bound to subcellular particles and is inactive or unavailable to the substrate. This bound enzyme can be rendered soluble *in vivo* during deadaptation, and *in vitro* either by aging the particles or by mechanical means.

The increase in the crypticity factor in vivo. The

TABLE 5

Analysis of the washed pellet fraction

A suspension of lyophilized maltose-grown cells was disintegrated as described in table 4. The pellet was washed 4 times and the supernatants from each wash were added to the original supernatant. The washed pellet was divided between two centrifuge tubes. Each pellet sample was resuspended to vol in M/15 phosphate buffer. One suspension was used to prepare a free amino acid pool (Halvorson and Spiegelman, 1952). The other pellet sample was allowed to age at 4 C for 24 hr, centrifuged, and a free amino acid pool also prepared from the pellet. Enzyme was measured immediately after collection of the fraction by the α-phenylglucoside method. Total protein was determined by the method of Folin and Ciocolteau (1927) and the free amino nitrogen by the method of Moore and Stein (1948).

Fraction	α-Glucosidase*	mg Protein/ml	Specific Activity†	NH_2 (N)‡
Supernatant	10.2	0.31	33.0	0.67
Washed pellet before aging	5.75			0
Washed pellet after aging	15.3	0.36	42.5	0.15
Residual pellet	4.15			0

* μMoles phenol/hr × ml.

† μMoles phenol/hr × mg. protein.

‡ μMoles/ml.

TABLE 6

Release of bound enzyme from the washed pellet fraction under conditions inhibiting protein synthesis

Three groups of washed pellets prepared from maltose-grown, lyophilized cells (see P_2, table 4) were suspended in M/15 phosphate buffer. The first served as a control. The second received *p*-fluorophenylalanine at a final concentration of 0.02 M. The third was irradiated for 180 sec. Following incubation at 30 C for 2 hr, the suspensions were centrifuged at 21,000 × G for 10 min and the α-glucosidase of the pellet and supernatant examined. The activities are calculated on the basis of 1 mg dry weight of cells.

Treatment	μMoles Phenol/Hr × Mg Dry Cells			Total
	Initial pellet	After release		
		Pellet	Supernatant	
Control	9.6	4.8	14.7	19.5
p-FPA	9.6	5.4	14.0	19.4
UV	9.6	4.8	14.7	19.5

increase in the crypticity factor during deadaptation cannot be explained by reaction 1, since under these conditions neither glucose fermentation nor α-glucosidase activity limits maltozymase. α-Glucosidase is measured as the total α-phenylglucoside hydrolyzing activity *in vitro*. Therefore, the increase in the crypticity factor during deadaptation can be explained by at least two alternative hypotheses: (1) α-glucosidase is not a member of the maltozymase sequence; (2) α-glucosidase is a member of the maltozymase sequence, but is not rate-limiting.

The evidence bearing on these two hypotheses will be discussed separately.

α-Glucosidase is not a member of the maltozymase sequence. The evidence which supports the hypothesis that α-glucosidase is a member of the maltozymase sequence can be summarized as follows:

1. The hydrolysis of maltose by α-glucosidase produces a substrate (glucose) which is handled by a constitutive system. Repeated attempts to detect phosphorylase or transferase activities or cofactor requirements for this enzyme have been negative. Gottschalk (1950), in a review of the literature, concluded that the hydrolysis of maltose by α-glucosidase is the initial step in maltose fermentation in yeast.

2. In genetic crosses, maltozymase activity has thus far always been associated with α-glucosidase activity. Furthermore, there are no reported cases of yeast fermentation of maltose in which α-glucosidase activity was not evident.

3. There is a parallel induction of α-glucosidase and maltozymase in inducible strains. During induction, the maltozymase activity is quantitatively explained by the α-glucosidase activity (Halvorson and Spiegelman, 1957).

From these observations, it appears highly unlikely that the entire α-glucosidase activity is excluded from the maltozymase sequence. However, the finding that the α-glucosidase activity is actually the summation of activities of a heterogeneous group of enzymes requires a more serious consideration. If a variable fraction of these α-glucosidases are members of the maltozymase sequence, then the total α-glucosidase activity would not always be proportional to maltozymase activity. Two types of α-glucosidase heterogeneity exist. In the first type, described here,

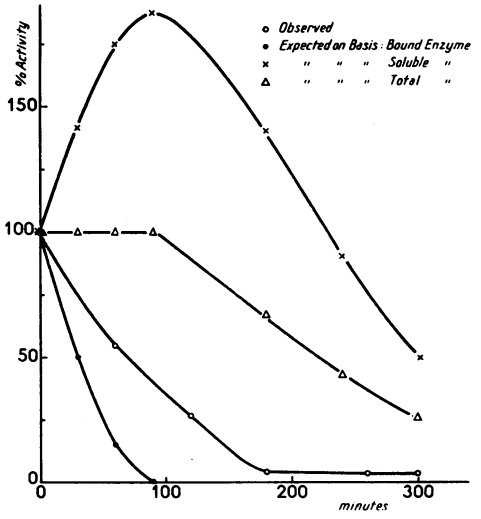

Figure 4. Examination of the possibility that α-glucosidase controls the rate of maltose fermentation during deadaptation. See figure 1 for experimental details. Bound enzyme was calculated on the basis of the enzyme increase during deadaptation. The rate of maltose fermentation is expressed as the percentage of the rate of adapted cells. The rates of maltose fermentation expected during deadaptation on the basis that this rate is controlled by bound enzyme, soluble enzyme, or total enzyme is expressed as the per cent of these components during deadaptation compared to their initial values in the adapted cell.

the α-glucosidase of induced cells is found in both a soluble and a bound form. An analysis of the expected maltozymase activity based on the assumption that it is determined by either the soluble, bound, or total α-glucosidase (figure 4) indicates that it is not proportional to any of these three. The second type of α-glucosidase heterogeneity assumed significance with the discovery that the activity could be separated into several different enzymes distinguished by their electrophoretic mobilities and substrate specificities (Mehlman and Spiegelman, *unpublished results*). The possibility that the observed crypticity could be explained by this second type of heterogeneity was tested by examining the behavior of α-glucosidase and maltozymase during deadaptation in *S. italicus* strain Y1225 which contains only the major α-glucosidase present in strain LK2G12 (Spiegelman, *personal communication*). The increase in crypticity factor during deadaptation of this strain (figure 5) removes the possibility that the phenomenon can be attributed to this type of α-glucosidase heterogeneity.

It must therefore be concluded that at the present time there is no direct evidence that the α-glucosidases, as measured by their hydrolytic activities, are not all members of the maltozymase sequence.

α-Glucosidase is a member of the maltozymase sequence, but is not rate-limiting. Previous evidence leads one to the conclusion that maltozymase may include as yet unrecognized components. The suggestion by Gottschalk (1950) that the cell membrane of yeast may determine the rate of fermentation provides an alternative explanation for the rate limitation during deadaptation. The recent demonstration of a specific and inducible mechanism for the internal accumulation of galactosides (galactoside permease) in *Escherichia coli* (Cohen and Rickenberg, 1955) strongly suggests the possibility that a similar enzymatic reaction might be involved in permeation by maltose itself. Since such a mechanism would explain not only the present data, but also many other cases of crypticity in yeast, a search was undertaken for a specific catalytic reaction leading to the internal accumulation of the substrate. In order to separate permeation from cell metabolism, a radioactive (C^{14}) gratuitous inducer for α-glucosidase, α-methylglucoside, was employed.

Early experiments indicated that α-methylglucoside was internally accumulated as such in yeast cells, and, furthermore, was not lost during

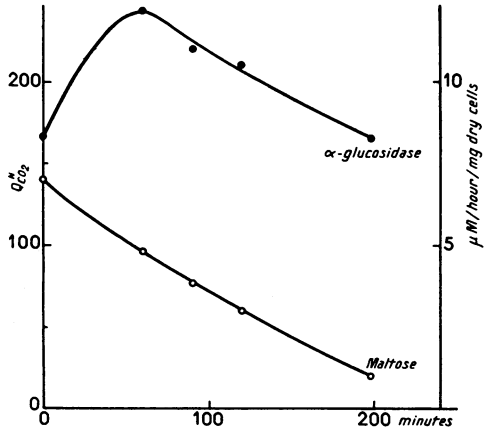

Figure 5. Kinetics of deadaptation of *S. italicus* strain Y1225. Deadaptation of maltose-grown cells was carried out under conditions identical to those described in figure 1.

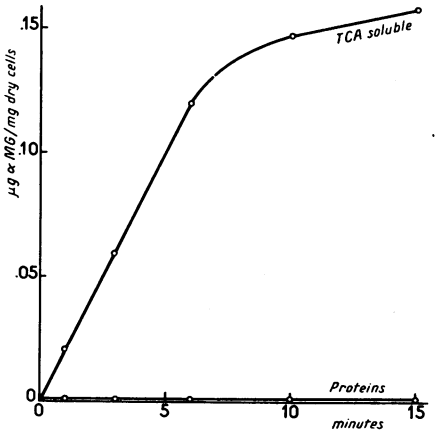

Figure 6. Kinetics of internal accumulation of C^{14} α-methyl glucoside. Maltose-grown cells were washed and suspended in buffer pH 4.5 to a density of 2.84 mg dry weight per ml. A suspension of 27 ml was shaken aerobically at 30 C. After 5 min, 3 ml of 10^{-2} M C^{14} α-methyl glucoside (0.354 mc/mole) was added. At intervals, 5 ml were removed, rapidly chilled, centrifuged and washed. The pools were extracted by adding 2 ml cold 5% TCA. After centrifuging and rewashing the pellet with cold 5% TCA, the proteins were solubilized in 7% NH_4OH. Aliquots of the samples were counted and from the known activity of the substrates, α-methyl glucoside accumulation (μg/mg dry cells) was calculated.

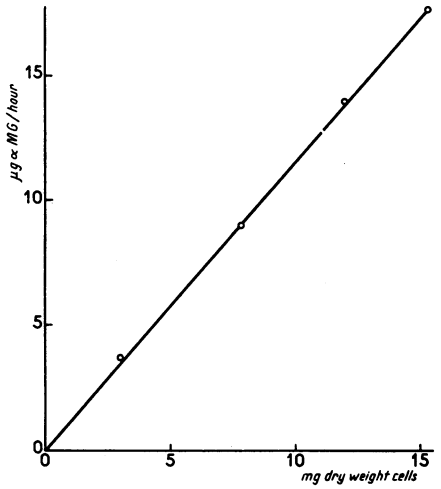

Figure 7. Relation between α-methyl glucoside accumulation and cell density. Flasks containing 4.5 ml of varying cell suspensions were preincubated at 30 C, following which 0.5 ml of 10^{-2} M C^{14} α-methyl glucoside was added. After 5 min the contents of the flask were filtered through a Co 5 membrane filter, washed with 15 ml of cold water and finally by a few drops of 10 per cent cold glycerol. The membranes were dried under an infrared lamp. The membranes were counted directly and from their activities, corrected for trace retention of α-methyl glucoside by the membrane, the rates of uptake were calculated.

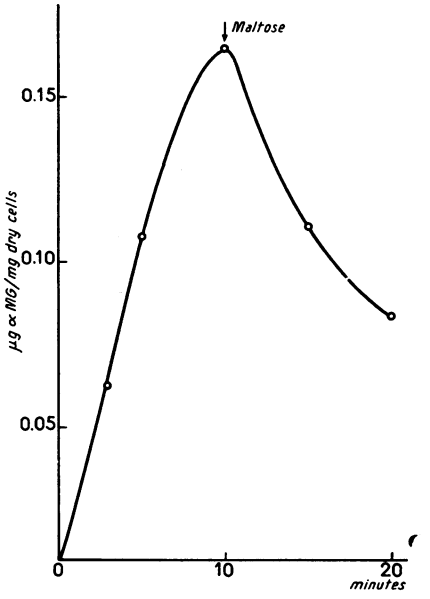

Figure 8. Displacement of α-methyl glucoside by maltose. To 27 ml of maltose-grown cells, preincubated for 5 min at 30 C, was added 3 ml of 10^{-1} M α-methyl glucoside (0.0354 mc/mole). After 3, 5 and 10 min, 5.0-ml samples were removed and the cells collected on membrane filters. At 10 min, 0.15 ml of 1 M maltose was added and 5.0-ml samples collected at 15 and 20 min. The accumulation of α-methyl glucoside (μg/mg dry cells) was calculated from their activities.

limited washing of the cells with cold water. Since the radioactivity is associated only with the pool fraction (figure 6), in subsequent experiments the washed intact cells were counted directly on membrane filters. The initial linear rate of inducer accumulation (figure 6) permits a direct measurement of the rate of the accumulation. This rate is directly proportional to the cell concentration (figure 7) and is completely inhibited by 5×10^{-3} M DNP, 10^{-2} M NaF or 10^{-2} M NaN_3. The accumulation process appears, therefore, to require energy. The reaction is reversible, the accumulated inducer being displaced by maltose (figure 8). The rate of accumulation per unit weight of yeast also depends upon the concentration of α-methyl-glucoside and follows Michaelian kinetics (figure 9). The dissociation constant calculated from these data (figure 9) is 0.12 M.

The permeation mechanism is specific (table 7). Of the glucosides, only maltose (4-glucose-α-glucoside) and to a lesser extent β-methyl-glucoside are inhibitory. It is interesting that

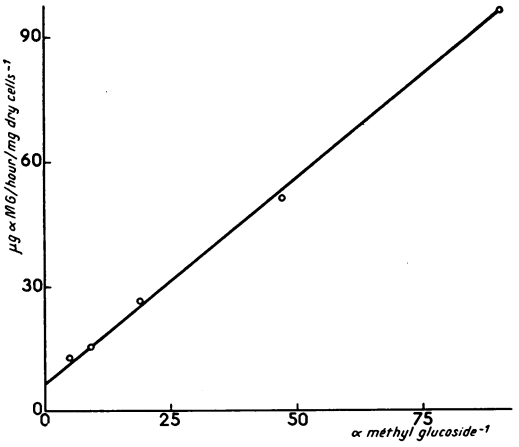

Figure 9. The relation between the concentration of α-methyl glucoside and the rate of its accumulation. See figure 7 for details. The rates were calculated from the amounts of α-methyl glucoside accumulated after incubation for 5 min.

TABLE 7

Specificity of permeation mechanism for α-methyl glucoside accumulation

All flasks contained 10^{-2} M α-methyl glucoside, substrates at concentration of 10^{-2} M, and 10 mg dry weight of cells. The total vol in each case was 5.0 ml. The suspensions were incubated for 5 min at 30 C, the cells collected on membrane filters. The rate of α-methyl glucoside accumulation was calculated from their radioactivities.

Substrates	μg α Mg/Hr × Mg dry cells
Control	0.97
Maltose (4-glucose-α-glucoside)	0.11
α-Phenyl glucoside	0.95
Trehalose (1-α-glucose-α-glucoside)	0.92
Cellobiose (4-glucose-β-glucoside)	0.97
β-Methyl glucoside	0.66
β-Methyl galactoside	0.82
Lactose (4 glucose-β-galactoside)	0.75
Melibiose (6-glucose-α-galactoside)	0.89
α-Methyl mannoside	0.87
Glucose	0.15
Galactose	0.64
Mannose	0.22
Fructose	0.26

TABLE 8

Competitive inhibition of α-methyl glucoside uptake by maltose

Five ml of maltose-grown cells (12.8 mg) were incubated at 30 C for 5 min in the presence of C^{14} α-methyl glucoside with or without maltose. The accumulation of α-methyl glucoside was determined as previously described. The inhibition by maltose was calculated as the per cent of that accumulated in the control suspensions containing only α-methyl glucoside.

α-Methyl Glucoside	Maltose	I/S	Control without Maltose
			%
0.010	0.0025	0.25	23
	0.005	0.50	6
0.020	0.005	0.25	28
	0.010	0.50	7
0.040	0.015	0.08	50
	0.010	0.25	22
	0.020	0.50	10

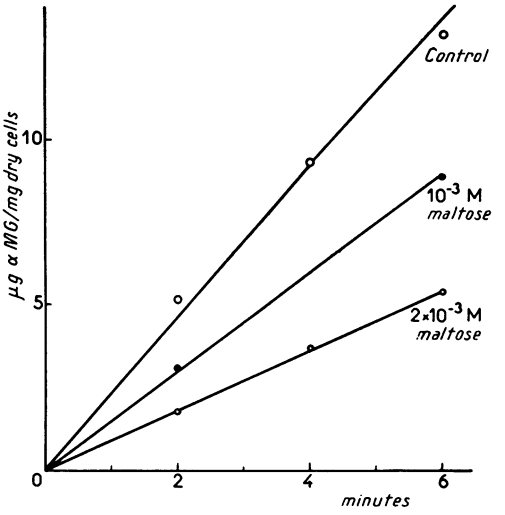

Figure 10. Kinetics of α-methyl glucoside accumulation in the presence of maltose. See text figure 7 for details.

α-phenylglucoside, an active substrate for the α-glucosidase, does not influence the accumulation process. The reaction is not inhibited by galactosides or by α-methyl-mannoside. The rate of α-methyl-glucoside accumulation is linear with respect to time and proportional to the concentration of maltose (figure 10). The competitive inhibition by maltose (table 8) further supports the hypothesis that maltose and α-methyl-glucoside are competing for some catalytic reaction. The dissociation constant for maltose calculated from these data (2.4×10^{-3} M) indicates that maltose has a complexing capacity for this system which is 50 times as great as that of α-methyl-glucoside. The inhibition by metabolized hexoses (table 7) was found to be noncompetitive.

The above evidence demonstrates the presence in this strain of a specific permeation mechanism for accumulating maltose within yeast cells. The demonstration that this permease is a component of the maltozymase sequence leading to maltose accumulation would require evidence that the permease acted upon maltose as a substrate, that its activity is distinctly separate from that of the first enzyme involved internally in maltose metabolism, and that the activity of maltozymase depends on the permease activity. The competitive inhibition of α-methylglucoside accumulation by maltose argues for the possibility that maltose forms a complex with this permease,

TABLE 9

Separation of the permeation mechanism from the activity of the α-glucosidase

The various strains were grown in complex medium containing either glucose or maltose as energy source. Log phase cells were harvested, washed and analyzed for their ability to accumulate C^{14} α-methyl glucoside. Parallel samples were lyophilized and assayed for α-glucosidase as previously described.

Strain	Grown on	Ability to Grow on Maltose	α-Glucosidase*	α-Mg† Accumulation
Saccharomyces cereviseae LK2G12	Maltose	+	15.1	0.93
	Glucose		1.2	0.83
Saccharomyces marcianus Hansen	Glucose	−	0	0.84
Saccharomyces fragilis Jong	Glucose	−	0	0.54

* μMoles phenol/hr × mg dry cells.
† μg α-mg/hr × mg dry cells.

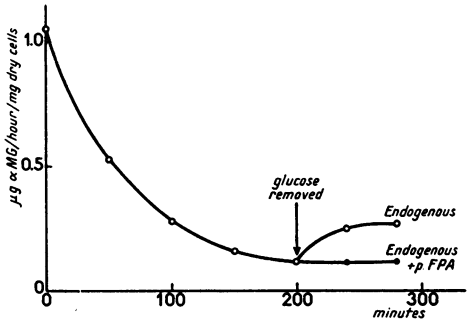

Figure 11. Loss of the permeation mechanism during deadaptation. A suspension of maltose grown cells (2.8 mg dry wt/ml) was incubated aerobically at 30 C with 3 per cent glucose. At intervals 5-ml aliquots were removed and the cells washed by centrifugation. After resuspension to original volume, the amount of C^{14} α-methyl glucoside accumulation after 5 min, was measured as previously described. After 200 min, the deadaptation suspension was divided, harvested by centrifugation, and resuspended to original volume in buffer. To one group was added *p*-FPA to a final concentration of 10^{-2} M. At 240 and 280 min, 5-ml aliquots of each were centrifuged, washed, and measured for permeation activity as described above.

although the actual internal accumulation of maltose has not been demonstrated. Furthermore, from the results in table 9, the activity of this permease is shown to be separated from the activity of the α-glucosidase. Not only is the system present in two maltose-negative strains which are devoid of α-glucosidase, but it is constitutive in the α-glucoside inducible strain grown on either glucose or pyruvate as the carbon source. In the latter case, α-glucosidase induction does not increase the level of the accumulating system. The dependence of maltozymase activity on permease activity is evident from the examination of these two activities during deadaptation. The results (figure 11) not only show a decrease in permease activity during deadaptation but also quantitatively account for the kinetics of the loss of maltozymase activity (figure 1). These results lead one to the conclusion that the glucoside permeation mechanism is involved in maltose accumulation and is a member of the maltozymase sequence.

Equation 1 describing maltozymase activity, should now be expressed:

$$\text{maltose exog.} \xrightarrow[k_1]{\text{permease}} \text{maltose endog.} \xrightarrow[k_2]{\alpha\text{-glucosidase}} \text{glucose} \xrightarrow[k_3]{} CO_2, \text{etc.} \qquad (2)$$

where the rate constants are k_1, permease; k_2, α-glucosidase and k_3, glucose fermentation. During induction, where glucose fermentation and the permease are constitutive, k_2 is rate-limiting and the over-all rate of CO_2 production can be controlled by the activity of the α-glucosidase. However, in fully induced cells and in particular during deadaptation, which leads to an increase in the crypticity factor, the reaction catalyzed by k_2 is no longer rate-limiting. In the latter condition the permease (k_1) becomes the rate-limiting step in maltose metabolism.

Conclusion: The development of crypticity cannot be directly attributed to changes in either soluble, bound or total α-glucosidase, α-glucoside heterogeneity or an alternate maltose-utilizing system. A search for another component in the maltozymase sequence revealed the presence of a specific catalytic mechanism for internally accumulating α-methyl-glucoside and probably the substrate maltose. The loss of this system during deadaptation directly furnishes an explanation for the crypticity that develops.

DISCUSSION

The production of CO_2 from maltose in yeast involves the collective action of the maltozymase system. At least part of this system involves the well characterized reactions of carbohydrate metabolism. In order to reveal other components of the maltozymase sequence, the maltozymase activity of cells incubated with glucose in nitrogen-free medium was examined. A study of the resulting loss of maltozymase activity (deadaptation) revealed that the phenomenon was complex. There are at least three components contributing to the over-all deadaptation: (1) Alpha-glucosidase is found in both a bound and a free form. During deadaptation the bound form is solubilized. (2) A permeation mechanism was discovered which is probably involved in the internal accumulation of the substrate. Under certain conditions, this mechanism is rate-limiting and results in the cell becoming cryptic towards maltose. (3) Both the α-glucosidase and permease activities are lost during deadaptation. We shall discuss these three phenomena in sequence.

1. One of the most interesting features to emerge from these studies was the demonstration of a bound form of the usually soluble α-glucosidase. When enzyme synthesis is retarded, either by the removal of the inducer during deadaptation, or at the completion of induction, there is release of enzyme from the bound to the soluble form. During induction of α-glucosidase, the enzyme appears more rapidly in the bound form than in the soluble form. One possible explanation for these findings is that the enzyme is bound to its site of synthesis. When further enzyme synthesis is stopped, there is a release of the recently synthesized molecules to the soluble form.

From this hypothesis one would expect that these particles exhibit the strong correlation observed between RNA synthesis and α-glucosidase synthesis (Spiegelman *et al.*, 1955). That such a correlation exists is suggested by the following observation. The incorporation of P^{32} into the nucleotides of a particulate RNA fraction, but not of the soluble RNA, is stimulated by α-glucosidase induction by α-methyl-glucoside. Whether such results indicate specific or secondary effects of the inducer are not known. Judging by properties observed thus far, these particles represent an interesting area for further study.

2. The problem of crypticity, especially towards carbohydrates, has long been recognized in yeast metabolism. The metabolism of maltose represents a typical example involving crypticity. Since Fisher (1895) first proposed that maltose is metabolized initially by the action of α-glucosidase, there have been conflicting observations indicating that maltose is not metabolized under conditions in which α-glucosidase would be expected to operate *in vivo* (Willstätter and Bamann, 1926; Leibowitz and Hestrin, 1945). These observations have led to the suggestion that α-glucosidase may exist on the cell wall of yeast (Myrbäck and Vasseur, 1943), or that the cell wall may represent an impermeable membrane for maltose (Gottschalk, 1950), or that specific permeases may be absent or rate-limiting for substrate entry in yeast (Monod, 1956). The present finding of a specific glucoside permeation mechanism, whose loss of activity during deadaptation leads to an increase in the crypticity factor, is in support of the latter hypothesis. Similar reactions could readily explain the crypticity in yeast towards raffinose (Davies, 1953) and sucrose (Leibowitz and Hestrin, 1945; Gros and Spiegelman, *unpublished results*).

The galactoside permease of *E. coli* has been shown to be under specific genetic control (Rickenberg *et al.*, 1956), and one would expect that the glucoside permeation mechanism in yeast is controlled similarly. If its substrate specificity differed from that of the enzyme acting initially upon the internally accumulated substrate, as was found in the present experiments, then the genetic loss or gain of such a permease could appear to control the metabolism of seemingly diverse biochemical reactions. Maltose fermentation has been shown to be under the control of 4 polymeric M genes (Winge and Roberts, 1950 *a*, *b*). M_1 controls the fermentation of both maltose and sucrose, and M_3 the fermentation of maltose alone. These findings could be resolved if the M_1 gene also controlled a permeation mechanism and if the permease worked on both maltose and sucrose. The possible control of a permease by M_1 would explain the observation that a yeast type containing M_3 required a longer period to acquire the ability to ferment maltose than did an M_1 genotype (Winge and Roberts, 1950 *a*, *b*). Similar claims for the existence of a series of alleles controlling the induction of an α-glucosidase capable of hydrolyzing 6 different oligosaccharides (Lindegren and Lindegren, 1953) may have a related basis involving a control of

the entry of both the inducer and the substrate.

The specific properties of the glucoside permease also provide an explanation for several problems previously encountered in the induction of maltozymase. In the presence of either galactose or glucose, the development of maltozymase is suppressed (Spiegelman and Dunn, 1947). Both galactose and glucose inhibit the functioning and also lead eventually to an inactivation of the permease. The resulting reduction in the internal concentration of the inducer decreases the further synthesis of maltozymase. Although the theoretically attractive hypothesis that such interactions represent competition between different enzyme-forming systems cannot be eliminated, definitive evidence that these competitions exist at the protein synthesizing level has not been adduced. Such evidence would require the use of nonmetabolized inducers whose interactions are not related to inhibitions of their respective permeases.

3. The basis of the inactivation of both the glucoside permeation mechanism and the α-glucosidase during deadaptation is not known. The observation of an inhibition of maltozymase deadaptation by DNP and azide (Spiegelman and Reiner, 1947) suggest that the inactivation process requires energy. The inactivation of these systems might be caused by toxic effects of the products of glucose metabolism, inhibition of their active centers, or degradation of the protein molecule itself. Although the present data do not permit a decision as to the latter two possibilities, the first one can be readily eliminated. Dialysis of deadapted extracts does not increase the α-glucosidase activity. Also, when deadapted cells are allowed to assimilate their endogenous reserves, only a slight increase in permease activity occurs (figure 11). This increase is inhibited by an amino acid analogue and probably represents limited protein synthesis from endogenous nitrogen reserves.

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SUMMARY

The metabolism of glucose by yeast cells grown in maltose in the absence of exogenous nitrogen leads to an increase *in vivo* of α-glucosidase, the development of crypticity towards its activity and the eventual metabolic destruction of enzyme.

Glucosidase is found both in a soluble form and bound to subcellular particles. During deadaptation there is a flow from the bound to the soluble form. The properties and kinetic behavior of the bound enzyme during induction suggest that it may be bound to its site of synthesis.

A glucoside permeation mechanism was found which leads to the internal concentration of α-methyl glucoside and which is competitively inhibited by maltose. The inactivation of the glucoside permease during deadaptation explains the development of crypticity of the intact cell to maltose metabolism.

The permease can be rendered inactive through inhibition and also probably by genetic loss. Such losses eliminate maltozymase activity and induced enzyme synthesis by suppressing the entry of the inducer. The significance of this permeation mechanism for the problems of competitive interactions and other yeast crypticities is discussed.

REFERENCES

COHEN, G. AND RICKENBERG, H. V. 1955 Étude directe de la fixation d'un inducteur de la β-galactosidase par les cellules d'*Escherichia coli*. Compt. rend., **240,** 466–468.

DAVIES, R. 1953 Enzyme formation in *Saccharomyces fragilis*. I. Invertase and raffinase. Biochem. J. (London), **55,** 484–497.

FISHER, R. 1895 Über den Einfluss der Configuration auf die Wirkung der Enzyme. III. Chem. Ber., **38,** 1429–1438.

FOLIN, O. AND CIOCOLTEAU, Y. 1927 On the tyrosine and tryptophan determinations in protein. J. Biol. Chem., **73,** 627–635.

GOTTSCHALK, A. 1950 α-D-Glucosidases. In *The enzymes*, Vol. I, pp. 551–583. Edited by J. B. Sumner and K. Myrbäck. Academic Press, Inc., New York.

HALVORSON, H. O. AND SPIEGELMAN, S. 1952 The inhibition of enzyme formation by amino acid analogues. J. Bacteriol., **64,** 207–221.

HALVORSON, H. O. AND SPIEGELMAN, S. 1954 On the role of the inducer in the synthesis of maltase in yeast. J. Bacteriol., **68,** 265–273.

HALVORSON, H. O. AND SPIEGELMAN, S. 1957 Kinetics of induced α-glucosidase synthesis in yeast. J. Gen. Physiol., (*accepted for publication*).

HELFERICH, B. AND SCHAFER, W. 1932 *Organic*

synthesis, Vol. I, pp. 384–386. Edited by A. Blatt and H. Gilman. John Wiley and Sons, Inc., New York.

KAPLAN, J. G. 1954 The alteration of intracellular enzymes. II. The relation between the surface and the biological activities of altering agents. J. Gen. Physiol., **38,** 197–208.

LIEBOWITZ, J. AND HESTRIN, S. 1945 Alcoholic fermentation of the oligosaccharides. Advances in Enzymol., **5,** 87–128.

LINDEGREN, C. G. AND LINDEGREN, G. 1953 The genetics of melezitose fermentation in *Saccharomyces*. Genetica, **26,** 430–444.

MONOD, J. 1956 Remarks on the mechanism of enzyme induction. In *Enzymes: Units of biological structure and function*, pp. 7–28. Academic Press, Inc., New York.

MOORE, S. AND STEIN, W. R. 1948 Photometric ninhydrin method for use in chromatography of amino acids. J. Biol. Chem., **176,** 367–388.

MYRBÄCK, K. AND VASSEUR, E. 1943 Über die Lactosegärung und die Lokalization der Enzyme in der Hefezelle. Z. physiol. Chem., **277,** 171–180.

RICKENBERG, H., COHEN, G., BUTTIN, G., AND MONOD, J. 1956 La galactoside-permease d'*Escherichia coli*. Ann. inst. Pasteur, **91,** 829–857.

ROBERTSON, J. 1955 An investigation of the α-glucosidase of yeast during deadaptation. Ph.D. Thesis: University of Michigan, Ann Arbor.

SPIEGELMAN, S. AND DUNN, R. 1947 Interactions between enzyme-forming systems during adaptation. J. Gen. Physiol., **31,** 153–173.

SPIEGELMAN, S. AND REINER, J. M. 1947 The formation and stabilization of an adaptive enzyme in the absence of its substrate. J. Gen. Physiol., **31,** 175–193.

SPIEGELMAN, S. AND HALVORSON, H. O. 1953 The nature of the precursor in the induced synthesis of enzymes. In *Adaptation in microorganisms*, pp. 98–131. Edited by R. Davies and E. F. Gale. University of Cambridge Press, Cambridge, England.

SPIEGELMAN, S., HALVORSON, H. O., AND BEN-ISHAI, N. 1955 Free amino acids and the enzyme-forming mechanism. In *Amino acid metabolism*, pp. 124–170. Edited by W. D. McElroy and H. B. Glass. Johns Hopkins Press, Baltimore, Md.

WILLSTÄTTER, R. AND BAMANN, E. 1926 Trennung von Maltase und Saccharase. Z. physiol. Chem., **151,** 273–285.

WINGE, O. AND ROBERTS, C. 1950*a* Identification of the gene for maltose fermentation in *Saccharomyces italicus*. Nature, **166,** 1114.

WINGE, O. AND ROBERTS, C. 1950*b* The polymeric genes for maltose fermentation in yeasts, and their mutability. Compt. rend. trav. lab. Carlsberg. Sér. physiol., **25,** 35–83.