Lactose Utilization and Hydrolysis in *Saccharomyces fragilis*

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SUMMARY

Sodium azide, 2,4-dinitrophenol and iodoacetate did not inhibit hydrolysis of lactose by cell-free preparations of *Saccharomyces fragilis* β -galactosidase, but with intact organisms fermentation and hydrolysis were inhibited to a similar extent. This suggests that these inhibitors may interfere with the transport of lactose into the cell. Galactose fermentation was inhibited by sodium azide and dinitrophenol to a much greater extent than was glucose or lactose fermentation. Hydrolysis of lactose and *o*-nitrophenyl- β -D-galactoside required potassium ion at an optimal concentration of 0.1M; ammonium ion also activated, but sodium ion was much less effective and, in presence of optimal concentrations of potassium ion, were inhibitory, as were calcium and zinc ions. Lithium, magnesium and manganese ions were without effect in presence of potassium optimal ion. The β -galactosidase activity of aqueous suspensions of acetone-dried organisms, or of cell-free extracts, decayed rapidly at 25°. This decay was prevented by addition of sodium phosphate or various potassium salts, but not by sodium or potassium chloride or by potassium sulphate. Sodium azide, arsenate or 2,4-dinitrophenol decreased the rate of decay and promoted partial recovery when decayed preparations were subsequently incubated with potassium phosphate. The enzyme was most stable in 0.1M-potassium phosphate (pH 7.0). A purified (237-fold) preparation of the β -galactosidase was obtained; this too decayed rapidly in water or potassium chloride solution but not in potassium phosphate solution.

INTRODUCTION

Monod & Cohn (1952) compared the properties of β -galactosidases from various species of micro-organisms and found that the enzymes from several strains of *Escherichia coli*, *Aerobacter aerogenes* and *Shigella sonnei* were indistinguishable by substrate or inhibitor specificity or by immunological behaviour, whereas they were clearly differentiated from the β -galactosidases of *Saccharomyces fragilis* and of some *Lactobacillus* strains. More recently, differences have been reported between the enzymes from strains of *E. coli* in respect to cation activation patterns (Kuby & Lardy, 1953; Wallenfels, Malhotra & Dabich, 1960), turn-over number, absorption spectra (Wallenfels, Zarnitz, Laule, Bender & Keser, 1959) and enzyme stability (Rickenberg, 1959). Little work has been reported on the β -galactosidase of *Saccharomyces fragilis* since the studies by Caputto, Leloir & Trucco (1948). The present paper describes some of the properties of lactose hydrolysis by β -galactosidase preparations from *S. fragilis* and by the intact organisms.

METHODS

Organisms, growth media and suspensions of organisms. The β -galactosidase-producing organism used throughout was a strain of *Saccharomyces fragilis* Jorgenson obtained from the Centraalbureau voor Schimmelcultures, Delft. *Saccharomyces mandshuricus* (Saito, 1916) was obtained from the Carlsberg Laboratory, Copenhagen. The basal yeast-extract medium was that of Davies, Faulkner, Wilkinson & Peel (1951). For some experiments a basal defined medium was used containing (per litre): $\text{NH}_4\text{H}_2\text{PO}_4$, 1 g.; $(\text{NH}_4)_2\text{HPO}_4$, 1 g.; KH_2PO_4 , 2 g.; 10 ml. trace element solution (Davies *et al.* 1951); biotin, 1 $\mu\text{g.}$; 200 $\mu\text{g.}$ each of calcium pantothenate, pyridoxin, nicotinic acid and thiamin hydrochloride; 1 mg. *mesoinositol*. After sterilization an appropriate source of carbon was added as indicated in the text. The medium was dispensed in Roux Bottles (150 ml./bottle) and inoculated with 0.5 ml. of a 24 hr. culture in basal yeast-extract medium + 2% (w/v) glucose. For inoculating cultures in defined medium the organisms were harvested, washed twice with sterile water and resuspended in sterile water to approximately the original volume. All cultures were incubated at 25°.

For enzymic tests *Saccharomyces fragilis* cultures were harvested by centrifugation, washed twice with water and suspended in water to a suitable concentration. Optical extinction of suspensions were measured with a Hilger Spekker absorptiometer and converted to mg. dry matter/ml. by means of a calibration curve. All experiments were done at 25°. Where concentrations of solutes are given as % this means % (w/v) in all cases.

Saccharomyces mandshuricus was grown at 25° for 40–48 hr in basal yeast-extract medium + 2% galactose, the organism harvested, washed twice in water and suspended in 0.2M-potassium phosphate + citric acid buffer (pH 6.0) containing 8 mM- NaN_3 , to give a final concentration of organism equivalent to about 100 mg. dry matter/ml. For experiments in which buffers other than potassium phosphate + citric acid were used, the organism was suspended in 8 mM- NaN_3 . The suspension was shaken aerobically for 2 hr at 25° to decrease endogenous CO_2 production to a negligible value. In presence of sodium azide oxidation and assimilation of sugar is inhibited and, under aerobic conditions, glucose and galactose are fermented almost quantitatively (95%) to ethanol and CO_2 .

Cell preparations. Preparations of disrupted *Saccharomyces fragilis* were obtained by shaking with glass beads (ballotini no. 12) in a Mickel shaker (Mickle, 1948) for 15 min. at 4° and filtering through a sintered glass filter (porosity no. 1). Cell-free extracts were obtained by centrifuging the broken organisms at 1000g for 15 min. Acetone-dried organisms were prepared by the method of Epps (1944).

Estimation of β -galactosidase activity. This was measured either (a) by the increase in reducing sugar, or (b) manometrically from the rate of CO_2 evolution when the β -galactosidase preparation was incubated aerobically with 0.075M-lactose in presence of 2 mM- NaN_3 and galactose-adapted *Saccharomyces mandshuricus*. The latter estimation was done in Warburg manometers containing (in a total volume of 4.0 ml.): 1.0 ml. *S. mandshuricus* suspension; 0.1M buffer (usually potassium phosphate + citric acid pH 6.0); 0.075M-lactose; in the side arm, the β -galactosidase preparation. Other additions are described in the text. The gas phase was air except where NaN_3 was omitted, in which case it was 95% (v/v) N_2 + 5% (v/v) CO_2 . In

presence of 2 mM- NaN_3 the rate of CO_2 evolution was linear up to 15 $\mu\text{l./min.}$ and the yield of CO_2 was 95 % of theoretical for conversion of 1 mole hexose to 2 moles ethanol + 2 moles CO_2 . Unless stated otherwise, the rates of CO_2 evolution have been divided by 89.6 and expressed as $\mu\text{moles lactose hydrolysed/mg. dry matter/hr.}$ One unit of β -galactosidase activity is defined as the amount of enzyme which hydrolyses 1 μmole lactose in 1 hr at 25° in 0.1 M-potassium phosphate + citric acid buffer (pH 6.0).

Hydrolysis of *o*-nitrophenyl- β -D-galactoside (ONPG) was determined by the method of Lederberg (1950) by using 5 mM-ONPG in 0.067M-potassium phosphate + citric acid buffer (pH 6.5), unless stated otherwise. Activities are expressed as $\mu\text{moles ONPG hydrolysed/mg. dry matter/hr.}$

RESULTS

Inducible fermentation of lactose and galactose

The ability of *Saccharomyces fragilis* to ferment lactose and galactose can be induced by growth on basal medium + lactose or galactose. When galactose was the substrate the resulting organisms fermented both lactose ($Q_{\text{CO}_2}^{\text{N}} = 240$) and galactose ($Q_{\text{CO}_2}^{\text{N}} = 233$) considerably faster than glucose ($Q_{\text{CO}_2}^{\text{N}} = 154$). When lactose was the substrate lactose was fermented more rapidly ($Q_{\text{CO}_2}^{\text{N}} = 233$) than galactose ($Q_{\text{CO}_2}^{\text{N}} = 173$) or glucose ($Q_{\text{CO}_2}^{\text{N}} = 182$). After growth in basal medium alone the organism fermented lactose and galactose only very slowly ($Q_{\text{CO}_2}^{\text{N}} = 4-8$), nevertheless the rates were usually slightly higher than for organisms grown in basal medium + 2 % glucose ($Q_{\text{CO}_2}^{\text{N}} = 1-12$). A possible cause of the low rate of fermentation of lactose would be a deficiency of β -galactosidase. However, *S. fragilis* grown on basal medium alone, or with the addition of 2 % glucose, possessed considerable β -galactosidase activity after acetone drying, in one instance ninety times the amount required for the observed rate of lactose fermentation and almost twice the amount required for the maximum fermentation rate observed with fully induced organisms (Table 1). *S. fragilis* grown in basal medium alone usually possessed a considerably higher latent β -galactosidase activity than organisms grown in basal medium + 2 % glucose, indicating repression of β -galactosidase synthesis by glucose. Davies (1956), who used *S. fragilis* grown in a chemostat under steady state conditions, observed that synthesis of β -galactosidase was strongly suppressed by glucose concentrations higher than 10^{-3}M.

With *Saccharomyces fragilis* grown in basal defined medium alone fermentation of glucose also was initially slow ($Q_{\text{CO}_2}^{\text{N}} = 30-60$), suggesting a deficiency in the glycolytic system which would also result in a low rate of galactose and lactose fermentation. Anaerobic incubation of such organisms for 2-4 hr in 0.1M-potassium phosphate + citric acid buffer (pH 6.0) containing 0.16M-glucose restored the glucolytic activity to normal ($Q_{\text{CO}_2}^{\text{N}} = 181-213$), but even after 6 hr in 3% galactose or lactose, fermentation of these sugars remained slow ($Q_{\text{CO}_2}^{\text{N}}$, less than 30). After 21 hr in presence of lactose the rate of fermentation of this sugar increased to $Q_{\text{CO}_2}^{\text{N}} = 146-254$, but for organisms similarly incubated in galactose $Q_{\text{CO}_2}^{\text{N}}$ (galactose) was only 14-37. Addition of small amounts of glucose (3 mM) and/or vitamin-free casein hydrolysate (0.7 mg./ml.) had no significant effect on the induction of lactose or galactose fermentation. Organisms which had been induced to ferment lactose ($Q_{\text{CO}_2}^{\text{N}} = 173$) by incubation with lactose for 21 hr were able to ferment galactose at a

much higher rate ($Q_{CO_2}^N = 89$) than organisms incubated with galactose for 21 hr ($Q_{CO_2}^N = 9$). A possible reason for this difference might be that synthesis of β -galactosidase and a transport system would be sufficient to enable the organisms to derive energy from lactose, whereas for galactose two or three enzymes would be required in addition to a transport system. Robichon-Szulmajster (1958) reported that galactokinase, galactose-1-phosphate-uridyl-transferase and uridyldiphosphate-galactose-4-epimerase are all inducible enzymes in *S. fragilis*, but Mills, Smith & Lockhead (1957) claimed that the epimerase was constitutive.

Table 1. *Lactose fermentation and β -galactosidase activity in *Saccharomyces fragilis**

Fermentation rates were measured manometrically at pH 6.0 in an atmosphere of 95 % (v/v) nitrogen + 5 % (v/v) CO_2 . β -Galactosidase activities were measured by the manometric method using acetone-dried organisms or, in one experiment, a crude Mickle extract. To permit direct comparison with β -galactosidase activities, fermentation rates are expressed as μ moles sugar fermented/mg. dry wt. organisms/hr, the values being calculated from the rates of CO_2 evolution by dividing by 44.8 (for glucose) or 89.6 (for lactose).

Growth medium	Period of growth (hr)	Fermentation rate (μ moles/mg./hr)		β -Galactosidase activity (μ moles/mg./hr)
		Glucose	Lactose	
Basal yeast extract (0.02 %) only	44	0.94	0.06	2.46
Basal yeast extract (0.02 %) only	68	1.50	0.09	8.05
Basal yeast extract (0.02 %) + 2 % glucose	38	4.12	0.13	1.52
Basal yeast extract (0.02 %) + 2 % glucose	43	5.20	0.05	2.42
Basal yeast extract (0.2 %) + 2 % lactose	25	5.20	5.42	17.43

* Mickle extract.

Table 2. *Effect of growth in tryptic digest of casein on induction of ability of *Saccharomyces fragilis* to ferment lactose*

S. fragilis was grown for 21 hr at 25° in basal yeast extract (0.02 %) medium supplemented with tryptic digest of casein as indicated. Organisms were harvested, washed and incubated under nitrogen in Warburg manometers with 0.075 M-lactose or glucose in 0.1 M-McIlvaine buffer (pH 4.5) at 25° and the rate of CO_2 production measured.

Incubation period (min.)		Casein added to growth medium as tryptic digest (%)				
		0	0.02	0.1	0.5	2.0
5	$Q_{CO_2}^N$ (glucose)	63	62	64	66	85
5	$Q_{CO_2}^N$ (lactose)	2	3	16	30	49
240	$Q_{CO_2}^N$ (glucose)	213	208	234	212	260
240	$Q_{CO_2}^N$ (lactose)	7	21	97	115	250

Addition of tryptic digest of casein to a modified basal yeast extract medium (0.02 % yeast extract) markedly increased the initial rate of lactose fermentation, and the increase in rate when washed uninduced organisms were subsequently incubated with lactose. The effect increased with increasing concentration of casein digest (Table 2). For all concentrations of casein digest the variation of $Q_{CO_2}^N$,

(lactose) with time had the form shown in Fig. 1 and the value of $Q_{CO_2}^N$ at the point of inflexion was a function of the casein digest concentration (Fig. 2). The effect was probably due to lactose present in the casein digest since a similar response was obtained by addition of lactose (10^{-4} to 10^{-1} %) to the basal defined growth medium, whereas an acid hydrolysate of vitamin-free casein was ineffective. The value of $Q_{CO_2}^N$ (lactose) at the point of inflexion probably gives a measure of the initial value of lactose fermenting capacity, the post-inflexion increase in $Q_{CO_2}^N$ resulting from new synthesis of rate-limiting components of the lactose fermenting system. The β -galactosidase activity of acetone-dried organisms also was increased by addition of small amounts of lactose to the basal defined growth medium, 10^{-4} M giving half maximum production (Fig. 3). Galactose was less effective than lactose at low concentrations but slightly more effective at high concentrations (28 mM).

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Fermentation of lactose by *Saccharomyces fragilis*. Organisms grown for 24 hr at 25° in basal yeast extract (0.02 %) medium + tryptic digest of casein as indicated. Manometers contained (in 3.5 ml.): 0.05 M McIlvaine buffer (pH 4.5), 0.075 M-lactose and equiv. 2 mg. dry wt. of *S. fragilis*. Gas phase N_2 . Curve A, no casein; curve B, 0.5 % casein; curve C, 2.0 % casein.

Fig. 2. Effect of addition of casein digest to growth medium on initial rate of lactose fermentation by *Saccharomyces fragilis*. The organism was grown for 24 hr at 25° in basal yeast extract (0.02 %) medium + tryptic digest of casein as indicated. The initial rate is the rate at the points of inflexion in Fig. 1.

Fig. 3. Effect of growth in presence of lactose or galactose on β -galactosidase activity of acetone-dried *Saccharomyces fragilis*. Organisms were grown for 48 hr at 25° in basal defined medium + lactose or galactose, as indicated. β -Galactosidase activity was estimated manometrically. Curve A, lactose; curve B, galactose.

Effect of inhibitors on lactose fermentation

The β -galactosidase of *Saccharomyces fragilis*, like that of *Escherichia coli*, is strongly activated by potassium ion (Caputto, Leloir & Trucco, 1948) but intact fully induced organisms have only about one-tenth the β -galactosidase activity of organisms disrupted under suitable conditions (Davies, 1956), although the potassium content of the organisms ($0.37 \mu\text{g. atoms/mg. dry matter}$, measured by flame photometry (Davies, Folkes, Gale & Bigger, 1953) is about equivalent to the concentration (0.1 M) required for maximum β -galactosidase activity with acetone-dried organisms or cell-free extracts. Because of the potassium requirement of *S. fragilis*

Table 3. *Effect of various treatments and inhibitors on utilization of lactose by Saccharomyces fragilis*

Saccharomyces fragilis was grown for 23 hr at 25° in basal yeast extract medium + 2 % lactose. Fermentations were carried out at 25° under nitrogen in Warburg manometers containing (in 1.0 ml.): 0.1 M-sodium or potassium phosphate + citric acid buffer (pH 6.0), 0.1 M-lactose, equiv. 5.0 mg. dry wt. *S. fragilis* (or equivalent amount of disrupted or acetone-dried organisms) and inhibitors as indicated. Incubation was continued until about one-third of the lactose had been fermented in the uninhibited control system. Organisms and protein were then precipitated with zinc sulphate + barium hydroxide and the supernatant fluids analysed for glucose, galactose and lactose by fermentation with uninduced and galactose-induced *S. mandshuricus*, using acetone-dried *S. fragilis* to hydrolyse residual lactose.

Inhibitor or treatment	Concn.	Buffer		(a) Lactose disappearing (μ mole/mg./hr)	(b) Lactose fermented (μ mole/mg./hr)	(c) $\frac{1}{2}$ (Glucose + galactose) accumulating (μ mole/mg./hr)	b/(b + c)
		Cation	Anions				
None		K ⁺	} Phosphate + citrate	3.05	2.51	0.13	0.95
None		Na ⁺		2.80	2.60	0.25	0.91
Iodoacetate	10 mM	K ⁺	} Phosphate + citrate	0.40	0.04	0.46	0.08
		Na ⁺		0.53	0.20	0.37	0.35
<i>p</i> -Chloromercuribenzoate	1 mM	K ⁺	} Phosphate + citrate	0.19	0.05	0.04	0.55
		Na ⁺		0.15	0	0.1	0
2,4-Dinitrophenol	3 mM	K ⁺	} Phosphate + citrate	0.74	0.66	0.09	0.88
		Na ⁺		1.18	1.19	0	1.00
NaN ₃	3 mM	K ⁺	} Phosphate + citrate	2.18	1.79	0.08	0.96
		Na ⁺		1.36	1.12	0.41	0.73
Toluene	—	K ⁺	} Phosphate + citrate	1.98	0.11	1.85	0.06
		Na ⁺		0.24	0	0.20	0
Aerosol OT	2 mg./mg. yeast	K ⁺	} Phosphate + citrate	2.71	0.37	2.10	0.15
		Na ⁺		0.16	0.04	0.40	0.09
CTAB	250 μ g./mg. yeast	K ⁺	} Phosphate + citrate	4.76	0	5.01	0
		Na ⁺		0	0	0	—
Polymyxin	250 μ g./mg. yeast	K ⁺	} Phosphate + citrate	5.33	0.29	4.58	0.06
		Na ⁺		0.93	0.63	0.11	0.85
Acetone	—	K ⁺	Phosphate	18.2	0	17.9	0
		Na ⁺	Phosphate	0.53	0	0.70	0
Mickle extract	—	K ⁺	} Phosphate + methylglyoxaline	23.8	0	24.1	0
		Na ⁺		0.59	0	0.50	0

β -galactosidase the effect of various treatments and inhibitors on lactose fermentation and hydrolysis by intact organisms was examined in buffer systems in which the cation was either all potassium or all sodium. With intact fully induced uninhibited organisms most of the lactose which disappeared could be accounted for by the CO_2 liberated (assuming a normal alcoholic fermentation) and there was little accumulation of glucose or galactose in the incubation mixtures with either sodium or potassium as the cation (Table 3). Of the inhibitors tested those (iodoacetate, *p*-chloromercuribenzoate, 2,4-dinitrophenol, sodium azide) which, so far as is known

Table 4. *Effect of inhibitors on production of CO_2 from glucose, galactose and lactose by Saccharomyces fragilis*

Saccharomyces fragilis was grown for 23 hr at 25° in basal yeast extract medium + 2 % lactose. Fermentation rates measured manometrically at pH 6.0 with a gas phase of 95 % (v/v) nitrogen + 5 % (v/v) CO_2 .

Inhibitor	$Q_{\text{CO}_2}^{\text{N}_2}$					
	Glucose		Galactose		Lactose	
	K	Na	K	Na	K	Na
None	200	181	159	137	204	155
NaN_3 , 0.3 mM	255	215	39	26	167	120
NaN_3 , 3 mM	208	167	10	9	87	46
None	319	297	233	210	356	289
2,4-Dinitrophenol, 0.3 mM	360	259	91	94	327	145
2,4-Dinitrophenol, 3 mM	86	67	68	53	72	19
None	216	174	207	183	330	264
Iodoacetate, 1.0 mM	168	126	159	114	330	180
Iodoacetate, 10 mM	15	0	9	0	51	54
None	255	—	252	—	263	—
<i>p</i> -Chloromercuribenzoate, 1.0 mM	120	—	36	—	4	—

are without disruptive effects on the integrity of the cell structures, inhibited CO_2 production and lactose disappearance to about the same extent, irrespective of whether the buffer cation was sodium or potassium; and again there was little accumulation of glucose or galactose. With the exception of *p*-chloromercuribenzoate (100 % inhibition of lactose hydrolysis at 0.63 mM), these substances at the concentrations used did not inhibit β -galactosidase activity of acetone-dried organisms or cell-free extracts. Sodium azide and 2,4-dinitrophenol at 0.3 mM, but none of the other substances tested, inhibited fermentation of galactose to a much greater extent than fermentation of glucose or lactose, irrespective of whether the buffer cation was sodium or potassium (Table 4). Fermentation of both galactose and lactose was more sensitive to inhibition by sodium azide (3 mM) than was fermentation of glucose, despite the fact that cell-free β -galactosidase activity was not inhibited. Inhibition of lactose and galactose fermentation by 2 mM-sodium azide was greater for organisms grown in basal yeast extract medium + 2 % lactose (lactose 68 %; galactose 74 %) than for organisms grown with 2 % galactose (lactose 10 %; galactose 57 %).

Treatments or substances which damage cell membrane structures (toluene, Aerosol OT, cetyltrimethylammonium bromide, polymyxin, acetone, disruption in Mickle disintegrator) inhibited CO_2 production from lactose but, provided potassium

ions were present, permitted hydrolysis at a rate which could be many times greater than that required to accommodate the rate of CO_2 production from lactose by intact, fully induced organisms (Table 8).

Effect of pH value on lactose fermentation and β -galactosidase activity

The effect of pH value on the rate of fermentation of lactose by intact *Saccharomyces fragilis* and on the rate of hydrolysis by acetone-dried organisms is shown in Figs. 4*a*, *b*. The fermentation rate at any pH value was similar in sodium or potassium phosphate buffers and was a maximum at pH 5.0–5.5. As with sucrose fermentation (Davies, 1953) there was a pronounced difference between the rates observed

Fig. 4*a*. Effect of pH value on fermentation of lactose by *Saccharomyces fragilis*. Curve A, potassium phosphate; curve B, sodium acetate; point C, sodium phosphate.

Fig. 4*b*. Effect of pH value on hydrolysis of lactose by acetone-dried *Saccharomyces fragilis*. Curve A, potassium buffer; curve B, sodium buffer.

Figs. 4*a*, *b*. Organisms were grown for 48 hr at 25° in basal yeast extract medium + 2 % lactose. Fermentation rates were measured in a system containing: 0.075 M-lactose, 0.05 M-phosphate as potassium or sodium phosphate of required pH value, + sufficient KHCO_3 or NaHCO_3 to give the same pH value in an atmosphere of 95 % (v/v) N_2 + 5 % (v/v) CO_2 and sufficient KCl or NaCl to give a final potassium or sodium ion concentration of 0.1 M. β -Galactosidase activities determined by using similar conditions + galactose-induced *S. mandshuricus* organisms without sodium azide.

with phosphate and acetate buffers at pH 5.0 and 5.5. In potassium phosphate buffers the rate of lactose hydrolysis by acetone-dried *S. fragilis*, and also by the purified enzyme preparation, showed a sharp maximum at pH 6.0–6.5, and in sodium phosphate buffers a broader maximum at pH 6.5. The rate of hydrolysis of ONPG by acetone-dried organisms showed a sharp maximum at pH 7.0 both in 0.1 M-sodium phosphate and 0.1 M-potassium phosphate. With either substrate and alkali metal ions the enzyme was almost inactive at pH 5.0. Caputto *et al.* (1948) reported maximum lactase activity at pH 6.7–6.8 for a different strain of *S. fragilis*.

Stimulation of β -galactosidase by cations

In these experiments hydrolysis of lactose was not estimated by the continuous *Saccharomyces mandshuricus* technique because of the possibility that thick suspensions of *S. mandshuricus* might remove some of the cations. Consequently hydrolysis was allowed to proceed for 30 min. in the absence of *S. mandshuricus* and the reaction then stopped by addition of zinc sulphate and barium hydroxide (Somogyi, 1945). The precipitate was centrifuged down and the glucose+galactose in the supernatant fluid estimated by fermentation with galactose-induced *S. mandshuricus* organisms in potassium phosphate+citric acid buffer (pH 4.5) containing 2 mM-sodium azide. It was established that, at the concentrations used, none of the

Fig. 5

Fig. 6

Fig. 5. Effect of concentration of acetone-dried *Saccharomyces fragilis* on β -galactosidase activity in absence of added potassium ion. The reactions were done in test-tubes containing 0.097 M-lactose and acetone-dried organisms as indicated, with and without 0.1 M-potassium (as acetate, pH 6.0). After incubation for 30 min. at 25° the free glucose+galactose was estimated by using galactose-induced *S. mandshuricus*. Curve A, no potassium; curve B, + potassium; point C, + boiled cell suspension at equiv. 6.0 mg./ml.

Fig. 6. Effect of concentration of K^+ , Na^+ and NH_4^+ on β -galactosidase activity of acetone-dried *Saccharomyces fragilis*. The reactions were done in test-tubes containing in 3.0 ml.: 0.097 M-lactose, 2 mg. acetone-dried organisms, and potassium, sodium or ammonium acetate (pH 6.0) at cation concentrations indicated. After 30 min. at 25°, protein was precipitated with $ZnSO_4 + Ba(OH)_2$, and the glucose+galactose in the supernatant fluid estimated with galactose-induced *S. mandshuricus*. Full lines, lactose; broken lines, ONPG. Curves A, potassium; curves B, sodium; curve C, ammonium.

cations inhibited fermentation of glucose or galactose by *S. mandshuricus*. Suspensions of acetone-dried *S. fragilis* containing more than 1 mg. dry matter/ml. of incubation system had appreciable β -galactosidase activity in the absence of added potassium ion and at 3 mg. dry matter/ml. the enzyme activity (1.45 units/mg. dry matter) was comparable with that observed in presence of 0.1 M-potassium acetate (2.46 units/mg.; Fig. 5). A boiled suspension of acetone-dried organisms at 6 mg./ml. was almost as good an activator as 0.1 M-potassium acetate. These observations can be explained by the fact that the potassium content of acetone-dried organisms was 0.87 μ g.atoms/mg. dry matter. The experiments on activation and inhibition of

β -galactosidase by cations were done with a concentration of acetone-dried organisms (0.67 mg./ml.) which was inactive in the absence of added cations.

The β -galactosidase of acetone-dried *Saccharomyces fragilis* was activated by potassium ion and, contrary to the observations of Caputto *et al.* (1948), to a less extent by ammonium ion. Sodium ion activated slightly, but lithium, calcium, magnesium, manganese and zinc (all as acetates, pH 6.0) at concentrations ranging from 10^{-3} to 10^{-1} M did not activate. The lack of activation by magnesium and manganese is at variance with the findings of Caputto *et al.* (1948). β -Galactosidase activity in sodium and potassium phosphate buffers was measured in three ways: (a) by continuous fermentation with *S. mandshuricus*; (b) by change in optical rotation; (c) by increase in reducing sugars. The values obtained were substantially the same (2.7, 1.6 and 0.3 units/mg. dry matter, respectively in 0.1 M-sodium phosphate buffer (pH 6.0); 16.9, 16.1 and 18.9 units/mg. in 0.1 M-potassium phosphate (pH 6.0).

The effect of cation concentration on β -galactosidase activation by potassium, ammonium and sodium is shown in Fig. 6. With potassium and ammonium ions maximum activation was achieved at 0.1 M; higher concentrations were less effective. Hydrolysis of ONPG by suspensions of acetone-dried lactose-induced *Saccharomyces fragilis* was stimulated by potassium ion and sodium ion (Fig. 6), the concentrations giving half maximum velocity being about 10^{-1} M for sodium and 10^{-2} M for potassium, although the maximum velocities determined by the method of Lineweaver & Burk (1934) were about the same. Hydrolysis of ONPG was stimulated slightly (50%) by 10^{-2} M- and 10^{-3} M-manganous chloride but not by magnesium chloride at 10^{-2} M or 10^{-1} M.

Table 5. *Inhibition of potassium-activated Saccharomyces fragilis β -galactosidase by other cations*

The reactions were done in test-tubes containing (in 3.0 ml.): 0.097 M-lactose, 0.1 M-potassium as acetate (pH 6.0, except for control without cations), 2 mg. acetone-dried *S. fragilis*, and other cations (as acetates, pH 6.0) as indicated. After 30 min. at 25°, protein was precipitated with $\text{ZnSO}_4 + \text{Ba(OH)}_2$, and the glucose + galactose in the supernatant fluid estimated with galactose-induced *S. mandshuricus*.

		β -Galactosidase activity (μ moles lactose hydrolysed/ mg./hr)	Inhibition of K^+ activated enzyme (%)
Cations			
None	—	1.1	—
	—	29.0	0
	+ Na^+ (10^{-2} M)	27.0	7
	+ Na^+ (10^{-1} M)	15.6	46
	+ Li^+ (10^{-2} M)	28.0	4
K^+ (10^{-1} M)	+ Mg^{++} (10^{-2} M)	29.2	0
	+ Ca^{++} (10^{-2} M)	14.0	42
	+ Zn^{++} (10^{-3} M)	4.8	83
	+ Zn^{++} (10^{-2} M)	0.6	98
	+ Mn^{++} (10^{-2} M)	28.2	3

Inhibition of potassium-activated β -galactosidase by other cations

The effect of various cations on the β -galactosidase activity of acetone-dried *Saccharomyces fragilis* in presence of 0.1 M-potassium ion is shown in Table 5. Zinc ion was strongly inhibitory, as observed by Caputto *et al.* (1948), and sodium and

calcium ions less so. Inhibition by zinc and calcium ions was reported for the β -galactosidase from *Escherichia coli* ML308 (Rickenberg, 1959) and from *E. coli* ML (Cohn & Monod, 1951), but Kuby & Lardy (1953) found calcium ion to be inert with the enzyme from *E. coli* K12. The degree of inhibition shown by sodium with the enzyme from *S. fragilis* (46% at 0.1 M-sodium) was very much less than was observed for lactose hydrolysis by the enzyme from *E. coli* ML (Cohn & Monod, 1951).

Stability of *Saccharomyces fragilis* β -galactosidase

The β -galactosidase activity of dry preparations of acetone-dried *Saccharomyces fragilis* decreases only very slowly at room temperature, 50% still remaining after 2 years, but in cell-free extracts or aqueous suspensions of acetone-dried organisms

Fig. 7a. Decay of β -galactosidase activity of acetone-dried *Saccharomyces fragilis* and in cell-free extracts at 25°. Enzyme preparations were incubated in various salt solutions for times shown and β -galactosidase activity then estimated by continuous manometric method in a system containing (in 3.0 ml.): 0.1 M-potassium (as acetate or phosphate, pH 6.0, or as chloride), 0.067 M 4(5)-methylglyoxaline-HCl (pH 6.0), 0.075 M-lactose, 2 mM- NaN_3 , acetone-dried galactose-induced *S. fragilis*, or cell-free extract equivalent to 0.5 mg. dry wt. organisms, and 100 mg. dry wt. galactose-induced *S. mandshuricus*. Curve A, acetone-dried organisms + potassium acetate; curve B, acetone-dried organisms + KCl; curve C, Mickle extract + potassium phosphate; curve D, Mickle extract + KCl.

Fig. 7b. Decay of β -galactosidase activity of acetone-dried *Saccharomyces fragilis* with *o*-nitrophenyl- β -D-galactoside (ONPG) as substrate. Acetone-dried galactose-induced organisms were suspended at 2 mg. dry wt./ml. in: (a) 0.1 M (with respect to phosphate) potassium phosphate pH 6.5; (b) 0.1 M-KCl; (c) water; the suspensions were then incubated at 25°. At the times shown samples (0.5 ml.) were withdrawn for estimation of β -galactosidase by the method of Lederberg (1950). Curve A, potassium phosphate; curve B, KCl; curve C, water.

the enzyme is unstable. Suspensions of acetone-dried organisms in water sometimes lost 80% of their activity in 5 min. at 25° and, frequently, such suspensions were inactive when tested in 0.1 M-KCl or in 0.067 M-4(5)-methylglyoxaline (pH 6.0) with

0.1M-KCl as sole source of potassium ion. This instability is in contrast to the relative stability of the β -galactosidases from *Bacillus megaterium* KM (Landman, 1957), *Escherichia coli* K12 (Lederberg, 1950) and *E. coli* ML (Cohn & Monod, 1951), although Rickenberg (1959) reported that the β -galactosidases from *E. coli* strains K12 and ML308 lost activity when diluted with water to protein concentrations less than 100 μ g./ml., a loss which was prevented by 1-0.2 mM-MnCl₂. Landman (1957) observed that the loss of activity which occurred when the *B. megaterium* enzyme was dialysed against ethylenediaminetetra-acetate (EDTA) and triple-distilled water could largely be prevented by the addition of 0.1 M-methionine to the dialysis system.

Decay of *Saccharomyces fragilis* β -galactosidase activity was observed with suspensions of acetone-dried organisms, cell-free extracts and also with the purified enzyme preparation, irrespective of the method used to estimate activity, and it

Fig. 8. Effect of potassium phosphate concentration on decay of β -galactosidase activity. Acetone-dried galactose-induced *Saccharomyces fragilis* was suspended at 2 mg. dry wt./ml. in potassium phosphate (pH 6.5; 0°) of the concentrations (with respect to phosphate) indicated, and incubated at 25°. Samples (1.0 ml.) were withdrawn at the times shown for estimation of β -galactosidase activity, with ONPG as substrate. Potassium phosphate concentrations: curve A, 0.2 M; curve B, 0.1 M; curve C, 0.033 M; curve D, 0.01 M; curve E, 0.005 M.

Fig. 9. Effect of pH value on decay of β -galactosidase activity at 25°. Acetone-dried galactose-induced *Saccharomyces fragilis* was suspended at 0° and 2 mg. dry wt./ml. in 33 mM (with respect to phosphate) potassium phosphate of the pH values indicated, and incubated at 25°. At the times shown samples (0.5 ml.) were withdrawn for estimation of β -galactosidase activity, with ONPG as substrate. Curve A, 15 min.; curve B, 30 min.; curve C, 60 min.

Fig. 10. Decay of purified β -galactosidase. Activity was estimated by the continuous manometric method in a system containing (in 3.0 ml.): 0.1 M-potassium as KCl or phosphate (pH 6.0), 5.45 μ g. dry wt. β -galactosidase preparation, 0.075 M-lactose, 100 mg. dry wt. galactose-induced *S. mandshuricus*, 2 mM-NaN₃, and, as indicated, 0.067 M-4(5)-methylglyoxaline-HCl (pH 6.0). Curve A, potassium phosphate only; curve B, potassium phosphate + methylglyoxaline; curve C, KCl + methylglyoxaline.

affected the ability to hydrolyse both lactose and ONPG (Fig. 7a, b). The extent of decay varied from one enzyme preparation to another and even between suspensions prepared on consecutive days from the same batch of acetone-dried organisms. It

was not obviously related to the conditions of growth, the age of the acetone-dried organisms, or the composition of the incubation system. The activity of crude cell-free extracts or suspensions of acetone-dried organisms did not decay in solutions (0.1 M with respect to anion; pH 6.0) of sodium phosphate, potassium phosphate, acetate, succinate, phthalate, maleate, or ammonium phosphate, but 0.1 M-potassium chloride, potassium sulphate or sodium chloride and also magnesium or manganous chloride at concentrations up to 10^{-2} M afforded no consistent protection, in contrast to the observations of Rickenberg (1959) with *Escherichia coli* β -galactosidase. Although activity of *S. fragilis* β -galactosidase was maintained in potassium succinate it was consistently only about 40 % of that observed with potassium phosphate.

The protective action of potassium phosphate was a function of its concentration (Fig. 8), of pH value (Fig. 9) and of temperature, decay being much slower at 0° (67 % residual activity after 2 hr at 0° in 5 mM-potassium phosphate pH 6.0; 3 % at 25° under similar conditions).

Table 6. *Effect of 2,4-dinitrophenol, sodium azide and sodium arsenate on β -galactosidase activity of acetone-dried Saccharomyces fragilis*

β -Galactosidase activity measured by the continuous manometric method, using double side-arm Warburg manometers containing *in the main compartment*: 1.0 ml. 0.2 M-4(5)-methylglyoxaline-HCl (pH 6.0), 0.5 ml. 20 % lactose, 0.9 ml. galactose-induced *S. mandshuricus* suspension (equiv. 100 mg. dry wt./ml.) and 0.3 ml. M-potassium as either phosphate (pH 6.0), chloride, or chloride containing 20 mM-2,4-dinitrophenol, sodium azide or sodium arsenate; *in side arm 1*: 0.5 mg. acetone-dried galactose-induced *S. fragilis* in 0.3 ml. 0.1 M-potassium, as either phosphate (pH 6.0), chloride, or chloride containing 2 mM-2,4-dinitrophenol, sodium azide or sodium arsenate; *in side arm 2*: 0.3 ml. M-KCl or M-potassium phosphate (pH 6.0). The gas phase was 95 % (v/v) nitrogen + 5 % (v/v) CO₂. Before tipping in the acetone-dried *S. fragilis* the manometers were shaken for 45 min. at 25° to eliminate endogenous CO₂ production. The contents of sidearm 2 was tipped 50 min. after that of side arm 1.

Inhibitor (2 mM):	None	None	2,4-Dinitro-phenol	Sodium azide	Sodium arsenate
Potassium salt present at time zero:	Phosphate	Chloride	Chloride	Chloride	Chloride
Potassium salt added at 50 min.:	Chloride	Phosphate	Phosphate	Phosphate	Phosphate
	μ moles lactose hydrolysed/hr/mg. acetone-dried organisms				
Initial activity	23.5	1.3	16.7	12.4	14.8
Activity at 50 min.	16.5	0.7	8.5	4.8	3.5
Activity 5 min. after adding second salt	16.0	1.7	10.4	7.6	4.5
Activity 30 min. after adding second salt	16.0	11.6	14.5	13.7	10.7

The purified enzyme preparation lost 90 % of its activity in 3 weeks at 4° and was inactive after 4 weeks, even when stored in 0.1 M-potassium phosphate (pH 7.0). In presence of 0.067 M-4(5)-methylglyoxaline-HCl (pH 6.0) + 0.1 M-KCl all activity was lost in 20 min. at 25°; in methylglyoxaline + 0.1 M-potassium phosphate (pH 6.0) the activity decreased to 25 % after 20 min., but in potassium phosphate alone there was no loss of activity in 20 min. (Fig. 10). With suspensions of acetone-dried organisms

0.067 M-methylglyoxaline was only slightly inhibitory (10–20 %) when potassium was present as phosphate, acetate or chloride and the rate of decay was not affected.

With suspensions of acetone-dried organisms and with Mickle extracts, sodium azide, sodium arsenate and especially 2,4-dinitrophenol (all at 1.75 mM) decreased the rate of decay. The incubation of partially decayed preparations with 0.1 M-potassium phosphate (pH 6.0) resulted in considerable recovery of activity (Table 6). Rickenberg (1959) reported that 50 mM-phosphate protected *Escherichia coli* β -galactosidase against loss of activity when dilute enzyme solutions were dialysed. With one preparation of acetone-dried *Saccharomyces fragilis*, for which the decay was less pronounced than usual, addition of cysteine (10 mM) in presence of 0.1 M-potassium phosphate (pH 6.0) increased the loss of activity after 90 min. at 25° from 0 to 83 %. Addition of boiled lactose-induced *S. fragilis* at ten times the concentration of acetone-dried organisms neither prevented decay nor stimulated recovery of decayed preparations.

Table 7. *Purification of Saccharomyces fragilis* β -galactosidase

β -Galactosidase activities are expressed as μ moles lactose hydrolysed/hr/unit log (I_0/I) at 278 m μ . The final preparation had log I_0/I (278 m μ) = 1.17 and contained 175 μ g. N/ml., corresponding to a β -galactosidase activity of 334 μ moles lactose hydrolysed/mg. N/min. at pH 6.0 and 25°.

Fraction	β -Galactosidase activity	Degree of purification	Recovery (%)
(1) Potassium phosphate extract of acetone-dried organisms	12.6	—	100
(2) Precipitate by 0.9 saturation with ammonium sulphate	75	6 times	92
(3) Adsorbed on $\text{Ca}_3(\text{PO}_4)_2$ gel, eluted and dialysed against 0.1 M-potassium phosphate (pH 6.0) at 4°	358	28 times	66
(4) Precipitate between 40–50 % acetone, adsorbed to and eluted from $\text{Ca}_3(\text{PO}_4)_2$ gel	975	77 times	41
(5) Precipitated with 55 % (v/v) acetone and dialysed against 0.1 M-potassium phosphate (pH 6.0) at 4°	8000	237 times	30

Purification of Saccharomyces fragilis β -galactosidase

A purified preparation of *Saccharomyces fragilis* β -galactosidase with an activity of 334 μ moles lactose hydrolysed/mg. N/min. was obtained by the following procedure (Table 7). A suspension of 19.1 g. acetone-dried organisms in 1 l. 0.1 M-potassium phosphate (pH 6.0) was incubated for 20 hr at 25° and centrifuged for 20 min. at 6000g. To the cell-free supernatant fluid was added cysteine (final concentration 10^{-2} M) and solid ammonium sulphate to 0.9 saturation. After standing for 3 hr at 4° the precipitate was centrifuged down (20 min. at 6000g), dissolved in 200 ml. 0.1 M-potassium phosphate (pH 6.0), 60 ml. calcium phosphate gel and 400 ml. water added and the mixture left for 15 min. at 4°. The gel was centrifuged down, suspended in 120 ml. 0.2 M- KH_2PO_4 and the mixture again centrifuged after 20 min. at 4°. The supernatant fluid was dialysed for 11 hr at 4° against 10 l. 0.1 M-potassium phosphate (pH 6.0) and then fractionated by addition of acetone at -10° . The

material which precipitated between 40 and 50 % (v/v) acetone was dissolved in 55 ml. 0.1 M-potassium phosphate (pH 6.0), adsorbed on 20 ml. calcium phosphate gel and eluted with 36 ml. 0.2 M-KH₂PO₄. The β -galactosidase was finally precipitated by addition of acetone at -10° to 55 % (v/v), the precipitate dissolved in 0.1 M-potassium phosphate (pH 6.0) and dialysed overnight against 0.1 M-potassium phosphate (pH 6.0). The final preparation contained 30 % of the initial β -galactosidase activity and had a specific activity of 334 μ moles lactose hydrolysed/mg. N/min. at 25° in 0.1 M-potassium phosphate (pH 6.0). The purification achieved from the crude extract was 237 times with respect to protein. The specific activity of the crystalline β -galactosidase from *Escherichia coli* ML309 is stated to be 127 μ moles ONPG hydrolysed/mg. protein/min. at 20° and that of the electrophoretically homogeneous enzyme from *E. coli* ML308 to be 146 μ moles ONPG hydrolysed/mg. protein/min. at 25° (Wallenfels & Malhotra, 1961). Assuming a Q_{10} of 2.0, these values are approximately equivalent, respectively, to 1200 and 913 μ moles ONPG hydrolysed/mg. N/min. at 25°.

Some properties of the purified β -galactosidase

The purified enzyme required potassium ion for activity, sodium ion being ineffective. Activity was rapidly lost in a system containing 0.1 M-KCl or 0.1 M-potassium phosphate (pH 6.0) + 0.067 M 4(5)-methylglyoxaline (pH 6.0), but not in 0.1 M-potassium phosphate alone (Fig. 10). The pH value for maximum activity was 6.2-6.4, measured by accumulation of glucose and galactose in a fixed time interval, or continuously by liberation of CO₂ in presence of *Saccharomyces mandshuricus*. The preparation did not react with rabbit antibodies prepared using purified *Escherichia coli* β -galactosidase as antigen.

DISCUSSION

It has been clearly established by the work of Monod, Cohn, Lederberg, Halvorson and others that β -galactosides are hydrolysed by *Escherichia coli* only after transport into the cell. Lactose utilization by *Saccharomyces fragilis* has not been studied so extensively, but the available data support the conclusion that a similar situation exists. The 10- to 20-fold increase in β -galactosidase activity observed when *S. fragilis* is disrupted in various ways (first reported by Caputto *et al.* 1948), coupled with the observations reported in the present paper and those of Sanches & Sols (1962) that only very small amounts of free glucose and galactose can be detected when lactose is metabolized under conditions which involve no obvious damage to the cell membranes, all suggest that transport into the *S. fragilis* cell may be the rate-limiting factor in lactose hydrolysis by this organism. Simultaneous inhibition of metabolism and hydrolysis of lactose by substances (sodium azide, 2,4-dinitrophenol, iodoacetate) which do not inhibit activity of the cell-free β -galactosidase suggests that these substances interfere with transport of lactose into the cell. Sodium azide (3 mM) does not inhibit glucose fermentation, but galactose fermentation is strongly inhibited even by 0.3 mM-azide and is also much more sensitive to inhibition by 2,4-dinitrophenol than is fermentation of glucose or lactose. This also may be due to an inhibition of transport of lactose into the cell.

The properties of the β -galactosidases of *Escherichia coli* and *Saccharomyces fragilis* are, in general, similar. Wallenfels, Malhotra & Dabich (1960) found that the

rate of hydrolysis of *o*-nitrophenyl- β -D-galactoside (ONPG) by the enzyme from *E. coli* ML309 was maximal at pH 6.6 for both the sodium-activated and the potassium-activated enzyme, whereas hydrolysis of lactose showed two maxima (pH 6.5 and 8.1) with the potassium-activated enzyme but only one (at pH 7.0) with the sodium-activated enzyme. In the case of *S. fragilis* β -galactosidase ONPG hydrolysis was maximal at pH 7.0 with both sodium- and potassium-activated enzyme, whereas lactose hydrolysis was maximal at pH 6.5 with the sodium-activated enzyme but at pH 6.0–6.5 with potassium activation. There was no evidence of a second maximum at pH 8 for hydrolysis of either lactose or ONPG by the potassium-activated enzyme. Other workers have reported pH optima for *S. fragilis* β -galactosidase which vary from pH 6.6 (van Dam, Revallier-Warffemius & van Dam-Schermerhorn, 1950) to pH 7.8 (Sanches & Sols, 1962). These differences may be due to strain variations since the pH optimum for ONPG hydrolysis by β -galactosidases from different strains of *Escherichia coli* varies from pH 6.6 to 7.3 (Wallenfels & Malhotra, 1961).

The preferential activation of *Saccharomyces fragilis* β -galactosidase by potassium ion when lactose is the substrate is similar to the behaviour of the enzyme from *Escherichia coli* ML strains, but hydrolysis of ONPG by the *S. fragilis* enzyme is also preferentially activated by potassium ion, in contrast to the behaviour of the *E. coli* enzymes. In presence of the optimal concentration of sodium ion, manganese and magnesium have been reported to stimulate hydrolysis of ONPG by the β -galactosidases of *E. coli* ML308 (Reithel & Kim, 1960) and *E. coli* K12 (Kuby & Lardy, 1953). A similar situation exists for the *S. fragilis* enzyme, but it is possible that the effect is not an activation of the enzyme but rather a protection from decay (Szabó & Davies, 1964). Rickenberg (1959) reported that 0.2–1.0 mM-MnCl₂ prevented the loss of activity which occurred when aqueous solutions of the β -galactosidase from *E. coli* ML308 were diluted to less than 100 μ g. protein/ml.

An absolute requirement for magnesium ion for the hydrolysis of ONPG, but not lactose, by *Escherichia coli* ML308 β -galactosidase was reported by Reithel & Kim (1960), based on the observation that ONPG hydrolysis was inhibited by 10⁻⁴M-EDTA, the inhibition being annulled by magnesium ion. A similar inhibition of ONPG hydrolysis by 10⁻⁴M-EDTA which is annulled by various metal cations, notably 10⁻⁴M-manganese and 10⁻⁴M-ferrous ions, has been observed with the *Saccharomyces fragilis* enzyme (Szabó & Davies, 1964). The annulment of inhibition might be due to removal of EDTA by chelation with the added cation, rather than a re-activation by some essential metal ion. As with the enzyme from *E. coli* ML308 (Reithel & Kim, 1960), hydrolysis of lactose by *S. fragilis* β -galactosidase was not significantly affected by magnesium or manganous ions, contrary to the findings of Caputto *et al.* (1948).

The reasons for the rapid loss of activity when the *Saccharomyces fragilis* β -galactosidase preparations, crude or purified, were incubated in KCl or NaCl solutions or in water, and for the protective effect of orthophosphate, arsenate, 2,4-dinitrophenol and sodium azide, are not clear. With crude preparations, degradation of β -galactosidase by proteolytic enzymes is a possibility, but this seems less likely for the purified enzyme preparation. Rapid decay of the purified enzyme preparation might be related to the presence of 4(5)-methylglyoxaline, since potassium phosphate protected in the absence but not in the presence of this compound. Decay

of the crude preparations, however, was not affected by the methylglyoxaline. Histidine, which has the same imidazole ring as 4(5)-methylglyoxaline, has been suggested as one group involved in β -galactosidase activity (Wallenfels & Malhotra, 1961).

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