4. La fraction de l'enzyme demeurant active en l'absence de Mg^{++}, oxyde les mêmes acides aminés et présente une activité optima à un pH différent (7.6); elle est activable par le tris(hydroxyméthyl)aminométhane.

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THE EFFECT OF METAL IONS AND PROTEINS ON THE STABILITY OF THE β-GALACTOSIDASE OF *ESCHERICHIA COLI*

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SUMMARY

It was found that the β-galactosidase of crude extracts of *Escherichia coli* lost activity when diluted to protein concentrations below 100 μg per ml unless protected by the manganous ion. A variety of extraneous proteins also protected the bacterial β-galactosidase against loss of activity.

The enzyme was completely inactivated by prolonged dialysis against H_2O. This inactivation appeared to be independent of the protein concentration. The presence during dialysis of any one of a number of salts protected β-galactosidase against loss of activity.

The following abbreviations have been used: β-g, β-galactosidase; ONPG, *o*-nitrophenyl-β-galactoside; NRS, normal rabbit serum; BSA, bovine albumin plasma fraction V (Armour Laboratories); phosphate buffer, sodium phosphate buffer, 0.05 *M*, pH 7; EDTA, (ethylenediamino) tetraacetic acid disodium salt.

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INTRODUCTION

The β-galactosidase (β-g) of *Escherichia coli* is a relatively stable enzyme. Thus less than 5 % loss of activity occurs when a concentrated preparation of purified β-g is incubated at 47° for 10 min[1]. However, at protein concentrations below 100 μg/ml, the enzyme loses its activity upon dilution not only at 47° but also at room temperature[2]. In this paper the kinetics of the loss of β-g activity upon dilution are described and evidence is presented that the manganous ion as well as a variety of proteins can protect the β-g of *E. coli* against loss of activity resulting from dilution. The protective role of the manganous ion was first suggested by its very striking ability to protect the highly labile β-g of *Paracolobactrum aerogenoides*[3].

MATERIALS AND METHODS

Strains and medium

Strains *E. coli* ML 308, K-12, and W-2244 were employed. ML 308 forms β-g constitutively; strain K-12 forms β-g inducibly; strain W-2244 does not form β-g under any conditions of growth tested.

MONOD's salts medium 56[1] was used. Lactose or glycerol was autoclaved separately and added to the cooled salts solution to a final concentration of 0.2 %. Cultures were grown with shaking or forced aeration at 37°.

Preparation of cell extracts

Bacteria in the exponential phase of growth were harvested by sedimentation, washed twice with distilled water and resuspended in water to a density of 10^{11} to $5 \cdot 10^{11}$ per ml, corresponding to approximately 20 to 100 mg protein per ml. The bacteria were disrupted by treatment for 30 min in a Raytheon 10 kc magnetostrictive oscillator. The extract was centrifuged for 60 min at 30,000 × *g* in a refrigerated centrifuge and the precipitate discarded. The supernatant fluid constituted the β-g preparation used in the experiments to be described. Such preparations could be stored for several months at —20° without detectable loss of enzymic activity. The extracts were equally stable at 3°. However, in order to avoid microbial decomposition, preparations were routinely stored at —20°. Dilutions were carried out at 3°.

Analytical methods

Bacterial density was measured turbidimetrically in a Klett-Summerson colorimeter using the 660 mμ filter. The protein content of extracts was determined by the method of LOWRY *et al.*[4]. β-g activity was determined by a modification[5] of the procedure described by LEDERBERG[6]. The reaction was carried out in a volume of 3.0 ml with a final concentration of 0.001 *M* ONPG and 0.05 *M* phosphate buffer. After incubation at 35° for an appropriate period of time (1–600 min) depending upon the activity of the extracts, 2.0 ml of 1 *M* K_2CO_3 were added to stop the reaction and to develop the color of the *o*-nitrophenol released. The amount of *o*-nitrophenol released was measured in a Klett-Summerson colorimeter using the 420 mμ filter. The values obtained are expressed as μmoles of ONPG hydrolyzed. Specific β-g activity is defined as the number of μmoles of ONPG hydrolyzed per minute per mg protein at 35°.

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RESULTS

Loss of β-g activity upon dilution of bacterial extracts and the protective effect of extraneous protein

An extract prepared from strain ML 308 was diluted to a concentration of 1000 μg of protein per ml in H_2O and then further diluted in phosphate buffer. The specific β-g activities of the dilutions are represented in Fig. 1. It is seen (curve A) that with dilution the specific activity decreases as a linear function of the logarithm of the protein concentration. Thus, the specific β-g activity of an extract assayed at 0.1 μg of protein per ml is only 55 % of the activity of the same extract assayed at 100 μg

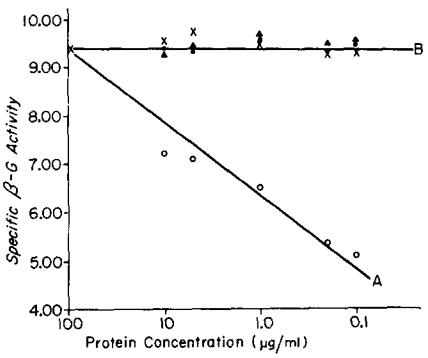

Fig. 1. The effect of dilution on the β-galactosidase activity of ML 308 extracts. Curve A. Extract diluted in 0.05 *M* sodium phosphate buffer. Curve B. Extract diluted in the presence of extraneous protein, as follows: –×–×–×– Normal rabbit serum diluted 100-fold in 0.05 *M* phosphate buffer and containing 590 μg of protein per ml. –▲–▲–▲– Bovine albumin plasma fraction V, 1000 μg per ml in 0.05 *M* phosphate buffer. –●–●–●– Gelatin, 1000 μg per ml in 0.05 *M* phosphate buffer.

protein per ml. Entirely analogous results were obtained upon repetition of this experiment employing independently prepared extracts of strain ML 308 and strain K-12. Dilution of the extracts necessitated a proportionately longer period of incubation with the substrate in order to obtain the hydrolysis of measurable amounts of ONPG. This prolonged incubation at 35° of dilute extracts (but not of more concentrated extracts) suggested that loss of enzymic activity might represent heat inactivation at that temperature. This possibility, however, was rendered unlikely by the finding that the percentage loss of specific β-g activity in a preparation that had been diluted to 1 μg of protein per ml was the same whether the enzyme was assayed at 35° or 3°. Furthermore, in other experiments extracts at protein concentrations ranging from 0.1 to 100 μg of protein per ml were pre-incubated at 35° for prolonged periods of time prior to the β-g assay. No significant loss of β-g activity other than the "dilution loss" described above occurred during pre-incubation at 35°, if the dilutions were carried out in phosphate buffer. (Pre-incubation at 35° for 30 min did, however, bring about an almost complete loss of β-g activity in preparations diluted to below 10 μg of protein per ml in H_2O.) It can be assumed then that the loss of specific β-g activity upon dilution in phosphate buffer is not due to heat inactivation of the enzyme.

The fact that the observed loss of enzymic activity upon dilution does not result from the removal of an endogenous cofactor is demonstrated in curve B, Fig. 1. When phosphate buffer was replaced as diluent by normal rabbit serum (NRS), bovine albumin plasma fraction V (BSA), or gelatin, no loss of β-g activity occurred upon dilution of extracts. That NRS stabilizes β-g activity was first observed in the course of serological experiments. An extract prepared from the *E. coli* Lac⁻ mutant W-2244 also completely protected ML 308 β-g against loss of activity by dilution.

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The effect of divalent metal ions on β-g activity at low protein concentrations

HU, WOLFE AND REITHEL[7] in the course of work that led to the crystallization of β-g from *E. coli* ML 308 found that the presence of Mg^{++} ($MgCl_2$ 0.01 *M*) was required for the retention of β-g activity. Earlier COHN AND MONOD[1] reported that Cu^{++}, Zn^{++}, Ca^{++}, Mn^{++}, Co^{++}, Fe^{++} and Sr^{++}, at the concentrations tested by them, inhibited β-g activity; Mg^{++} at a concentration of $2 \cdot 10^{-2}$ *M* gave less than 10% stimulation. KUBY AND LARDY[8] have reported that "Mg^{++} and Mn^{++} activate (β-g) slightly in a system fully activated by Na^{+}". In view of the very striking stabilization of the labile β-galactosidase of *Paracolobactrum aerogenoides* by the manganous ion[3], it was decided to test the effect of this cation and also other divalent metal ions on the β-g activity of *E. coli* ML 308 extracts at low protein concentrations. ML 308 extracts were diluted to 1000 μg of protein per ml in H_2O and then further diluted in the presence of the cation to be tested. In some experiments 0.1 *M* tris (hydroxymethyl-aminomethane) buffer at pH 7, to which NaCl had been added to a final concentration of 0.05 *M*, was substituted for the phosphate buffer. No significant difference was found between the β-g activity of preparations assayed in phosphate or in tris buffer. The effects of divalent metal ions on β-g activity are presented in Table I. β-g activities are reported as percentage figures since different enzyme preparations differing somewhat in specific β-g activity were employed in individual experiments. The activity of extracts diluted in NRS is given a value of 100%. Inspection of Table I shows that Mn^{++} at concentrations between $2 \cdot 10^{-4}$ *M* and 10^{-3} *M* stabilizes β-g against loss by dilution to the same extent as do NRS and other proteins. At a concentration of 10^{-2} *M* and above, Mn^{++} is inhibitory. Mg^{++} at concentrations below 10^{-1} *M* has no effect on β-g activity; at higher concentrations it is inhibitory. Cr^{++} may have a slight stabilizing effect. The other divalent cations tested inhibit β-g activity*.

Reversibility of dilution inactivation of β-g activity

Table II shows that the decrease of β-g activity resulting from the dilution of extracts into phosphate buffer can be reversed completely by the addition of NRS or Mn^{++}. Even after a 90 min period of storage at 3°, enzyme activity can be restored by the addition of NRS or Mn^{++}.

The role of protein in the stabilization of β-g

The fact that both Mn^{++} and proteins can stabilize β-g against loss of activity at high dilutions suggested the possibility that the proteins might act as carriers of the metal ion. Alternatively, it might be postulated that the mechanism of protection by extraneous protein does not involve the presence of, or participation by, metal ions. The following experiment was designed to explore these possibilities. One aliquot of NRS was dialyzed exhaustively against H_2O; another aliquot was pretreated with 10^{-2} *M* EDTA and then dialyzed against H_2O. A third aliquot of NRS was ashed. The three aliquots and a fourth aliquot of untreated NRS were diluted (resuspended in the case of the ashed NRS) in phosphate buffer to a concentration corresponding to a

* The Na^{+} ion had no effect on β-g activity when added at concentrations between 10^{-3} and 10^{-1} *M*. The sodium requirement for the hydrolytic activity of the enzyme was met by the presence of sodium in the buffer.

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TABLE I

THE EFFECT OF DIVALENT METAL IONS ON THE β-GALACTOSIDASE ACTIVITY OF DILUTE *E. coli* EXTRACTS

The final protein concentration of extracts diluted in the presence of Mn^{++}, Mg^{++}, and no added divalent metal ion was 10 μg of protein per ml. The final protein concentration of extracts diluted in the presence of the other metal ions was 5 μg of protein per ml. The extracts were diluted in 0.05 *M* sodium phosphate or 0.1 *M* tris (0.05 *M* with respect to Na^+) buffer containing the metal ion. The salts employed were $MnCl_2 \cdot 4H_2O$; $MgCl_2 \cdot 6H_2O$; $ZnCl_2$; $NiCl_2 \cdot 6H_2O$; $CaCl_2$ and chromium trioxide CrO_3.

Metal ion added	*Concentration*	*β-g activity (Expressed as % of activity found when extract diluted in NRS)*
None	—	81
Mn^{++}	$2 \cdot 10^{-4}$ *M*	100
Mn^{++}	$5 \cdot 10^{-4}$ *M*	100
Mn^{++}	10^{-3} *M*	100
Mn^{++}	10^{-2} *M*	80
Mn^{++}	$2 \cdot 10^{-2}$ *M*	41
Mn^{++}	10^{-1} *M*	0
Mg^{++}	$5 \cdot 10^{-4}$ *M*	80
Mg^{++}	10^{-3} *M*	81
Mg^{++}	10^{-2} *M*	81
Mg^{++}	$5 \cdot 10^{-2}$ *M*	80
Mg^{++}	10^{-1} *M*	0
None	—	77
Zn^{++}	10^{-5} *M*	31
Zn^{++}	10^{-4} *M*	3
Zn^{++}	10^{-3} *M*	3
Ni^{++}	10^{-5} *M*	66
Ni^{++}	10^{-4} *M*	38
Ni^{++}	10^{-3} *M*	5
Ca^{++}	10^{-5} *M*	67
Ca^{++}	10^{-4} *M*	54
Ca^{++}	10^{-3} *M*	38
Cr^{++}	10^{-4} *M*	85

TABLE II

REVERSIBILITY OF THE DILUTION LOSS OF β-GALACTOSIDASE ACTIVITY

ML 308 extract was diluted to 1 μg protein per ml in phosphate buffer, NRS (1/100 in phosphate buffer), and Mn^{++} ($2 \cdot 10^{-4}$ *M* in phosphate buffer) respectively. 90 min later the NRS and Mn^{++} (at ten times their final concentration) were added to the preparation that had been diluted in buffer only. The values given are compensated for the 10% dilution resulting from the addition of the Mn^{++} and NRS.

Time of assay	*Additions to diluted enzyme preparation*	*Specific β-g activity x 10^2 of extract diluted in*		
		Buffer	*NRS*	*Mn++*
1. Immediately after dilution	None	540	700	700
2. Diluted extracts kept at 3° for 90 min	None	520	700	700
3. Diluted extracts kept at 3° for 90 min	Mn^{++}, $2 \cdot 10^{-4}$ *M* (final concn.)	730	—	—
4. Diluted extracts kept at 3° for 90 min	NRS, 1/100 (final concn.)	750	—	—

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1:100 dilution of the original NRS. ML 308 extract was then diluted to a concentration of 1 μg of extract protein per ml in the four NRS preparations and assayed for β-g activity. Table III shows that exhaustive dialysis did not deprive NRS of its stabilizing activity. Dialysis preceded by EDTA treatment reduced the stabilizing effect of NRS. It was noted that there was a precipitate in the EDTA-treated, dialyzed NRS preparation following dialysis. This indicates at least partial denaturation of the NRS and explains the lowered stabilizing activity of this preparation. Ashing abolished the stabilizing activity of NRS completely.

TABLE III

THE EFFECT OF EDTA-TREATMENT, DIALYSIS, AND ASHING ON THE ABILITY OF NRS TO STABILIZE THE β-GALACTOSIDASE ACTIVITY OF DILUTE EXTRACTS

A ML 308 extract was diluted to a concentration of 100 μg of protein per ml in H_2O and then further diluted 100-fold to a concentration of 1 μg of extract protein per ml in the diluents as listed in the table. Diluents: NRS was diluted 10-fold in H_2O; 5 ml of the 1:10 dilution were then treated with 10^{-2} *M* EDTA (final concentration) at room temperature for 2 h. The EDTA-treated aliquot and another untreated aliquot of 1:10 NRS were then dialyzed against five 1000 ml changes of H_2O over a 48-h period at 3°. Following dialysis the NRS was diluted 10-fold in phosphate buffer to a final NRS concentration of 1:100. An undialyzed aliquot of NRS was also diluted 100-fold in buffer. Ashed NRS was suspended in buffer at a concentration corresponding to a 100-fold dilution of the original NRS.

Diluent	*Specific β-g activity x 10^3*
Phosphate buffer	670
NRS, untreated	950
NRS, dialyzed	950
NRS, EDTA-treated, dialyzed	820
NRS, ashed	670

The effect of dialysis on the β-g activity of ML 308 extracts

COHN AND MONOD[1] had noted that a 14-fold purified *E. coli* β-g preparation was irreversibly inactivated by prolonged dialysis against H_2O. This finding was confirmed employing an ML 308 preparation purified approximately 15-fold by acetone fractionation[9] and stored in 1% gelatin. This stock preparation was diluted 200-fold

TABLE IV

THE EFFECT OF DIALYSIS ON THE β-GALACTOSIDASE ACTIVITY OF ML 308 EXTRACTS

Five ml aliquots were dialyzed against five 1000 ml changes of H_2O, buffer, Mn^{++}, etc. at 3° over a 48-h period. The activity values presented in the table were obtained by diluting the dialyzed extracts ten-fold into NRS. In Expt. 2 the activities of the dialyzed extracts were compared after ten-fold dilution (final extract concentration of 10 μg of extract protein per ml) in buffer; NRS; $2 \cdot 10^{-4}$ *M* Mn^{++}; $2 \cdot 10^{-3}$ *M* Mg^{++}. The activities of the aliquots diluted in buffer or in Mg^{++} were approximately 20% lower than the activities of aliquots diluted in NRS or in the presence of Mn^{++}.

Expt. No.	*β-g Activity (Activity of undialyzed controls = 100) Dialysis against*				
	H_2O	*Phosphate buffer $5 \cdot 10^{-4}$ M*	*$MnCl_2$ 10^{-3} M*	*$MgCl_2$ 10^{-3} M*	*NaCl $5 \cdot 10^{-4}$ M*
1	25	—	108	—	—
2	0	100	100	93	90

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in water. 5 ml aliquots of the diluted preparation were dialyzed exhaustively against H_2O and 10^{-3} *M* Mn^{++} respectively. Table IV, Expt. 1, shows that Mn^{++} completely protects β-g against loss of activity during dialysis; a 75% loss of activity was suffered by the same enzyme preparation when dialyzed against H_2O. In another experiment (Table IV, Expt. 2) aliquots of a crude ML 308 extract at 100 μg of protein per ml were dialyzed exhaustively against H_2O; 10^{-3} *M* $MnCl_2$; 10^{-3} *M* $MgCl_2$; $5 \cdot 10^{-2}$ *M* sodium phosphate buffer, pH 7; and $5 \cdot 10^{-2}$ *M* NaCl. In this experiment the loss of β-g activity upon dialysis against H_2O was complete while all the metal ions tested afforded essentially complete protection. In still other experiments it was found that β-g inactivation occurred, even when concentrated extracts were dialyzed against H_2O and also that lactose, the substrate of the enzyme, and galactose, one of the hydrolysis products, at concentrations of 10^{-3} *M* in H_2O did not protect the enzyme.

The effect of Mn^{++} and Mg^{++} on the heat stability of ML 318 β-g at a protein concentration of 100 μg per ml

The effect of Mn^{++} and Mg^{++} on the stability of β-g in crude extracts at 50° was studied. Fig. 2 shows that under the conditions of the experiment Mn^{++} affords complete protection against heat inactivation while Mg^{++} exerts some protective effect. In experiments not described here it was found that heat inactivation of the β-g of extracts at a protein concentration of 100 μg per ml was extremely rapid if the extracts were diluted in H_2O instead of phosphate buffer.

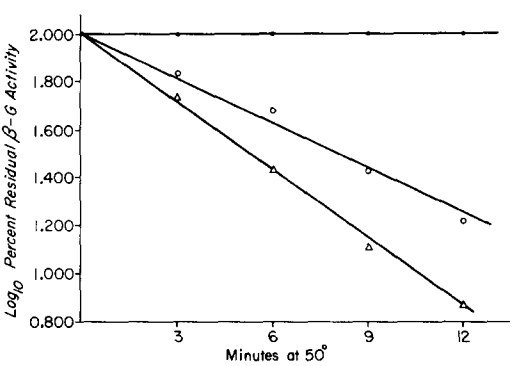

Fig. 2. The effect of Mn^{++} and Mg^{++} on the stability of ML 308 β-galactosidase at 50°. ML 308 extract was diluted to a concentration of 1000 μg of protein per ml in H_2O. The diluted extract was kept in an ice-water bath. 1-ml aliquots of this diluted extract were added at 0 time to 9-ml aliquots of buffer, of buffer containing 10^{-3} *M* Mn^{++}, and of buffer 10^{-3} *M* with respect to Mg^{++} respectively. The 9-ml aliquots had been kept at 55.5° prior to the addition of the 1-ml extract aliquots. The diluted extracts (now containing 100 μg of protein per ml and at 50°) were transferred to a 50° waterbath. 1-ml samples were taken at 3 min intervals and pipetted into 9 ml of buffer $6 \cdot 10^{-4}$ *M* with respect to Mn^{++}, and kept in an ice-water bath. The diluted samples were then assayed for β-g activity at a final concentration of 10 μg of protein per ml. –△–△– Extract diluted in buffer only. –●–●– Extract diluted in buffer, 10^{-3} *M* with respect to Mn^{++}. –O–O– Extract diluted in buffer, 10^{-3} *M* with respect to Mg^{++}.

DISCUSSION

COHN AND MONOD[1] have shown that purified *E. coli* β-g is inactivated irreversibly by prolonged dialysis against H_2O. The present work shows that the β-g activity of crude as well as of purified preparations is destroyed by dialysis against H_2O. The presence of Mn^{++}, Mg^{++}, or Na^{+} afforded essentially complete protection against inactivation during prolonged dialysis. The possibility that the anions, *i.e.* phosphate or chloride, were responsible for the protective effect cannot be excluded.

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A more specific Mn^{++} requirement for β-g activity became apparent at low protein concentrations. Thus an extract dialyzed exhaustively against sodium phosphate buffer was fully as active as an extract dialyzed against Mn^{++}, when assayed at protein concentrations of 100 μg per ml or above. However, dilution to and assay at 10 μg of protein per ml resulted in an approximately 20 % decrease in β-g activity, when the dilution was carried out in sodium phosphate buffer. The decrease in β-g activity appeared to follow the kinetics of a first order reaction (loss of activity was a linear function of the logarithm of the protein concentration). The decrease in β-g activity could be reversed completely by the addition of Mn^{++} or by the addition of a variety of proteins such as NRS, BSA, or gelatin. Mg^{++} or Na^{+}, alone, did not restore full enzyme activity.

Loss of enzyme activity at low protein concentrations and stabilization by proteins, such as gelatin and serum albumin, has been reported for a number of enzymes[10–12]. It is generally assumed that surface denaturation is responsible for the loss of enzyme activity at low protein concentrations and that extraneous protein protects the enzyme by competing with it for the liquid–gas interphase at which denaturation presumably occurs. The stabilization of β-g by Mn^{++} suggests that this cation plays a role in maintaining the intactness of the secondary or tertiary structure of the enzyme molecule. Whether or not Mn^{++} forms an integral part of the native β-g molecule may only be decided by examination of crystalline β-g.

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