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THE INFLUENCE OF MONOVALENT CATIONS AND HYDROSTATIC PRESSURE ON β-GALACTOSIDASE ACTIVITY*

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SUMMARY

Monovalent cation activation of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was investigated utilizing *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose as substrates. With these substrates the affinity of β-galactosidase for Na^+ was found to be higher than for K^+. Activation of *p*-nitrophenyl-β-D-galactopyranoside hydrolysis by K^+ was inhibited by Na^+, while the activation of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis by Na^+ was stimulated by K^+.

Hydrostatic pressure stimulated the rate of *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose hydrolysis in the presence of saturating levels of Na^+ and substrate. In contrast, activation by K^+ with these substrates was consistently depressed by the application of hydrostatic pressure. These results are interpreted on the basis of volume change of an activated enzyme–substrate complex. It was concluded that a decrease in the volume of the complex occurs in the presence of Na^+, while the converse occurs in the presence of K^+, and that the mechanism of hydrolysis is different in the case of Na^+-activated as opposed to K^+-activated substrate hydrolysis.

INTRODUCTION

The activation of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from *E. coli* by monovalent cations previously has been demonstrated[1-9]. When lactose, *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-arabinopyranoside were used as the substrates, maximal activation was obtained in the presence of K^+. When *o*-nitrophenyl-β-D-galactopyranoside or *o*-nitrophenyl-β-D-arabinopyranoside was used as the substrate, the greatest activation was observed from the addition of Na^+. It appears that the position of the nitro group on the aglycone is critical in determining whether Na^+ or K^+ activates to the greatest extent. The affinity of β-galactosidase for any one of the above substrates was altered by the addition of the monovalent cations and was greatest in the presence of Na^+ (ref. 9).

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Several hypotheses have been suggested to explain monovalent cation activation of β-galactosidase. According to one proposal, monovalent cation activation resembles the modification of enzyme activity caused by changes in H^+ concentration[4]. NEVILLE AND LING[8], utilizing graphical methods of DIXON[10], have determined (a) the dissociation constants for the substrate, *o*-nitrophenyl-β-D-galactopyranoside, in the presence of several Na^+ concentrations and (b) the dissociation constant for Na^+ in the presence of several substrate concentrations. They found that a change in the dissociation constant for *o*-nitrophenyl-β-D-galactopyranoside produced by the addition of Na^+ was proportional to the change in the dissociation constant for Na^+ resulting from the addition of *o*-nitrophenyl-β-D-galactopyranoside. They also suggested that the monovalent cations were not reacting with the uncharged substrate and that a reaction of cations with product would not be important because initial rates were measured in the assays. Another postulated mode of activation is that monovalent cations surround a negatively charged species near an imidazolium group on the enzyme and that protection of this group from the negatively charged species facilitates an increased dissociation resulting in a shift of the pK toward a more acidic position[9].

This paper presents the results of an investigation concerning the mechanism of Na^+ and K^+ activation of β-galactosidase. The effect of hydrostatic pressure on this cation activation was also investigated with the intent of providing some insight into the mechanisms involved.

MATERIALS AND METHODS

Preparation of β-galactosidase

Cultures of *Escherichia coli*, ML 308, constitutive for β-galactosidase, were a generous gift from Mr. Richard Newton (Department of Chemistry, University of Oregon, Eugene, Oreg.). The cells were grown overnight at 37° in a 100-l fermentor. Each liter of the culture medium contained 13.2 g $Na_2HPO_4 \cdot 7H_2O$, 3.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.4 g $MgSO_4$ and 10 ml of glycerol. After harvesting with a Sharples centrifuge, the cells (700 g wet weight) were suspended in 2 l of Buffer A which contained 0.05 M acetic acid, 0.028 M thioglycollic acid, 0.01 M $MgCl_2$, and solid Tris (pH 7.0) (Trizma, Sigma Chemical Company, St. Louis). The cellular suspension was stored at −90° until utilized.

All purification experiments were performed at 0–5° unless otherwise designated. Part of the cellular suspension (850 ml) was broken by means of a French press with a pressure of approx. 8 tons/inch^2. A crude extract was obtained by centrifugation of the macerated cells for 1 h at 34 000 × g. The initial steps in the purification scheme were performed according to the procedure of HU *et al.*[11] which involved a streptomycin sulfate treatment, $(NH_4)_2SO_4$ precipitation, and DEAE-cellulose column chromatography. The fractions of high specific activity from the DEAE-cellulose column were precipitated by adding 2 vol., in relation to the volume of eluate, of a saturated $(NH_4)_2SO_4$ solution. The precipitate was dissolved in 55 ml of Buffer B (0.01 M thioglycollic acid and 0.01 M $MgCl_2$ adjusted to pH 7.7 with solid Tris) and dialyzed overnight against Buffer B. The final solution was added to a Sephadex G-200 column (2.5 cm × 35 cm) and eluted with Buffer B by the reverse-flow method. The active fractions eluting directly from the column were then applied to a DEAE-Sephadex

G-50 column (2.5 cm × 35 cm) and eluted with 500 ml of a linear NaCl gradient (0–5%) prepared in Buffer B. The fractions containing β-galactosidase of the highest specific activity were combined, and 2 vol. of satd. $(NH_4)_2SO_4$ solution were added. The resultant precipitate was dissolved in 0.033 M sodium phosphate buffer (pH 6.0) containing 5% NaCl. The protein concentration of this solution was 20 mg/ml. Upon the slow addition of saturated $(NH_4)_2SO_4$ the fraction precipitating between 27 and 31% of saturation was collected by centrifugation and stored at −20° in 50% saturated $(NH_4)_2SO_4$ in Buffer B.

Prior to use, the enzyme preparation was dialyzed overnight against 250 ml of 0.1 M histidine (free base, L form, Sigma), with two changes of the dialysis solution. The histidine was recrystallized from a saturated aqueous solution by the addition of 2 vol. of redistilled absolute ethanol. A histidine buffer was chosen because a solution of the free base has a pH of 7.6 without the addition of an acid or base, and it did not appear to affect the activity of the enzyme. An appropriately diluted preparation of the enzyme and a 0.1 M histidine buffer system were used in all experiments unless indicated otherwise. In all experiments the glassware was rinsed and the reagents were prepared in glass-distilled deionized water. In those experiments in which low levels of Na^+ were added, experimental glassware was washed with 3 M HCl and rinsed with glass-distilled deionized water.

Assays for β-galactosidase

Routine assays during the enzyme purification scheme were performed at 22° utilizing Buffer A, an appropriately diluted enzyme, 2.3 mM *o*-nitrophenyl-β-D-galactopyranoside (Sigma), and 0.1 M NaCl. The enzymatic activity in this reaction mixture (3.0 ml) was determined spectrophotometrically by the change in absorbance at 410 mμ. Protein concentration was determined on the basis of absorbance at 260 and 280 mμ (ref. 12).

Assays for monovalent cation activation of the hydrolysis of either *o*-nitrophenyl- or *p*-nitrophenyl-β-D-galactopyranoside (Sigma) by β-galactosidase were conducted in a reaction containing the histidine buffer, the substrate at the desired concentration, the appropriate amount of monovalent cation chloride, and the enzyme in a total volume of 3.0 ml. The activity was measured by the change in absorbance per min at 410 mμ at 22° using a Cary Model 11 spectrophotometer.

When lactose was used as the substrate, each reaction mixture was incubated at 22° utilizing a substrate concentration at 45 mM unless indicated otherwise, the appropriate amount of cation chloride, and enzyme. The mixture (0.3 ml) was incubated for 10 min and the reaction was stopped by placing it in boiling water for 5 min. Enzymatic activity was followed by the measurement of the production of free glucose present with the Glucostat reagent (Worthington Biochemical Corporation, Reinhold, N.J.).

Hydrostatic pressure experiments

The experimental apparatus for hydrostatic pressure experiments and its operation were essentially as previously described[13]. The pressure bombs were equilibrated to 5° before use, and all pressure experiments unless otherwise indicated were performed at this temperature. The concentrations of substrate and cation chlorides were as given in Figs. 2, 3 and 4 *plus* Tables III and IV. The incubation mixture and

the diluted enzyme at 0–1° were mixed immediately before incubation and transferred to a 10 mm × 75 mm Pyrex culture tube. The reaction mixture of 4 ml completely filled each tube. A nichrome wire extending into each reaction mixture and hooked over the edge of each of the tubes facilitated the escape of a portion of the reaction solution that occurred upon the insertion of a Neoprene stopper. Tubes were transferred immediately to bombs, the bombs were closed, and the desired pressure applied. This entire operation was completed within 2–3 min. The bombs and the tubes containing the incubation mixtures were then transferred to a water bath at 5°. After incubation for 1 h, the pressure was checked and released. In those experiments in which *o*-nitrophenyl- and *p*-nitrophenyl-β-D-galactopyranoside were used as the substrate, the absorbance at 410 mμ was determined immediately. When lactose was used as the substrate the reactions were stopped by placing the tubes in boiling water for 5 min. The free glucose in a 0.3-ml aliquot was assayed as described previously.

RESULTS

Preparation of β-galactosidase

The specific activity of the purified enzyme assayed in the histidine buffer system at 22° in the presence of 100 mM NaCl and 2.3 mM *o*-nitrophenyl-β-D-galactopyranoside was 105 μmoles *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg protein. The specific activity as measured in the phosphate buffer system at 22° utilized by HU *et al.*[11] was not appreciably increased. Specific activities (μmoles *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg protein) which have been reported previously for a homogenous preparation include those of HU *et al.*[11] of 146 when assayed at 25°, and COLBY AND HU[14] of 160 when assayed at 30°.

Acrylamide gel disc electrophoresis[15] of the enzyme preparation in 0.05 M Tris and 0.38 M glycine (pH 8.3) revealed two main bands and four minor bands after fixing with acetic acid and staining with amido black[16]. Exposure of some unfixed and unstained gels to a buffered *o*-nitrophenyl-β-D-galactopyranoside solution containing 0.1 M NaCl revealed a yellow color in the areas of a centrally located main band and in another band that remained near the origin. It is estimated that these two bands compose from 80 to 90% of the protein in the gel. It seems possible that the slower moving active band represents a polymeric form of the enzyme since multiple species of this enzyme have been detected[17] by use of the ultracentrifuge.

Monovalent cation activation

The Michaelis–Menten constants for the substrates *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose are given in Table I. In all instances the affinity in the presence of Na^+ was considerably greater than that in the presence of K^+. The apparent dissociation constants for Na^+ and K^+, and for the same substrates are given in Table II. With all substrates tested the values obtained for Na^+ are lower than corresponding values observed for K^+.

For all the substrates utilized, appreciable hydrolysis occurred without the addition of exogenous monovalent cations. This hydrolysis in the absence of added cations could be due to either small amounts of contaminating cations or the lack of an absolute cation requirement, although on the basis of Na^+ and K^+ measurements made with a flame photometer the former appears to be more probable. The apparent

TABLE I

THE APPARENT MICHAELIS–MENTEN CONSTANTS OF β-GALACTOSIDASE FOR THE SUBSTRATES *o*-NITROPHENYL-β-D-GALACTOPYRANOSIDE, *p*-NITROPHENYL-β-D-GALACTOPYRANOSIDE, AND LACTOSE IN THE PRESENCE OF Na^+ AND K^+

The enzymatic assays were performed as described in MATERIALS AND METHODS. The respective constants were determined from Lineweaver–Burk double reciprocal plots.

Substrate	*Cation*	*Cation concn. (mM)*	K_m *(mM)*
o-Nitrophenyl-β-D-galactopyranoside	K^+	5	0.54
	Na^+	2.5	0.27
p-Nitrophenyl-β-D-galactopyranoside	K^+	22.5	0.77
	Na^+	1	0.13
Lactose	K^+	20	5.3
	Na^+	5	0.58

K_A values, mentioned above and calculated on the basis of added cations, are low and this high affinity could account for the observed substrate hydrolysis in the absence of cation additions.

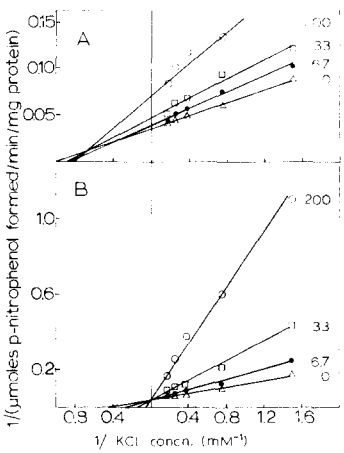
A study was conducted to determine the effect of Na^+ at different concentrations on K^+ activation of the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside by β-galactosidase. A double reciprocal plot of these results (Fig. 1) yields either a mixed type or competitive inhibition depending upon the method of presenting these results If the results are plotted without substracting the endogenous rate (Fig. 1A) the mixed inhibition is observed. If the endogenous rate is substracted from each value the plotted data illustrate a competitive type of inhibition (Fig. 1B). When *o*-nitrophenyl-β-D-galactopyranoside was utilized as the substrate in presence of Na^+ as an activator cation, the addition of K^+ increased rather than decreased the rate of hydrolysis. The increase in rate obtained by adding the two cations together was not equivalent to the increase obtained in adding the cations separately.

TABLE II

THE APPARENT DISSOCIATION CONSTANTS OF β-GALACTOSIDASE FOR THE CATIONS K^+ AND Na^+ UTILIZING *o*-NITROPHENYL-β-D-GALACTOPYRANOSIDE, *p*-NITROPHENYL-β-D-GALACTOPYRANOSIDE, AND LACTOSE AS SUBSTRATES

The enzymatic assays were performed as described in MATERIALS AND METHODS. The respective dissociation constants were determined from Lineweaver–Burk double reciprocal plots.

Substrate	*Substrate concn. (mM)*	*Cation*	K_A *(mM)*
o-Nitrophenyl-β-D-galactopyranoside	10	K^+	0.07
	10	Na^+	0.01
p-Nitrophenyl-β-D-galactopyranoside	10	K^+	1.1
	10	Na^+	0.01
Lactose	45	K^+	4.8
	45	Na^+	0.23



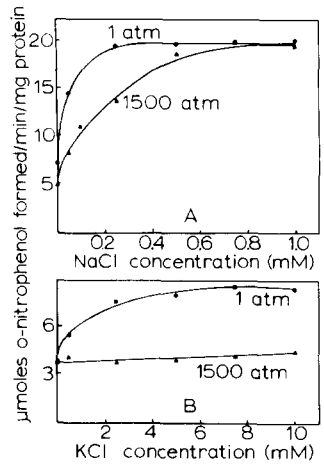


Fig. 1. The inhibition by Na^+ of the K^+ activation of β-galactosidase hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside. Assay and incubation conditions were as described in MATERIALS AND METHODS with the substrate concentration at 10 mM. A. The inverse plot of the results as they were obtained. B. The inverse plot of results where the rate in the absence of cation additions was subtracted from each value. The numerical values to the right of each line are molar Na^+ concentrations added to the respective incubation mixtures.

Fig. 2. The effect of hydrostatic pressure on the Na^+ (A) and K^+ (B) activated hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside by β-galactosidase. The incubation mixture (4 ml) consisted of 0.1 M histidine, 2.3 mM *o*-nitrophenyl-β-D-galactopyranoside, 5 mM dithiothreitol, the enzyme preparation (0.3 μg protein), and the appropriate cation chloride addition. The assay mixtures were incubated at 5° for 1 h either at 1 or 1500 atm pressure. Activity was determined on the basis of the change in absorbance at 410 mμ.

TABLE III

THE EFFECTS OF HYDROSTATIC PRESSURE ON THE RATE OF β-GALACTOSIDASE HYDROLYSIS OF *o*-NITROPHENYL-β-D-GALACTOPYRANOSIDE AND *p*-NITROPHENYL-β-D-GALACTOPYRANOSIDE

The incubation mixture (4 ml) for the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside consisted of the substrate, 5 mM dithiothreitol, the appropriate cation chloride addition and the enzyme. The assay mixtures were incubated at 5° for 1 h at the designated pressure. Activity was determined on the basis of the change in absorbance at 410 mμ. The volume change (ΔV^*) was calculated on the basis of the rate at atmospheric pressure and the rate at the applied pressure.

Substrate	*Substrate concn. (mM)*	*Cation*	*Cation concn. (mM)*	*Pressure (atm)*	ΔV^* *(cm³/mole)*
o-Nitrophenyl-β-D-galactopyranoside	2.3	K^+	10	1500	+10.1
	10	K^+	30	1500	+16.9
	2.3	Na^+	1	1500	0
	10	Na^+	3.3	1500	− 4.2
p-Nitrophenyl-β-D-galactopyranoside	2.3	K^+	20	1500	+20.4
	10	K^+	30	500	+ 4.2
	10	K^+	30	1000	+ 8.6
	10	K^+	30	1500	+17.9
	2.3	Na^+	1	1500	− 6.8
	10	Na^+	3.3	1500	− 5.0

Hydrostatic pressure experiments

A series of experiments were conducted to determine the effects of hydrostatic pressure on the monovalent cation activation of the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside and *o*-nitrophenyl-β-D-galactopyranoside. At the beginning of these experiments it was established that the rate of hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside in presence of Na^+ at 1500 atm was linear for a period of over 1 h. Also in preliminary experiments it was demonstrated that the inhibition of the K^+ activation of the hydrolysis of *p*-nitrophenyl-β-D-galactosidase by hydrostatic pressure was reversible because hydrolysis in a reaction inhibited by pressure continued at a normal rate when the pressure was released.

The effects of hydrostatic pressure on the enzymatic hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose in the presence of Na^+ and K^+ are shown in Figs. 2, 3 and 4 and Table III. Each value represents an average of three replications. In the case of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis pressure did not affect the rate in the presence of saturating levels of Na^+ and 2.3 mM substrate (Fig. 2) but pressure increased the rate at 10 mM substrate (Table II). The effect of pressure on the K^+ activation of lactose hydrolysis (Fig. 4) was not as pronounced as the effect on the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside but inhibition by pressure was still evident.

Since all of the preceeding pressure experiments were conducted at 5°, experiments were also performed at 25° using *p*-nitrophenyl-β-D-galactopyranoside as the substrate. Pressure effects at 25° were similar to those observed at 5° except that the turnover rate of the enzyme was increased.

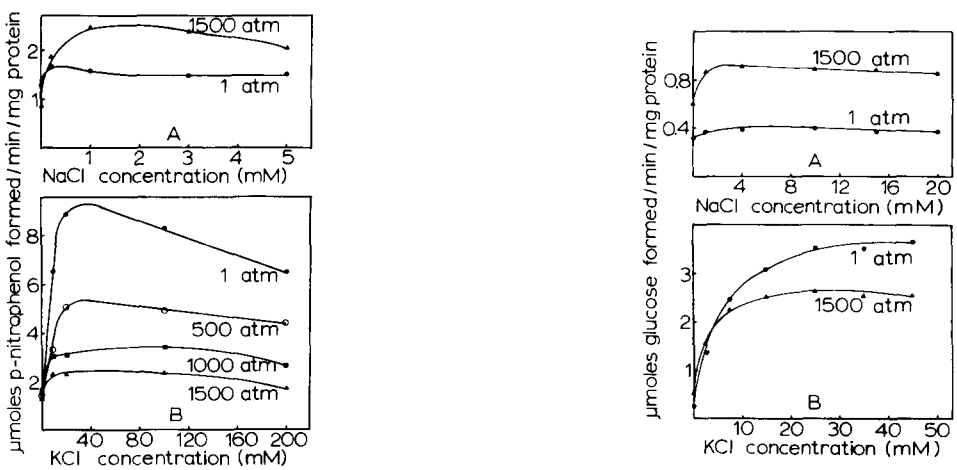


Fig. 3. The effects of hydrostatic pressure on the Na^+ (A) and K^+ (B) activated hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside by β-galactosidase. Conditions were similar to those described in Fig. 2 except that *p*-nitrophenyl-β-D-galactopyranoside (2.3 mM) was used as the substrate, the enzyme preparation consisted of 0.72 μg protein, and the pressure was varied as indicated.

Fig. 4. The effect of hydrostatic pressure on the Na^+ (A) and K^+ (B) activated hydrolysis of lactose by β-galactosidase. The conditions were similar to those described in Table II with the exception that lactose (45 mM) was substituted for *o*-nitrophenyl-β-D-galactopyranoside and the enzyme preparation consisted of 72 μg protein (A) or 3 μg protein (B). At the end of the incubation period and the release of the pressure the reaction was stopped by boiling for 5 min. A 0.3-ml aliquot was removed and assayed for glucose as indicated in MATERIALS AND METHODS.

Enzyme–substrate volume change

LAIDLER[18,19] has postulated that a volume change accompanies the transformation of the enzyme–substrate complex into an activated complex. Under conditions in which the enzyme is saturated with substrate it is this transformation which is postulated to occur just prior to hydrolysis, that is affected by pressure. The computation of the volume change was made by utilizing Eqn. 5 as given in the DISCUSSION. By the use of Eqn. 5 the volume changes of the activated enzyme–substrate complexes were calculated and the results are presented in Tables III and IV.

TABLE IV

THE EFFECTS OF HYDROSTATIC PRESSURE ON THE ENZYMATIC HYDROLYSIS OF LACTOSE BY β-GALACTOSIDASE

The assay conditions were as described in Fig. 4.

Cation addition	*Cation concn. (mM)*	ΔV^* *(cm³/mole)*
K^+	45	+ 4.5
Na^+	20	−12.0

DISCUSSION

It has been reported[8,9,20,21] that the rate limiting step in the β-galactosidase reaction is the dissociation of the enzyme–substrate complex into enzyme and products. This conclusion is based on the demonstration of the independence of the K_m and the v_{max} by cation activation studies[7] and by comparing the v_{max} with the K_m at several temperatures[20]. Results of stop-flow experiments[9,21] with several substrates further substantiate this conclusion. Since the formation of products is rate limiting and since stimulation was attained at saturating substrate concentrations, these results indicate that Na^+ and K^+ are involved in the dissociation of the enzyme–substrate complex into enzyme and products. The K_m of the enzyme for substrate also is influenced by Na^+ and K^+ (Table I and see ref. 9) and this indicates involvement of cations in the formation of the enzyme–substrate complex.

The K_A values (Table II) calculated for Na^+ and K^+ may only be relative values since, as reported by NEVILLE AND LING[8] for the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside, Na^+ can react with either the enzyme or the enzyme–substrate complex. If this were valid for all the activation studies conducted here the apparent K_A values would be less than the true K_A values due to the alteration of the equilibrium between the enzyme and metal and the enzyme–metal complex[22].

It would appear from the higher affinity of the enzyme for Na^+ than for K^+ (indicated by the kinetic determinations and the inhibition experiments) that Na^+ is bound to the enzyme in preference to K^+ and at probably the same site(s). This would indicate, as pointed out by USSING[23] that the association between the enzyme and the particular cation is occurring on the basis of the crystal or ionic radius rather than the hydrated radius. From this point of view a smaller atom, *i.e.* Na^+, would be more tightly bound or more closely associated with a negative component of the protein than a larger particle, *i.e.* K^+, of the same charge.

In the presence of high substrate and Na^+ concentrations the application of hydrostatic pressure increased in the rate of hydrolysis of all substrates tested. The rate of hydrolysis of all substrates in the presence of optimum levels of K^+ was decreased by pressure. These results can be interpreted according to the proposals of LAIDLER[18,19] which include the following rational.

Many enzymatic reactions proceed *via* an activated enzyme–substrate complex which occurs immediately prior to product formation. Furthermore, in some of these enzymatic reactions the dissociation of the enzyme–substrate complex into enzyme and products is the rate limiting step. At substrate concentrations which are considerably greater than the saturation levels for the enzyme formation of the enzyme–substrate complex would not be influenced by pressure. Under these conditions and at atmospheric pressure the rate would be related to total enzyme concentration ($[E_t]$) by the proportionally constant k_0 which can be expressed as follows:

$$v_0 = k_0[E_t] \tag{1}$$

The equation for the specific rate constant[24] of any elementary process influenced by pressure always can be written as follows:

$$k_p = k_0 e^{-P\Delta V^*/RT} \tag{2}$$

where k_0 is the rate at zero pressure, k_p is the rate at pressure P, R is the gas constant, T is the absolute temperature, and ΔV^* is the volume change accompanying the transformation. Under the specific conditions mentioned above the velocity at pressure P is given as the following:

$$v_p = k_p[E_t] \tag{3}$$

where k_p is the rate constant for the formation of products at the designated pressure. By combining Eqns. 2 and 3 an expression of the enzymatic reaction rate at pressure P can be obtained.

$$v_p = k_0[E_t]e^{-P\Delta V^*/RT} \tag{4}$$

consequently from Eqns. 1 and 4:

$$v_p = v_0 e^{-P\Delta V^*/RT} \tag{5}$$

This equation then represents a means by which the volume change of the activated enzyme–substrate complex can be calculated based on the rates in the presence of specific pressures. From this equation the volume change accompanying the formation of the activated complex can be calculated when both the substrate and the cation are at saturating levels. It is this volume change that is measured in the β-galactosidase reaction during saturating Na^+ and K^+ concentrations and that is correspondingly affected as the rates are either increased or decreased by hydrostatic pressure.

It is reported[18,19,24] that increasing pressure decreases the volume of the enzyme–substrate complex. In the experiments reported above, Na^+ activation was increased by pressure. In this case the volume of the activated enzyme–substrate–cation complex would be expected to be less in the presence of Na^+ than in its absence (Tables III and IV). The converse must be true for K^+ activation of the hydrolysis of all the substrates tested, since the application of pressure decreased the activation by K^+. Consequently, K^+ activation must occur with an increase in volume of the activated enzyme–sub-

strate–cation complex (Tables III and IV). Because a decrease in volume occurs upon ionization and an increase in volume occurs upon unfolding or association of ionized species[24], it is conceivable that Na^+ activation proceeds with an increase in ionization, while K^+ activation occurs with an increase in unfolding or an increased association of charged species.

Although the results of this investigation do not allow the prediction of a specific model for monovalent cation activation of β-galactosidase, they aid in the interpretation of results of other investigations. It is evident that both Na^+ and K^+ influence the formation of the enzyme–substrate complex as well as the hydrolysis of the substrate. During the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside, Na^+ can react with either the enzyme or the enzyme–substrate complex[8]. Multiple sites may be involved since a lower concentration of Na^+ is required for saturation than K^+ although these results could be explained on the basis of the higher affinity of the enzyme for Na^+. During the formation of the activated enzyme–substrate complex as measured by the influence of hydrostatic pressure, the mechanism of activation by Na^+ and K^+ appears to be different. A decrease in volume accompanies the formation of the complex in the presence of Na^+ while an increase in volume occurs in the presence of K^+.

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