specific inhibition by denaturation. Even with protein-reactive substrate analogs nonspecific inhibition must be carefully excluded by evidence for a stoichiometric relationship between incorporation of reagent and decrease of enzymatic activity.

In general, activation by a covalently bound reagent should give more confidence in a specific reaction than inhibition. This singular advantage seems to apply to the reaction of the protein reactive thioether analog of 5′-AMP and phosphorylase *b*. In view of the single labeled peptide in peptide fingerprints of the covalently activated enzyme, the possibility of a nonspecific activation seems rather remote.

In current experiments, we are trying to isolate homogeneously labeled phosphorylase *b* from the mixture of native enzyme and nucleotide derivatized enzyme after covalent activation reactions carried to about 25% of maximal enzymatic activity. This purification is an essential condition for the study of the kinetics of the labeled enzyme in comparison to 5′-AMP-activated phosphorylase *b*. Future experiments will aim at the isolation of the nucleotide-bound peptide from the peptic digest of this material for the determination of its sequence.

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A Common Intermediate in the Hydrolysis of β-Galactosides by β-Galactosidase from *Escherichia coli*†

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ABSTRACT: Kinetic data are presented which evidence the formation of a common intermediate during the hydrolysis of various aryl β-galactosides by β-galactosidase. Eight aryl β-galactosides were hydrolyzed in the presence of 0.247 M methanol. Methanol acts as a galactose acceptor in competition with water. The ratio of products (substituted phenol: methyl β-galactoside) was 3.01 ± 0.08 for these eight substrates. A similar experiment was done with four β-galactosides using 0.171 M ethanol as the acceptor. Again the ratio of products (substituted phenol:ethyl β-galactoside) was constant, 9.02 ± 0.25. If a series of substrates which vary only in the identity of the leaving group reacts in such a way as to produce a common intermediate that in turn reacts with two acceptors, water and an added acceptor, the ratio of products will be the same regardless of the leaving group. Alternatively, if no common intermediate is formed then the presence of the leaving group will influence the relative ability of two substances to serve as acceptors. Consequently, in the latter case the ratio of products will depend on the leaving group. Since a constant ratio of products was obtained with both methanol and ethanol as the extra acceptor, it may be concluded that a common intermediate is involved in the reaction mechanism for the enzymic hydrolysis of β-galactosides.

As in the case of other glycosidases, there is considerable interest in whether a common enzymic intermediate is formed during the hydrolysis of different galactosides by β-galactosidase. In nonenzymic hydrolysis of glycosides the nature of the intermediate depends on the conditions of the hydrolysis. In acid the hydrolysis proceeds through a carbonium ion intermediate (BeMiller, 1967), whereas in base an intramolecular displacement reaction probably occurs (Ballou, 1954; Gasman and Johnson, 1966; Piszkiewicz and Bruice, 1968). Both these processes seem to have analogous counterparts in enzymic systems. Thus a stabilized carbonium ion is favored

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for lysozyme (Blake *et al.*, 1967; Phillips, 1967; Dahlquist *et al.*, 1965, 1969) and a covalent glucosyl enzyme for sucrose phosphorylase (Voet and Abeles, 1970).

There are a number of observations that relate to the question of the existence of a common intermediate in reactions mediated by β-galactosidase or that are pertinent to the experimental basis for this paper. The existence of trans galactosidation reactions with various acceptors including methanol and ethanol has been demonstrated previously (Shifrin and Hunn, 1969; Viratelle *et al.*, 1969; Wallenfels and Malhotra, 1961). Shifrin and Hunn (1969) showed that with increasing concentrations of methanol, ethanol, and 1-propanol the enzymatic activity as measured by the release of *o*-nitrophenol from *o*-nitrophenyl β-galactoside first increased quite markedly to a maximum value and then markedly decreased. Furthermore, they found that "neither methanol nor ethanol dissociate the subunits of the active tetramer nor induce conformational changes in the protein as measured by sedimentation velocity, ultraviolet absorption spectroscopy, and fluorescence." These authors used methanol concentrations up to 10 M and ethanol concentrations up to 7 M.

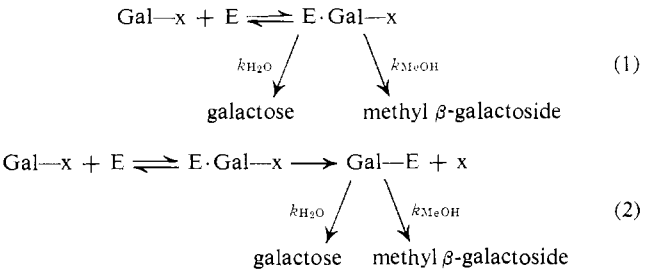
Viratelle *et al.* (1969) reported that the maximum velocity and the K_m increase when methanol is added but these values tend to approach a limiting value as the methanol concentration is increased. They concluded that the leveling off effect was consistent with the formation of an intermediate complex that forms after the Michaelis complex and is susceptible to nucleophilic attack by water or alcohol. The leveling off effect can be explained by a change in the rate-limiting step. That is, as the breakdown of the intermediate is increased by increasing concentrations of alcohol the formation of the intermediate becomes rate determining. Shifrin and Hunn do not find that the velocity of the reaction approaches a limiting value as the concentration of alcohol is increased. However, there would appear to be a limiting value if experiments were restricted to one side of the maximum. The interpretation of Viratelle *et al.* that the apparent limiting velocity may be a kinetic effect arising from the participation of the alcohol as an acceptor may be correct but it is uncertain. Such a limiting effect if it is of kinetic origin can also be interpreted as indicating a binding site for methanol

Viratelle *et al.* also found that the ratio of methyl β-galactoside to galactose formed as products was 2.56 ± 0.61 in the case of *o*-nitrophenyl β-galactoside and 1.96 ± 0.19 in the case of phenyl β-galactoside. They thought these measured values were similar enough to suggest that the actual ratios were precisely the same, as they must be if there is a common intermediate (Epand and Wilson, 1963).

In this paper we use the method of a constant ratio of products, independent of the leaving group, in a series of substrates, to show that a common intermediate is formed during the hydrolysis of substrates by β-galactosidase. This method depends upon the observation of others that methyl β-galactoside and ethyl β-galactoside are formed in addition to galactose when β-galactosides are hydrolyzed by β-galactosidase in the presence of the corresponding alcohols.

In this method there is no comparison of rates with and without methanol and therefore it is not necessary to make the assumption that methanol functions only as an acceptor. Thus the method is valid even if methanol should have a solvent effect that changes the conformation of the protein.

In this paper we present evidence for the occurrence of a common intermediate based upon the ratio of the products. Consider the two schemes for simultaneous hydrolysis and trans galactosidation with methanol as an acceptor



where Gal—x is a β-galactoside with leaving group x, E·Gal—x is the Michaelis complex between enzyme and substrate, Gal—E is the possible common intermediate, *e.g.*, a galactosyl enzyme or a galactosylcarbonium ion–enzyme complex. We have to decide whether the reaction with water and methanol stems from the Michaelis complex or from a common intermediate. It is immediately evident that if the common intermediate (should it form) has a reasonable lifetime, that is a lifetime long enough for x to diffuse away, the nature of x cannot influence the ratio of the products, β-galactose:methyl β-galactoside. Thus the same ratio will be obtained with different substrates. On the other hand, if a common intermediate is not formed the nature of x will surely influence the ratio of products and different ratios will be obtained with different substrates. In accordance with this rationale we have measured the ratio of products for a series of substrates in the presence of two different acceptors, 0.247 M methanol and 0.171 M ethanol. This method has been used previously for demonstrating a common intermediate in reactions catalyzed by chymotrypsin (Epand and Wilson, 1963) and by alkaline phosphatase (Barrett *et al.*, 1969).

In this work we measured two products: the substituted phenol (x) and methyl β-galactoside. Galactose was obtained by difference. The concentration of substituted phenol was conveniently measured by spectrophotometry since in all cases there was a sizeable difference spectrum between the substrate and the phenol formed as a product. The concentration of methyl β-galactoside was measured radiometrically using ^{14}C-labeled methanol. Methanol was separated from methyl β-galactoside by lyophilization of the reaction mixture.

Results

The substrates used in these experiments are listed in Table I along with some of their kinetic properties measured in this work. Kinetic parameters are also given by others for some of these substrates for various conditions of pH, temperature, and composition of the medium (Kuby and Lardy, 1953; Tenu *et al.*, 1971; Wallenfels and Malhorta, 1960). Even though one of the products, methyl β-galactoside, can also be a substrate, it was not significantly hydrolyzed in these trans galactosidation reactions, because β-galactosidase has a very poor affinity for this substrate (Kuby and Lardy, 1953; Tenu *et al.*, 1971; Wallenfels and Malhorta, 1961). The experiments were conducted with substrate concentrations of 4.6×10^{-2} M. This concentration is 300 times higher than the K_m of the poorest substrate, *p*-chlorophenyl galactoside, whereas the final concentration of methyl galactoside formed in the reaction was always less than its K_m.

The ratio of methyl β-galactoside:galactose using *o*-nitrophenyl β-galactoside as the substrate was found to be linear with respect to methanol concentration up to at least 0.5 M,

TABLE I: The Kinetic Parameters for the β-Galactosides in Tris-HCl Buffer (pH 7.50), Containing 0.01 M $MgCl_2$–0.1 M NaCl at 25°.

β-Galactoside	pK_a[a] of Phenol	$K_m \times 10^4$ (M)	V_{max} (μmoles/min per mg of Enzyme)	V_{max}/K_m (min^{-1}/mg of Enzyme)
o-Nitrophenyl	7.21	1.4	360	2570
m-Nitrophenyl	8.27	1.1	327	2970
m-Chlorophenyl	9.02	1.0[b]	180	1800
p-Nitrophenyl	7.15	0.52	69	1330
Phenyl	10.0	1.5[b]	40	270
p-Methoxyphenyl	10.21	1.6[b]	33	210
p-Chlorophenyl	9.42	0.78[b]	7.5	96
p-Bromophenyl	9.32	0.58[b]	7.0	121
Methyl	15.5	90[b]	*c*	~4.4

[a] Albert and Serjeant (1962). [b] Measured as the K_I. [c] The V_{max} for methyl β-galactoside is approximately the same as for phenyl β-galactoside (Kuby and Lardy, 1953; Viratelle *et al.*, 1971).

twice the concentration used in this study. This linearity suggests that either methanol is not bound or is only loosely bound to the enzyme. As a consequence the actual ratio of the rate constants for methyl β-galactoside and galactose formation can be calculated easily, *i.e.*

$$k_{MeOH}/k'_{H_2O} = 1/(R - 1)(MeOH)$$

where R is the ratio of substituted phenol to methyl β-galactoside formed as products. The concentration of water is contained in the constant k'_{H_2O}.

The ratios of substituted phenol to methyl β-galactoside after partial enzymatic hydrolysis in the presence of 0.247 M methanol are listed for eight substrates in Table II. Each value represents at least three experimental determinations. The values for the different substrates are the same within experimental error. The average ratio of substituted phenol to methyl β-galactoside is 3.01 ± 0.14 and the average value of k_{MeOH}/k'_{H_2O} is 2.03 ± 0.14. Similar values for k_{MeOH}/k'_{H_2O} were obtained by Viratelle *et al.* (1969) who measured the rates of formation of galactose and methyl β-galactoside in the presence of methanol. They obtained k_{MeOH}/k'_{H_2O} ratios of 2.56 ± 0.61 and 1.96 ± 0.19 for *o*-nitrophenyl β-galactoside and phenyl β-galactoside, respectively.

The ratios of substituted phenol to ethyl β-galactoside after a small amount of enzymatic hydrolysis in the presence of 0.171 M ethanol are listed for four substrates in Table III. Again each value represents at least three experimental determinations. The value of k_{EtOH}/k'_{H_2O} is constant within this series. The average value of k_{EtOH}/k'_{H_2O}, 0.731 ± 0.041, is about one-third that of k_{MeOH}/k'_{H_2O} but on a molar basis EtOH is still a considerably better nucleophile than water.

Discussion

The purpose of this study was to determine whether the ratio of products, galactose to alkyl β-galactoside, formed in the presence of methanol and ethanol acting as acceptors, is independent of the nature of the galactoside leaving group of substrates in reactions mediated by β-ga'actosidase. The ratios presented in Tables II and III are independent of the leaving group. These results are consistent with the attack of the nucleophiles on an enzymic intermediate common to all substrates.

TABLE II: Ratio of Products in 0.247 M Methanol in Tris-HCl Buffer (pH 7.50), Containing 0.01 M $MgCl_2$–0.1 M NaCl at 25°.

β-Galactoside	Substituted Phenol/Methyl β-Galactoside	k_{MeOH}/k'_{H_2O}
o-Nitrophenyl	3.06 ± 0.05	1.97 ± 0.05
m-Nitrophenyl	3.08 ± 0.14	1.96 ± 0.13
m-Chlorophenyl	2.96 ± 0.23	2.08 ± 0.24
p-Nitrophenyl	3.04 ± 0.04	1.99 ± 0.05
Phenyl	3.10 ± 0.22	1.94 ± 0.20
p-Methoxyphenyl	2.89 ± 0.12	2.14 ± 0.13
p-Chlorophenyl	2.91 ± 0.14	2.13 ± 0.16
p-Bromophenyl	3.02 ± 0.21	2.02 ± 0.16
Average	3.01 ± 0.08	2.03 ± 0.08

The constant ratio of products cannot be attributed to an inability of the enzyme to recognize differences in the leaving group of the substrates. This enzyme does recognize differences in the leaving group as indicated in Table I where it is shown that the second-order rate constant for the hydrolysis, V_{max}/K_m, varies by a factor of 30 for the substrates used in this study.

Methanol (and also ethanol) is a far more effective nucleophile than water; 0.247 M methanol is one-half as effective as 55 M water. Clearly, this is a consequence of the totality of interactions of the methanol molecule with that enzymic species with which it reacts. If this enzymic species were to contain the leaving group as part of the structure as would be the case if the enzymic species were the Michaelis complex, then the nature of the leaving group would surely influence the ability of methanol to compete with water and the ratio of products would not be invariant but would vary from substrate to substrate. This argument makes it highly probable that a constant ratio of products is a sufficient condition for the formation of a common intermediate. Thus our finding of an invarient ratio of products is strong evidence for the

TABLE III: Ratio of Products of 0.1713 M Ethanol in Tris-HCl Buffer (pH 7.50), Containing 0.01 M $MgCl_2$–0.1 M NaCl at 25°.

β-Galactoside	Substituted Phenol/Ethyl β-Galactoside	k_{EtOH}/k'_{H_2O}
m-Nitrophenyl	9.08 ± 0.16	0.722 ± 0.014
p-Nitrophenyl	8.66 ± 0.58	0.764 ± 0.059
Phenyl	9.18 ± 0.69	0.717 ± 0.061
p-Methoxyphenyl	9.17 ± 0.36	0.719 ± 0.028
Average	9.02 ± 0.25	0.731 ± 0.022

formation of an intermediate that is common to all substrates.

The second-order rate constant, V_{max}/K_m, is the rate constant for the formation of this intermediate. Except for the *o*-nitro derivative, our series of substrates is a group that in nonenzymic reactions usually has the quality of showing an approximate linear relationship between the pK_a of the leaving group and the logarithm of the rate constant for the reaction. In our enzymic reaction no such relationship exists; indeed, the rate constants do not form even a monotonic sequence with respect to the pK_a of the leaving group. However all vestiges of such a relationship are not entirely submerged because it is apparent that the substrates with the better leaving groups ($pK_a \leq 9$) react with the enzyme distinctly more rapidly than do the others.

From our results it is not possible to know the nature of the common intermediate. Either a stabilized carbonium ion or a galactosyl enzyme is a reasonable possibility that is consistent with our findings.

A different argument based on the constant ratio of products supports the concept of a common intermediate because it rules out the simpler scheme, (1). This scheme predicts that upon the addition of methanol the rate of utilization of substrate (production of ROH) at high substrate concentration will be increased by the factor $R/(R - 1)$. Since R is the same for all substrates, the maximum velocity should be increased by the same factor for all substrates. This is not the case. The rate of utilization of *o*-nitrophenyl β-galactoside is markedly increased by low concentrations of methanol, yet the rate of utilization of phenyl β-galactoside is not discernably affected (Viratelle *et al.*, 1969). However, this last argument is based upon the assumption that the only important effect of methanol arises from its ability to serve as an acceptor. Also, this argument does not rule out a scheme in which there is a rate influencing conformational change: $E \cdot S \rightarrow E' \cdot S$.

Experimental Section

Materials

The *o*-, *m*-, and *p*-nitrophenyl and phenyl β-galactosides were purchased from Sigma. The *m*-chloro-, *p*-chloro-, *p*-bromo-, and *p*-methoxyphenyl β-galactosides were synthesized (Csürös *et al.*, 1964). These galactosides were crystalline white products with the appropriate physical properties. After preliminary purification, all the phenols were further purified by sublimation. The [^{14}C]methanol and [1-^{14}C]ethanol were obtained from New England Nuclear. These were diluted to the required specific activity using Spectrograde alcohols. Partially purified β-galactosidase from *Escherichia coli* (approximately 80% pure) was obtained from Worthington Chemical Co.

Methods

Extinction Coefficients. Extinction coefficients of the substituted phenols and phenyl β-galactosides were determined in 1.0 M sodium carbonate buffer (pH 10.5) at the appropriate wavelength in a Zeiss spectrophotometer immediately following purification of the compounds.

Ratio of Products. All experiments were carried out at 25° in 0.05 M Tris-HCl buffer (pH 7.50), containing 0.01 M $MgCl_2$, 0.1 M NaCl, 4.6×10^{-2} M substrate, and either [^{14}C]methanol or [^{14}C]ethanol (the methanol or ethanol concentration was less than 1%). Enough β-galactosidase was added to utilize 10–50% of the substrate in 1–2 hr. The total substrate utilized was determined by measuring the amount of substituted phenol that was liberated. This was done by taking an aliquot of the reaction mixture and diluting it at least 40 times with 1.0 M sodium carbonate buffer (pH 10.5) and then measuring the absorption. The amount of alkyl β-galactoside was measured radiometrically as the [^{14}C]alkyl β-galactoside by taking a 0.5-ml sample of the reaction mixture and adding it to a scintillation vial in a boiling-water bath. The water and excess methanol or ethanol were removed by lyophilization. The residue was redissolved in 0.5 ml of distilled water and 20 ml of scintillation fluor and solubilizer was added. The radioactivity was measured in a Beckman scintillation counter. Suitable controls were run simultaneously. The amount of alkyl galactoside was calculated from the specific activity of the alcohol. The amount of galactose was calculated by subtracting the amount of alkyl galactoside from the total amount of substrate utilized.

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