Catalytic Consequences of Experimental Evolution. Part 2.† Rate-limiting Degalactosylation in the Hydrolysis of Aryl β-D-Galactopyranosides by the Experimental Evolvants *ebg*[a] and *ebg*[b]

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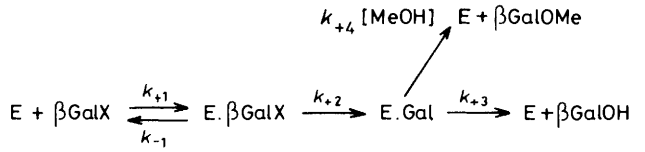
The build-up of a galactosyl-enzyme intermediate in the hydrolysis of 2-naphthylgalactoside by *ebg*[b] enzyme has been observed by fluorescence in a stopped-flow apparatus. Degalactosylation has been shown to be rate limiting in the steady-state hydrolysis of 3,4-dinitrophenylgalactoside by both *ebg*[a] and *ebg*[b] enzymes by nucleophilic competition with methanol. The implications of this slowing down of degalactosylation as a consequence of experimental evolution of the gene are discussed.

The description of the phenomenon of enzyme catalysis in terms of physical organic chemistry is a subject of some current interest,[1] and considerable progress has been made in accounting for the catalytic efficacy of a number of enzymes isolated from modern organisms.[1,2] The processes by which modern enzymes attained this efficacy are less well understood. A detailed analysis of the free energy profile for the interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by the triose phosphate isomerase of rabbit muscle [3] led to the proposal that this key metabolic enzyme of an advanced organism had reached ' evolutionary perfection ' since the reaction rate in one direction was limited by the diffusion together of enzyme and substrate.‡ It was further proposed [4] that this state could be approached by three evolutionary changes of increasing improbability: (1) uniform stabilisation of all enzyme-bound intermediates and transition states; (2) selective changes in the relative stabilities of enzyme-bound intermediates; (3) selective stabilisation of transition states for individual catalytic steps.

These proposals are at the time of writing the only ideas on the evolution of enzyme activity subject to experimental test. Since it is only rarely possible to relate changes in the amino-acid sequence of an enzyme to their kinetic consequences, sequence data on related enzymes of different modern species are of limited value in understanding this aspect of evolution. Furthermore, it is in principle impossible to know the selection pressures which produced the observed differences in modern organisms.

Because of their short generation time it is possible to observe evolution in bacteria in the laboratory.[5] The selection pressures that produced observed changes can then be known absolutely. Moreover, where this selection pressure is applied to an enzyme, the ancestral and evolved enzyme can be isolated and subjected to chemical investigation.

The bacterial system best suited to this type of study is the *ebg* gene in *Escherichia coli K12*.[6] Ordinarily, when this bacterium grows on lactose, the initial stages of lactose utilisation are brought about by proteins coded for by the genes grouped together in the *lac* operon.[7] The product of the Y gene transports lactose into the cell and the *lacZ* β-galactosidase hydrolyses it to glucose and galactose. The system is controlled by a repressor protein, coded for by the i gene, which binds to the DNA and physically blocks its transcription, except in the presence of a β-galactopyranosyl derivative, typically in the laboratory isopropyl 1-thio-β-D-galactopyranoside.



Scheme. k_{+3} is the pseudounimolecular rate constant for reaction with water

It is possible to obtain mutant bacteria in which the *lacZ* gene is absent. These mutants are unable to grow on lactose, but since in the presence of isopropyl thiogalactoside the synthesis of the *lacY* protein can be induced, galactosides can get into the cell. If these mutant cells are incubated on a medium containing lactose as only possible carbon source, some colonies begin to grow. Genetic examination reveals that they owe this capacity to *ebg* gene products of improved catalytic efficacy; the *ebg* gene is remote from the *lac* operon.[8] A linear relationship exists between growth rate of the experimentally evolved bacterial strain and k_{cat}/K_m for lactose hydrolysis by the purified *ebg* enzyme.[8]

In fact two types of point mutation giving rise to an adequately effective enzyme can be discerned.[9] Class I is obtained with 90% frequency when selection is carried out on lactose: a typical gene product is *ebg*[a] enzyme. Class II is obtained with 10% frequency when selection is carried out on lactose, and exclusively when selection is carried out on lactulose [4-*O*-(β-D-galactopyranosyl)-D-fructose]: a typical gene product is *ebg*[b] enzyme. The sites of the class I and class II point mutations are separated by *ca.* ⅓ of the total length of the *ebg* enzyme gene (changes in regulatory genes are also involved).[9]

In the preceding paper [10] we present evidence that the kinetic mechanism of the wild-type enzyme is a simple three-step one, in which a galactosyl enzyme intermediate can transfer a β-D-galactopyranosyl residue to either water or methanol (Scheme). We now show that the process of experimental evolution has slowed down the hydrolysis of the galactosyl enzyme in the case of both *ebg*[a] and *ebg*[b], such that it limits the rate of hydrolysis of aryl galactosides by these evolvants.

Results and Discussion

A *Observation of a Pre-steady-state ' Burst ' of Aglycone in Hydrolysis of 2-Naphthyl β-D-Galactopyranoside by* ebg[b].—

† Part 1, ref. 10.
‡ The rate in the other direction is then determined by the overall equilibrium constant.

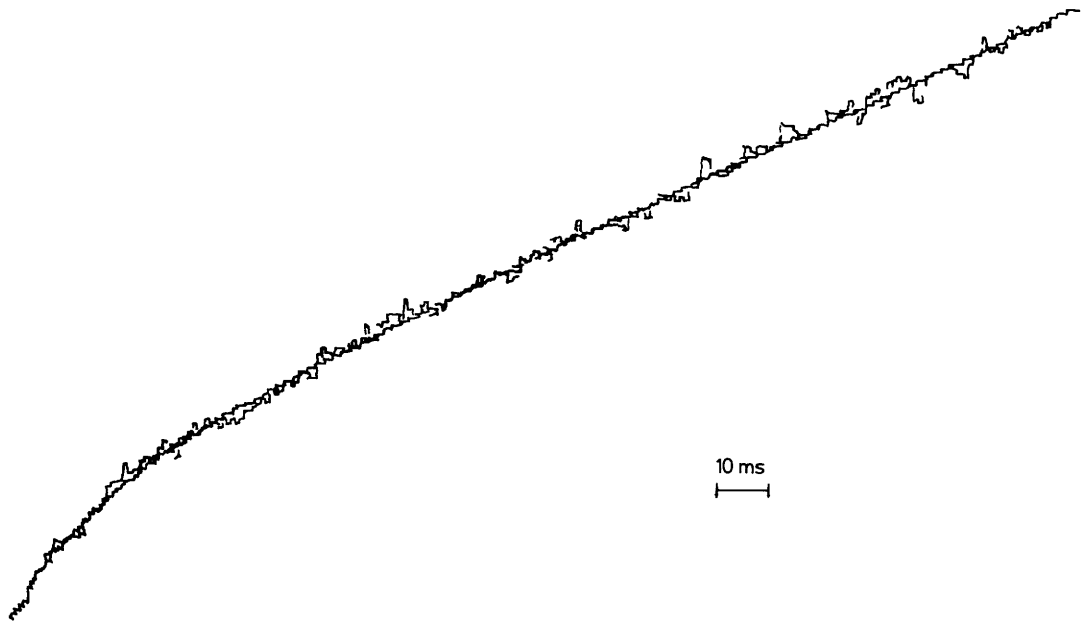


Figure 1. Increase of fluorescence consequent on rapidly mixing equal volumes of 2-naphthylgalactoside (6.63mM) and *ebg*^{b} (*ca.* 1.4 mg ml^{-1}), in a stopped flow apparatus at 25 °C

If the reaction of enzyme and substrate is observed on a time-scale comparable with $1/k_{+2}$, and if, further, changes in [HX] comparable with $[E]_0$, the total concentration of active sites can be observed, then the approach to the steady state can be measured if $k_{+2} \geqslant k_{+3}$. Three parameters are obtained from such an experiment, usually carried out by stopped flow techniques.[11]

(i) The rate constant for the approach to the steady state given by equation (1)

$$k_{+3} + \frac{k_{+2}k_{+3}\,[S]}{(k_{+2} + k_{+3})K_m + k_{+3}[S]} \qquad (1)$$

(ii) The amplitude of the ' burst ', π, where equation (2) holds

$$\pi = \left(\frac{k_{+2}}{k_{+2} + k_{+3}}\right)^2 \left(\frac{[S]}{[S] + K_m}\right)^2 [E]_0 \qquad (2)$$

(iii) The steady state rate V where equation (3) applies

$$V = \left(\frac{k_{+2}}{k_{+2} + k_{+3}}\right) k_{+3}\,[E]_0 \left(\frac{[S]}{[S] + K_m}\right) \qquad (3)$$

$$\text{whence } \left(\frac{[S]}{[S] + K_m}\right)\frac{V}{\pi} = k_{+2}\left(k_{+2} + k_{+3}\right) \qquad (4)$$

Attempts to detect a ' burst ' in the hydrolysis of 4-nitrophenyl β-D-galactopyranoside ([S] 1.00mM; K_m 0.27 × 10^{-3} mol l^{-1}) [12] by monitoring optical transmission at 415 nm in the observation chamber of a stopped-flow apparatus failed. This was tentatively ascribed to k_{+2} being too fast, such that the transient was over in the dead-time of the instrument (*ca.* 3 ms). Change of substrate to 2-naphthyl galactoside ([S] 3.32mM; K_m 0.73 × 10^{-3} mol l^{-1}),[12] and the method of detection to fluorescence, enabled a ' burst ' to be observed (Figure 1). The displayed data are the computer average for four pushes, and give k_{+3} 13.3 and k_{+2} 16.2 s^{-1}; the average of three pushes with the same enzyme and a 0.1 s time-base gave k_{+3} 11.3 and k_{+2} 12.6 s^{-1}. A largely denatured sample of enzyme, at a protein concentration approximately seven-fold greater than that of the previous experiments, but at $\frac{1}{5}$ the signal attentuation, gave k_{+3} 15.5 and k_{+2} 55.5 s^{-1} from the average of six pushes. The value of k_{+2} is very sensitive to the curve-fitting procedure and errors are amplified by the method of calculation, so no conclusions can be drawn from the variation in this parameter. However, the value of k_{+3} is fairly firm.

It is conventional in this type of study to demonstrate that the size of π is that predicted from the known concentration of enzyme active sites. However, calibration of fluorescence responses and measurement of the concentration of enzyme active sites [13] is a problematic proceeding, and we here conduct an essentially equivalent calculation.

Since log k_{cat}/K_m for a series of aryl galactosides is correlated with aglycone pK_a with a β_{lg} of −0.31,[12] k_{cat} for 4-nitrophenyl β-D-galactopyranoside is k_{+3} (with 2-naphthol as a leaving group $k_{+2} > k_{+3}$; with the more acidic 4-nitrophenol $k_{+2} \gg k_{+3}$). Freshly isolated *ebg*^{b} enzyme gives a $V_{max.}$ of 7.62 × 10^{-5} mol s^{-1} g^{-1} with 4-nitrophenyl galactoside. This value, together with the average k_{+3} value obtained from the stopped-flow experiment (13.4 ± 2) gives a molecular weight per active site of 176 000 ± 26 000 in fair agreement with Hall's reported value of 120 000.[8] Certainly, given that the enzyme as isolated will always be less than 100% active, there is little evidence for ' half of the sites ' reactivity.

B *Effect of Methanol and Dioxan on the Steady-state Kinetic Parameters for Hydrolysis of* 3,4-*Dinitrophenyl* β-D-*Galactopyranoside by* ebg^{a} *and* ebg^{b}.—The steady state kinetic parameters for the Scheme are given by equations (4) and (5).[14]

$$k_{cat} = \frac{k_{+2}(k_{+3} + k_{+4}[MeOH])}{k_{+2} + k_{+3} + k_{+4}[MeOH]} \qquad (4)$$

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}}(k_{+3} + k_{+4}[MeOH]) \qquad (5)$$

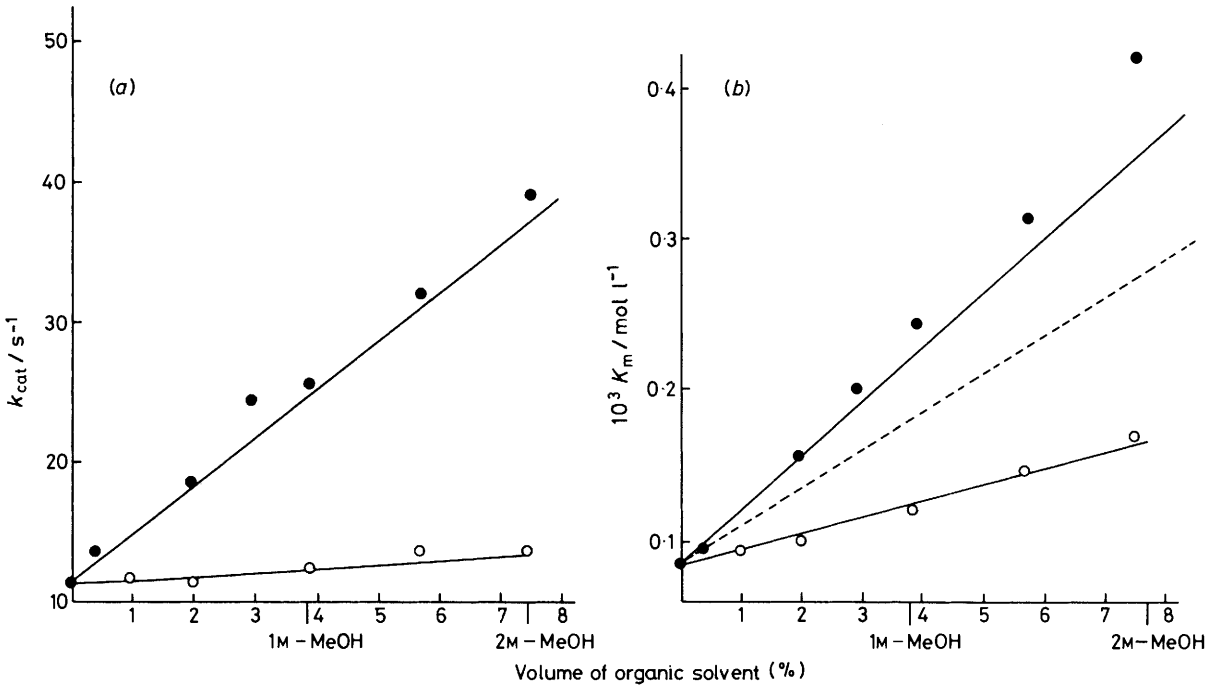


Figure 2. Effect of methanol (●) and dioxan (○) on (a) k_{cat} and (b) K_m for hydrolysis of 3,4-dinitrophenyl galactoside by *ebg*^{b}. The solid line through the methanol points in (a), and the broken line in (b), show the variation in these parameters calculated from the value of k_{+4}/k_{+3} measured by g.l.c. of the methanolysis products and the assumption that $k_{+2} \gg k_{+3}$. The solid line through the methanol points in (b) is calculated on the assumption that the effect of methanol on K_m is caused both by nucleophilic competition and by a solvent effect identical to that caused by the same volume of dioxan

Thus, if $k_{+2} \geqslant k_{+3}$, a linear relationship should be observed between both k_{cat} and K_m and [MeOH]. Moreover, the increase in k_{cat} and K_m should be quantitatively accounted for by k_{+4}/k_{+3}, independently measured. This is partitioning ratio of the galactosyl enzyme between water and methanol and, as described in the previous paper, can be measured by g.l.c. of the trimethylsilylated products of enzymic hydrolysis of a galactoside which is a better substrate than methyl galactoside.

Ten analyses of five *ebg*^{b}-catalysed hydrolyses of 3,4-dinitrophenyl galactoside at 1.98 and 0.99M concentrations of methanol gave a value of k_{+4}/k_{+3} of 1.14 ± 0.17 l mol^{-1}. No time-dependence of this ratio was observed, *i.e.* methyl galactoside is a sufficiently poor substrate that its hydrolysis by the enzyme is unimportant. A similar experiment with *ebg*^{a}, gave a value of 1.86 ± 0.23 l mol^{-1} (12 analyses of six hydrolyses). These figures mean that, whereas the wild-type galactosyl-enzyme intermediate has only a seven-fold preference for methanol on a molar basis, that from the evolvant *ebg*^{a} has a 102-fold preference, and that from the evolvant *ebg*^{b} a 63-fold preference.

Figure 2 shows the effect of methanol and dioxan on Michaelis–Menten parameters for hydrolysis of 3,4-dinitrophenyl galactoside by *ebg*^{b}. It is clear that dioxan has little effect and that therefore the observed effect with methanol is not caused simply by the addition of an organic solvent. Moreover, k_{cat} and K_m increase with methanol concentration in a way which is quantitatively accounted for by the measured value of k_{+4}/k_{+3}. Therefore the observed increases of k_{cat} and K_m are caused by the faster reaction of the galactosyl-enzyme with added methanol than with water.

The demonstration of rate-limiting degalactosylation by both pre-steady-state measurements and nucleophilic competition with methanol validates the latter technique for use with other *ebg* enzymes. Figure 3 shows the effect of methanol and dioxan on Michaelis–Menten parameters for hydrolysis of 3,4-dinitrophenyl galactoside by *ebg*^{a} enzyme. Again, the data clearly show that k_{cat} for this substrate represents k_{+3}.

Experimental evolution of the gene coding for the enzyme has thus resulted in a slowing down of the rate of hydrolysis of the galactosyl-enzyme intermediate, from a minimum value of 186 s^{-1} [10] for the wild-type enzyme to 11.6 s^{-1} for *ebg*^{b} and 14.8 s^{-1} for *ebg*^{a} (at 25 °C).

C *Implications for the Evolution of Enzyme Catalytic Activity (including that of Antibiotic Detoxifying Enzymes).*—Since the growth rates for various *lacZ*$^-$ mutants correlate with k_{cat}/K_m for lactose hydrolysis by the appropriate *ebg* enzyme,[8] and since this parameter does not contain k_{+3}, no evolutionary disadvantage accrues to the organism if the hydrolysis of the galactosyl enzyme becomes slower. This is a molecular example of a common feature of evolution, that selection for one character can result in a loss of effectiveness in a non-critical character. Biologically, the changes can be regarded as a type of pleiotropy: a single genetic change has resulted in changes in two characters of the phenotype, k_{+2} and k_{+3}, for the enzyme-catalysed hydrolysis.

Albery and Knowles [4] proposed that the easiest evolutionary change, in the free energy profile of the catalysed reaction would be a uniform increase in binding of all internal states. Such a change is illustrated in Figure 4: it would not alter the rates of the various unimolecular processes of bound intermediates. However, we find that in both the cases so far examined where a point mutation results in a catalytically improved enzyme, the rates of unimolecular processes do alter. This proposal must therefore be regarded as at best of limited validity.

A common mechanism of antibiotic resistance in bacteria is the plasmid transfer of genes coding for detoxifying enzymes.[15] One type of β-lactamase, indeed, is a simple two-step hydro-

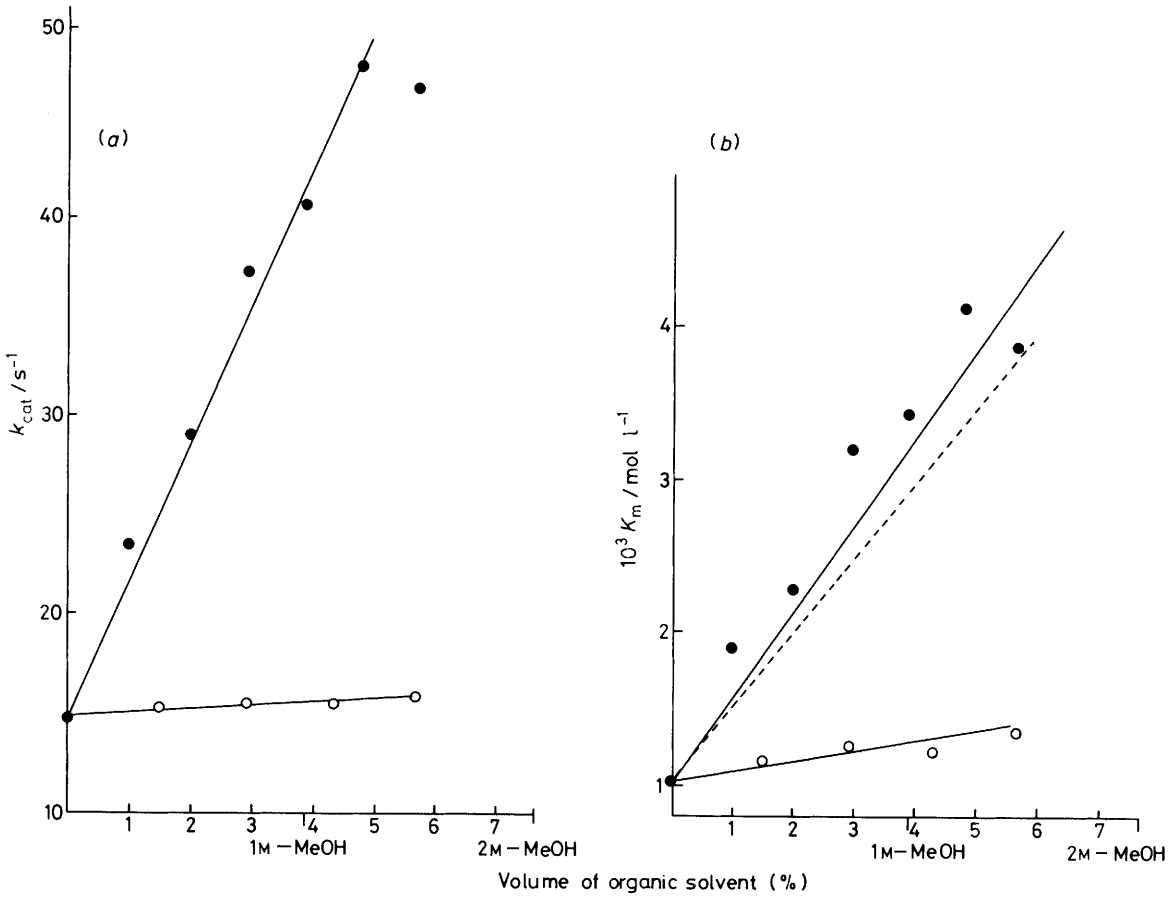


Figure 3. Effect of methanol (●) and dioxan (○) on (a) k_{cat} and (b) K_m for hydrolysis of 3,4-dinitrophenyl galactoside by *ebg*[a]. The solid line through the methanol points in (a), and the broken line in (b) show the variation in these parameters calculated from the value of k_{+4}/k_{+3} measured by g.l.c. of the methanolysis products and the assumption that $k_{+2} \gg k_{+3}$. The solid line through the methanol points in (b) is calculated on the assumption that the effect of methanol on K_m is caused both by nucleophilic competition and by a solvent effect identical to that caused by the same volume of dioxan

lase kinetically similar to the *ebg* enzymes.[16] The intensive search for, and widespread application of, structurally modified penicillins and cephalosporins inert, or at least less susceptible, to wild-type β-lactamases, in the present context can be regarded as a vast, unplanned scale-up of the laboratory experiments in bacterial evolution, described in ref. 5. The demonstration that there are *E. coli* strains in which spontaneous point mutations occur producing *ebg* enzymes of dramatically different catalytic characteristics from the wild-type, and the now widespread distribution of wild-type β-lactamase genes in bacteria, together make it likely that structurally modified β-lactam antibiotics will have only a very short effective life, before the β-lactamase gene(s) evolve to cope with them.

Experimental

ebg[a] and *ebg*[b] enzymes were isolated from the strains, and by the methods described by Hall.[9] Steady-state kinetic measurements were made on a Unicam SP8-200 spectrophotometer, at wavelengths described in the previous paper; $V_{max.}$ and K_m values were calculated from linear least-squares treatment of *V versus V*/[S] plots. Substrates were as described.[10] Methanol and dioxan were distilled before use. All data pertain to 0.125M-potasium phosphate buffer, pH 7.5, 5mM in magnesium chloride, 25μM in 2,2′-bipyridyl at 25.0 °C.

Analysis of Products of Enzymic Hydrolyses in the Presence of Methanol.—Solutions (5mM) of 3,4-dinitrophenyl β-D-galactopyranoside in buffer containing either 0.99 or 1.98M-methanol were hydrolysed with enzyme to between 20 and 70% conversion. The reaction was then stopped by heating at 80 °C for 5 min, and the solutions were dried in a vacuum desiccator over P_2O_5 for 2 weeks. The residue was trimethylsilylated with a mixture of pyridine, hexamethyldisilazane, and trimethylsilyl chloride (5 : 2 : 1 v/v) the suspension being shaken for 1 h. The trimethylsilyl derivatives of α- and β-galactose, and methyl β-D-galactoside, were analysed in a Pye GCD gas chromatograph (N_2 elution), at a temperature of 175 °C using a 2 m column of SE 33 stationary phase. Relative retention times were 1.06_5 : 1.26 : 1. Peaks were quantitated by triangulation, and identical molar flame ionisation response factors were assumed.

Pre-steady-state Measurements.—The stopped flow fluorimeter was described by Bagshaw *et al.*[17] Aglycone fluorescence was excited at 340 nm from a double monochromator with a 10 nm half bandpass, and fluorescent light was viewed through a Schott KV 393 cut-off filter. Data was captured by a Datalab OL905 transient recorder and transferred to cassette tape. The results from several experiments were averaged, and the averaged traces analysed, using a program on a PET microcomputer similar to that described by Attwood.[18]

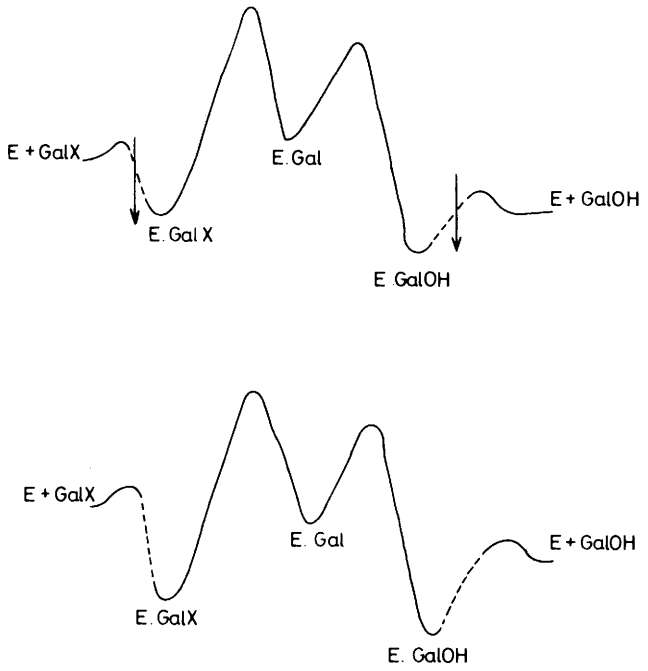


Figure 4. Effect of a uniform increase in binding of all internal states. This process, considered by Albery and Knowles [4] to be the easiest evolutionary change to make, does not alter values of k_{+2} or k_{+3}

Acknowledgements

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