Catalytic Consequences of Experimental Evolution. Part 3.[1] Construction of Reaction Profiles for Hydrolysis of Lactose by *ebg*°, *ebg*ª, and *ebg*ᵇ Enzymes *via* Measurement of the Enzyme-catalysed Exchange of D-[1-^{18}O]Galactose by ^{13}C Nuclear Magnetic Resonance Spectroscopy †

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Rate-constants at 37 °C for the exchange of D-[1-^{18}O]galactose with solvent, catalysed by the wild-type second β-galactosidase of *E. coli*, *ebg*°, and the experimental evolvants *ebg*ª and *ebg*ᵇ have been measured. These have been used with other data to construct substantially complete free-energy profiles for the wild-type enzyme and the two evolvants selected for catalytic efficiency on lactose. The consequences of the *ebg*° ⟶ *ebg*ª change are not simple, but the *ebg*° ⟶ *ebg*ᵇ change can be regarded as largely a stabilisation of the galactosyl-enzyme intermediate, considered to be the second most likely evolutionary change in a free-energy profile by W. J. Albery and J. R. Knowles, *Biochemistry*, 1976, **15**, 5631.

In previous papers,[1,2] we have advanced evidence that the *ebg* β-galactosidases, products of the *ebgA* gene, of *Escherichia coli K12*, work by a three-step mechanism involving a galactosyl-enzyme intermediate [equation (1)]. For this

$$E + \beta GalX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E.\beta GalX \xrightarrow[-HX]{k_{+2}} E.Gal \xrightarrow{k_{+3}} E + \beta GalOH \quad (1)$$

scheme the steady-state kinetic parameters k_{cat} and K_m are given by equation (2).

$$k_{cat} = \frac{k_{+2}k_{+3}}{k_{+2} + k_{+3}}; \; K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \frac{k_{+3}}{k_{+2} + k_{+3}} \quad (2)$$

Artificial selection pressure, directed towards maximising the rate of hydrolysis of lactose [3] under physiological conditions ([S] ≤ K_m), results in the emergence of spontaneous point mutants of *E. coli* in the *ebgA* gene, which produce altered *ebg* enzymes having this parameter augmented at the expense, in the case of *ebg*ª and *ebg*ᵇ, of a slowing down of k_{+3}.[2]

The kinetic scheme above, with the inclusion of a β-D-galactopyranose–enzyme complex, which is not kinetically significant in the reaction scheme as written, corresponds to the free energy profile of Figure 1. A decrease in k_{+3} could correspond either to a raising of ‡3, a lowering of the galactosyl-enzyme intermediate, or some combination of the two. It is possible, experimentally, to make these distinctions if the reaction running from right to left of Figure 1 could be studied. Such a reaction is merely the scheme of equation (1) with X = OH, or, to enable the reaction to be followed, ^{18}OH. A convenient way for ^{18}O exchange to be followed is *via* the ^{18}O isotope shift on the ^{13}C n.m.r. signal of the attached carbon.[4-6] We now report studies along these lines, which, with ancillary measurements, provide for the first time an experimental basis for any discussion of the evolution of enzyme function in terms of the free energies of intermediates and transition states.

† Presented at the Third International Conference on the Mechanisms of Reactions in Solution, Canterbury, Kent, 5—9 July 1982, Abstract B 13.

Experimental

Materials.—D-[1-^{18}O]Galactose (*ca.* 70% enriched) was made by heating D-galactose (2.0 g) with $H_2{}^{18}O$ (71.3% atom % ^{18}O; Prochem Ltd., lot no. 62 × 158) (1.0 ml) for 1.5 h on a steam-bath. After cooling, ethanol was added and the labelled galactose crystallised out as a mixture of α and β anomers, m.p. 157—165 °C. Substrates and *ebg* enzymes are as described in previous papers,[1,2] although some experiments were carried out with an *ebg*° fraction only *ca.* 10% pure, with no detectably different results. The frozen stock solutions were dialysed against standard *ebg* buffer before use. This buffer consisted of 0.125M-potassium phosphate, pH 7.5, 5mM in magnesium chloride, and 25μM in 2,2'-bipyridyl.[1]

Spectrophotometric Kinetic Measurements.—These were made, at 390 nm for 3,4-dinitrophenyl galactoside and at 400 nm for 4-nitrophenyl galactoside, in a Unicam SP 1800 spectrophotometer, fitted with a thermostatted cell-block through which water, maintained at 25.0 °C (for enzyme assay) or at 37.0 °C, was circulated by a Tecam Tempunit pump. Concentrations of enzyme active sites were estimated from enzyme assays against 5mM-4-nitrophenyl galactoside, the specific activity of freshly isolated enzyme, and an assumption of a molecular weight per active site of 1.5×10^5: pre-steady state measurements [2] have shown this to be not seriously incorrect.

K_I values for galactose were estimated from initial rate measurements at each of five substrate concentrations, and five inhibitor concentrations, including zero. Data were processed on a Hewlett-Packard 9821A calculator using a program written by Dr. P. J. England, and the inhibition was shown to be strictly competitive.

^{18}O-Exchange Measurements.—Measurements were made on solutions containing labelled galactose (*ca.* 100 mg), D_2O (as lock) (0.1 ml), buffer, and dialysed *ebg* enzyme solution, to a total volume of 1.0 ml. The enzyme was assayed before and after each run and enzyme concentrations were maximally 6 mg ml^{-1}.

The natural-abundance ^{13}C spectra were obtained at 37 °C on a JEOL FX200 spectrometer operating at 50.1 MHz in the Fourier transform mode. A spectral width of 200 Hz was

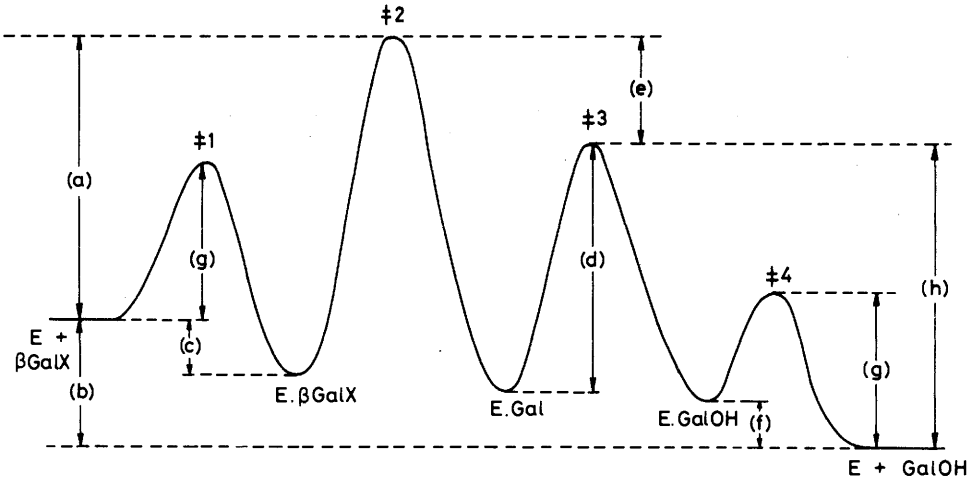

Figure 1. Reaction profile for the kinetic scheme of equation (1), illustrating how it can become experimentally accessible by measurement of the following parameters (in the case of lactose). (a) k_{cat}/K_m for lactose; (b) equilibrium constant for lactose hydrolysis; (c) K_s for lactose; (*d*) k_{+3}; (e) selectivity of the galactosyl-enzyme between water and O(4) of glucose; (f) K_s for galactose; (g) diffusional barrier to binding of ligands to enzyme (commonly *ca.* 10^7 M^{-1} s^{-1}); and (h) k_{cat}/K_m for hydrolysis of [1-^{18}O] galactose.

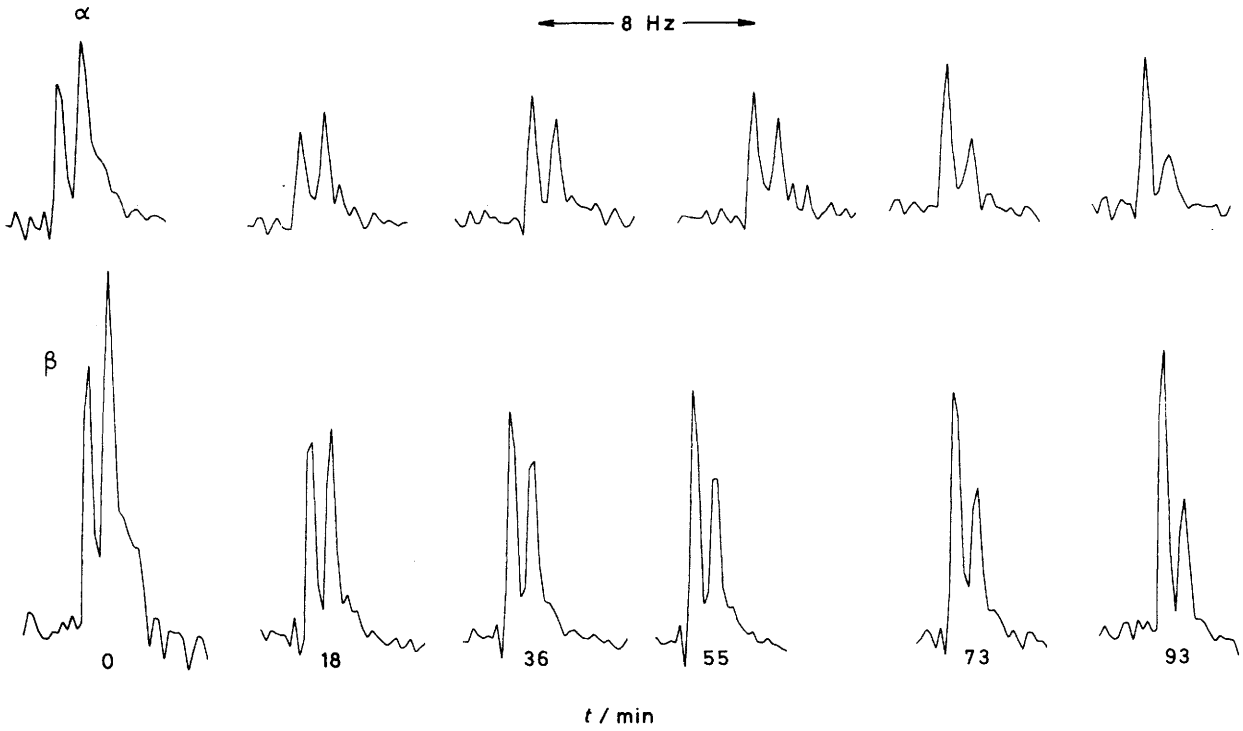

Figure 2. Natural abundance ^{13}C resonances of C(1) of α(top) and β(bottom) D-[1-^{18}O]galactopyranose, in the presence of *ebg*^{a} as a function of time. Chemical shifts are 94.3 and 90.2 p.p.m., respectively, downfield with respect to external Me_4Si.

sufficient to cover the signals of the anomeric carbons of both α-pyranose and β-pyranose isomers, and typically 2K data points were accumulated from a total of 200 transients, giving an accumulation time for each spectrum of *ca.* 17 min. For each reaction some 6—8 spectra were accumulated, and processed by zero filling to 8K points and resolution enhancement to give acceptable resolution of the peaks corresponding to ^{16}O and ^{18}O isotopomers. Under the conditions of the experiment differential nuclear Overhauser and T_1 effects between isotopomers are likely to be negligible: this implies T_2 values for isotopomers are identical, and hence that the resolution enhancement procedures will not appreciably affect intensity measurements. The rate constants for the exchange reaction were determined by comparison of the observed spectra with simulated spectra calculated from first-order kinetics, and are estimated to be accurate to ±20%, k_{cat} values are calculated from duplicate k_{obs} measurements.

Results and Discussion

Figure 2 displays the resonance of the anomeric carbon atom of both α- and β-D-[1-^{18}O]galactopyranose, as it changes with time in the presence of *ebg*^{a} enzyme. It is clear that washout of ^{18}O is slow compared with interconversion

Table. Kinetic parameters for hydrolysis at 37 °C by *ebg* enzymes

Substrate	Parameter	*ebg*°	*ebg*^{a}	*ebg*^{b}
3,4-Dinitropheny-D-galactopyranoside	k_{cat}/s^{-1}	442	39	43
	K_m/mM	6 ± 1	0.88 ± 0.03	0.11 ± 0.01
D-[1-^{18}O]Galactose	k_{cat}/s^{-1} [a]	150	0.8	40
	K_m/mM [b]	27 ± 2	7.8 ± 0.2	15.1 ± 0.5

[a] Blank rate 2.2×10^{-5} s^{-1}; for calculations of k_{cat}, k_{obs} was minimally twice this. [b] K_i for mutarotated solution of galactose

of anomers of the sugar. The isotope shifts, 0.016 and 0.013 p.p.m. upfield for the heavy isotopomer of the α- and β-pyranose forms, respectively, are, within experimental error, the same as those reported by Risley and van Etten[6] for α- and β-D-glucopyranose (0.018 and 0.016 ± 0.002, respectively), and large enough to enable ^{18}O exchange to be followed.

The kinetics of ^{18}O exchange in this system are predicted to be first order, irrespective of substrate concentration, since unlabelled material acts as a competitive inhibitor of the exchange process, of K_i the same as K_m for substrate, *i.e.* equation (3) holds. K_m was estimated, as a K_i, from inhibition of

$$k_{obs} = k_{blank} + \frac{k_{cat}\,[E]_o}{(K_m + [\text{galactose}])} \qquad (3)$$

the hydrolysis of 3,4-dinitrophenyl β-D-galactopyranoside by *ebg*°, and of 4-nitrophenyl β-D-galactopyranoside by *ebg*^{a} and *ebg*^{b}. The K_i values we use refer to a fully mutarotated solution of D-galactose, which contains 63 ± 2% of the β-pyranose isomer.[7] On the assumption that other forms neither bind nor react, this introduces a constant 0.31 kcal error into the free energy of the E–β-galactopyranose complex, and ‡3, but does not alter k_{cat}. This is immaterial in discussions of changes in the free-energy profile as a consequence of evolution.

Degalactosylation, (the k_{+3} step), has been shown to be rate determining in the hydrolysis of 3,4-dinitrophenyl β-D-galactopyranoside by both *ebg*^{a} and *ebg*^{b} at 25 °C.[2] k_{cat} Values for aryl galactosides only fall below the value of k_{+3} when the aglycone pK_a exceeds 8:[8] therefore it is unlikely that a change in temperature of only 12 °C would make k_{+2} rate determining. We therefore take k_{cat} for 3,4-dinitrophenyl β-D-galactopyranoside at 37 °C as representing k_{+3} for *ebg*^{a} and *ebg*^{b} (see Table).

Degalactosylation is not kinetically accessible in the case of *ebg*°, a minimum value being given by k_{cat} for 3,4-dinitrophenyl β-D-galactopyranoside. This is sufficiently faster than k_{cat} for D-[1-^{18}O]galactose that the latter can be taken as k_{+2} for galactose. However, we can provide only a lower limit to the free energy of the galactosyl-enzyme intermediate.

In the case of D-[1-^{18}O]galactose hydrolysed by *ebg*^{b}, the similarity of k_{cat} and k_{+3} indicates that $k_{+2} \gg k_{+3}$, and that galactose is bound, not as the non-covalent enzyme galactose complex, but as the galactosyl enzyme. As a consequence we cannot locate the *ebg*^{b} galactose complex on the reaction profile, other than from the relationship $K_m < K_s$. In the case of the *ebg*^{a}-catalysed hydrolysis of D-[1-^{18}O]galactose, by contrast $k_{+2} \ll k_{+3}$, so both the galactosyl *ebg*^{a} and *ebg*^{a}–galactose complexes can be located.

Michaelis–Menten parameters for lactose,[9] combined with the values of k_{+3} here described, enable k_{+2} and K_s for lactose hydrolysis to be calculated from equation (2). These parameters pertain to the fully mutarotated equilibrium mixture.

Construction of the free-energy profiles then requires only choice of a thermodynamic standard state, an estimate of the diffusional barrier to the binding of lactose and galactose to the enzyme, and an estimate of the standard free energy of hydrolysis of lactose. We chose the thermodynamic standard state as 37 °C,[3] the temperature of the evolutionary experiments, and 53mM, the best estimate of the intracellular concentration of lactose in a growing cell. (The adoption of the physiological concentration of each individual sugar as the standard state for its bimolecular reaction with E or E.Gal would be more appropriate, but the concentrations of glucose and galactose in *E. coli* cells growing on lactose are not available.)[10] A value of 10^7 M^{-1} s^{-1} for the diffusional barrier was chosen for illustrative purposes, this value being typical for enzyme–small ligand binding processes.[11] It is clear that, whatever the true value, with such evolutionary imperfect enzymes,[12] diffusion together of enzyme and substrate is not kinetically significant.

There appears to be no experimental value for the free energy of hydrolysis of lactose. A value of −4 kcal mol^{-1} (standard state 1M, 37 °C activity of pure water taken as 1.0) has been estimated for the standard free energy of hydrolysis of maltose, *via* some phosphorolysis reactions,[13] which are not possible with lactose. Cellobiose, with, like lactose, a β(1⟶4) pyranose link, has been estimated to be 0.86 kcal $mole^{-1}$ more stable than maltose.[14] We take the standard free energy of hydrolysis of lactose as −3.14 kcal mol^{-1} (standard state M).

These data together give the free-energy profiles of Figure 3. There are a couple of internal checks it is possible to make. First, k_{cat} for [1-^{18}O]galactose in no case exceeds k_{+3}. Second, hydrolysis of 4-nitrophenyl β-D-galactopyranoside by *ebg*^{a} in the presence of 1.0M-glucose gives no detectable lactose.[15] This accords with previous studies on the transferase activity of the *ebg* enzymes,[16] and corresponds to a free-energy difference between ‡2 and ‡3 of >3.7 kcal mol^{-1}.

The profiles of Figure 3, pertaining to a wild-type and two experimentally evolved enzymes, can be directly compared with the predictions of Albery and Knowles [17] about the likely changes in a free-energy profile of an enzyme in the course of evolution. These were considered to be, in order of decreasing probability: (i) a uniform increase or decrease in binding of all internal states; (ii) selective alterations in the stabilisation of bound intermediate(s); and (iii) selective acceleration of individual steps.

It is clear that neither the *ebg*° ⟶ *ebg*^{a} change, nor the *ebg*° ⟶ *ebg*^{b} change, corresponds to (i). The *ebg*° ⟶ *ebg*^{b} change, however, could conceivably be attributed to (ii), *i.e.* the selective stabilisation of the glycosyl enzyme, with lesser effects of ‡2 and ‡3. There is some evidence from structure–reactivity correlations[8] that this change is associated with more effective application of acidic assistance to aglycone departure at ‡2. If an acid catalytic group, AH, were to be made slightly more acidic, then A^- would be a slightly weaker base, and the hydrolysis of the galactosyl enzyme, presumably involving the general base catalysis of the attack of water on the galactosyl enzyme, would be slower. The galactosyl enzyme, in which HA would be ionised, would be stabilised, as is observed.

The changes consequent upon the evolution of *ebg*° to *ebg*^{a} do result in an equalisation of the energies of inter-

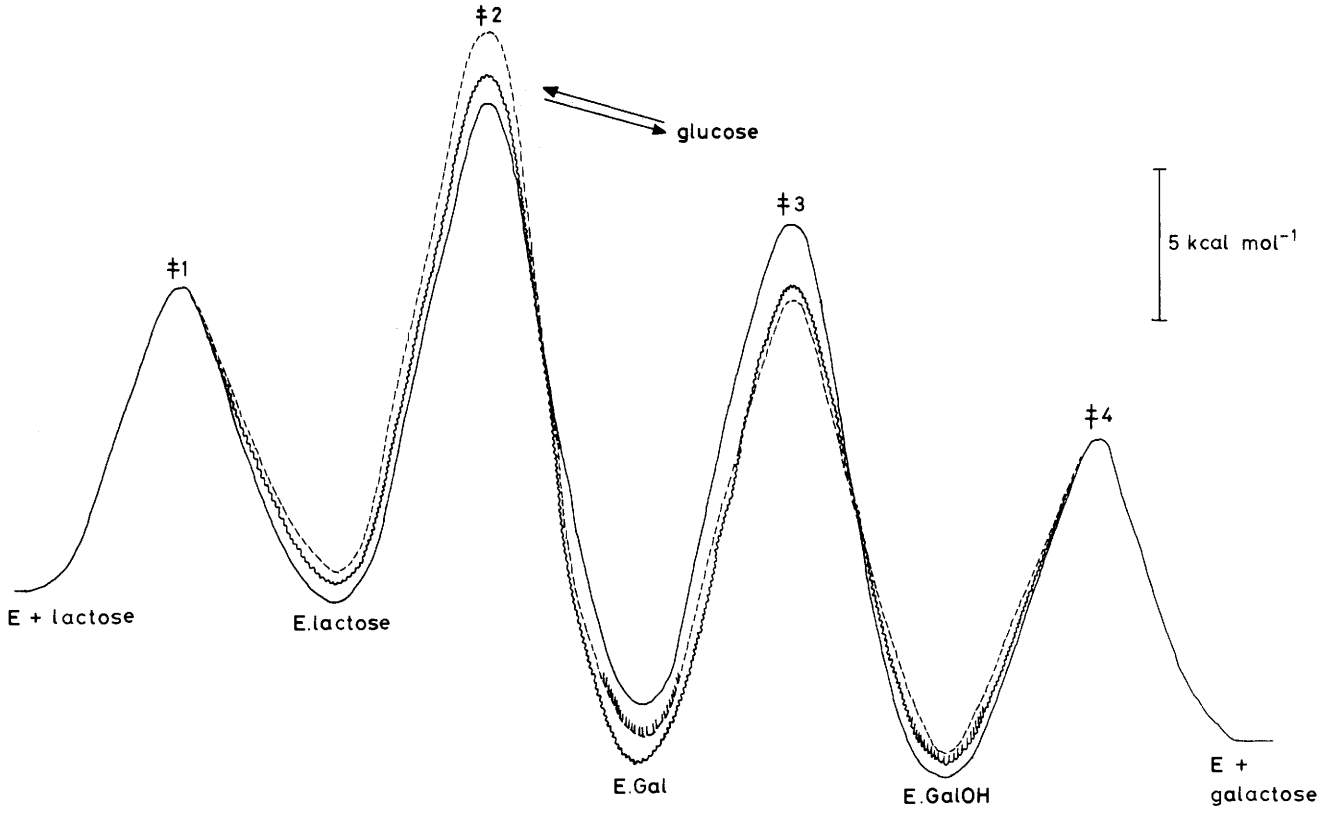

Figure 3. Free-energy profile for hydrolysis of lactose by: dashed line, *ebg*^{o}; continuous line, *ebg*^{a}; and wavy line, *ebg*^{b}. Vertical hatching denotes a lower limit.

mediates on either side of the rate-determining transition state (‡2), and thus appear to be described by Albery and Knowles' second change. However, for ‡2 to be lowered by the Hammett-type relationships suggested by these authors, ‡2 would have to resemble E.lactose rather than E.Gal, and thus be affected more by the lowering of E.lactose than the raising of E.Gal. However, structure–reactivity correlations with synthetic substrates indicate, at least for *ebg*^{o}, just the opposite, a high degree of cleavage of the Gal–X bond at ‡2.[1] The *ebg*^{o} ⟶ *ebg*^{a} change thus fits none of Albery and Knowles' categories.

The only feature common to the free-energy profiles of *ebg*^{a} and *ebg*^{b} *vis à vis* that of *ebg*^{o} is that ‡2 is lowered. Since, under the conditions of the biological selection experiments, [S] ≤ K_m, fitness will depend on k_{cat}/K_m (the dominating term in the 'efficiency function'[17] in this case, since glucose and galactose do not accumulate).

The mechanism whereby k_{cat}/K_m is increased is a single amino acid change, out of a total of ~10^3 per subunit.[3] There are thus at most around 2×10^4 amino acid changes possible, but only 40% of these are caused by change of a single nucleotide 'letter' in the *ebgA* gene. There is no compelling reason why this limited repertoire of amino acid changes should oblige the physical scientist by causing a rate enhancement attributable to a single clear-cut physico-chemical phenomenon. There is, in short, no necessity for chance mutations to do other than what they are selected for.

Acknowledgements

We thank the S.E.R.C. for personnel support and purchase of the n.m.r. spectrometer, and Professors W. J. Albery and J. R. Knowles for enlightening correspondence.

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Received 23rd *February* 1983; *Paper* 3/292

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