lized as before, and then, without the usual preliminary incubation, were immediately subjected to sectioning, enzyme maceration, and teasing apart. Clusters of endophyte filaments were mechanically separated by filtration, repeatedly transferred by micropette until free of cell debris, and then pipetted into 6B broth in 6-cm petri plates; the plates were sealed with Parafilm and incubated in the dark at 25°C. Within 3 weeks, a small number of the clusters had formed; these were typical colonies of the actinomycete. These colonies could be subcultured on yeast-extract medium. Thus, the same isolation procedure has been utilized successfully both in the initial isolation and in the subsequent recovery of the same organism, demonstrating unequivocally the effectiveness of the technique and the certainty of the identity of the microorganism.

The current taxonomic status of the actinomycetous root nodule endophytes is felt by many authors to be quite uncertain (*1, 19, 23*). Becking (*24*) applied the generic name *Frankia* to the endophytes of nonleguminous nodules and designated specific epithets referable to the host species or to original designations. According to his taxonomic treatment, the *Comptonia* isolate would be referable to *Frankia brunchorstii*. With the successful establishment of this organism in pure culture, we prefer to reserve judgment on the appropriate scientific designation. Better defined chemotaxonomic characters of the *Comptonia* isolate will help to establish its affinities with the known groups of actinomycetes and perhaps to clarify its taxonomic position (*25*).

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13. Composition of M-3 (grams per liter): $CaCO_3$, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $MnSO_4 \cdot H_2O$, 0.025; H_3BO_3, 0.10; $ZnSO_4 \cdot 7H_2O$, 0.010; $Na_2MoO_4 \cdot 2H_2O$, 0.00025; $CuSO_4 \cdot 5H_2O$, 0.000025; Difco yeast extract, 0.5; Edamin, 1.0; thiamin hydrochloride, 0.0001; nicotinic acid, 0.0005; pyridoxine hydrochloride, 0.0001; mannitol, 1.0; sucrose, 20.0; agar, 10.0; *p*H adjusted to 7.0.
14. B-D medium [P. Goforth and J. G. Torrey, *Am. J. Bot.* **64**, 475 (1977)] used as a base supplemented with 1 m*M* each of L-glutamic acid, L-aspartic acid, glycine, L-arginine, L-asparagine, L-glutamine, and urea, and (per liter) 2.0 mg of naphthaleneacetic acid and 1 μg of zeatin.
15. These hydrolytic enzymes of the cell wall were manufactured by Yakult Biochemical Co., Inc., of Japan.
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17. Stock cultures are maintained in 0.5 percent Difco yeast extract in distilled water at *p*H 6.4 in unshaken flasks or test tubes 5 to 6 cm deep with medium. Subculture is effected by crushing washed colonies in a tissue homogenizer with distilled water and inoculation of new cultures with a drop of the suspension. Cultures 4 to 6 weeks old are routinely used for subculture or plant inoculation.
18. Medium composition (milligrams per liter): $CaCl_2 \cdot 2H_2O$, 200; KH_2PO_4, 200; $MgSO_4 \cdot 7H_2O$, 200; NaCl, 100; H_3BO_3, 1.5; $ZnSO_4 \cdot 7H_2O$, 1.5; $MnSO_4 \cdot H_2O$, 4.5; $NaMoO_4 \cdot 2H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.04; thiamin hydrocholoride, 0.1; nicotinic acid, 0.5; pyridoxine hydrochloride, 0.5; and 10 m*M* succinic acid, 2 m*M* L-glutamine, 0.5 m*M* myoinositol, and 0.1 m*M* Fe · EDTA; *p*H 6.4.
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25. The *Comptonia* isolate described here has been used successfully in our laboratory to induce the formation of root nodules with high acetylene-reduction activity in seedlings of *Myrica gale* and *M. cerifera* and in the laboratory of M. Lalonde, Kettering Research Laboratory, Yellow Springs, Ohio (personal communication) in seedlings of *Alnus crispa* and *A. glutinosa*.
26. Supported in part by NSF research grant BMS 74-20563 and by the Maria Moors Cabot Foundation for Botanical Research of Harvard University. We thank J. Tjepkema for assistance in the microbiological aspects of this work and S. LaPointe for greenhouse care.

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Membrane Enzymes: Artifacts in Arrhenius Plots Due to Temperature Dependence of Substrate-Binding Affinity

Abstract. *For the membrane sodium-stimulated magnesium-adenosinetriphosphatase of* Acholeplasma laidlawii *B both the* V_{max} *and* K_m *values in the Michaelis equation vary strongly with temperature. Simulations of Arrhenius plots show that an enzyme with a temperature-dependent* K_m *can yield a variety of Arrhenius plot artifacts, most notably erroneous "breaks," if activity is assayed at a fixed substrate concentration.*

In studies of membrane-bound enzymes, Arrhenius plots of the temperature dependence of enzyme activity have often been utilized to correlate the activity of the enzyme with the phase state of the membrane lipids. In particular, any abrupt changes in the measured activation energies E_a are often attributed to a lipid phase transition; it has been suggested that such breaks in Arrhenius plots arise from a change in the enzyme conformation or in the nature of the rate-limiting step of the overall catalyzed reaction (*1*). In a study of the membrane Mg^{2+}-adenosinetriphosphatase of *Acholeplasma laidlawii* B (*2*), we have found that the variation with temperature of its substrate-binding affinity can strongly influence the behavior of Arrhenius plots, altering the measured E_a values and break temperatures and even creating artifactual breaks in some cases if enzyme activity is assayed at a single fixed substrate concentration. As we will demonstrate in this report, such effects can be so pronounced that they should be accorded more serious consideration in experimental design than is generally the case at present.

Acholeplasma laidlawii B cells were cultured as described elsewhere (*3*) in the presence of oleic, elaidic, or palmitic acid to replace approximately 70 percent of the normal membrane fatty acids with the exogenous species, thus producing three cell populations of widely varying membrane fluidity (*4*). Cells were harvested and membranes were prepared by osmotic lysis as described elsewhere (*5*). To assay the adenosinetriphosphatase activity, membranes (100 to 200 μg of protein per milliliter) were incubated with 50 m*M* NaCl, 15 m*M* $MgSO_4$, 50 m*M* tris buffer (*p*H 7.4), and various concentrations of adenosine triphosphate (ATP) at various temperatures; portions of the reaction mixture were quenched by mixing with an equal volume of 0.5 percent dodecyl sulfate solution and assayed for phosphate by the method of Atkinson *et al.* (*6*). Protein was assayed by the method of Hartree (*7*).

To ensure that all factors other than ATP concentration were optimal in our assays, we began by reexamining the ionic requirements of the enzyme. As previously reported (*2*), the enzyme has an absolute requirement for Mg^{2+}. How-

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ever, we found the measured activation constant for Mg^{2+} in the presence of moderate levels of ATP to correspond closely to the measured K_m value for ATP determined in the presence of excess Mg^{2+} over a wide range of temperatures, which suggests that Mg^{2+} is required to generate the true substrate, Mg-ATP, and not to activate the enzyme directly. The enzyme was also found to be stimulated approximately two- to threefold by Na^+ but not by K^+ or tris-H^+ ions; the dependence of reaction rate on Na^+ concentration is complex and suggests that at least two sodium ions are required for full stimulation. The Mg^{2+} and Na^+ concentrations finally adopted as standard in subsequent assays (15 mM and 50 mM, respectively) gave maximal activity at all temperatures.

When the rate of the catalyzed reaction was studied as a function of ATP concentration, we found that at low to moderate ATP concentrations (up to at least $4 \times K_m$) the dependence of rate on ATP concentration is hyperbolic, while at high concentrations there is strong substrate inhibition. Values of K_m and V_{max} were therefore determined from the low-ATP portions of Eadie-Hofstee plots (of the measured velocity v versus v/[ATP]); and values of the inhibitor constant K_i from the high-ATP portions. These parameters are given in Table 1 for one set of experiments. The temperature ranges studied encompass most of the calorimetrically determined phase transition range in palmitate- and elaidate-enriched membranes and lie well above the phase transition in oleate-enriched membranes (*4*); the temperature dependences of K_m and K_i, which are observed for all three membrane preparations, are therefore not simply due to a membrane phase transition. From van't Hoff plots, effective average ATP binding enthalpies ΔH_B^{eff} of -17, -19, and -44 kcal mole^{-1} are calculated for membranes enriched in elaidate, oleate, and palmitate, respectively, over the temperature ranges examined. Arrhenius plots of extrapolated V_{max} values exhibit breaks for palmitate- and elaidate- but not oleate-enriched membranes, consistent with the results of de Kruyff *et al.* (*8*), who, however, reported break temperatures much lower than those we determined (see Table 1). The lower break temperatures reported by these authors appear to be due to an artifactual shift in the measured break temperature which can occur when K_m varies with temperature and activity is assayed at a single substrate concentration (see below). The combined effects of varying K_i and K_m on the reaction velocity at 5 mM ATP (as a fraction of V_{max}) are illustrated in Table 1 for the three membrane preparations at various temperatures. The ratio v(5 mM)/V_{max} steadily declines as the temperature rises, an effect which causes Arrhenius plots of v(5 mM) to curve downward at higher temperatures even when E_a is constant. This phenomenon is discussed in more detail below.

In early experiments, we found that Arrhenius plots derived from measurements of enzyme activity at a fixed concentration of ATP (1 mM) exhibited strong downward curvature, even in oleate-supplemented membranes where no phase transition occurs in the temperature range studied; this curvature proved to be primarily due to the temperature dependence of K_m. To assess more fully the effect of a temperature dependence of K_m on an Arrhenius plot of reaction rate at a fixed substrate concentration, we carried out some simulations of Arrhenius plots using enthalpic parameters (E_a and ΔH_B^{eff}) comparable to those determined in our experiments.

In one set of simulations, we assumed that the enzyme E_a value changed abruptly at some temperature and considered the effect of various values of ΔH_B^{eff} and substrate concentration (relative to K_m) on the shape of the Arrhenius plot. One common result was that shown in Fig. 1a: for even modest values of

Table 1. Kinetic parameters at various temperatures for adenosinetriphosphatase of cell membranes enriched in oleic, elaidic, or palmitic acids. Values in columns 3 and 4 are means ± standard deviations; N.D., not determined.

Fatty acid supplement	Temperature (°C)	K_m (mM)	V_{max} (nmole mg^{-1} min^{-1})	K_i (mM)	v(5 mM)/V_{max}	Break temperature (°C)
Oleate	15	0.051 ± 0.008	35 ± 2	8.3	0.62	
	22.5	0.165 ± 0.014	83 ± 5	5.6	0.52	
	30	0.51 ± 0.05	197 ± 5	3.7	0.41	
	37.5	0.55 ± 0.03	352 ± 21	2.3	0.30	
Elaidate	15	0.099 ± 0.013	14.5 ± 1.3	7.4	0.63	22–24 (16)*
	19.2	0.189 ± 0.016	64 ± 4	5.1	0.52	
	30	0.53 ± 0.02	325 ± 12	4.5	0.45	
	37.5	0.78 ± 0.08	500 ± 16	2.7	0.33	
Palmitate	22.5	0.015 ± 0.003	13.9 ± 1.0	N.D.	0.95	27–29 (18)*
	27.5	0.224 ± 0.026	59 ± 4	9.9	0.71	
	32.5	0.44 ± 0.02	173 ± 14	6.8	0.55	
	37.5	0.54 ± 0.02	303 ± 14	6.7	0.54	

*Values in parentheses are the results of de Kruyff *et al.* (*8*).

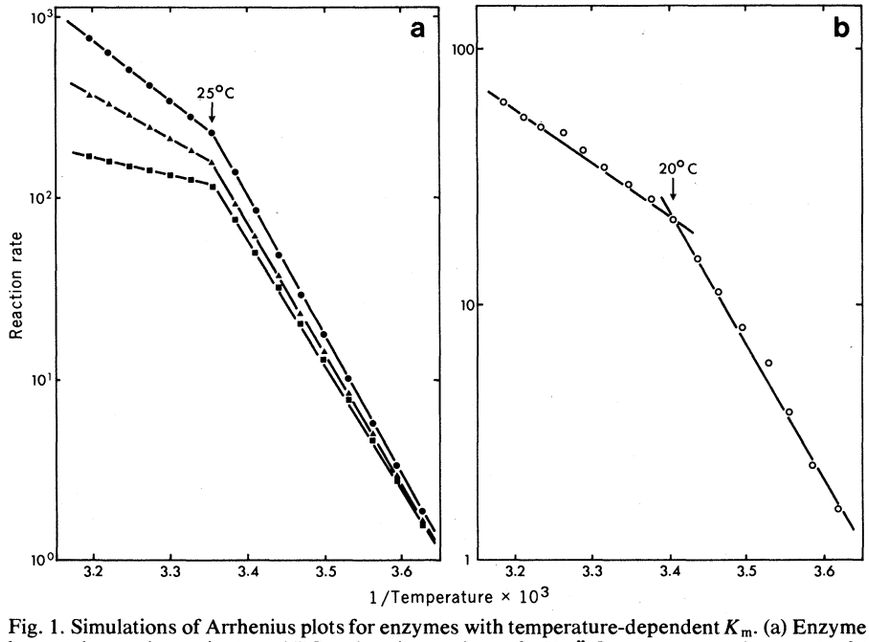

Fig. 1. Simulations of Arrhenius plots for enzymes with temperature-dependent K_m. (a) Enzyme has an abrupt change in E_a at 25°C and various values of ΔH_B^{eff}. Parameters used were: E_a, 35 kcal mole^{-1} ($T < 25$°C); E_a, 15 kcal mole^{-1} ($T > 25$°C); K_m, 1 mM at 5°C; substrate concentration, 20 mM; and ΔH_B^{eff}, 0 (●), -10 (▲), or -15 kcal mole^{-1} (■). (b) Simulation of an Arrhenius plot for an enzyme with constant E_a assayed at a constant substrate concentration. Parameters used were: E_a, 30 kcal mole^{-1}; ΔH_B^{eff}, -20 kcal mole^{-1}; K_m, 1 mM at 5°C; and substrate concentration, 20 mM; the standard error of the assay is ± 5 percent.

ΔH_B^{eff}, the values derived for E_a, particularly above the break, were sharply reduced from the true values. In the particular case shown, the E_a value that would be determined above the break is only 5.3 kcal mole^{-1} when ΔH_B^{eff} is -15 kcal mole^{-1}, while the true E_a value in this temperature region is 15 kcal mole^{-1}. Another frequently observed result was that the break was shifted to appreciably lower temperatures than the true break (in E_a for V_{max}) when somewhat lower substrate concentrations (two to ten times the K_m at 5°C) were employed.

In a second set of simulations, we assumed E_a to be constant and ΔH_B^{eff} to be finite and negative over the temperature range of interest. In such cases, a wide range of combinations of E_a, ΔH_B^{eff}, and substrate concentration yield strongly curved Arrhenius plots; when there is any significant amount of experimental scatter of the data points, such curves can convincingly mimic biphasic, linear Arrhenius plots. One example of such behavior is shown in Fig. 1b, where values for E_a and ΔH_B^{eff} comparable to those determined for the adenosinetriphosphatase in oleate-enriched membranes were employed.

These simulations indicate that a variety of errors, most notably artifactual breaks, can arise in Arrhenius plots when temperature-dependent increases of K_m are not accounted for; similar results are found if K_m decreases with increasing temperature, except that E_a values tend to be over- rather than underestimated in this case. To avoid such errors, all of the relevant kinetic parameters of the membrane-bound enzyme under investigation should be determined for at least three temperatures which encompass the entire temperature range to be studied. If it proves impossible to derive a single set of optimal conditions for all temperatures, V_{max} should be determined by means of Eadie-Hofstee or Lineweaver-Burk plots at all temperatures studied, with careful attention to other parameters such as ion affinity constants or pH variations with temperature. While a few reports of a temperature dependence of K_m or of a substantial enthalpy of substrate binding to membrane enzymes have appeared (*9*), the magnitude and significance of this effect do not seem to be generally appreciated in the existing literature, where the temperature dependence of enzyme kinetic parameters other than V_{max} is frequently neglected when Arrhenius plots for membrane enzymes are interpreted.

The results reported here clearly demonstrate that serious errors in the interpretation of Arrhenius plots can arise if temperature variations in substrate-binding affinity are not taken into account when determining the effect of temperature on the rate of the catalyzed reaction. As the relationship of membrane fluidity to the activity of membrane enzymes is at present an area of considerable interest and controversy, such errors in interpretation may seriously hamper subsequent attempts to develop realistic models of the nature of enzyme-membrane interactions.

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Sulfhydryl Groups and the Monodeiodination of Thyroxine to Triiodothyronine

Abstract. *Sulfhydryl reagents exert a profound influence on the monodeiodination of thyroxine to triiodothyronine by rat and sheep tissues in vitro. A marked dithiothreitol-induced increase in the monodeiodination by fetal sheep liver homogenates suggests that the characteristically low conversion in fetal tissues is related more to the status of sulfhydryl groups than to a deficiency of the monodeiodinating enzyme.*

3,5,3′-Triiodothyronine (T_3) is characteristically present at a much lower concentration in the circulation of the fetus than in the adult. Immediately after birth, however, there is a marked increase in serum T_3 (*1*). This increase in the availability of T_3, a very potent thyromimetic agent, may play a vital role in maintaining the body temperature of the newborn when the warm intrauterine environment is no longer available. The lower serum T_3 concentration in the fetus than in the adult is due to a lower rate of conversion of thyroxine (T_4) to T_3 in extrathyroidal tissues (*2*). Recent studies indicate that this process of outer-ring monodeiodination of T_4 to T_3 is enzymatic in nature (*3*). The extrathyroidal tissues of the fetus, such as liver, have also been shown to convert T_4 to T_3 in vitro to a much lesser extent than the tissues of the adult or the newborn; kinetic studies of the conversion with liver tissue indicate significant differences in the maximum rate of the reaction, V_{max}, as defined by the Michaelis equation, and not in the Michaelis constant, K_m (*4*). However, it is not known whether reduced conversion of T_4 to T_3 in the fetus is related primarily to a deficiency in the amount of the putative iodothyronine monodeiodinase or to inactivation of the existing enzyme—for example, through a conformational change in the enzyme which may not permit appropriate activity. The study reported here demonstrates that sulfhydryl groups of tissues are essential to their ability to monodeiodinate T_4 to T_3. The data also suggest that T_4 outer-ring monodeiodinase is plentiful in the tissues of the fetus but is

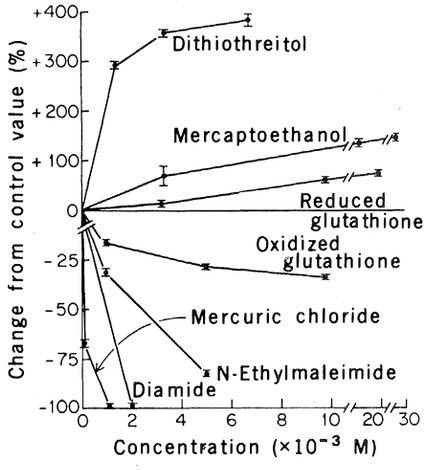

Fig. 1. Effect of various sulfhydryl reagents on monodeiodination of T_4 to T_3 by rat liver homogenate. The T_3 produced in the absence of a sulfhydryl agent (control) was arbitrarily assigned a value of 100, and the product in the presence of a sulfhydryl reagent was expressed as a percentage of the control value; each result is the mean ± standard error (S.E.) for triplicate or quadruplicate determinations. *N*-Ethylmaleimide and mercuric chloride were obtained from Baker Chemical Company. All other reagents were obtained from Sigma Chemical Company.

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