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TETRAHEDRON PERSPECTIVE NUMBER 4

COINCIDENCES, DECARBOXYLATION, AND ELECTROSTATIC EFFECTS

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Abstract—Electrostatic effects promote the efficiency of acetoacetate decarboxylase and of mandelate racemase by reducing the p*K*s of their essential lysine residues by about 5 p*K* units.

The opportunity to present a *Perspective* for Tetrahedron allows me to review some of my past. One of my proudest recollections at Harvard is of the colloquium at which I spoke in 1966: at that time, Ed Dennis, who is now a Professor at UCSD, and I had just discovered the pseudorotation[1] at phosphorus that accompanies the hydrolysis of cyclic phosphate esters. That was, however, many years ago. In general, the question properly asked of any chemist is, "What have you done for us lately?", and I haven't done any significant research lately. I remember with dismay a lecture that Frank Whitmore gave, perhaps 50 years ago, at the University of Chicago. Whitmore was a pioneer in demonstrating the role of carbonium ions in molecular rearrangements. The faculty and the graduate students at Chicago looked forward to his lecture, but it turned out to be a sad occasion; he had not kept up with the field he had helped to create, and we all knew more about carbonium ion rearrangements than he did. If I attempted to write here about pseudorotation or photoaffinity labelling[2] or molecular mechanics[3] or hydrogen transfer,[4] I would risk imitating Whitmore.

Therefore, I decided to write about coincidences in research, or more precisely coincidences in my own research, in the hope that this aspect of the past will provide a new perspective on old work. Some of my research and that of my coworkers at Harvard involved the mechanism of the enzymatic decarboxylation of acetoacetic acid, and the mechanism for that process turned out to depend strongly on electrostatic effects. My early research at the University of Chicago concerned the physical–organic chemistry of electrostatic effects, and this subject provides an introduction to this *Perspective*.

After my Ph.D. at Harvard and a postdoctoral at Columbia, I was fortunate enough to secure, in 1936, an appointment at the University of Chicago as an independent Research Associate, one step down in rank from an Instructor, which was one step down from an Assistant Professor, which is the lowest rank that exists today. With the characteristic modesty of the young, I planned to solve the entire problem of the mechanism of enzyme action at a single stroke. While carrying out experiments directed toward this somewhat flawed project, I was acutely aware of my deficiencies in physics, and therefore audited the sophomore course in Electricity and Magnetism, and did all the problem sets and took all the exams along with the physics concentrators.

During that year, and perhaps in part because of that course, I became interested in the quantitative aspects of electrostatic effects in organic chemistry. A paper on the electrostatic effect of negative charge on the strengths of polyacids, had been published by Niels Bjerrum,[5] and a paper on the electrostatic effects of dipoles such as the carbon–chlorine bond on the strengths of substituted organic acids had been published by Arnold Eucken,[6] but their papers were

simultaneously simple, elegant, and contradictory. That contradiction created both a problem and an opening for research.

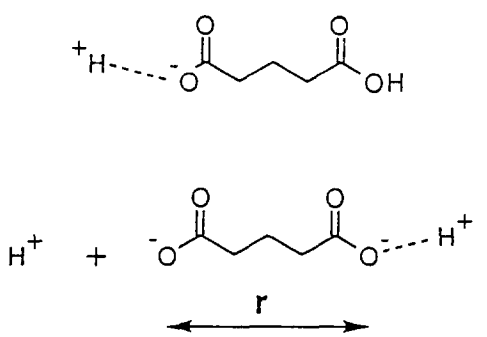
COINCIDENCE NUMBER 1

After taking the course in Electricity and Magnetism, I could see the problem clearly, but was incapable of doing anything about it. A great physical chemist, John Kirkwood, had published some complicated mathematical papers on electrostatics as applied to the activity coefficients of dipolar compounds, such as aminoacids, but they baffled me.[7] Coincidence came to the rescue. I was too low on the totem pole to hear anything about negotiations for new staff, but the Chemistry Department at the University of Chicago was at that same time negotiating with Kirkwood to come there. He had been an Assistant Professor at Cornell, he came to Chicago in 1937 as an Associate Professor, and returned to Cornell the next year as a full Professor. But his one year at Chicago was a window of opportunity for me.

I took my problem to Kirkwood. He saw that we could modify his equations—the ones that baffled me—to overcome my difficulties. He assigned me Byerly's "Elementary Treatise on Fourier's Series and Spherical Harmonics", and I took a month off to teach myself some mathematics which, shamefully, I have now mostly forgotten. Then Kirkwood and I—with strong emphasis on Kirkwood—developed a theory to quantify electrostatic effects in organic chemistry, a theory with no arbitrary parameters that, although still crude, reconciled Bjerrum and Eucken, and presented a unified set of equations for the effects of both charges and dipoles. The theory has stood the test of time for more than half a century.[8]

There was no way in which I could have participated in this research without the course in Electricity and Magnetism; there is no way in which I would have attempted the project if Kirkwood hadn't happened to come, for that crucial year, to the University of Chicago. This is coincidence number 1.

The theory explains the magnitude of electrostatic effects. In the ionization of an acid, a proton is removed against the electrostatic force created by the incipient negative charge that is then left behind on the anion. When a second proton is removed from a symmetrical polybasic acid, the second proton must be removed not only against a similar force, but also against the additional force created by the negative charge left over from the first ionization. Thus the ionization of the second



The ionization of glutaric acid.

proton that is removed, for example, in the ionization in water of the monoanion of glutaric acid or of $H_2PO_4^-$, takes place in the presence of a residual negative charge. Because of the extra electrostatic work required to remove a proton from this anionic residue, the second ionization constant of phosphoric acid (for example) is less than the first; for phosphoric acid, it is in fact less

by a factor of about 10^5. The example of phosphoric acid provides a measure of the possible magnitude of electrostatic effects.

Bjerrum calculated the work of removing the second proton from a symmetrical dibasic acid in the electrostatic field of the negative charge left over from the first ionization as

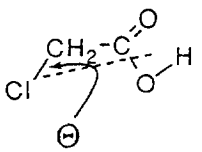
$\Delta\Delta F = Ne^2/D\ r = RT\ Ln(K_1/4K_2)$,

where N is Avagadro's number, e the electronic charge, r the distance between the protons of the dibasic acid, and D the dielectric constant of water; a statistical factor of four must also be included, but is relatively unimportant.

Eucken published the corresponding electrostatic formula for the effect of a dipole on the ionization constant of a substituted acid, that is

$\Delta\Delta F = Ne\ \mu\ \cos\Theta/D\ r^2 = RT\ Ln\ (K_{substituted}/\ K_{unsubstituted})$,

where μ is the dipole moment of the substituent, Θ is the angle the moment makes with the line joining its center to the ionizing proton, and D is 1.00 , that is the dielectric constant of empty space.

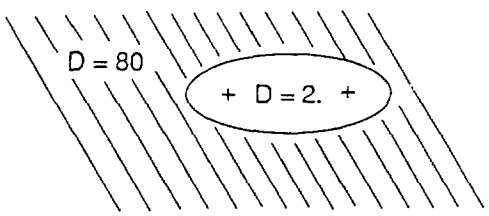


Chloroacetic acid.

Neither value of the dielectric constant, that for water (Bjerrum) nor that for empty space (Eucken), worked very well, but certainly we couldn't have both. In particular, Bjerrum's formula was wildly wrong for small molecules like phosphoric acid; it grossly underestimated the electrostatic effects.

KIRKWOOD MODEL

Kirkwood and I introduced a model in which the charges or dipoles were contained in a sphere or prolate ellipsoid of low dielectric constant, representing the molecule, surrounded by water of dielectric constant 80. We then worked out the electrostatics by classical physics.



Kirkwood–Westheimer model.

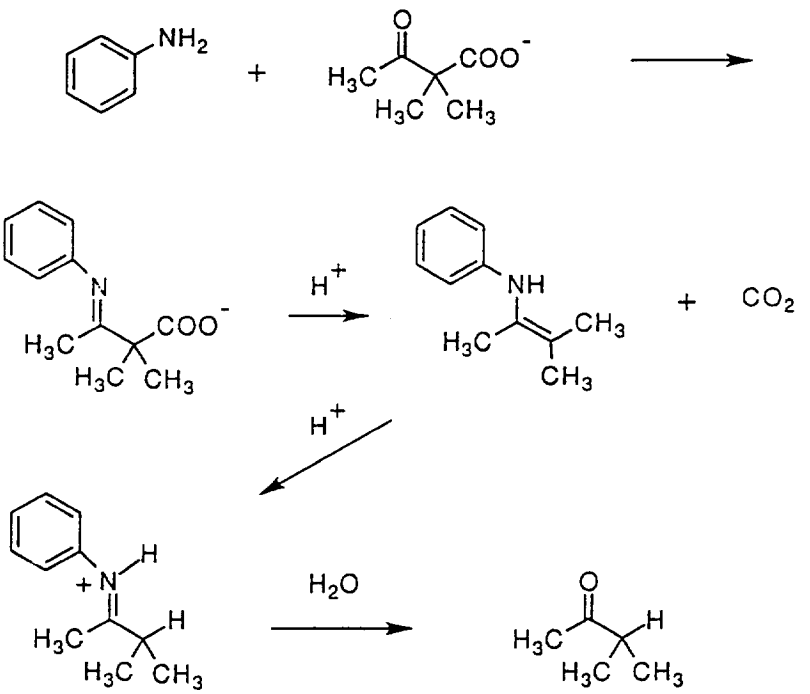
Our model is obviously simplified, but less crude than placing the charges directly in water as Bjerrum did, or in empty space as Eucken did, and it works remarkably well, considering how crude it is. At least it has the advantage of consistency for both charges and dipoles. The equations have the same form as those of Bjerrum and of Eucken, but with an effective dielectric constant, D_E, substituted for the dielectric constant in those equations; all the mathematical complexity relates to the calculation of the effective dielectric constant.

Much more recently, other investigators have sought simpler equations than ours with which to calculate the effective dielectric constant for charges immersed in proteins. They have come up with

semi-empirical formulas that are useful and quite satisfactory for this important application.[9] Large electrostatic effects dominate the chemistry of the enzyme, acetoacetate decarboxylase, discussed later in this paper.

DECARBOXYLATION

My interest in decarboxylation had been stimulated by some research at the University of Chicago[10] with oxaloacetate, and that interest carried over to research at Harvard, where my coworkers and I determined the mechanism of the enzymic decarboxylation of acetoacetic acid.[11] A mechanism for the decarboxylation of dimethylacetoacetic acid, with catalysis by aniline, had been postulated by K. J. Pedersen.[12]

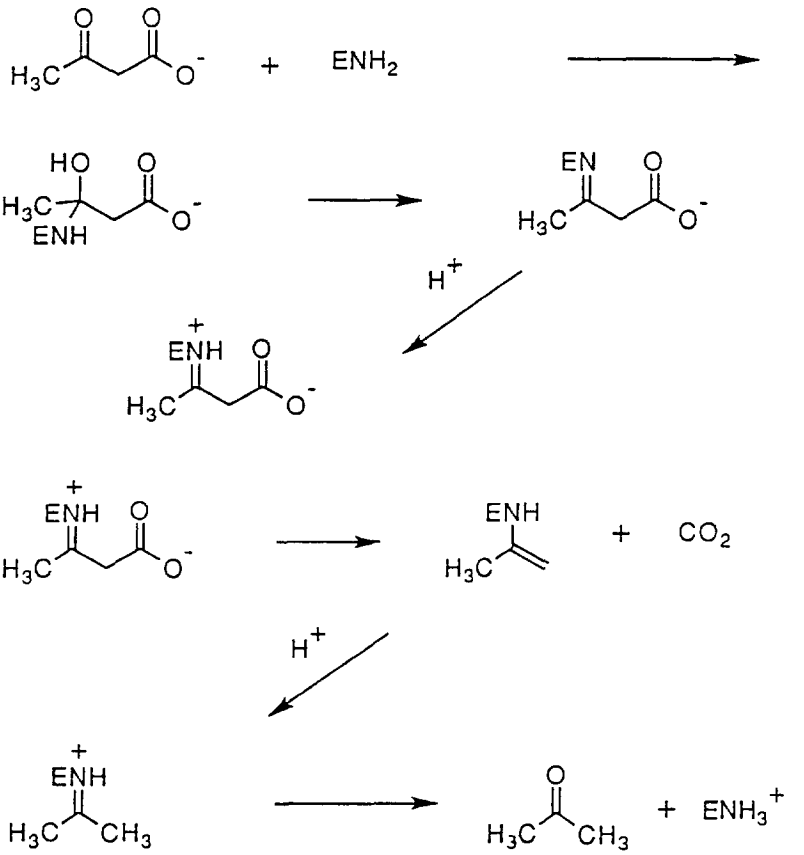


K. J. Pederson, 1938

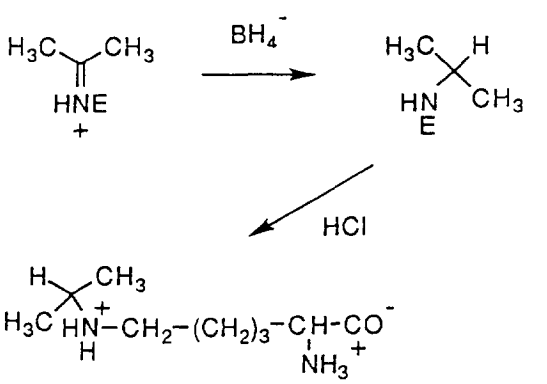
The enzymatic reaction is a simple one, and the enzyme was once industrially important in fermentation to make acetone. We showed that the enzymatic decarboxylation takes place by way of an enamine, according to the equations shown opposite.

TRAPPING AN INTERMEDIATE

The definitive experiments that demonstrated this mechanism were conducted by Irwin Fridovich, a Professor of Biochemistry at Duke, while he was on sabbatical at Harvard. Fridovich carried out the enzymatic decarboxylation in the presence of sodium borohydride.[13] Borohydride alone does not affect the activity of the enzyme, but in the presence of substrate it inactivates it.



With 3-^{14}C-acetoacetate, borohydride incorporates one atom of radiocarbon for each active site in the enzyme. It traps, and thereby demonstrates the reality, of the postulated enamine, the enamine of acetone, shown in the equations below. The modified reduced protein was then hydrolyzed to allow the identification of the product as ε-isopropyl lysine.



The mechanism parallels that which Kai Pedersen had postulated, decades earlier, for the catalysis by aniline of the non-enzymic decarboxylation of dimethylacetoacetic acid. Using radioactive acetoacetate and reduction, Richard Laursen,[14] now a Professor at Boston University, isolated the peptide that contains the essential lysine, and showed that, in the neighborhood of the active site, the enzyme has the sequence (in the one letter code where K stands for lysine), as follows:

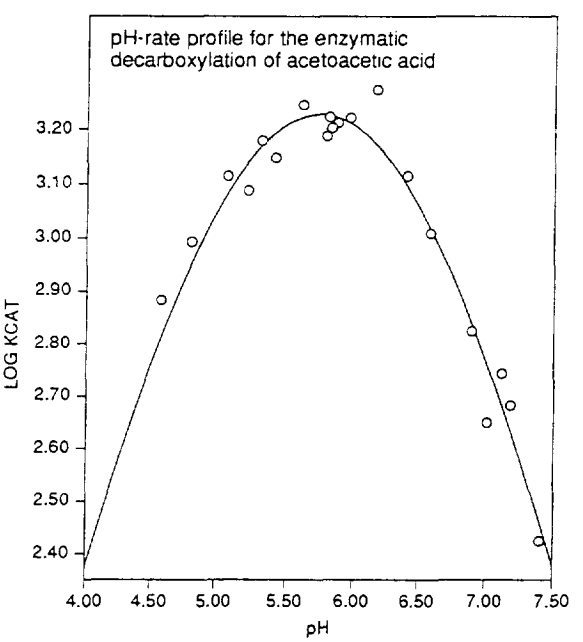
....ELSAYP*K**KLGYPKLFVDSDT....

The starred lysine, number 115 in the sequence,[15] is the active one; this is the lysine that is isopropylated in the borohydride reduction.

THE p*K* OF LYSINE

But this formulation presents a problem. The p*K* of the protonated ε-amino group of free lysine is 10.5. The enzyme is active around pH 6, where the ε-amino group of a lysine residue should be almost completely protonated. And of course a free amino group, and not an ammonium salt group, is required as a nucleophile for the formation of an enamine. Granted, at pH 6, three parts in 10^5 of lysine is present as the free amine, but it's hard to believe that an enzyme, that according to Jeremy Knowles' principle[16] should be perfect, would operate under such a disadvantage.

Of course, it does not. The p*K* of the essential lysine is shifted, in acetoacetate decarboxylase, from 10.5 to 5.6. Two points require elucidation. First, just what is the experimental evidence that the p*K* has been shifted in this manner? Second, granted that the p*K* has been so shifted, what has caused the shift? But first, the evidence of the shift. This has to come first; nothing is so embarrassing in science as a beautiful explanation for something that is not so. In this case, however, the extraordinary fact is real.



For many years, biochemists thought that they could determine the pK of groups at the active sites of enzymes by determining the pH-rate profile of their kinetics; the mid-points in the profiles presumably indicated the pKs of the essential aminoacids.

That method, although usually valid,[17] is not without pitfalls. Specifically, if the enzyme carries out a multistep process (as in the decarboxylation of acetoacetic acid), and if the various intermediates are not all at the same level of protonation (as is the case in our decarboxylation) then an apparent ionization constant determined from the kinetics of the overall process can be multiplied by a factor of unknown magnitude, a factor that derives from the quotient of various protonation equilibria and rate constants. This trap was pointed out for the general case by T. C. Bruice,[18] and was illustrated in our lab by detailed equations related to the enzymatic decarboxylation of acetoacetic acid.[19]

$$V_{max} = \frac{V_1}{\frac{K_1}{(H^+)} + 1 + \frac{(H^+)}{K_2}} \qquad \text{where}$$

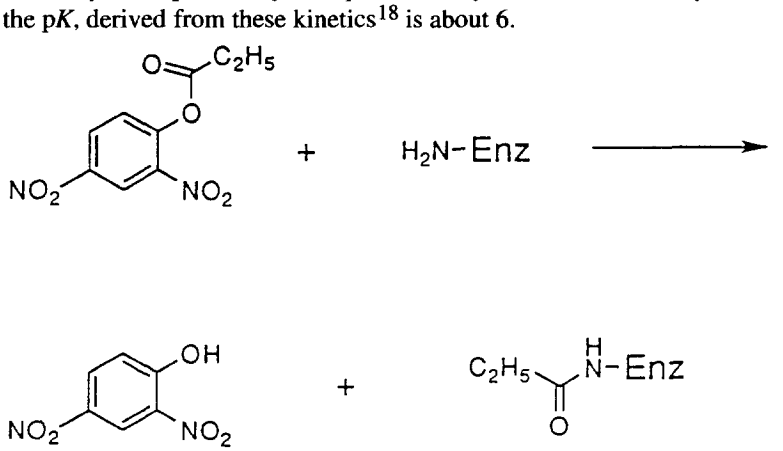
$$K_1 = \frac{K_{ES} + k_2 / k_3}{1 + k_2 / k_3 + k_2 / k_2 K_{ES'}} \qquad K_2 = \frac{1 + k_3 / k_4 + k_2 / k_3 K_{ES'}}{k_2 / k_4 K_{ES'}}$$

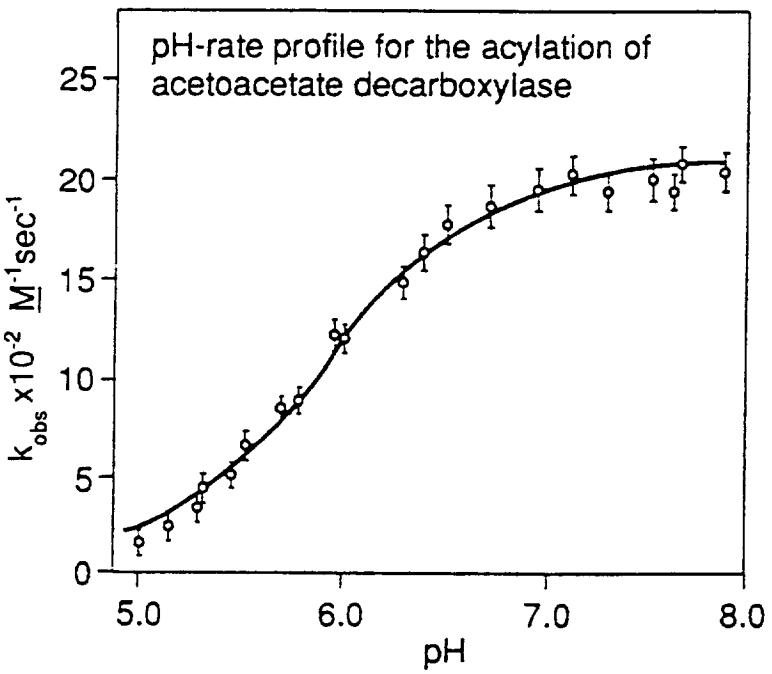
Obviously, the constants, K_1 and K_2, extracted from the pH-rate profile, are not related in any simple fashion to the desired pKs of the amino acids of the active site.

DETERMINATION OF THE pK

But it proved possible, by two independent methods, to determine the pK of the essential lysine in acetoacetic acid decarboxylase. These methods included the reporter group technique invented by D. E. Koshland,[20] and a kinetic method based on a single-step reaction that avoids the fatal pitfall of multistep processes. The latter method involves the pH-rate profile of the acylation of the enzyme by a dinitrophenyl ester.[21] The enzyme is inactivated at the same rate as dinitrophenol is released, with no intermediates in the process.

The enzyme is acylated specifically and quantitatively on the active-site lysine, number 115, in the sequence; the pK, derived from these kinetics[18] is about 6.



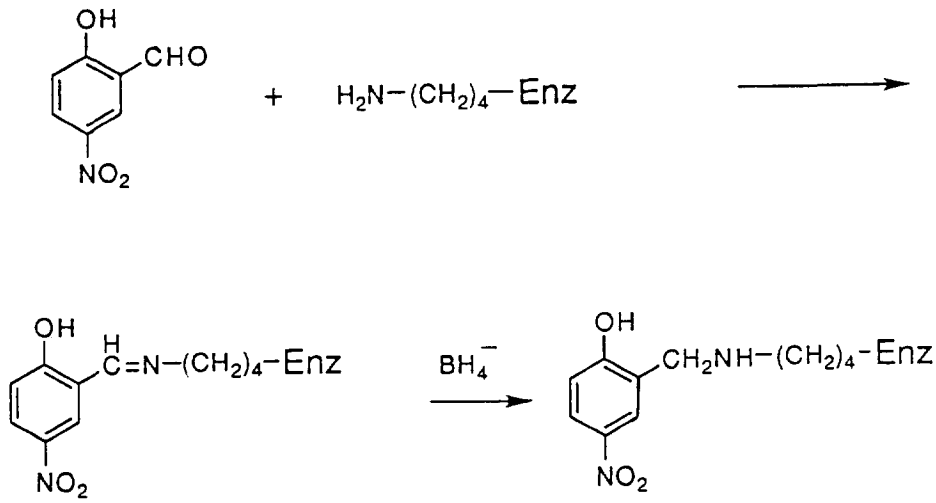


THE REPORTER GROUP METHOD

The reporter group method involved forming a Schiff base between the active lysine and nitrosalicylaldehyde, followed by borohydride reduction. This locks the nitrophenol chromophore at the active site; provided that no major conformational change has occurred, the chromophore is then in the same environment as the original lysine 115. Then one can determine the p*K* of the phenol spectrophotometrically; it 'reports' on the environment at the active site. Furthermore, fortunately, the spectrum of the phenol is slightly altered by the ionization of the amine, and spectrophotometry is so sensitive that the small shift in the absorption of the nitrophenol allows the determination of the p*K* of the amine, too.[22]

The phenolic hydroxyl should be in the general neighborhood of the original amino group of the lysine; the amine should be almost exactly there. Both methods, kinetic and thermodynamic, showed that the lysine actually has a p*K* of 5.6 (just as one would fortuitously have concluded from its pH-rate profile). The ionization constant had been shifted by a factor of nearly 100,000!

Knowles' principle of enzymatic perfection would suggest that such a shift should have occurred, in fact must have occurred. As early as 1971, Kokesh[21] had discussed the evolutionary advantage of a low p*K* for the essential lysine in acetoacetate decarboxylase, i.e. evolution would select for mutants of *Clostridium acetobutylicum* that somehow had made a protein in which the p*K* of the active lysine had been lowered so as to present acetoacetate with a free amino group. And what had caused this enormous shift? It is most probably electrostatic in origin. It would be only decent, only a reasonable coincidence, if that would be the way it would work out in my lab.



Chemistry of forming reporter group.

p*K*s FROM THE REPORTER GROUP

p*K* OF 2-HYDROXY-5-NITRO-*N*-METHYL-BENZYLAMINE		5.9
p*K* OF PHENOLIC RESIDUE OF REPORTER GROUP		2.4
	DIFFERENCE	3.5
p*K* OF LYSINE		10.5
p*K* OF AMINO RESIDUE OF REPORTER GROUP[23]		6.0
	DIFFERENCE	4.5

ELECTROSTATICS AGAIN

The active lysine is adjacent, in the aminoacid sequence, to a second lysine. Granted, if the active site was part of a helical structure or a β-sheet, the amino groups on adjacent lysine residues would be far apart. But if the lysines are part of a region of random coil, as is typical of the active sites of enzymes, the active amino group could be close in space to the ammonium salt group of the second lysine residue. If an ammonium salt group is really close to the essential amine residue of the enzyme, it would sharply lower the p*K* of that lysine, since a positive charge would repel a proton that approached the second amino group. And if the ammonium salt group is held close to the second amino group, the effect could be enormous. It could be comparable in magnitude,

although of course opposite in sign, to the electrostatic effect on the second ionization constant of phosphoric acid.

We postulated that this was indeed the case. Regrettably, neither we nor anyone else has X-ray evidence that such is (or is not) truly so. Gordon Hamilton,[11] who is now Professor of Chemistry at Penn State, had crystallized the enzyme right at the beginning of our investigations, but the crystals are extremely thin, and unsuitable for X-ray analysis. However, John Gerlt, now a Professor of Chemistry at the University of Illinois who received his Ph.D. with me in 1974, offers other evidence for the validity of this assignment.

Gerlt, in unpublished work when he was at the University of Maryland, has used site directed mutagenesis to substitute other amino acids for lysine 116, the amino acid adjacent to the essential lysine.[24] The mutant enzymes prepared by these substitutions show rate constants less by a factor of about 50 than that of the wild type; in these mutants, the p*K* of lysine 115 is so high that Gerlt and his coworkers have so far been unable to measure it because the enzyme is denatured in strongly alkaline solutions.

A mutant where cysteine replaces lysine 116, *K116C*, is one of the proteins with lowered enzymatic activity; when it is treated with bromoethylamine, the sulfhydryl group of the cysteine presumably displaces the bromine of the bromoethylamine and so extends the cysteine side-chain by two carbon atoms and an amino group. The reaction should put an amino group back in almost the same position as that of lysine 116 in the native enzyme. The enzymic activity of this modified mutant returns to half that of the wild type, and the p*K* of lysine 115 is lowered by the new amino group. These data show that lysine 116 perturbs the p*K* of lysine 115, as postulated, presumably by an electrostatic effect.

The diminution of the rate by a factor of only 50 despite the large change in p*K* demands explanation. Of course, one would not have expected the rate to fall by a factor of 10^5, the factor for the change in ionization constant. A stronger base will be a better nucleophile so the change in rate on lowering the p*K* of the base will be less than the change in ionization constant. The relationship between rate and basicity is presumably controlled by a Bronsted factor. If this factor for the reaction is 0.33, the rate would have been diminished by a factor of $100{,}000^{0.33}$, or about 50. Perhaps a Bronsted factor of 0.33 and a rate factor 50 are not unreasonable. In any event, the data show that lysine 116 controls the p*K* of lysine 115.

COINCIDENCE NUMBER 2

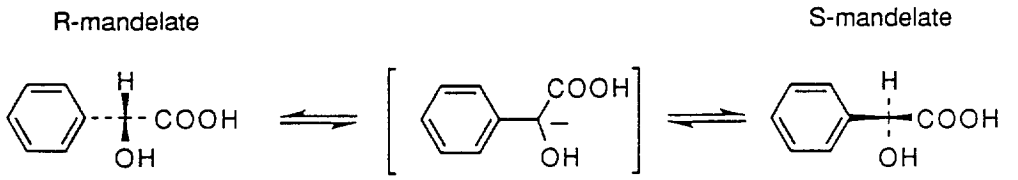
That the mechanism of action of our enzyme should depend critically on the magnitude of an electrostatic effect, so that the work I had done many years earlier on an entirely different and apparently unrelated subject should be essential to our later investigation is, I submit, coincidence number 2.

COINCIDENCE NUMBER 3

So much for the older work from my lab, and Gerlt's addition to it. The next coincidence depends on some of the research of two of my former collaborators: John Gerlt and George Kenyon, who is now a Professor and Dean at the University of California Medical School at San Francisco. They have carried out brilliant research on the mechanism of action of mandelate racemase, and by coincidence, this research turns out to be relevant to my story.

MANDELATE RACEMASE

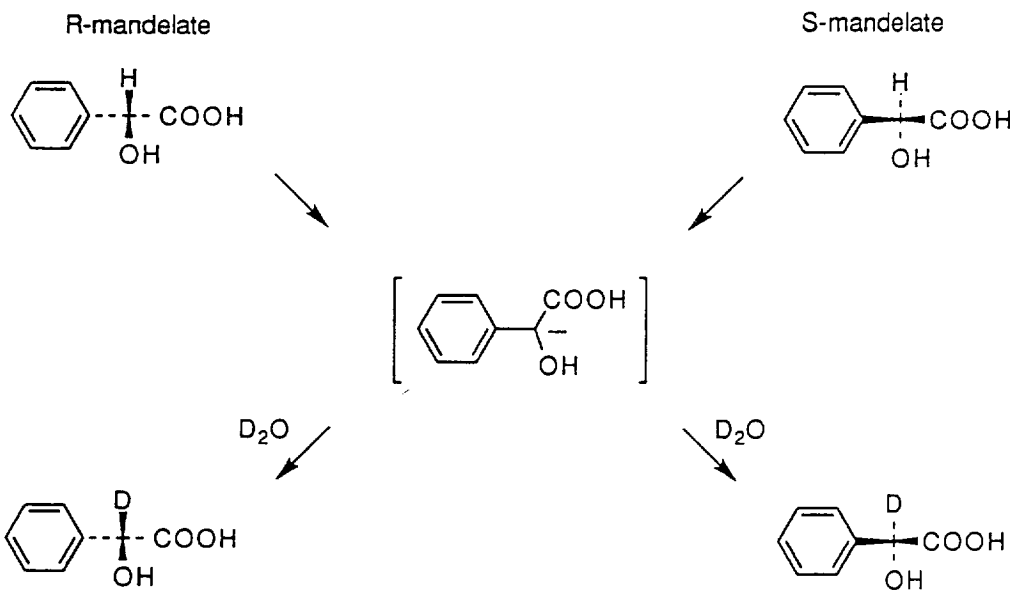
Mandelate racemase catalyzes the conversion of either *R*- or *S*-mandelic acid to the racemic mixture.[25]



Mandelate racemase.

In research on this enzyme, Kenyon, Gerlt and their coworkers[26] established that a histidine residue and a lysine residue are essential for enzyme activity, and they showed that the racemization proceeds by way of an anion, i.e. the anion, shown above, that is produced when the proton on the asymmetric carbon is ionized.

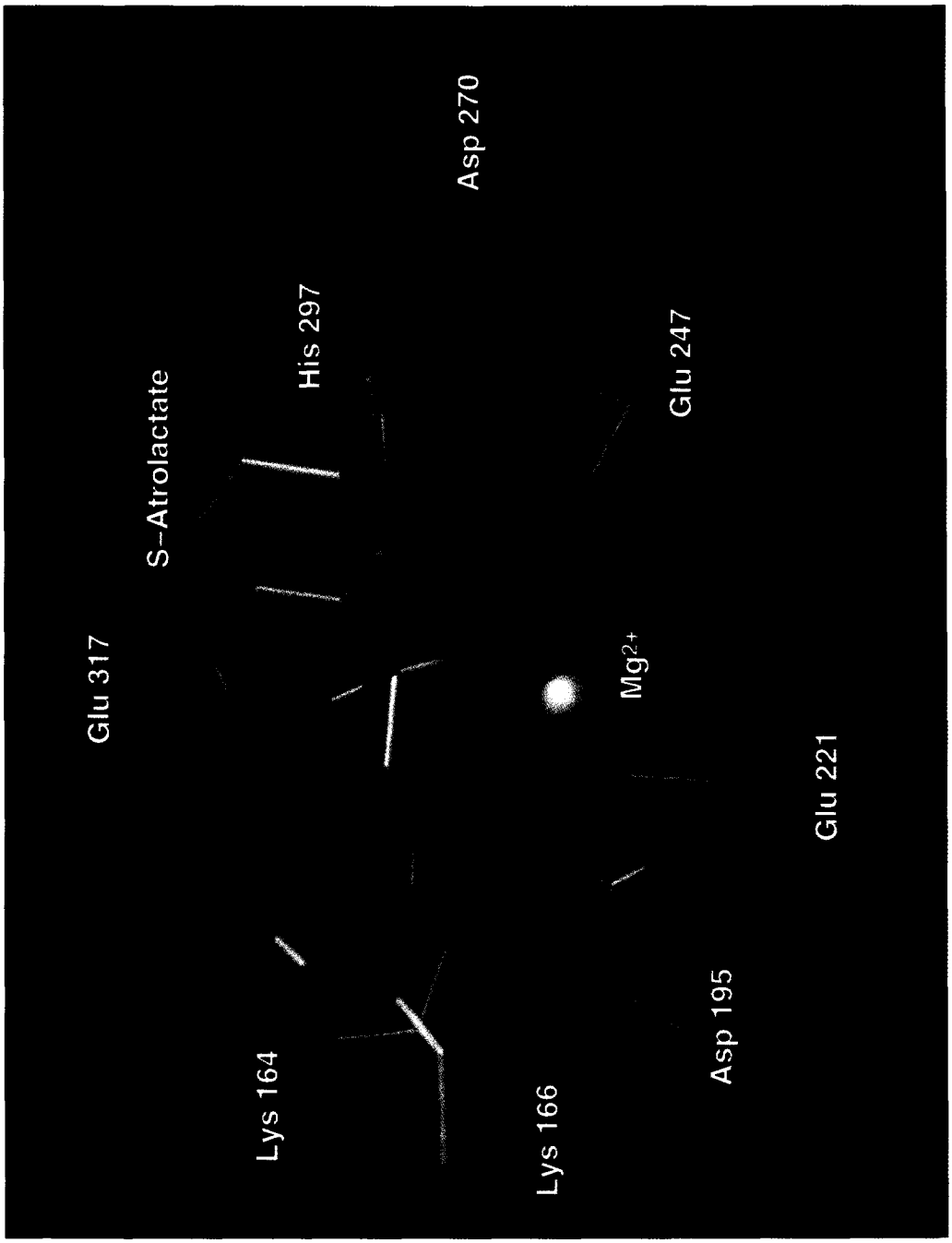
In support of a mechanism that requires an anionic intermediate, the enzyme catalyses the exchange of deuterium for hydrogen when it acts on either *R*- or *S*-mandelic acid in D_2O as solvent.



Mandelate racemase. Hydrogen deuterium exchange.

The enzyme has been crystallized, and Gregory Petsko, at Brandeis, collaborated with Kenyon and Gerlt to determine the X-ray structure of the enzyme.[27] The critical portion of the active site is shown overleaf.

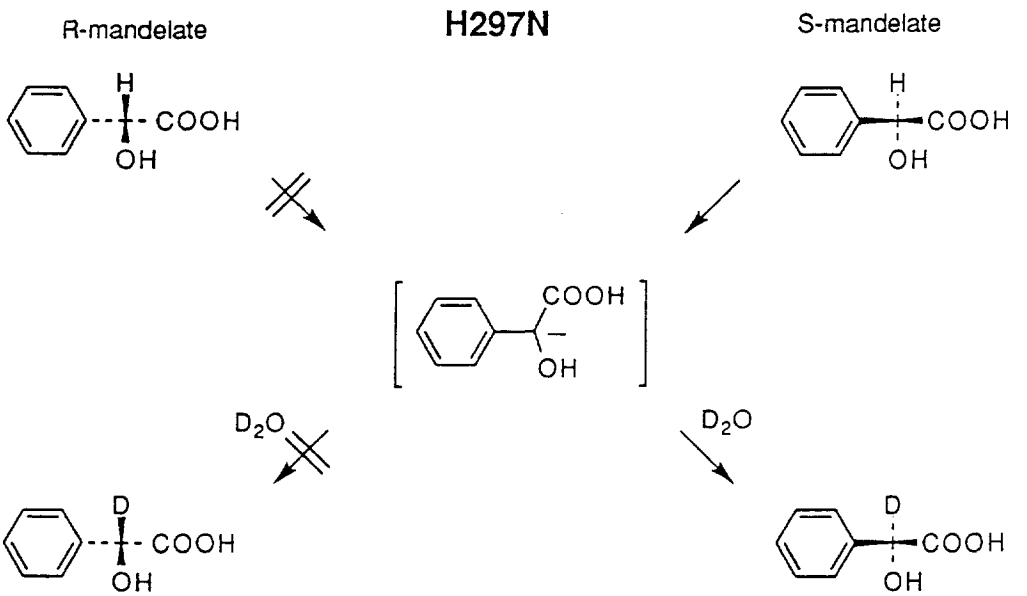
Lysine 166 lies on one side of the substrate and histidine 297 on the other. In the racemization, the essential lysine residue removes a proton from *S*-mandelate, and a histidinium ion pushes a proton back to form *R*-mandelate, whereas the essential histidine removes a proton from *R*-mandelate, and a lysinium ion puts one back on the other side. [28]



X-Ray structure

SITE-DIRECTED MUTAGENESIS AGAIN

This mechanism, strongly suggested by much chemistry and by the X-ray picture, has been verified by the application of site-directed mutagenesis. Gerlt and his coworkers used that technique to remove the essential histidine from mandelate racemase, and replace it by an asparagine; in other words, they prepared *H297N*, where H is the single letter code for histidine, and N is the single letter code for asparagine.[29] X-Ray analysis shows that the mutant protein has essentially the same conformation as the native one but the new protein, *H297N*, is totally inactive as a mandelate racemase. That is as it should be, since in the Kenyon–Gerlt mechanism, histidine is essential for racemization. On the other hand, in D_2O as solvent, the enzyme should, and does, exchange the essential hydrogen for deuterium in the *S*- but not in the *R*-enantiomer; the rate of exchange is comparable to that of racemization by the native enzyme. The essential lysine is in place in the mutant protein, and ionizes the substrate. In D_2O, lysine exchanges its ionizable protons for deuterons, and puts one of these back on the asymmetric carbon on the same side as that from which lysine base had removed a proton. Hydrogen–deuterium exchange then occurs without racemization. This experiment strongly supports the mechanism offered for the enzymic racemization of mandelate.



At the start of my scientific lifetime, no one had the foggiest notion how any enzymic reaction takes place. In relatively few years—one lifetime—the scientific community can now claim that the mechanism of an enzymic reaction—that of mandelate racemase—is as well or better understood than that of any non-enzymic reaction in solution. That is a startling and gratifying statement.

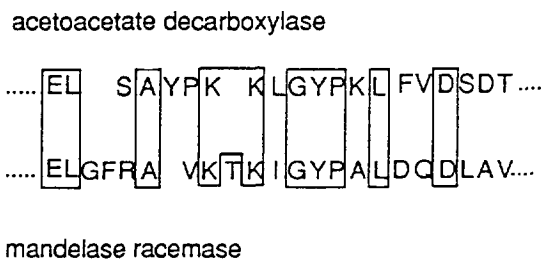
THE p*K* PROBLEM AGAIN

But of course, readers will undoubtedly already have homed in on a major difficulty with the Kenyon–Gerlt mechanism. At pH 7, where mandelate racemase is active, the ε-amino group of lysine should be almost completely protonated. The mechanism requires free basic lysine to remove

the critical proton for the racemization, and at pH 7, there should be almost none of it. Unless, of course, the p*K* of that lysine, lysine 166, has been shifted, presumably, by an electrostatic effect to a much lower value than normal. Kenyon, Gerlt, and their collaborators have determined the p*K* of that lysine and it is indeed around 6. The lysine does exist as the free base in neutral solution, as it should for the mechanism as outlined. Admittedly Kenyon and Gerlt determined the p*K* of the lysine by kinetics, and the method is not necessarily correct, but their reaction does not involve multiple steps, and so their determination is probably valid. They used the same method to determine the p*K* of the active site base in the mutant enzyme, *H297N*, where no histidine is present, and again found a p*K* of 6. This p*K* is almost certainly that of lysine 166. Furthermore, the X-ray structure of the enzyme shows an ammonium salt group of a second lysine—lysine 164—close to the working end of the first,[26] just as it should be to produce a large electrostatic effect.

The type of reaction catalyzed by mandelate racemase is totally unrelated to that catalyzed by acetoacetate decarboxylase. In one reaction, lysine functions as a base, in the other as an amine to react with a carbonyl group and form an enamine. For entirely different reasons, both enzymes need a low p*K* of the essential lysine, and both show that p*K*.

The sites around the critical lysines of the two enzymes turn out to be quite similar, with 50% homology, in sequence. These sequences, in one-letter code, are shown below.



Active sites for acetoacetate decarboxylase and mandelate racemase.

In mandelate racemase, a second lysine is again close in sequence to the active one; it is separated by one aminoacid residue. But here one has even better evidence for an electrostatic effect; the X-ray picture of the enzyme shows that the business ends of the two lysines, 164 and 166, are close together. Here one can be reasonably sure that the low p*K* of the lysine residue is primarily electrostatic in origin.

Two entirely different enzymes, operating by entirely different mechanisms, both require a large shift in p*K* for a lysine residue. The similarity in sequence cannot arise because of similarity in mechanism, but perhaps this sequence is one that will generally be found in all of those enzymes where a critical part of the mechanism is the lowering of the p*K* of a lysine residue.[30] One example was discovered in my lab, and of all the people working on enzyme mechanisms, it had to be two of my former students who found a second example of this huge shift in the p*K* of a lysine residue. This is coincidence number 3.

COINCIDENCES

What about coincidences? Of course there are bound to be some in the world—but the number of coincidences is statistically controlled. Aren't there too many in this story? I believe that there are. But if these are not coincidences, what are they? Well, let me begin by destroying the major coincidence. The shift of p*K*s by electrostatic effects is common.[8] A large change will occur with

lysine, because it has a pK far from neutrality, and the shift in pK discussed here is as large as or perhaps larger than any other of which I am aware.

In particular, the pK of the Schiff base between a lysine and retinal is widely different in a model compound and in bacteriorhodopsin,[31] and the proton pump in bacteriorhodopsin probably depends on the shift in this pK on absorption of light.[32] One may speculate on the possibility that the same type of electrostatic effects that operate in acetoacetate decarboxylase and mandelate racemase are operative here.

The shift of pKs of various aminoacids occurs in many enzymes. Here are a few examples. The shift of the pK of an essential histidine in thiomethyl papain by about 4 pK units has been determined by NMR by Shafer and his coworkers;[33] the pK of the essential cysteine is shifted by a like amount. A pK shift of 1 log unit for histidine in subtilisin has been measured and ascribed to electrostatic effects by Fersht and his collaborators,[34] and small shifts in the pKs of histidines in ribonuclease were reported as early as 1969 by Jardetsky and his coworkers.[35] Kirsch has ascribed a shift in the pK of an aldimine in aspartate transaminase to a hydrogen bond to tyrosine.[36] Karplus has correctly calculated the changes in pKs of histidine in azurin[37] from the electrostatic effect on oxidizing the copper in the protein from Cu^{+} to Cu^{2+}; he ascribed an internal dielectric constant of 4 to the protein, and then modeled his electrostatic calculation on the methods pioneered by Kirkwood.[6]

As evolution drives enzymes to perfection, according to Knowles' principle,[16] one of the easiest adjustments is that of pKs. Proteins are filled with charged groups, and not much in the way of mutation is required to push a charge into the required position to affect the pK of an adjacent group. The literature shows that the probability of finding a modified pK is great, and the probability of finding a strongly modified pK for a lysine may depend primarily on the probability of finding that an uncharged lysine is needed in the active site. Perhaps it was a coincidence that two former collaborators happened upon an enzyme that required such a lysine. But probably every—or almost every—uncharged lysine in any active site has a highly shifted pK, and the probability that some of my former students would come upon an example primarily depended on the fact that they are active investigators of enzyme mechanisms.

Well, what about the coincidence that I learned a little electrostatics, and then it turned out that electrostatics was just exactly what was needed? That isn't much of a coincidence, either. Anything whatsoever that one learns in science will turn out to be useful, and quite often just what is needed. In scientific research, we need everything. No one can possibly know more than a small fraction of the immense body of science. Anything and everything that anyone learns in science will almost always have an application to his or her own research sooner or later, and usually sooner. It is not even close to a coincidence that electricity and magnetism could be applied in physical–organic chemistry.

There is one real coincidence in my story, one piece of genuine good luck. It was a coincidence that Kirkwood showed up at the University of Chicago just exactly when I needed him. But if he hadn't, it is less likely but still possible that I would have found him at Cornell, or found someone in the physics department at Chicago who would have helped me. And if not, and if I hadn't worked on that problem in electrostatic effects, sometime in my career that course in electrostatics would have been just what I needed for some other problem. One simply can't know too much science. Electrostatic effects are prevalent in physical–organic chemistry, and therefore in enzymology.

A SYNTHETIC DECARBOXYLASE

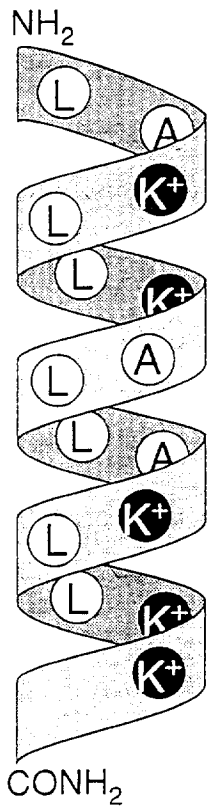
In a sense, that should be the end of this paper; it is the end of my discussion of coincidences. But it is not the end of the discussion of electrostatic effects, or decarboxylation, or of enamines in enzymology. Perhaps the only truly 'synthetic enzyme' so far prepared is a 14-unit polypeptide,

designed and synthesized by Steven Benner and his coworkers,[38] a polypeptide that catalyzes the decarboxylation of a β-ketoacid. Its active site consists of a lysine amino group, and the pK of that amino group is displaced by spacially adjacent lysine residues. The pK is displaced by only 1.5 pK units, rather than almost 5 units, as in acetoacetate decarboxylase and mandelate racemase, but then nature has made more mutants than Benner has—so far. The polypeptide catalyzes the decarboxylation of oxaloacetate by an enamine mechanism.

The 'synthetic enzyme', if I may call it that, required the preparation of a sequence that folds into an amphiphilic helix, where one side of the helix is hydrophilic and one is hydrophobic; the hydrophilic side is loaded with lysine residues. Benner's 'enzyme' has the sequence:

$$RNH\text{–}L\text{–}A\text{–}L\text{–}L\text{–}K\text{–}A\text{–}L\text{–}L\text{–}A\text{–}K\text{–}L\text{–}L\text{–}K\text{–}K\text{–}CONH_2$$

where the R-group is acetyl, and he has shown by circular dichroism and two dimensional NMR that it is partially helical; as a helix, the peptide would look like this:



Benner's helix.

The polypeptide is a catalyst for the decarboxylation of oxaloacetate. The substrate binds to the polypeptide, and the reaction shows Michaelis–Menten kinetics, with K_M of around 20 mM. The binding must be electrostatic, since acids such as acetoacetic, with only one negative charge are not inhibitors, and a polypeptide where three of the lysine residues have been replaced by neutral amino acids does not bind oxaloacetate.

The rate of decarboxylation is increased by a factor of about 10^4 over that of the reaction catalyzed by a simple amine. The lowest pK of any of the lysines is 8.9. Benner's 'enzyme' increases

the rate of the reaction for which it was designed by about the same factor as do the earliest catalytic antibodies prepared by Richard Lerner and Peter Schultz.[39] They have developed that methodology beautifully, and although their antibodies have greater catalytic activity and range than Benner's catalyst, the two advances are complementary, not competetive. Benner designed his 'enzyme', whereas Lerner and Schultz found a way to induce nature to produce theirs. We know how Benner's works; we are just now finding that out for catalytic antibodies. Nevertheless, Benner's 'enzyme' is a long way from ideal. Put aside the obvious fact that his 'enzyme' increases the rate of his decarboxylation by a factor of less than 10^4 as compared to catalysis by simple amines, whereas any self-respecting enzyme increases the rate of the reaction that it catalyzes by a factor of 10^9 or more. Benner's reaction is too easy, with a high spontaneous rate. In addition, the decarboxylation of oxaloacetate can be catalyzed by polyvalent cations,[10] without proteins.

Nevertheless, Benner has synthesized a polypeptide that was designed to fold into a helix, that was designed to present a pK shift in its catalytic amino group, was designed to catalyze a particular chemical reaction by a specific mechanism, and it does all of these things—not especially well, but it does them. The rational design of catalytic peptides has been one of the major objectives of physical–organic chemistry; it is essentially the problem I set myself—and spectacularly failed to solve—in 1936. In a rather preliminary way, Benner has managed it. I am confident that in the next few years, he and others will achieve this major objective of enzymology: the rational synthesis of excellent catalysts. As they do so, once in a while they may benefit from a friendly coincidence.

Acknowledgment—Finally, and obviously, I have leaned heavily on the work, published and unpublished, of my former collaborators for the material of this manuscript. It goes almost without saying, but it is worth saying anyway, that they and I learned chemistry together, and that I am immensely grateful to them for providing me with the chemistry that I have presented here.

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