Mechanism of the Chain Extension Step in the Biosynthesis of Fatty Acids†

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ABSTRACT: The chain extension step in the enzymatic synthesis of fatty acids by fatty acid synthase, involving a formal Claisen condensation of thio esters, has been clarified by theoretical calculations for model systems, using the modified neglect of diatomic overlap and Austin Model 1 parametric self-consistent field molecular orbital procedures. The reaction involves a free carbanion, formed by decarboxylation of a malonate ion. Formation of the carbanion and condensation with the fatty acid thio ester are not concerted. The decarboxylation is strongly endothermic. It is brought about by electrostatic interaction (field effect) with an ammonium ion derived from an adjacent lysine residue, the ions being far enough apart to inhibit proton transfer between them. Proton transfer would lead to an enol that is predicted not to be able to undergo the Claisen condensation. The formation of the ammonium ion is considered in terms of the pK_a of the relevant groups. The bearing of this work on a recent interpretation of the activity and selectivity of enzyme reactions is discussed, and some misunderstandings concerning this interpretation are clarified.

The high rates and specificities of enzyme reactions have puzzled enzyme chemists for many years. While the activity of many metalloenzymes may be attributable to the special catalytic properties of transition metals, no such simple explanation is available in the case of typical "organic" enzymes such as chymotrypsin or indeed in the case of metalloenzymes containing group II metals, e.g., zinc.

Previous interpretations of enzyme mechanisms had been based on the assumption that enzyme reactions are no different from other reactions in solution and can be interpreted in similar terms. Dewar and Storch (1985b) have recently suggested that this model may not represent the real situation in an enzyme reaction. The acceleration of a reaction by an enzyme may be due primarily to the exclusion of water from between the groups involved in the reaction, in the enzyme and substrate, when the substrate is adsorbed in the active site of the enzyme. The condensed form in which these ideas were presented has, however, led to misunderstandings that a subsequent account (Dewar, 1986) has apparently failed to dispel. The following summary should clarify the situation.

Theoretical studies (Dewar & Storch, 1985a) have shown that various anionic nucleophiles react with esters or amides in the gas phase to form tetrahedral adducts, exothermically and without activation. Since these conclusions have been confirmed by subsequent high-level ab initio calculations (Weiner et al., 1985; Madura & Jorgensen, 1986; Ewig & Van Wazer, 1986), there can be little doubt that they are correct. The barriers to nucleophilic substitution at carbonyl carbon in solution ($B_{AC}2$ reactions) must then be due *entirely* to some action by the solvent.

Consider a "normal" (intrinsic barrier; IB) reaction between an ion and a neutral molecule, i.e., one where the activation barrier corresponds to the energy needed to bring about changes in bonding involved in forming the transition state (TS). Once the reactants are in contact, any further changes in geometry involved in forming the TS are small. If the reaction is carried out in solution, the corresponding change in solvation energy should then also be small, corresponding to the difference in solvation energy between two species with similar sizes, shapes, and ionic charge, differing only in the way the ionic charge is distributed. Indeed, calculations (Dewar & Carrion, 1984) for a number of S_N2 halide-exchange reactions have indicated that the barriers in the gas phase and solution, starting from the contact pair in each case, are similar.

The same should clearly be true for the $B_{AC}2$ reactions discussed above. In solution, once the reactants have formed a contact pair, addition to form the tetrahedral adduct should take place without activation, as it does in the gas phase. The solvent should play only the same minor role that it does in a normal IB reaction. The barrier to such a $B_{AC}2$ reaction in solution must then occur during association of the reactants. The only source of such a barrier is the energy needed to partially desolvate the ion in order to make room for the other reactant to approach. The exact mechanism involved in generating the barrier is not relevant in the present connection. The essential point is that the barrier in such a desolvation barrier (DSB) reaction is due solely to the energy needed to remove solvent from between the reactants so that they can approach one another.

When a proper substrate is adsorbed in the active site of an enzyme, the groups involved in the subsequent enzyme reaction must unquestionably be in contact, without molecules of water intervening. If water were trapped between them, it would clearly prevent or inhibit the reaction. The reaction will then take place in the same way as an analogous reaction in solution, *starting with the reactants in contact*. As we have seen, such a reaction takes place in a manner analogous to a similar reaction in the gas phase. If the latter involves no activation, the same should be true for the enzyme reaction. Now the rate-determining step in the cleavage of peptides by serine-type proteases involves a $B_{AC}2$ reaction between an amide carbonyl in the peptide and an alkoxide ion, generated by an endothermic proton transfer from serine to an aspartate ion, within the enzyme. Since the $B_{AC}2$ reaction should take place exothermically and without activation, it should act as a driving force for the proton transfer, both reactions taking place in a single concerted step. The rate of the enzyme

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reaction can be explained quantitatively (Dewar & Storch, 1985b), the calculated overall heat of reaction being ca. 10 kcal/mol, while the observed rate corresponds to a free energy of activation of ca. 12 kcal/mol. The fact that the rate is faster by ca. 13 orders of magnitude than an analogous uncatalyzed reaction in solution can thus be explained without any need to invoke any special behavior on the part of the enzyme. The acceleration would indeed be very much greater were it not for the endothermicity of the proton transfer involved in generating the serinoxide ion. According to this interpretation, the enzyme acts not as a catalyst but as an antiinhibitor. It eliminates the inhibition of the reaction by water. If the rate is compared with that of a corresponding reaction in the absence of water, i.e., in the gas phase, there is no significant acceleration.

Apart from the calculations referred to above (Dewar & Storch, 1985b), strong support for this interpretation has been provided by detailed theoretical calculations for trypsin by Weiner et al. (1986) and by experimental studies of chymotrypsin models (Mallick et al., 1984). Particularly striking evidence was provided by the behavior of a model where the components of the catalytic triad and substrate were locked together in contact by attachment to a rigid support. This underwent a chymotrypsin-like hydrolysis almost as fast as the enzyme reaction itself, even though the reaction was carried out in water so that the reactants were completely surrounded by water. The acceleration must clearly have been due simply to the exclusion of water from between the groups involved in the reaction. There seems therefore little doubt that the interpretation of serine-type peptidases by Dewar and Storch (1985b) was correct.

The phrase they used ("exclusion of water from the active site") has, however, been misunderstood. This referred *only* to extraneous molecules of water that play no direct role in the reaction, either by taking part in it as reactants or by forming part of the active site. Inessential water needs to be, and is, excluded when a proper substrate is adsorbed. Failure to exclude such water will lead to a decrease in rate, due to solvent sticking to the reaction centers. This will happen in the case of a potential substrate that does not fit the active site closely. If the corresponding reaction takes place at all, it will take place much more slowly. The selectivity of serine peptidases for specific kinds of peptide can be explained in this way, again without any need for any special assumptions. Water is efficiently excluded from between the reaction centers only in the case of a proper substrate of the enzyme in question.

These remarks should also correct misunderstanding of the analogy drawn by Dewar and Storch (1985b) between enzyme reactions and reactions in the gas phase. In both cases the reactants can associate without hindrance. Both thus avoid the desolvation barriers encountered by ionic reactions in solution. Since the solvent has only a relatively minor effect on the rate of a reaction in which charge is conserved, once the reactants have associated, "solvation" of the reaction center in an enzyme reaction by the enzyme itself is not expected to have a large effect on the rate. In assessing possible mechanisms for an enzyme, it can then be assumed that the rate of a given possible step will be comparable with that of an analogous reaction in the gas phase.

Note that this assumption does *not* imply that the solvation energies are negligible, only that they do not vary to any large extent once the reactants have associated. Since an ionic reaction involves migration of charge from one atom to another, any stabilization of the charge in the reactants needs to be balanced by a corresponding stabilization in the transition state and products. It seems, however, likely that the corresponding changes in energy will, in most cases, be similar, a conclusion supported in the case of the serine proteases by the calculations and other evidence indicated above. This point needs to be stressed in view of a recent approximate valence bond treatment by Warshell and Russel (1986), which implied that the acceleration of peptide cleavage is due simply to more effective stabilization of the transition state by charged groups in the enzyme. As indicated above, reliable calculations indicate that there is *no* transition state to stabilize and that the rate of the reaction corresponds in any case to that expected for an analogous system in which there are *no* stabilizing interactions.

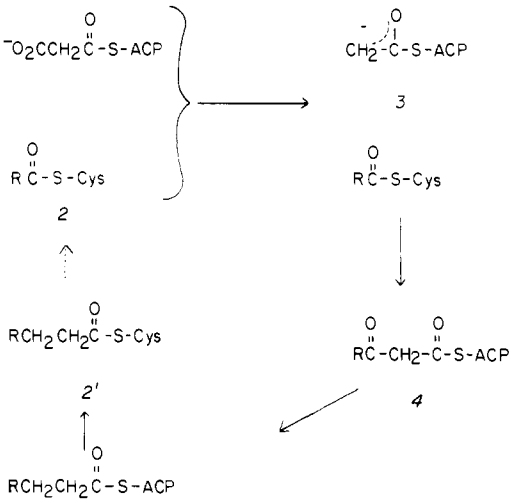
Note in this connection that electrostatic interactions are likely to be much stronger in the case of an enzyme reaction than in an analogous reaction in a polar solvent because of the lower dielectric constant. This, as Dewar and Storch (1985b) pointed out, is another respect in which enzyme reactions resemble reactions in the gas phase. The difference may become important if a specific electrostatic interaction plays a direct role in a reaction, the corresponding polar group acting as a specific reactant. As will appear presently, the enzyme reaction studied here seems to exemplify this situation.

Further misunderstanding has arisen from a seeming implication in the original paper (Dewar & Storch, 1985b) that a DSB reaction necessarily lacks an intrinsic barrier and that a corresponding enzyme reaction should therefore take place without activation. This is clearly not the case (Dewar, 1986). The only requirement for a reaction to be of DSB type is that desolvation be rate-determining. There is no reason why the reaction should not involve a second (intrinsic) barrier, provided it is lower than the one corresponding to desolvation. The intrinsic barrier will naturally survive in an enzyme reaction and will correspondingly limit its rate.

The evidence indicated above seems to leave little doubt that the activity of serine proteases can be explained in terms of elimination of water. Whether or not the same situation holds in the case of other enzymes remains to be seen. However, this interpretation should clearly be given precedence unless and until the need for additional assumptions has been established. The first question to ask concerning an enzyme reaction is not "why is it so much faster than an analogous reaction in aqueous solution?" but "why is the analogous reaction in aqueous solution so slow?". Indeed, one of the attractive features of the interpretation of enzymes as antiinhibitors rather than catalysts is the explanation it gives for one of the most puzzling features of biological systems, i.e., the coexistence in a cell of a very large number of diverse molecules, often potentially very reactive, without reactions taking place between them. They coexist without reacting because the reactions that might take place are inhibited by water. Enzymes operate by creating a microenvironment where the inhibiting water is absent.

Interpretations of the rates of organic reactions in solution are based on the rates of reaction of simple models and the effects of substituents on those rates. When the same procedure is applied to an individual step in an enzyme reaction, the model reaction should be a reaction in the gas phase, not in solution. Since experimental data concerning ionic reactions in the gas phase are scarce and hard to come by, this is an area where theoretical calculations could be very useful. Indeed, it is an area where theoretical calculations should be particularly effective, referring as they do to reactions of isolated reactants, i.e., to reactions in the gas phase. Theoretical treatments of reactions in solution are hampered by the dif-

Scheme I



ficulty of assessing the role of the solvent.

To be useful in this connection, a theoretical treatment must be sufficiently accurate. This restricts the choice to ab initio treatments or the parametric treatments [MINDO/3 (Bingham et al., 1975), modified neglect of diatomic overlap (MNDO) (Dewar & Thiel, 1977), Austin Model 1 (AM1) (Dewar et al., 1985)] that have been developed by our group. It must be possible to carry out calculations for realistic models of the reactions in question, which commonly involve quite large molecules. Ideally, one would like to study models incorporating all the relevant features of the active site. Here problems arise due to the amount of computing time needed. Tests (Dewar & Storch, 1985a) have shown that our treatments, in particular AM1, are comparable in accuracy with ab initio ones that require thousands of times more computing time. There is clearly no point in using the latter. Simpler ab initio procedures are less accurate than ours and still need far more computing time. While high-level ab initio methods are certainly better than ours, they are currently restricted to reactions of very small molecules.

Our procedures are thus the methods of choice for studies of reactions in cases where they are suitable. Extensive tests and applications to very many reactions[1] (Dewar et al., 1986; Dewar & Ruiz, 1987) have indicated their likely performance in a wide variety of situations. Applications of MINDO/3 and MNDO to biological systems were, however, restricted by their inadequate description of hydrogen bonds. The failing that led to this has been overcome in a new third generation treatment, AM1, which also seems to be generally superior to its predecessors. Calculations for two enzyme reactions (Dewar & Storch, 1985b; Dewar & Ruiz, 1987) have, moreover, given satisfactory results. Here we report an AM1 study of the basic reactions involved in the chain extension step in the biosynthesis of fatty acids.

Biosynthesis of Fatty Acids

The key step in the enzymatic synthesis of a fatty acid involves extension of an alkanoic acid by two carbon atoms by a formal Claisen condensation. Extensive studies[2] of the way this is brought about have led to the following conclusions (see Scheme I).

(a) The two-carbon unit is supplied in the form of a monothio ester (**1**) of monomalonate ion, the thiol function being derived from acyl carrier protein (ACP) in nonassociated synthases and ACP-type functions in associated ones.

(b) The growing carboxylic acid is also present as a thio ester (**2**) formed by the thiol of a cysteine unit in the active site of the enzyme.

(c) The carbanion (**3**) involved in the chain extension is formed by decarboxylation of **1**.

(d) No primary isotope effect is observed if **1** is replaced by the corresponding derivative of dideuteriomalonic acid.

(e) No tritium is incorporated when the reaction is carried out in tritiated water.

(f) Claisen condensation of **3** with **2** leads to an ACP β-keto ester (**4**). The new CC bond is formed stereospecifically, corresponding to attack trans to the leaving CO_2 group in **1**.

(g) Other parts of the enzyme, or enzyme complex, then reduce the β-carbonyl group in **4** to methylene. The ACP thio ester of the resulting saturated acid then transfers the acyl group to the cysteine thiol to form a new enzyme thio ester (**2**), ready for attachment of another acetic acid residue. We will not be concerned here with these subsequent steps.

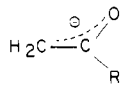
(h) The active site contains a lysine residue whose terminal amino group seems to be involved in some way in the reaction.

(i) The native enzyme, in the absence of acetyl-CoA, i.e., with the free thiol group of cysteine (cysteine thiol) (see **2**) decarboxylates **1** only very slowly. Treatment of the enzyme with iodoacetamide destroys its activity by carboxamidomethylating the thiol group of cysteine (**5**). While the inhibited enzyme cannot synthesize fatty acids, it decarboxylates **1** very rapidly, the activity being ca. 60% that involved in the synthesis of fatty acids by the native enzyme in the presence of both acetyl-CoA and malonyl-CoA (Kresze et al., 1977).

H_2NCOCH_2 S Cys

5

The failure to observe a primary isotope effect in the reaction of dideuteriomalonyl-CoA and the lack of tritium exchange when the reaction is carried out in tritiated water have been taken as evidence that **2** cannot be formed as a free anion during the reaction and that decarboxylation and condensation must therefore occur together in a single concerted step. The stereospecificity of CC bond formation[2] is also consistent with a concerted mechanism. These arguments are, however, flawed. Under the conditions of an enzyme reaction, water is expected to be absent from the active site. Tritium exchange would not therefore be expected, irrespective of the mechanism of the condensation, and similar comments apply to the lack of a deuterium isotope effect. Furthermore, if **3** were formed as a stable intermediate, it would be expected to retain its stereochemistry because internal rotation about the H_2C–C bond will be inhibited by a resonance interaction, **3** being a vinyloxy anion, **6**.



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An alternative possibility[2b] is that decarboxylation of **1** is brought about by proton transfer from the ammonium group of the lysine, leading to a vinyl alcohol, **7**. This could then undergo Claisen condensation with **2** with return of the proton to the lysine.

Our object was to distinguish between these various possibilities by studying the energetics of appropriate models.

[1] For an extensive bibliography of MNDO and MINDO/3 calculations, see Clark (1985).

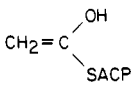
[2] For recent reviews, see (a) Bűlock (1979), (b) Wakil and Stoops (1983), (c) Simpson (1980), and (d) Wakil et al. (1983).

Procedure

The calculations were carried out by using the standard AM1 model (Dewar et al., 1985) as implemented in the AMPAC package of computer programs.[3] These are designed for easy use by chemists and contain a wide range of options for various types of calculations. Geometries were completely optimized in all cases, without making any assumptions apart from constraints needed to mimic the placement of groups in the enzyme reactions. Transition states were located by the reaction coordinate method (Dewar & Kirschner, 1971) or a new procedure developed here (Dewar et al., 1984) and refined by minimizing the scalar gradient of the energy (Komornicki & McIver, 1971, 1972). Stationary points were characterized by calculating force constants (Komornicki & McIver, 1971, 1972).

Results and Discussion

According to our suggestion (Dewar & Storch, 1985b) concerning the mechanisms of enzyme reactions, analogous processes in the gas phase must take place at least as fast as the enzyme reaction itself. Calculations for simple model systems should then serve to eliminate many possible candidates on the grounds that they are inherently too slow. In the work reported here we have studied simple models of the possible reactions involved in the chain extension step of fatty acid synthase, replacing **1** and **2** by the corresponding methyl esters (**8** and **9**) and ϵ-protonated lysine by methylammonium



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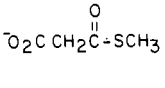

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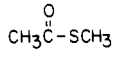

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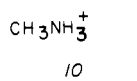

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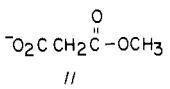

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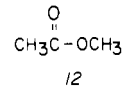

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ion (**10**). Calculations for **8** and **9** had to be carried out by using MNDO because AM1 parameters are not yet available for sulfur. In cases where the effects of hydrogen bonding needed to be assessed, AM1 calculations were carried out for the corresponding oxygen species (**11** and **12**). The relationship of their reactions to those of the sulfur analogues was established by parallel MNDO calculations.

Since all current theoretical procedures are essentially empirical (Dewar, 1985), the first step in any theoretical study should be to test the procedure being used by appropriate comparisons with experiment. Table I compares with experiment the heats of formation calculated by MNDO and AM1 for the various molecules used in our simulations. The agreement is within the usual limits of error expected for MNDO and AM1 [and also, incidentally, for the 6-31G* ab initio model (Dewar & Storch, 1985a)] except for carbon dioxide, which presents problems in all theoretical treatments, ab initio as well as ours (Dewar et al., 1985). Note the estimated errors for the carboxylate ions. These are based on the errors for the corresponding conjugate acids and on the average errors in deprotonation enthalpies[4] (DPE) calculated

Table I: Comparison with Experiment of MNDO and AM1 Calculated Heats of Formation (kcal/mol)

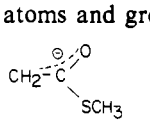
molecule	exptl	MNDO	error	AM1	error
CH_3COSCH_3	−47.8[a]	−51.0	−3.2		
$^-CH_2COSCH_3$		−54.6			
$CH_3CO_2CH_3$	−98.4[b]	−93.6[c]	4.8	−96.4[d]	2.0
$^-CH_2CO_2CH_3$	−94.6[e]	−90.4	4.2	−94.2[f]	0.4
$HO_2CCH_2COSCH_3$	−136.2[a]	−135.7	0.5		
$^-O_2CCH_2COSCH_3$		−154.5	(10.5)[k]		
$HO_2CCH_2CO_2CH_3$	−186.7[a]	−177.7	9.0	−182.8	3.9
$^-O_2CCH_2CO_2CH_3$		−192.3	(19.0)[k]	−207.4	(9.9)[k]
CH_3NH_2	−5.5[b]	−7.5[c]	−2.0	−7.4[d]	−1.9
$CH_3NH_3^+$	147.6[g]	161.8[h]	14.2	148.7[f]	1.1
HCO_2H	−90.5[b]	−92.7[c]	−2.2	−97.4[d]	−6.9
HCO_2^-	−112.5[e]	−101.7[i]	10.8	−110.0[f]	2.5
CO_2	−94.1[j]	−75.4[c]	18.7	−79.8[d]	14.3

[a] ΔH_f estimated by using Benson's group additivity method (Benson, 1976). The contribution for S(CO)(C) was estimated as S(CO)(H) [S(C)$_2$–S(C)(H)] + [S(C)(C$_d$)–S(C)$_2$]. [b] See Pedley and Rylance (1977). [c] See Dewar and Thiel (1977). [d] See Dewar et al. (1985). [e] ΔH_f calculated by using the experimental values of the DPE (Bartmess, 1987) and ΔH_f (Bartmess, 1987) of the protonated form and the experimental value for $\Delta H_f(H^+)$ (Stull & Prophet, 1971). [f] See Dewar and Dieter (1986). [g] ΔH_f calculated by using the experimental values for the proton affinity (Lias et al., 1984) and ΔH_f (Bartmess, 1987) of the deprotonated form and the experimental value for $\Delta H_f(H^+)$ (Stull & Prophet, 1971). [h] See Olivella et al. (1984). [i] See Dewar and Rzepa (1978). [j] See Wagman et al. (1982). [k] Estimated by using the known tendency of MNDO and AM1 to overestimate the DPEs of aliphatic carboxylic acids by 10 and 6 kcal/mol, respectively (Dewar & Dieter, 1986; Olivella et al., 1984).

(Dewar & Dieter, 1986; Olivella et al., 1984) for a number of other carboxylic acids for which experimental data are available. This emphasizes one of the advantages of our procedures, i.e., the fact that so many calculations have been carried out by using them that the likely error in any new application can usually be predicted with reasonable assurance.

(*A*) *Role of the Sulfur Atoms.* The use of thio esters (**1** and **2**) in place of normal esters in the enzyme reaction is interesting. It has been explained in terms of greater $+E$ (electron attracting conjugative) activity on the part of the (alkylthio)carbonyl (COSR) group compared with that of alkoxycarbonyl (COOR), due to the resonance interaction between sulfur and carbonyl in COSR being less than that between oxygen and carbonyl in COOR. Decarboxylation of **1** should thus be easier than decarboxylation of a corresponding malonate, and addition of the resulting anion (**3**) to the carbonyl group of **2** should likewise be easier than addition of **3** to the corresponding acetate. While this argument is certainly reasonable and while thio esters certainly seem to react faster with nucleophiles in solution, no gas-phase comparisons of acidities have been reported for analogous esters and thio esters. As the results in Table I show, the deprotonation energy of **9** to **13** is indeed predicted to be significantly less than that of **12** to **14**, the difference being 6.8 kcal/mol. Since the MNDO DPE for **12** agrees almost exactly with experiment [370.4 vs. 371.0 kcal/mol (Olivella et al., 1984)], this estimate is probably fairly reliable.

Table II compares the calculated formal charges at the relevant atoms and groups in **9**, **12**, **13**, and **14**. The last



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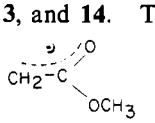

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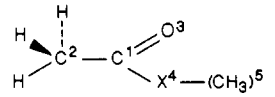
column shows the change in formal charge on passing from

[3] Available from the Quantum Chemistry Program Exchange (QCPE), Department of Chemistry, Indiana University.

[4] The deprotonation enthalpy (DPE) of molecule BH is defined as the heat of the reaction BH → B$^-$ + H$^+$.

Table II: Calculated Atomic Charges for Methyl Acetate, Methyl Thiolacetate, and Their Conjugate Bases Resulting from Loss of an α-H^+

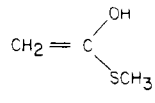


	AM1			MNDO		
	neutral	anion	change	neutral	anion	change
X = O						
C^1	0.30	0.34	0.04	0.35	0.38	0.03
C^2	−0.22	−0.64	−0.42	0.05	−0.50	−0.55
O^3	−0.35	−0.58	−0.23	−0.36	−0.61	−0.25
O^4	−0.28	−0.35	−0.07	−0.35	−0.39	−0.04
$(CH_3)^5$	0.21	0.07	−0.14	0.23	0.11	−0.12
X = S						
C^1				0.14	0.22	0.08
C^2				0.01	−0.46	−0.47
O^3				−0.29	−0.56	−0.27
S^4				0.03	−0.16	−0.19
$(CH_3)^5$				0.03	−0.07	−0.10

the acid to the anion. Note that the change in the case of the carbonyl oxygen is only a little greater for **9** than for **12**. The greater acidity of **9** is therefore *not* due to increased $+E$ activity on the part of carbonyl because this would lead to an increased diversion of negative charge from the methylene carbon atom to the carbonyl oxygen. While the increase in negative charge on the methylene carbon is less for **9** → **13** than for **12** → **14**, the extra charge in the case of **9** is diverted to *sulfur*, the increase in negative charge on sulfur in **9** → **13** being much greater than that on the corresponding oxygen atom in **12** → **14**. Therefore, the greater stability of **13**, relative to **14**, is due not to a smaller resonance interaction between sulfur and carbonyl but to the greater ability of divalent sulfur to accept negative charge. The extra stabilization involves transfer of charge from carbon to sulfur, not to the carbonyl oxygen.

(*B*) *Decarboxylation.* We next studied the decarboxylation of **8**. The reaction is predicted (Table I) to be endothermic by 24.5 kcal/mol and was found to involve an activation barrier, the transition state (TS) being higher in energy than **8** by 29.0 kcal/mol. The situation is of course complicated by the errors (Table I) in the MNDO heats of formation (ΔH_f) for CO_2 and carboxylate ions. Since these partially cancel in the present connection, the net effect should be to make the calculated heat of decarboxylation too positive by 8 kcal/mol. While the error in the corresponding TS is uncertain, it is likely to be similar. The activation energy for decarboxylation is therefore unlikely to be less than 20 kcal/mol, which is too high for a step in an enzyme reaction, even with a favorable frequency factor. Therefore, **1** is not expected to decarboxylate without assistance even when the carboxylate group is stripped of water. Such solvation will of course further inhibit decarboxylation in solution.

As noted above, it has been suggested that decarboxylation of **1** in the enzyme is promoted by transfer of a proton from the protonated lysine. We therefore studied the effect of adding a proton to the $COSCH_3$ carbonyl in **8**. Attempts to optimize the resulting structure led, however, directly to the enol (**15**) of **9**, the reaction being exothermic by no less than



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45.8 kcal/mol. Protonation of **8** not only makes decarboxylation exothermic but also removes the activation barrier.

As also noted above, one of the mechanisms suggested on the basis of isotopic studies postulates a one-step reaction involving concerted elimination of CO_2. To check this, we studied the corresponding reaction of **8** with **9**. No path of this kind could be found with an activation energy <50 kcal/mol. The easiest path was a two-step one, involving prior decarboxylation of **8**. These results seem to exclude concerted decarboxylation in the enzyme reaction.

Next we examined the effect of a neighboring methylammonium ion (**10**) on the decarboxylation of **11**, using AM1. The overall reaction, which included transfer of a proton from **10** to the carbonyl oxygen of **11**, was now exothermic and exergonic (ΔH, −20.9 kcal/mol; ΔG, −30.9 kcal/mol), and the calculated activation barrier (10.7 kcal/mol) was much less than in the absence of **10**. However, decarboxylation and proton transfer were *not* synchronous. Indeed, the reaction was not even concerted. The structure of the TS corresponded solely to elimination of CO_2, the C–CO_2 bond length being 2.06 Å and transfer of hydrogen not having even begun. The transfer of a proton from **10** occurred later in the reaction when the C–CO_2 distance was 3.9 Å. When the errors in the AM1 energies for carboxylate ions and carbon dioxide were taken into account, the reaction, prior to proton transfer, was thermoneutral. However, the entropy term made it exergonic. The assistance of decarboxylation by **10** is not therefore connected with proton transfer. It must be due entirely to the electrostatic effect of the positive charge on the ammonium ion.

As a check, we repeated the calculations for the decarboxylation of **11**, using AM1 but replacing **10** by a disembodied positive charge. AMPAC has an option for including an arbitrary number of such charges ("sparkles"). The sparkle was placed in the same position as the corresponding proton in the **11**–**10** system just prior to proton transfer, 1.85 Å from the carbonyl oxygen. The heat of activation for decarboxylation was reduced to 7.2 kcal/mol. The calculation was then repeated with the O-sparkle distance increased to 2.25 Å. The activation energy increased only to 12.0 kcal/mol. Since AM1 probably overestimates the activation energy for decarboxylation by the same amount (ca. 8 kcal/mol) and for the same reason (see above) as MNDO, it seems clear that the electrostatic effect of the protonated lysine would be sufficient in itself to bring about decarboxylation.

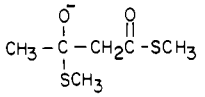
These calculations referred to decarboxylation of the normal ester (**11**). In order to estimate the effect of sulfur, analogous MNDO calculations were carried out for the decarboxylations of **8** and **11**, promoted by methylammonium ion. To prevent hydrogen migration, the distance between the ammonium hydrogen and the carbonyl oxygen was held at the distance optimized in the AM1 calculation for **11**. This should ensure cancellation of any errors due to the inadequate treatment of hydrogen bonds in comparing **8** with **11**. The activation barrier for **8** was found to be less than that for **11** by 3.6 kcal/mol. Assuming the difference to be the same for AM1 as for MNDO, the AM1 enthalpy of activation for decarboxylation of **8** in the presence of **10** is estimated to be 7.1 kcal/mol.

This result raises another important point. In the case of reactions in solution, the electrostatic effects of charged groups are greatly attenuated by solvation. No such attenuation should occur during an enzyme reaction. Charged groups in an enzyme may therefore have dramatic effects without themselves being directly involved in the reaction.

(*C*) *Condensation.* As noted above, our calculations seem to eliminate any possibility of the condensation of **1** with **2** taking place as a simple concerted process. It also seems clear

that the decarboxylation of **8** requires assistance by the protonated lysine moiety.

A possible mechanism for the enzyme reaction would then involve protonation/decarboxylation of **1** to the enol tautomer (**7**) of **2**, which might then condense with **2**. To test this, we studied the reaction of **15** with **9**. No addition took place. The only way reaction can occur is by removal of the proton from **15** to generate **13**. According to MNDO, **13** reacts exothermically (ΔH −2.6 kcal/mol) with **9** to form the adduct **16**, the corresponding activation energy being 15.3 kcal/mol.



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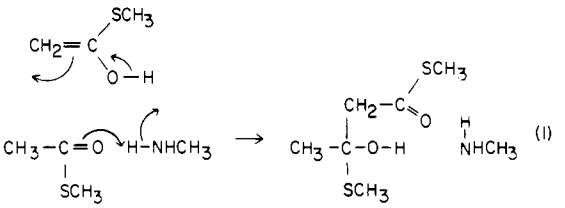
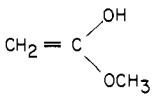
Since the deprotonation of **15** by **10** is strongly endothermic, **15**, once formed, cannot react in any reasonable way with **9**.

Protonation of **16** by **10** should be strongly exothermic. It is therefore possible that deprotonation of **15**, condensation with **9**, and protonation of the adduct (**16**) all take place in a single concerted step, methylamine acting in effect as a proton carrier; i.e.



(I)

However, we were unable to find a TS for such a process, although we searched the potential energy surface in some detail. We feel confident that no concerted path of this kind exists, the reaction taking place stepwise. In any case we feel certain that if such a path does exist, it involves a very high activation barrier. Since the intermediates in the proposed mechanism involve hydrogen bonds, we repeated the calculation for the corresponding sulfur-free system, **17**–**12**, using AM1. Again, we could locate no concerted path for the reaction.



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Only one alternative seems left, i.e., decarboxylation of **8** followed by condensation of the resulting anion (**13**) with **9**, *without* protonation of the intermediates. Our calculations indicate that the positive charge could bring about decarboxylation of **8** even at distances large enough to inhibit rapid proton transfer. A further possibility was that the decarboxylation, when assisted by a neighboring molecule of **10**, might be further assisted by the dipole field of an adjacent molecule of **9**, even though **9** had proved insufficient to promote decarboxylation on its own. This possibility was studied in the case of the analogous sulfur-free system to avoid complications from the overestimation of nonbonded repulsions in MNDO. As noted above, the AM1 barrier to decarboxylation of **11**, assisted by **10**, was 10.7 kcal/mol. When the calculation was repeated with a molecule of **12** present, oriented so that its dipole field would be expected to assist the reaction, the barrier in fact fell to 5.2 kcal/mol.

(*D*) *Role of Lysine.* Our last concern was with a minor point related to the manner in which the lysine moiety operates. It has been suggested[2] that the amino group of the lysine is initially neutral and that it becomes protonated during acylation of the enzyme to **2** by the proton liberated from the thiol group of the cysteine. Now the pK_a of the corresponding ammonium ion and cysteine in water are 10.8 and 8.1, respectively, implying that lysine should be present in the free enzyme as the ammonium ion, not the free base, under physiological conditions and that the cysteine thiol should likewise be present as such, not as the thiolate anion. However, the NH_3^+ and SH groups must be close together in the enzyme if they are to cooperate in the way suggested. The electrostatic interaction between them should then greatly increase the acidity of the SH group.[2b] Since its pK_a is initially 8.1, it should then ionize, lysine and cysteine being present as an ammonium salt. This result illustrates another major difference between gas-phase chemistry and solution chemistry, i.e., the fact that the relative strengths of acids and bases are commonly quite different in solution and in the gas phase. That is certainly the case here. Thus, while the process

$$CH_3NH_3^+ + CH_3S^- \rightarrow CH_3NH_2 + CH_3SH \qquad (2)$$

is weakly endothermic in the presence of water, it is exothermic in the gas phase by ca. 150 kcal/mol.[5] In the enzymatic acylation of the enzyme to **2**, adsorption of the substrate requires elimination of water. While the energy needed to desolvate ions in water is large, here it is supplied by the exothermicity of the reverse proton transfer of eq 2. Thus, when acylation is about to take place, the relevant amino and thiol groups are expected to be present as such, as has been postulated, contrary to what would be expected on the basis of analogies with solution chemistry.

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[5] The observed (Bartmess, 1987) DPE of CH_3SH is 359.0 kcal/mol, while the observed (Lias et al., 1984) proton affinity of CH_3NH_2 is 214.1 kcal/mol ($H^+ + CH_3NH_2 \rightarrow CH_3NH_3^+$).

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Binding of Glycogen, Oligosaccharides, and Glucose to Glycogen Debranching Enzyme†

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ABSTRACT: The binding of glucose and a series of oligosaccharides to glycogen debranching enzyme was determined by the ability of the saccharides to decrease the rate of reaction of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoate) (DTNB). At pH 7.2, the strength of binding increases with chain length from glucose to maltotriose to maltopentaose but not to maltohexaose, and the free energies for binding of the oligosaccharides suggest subsites of equivalent affinities for the four glucose units following the initial reducing moiety. The rate of reaction of DTNB with enzyme saturated with saccharide is the same for all compounds, suggesting that all the saccharides, including gluose, induce the same conformational state. The site of binding may be that which binds the α-1,6-linked side chain of the natural limit dextrin substrate. At pH 8.0, this site exhibits similar characteristics, but an additional site, which may bind the four terminal glucose units of the main chain of the natural substrate, is manifested and exhibits different characteristics, including a very low affinity for glucose itself. The binding of glycogen to the debranching enzyme was monitored by centrifugal separation from the protein and exhibits a much lower dissociation constant than that for the oligomers, suggesting that branched polymers have more than one set of subsites.

Amylo-1,6-glucosidase/4-α-glucanotransferase (glycogen debranching enzyme) (EC 3.2.1.33 + EC 2.4.1.25) is a monomeric enzyme of about 165-kilodalton (kDa)[1] molecular mass that encompasses both glucosidase and transferase activities on a single polypeptide chain (Brown & Brown, 1966; Nelson et al., 1979). Following extensive studies with reversible inhibitors and a catalytic site directed irreversible inhibitor, Nelson and colleagues (Nelson et al., 1979; Gillard & Nelson, 1977; Gillard et al., 1980) envisioned this enzyme as possessing a single, overlapping or strongly interacting polymer binding site(s) flanked on one side by a glucosidase site and on the other by the transferase site.

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[1] Abbreviations: α-GSD, α-glucosyl α-Schardinger dextrin; φ-LD, glycogen limit dextrin from the action of phosphorylase on glycogen; DTNB, 5,5′-dithiobis(2-nitrobenzoate); G, glucose; MT, maltotriose; MP, maltopentaose; MH, maltohexaose; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; Nojirimycin, 5-amino-5-deoxy-D-glucopyranose; kDa, kilodalton(s).

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