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THE ESSENTIAL THIOL GROUP OF PROPIONYL-CoA CARBOXYLASE

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SUMMARY

The function of the essential thiol group in propionyl-CoA carboxylase (propionyl-CoA:CO_2 ligase (ADP), EC 6.4.1.3) has been investigated kinetically with the use of *N*-ethylmaleimide as the thiol blocking reagent. The inhibition of the enzyme was first order with respect to time and inhibitor concentration. From the data obtained it was concluded that only one molecule of *N*-ethylmaleimide reacted with the enzyme. The rate of inhibition remained constant between pH 7.0 and 8.0 then increased very rapidly with increasing pH. The inflection point at pH 8.2 in the pK_m/pH plots indicated that the thiol group was involved in forming the enzyme–propionyl-CoA complex. This was confirmed by a kinetic analysis of the chemically modified enzyme which showed that the apparent K_m value for propionyl-CoA increased while the values for ATP and HCO_3^- remained constant. By measuring the rate constant of the inactivation process in the presence of varying propionyl-CoA concentrations, it was concluded that the inhibitor could not react with the enzyme–propionyl-CoA complex. The dissociation constant for the complex was 0.25 mM.

INTRODUCTION

Previous studies carried out with pig heart propionyl-CoA carboxylase (propionyl-CoA:CO_2 ligase (ADP), EC 6.4.1.3) have indicated that the enzyme is dependent on the presence of at least one free sulphydryl group for enzymic activity[1]. The evidence for this is that reduced glutathione is necessary to maintain enzymic activity and that sulphydryl reagents such as *N*-ethylmaleimide, iodoacetamide and *p*-hydroxymercuribenzoate inactivated the enzyme. Indications are that the cysteine residue may be at or near the active centre of the enzyme since HEGRE AND LANE[2] using liver propionyl-CoA carboxylase showed that propionyl-CoA and propionylpantetheine partially protected against the inactivation process. Other components of the reaction mixture did not protect against the sulphydryl group inhibitors; in fact, ATP and Mg^{2+} appeared to increase the rate of inactivation.

The sulphydryl groups of many enzymes are known to react with sulphydryl reagents yielding a catalytically inactive product. In most cases it is not clear whether

an essential group in the active site has been blocked or whether the reaction with a group or groups elsewhere in the protein has caused conformation changes that have led to an inactive enzyme. This report describes a series of studies showing that the cysteine residue is involved in the reaction sequences and is a binding amino acid for propionyl-CoA rather than a catalytic or structural residue.

METHODS AND MATERIALS

Propionyl-CoA carboxylase was purified from pig heart by a method modified from that described by TIETZ AND OCHOA[1]. Enzyme assays were performed using the $^{14}CO_2$ fixation method[1]. The reaction mixtures were incubated at 30° and enzymic activity terminated by the addition of 0.4 vol. of 10% trichloroacetic acid. After centrifuging, aliquots of the protein-free supernatants were dried on Whatman 3MM paper discs[3] and counted in a Packard Tri-Carb scintillation spectrometer. Each assay was counted in triplicate. All double reciprocal graphs and straight lines were determined by the method of least squares.

Barium [^{14}C]carbonate was obtained from the Radiochemical Centre, Amersham, Bucks, U.K., and $^{14}CO_2$ distilled from $HClO_4$ into an equivalent amount of NaOH. Reduced glutathione, ATP and CoA were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Other reagents were analytical grade. Propionyl-CoA was prepared by the method of SIMON AND SHEMIN[4]; the propionyl-CoA was treated chromatographically by the method of ZETTERSTROM AND LJUNGGREN[5], and the purified compound eluted with 1 μM EDTA (pH 7.6). The concentration of the solution was adjusted to the required molarity using the molar absorptivity at 259 mμ.

RESULTS

Order of inactivation with respect to time and N-ethylmaleimide concentration

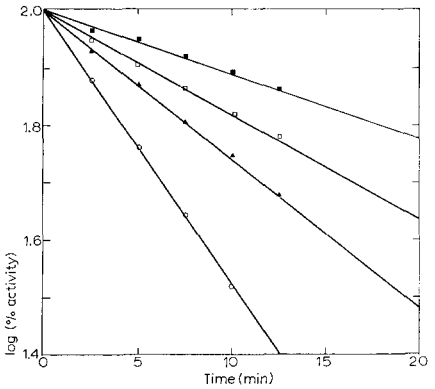
Fig. 1 shows the percentage of propionyl-CoA carboxylase activity plotted as a function of time on a semi-log scale for various *N*-ethylmaleimide concentrations. The linearity of the plot down to 20% of the initial enzymic activity indicates that the inactivation process approximates first-order kinetics with respect to time at any fixed concentration of this inhibitor. The same data replotted and presented in Fig. 2 shows that the inactivation process is first order with respect to the *N*-ethylmaleimide concentrations used in this investigation.

Since the plot of log of enzymic activity against time is linear during the loss of a major portion of the activity (Fig. 1) then the initial slope of such a plot gives an approximation of the first-order rate constant k. LEVY, LEBER AND RYAN[6] showed that the apparent first-order rate constant depends on the concentration of the inhibitor and may be expressed by the following equation,

$$k = k' \,[\text{inhibitor}]^n$$

where n is a number equal to the average order of the reaction with respect to the concentration of the inhibitor and k' is some function of inhibitor concentration. Taking logarithms of both sides of the equation leads to:

$$\log k = \log k' + n \log [\text{inhibitor}]$$



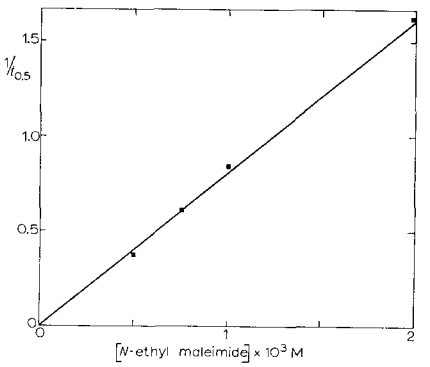


Fig. 1. The rate of inhibition of propionyl-CoA carboxylase with varying concentrations of *N*-ethylmaleimide. Enzyme (200 μg) was incubated with *N*-ethylmaleimide contained in 1 ml of 0.05 M Tris–HCl buffer (pH 8.0) at 22°. Aliquots of 0.05 ml were taken at various time intervals and assayed. The reaction mixture also contained 1% bovine serum albumin and reduced glutathione 0.01 M. The concentrations of *N*-ethylmaleimide used were 2 mM (○—○), 1 mM (▲—▲), 0.75 mM (□—□) and 0.5 mM (■—■). Log percent activity was plotted against time. Appropriate controls were necessary since enzymic activity decreased slightly under these conditions.

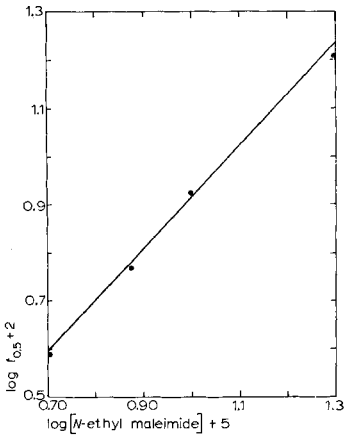
Fig. 2. The pseudo first-order kinetics of the inactivation with respect to *N*-ethylmaleimide. The half-time ($t_{0.5}$) was obtained from the data shown in Fig. 1. The reciprocal of the half-time was plotted against *N*-ethylmaleimide concentration.

Therefore, the order of the reaction can be experimentally estimated by determining k at a number of different inhibitor concentrations and a plot of log k against log of inhibitor concentration will have a slope of n.

Fig. 3 shows the values obtained in Fig. 1 plotted in this manner. The reciprocal of the half-time for inactivation has been used rather than k. SCRUTTON AND UTTER[7] point out that although this process introduces a constant it has no effect on the slope. The points fit a straight line with a slope of 1.0. This is taken to indicate that when inactivation occurs, only one molecule of *N*-ethylmaleimide reacts with one mole of propionyl-CoA carboxylase. Using a similar technique, LEVY, LEBER AND RYAN[6], concluded that 3 moles of 2,4-dinitrophenol bind to 1 molecule of myosin causing inactivation. SCRUTTON AND UTTER[7] obtained a value of 1.4 for the slope of a similar plot when they studied the inactivation of pyruvate carboxylase by avidin. They concluded that at least 2 molecules of avidin were involved in the process.

Effect of pH on the apparent K_m and $v_{max.}$ values

Apparent Michaelis constants were calculated from a series of double reciprocal plots obtained at various pH values using propionyl-CoA as the variable substrate. Fig. 4 shows a plot of log ($1/K_m$) against pH. The important feature of the pH profile is the diminution of the pK_m in the pH range where the enzyme exhibits maximum activity. This indicates a pH dependence of K_m when $v_{max.}$ or velocity (v) at high substrate concentration is independent of pH. From the theory of DIXON[8], the inflection at pH 8.2 is due to the change in ionization of the enzyme–substrate complex. This interpretation is applicable if the enzyme forms a single enzyme–substrate intermediate. If, on the other hand, a number of enzyme–substrate com-



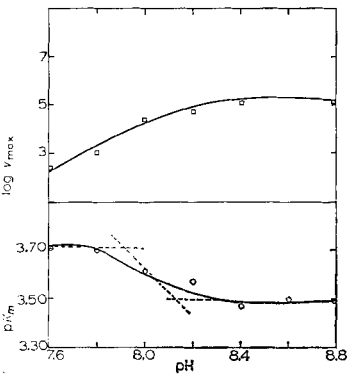


Fig. 3. Determination of the order of the reaction between propionyl-CoA carboxylase with respect to *N*-ethylmaleimide. The data were those of Fig. 1 plotted as $\log_{10}$ of the reciprocal of the half-time of inactivation against $\log_{10}$ concentration of *N*-ethylmaleimide.

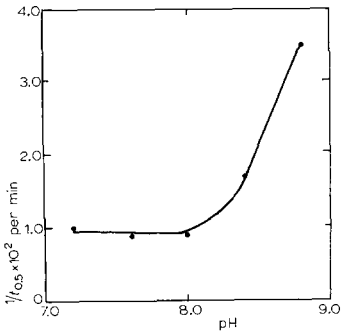
Fig. 4. Apparent K_m for propionyl-CoA calculated at different pH values. The reaction mixture was as described in METHODS AND MATERIALS and Tris–HCl buffer was used throughout. The reaction mixtures were incubated with 15 μg enzyme (spec. act. 2.0) for 10 min at 30°. The K_m and $v_{max.}$ values for propionyl-CoA were calculated from Lineweaver–Burk plots.

plexes are formed, the experimentally derived value for K_m then represents a complex kinetic function and the change in K_m with pH may instead reflect a change in the rate controlling step.

Effect of pH on the rate of loss of activity

Although previous workers have indicated that a reactive sulphydryl group is required for propionyl-CoA carboxylase activity, the pK_a value of 8.2 obtained in Fig. 4 could not be taken as positive proof that the ionization was due to the SH group. The pK_a value of an SH group is markedly dependent on the electric field present, that is, on the vicinal ionic groups. The pK_a value for the SH group may vary between 7.2 and 8.5 when it is situated near a positively charged group[9]. The value may vary between 8.5 and 9.2 if there is no electric field in the vicinity of the group while if the group is in close proximity to a negatively charged group, the pK_a value may vary between 9.2 and 10.2. Therefore, to positively identify the ionization at pH 8.2 as being due to a sulphydryl group, a series of experiments were carried out to determine the rate at which the thiol reagent (*N*-ethylmaleimide) would react with the enzyme at varying pH values. The rationale here is that the rate at which *N*-ethylmaleimide reacts with the thiol group will depend on the state of ionization of this group.

Alkylation of the enzyme followed first order kinetics with respect to inhibitor concentration (Fig. 2). Therefore, the apparent rate constant for the loss of enzymic activity was determined over the pH range from 7.2 to 8.8 (Fig. 5). It can be seen that when the rate constant (or $1/t_{0.5}$) of inactivation of propionyl-CoA carboxylase activity by *N*-ethylmaleimide was plotted as a function of pH, the rate of inactivation remained constant as the pH increased to pH 8.0. At higher pH values the rate of inactivation increased rapidly. The increase in the rate of inactivation at



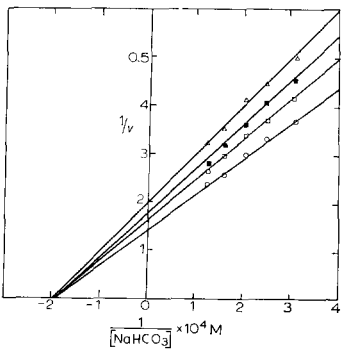


Fig. 5. Rate of inhibition of propionyl-CoA carboxylase with *N*-ethylmaleimide at varying pH values. The inhibitor procedure was as described in Fig. 1 using a concentration of *N*-ethylmaleimide 0.1 mM. Tris–HCl buffers were used throughout. The reciprocal of half-time of inactivation was plotted against pH.

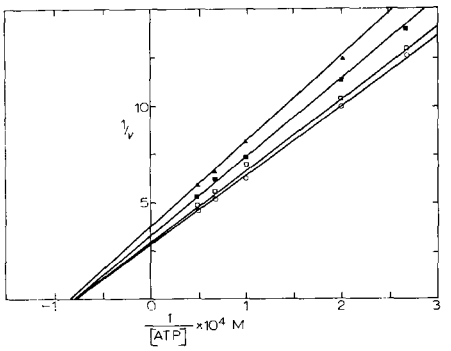
Fig. 6. Kinetic constants for $NaHCO_3$ in the chemically modified enzyme. Enzyme (600 μg) was incubated in 1 ml containing 0.1 mM *N*-ethylmaleimide in 0.05 M Tris–HCl (pH 8.0) at 22°. Aliquots of the enzyme (0.2 ml) were removed at 0, 5, 10, 15 min and the modification stopped by dilution in 1% bovine serum albumin and 0.01 M reduced glutathione. The modified enzyme was then assayed using varying amounts of $NaHCO_3$. Double reciprocal plots of data obtained after modification for time 0 (○—○); 5 min (□—□); 10 min (■—■); and 15 min (△—△).

pH values above 8.0 indicates that the reactive amino acid residue is undergoing an ionization change at this point and that the inhibitor reacts more readily with the ionized form. VIRDEN AND WATTS[10] obtained a similar result when they inhibited arginine kinase with iodoacetamide. Furthermore, HOLLAWAY, MATHIAS AND RABIN[11] found that the free thiol group in ficin reacts with an equimolar concentration of iodoacetamide at a rate too fast to measure above pH 8.2.

Effect of chemical modification of the active site

ZERNER AND BENDER[12] and KNOWLES[13] have shown that kinetic studies may define the role of a particular amino acid in an enzymic reaction. Chemical modification of binding amino acids may cause changes in the apparent K_m value for a particular substrate, while modification of a catalytic amino acid, *i.e.*, those residues involved in breaking down the enzyme–substrate complex, will exert changes in the apparent $v_{max.}$. The apparent K_m and $v_{max.}$ values of propionyl-CoA carboxylase were determined for HCO_3^-, ATP and propionyl-CoA with the enzyme in the native state and again after various stages of inactivation using *N*-ethylmaleimide as the thiol blocking reagent. *N*-Ethylmaleimide has advantages as the modifying reagent because it alkylates cysteine residues irreversibly and therefore the modifications avoided the complications arising from the use of reversible inhibitors. Furthermore, only 1 molecule of this reagent reacts per molecule of enzyme as shown above.

From the data shown in Figs. 6, 7 and 8, it can be seen that the apparent K_m values for HCO_3^- and ATP are unchanged in the modified enzyme but the apparent K_m for propionyl-CoA increases as more of the enzyme is inactivated. This observation implies that although the velocity of the reaction decreases at fixed substrate concentrations of propionyl-CoA, the $v_{max.}$ does not change, *i.e.*, the substrate concentration for maintaining half maximal velocity increases as more of the thiol reagent reacts with the enzyme.



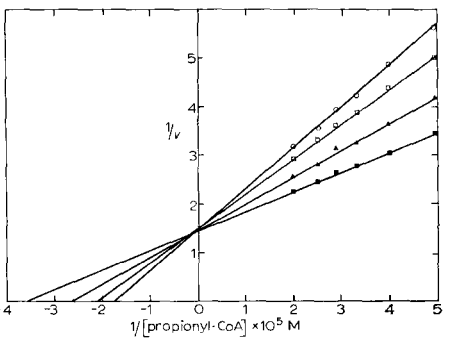


Fig. 7. Kinetic constants for ATP in the chemically modified enzyme. The enzyme was modified as described in Fig. 6. The enzyme was assayed using varying amounts of ATP. Double reciprocal plots of data obtained after modification for time 0 (○—○); 5 min (□—□); 10 min (■—■); and 15 min (▲—▲).

Fig. 8. Kinetic constants for propionyl-CoA in the chemically modified enzyme. Modification of the enzyme was as described in Fig. 6. The enzyme was assayed using varying amounts of propionyl-CoA. Double reciprocal plots of data obtained after modification for time 0 (■—■); 5 min (▲—▲); 10 min (□—□); and 15 min (○—○).

It could be argued that the changes observed in the $v_{max.}$ for ATP and HCO_3^- (Figs. 6 and 7) reflect a modification of catalytic residues involved in the breakdown of enzyme–substrate complexes involving these substrates. However, the fact that only 1 molecule of *N*-ethylmaleimide reacts with the enzyme and that ATP stimulates the rate of inactivation due to *p*-chloromercuribenzoate tends to lessen this possibility. The most likely explanation for the decrease in $v_{max.}$ when either ATP or HCO_3^- is the variable substrates is that the fixed propionyl-CoA concentration is no longer saturating in the modified enzyme. A further characteristic of the inhibition by covalently bound *N*-ethylmaleimide is that it introduces a new side chain into the active site and the change in $v_{max.}$ may be as much due to the presence of this side chain. This explanation could also apply to the observed reduction in the rate of the propionyl-CoA carboxylase catalysed ATP $\leftrightharpoons$ P_i exchange in the presence of *p*-chloromercuribenzoate[14].

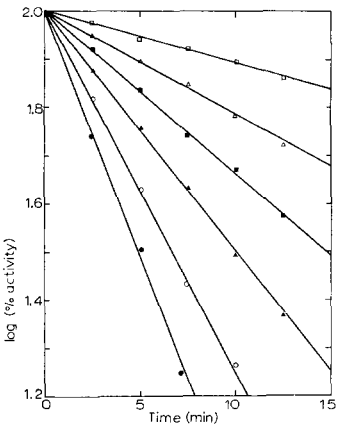
Propionyl-CoA protection against N-ethylmaleimide inhibition

Previous reports of protection by propionyl-CoA against thiol group reagents[2] indicated that propionyl-CoA may be bound to a cysteine residue. In this communication additional evidence both from the pK_m/pH profile and the change in the apparent pK_m value for propionyl-CoA in the chemically modified enzyme implicates a cysteine residue at the CoA ester binding site. Further kinetic proof was obtained by determining the ratio of the rate constants for the inactivation of the free enzyme and the enzyme–propionyl-CoA complex. This was accomplished by measuring the inactivation rate constant in the presence of varying concentrations of propionyl-CoA (Fig. 9).

SCRUTTON AND UTTER[7] have used this technique to demonstrate that avidin cannot react with the pyruvate carboxylase–ATP complex. They derived the equation,

$$\frac{V_a}{V_o} = \frac{k_2}{k_1} + K_a \frac{(1 - V_a/V_o)}{A}$$

where V_a and V_o represent, respectively, the pseudo first-order rate constants for inactivation in the presence and absence of A, the substrate, *i.e.*, propionyl-CoA; k_1 and k_2 are the fractional order rate constants for inactivation of free enzyme and enzyme–propionyl-CoA complex; and K_a the dissociation constant for A. When the ratios of (V_a/V_o) of pseudo first-order rate constants for inactivation in the presence and absence of A are plotted against $(1-V_a/V_o)/A$, the intercept represents k_2/k_1, the ratio of the fractional order rate constants for the reaction between free enzyme and enzyme–substrate complex with inhibitor. The slope is K_a. Such a plot derived from the data obtained in Fig. 9 is shown in Fig. 10.



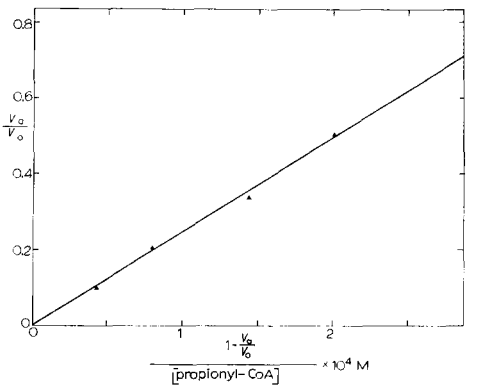


Fig. 9. Protection of propionyl-CoA carboxylase by propionyl-CoA against *N*-ethylmaleimide inhibition. The enzyme was modified with *N*-ethylmaleimide, 1 mM as described in legend for Fig. 1 in the presence of varying concentrations of propionyl-CoA. The concentrations of propionyl-CoA used were 0 (●—●); 0.25 mM (○—○); 0.5 mM (▲—▲); 1.0 mM (■—■); 1.5 mM (△—△); and 2.0 mM (□—□).

Fig. 10. Determination of the K_a (dissociation constant of enzyme–propionyl-CoA complex). The $t_{0.5}$ for protected (V_g) and unprotected (V_o) enzyme inhibited as described in Fig. 9 were replotted

$$\frac{V_a}{V_o} \text{ against } \frac{(1-V_a/V_o)}{[\text{propionyl-CoA}]}$$

From the fact that the line in Fig. 10 passes through the origin it is concluded that *N*-ethylmaleimide cannot combine with the enzyme–propionyl-CoA complex *i.e.*, k_2 is very small compared with k_1. The dissociation constant, K_a is calculated at 0.25 mM.

DISCUSSION

Evidence has been presented in the preceding section that propionyl-CoA carboxylase contains an essential sulphydryl group which is involved in binding propionyl-CoA to the active site. The sulphydryl groups of many enzymes are known to react with sulphydryl reagents yielding a catalytically inactive product. In most cases it is not clear whether the sulphydryl group at the active site has been blocked or whether the reaction with a group elsewhere has caused conformation changes that have led to an inactive enzyme. WEBB[15] has pointed out that it is difficult to distinguish whether the exposed reactive group (a) is at the active site and functional in the reaction mechanism, (b) is at the active site but non-functional, (c) is vicinal to the

active site, or (d) reacts and alters the enzyme structure. Proof of the functional role of reactive groups then must come from evidence other than inhibition or protection experiments since the demonstration of inhibition by sulphydryl reagents indicates at best that one or more cysteine residues are sufficiently near the active site to interfere with the catalysis, either directly or by structural changes.

In the present investigation we have used several approaches to implicate a cysteine residue in the reaction mechanism of propionyl-CoA carboxylase. Chemical modification of the enzyme using a sulphydryl reagent indicated that the binding of propionyl-CoA was affected, while the binding of the other two substrates remained unchanged. The protection experiments reported here as well as those of HEGRE AND LANE[2] and KAZIRO, LEONE AND OCHOA[14] indicated that propionyl-CoA rather than ATP or HCO_3^- was involved in reacting with the cysteine residue. Support for this conclusion was obtained from the pH studies which indicated that the propionyl-CoA–enzyme complex has a pK_a value of 8.2.

CECIL[16] has pointed out that the high reactivity of the SH groups in enzymes has led investigators to postulate their participation at the active site. In very few instances, however, has it been shown that the SH groups form part of the active site and become involved in the catalytic sequence. The four instances where it is reasonably certain that a SH is involved in an enzymic reaction[17–21] have one feature in common, *i.e.*, they all form *S*-acyl derivatives. However, HEGRE AND LANE[2] tried unsuccessfully to show that an acylpropionyl-CoA carboxylase was formed. Also, VIRDEN AND WATTS[10] concluded that at least one of the five thiol groups of arginine kinase is essential for activity and it probably forms part of the catalytic mechanism. From their evidence it would appear that an *S*-acyl derivative was not formed.

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REFERENCES

1 A. TIETZ AND S. OCHOA, *J. Biol. Chem.*, 234 (1959) 1394.
2 C. S. HEGRE AND M. D. LANE, *Biochim. Biophys. Acta*, 128 (1966) 172.
3 C. H. WANG AND D. E. JONES, *Biochem. Biophys. Res. Commun.*, 1 (1959) 203.
4 E. J. SIMON AND D. J. SHEMIN, *J. Am. Chem. Soc.*, 75 (1953) 2520.
5 R. ZETTERSTROM AND M. LJUNGGREN, *Acta Chem. Scand.*, 5 (1951) 291.
6 H. M. LEVY, P. D. LEBER AND E. M. RYAN, *J. Biol. Chem.*, 238 (1963) 3654.
7 M. C. SCRUTTON AND M. F. UTTER, *J. Biol. Chem.*, 240 (1965) 3714.
8 M. DIXON, *Biochem. J.*, 55 (1953) 161.
9 J. L. WEBB, *Enzyme and Metabolic Inhibitors*, Vol. 1, Academic, New York, 1st Ed., 1963, p. 703.
10 R. VIRDEN AND D. C. WATTS, *Biochem. J.*, 99 (1966) 162.
11 M. R. HOLLAWAY, A. P. MATHIAS AND B. R. RABIN, *Biochim. Biophys. Acta*, 92 (1964) 111.
12 B. ZERNER AND M. L. BENDER, *J. Am. Chem. Soc.*, 86 (1964) 3669.
13 J. R. KNOWLES, *Biochem. J.*, 95 (1965) 180.
14 Y. KAZIRO, E. LEONE AND S. OCHOA, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 1319.
15 J. L. WEBB, *Enzyme and Metabolic Inhibitors*, Vol. 2, Academic, New York, 1st Ed., 1966, p. 647.
16 R. CECIL in H. NEURATH, *The Proteins*, Vol. 1, Academic, New York, 2nd Ed., 1963, p. 380.
17 F. LYNEN, *Federation Proc.*, 20 (1961) 941.
18 I. KRIMSKY AND E. RACKER, *Science*, 122 (1955) 319.
19 O. J. KOEPPE, P. D. BOYER AND M. P. STULBERG, *J. Biol. Chem.*, 219 (1956) 569.
20 E. L. SMITH, *J. Biol. Chem.*, 233 (1958) 1392.
21 E. E. CLIFFE AND S. G. WALEY, *Biochem. J.*, 79 (1961) 475.