Equilibria and Substrate Specificity Studies with Alcohol Dehydrogenases

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While secondary alcohols and NAD^+, and ketones and NADH react much slowly than the ethanol–acetaldehyde system in the presence of yeast and liver alcohol dehydrogenases, by starting near equilibrium concentrations, it has been shown that expected thermodynamic equilibria are obtained. With liver ADH, cycloalkanols are remarkably reactive, while with yeast ADH those with six or more carbon atoms in the ring are unreactive. Horse liver ADH shows stereospecificity for 2-octanol, but no stereospecificity for 2-butanol. On the other hand, yeast ADH shows the same stereoselectivity for both alcohols. In the presence of yeast ADH, R(−)-isomers of 2-butanol, 2-octanol and 1-methoxy-2-propanol are not reactive, comparing to the big reactivity of *S*(+)-isomers of these alcohols, but *R*(−)-isomer of propylene glycol is also slightly reactive as *S*(+)-isomer. Further, the reactivity of secondary alcohols with NAD^+ in the presence of liver ADH is following, 2-octanol>2-butanol>2-propanol. These results suggest that horse liver ADH is much less sterically hindered at its active site than yeast ADH and that not only the steric factor but also hydrophobic bonding between the end alkyl group of alcohol and the hydrophobic region of enzyme might be another important factor to determine stereospecificity and reactivity of enzyme.

The stereoselective reduction of acetol to *R*(−)-propylene glycol by fermenting yeast was reported many years ago by Levene and Walti.[1] To improve the convenience of workup, we studied the fermenting yeast reduction of acetol benzoate; to our surprise, the product was the benzoate of *S*(+)-propylene glycol.[2]

The pure enzyme, yeast alcohol dehydrogenase (yeast ADH), has been shown to be stereoselective in the interconversion of ethanol and acetaldehyde.[3] It was the purpose of this investigation to determine whether the enzyme was also stereoselective for ketone reduction and, especially, whether this could explain the reversal of the steric sense of reduction for acetol and acetol benzoate. Kaplan and Van Eys[4] have shown that yeast ADH is indeed stereoselective in oxidizing only the *S*(+)-isomers of 2-butanol and 2-octanol. In this case, the isolated enzyme result accords with the observation that fermenting yeast reduced 2-octanone to the *S*(+)-enantiomer.[5] The absolute optical specificity of the yeast enzyme towards 2-alkanols and its unreactivity towards 3-pentanol, 3-hexanol and 3-heptanol were explained by its inability to accept alkyl groups larger than methyl in the unbound positions in a viable ternary complex.[6] Liver ADH, however, has been reported to lack such stereoselectivity.[7]

While Burton and Wilson[8] have established the equilibrium for 2-propanol–acetone ($K_{eq}=7.19\times10^{-9}$) by starting with mixtures containing three of the four

$$NAD^+ + R_2CHOH \overset{YADH}{\rightleftharpoons} NADH + R_2C{=}O + H^+$$

$$K_{eq} = \frac{[NADH][R_2C{=}O][H^+]}{[NAD^+][R_2CHOH]}$$

organic components, there are reports that cyclic ketones and higher homologs of 2-butanone are not reduced by either yeast or liver alcohol dehydrogenases.[9–11]

Preliminary investigations of the propylene glycol–acetol system were therefore initiated from the alcohol oxidation side.[12] In this early work, 2-hydroxypropyl benzoate was found inactive to enzymic oxidation. 1-Methoxy-2-propanol was, however, reactive. Furthermore, it was shown that methoxyacetone was stereoselectively reduced by fermenting yeast to *S*(+)-1-methoxy-2-propanol. The methoxy group thus provides the same reversal sense as does the benzoate group.

While these preliminary studies[12] showed that oxidation of 1,2-propanediol with yeast ADH did proceed, the reactions were much slower than for ethanol oxidation. Furthermore, the reactions stopped in several hours far short of true equilibrium. It has now been shown that this was due to inactivation of the enzyme during the time required for the slow oxidation of glycol derivatives.

By starting with reaction mixture near equilibrium, it has been possible to show that thermodynamic equilibrium is reached. Based on equilibrium constants and optical density changes at 340 nm, the factors determining stereospecificity of enzymes are discussed.

Experimental

Twice-crystallized yeast ADH and β-NAD⁺ were purchased from Sigma Chemical Co. and further purified. Once-crystallized horse liver ADH was purchased from Worthington Biochemicals and purified in the same way as yeast ADH.

Commercial NAD⁺ (grade III) was further purified by DEAE cellulose column chromatography.[13]

S(+)-1,2-Propanediol was prepared either through yeast reduction of acetol benzoate[α]$_D^{30}$=+26.2 °C (*C*=1, $CHCl_3$), or from *S*(+)-lactic acid by lithium alminum hydride reduction,[14], [α]$_D^{25}$=+29.10 (*C*=1, $CHCl_3$).

Acetol[15] was converted to *R*(−)-1,2-propanediol, [α]$_D^{25}$=−27.7 (*C*=1, $CHCl_3$) by yeast fermentation.

S(+)-and R(−)-Octanol, [α]$_D^{25}$=+8.37 and −8.66, respectively (*C*=5, 95% ethanol), were prepared by Ingersoll's procedure.[16] *S*(+)-2-Butanol was similarly prepared.[16]

Measurement of Equilibrium Constant. Solutions of different composition were prepared by mixing 1.0 ml of variable mol ratio of alcohol to ketone solutions and 1.90 ml of mixed coenzyme solution (NAD^+ and NaDH in tris buffer) in test tubes. A blank was also made up from 1.0 ml of water and

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1.9 ml of the same coenzyme solution.

These solutions were kept standing for an hour in the 25.0 °C constant temperature bath. Preserved horse liver ADH in the frozen state was diluted with phosphate buffer (near 0 °C). This diluted ADH solution (0.1 ml each) was added to each solution at 25 °C.

Just after adding ADH, the optical density of the blank solution was measured at 340 nm. All the solutions were kept standing at 25.0 °C, usually for 4 h, and then the optical density of each solution was measured at 340 nm. At the same time, the pH of the solution was measured. The amount and direction of shift from the initial concentrations was calculated from the difference of optical density of each solutions from that of the blank.

Results and discussion

Equilibrium Constants. A typical example of the experimental data used to calculate equilibrium constant K_{eq} is summarized in Table 1. Values thus obtained are listed in Table 2.

From the equilibrium constants in Table 2, the standard free energy change for the reaction is readily estimated ($\Delta G = -RT \ln K_{eq}$). With the known $\Delta G^0 = 5.22$ kcal/mol for the NAD$^+$–NADH couple,[8] this leads to values for the ketone–alcohol couples listed in Table 2.

In these experiments we measured the amount of alcohol oxidized by NAD$^+$ through increase in absorbance at 340 nm, a λ_{max} for NADH. It has, however, been shown that ketones, especially acetol and methoxyacetone, undergo a base catalyzed reaction with NAD$^+$ producing compounds with a λ_{max} near 340 nm.[17] In Fig. 1, data on the reaction of several ketones with

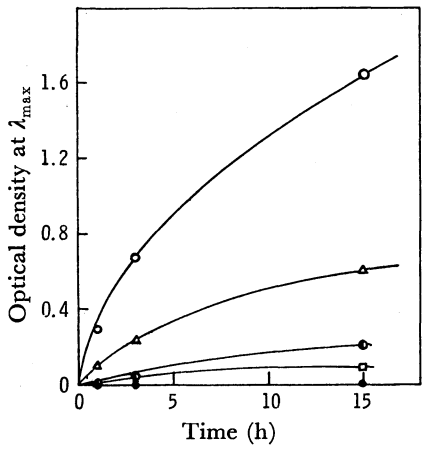


Fig. 1. Rate of reaction of NAD$^+$ and ketones at pH 9; initial concentration of NAD$^+$, 6.2×10^{-4}M; ○ acetol, 1.03×10^{-2} M; △ methoxyacetone, 6.90×10^{-3} M; ◐ cyclohexanone, 6.90×10^{-3} M; □ cyclopentanone, 3.30×10^{-3} M; ● acetone, 6.90×10^{-3} M; Tris buffer, 0.02 M; 25.0°C; no ADH. (No reaction at pH 7.0 under the same conditions.) λ_{max}: 340—345 nm.

Table 1. Experimental data used to estimate K_{ep} for the $S(+)$-2-butanol–2-butanone system with horse liver ADH[a]

[ROH][b]	[ketone]/[ROH]	OD[c]$_{340nm}$	ΔOD	K'[d]
5.56	0.075	1.31	+0.12	
5.22	0.15	1.275	+0.085	5.7×10^{-9} (K'_A)
4.14	0.45	1.16	−0.03	1.4×10^{-8} (K'_B)
3.15	0.90	1.03	−0.16	

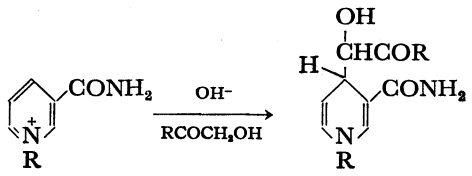
a) 25°C, $[NAD^+]_0 = 5.87\times10^{-4}$ M; $[NADH]_0 = 2.09\times10^{-4}$ M; [ADH] $= 1.5\times10^{-7}$ N; Tris chloride buffer, 0.02 M. b) $\times10^{-3}$ M. c) Optical density at 340 nm after 15 h; pH=7.01. d) $K' =$ [NADH][2-Butanone][H$^+$]/[NAD$^+$][2-Butanol] at 15 h; $K_{eq} = \{K_A + \Delta OD_A\ (K_B - K_A)\}/(\Delta OD_A + \Delta OD_B \cdot p)$ where $p = [Ketone]_B[ROH]_A/[ROH]_B[Ketone]_A$.

Table 2. Summary of equilibrium constants of various alcohol–ketone systems with yeast and horse liver ADH

Alcohol–Ketone System	Catalyst		Average
	Yeast ADH	Liver ADH	
2-Propanol–acetone	8.6×10^{-9}	8.5×10^{-9}	8.6×10^{-9}
(+)-2-Butanol–butanone	1.2×10^{-8}	9.7×10^{-9}	1.1×10^{-8}
dl-2-Butanol–butanone[a]	(5.1×10^{-9})	(7.1×10^{-9})	—
(+)-2-Octanol–octanone	2.7×10^{-8}	3.1×10^{-8}	2.9×10^{-8}
dl-2-Octanol–octanone[a]	(1.5×10^{-8})	(1.2×10^{-8})	—
(−)-2-Octanol–octanone	N. R.	N. R.	
Cyclopentanol–cyclopentanone	3.5×10^{-8}	3.7×10^{-8}	3.6×10^{-8}
Cyclohexanol–cyclohexanone	N. R.	3.6×10^{-10}	3.6×10^{-10}
dl-1-Methoxy-2-propanol–methoxyacetone	4.7×10^{-11}	1.0×10^{-10}	7.4×10^{-11}
(+)-1,2-Propanediol–acetol[b]	8.9×10^{-11}	9.3×10^{-11}	9.1×10^{-11}
dl-1,2-Propanediol–acetol[a]	7.5×10^{-11}	1.1×10^{-10}	9.3×10^{-11}
(−)-1,2-Propanediol–acetol[b]	7.9×10^{-11}	7.7×10^{-11}	7.8×10^{-11}

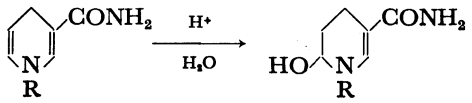
a) K_{ap} using total ROH; since only one enantiomer reacts, the values in parentheses are not proper K_{eq}.
b) To obtain the equilibrium data, acetol was used, although the real system may involve lactaldehyde as well.

NAD^+ at pH 9.1 are summarized.



The ΔOD for acetol and methoxyacetone are so great that the values for K_{eq} would be seriously in error at this pH. At pH 7, there was no increase in OD, even for acetol.

NADH is also known to react with acids[18] to lose optical density at 340 nm.



We found that NADH was rapidly destroyed at pH 6 (*ca.* 20—25% in 19 h, 25°C) and even at pH 7, about 6% reacts in 19 h. The presence of either yeast or liver ADH markedly enhances NADH stability, less than 1% disappearing in 19 h at 25°C. We therefore chose pH 7 as the most suitable pH, both for NADH stability to acid and NAD^+ stability to ketone.

Table 3. The standard free energy changes for various alcohol–ketone systems

Alcohol–ketone system	ΔG° (kcal mol^{-1})	ΔG° (lit)
2-Propanol–acetone	5.8	5.89(8), 6.0(19)(20)
2-Butanol–2-butanone[a]	5.7	5.7(19), (20)
2-Octanol–2-octanone[a]	5.1	not available
Cyclopentanol–cyclopentanone	5.0	5.7(19)
Cyclohexanol–cyclohexanone	7.7	7.5(19)
1-Methoxy-2-propanol–methoxyacetone	8.7	8.8(19)
1,2-Propanediol–acetol	8.6	not available

a) K_{eq} for $S(+)$-isomers were used to calculate ΔG, since these are reactive isomers.

The equilibrium data in Table 2 and 3 are in reasonably satisfactory agreement with earlier data obtained by chemical equilibration[19,20] and with the YADH equilibration for 2-propanol–acetone.[8] The fact that yeast and liver ADH give values essentially the same is support for the conclusion that the procedure used has given true equilibrium. The catalyst should, of course, contribute only to the rate and, at the low relative concentrations used, should not influence the equilibrium position.

Stereospecificity of Liver and Yeast ADH for 2-Butanol and 2-Octanol. The data in Table 2 support the earlier proposal[4] that yeast ADH is stereoselective for the $S(+)$-isomers of 2-butanol and 2-octanol, since the "apparent" K_{eq} for the racemates is nearly half of K_{eq} for the $S(+)$-isomers. We found, however, that liver ADH shows optical specificity for 2-octanol and even for 2-butanol it seems to show small optical specificity. Stereospecificity of horse liver ADH for 2-octanol was confirmed by the initial rate of oxidation of secondary alcohols, but that for 2-butanol was not clear as shown in Table 4.

Table 4. Initial rate of oxidation reaction of various alcohols with NAD^+ in the presence of horse liver ADH.

Alcohol	ΔOD at 340/min
2-Propanol	0.012
(+)-2-Butanol	0.016
dl-2-Butanol	0.016
(+)-2-Octanol	0.048
dl-2-Octanol	0.024
(−)-2-Octanol	0.004
(+)-1,2-Propanediol	0.024
dl-1,2-Propanediol	0.018
(−)-1,2-Propanediol	0.016
1-Methoxy-2-propanol	0.024

Initial conc.: NAD^+, 6×10^{-3} M; horse liver ADH, 1.5×10^{-7} N; Tris chloride buffer, 0.02 M; 25.0 °C; pH, 7.1—7.3.

Stereospecificity of ADHs for 1-Methoxy-2-propanol and 1,2-Propanediol. Yeast ADH is probably stereoselective for 1-methoxy-2-propanol, because the "apparent" K_{eq} of yeast ADH is just half of that of horse liver ADH.

As for the motivating reason for this investigation, there appears to be no stereoselection between the $S(+)$- and $R(-)$-1,2-propanediol enantiomers in the presence of ADHs and NAD^+.

There is one complication with propylene glycol oxidation in that it can oxidize at either the primary alcohol group to give lactaldehyde or the secondary to give acetol. Huff[21] reports that liver ADH oxidizes 1,2-propanediol preferentially to lactaldehyde while Tjioe[12] detected acetol (as the 2,4-dinitrophenylhydrazone) using yeast ADH. If the two equilibria for the glycol are established independently, then the overall observed K_{eq} should be $K_{10}+K_{20}$. If we assume that the K_{10} is approximately the same as for ethanol (1.2×10^{-11}) and for K_{20} approximately the same as for 1-methoxy-2-propanol (1.0×10^{-10}), the sum (1.1×10^{-10}) is close to the experimental values for propylene glycol.

Propylene glycol shows different character comparing to other examined secondary alcohols on the points that $R(-)$-isomer shows the same low reactivity as the $S(+)$-isomer even in the presence of yeast ADH. This result is surprising in view of the fact that whole yeast cell catalyze the reduction of acetol to only $R(-)$-1,2-propanediol and the reduction of methoxy acetone and acetol benzoate to $S(+)$-isomer of 1-methoxy-2-propanol and 2-hydroxypropyl benzoate, respectively.[2,12,15]

Effect of Ring Size on Substrate Specificity of ADHs for Cyclic Secondary Alcohols. The reactivity of various cyclic alcohols was examined from optical density changes with horse liver ADH and yeast ADH. Table 5 shows the optical density change of cycloalkanols after 3 hours of oxidation reaction. With horse liver ADH, all the cyclic alkanols show big reactivities which is almost independent of ring size. In the presence of yeast ADH, reactivity of cycloalkanol decreases with

Table 5. Effect of ring size of cyclic alcohols on substrate specificity of alcohol dehydrogenase[a)]

Cycloalkanol	Yeast ADH		Horse liver ADH	
	pH 9.0	pH 7.2	pH 9.0	pH 7.2
C_4	2.0	1.5	1.9	1.5
C_5	0.55	0.4	1.9	1.2
C_6	0	0	1.8	0.8
C_8	0	0	1.9	1.8
C_{12}	0	0	0.2	0.1

a) Initial concentration: NAD^+, 2.0×10^{-4} M; Cyclic alcohols 6.0×10^{-3} except cyclododecanol, about 10^{-3} M; Yeast ADH, 8.4×10^{-7} N; Horse liver ADH, 5.2×10^{-7} N; Tris chloride buffer, 0.02 M, 25.0°C; Unit, ΔOD (340 nm) at 3 h.

increasing ring size and when the number of ring carbon becomes more than 6, it becomes completely non-reactive.

These results indicate that the active site of yeast ADH is much more sterically hindered than horse liver ADH.[6)]

Factors to Determine an Optical Specificity of ADHs for Secondary Alcohols. Dalziel and Dickinson[6,7)] explained the lack of stereospecificity of liver ADH for secondary alcohols with the absence of steric hindrance of the binding site of enzyme.

However, we noted that liver ADH also exhibits optical specificity for higher secondary alcohols even though steric hindrance at the active site of liver ADH is estimated to be small. Therefore, another factor should be considered to explain stereospecificity of enzymes in addition to steric hindrance.

Sigman[22)] determined the dissociation constants $K_{e,s}(=[E][S]/[ES])$ for complexes of liver ADH with various primary alcohols by measuring the diminution that complexes cause in the intensity of enzyme-bipyridine spectrum and showed that bigger alkyl groups interact more strongly with lipophilic site of enzyme.

A plot of initial rate (ΔOD/min) of oxidation reaction of various secondary alcohols shown in Table 4 *vs.* $1/K_{e,s}$ of primary alcohols obtained by Sigman shows that initial rate of oxidation reaction increases with the increases of $1/K_{e,s}$ as shown in Fig. 2. Thus, the reactivity of the alcohols is much influenced by the hydrophobic interaction of end-alkyl group of alcohols with enzyme.

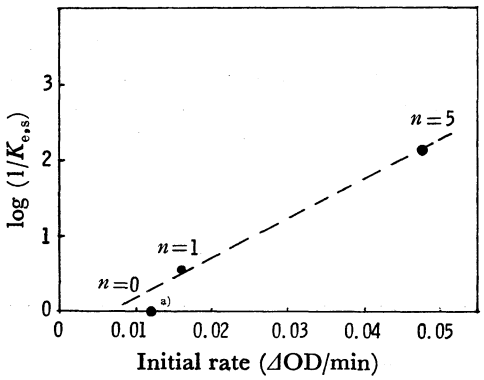


Fig. 2. Plots of the reciprocal of the dissociation constants log $(1/K_{e,s})$ of primary alcohol, $CH_3(CH_2)_n$-OH *vs.* initial rate of oxidation (ΔOD/min) of secondary alcohol, $CH_3(CH_2)_n\rangle CHOH$ (CH_3)

a) $K_{e,s}$ of CH_3OH was estimated to be 1.0 from Sigman's data.[22)]

Stereospecificities of alcohol dehydrogenases for secondary alcohols are summarized in Table 6, which shows a common tendency to three enzymes; $S(+)$-isomers are more reactive in secondary alcohols whose end groups are both normal alkyl or aromatic and $R(-)$-isomers are more reactive or reactive as $S(+)$-isomer in secondary alcohols where one of the end groups is hydroxyl.

If we apply the idea of hydrophobic bonding between substrate and lipophilic site of the enzyme in addition to steric hindrance, we can explain well the optical specificity of the enzymes; thus for 2-butanol, 2-octanol, 1-methoxy-2-propanol and 2-hydroxypropyl benzoate, the C_2H_5–, C_6H_{13}–, CH_3– of $-CH_2OCH_3$ and C_6H_5– of $-CH_2OCOC_6H_5$ group, respectively, binds more strongly with the lipophilic site of the enzyme than does the CH_3-group attached to α-carbon. Hexyl group binds more strongly with lipophilic site of enzyme than ethyl group and this might be a reason why liver ADH shows clear stereospecificity to 2-octanol, in spite it shows small stereospecificity to 2-butanol.

For 1,2-propanediol, the CH_2OH group binds more weakly with the enzyme than CH_3– group attached to α-carbon. As Tsai[23)] observed that the introduction of polar groups such as amino, carbonyl or halogen to primary alcohols renders substrates inactive or inhibitory, generally the introduction of polar groups such as –OH, $-NH_2$, –COOH, $-NO_2$ or halogen to the

Table 6. Reactive optical isomer of various secondary alcohols in the presence of ADHs and that produced from ketone in the presence of yeast cell

Sec. alcohol	Enzyme and Coenzyme		
	Yeast ADH and NAD^+	Horse liver ADH and NAD^+	Yeast cell
2-Butanol	(+)	(+) and (−)	mostly (+)[a)]
2-Octanol	(+)	(+)	no data
1-Methoxy-2-propanol	(+) ?	(+) and (−)	(+)[12)]
1,2-Propanediol	(+) and (−)	(+) and (−)	(−)[15)]
2-Hydroxypropyl benzoate	no data	no data	(+)[2)]

a) According to our experiments, from 20 g of 2-butanone 0.48 g of 2-butanol, which consists of about 80% of (+)-isomer and $[\alpha]_D^{25}=+8.8°$ in 95% ethanol, was obtained in the presence of sugar and yeast cell.

end of one of the alkyl group of secondary alcohol seems $R(-)$-isomer make more reactive or as reactive as $S(+)$-isomer. Although $S(+)$-1,2-propanediol is as reactive as $R(-)$-isomer in the presence of liver ADH, in the presence of yeast cell $S(+)$-isomer losses its reactivity. Further study is necessary to elucidate above difference.

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References

1) P. A. Levene and A. Walti, *J. Biol. Chem.*, **68**, 415 (1926).
2) N. Shieh and C. C. Price, *J. Org. Chem.*, **24**, 1169 (1959).
3) F. A. Loewus, F. H. Westheimer, and B. Vennesland, *J. Amer. Chem. Soc.*, **77**, 3391 (1955).
4) J. Van Eys and N. O. Kaplan, *ibid.*, **79**, 2782 (1957).
5) C. Newberg and F. F. Nord, *Ber.*, **52**, 2237 (1919).
6) F. M. Dickinson and K. Dalziel, *Biochem. J.*, **104**, 165 (1967).
7) F. M. Dickinson and K. Dalziel, *Nature*, **214**, 31 (1967).
8) K. Burton and T. H. Wilson, *Biochem. J.*, **54**, 86 (1953).
9) W. A. Buhler, *Arch. Biochem. Biophys.*, **62**, 173 (1956).
10) A. D. Winer, *Acta Chem. Scand.*, **12**, 1695 (1958).
11) A. D. Merritt and C. M. Tompkins, *J. Biol. Chem.*, **234**, 2778 (1959).
12) G. B. Tjioe, "Rate and Equilibria Studies of Enzymatic Oxidation of Propylene Glycol and 1-Methoxy-2-propanol," Ph. D. Thesis, University of Pennsylvania (1968).
13) A. D. Winer, *J. Biol. Chem.*, **239**, 3598 (1964).
14) C. C. Price, M. K. Akkapeddi, B. T. Debona, and B. C. Furie, *J. Amer. Chem. Soc.*, **94**, 3964 (1972).
15) P. A. Levene and A. J. Walti, *Org. Syn.*, **10**, 1 (1930).
16) A. W. Inersoll, "Organic Reactions," Vol. II, John Wiley & Sons (1944), p. 376.
17) R. M. Burton and N. O. Kaplan, *J. Biol. Chem.*, **206**, 283 (1954) and **211**, 447 (1954).
18) E. Haas, *Biochem. Z.*, **288**, 123 (1936); P. Karrer and F. J. Stare, *Helv. Chim. Acta*, **20**, 418 (1937); P. Karrer and F. W. Kahnt, *ibid.*, **21**, 223 (1938).
19) H. Adkins, R. M. Elofson, A. G. Rossow, and C. C. Robinson, *J. Amer. Chem. Soc.*, **71**, 3672 (1949).
20) R. A. Day, Jr., A. E. Robinson, Jr., J. M. Bellis, and S. B. Till, *ibid.*, **72**, 1379 (1950).
21) E. Huff, *Biochim. Biophys. Acta*, **48**, 506 (1961).
22) D. S. Sigman, *J. Biol. Chem.*, **242**, 3815 (1967).
23) C. S. Tsai, *Can. J. Biol. Chem.*, **46**, 381 (1968).