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EQUILIBRIUM KINETIC STUDY OF THE MECHANISM OF MITOCHONDRIAL AND SUPERNATANT MALATE DEHYDROGENASES OF BOVINE HEART

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SUMMARY

The kinetics at equilibrium have been examined for mitochondrial and supernatant malate dehydrogenases (L-malate: NAD$^+$ oxidoreductase, EC 1.1.1.37) from bovine heart at catalytic concentration by means of isotopic exchange of reactants to compare their catalytic mechanisms. In accord with previous studies of lactate, alcohol and pig heart mitochondrial malate dehydrogenases the rate of substrate interchange exceeded the NAD$^+ \rightleftharpoons$ NADH rate, in some cases by more than 100-fold, except at lowest substrate concentration. At pH 8.0 a marked depression in the NAD$^+ \rightleftharpoons$ NADH rate to near zero without inhibition in the oxaloacetate $\rightleftharpoons$ malate rate was compatible with a compulsory binding order with substrate binding to *E* (enzyme)–coenzyme complexes, but not to free enzyme. At pH 9.0 only a moderate depression in the NAD$^+ \rightleftharpoons$ NADH rate was found, indicating a partially compulsory pathway in which coenzyme dissociation occurs from both *E*–coenzyme and *E*–coenzyme-substrate complexes, but is more rapid from the binary complex. Kinetics with variable concentration of non-reactive pairs of substrate and coenzyme suggested the formation of the abortive complex *E*–NADH–malate, but not *E*–NAD$^+$–oxaloacetate. The initial rate of reduction of oxaloacetate was in all cases greater than the equilibrium rates. Minimum estimates were obtained for some dissociation constants.

Despite considerable structural differences, the similar kinetic behavior at equilibrium of the mitochondrial and supernatant enzymes suggests a uniform catalytic mechanism which includes a compulsory binding order at pH 8.0 with coenzyme binding prior to substrate, a partially compulsory pathway at pH 9.0, non-rate-limiting chemical transformation, and formation of the abortive complex, *E*–NADH–malate. This mechanism is also in accord with that previously found with pig heart mitochondrial malate dehydrogenase.

INTRODUCTION

Malate dehydrogenase (L-malate: NAD$^+$ oxidoreductase, EC 1.1.1.37) has been shown to occur in two forms which differ in physical, catalytic and immunological properties and cellular location[1–7] Initial rate kinetic studies[8–12] have suggested the presence of a compulsory binding order mechanism for pig heart and bovine heart mitochondrial malate dehydrogenases with coenzyme binding prior to substrate Kinetic study at equilibrium by means of isotopic exchange, which has been shown to be particularly useful for elucidation of compulsory pathways and of the nature of the rate-limiting step[13–16], has confirmed this mechanism for pig heart mitochondrial malate dehydrogenase[17] Equilibrium kinetic study of bovine heart malate dehydrogenases, undertaken to determine whether a similar mechanism exists for supernatant and mitochondrial forms of this enzyme, indicates that the mechanisms are similar

METHODS

The materials and techniques for equilibrium kinetic study have been described[17] A crystalline preparation of bovine heart supernatant malate dehydrogenase was generously made available by Dr Sasha Englard and bovine heart mitochondrial malate dehydrogenase was kindly supplied by Dr Lewis Siegel Specific activity was 208 μmoles per mg protein per min for supernatant enzyme and 101 μmoles per mg protein per min for mitochondrial enzyme, assayed from the NADH side Enzyme activity was determined by obtaining the initial rate of change in absorbance at 340 mμ at 25° (refs 17 and 18)

RESULTS

Increasing substrate concentration while maintaining equilibrium at pH 8 0 resulted in an increase followed by a sharp decrease in the $NAD^+ \rightleftharpoons NADH$ rate catalyzed by mitochondrial (Fig 1) and supernatant (Fig 2) bovine heart malate dehydrogenase The malate $\rightleftharpoons$ oxaloacetate rate was more than 20 times greater than the $NAD^+ \rightleftharpoons NADH$ rate at highest substrate concentration (0 67 M malate, 67 mM oxaloacetate)

Increasing concentration of the non-reactive pair NAD^+ and oxaloacetate

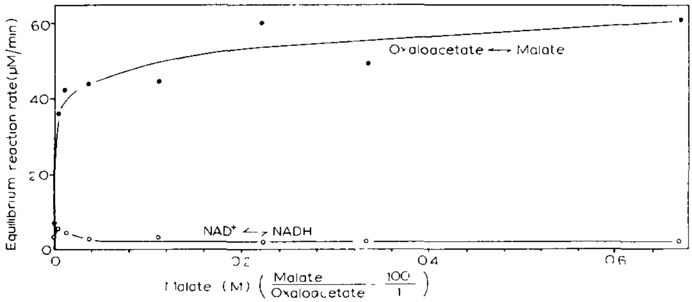

Fig 1 Effect of malate and oxaloacetate concentration on the oxaloacetate $\rightleftharpoons$ malate and $NAD^+ \rightleftharpoons NADH$ reaction rates at equilibrium catalyzed by bovine heart mitochondrial malate dehydrogenase at pH 8 0 Reaction mixtures contained 7 28 mM NAD^+, 73 2 μM NADH, 1 8 μg/ml of enzyme, and malate and oxaloacetate as shown in 71 mM Tris–HNO_3 at 1° The initial rate of oxaloacetate reduction with NADH was 41 times higher than the maximum $NAD^+ \rightleftharpoons NADH$ rate and 3 6 times higher than maximum malate $\rightleftharpoons$ oxaloacetate rate

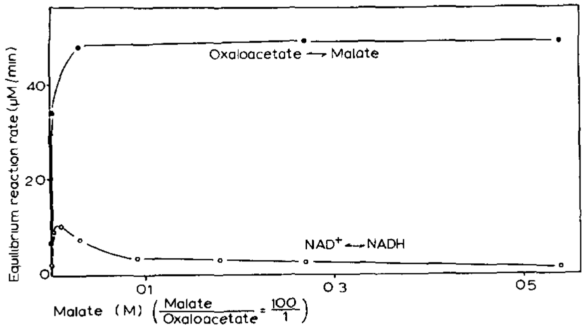

Fig 2 Effect of malate and oxaloacetate concentration on the oxaloacetate $\rightleftharpoons$ malate and NAD$^+$ $\rightleftharpoons$ NADH reaction rates at equilibrium catalyzed at pH 8 0 by bovine heart supernatant malate dehydrogenase (1 1 μg/ml, conditions as in Fig 1) Initial rate of oxaloacetate reduction was 25 times the maximum NAD$^+$ $\rightleftharpoons$ NADH rate and 5 2 times maximum malate $\rightleftharpoons$ oxaloacetate rate

resulted in an increase followed by nearly total inhibition in the NAD$^+$ $\rightleftharpoons$ NADH rate with both mitochondrial (Fig 3) and supernatant (Fig 4) enzyme The oxaloacetate $\rightleftharpoons$ malate rate simultaneously rose to a maximum without any inhibition These results, previously found with pig heart mitochondrial malate dehydrogenase, are compatible with a compulsory binding order with coenzyme binding prior to substrate These

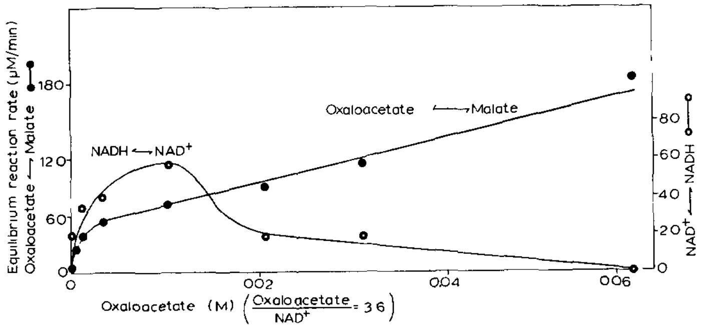

Fig 3 Effect of oxaloacetate and NAD$^+$ concentration on oxaloacetate $\rightleftharpoons$ malate and NAD$^+$ $\rightleftharpoons$ NADH reaction rates at equilibrium catalyzed by bovine heart mitochondrial malate dehydrogenase at pH 8 0 Reaction mixtures contained 258 mM malate, 7 23 μM NADH, 4 4 μg/ml of enzyme, NAD$^+$ and oxaloacetate as indicated in the figure in 71 mM Tris–HNO$_3$ at 1° The initial rate of reduction of oxaloacetate was 63 times greater than maximum NAD$^+$ $\rightleftharpoons$ NADH rate and 2 1 times greater than maximum oxaloacetate $\rightleftharpoons$ malate rate

findings do not support the formation of the abortive complex E(enzyme)–NAD–oxaloacetate since in that case both reaction rates would have decreased as substrate concentration was raised, since active sites would be removed from the catalytically active pool[13,17,19]

On the other hand evidence for formation of the abortive complex E–NADH–malate was obtained by varying the concentration of NADH and malate At elevated substrate concentration both the NAD$^+$ $\rightleftharpoons$ NADH and oxaloacetate $\rightleftharpoons$ malate rates were inhibited (Figs. 5 and 6).

The effect of concentration was also tested at pH 9 0 (Figs 7 and 8) In contrast

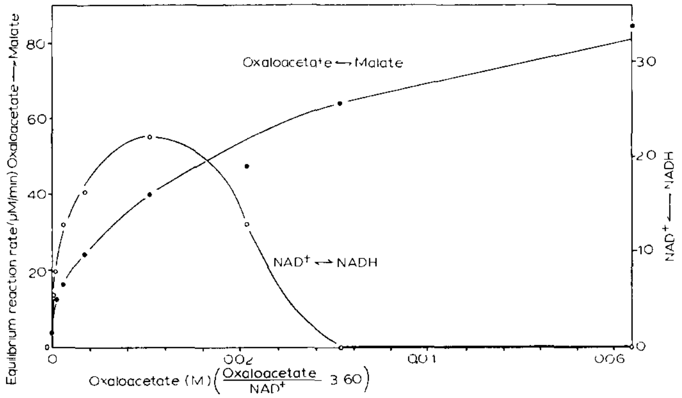

Fig 4 Effect of oxaloacetate and NAD$^+$ concentration on the oxaloacetate ⇌ malate and NAD$^+$ ⇌ NADH reaction rates at qeuilibrium catalyzed by bovine heart supernatant malate dehydrogenase at pH 8 0 (1 1 µg/ml of enzyme, conditions as in Fig 3) Initial rate of oxaloacetate reduction was 91 times maximum NAD$^+$ ⇌ NADH and 2 5 times greater than maximum oxaloacetate ⇌ malate rates

to results at pH 8 0 the depression in the NAD$^+$ ⇌ NADH rate at elevated malate and oxaloacetate concentration was only about 40–50% for mitochondrial and supernatant enzymes The disparity between oxaloacetate ⇌ malate and NAD$^+$ ⇌ NADH rates at saturating malate and oxaloacetate concentration was only about 6-fold for the mitochondrial enzyme and less than 2-fold for supernatant enzyme A decrease occurred in the oxaloacetate ⇌ malate rate similar to that noted with pig heart malate dehydrogenase at pH 9 0 (ref 17) and to the pyruvate ⇌ lactate rate with bovine heart lactate dehydrogenase at pH 9 7 (ref 14)

Plots of $1/R'$ *vs* $1/[S]$ yield minimum estimates for dissociation constants[13–15]

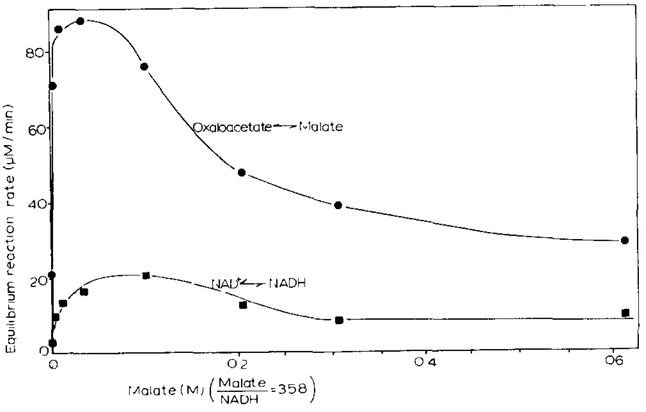

Fig 5 Effect of malate and NADH concentration on oxaloacetate ⇌ malate and NAD$^+$ ⇌ NADH reaction rates at equilibrium catalyzed by bovine heart supernatant malate dehydrogenase at pH 8 0 Reaction mixtures contained 258 µM oxaloacetate, 7 19 mM NAD$^+$, 2 2 µg/ml of enzyme, and malate and NADH as indicated in 71 mM Tris–HNO$_3$ at 1° Initial rate of oxaloacetate reduction was 5 1 times the maximum malate ⇌ oxaloacetate rate and 22 times maximum NAD$^+$ ⇌ NADH rate

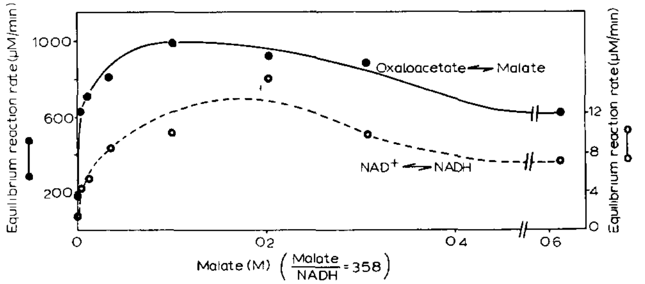

Fig 6 Effect of malate and NADH concentration on oxaloacetate ⇌ malate and NAD+ ⇌ NADH reaction rates at equilibrium catalyzed by bovine heart mitochondrial malate dehydrogenase at pH 8 0 (2 2 μg/ml of enzyme, conditions as in Fig 5) Initial rate of oxaloacetate reduction was 3 4 times the maximum malate ⇌ oxaloacetate rate and 21 times the maximum NAD+ ⇌ NADH rate

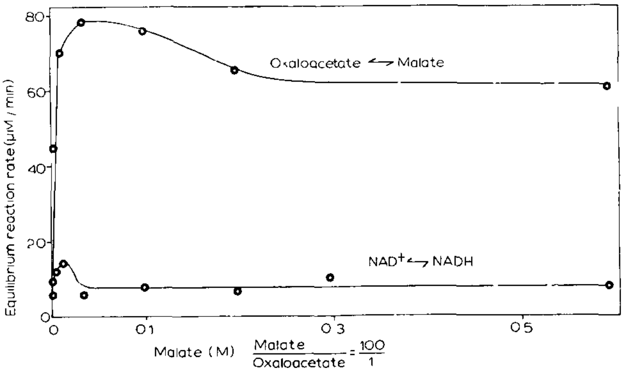

Fig 7 Effect of malate and oxaloacetate concentration on oxaloacetate ⇌ malate and NAD+ ⇌ NADH reaction rates at equilibrium catalyzed by bovine heart mitochondrial malate dehydrogenase at pH 9 0 Reaction mixtures contained 7 18 mM NAD+, 721 μM NADH, 1 8 μg/ml of enzyme and malate and oxaloacetate as indicated in 213 mM Tris–HNO_3 at 1° Initial rate of oxaloacetate reduction was 2 4 times the maximum malate ⇌ oxaloacetate rate and 15 times maximum NAD+ ⇌ NADH rate

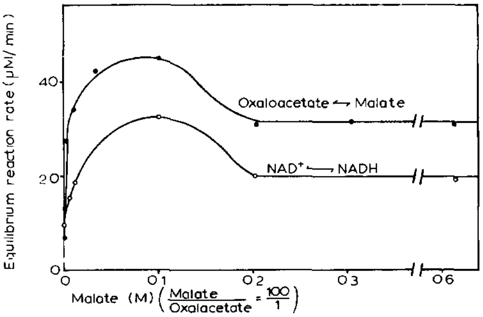

Fig 8 Effect of malate and oxaloacetate concentration on oxaloacetate ⇌ malate and NAD+ ⇌ NADH reaction rates at equilibrium catalyzed by bovine heart supernatant malate dehydrogenase at pH 9 0 (1 1 μg/ml of enzyme, conditions as in Fig 7) The initial rate of reduction of oxaloacetate was 2 3 times the maximum malate ⇌ oxaloacetate rate and 4 3 times maximum NAD+ ⇌ NADH rate

TABLE I

ESTIMATES OF MINIMUM VALUES FOR DISSOCIATION CONSTANTS FROM $1/R'-1/S$ PLOTS FOR BOVINE HEART MITOCHONDRIAL AND SUPERNATANT MALATE DEHYDROGENASES

Fraction	$K_{oxaloacetate} \times 10^6$		$K_{NAD^+} \times 10^4$ (*pH 8 0*)	$K_{NADH} \times 10^6$ (*pH 8 0*)
	pH 8 0	*pH 9 0*		
Mitochondrial	10 1	6 43	2 79	4 94
Supernatant	29 6	9 60	1 98	4 56

which are given in Table I The values approximate reported K_m and dissociation constant figures[6,12,20,21] K_{NADH} is about 10 times less than reported K_m and 10 times greater than the dissociation constant reported for supernatant enzyme[20] The dissociation constants for both NAD^+ and NADH are within an order of magnitude less than reported values for the mitochondrial enzyme[12]

DISCUSSION

There has been considerable evidence that mitochondrial and supernatant forms of malate dehydrogenase are different proteins with differing properties The two enzymes are generally separable on the basis of charge They differ in amino acid composition, including the absence of tryptophan in the supernatant form[5], number of coenzyme molecules bound per mole of enzyme[20,22], number of titratable sulfhydryl groups[5] and antigenicity[7] The effect of citrate, fumarate[23], ions[5,24–26], *p*-mercuribenzoate, oxaloacetate[5], and coenzyme analogues[7] on enzymatic activity also differ in the two forms Despite these considerable diversities the kinetic behavior at equilibrium of supernatant and mitochondrial malate dehydrogenases from bovine heart suggests that the basic reaction mechanism of the structurally distinct enzymes which catalyze the same reaction is similar Further, the mechanism is not distinguishable from that exhibited by the mitochondrial enzyme from pig heart[17]

The data obtained for the interconversion of oxaloacetate and malate and of NAD^+ and NADH in free solution at equilibrium in the presence of catalytic quantities of supernatant or mitochondrial malate dehydrogenase from bovine heart suggest the presence of a compulsory binding order at pH 8 0 such that prior binding of NAD^+ or NADH is required for the binding of malate or oxaloacetate (Fig 9) Increasing

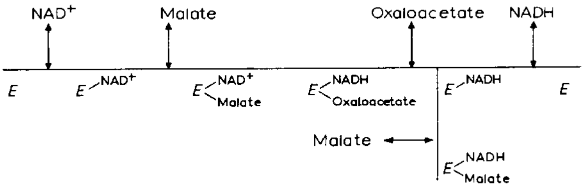

Fig 9 Compulsory order mechanism for malate dehydrogenase catalysis with one abortive complex, depicted for one catalytic center Binding of NADH allows the binding of oxaloacetate to form the reactive complex *E*–NADH–oxaloacetate, or malate to form the unreactive complex *E*–NADH–malate

concentration of malate and oxaloacetate at pH 8.0 resulted in a marked fall in the $NAD^+ \rightleftharpoons NADH$ interchange while the oxaloacetate ⇌ malate rate increased to a maximum Further, when the concentration of oxaloacetate and NAD^+ was increased to the extent that the levels of NAD^+ were similar to those used in experiments with variable oxaloacetate and malate but 10 times higher in oxaloacetate concentration, a complete inhibition in the $NAD^+ \rightleftharpoons NADH$ interchange occurred This finding adds additional support for the compulsory binding order mechanism[13]

HARADA AND WOLFE[27] have recently proposed a reciprocating compulsory order mechanism for pig heart mitochondrial malate dehydrogenase in which two catalytic centers function alternately in a coordinated fashion Equilibrium kinetic experiments with pig heart mitochondrial malate dehydrogenase failed to support the reciprocating mechanism since an expected decrease in the oxaloacetate ⇌ malate rate at highly saturating concentrations of oxaloacetate and malate, NAD^+ and oxaloacetate, and NAD^+ and NADH did not occur[17] The present data for similar reasons do not support such a mechanism for the mitochondrial and supernatant enzymes from bovine heart. This conclusion is supported by the apparent similarity in catalytic mechanism of mitochondrial and supernatant enzyme despite recent evidence for only one coenzyme binding site for the supernatant enzyme[20] which would preclude a mechanism requiring coordinated action of two catalytic centers on each enzyme molecule.

CASSMAN AND ENGLARD[28] have proposed a mechanism for bovine heart supernatant malate dehydrogenase based on initial rate kinetics at pH 8.5 in which the binary complex E–oxaloacetate and binary E–coenzyme complexes may occur but the complex E–malate does not occur Similar analysis done for mitochondrial malate dehydrogenase from pig[8] and bovine[12] heart was compatible with a compulsory reaction pathway in which neither E–oxaloacetate nor E-malate was formed. The present experiments of kinetics at equilibrium do not indicate a basic difference in reaction mechanism between the two forms of malate dehydrogenase from bovine heart If the complex E–malate does not form with supernatant enzyme, it might be expected that high malate concentration would be more effective than oxaloacetate in blocking coenzyme dissociation, and therefore the $NAD^+ \rightleftharpoons NADH$ rate The present data at equilibrium suggest that oxaloacetate is more effective than malate in this inhibition.

The $NAD^+ \rightleftharpoons NADH$ rate is much less inhibited at saturating substrate concentration at pH 9.0 than at 8.0 This finding is compatible with a partially compulsory mechanism at the higher pH with rate-limiting dissociation more rapid from binary E–coenzyme than from ternary E–coenzyme–substrate complexes. A hump occurred in the oxaloacetate ⇌ malate rate at pH 9.0 analogous to that previously obtained with lactate dehydrogenase and pig heart mitochondrial malate dehydrogenases[14,17] Binding of substrate to the enzyme at a control site with modification by conformation change of rate-limiting dissociation of substrate from a catalytic center could account for this behavior A similar phenomenon may conceivably affect the $NAD^+ \rightleftharpoons NADH$ rate at elevated substrate concentration

The data on increasing concentration of a potential abortive complex pair are compatible with the formation of the ternary complex E–NADH–malate but not E–NAD^+–oxaloacetate[13,14,17] (Fig 9) Recent initial rate kinetic studies with the mitochondrial enzyme from bovine heart failed to detect either complex, although the conditions used differ from those of the present study[12] It is not possible to rule out completely a less likely explanation for the decrease of both oxaloacetate ⇌ malate and NAD$^+ \rightleftharpoons$ NADH rates at elevated concentrations of malate and NADH, namely a decrease in rate-limiting dissociation steps in both interchanges to a similar degree due to binding of ligand at a modifier site

The marked disparity between the oxaloacetate $\rightleftharpoons$ malate and NAD$^+ \rightleftharpoons$ NADH rates except at lowest substrate concentrations suggests that dissociation rather than chemical transformation steps are rate-limiting[13,14] The disparity in rate is decreased by about 65% to about 6–8-fold for mitochondrial malate dehydrogenase at pH 9 0, and from about 15- to less than 2-fold for the supernatant enzyme catalyzed reaction, suggesting that chemical transformation may be approaching a rate-limiting condition for the supernatant enzyme

As previously observed for all dehydrogenases studied, the initial rate of substrate reduction is greater than both equilibrium rates, from which deductions may be made concerning certain rate constants[17]

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REFERENCES

1 A Dalbruck, H Schimassek, K Bartsch and T Bucher, *Biochem Z* , 331 (1959) 297
2 A Delbruck, E Zebe and T Bucher, *Biochem Z* , 331 (1959) 273
3 T Wieland, G Pfleiderer, I Haupt and W Worner, *Biochem Z* , 332 (1959) 1
4 C J R Thorne, *Biochim Biophys Acta*, 42 (1960) 175
5 L Siegel and S Englard, *Biochim Biophys Acta*, 54 (1961) 67
6 S Englard and H H Breiger, *Biochim Biophys Acta*, 56 (1962) 571
7 G B Kitto and N O Kaplan, *Biochemistry*, 5 (1966) 3966
8 D N Raval and R G Wolfe, *Biochemistry*, 1 (1962) 263
9 D N Raval and R G Wolfe, *Biochemistry*, 1 (1962) 1118
10 D N Raval and R G Wolfe, *Biochemistry*, 1 (1962) 1112
11 D N Raval and R G Wolfe, *Biochemistry*, 2 (1963) 220
12 E Heyde and S Ainsworth, *J Biol Chem* , 243 (1968) 2413
13 P D Boyer and E Silverstein, *Acta Chem Scand* , 17 (1963) S195
14 E Silverstein and P D Boyer, *J Biol Chem* , 239 (1964) 3908
15 E Silverstein and P D Boyer, *J Biol Chem* , 239 (1964) 3901
16 H J Fromm, E Silverstein and P D Boyer, *J Biol Chem* , 239 (1964) 3645
17 E Silverstein and G Sulebele, *Biochemistry*, 8 (1969) 2543
18 B L Horecker and A Kornberg, *J Biol Chem* , 175 (1948) 385
19 E Silverstein, Ph D Thesis, University of Minnesota, 1963
20 M Cassman and S Englard, *J Biol Chem* , 241 (1966) 787
21 F C Grimm and D G Doherty, *J Biol Chem* , 236 (1961) 1980
22 C J R Thorne and N O Kaplan, *J Biol Chem* , 238 (1963) 1861
23 C Cennamo, G Montecuccoli and G Konig, *Biochim Biophys Acta*, 139 (1967) 514
24 D J Blonde, E J Kresack and G W Kosicki, *Can J Biochem* , 45 (1967) 641
25 B K Joyce and S Grisolia, *J Biol Chem* , 236 (1961) 725
26 L Siegel, *Biochemistry*, 6 (1967) 2261
27 K Harada and R G Wolfe, *J Biol Chem* , 243 (1968) 4131
28 M Cassman and S Englard, *J Biol Chem* , 241 (1966) 793