Metabolic Control and Structure of Glycolytic Enzymes. VI. Competitive Inhibition of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by Cyclic Adenosine Monophosphate, Adenosine Triphosphate, and Other Adenine-Containing Compounds*

Shih Tzy Yang† and W. C. Deal, Jr.

ABSTRACT: The existence and magnitude of effects of adenine nucleotides on the catalytic activity and the structure of yeast glyceraldehyde 3-phosphate dehydrogenase were evaluated for their significance to metabolic controls. The results also led to an elucidation of the principal features of the mechanism of binding of nicotinamide–adenine dinucleotide to the enzyme. The two nucleotide moieties in nicotinamide–adenine dinucleotide were found to have almost totally separate functions, the adenine nucleotide moiety being mainly responsible for binding, and the nicotinamide nucleotide moiety for catalysis. Thus, glyceraldehyde 3-phosphate dehydrogenase was inhibited by the following compounds (K_i values in parentheses): adenine (10.2 mM), adenosine (2.1 mM), adenosine 5′-monophosphate (1.1 mM), adenosine diphosphate (1.5 mM), adenosine triphosphate (2.5 mM), adenosine 3′,5′-cyclic monophosphate (0.11 mM), and pyridine 3-aldehyde nicotinamide–adenine dinucleotide (0.27 mM). In each case, the inhibition was competitive with respect to nicotinamide–adenine dinucleotide (K_m = 0.18 mM). Glyceraldehyde 3-phosphate dehydrogenase was not inhibited by nicotinamide mononucleotide. Therefore, the adenine nucleotide moiety was bound, and was essential for binding, while the nicotinamide was neither. The 6-amino group of the adenine moiety and 2′-hydroxyl group of the adenine-linked ribose moiety were essential for binding. A 5′-phosphate linked to adenosine greatly aided the binding, if it was a diester. The inhibition was fairly specific for purines, especially for adenine-containing compounds. Thus, the compounds 2′-deoxyadenosine, 2′-deoxyadenosine 5′-monophosphate, 2′-deoxyadenosine 5′-triphosphate, adenosine 2′-monophosphate, adenosine 3′-monophosphate, adenosine 2′,3′-cyclic monophosphate, nicotinamide–adenine dinucleotide phosphate, inosine, inosine 5′-monophosphate, nicotinamide–6-deaminoadenine dinucleotide, nicotinamide mononucleotide, guanosine triphosphate, uridine triphosphate, and cytidine triphosphate gave little or no inhibition. The adenosine 3′,5′-cyclic monophosphate was an extremely potent inhibitor and was bound to the enzyme even more tightly than nicotinamide–adenine dinucleotide. The inhibition of glyceraldehyde 3-phosphate dehydrogenase by adenosine 3′,5′-cyclic monophosphate, adenosine triphosphate, and other adenine-containing compounds merits consideration as a mechanism for control of glyceraldehyde 3-phosphate dehydrogenase activity. Binding of adenine-containing compounds may also be important in control of the level of glyceraldehyde 3-phosphate dehydrogenase.

Glyceraldehyde 3-phosphate dehydrogenase constitutes about 19% of the total soluble protein in yeast (Krebs *et al.*, 1953) and about 10% in rabbit muscle (Cori *et al.*, 1948). The natural abundance of glyceraldehyde 3-phosphate dehydrogenase is something of an enigma. Its maximal specific activity is relatively high, so that apparently does not provide a need for its abundance. However, it has been pointed out (Cori *et al.*, 1948) that at the low pH found in muscle cells, the catalytic activity of glyceraldehyde 3-phosphate dehydrogenase would be only a fraction of its maximal activity at higher pH values. This may explain the abundance of this enzyme in muscle, but it does not explain its abundance in yeast.

How the activity and level of glyceraldehyde 3-phosphate dehydrogenase are controlled, and how this control relates to the control of glycolysis are major questions. In an effort to evaluate the possibility of control of the glycolytic pathway at the glyceraldehyde 3-phosphate dehydrogenase step, we undertook a systematic examination of the effect of a series of important metabolites and related compounds on the activity and structure of glyceraldehyde 3-phosphate dehydrogenase from both yeast and rabbit muscle sources.

In making a decision on which metabolites were to be analyzed initially, we considered the results of previous studies on the control of carbohydrate metabolism. In mammals and in some microorganisms the metabolism of carbohydrates has been shown to be controlled by adenine nucleotides at several physiologically "one-way" steps, *i.e.*, phosphorylase, glycogen synthetase, phosphofructokinase, fructose 1,6-diphosphatase, and pyruvate kinase (for reviews, see Atkinson, 1965, 1966; Stadtman, 1966; Wood, 1966). Accordingly, we began the analysis with studies of the effect of adenine nucleotides on yeast glyceraldehyde 3-phosphate dehydrogenase structure and activity, and later broadened the study to include other adenine-containing compounds.

We have found that a series of adenine-containing com-

* From the Department of Biochemistry, Michigan State University, East Lansing, Michigan. *Received January 8, 1969.* Paper V in this series is the work of Stancel and Deal (1968). Supported in part by grants from the National Institute of General Medical Sciences, U. S. Public Health Service (GM-11170), and the Michigan Agricultural Experiment Station (Hatch 932; Publication No. 4499).

† Present address: Wilson Laboratories, Chicago, Ill. 60609.

TABLE I: Summary of Inhibition of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by Various Compounds.[a]

Compound	K_i (mM)	Inhibition (%) 1 mM	5 mM	10 mM
Strong Inhibitor Class				
3′,5′-Cyclic AMP	0.11	70	94	100
Pyridine 3-aldehyde-NAD	0.27	50	94	100
Moderate Inhibitor Class				
5′-AMP	1.1	18	50	64
ADP	1.5	14	44	58
ATP	2.5	10	38	50
Adenosine	2.1	8	34	52
Weak Inhibitor Class				
dATP		4	21	30
dAMP		4	17	30
2′-Deoxyadenosine		4	15	30
GTP		0	25	40
CTP		0	12	24
UTP		0	12	24
Adenine	10.2	0	14	
2′-AMP		0	8	20
3′-AMP		0	14	20
NADP		0	16	27
5′-IMP		0	5	10
Inosine		0	5	10
2′,3′-Cyclic AMP[b]		0	0	0
Ribose 5-phosphate[b]		0	0	0
NMN[b]		0	0	0

[a] Data taken from Figures 1 to 7, and grouped into three classes according to effectiveness of inhibition. [b] Noninhibitory.

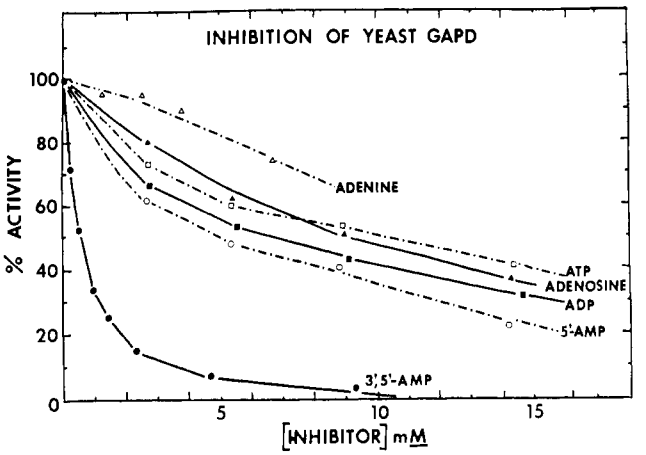

FIGURE 1: Effects of adenine, adenosine, 5′-AMP, ADP, ATP, and 3′,5′-cyclic AMP on the enzymic activity of yeast glyceraldehyde 3-phosphate dehydrogenase. The activity was assayed at 25° in a 33 mM glycylglycine buffer (pH 7.8) containing 0.1 M KCl, 6.6 mM sodium arsenate, 6.6 mM cysteine, 0.5 mM glyceraldehyde 3-phosphate, 0.77 mM NAD, and 2.2 × 10^{-5} mM yeast glyceraldehyde 3-phosphate dehydrogenase. See Methods for details.

pounds produce three distinct (but apparently related) effects on the enzymic activity and structure of yeast glyceraldehyde 3-phosphate dehydrogenase. They are: (a) an *instantaneous inhibition of enzymic activity* produced by adenine, adenosine, AMP, ADP, ATP, and 3′,5′-cyclic AMP, due to their competition with NAD for the NAD binding site on the enzyme (the present work); (b) a *slow loss of enzymic activity* produced by AMP, ADP, and ATP, due to a *slow, marked* structural change (inactivation and dissociation into subunits) induced upon binding of these nucleotides to the enzyme (Stancel and Deal, 1968; Yang and Deal, 1969); and (c) a *very rapid loss of activity*, produced by ADP and ATP in the presence of chymotrypsin, due to very *rapid, subtle*, structural changes induced upon binding these nucleotides to the enzyme (Yang and Deal, 1969). Analysis and interpretation of these results has provided not only an evaluation of the existence and magnitude of effects of adenine nucleotides on the activity and structure of yeast glyceraldehyde 3-phosphate dehydrogenase, but also with considerable information about the mechanism of binding of NAD to this enzyme.

Comparative studies with rabbit muscle glyceraldehyde 3-phosphate dehydrogenase have shown it to also be inhibited by adenine-containing nucleotides (S. T. Yang and W. C. Deal, in preparation) and dissociated into subunits by ATP (Constantinides and Deal, 1968a), ammonium sulfate (Constantinides and Deal, 1967), and KCl (Constantinides and Deal, 1968b).

Results

Inhibition of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by Adenine, Adenosine, AMP, ADP, ATP, and 3′,5′-Cyclic AMP. As shown in Figure 1, all the adenine compounds in the series listed above inhibited yeast glyceraldehyde 3-phosphate dehydrogenase activity. According to their effectiveness of inhibition (at an NAD concentration[1] of 0.77 mM) these inhibitors were roughly divided into three classes: (a) *the strong inhibitor class*, which consisted of only 3′,5′-cyclic AMP, which produced 50% inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase activity at 0.5 mM concentration; (b) *the moderate inhibitor class*, which included adenosine, AMP, ADP, and ATP, which produced 50% inhibition at 5–10 mM concentrations; and (c) *the weak inhibitor class*, which consisted of only adenine, which produced about 20% inhibition of activity at 5 mM concentration[1] (also see Table I). As will be seen later (Figures 3–6 and Table I), other purine and pyrimidine compounds, including 2′-AMP, 3′-AMP, 2′,3′-cyclic AMP, deoxyadenosine, dAMP, dATP, inosine, IMP, GTP, CTP, UTP, and NADP, were either noninhibitory or belonged to the weak inhibitor class.

Kinetics of Inhibition of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by Adenine, Adenosine, AMP, ADP, ATP, and 3′,5′-Cyclic AMP. Since there are structural features common to the inhibitors shown in Figure 1 and NAD, we ex-

[1] It should be noted that the NAD concentration, 0.77 mM, used in these inhibition experiments (Figures 1 and 3–7) was not the K_m concentration for NAD, so the inhibitor concentration giving 50% inhibition is not equal to K_i. The K_m for NAD was determined to be 0.18 mM (Figure 2) under the present activity assay conditions (see Methods).

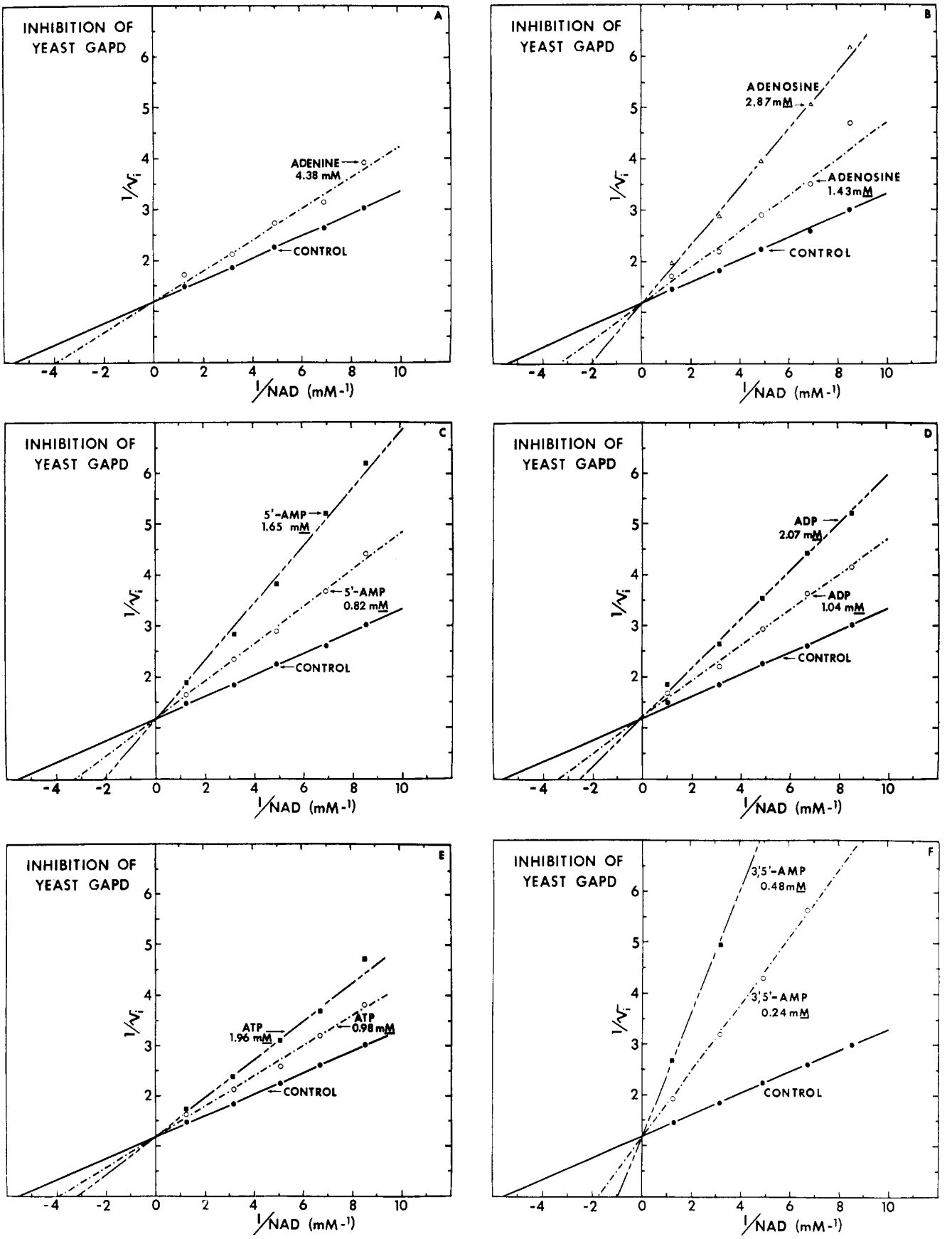

FIGURE 2: Lineweaver–Burk plots of the inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase by adenine (A), adenosine (B), 5′-AMP (C), ADP (D), ATP (E), and 3′,5′-cyclic AMP (F) at concentrations indicated, at 25° and pH 7.8. Glyceraldehyde 3-phosphate dehydrogenase activity was assayed as described in the legend of Figure 1.

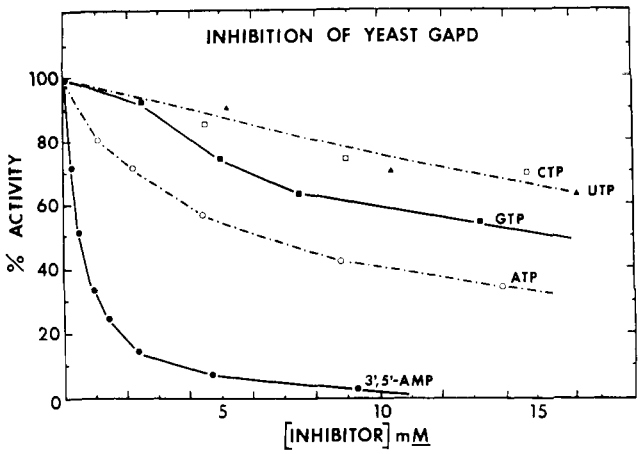

FIGURE 3: Effects of GTP, UTP, and CTP on the enzymic activity of yeast glyceraldehyde 3-phosphate dehydrogenase. The data for ATP and 3′,5′-cyclic AMP (from Figure 1) are included for comparison. See the legend of Figure 1 and Methods for conditions of activity assay.

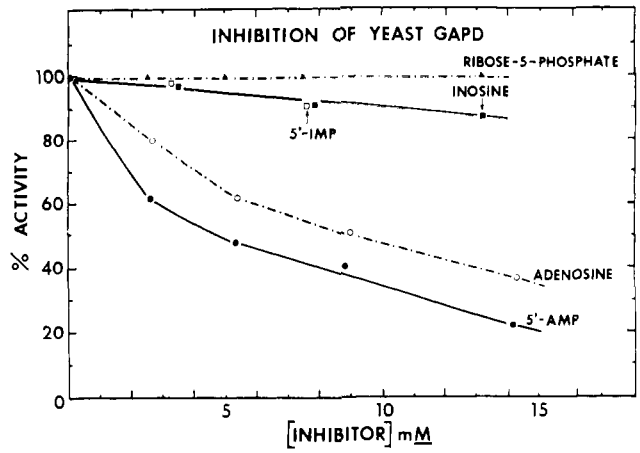

FIGURE 4: Effects of inosine, IMP, and ribose 5-phosphate on the enzymic activity of yeast glyceraldehyde 3-phosphate dehydrogenase. Data for adenosine and AMP (from Figure 1) are included for comparison.

amined the effect of NAD concentration on the inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase by these inhibitors, to see whether NAD and the nucleotides were binding at the same site. The Lineweaver–Burk plots are shown in Figure 2.

The inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase by adenine (Figure 2A) appeared to be competitive with respect to NAD concentration. The apparent inhibition constant, K_i, for adenine calculated from this plot was 10.2 mM, which was about 56 times higher than the value of 0.18 mM for the apparent Michaelis constant, K_m, for NAD.

Figure 2B shows that at two different concentrations of adenosine, the inhibition was competitive with respect to NAD. Thus, the same mechanism appeared to be operating in the inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase by both adenine and adenosine. Furthermore, the K_i of 2.11 mM for adenosine was about one-fifth that for adenine, which suggested that the ribose moiety caused adenosine to bind more strongly than adenine to the enzyme.

Figures 2C–E show the inhibition of glyceraldehyde 3-phosphate dehydrogenase by AMP, ADP, and ATP, respectively, also at two different concentrations for each inhibitor. Like adenine and adenosine, all three of these compounds were competitive with respect to NAD. The values of K_i were 1.1, 1.5, and 2.5 mM for AMP, ADP, and ATP, respectively. The similarity in values for these constants and that for adenosine (2.11 mM) suggested that attachment of a monophosphate, diphosphate or triphosphate at the 5′ position of adenosine had no pronounced effect on its binding to the NAD site of the enzyme.

However, 3′,5′-cyclic AMP was a far better inhibitor than any of the other compounds tested (Figures 1 and 2F). It was bound to yeast glyceraldehyde 3-phosphate dehydrogenase even more tightly than the coenzyme, NAD, as shown by the fact that the K_i for 3′,5′-cyclic AMP was 0.11 mM, while the K_m for NAD was 0.18 mM. This suggested that the 5′-phosphate group greatly aided the binding, but only if the terminal phosphate was a diester. This idea was supported by later evidence that free terminal phosphates on adenosine phosphates have strong destabilizing effects on glyceraldehyde 3-phosphate dehydrogenase, resulting from electrostatic repulsions (Yang and Deal, 1969). Like the other compounds, the inhibition by 3′,5′-cyclic AMP was also competitive with respect to NAD (Figure 2F).

Inhibition of Yeast Glyceraldehyde 3-Phosphate Dehydrogenase by Other Purine and Pyrimidine Compounds. Figure 3 shows the effects of GTP, CTP, and UTP on yeast glyceraldehyde 3-phosphate dehydrogenase activity. The data for ATP and 3′,5′-cyclic AMP are also included for comparison. GTP, a close purine analog to ATP, exhibited some inhibition throughout the concentration range tested. However, the inhibition was much less than that of ATP, and negligible compared with that of 3′,5′-cyclic AMP. Hence, the 6-amino group of the adenine moiety appeared to be of prime importance in the binding of ATP to the NAD binding site of the enzyme. There was very little inhibition by either CTP or UTP, particularly at low concentrations. It was, therefore, concluded that the inhibition was generally specific for purine compounds, and particularly specific for adenine compounds.

Binding Dependence upon the 6-Amino Group of Adenine. To further analyze the importance of the 6-amino group in the binding of NAD or NAD inhibitors to yeast glyceraldehyde 3-phosphate dehydrogenase, inosine and IMP (the respective 6-deamino analogs of adenosine and AMP, respectively), were tested for their inhibition effect. The results are shown in Figure 4, along with the data for adenosine and AMP for comparison. Neither inosine nor IMP significantly inhibited yeast glyceraldehyde 3-phosphate dehydrogenase activity.

Pullman *et al.* (1952) and Stockell (1959) have shown that 6-deamino-NAD is bound to glyceraldehyde 3-phosphate dehydrogenase and serves as a cofactor of the glyceraldehyde 3-phosphate dehydrogenase reaction. Under our assay conditions, 6-deamino-NAD had a K_m of 13.3 mM, which was only about $^1/_{74}$ that of NAD, while its V_m was about the same as that of NAD. These results clearly illustrated the importance of the 6-amino group of the adenine moiety for the binding of NAD and its competitive inhibitors at the NAD binding site of the enzyme.

Binding Dependence upon a Free 2′-Hydroxyl on the Adenine-Linked Ribose. Since adenosine, AMP, ADP, and ATP seemed

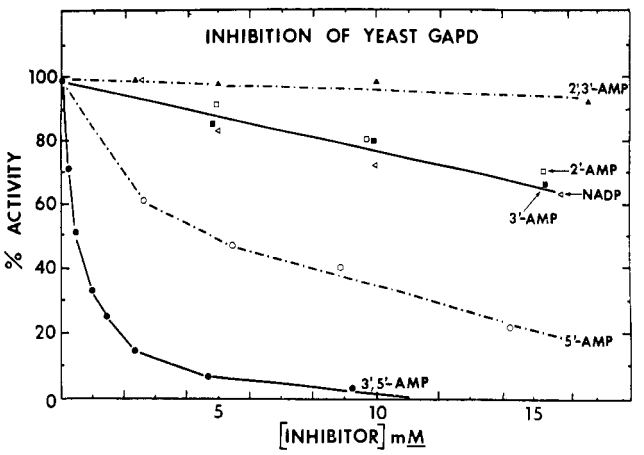

FIGURE 5: Effects of 2'-AMP, 3'-AMP, 2',3'-cyclic AMP, and NADP on the enzymic activity of yeast glyceraldehyde 3-phosphate dehydrogenase. Data for ATP and 3',5'-cyclic AMP (from Figure 1) are included for comparison.

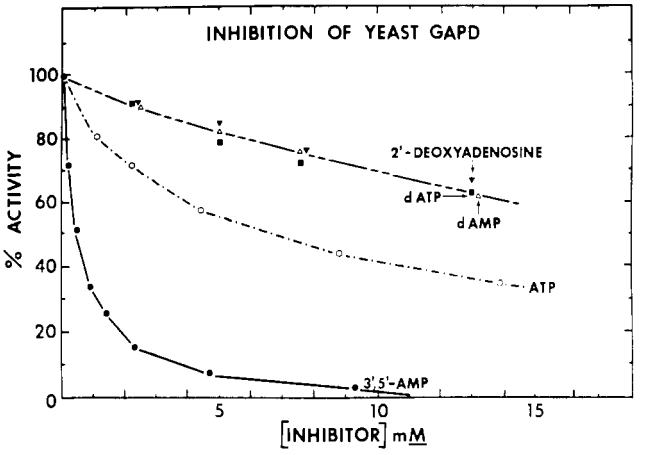

FIGURE 6: Effects of 2'-deoxyadenosine, dAMP, and dATP on the enzymic activity of yeast glyceraldehyde 3-phosphate dehydrogenase. Data for ATP and 3',5'-cyclic AMP (from Figure 1) are included for comparison.

to differ very little from one another in their binding affinities to yeast glyceraldehyde 3-phosphate dehydrogenase, and since adenosine had a higher binding affinity for the enzyme than adenine, it was evident that the ribose moiety played an important part in the binding of NAD and its competitive inhibitors to the enzyme. However, ribose by itself appeared to have no binding affinity to the NAD binding site of the enzyme, since ribose 5-phosphate failed to inhibit the yeast glyceraldehyde 3-phosphate dehydrogenase activity at concentrations as high as 13 mM (Figure 4). The binding of ribose, therefore, apparently required the simultaneous presence of an adenine moiety. Thus, there appeared to be a cooperative interaction between the adenine and ribose components of adenosine.

To determine which, if any, positions on the ribose ring were critical for binding, we made a study of the relative importance of the free hydroxyl groups on the 2'- and 3'-carbons of ribose. For this study several compounds, including 2'-AMP, 3'-AMP, and 2',3'-cyclic AMP, were examined for their ability to inhibit the activity of yeast glyceraldehyde 3-phosphate dehydrogenase. As seen in Figure 5, 2'-AMP and 3'-AMP had only slight inhibition, and 2',3'-cyclic AMP was totally without effect. These results, together with the fact that 3',5'-cyclic AMP was strongly inhibitory, strongly indicated a requirement for an unoccupied 2'-hydroxyl group for the binding of NAD or its inhibitors to the enzyme. The importance of an unoccupied 2'-hydroxyl group was also illustrated by the lack of inhibition produced by NADP (Figure 5), which differs from NAD only by having a phosphate ester at the 2'-carbon of the adenine-linked ribose.

To test for a positive contribution to binding from the 2'-hydroxyl group, 2'-deoxyadenosine, dAMP, and dATP were examined for inhibition. As seen in Figure 6, none exerted any significant inhibition. In fact, the absence of a free 2'-hydroxyl group, as in 2'-AMP, NADP, 2'-deoxyadenosine, dAMP, and dATP, caused the inhibition curves to become more like that of adenine (Figures 5 and 6). This suggested that substitution, or removal, of this 2'-hydroxyl group eliminated the contribution of the ribose moiety to the binding. Figure 6 also shows that 5'-diphosphate and triphosphate groups made no positive contributions to binding in the 2'-deoxy derivatives, since the data for the deoxynucleoside phosphates fell on the same line as the deoxynucleoside.

Lack of Binding Dependence upon the Nicotinamide Mononucleotide Moiety. The inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase by pyridine 3-aldehyde-NAD$^+$ and NMN is shown in Figure 7. Pyridine 3-aldehyde-NAD is a potent inhibitor of glyceraldehyde 3-phosphate dehydrogenase enzymes from both yeast and rabbit muscle (Kaplan *et al.*, 1956). From a Lineweaver–Burk plot, we found the K_i for pyridine 3-aldehyde-NAD to be 0.27 mM, which is comparable with the K_m of 0.18 mM for NAD. This apparently excluded the possibility that the amide group of the nicotinamide moiety participated significantly in the binding of NAD to the enzyme. Furthermore, the failure of NMN to compete with NAD for the NAD binding site also seemed to exclude the possibility that any portions of the NMN moiety of NAD had a major role in the binding of the nucleotide to the enzyme.

Discussion

Binding and Catalytic Components of NAD. The functioning of NAD as a coenzyme involves two operations: (1) binding to the enzyme, and (2) actual catalysis. The data in Table I indicate that for yeast glyceraldehye 3-phosphate dehydrogenase, the relationship between NAD structure and function is quite clear-cut, in that one of the two nucleotides, the adenine nucleotide, is primarily involved in binding, while the other, the nicotinamide nucleotide, is involved in catalysis.

In particular, it is concluded that neither the nicotinamide nucleotide nor the pyrophosphate group is necessary for, or strongly involved in, the binding. The adenosine-linked 5'-phosphate greatly aids the binding if it is not a free terminal phosphate, or linked to a free terminal phosphate. But even it is not essential for binding; adenosine apparently provides a substantial portion of the binding force. A structural component which seems to be absolutely essential is the adenosine-2'-hydroxyl. However, a free adenosine-3'-hydroxyl is not essential, since 3',5'-AMP binds even more tightly than the

natural coenzyme, NAD. But substitution at the 3′ position is limited, since 3′-AMP does not bind. There is a pronounced specificity for adenine as the nucleotide base, over other purines or pyrimidines.

Other positions and components of NAD have previously been postulated to be important in binding. Windmueller and Kaplan (1961) concluded that the 1 position of adenine was even more critical than the 6-amino for catalytic activity. Stockell (1959) concluded that the 6-amino group of adenine and the amide group of the pyridine moiety were important, but not absolutely necessary, for the binding of NAD. Stockell's dissociation constants are much smaller than our K_m values for corresponding compounds. The reasons for such differences have been discussed elsewhere (Velick and Furfine, 1963).

Other workers (Racker and Krimsky, 1952; Velick, 1953; Racker, 1965) have stressed the importance of a spectrally active complex between position 4 on the NAD pyridine ring and a sulfhydryl group on the enzyme, although recognizing that more than one segment of NAD was involved in the binding.

We suggest that the pyridine–enzyme complex is the result of, not the cause of, the binding of NAD to the enzyme. This idea is supported by our results indicating that 3′,5′-cyclic AMP, which has no nicotinamide ring, binds even better than NAD. It is further supported by the observation that pyridine 3-aldehyde-NAD, which does not form the spectrally active complex, also binds better than NAD to rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Kaplan *et al.*, 1957). These two observations raise the possibility that formation of the spectrally active complex may even decrease the binding ability of the NAD somewhat, rather than aiding it.[2]

In our view, the purpose of forming the spectrally active complex is to make NAD more reactive catalytically: the 4 position on the pyridine ring is known to be the site of catalytic function, the acceptance of hydride ions. Other studies have analyzed the structural requirements of NAD for catalysis (see Anderson and Kaplan, 1959, and references therein).

Is Binding by the Adenine Nucleotide Portion of NAD a General Property of Dehydrogenases? The importance of the adenine ring in the binding of NAD has been demonstrated for alcohol dehydrogenases from yeast (Wallenfels *et al.*, 1957; Hoch *et al.*, 1960; Anderson and Reynolds, 1965; Anderson *et al.*, 1965) and from liver (Yonetani, 1963; Theorell and Yonetani, 1964; Theorell, 1964). It has also been demonstrated to play an important role in the binding of NAD to rabbit muscle α-glycerophosphate dehydrogenase (Kim and Anderson, 1968). However, "multiple inhibition analysis" (Yonetani and Theorell, 1964) has indicated the involvement of different regions of NAD in binding these enzymes (Yonetani and Theorell, 1964; Anderson and Reynolds, 1965; Fonda and Anderson, 1967; Kim and Anderson, 1968).

Our results and those of previous workers suggest a new dimension in adenine nucleotide control of metabolism:

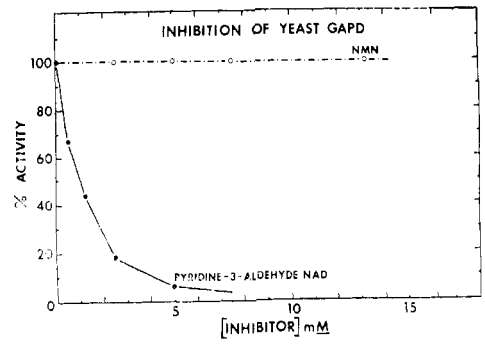

FIGURE 7: Effects of nicotinamide mononucleotide and pyridine 3-aldehyde-NAD on the enzymic activity of yeast glyceraldehyde 3-phosphate dehydrogenase.

control of certain dehydrogenases (and, perhaps, other enzymes with adenine-containing coenzymes). These results may also have a bearing on the evolution of certain compounds in such a way as to contain adenine, or adenosine and, as a result, to elevate the status and importance of adenine-containing compounds in metabolism and its control.

Inhibition of Glyceraldehyde 3-Phosphate Dehydrogenase by Adenine Nucleotides and Significance for Metabolic Control. The discovery that 3′,5′-cyclic AMP is an extremely potent inhibitor of glyceraldehyde 3-phosphate dehydrogenase raises the question of how this effect might be involved in metabolic control in yeast and other forms of life. The following working hypothesis provides a possible mechanism for such control. It invokes a glyceraldehyde 3-phosphate dehydrogenase controlled FDP condensor, with coordinated control of phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, and pyruvate kinase by 3′,5′-cyclic AMP and FDP. It has oscillatory characteristics and could explain the effects of 3′,5′-cyclic AMP on yeast observed by Chance and coworkers (Chance and Schoener, 1964; Chance *et al.*, 1965).

Consider a cell in a state with ATP relatively high and FDP and glycolytic flux low. Since ATP inhibits pyruvate kinase and the level of FDP is too low to activate the enzyme, the flux of the triose phosphate metabolites of glycolysis is slow and their levels are relatively high. In particular, the level of 1,3-diphosphoglyceric acid is high enough to produce significant inhibition of glyceraldehyde 3-phosphate dehydrogenase; but FDP does not build up, because phosphofructokinase is also inhibited and makes very little FDP.

Assume that the level of cyclic AMP is then increased markedly by some means. Phosphofructokinase is activated and FDP production is drastically increased. But since glyceraldehyde 3-phosphate dehydrogenase is markedly inhibited, FDP utilization is not increased immediately, at least not to the same extent as FDP production. Therefore, the FDP level rises. Thus, glyceraldehyde 3-phosphate dehydrogenase acts like a condensor, to cause a build-up of FDP.

Pyruvate kinase is postulated to discharge the glyceraldehyde 3-phosphate dehydrogenase controlled FDP condensor. When the FDP level gets high enough, pyruvate kinase is activated, thereby quickly decreasing the level of 1,3-diphosphoglycerate. Consequently, glyceraldehyde 3-phosphate dehydrogenase becomes very active, the FDP level is lowered, and the NADH level is increased. This causes some product inhibition of glyceraldehyde 3-phosphate dehydro-

[2] The fact that ADP does not bind as well as NAD does not exclude this possibility. The additional negative charge on the terminal phosphate has been shown to have a destabilizing effect on yeast glyceraldehyde 3-phosphate dehydrogenase (see Figure 4 of Yang and Deal, 1969).

genase. Also, as the FDP is lowered, pyruvate kinase becomes deactivated, resulting in a build-up of 1,3-diphosphoglycerate and an additional inhibition of glyceraldehyde 3-phosphate dehydrogenase. This brings us back to the initial stages of the process, which can then be repeated. In this model, any intermittent release of a fixed amount of 3,5′-cyclic AMP could cause a continuous oscillation. Also, AMP might produce effects qualitatively similar to those of 3′,5′-cyclic AMP.

Chance and coworkers also found that in cell-free yeast extracts, 3′,5′-cyclic AMP increased the steady-state NAD level and accelerated the rate of NADH oxidation. They attributed these effects to a possible activation at a site between 1,3-diphosphoglycerate kinase and alcohol dehydrogenase, and at another unknown glycolytic site. We have found that 3 mM 3′,5′-cyclic AMP does not activate yeast alcohol dehydrogenase at an NADH concentration of 0.1 mM, excluding this as a main control site. But inhibition of glyceraldehyde 3-phosphate dehydrogenase by 3′,5′-cyclic AMP could lead to a decreased rate of NAD reduction and a corresponding increase in the steady-state level of NAD.

Inhibition of glyceraldehyde 3-phosphate dehydrogenase by 3′,5′-cyclic AMP could also be part of the explanation for the accelerated rate of NADH oxidation. Added 3′,5′-cyclic AMP would activate phosphofructokinase (counteracting ATP inhibition) and inhibit glyceraldehyde 3-phosphate dehydrogenase, thereby increasing the FDP level greatly. The high FDP and the 3′,5′-cyclic AMP would stimulate pyruvate kinase to produce more pyruvate and consequently accelerate the rate of NADH oxidation by alcohol dehydrogenase.

This postulated model is based on the following observations by previous workers. Embryonic pyruvate kinase is strongly activated by either FDP or 3′,5′-cyclic AMP, and the activation is synergistic when both are present (Milman and Yurowitzki, 1967). Phosphofructokinases from liver fluke and from pig heart are inhibited by ATP but the inhibition is reversed by 3′,5′-cyclic AMP (Mansour and Mansour, 1962; Mansour, 1963).

In Vivo Levels. The adenine-containing compounds which seem to merit consideration as regulatory inhibitors *in vivo* are 3′,5′-cyclic AMP and ATP. For the yeast system at least, all the others have K_i values greater than 1 mM and *in vivo* concentrations considerably less than this (Betz and Moore, 1967). Since 3′,5′-cyclic AMP, ATP, and NAD compete for the same binding site, their concentrations and binding constants, as well as the glyceraldehyde 3-phosphate dehydrogenase concentration, will determine whether these nucleotides are involved in regulating glyceraldehyde 3-phosphate dehydrogenase enzymes in various forms of life. Although rabbit muscle glyceraldehyde 3-phosphate dehydrogenase binds these nucleotides less tightly than yeast glyceraldehyde 3-phosphate dehydrogenase (S. T. Yang, and W. C. Deal, in preparation), it seems likely that glyceraldehyde 3-phosphate dehydrogenase enzymes from various sources with metabolic capabilities similar to those in yeast will more closely resemble yeast glyceraldehyde 3-phosphate dehydrogenase than rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. Assuming the *in vivo* yeast glyceraldehyde 3-phosphate dehydrogenase weight concentration to be about 5 mg/ml, its molar concentration is about 4×10^{-5} M.

In connection with our previous discussion of the experiments of Chance and coworkers (Chance and Schoener, 1964; Chance *et al.*, 1965), we have pointed out that 3′,5′-cyclic AMP concentrations in the range of 0.5 mM would significantly inhibit glyceraldehyde 3-phosphate dehydrogenase. However, evaluation of the significance of glyceraldehyde 3-phosphate dehydrogenase inhibition by 3′,5′-cyclic AMP as a regulatory mechanism must await a knowledge of its levels *in vivo*.

The ATP concentration in most cells is probably considerably greater than the K_i of 2.5 mM which we found for yeast glyceraldehyde 3-phosphate dehydrogenase. Estimates of the levels of ATP have ranged from 1 to 2 mM for yeast (Betz and Moore, 1967) to 7 to 8 mM for guinea pig heart (Mansour, 1963).

The average NAD concentration in cells such as liver and yeast is probably near the value of 0.6 mM estimated for rat liver (Jacobson and Kaplan, 1957). There are difficulties in obtaining the free NAD concentration (see Yang and Deal, 1969). Since the K_m for NAD is 0.18 mM for yeast glyceraldehyde 3-phosphate dehydrogenase, inhibition by ATP would not be appreciable until the NAD concentration fell considerably below the K_m value. It seems likely that the NAD concentration fluctuates considerably with marked changes in the availability of fuel supplies, so this might be significant. The inhibition by 3,5′-cyclic AMP would be significant if the 3′,5′-cyclic AMP concentration was as great as 0.1 mM (or, if the NAD concentration were lower, at even lower concentrations).

Materials and Methods

Nucleotides. Adenine, adenosine, AMP, ADP, ATP, 3′,5′-cyclic AMP, 2′-AMP, 2′,3′-cyclic AMP, NMN, and ribose 5-phosphate were obtained from the Sigma Biochemical Co., while 2′-deoxyadenosine, dAMP, dATP, GTP, CTP, UTP, NAD, deamino-NAD, and NADP were products of the P-L Biochemicals, Inc.

Nucleotides and other compounds used in the inhibition studies were dissolved in 0.05 M glycylglycine buffer (pH 8.5), containing 0.15 M KCl and 0.01 M sodium arsenate. The pH of each resulting solution was carefully adjusted to 8.5 and the solution was used immediately. Concentrations of the nucleotides and other compounds were determined spectrophotometrically.

Enzyme Preparation and Assay. Glyceraldehyde 3-phosphate was obtained from Sigma as the diethylacetal barium salt and converted into the free acid as previously described (Deal, 1969). NAD was obtained from P-L Biochemicals, Milwaukee.

Three- or four-times-crystallized yeast glyceraldehyde 3-phosphate dehydrogenase was prepared from Red Star bakers yeast as previously described (Deal, 1969) and stored as a crystalline ammonium sulfate suspension at a concentration of 5 mg/ml at 0°. For use, a stock enzyme solution was prepared by directly diluting the crystalline suspension 100-fold with a 0.04 M imidazole buffer (pH 6.9), containing 0.12 M KCl, 0.006 M cysteine, and 0.08 mM EDTA. The enzyme solution thus prepared was stable for up to a 1 week with no appreciable loss of enzymic activity when kept at 0°.

Yeast glyceraldehyde 3-phosphate dehydrogenase activity was measured spectrophotometrically with a Gilford Model 2000 recording spectrophotometer by following the initial velocity of increase in absorbance at 340 mμ due to produc-

tion of NADH. For the study of instantaneous inhibition of yeast glyceraldehyde 3-phosphate dehydrogenase by nucleotides and other compounds (see Figures 1 and 3–7), the previously published standard procedure of activity assay (Deal, 1969) was slightly modified as follows. First, a 265-μl portion of a 0.05 M glycylglycine buffer containing 0.15 M KCl, 0.01 M sodium arsenate, and varying concentrations of nucleotide inhibitor at pH 8.5, was added to a microcuvet of 0.4-ml volume maintained at 25°. Then successive additions, with mixing, were made of the following: (1) 10 μl of an aqueous 0.0308 M NAD solution (pH 6), (2) 50 μl of an aqueous 0.038 M cysteine hydrochloride solution, (3) 50 μl of an aqueous 0.004 M glyceraldehyde 3-phosphate solution and, finally (4) 25 μl of enzyme solution. The enzyme solution was always added in the last step to initiate the enzymic reaction.[3] The final reaction mixture (0.4 ml) had a pH of 7.8 and contained 0.77 mM NAD, 6.6 mM sodium arsenate, 0.5 mM glyceraldehyde 3-phosphate, 2.2×10^{-5} mM yeast glyceraldehyde 3-phosphate dehydrogenase, 0.1 M KCl, and 33 mM glyclyglycine buffer. The increase of absorbance at 340 mμ was recorded for 30 sec to 1 min, during which time a linear reaction rate was always observed.

To obtain the Lineweaver–Burk plots, as shown in Figure 2, the activity assays were carried out as described above except that the NAD concentration in the reaction mixture was varied between 0.12 and 0.82 mM.

All the other chemicals and reagents used in this study were either reagent grade or the highest quality available.

References

Anderson, B. M., and Kaplan, N. O. (1959), *J. Biol. Chem. 234*, 1226.

Anderson, B. M., and Reynolds, M. L. (1965), *Arch. Biochem. Biophys. 111*, 1.

Anderson, B. M., Reynolds, M. L., and Anderson, C. (1965), *Biochim. Biophys. Acta 99*, 46.

Atkinson, D. E. (1965), *Science 150*, 851.

Atkinson, D. E. (1966), *Ann. Rev. Biochem. 35*, 85.

Betz, A., and Moore, C. (1967), *Arch. Biochem. Biophys. 120*, 268.

Chance, B., and Schoener, B. (1964), *Biochem. Biophys. Res. Commun. 17*, 416.

Chance, B., Schoener, B., and Elsaesser, S. (1965), *J. Biol. Chem. 240*, 3170.

Constantinides, S. M., and Deal, W. C., Jr. (1967), 154th National Meeting of the American Chemical Society, Chicago, Ill., Abstract No. C-198.

Constantinides, S. M., and Deal, W. C., Jr. (1968a), 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Abstract No. 71.

Constantinides, S. M., and Deal, W. C., Jr. (1968b), *Federation Proc. 27*, 522.

Cori, G. T., Selin, M. W., and Cori, C. F. (1948), *J. Biol. Chem. 173*, 605.

Deal, W. C., Jr. (1969), *Biochemistry 8*, 2795 (this issue; preceding paper).

Fonda, M. L., and Anderson, M. L. (1967), *Arch. Biochem. Biophys. 120*, 49.

Hoch, G. L., Martin, R. G., Wacker, W. E. C., and Vallee, B. L. (1960), *Arch. Biochem. Biophys. 91*, 166.

Jacobson, K. B., and Kaplan, N. O. (1957), *J. Biol. Chem. 226*, 603.

Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E. (1956), *J. Biol. Chem. 221*, 833.

Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E. (1957), *Arch. Biochem. Biophys. 69*, 441.

Kim, S. J., and Anderson, B. M. (1968), *J. Biol. Chem. 243*, 3351.

Krebs, E. G., Rafter, G. W., and Junge, J. M. (1953), *J. Biol. Chem. 200*, 479.

Mansour, T. E. (1963), *J. Biol. Chem. 238*, 2285.

Mansour, T. E., and Mansour, J. M. (1962), *J. Biol. Chem. 237*, 629.

Mendicino, J., and Vasarhely, F. (1963), *J. Biol. Chem. 238*, 3528.

Milman, L. S., and Yurowitzki, Y. G. (1967), *Biochim. Biophys. Acta 146*, 298.

Pullman, M. E., Colowick, S. P., and Kaplan, N. O. (1952), *J. Biol. Chem. 194*, 593.

Racker, E. (1965), Mechanisms in Bioenergetics, New York, N. Y., Academic, p 20.

Racker, E., and Krimsky, I. (1952), *Nature 169*, 1043.

Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.

Stancel, G., and Deal, W. C., Jr. (1968), *Biochem. Biophys. Res. Commun. 31*, 398.

Stockell, A. (1959), *J. Biol. Chem. 234*, 1286, 1293.

Theorell, H. (1964), *Bull. Soc. Chim. Biol. 46*, 1533.

Theorell, H., and Yonentani, T. (1964), *Arch. Biochem. Biophys. 106*, 252.

Velick, S. F. (1953), *J. Biol. Chem. 203*, 563.

Velick, S. F., and Furfine, C. (1963), *Enzymes 7*, 243.

Wallenfels, K., Sund, H., and Diekmann, H. (1957), *Biochem. Z. 329*, 48.

Windmueller, H. G., and Kaplan, N. O. (1961), *J. Biol. Chem. 236*, 2716.

Wood, W. A. (1966), *Ann. Rev. Biochem. 35*, 521.

Yang, S. T., and Deal, W. C., Jr. (1969), *Biochemistry 8*, 2814 (this issue; following paper).

Yonetani, T. (1963), *Acta Chem. Scand. 17*, 596.

Yonetani, T., and Theorell, H. (1964), *Arch. Biochem. Biophys. 106*, 243.

[3] Ordinarily glyceraldehyde 3-phosphate is always added last (Deal, 1969) since it seems to decompose (see also Cori *et al.*, 1948). But for these studies, it was desirable to add enzyme last to ensure against inactivation before measurement of activity.