BBA 65908

KINETIC EFFECTS OF UREA ON THE ACTIVATION OF AGED GLYCOGEN PHOSPHORYLASE *a* BY ADENOSINE 5'-PHOSPHATE

FERMÍN SAGARDÍA* AND JAMES W. GREEN

Department of Physiology and Biochemistry, Rutgers, The State University, New Brunswick, N.J. (U.S.A.)

(Received January 21st, 1969)

SUMMARY

Previous observations have indicated that the sigmoid curve of rate *versus* P_i concentration obtained with aged preparations of phosphorylase *a* (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1), is abolished by the activator AMP. The effects of urea on the kinetics of the enzymatic reaction were investigated in order to help clarify the relationship between structure and function. Urea also abolished the sigmoid curve although at concentrations 10 000-fold higher than AMP. However, although $2 \cdot 10^{-5}$ M AMP reduced the apparent K_m for P_i, 0.3 M urea was a competitive inhibitor with respect to P_i, and 1.0 M urea caused inhibition of the mixed type. Mixed inhibition experiments with urea and arsenate, a true competitor of P_i, and the reduction of the K_m for AMP by both urea and arsenate indicated that urea indeed competed with P_i. Urea did not affect the V for AMP, whereas arsenate reduced it. The data indicated that AMP protected the enzyme against urea inhibition by reducing the affinity of the enzyme for urea. It is suggested that aging causes changes in the structure of the enzyme which allow easier demonstration of homotropic interactions.

INTRODUCTION

Considerable attention has recently been given to the role of AMP in the activation of phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1) In both the *a* and *b* forms, AMP increases the affinity of the enzyme for the substrates[1–5], and it has been suggested that AMP alters the conformation of the enzyme. Thus, AMP favors the dissociation of the tetrameric *a* form into a dimer which is more active than the native enzyme[6]. Other agents which affect the activity of the enzyme alter its state of aggregation[7]. Conformational effects have also been proposed, based on studies of pH and temperature effects[8], of the interaction with antibody fragments[9], of the binding of bromthymol blue by phosphorylase *b* (ref. 10) and of the optical rotatory dispersion of the enzymes[11].

* Present address: Department of Microbiology, University of Puerto Rico School of Medicine, San Juan, Puerto Rico. Send correspondence to senior author.

In a preliminary communication it was stated that under some conditions the saturation curve of aged preparations of phosphorylase *a* with P_i was sigmoid in the absence of AMP but that in its presence the curve was hyperbolic. Also it was shown that high concentrations of arsenate in the absence of AMP abolished the sigmoid curve and that this effect of arsenate[5] was specific. Here are presented further observations on these phenomena. In particular, the effect of urea on the kinetic parameters of the enzymatic reaction are analysed, since urea is known to affect protein conformation in many cases, particularly that of phosphorylase *b* (ref. 12).

EXPERIMENTAL

Phosphorylase a assays

The enzyme was assayed in the direction of glycogen breakdown. The formation of glucose 1-phosphate from glycogen and P_i was followed by measuring the reduction of $NADP^+$ to NADPH in the presence of excess phosphoglucomutase and glucose-6-phosphate dehydrogenase. Except where otherwise indicated the composition of the reaction mixture was 10 mM Tris, 10 mM cysteine, 10 mM $MgCl_2$, 0.24 mM $NADP^+$, 1.0 mg/ml glycogen, 10 mM P_i, 0.3 unit each of glucose-6-phosphate dehydrogenase and phosphoglucomutase and $2 \cdot 10^{-5}$ M AMP, when present. The amount of phosphorylase *a* ranged from 1.6 to 5.0 μg depending on the activity of the preparation and on the particular experiment to be performed. The final volume of the system was 3.0 ml at pH 7.5. The reaction was started by adding the enzyme, and after a lag of 2–3 min, it became a linear function of time. The rate was recorded for 5 min in the linear region using a Beckman-DU spectrophotometer with an attached linear-log Varicord recorder (Model 43, Photovolt Corp.). Initial velocity conditions were maintained throughout the reaction period by keeping the measured rates below 0.02 μmole NADPH per min. In order to eliminate any inhibitory effects which the factors studied had on the auxiliary enzymes of the assay system, these factors were added to the system in absence of phosphorylase *a*. The capacity of the system was then tested by adding glucose 1-phosphate to a final concn. of 0.006 M. When necessary, the amounts of the auxiliary enzymes were increased so that their effective activities remained at 0.3 units each. The results were calculated on the basis of μmoles of NADPH formed per mg of protein per min equals one unit.

Twice crystallized phosphorylase *a*, was purchased from Worthington Biochemical Corporation as a suspension of crystals in $(NH_4)_2SO_4$. The various preparations used had aged several weeks at 5°. The protein was collected by centrifugation and was dissolved in water as needed. The concentration of dissolved phosphorylase was determined from the data of VELICK AND WICKS[13] by using a molar extinction coefficient of $5.8 \cdot 10^5$ at 280 mμ. Phosphoglucomutase and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company or from Calbiochem as suspensions in $(NH_4)_2SO_4$ or as powders. After reconstituting them when needed, they were centrifuged and dissolved in water. $NADP^+$ and AMP were purchased from Sigma Chemical Co. Glycogen was obtained from Fisher Scientific Company. Since AMP was a contaminant in the glycogen, a 5% solution was purified by treating it twice with activated charcoal in the cold, followed by centrifugation and then by filtration. Before and after treatment, aliquots were treated with 1 M HCl until the glycogen was hydrolyzed, and their absorbances were measured at 259 mμ. No de-

tectable absorption remained after the charcoal treatment, whereas strong absorption was observed prior to it. A similar purification procedure has been used by HELMREICH AND CORI[1].

RESULTS

As previously reported, the sigmoid curve obtained when the velocity of the reaction is plotted against the concentration of P_i can be abolished specifically at low concentrations by the activator AMP and by the inhibitor arsenate, both of which have effects on the K_m for P_i (ref. 5). Na_2SO_4, a noncompetitive inhibitor at the 0.01 M concentration (Fig. 1), does not have a similar effect (Fig. 2). In our previous commu-

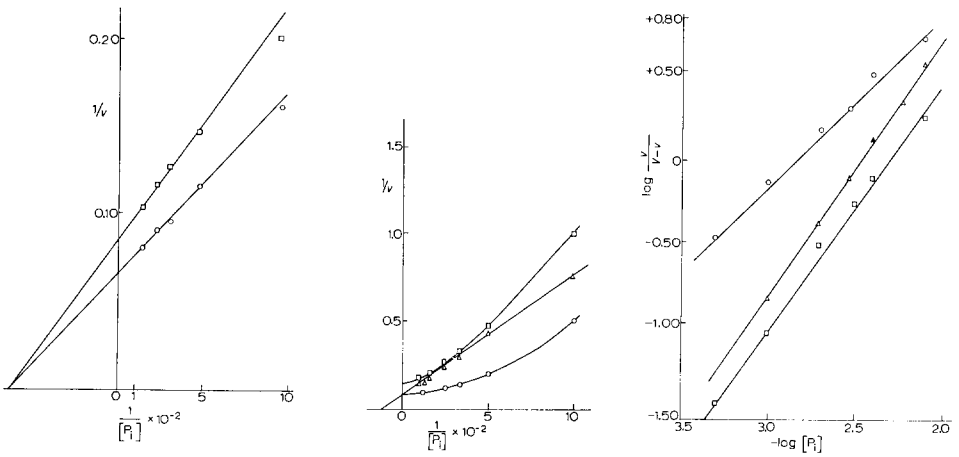

Fig. 1. The effects of 0.01 M Na_2SO_4 on the rate dependence of the reaction with respect to P_i in the presence of $2 \cdot 10^{-5}$ M AMP. ○, control; □, with SO_4^{2-}. K_m for both equals $1.7 \cdot 10^{-3}$ M. V is decreased from 15.1 to 12.0 by SO_4^{2-}.

Fig. 2. The effects of 0.01 M Na_2SO_4 and 0.01 M sodium arsenate on the rate dependence of the reaction with respect to P_i in the absence of AMP. ○, control; □, with SO_4^{2-}; △, with arsenate. K_m in the presence of 0.01 M arsenate is $12 \cdot 10^{-3}$ M P_i. V is reduced by SO_4^{2-} from 12.0 to 7.1.

Fig. 3. The effects of AMP and Na_2SO_4 on a plot of the data according to Equation 1. Slope of the line equals n. ○, in the presence of $2 \cdot 10^{-5}$ M AMP, n equals 0.99; □, no additions, n equals 1.44; △, with 0.01 M SO_4^{2-} and no AMP, $n = 1.48$. The enzyme used for the experiment with SO_4^{2-} was from a different lot and had a different specific activity from the lot used for the other two curves.

nication it was stated that SO_4^{2-} increases the sigmoid character of the curve. This implies that SO_4^{2-} is a negative effector and thus modifies interactions of substrates. However, this apparent effect is due to the relative distortions of the curves in Lineweaver–Burk plots. An analysis of the data in terms of the equation:

$$\log v/V - v = n \log [S] + \log K \qquad (1)$$

proposed by MONOD *et al.*[14], for the case of sigmoidal rate curves, shows that the value of n is not changed by SO_4^{2-} (Fig. 3). ATKINSON *et al.*[15] have indicated some of the possible pitfalls in the interpretation of the data when sigmoid rate curves are obtained. Confidence in the positive effect of AMP is given by the values of Fig. 3 which show that in its presence the value of n approaches 1.0. In addition, it should be mentioned

TABLE I

EFFECT OF INCREASING UREA CONCENTRATIONS ON THE ACTIVITY OF PHOSPHORYLASE *a*

Urea was added directly to the reaction mixture. The reaction was started with enzyme and measured as described in EXPERIMENTAL.

Urea (molar concn.)	*Velocity of reaction*		*Ratio* v_1/v_2
	No AMP v_1	$2 \cdot 10^{-5}$ *M AMP* v_2	
0	10.22	11.83	0.86
0.3	4.35	9.10	0.48
0.6	3.78	8.37	0.45
0.9	0.40	6.36	0.06
1.2	0.00	3.78	0
1.5	0.00	1.45	0

that the effects of 0.01 M arsenate on the sigmoid curve are present down to a very low concentration of phosphate in relation to the K_m obtained in that experiment. Theoretically, a lower concentration of phosphate could result in a higher rate in the presence of arsenate, as happens with aspartate and maleate in aspartate transcarbamylase[16]. However, this could not be tested as we were working at the lower limits of accuracy of our method for the determination of the reaction velocity.

Judged by the low concentrations at which they are effective, both AMP and arsenate are very specific effectors. However, it is possible to alter the behavior of the enzyme by using higher concentrations of apparently less specific agents such as increased ionic strength or urea. Addition of 0.1 M NaCl in the absence of AMP abolishes the sigmoid curve but also results in a marked, competitive inhibition with K_m of $15 \cdot 10^{-3}$ M P_i. This value is about three times the apparent affinity constant in the absence of NaCl. In the presence of AMP when 0.1 M NaCl is added, a competitive effect also occurs with an increase in the K_m from $1.5 \cdot 10^{-3}$ M to $5 \cdot 10^{-3}$ M P_i (ref. 5). Similar effects are observed when Tris is used to increase the ionic strength.

In view of the results caused by high ionic strength, it was decided to investigate

TABLE II

REVERSIBILITY OF INHIBITION BY UREA

Phosphorylase *a* was exposed to 1.5 M urea for 5 min and assayed as described. For assay in the absence of added urea, the final concentration of this agent carried over with the enzyme was 0.1 M. For assay in 1.5 M urea, enough of the concentrated 6.0 M solution of this agent was added to the reaction mixture to account for 1.4 M urea. Enzyme unexposed to urea also was assayed in the presence of added 0.1 M urea.

Urea (molar concn.)		*Velocity of reaction*		*Ratio* v_1/v_2
Enzyme exposed to	*Assayed in*	*No AMP* v_1	$2 \cdot 10^{-5}$ *M AMP* v_2	
1.5	0.1	7.65	9.50	0.80
1.5	1.5	0.00	0.88	0.00
0	0.1	8.62	10.95	0.79

the effects of urea, an agent which is known to change the structure of enzymes. Table I shows that the presence of AMP appears partially to protect the enzyme against the inhibition by urea. AMP also protects phosphorylase *b* against urea[12]. The data from Table II show that the inhibition by urea is reversible. Although there was some loss of activity, the enzyme which was recovered had the same ratio of activities both in the absence and in the presence of AMP.

Fig. 4 presents the effects of 0.3 M urea on the kinetics with respect to phosphate in the absence of AMP. The kinetics of reaction are normalized, and the K_m for P_i is $11 \cdot 10^{-3}$ M. Since the inhibition by urea is similar to that of NaCl, the effects of AMP were studied. Fig. 5 shows that 0.3 M urea indeed is competitive, but that 1.0 M urea causes a mixed type of inhibition. The question remains whether at low concentrations urea is acting as a fully or partially competitive inhibitor.

Kinetic K_m values reported for phosphorylase *a* for AMP of $0.5 \cdot 10^{-6}$–$2 \cdot 10^{-6}$ M (refs. 1, 2, 17) agree remarkably well with the dissociation constant obtained from

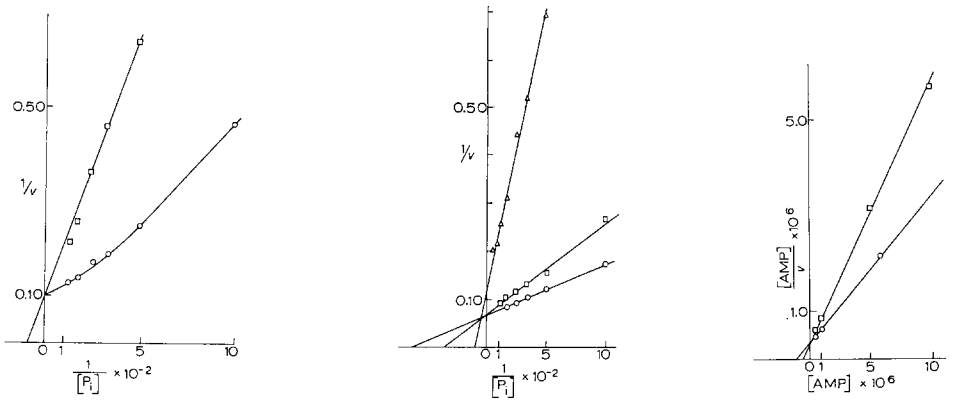

Fig. 4. Effects of 0.3 M urea on the Lineweaver–Burk plot with variable P_i concentration in the absence of AMP. ○, control; □, with 0.3 M urea. K_m in the presence of urea equals $11 \cdot 10^{-3}$ M P_i.

Fig. 5. Effects of 0.3 M and 1.0 M urea on the Lineweaver–Burk plot with a variable P_i concentration in the presence of $2 \cdot 10^{-5}$ M AMP. ○, control; □, 0.3 M urea; △, 1.0 M urea. The K_m is increased by the two concentrations of urea from $1.7 \cdot 10^{-3}$ to $3.0 \cdot 10^{-3}$ to $11 \cdot 10^{-3}$ M P_i. 1.0 M urea reduces the V from 15.1 to 9.1.

Fig. 6. Plot of [AMP]/*v versus* [AMP] for activation and the effects of 0.01 M sodium arsenate. ○, control; □, 0.01 M arsenate. K_m is reduced from $1.0 \cdot 10^{-6}$ M to $0.6 \cdot 10^{-6}$ M, and V from 3.3 to 1.8.

ultracentrifugation experiments of mixtures of protein and AMP[18]. The same is true for phosphorylase *b* (K_m $3 \cdot 10^{-5}$–$5 \cdot 10^{-5}$ M). Although the conditions were somewhat different in each experiment, it can be assumed that K_m values, determined kinetically, represent fairly accurately the dissociation constant of the enzyme for AMP. The difference between the activity at each concentration of AMP and the activity without AMP was taken as v. Because of the relatively small differences that were measured, the data were more variable than in the previous experiments. The more precise plots of $[S]/v$ *versus* $[S]$, instead of $1/v$ *versus* $1/[S]$ (ref. 19), were used.

The K_m for AMP is $1.0 \cdot 10^{-6}$ M with a V in this particular preparation of 3.3 units. The effect of 0.01 M arsenate, as seen in Fig. 6, is uncompetitive; that is, the K_m is reduced in half, but there is also a 45% inhibition of V. The effects of urea are shown in

Fig. 7. Increasing concentrations of urea progressively increase the binding of AMP but in this case, there is no effect on the V for AMP. Both 0.01 M Na_2SO_4 and 0.1 M NaCl act as noncompetitive inhibitors, causing 30 and 64% inhibitions of V, respectively (Fig. 8).

Uncompetitive inhibition by arsenate can be explained on the basis of observations previously made. AMP increases the affinity of phosphorylase *a* for arsenate[8]. Increasing the concentration of P_i causes a moderate decrease in the K_m for AMP[2]. The data presented here show that binding of arsenate at the active site causes the same effect, namely an increase in the affinity for AMP, as P_i binding.

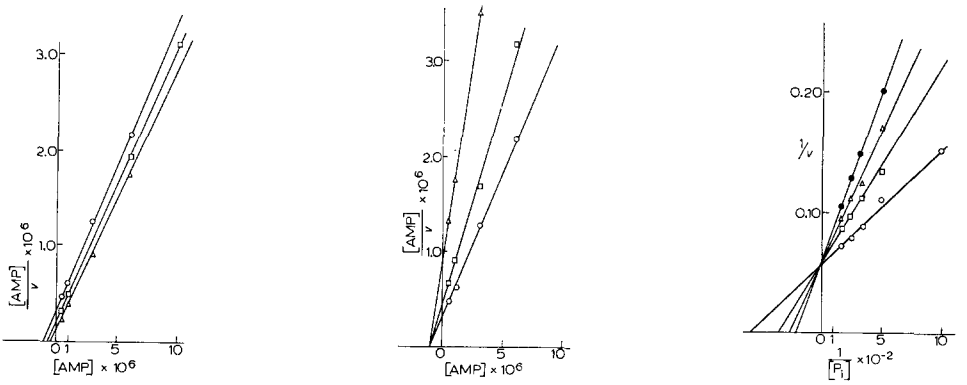

Fig. 7. The effects of 0.3 M and 1.0 M urea on plots [AMP]/v *versus* [AMP] for activation. ○, control; □, 0.3 M urea; △, 1.0 M urea. V equals 3.2, 3.3 and 3.6, respectively. K_m is reduced by urea from $1.0 \cdot 10^{-6}$ to $0.6 \cdot 10^{-6}$ to $0.3 \cdot 10^{-6}$ M.

Fig. 8. The effects of 0.01 M Na_2SO_4 and 0.1 M NaCl on plots of [AMP]/v *versus* [AMP] for activation. ○, control; □, 0.01 M SO_4^{2-}; △, 0.1 M NaCl. K_m is unchanged at $1.0 \cdot 10^{-6}$ M. V equals 3.3, 2.3 and 1.2, respectively.

Fig. 9. Effects of mixed inhibition with 0.3 M urea and 0.01 M sodium arsenate on the rate dependence of the reaction with respect to P_i in the presence of $2 \cdot 10^{-5}$ M AMP. ○, control; □, 0.3 M urea; △, 0.01 M arsenate; ●, 0.3 M urea and 0.01 M arsenate. K_m calculated assuming that both inhibitors bind at the same site for the mixture using the values obtained from the first three curves, $5.1 \cdot 10^{-3}$ M P_i.

Both urea and NaCl behave as competitive inhibitors of phosphate. Their different behaviors toward AMP binding, however, suggest that the mechanism of the inhibition is different. The urea effect at low concentrations may be due to its binding at the phosphate site. At higher concentrations additional effects counteracted by AMP must be present. NaCl, on the other hand, probably does not have a direct effect at the phosphate site; that is, because it does not affect the K_m for AMP it is a partially competitive inhibitor.

In order to test the assumption that urea at low concentration binds at the phosphate site, a mixed inhibition experiment with arsenate and urea was performed. If two competitive inhibitors, A and B, act at the same site, the K_m for the substrate will be increased by $(1 + [A]/K_a + [B]/K_b)$ when both are present simultaneously. If they act at two separate sites but have a fully competitive effect so that no *EAS* or *EBS* complexes are formed, the slope is increased by $(1 + [A]/K_a) \cdot (1 + [B]/K_b)$ (ref. 20).

Fig. 9 shows the mixed inhibition experiment using 0.3 M urea and 0.01 M arsenate. The theoretical value for the first case was $5.1 \cdot 10^{-3}$ M P_i, which also was obtained experimentally, indicating that urea is a true competitor of phosphate. However, there remains the possibility that at low concentrations urea is a partially competitive inhibitor. The rate equation obtained for this case is too complex for direct experimental testing. It is not possible to determine whether urea is a partially competitive inhibitor by varying the inhibitor concentration at a fixed substrate concentration[21] because, at higher concentrations, inhibition by urea is of the mixed type, as shown in Fig. 5. This eliminates the possibility of observing a leveling off in the curve of *v versus* urea concentration, as would be expected if urea were a partially competitive inhibitor.

Similar experiments were performed with NaCl and arsenate and with NaCl and urea. It was found, however, that when NaCl was present with the other inhibitors, the data became very inconsistent. Apparently, the structural effects caused by NaCl make the enzyme unstable.

DISCUSSION

The evidence presented indicates that with phosphate as a substrate, aged preparations of glycogen phosphorylase *a* exhibit cooperative effects, which are abolished specifically by $2 \cdot 10^{-5}$ M AMP or by 10 000-fold higher concentrations of urea. These effects cannot be related to changes in the state of aggregation of the enzyme because: (a) the effects observed do not require preincubation; (b) at the concentration used here phosphorylase *a* exists as a dimer[6]. They cannot be related either to the possible presence of phosphorylase *b* in the preparation, because the preparation is saturated at $2 \cdot 10^{-5}$ M AMP, whereas the K_m value of phosphorylase *b* for the nucleotide is higher[22]. These effects, therefore, may be due either to an allosteric transition[14] or to changes in the mechanism of the reaction[23]. Although the evidence does not allow a decision on the underlying causes of the effects observed, it clearly indicates a difference in the behavior of aged and freshly prepared phosphorylase and can be more easily interpreted on the basis of an allosteric transition.

It has been recently suggested that the difference in affinities of substrates and AMP for the distinct configurational states of the enzymes is greater in phosphorylase *b* than in *a* and that this accounts for the fact that homotropic interactions can be observed more readily with the *b* form[24]. The phosphorylase *a* preparations used here were extracted from rabbit muscle and had aged several weeks before they were used. This was in contrast to the many studies performed with freshly prepared enzyme by direct conversion from phosphorylase *b*. We suggest that aging causes structural changes which permit an easier demonstration of homotropic interactions in the *a* form, the capability for which is present in the "native" molecule. Although freshly crystallized preparations of phosphorylase *a* do not show complex kinetics of binding to antibody fragments, aged preparations show sigmoidal behavior. AMP apparently counteracts the aging effects[9]. Recent experiments also indicate that the dissociation constant for AMP and the enzyme is larger in aged than in fresh preparations[25].

If site–site interactions are taking place, it appears that agents which affect the K_m for P_i can abolish the interactions. Urea would seem to accomplish this by favoring a more "open" configuration of the enzyme, since a reduction in the K_m for AMP is

observed. If AMP activates the enzyme by increasing the accessibility of the active site to all possible ligands, an increased affinity for urea may be observed. As far as the present data can be interpreted, this is not the case. The K_i for urea in the presence or absence of AMP cannot be determined from plots of $1/v$ *versus* $[I]$ from the data in Table I, because moderately high concentrations of urea cause mixed inhibition (Fig. 5), and these plots are not linear in this case. However, the K_i for urea in the presence of AMP can be calculated from the data in Fig. 5 with 0.3 M urea using the relation K_m (inhibited) $= K_m$ (control) $\times (1 + [I]/K_i)$. The value obtained is approx. 0.4 M. Assuming that from Fig. 4 the K_i can be similarly calculated from the data without AMP and assuming a value for K_m (control) of 0.004 M, which is the P_i concentration needed to half saturate the enzyme[5], the K_i calculated is approx. 0.2 M. Thus, although AMP increases the affinity of substrates for the active site, it does not cause an increase in the affinity of urea for the enzyme. The effects of AMP on the enzyme appear to be of a specific kind which yield a form with more affinity for the substrates and with less affinity for the inhibitor.

ACKNOWLEDGMENT

This work was supported by Grant GM-T866 from U.S. Public Health Service.

REFERENCES

1 E. HELMREICH AND C. F. CORI, *Proc. Natl. Acad. Sci. U.S.*, 51 (1964) 131.
2 O. H. LOWRY, D. W. SCHULZ AND J. V. PASSONNEAU, *J. Biol. Chem.*, 239 (1964) 1947.
3 N. B. MADSEN, *Biochem. Biophys. Res. Commun.*, 15 (1964) 390.
4 H. E. MORGAN AND A. PARMEGGIANI, *J. Biol. Chem.*, 239 (1964) 2440.
5 F. SAGARDÍA, *Biochem. Biophys. Res. Commun.*, 17 (1964) 383.
6 J. H. WANG AND D. J. GRAVES, *Biochemistry*, 3 (1964) 1437.
7 J. H. WANG, M. L. SHONKA AND D. J. GRAVES, *Biochem. Biophys. Res. Commun.*, 18 (1965) 131.
8 E. HELMREICH AND C. F. CORI, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 647.
9 M. C. MICHAELIDES, R. SHERMAN AND E. HELMREICH, *J. Biol. Chem.*, 239 (1964) 4171.
10 A. ULLMAN, P. R. VAGELOS AND J. MONOD, *Biochem. Biophys. Res. Commun.*, 17 (1964) 86.
11 J. L. HEDRICK, *Arch. Biochem. Biophys.*, 114 (1966) 216.
12 W. W. APPLEMAN AND E. H. FISCHER, *Federation Proc.*, 21 (1962) 83.
13 S. F. VELICK AND L. F. WICKS, *J. Biol. Chem.*, 190 (1951) 741.
14 J. MONOD, J. P. CHANGEUX AND F. JACOB, *J. Mol. Biol.*, 6 (1963) 306.
15 D. E. ATKINSON, J. A. HATHAWAY AND E. C. SMITH, *Biochem. Biophys. Res. Commun.*, 18 (1965) 1.
16 J. C. GERHART AND A. B. PARDEE, *Cold Spring Harbor Symp. Quant. Biol.*, 28 (1963) 491.
17 C. F. CORI, G. T. CORI AND A. A. GREEN, *J. Biol. Chem.*, 151 (1943) 39.
18 N. B. MADSEN AND C. F. CORI, *J. Biol. Chem.*, 224 (1957) 899.
19 G. N. WILKINSON, *Biochem. J.*, 80 (1961) 324.
20 J. L. WEBB, *Enzyme and Metabolic Inhibitors*, Vol. 1, Academic Press, New York, 1963, p. 489.
21 M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, New York, 1958, p. 22.
22 G. T. CORI AND C. F. CORI, *J. Biol. Chem.*, 135 (1940) 733.
23 J. R. SWEENEY AND J. R. FISHER, *Biochemistry*, 7 (1968) 561.
24 L. L. KASTENSCHMIDT, J. KASTENSCHMIDT AND E. HELMREICH, *Federation Proc.*, 26 (1967) 560.
25 E. HELMREICH, M. C. MICHAELIDES AND C. F. CORI, *Biochemistry*, 6 (1967) 3695.