Matsuo, K., and Tsuboi, M. (1966), *Bull. Chem. Soc. Japan 39*, 347.
McPhie, P., and Gratzer, W. B. (1966), *Biochemistry 5*, 1310.
Norland, K. S., Fasman, G. D., Katchalski, E., and Blout, E. R. (1963), *Biopolymers 1*, 277.
Olins, D. E., Olins, A. L., and von Hippel, P. (1967), *J. Mol. Biol. 24*, 157.
Olins, D. E., Olins, A. L., and von Hippel, P. (1968), *J. Mol. Biol. 33*, 265.
Oriel, P. J. (1966), *Arch. Biochem. Biophys. 115*, 577.
Sarkar, P. K., and Yang, J. T. (1965), *J. Biol. Chem. 240*, 2088.
Sober, H. A., Schlossman, S. F., Yaron, A., Latt, S. A., and Rushizky, G. W. (1966), *Biochemistry 5*, 3608.
Spitnik, P., Lipshitz, R., and Chargaff, E. (1955), *J. Biol. Chem. 215*, 765.
Stedman, E., and Stedman, E. (1950), *Nature 166*, 780.
Szer, W. (1966a), *Biochem. Biophys. Res. Commun. 22*, 559.
Szer, W. (1966b), *J. Mol. Biol. 16*, 585.
Tinoco, I., Woody, R. W., and Bradley, D. F. (1963), *J. Chem. Phys. 38*, 1317.
Ts'o, P. O. P., and Bonner, J. (1964), *in* The Nucleohistones, Bonner, J., and Ts'o, P. O. P., Ed., San Francisco, Calif., Holden-Day, p 367.
Tsuboi, M. (1967), *in* Conformation of Biopolymers, Vol. 2, Ramachandran, G. N., Ed., New York, N. Y., Academic, p 689.
Tsuboi, M., Matsuo, K., and Ts'o, P. O. P. (1966), *J. Mol. Biol. 15*, 256.
Warshaw, M. M., Bush, C. A., and Tinoco, I., Jr. (1965), *Biochem. Biophys. Res. Commun. 18*, 633.
Zamenhof, S., and Chargaff, E. (1963), *Methods Enzymol. 3*, 702.

Studies on the Catalytic Mechanism of *Escherichia coli* Succinic Thiokinase*

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ABSTRACT: The coenzyme A analog, desulfocoenzyme A, does not significantly affect the rate of succinyl phosphate formation from phosphorylated succinic thiokinase (succinate:coenzyme A ligase (adenosine diphosphate), EC 6.2.1.5).

The effector action of desulfocoenzyme A seems to require the presence of adenosine triphosphate. Phosphoryl-enzyme is shown to be an intermediate in succinyl phosphate formation from adenosine triphosphate and succinate, and additional evidence is presented supporting the intermediary role of phosphoryl-enzyme in the over-all catalytic mechanism of the enzyme.

Succinic thiokinase catalyzes the formation of succinyl phosphate from ATP and succinate (Nishimura and Meister, 1965). This reaction is stimulated by the coenzyme A analog, desulfo-CoA[1] (Grinnell and Nishimura, 1969b). Since the formation of succinyl phosphate from phosphoryl-STK[1] and succinate was also known to occur (Nishimura, 1967), it was of interest to investigate the influence of the CoA analog on reactions involving phosphoryl-STK. We have studied the effect of desulfo-CoA on the rate of succinate phosphorylation by STK-P at various succinate concentrations, and measured the rate of STK-P $\rightleftarrows$ ATP exchange in the presence and absence of desulfo-CoA. Additional studies on the formation of succinyl phosphate from ATP and succinate are also reported. Possible mechanisms of stimulation of succinyl phosphate formation by desulfo-CoA are discussed.

Further evidence for the participation of STK-P in the catalytic mechanism of succinic thiokinase is also presented. In the presence of all substrates, the initial rates of the reactions, STK-P $\rightarrow$ P_i and ATP $\rightarrow$ P_i, have been measured simultaneously by dual-radioisotope experiments using [^{32}P]-STK-P and [γ-^{33}P]ATP.

Experimental Section

Materials. [2,3-^{14}C]Succinic acid, [^{32}P]P_i, and [^{33}P]P_i were purchased from New England Nuclear Corp. [γ-^{32}P]ATP and [γ-^{33}P]ATP were synthesized enzymatically (Glynn and Chappell, 1964). All labeled ATP and P_i used in this investigation was purified by DEAE-cellulose chromatography (Wehrli *et al.*, 1965).

Enzyme. Succinic thiokinase was isolated and assayed as described previously (Grinnell and Nishimura, 1969a).

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[1] Abbreviations used are: CoAH, desulfocoenzyme A; STK, succinic thiokinase; STK-P, phosphorylsuccinic thiokinase.

TABLE I: Effect of Desulfo-CoA on Succinyl-P Formation from STK-P at Various Succinate Concentrations.[a]

Succinate (mM)	CoAH	Rate[b]
0.63	−	15
0.63	+	27
12.5	−	51
12.5	+	63
125.0	−	278
125.0	+	304

[a] The reaction mixtures contained Tris-HCl (2.0 μmoles, pH 7.2), $MgCl_2$ (200 mμmoles), 1200 cpm of [^{32}P]STK-P (0.15 mμmole, 7.7 units), succinate (as specified), and CoAH (3.3 mμmoles where specified). The final volume was 0.04 ml. Incubation times were from 30 sec to 2 min at 37°. The reactions were stopped by addition of 0.005 ml of 5 mM $HgCl_2$. Aliquots of the reaction mixtures were subjected to electrophoresis in the Tris-acetate buffer system and analyzed as described in the Experimental Section. [b] (mμmoles/min per unit) × 10^4.

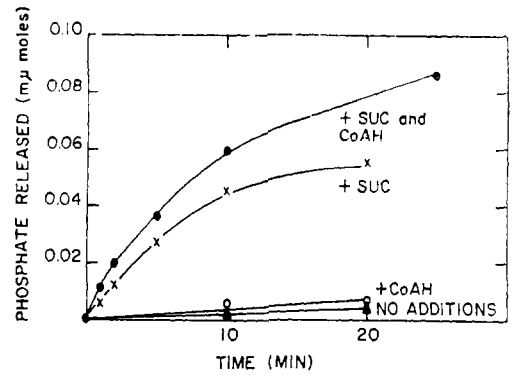

FIGURE 1: Time dependence of release of succinyl phosphate or phosphate from phosphoryl-STK. The reaction mixtures contained Tris-HCl (2 μmoles, pH 7.2), $MgCl_2$ (200 mμmoles), and 10,400 cpm of [^{32}P]STK-P (0.177 mμmole, 10 units). Succinate (10 mμmoles) and CoAH (2.5 mμmoles) were added where indicated. The final volume was 0.04 ml. After incubation at 37° for the specified times the reactions were stopped by the addition of 0.005 ml of 5 mM $HgCl_2$. Aliquots of the reaction mixtures were subjected to electrophoresis in the Tris-maleate buffer system and analyzed as described in the Experimental Section.

The specific activity of enzyme used in experiments reported in this paper was 800–1000.

Purification and Isolation of Radioactive Phosphoryl-enzyme. The procedure for phosphorylation of the enzyme by ATP was essentially that employed by Upper (1964). The conditions of the incubation of [γ-^{32}P]ATP or [γ-^{33}P]ATP with enzyme were those previously shown to yield maximal phosphorylation (Grinnell and Nishimura, 1969a). Phosphoryl-STK was isolated as has been described (Nishimura, 1967).

Paper Electrophoresis. Whatman No. 3MM paper strips (2.54 × 75 cm) were moistened with appropriate buffer and placed on a Savant 30-in. flat-plate electrophoresis unit, cooled at 0°, where samples were applied to the paper. For the separation of succinate, succinyl phosphate, and the phosphoryl-enzyme, the Tris-acetate buffer system of Cha *et al.* (1967) was used. Samples were subjected to 33 V/cm for 90 min. For the separation of succinyl phosphate, P_i, ATP, and phosphoryl-enzyme, a modification of the Tris-maleate buffer system described by Nishimura and Meister (1965) was employed. The buffer consisted of 0.05 M Tris adjusted to pH 7.0 by addition of maleic acid. Samples were subjected to 54 V/cm for 60 min.

Radioactive Measurements. Radioactivity on paper electropherograms was measured in a Nuclear-Chicago Actigraph III scanner equipped with scaler. The radioactive areas of the strips were cut out and placed in scintillation vials containing 5 ml of Liquifluor (obtained from New England Nuclear Corp.)–toluene solution. This procedure is similar to that described by Wang and Jones (1959). The vials were counted in a Nuclear-Chicago liquid scintillation spectrophotometer using settings calibrated for simultaneous determination of the radioisotopes ^{32}P and ^{33}P, or using settings calibrated for the radioisotope ^{14}C. The facilities of the New England Medical Center Hospital Computer Applications Center were used in computing the amounts of radioactivity in dual-radioisotope experiments.

Results

Studies on Phosphoryl-enzyme. The time dependence of succinate phosphorylation by phosphoryl-STK is shown in Figure 1. The coenzyme A analog, desulfo-CoA, stimulated the reaction only slightly, even when added at high concentrations (1.25 mM). In the absence of succinate, desulfo-CoA had little effect on the enzyme. The minimal influence of desulfo-CoA on reactions involving STK-P was surprising. The analog was reported to be an activator of succinyl phosphate synthesis from ATP and succinate in a previous communication (Grinnell and Nishimura, 1969b). Furthermore, desulfo-CoA induced an ATPase activity in the enzyme when added at high concentrations.

As the level of succinate was increased, the per cent increase in the rate of succinyl phosphate synthesis caused by the addition of desulfo-CoA diminished (Table I). The rate of the reaction, at high succinate concentrations, was greater than would be expected from Michaelis–Menten kinetics. Substrate activation by succinate has been reported previously by Cha and Parks (1964), who used the enzyme isolated from pig heart. They observed activation of the formation of succinyl-CoA from GTP, CoA, and succinate.

The possibility that the formation of succinyl phosphate from ATP involved direct phosphorylation of succinate was considered by Nishimura and Meister (1965). This reaction would be distinct from succinyl phosphate formation from STK-P as there would be no intermediation of the phosphoryl-enzyme. Two different paths of succinyl phosphate formation might have explained the differential effects of desulfo-CoA on those reactions involving ATP and enzyme and those involving phosphoryl-enzyme. To test the possibility that succinyl phosphate formation from ATP involved direct phosphorylation of succinate, the dual-radioisotope experiment described in Figure 2 was performed. [^{32}P]STK-P and [γ-^{33}P]ATP were incubated with succinate under conditions which resulted in succinyl phosphate formation. The amounts of ^{32}P and ^{33}P in the product were determined. The ratio of

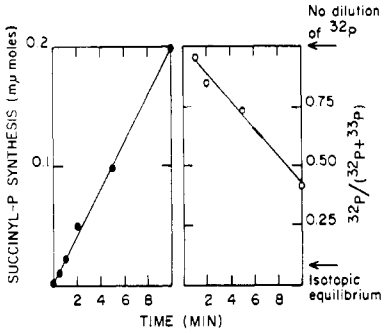

FIGURE 2: Origin of phosphorus in succinyl phosphate. The reaction mixtures contained Tris-HCl (2 μmoles, pH 7.2), $MgCl_2$ (200 mμmoles), succinate (150 mμmoles), 37,300 cpm of [γ-^{33}P]ATP (12 mμmoles), and 2970 cpm of [^{32}P]STK-P (0.3 mμmole, 15.4 units). The final volume was 0.04 ml. After incubation at 37° for the specified times the reactions were stopped by the addition of 0.005 ml of 5 mM $HgCl_2$. Aliquots of the reaction mixtures were subjected to electrophoresis in the Tris-maleate buffer system and analyzed as described in the Experimental Section.

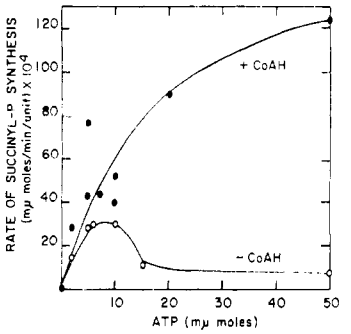

FIGURE 4: ATP dependence of succinyl phosphate formation. The reaction mixtures contained Tris-HCl (2 μmoles, pH 7.2), $MgCl_2$ (200 mμmoles), [2,3-^{14}C]succinate (77 mμmoles, specific activity 10.6), enzyme (11.5 units), and ATP as indicated. CoAH (5 mμmoles) was added where specified. The final volume was 0.04 ml. After incubation at 37° for 20 min the reactions were stopped by chilling. Aliquots of the reaction mixtures were subjected to electrophoresis in the Tris-acetate buffer system and analyzed as described in the Experimental Section.

counts of ^{32}P:(^{32}P + ^{33}P) in succinyl phosphate served as an index to the origin of the phosphate. If phosphoryl-enzyme were the source this ratio would be 1.0; if ATP were the source, this ratio would be zero; and if isotopic equilibrium were attained, this ratio would be 0.072 based on the specific activities of the starting materials.

At early times the succinyl phosphate that was formed was predominantly labeled with ^{32}P, indicating that phosphoryl-enzyme was the source of phosphate in succinyl phosphate. The decrease in the ratio results primarily from the exchange reaction between STK-P and ATP phosphate that occurs simultaneously with succinyl phosphate synthesis. The total amount of succinyl phosphate formed was comparable to that expected if the incubation contained enzyme instead of phosphoryl-enzyme. On the basis of this experiment, the possibility that there were two different paths of succinyl phosphate formation (one not involving the phosphoryl-enzyme) was ruled out.

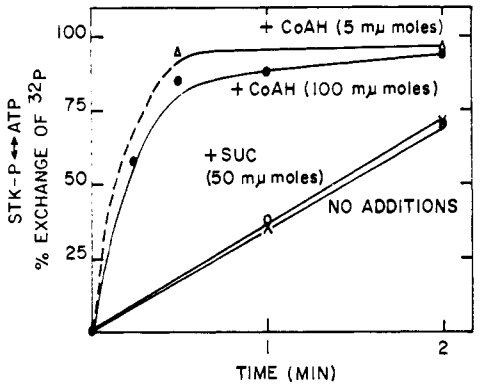

FIGURE 3: STK-P ⇄ ATP exchange. The reaction mixtures contained Tris-HCl (2.0 μmoles, pH 7.2), $MgCl_2$ (200 mμmoles), 40,000 cpm of [γ-^{33}P]ATP (12.0 mμmoles), and 1500 cpm of [^{32}P]/STK-P (0.15 mμmole, 7.7 units). Succinate and CoAH were added as indicated. The final volume was 0.04 ml. After incubation at 37° for the specified times, the reactions were stopped by the addition of 0.005 ml of 5 mM $HgCl_2$. Aliquots of the reaction mixtures were subjected to electrophoresis in the Tris-maleate buffer system and analyzed as described in the Experimental Section.

When the dual-radioisotope experiment was carried out with desulfo-CoA added, 98% of the radioactivity in the succinyl phosphate product after 1-min incubation was ^{32}P, which was similar to the result obtained in the absence of the analog (see above). However, the rate of exchange of [^{32}P]-STK-P with ATP was stimulated. This effect did not require succinate and is shown in Figure 3.

Effect of ATP Concentration on Succinyl Phosphate Formation. The relative inactivity of desulfo-CoA in the synthesis of succinyl phosphate from STK-P and succinate, as compared to its effector role in succinyl phosphate formation from ATP, succinate, and enzyme, is not explainable by the mechanisms of succinyl phosphate formation previously considered. An alternative explanation for this observation was that the presence of adenine nucleotide (ATP or ADP) produced a state of the enzyme that was desulfo-CoA sensitive. Figure 4 shows the ATP dependence of succinyl phosphate formation (from ATP and succinate) in the presence and absence of desulfo-CoA. At low levels of ATP there was little difference in the rate of formation between incubations with, or without, desulfo-CoA. At higher concentrations, however, the desulfo-CoA effect became pronounced. The implications of this experiment will be considered in detail in the Discussion.

Further Evidence for the Role of Phosphoryl-enzyme as a Catalytic Intermediate. If the phosphorylated form of STK is a covalent catalytic intermediate in the over-all reaction catalyzed by this enzyme, then two kinetic criteria must be met. The rate of formation of STK-P and the rate of dephosphorylation of this enzyme species must both occur at rates as fast as, or faster than, the rate of the over-all reaction (Bridger *et al.*, 1968). The first of these criteria has been elegantly demonstrated (Bridger *et al.*, 1968). Using [^{32}P]-STK-P and [γ-^{33}P]ATP, in the presence of all other substrates, we have simultaneously measured the rates of the reactions STK-P → P_i and ATP → P. The unavailability of stopped-flow equipment made it necessary for these studies to be carried out at low temperature. One advantage of this restraint was that no phosphate exchange occurred between

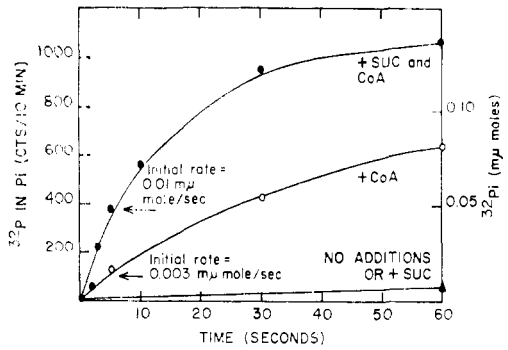

FIGURE 5: Rate of STK-P → P_i reaction. The reaction mixtures contained Tris-HCl (2 μmoles, pH 7.2), MgCl (200 mμmoles), 42,000 of [γ-^{33}P]ATP (12 mμmoles), and 1600 cpm of [^{32}P]STK-P (0.15 mμmole, 7.7 units). Succinate (2 mμmoles) and CoA (2 mμmoles, containing 4 mμmoles of dithiothreitol) were added where specified. The final volume was 0.04 ml. After incubation at −4° for the indicated times, the reactions were stopped by the addition of 0.005 ml of 5 mM $HgCl_2$. Aliquots of the reaction mixtures were subjected to electrophoresis in the Tris-maleate buffer system and analyzed as described in the Experimental Section.

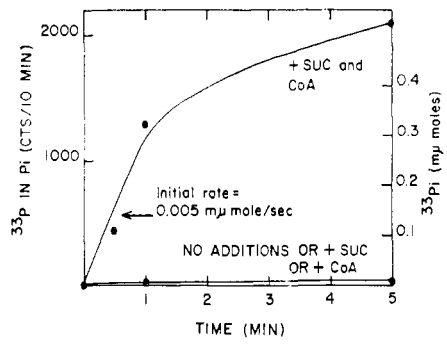

FIGURE 6: Rate of ATP → P_i reaction. Details are given under Figure 5.

STK-P and ATP under the reaction conditions. The rate of the STK-P → P_i reaction (Figure 5) was significantly faster than the rate of the ATP → P_i reaction (Figure 6), which is consistent with the proposed role of STK-P as a covalent catalytic intermediate in the over-all reaction. The nonsuccinate-dependent release of phosphate from STK-P (Figure 5) probably reflects formation of the high-energy nonphosphorylated form of the enzyme, which has been described in detail (Moyer *et al.*, 1967; Cha *et al.*, 1967).

Discussion

The studies reported in this communication indicate that formation of succinyl phosphate from phosphoryl-enzyme (reaction 1) is not greatly stimulated by desulfo-CoA.

$$\text{STK-P} + \text{succinate} \overset{Mg^{2+}}{\rightleftharpoons} \text{STK} + \text{succinyl phosphate} \quad (1)$$

$$\text{ATP} + \text{succinate} \overset{\text{STK}, Mg^{2+}}{\rightleftharpoons} \text{ADP} + \text{succinyl phosphate} \quad (2)$$

On the contrary, the synthesis of succinyl phosphate from ATP and succinate (reaction 2) required desulfo-CoA for significant activity (Grinnell and Nishimura, 1969b). The differential effects of desulfo-CoA on reactions 1 and 2 are incompatible with the previous suggestion that desulfo-CoA influences the binding of succinate to the enzyme or of succinyl phosphate formation on the enzyme surface. Moreover, the possibility that reactions 1 and 2 proceed by different paths is discounted by the dual-radioisotope experiments with [^{32}P]STK-P and [γ-^{33}P]ATP. Results of these experiments and the kinetic data represented in Figures 5 and 6 support the conclusions that phosphoryl-enzyme is an intermediate in reaction 2 and in the over-all reaction catalyzed by STK.

Figure 4 shows that desulfo-CoA has little effect on the rate of succinyl phosphate formation from ATP and succinate at low ATP concentrations (*ca.* 0.1 mM). However, high concentrations of ATP cause marked inhibition unless desulfo-CoA is present.[2] This suggests that the effect of desulfo-CoA may be concerned with the reversal of an inhibitory effect of ATP or ADP.[3]

Other lines of evidence point to interactions between CoA and adenine nucleotide that are not predicted by any of the mechanisms proposed for the STK reaction. The ADP ⇄ ATP exchange reaction is stimulated by succinyl-CoA, CoA, and to a limited extent desulfo-CoA (Grinnell and Nishimura, 1969a; Bridger *et al.*, 1968). At high desulfo-CoA concentrations (*ca.* 2.0 mM) an ATPase activity is induced in STK. This also occurs with CoA in place of desulfo-CoA, although not as well (Grinnell and Nishimura, 1969b). Finally, in Figure 3, it was seen that low concentrations of desulfo-CoA (0.125 mM) stimulated STK ⇄ ATP exchange and that 20-fold higher desulfo-CoA levels showed little difference in effect. Under comparable conditions, CoA (0.025 mM) caused such a rapid release of P_i from STK-P that the exchange reaction could not be measured. However, CoA does stimulate this exchange according to Bridger *et al.* (1968).

In reactions catalyzed by *E. coli* succinic thiokinase, ATP is the source of high-energy phosphate. ATP may also have an effector role similar to that of desulfo-CoA. For instance, Bridger *et al.* (1968) have reported synergistic effects of ATP, but not ADP, upon succinate ⇄ succinyl-CoA exchange. As shown in Figure 4, the maximum rate of succinyl phosphate formation in the presence of desulfo-CoA (2.0 mM succinate and 1.25 mM ATP) was 123 × 10^{-4} mμmole/min per unit. If ATP is an effector molecule required for maximal activity, one would expect that in comparable experiments using phosphoryl-enzyme (thus omitting ATP) lower rates of succinyl phosphate formation would be observed. This was seen in Table I. Even at succinate levels much higher than 2.0 mM (12.5 mM), in the presence of desulfo-CoA, the rate of succinyl phosphate formation was only 63 × 10^{-4} mμmole/min per unit. This does suggest an effector role for ATP.

In summary, it would appear that the effector action of

[2] In previous studies, concentrations of ATP were employed at which a desulfo-CoA requirement could be seen (Grinnell and Nishimura, 1969b).

[3] Hildebrand and Spector (1969) reported that desulfo-CoA stimulated ATP synthesis from succinyl phosphate and ADP catalyzed by succinic thiokinase. ATP synthesis was measured by coupled reaction with hexokinase; therefore, there was essentially only free ADP in the reaction mixture. If desulfo-CoA action is related to the presence of adenine nucleotide it is probably interacting with ADP.

desulfo-CoA in succinyl phosphate formation is related to an interaction of desulfo-CoA and ATP (or ADP) with the enzyme, and furthermore, that ATP may also have an effector function. No evidence has as yet been presented indicating that succinic thiokinase is an allosteric enzyme, *i.e.*, no homotropic or heterotropic binding influences have been observed. However, it appears likely that there is some site on the enzyme, not necessarily the active site, that interacts with CoA or desulfo-CoA and that this interaction is modified by ATP or ADP. When this site is activated, succinate thiokinase is in its most reactive form.

Whether the molecular mechanism of succinic thiokinase activation is by induced fit, which has been suggested as a possible explanation for substrate synergism with this enzyme (Bridger *et al.*, 1968), or by some other process, is unclear. The mechanism may be functionally related to the control of the amount of enzyme in the phosphorylated form. Although CoA does not affect the maximum phosphorylatability of STK (Grinnell and Nishimura, 1969a), succinyl-CoA does exhibit such influence (Ramaley *et al.*, 1967). Furthermore, Bridger *et al.* (1968) have estimated the amount of enzyme in the phosphorylated form when the over-all reaction is at steady state, and this value varied significantly depending upon whether net ATP or succinyl-CoA synthesis was occurring. In the presence of succinyl-CoA, ADP, and P_i (ATP synthesis) the steady-state STK-P level was about eight times lower than in the presence of succinate, CoA, and ATP (succinyl-CoA synthesis). Studies using ATP analogs, such as adenosine 5′-methylenediphosphonate, may provide further information on the effector relationships of CoA and ATP to the enzyme.

References

Bridger, W. A., Millen, W. A., and Boyer, P. D. (1968), *Biochemistry 7*, 3608.

Cha, S., Cha, C.-J. M., and Parks, R. E., Jr. (1967), *J. Biol. Chem. 242*, 2582.

Cha, S., and Parks, R. E., Jr. (1964), *J. Biol. Chem. 239*, 1968.

Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90*, 147.

Grinnell, F. L., and Nishimura, J. S. (1969a), *Biochemistry 8*, 562.

Grinnell, F. L., and Nishimura, J. S. (1969b), *Biochemistry 8*, 568.

Hildebrand, J. G., and Spector, L. B. (1969), *Federation Proc. 28*, 44.

Moyer, R. W., Ramaley, R. F., Butler, L. G., and Boyer, P. D. (1967), *J. Biol. Chem. 242*, 4299.

Nishimura, J. S. (1967), *Biochemistry 6*, 1094.

Nishimura, J. S., and Meister, A. (1965), *Biochemistry 4*, 1457.

Ramaley, R. F., Bridger, W. A., Moyer, R. W., and Boyer, P. D. (1967), *J. Biol. Chem. 242*, 4287.

Upper, C. D. (1964), Ph.D. Dissertation, University of Illinois, Urbana, Ill.

Wang, C. H., and Jones, D. E. (1959), *Biochem. Biophys. Res. Commun. 1*, 203.

Wehrli, W. E., Verheyden, D. L. M., and Moffatt, J. G. (1965), *J. Am. Chem. Soc. 87*, 2265.