FAILURE OF ARSENATE TO UNCOUPLE THE PHOSPHOTRANSACETYLASE SYSTEM IN CLOSTRIDIUM ACIDIURICI

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ABSTRACT

SAGERS, RICHARD D. (Brigham Young University, Provo, Utah), MOSHE BENZIMAN, AND SIGRID M. KLEIN. Failure of arsenate to uncouple the phosphotransacetylase system in Clostridium acidiurici. J. Bacteriol. 86:978–984. 1963.—The conversion of pyruvate to acetyl phosphate by extracts of Clostridium acidiurici required coenzyme A (CoA), an electron-carrier system (ferredoxin and nicotinamide adenine dinucleotide), and a divalent metal. Other cofactors may be involved but are not presently defined. The metal activates the system transferring acetyl units between CoA and phosphate. Acetyl CoA could be generated from pyruvate or from acetyl phosphate, but in both cases arsenate failed to uncouple the high-energy acyl compounds. The rate of acetyl transfer between acetyl phosphate and CoA was markedly decreased by 0.005 M arsenate, but the generation of acetyl CoA from pyruvate was essentially unaffected until the arsenate concentration exceeded 0.02 M. Close agreement was observed between the amount of pyruvate utilized and the amount of acetyl phosphate formed, both in the presence and absence of arsenate. The CoA-dependent exchange of $\text{P}^4\text{O}_4^{3-}$ with acetyl phosphate proceeded at a rate approximately one-eightieth of the rate of acetylation of CoA, indicating an equilibrium value for the phosphotransacetylase reaction similar to that observed for Clostridium kluyveri. The failure of arsenate to uncouple the $C. \text{acidiurici}$ enzyme may indicate a high degree of specificity in relation to the acetyl unit acceptor, giving preference to phosphate over arsenate.

Uncoupling of high-energy acetyl compounds by arsenate has been employed in the assay for phosphotransacetylase from a number of bacterial species (Stadtman, Novelli, and Lipmann, 1951). In this reaction, acetyl phosphate serves as a donor for the acetylation of coenzyme A (CoA). In the presence of arsenate, CoA transfers its acyl group to arsenate forming an unstable complex which immediately decomposes. The rate of the reaction may be determined quantitatively by measuring the rate of acetyl phosphate disappearance.

In the catabolism of uric acid by Clostridium acidiurici, acetate is an end product with pyruvate occurring as an intermediate (Sagers, Benziman, and Gunsalus, 1961), and the degradation of pyruvate is dependent on CoA. It was postulated that acetate generation might involve the formation of acetyl CoA and acetyl phosphate, and that a phosphotransacetylase might be present. When cell-free extracts of $C. \text{acidiurici}$ were tested for phosphotransacetylase activity by the arsenolysis assay by use of catalytic amounts of CoA, no measurable activity was observed. It was determined that acetyl CoA was generated from acetyl phosphate or from pyruvate at a rapid rate when substrate amounts of CoA were added to the system. The addition of arsenate, however, did not result in an overall disappearance of high-energy acetyl groups as would be expected. The purpose of the present study was to define the catabolism of pyruvate by $C. \text{acidiurici}$, and to determine the properties of the enzyme responsible for the transfer of acetyl groups between CoA and phosphate. A preliminary report of portions of this work has appeared (Sagers and Klein, 1962).

MATERIALS AND METHODS

Cells of $C. \text{acidiurici}$ were grown, and extracts were prepared as described previously (Benziman, Sagers, and Gunsalus, 1960), with the exception that a newly designed cell homogenizer was employed (Sagers, 1962). A protein fraction rich in phosphotransacetylase activity was obtained after protamine sulfate treatment of crude extracts and fractional precipitation with crystal-
line ammonium sulfate. An 18-ml amount of 1% aqueous protamine sulfate was added to each 100 ml of extract (pH 6.5) containing 8 to 10 mg of protein per ml. After stirring for 15 min, the precipitate was removed by centrifugation and discarded. Ammonium sulfate fraction A, precipitating between 12-fold purified phosphotransacetylase units and 1.0 saturation, showed a 12-fold increase in specific activity. This fraction contained 18% of the protein and 40% of the phosphotransacetylase units and was used in subsequent experiments where the partially purified enzyme was preferred. A unit of phosphotransacetylase activity is defined as the amount of enzyme required to catalyze the disappearance of 1 μmole of acetyl phosphate in 5 min with pantetheine acting as acetyl acceptor.

Because the C. acidiurici phosphotransacetylase does not catalyze the decomposition of acetyl CoA by arsenate as observed for the corresponding enzyme from C. kluyveri, Escherichia coli, (Stadtman et al., 1951), and Diplococcus glycinophilus (Klein and Sagers, 1962; Sagers and Klein, 1962), it was necessary to measure the transfer of acetyl groups more directly. This was accomplished by the addition of substrate levels of CoA or pantetheine, and determination of the enzymatic transfer of acetyl groups from acetyl phosphate to these acceptors. The heat- and acid-stable thioesters (Stadtman, 1952) thus generated were measured by the hydroxamate method mentioned below after heating samples of reaction mixtures in 0.1 N HCl for 6 min to destroy the acetyl phosphate. In cases where large quantities of acetyl acceptor were required (as during the fractionation procedure), pantetheine was substituted for CoA. This compound was less efficient (5 to 10%) than CoA as an acetyl acceptor, but, because of the high specific activity of the phosphotransacetylase, satisfactory quantities of acetyl pantetheine were readily generated during the short reaction periods.

Acetyl phosphate, acetyl CoA, and acetyl pantetheine were determined colorimetrically by the hydroxamate method of Lipmann and Tuttle (1945). Acetyl CoA was visualized on paper chromatograms according to the method of Stadtman (1952). Reduced glutathione, which was added to the enzyme fraction prior to storage and to some reaction mixtures, was entirely ineffective as an acetyl unit acceptor under the conditions employed.

Pyruvate was determined by the direct method of Friedemann and Haugen (1943).

Incorporation of inorganic P32 into acetyl phosphate was determined by the selective calcium precipitation method of Lipmann and Tuttle (1944), and radioactivity measurements were made with a Tracerlab TGC-14 detector and Compumatic II scaler or with a Packard Tri-Carb model 314 EX liquid scintillation spectrometer.

Acetyl phosphate was prepared as the dilithium salt by the method of Stadtman and Lipmann (1950).

Pantetheine was prepared from pantetheine (Nutritional Biochemicals Corp., Cleveland, Ohio) by sodium borohydride reduction in aqueous solution until no further increase in sulfhydryl groups could be detected. The solution was saturated with potassium carbonate, the pantetheine extracted with absolute ethanol, and the latter removed by vacuum distillation at 30 C. The pantetheine was redissolved in water and stored in the frozen state until used.

CoA was obtained from Pabst Laboratories, Milwaukee, Wis.

Enzymatic reactions, carried out in final volumes of 1.0 ml, were initiated by adding the substrate after a 5-min period of enzyme activation and temperature equilibration, and stopped either by adding 1 ml of 2.5 X 10^-3 M neutral p-hydroxymercuribenzoate or by placing the samples in an ice bath and adding 0.5 ml of cold 5% trichloroacetic acid.

**RESULTS AND DISCUSSION**

The conversion of pyruvate to acetyl phosphate by extracts of C. acidiurici requires CoA, ferredoxin (Valentine, Brill, and Sagers, 1963), an electron acceptor (nicotinamide adenine dinucleotide or a dye), and a divalent metal. Other cofactors, possibly thiamine pyrophosphate and lipoic acid, may be involved (Sagers et al., 1961), but precise requirements for additional factors have not yet been determined. Figure 1 shows that the accumulation of acetyl phosphate is stoichiometric with the amount of pyruvate used when P32-labeled inorganic phosphate uptake, in conjunction with acetyl hydroxamate measurement, is used as an indicator for acetyl phosphate formation.

Activation by CoA of the system converting pyruvate to acetyl phosphate is shown in Table 1.
addition of metal-binding reagents, this inhibition was overcome by supplementing the extracts with a purified phosphotransacetylase fraction from C. kluyveri. This fraction alone was incapable of converting pyruvate to acetyl phosphate, but was active in the conversion of acetyl CoA to acetyl phosphate. It was apparent, therefore, that the action of the ferrous iron was to activate a phosphotransacetylase present in extracts of C. acidiurici.

To confirm the site of action of the divalent metal and to study other properties of the enzyme responsible for the transfer of acetyl groups between CoA and phosphate, the partially purified fraction described in Materials and Methods was used. During storage or during the fractionation procedures employed, the phosphotransacetylase activity entirely disappeared from the extracts and fractions. Reactivation was readily accomplished, however, by the addition of ferrous iron (Fig. 2). The marked dependency of the C.

Table 2 indicates that this conversion is further dependent on a divalent metal. Ferrous iron proved to be more effective than other metals tested for activation of this system. Although it was noted that the formation of acetyl phosphate from pyruvate by C. acidiurici extracts was inhibited by omission of ferrous iron or by the addition of metal-binding reagents, this inhibition was overcome by supplementing the extracts with a purified phosphotransacetylase fraction from C. kluyveri. This fraction alone was incapable of converting pyruvate to acetyl phosphate, but was active in the conversion of acetyl CoA to acetyl phosphate. It was apparent, therefore, that the action of the ferrous iron was to activate a phosphotransacetylase present in extracts of C. acidiurici.

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Acidiurici phosphotransacetylase on a divalent metal differs from observations we made with extracts of C. kluyveri, C. butyricum, and D. glycinophilus in which no metal requirement is apparent.

A further difference between the C. acidiurici phosphotransacetylase and the enzyme in other organisms became apparent when it was observed that addition of arsenate to extracts of C. acidiurici (supplemented with CoA, Fe++, and an electron-transport system) did not result in net disappearance of acetyl phosphate when assays were carried out according to the method of Stadtman et al. (1951). It was determined, however, that acetyl units could be transferred to CoA either from acetyl phosphate or from pyruvate in the presence of arsenate by use of substrate amounts of CoA and measurement of the accumulation of acetyl thioester. It was then desired to determine whether acetyl phosphate could be generated from pyruvate in the presence of arsenate by use of catalytic amounts of CoA. The apparent rate of degradation of pyruvate as well as the formation of acetyl phosphate was markedly inhibited by increasing amounts of arsenate (Fig. 3); it is significant to note that even in the presence of relatively high concentrations of arsenate, acetyl phosphate could be generated. It should further be noted that the pyruvate utilization and acetyl phosphate formation curves are superimposable on each other. This is contrary to what might be expected if arsenate were acting as an uncoupling agent. Under the latter conditions, the rate of pyruvate utilization would increase with increasing amounts of arsenate, even though acetyl phosphate would not accumulate. We performed such experiments with extracts of D. glycinophilus, wherein addition of arsenate increased the rate of pyruvate utilization (Klein and Sagers, 1962). Furthermore, the cation effects described by Stadtman (1952a) were observed with D. glycinophilus, but such effects were not apparent with C. acidiurici extracts.

Evidence that arsenate exerts its inhibitory effect on the phosphotransacetylase to a greater extent than on the system converting pyruvate to acetyl CoA is indicated in Fig. 4. In this case, acetyl CoA accumulation continued essentially unretarded at arsenate concentrations which produced marked inhibition of acetyl phosphate formation. In Fig. 4 CoA was supplied in substrate amounts, whereas in Fig. 3 it was supplied only in catalytic amounts.

**Fig. 2.** Iron activation of acetyl transfer between acetyl phosphate and CoA. Reaction mixtures (1 ml) contained (in μmoles): tris(hydroxymethyl)aminomethane (pH 8.0), 100; ethylenediaminetetraacetaete, 0.1; CoA, 20; acetyl phosphate, 20; ferrous sulfate, as indicated. Protein fraction A was equivalent to 0.07 mg of protein. Reaction time, 1 min at 36 C.

**Fig. 3.** Pyruvate utilization and acetyl phosphate formation as a function of arsenate concentration. Reaction mixtures (1 ml) contained (in μmoles): KH₂PO₄ (pH 8.0), 100; thiamine pyrophosphate, 0.1; nicotinamide adenine dinucleotide, 0.1; CoA, 0.2; triphenyltetrazolium, 10; FeSO₄, 2; potassium pyruvate, 20; potassium arsenate as indicated. Crude extract was equivalent to 1.2 mg of protein; 10-min reaction at 37 C.
The function of pyruvate was studied in the presence and absence of arsenate. Reaction mixtures (1 ml) contained (in μmoles): KHPO₄ (pH 8.0), 100; CoA, 0.2; thiamine pyrophosphate, 0.1; nicotinamide adenine dinucleotide, 0.1; Fe⁺⁺, 2; potassium pyruvate, 40; triphenyltetrazolium, 40; potassium arsenate, where added, 40. Crude extract was equivalent to 1.3 mg of protein; temperature, 37 C.

When the generation of acetyl phosphate from pyruvate as a function of time was studied (Fig. 5), a rapid rate was observed in the absence of arsenate. The generation of acetyl phosphate also proceeded in the presence of arsenate, although at a slower rate. In both cases, the amount of pyruvate disappearing was equal to the amount of acetyl phosphate generated. Thus, arsenate can be considered as an inhibitor but not an uncoupler in the conversion of pyruvate to acetyl phosphate by extracts of this organism.

In studies of the phosphotransacetylase reaction in the direction of acetyl CoA generation with acetyl phosphate as the acyl donor, either CoA or pantethine could be used as acetyl acceptors. In such experiments, the total acyl concentration was determined by the hydroxame.
To obtain a measurable rate of phosphate exchange, the amount of enzyme was increased ten times over that used for measuring the rate of acetyl CoA generation. The conditions were essentially the same as those shown in Fig. 7, except arsenate was omitted and 20 μmoles of KHP$^{32}$O$_4$ added. The rate of acetyl CoA acetylation was 1680 μmoles per hr per mg of protein, whereas the rate of phosphate exchange (the reverse reaction) was 21 μmoles per hr per mg of protein, or about one-eighth the rate of acetyl CoA formation. Thus, the equilibrium of the reaction in C. acidiurici preparations appears to compare favorably with that reported for C. kluyveri (Stadtman et al., 1951), and does not readily explain the failure of arsenate to uncouple the reaction. Furthermore, the demonstration of acetyl phosphate generation from pyruvate in the presence of arsenate indicates the inability of arsenate to substitute for acetyl phosphate under the conditions of these experiments. It seems possible that the divalent metal increases the specificity of the reaction in relation to the acetyl acceptor, with preference given to phosphate over arsenate in the reaction. Exploration of this hypothesis, which requires more detailed study of the properties of the enzyme together with the steric and thermodynamic relationships among the reactants involved, is currently in progress.

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**Literature Cited**


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