Integrated Enzyme Activity in Soluble Extracts of Heart Muscle

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THE CELLS OF PRACTICALLY ALL ANIMAL TISSUES contain a complex of enzymes, probably several hundred in number, which are so interdigitated structurally and functionally that the complex can be treated as if it were a single entity. The structural unit of this complex of enzymes has been identified as the mitochondrion (*1–3*), and the term "cyclophorase" has been applied to the functional or enzymatic activities exhibited by the constituent enzymes (*4, 5*). The cyclophorase-mitochondrial system catalyzes among other processes (*a*) the complete oxidation of members of the citric acid cycle, (*b*) the complete oxidation of fatty acids (*6*) and certain amino acids (*7–9*), (*c*) oxidative phosphorylation (10), and (*d*) various synthetic functions, such as the synthesis of hippuric acid (*11*) and citrulline (*12*). When mitochondria are prepared under proper conditions, they contain the full complement of enzymes and coenzymes necessary for bringing about each of the above-mentioned complicated series of reactions. One of the most fascinating problems of enzyme chemistry is that of elucidating the chemical organization that underlies the arrangement of the constituent mitochondrial enzymes, and recognizing the operational principles that underlie the reactions catalyzed by the complex.

Recently a nonmitochondrial system from pig heart has been reconstructed which catalyzes all the reactions characteristic of the organized complex (*13*). The heart system consists of three parts: a particulate nonmitochondrial fraction, a group of soluble enzymes, and a group of nucleotide coenzymes. The major components of the coenzyme group are di- and triphosphopyridine nucleotides (DPN and TPN), coenzyme A (CoA), and adenosinetriphosphate (ATP). The particulate fraction appears to supply mainly the enzymes necessary for interaction with molecular oxygen. When oxygen is replaced by some other suitable electron acceptor, many of the reactions carried out by the original heart system can now be duplicated with soluble enzymes in the presence of the necessary coenzymes. A measure of progress has been made in documenting the properties of some of these soluble enzymes. The rest of this article will be devoted to a consideration of some basic properties of the pyruvic and α-ketoglutaric oxidases, acyl coenzyme A deacylase, DPN cytochrome reductase, and the group of enzymes that collectively implement oxidative phosphorylation and the activation of acetate, acetoacetate, and fatty acids. Much of the complexity of the mitochondrial unit is still mirrored in some of these soluble enzymes.

Pyruvic and α-ketoglutaric oxidases. Chronologically the discovery of the method for solubilizing the pyruvic oxidase by Jagannathan preceded all other studies referred to here. He found that the oxidase could be extracted by multiple freezing and thawing of the particulate fraction (microsomal) of pigeon breast muscle. The systematic exploration and isolation of this oxidase was undertaken by Jagannathan and Schweet (*14*). They have obtained the oxidase of pigeon breast muscle in homogenous form, as shown by Schweet *et al.* (*15*) in electrophoresis and ultracentrifuge studies. It has a molecular weight of about 4 million. The oxidase catalyzes (*a*) the oxidation of pyruvate to acetate and carbon dioxide; (*b*) the dismutation of diacetyl to acetoin, acetate, and carbon dioxide; and (*c*) the anaerobic conversion of pyruvate to acetoin and carbon dioxide. As isolated, the oxidase requires only the addition of cocarboxylase for activity, although in some preparations a partial requirement for magnesium ions is also demonstrable. The addition of no other coenzyme is needed for the oxidation of pyruvate to acetate when ferricyanide or methylene blue is used as an electron acceptor.

Subsequently Sanadi and Littlefield (*16*) isolated the α-ketoglutaric oxidase of pig heart muscle in homogenous form. The enzyme was also extracted from the particulate fraction by alternate freezing and thawing. The molecular weight of the oxidase is in the same range as that of the pyruvic oxidase of pigeon breast muscle—i.e., about 2 million. Two catalytic activities accompany the oxidase at all stages of purity: the oxidation of α-ketoglutarate to succinate and the anaerobic decarboxylation of α-ketoglutarate to succinsemialdehyde. No additional components are needed for the reaction of the α-ketoglutaric system with ferricyanide or indophenol.

Jagannathan and Schweet (*14*) have been able to link the soluble pyruvic oxidase system of pigeon breast muscle with the sulfanilamide acetylation system of Lipmann (*17*) through CoA as the intermediary coenzyme. Under these conditions the net reaction is the oxidative acetylation of sulfanilamide by pyruvate. A similar link was effected by Sanadi and Littlefield between the α-ketoglutaric oxidase and the Lipmann acylation system, leading to the oxidative succinylation of sulfanilamide by α-ketoglutarate through CoA as the linking coenzyme (*18*). Other

acyl acceptor systems can be used in place of the Lipmann sulfanilamide acylase. Thus, Gergely and Hele (*19*) and Korkes *et al.* (*20*) linked pyruvic oxidase with the Stadtmann transacetylase (*21*) system of *Cl. Kluyverii* and accomplished the oxidative acetylation of inorganic phosphate by pyruvate. These observations point to the following formal sequence of reactions:

(1) Pyruvate $\xrightarrow{-2e}$ (oxidase + CoA) acetyl CoA $\begin{cases} \nearrow \text{acetyl sulfanilamide} \\ \searrow \text{acetyl phosphate} \end{cases}$

(2) α-Ketoglutarate $\xrightarrow{-2e}$ (oxidase + CoA) succinyl CoA $\longrightarrow$ succinylsulfanilamide

The fact that indophenol and ferricyanide can be used as electron acceptors in the assay systems for pyruvic and α-ketoglutaric oxidases, and that with these acceptors no additional cofactors are needed for maximal activity other than cocarboxylase and magnesium ions, has led to a good deal of confusion which has not yet been entirely cleared up. These two oxidases are also capable of interacting with DPN, and for this interaction CoA is an essential requirement, as was shown by the Ochoa group (*20*) and in our own laboratory (*13*). Sanadi and Littlefield (*22*) have now clarified the nexus between DPN reduction and CoA requirement. They have shown that, when CoA is added to the α-ketoglutaric oxidase system, as much DPN is reduced as CoA disappears. This would suggest the following formal reaction sequence:

(3) α-Ketoglutarate + CoA + $DPN^+ \longrightarrow$ succinyl CoA + DPNH + CO_2 + H^+

Succinyl CoA has been identified by various diagnostic reactions (hydroxamic reaction, absence of mercaptan reaction, direct succinylation of sulfanilamide) and isolated from the reaction mixture in a relatively pure state. This represents the first unequivocal demonstration and isolation of an acyl coenzyme A compound formed as an intermediate in the oxidation of a keto acid. Such evidence could be interpreted to mean that both DPN and CoA are the prosthetic groups of α-ketoglutaric oxidase—the former acting as electron acceptor and the latter as succinyl acceptor.

When highly purified pyruvic oxidase free of lactic dehydrogenase is tested under the above conditions, a similar relation has been found between DPN reduction and CoA concentration; as expressed by the following reaction sequence (*22*):

(4) Pyruvate + CoA + $DPN^+ \longrightarrow$ acetyl CoA + DPNH + CO_2 + H^+

Both the α-ketoglutaric and pyruvic oxidases require at least four prosthetic groups to catalyze the oxidative decarboxylation of their substrates: (1) diphosphothiamine, (2) magnesium ions,[1] (3) pyridine nucleotide, and (4) coenzyme A. The first and second are concerned with the decarboxylation of pyruvate or α-ketoglutarate to an intermediate at the aldehyde level. The third is concerned with the electron transfer by which the aldehyde intermediate is oxidized to the acyl CoA derivative, and the fourth is concerned with acyl transfer. Each of these components appears to be highly specific for its particular function. Diphosphothiamine is found bound to the respective enzymes, whereas DPN and CoA are usually completely dissociated.

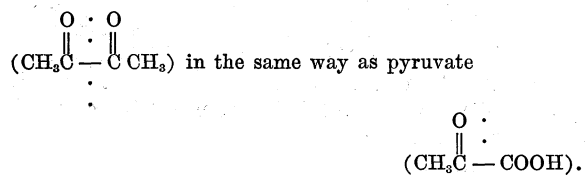
Recent studies by Gunsalus on the pyruvic oxidase systems of various bacteria have led to the discovery that the growth factor "protogen" is an essential constituent of these systems (*23*). The active principle has now been isolated by Reed *et al.* (*24*) in crystalline form and renamed α-lipoic acid. Considerable amounts of this factor have been found to be present in homogenous preparations of both the pyruvic and α-ketoglutaric oxidases of animal tissues. Thus a fifth prosthetic group, with function as yet unknown, has also to be brought into the complete picture for the pyruvic and α-ketoglutaric oxidases.

How can one explain the interaction of the α-ketoglutaric and pyruvic oxidases with indophenol or ferricyanide in absence of both CoA and DPN? There is the possibility that these oxidases contain in bound form a sufficient amount of both DPN and CoA to permit interaction at maximal velocity with ferricyanide or indophenol. However, analyses of the purified α-ketoglutaric or pyruvic oxidases have failed to disclose significant amounts either of DPN or CoA.

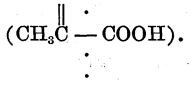
The isolation of the pyruvic oxidase in homogenous form has made possible the solution of two problems that have been shelved for some years. It has been known that animal tissues contain enzymes that catalyze the formation of one molecule of acetoin from two molecules of pyruvate (*25*), and the dismutation of diacetyl to acetoin, acetate, and CO_2 (*26*). Both these enzymatic activities have been found to be associated with the pyruvic oxidase of pigeon breast muscle from the first crude extracts to the stage of homogeneity. The mechanism of acetoin formation would appear to be as follows:

(5) Pyruvate + enzyme $\longrightarrow$ aldehyde – enzyme + CO_2
(6) 2 Aldehyde – enzyme $\longrightarrow$ acetoin + 2 enzyme

According to Schweet (*27*), the dismutation of diacetyl may be formulated as follows: diacetyl is cleaved at the carbon—carbon bond



$(CH_3C(=O)\cdot\cdot C(=O)CH_3)$ in the same way as pyruvate



$(CH_3C(=O)COOH)$.

That is to say, diacetyl is acted upon as if it were a substituted pyruvic acid. The cleavage of pyruvate yields an aldehyde enzyme product and CO_2. The

[1] The requirement of the α-ketoglutaric oxidase for magnesium ions is as yet inconclusive.

cleavage of diacetyl yields an aldehyde enzyme product and acetate. The former can undergo condensation to form acetoin, whereas the latter can be hydrolyzed to acetic acid. In line with this hypothesis is the observation that in the presence of ferricyanide, diacetyl is converted almost quantitatively to acetate, since the aldehyde enzyme product can be further oxidized to acetate. Thus the dismutation of diacetyl takes place only in the absence of an added electron acceptor.

Acyl coenzyme A deacylase. The purified α-ketoglutaric oxidase in the presence of substrate does not interact with DPN. Addition of a catalytic amount of CoA leads to no more reduction of DPN than is stoichiometric with the CoA added. Gergely, Hele, and Ramakrishnan (*28*) have found that when an extract of pig heart is added to the α-ketoglutaric oxidase system in the presence of catalytic amounts of CoA, DPN is rapidly and completely reduced. This extract contains an enzyme that has now been brought to a stage of high purity, and that has been shown to catalyze the following reaction:

(7) Succinyl CoA + H_2O ⟶ succinate + CoA

The enzyme thus has the character of an acyl CoA deacylase. Although the enzyme has been invaluable to the enzyme chemist in that it has facilitated the study of the interaction of the α-ketoglutaric oxidase with DPN, there is difficulty in understanding the physiological function of an enzyme that, in effect, nullifies the conservation of oxidative energy. Deacylase stands in the same relation to acyl CoA as ATP-ase does to ATP.

The presence of a second acyl coenzyme A deacylase specific for acetyl coenzyme A has also been demonstrated in crude extracts of pig heart (*29*). This enzyme catalyzes the reaction

(8) Acetyl CoA + H_2O + ⟶ acetate + CoA

There are indications of the presence in heart extract of a deacylase that is active on acetoacetyl and β-ketocaproyl CoA (*30*).

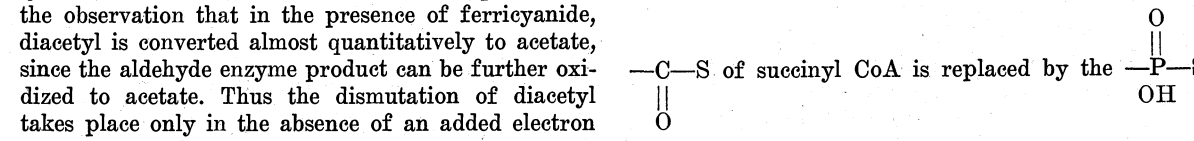
Oxidative phosphorylation. The purified α-ketoglutaric oxidase does not require inorganic phosphate, nor is the oxidation of α-ketoglutarate by suitable electron acceptors accompanied by the esterification of inorganic phosphate. However, when a soluble extract of pig heart muscle is added to the purified oxidase, the oxidation of α-ketoglutarate can now be coupled to esterification of inorganic phosphate (*31, 13*). The evidence is consistent with the following postulated reaction sequence:

(9) Succinyl CoA + inorganic phosphate $\overset{\text{phosphorylating enzyme}}{\rightleftharpoons}$ succinate + phosphoryl CoA

(10) Phosphoryl CoA + ADP $\overset{\text{transphosphorylase}}{\rightleftharpoons}$ CoA + ATP

(11) ATP + glucose $\overset{\text{hexokinase}}{\longrightarrow}$ ADP + glucose-6-phosphate

The link with inorganic phosphate is brought about by an enzyme that catalyzes the replacement of a succinyl by a phosphoryl group. Assuming the functional group of CoA to be SH (Lynen), then the



of phosphoryl CoA. The high energy bond is conserved during the replacement reaction, as represented in the following formal equation, in which the Lipmann symbol ~ denotes a high energy bond:

(12) Succinyl ~ CoA + inorganic phosphate ⟶ phosphoryl ~ CoA + succinate.

The successful reconstruction of oxidative phosphorylation requires the separation of the phosphorylating enzyme from acyl coenzyme A deacylase. The latter decomposes succinyl CoA before the replacement reaction can take place and thus makes impossible the esterification of inorganic phosphate.

Kaufman, in Ochoa's laboratory (*31*), has attempted to reverse the above reaction sequence by starting with succinate and ATP in the presence of both the phosphorylating and transphosphorylating enzymes supplemented with CoA. The net reaction should be a CoA-dependent dephosphorylation of ATP to ADP and inorganic phosphate. Although this dephosphorylation does indeed proceed, the mechanism appears not to involve the reversal of Reactions (9) and (10).

The systematic purification and isolation of the enzymes involved in oxidative phosphorylation are being carried on in our laboratory by Sanadi and Littlefield (*32*). The application of low temperature alcohol fractionation has proved very useful as a means of separating them from acyl coenzyme A deacylase.

The system whose reaction sequence is represented in Equations (9) to (11) bears close analogies to its counterpart in the mitochondrial system. The oxidation process will come to a halt unless inorganic phosphate is present, since the deacylation of the oxidase is dependent upon replacement of the acyl group by inorganic phosphate. Furthermore, the oxidation leads to the formation of ATP, thus in effect converting oxidative energy into phosphate bond energy.

In the mitochondrial system, the oxidation of each member of the citric acid cycle, the oxidation of β-hydroxybutyrate and glutamate and the interaction of DPNH with flavin, and of reduced flavin with cytochrome are all linked with phosphate esterification. Much remains to be done on these different facets of the problem of oxidative phosphorylation. However, the experimental door is now slightly ajar and appears not too resistant to being opened further.

Activation of acetate. A soluble enzyme has been prepared from extracts of pig heart muscle in purified form which, in the presence of CoA and magnesium ions, catalyzes the following reaction (*30*):

(13) Acetate + ATP + hydroxylamine $\xrightarrow[Mg]{CoA}$ acethydroxamic acid + inorganic pyrophosphate + AMP

This over-all process presumably is brought about by three consecutive reactions:

(14) ATP + CoA ⟶ AMP + pyrophosphoryl CoA

(15) Pyrophosphoryl CoA + acetate ⟶ acetyl CoA + inorganic pyrophosphate

(16) Acetyl CoA + hydroxylamine ⟶ acethydroxamic acid + CoA

The enzyme that catalyzes Reaction (15) is active toward propionate but not butyrate. Sodium but not potassium ions inhibit the activity of this enzyme.

Inspection of Equations (14) and (15) discloses that the activation of acetate is not the reverse of oxidative phosphorylation accompanying the oxidation of pyruvate:

(17) Pyruvate + CoA + DPN ⟶ acetyl CoA + DPNH + CO_2 + H^+

(18) Acetyl CoA + inorganic phosphate ⟶ phosphoryl CoA + acetate

(19) Phosphoryl CoA + ADP ⟶ CoA + ATP.

Acetyl CoA can be shown to be formed as the product of oxidation of pyruvate and of the interaction of ATP, CoA, and acetate. The intermediary formation of phosphoryl CoA in the former case and of pyrophosphoryl CoA in the latter case has still to be established by direct isolation, although there is much supporting evidence for intermediaries.

Activation of acetoacetate. Soluble extracts of pig heart muscle contain enzymes which rapidly catalyze the following over-all reaction (*30*):

(20) Acetoacetate + α-ketoglutarate + 3 DPN^+ + 2 malate ⟶ 2 citrate + CO_2 + 3 DPNH + CO_2 + 3 H^+

This appears to be made up of the following constituent reactions:

(21) Succinyl CoA + acetoacetate ⟶ acetoacetyl CoA + succinate

(22) Acetoacetyl CoA + CoA ⟶ 2 acetyl CoA

(23) 2 Malate + DPN^+ ⟶ 2 oxalacetate + 2 DPNH + 2 H^+

(24) 2 Acetyl CoA + 2 oxalacetate ⟶ 2 citrate + 2 CoA

Acetoacetate can be replaced by other β-ketoacids such as β-ketovalerate and β-ketocaproate. The activity declines with increasing chain length and is essentially zero toward β-ketooctanoate.

Two simpler and more direct test systems have been devised for demonstrating the formation of acetoacetyl CoA. The breakdown of ATP (ADP) to ADP (AMP) and inorganic P can be catalyzed by small (catalytic) amounts of acetoacetate in the presence of the appropriate heart enzyme fraction. The reaction sequence involves the formation of acetoacetyl CoA and its breakdown by an acyl coenzyme A deacylase. A small amount of acetoacetate can thus bring about the dephosphorylation of a large amount of ATP. Other β-ketoacids such as β-ketovaleric and β-ketocaproic acids can replace acetoacetic acid. This ATP-ase type of test system has also been useful in demonstrating acyl coenzyme A formation from acetic and propionic acids.

Under appropriate conditions the activation of acetoacetate can be followed by this reaction:

(25) Acetoacetate + ATP + hydroxylamine $\xrightarrow{CoA}$ acetoacethydroxamic acid + AMP + inorganic pyrophosphate

This has proved to be the simplest assay system for the activating enzyme.

Oxidation of fatty acids. Fatty acid oxidation as carried out in soluble extracts of pig heart may be resolved into three component processes (*33*): (1) the formation of the fatty acyl CoA compound starting with ATP, CoA, and fatty acid; (2) the β-oxidation of this compound to the corresponding β-ketoacid; and finally (3) the cleavage of the β-ketoacid CoA compound to acetyl CoA and an acyl CoA with 2 carbon atoms less than the parent fatty acid. There are at least three physiological mechanisms for forming the fatty acyl CoA compound. The first involves replacement of the pyrophosphoryl group in pyrophosphoryl CoA by a fatty acid. The second involves Reaction (22) in which the higher β-ketoacid is cleaved into acetyl CoA and fatty acyl CoA. The third involves a replacement of the succinyl group in succinyl CoA by a fatty acid—*cf.* Reaction (21).

A group of enzymes is involved in the activation, respectively, of fatty acids, α-β unsaturated acids, and β-hydroxyacids. Thus, one enzyme derived from pigeon liver and pig heart catalyzes the interaction of butyrate and caproate with hydroxylamine in the presence of ATP and CoA. Octanoate is acted upon relatively slowly by this enzyme. Crotanate and β-hydroxybutyrate are essentially inactive as substrates.

The enzymes concerned in the activation of fatty acids and in the disposal of β-ketoacids have posed no special technical difficulties. However, the enzymes concerned in the β-oxidation of activated fatty acids have proved to be exceptionally labile.

Role of coenzyme A. Considering the pivotal role of coenzyme A in practically all the important mitochondrial reaction sequences, it is appropriate to note that the recent advances in reconstructing mitochondrial activities in soluble extracts are mere extensions and applications of the fundamental studies of Lipmann (*34*) who, following his codiscovery of coenzyme A with Nachmansohn and Machado (*35*), worked out the operational principles of acyl transfer reactions. Many of the enzymatic mechanisms that have been considered in this article were anticipated and predicted by Lipmann, and a good deal of our contemporary terminology reflects the influence of his thinking. The elegant studies of fatty acid oxidation and syntheses in *Cl. Kluyverii* by Barker and Stadtmann (*36*) have also contributed greatly to our present knowledge of the mechanism of acyl transfer reactions involving coenzyme A. More recently Stern and Ochoa (*37*) have made an important advance in isolating the enzyme that catalyzes the condensation

of acetyl CoA and oxalacetic acid to form citric acid. Finally, Lynen (*38*) has pointed up the SH group of CoA as the group involved in acyl transfer reactions. These advances were all essential preliminaries to the successful reconstruction of oxidative phosphorylation and fatty acid oxidation in soluble extracts.

Consideration of any reaction sequence involving CoA reveals why it has been extremely difficult to resolve these sequences into a series of one-step reactions. For example, the activation of acetate involves the formation of pyrophosphoryl CoA and then a replacement reaction to form acetyl CoA. To study the latter reaction, pyrophosphoryl CoA must be provided as a substrate, whereas in the over-all reaction pyrophosphoryl CoA is acting catalytically. The substrates of a considerable number of enzymes are derivatives of CoA, and the only way these enzymes can be studied with reference to the particular reaction catalyzed is to supply the CoA derivative in substrate amounts. Until recently, it was not feasible to carry out such experiments. But with the development of a rapid and simple method by Beinert *et al.* (*39*) for isolating highly purified coenzyme A in good yield, the way is now open for the large-scale routine isolation of the biologically important derivatives of CoA, such as phosphoryl, acetyl, and succinyl CoA. The availability of these derivatives should make it possible to investigate one enzymatic reaction at a time.

The interdependence of reactions involving acyl transfer through CoA may help to clarify the necessity for an organized structure like the mitochondrion. It may well be that structural contiguity of enzymes makes for greater efficiency in executing reaction sequences in which activated molecules are transferred from one enzyme to another.

DPN cytochrome reductase. The interaction of DPNH with cytochrome *c* is a process that hitherto has been demonstrable only in particulate preparations. Edelhoch, Hayaishi, and Teply (*40*) have solubilized DPN cytochrome reductase, starting with a particulate fraction of pigeon breast muscle. More recently Mahler, Vernon, and Sarkar (*41*) have achieved the same result with pig heart muscle and have succeeded in obtaining DPN cytochrome reductase in homogenous form.

There are two categories of enzymes involved in the oxidation of DPNH: (*a*) the diaphorases, which catalyze the oxidation of DPNH by various nonphysiological electron acceptors; and (*b*) the reductases, which catalyze the oxidation of DPNH by cytochrome *c*. The relationship between these two types of enzymes has been obscure. Electrophoretic studies of cytochrome reductase have established that both diaphorase and reductase activities are associated with the same flavoprotein component. Diaphorase activity would thus appear to be a consequence of the partial inactivation of the reductase enzyme.

Cytochrome reductase is a flavoprotein with a molecular weight of about 70,000, based on spectrophotometric flavin analyses and physical measurements. The flavin prosthetic group is probably not identical either with flavin adenine dinucleotide or flavin monophosphate, though under certain conditions it can give rise to the latter.

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