# ALPHA-HYDROXY ACID OXIDASES IN SUBCELLULAR FRACTIONS FROM RAT KIDNEY

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It has been shown, by means of gel electrophoresis and staining with histochemical techniques, that rat kidney homogenates exhibit more than five molecular species with lactate dehydrogenase activity (Buta, Conklin and Dewey, 1966). One of the additional forms is able to react even in the absence of the coenzyme NAD. It was suggested that this peculiar fraction would be an alpha-hydroxy-acid oxidase. The localization of this enzyme in renal microbodies has been reported by Allen and Beard (1965).

We have studied the distribution of the enzyme in rat kidney subcellular fractions and found evidence of molecular heterogeneity.

## METHODS

Homogenates from adult rat kidney in water (1:5 weight/volume) were submitted to 20,000 g for 20 minutes at 4° C and the supernatant used for electrophoretic studies.

Cellular fractions from adult rat kidney were obtained by the usual technique of differential centrifugation in 0.25 M sucrose, 0.0025 M Tris pH 7.4. The mitochondrial fraction was washed three times with 0.25 M sucrose, and then lysed with one volume of distilled water and repeated freezing and thawing.

Electrophoresis on starch gel was performed using the vertical device

of Smithies (1959). The borate buffer proposed by Smithies was usually employed; occasionally, other systems were also used: Tris, citric - borate (Poulik, 1957), phosphate (Koen and Shaw, 1965), and Tris, boric EDTA (Markert and Faulhaber, 1965). Staining for lactate dehydrogenase was performed by the method used by Zinkham and col. (1964).

Enzymatic activity was determined by a method adapted from that of Nachlas and col. (1960) for succinic dehydrogenase, using nitro-blue tetrazolium. Total protein concentration, by measuring optical densities at 260 and 280 mμ.

## RESULTS AND DISCUSSION

After starch gel electrophoresis and staining for lactate dehydrogenase, total homogenates of adult rat kidney show a pattern composed by six fractions (Fig. 1). Five of them correspond to the LDH isozymes common to most tissues, and the sixth migrates between the areas of LDH-2 and LDH-3. We have not obtained patterns with seven bands like those described by Buta and col. (1965). The additional fraction can be demonstrated also by omitting the coenzyme NAD from the reagent mixture. In such a case, LDH isozymes do not stain, but the formazan precipitation on the extra band show the same intensity.

Mitochondrial extracts exhibit a faint LDH-1, and very weak LDH-2 and LDH-3 bands. An area of intense staining appears between LDH-2 and LDH-3 (Fig. 1). The pattern for the supernatant remaining after separation of microsomes, is similar to that described for renal homogenates.

Like the additional area in supernatant and total homogenate, the extra band in mitochondria does not need added NAD to react, but it differs in

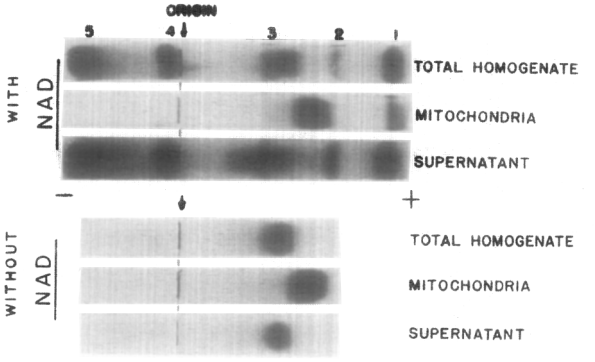


Figure 1. - Electrophoretic patterns on starch gel. Staining for lactate dehydrogenase. Numbers at the top indicate the position of LDH isozymes. Lower part of the photograph shows patterns from the other half of the gel stained without NAD.

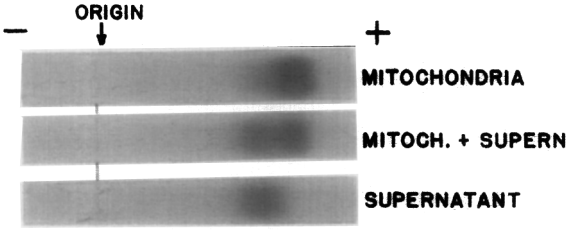


Figure 2. - MITOCHONDRIA + SUPERN. is a 1 : 1 mixture of mitochondrial extract and supernatant remaining after microsome separation. Staining for LDH except NAD.

electrophoretic mobility. This difference persisted with the various buffers and electrophoretic systems used, or when mixtures of mitochondrial extract and supernatant were electrophoresed (fig. 2). Both bands were always shown as two distinct fractions.

Separation of microbodies with the method utilized by Allen and Beard (1965) demonstrated that the band in supernatant described here is identical to that shown by their preparations.

Both bands reacted better when alpha-OH-acids of longer carbon chain were substituted for lactate in the staining mixture. Alpha-OH-valerate and alpha-OH-butyrate gave the most intense staining on the starch. Certain amino acids, specially L-leucine, can be used as substrates by the enzyme but at a lower rate. No activity could be detected against D-amino acids.

Specific activity with lactate was five fold higher in mitochondrial preparations than in supernatant. Table I shows the relative activity of the two fractions against several substrates. The difference in reactivity with amino acids between the mitochondrial and supernatant enzymes, is another evidence in favor of the diversity of the two fractions.

Attempts to separate the bound coenzyme, such as adsorption on activated charcoal columns, treatment with concentrated phosphate buffer, and dialysis, which usually split pyridine nucleotides, failed to inactivate the enzyme.

Although we do not have a direct evidence as to the nature of the prosthetic group involved, the catalytic properties exhibited by the two enzymes strongly suggest that they are alpha-hydroxy-acid oxidases and, therefore, flavoproteins. Alpha-OH-acid oxidase from rat kidney was

TABLE I

ACTIVITY OF ALPHA HYDROXY ACID OXIDASES
FROM RAT KIDNEY AGAINST DIFFERENT SUBSTRATES
( Relative values expressed in percentage )

| | MITOCHONDRIA | SUPERNATANT |
|---|---|---|
| DL-LACTATE | 100 | 100 |
| DL-α-OH BUTYRATE | 123 | 104 |
| DL-α-OH VALERATE | 145 | 120 |
| L-LEUCINE | 20 | 40 |
| DL-LEUCINE | 15 | 35 |
| DL-METHIONINE | 18 | 31 |
| DL-NORLEUCINE | 15 | 26 |
| DL-ISOLEUCINE | 8 | 24 |

identified by Blanchard and col. (1946). These investigators demonstrated that the prosthetic group is flavin mononucleotide.

Although kidney is apparently the organ in which alpha-OH-acid oxidase is present in higher concentration, we have found that other rat tissues exhibit also the enzyme. Liver mitochondria, for example, show the same isozyme as kidney mitochondria.

Molecular heterogeneity of enzymes is a very common and well documented phenomenon; numerous enzymes have been demonstrated to exist in multiple molecular forms. The evidence presented here indicates that alpha-hydroxy-acid oxidase exhibit also isozymic forms. Whether they represent entirely different proteins or have some common structural feature (e. g. polypeptide subunits) remains to be studied.

The demonstration of these enzymes by the usual histochemical techniques for lactate dehydrogenase, contributes with additional bands to the LDH pattern, simulating subfractionation. This possibility is to be considered in some cases of LDH-subbanding.

As the metabolic role of alpha-OH-acid oxidase is not well understood, it is not possible to assign a functional signification to the findings reported here. However, the demonstration of different molecular forms of an enzyme specifically associated with subcellular organelles or fractions, is of great biological interest. Although catalysing the same reaction, these enzymes must be integrated in different metabolic pathways.

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