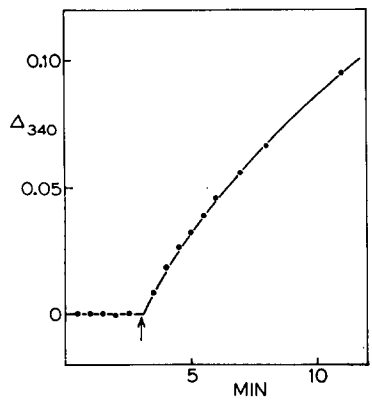

Fig. 1. Partial resolution of KG oxidase. Microcells (1-cm light path) contained in a total vol. of 1.0 ml: 0.06 *M* phosphate, pH 7.4; $4.5 \cdot 10^{-5}$ *M* CoA; $3 \cdot 10^{-3}$ *M* cysteine; $3 \cdot 10^{-4}$ *M* DPN and 3 μg of a fraction eluted from a column in front of the KG oxidase (see text). At the time shown by the arrow 8 μg purified diaphorase was added. An identical result was obtained by reversing the order of addition of the two protein solutions. Temp., 25°.

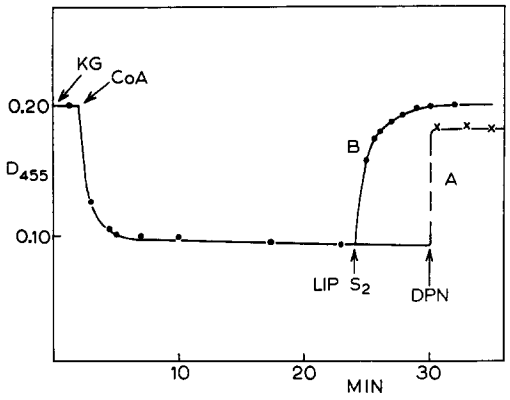

Fig. 2. The anaerobic reduction and re-oxidation of flavin in the KG oxidase complex. Two anaerobic cells were used containing 0.06 *M* phosphate, pH 7.4, and 10 mg KG oxidase in a final volume of 3.0 ml. Reduction was obtained by the addition of 0.15 μmole CoA (reduced with cysteine) and 2 μmoles KG. Reoxidation was obtained on the addition in cell (A) of 2 μmoles DPN or (B) 4 μmoles lip S_2. Temp., 4°.

diaphorase in the KG oxidase system of pig heart suggest very strongly that the physiological role of diaphorase is as the lipoic dehydrogenase of keto acid oxidation rather than as a component of the electron-transport chain in DPNH oxidation.

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Labeling of the pH-5 enzyme with adenosine triphosphate

In a previous communication from this laboratory[1] we have shown that the exchange between ATP and ^{32}P-labeled inorganic pyrophosphate by the pH-5 enzyme from rabbit liver is sensitive to ribonuclease. To obtain more direct evidence concerning the role of RNA in this reaction, ^{32}P labeling of the pH-5 enzyme with [$\beta\gamma$-$^{32}P_2$]ATP was studied.

[$\beta\gamma$-$^{32}P_2$]ATP was prepared by oxidative phosphorylation with rat-liver mitochondria in the presence of $H_3{}^{32}PO_4$, and purified twice by chromatography on

Abbreviations: ATP, adenosine triphosphate; PP, inorganic pyrophosphate; RNA, ribonucleic acid.

Dowex 1, employing the method of COHN AND CARTER[2]. The pH-5 enzyme and [$^{32}P_2$]PP were prepared as described in previous papers[1,3].

When the pH-5 enzyme was incubated with [$\beta\gamma$-$^{32}P_2$] ATP and Mg^{++}, ^{32}P-activity remained in the precipitate containing RNA and protein, after washing 8–10 times with cold 6 % $HClO_4$ or dilute acetate buffer (pH 5). This labeling was dependent on Mg^{++} concentration, the maximum being obtained above 5 mM. The fractionation of ^{32}P-labeled pH-5 enzyme was carried out by the modified method of KIRBY[4]. A typical example is shown in Table I. As shown in this table, specific activity (per mg dry weight) was the highest in the RNA fraction and the lowest in the protein fraction.

TABLE I

SPECIFIC ACTIVITY OF RNA AND PROTEIN FRACTION OF THE ^{32}P-LABELED pH-5 ENZYME

0.15 μmole [$\beta\gamma$-$^{32}P_2$]ATP (161,500 counts/min), 50 μmoles $MgCl_2$, 150 mg enzyme protein in a final vol. of 10 ml. Incubation at 37° for 5 min. RNA was fractionated according to the method of HOAGLAND *et al.*[10].

Reaction time (min)	*Specific activity (counts/min/mg)*		
	Protein-RNA	*Protein*	*RNA*
0	14.0	5.1	114
5	66.0	51.0	750

The labeled RNA fraction was then subjected to paper chromatography according to the modified procedure of MAGASANIK *et al.*[5] (butyric acid and NH_4OH as solvent, pH 3.5), by which nucleic acid remained immovable while acid-soluble phosphate compounds were developed. The fact that radioactivity was present only at the origin indicated that the radioactivity of this fraction was probably due to polynucleotide and not to contamination by acid-soluble phosphate compounds.

The specific activity of the pH-5 enzyme was decreased by the addition of unlabeled ATP and the decrease was dependent on NaF, although high concentrations of NaF inhibited the labeling. Furthermore, Fig. 1 shows that the specific activity of the pH-5 enzyme decreased and the total activity of PP increased on the addition of an amino acid mixture (containing 12 amino acids as described in the previous paper[3]) in the presence of a suitable amount of NaF. This labeling was depressed by the addition of PP.

The possibility of a reversal of this reaction, *i.e.*, labeling of the enzyme with [$^{32}P_2$]PP, was then examined. It was found that the enzyme was also labeled to a small extent with [$^{32}P_2$]PP but not with $H_3{}^{32}PO_4$. The labeling increased as the Mg^{++} concentration increased up to 5 mM. ATP was necessary for the labeling of the enzyme with [$^{32}P_2$]PP, although high concentrations of ATP caused a dilution in the labeling. It was further noted that the labeling also increased in the presence of the amino acid mixture.

It is generally supposed that an intermediate in the amino acid activation is adenyl amino acid, and current evidence indicates that this may be true in the activation of tryptophan[6,7], leucine[8], and methionine[9]. However, the present results support the view that complex formation between the terminal PP of ATP and the pH-5 RNA may occur in the activation of at least some amino acids by the pH-5

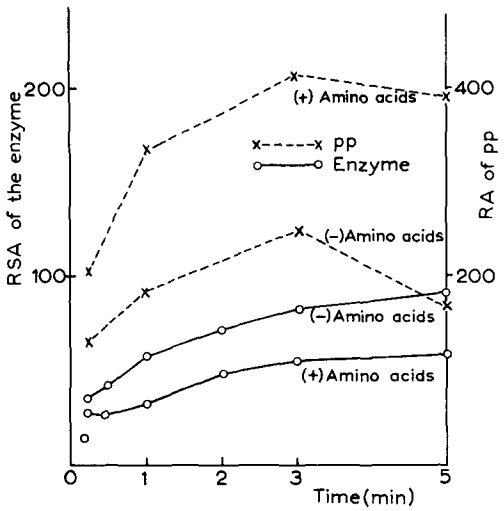

Fig. 1. Effect of amino acids on the ^{32}P labeling of the pH-5 enzyme. 10 mμmoles [$\beta\gamma$-$^{32}P_2$]ATP, 5 μmoles $MgCl_2$, 10 μmoles NaF, 5 μmoles of each of twelve amino acids, 6.2 mg enzyme protein, in a final vol. of 1 ml. Incubation at 10°.

$$RSA = \frac{\text{specific activity of enzyme (counts/min/mg)}}{\text{activity of ATP (counts/min)}} \cdot 10^4$$

$$RA = \frac{\text{activity of PP (counts/min)}}{\text{activity of ATP (counts/min)}} \cdot \frac{10^4}{\text{amount of enzyme (mg)}}$$

enzyme. The bound PP may then be substituted by amino acid or adenyl amino acid and, as a result, amino acid binds with the pH-5 RNA, as was shown by HOAGLAND *et al.*[10], the present authors[1], and others[11–15].

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