THE MECHANISM OF ACTION OF UDPG DEHYDROGENASE*

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In 1954 Strominger et al. demonstrated the NAD-linked conversion of UDPG[1] to UDPGA, catalyzed by an enzyme preparation from calf liver. Since attempts to demonstrate the involvement in the reaction of a compound resulting from the transfer of a single pair of electrons were negative and since reasonable stoichiometry between $NADH_2$ and UDPGA was observed throughout the reaction, it was concluded that any intermediate in the 4 electron transfer reaction must remain tightly bound to the enzyme (Maxwell et al., 1956).

Recently, Simonart et al. (1966) have presented kinetic and chemical evidence that the oxidation occurs in a stepwise fashion with the formation of an unidentified intermediate which subsequently is converted to UDPGA. In both these studies a partially-purified enzyme of relatively low specific activity was employed. We now have reinvestigated the dehydrogenation of UDPG with a homogeneous enzyme of high specific activity and have not been able to demonstrate an intermediate. Furthermore, we have been able to explain the anomolous kinetics of the reaction on the basis of product inhibition. Details are presented in this paper.

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1. The following abbreviations are used: uridine diphosphate-D-glucose, UDPG; uridine diphosphate-D-galactose, UDPGal; uridine diphosphate-D-glucuronic acid, UDPGA.

Materials and Methods. All unlabelled nucleotides were commercial products. UDPG uniformly labelled with ^{14}C in the D-glucosyl moiety was obtained from New England Nuclear. Descending chromatography was carried out on Whatman 3 MM paper in ethanol: 0.1 M ammonium acetate, 0.002 M EDTA, pH 7.0 (7:3) for 48 hours at 25° (Paladini and Leloir, 1952) and in ethanol: butanone: 0.5 M morpholinium tetraborate, pH 8.6 (7:2:3) (Carminatti et al., 1965). Nucleotides were located on chromatograms by visual inspection of ultraviolet quenching at 254 mμ. Radioactive compounds were located with a Nuclear Chicago 4 TT strip scanner. ^{14}C was counted in the Packard Model 3375 Tri-Carb Liquid Scintillation Spectrometer at maximum efficiency. 4 x 5 cm areas of chromatograms containing radioactive material were counted in vials containing scintillation fluid consisting of 5 g of PPO and 0.1 g of POPOP per liter of toluene. Appropriate areas containing no radioactive material were used as blanks.

Assays for UDPG dehydrogenase activity were performed at 30° in 1 ml of 0.1 M glycine, pH 8.7, containing 1 μmole each of NAD and UDPG. The increase of absorbance at 340 mμ was followed in the Gilford Multiple Sample Recorder.

Results. UDPG dehydrogenase was purified from fresh beef liver homogenate by extraction at pH 4.9, ammonium sulfate fractionation between 0.30 and 0.50 saturation, treatment at 60° and pH 4.9 for 1 minute, refractionation with ammonium sulfate between 0.35 and 0.45 saturation, titration with calcium phosphate gel, chromatography on carboxymethyl cellulose, and finally chromatography on Sephadex G-200. The purification, which is summarized in Table 1, will be reported in detail elsewhere (Zalitis and Feingold, in preparation). The product, which has approximately twice the specific activity of the preparation obtained by Wilson (1965), gives a single band upon electrophoresis in polyacrylamide gel at pH 8.9 (Davis, 1964). It yields a single symmetrical peak upon boundary sedimentation in the Spinco Model E ultracentrifuge; in addition, zone sedimentation of the enzyme in a sucrose gradient gives a symmetrical peak with complete coincidence of patterns for

Table 1

PURIFICATION OF UDPG DEHYDROGENASE

Fraction	Specific Activity*	Purification (-fold)	% Recovery
pH 4.9 extract	0.006	1	100
$(NH_4)_2SO_4$	0.024	4	82
Heat	0.032	5	57
$(NH_4)_2SO_4$	0.069	12	54
Calcium phosphate gel	0.35	60	29
CM cellulose	1.14	190	15
Sephadex G-200	3.40	570	13

* Units per mg protein at 30 C.

enzyme activity and total protein.

Simonart et al. (1966) ascribe the kinetics obtained for the enzymatic dehydrogenation of UDPG, which are not in accord with expectations for a typical two-substrate reaction, to a two-step mechanism. Neufeld and Hall (1965) have shown that one of the products of the dehydrogenation, UDPGA, is an inhibitor competitive with UDPG (K_i = 0.05 mM) and non-competitive with NAD. $NADH_2$ likewise is an inhibitor of beef liver UDPG dehydrogenase, strictly competitive with $NADH_2$ (K_i = 0.006 mM) (Fig. 1). Thus the kinetics of the reaction can be explained without invoking a two-step mechanism, for as has been pointed out by Webb (1963), if a reaction product is an inhibitor and its K_i is small, the reaction rate may drop very rapidly after the reaction has started.

In order to investigate whether a demonstrable intermediate is formed during the dehydrogenation, the stoichiometry of the reaction was examined

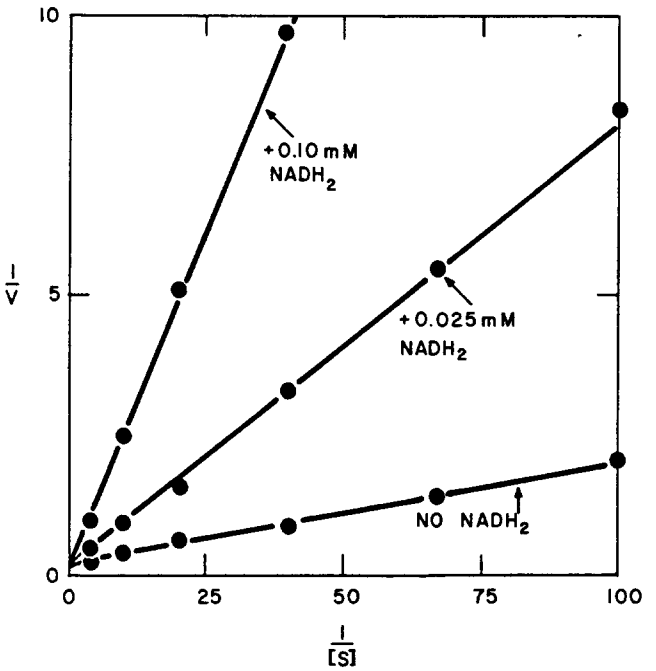

Figure 1. Inhibition of UDPG dehydrogenase by $NADH_2$. Conditions of assay are as described in the text with the exception that the NAD concentration was varied as indicated. V = mμmoles UDPG oxidized/minute; S = mM NAD.

in detail. As shown in Fig. 2, there is exact correspondence between the quantities of $NADH_2$ and UDPGA formed as well as between the UDPG consumed and the UDPGA produced. Duplicate samples removed from the reaction mixture at times 0, 2, 5 and 80 minutes were inactivated and chromatographed as described in the Figure; the radioactive areas were located, eluted, and rechromatographed in the solvent of Carminatti et al. (1965). UDPG, UDPGal, UDPGA and UMP were run simultaneously as standards to test for the efficacy of separation. No radioactive compounds other than those with the chromatographic mobilities of UDPG and UDPGA were present in any sample. Since no intermediate can be demonstrated and since there is precise stoichiometry during the conversion of UDPG to UDPGA, it must be concluded that any compound of intermediate oxidation level remains tightly bound to the enzyme throughout the reaction.

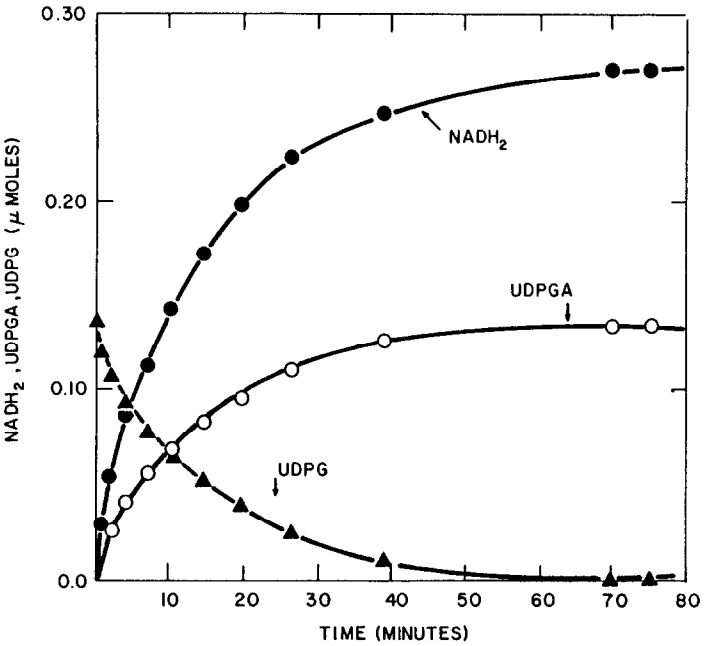

Figure 2. Time course of UDPG dehydrogenase action. Reaction mixtures at 30° in 1 ml of 0.1 M glycine buffer, pH 8.7, contained 0.135 μmoles ^{14}C-UDPG (2.2 x 10^6 cpm), 1 μmole NAD and 0.01 units of enzyme. At times indicated 25 μl samples were inactivated at 100° for 1 minute and after dilution with a mixture of unlabelled UDPG and UDPGA were separated chromatographically in the solvent system of Paladini and Leloir (1952). Areas containing UDPG and UDPGA were excised and counted as described. That active enzyme still was present at the end of the reaction was shown by addition of 1 μmmole unlabelled UDPG to the reaction mixture resulting in a corresponding increase of $NADH_2$.

UDPG dehydrogenase is relatively unspecific for the nucleoside portion of the substrate but is highly specific for the glucosyl moiety (Table 2). Were the uracil base obligately involved in the dehydrogenation, no activity would be expected with adenosine diphosphate glucose, cytidine diphosphate glucose, or guanosine diphosphate glucose. By the same token, UDPGal and uridine diphosphate mannose might be expected to participate in the initial oxidation postulated by Simonart et al. (1966), but as can be noted in Table 2, they are completely inactive. These results are further presumptive evidence against direct involvement of the uracil moiety of UDPG in the action of UDPG dehydrogenase.

Table 2

SPECIFICITY OF UDPG DEHYDROGENASE FOR SUGAR NUCLEOTIDES

Substrate	Relative Rate	K_m(mM)
Uridine diphosphate glucose	100.0	0.013
Thymidine diphosphate glucose	8.5	2.0
Cytidine diphosphate glucose	0.3	6.0
Guanosine diphosphate glucose	0.1	10.0
Adenosine diphosphate glucose	0.03	20.0
Uridine diphosphate galactose	0	-
Uridine diphosphate mannose	0	-

Reaction mixtures at 30° in 1 ml of 0.1 M glycine buffer, pH 8.7, contained 2 μmoles each sugar nucleotide and NAD. The reaction was started by addition of 4.5 mg of enzyme (3 μg in reaction mixtures containing uridine diphosphate glucose and thymidine diphosphate glucose). Increase of absorbance at 340 mμ was followed.

Km values were determined under identical conditions except that the quantities of sugar nucleotide were varied appropriately.

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