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Analogs of Uridine Diphosphate Glucose in the Reaction with UDPG Dehydrogenase

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In connection with elucidating the mechanism of enzymatic reactions in which nucleoside diphosphate sugars participate, we studied the behavior of synthetic analogs of UDPG* with a modified uracil nucleus ([1]) in the corresponding transformations. It was of particular interest to determine whether the requirements for the presence of certain groups in the uracil nucleus are common to different enzymes, or whether different groupings in the heterocyclic nucleus of NDPS are essential for each enzyme. In the present communication we give data on the study of some analogs of UDPG in the oxidation reaction of the monosaccharide contained in the NDPS to a glucuronic acid residue in the presence of UDPG dehydrogenase. The oxidation reaction proceeds according to the equation

$$\text{UDPG} + 2\,\text{NAD} \rightarrow \text{UDP-glucuronic acid} + 2\,\text{NADH}_2.$$

We studied the influence of changes in the uracil nucleus of the analogs on the maximum initial rate of the reaction, which made it possible to evaluate the influence of these changes on the formation of the final product of the reaction –NDP-glucuronic acid. Application of the method of "mixed substrates" ([2]) made it possible to judge the ability of UDPG analogs to form enzyme-substrate complexes during the reaction. As a result of the work carried out, it was established that 3-N-methyl-UDPG and CDPG are not capable of being oxidized to the corresponding nucleotide diphosphate glucuronic acids, whereas 6-aza-UDPG is oxidized to 6-aza-UDP-glucuronic acid. The enzymatic preparation of UDPG dehydrogenase was obtained by us by the method of Strominger ([3]) from acetone powder of calf liver. The preparation obtained after five stages of purification had a specific activity equal to 60-90 units** per 1 mg of protein. A preparation of this degree of purification contains no admixture of nonspecific dehydrogenases and is usually used for determining the purity of UDPG preparations ([4]). The activity of the enzymatic preparation was determined by the increase in optical density at 340 mμ accompanying the formation of the reduced form of NAD ([5]). In addition, the activity of the enzymatic preparation was determined from the increase in glucuronic acid by the colorimetric method of Dische ([6]). In determining the activity of the enzymatic preparation, the reaction mixture contained, in addition to the enzyme, $0.6\,\mu M$ UDPG, $5\,\mu M$

NAD in 3 ml of 0.1 M glycine buffer, pH 8.75; the reaction was carried out at room temperature.

As was mentioned above, in studying analogs in this enzymatic reaction we used, for comparing their activity with uridine diphosphate glucose, the value of the maximum initial rate ($V_{\max}$). Therefore we determined a series of kinetic characteristics of the obtained

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* Abbreviations used in the article: UDPG—uridine diphosphate-α-D-glucopyranose, CDPG—cytidine diphosphate-α-D-glucopyranose, NAD—nicotinamide adenine dinucleotide, NDPS—nucleoside diphosphate sugar.

** One unit of enzyme activity was taken as that amount of enzyme required to increase the optical density at 340 mμ by 0.001 per minute under the experimental conditions.

preparation of UDPG dehydrogenase. The dependence of the initial rate on protein concentration was linear.

The dependence of the initial reaction rate on the concentration of UDPG showed that, under our experimental conditions, $1.2\,\mu M$ UDPG is a saturating concentration. UDPG analogs were studied under the standard conditions described above, at a concentration of 1.5—$2\,\mu M$; if no appreciable increase in optical density at 340 mμ occurred, the concentration of the analog was increased to 4—5 μM, and the enzyme concentration was increased 2—3-fold in comparison with the usual one (60—90 units). The absence of a change in absorption at 340 mμ under these conditions as well indicated inactivity of the analogs.

Fig. 1. Kinetics of the oxidation reaction by UDPG dehydrogenase. *I* —in the presence of a saturating concentration of UDPG ($2\,\mu M$); *II* —in the presence of saturating concentrations of UDPG ($2\,\mu M$) and 6-aza-UDPG ($4.9\,\mu M$); *III* —in the presence of a saturating concentration of 6-aza-UDPG. The samples contained $5\,\mu M$ NAD, $920\,\gamma$ of protein in 3 ml of 0.1 M glycine buffer, pH 8.75; incubation t 22°C.

In the study of 6-aza-UDPG, a noticeable increase in absorption was found in the presence of $1.5\,\mu M$ 6-aza-UDPG, and the saturating concentration of 6-aza-UDPG under the experimental conditions described above was $5\,\mu M$. A change in the reaction kinetics showed that the initial reaction rate at a saturating concentration of 6-aza-UDPG was 30% of the initial reaction rate at a saturating concentration of UDPG*. Since 6-aza-UDPG is a synthetic analog, it was natural to think that the oxidation reaction of 6-aza-UDPG and UDPG is catalyzed by one and the same enzyme. To confirm this assumption we applied the method of "mixed substrates." It turned out that the total rate upon introducing saturating amounts of UDPG and 6-aza-UDPG into the sample was intermediate between the values of the rates observed in experiments with the same concentrations of substrates taken separately. The kinetic curve for the

mixture of substrates lay between the curves for each substrate (Fig. 1), which confirms the assumption that the 6-aza analog is capable of forming an enzyme-substrate complex with UDPG dehydrogenase. The ratio of the concentration of formed $NADH_2$, calculated on the basis of optical density, to the concentration of the resulting glucuronic acid, determined colorimetrically by the Dische method, was 2 : 1 for 6-aza-UDPG, i.e., the same as for UDPG.

Fig. 2. Kinetics of the oxidation reaction by UDPG dehydrogenase in the presence of UDPG ($2\,\mu M$) and CDFG ($5\,\mu M$). 1 –CDFG and UDPG; 2 – UDPG. The sample contained $5\,\mu M$ NAD, $920\,\gamma$ of protein in 3 ml of 0.1 M glycine buffer, pH 8.75; incubation t 22°C.

6-aza-UDPG-glucuronic acid was identified by twofold successive paper chromatography in the systems ethanol–0.5 M ammonium acetate (5 : 2) at pH 7.5 (system I) and ethanol–0.5 M ammonium acetate (5 : 2) at pH 3.8 (system II); in system I $R_{\text{UDPG}} = 0.29$, and in system II $R_{\text{UDPG}} = 0.6$. As can be seen from the data of Table 1, unlike 6-aza-UDPG, 3-N-methyl-UDPG and CDFG in this enzymatic system proved inactive.

* *Note added in proof.* A paper (10) has recently been published devoted to the study of the interaction of 6-azauridine diphosphate glucose and 5-fluorouridine diphosphate glucose with UDPG dehydrogenase. The results obtained agree well with ours.

Table 1

Change in optical density over time at different concentrations of UDPG analogs

Name of analog	Concentration, μM	Change in optical density *	Change in optical density *	Change in optical density *	Change in optical density *	Change in optical density *
		2 min	4 min	6 min	8 min	10 min
Control sample	Does not contain	0.030	0.045	0.050	0.060	0.020
CDPG	1.37	0.020	0.030	0.045	0.050	0.050
CDPG	2.74	0.025	0.035	0.040	0.055	0.050
CDPG	4.11	0.040	0.040	0.045	0.060	0.060
3-N-methyl-UDPG	1.25	0.010	0.015	0.025	0.030	0.040
3-N-methyl-UDPG	3.70	0.020	0.020	0.025	–	0.040
UDPG	1.70	0.165	0.305	0.415	0.505	0.645

* Difference between the optical density at the moment of measurement and the initial optical density.

No increase in absorption at 340 mμ occurred at various concentrations of these analogs. Addition to the sample of cytidine diphosphate glucose together with UDPG at a concentration 1.5 times higher than the concentration of UDPG did not change the reaction rate (Fig. 2).

Considering the results presented above from the point of view of the hypothesis proposed earlier concerning the presence of a secondary structure in nucleoside diphosphate sugars, held together by hydrogen bonds between the uracil residue and glucose [7], it should be noted that, as in the reaction we studied for the formation of sucrose from UDPG and fructose in the presence of the enzyme sucrose-UDP-transglucosylase [8], 3-N-methyl-UDPG and CDPG proved to be inactive. This indicates the necessity of the grouping $N_{(3)}H—C_{(4)} = X$ in the uracil nucleus in order for an analog of UDP-glucose to be capable of participating in both enzymatic systems studied. The ability of 6-aza-UDPG to enter into the oxidation reaction, shown in this work, is consistent with this conclusion, since replacement of $C_{(6)}$ by nitrogen in the uracil nucleus should not substantially affect the ability of an NDP-sugar to form a secondary structure. Recent literature data on the ability of pseudouridine diphosphate glucose to enter into enzymatic reactions of UDP-glucose [9] also confirm our conclusions.

Thus, the data presented in this work, as well as data from the study of the reaction of sucrose biosynthesis, are consistent with the previously expressed idea of the relationship between the structure and function of NDPS and of the role of the secondary structure of NDPS in enzymatic reactions involving NDPS. At the same time, it should be borne in mind that the results obtained may be explained by the fact that the grouping $N_{(3)}H—C_{(4)} = X$ is necessary not so much for the formation of the secondary structure as for the binding of NDPS and, in particular, of the UDPG analogs studied, to the active center of the enzyme. A final decision on this question can be made only on the basis of study of a large number of UDPG analogs in a whole series of enzymatic systems and of simultaneous physicochemical investigation of the corresponding nucleoside diphosphate sugars.

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Note: Figure translations are in progress. See original paper for figures.