Biosynthesis of Uridine Diphosphate D-Xylose. III. Uridine Diphosphate D-Glucose Dehydrogenase of *Cryptococcus laurentii**

Helmut Ankel,† Else Ankel,† and David Sidney Feingold‡

ABSTRACT: Uridine diphosphate D-glucose dehydrogenase (EC 1.1.1.22) from *Cryptococcus laurentii* has been partially purified and its properties have been investigated. Uridine diphosphate D-glucose is a substrate; the diphosphoglucose derivatives of adenosine, cytidine, guanosine, and thymidine are not substrates. Nicotinamide–adenine dinucleotide (NAD), 3-acetylpyridine–AD, or thionicotinamide–AD are active as hydrogen acceptors; α-NAD, deamino-NAD, 3-formylpyridine–AD, 3-propionylpyridine–AD, and ethylnicotinate–AD are inactive. The pH optimum is in the range 7.3–7.8; K_m is 0.6 mM for both uridine diphosphate D-glucose and NAD. The enzyme is strongly and specifically inhibited by uridine diphosphate D-xylose; the inhibition can be reversed by increased concentrations of either uridine diphosphate D-glucose or NAD. These observations as well as kinetic studies show that the inhibition is allosteric.

In *Cryptococcus laurentii* UDPXyl[1] is formed by decarboxylation of UDPGA (Ankel and Feingold, 1966). UDPG and UDPXyl, but not UDPGA, have been isolated from *Cr. laurentii* (Ankel *et al.*, 1964), leaving unsettled the question of whether UDPGA is formed by the organism. This has now been resolved by the demonstration and partial purification of UDPG dehydrogenase of *Cr. laurentii*.

Experimental Section

Materials. The sources of the following materials have been described previously (Ankel and Feingold, 1965): enzyme grade ammonium sulfate, Sephadex, NAD, $NADH_2$, NADP, and UDPGA (uniformly labeled with ^{14}C in the glycosyl moiety, specific activity 50 μc/μmole). UDPXyl was purchased from Calbiochem, and UDPG from Sigma. Analogs of NAD were donated by Dr. N. O. Kaplan, Brandeis University. ADPG, CDPG, and TDPG were gifts from Dr. E. F. Neufeld, National Institutes of Health; GDPG was a gift from Dr. Susana Passeron, Instituto De Investigaciones Bioquimicas, Buenos Aires.

Cr. laurentii (NRRL Y-1401) was obtained from the Northern Regional Research Laboratories, U. S. Department of Agriculture, Peoria, Ill. UDPGA carboxy-lyase from wheat germ was obtained as described previously (Ankel and Feingold, 1965).

Methods. Paper chromatographic and electrophoretic methods as well as analytical techniques used have been described in detail elsewhere (Feingold *et al.*, 1964; Ankel *et al.*, 1964; Ankel and Feingold, 1965, 1966). UDPG dehydrogenase was assayed essentially as described by Strominger *et al.* (1957) (see Feingold *et al.*, 1964). Assay mixtures contained 2 μmoles of UDPG, 5 μmoles of NAD, and enzyme in a total volume of 1 ml of 0.1 M sodium and potassium phosphate buffer, pH 7.5. Since the enzyme is rapidly inactivated under assay conditions, it was added last. Absorbancy increase was measured in an automatic recording spectrophotometer equipped with a device to keep the temperature of the reaction mixture at 30°. A unit of enzyme activity is defined as the amount of enzyme required to produce 2 μmoles of $NADH_2$/min at 30°.

Enzyme Purification. The following buffers were used: buffer A, 0.1 M sodium phosphate, pH 7.0, containing 0.5 g of EDTA and 0.3 g of glutathione/l.; buffer B, which had the same composition except that the sodium phosphate concentration was 0.01 M. Unless otherwise noted, all procedures were carried out at 0–4°. A cell-free extract of *Cr. laurentii* was prepared as previously described (Ankel and Feingold, 1966); it was centrifuged at 28,000*g* for 20 min and the precipitate

* From the Microbiology Section, Department of Biology, University of Pittsburgh, Pittsburgh, Pennsylvania. *Received March 28, 1966.* Supported by grants from the U. S. Public Health Service (GM 08820), and the Health Research and Service Foundation, Pittsburgh, Pa. (H-14).

† Present address: Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisc.

‡ Career development grant (1K3-GM-28,296-01) awardee of the U. S. Public Health Service.

[1] The following abbreviations are used: uridine 5′-(α-D-xylopyranosyl pyrophosphate), UDPXyl; uridine 5′-(β-L-arabinopyranosyl pyrophosphate), UDPAra; uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate), UDPGA; guanosine 5′-(α-D-mannopyranosyl pyrophosphate), GDPM; adenosine, cytidine, guanosine, thymidine, and uridine 5′-(α-D-glucopyranosyl pyrophosphate), ADPG, CDPG, GDPG, TDPG, and UDPG, respectively; nicotinamide–adenine dinucleotide, NAD; reduced NAD, $NADH_2$; NAD phosphate, NADP; *p*-mercuriobenzoate, PCMB; uridine monophosphate, UMP. The following trivial names are used for enzymes which have been assigned systematic names by the Commission on Enzymes of the International Union of Biochemistry, 1961; orthophosphoric diester phosphohydrolase (EC 3.1.4.1), phosphodiesterase; orthophosphoric monoester phosphohydrolase (EC 3.1.3.2), acid phosphatase; UDP glucose:NAD oxidoreductase (EC 1.1.1.22), UDPG dehydrogenase.

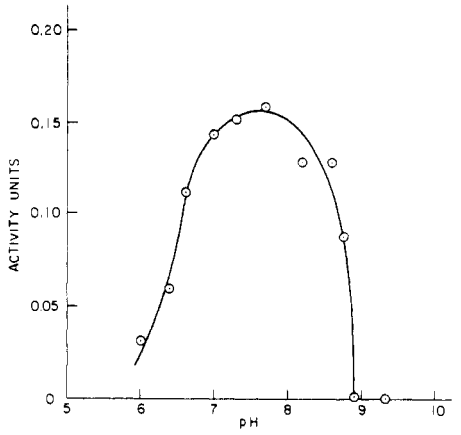

FIGURE 1: Dependence of Activity on pH. The conditions of assay are those described except that pH and buffer were varied as indicated in the text.

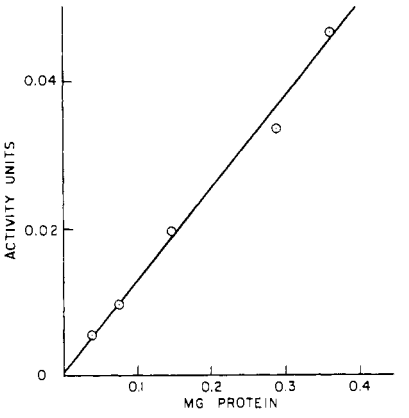

FIGURE 2: Dependence of reaction rate on protein concentration. Assay conditions are as described in the text.

was discarded. The turbid supernatant fluid was centrifuged at 105,000*g* for 1 hr; the resultant clear supernatant solution (crude supernatant) contained all the dehydrogenase activity. Solid $(NH_4)_2SO_4$ (57.5 g) was added to the crude supernatant (345 ml) to 0.3 saturation. The precipitate was discarded and the supernatant fluid was brought to 0.8 saturation by addition of 117 g of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in sufficient buffer A to yield 25 ml of solution [$(NH_4)_2SO_4$ I]. This solution was chromatographed on a column 3.6 cm in diameter containing 700 ml of Sephadex G-100 equilibrated with the same buffer, which was also used as eluent; fractions of 2–3 ml were collected. Enzyme-containing fractions (total volume 62 ml) were pooled (Sephadex-100) and the protein was precipitated from them by the addition of 33.1 g of solid $(NH_4)_2SO_4$ to 0.8 saturation [$(NH_4)_2SO_4$ II]. The precipitate was taken to a volume of 15 ml with buffer A and chromatographed in the same way on a column containing 700 ml of Sephadex G-200. Active fractions (total volume 100 ml) were pooled (Sephadex-200) and 37.1 g of solid $(NH_4)_2SO_4$ was added to 0.6 saturation. The precipitate was taken to a volume of 4.8 ml with buffer A [$(NH_4)_2SO_4$ III] and chromatographed on a column 2 cm in diameter containing 200 ml of Sephadex G-25 equilibrated with buffer B, which also was used as eluent. Enzyme-containing fractions were pooled (Sephadex-25). Chromatography on Sephadex G-25 in buffer B extensively inactivates contaminating UDPGA carboxy-lyase, which is unstable in low ionic strength phosphate buffer (Ankel and Feingold, 1966). In crude supernatant the ratio of carboxy-lyase:dehydrogenase activity is *ca.* 1:1, while in Sephadex-25 it is *ca.* 1:10. In addition, Sephadex G-25 chromatography removes $(NH_4)_2SO_4$, which inhibits the dehydrogenase. The activity of the purified enzyme was 0.4 unit/mg of protein. As can be noted from Table I, sevenfold purification was accomplished, with recovery of 27% of the initial activity.

Stability. The enzyme was stable for at least 2 months when stored frozen at −10°. It was markedly unstable at higher temperatures, losing 95% of its initial activity after 3 min at 30°. Forty per cent of the initial activity was retained under the same conditions in the presence of 2 mM UDPG, and 20% was retained in the presence of 1 mM NAD. Neither NADP nor EDTA had any effect on stability.

TABLE I: Summary of Purification of UDPG Dehydrogenase.

Fraction	Volume (ml)	Total[a] Units	Sp Act. (Units/mg[a] × 10^2)	Recovery (%)[a]
Crude ext	345	21.4	2.0	100
$(NH_4)_2SO_4$ I	25	(10.3)	(1.9)	(48)
Sephadex-100	62	13.7	4.5	64
$(NH_4)_2SO_4$ II	15	(6.7)	(3.0)	(31)
Sephadex-200	100	8.7	11.0	41
$(NH_4)_2SO_4$ III	4.8	(5.3)	(10.0)	(25)
Sephadex-25	20	5.9	14.0	27

[a] Figures in parentheses are activities measured in the presence of inhibitory $(NH_4)_2SO_4$.

Reaction Products. Enzyme reaction mixtures contained 2 μmoles of UDPG, 5 μmoles of NAD, and 0.5 mg of enzyme in buffer B in a total volume of 0.35 ml. After 2 hr at 25° the reaction products were separated by paper electrophoresis at pH 5.8. In addition to a little residual NAD and UDPG, two major products were present: one had the mobility of authentic $NADH_2$ and fluoresced under ultraviolet illumination; the other had the mobility of UDPGA and was ultraviolet absorbing. This compound was eluted from the paper and further characterized. Its absorption spectrum at pH 7.0 was identical with that of authentic UDPGA and it had the mobility of authentic ^{14}C-labeled UDPGA upon coelectrophoresis at pH 3.6 and 5.8. A portion of

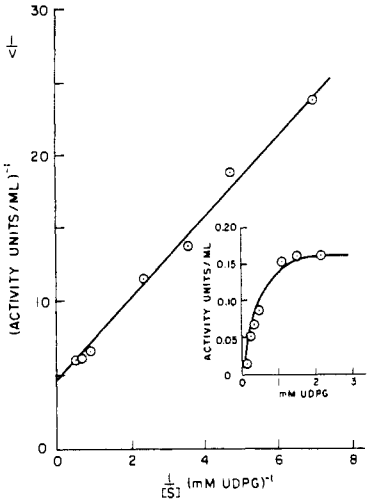

FIGURE 3: Dependence of reaction rate on UDPG concentration. Assay conditions are those described in the text, except that the concentration of UDPG was varied.

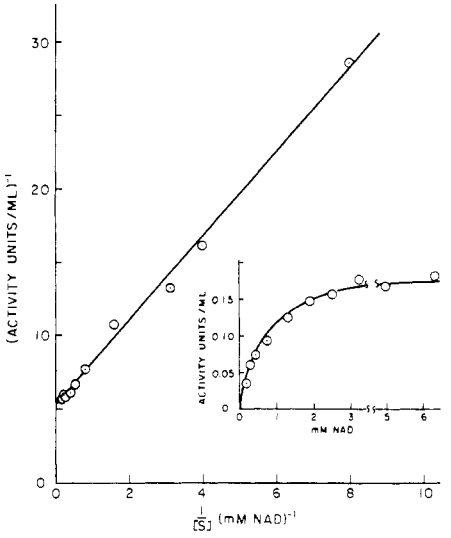

FIGURE 4: Dependence of reaction rate on NAD concentration. Assay conditions are those described in the text, except that the concentration of NAD was varied.

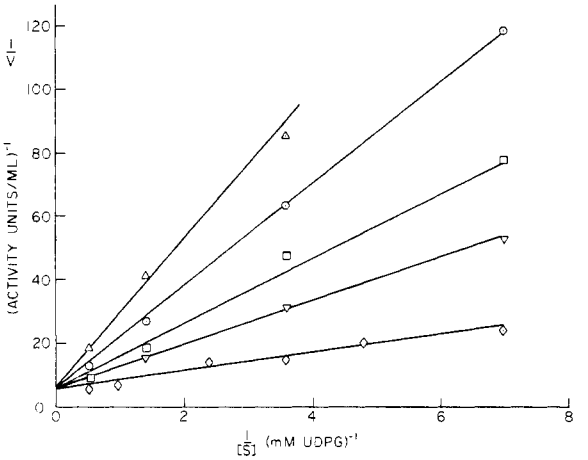

FIGURE 5: Inhibition of UDPG dehydrogenase activity by UDPGA. Reciprocal plot of reaction rate *vs.* UDPG concentration at the following concentrations of UDPGA: —◇—, no UDPGA added; —▽—, 0.35 mM; —□—, 0.7 mM; —○—, 1.4 mM; —△—, 3.5 mM. The assay conditions are those described in the text except that the concentration of UDPG was varied. UDPGA was added to complete reaction mixtures prior to the addition of enzyme.

the reaction product was mixed with authentic ^{14}C-labeled UDPGA and hydrolyzed with phosphodiesterase. Electrophoresis at pH 5.8 gave a single ultraviolet-absorbing spot with the electrophoretic mobility of UMP and an organic phosphate (revealed with the molybdic acid spray of Bandurski and Axelrod, 1951) that coincided with the radioactive D-glucosyluronic acid phosphate. In a separate set of experiments treatment with phosphodiesterase was followed by treatment with acid phosphatase and the products were separated by electrophoresis as before. The spot obtained with *p*-anisidine phosphate spray (Feingold *et al.*, 1958) coincided exactly with radioactive D-glucuronic acid. Another portion of the reaction product was incubated with 0.2 mg of purified UDPGA carboxy-lyase from wheat germ (Ankel and Feingold, 1965) at pH 7.0 in buffer B. There was complete conversion to an ultraviolet-absorbing compound with the electrophoretic mobility of UDPXyl at pH 5.8. This compound was treated successively with phosphodiesterase and acid phosphatase; carbohydrates released were separated by two-dimensional chromatography in H_2O-saturated phenol followed by 1-propanol–ethyl acetate–water (7:1:2, v/v) and revealed with *p*-anisidine. Only one sugar, with the mobility and characteristic red reaction of xylose, was present in the hydrolysate. These data establish the identity of the initial reaction product as uridine 5′-(D-glucopyranosyluronic acid pyrophosphate).

Optimum pH. The optimum pH was determined using 0.1 M phosphate buffer between 6 and 7.7, and 0.1 M glycine–sodium hydroxide buffer between 8.2 and 9.6. As shown in Figure 1, the pH optimum is in the range of 7.3–7.8.

Effect of Enzyme Concentration. Under the conditions of assay the reaction is linear for at most 1 min. A linear relation exists between activity and amount of protein (Figure 2).

Effect of Substrate Concentration. The effect of UDPG concentration on reaction rate is shown in Figure 3 and that of NAD concentration in Figure 4. The apparent K_m values at 30°, determined according to Lineweaver and Burk (1934) are 0.6 mM for both UDPG and NAD.

Substrate Specificity. Reaction mixtures in which UDPG was replaced by 0.5 μmole of α-D-glucopyrano-

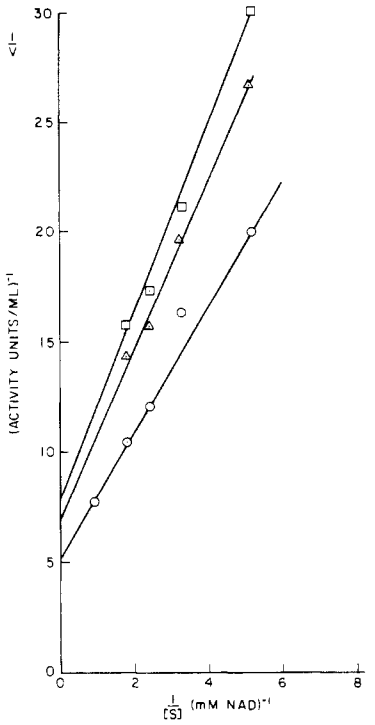

FIGURE 6: Inhibition of UDPG dehydrogenase activity by UDPGA. Reciprocal plot of reaction rate *vs.* NAD concentration at the following concentrations of UDPGA: —⊙—, no UDPGA added; —△—, 2 mM; —⊡—, 4 mM. The assay conditions are those described in the text except that the concentration of NAD was varied. UDPGA was added to complete reaction mixtures prior to addition of enzyme.

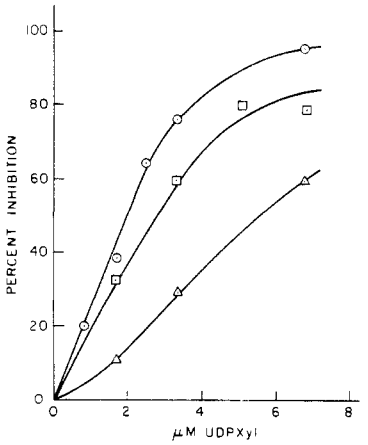

FIGURE 7: Inhibition of UDPG dehydrogenase activity as a function of UDPXyl concentration. The conditions of assay are those described in the text except that the following concentrations of UDPG were employed: —△—, 0.14 mM; —⊡—, 0.28 mM; —⊙—, 0.43 mM. UDPXyl was added to complete reaction mixtures prior to the addition of enzyme. Per cent Inhibition = 100 (activity in absence of UDPXyl − activity in presence of UDPXyl)/activity in absence of UDPXyl.

syl phosphate, ADPG, CDPG, GDPG, TDPG, or GDPM did not yield $NADH_2$ when incubated with 0.4 mg of enzyme under the conditions of assay, nor was there inhibition of enzyme activity when an equal amount of UDPG was subsequently added. In reaction mixtures in which NAD was replaced by 0.2 μmole of NADP, α-NAD, deamino-NAD, 3-formylpyridine–AD, 3-propionylpyridine–AD, or ethyl nicotinate–AD, no reduction was observed. However, reduction occurred when 0.2 μmole of 3-acetylpyridine–AD or thionictotinamide–AD was used instead of NAD. Relative reaction rates were: NAD, 100; 3-acetylpyridine–AD, 30; thionicotinamide–AD, 20.

Inhibition. The following have no effect on enzyme activity (tested under conditions described for the assay): 1 mM $MgSO_4$, 1 mM $ZnSO_4$, 1 mM $MnSO_4$, 10 mM EDTA, 3 mM UTP, UDP, ATP, 2 mM α-D-glucopyranosyl phosphate, α-D-xylopyranosyl phosphate, or α-D-mannopyranosyl phosphate. *Ca.* 50% inhibition is obtained with 8 mM AMP, 4 mM ADP, and 1 mM UDPAra. PCMB (0.5 μmole/mg of protein) inhibits 70% without preincubation.

As can be seen from Figure 5, the enzyme is inhibited by UDPGA, the product of the reaction; inhibition is competitive with UDPG (K_i = 0.5 mM) and non-competitive with NAD (K_i = 5 mM) (Figure 6). While inhibition by UDPGA is marked only at relatively high concentrations of inhibitor, much stronger inhibition is obtained with UDPXyl. UDPXyl inhibits 50% at a concentration of 0.01 mM when the UDPG concentration is 2 mM, and (Figure 7) at a concentration of 2.8 μM when the UDPG is 280 μM. Reciprocal plots of reaction velocity *vs.* UDPG concentration at varying inhibitor concentrations result in a family of curves which show an upward inflection with increasing 1/[S] values (Figure 8). Figure 9 shows a similar family of curves for reciprocal plots of reaction velocity *vs.* NAD concentration at varying UDPXyl levels. These findings indicate that inhibition by UDPXyl can be overcome by increasing concentrations of either substrate.

Discussion

The UDPG dehydrogenase of *Cr. laurentii* differs somewhat in pH optimum and K_m from the pea (Strominger and Mapson, 1957) and liver (Strominger *et al.*, 1957, 1960) dehydrogenases: pH optima are in the range of 7.3–7.8, 9.0, and 8.7, respectively; K_m values for UDPG are 0.6, 0.07, and 0.02 mM, respectively; for NAD they are 0.6, 0.1, and 0.1 mM, respectively. These differences notwithstanding, there is a striking similarity of all three dehydrogenases in respect to patterns of inhibition by sugar nucleotides. *Cr. laurentii* UDPG dehydrogenase is inhibited by the reaction product, UDPGA; the inhibition is competitive with UDPG, K_i being 0.5 mM for the yeast enzyme (Figure 5), and 0.3 and 0.05 mM for the pea and liver enzymes, respectively

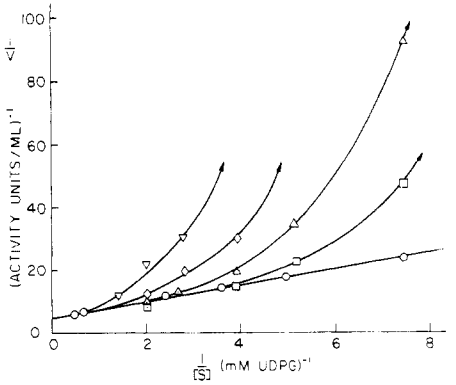

FIGURE 8: Dependence of reaction rate on UDPG concentration at different concentrations of UDPXyl. The conditions of assay are those described in the text except that the concentration of UDPG was varied. UDPXyl was added to complete reaction mixtures prior to addition of enzyme. UDPXyl concentration: —⊙—, no UDPXyl added; —⊡—, 6.5 μM; —△—, 9.2 μM; —◇—, 12.0 μM; —▽—, 17.0 μM.

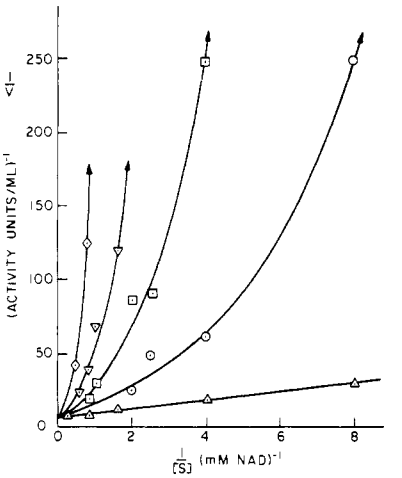

FIGURE 9: Dependence of reaction rate on NAD concentration at different concentrations of UDPXyl. The conditions of assay are those described in the text except that the concentration of NAD was varied. UDPXyl was added to complete reaction mixtures prior to addition of enzyme. UDPXyl concentration: —△—, no UDPXyl added; —⊙—, 27 μM; —⊡—, 54 μM; —▽—, 81 μM; —◇—, 135 μM.

(Neufeld and Hall, 1965). The inhibition is noncompetitive with NAD in all three cases (Figure 6; Neufeld and Hall, 1965). UDPG dehydrogenase from *Cr. laurentii* is specifically and potently inhibited by UDPXyl (Figure 7). UDPAra, which differs from UDPXyl only in the configuration at C-4 of the glycosyl moiety, is far less inhibitory; a concentration of 1.0 mM is required to achieve 50% inhibition under the conditions used for enzyme assay, whereas 0.01 mM UDPXyl inhibits 50% under the same conditions.

UDPXyl is not a simple competitive inhibitor of UDPG dehydrogenase, since reciprocal plots of velocity *vs.* UDPG concentration (Figure 8) or NAD concentration (Figure 9) in the presence of UDPXyl depart progressively from linearity with increasing UDPXyl concentration. These results, which are similar to those of Neufeld and Hall (1965), are indicative of an allosteric effect (Monod *et al.*, 1963) of UDPXyl on UDPG dehydrogenase. This is further substantiated by the sigmoid nature of the inhibition curve (Figure 7), which shows that a cooperative interaction exists between several inhibitor-binding sites on the enzyme (Monod *et al.*, 1963). The apparent number of such sites, calculated from the data of Figure 7 by the graphical estimation method of Taketa and Pogell (1965), is 1.7, which compares with values of 1.5 for the pea and 2.3 for the liver dehydrogenase (Neufeld and Hall, 1965).

The extracellular polysaccharide of *Cr. laurentii* probably consists of a branched backbone of D-mannosyl residues to which D-xylosyl and D-glucuronosyl moieties are attached in a terminal position (Abercrombie *et al.*, 1960). It has been suggested that GDPM, UDPXyl, and UDPGA are ultimate precursors of the polysaccharide; however, UDPGA was not isolated from *Cr. laurentii*, although GDPM, UDPG, and UDPXyl were (Ankel *et al.*, 1964). The ratio of UDPG:UDPXyl was in the order of 100:1 (H. Ankel and D. S. Feingold, unpublished results). This can be explained by the observed feed-back inhibition of UDPG dehydrogenase by UDPXyl, which would decrease the UDPGA concentration to a level too low to be demonstrated readily and cause UDPG to pile up.

UDPXyl formation itself is dependent upon the intracellular ratio of $NADH_2$:NAD, which affects the activity of UDPGA carboxy-lyase (Ankel and Feingold, 1966). These separate but interrelated controls, acting in concert, probably serve to regulate the relative concentrations of the glycosyl donors UDPGA and UDPXyl during polysaccharide synthesis. Utilization of UDPXyl would lower its concentration and therefore permit formation of UDPGA and $NADH_2$; however, decarboxylation of UDPGA would be inhibited by the increased $NADH_2$:NAD ratio, which would maintain the concentration of UDPGA at a level necessary for glycosyl transfer.

It is noteworthy that UDPXyl has been shown to be an allosteric inhibitor of every UDPG dehydrogenase investigated to date. In higher plants and *Cr. laurentii* UDPXyl doubtless functions as a feed-back inhibitor of an enzymic step which leads to its own synthesis. Neufeld and Hall (1965) have postulated a similar role for it in vertebrates. This has been substantiated by recent work with enzyme preparations from hen oviduct, in which have been demonstrated a soluble UDPXyl-inhibitable UDPG dehydrogenase (A. Bdolah and D. S. Feingold, unpublished experiments), a particulate UDPGA carboxy-lyase (Bdolah and Feingold, 1965), and a particulate UDPXyl:acceptor xylosyltransferase (Grebner *et al.*, 1966). It seems likely that UDP sugar-

linked pathways leading to glycosidically bound D-xylose are much more widely distributed in nature than was previously thought, and may be ubiquitous.

Acknowledgments

We are grateful to Drs. Elizabeth Neufeld and Alan Mehler for stimulating discussion and helpful criticism. Our thanks are also due to Mr. John S. Schutzbach for skillful technical assistance.

References

Abercrombie, M. J., Jones, J. K. N., Lock, M. V., Perry, M. B., and Stoodley, R. J. (1960), *Can. J. Chem. 38*, 1617.

Ankel, H., Farrell, D. G., and Feingold, D. S. (1964), *Biochim. Biophys. Acta 90*, 397.

Ankel, H., and Feingold, D. S. (1965), *Biochemistry 4*, 2468.

Ankel, H., and Feingold, D. S. (1966), *Biochemistry 5*, 182.

Bandurski, R., and Axelrod, B. (1951), *J. Biol. Chem. 193*, 405.

Bdolah, A., and Feingold, D. S. (1965), *Biochem. Biophys. Res. Commun. 21*, 453.

Feingold, D. S., Neufeld, E. F., and Hassid, W. Z. (1958), *J. Biol. Chem. 233*, 783.

Feingold, D. S., Neufeld, E. F., and Hassid, W. Z. (1964), *Modern Methods Plant Analy. 7*, 474.

Grebner, E. E., Hall, C. W., and Neufeld, E. F. (1966), *Biochem. Biophys. Res. Commun. 22*, 672.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Monod, J., Changeaux, J. P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.

Neufeld, E. F., and Hall, C. W. (1965), *Biochem. Biophys. Res. Commun. 19*, 456.

Strominger, J. L., and Mapson, L. W. (1957), *Biochem. J. 66*, 567.

Strominger, J. L., Maxwell, E. S., Axelrod, J., and Kalckar, H. M. (1957), *J. Biol. Chem. 224*, 79.

Strominger, J. L., Okazaki, R., and Okazaki, T. (1960), *Enzymes 7*, 161.

Taketa, K., and Pogell, B. M. (1965), *J. Biol. Chem. 240*, 651.