could be imagined that this change tends to pull the substrate-binding site, and thus the bound substrate, away from the hydrolytic center. This effect is absent for the glycine derivatives which lack a hydrophobic substituent at their α-carbon atom.

Inhibition experiments with a series of aliphatic homologs showed that a primary amino group is preferred and better bound by the aminopeptidase than a carboxyl or an amide group. Thus amines produce competitive inhibition, whereas a free carboxyl group lowers the binding affinity considerably. The amino acid has to have the L configuration and needs a primary amino group for the optimal hydrolysis and binding of its derivatives. The primary amino group has to be in the α position to the carboxyl group since in the β position no hydrolysis occurs. The strong affinity of the α-amino group for the enzyme is significantly reduced by an amide nitrogen in the β position (asparagine and β-alanine amide). Inhibition of aminopeptidase by amino acids is therefore a mixed form of competitive and uncompetitive inhibition but with the former type dominating.

The Michaelis constant of aminopeptidase which is ten times smaller for amino acid *p*-nitroanilides than for the corresponding amides demonstrates that there should be also a binding site for the penultimate amino acid. The binding site for the penultimate amino acid can influence the hydrophobic binding site, as can be seen from inhibition constants of the carboxylic acids and α-amino acids compared to the corresponding amides.

Thus aminopeptidase must contain at least two binding sites for the N-terminal amino acid of peptides (one for the primary amino group, a second for the hydrophobic residue at the α-carbon atom), and a third for the penultimate amino acid in a peptide and its derivatives.

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Biosynthesis of Uridine Diphosphate D-Xylose. II. Uridine Diphosphate D-Glucuronate Carboxy-lyase of *Cryptococcus laurentii**

Helmut Ankel† and David Sidney Feingold‡

ABSTRACT: Uridine diphosphate D-glucuronate carboxy-lyase of *Cryptococcus laurentii* has been partially purified and its properties have been investigated. Uridine diphosphate D-xylose and carbon dioxide are the only products of enzyme action. The pH optimum is between 7.0 and 7.5.

K_m for uridine diphosphate D-glucuronic acid is 1.1 $\times$ 10^{-3} M; the enzyme has an absolute and specific requirement for nicotinamide–adenine dinucleotide (NAD); K_a = 3 $\times$ 10^{-6} M. Reduced nicotinamide–adenine dinucleotide inhibits competitively with NAD; K_i = 2 $\times$ 10^{-6} M. The enzyme is inhibited by *p*-mercuribenzoate; the inhibition is reversed by cysteine. Label is retained when uridine diphosphate D-glucuronate-3-*t* or -4-*t* is decarboxylated by the enzyme.

A number of species of the genus Cryptococcus produce extracellular polysaccharides which contain D-xylosyl moieties (Benham, 1956); that secreted by *Cryptococcus laurentii* (NRRL Y-1401) consists of D-mannose, D-xylose, and D-glucuronic acid in the approximate molar ratio 5:2:1 (Abercrombie *et al.*, 1960; see also Jeanes *et al.*, 1964). In higher plants it has been shown that D-glucuronosyl and D-xylosyl moieties

* From the Microbiology Section, Department of Biology, University of Pittsburgh, Pittsburgh, Pennsylvania. *Received September 14, 1965.* Supported by a grant from the U. S. Public Health Service (GM 08820). Some of the results presented here were reported in preliminary communications (Ankel and Feingold, 1964, 1965).

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‡ Research Career Development Award (1-K3-GM-28, 296-01) grantee of the National Institutes of Health, U. S. Public Health Service.

are produced, at least in part, by conversion of UDPG[1] to UDPGA and decarboxylation of the latter to UDP-Xyl (Hassid, 1962). The presence of a similar pathway in *Cr. laurentii* is indicated by the isolation of UDPG and UDPXyl from cells of the organism (Ankel *et al.*, 1964) as well as by the demonstration of D-xylosyl transfer from UDPXyl to acceptor, catalyzed by an enzyme preparation from *Cr. laurentii* (Cohen and Feingold, 1964). The enzyme responsible for formation of UDPXyl in higher plants, UDPGA carboxy-lyase, has been purified from wheat germ and some of its properties determined. The wheat germ enzyme requires no cofactors for activity; however, it contains tightly bound nicotinamide–adenine dinucleotide (NAD), which is only released upon denaturation (Ankel and Feingold, 1965). We have now demonstrated and partially purified the UDPGA carboxy-lyase of *Cr. laurentii* and shown it to have an absolute requirement for NAD and to be inhibited by reduced NAD.

Experimental Section

Materials. The sources of the following materials have been described previously (Ankel and Feingold, 1965): enzyme grade ammonium sulfate, Sephadex, NADase, NAD, $NADH_2$, NADP, $NADPH_2$, UDPGA; also UDPGA, D-glucuronic acid 1-phosphate, D-glucuronic acid, UDP-D-galacturonic acid (all uniformly labeled with ^{14}C in the glycosyl moiety, specific activity 50 μcuries/μmole); UDPGA-3-*t* and UDPGA-4-*t* (7×10^6 and 4×10^6 cpm/μmole, respectively, counted at 18% efficiency). Biogel P-200 was purchased from Bio-Rad Corp. and UDPXyl from Calbiochem. Analogs of NAD were generously donated by Dr. N. O. Kaplan, Brandeis University.

Cr. laurentii (NRRL Y-1401) was obtained from the Northern Regional Research Laboratories, U. S. Department of Agriculture, Peoria, Ill. A strain of *Cr. diffluens* (Y-1402 CBS 10672) was purchased from the National Type Culture Collection, Washington, D. C. *Tremella mesenterica* (NRRL Y-6151) was likewise obtained from Northern Regional Research Laboratories. These organisms were maintained on slants of potato–dextrose–yeast extract.

Methods. Chromatography of sugars was performed on Whatman No. 1 paper, using water-saturated phenol in the first dimension and 1-propanol–ethyl acetate–water (7:1:2, v/v) in the second dimension. Nucleotides were chromatographed on paper in ethanol–1 M ammonium acetate, pH 7.0 (7:3, v/v) (Paladini and Leloir, 1952), and two-dimensionally on a cellulose thin layer, using 1-butanol–acetone–acetic acid–5% aqueous ammonia–water (3:5:2.5:1:5:1, v/v) in the first dimension and 1-propanol–HCl–water (82:22:23, v/v) in the second (Randerath, 1962). Gas–liquid partition chromatography of the trimethylsilyl derivatives of carbohydrates was performed by the method of Sweeley *et al.* (1963). Paper electrophoresis was done on oxalic acid washed sheets of Whatman 3MM or No. 1 paper either at pH 3.6 or 5.8 (Feingold *et al.*, 1964), or in 0.05 M sodium borate, pH 9.2, using the solvent-cooled high-voltage GME Electrophorator. Nucleotides were located on paper and thin layers by visual observation under short-wave (254 mμ) ultraviolet illumination. Carbohydrates were revealed with *p*-anisidine phosphate (Feingold *et al.*, 1958). ^{14}C-Labeled compounds were located by radioautography and counted after elution from paper in a Packard Model 527 liquid scintillation spectrometer, using the scintillator solution described by Bray (1960). Protein was estimated by the method of Lowry *et al.* (1951). Acid-labile phosphate was determined after hydrolysis with 1 N HCl for 10 min at 100°, total phosphate according to Rockstein and Herron (1951), and reducing sugar by the method of Parke and Johnson (1949). The value 9.9×10^3 was used for the molar absorptivity of uridine (Ploeser and Loring, 1949). UDPGA carboxy-lyase was assayed as described previously except that enzyme reaction mixtures contained, in addition, 10 μl of 0.01 M NAD; activity units are defined as described previously (Ankel and Feingold, 1965).

Growth of Cells. Cryptococcus laurentii (Northern Regional Research Laboratories Strain Y-1401) was grown in the following medium: (Mager, 1947) 2% (w/v) glucose, 0.1% urea, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 5H_2O$, and 0.2 mg/l. thiamine HCl. Starter culture was prepared by inoculating from a slant of the organism into 100 ml of medium contained in a 500 ml erlenmeyer flask equipped with two stainless-steel baffles and a gauze closure. The culture was shaken on a New-Brunswick gyratory shaker (300 oscillations/min) at 28°. Generally after 24 hr the culture gave a Klett reading of about 400 (filter 42). It was used to inoculate fresh flasks of culture medium at the rate of 5%; these flasks were shaken at 28° as described. After 16–18 hr the cultures gave a Klett reading of 400–500. The cells were spun down and washed once with 0.1 M phosphate buffer, pH 7.0, 5 mM in mercaptoethanol and 2 mM in EDTA.

Preparation of Cell-Free Extracts. (All subsequent operations were performed at 0–4°.) The packed, washed cells were suspended in about an equal volume of 0.1 M sodium and potassium phosphate buffer, pH 7.0, containing 0.5 ml of mercaptoethanol and 0.5 g of EDTA/l. (buffer). The cells were disrupted by sonification in a cooling cell (Rosset, 1965) with the Branson

[1] The following abbreviations are used: uridine-5′-(α-D-glucopyranosyl pyrophosphate), UDPG; uridine-5′-(α-D-xylopyranosyl pyrophosphate), UDPXyl; uridine-5′-(α-D-glucopyranosyluronic acid pyrophosphate), UDPGA; uridine-5′-(α-D-galactopyranosyl pyrophosphate), UDPGal; uridine-5′-(α-D-*xylo*-hexopyranosyluronic acid-4-ulose pyrophosphate), UDP-4-keto-GA; uridine-5′-(β-L-*threo*-pentopyranosyl-4-ulose pyrophosphate), UDP-4-keto-Xyl; *p*-mercuribenzoate, PCMB; NAD, nicotinamide–adenine dinucleotide; $NADH_2$, reduced NAD; NADP, NAD phosphate. The folowing trivial names are used for enzymes which have been assigned systematic names by the Commission on Enzymes of the International Union of Biochemistry, 1961: β-D-glucose:O_2 oxidoreductase (EC 1.1.3.4), glucose oxidase; UTP:α-D-xylose 1-phosphate uridylyl transferase (EC 2.7.7.11), UDPXyl pyrophosphorylase; NAD glycohydrolase (EC 3.2.2.5), NADase.

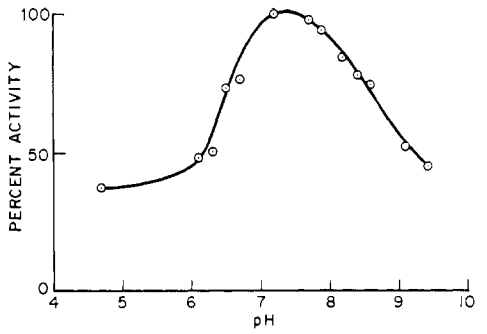

FIGURE 1: Dependence of activity on pH. The experimental conditions are the same as in the enzyme assay except that the buffer and pH are varied as indicated in the text. Since the activity in phosphate buffer at pH 7.2 is 15% less than in glycylglycine buffer of the same pH, activities measured in phosphate buffer are plotted as per cent of the activity at pH 7.2 in phosphate buffer, while activities measured in glycylglycine buffer are plotted as per cent of the activity at pH 7.2 in glycylglycine buffer.

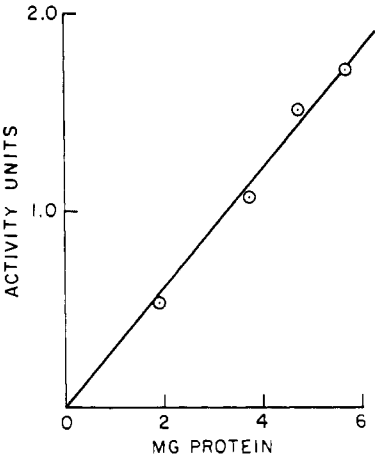

FIGURE 2: Dependence of reaction rate on enzyme concentration. Experimental conditions are those described in the text for the assay. The enzyme preparation used had a specific activity of 0.3.

Sonifier at an output of 11 amp for 6 min, the suspension being kept below 6° during sonification by immersion of the cell in an ice bath. The broken cell suspension was centrifuged at 28,000 × *g* for 20 min and the precipitate discarded. The turbid supernatant fluid was then spun at 105,000 × *g* for 60 min. The resulting clear supernatant solution (crude supernatant) contained all the carboxy-lyase activity.

Enzyme Purification. Crude supernatant (188 ml) was efficiently stirred and 9.9 ml of 0.5 M $MnCl_2$ was added. Stirring was continued for 5 min after addition and the precipitate was spun down and discarded ($MnCl_2$ supernatant). Solid $(NH_4)_2SO_4$ was added to the $MnCl_2$ supernatant to 40% saturation. The precipitate was spun out and discarded, and the supernatant solution was brought to 60% saturation by addition of solid $(NH_4)_2SO_4$. The precipitate was dissolved in sufficient buffer to yield 5 ml of solution (ammonium sulfate fraction). This solution was placed on a 2.5 cm in diameter column containing 100 ml of Sephadex G-100 equilibrated with buffer. The column was eluted with buffer, 1-ml fractions being collected. Enzyme-containing fractions were pooled and protein was precipitated by addition of solid $(NH_4)_2SO_4$ to 65% saturation. The precipitated protein was dissolved in a minimal volume of buffer to yield 2 ml of solution (Sephadex filtrate). This solution was chromatographed in an identical manner on a column containing 100 ml of Biogel-200. Active fractions were pooled, and solid $(NH_4)_2SO_4$ was added to 65% saturation. The precipitate was dissolved in a minimal volume of buffer to yield 1 ml of purified enzyme (Biogel filtrate). The purification is summarized in Table I.

Reaction Products. UDPGA (5.0 μmoles) was incubated with 0.5 mg of enzyme in the presence of NAD in a total volume of 0.25 ml containing 0.2 ml of buffer.

TABLE I: Purification of UDPGA Carboxy-lyase.

Fraction	Volume (ml)	Total Units	Spec Activity (units/mg × 10^2)	Recovery (%)
Crude extract	188	8.4	3	100
$MnCl_2$ supernatant	184	8.3	3	98
$(NH_4)_2SO_4$ fraction	5	6.0	10	72
Sephadex filtrate	2	4.7	23	56
Biogel filtrate	1	3.6	60	43

After 2 hr at 37° the mixture was subjected to paper electrophoresis on Whatman 3MM paper at pH 5.8. Only one band, which had the mobility of UDPXyl, was present. This material was eluted and further characterized. ^{14}C-Labeled UDPGA was similarly converted to a reaction product with the electrophoretic mobility of UDPXyl. This radioactive reaction product was shown to be UDP-pentose by coelectrophoresis with authentic unlabeled UDPXyl at pH 3.6 and 5.8 and by cochromatography in ethanol–ammonium acetate.

The nonradioactive reaction product had the typical ultraviolet absorption spectrum of uridine nucleotides at pH 2, 6, and 11. Acid hydrolysis (0.01 N HCl, 100°, 15 min) yielded three ultraviolet-absorbing compounds with the paper-electrophoretic mobilities at pH 3.6 and 5.8 of UDPXyl, UDP, and UMP, respectively. The spot corresponding in mobility to UDP was eluted and a portion of the material was again hydrolyzed as above, yielding a nucleotide with the paper-electrophoretic

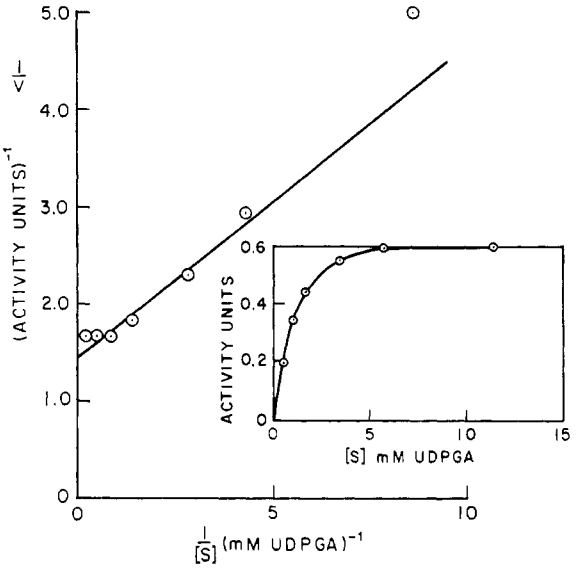

FIGURE 3: Dependence of reaction rate on substrate concentration. Experimental conditions are those described in the text for the assay except that the concentration of UDPGA was varied. Velocity is expressed as units per ml of enzyme solution containing 6 mg/ml of protein, specific activity 0.1 (ammonium sulfate fraction).

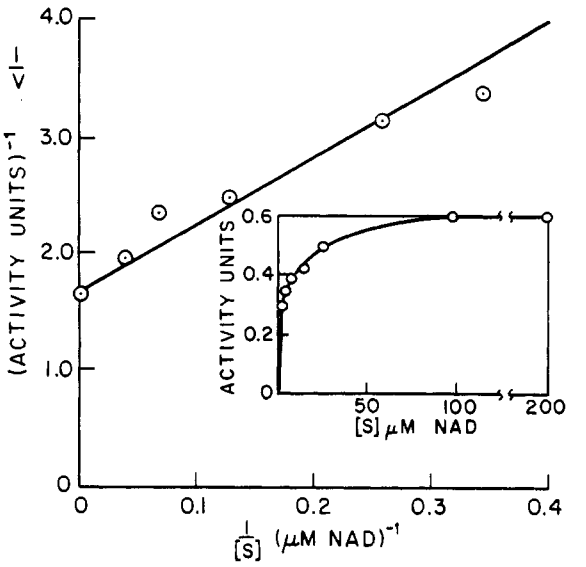

FIGURE 4: Dependence of reaction rate on NAD concentration. Experimental conditions are those described in the text for the assay, except the concentration of NAD was varied. Velocity is expressed as units per ml of enzyme solution containing 6 mg/ml of protein, specific activity 0.1 (charcoal-treated ammonium sulfate fraction).

mobility of UMP at pH 3.6 and 5.8, and the R_F of authentic UMP upon two-dimensional chromatography on a cellulose thin layer (Randerath, 1962).

Analysis of the reaction product gave uridine–acid-labile phosphate–total phosphate–reducing sugar, 1.0:1.1:2.2:1.1. The identity of the sugar portion of the reaction product was established as follows. The sugar released by acid hydrolysis (0.1 N HCl, 100°, 15 min) had the R_F of authentic xylose upon two-dimensional paper chromatography; it had the mobility of xylose upon paper electrophoresis at pH 9.2 and the characteristic retention time of xylose upon gas–liquid partition chromatography of the trimethylsilyl derivative. In addition, the radioactive compound released by acid hydrolysis of the ^{14}C-labeled reaction product was identical with authentic, unlabeled xylose by two-dimensional paper chromatography and by paper electrophoresis at pH 9.2.

The xylosyl moiety was shown to be the D isomer by oxidation to D-xylonic acid with glucose oxidase as described previously (Ankel *et al.*, 1964). The configuration of the linkage of the D-xylosyl moiety was established with UDPXyl pyrophosphorylase (Feingold *et al.*, 1964). Decarboxylation product (0.1 μmole) was incubated at 37° for 1 hr with an excess of sodium pyrophosphate, $MgCl_2$, and enzyme at pH 7.0, and the mixture was subjected to paper electrophoresis at pH 5.8. UTP formed in the reaction was demonstrated by its characteristic electrophoretic mobility. The other reaction product, α-D-xylopyranosyl phosphate, was demonstrated by its electrophoretic mobility at pH 3.6 and 5.8 and by its reaction with the reagent of Bandurski and Axelrod (1951) for organic phosphate. These data establish the structure of the product of decarboxylation of UDPGA by the *Cr. laurentii* enzyme as uridine-5′-(α-D-xylopyranosyl pyrophosphate). The identical compound has been isolated from *Cr. laurentii* (Ankel *et al.*, 1964). Carbon dioxide released during the reaction was demonstrated qualitatively, using methods described previously (Ankel and Feingold, 1965).

Optimum pH. The optimum pH was determined with 0.1 M acetate buffer between 4.7 and 6.1, 0.1 M phosphate buffer between 6.3 and 7.4, 0.2 M glycylglycine–NaOH buffer between 7.2 and 8.6, and $NaHCO_3$–Na_2CO_3 buffer between pH 9.1 and 9.4. As can be seen from Figure 1, the pH optimum is between 7.0 and 7.5.

Effect of Enzyme Concentration. The reaction is linear for at least 6 min under the conditions of assay. Activity varies linearly with enzyme concentration (Figure 2).

Effect of Substrate Concentration. In Figure 3 the effect of UDPGA concentration on reaction rate is shown. The apparent K_m value at 37°, determined according to Lineweaver and Burk (1935), is 1.1×10^{-3} M.

Substrate Specificity. UDP-D-galacturonic acid, pseudo-UDPGA, D-glucuronic acid 1-phosphate, or D-glucuronic acid was incubated with enzyme and the reaction mixtures were subjected to paper electrophoresis at pH 3.6 and 5.8. In each case the electrophoretic patterns with enzymes and boiled enzyme controls were identical, indicating no activity with these compounds. In an identical reaction mixture containing UDPGA there was complete conversion to UDPXyl.

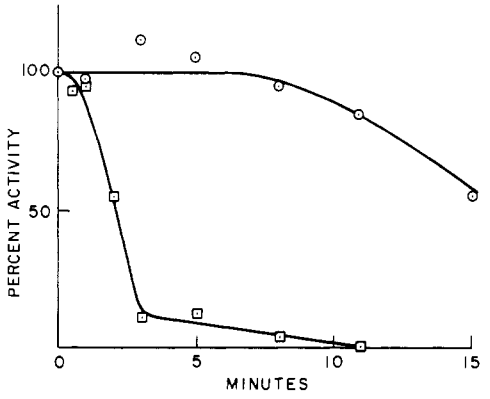

FIGURE 5: Protection of UDPGA carboxy-lyase by NAD. The enzyme was held at 50°: O——O in the presence of 10^{-3} M NAD, ⊡——⊡ no NAD. After the indicated time intervals samples were withdrawn and assayed for activity as described in the text.

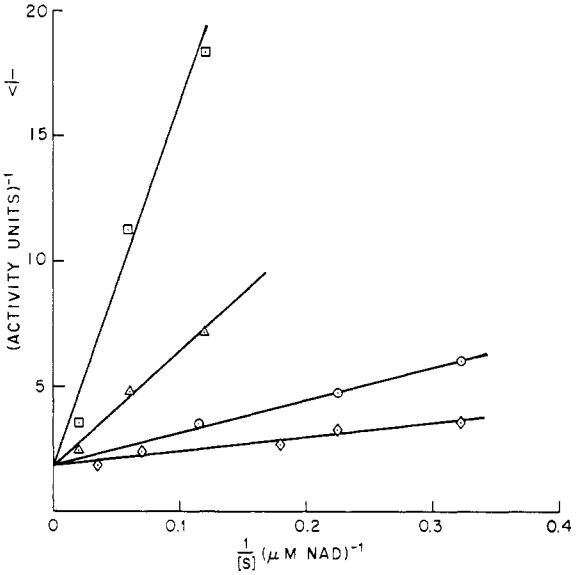

FIGURE 6: Dependence of reaction rate on NAD concentration in the presence of increasing concentrations of $NADH_2$. Experimental conditions are the same as described for Figure 4, except that $NADH_2$ was added to the reaction mixtures as indicated; ⊙——⊙ 2.3 μM $NADH_2$, △——△ 8.2 μM $NADH_2$, ⊡——⊡ 41.0 μM $NADH_2$, ◇——◇ no $NADH_2$.

NAD Requirement. Ammonium sulfate fraction (1.0 ml), containing 20 mg of protein of specific activity 0.2, was mixed with 0.6 ml of a suspension of 150 mg of acid-washed Norit A in buffer. After 5 min at 4° the charcoal was spun out and discarded. Assays in the presence of added NAD were performed on the enzyme before as well as after the absorption procedure; in each case the specific activity was unchanged. However, when NAD was omitted from the assay mixtures the untreated enzyme had a specific activity of 0.04 and the charcoal-treated enzyme had no detectable activity. In addition, treatment of the ammonium sulfate fraction (0.05 ml) with 100 μg of NADase in a total volume of 0.28 ml of buffer for 10 min at 37° completely abolished enzyme activity. The enzyme was not reactivated by 0.001 M NAD which had been pretreated with NADase. These data show that partially purified preparations of UDPGA carboxy-lyase contain NAD and that the enzyme requires NAD for activity. Loss of activity after NADase treatment shows that the cofactor is NAD and not some impurity in the sample of NAD used.

The NAD requirement appears to be specific, since NAD could not be replaced by NADP, NAD analogs, or an equimolar mixture of adenosine monophosphate and nicotinamide mononucleotide. The effect of NAD concentration on reaction rate is shown in Figure 4. The apparent K_a at 37°, determined by the method of Lineweaver and Burk (1934), is 3×10^{-6} M.

NAD protects the enzyme from heat denaturation. When ammonium sulfate fraction was held at 50° for 3 min 90% of the activity was lost and could not be restored by addition of NAD. However, full activity could be retained (and a twofold purification achieved) by holding ammonium sulfate fraction at 50° for up to 8 min in the presence of 0.001 M NAD (Figure 5). At 60°, even in the presence of 0.001 M NAD, activity was completely lost in 2 min. Heat-purified enzyme was unstable and could not be purified further by the procedure described for enzyme purification without loss in the next step of at least 80% of the initial activity. The protective effect of NAD was much less marked with more highly purified enzyme. Thus Sephadex filtrate was 50% inactivated at 45° for 5 min, or at 50° for 3 min, even in the presence of 0.001 M NAD.

Stability. The ammonium sulfate fraction can be stored at −20° for at least 3 weeks with no loss of activity. Progressive inactivation resulted from repeated freezing and thawing. After Sephadex filtration the enzyme was relatively unstable and rapidly lost activity, whether at −20° or at 4°, nor was it protected by NAD from heat denaturation. Buffer composition and concentration also affected enzyme stability. If after precipitation with ammonium sulfate the enzyme was taken up in 0.1 M Tris or 0.01 M phosphate buffer (both pH 7.0, containing 0.5 g of EDTA and 0.5 ml of mercaptoethanol per l.) activity was lost; it was not restored by transferring the inactive carboxy-lyase back to buffer. The activity of 90%-inactivated enzyme resulting from Sephadex filtration of heat-treated enzyme or from heat treatment of Sephadex filtrate could not be restored by 0.001 M NAD.

Inhibitors. UDPGA carboxy-lyase is inhibited by $NADH_2$. As can be seen from Figure 6, the inhibition is strictly competitive with respect to NAD; the apparent K_i at 37° is 2×10^{-6} M. The inhibition is not dependent on UDPGA concentration, which is illustrated in Figure 7. NAD analogs in which nicotinamide is replaced by the various moieties are also in-

TABLE II: Inhibition of UDPGA Carboxy-lyase by NAD Analogs.

Analog	K_i (M × 10^6)
3-Formylpyridine-AD	10
3-Acetylpyridine-AD	12
3-Propionylpyridine-AD	13
Thionicotinamide-AD	16
Adenosine monophosphate	28
α-NAD	130

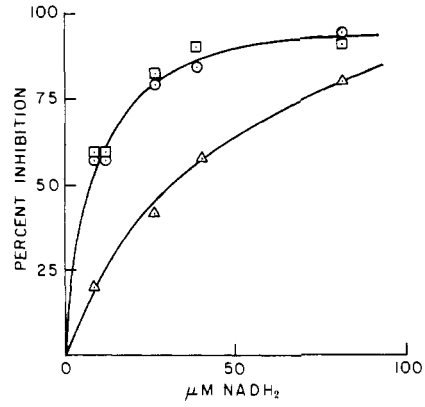

FIGURE 7: Per cent inhibition *vs.* increasing concentrations of $NADH_2$. Experimental conditions are as described in the text for the assay except that NAD, $NADH_2$, and UDPGA concentrations were varied as indicated; top curve: ⊡——⊡ 70 mM UDPGA, 9 μM NAD; ⊙——⊙ 2.3 mM UDPGA, 9 μM NAD; bottom curve: △——△ 2.3 mM UDPGA, 43 μM NAD.

hibitors competitive with NAD; this is shown in Table II. No inhibitory effect is exerted by any of the following: deamino-NAD, NADP, ADP, or NMN. The following compounds are inhibitors competitive with UDPGA: UMP, $K_i = 2 \times 10^{-3}$ M; UDPG, $K_i = 2 \times 10^{-3}$ M; and UDP, $K_i = 0.2 \times 10^{-3}$ M. UDPXyl and UDP-L-arabinose have no inhibitory effect at a concentration of 0.001 M, nor do the following have an effect on enzyme activity: 0.01 M Zn^{2+}, Mg^{2+}, or 0.1 M EDTA.

UDPGA carboxy-lyase from wheat germ is progressively and irreversibly inactivated by PCMB; neither cysteine, NAD, nor both could restore activity to enzymes which had been exposed to the inhibitor (Ankel and Feingold, 1965). On the other hand, PCMB inactivation of the UDPGA carboxy-lyase of *Cr. laurentii* can be reversed by cysteine. This is shown in the following series of experiments. Sephadex filtrate (1 ml, specific activity 0.2) was dialyzed against 1 l. of buffer (without mercaptoethanol) for 24 hr, the buffer solution being changed after 12 hr. The dialyzed enzyme was brought to a final volume of 2 ml. The dialyzed enzyme (50 μl, specific activity 0.1) was treated with 5 μl of 0.001 M PCMB at 25° for 10 min, 10 μl each of 0.03 M UDPGA and 0.01 M NAD was then added, and incubation was continued for 10 additional min. Glacial acetic acid (60 μl) was then added to stop the reaction, the mixture was held at 100° for 15 min, and D-xylose was determined in the hydrolysate as described. Enzyme activity could be recovered completely in reaction mixtures from which PCMB had been omitted; no activity could be recovered from mixtures which contained PCMB. Inclusion of 10 μl of 0.01 M NAD in the initial incubation mixture did not protect the enzyme; however, when 10 μl of 0.1 M cysteine was added to such mixtures at the end of the initial 10-min incubation, 90% of the activity could be recovered. Omission of NAD from the initial incubation mixture and addition of cysteine after 10 min permitted recovery of 70% of enzyme activity.

Reaction Mechanism. When UDPGA-4-*t* or UDPGA-3-*t* is converted to UDPXyl by UDPGA carboxy-lyase from wheat germ, there is no exchange of label with the medium (Ankel and Feingold, 1965). The same experiment was carried out with enzyme from *Cr. laurentii*. Reaction mixtures contained UDPGA-4-*t* or UDPGA-3-*t* and 10–20 μg of enzyme at pH 7.0 in a total volume of 30 μl. Other reaction mixtures contained NAD, or NAD and $NADH_2$ (0.03 and 0.005 μmole). These were worked up and counted exactly as described in the previous publication. Tables III and IV show that there was no loss of label during conversion of UDPGA-3-*t* or -4-*t* to UDPXyl. These results are similar to those obtained with the UDPGA carboxy-lyase of wheat germ (Ankel and Feingold, 1965).

TABLE III: Distribution of Label in UDPGA and UDP-Xyl Isolated from Reaction Mixtures Containing UDPGA-4-*t*.

Reaction Mixtures (cpm)	UDP-Xyl (cpm)	UD-PGA (cpm)	Total (cpm)	% of Con-trol
Control (boiled enzyme)	33	723	756	100
No cofactors	52	719	771	102
Plus NAD	365	308	673	89
Plus NAD and $NADH_2$	400	405	805	106

UDPXyl Formation by Other Organisms. Crude extracts prepared from cells of *Cr. diffluens* and *Tremella mesenterica* both had demonstrable UDPGA carboxy-lyase activity, which was abolished by treatment with NADase. These observations show that the UDPGA carboxy-lyases of these organisms are, at least in their NAD requirement, similar to that of *Cr. laurentii*.

TABLE IV: Distribution of Label in UDPGA and UDP-Xyl Isolated from Reaction Mixtures Containing UDPGA-3-*t*.

Reaction Mixtures (cpm)	UDP-Xyl (cpm)	UDPGA (cpm)	Total (cpm)	(%) of Control
Control (boiled enzyme)	54	1615	1669	100
No cofactors	115	1705	1820	109
Plus NAD	1494	285	1779	106
Plus NAD and $NADH_2$	1400	227	1627	97

Discussion

UDPGA carboxy-lyase of wheat germ contains tightly bound NAD but is not affected by exogenous NAD or $NADH_2$ (Ankel and Feingold, 1965), while the enzyme isolated from *Cr. laurentii* requires NAD for activity and is inhibited by $NADH_2$. As was pointed out in the previous publication, in this regard UDPGal-4-epimerases from yeast and liver resemble UDPGA carboxy-lyase from wheat germ and *Cr. laurentii*, respectively. It has been proposed that the epimerization mechanism involves a reversible oxidoreduction between NAD and carbon 4 of the hexosyl moiety. Direct evidence for this view is lacking, and in fact tritium from free NAD or $NADH_2$ is not incorporated into the product when the epimerization is catalyzed by either the liver (Maxwell, 1957) or yeast epimerase (Maxwell and de Robichon-Szulmajster, 1960) nor is there loss of label when the yeast enzyme is used to catalyze the epimerization of UDPG-4-*t* (Bevill *et al.*, 1963). de Robichon-Szulmajster (1961) has proposed that the uracil moiety itself is involved in the reaction as a reversible hydrogen acceptor, and that the same H atom transferred to NAD from carbon 4 in the oxidation step of the reaction is replaced in the reduction, thus explaining the observed lack of incorporation or exchange of tritium. Lately Wilson and Hogness (1964) have presented spectroscopic evidence that enzyme-bound $NADH_2$ is formed when UDPG is added to highly purified UDPGal-4-epimerase of *E. coli*, which contains tightly bound NAD.

Some of the results presented in this paper, namely the effect of NAD and $NADH_2$ and the retention of label upon conversion of UDPGA-4-*t* to UDPXyl, are analogous to those found by Maxwell (1957) and Bevill *et al.* (1963), respectively, with UDPGal-4-epimerase from calf liver. In decarboxylation of an acid the carboxyl group leaves one pair of electrons behind on the remaining residue. The β-carbonyl group in β-keto acids facilitates loss of carbon dioxide by acting as an electron sink, stabilizing the remaining residue after loss of carbon dioxide (Westheimer and Jones, 1941). Therefore, the hypothesis that UDP-4-keto-GA is an intermediate in the decarboxylation reaction is an attractive one.

The reaction sequence, which is similar to that suggested by Wilson and Hogness (1964), would then be

$$\text{NAD–E} + \text{UDPGA} \rightleftharpoons \text{NAD–E–UDPGA} \quad (1)$$

$$\text{NAD–E–UDPGA} \rightleftharpoons \text{NADH}_2\text{–E–I} \quad (2)$$

$$\text{NADH}_2\text{–E–I} \longrightarrow \text{NADH}_2\text{–E–II} + \text{CO}_2 \quad (3)$$

$$\text{NADH}_2\text{–E–II} \rightleftharpoons \text{NAD–E–UDPXyl} \quad (4)$$

$$\text{NAD–E–UDPXyl} \rightleftharpoons \text{NAD–E} + \text{UDPXyl} \quad (5)$$

in which I is UDP-4-keto-GA and II is UDP-4-keto-Xyl. This model explains the observed retention of label when UDPGA-4-*t* is converted to UDPXyl by UDPGA carboxy-lyase from either wheat germ or *Cr. laurentii* if it is assumed that there is no exchange between bound and free $NADH_2$. This assumption is substantiated by the tight binding of NAD in wheat germ carboxy-lyase (Ankel and Feingold, 1965), the low K_s for NAD (3×10^{-6} M) of cryptococcal carboxy-lyase, and the observed greater tenacity of $NADH_2$ than NAD binding to dehydrogenases (Velick, 1961). It should be noted that, as far as the proposed reaction mechanisms are concerned, both UDPGal-4-epimerase and UDPGA carboxy-lyase are dehydrogenases.

The protective action of NAD against heat denaturation is further evidence for its role in the catalytic action of the carboxy-lyase. Protection by NAD from denaturation has been described for a number of dehydrogenases. NAD protects bovine heart lactic dehydrogenase (Pfleiderer *et al.*, 1957), yeast (Racker, 1950), and liver (Yonetani and Theorell, 1962) alcohol dehydrogenase, and glyceraldehyde phosphate dehydrogenase (Shifrin *et al.*, 1959) from denaturation by heat.

The kinetic data and the close structural similarity between NAD and $NADH_2$ make it seem likely that these two substances compete for the same site on the enzyme surface. If NAD and $NADH_2$ were acting as allosteric effector and inhibitor, respectively, the inhibitory effect of $NADH_2$ would be expected to vary with UDPGA concentration (Monod *et al.*, 1965). That this does not occur is shown in Figure 7, making it seem unlikely that an allosteric effect is involved.

The high specificity of UDPGA carboxy-lyase for NAD is noteworthy. None of the analogs tested activated the enzyme; many inhibited competitively with NAD, although in no case was the K_i lower than that of $NADH_2$. Maxwell (1957) noted that neither α-NAD nor 3-formylpyridine-AD could replace NAD in the liver enzyme-catalyzed 4-epimerization, and that 3-acetylpyridine-AD inhibited. With most other dehydrogenases, NAD analogs such as 3-acetylpyridine-AD, which differ from NAD only in the amide group, are active as hydrogen acceptors, while other types of analogs do not function as hydrogen acceptors and

frequently act as competitive inhibitors of NAD (Anderson and Kaplan, 1959).

The ratio of $NADH_2$ to NAD in *Cr. laurentii* conceivably could play a role in controlling the concentration of UDPXyl. Robinson *et al.* (1963) have shown that UDPGal-4-epimerase could be demonstrated in L cells only when the assay was performed in the presence of added NAD, and suggested that UDPGal formation is inhibited in these cells by the high $NADH_2$/NAD ratio. In *Cr. laurentii* a similar mechanism may be operative, increasing the activity of UDPGA carboxy-lyase when the $NADH_2$/NAD ratio is low, decreasing it when it is high. It is of interest that the UDPGA carboxy-lyase of wheat germ is not subject to this control, being unaffected by either NAD or $NADH_2$ (Ankel and Feingold, 1965).

Acknowledgments

We are indebted to Drs. R. Bentley and C. Sweeley for the gas-chromatographic identification of D-xylose and to Mr. John S. Schutzbach for skillful technical assistance. Thanks are also due to Drs. G. Ashwell, W. Z. Hassid, N. O. Kaplan, and S. Kirkwood for generous gifts of materials.

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