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Biosynthesis of Uridine Diphosphate D-Xylose. I. Uridine Diphosphate Glucuronate Carboxy-lyase of Wheat Germ*

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ABSTRACT: Uridine disphosphate D-glucuronate carboxy-lyase from wheat germ has been purified 350-fold. The only products of enzyme action are uridine diphosphate D-xylose and CO_2. The enzyme has a pH optimum between 6.8 and 7.0. K_m for uridine disphosphate D-glucuronate is about 3×10^{-4} M.

Wheat germ carboxy-lyase is neither activated by nicotine-adenine dinucleotide (NAD) nor inhibited by reduced NAD ($NADH_2$), in contrast to the analogous enzyme from *Cryptococcus laurentii*. However, NAD is released by wheat germ enzyme upon heat denaturation.

Polymers of D-xylose abound in higher plants. It was long suspected that the metabolic pathway in plants leading from hexose to pentose involved C-6 decarboxylation. This was substantiated by studies of many workers using either intact plants (Altermatt and Neish, 1956; Neish, 1958; Loewus *et al.*, 1958) or plant slices (Slater and Beevers, 1958). In these experiments the tissue was supplied with a suitably labeled hexose or uronic acid and the distribution of label in the pertinent metabolic products was determined. Involvement of sugar nucleotides in this conversion was suggested by the isolation of a mixture of uridine 5′-(α-D-xylopyranosyl pyrophosphate), uridine 5′-(β-L-arabinopyranosyl pyrophosphate), uridine 5′-(α-D-glucopyranosyl pyrophosphate), and uridine 5′-(α-D-galactopyranosyl pyrophosphate) (UDPXyl, UDPAra, UDPG, and UDPGal, respectively)[1] from mung bean seedlings by Ginsburg *et al.* (1956); uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate) (UDPGA) was isolated from the same source by Solms and Hassid (1957). Subsequently it was shown that UDPXyl was formed by the decarboxylation of UDPGA. This was demonstrated by use of partially purified extracts from mung beans (Feingold *et al.* 1960) and other plants. These preparations were of low activity and were always contaminated by UDPAra-4-epimerase.

Recently we have demonstrated and partially purified the UDPGA carboxy-lyase of *Cryptococcus laurentii* (Ankel and Feingold, 1964). This enzyme has an absolute requirement for catalytic quantities of nicotinamide-adenine dinucleotide (NAD) and is inhibited by reduced NAD ($NADH_2$). We have now reinvestigated the plant enzyme in order to compare it with the carboxy-lyase from *Cryptococcus laurentii*. In this paper we describe the partial purification and properties of the UDPGA carboxy-lyase of wheat germ.

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[1] The following abbreviations are used: nicotinamide-adenine dinucleotide, NAD; reduced NAD, $NADH_2$; uridine monophosphate, UMP; uridine diphosphate, UDP; uridine triphosphate, UTP; thymidine diphosphate, TDP; uridine, adenine, cytosine, and guanine 5′-(α-D-glucopyranosyl pyrophosphate), UDPG, ADPG, CDPG, and GDPG, respectively; uridine 5′-(α-D-xylopyranosyl pyrophosphate), UDPXyl; uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate), UDPGA; uridine 5′-(α-D-galactopyranosyluronic acid pyrophosphate), UDPGalA; uridine 5′-(β-L-arabinopyranosyl pyrophosphate), UDPAra; 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. 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: β-D-glucose:O_2 oxidoreductase (EC 1.1.3.4), glucose oxidase; D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating) (EC 1.2.1.12), GAP dehydrogenase; UTP:α-D-xylose 1-phosphate uridylyl transferase (EC 2.7.7.11), UDPXyl pyrophosphorylase; NAD glycohydrolase (EC 3.2.2.5), NADase; orthophosphoric diester phosphohydrolase (EC 3.1.4.4), phosphodiesterase. *p*-Mercuribenzoate is abbreviated PCMB.

Experimental Section

Materials. Raw wheat germ was obtained from Walnut Acres, Penn's Creek, Pa. Sephadex G-75 and G-100 were purchased from Pharmacia Corp. β-D-Glucose:O_2 oxidoreductase (glucose oxidase) was a product of Worthington Biochemicals. UTP:α-D-xylose 1-phosphate uridylyl transferase (UDPXyl pyrophosphorylase) was prepared as described by Feingold *et al.* (1964). UDPGA, DL-glyceraldehyde 3-phosphate, NAD, NAD phosphate (NADP), $NADH_2$, $NADPH_2$, NAD glucohydrolase (NADase), and D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating) (GAP dehydrogenase) were products of Sigma Chemical Co. Pseudo-UDPGA was generously given by Dr. Y. Kanai, Takeda Chemical Industries, Ltd., Osaka, Japan. U-^{14}C-Labeled D-glucopyranosyl phosphate (50 μc/μmole) was purchased from California Biochemical Co. This compound was converted to ^{14}C-labeled UDPGA, D-glucuronic acid 1-phosphate, and D-glucuronic acid as previously described (Feingold *et al.*, 1960). α-D-Glucopyranosyl phosphate-3-*t* (a gift from Drs. G. Ashwell and O. Gabriel) was converted to UDPGA-3-*t* (7×10^6 cpm/μmole at 18% efficiency). UDPG-4-*t* (4×10^6 cpm/μmole at 18% efficiency), kindly given by Dr. S. Kirkwood, University of Minnesota, was used to make UDPGA-4-*t*. Uridine 5′-(α-D-galactopyranosyluronic acid pyrophosphate) (UDPGalA) labeled with ^{14}C in the D-galacturonosyl moiety was a gracious gift from Dr. W. Z. Hassid, University of California at Berkeley. UDPXyl labeled with ^{14}C in the D-xylosyl moiety was prepared from ^{14}C-labeled UDPGA using a partially purified UDPGA carboxy-lyase from *Cryptococcus laurentii* (Ankel and Feingold, 1964). Calcium phosphate gel was purchased from Nutritional Biochemicals Corp. Ammonium sulfate, enzyme grade, was a product of Mann Research Laboratories. Two buffer solutions were used throughout the enzyme purification: A, 0.1 M sodium and potassium phosphate, pH 7.0, containing 0.5 g of EDTA and 0.5 ml of mercaptoethanol/l.; B, 0.01 M sodium and potassium phosphate, pH 7.0, with the same EDTA and mercaptoethanol concentration as buffer A.

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. Paladini and Leloir's (1952) solvent, 95% ethanol–1 M ammonium acetate, pH 7.0 (7:3, v/v), was used for nucleotides. Paper electrophoresis was done on oxalic acid washed sheets of Whatman 3MM or No. 1 paper at either pH 3.6 or 5.8 (Feingold *et al.*, 1964), using the solvent-cooled high-voltage GME Electrophorator. Nucleotides were located on paper by visual observation under short-wave (254 mμ) ultraviolet illumination. Carbohydrates were revealed with *p*-anisidine phosphate (Feingold *et al.*, 1958). Organic phosphate was detected by the method of Bandurski and Axelrod (1951). ^{14}C-Labeled compounds were 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).

UDPGA carboxy-lyase was assayed as follows. Reaction mixtures contained 0.03 M UDPGA, 0.04 ml; buffer A, 0.26 ml; and enzyme, suitably diluted, 0.3 ml. Incubation was at 37°. At appropriate times 0.1-ml samples were inactivated by addition to tubes containing 0.06 ml of glacial acetic acid. UDPXyl present in these samples was hydrolyzed at 100° for 15 min. Xylose released was determined by an adaptation of the method of Roe and Rice (1948). A solution (0.65 ml) of 2% *p*-bromoaniline in glacial acetic acid saturated with thiourea was added to each tube and the tubes were held at 70° for 10 min. They were then left at room temperature in the dark for at least 60 min. Any precipitate present was removed by centrifugation and the absorbance was read at 520 mμ. A standard curve was run with each determination, using quantities of D-xylose ranging from 0.05 to 0.3 μmole/sample. A unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmole of UDPXyl/min.

Enzyme Purification. All operations were performed at 0–4° except as otherwise noted. Centrifugation of protein precipitates was performed at 10,000*g* for 10–20 min. Raw wheat germ (250 g) was stirred with 1 l. of buffer A for 1 hr. The thick slurry was centrifuged, yielding 700 ml of dark, turbid supernatant fluid (crude extract). This was efficiently stirred, and 0.5 M $MnCl_2$ was rapidly added to a final concentration of 0.01 M. After stirring for 5 additional min the heavy, greenish precipitate was spun down and discarded. The pH of the turbid supernatant fluid ($MnCl_2$ supernatant) was brought to 7 with 7.5 N NH_4OH. To this solution (640 ml) was added 148 g of $(NH_4)_2SO_4$, and the precipitate was discarded. The supernatant fluid (550 ml) was treated with 107 g of $(NH_4)_2SO_4$, the precipitate was removed by centrifugation and taken up in buffer A to a final volume of 140 ml [$(NH_4)_2SO_4$-I]. At this point the protein concentration should be 120–160 mg/ml. The solution (in a beaker) was placed in a 60° bath and gently stirred mechanically until its temperature rose to 50°. The beaker was then immediately placed in a bath at 50° and stirred mechanically for 5 min. At the end of this time the beaker was transferred to an ice bath; stirring was continued until the temperature fell to 4°. After removal of denatured protein by centrifugation the clear, brown, supernatant fluid (heat-step supernatant) was placed on a 4-cm diameter column containing 500 ml of Sephadex G-75 equilibrated in buffer B. The column was eluted with buffer B, 50–60 5-ml fractions being collected. Enzyme emerged over a range of about 30 fractions. Usually only tubes which contained precipitated protein showed enzyme activity. The enzyme-containing fractions were pooled (Sephadex-I), and the precipitate was spun down and discarded. To the clear, brown, supernatant fluid (120 ml) 36 g of $(NH_4)_2SO_4$ was added and the precipitate was discarded. To the supernatant fluid (130 ml) was added 8.5 g of $(NH_4)_2SO_4$; the precipitated protein was taken up in buffer B to a final volume of 8 ml [$(NH_4)_2SO_4$-II]. This solution was chromatographed on 150 ml

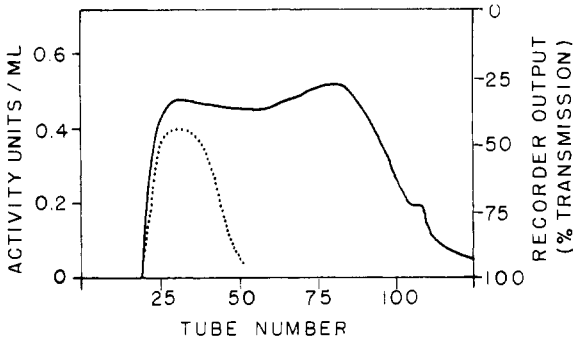

FIGURE 1: Chromatography of gel supernatant on Sephadex G-100. Gel supernatant (35 ml, specific activity 0.045) was added to 800 ml of Sephadex G-100 (equilibrated with buffer B, contained in a column 3.5 cm in diameter). The column was eluted with buffer B at the rate of 0.5 ml/min. Per cent transmission (solid curve) at 280 mμ was determined continuously with a GME ultraviolet absorption meter. Fractions of 3 ml were collected. Enzyme activity (dotted curve) in each fraction was determined as follows. Eluate (50 μl) was added to 10 μl of UDPGA. After 15 min at 37° UDPXyl formed was determined as described in the text. Fractions 21–38 were pooled and fractionated with $(NH_4)_2SO_4$ as described in the text to yield 10 ml of enzyme (Sephadex-III) of specific activity 0.56.

of Sephadex G-100 (equilibrated with buffer B, contained in a column 2 cm in diameter) using buffer B as eluent and collecting 3-ml fractions. The enzyme emerged in 8 fractions (Sephadex-II). The protein concentration was adjusted to 11 mg/ml with buffer B, and $Ca_3(PO_4)_2$ gel (3 mg dry wt/mg of protein) was added with stirring. After stirring for an additional 5 min the gel was spun down and discarded. To the supernatant liquid (52 ml) was added 15.5 g of $(NH_4)_2SO_4$ and the resultant precipitate was taken to a final volume of 3.4 ml in buffer B (gel supernatant). This solution was chromatographed on Sephadex G-100 as described above. Enzyme emerged in three successive fractions. These were pooled and protein was precipitated by addition of 3 g of $(NH_4)_2SO_4$. The precipitated enzyme was taken up in 1 ml of buffer B to yield a colorless solution with a protein concentration of 2.5 mg/ml (Sephadex-III). An illustration of the chromatography, carried out on a 10-fold scale, is given in Figure 1. The results, which are identical with those described here, attest to the reproducibility of the procedure.

The activity of the purified enzyme was 0.56 unit/mg of protein. The enzyme solution could be stored frozen for several months without loss of activity. As can be seen from Table I, a purification of 350-fold was achieved, with about 2% recovery.

Reaction Products. Uridine diphosphate pentose (UDP-pentose) previously has been shown to be a product of UDPGA decarboxylation catalyzed by crude extracts of wheat germ (Feingold *et al.*, 1963, 1964). Further characterization of the reaction products

TABLE I: Purification of UDPGA Carboxy-lyase.

Fraction[a]	Volume (ml)	Total Units	Specific Activity (units/mg × 10^3)	Recovery (%)
Crude extract	700	88	1.6	100
$MnCl_2$ supernatant	640	80	1.6	91
$(NH_4)_2SO_4$-I	140	64	3.0	73
Heat step supernatant	85	34	3.5	39
Sephadex-I	120	17	5.6	19
$(NH_4)_2SO_4$-II	8	11	10.0	13
Sephadex-II	25	7.0	14.0	8
Gel supernatant	3.4	4.4	46.0	5
Sephadex-III	1.1	1.8	560.0	2

[a] See text for a description of the fractions.

of the purified UDPGA carboxy-lyase was carried out as follows. Enzyme reaction mixtures contained 2.5 μmoles of UDPGA, 0.2 mg of enzyme, and 0.1 ml of buffer B in a total volume of 0.13 ml. A comparable reaction was run using ^{14}C-labeled UDPGA. After 1 hr at 37° the nucleotide reaction product was isolated by paper electrophoresis at pH 5.8. Only one ultraviolet-absorbing or radioactive compound was present in reaction mixtures containing unlabeled or labeled UDPGA, respectively. Unlabeled reaction product was shown to be identical with authentic ^{14}C-UDPXyl by coelectrophoresis at pH 3.6 and 5.8 and by cochromatography in the solvent of Paladini and Leloir (1952). Contamination of previous preparations of UDPGA carboxy-lyase by UDPAra-4-epimerase has yielded mixtures of UDPXyl and UDPAra from UDPGA (Feingold *et al.*, 1960, 1963, 1964). Absence of such contamination in the purified enzyme was shown by the presence of only one radioactive compound, which cochromatographed with authentic D-xylose, upon two-dimensional paper chromatography of the hydrolysate (0.1 N HCl, 100°, 15 min) of the UDP-pentose produced from ^{14}C-labeled UDPGA. Experimental conditions would have permitted detection of less than 1% of L-arabinose in the hydrolysate. These results show that the purified UDPGA carboxy-lyase does not contain UDPAra-4-epimerase. 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). Confirmation of the structure of the decarboxylation product was afforded by UDPXyl pyrophosphorylase from mung beans (Feingold *et al.*, 1964). Decarboxylation product (0.1 μmole) was incubated with an excess of sodium pyrophosphate, $MgCl_2$, and enzyme at pH 7.0. The reaction products were separated by paper electrophoresis at pH 5.8. Uridine triphosphate (UTP) formed in the reaction was demonstrated by its characteristic electrophoretic mobility.

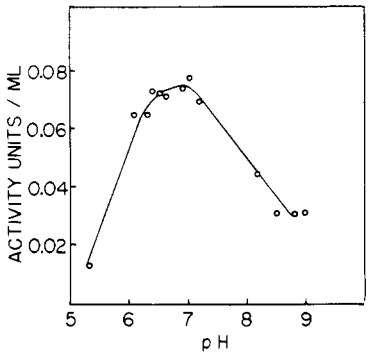

FIGURE 2: Dependence of activity on pH. The experimental conditions were the same as in the enzyme assay except that the buffer and pH were varied as indicated in the text.

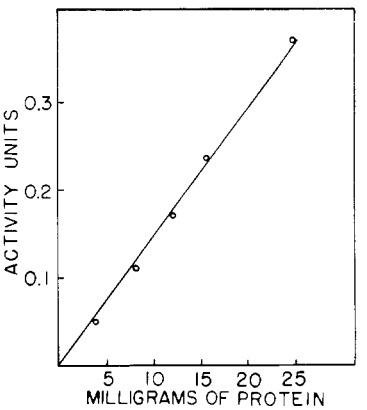

FIGURE 3: Dependence of reaction rate on enzyme concentration. Experimental conditions are those described in the text for the assay.

The α-D-xylopyranosyl phosphate formed in the reaction was shown by its electrophoretic mobility and its reaction with molybdic acid spray for organic phosphate (Bandurski and Axelrod, 1951). In addition, treatment of the decarboxylation product with snake venom phosphodiesterase at pH 8.8 released an ultraviolet-absorbing compound with the characteristic electrophoretic mobility at pH 5.8 of uridine monophosphate (UMP). These results establish the identity of the decarboxylation product as uridine 5′-(α-D-xylopyranosyl pyrophosphate).

The loss of C-6 as CO_2 was shown as follows. UDPGA-^{14}C (8×10^5 cpm) was incubated in a sealed Conway dish at pH 7.0 with 50 μg of enzyme in a total volume of 0.35 ml. The center well contained a small sheet of filter paper wet with 0.1 ml of 1 N NaOH. After 4 hr at 25° the contents of the dish were subjected to paper electrophoresis at pH 5.8. Only one radioactive substance, with the mobility of UDPXyl, was present. This material was eluted in 0.8 ml of water. The filter paper from the center well of the Conway dish was eluted in 0.6 ml of 0.1% NH_4OH. Aliquots of both solutions were counted, that from the electrophoregram yielding 4.40×10^5 cpm and that from the center well yielding 1.00×10^5 cpm. The sum of these activities, 5.40×10^5 cpm, represents 68% recovery of total label. These data, while not strictly quantitative, show that the carboxyl carbon of UDPGA is released as CO_2.

Contaminating Activities. Nucleoside diphosphate sugar pyrophosphorylase activity in the purified enzyme was tested for using ^{14}C-labeled sugar phosphate and various nucleoside triphosphates. After a suitable time interval the reaction mixtures were subjected to paper electrophoresis and the separated products were located by autoradiography. In all cases enzyme incubation mixtures and boiled-enzyme controls were identical. No UDPG, UDPXyl, GDPG, ADPG, or UDPGA pyrophosphorylase could be demonstrated. The purified enzyme was also free from UDPGalA-4-epimerase, tested with either UDPGA or UDPGalA labeled with ^{14}C (Feingold *et al.*, 1964).

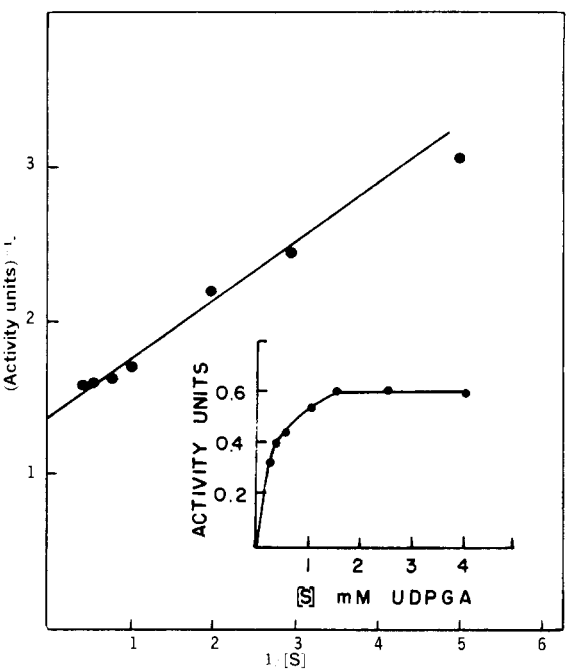

FIGURE 4: Dependence of reaction rate on substrate concentration. Experimental conditions are those described in the text for the assay, except that the substrate concentration was varied.

Optimum pH. The optimum pH was determined using 0.1 M sodium acetate buffer between 3.6 and 5.3, 0.1 M phosphate buffer between 6.1 and 7.2, and 0.1 M Tris-NaOH buffer between pH 8.2 and 9.0. As can be seen in Figure 2, UDPGA carboxy-lyase has an optimum at pH 6.8–7.0.

Effect of Enzyme Concentration. Under the conditions of assay the reaction is linear for at least 6 min. A linear relation obtains between activity and amount of protein (Figure 3).

Effect of Substrate Concentration. The effect of UD-

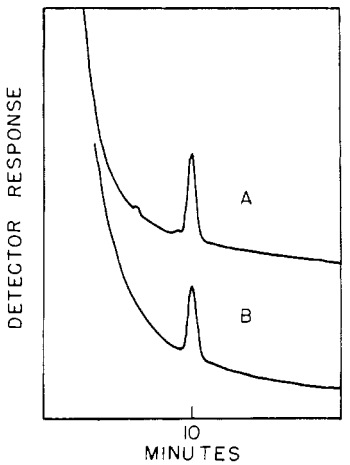

FIGURE 5: Gas chromatography of trimethylsilyl derivatives of the hydrolysates of heat-supernatant component (curve A) and of authentic NAD (curve B).

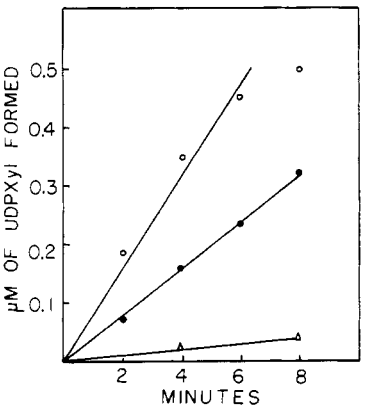

FIGURE 6: Activation of UDPGA carboxylyase of *Cr. laurentii* by heat supernatant or NAD. All reaction mixtures (37°) contained 50 μl (0.08 unit) of partially purified UDPGA carboxy-lyase from *Cr. laurentii* (Ankel and Feingold, 1964) in buffer A and 20 μl of 0.03 M UDPGA. Enzyme activity was determined as described in the text, using 50-μl samples. Reaction mixtures contained, in addition, 0.23 ml of 2×10^{-4} M NAD, —o—; 0.23 ml of heat supernatant, —●—; and either 0.23 ml of water or 0.23 ml of heat supernatant previously treated for 10 min with 100 μg of NADase, —△—.

PGA concentration on reaction rate is shown in Figure 4. The apparent K_m value at 37°, determined according to Lineweaver and Burk (1934), is about 3×10^{-4} M.

Substrate Specificity. UDPGalA, pseudo-UDPGA, D-glucuronic acid 1-phosphate, or D-glucuronic acid were incubated with enzyme and the reaction mixtures were subjected to paper electrophoresis at pH 3.6 and 5.8. In all cases enzyme reaction mixtures and boiled enzyme controls were identical.

Inhibition. The following have no effect on enzyme activity: 0.01 M Zn^{2+}, Mg^{2+}, or 0.1 M EDTA. In addition, 0.001 M NAD, NADP, $NADH_2$, and $NADPH_2$ neither stimulate nor depress enzyme activity. Incubation with NADase or treatment with charcoal does not affect enzyme activity, nor does dialysis for 24 hr at 4° against a 2×10^3-fold volume of 5×10^{-3} M mercaptoethanol. Carbonyl reagents such as hydrazine, phenylhydrazine, or semicarbazide (all 4×10^{-2} M) do not influence enzyme activity or the nature of the product.

The enzyme is inhibited by a number of uridine compounds; the most effective is UDP, which inhibits 60% at concentrations 0.1 that of substrate. This is shown in Table II.

Bound NAD. Since UDPGA carboxy-lyase from *Cr. laurentii* has an absolute requirement for NAD (Ankel and Feingold, 1964), wheat germ enzyme was examined for bound nucleotide. Purified carboxy-lyase was dialyzed for 24 hr against 1500 times its volume of water, yielding 3.7 ml of enzyme solution containing 5.7 mg of protein/ml. This solution was held at 100° for 4 min and denatured protein was spun down and discarded. The supernatant fluid ("heat supernatant"), which had an absorbance maximum around 260 mμ at pH 1, 7, and 11, was examined for the presence of NAD. A 0.5-ml portion of the heat supernatant was taken to dryness at 30°, taken up in 20 μl of 0.1 M sodium acetate, pH 4.5, and hydrolyzed at 100° for 16 hr according to Butcher and Westheimer (1955).

TABLE II: Inhibition of UDPGA Carboxy-lyase by Uridine Compounds.[a]

Inhibitor	Concn (mM)	% Inhibition[b]
UMP	2	60
UDP	0.3	60
	2	85
UDPG	2	40
UDPAra	1	20
UDPXyl	0.2	20
	2	50

[a] The experimental conditions were the same as in the enzyme assay except that reaction mixtures contained the indicated concentrations of inhibitor. [b] The rate in the absence of inhibitor is taken as 100.

After deionization with mixed-bed ion-exchange resins the hydrolysate was taken to dryness. As a control, 6×10^{-3} μmole of NAD in 0.5 ml of solution was treated identically. The deionized hydrolysates were examined by gas–liquid partition chromatography of their trimethylsilyl derivatives (Sweeley *et al.*, 1963). Each contained a single carbohydrate component with a retention time identical with that of D-ribose which had been put through the same procedure. The curves for the heat-supernatant component and for authentic NAD are given in Figure 5.

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

Reaction Mixtures	UDPXyl (cpm)	UDPGA (cpm)	Total cpm	% of Control
Control (boiled enzyme)	6	601	607	100
No cofactors	478	134	612	101
Plus NAD	550	75	625	103
Plus $NADH_2$	577	102	679	112
Plus NAD and $NADH_2$	496	95	591	97

These data suggested that heat supernatant contained NAD. Accordingly, 30 μl of a mixture containing 30 μg of GAP dehydrogenase, 0.4 μmole of DL-glyceraldehyde phosphate, and 3 μmoles of sodium arsenate, pH 6.5, were added to 0.3 ml of heat supernatant, and absorbance change at 340 mμ was noted. In an identical experiment in which 0.3 ml of 5 μM authentic NAD was tested before and after heating at 100° for 4 min, 70% of the NAD survived the heat treatment. Calculation of the NAD content of heat supernatant from the absorbance value, corrected for loss during heating, yielded 9.5×10^{-7} mole of NAD/ml. The enzyme preparations therefore contained 1 mole of bound NAD/600,000 g of protein. Heat supernatant could also be used to activate the NAD-requiring UDPGA carboxy-lyase of *Cr. laurentii;* these results are presented in Figure 6. From the activities obtained, a value of 1 mole of NAD/760,000 g of wheat germ enzyme can be calculated, in reasonable agreement with the value found using GAP dehydrogenase.

Maxwell and deRobichon Szulmajster (1960) have been able to reactivate *p*-mercuribenzoate-(PCMB) inactivated UDPGal-4-epimerase from yeast with cysteine and NAD. Procedures described by these authors were employed in attempts to reactivate PCMB-treated UDPGA carboxylyase.

Purified enzyme (0.5 ml) was dialyzed overnight against 1 l. of 0.01 M phosphate buffer, pH 7.0, containing 0.5 g of EDTA/l. The dialyzed enzyme was diluted to 5 ml with the same buffer. The enzyme was inactivated by incubation of 50-μl samples with 10 μl of 2.5×10^{-4} M PCMB at 25° for 10 min. The following were then added to separate incubation mixtures: 10 μl of 0.2 M cysteine, 10 μl of 5 mM NAD, or 10 μl of each of the foregoing. After 10 more min at 37°, 10 μl of 3×10^{-2} M UDPGA was added to each reaction mixture and incubation was continued at 37° for 10 additional min. The reaction mixtures were then treated with 50 μl of glacial acetic acid and held at 100° for 15 min to hydrolyze any UDPXyl formed; the hydrolysates were assayed for xylose by the method of Roe and Rice (1948). No UDPXyl formation could be detected in any of the reaction mixtures, showing that the treatment with cysteine and NAD failed to reactivate the enzyme. In another experiment, PCMB-treated enzyme was held at 37° for 10 min, UDPGA and cysteine were added, the mixture was held at 37° for 10 min, NAD then was added, and incubation was continued for 10 additional min. This treatment likewise did not reactivate the carboxy-lyase. Substitution of 0.1 M mercaptoethanol for cysteine or 20 μl of heat supernatant for NAD in the above experiments also had no activating effect; neither did addition of 10 μl of 0.01 M $ZnSO_4$, $MgCl_2$, or $MnCl_2$ together with NAD after cysteine addition (EDTA-free enzyme was used in this set of experiments). As a control the procedure was performed without PCMB. Mixtures containing cysteine or cysteine plus NAD retained full activity; 50% of initial activity was recovered in the mixture which contained only NAD. Simultaneous addition of PCMB and cysteine to the enzyme prevented inactivation. Thus the quantity of cysteine used in the experiments described was sufficient to bind all of the PCMB present and protect the enzyme. Addition of cysteine 1 min after PCMB permitted recovery of at least 90% of the initial activity. Forty per cent of the initial activity could be recovered when cysteine was added 5 min after PCMB, and less than 5% when added 10 min after PCMB. Attempts to reactivate partially inactivated enzyme by the methods described were unsuccessful. The inactivation of UDPGA carboxy-lyase by PCMB seems to be a progressive, irreversible process, at least under the experimental conditions used. In this respect the carboxy-lyase differs from UDPGal-4-epimerase. However, a number of cases are known where cysteine or reduced glutathione does not reactivate PCMB-treated NAD-linked dehydrogenases, *i.e.*, malic dehydrogenase of heart (Wolfe and Neilands, 1956) and NAD cytochrome *c* reductase of muscle (Lehman and Nason, 1956).

Reaction Mechanism. The presence of tightly bound NAD in UDPGA carboxy-lyase from wheat germ and the ease of decarboxylation of β-keto acids suggested that uridine 5′-(α-D-*xylo*-hexopyranosyluronic acid-4-ulose pyrophosphate) (UDP-4-keto-GA) might be a transient reaction intermediate. This possibility was tested by determining whether UDPGA-4-*t* retained its label upon conversion to UDPXyl. In a typical experiment, 20 μl of UDPGA-4-*t* (650 cpm) was incubated with 12 μg of enzyme at pH 7.0 in a total volume of 30 μl for 30 min. Other reaction mixtures contained, in addition, NAD, $NADH_2$, or both (0.03 μmole each). The reaction was stopped by holding the mixture at 100° for 5 min. A control was run with boiled enzyme.

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

Reaction Mixtures	UDPXyl (cpm)	UDPGA (cpm)	Total cpm	% of Control
Control (boiled enzyme)	33	1605	1638	100
No cofactors	1585	150	1735	106
Plus NAD	1693	108	1801	110
Plus $NADH_2$	1638	140	1778	108
Plus NAD and $NADH_2$	1695	185	1880	115

Unlabeled UDPGA and UDPXyl, 0.05 μmole each, were then added to reaction mixtures and control as ultraviolet-absorbing markers; the nucleotides were separated by paper electrophoresis at pH 5.8. The UDPGA and UDPXyl were visually located, eluted, and made up to 1 ml final volume. This solution was added to 20 ml of scintillation solution and counted. Results are presented in Table III. As can be seen from the table, all of the label could be recovered in unreacted UDPGA and product UDPXyl. Similarly, UDPGA-3-*t* retained its label upon conversion to UDPXyl in an identical series of experiments. These results are presented in Table IV.

Discussion

Previous enzyme preparations from higher plants produced a mixture of UDPXyl and UDPAra from UDPGA (Feingold *et al.*, 1960, 1964). Kinetic studies with such preparations indicated that UDPXyl was the product of the decarboxylation and that the observed results were due to the presence of UDPAra-4-epimerase. We have now shown that UDPXyl is the only sugar nucleotide formed from UDPGA by the purified enzyme, thus confirming earlier work.

We have also confirmed previous observations that UDPGalA is not decarboxylated by UDPGA carboxy-lyase. UDPGalA is present in higher plants (Neufeld and Feingold, 1961), and its role as an immediate precursor of UDPAra frequently has been postulated. However, to date there has been no evidence to substantiate such a view.

UDPGA carboxy-lyase of *Cr. laurentii* has an absolute requirement for NAD and is inhibited by $NADH_2$ (Ankel and Feingold, 1964, 1965). Wheat germ enzyme, on the other hand, is neither activated by added NAD nor inhibited by $NADH_2$. UDPGal-4-epimerase isolated from mammalian liver is markedly stimulated by NAD and inhibited by $NADH_2$, but UDPGal-4-epimerase isolated from D-galactose-adapted *Saccharomyces fragilis* is not affected by exogenous NAD or $NADH_2$ (Maxwell, 1961). A striking similarity exists in the behavior of the 4-epimerases and the decarboxylyases toward NAD and $NADH_2$. The interconversion of UDPG and UDPGal is thought to involve oxido reduction at C-4 (Maxwell, 1961; Wilson and Hogness, 1964), although it has not yet been possible to demonstrate the existence of the postulated intermediate, uridine 5′-(α-D-*xylo*-hexopyranosyl pyrophosphate). The decarboxylation of UDPGA may involve a similar oxidized intermediate, UDP-4-keto-GA, which would readily decarboxylate. Subsequent stereospecific reduction of the decarboxylation product, uridine 5′-(β-L-*threo*-pentopyranosyl-4-ulose pyrophosphate) (UDP-4-keto-Xyl), would yield UDPXyl. If NAD and $NADH_2$ are intermediates in the reaction catalyzed by the wheat germ carboxy-lyase, they must be very tightly bound to the enzyme, resisting the action of NADase and charcoal. Tight binding would also explain the retention of tritium label at C-4 during the decarboxylation, since exchange of $NADt_2$ formed with free $NADH_2$ present in the reaction mixture would yield unlabeled UDPXyl. Retention of tritium label at C-3 is not necessarily inconsistent with a reaction mechanism involving a 4-keto compound. Gabriel and Ashwell (1964) have shown that thymidine diphosphate (TDP) glucose-3-*t* is converted to TDP-4-keto-6-deoxyglucose by an enzyme preparation from *Pseudomonas-aeruginosa* with complete loss of label; however, there is complete retention of label in the product when the reaction is catalyzed by an enzyme from *Xanthomonas campestris*.

The demonstration of NAD strongly bound to wheat germ UDPGA carboxy-lyase makes it seem likely that the dinucleotide is necessary for enzyme activity. However, this cannot be shown with certainty until it is possible to inactivate the enzyme by removal of NAD and to restore activity by its addition.

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Reaction of 4-Formyl-1-methylpyridinium Iodide Oxime with Isopropyl Methylphosphonofluoridate*

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ABSTRACT: The reaction of isopropyl methylphosphonofluoridate (GB) with 4-formyl-1-methylpyridinium iodide oxime (4-PAM), a model treatment compound for anticholinesterase poisoning, in near-neutral aqueous solution yields *O*-(isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime (4-PPAM). Its rate of formation is defined by the relationship $dx/dt = k[GB][4\text{-PAM anion}]$, where $k = 885 \pm 70$ M^{-1} min^{-1}, 30°, pH 7.6, 0.1 M KNO_3. *O*-(Isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime is quite stable in acidic and neutral aqueous solution. Its decomposition in alkaline solution is defined by the relationship $dx/dt = k[OH^-][4\text{-PPAM}]$, where $k = 86 \times 10^3$ M^{-1} min^{-1}, 30°, 0.1 M KCl.

O-(Isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime is a potent anticholinesterase. Its decomposition in near-neutral aqueous solution is speeded by imidazole, 4-formyl-1-methylpyridinium iodide oxime, bicarbonate, and two hydroxamic acids.

In the past two decades the class of organophosphorus compounds demonstrating anticholinesterase properties has become increasingly important. Among these we find a wide range of insecticides, certain medicinals, and the chemical compounds known as nerve agents. These compounds exhibit a spectrum of pharmacological effects; however, their principal actions closely resemble those of the neurohumor acetylcholine. This, together with their ability to rapidly and irreversibly inhibit the enzyme acetylcholinesterase, has suggested that their toxic action is a direct result of the inhibition of this enzyme. Inhibition of the enzyme has been shown to involve the actual phosphorylation of the enzyme, presumably at its active site. In general, the anticholinesterases are relatively resistant to aqueous hy-

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