BIOSYNTHESIS OF UDP-APIOSE AND APIIN

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ABSTRACT

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By

Ronald Ross Watson

A method was developed for synthesizing the product of the reaction catalyzed by UDPGA cyclase. The reaction product was synthesized from the substrate of the enzyme, UDP-glucuronic acid, using purified UDPGA cyclase isolated from Lemna minor. Catalytic quantities of NAD^+ were required for the synthesis of the reaction product. The reaction product was extensively characterized. It migrated on paper in four different solvents with an R_F of uridine 5'-(α-D-xylopyranosyl pyrophosphate), UDP-xylose, and had the same mobility as UDP-xylose when subjected to paper electrophoresis at pH 5.8. When [^{3}H]UDP-[U-^{14}C]glucuronic acid was used as the substrate, the ^3H:^{14}C ratio in the reaction product was that expected if D-apiose remained attached to the uridine. The reaction product yielded [^{3}H]uridine 5'-diphosphate ([^{3}H]UDP) and D-[U-^{14}C]-apiose when hydrolyzed at pH 2 and 100° for 15 minutes. When boiled at pH 8.0 for 5 minutes, it phosphorylated intramolecularly, yielding [^{3}H]uridine 5'-monophosphate ([^{3}H]UMP) and α-D-[U-^{14}C]apio-D-furanosyl cyclic 1:2-phosphate. The reaction product served as the [U-^{14}C]apiosyl donor in the enzymatic synthesis of apiin from 7-(4',5,7-trihydroxyflavonyl)O-β-D-glucopyranoside. The other product of this reaction was identified as [^{3}H]UDP. These results established that the reaction product is uridine 5'-(α-D-apio-D-furanosyl pyrophosphate), UDP-apiose. Through the

use of purified UDPGA cyclase UDP-apiose could be prepared in approximately 50% yield with a radiochemical purity of at least 60%. Stability studies showed that UDP-apiose was hydrolyzed to UMP and α-D-apio-D-furanosyl cyclic-1:2-P by exceedingly mild alkaline conditions. In a solution which contained 0.1 nmoles of UDP-[U-^{14}C]apiose approximately one-third of the UDP-[U-^{14}C]apiose was hydrolyzed in 2 hours at pH 8.0 and 25°. Stability studies showed that UDP-apiose was hydrolyzed by exceedingly mild acid conditions (pH 4 and 25°). UDP-Apiose was stored for months without degradation at pH 5-6 at -20° in a solution 50% ethanol by volume. UDP-xylose was not degraded by mild acid or alkaline conditions.

The purified duckweed UDPGA cyclase contained UDPGA decarboxylase which formed [^{3}H]UDP-[U-^{14}C]xylose from [^{3}H]UDP-[U-^{14}C]glucuronic acid and NAD^{+}.

D-Apiose was shown to be transglycosylated from UDP-apiose in a reaction catalyzed by apiin synthase. This enzyme catalyzed the transglycosylation of D-apiose from UDP-apiose forming apiin. Apiin synthase [UDP-apiose: 7-(4',5,7,-trihydroxyflavonyl) O-β-D-glucopyranoside, D-apiose transferase] was isolated from parsley and purified 45 fold. During purification it was separated from the parsley UDPGA cyclase. The substrates of the reaction, UDP-apiose and 7-(4',5,7-trihydroxyflavonyl) β-glucopyranoside were identified. Apiin and UDP were identified as the products of the reaction. The pH optimum for apiin synthase is 7.6-8.4. The rate of formation of apiin increased linearly with increasing amounts of apiin synthase or increasing incubation. The K_m for 7-(4',5,7-trihydroxyflavonyl) O-β-D-glucopyranoside is 7.0×10^{-5} M.

The K_m for UDP-apiose is 0.6 x 10^{-5} M. Apiin synthase occurs primarily in the leaves. None of 12 compounds tested which contained metal ions increased apiin synthase activity. Five compounds which chelate various metal ions were incubated with apiin synthase and none inhibited it. Sulfhydryl reagents inhibited apiin synthase when incubated with it at 1 mM final concentrations. All (100%) of the apiin synthase activity was lost when p-chloromercuribenzoate, 75% with iodoacetamide and less with oxidized glutathione and N-ethylmaleimide. Uridine, UMP, UDP, UTP, UDP-galactose, UDP-glucuronic acid and UDP-xylose inhibited apiin synthase to some extent, but UMP and UDP inhibited it most at the lower concentrations (1 x 10^{-6} and 1 x 10^{-5} M). Apiin synthase was also isolated from Digitalis purpurea (foxglove) as shown by the formation of a compound with the R_f of apiin from UDP-apiose and 7-(4',5,7-trihydroxyflavonyl) O-β-D-glucopyranoside. Enzyme isolated from foxglove was necessary for the formation of the compound with the R_f of apiin.

A method for isolating and purifying UDPGA cyclase from Petroselinin crispum (parsley) is described. It was purified 93 fold from parsley leaves. The pH optimum was 8.0-8.3 and the K_m was 0.33 x 10^{-5} M for UDP-glucuronic acid. Exogenous NAD^+ was required to synthesis any UDP-apiose. Optimum synthesis was obtained with 1-2 x 10^{-3} M NAD^+. UDPGA decarboxylase activity was found with purified UDPGA cyclase. The pH for optimum activity of parsley UDPGA decarboxylase activity was 8.0-8.2. UDP-xylose inhibits UDPGA decarboxylase more than UDPGA cyclase and at lower concentrations (1 x 10^{-6} M). Exogenous NAD^+ was not required to synthesize 65% of the amount of UDP-xylose formed with the optimum amount of NAD^+.

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By

Ronald Ross Watson

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VITA

Ronald Ross Watson was born in Texas in December of 1942. He was raised and educated in Moscow, Idaho. He graduated from Moscow High School in 1961. That same year he began his Sophomore year at the University of Idaho which was interrupted to preform service for the Church of Jesus Christ of Latter-day Saints in Chile. After two and one-half years in Chile he returned obtaining his B.S. in Chemistry from Brigham Young University on May 27, 1966. That significant day in May marked his marriage to Anita Ann Hebert and the start of their journey to the East for further study. After four and one-half years at Michigan State University they are continuing their trek eastward to Harvard University where Ronald will participate with Drs. G. Edsall and A. B. MacDonald in research.

" . . . . . There is in science quite a gap between belief and certainty. But would one ever have the patience to wait and establish the certainty if the inner conviction was not already there [Jaques Monod (75)] "Truth is the knowledge of things as they are, and as they were, and as they are to come." [Joseph Smith (74)]

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Finally, my most sincere thanks and love to my wife Anita for her continued support. My thanks for her understanding as she did without some of the material benefits of a working husband as well as being along way from home and family. I appreciate her wisdom in ordering the important phases of our family life, her love and her encouragement.

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PART 4.

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ABBREVIATIONS

UDP-glucuronic acid	uridine 5'-(α-D-glucopyranosyl-uronic acid pyrophosphate)
UDP-apiose	uridine 5'-(α-D-apio-D-furanosyl pyrophosphate)
UDP-galacturonic acid	uridine 5'-(α-D-galactopyranosyl-uronic acid pyrophosphate)
UDP-galactose	uridine 5'-(α-D-galactopyranosyl pyrophosphate)
UDP-xylose	uridine 5'-(α-D-xylopyranosyl)
UDP-pentose	uridine 5'-(pentosyl pyrophosphate)
UMP	uridine 5'-phosphate
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate
α-D-apio-D-furanosyl cyclic-1:2-P	α-D-apio-D-furanosyl cyclic 1:2-phosphate
7GA	7-(4',5,7-trihydroxyflavonyl) O-β-D-glucopyranoside
NAD^+	nicotinamide adenine dinucleotide
Tris-HCl	tris (hydroxymethyl) amino methane-HCl
Tris-acetate	tris (hydroxymethyl) amino methane-acetic acid
EDTA	ethylene diamine tetraacetic acid
dpm	disintegrations per minute

The following trivial names are used for enzymes which have been assigned systematic names: UDP-glucose pyrophosphorylase, UTP: α-D-glucose-1-phosphate uridyltransferase (EC 2.7.7.9); inorganic pyrophosphatase, pyrophosphate phosphohydrolase (EC 3.6.1.1) and UDP-glucose dehydrogenase, UDP-glucose: NAD^+ oxidoreductase (EC 1.1.1.22).

PART 1

CHARACTERIZATION AND BIOSYNTHESIS OF D-APIOSE AND OF COMPOUNDS CONTAINING D-APIOSE

Occurrence of D-Apiose in Nature. -- At the turn of the century the first branched-chain sugar was isolated (1). Its discoverer, Vongerichten, named the unusual sugar apiose (2,3). In the 55 years following the discovery of D-apiose few studies of the sugar were reported. For many years D-apiose was considered a rare sugar and a curiosity. During the last 15 years considerable work on the chemistry and biochemistry of D-apiose has appeared. The chemistry of this branched-chain sugar and a number of its synthetic derivatives has been investigated and reviewed elsewhere (4,5). Extensive characterization has shown D-apiose to be 3-C-hydroxymethyl-aldehydo-D-glycero-tetrose (4). The 4 carbon atoms of D-apiose constituting part of the furanose ring have been numbered 1 to 4 starting with the anomeric carbon. With D-apiose in the furanose ring the carbon atom of the free hydroxymethyl group is numbered 3' (Fig. 1).

Research published during the last 10 and particularly the last 5 years has shown that D-apiose occurs in a large number of different plants from many families. Duff (6) investigated the hydrolysates from 175 plants belonging to many families and found D-apiose in about 60% of the plants. Duff discerned no taxonomic pattern in the plants giving a negative test for the sugar. Therefore a random sample of these plants was taken and re-examined more carefully. All were found to contain traces of the sugar encouraging him to suggest that all of the plants which

originally did not give a positive test for D-apiose did in fact contain the sugar. Duff detected D-apiose by hydrolyzing plant material with sulfuric acid, neutralizing the hydrolysate and then chromatogramming the hydrolysate on paper. The sugars present were visualized by spraying the chromatogram with benzidine-trichloroacetic acid. Other investigators have found D-apiose in various plants. Van Beusekom (7), for example, did a study with 27 species of monocotyledons and found D-apiose in 10 of them. Appreciable amounts of D-apiose were found only in the hydrolysates of Lemnaceae, and species of Hydrocharitaceae, Potamogetonaceae and Zannichelliaceae which grow in the sea or in brackish water.

No report of the occurrence of D-apiose outside of the plant kingdom has yet appeared. A bacterial mutant has been isolated which metabolizes D-apiose. Neal and Kindel (8) isolated a mutant of Aerobacter aerogenes PRL-R_3 which used D-apiose as a sole source of carbon. A new enzyme was isolated, D-apiose reductase, which catalyzes the NAD^+-dependent reduction of D-apiose to D-apiitol.

Characterization of Compounds Containing D-Apiose. -- In Posidonia australis (70), Tilia sp. (9), Zostera marina (9,10,11), Lemna gibba (12), L. minor (6,12-16), Z. nana (6), Z. pacifica and Phyllospadix (11) D-apiose appears to be primarily a component of polysaccharides. In the case of Z. marina (10,11), T. sp. (9), L. minor (6,13-16), Z. pacifica and Phyllospadix (11) a polysaccharide fraction

or fractions were isolated and shown to contain D-apiose. Beck (14,15) reported the isolation of two apiogalacturonans from L. minor which contained 28% and 25% D-apiose. One of these polysaccharides was reported to also contain D-xylose and D-galactose. Hart and Kindel (16) isolated cell walls of L. minor that contained 83% of the total D-apiose present in the intact plant. The cell wall polysaccharides containing D-apiose that were solubilized all appeared to be of one general type, namely apiogalacturonans. Some of the polysaccharides were further purified and characterized. D-Apiose in these polysaccharides was found in a disaccharide, O-β-D-apio-(?)-furanosyl-(1→3')-D-apiose. The disaccharides were attached as side chains to the galacturonan (17).

D-Apiose has been isolated glycosidically linked to certain small molecular weight compounds. The small molecular weight types of compounds that have been isolated are isoflavone, phenolic, and flavone glucosides. The parsley plant, Petroselinum crispum, contains at least two D-apiose flavonoids, the principal one is apiin [7-(4',5,7-trihydroxyflavonyl) O-β-D-apio-D-furanosyl-(1→2)-β-D-glucopyranoside]. Apiin is also the principal flavonoid compound in the stems and leaves of this plant (18).

Recently the results obtained by several workers have led to the elucidation of the structure of apiin. After

methylation of apiin followed by hydrolysis, Hemming and Ollis (19) isolated a tri-O-methyl derivative of D-apiose and 3,4,6-tri-O-methyl-D-glucose. From these data, they concluded that the linkage between the two sugars was from carbon atom 1 of D-apiose to carbon atom 2 of the glucose moiety. Halyalker, Jones and Perry (20) in 1965 investigated the stereochemistry at carbon atoms 1 and 3 of the D-apiose moiety. They also methylated apiin and isolated the resulting tri-O-methyl-D-apiose. The compound was identical by paper, thin layer and gas-liquid chromatography, optical rotation, and infrared spectra, to chemically synthesized, 2,3,3'-tri-O-methyl-D-apio-D-furanose. This shows that D-apiose-D-furanose is the naturally occurring form, at least in parsley. Periodate oxidation data also indicated that the hydroxyl groups at carbon atoms 2 and 3 were in the cis configuration. From a knowledge of the molecular rotation of 7GA[1] and apiin and by application of the molecular rotation theory of Klyne (21) these workers concluded that the stereochemistry of the linkage between carbon atom 1 of D-apiose and carbon atom 2 of D-glucose was β. Therefore, apiin is 7-(4',5,7-trihydroxyflavonyl) O-β-D-apio-D-furanosyl-(1→2)-β-D-glucopyranoside.

Besides apiin there is two and perhaps more flavonoid glycosides in parsley which contain D-apiose.

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[1]Abbreviations are listed on page xiii.

TABLE I: Small molecular weight compounds containing D-apiose

Compound Containing D-Apiose	Aglycone (Trivial Name)	Aglycone* ("Systematic" Name)	Plant Source
Apiin	Apigenin	4',5,7-Trihydroxy-flavone	P. crispum, A. graveolens, Chrysanthemum leucanthemum, C. uliginosum, C. maximum, Cuminum cyminum, Bellis perennis, Anthemis nobilis, C. frutescens, Matricaria chamomilla, M. inodora, Centaurea scabiosa, C. cyanus, Serratula coronata, Eckinops gmelini, and Vicia hirsuta
Petroselinin (existence not conclusively established)	Desmotin	4'-Methoxy-3',5,7-trihydroxyflavone	P. crispum (4)
"Glucoapiosylapigenin"**	Apigenin	4',5,7-Trihydroxy-flavone	Digitalis purpurea
Graveolbioside A	Luteolin	3',4',5,7-Tetra-hydroxyflavone	Parsley (18) and Apium graveolens
Graveolbioside B	Chrysoeriol	3'-Methoxy-4',5,7-trihydroxyflavone	A. graveolens and P. hortense
Lanceolarin	Biochanin-A	4'-Methoxy-5,7' dihydroxyisoflavone	Dalbergia lanceolaria
Furcatin	p-Vinylphenol	p-Vinylphenol	Viburnum furcatum

*Flavone = 2-phenylbenzopyrone; Isoflavone = 3-phenylbenzopyrone

**Characterized only by acid hydrolysis and subsequent identification of the sugar and flavone components by paper chromatography. The parent compound was not characterized.

Vongerichten (1,2,86) obtained from the leaves of P. crispum what he believed was a crude mixture of two glycosides. This was shown by hydrolyzing the mixture and obtaining two aglycones which were partially characterized as apigenin (4',5,7-trihydroxyflavone) and desmotin (3',5,7-trihydroxy-4'-methoxyflavone)(4). Later Gupta and Seshadri (45) isolated crystalline apiin from P. crispum, moss-curled variety, and obtained only one glycoside containing D-apiose. The D-apiose containing compound was well characterized and found to be apiin. It was not characterized by paper chromatography. They found that some of the apiin isolated had a melting point 10° lower than authentic apiin and was otherwise identical in every other aspect tested. Shortly thereafter Nordstrom, Swain and Hamblin (18) isolated flavonoid glycosides containing D-apiose from parsley. They extensively characterized the two glycosides they obtained after separating them from each other by paper chromatography. They identified the aglycone portions by their R_F values, color reactions, u.v. spectra in alcohol and other tests as apigenin and luteolin (3',4',5,7-tetrahydroxyflavone). They also extensively characterized the glycosides before and after hydrolysis as apiin and the apiosyl glucoside of luteolin. In 1966 Grisebach and Bilhuber (60) partially characterized the two glycosides they isolated from P. hortense Hoffm. (parsley). The major component was identified as apiin and the minor component was identified from its hydrolytic and spectral properties

as the apiosyl glucoside of chrysoeriol. The apparent discrepancy between these last two studies may be that the flavonoids were isolated from different species of parsley. Nordstrom and Swain and Hamblin (18) did not identify the specie of parsley they tested although they most probably used P. crispum as they were reinvestigating the work of Vongerichten (1,2,86) who investigated the flavonoid glycosides from P. crispum. Therefore the flavone glycosides containing D-apiose in P. crispum are apiin (18,45) and small amounts of apiosyl glucoside of luteolin (18). The flavone glycosides containing D-apiose in P. hortense are primarily apiin and some apiosyl glucoside of chrysoeriol (60).

Recently, D-apiose was found in glycosidic combination with a flavone in Digitalis purpurea (foxglove)(23). This compound had all the components of apiin i.e. D-apiose, D-glucose and apigenin but it was not further characterized. Apiin has also been isolated from the white flowers of Chrysanthemum uliginosum, C. leucanthemum, C. maximum, Bellis perennis (English daisy), and Anthemis nobilis by Wagner and Kirmayer (24). The yield of apiin was 1-3%. Apiin was mixed with authentic apiin isolated from P. crispum and then identified by its mixed melting point. The isolated apiin and its acid hydrolysis products were also identified by paper chromatography. Apiin was isolated from the flowers of C. frutescens, Matricaria chamomilla, M. inodora, Centaurea scabiosa, C. cyanus,

Serratula coronata, and Eckinops gmelini. The apiin isolated from these last seven plants was identified only by paper chromatography in one solvent. Apiin has been isolated from Vicia hirsuta (vetch) by Nakaoki, Morita, Motosume, Hiraki and Takeuchi (25). Some workers in India isolated apiin from an ethanol extract of one of five species tested, Cuminum cyminum (78).

In addition to the parsley flavonoids, and the plants containing apiin, D-apiose-containing flavonoid glucosides have been found in Apium graveolens (celery) (22,76,79). Farooq, Gupta, Kiamuddin, Rahman and Seshadri (22) isolated from celery the two principal glucosides containing D-apiose, graveolbioside A and B. Both have the structure of apiosyl-glucosyl-7-aglycone. The aglycone portion of graveolbioside A is luteolin and that of graveolbioside B is chrysoeriol, the 3' methyl ester of luteolin (See Table I for structures).

Malhotra, Marti and Seshadri (26) reported the occurrence of an apiosyl glucoside of biochanin-A, lanceolarin, in the root-bark of the tree Dalbergia lanceolaria. Biochaninin-A is an isoflavone.

D-Apiose has been identified glycosidically linked to D-glucose which in turn is glycosidically linked to a non-flavonoid aglycone. Hattori and Imaseki (27) isolated this compound from Viburnum furcatum Blume. They showed that the new compound, furcatin, was p-vinylphenyl O-(?)-D-apio-(?)-furanosyl-(1→6)-β-D-glucopyranoside. Imaseki and Yamamoto (28) later isolated a glycosidase from

V. furcatum which hydrolyzes furcatin into the aglycone and O-(?)-D-apio-(?)-furanosyl-(1→6)-β-D-glucose. The addition of alcohols to the mixture containing the glycosidase and furcatin allowed the glycosidase to transfer the apiosyl-glucose residue from furcatin to the alcohols, thereby forming alkyl O-D-apio-furanosyl-(1→6)-glucosides, instead of transferring the residue to water.

In vivo Biosynthesis of D-Apiose.-- D-Apiose biosynthesis has been investigated only in P. crispum, L. gibba G_3, and L. minor. Enzymes isolated from these species were used for the biosynthetic studies to be discussed in this thesis. Enzymes isolated from several other plants have been found to convert UDP-glucuronic acid into UDP-apiose as measured by the formation of α-D-apio-D-furanosyl cyclic-1:2-P after intramolecular phosphorylation (Part 2) and by the release of D-apiose after acid hydrolysis of the incubation mixture.[2] These plants are Medicago sativa L. (alfalfa), Trifolium repens L. (white clover) and Trifolium protense L. (red clover).[2]

The biosynthetic studies to date support the principal portions of a hypothesis proposed for the synthesis of D-apiose and apiin by Grisebach and Dobereiner (30). They postulated that D-apiose was formed as part of a sugar nucleotide by a decarboxylation reaction which used a nucleoside diphosphate glucuronic acid as the substrate. They further postulated that a rearrangement reaction must

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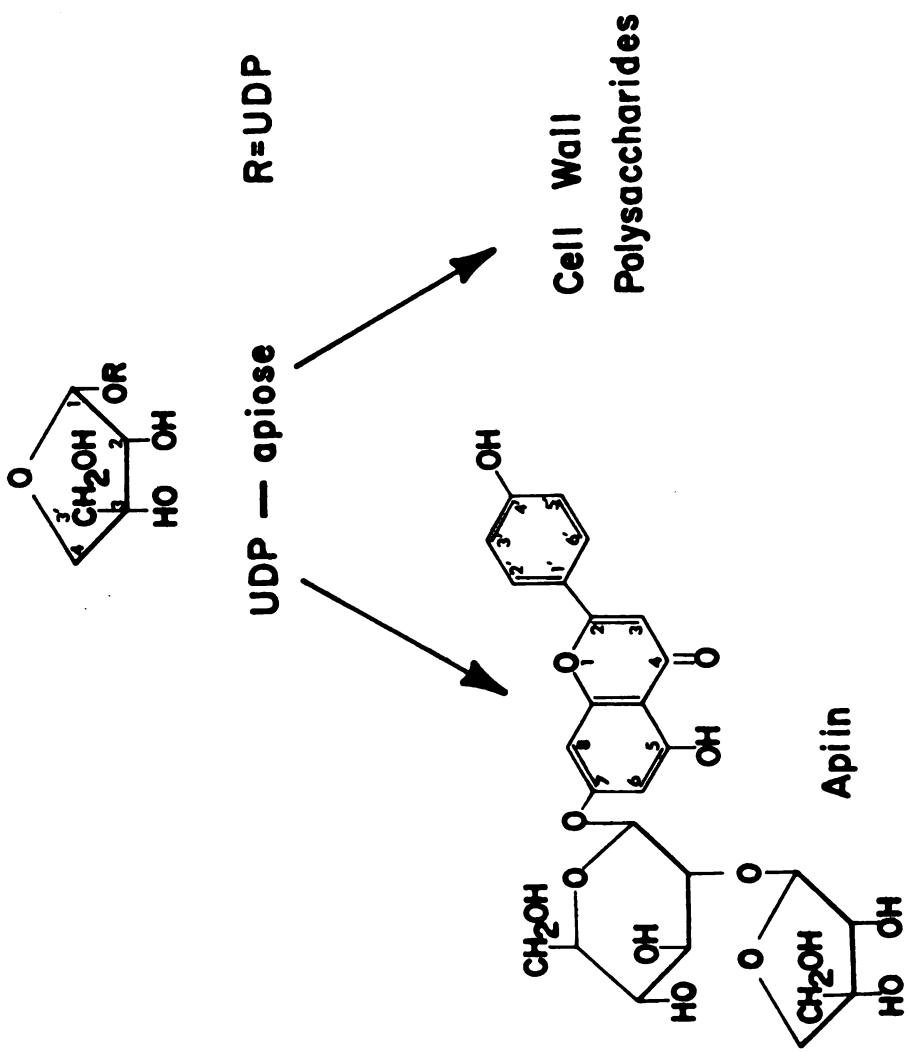
[2]Gustine, D. L., Watson, R. R. and Kindel, P. K., manuscript in preparation.

Fig. 1. Transglycosylation of D-apiose from UDP-apiose. D-Apiose is transglycosylated to 7GA, an acceptor molecule, to form apiin. This is shown by the data presented in Part 2 and 4. A pathway for the formation of glycosidically linked D-apiose in polysaccharides from UDP-apiose has been proposed by Roberts, Shah and Loewus (35). It is shown in this figure.


R=UDP
UDP — apiose
Cell Wall
Polysaccharides
Apiin

also occur in order to form the branched-chain D-apiose from this intermediate. This latter suggestion is based upon a hypothesis earlier presented by Baddiley, Blumson, Di Girolano and Di Girolano (37) for the rearrangement of an intermediate in the biosynthesis of L-rhamnose to form streptose, another branch-chain sugar. The NDP-apiose formed would be involved in the synthesis of polysaccharides and glycosides such as spiin. Grisebach's hypothesis was initially tested by carrying out isotope incorporation experiments with parsley and duckweed. The experiments of Grisebach as well as others, including those reported in this thesis, have confirmed this hypothesis.

Grisebach and Dobereiner (30,31) gave radioactive precursors to young parsley shoots. [^{14}C]Formate was administered to the stems and although the percentage of radioactivity in D-apiose was high, the nonspecific incorporation of [^{14}C]formate into D-glucose showed that [^{14}C]formate was not functioning as a one carbon donor and that randomization of ^{14}C had occurred. These above results and additional experiments with [1-^{14}C]acetate suggested that one and two carbon units do not participate directly in the biosynthesis of D-apiose. The specific activities of D-apiose and of D-glucose from apiin were about equal when either D-[U-^{14}C]glucose or D-[3,4-^{14}C]glucose were precursors (30). Almost half of the radioactivity from D-[3,4-^{14}C]glucose was found in carbon atoms 3 and 3' of D-apiose, thus carbon atom 3' comes from either carbon atom 3 or 4 of D-glucose. This

observation suggested that D-apiose does not occur in a free (aldehyde) form during the biosynthesis of apiin. If it did, randomization of ^{14}C activity between carbon atoms 3' and 4 of D-apiose would be expected to occur. To further test his hypothesis, Grisebach fed D-[6-^{14}C,4-^{3}H]glucose to parsley shoots. About 70% of the tritium was located at carbon atom 3' of D-apiose. This result could be explained by the assumption that hydrogen is removed from carbon atom 4 of UDP-glucuronic acid by coenzyme-enzyme complex and that the same coenzyme-enzyme is used for the reduction of the transient carbonium ion Grisebach postulates is formed during the rearrangement step.

Additional support for his own hypothesis was obtained by Grisebach and Sandermann in experiments with D-[U-^{14}C] glucuronic acid (32). The results showed a very large incorporation of ^{14}C from D-glucuronic acid into the D-apiose (92%) and very little incorporation into the D-glucose (5%) and the apigenin (3%) of apiin. In contrast, with D-[6-^{14}C]glucuronic acid as precursor, the incorporation into D-apiose is much lower. This is to be expected since **carbon atom six was postulated to be lost as [^{14}C]CO_2. This would be true even if refixation of [^{14}C]CO_2 by photosynthesis occurred.**

Mendicino and Picken (13,33) independently studied the biosynthesis of D-apiose in P. crispum and in L. minor. They compared the relative extent of incorporation of various precursors into D-apiose. On the basis of their findings that [2-^{14}C]acetate, [$^{14}CH_3$]methionine and

[3-^{14}C]serine were incorporated into D-apiose, Mendicino and Picken suggested that the transfer of a one carbon group might be involved in D-apiose biosynthesis. In these experiments there was incorporation of ^{14}C into D-glucose. The potential carbon atom 1 donor compounds were metabolized and that ^{14}C had randomized in this experiment. This cast doubt upon the conclusion that one carbon units participate in the biosynthesis of D-apiose. If they did participate the incorporation of ^{14}C from one carbon unit precursors into the branched-chain sugar should have occurred with little incorporation into other sugars. The work of Grisebach, reviewed above, showed that a one carbon unit is not important in the biosynthesis of D-apiose as does the work reported in this thesis. Mendicino and Picken also found that carbon atoms 1 through 5 of D-glucose were converted into D-apiose.

In studies with whole parsley plants in which various ^{14}C-containing compounds were metabolized, Mendicino and Picken found that the specific activity of the apiin isolated was ten times higher in the roots than the leaves. Since the greatest concentration of apiin is in the leaves they suggested that it was synthesized in the roots and transported to the leaves. In contrast to these findings and conclusions, Grisebach (34) and Grisebach and Dobereiner (31) have reported that little if any difference existed in the incorporation of D-glucose into D-apiose of apiin in parsley with or without roots. Mendicino and Picken (13) also

found that free D-apiose was not utilized by the parsley plant. When D-[U-^{14}C]apiose was administered to the plant negligible incorporation into apiin was observed.

Roberts, Shah and Loewus (35) studied D-apiose biosynthesis in P. crispum and L. gibba G_3 with isotopic incorporation experiments utilizing myo-inositol. Myo-inositol is an effective precursor of D-glucuronic acid in higher plants and is rapidly and specifically utilized in the biosynthesis of uronic acid residues (71,72). [2-^{14}C]Myo-inositol is oxidized in plant to D-[5-^{14}C]glucuronic acid. Roberts, Shah and Loewus (35) observed that [2-^{14}C]myo-inositol functions in the biosynthesis of D-apiose- and D-xylose-containing polysaccharides of L. gibba G_3. They also found that leaves of P. crispum utilized [2-^{3}H]myo-inositol for the biosynthesis of D-apiose of apiin. Apiin isolated from P. crispum after the isotope incorporation study contained ^{3}H only in the D-apiose moiety. Periodate oxidation of this D-apiose showed that 94% of the ^{3}H in D-apiose was located in carbon atoms 3' and 4. Experiments by other workers discussed above showed that radioactivity located at carbon atoms 3 or 4 of D-glucose becomes carbon atoms 3 or 3' of D-apiose. The experiments of Roberts, Shah and Loewus can then be interpreted as showing that carbon atom 4 of D-apiose obtained from [2-^{3}H]myo-inositol is equivalent to D-[5-^{3}H]glucuronic acid. The results they obtained independently provided direct evidence for a pathway of D-apiose biosynthesis involving D-glucuronic acid metabolism.

When L-[1-^{14}C]arabinose was supplied to L. gibba G_3, both L-arabinosyl and D-xylosyl units of cell wall polysaccharides contained ^{14}C, but no ^{14}C was found in D-apiose (35). Some plants contain an epimerase which can convert L-arabinose to D-xylose (UDP-L-arabinose ⇄ UDP-D-xylose). The work of Roberts, Shah and Loewus showed that there was no mechanism for converting D-xylose or L-arabinose to D-apiose in L. gibba G_3.

The in vivo biosynthetic studies of D-apiose in L. minor by Beck and Kandler (36) confirm those already described above. They studied the patterns of ^{14}C incorporation into D-glucose, D-xylose and D-apiose obtained after administering D-[U-^{14}C]glucose, D-[1-^{14}C]glucose, D-[2-^{14}C]glucose and D-[6-^{14}C]glucose. They found very significant incorporation of ^{14}C into the three sugars studied. The ^{14}C of [$^{14}CH_3$]methionine, however, was incorporated only slightly into these sugars. Furthermore, D-apiose did not contain more ^{14}C than D-xylose as would have been expected if the addition of a one carbon unit was an important step in the biosynthesis of D-apiose but not in D-xylose biosynthesis. D-Xylose has been shown to be synthesized from UDP-glucuronic acid in plants by a decarboxylation reaction (34,71,77). Beck and Kandler (12) isolated D-apiose, D-xylose and D-glucose from L. minor polysaccharides and determined the distribution of radioactivity in each carbon atom. The results showed that both D-apiose and D-xylose were derived from D-glucose. They (12) also

found that during photosynthesis $^{14}CO_2$ was incorporated into D-apiose and D-xylose. The incorporation of $^{14}CO_2$ into D-xylose and D-apiose during the first two hours was very similar and yet different from other sugars synthesized. He therefore suggested that the biosynthesis of D-apiose and D-xylose was part of the same biosynthetic pathway.

Very recently in vitro studies of the biosynthesis of D-apiose, UDP-apiose and apiin have been reported. This literature will be reviewed and cited as necessary in the rest of the dissertation.

PART 2

BIOSYNTHESIS AND CHARACTERIZATION OF UDP-APIOSE

INTRODUCTION

Cell-free systems from Lemna minor (duckweed) and Petroselinin crispum (parsley) have been described which carry out the biosynthesis of D-apiose (3-C-hydroxymethyl-aldehydo-D-glycero-tetrose) from UDP-glucuronic acid (38, 39). Although not fully characterized the product of the reaction was reported to be UDP-apiose (38, 40). UDP-apiose was not found when assay mixtures were incubated, subjected to a brief heat treatment and chromatographed under slightly alkaline conditions (39). Under these conditions a single compound containing D-apiose was formed which proved to be neither UDP-apiose, a D-apiose phosphate monoester nor free D-apiose. This compound has been identified as α-D-apio-D-furanosyl cyclic-1:2-P and has been shown to be formed non-enzymatically from UDP-apiose[1] under exceedingly mild conditions (42). The tentative identification of this compound has recently been reported by Sandermann and Grisebach (40).

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[1]Although not established, the configuration at carbon atom 3 of the D-apiose moiety in UDP-apiose and in α-D-apio-D-furanosyl cyclic-1:2-P is assumed to be D as in apiin (20). The configuration at carbon atom 1 of α-D-apio-D-furanosyl cyclic-1:2-P must be α in order for this sugar cyclic-1:2-phosphate to exist. This was shown clearly by using Framework Molecular Models of the above compounds made from components manufactured to scale.

Apparently a single enzyme catalyzes the conversion of UDP-glucuronic acid to UDP-apiose and CO_2[2]. This enzyme has been purified about 55-fold from L. minor and has been given the common name UDPGA cyclase[2]. Only through the use of purified UDPGA cyclase has it been possible to prepare UDP-apiose of sufficiently high radiochemical purity to permit complete characterization of this compound. A preliminary account of this work has been presented (42).

The purified UGPGA cyclase contains UDPGA decarboxylase. This will be indicated by characterization of one of the two sugar nucleotides formed from UDP-glucuronic acid, UDP-xylose.

In this Part, two things will be described: 1) a method for the enzymatic synthesis of UDP-apiose from UDP-glucuronic acid and 2) an extensive characterization of one of the products of the UDPGA cyclase reaction and its identification as UDP-apiose. The other product presumably is CO_2.

MATERIALS AND METHODS

Materials. -- UDP-glucuronic acid, UDP-xylose, UMP, UDP, UTP, and NAD^+ were obtained from P-L Biochemicals, Inc. D-Apiose was isolated as described elsewhere (8). Framework Molecular Models were manufactured by Prentice-Hall, Inc. UDP[U-^{14}C]glucuronic acid (233 mCi/mmole) and the dipotassium salt of α-D-[U-^{14}C]glucose-1-phosphate (194 mCi/mmole) were

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[2]Gustine, D. L., Watson, R. R. and Kindel, P. K., manuscript in preparation

obtained from New England Nuclear Corp. The tetralithium salt of [5-^{3}H]UTP (12.1 Ci/mmole) was purchased from Schwarz/Mann. Crystalline calf liver UDP-glucose pyrophosphorylase (93 units/mg protein) was a gift from Dr. J. A. Boezi. One unit of enzyme will form 1 µmole of α-D-glucose 1-phosphate (NADPH) per minute at 25° and pH 7.8 (61). Crystalline yeast inorganic pyrophosphatase, Type II (approx. 250 units/mg protein) was purchased from Sigma Chemical Co. One unit of enzyme will liberate 1 µmole of inorganic orthophosphate per minute at 25° and pH 7.2. Partial purified calf liver UDPG dehydrogenase was a gift from Dr. R. G. Hansen. One unit of enzyme will oxidize 1 µmole of UDP-glucose per minute at 25° and pH 8.7. Purified Escherichia coli alkaline phosphatase, Code BAPC (35 units/mg protein) was purchased from Worthington Biochemical Corp. One unit of enzyme will liberate 1 µmole of p-nitrophenol per minute at 25°. "7GA" was isolated from crystalline "apiin" by acid hydrolysis, collected by suction filtration, washed thoroughly with distilled water and recrystallized from 95% aqueous ethanol. Crystalline "apiin" was isolated from parsley seeds by the method of Gupta and Seshadri (45). Although crystalline, it was a mixture, hence the quotation marks (8).

General Methods. -- Electrophoresis of UDP-apiose, UDP-xylose, D-apiose and α-D-apio-D-furanosyl cyclic-1:2-P was carried out on unwashed Whatman No. 3MM paper at 4°. The buffer employed was 0.2 M ammonium acetate, pH 5.8. Electrophoresis was performed at 30 volts per cm with a

Pherograph Original Frankfurt, Type 64 (distributed by Brinkmann Instruments, Inc., Westbury, N.Y.). Radioactivity was detected on chromatograms with a Packard radiochromatogram scanner, Model 7201 (Packard Instrument Co.). All other radioactivity measurements were made with a Packard Tri-Carb liquid scintillation counter, Model 3310, employing either (A) a scintillation solution made as described by Bray (84) or (B) 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene in reagent grade toluene (4 g/l.). When using solution B the radioactive compounds were counted directly on paper by cutting out the appropriate portions of the chromatogram and completely immersing them in this solution. The counting efficiencies with solutions A and B were 79 and 60%, respectively. Duckweed was cultured as described by Kindel and Watson[3].

Paper Chromatography. -- Paper chromatography was by the descending technique and was carried out with washed Whatman No. 3MM paper at 4° unless otherwise stated. The paper was washed with 0.1 M citric acid followed by distilled water. Unwashed Whatman No. 3MM paper impregnated with 2.5% neutralized polyethylenimine was used with solvent F (46). The following solvents were employed: (A) acetone-ethanol-2-propanol-H_2O (3:1:1:2, v/v); (B) 1-propanol-ethyl acetate-H_2O (7:1:2, v/v); (C) acetone-ethanol-2-propanol-0.05 M borate buffer, pH 8.6, (3:1:1:2, v/v) (47); (D) 75% aqueous

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[3]Kindel, P. K. and Watson, R. R., manuscript in preparation

ethanol; (E) ethyl acetate-H_2O-acetic acid-formic acid (18:4:3:1, v/v); (F) 0.3 M LiCl; (G) 95% aqueous ethanol-1.0 M ammonium acetate, pH 7.5, (7:3, v/v), (H) 1-butanol-acetic acid-H_2O (4:1:5, v/v, upper phase); (I) ethanol-methyl ethyl ketone-0.5 M morpholinium tetraborate, pH 8.6, in 0.01 M EDTA (70:20:30, v/v)(90).

Preparation and Assay of UDPGA Cyclase and Apiin Synthase. -- UDPGA cyclase was purified from duckweed. The purification and assay procedures were modifications[2] of those described by Gustine (41). One unit of enzyme has been defined as that amount which will form 1 µmole of UDP-apiose per minute under the conditions of the standard assay. Apiin synthase was purified from parsley leaves and assayed by procedures described in Part 3. One unit of enzyme has been defined as that amount which will form 1 µmole of apiin per minute under the conditions of the assay.

Preparation of [^{3}H]UDP-[U-^{14}C]glucuronic Acid. -- [^{3}H]UDP-[U-^{14}C]glucose was prepared from [^{3}H]UTP and α-D-[U-^{14}C]glucose 1-phosphate by a modification of the procedure of Fitzgerald and Ebner (51) and was converted to [^{3}H]UDP-[U-^{14}C]glucuronic acid by the procedure of Castanera and Hassid (52). The incubation mixture contained 1.4 nmoles of [^{3}H]UTP (16.9 µCi), 10.3 nmoles of α-D-[U-^{14}C]glucose-1-phosphate (2.0 µCi), 7 nmoles of UTP, 1.8 µmoles of magnesium

acetate, 25 μmoles of glycine-NaOH buffer, pH 8.7, 5.4 units of UDP-glucose pyrophosphorylase and 1.2 units of inorganic pyrophosphatase in a volume of 0.48 ml. The mixture was incubated 60 minutes at 25° and then 3 nmoles of UTP and 2.7 units of UDPG pyrophorylase were added making the final volume 0.5 ml. The reaction was incubated an additional 30 minutes at 25° and then heated for 1 minute at 100°. To the cooled solution were added 0.4 μmole of NAD^+ and 0.05 units of UDP-glucose dehydrogenase. After incubating for 1 hour at 25° the mixture was heated for 1 minute at 100°. To establish that [^{3}H]UDP-[U-^{14}C]glucuronic acid had been formed and to determine its purity, small portions of the preparation were examined by paper chromatography with solvents B, C, F and G. Only [^{3}H]UDP-[U-^{14}C]glucuronic acid and a small amount of α-D-[U-^{14}C]glucose were observed. The latter compound had been present in the commercial α-D-[U-^{14}C]glucose 1-phosphate. It does not contaminate the final product, [^{3}H]UDP-[U-^{14}C]apiose, since it is separated from the UDP-pentoses during paper chromatography in solvent A. None of the radioactive substrates remained since all the [^{3}H]UTP had been converted to product when it was the limiting substrate and when the subsequent addition of UTP made α-D-[U-^{14}C]glucose-1-phosphate limiting, this radioactive sugar phosphate was all converted to product. [^{3}H]UDP-[U-^{14}C]glucuronic acid was converted to [^{3}H]UDP-[U-^{14}C]apiose as described in the Results.

RESULTS

Identification of the Product of the Reaction Catalyzed by UDPGA Cyclase. -- The assay procedure which has been used to detect UDPGA cyclase involved incubating the enzyme with UDP-[U-^{14}C]glucuronic acid and NAD^+ in sodium phosphate buffer, pH 8.0, for 5 minutes at 25°, heating the assay mixture briefly at 100° and then chromatographing the mixture in solvent G (39). With this procedure Gustine and Kindel (39) found that all the D-[U-^{14}C]apiose that was synthesized by duckweed UDPGA cyclase was found in a compound they called Compound III. They showed that Compound III was neither UDP-[U-^{14}C]apiose, a D-[U-^{14}C]apiose phosphate monoester nor free D-[U-^{14}C]apiose. Later Kindel[3] showed that if the assay mixture was not heated but immediately cooled to 4°, spotted and chromatographed in solvent G at this same temperature, little Compound III was obtained. Instead a D-[U-^{14}C]apiose-containing compound with an R_F of UDP-xylose was obtained. He also showed that this new compound was converted to Compound III when heated at 100° in pH 8.0 buffer. These results showed that the true product of the reaction was not Compound III but was a compound which migrated with an R_F of UDP-xylose. He also showed that the reaction product could be converted to Compound III. Similar results were obtained with enzyme isolated from parsley and are described in Part 4. Compound III was subsequently identified as α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P[3].

I had previously observed that parsley UDPGA cyclase was necessary to synthesize apiin. Presumably it formed an unidentified D-apiose donor molecule from UDP-glucuronic acid. The reaction synthesizing apiin also required the presence of "7GA" and was catalyzed by parsley apiin synthase. I believed that this D-apiose donor molecule could be UDP-apiose which had been postulated to function as a D-apiose donor molecule in this glycosidic reaction (34).

A method for preparing the D-[U-^{14}C]apiose containing reaction product enzymatically from UDP-[U-^{14}C]glucuronic acid was developed and is described.

An incubation mixture containing 11 nmoles of UDP-[U-^{14}C]glucuronic acid (5,104,000 dpm) 0.4 µmole of NAD+ 10 µmoles of sodium phosphate buffer, pH 8.0, 20 nmole of EDTA, and 14.5 milliunits of duckweed UDPGA cyclase (2.4 mg) purified through the DEAE-Sephadex step (41) in a total volume of 0.4 ml was prepared. The mixture was incubated 15 minutes at 25° and then cooled to 4°. The remaining steps were carried out at 4°. The incubation mixture was streaked on washed Whatman No. 3MM paper over 5 cm and the paper was developed in solvent A for 16 hours. Without having been dried, the chromatogram was cut and that portion with an R_F of UDP-xylose was eluted with 3.2 ml of solvent A. This solution had a pH of approximately 6.0 and contained the reaction product. It was concentrated in 0.5 hour to 1.0% of the starting volume by rotary evaporation under reduced pressure and at 25°. From

radioactivity measurements, the yield of reaction product plus UDP-[U-^{14}C]xylose in this particular preparation was 79%. Paper chromatography of aliquots of the solution containing the reaction product in solvents B and C at 4° together with an examination of the original chromatogram showed that 10% of the starting radioactivity was present in α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P (Compound III) of which 3% had separated from the UDP-[U-^{14}C]apiose during chromatography in solvent A and 7% was formed during elution of the reaction product. During elution of the reaction product D-[U-^{14}C]apiose was formed to the extent of 5% from UDP-[U-^{14}C]apiose. No UDP-[U-^{14}C]glucuronic acid was detected. Where appropriate, percent figures are corrected to take into account the loss of carbon atom 6 of glucuronic acid as [^{14}C]CO_2. No attempt was made to account for the remainder of the starting radioactivity. Chromatography in solvent A separated the reaction product from NAD^+. An aliquot of the solution containing the reaction product was made 0.05 M with respect to sodium phosphate buffer, pH 8.0, heated for 4 minutes at 100° and the hydrolysis products chromatographed on paper in solvent G. Measuring the amount of α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P formed showed that 54% of the radioactivity was in the reaction product and 34% was in UDP-[U-^{14}C]xylose. The remainder was in the compounds mentioned above. An alternative procedure for determining the amount of reaction product present was to adjust an aliquot of the solution to

pH 2 with HCl, heat the solution for 15 minutes at 100°, chromatograph the hydrolysis products on paper in solvent E and then measure the ^{14}C content of D-[U-^{14}C]apiose and D-[U-^{14}C]xylose formed. Both procedures gave comparable results. The reaction product was stored at -20° in 50% aqueous ethanol. Reaction product prepared in this way was used for the characterization experiments. In this paper reaction product refers to the product of the reaction catalyzed by UDPGA cyclase, i.e., UDP-apiose. The UDP-[U-^{14}C]xylose present in the reaction product is formed in the reaction catalyzed by UDPGA decarboxylase. Reaction product prepared in this manner was used in all of the following experiments. Rechromatography of the reaction product in solvent B proceeded with less than 2% degradation of the reaction product. After chromatography in solvent B at 4° for 24 hours UDP-[U-^{14}C]apiose was eluted with 0.001 M sodium phosphate, pH 6.0 which was 50% ethanol by volume. This step removed the UMP, α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and D-[U-^{14}C]apiose which contaminates the reaction product and UDP-[U-^{14}C]xylose after chromatography in solvent A as described above. Elution with sodium phosphate buffer prevented the degradation of the reaction product to D-apiose and D-apio-D-furanosyl cyclic-1:2-P. Degradation often occurred when the reaction product was eluted with solvent A.

The reaction product and UDP-[U-^{14}C]xylose obtained migrated on paper at 4° as a single peak in solvents A-D. In each solvent they had the same R_F as UDP-xylose. When

subjected to paper electrophoresis at pH 5.8, the mobility of the reaction product was the same as UDP-xylose. An essentially identical preparation of the reaction product as that described above was carried out except that [^{3}H]UDP-[U-^{14}C]glucuronic acid was used. The reaction product was isolated and its ^{3}H:^{14}C ratio was that expected if the D-[U-^{14}C]apiose and D-[U-^{14}C]xylose each remained attached to the uridine and one carbon atom of the D-[U-^{14}C]glucuronic acid moiety was lost as [^{14}C]CO_2.

Doubly labeled reaction product (0.07 nmoles) in 0.25 ml of 0.01 N HCl was heated for 15 minutes at 100°. The hydrolysis mixture, UDP, UMP, D-xylose and D-apiose were spotted separately on paper and the paper was developed in solvent C at 4°. Four radioactive hydrolysis products were obtained. Starting from the origin, these compounds were numbered 1 to 4. The compounds were eluted from the chromatogram with water at 4°. Compounds 3 and 4 were eluted together.

A portion of each of the products from acid hydrolysis was used for determining the isotope content and ^{3}H:^{14}C ratio. The remainder was used for identification. The results are seen in Table II. In solvent C compounds 1 and 2 had the same R_F as UDP and UMP, respectively. In solvents F and G compounds 1 and 2 migrated as single peaks and co-chromatographed with UDP and UMP, respectively. On this basis they were identified as [^{3}H]UDP and [^{3}H]UMP.

TABLE I: The ratio of ^{3}H:^{14}C in the product from the reaction catalyzed by UDPGA cyclase with [^{3}H]UDP-[U-^{14}C]glucuronic acid as the substrate*.

Compound	Ratio of ^{3}H:^{14}C
[^{3}H]UDP-[U-^{14}C]glucuronic acid	8.91
Reaction product	10.6 (observed)
	10.4 (calculated)**

*The incubation mixture contained 1.57 nmole of [^{3}H]UDP-[U-^{14}C]glucuronic acid, 0.55 µmole of NAD^{+}, 25.5 µmoles of sodium phosphate buffer, pH 8.0, 0.055 µmole of EDTA, 10 µmoles of β-mercapto-ethanol, 3 milliunits of duckweed UDPGA cyclase (2.0 mg) purified through the DEAE-Sephadex step in a total volume of 0.55 ml. The mixture was incubated for 25 minutes at 25° and then the reaction product was isolated as described in the Results. After paper chromatography in solvent A, no α-D-[U-^{14}C]apio-D-furanosyl cyclic -1:2-P or [^{3}H]UDP-[U-^{14}C]glucuronic acid could be detected in the reaction product. A 1% contamination of either would have been detected. Acid hydrolysis of the reaction product, as described in the Results, showed that 71% of the ^{14}C was in the reaction product while 29% was in [^{3}H]UDP-[U-^{14}C]xylose. A portion of the reaction product was rechromatographed on paper in solvent C at 4° and the paper was scanned. The reaction product (with contaminating [^{3}H]UDP-[U-^{14}C]xylose) was eluted with water and its ^{3}H:^{14}C ratio was determined. This rechromatography was necessary in order to remove contaminating [^{3}H]UMP. This latter compound presumably was formed by alkaline hydrolysis during the preparation of the reaction product. Of the total tritium present in the solution containing the reaction product 7.8% was in [^{3}H]UMP. A portion of the [^{3}H]UDP-[U-^{14}C]glucuronic acid was chromatographed in solvent B in order to separate it from the contaminating α-D-[U-^{14}C]glucose. After the paper was scanned the [^{3}H]UDP-[U-^{14}C]glucuronic acid was eluted with water and the ^{3}H:^{14}C ratio was determined.

**Calculated assuming 1/6 of the ^{14}C in [^{3}H]UDP-[U-^{14}C]glucuronic acid was released as [^{14}C]CO_2.

TABLE II: Products obtained on acid hydrolysis of doubly labeled reaction product and their ^{3}H:^{14}C ratios.

Compound	Ratio of ^{3}H:^{14}C	^{3}H (%)	^{14}C (%)
Doubly labeled reaction product*	10.6	100	100
Products from acid hydrolysis**			
UDP at origin	549	21.2	0.4
UDP	1028	64.7	0.6
UMP	109	10.3	1.0
D-apiose and D-xylose	0.005	0.1	95.0
		96.3	97.0

*The doubly labeled reaction product was the same as that used in Table I. The starting percent of ^{3}H and ^{14}C is based on that from both the reaction product and the contaminating [^{3}H]-[U-^{14}C]xylose.

**The products of hydrolysis were identified as described in the Results. The [^{3}H]UMP present in the reaction product was taken into account in calculating the values for UMP.

Compounds 3 and 4 were only partially resolved by solvent C. Both authentic D-apiose and D-xylose had migrated to the same area as these two compounds. Rechromatography in solvent E with authentic D-apiose and D-xylose showed that compound 3 was D-[U-^{14}C]xylose and compound 4 was D-[U-^{14}C]apiose. Frequently, some radioactive material remained at the origin or extended from the origin to compound 1. Co-chromatography in solvents F and G showed that this material was [^{3}H]UDP. The amount of this material could be reduced, and in some cases was completely eliminated, by adding carrier UDP to the hydrolysate before chromatography in solvent C. Control experiments

were run in which the reaction product in water at pH 5-6 was kept at 4° for the duration of the experiment and then was chromatographed in solvent C. No free D-[U-^{14}C]apiose or D-[U-^{14}C]xylose could be detected in the controls and less than 2% of the ^{3}H migrated with the R_F of [^{3}H]UMP. These data established that the products from the acid hydrolysis of the reaction product were [^{3}H]UDP and D-[U-^{14}C]apiose. These data and others presented earlier established that the reaction product contained [^{3}H]UDP-[U-^{14}C]xylose since [^{3}H]UDP and D-[U-^{14}C]xylose were obtained after acid hydrolysis. UDPGA decarboxylase which contaminates the UDPGA cyclase would account for the formation of [^{3}H]UDP-[U-^{14}C]xylose.

Doubly labeled reaction product (0.077 nmoles) in 0.1 ml of 0.07 M sodium phosphate buffer, pH 8.0, was heated for 5 minutes at 100°. This mixture, UMP and UDP-xylose were spotted separately on paper and the paper was developed in solvent G at 22° for 48 hours. Three radioactive products were obtained, one of which was allowed to run off the paper. Chromatography for 48 hours was needed in order to separate [^{3}H]UMP and [^{3}H]UDP-[U-^{14}C]xylose. The compounds, including [^{3}H]UDP-[U-^{14}C]xylose were eluted from the chromatogram with water at 4°. A portion of each was used for determining the isotope content and the ^{3}H:^{14}C ratio. The remainder was used for identification. The results are seen in Table III. There was no detectable breakdown of [^{3}H]UDP-[U-^{14}C]xylose as evidenced by its complete recovery and unchanged ^{3}H:^{14}C ratio. The two compounds remaining on the paper had the same R_F values

as the UMP and UDP-xylose standards. In solvent F one compound, [^{3}H]UMP, migrated as a single radioactive peak and co-chromatographed with UMP. In solvent F [^{3}H]UDP-[U-^{14}C]xylose migrated as a single radioactive peak and co-chromatographed with UDP-xylose. Rechromatography of the material which ran off the chromatogram showed that it migrated as a single peak in solvent B and had the same R_F as α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. When this compound was hydrolyzed with acid by the procedure previously used with Compound III (39) and the ethanol-soluble material chromatographed on paper in solvent E, a single radioactive peak co-chromatographing with D-apiose was obtained. The controls were the same as those described above for the acid hydrolysis of the reaction product. No [^{3}H]UMP and no α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P were detected on the chromatogram. These data established that the products from the alkaline hydrolysis of the reaction product were [^{3}H]UMP and α-D-[U-^{14}C]apio-D-furanosyl-cyclic-1:2-P. They further showed that [^{3}H]UDP-[U-^{14}C]xylose was not degraded.

The reaction product acted as a D-[U-^{14}C]apiose donor. However, [^{3}H]UDP-[U-^{14}C]xylose and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P did not act as D-[U-^{14}C]xylose and D-[U-^{14}C]-apiose donors in the reaction catalyzed by apiin synthase using "7GA" as the accepting compound. These results are shown by the data in Table IV. When doubly labeled reaction product and "7GA" were incubated together with apiin synthase, two products were obtained. They had the same R_F values in

TABLE III: Products obtained on alkaline hydrolysis of doubly labeled reaction product and their ^{3}H:^{14}C ratios.

Compound	Ratio of ^{3}H:^{14}C	^{3}H (%)	^{14}C (%)
Doubly labeled reaction product*	10.6	100	100
Products from alkaline hydrolysis**			
UMP	418.0	61.6	1.5
α-D-apio-D-furanosyl cyclic-1:2-P	0.01	0.1	57.7
Other compounds			
UDP-xylose	10.2	35.4	36.2
		97.1	95.4

*The doubly labeled reaction product was the same as that used in Table I. The starting percent of ^{3}H and ^{14}C is based on that from both the reaction product and the contaminating [^{3}H]UDP-[U-^{14}C]xylose.

**The products of hydrolysis were identified as described in the Results. The [^{3}H]UMP present in the reaction product was taken into account in calculating the values for UMP.

solvent G as the UDP and apiin which had been spotted separately on the paper. In solvent F the material with an R_F of UDP migrated as a single peak and co-chromatographed with authentic UDP. In solvent H the material with an R_F of apiin migrated as a single peak and co-chromatographed with apiin. When the product with an R_F of apiin was hydrolyzed with acid by the procedure previously used with Compound III (39) and the ethanol-soluble material chromatographed on paper in solvent E, a single radioactive peak was obtained which co-chromatographed with D-apiose. These data identified UDP and apiin as the products

of the reaction catalyzed by apiin synthase. Two other compounds were present on the original chromatogram in addition to [^{3}H]UDP-[U-^{14}C]xylose. These two compounds had the same R_F values as UMP and α-D-apio-D-furanosyl cyclic-1:2-P. In solvent G after 18 hours at 22°, UMP and UDP-xylose have approximately the same R_F. When the material migrating with an R_F of UMP and UDP-xylose was rechromatographed on paper in solvent F for 4 hours, part of the radioactivity co-chromatographed with UMP while the remainder co-chromatographed with UDP-xylose. In solvent B the material with an R_F of α-D-apio-D-furanosyl cyclic-1:2-P migrated as a single peak and chromatographed with the same R_F as the authentic compound. When this radioactive compound was hydrolyzed with acid by the procedure previously used with Compound III (39) and the ethanol-soluble material chromatographed on paper in solvent E, a single radioactive peak with the same R_F as D-apiose was obtained. These data established that the two compounds were [^{3}H]UMP and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. Alkaline hydrolysis of unreacted doubly-labeled reaction product accounts for the formation of these two compounds. The following three control experiments were run: (1) with heated apiin synthase; (2) with heated reaction product; (3) with both heated apiin synthase and heated reaction product. The incubation mixtures for the controls were identical to those described in Table IV except that apiin synthase or doubly-labeled reaction product or both were heated for 2 minutes at 100° and pH 8.0 before being added

TABLE IV: Products obtained on incubation of doubly labeled reaction product and "7GA" with apiin synthase and their $^{3}H:^{14}C$ ratios*

Compound	Ratio of $^{3}H:^{14}C$	^{3}H (%)	^{14}C (%)
Reactant			
Doubly labeled reaction product**	10.6	100	100
Products			
Apiin	0.1	0.4	54.8
UDP	5512.0	50.2	0.1
Other compounds			
α-D-apio-D-furanosyl cyclic-1:2-P	0.9	1.4	14.9
UDP-xylose and UMP	16.7	44.1	28.3
		96.1	98.1

*The incubation mixture contained 0.0133 nmole of doubly labeled reaction product, 0.015 μmole of "7GA", 7.5 μmoles of sodium phosphate buffer, pH 8.0, and 0.1 milliunit of apiin synthase (0.36 mg) in 0.15 ml. The mixture was incubated 5 minutes at 25° and then heated for 2 minutes at 100°. The incubation mixture, UDP, UMP, α-D-[U-^{14}C]-apio-D-furanosyl cyclic-1:2-P, and apiin were spotted separately on paper and the paper was developed in solvent G for 18 hours at 22° and scanned. The various radioactive compounds were eluted at 4° with 75% aqueous ethanol. A portion of each was used for determining the isotope content and $^{3}H:^{14}C$ ratio. The remainder was used for identification. The compounds were identified as described in the Results. The [^{3}H]UMP present in the reaction product was taken into account in calculating the values for UMP.

**The doubly labeled reaction product was the same as that used in Table I. The starting percent of ^{3}H and ^{14}C is based on that from both the reaction product and the contaminating [^{3}H]UDP-[U-^{14}C]xylose.

to their respective mixtures. The incubation mixtures were treated and the per cent of each isotope in a compound was determined as described in Table IV. A value of 100% was assigned to both the ^{14}C and ^{3}H content of the doubly-labeled reaction product. The ^{14}C and ^{3}H content of the following compounds was: apiin (^{14}C-2.1%, ave. of 1.6, 2.3, 2.5%; ^{3}H-0.6%, ave. of 0.0, 0.4, 1.3%), UDP (^{14}C-0.5%, ave. of 0.0, 0.0, 1.4; ^{3}H-3.9%, ave. of 2.1, 2.3, 7.2%), α-D-apio-D-furanosyl cyclic-1:2-P (^{14}C-57%, ^{3}H-2.0%); UMP (^{14}C-0%; ^{3}H-53%), UDP-xylose (^{14}C-35%; ^{3}H-36%).

Stability of UDP-[U-^{14}C]apiose. -- The stability of UDP-[U-^{14}C]apiose was greatest at pH 5-6 (Fig. 1). Some hydrolysis occurred even at pH 4 and 7. Under the conditions used no significant hydrolysis of UDP-[U-^{14}C]xylose was detected at any pH except possibly a small amount at pH 2 and 11. Hydrolysis of UDP-[U-^{14}C]apiose to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and UMP occurred rapidly at pH 8 and 100°, being virtually complete after 2 minutes (Fig. 2). There was no detectable hydrolysis of UDP-[U-^{14}C]xylose even during the longest heating periods. This was established by hydrolyzing the compounds with an R_F of α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and UDP-[U-^{14}C]xylose with acid from those incubation mixtures that were heated for time periods longer than 2 minutes. From the former only D-[U-^{14}C]apiose was obtained and from the latter only D-[U-^{14}C]xylose. Hydrolysis of UDP-[U-^{14}C]apiose to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and UMP occurred much more slowly at pH 8 and

Fig. 1. The extent of hydrolysis of UDP-[U-^{14}C]apiose after 90 minutes at 25° as a function of pH. -- Solutions at 4° were prepared which contained 0.036 nmole of UDP-[U-^{14}C]apiose (11,800 dpm) and 0.020 nmole of UDP-[U-^{14}C]xylose (5,900 dpm). To each was added a buffer solution which contained 1.8 μmoles of H_3BO_3, 1.8 μmoles of sodium dihydrogen phosphate, 2.3 μmoles of HCl and varying amounts of NaOH. The buffer solutions were at either pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 or 11.0. The final volume of each sample solution was 0.15 ml. After incubation at 25° for 90 minutes the solutions were cooled to 4° and then spotted on washed paper at the same temperature. The paper was developed at 4° with solvent B for 30 hours, scanned and the appropriate radioactive areas were cut out and counted in scintillation solution B. Per cent hydrolysis is the per cent of the UDP-[U-^{14}C]apiose used which was converted to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P or D-[U-^{14}C]apiose. All samples were corrected for the α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and D-[U-^{14}C]apiose already present in the UDP-[U-^{14}C]apiose solution before incubation. To make these corrections control solutions identical to the sample solutions described above except that the buffer solution was replaced by water, were prepared. The solutions were kept at pH 5-6 and 4° for the duration of the experiment and then treated as described above. Data presented in the Results show that no measurable hydrolysis of UDP-[U-^{14}C]apiose occurs at pH 6 and 4° in 90 minutes.

(O ····· O ····· O) α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P

(● ——— ● ——— ●) D-[U-^{14}C]apiose

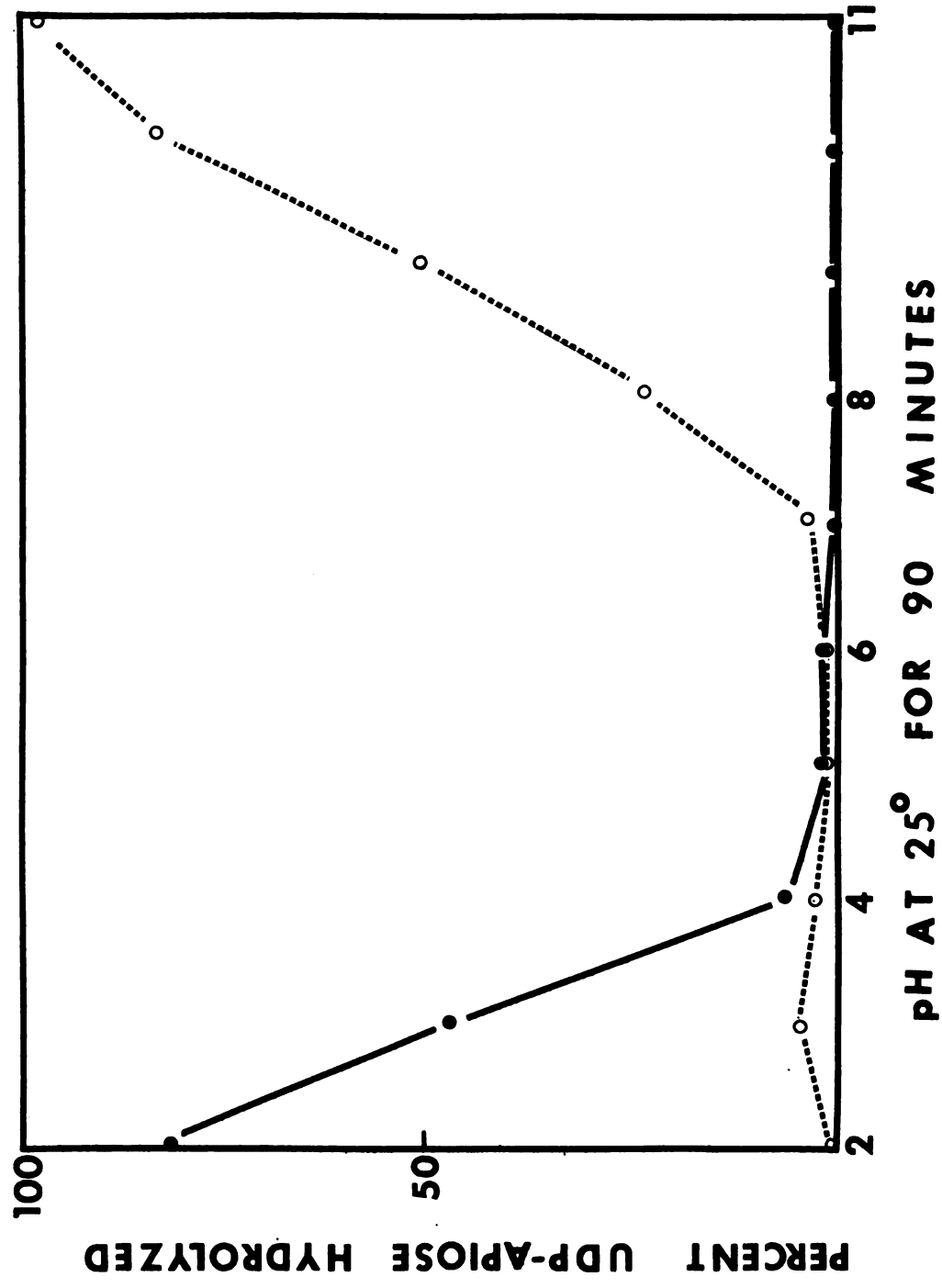
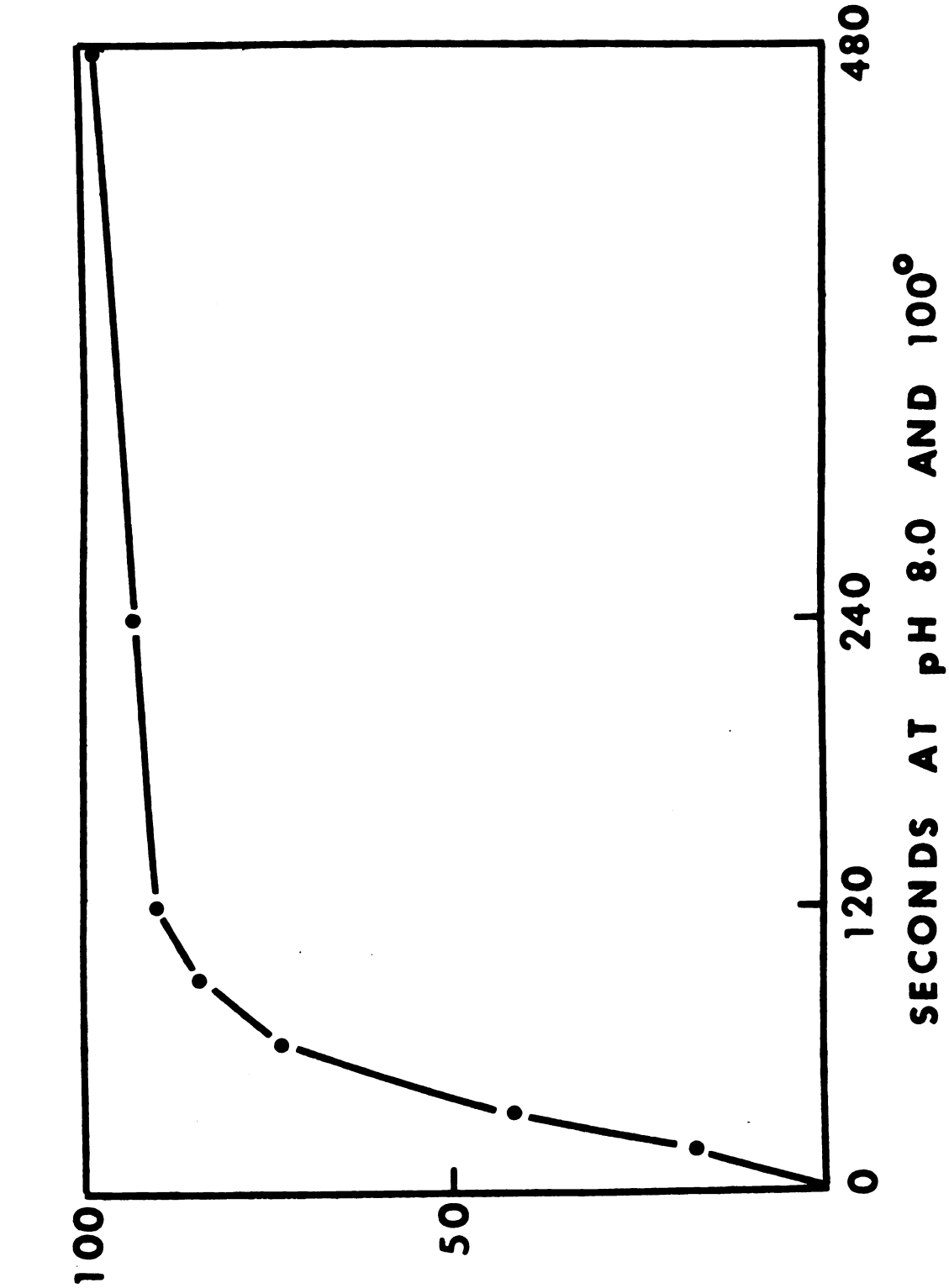

PERCENT UDP-APIOSE HYDROLYZED
100
50
2
4
6
8
11
pH AT 25° FOR 90 MINUTES

Fig. 2. The rate of hydrolysis of UDP-[U-^{14}C]apiose to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and UMP at pH 8.0 and 100^{0} with time. -- Solutions at 4^{o}, each containing 0.35 nmoles of UDP-[U-^{14}C]apiose (26,600 dpm), 0.38 nmoles of UDP-[U-^{14}C]xylose (28,900 dpm), 5 μmoles of sodium phosphate buffer, pH 8.0 and 2.5 nmoles of EDTA in a final volume of 0.1 ml, were preincubated 2 minutes at 25^{o}. Then they were incubated at 100^{o} for 0, 15, 30, 60, 90, 120, 240 or 480 seconds. Solutions were started sequentially and each was removed at the appropriate time and kept at 4^{o} until the last one was removed. The zero-time sample was preincubated at 25^{o} and then kept at 4^{o} and pH 8.0 for the duration of the experiment (approximately 20 minutes). Then all the solutions were adjusted to pH 5-6 and were spotted on washed paper at 4^{o}. The paper was developed at 4^{o} with solvent C for 17 hours, scanned and the appropriate radioactive areas were cut out and counted in scintillation solution B. Per cent hydrolysis is the per cent of the UDP-[U-^{14}C]apiose used which was converted to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. All samples were corrected for the α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P present in the zero-time sample. To determine if any hydrolysis had occurred in the zero-time sample during the experiment control solutions, identical to those described above but at pH 6.0, was prepared and treated as described above for the control solutions. The data obtained from the controls showed there was no detectable hydrolysis in the zero-time sample.

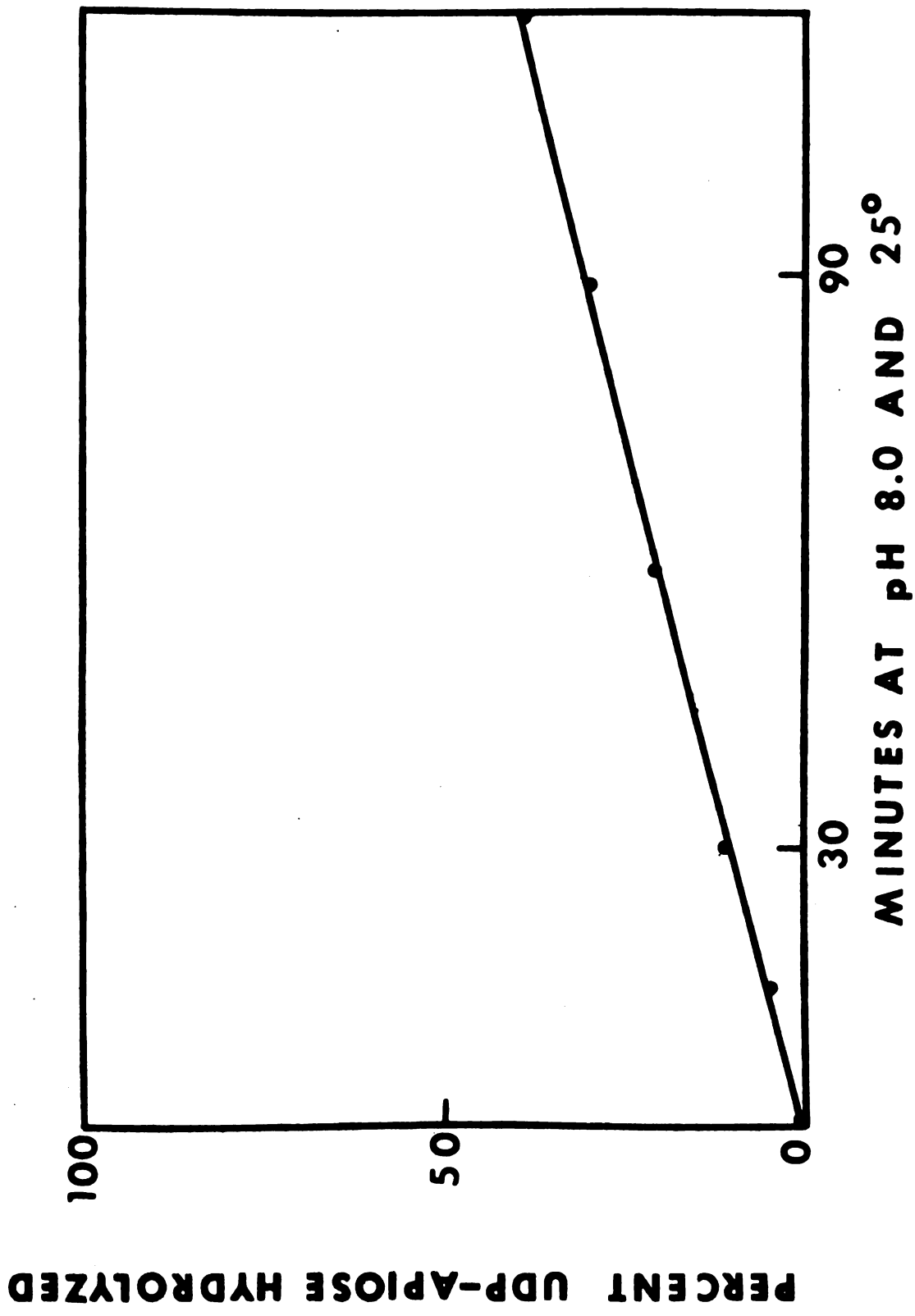

PERCENT UDP-APIOSE HYDROLYZED
100
50
0
120
240
480
SECONDS AT pH 8.0 AND 100°

25° than at pH 8 and 100° (Figs. 1 and 2). Some hydrolysis occurred even at pH 8 and 4° (Fig. 3). Stability of UDP-[U-^{14}C]apiose at 4° and pH 6.0 was determined. Each of three incubation mixtures contained 0.23 nmoles of UDP-[U-^{14}C]apiose (17,600 dpm), 0.24 nmoles of UDP-[U-^{14}C]-xylose (18,500 dpm), 4.8 μmoles of sodium phosphate buffer, pH 6.0, and 2.4 nmoles of EDTA in a total volume of 0.05 ml. After incubation for 5 hours at 4° the mixtures were spotted and chromatographed on washed Whatman No. 3MM paper in solvent B for 26 hours. The results obtained were compared to those obtained from three identical incubation mixtures which were made up 5 hours later. UDP-[U-^{14}C]apiose for this latter set of incubation mixtures was kept at -20° in solvent A during the 5 hours that the first set of incubation mixtures was incubated at 4°. The amount of radioactivity which migrated with an R_F of D-[U-^{14}C]apiose and α-D-[U-^{14}C]-apio-D-furanosyl cyclic-1:2-P from both sets of incubation mixtures was measured. The data showed that no detectable hydrolysis of UDP-[U-^{14}C]apiose occurred during the 5 hours at pH 6.0 and 4°. Hydrolysis of 1% or more of the UDP-[U-^{14}C]-apiose would have been detected.

The stability of UDP-[U-^{14}C]apiose was determined at pH 5.5 and in 50% ethanol by volume. This was done by storing the sugar nucleotide at -20° for 2 months as described in Table V. It was not hydrolyzed to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P or to D-[U-^{14}C]apiose during the storage period.

The stability of UDP-[U-^{14}C]apiose at 25° and pH 6.0 was determined to check the feasibility of concentrating solutions

Fig. 3. The rate of hydrolysis of UDP-[U-^{14}C]apiose to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and UMP at pH 8.0 and 25° with time. -- Solutions at 4°, each containing 0.099 nmoles of UDP-[U-^{14}C]apiose (7,550 dpm), 0.11 nmoles of UDP-[U-^{14}C]xylose (8,200 dpm), 7.5 μmoles of sodium phosphate buffer, pH 8.0, and 3.75 nmoles of EDTA in a final volume of 0.15 ml, were incubated at 25° for 0, 15, 30, 60 or 120 minutes and then were brought to 4°. All solutions were started at the same time and each was removed at the appropriate time and kept at 4° until the last one was removed. Then the solutions were adjusted to pH 5-6 and were spotted on washed paper at 4°. The paper was developed at 4° with solvent C for 17 hours, scanned, and the appropriate radioactive areas were cut out and counted in scintillation solution B. Per cent hydrolysis is the per cent of the UDP-[U-^{14}C]apiose used which was converted to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. All samples were corrected for the α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P present in the zero-time sample. To determine if any hydrolysis had occurred in the zero-time sample during the experiment a control solution, identical to those described above but at pH 6.0, was prepared in duplicate. The two control solutions were kept at 4° for the duration of the experiment and then treated as described above. The data obtained from the controls showed that 5.5% of the UDP-[U-^{14}C]apiose hydrolyzed in the zero-time sample.


PERCENT UDP-APIOSE HYDROLYZED
100
50
0
30
90
MINUTES AT pH 8.0 AND 25°

of it under reduced pressure with little or no degradation. The degradation of UDP-[U-^{14}C]apiose under these conditions yielded both D-[U-^{14}C]apiose and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P about equally throughout the course of the experiment (Table VI). After 1 hour 1.2% of the UDP-[U-^{14}C]-apiose was degraded. After 5 hours 3.0% was degraded to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and 3.2% was degraded to D-[U-^{14}C]apiose. The data show that the degradation of UDP-[U-^{14}C]apiose to D-[U-^{14}C]apiose and to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P is each linear with time at 25° and pH 6.0.

Solvents A, B, C, D, and I effectively prevented the appearance of α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and D-[U-^{14}C]apiose from UDP-[U-^{14}C]apiose during chromatography at 4°. Solvents F and G permitted the degradation of some of the sugar nucleotide during chromatography at 4° to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P.

TABLE V: The stability of UDP-[U-^{14}C]apiose at -20° and at pH 5.5*

Days	% UDP-[U-^{14}C]apiose converted to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P	% UDP-[U-^{14}C]apiose converted to D-[U-^{14}C]apiose	Total % UDP-[U-^{14}C]apiose degraded
0	+0.00	+0.00	+0.00
10	+0.04	+0.23	+0.27
14	-0.21	+0.34	+0.13
21	-0.47	+0.34	-0.13
29	-0.11	-0.20	-0.31
41	+0.39	+0.13	+0.52
60	-0.23	+0.12	-0.11

*The storage mixture contained 1.97 nmoles of UDP-[U-^{14}C]apiose (147,400 dpm) in each ml of a mixture of equal volumes of 0.04 mM Tris-acetate, pH 5.5 and absolute ethanol. The mixture was stored at -20°. At the time intervals indicated, 2-100 µl portions were removed, spotted on washed paper at 4° and chromatographed in solvent B for 30 hours at the same temperature. The radioactivity migrating with an R_F of D-[U-^{14}C]apiose and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P was measured and the average value for the 2-100 µl portions was recorded. The values for these two compounds in the zero-time samples were set equal to zero. Some fluctuation in the values of these two compounds during the course of the experiment was expected as the amount of them in the zero-time samples was relatively high (1.8%) compared to the amount formed upon storage.

TABLE VI: Hydrolysis of UDP-[U-^{14}C]apiose at pH 6.0 and 25° with time*.

Time (minutes)	UDP-[U-^{14}C]apiose remaining (%)	α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P (%)	D-[U-^{14}C]-apiose (%)
0	100	0.0	0.0
30	99.5	0.1	0.4
60	97.9	0.5	0.7
90	97.3	1.7	1.0
120	97.2	1.5	1.3
210	96.3	2.2	1.5
300	93.8	3.0	3.2

*Solutions at 4°, each containing 0.35 nmole of UDP-[U-^{14}C]apiose (26,600 dpm), 0.38 nmole of UDP-[U-^{14}C]xylose (28,900 dpm) and 5 μmoles of sodium phosphate buffer, pH 6.0, and 2.5 nmole of EDTA in a final volume of 0.1 ml, were incubated at 25° for 0, 30, 60, 90, 120, 210, or 300 minutes and then were brought to 4°. All solutions were started at the same time and each was removed at the appropriate time and kept at 4° until the last one was removed. The solutions were spotted on washed paper at 4°. The paper was developed at 4° with solvent B for 30 hours, scanned, and the appropriate radioactive areas were cut out and counted in scintillation solution B. Hydrolysis was measured by the appearance of α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P or D-[U-^{14}C]apiose or both. Per cent hydrolysis is the per cent of the UDP-[U-^{14}C]apiose used which was converted to α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and D-[U-^{14}C]apiose as determined by radioactivity measurements. All samples were corrected for the α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and D-[U-^{14}C]apiose present in the zero-time sample. Data presented above show that no hydrolysis of UDP-[U-^{14}C]apiose occurred in the zero-time sample during the course of the experiment.

DISCUSSION

One of the richest sources of D-apiose is in the cell wall polysaccharides of duckweed. Some of these polysaccharides have recently been isolated and characterized (15-17). Hart and Kindel (17) found D-apiose glycosidically linked to polygalacturonic acid (apiogalacturonans). As the biosynthesis of polysaccharides appears to involve transglycosylation reactions from sugar nucleotides (68) similar reactions could be expected to occur in the biosynthesis of apiogalacturonans of duckweed.

The search for a sugar nucleotide containing D-apiose produced several preliminary and inconclusive communications. Sandermann and Grisebach (69) isolated sugar nucleotides from P. crispum and obtained 7250 µmoles of ultraviolet absorbing material. These were hydrolyzed with acid liberating the sugars of the sugar nucleotides. One of the sugars (0.15 µmole) migrated with the R_F of D-apiose. These data, they said, showed the presence and existence of a sugar nucleotide containing D-apiose. Sandermann, Tisue and Grisebach (38) later reported the isolation of a cell-free extract from duckweed which formed several radioactive compounds from UDP-[U-^{14}C]glucuronic acid. These compounds migrated with the R_F of UDP-xylose. The cell-free extract was incubated with UDP-[U-^{14}C]glucuronic acid (1,100,000 dpm) and NAD^+ for 4 hours and 20 minutes at 25°. Then the UDP-pentoses (14,500 dpm) formed during incubation were isolated by paper chromatography and electrophoresis. They were hydrolyzed

with acid and the liberated pentoses were isolated. One of these pentoses (260 dpm) migrated with the R_F of D-apiose. As reported by Grisebach and co-workers, the amount of UDP-apiose formed (38) or isolated (69) was quite small and the possibility that they were a contamination or an artifact can not be easily discounted. In both reports very limited data were used to show the existence of the new compound.

Independently Gustine and Kindel (39) reported the isolation of a cell-free extract from duckweed which converted 22% of the substrate, UDP-[U-^{14}C]glucuronic acid, into a D-apiose containing compound. No UDP-apiose was isolated under the conditions they carried out their experiments. As mentioned in the Results of Part 2 of this thesis, Kindel afterwards found that much less of this D-apiose containing compound (later identified as α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P) was formed in incubation mixtures which were not boiled at pH 8 and which were chromatographed at 4°. He postulated that α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P was the degradation product of UDP-[U-^{14}C]apiose rather than the primary enzymatic product of the reaction with UDP-[U-^{14}C]glucuronic acid catalyzed by the extract from duckweed. Early work by Paladini and Leloir (62) supported this hypothesis. They studied the base catalyzed intramolecular phosphorylation of UDP-glucose and found that UDP-glucose would phosphorylate intramolecularly above pH 8. If UDP-apiose was formed by the extract and if it was more sensitive than UDP-glucose to lower pH then it might phosphorylate intramolecularly at pH 8.

I had previously made an observation that suggested that UDP-apiose was being formed by parsley UDPGA cyclase from UDP-glucuronic acid. I observed that parsley UDPGA cyclase was necessary to synthesize apiin through its formation of an unidentified D-apiose donor molecule. This reaction also required the presence of "7GA" and was catalyzed by parsley apiin synthase.

The above observations suggested that UDP-apiose was formed from UDP-glucuronic acid by enzymes isolated from duckweed and parsley. This was unequivocally established by the isolation of all the sugar nucleotides formed from [^{3}H]UDP-[U-^{14}C]glucuronic acid by purified duckweed UDPGA cyclase and duckweed UDPGA decarboxylase and their subsequent characterization. A very mild isolation and partial purification procedure was devised which separated the sugar nucleotides from the other substances present in the incubation mixture (NAD^+, UMP, α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P and duckweed enzymes).

The presence of base, sugar and phosphate in the reaction products (sugar nucleotides) was demonstrated in the following five ways, which showed that they were sugar nucleotides. First, the reaction products were found to contain the expected amounts of ^{3}H and ^{14}C based upon the amounts of ^{3}H and ^{14}C present in the substrate and assuming that 1/6 of the ^{14}C in the substrate was lost as [^{14}C]CO_2. These data show that the assumption that the uridine portion remained attached to the sugar portion. Second, both of these sugar

nucleotides had the same R_F as authentic UDP-xylose in a variety of paper chromatographic systems. Third, mild acid hydrolysis (pH 2) yielded [^{3}H]UDP, a little [^{3}H]UMP, D-[U-^{14}C]xylose and D-[U-^{14}C]apiose. Paladini and Leloir (62) had observed that mild acid hydrolysis (pH 2) was a good indication of the presence of a sugar nucleotide. The glycosyl phosphate linkage of a sugar nucleotide is less stable than that of the corresponding glycosyl phosphate. For example, D-glucose can be quantitatively split from UDP-glucose by heating it at 100° for 15 minutes in 0.01 N hydrochloric acid (62), whereas complete cleavage of D-glucopyranosyl 1-phosphate to D-glucose and inorganic phosphate requires similar treatment in N acid (63). The ΔG° of hydrolysis of the D-glucopyranosyl 1-phosphate bond of UDP-glucose has been calculated to be approximately -7600 calories at pH 7.4 (64), whereas that of D-glucopyranosyl 1-phosphate is -4800 calories at pH 8.5 (65). Fourth, the product of intramolecular phosphorylation of one of the two reaction products resulted in the formation of [^{3}H]UMP and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. The reaction product containing D-[U-^{14}C]xylose like a similar pyranoid sugar nucleotide, UDP-glucose (62), did not phosphorylate intramolecularly at pH 8 and 100°. This reaction product was recovered and its ^{3}H:^{14}C ratio was unchanged. Fifth, apiin synthase was employed to further characterize one of the reaction product as [^{3}H]UDP-[U-^{14}C]-apiose by showing that it would function as a D-apiose donor

molecule in a glycosidic reaction. A reaction mixture was prepared with [^{3}H]UDP-[U-^{14}C]xylose, [^{3}H]UDP-[U-^{14}C]apiose, "7GA" and apiin synthase. [^{3}H]UDP-[U-^{14}C]apiose did react to form [^{14}C]apiin. This was shown by the formation of [^{3}H]UDP and [^{14}C]apiin and by the disappearance of [^{3}H]UDP-[U-^{14}C]apiose. The experiment also shows that the products of the reaction catalyzed by apiin synthase are UDP and apiin. [^{3}H]UDP-[U-^{14}C]xylose did not react in the above incubation mixture. This was shown by the recovery of the added [^{3}H]UDP-[U-^{14}C]xylose and its unchanged ^{3}H:^{14}C ratio. A reaction mixture was prepared with [^{3}H]UDP-[U-^{14}C]xylose [^{3}H]UMP, α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P, "7GA" and apiin synthase. After incubation the above radioactive compounds were recovered unchanged. Radioactive compounds were not observed with the R_F values of the expected products of the above transglycosylation reaction i.e. [^{3}H]UDP and [^{14}C]-apiin. These results indicate that UDP-[U-^{14}C]xylose did not react in the reaction catalyzed by apiin synthase. The above results which show that apiin and UDP are formed from "7GA" and UDP-apiose by apiin synthase agree with those of various glycosylation reactions with sugar nucleotides (65,68). UDP has not been identified before as a product of transglycosylation reactions where the substrates were a nucleoside diphosphate sugar and a flavonoid or similar phenolic compound (68,54,55,57).

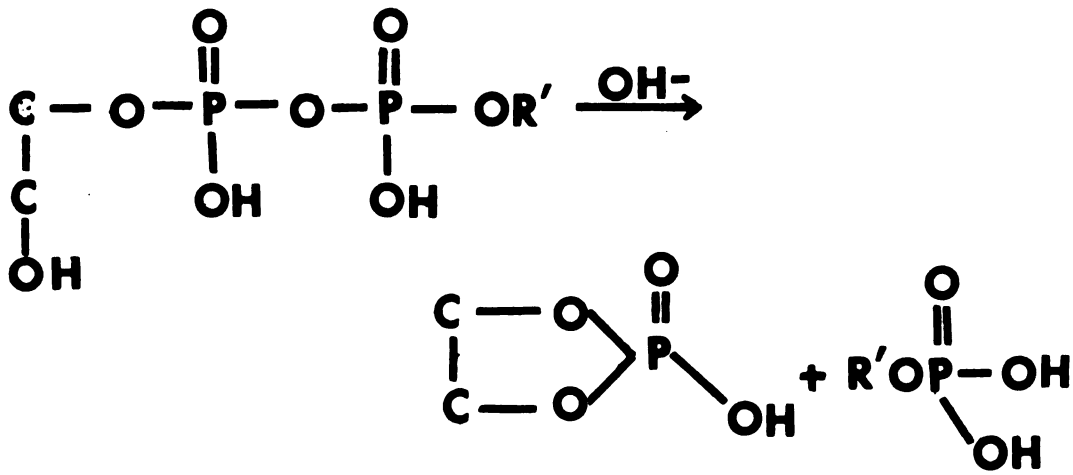
The final proof of the proposed structure of UDP-apiose [uridine-5' (α-D-apio-D-furanosyl-pyrophosphate)] will require

its chemical synthesis.

The stability of UDP-xylose and UDP-apiose was investigated in both acidic and alkaline solutions. UDP-xylose was stable under the conditions tested except at pH 2 and 100°. The hydrolysis properties of both UDP-xylose and UDP-apiose at pH 2 and 100° are those that are characteristic of sugar nucleotides (65). UDP-apiose was unstable under most conditions tested. Optimum conditions for storing UDP-apiose without degradation are -20°, pH 5 to 6 and in a solution which is 50% ethanol by volume. The other organic solvents used to store UDP-apiose at -20° were 50% acetone by volume and solvent A. UDP-apiose stored at -20°, in solvent A and at pH 5 to 6 was stable for months. At 25° and below pH 5 acid hydrolysis of UDP-apiose occurred. At 25° and at pH values above 6 alkaline hydrolysis (phosphorylates intramolecularly) occurred. Lowering the temperature retarded hydrolysis at these pH values. At pH 8.0, for example, alkaline hydrolysis at 100° was nearly complete after 2 minutes. At 25° it was 40% complete after 90 minutes and at 4° it was 5.5% complete after 90 minutes. At all 3 temperatures and pH 8 some hydrolysis occurred.

The above reaction is an example of reactions which occur with some sugar nucleotides in the presence of alkali. The result is a nucleoside monophosphate and a cyclic 1:2-phosphate of the sugar. This type of reaction occurs if the hydroxyl group on carbon atom 2 of the sugar is located sufficiently close to the phosphorus atom on carbon atom 1 of the sugar residue to permit the formation of a 5-membered ring (67).

Khorana, Tener, Wright and Moffatt (67) called this type of a reaction intramolecular phosphorylation and represented the general reaction as given below.



Intramolecular phosphorylation has been found to occur in UDP-glucuronic acid in NH_4OH (50,66). Basic hydrolysis (intramolecular phosphorylation) at pH 8 of [^{3}H]UDP-[U-^{14}C]xylose and [^{3}H]UDP-[U-^{14}C]apiose revealed that only [^{3}H]UDP-[U-^{14}C]xylose was stable at pH 8. [^{3}H]UDP-[U-^{14}C]apiose was degraded to [^{3}H]UMP and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. Both UDP-apiose and UDP-xylose would be expected to form a cyclic sugar phosphate because of the stereochemistry at carbon atom 1 and 2 of the sugars. However, this was not expected under the mild conditions that resulted in the basic hydrolysis of UDP-apiose (4° and pH 8) since Leloir and Paladini (62) did not observe intramolecular phosphorylation with UDP-glucose except under more severe

conditions, i.e. higher pH and temperature. UDP-glucose, a compound with a pyranoid ring and with the same stereochemistry as UDP-xylose, is quite stable at pH 8. After 18 hours at 18° and pH 8, UDP-glucose is undegraded (62). However, it did begin to cyclize at a more alkaline pH and 100°.

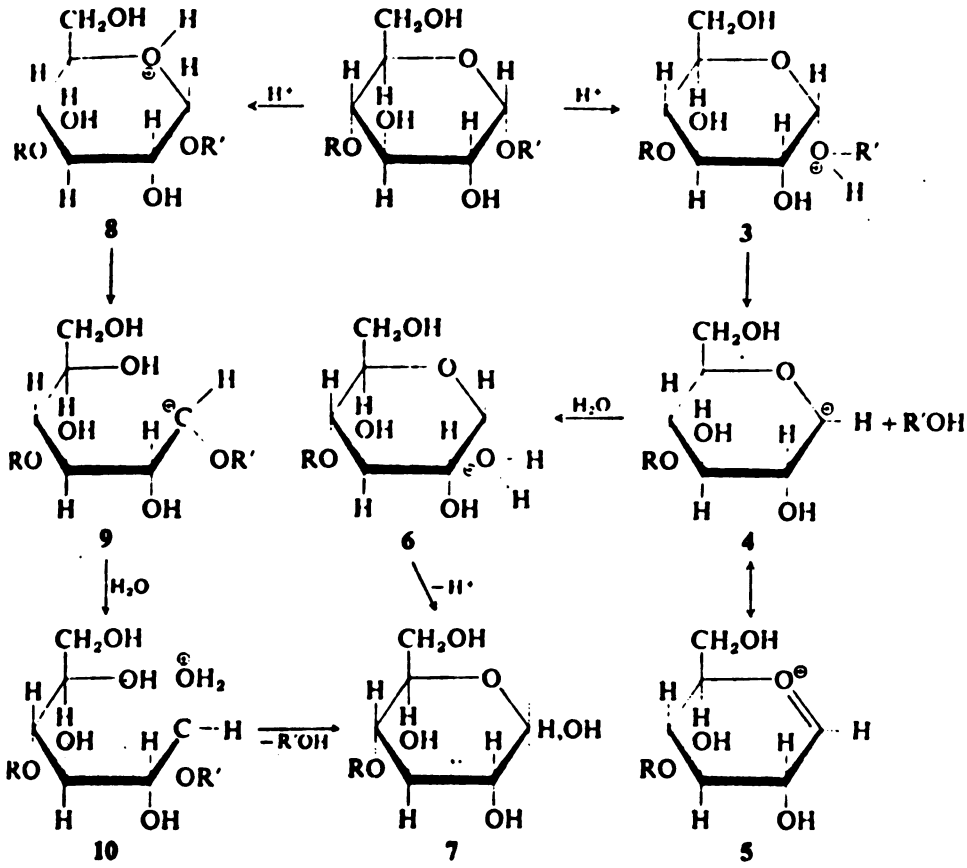
The tendency of UDP-apiose to phosphorylate intramolecularly at pH 8 has recently been mentioned by Sandermann and Grisebach (40). They suggested that the ready formation of α-D-apio-D-furanosyl cyclic-1:2-P could be explained by its great stability. This stability is due to the presence of two condensed 5-membered rings which would be in contrast to the cyclic 1:2-phosphate of D-xylose and of D-glucose. These latter compounds have a 5-membered ring condensed to a 6-membered ring. The facile degradation of 5-phospho-ribofuranosyl-pyrophosphate was cited as an analogous reaction resulting in the formation of two condensed 5-membered rings.

Besides the suggestion of Sandermann and Grisebach (40) another reason for the unusual sensitivity of UDP-apiose to basic conditions can be suggested. It would also explain the unusual sensitivity of UDP-apiose to acidic conditions which the suggestion of Sandermann and Grisebach (40) does not explain. (They did not report investigating the stability of UDP-apiose in acidic conditions nor its isolation and characterization.) My suggestion (which will be explained in more detail later) is that a pyranose sugar of a sugar nucleotide requires more energy to change into the best conformation for intramolecular phosphorylation of the sugar

nucleotide or acid hydrolysis of the glycosidic linkage than does a furanose sugar of a sugar nucleotide. The amount of energy necessary to convert the sugar into the ideal conformation for the greatest reactivity of the sugar nucleotide affects the sugar nucleotide's lability in acidic and basic solutions.

My suggestion can be readily understood from the information supplied by studies on the hydrolysis of the glycosidic linkages of oligosaccharides. The glycosidic linkages of oligosaccharides are readily hydrolyzed by acids, but are relatively stable in alkaline conditions. Acid hydrolysis of oligosaccharides occurs, as with other glycosides, by fission of the bond between carbon atom 1 of the glycosyl residue and the glycosidic oxygen atom (91). The mechanism of the acid-catalyzed hydrolysis of pyranosides has been extensively investigated. Little information is available on the hydrolysis of furanosides by acid. Pazur and Gordon (92) and Aspinall and Telfer (93) reported that glycosidic linkages involving furanose residues were extremely acid-labile in polysaccharides. Shafizadeh (82) says that there are two possible mechanisms consistent with the observations stated above and other observations which would explain the acid-catalyzed hydrolysis of pyranosides. "They are shown in formulas [3] to [10] (Fig. 4). One mechanism involves rapid reversible protonation of the glycosidic oxygen atom to yield the protonated oligosaccharide [3] which undergoes a slow unimolecular decomposition to a stable monosaccharide

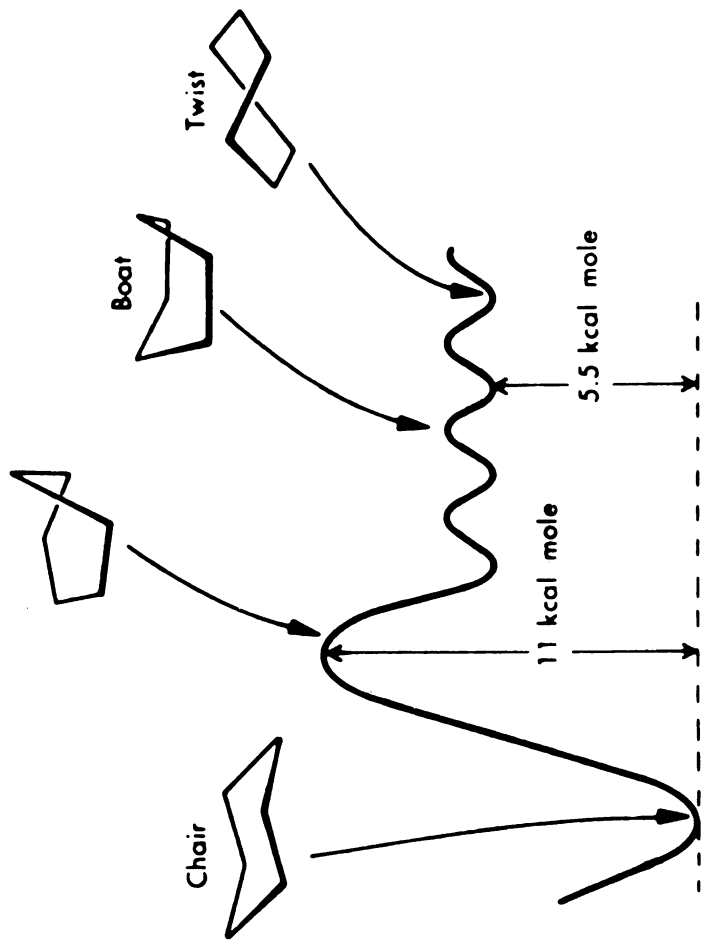
Fig. 4. Two possible mechanisms for the acid hydrolysis of glycosidic linkages between pyranoses (88).


CH2OH
RO
OR'
OH
H
8
3
9
6
4
10
7
5
H+
H2O
-H+
-R'OH
H + R'OH

and a cyclic carbonium ion [4]. Nucleophilic addition of water to the electron-deficient carbon center yields a protonated reducing sugar [6], and subsequent loss of a proton yields the expected hydrolytic products [7]." A possible, but not widely accepted alternative mechanism, Shafizadeh (82) says, "would involve protonation of the ring oxygen atom of the glycosyl moiety to yield a protonated oligosaccharide [8] followed by the cleavage of the pyranose ring to give an acyclic carbonium ion [9]. Nucleophilic addition of water on the acyclic ion would yield an unstable intermediate [10], which would predictably eliminate a monosaccharide residue to yield an aldehydo-monosaccharide. Rearrangement of the latter into the pyranose structure would yield the final hydrolytic product [7]." Indirect evidence from experiments on the rates of hydrolysis of D-xylose derivatives supports the first mechanism as being operative in the hydrolysis of these compounds (94). The relative rates of hydrolysis of pyranosides in terms of the first mechanism have been explained by conformational effects and intramolecular steric interactions (95).

Pazur (88) says, "the rate-determining step is the formation of the carbonium ion, which is considered to exist in a "half-chair" conformation." The most unstable conformation of cyclohexane, a compound which is very similar in conformation to the various pyranoses discussed, is the "half-chair" conformation (96). About 5.5 kcal/mole is required to change from the stable, "chair" conformation to

Fig. 5. Torsional isomerism in cyclohexane (96).


Twist
Boat
5.5 kcal mole
11 kcal mole
Chair

the "twist" conformation. This means that at 25° there are 10,000 "chair" conformations for each "twist" conformation (96). About 0.5 kcal/mole is required to change from the "twist" conformation to the "boat" conformation (Fig. 5). However, to change from "chair" conformation to the "half-chair" conformation requires 11 kcal/mole (96). This is because formation of the "half-chair" conformation involves a small rotation about the carbon atoms 2 to 3 and carbon atom 3 to 4 bonds. The rate of acid hydrolysis is dependent primarily on the extent of the interaction of the equatorial substituents on carbon atom 2 relative to carbon atom 3 and on carbon atom 4 relative to carbon atom 5. These groups eclipse each other in reaching the transition state for the reaction--the "half-chair" conformation (88).

In cyclopentane, a furanose, the change from "envelope" conformation to "half-chair" conformation and back again involves almost no change in potential energy (89). In substituted cyclopentanes one form or the other may have greater stability sometimes by as much as 2 kcal/mole (89). Even so this difference is much smaller than that observed in cyclohexane when it changed from the "chair" to "half-chair" conformation. Actually the shape of cyclopentane is not fixed. The individual carbon atoms move up and down at right angles to the average plane of the ring in such a manner as to cause the irregularity or puckering to move around the ring (89).

The above observations explain the acid lability of UDP-apiose resulting in its' hydrolysis under mild conditions

(pH 4 and 25°). The comparative stability of UDP-glucuronic acid, UDP-xylose and UDP-glucose to mild acid hydrolysis is easily understood. Apparently in each case the amount of energy required to reach the rate limiting or critical reaction conformation or intermediate determines the reactivity of the sugar nucleotide. UDP-glucuronic acid, UDP-xylose and UDP-glucose, require a large amount of energy to change the conformation of the sugars from the stable, "chair" conformation to the critical, unstable, reaction conformation. Some of the D-apiose of UDP-apiose either exists in the critical, reactive conformation ("half-chair") or requires very little energy to bring D-apiose into this conformation.

An examination of Framework Molecular models representing UDP-apiose and UDP-xylose reveals that UDP-apiose can readily phosphorylate intramolecularly. In sugar nucleotides free rotation about phosphorus to oxygen to phosphorus bonds is restricted by steric hinderance. Thus, the models reveal that UDP-xylose must change from the more stable "chair" conformation to the less stable "half-chair" conformation (96,89) to bring the hydroxyl group on carbon atom 2 to its closest position to the β phosphorus atom of the sugar nucleotide so that intramolecular phosphorylation is more likely to occur. The hydroxyl group of carbon atom 2 of the stable, "half-chair" form of D-apiose in UDP-apiose is much closer to the β phosphorus atom than it is in the stable, "chair" form of D-xylose in UDP-xylose. In fact the "half-chair" conformation and the "envelope" conformation of furanoses change back and

forth and back again via intermediate asymmetric arrangements which involve no substantial change of potential energy (89). The change from one form of D-apiose to the other involves little movement of the hydroxyl group on carbon atom 2 away from the β phosphorus atom. These facts support my suggestion that the furanoid ring of D-apiose is important in causing instability at alkaline pH values as well as acidic pH values.

PART 3

PURIFICATION AND CHARACTERIZATION OF APIIN SYNTHASE FROM *P. crispum*

INTRODUCTION

A wide variety of phenolic compounds exist in higher plants including the flavonoids and related compounds. Flavonoids normally occur in higher plants not as free phenolic compounds but rather in an amazing variety of glycosides containing mono- or oligosaccharides (53,68).

Barber (54) first described the glycosylation of a flavonoid, quercetin (3',4',3,5,7-pentahydroxyflavone), in which the glycosyl donor was UDP-glucose. The product of the reaction was 3-(3',4', 3,5,7-pentahydroxyflavonyl) O-β-D-glucopyranoside. The reaction was catalyzed by a cell-free extract from Phaseolus aureus (mung bean). Further incubation of the enzyme preparation in the presence of TDP-L-rhamnose catalyzed the transfer of L-rhamnose to form 3-(3',4',3,5,7-pentahydroxyflavonyl) O-β-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside. Barber (55) later reported the glycosylation of quercetin by UDP-L-rhamnose to quercetrin, 3-(3', 4',3,5,7-pentahydroxyflavonyl) O-β-L-rhamnopyranoside. This reaction was catalyzed by a cell-free extract from Leucaena glauca. Miles and Hagen (48) reported the formation of the 3-monoglucoside of kaempferol, quercetin and myricetin from UDP-glucose by a cell-free extract of Impatiens balsamina. Marsh (49) reported the formation of 3-(3',4',3,5,7-pentahydroxyflavonyl) O-β-D-glucopyranosyluronic acid from UDP-glucuronic acid and quercetin by an extract of Phaseolus vulgaris (French bean).

In 1970 there were two preliminary reports on the enzymatic glycosylation of another flavonoid. Watson and Kindel (56) reported the purification of an enzyme from P. crispum which catalyzed the transfer of D-apiose (3-C-hydroxymethyl-aldehydo-D-glycero-tetrose) from UDP apiose to

7-(4'5,7-trihydroxyflavonyl) O-β-D-glucopyranoside to form apiin (Fig. 1, Part 1). They gave the common name apiin synthase to this enzyme. Ortmann, Sandermann and Grisebach (57) also reported the isolation from P. hortense of a cell-free extract forming the same compound.

I report here the partial purification and characterization of apiin synthase. The substrates and products of the reaction catalyzed by apiin synthase are identified. Kindel[1] had previously obtained evidence for the existence of this enzyme in cell-free extracts of P. crispum and established that one of the products of the reaction was apiin. UDP-apiose:7-(4'-5,7-trihydroxyflavonyl) β-O-D-glucopyranoside, D-apiose transferase is the systematic name Watson and Kindel[2] suggest for apiin synthase.

MATERIALS AND METHODS

Materials. -- NAD^+ was obtained from Sigma Chemical Co. UDP-[U-^{14}C]glucuronic acid was obtained from New England Nuclear Corp. Sephadex G-25, G-100 and DEAE-Sephadex A50 were obtained from Pharmacia Fine Chemicals, Inc. and polyethylenimine was obtained from Dow Chemical Co. "7GA" was isolated from crystalline "apiin" and recrystallized from 95% aqueous ethanol (8). Crystalline "apiin" was isolated from parsley seeds by the method of Gupta and Seshadri (45). Although crystalline, it was a mixture, hence the quotation marks for both it and "7GA" (8).

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[1]Kindel, P. K., unpublished data.

[2]Watson, R. R. and Kindel, P. K., manuscript in preparation

UDPGA cyclase was isolated from L. minor (41). α-D-[U-^{14}C]-Apio-D-furanosyl cyclic-1:2-P[1] and UDP-[U-^{14}C]apiose were formed enzymatically from UDP-[U-^{14}C]glucuronic acid (Part 2). D-Apiose was isolated from "apiin" (8). All other materials used were of the highest quality available from commercial sources. The parsley used to obtain apiin synthase and UDPGA cyclase was P. crispum, moss-curled variety. They were mature (8-12 months of age) plants grown in a greehnouse from seeds obtained from Ferry-Morse Seed Co., Fulton, Ky. Bovine serum albumin was purchased from Research Products Division, Miles Laboratories, Inc. UMP, UDP, UTP, UDP-glucuronic acid, UDP-galactose and UDP-xylose were from Sigma Chemical Co. Diaflo cells and UM-10 Diaflo membranes were purchased from the Amicon Corporation.

General Methods. -- Paper chromatography was by the descending technique and was carried out with Whatman No. 3MM paper. The paper used with solvents A and E was treated with 2.5% neutralized polyethylenimine as described by Verachtert, Bass, Wilder and Hansen (46). The paper used with solvents B-D and F-H was washed with 0.1 M citrate and then with distilled water. The following solvents were employed: (A) 0.3 M LiCl, (B) 95% aqueous ethanol-1.0 M ammonium acetate, pH 7.5 (7:3, v/v), (C) 1-butanol-acetic acid-H_2O (4:1:5, v/v, upper phase), (D) 1-propanol-ethyl acetate-water (7:1:2, v/v), (E) 0.5 M LiCl, (F) pyridine-ethyl acetate-acetic acid-water (5:5:1:3, v/v), (G) ethyl acetate-acetic

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[1]Kindel, P. K. and Watson, R. R., manuscript in preparation

acid-formic acid-water (8:4:1:3, v/v) and (H) 2-propanol-water (9:1, v/v).

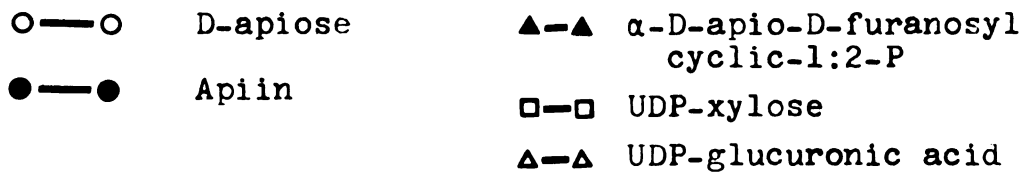
Radioactivity was detected on chromatograms with a radiochromatogram scanner, Model 7201 (Packard Instrument Co.). All other radioactivity measurements were made with a Tri-carb liquid scintillation counter, Model 3310, employing either: (A) a scintillation solution made as described by Bray (84) or (B) 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]thiophene in reagent grade toluene (4 gm/l). The portion of the chromatogram containing a radioactive compound was cut out and completely immersed in solution B in a scintillation vial and counted. The counting efficiencies with solution A and B were 79 and 60%, respectively.

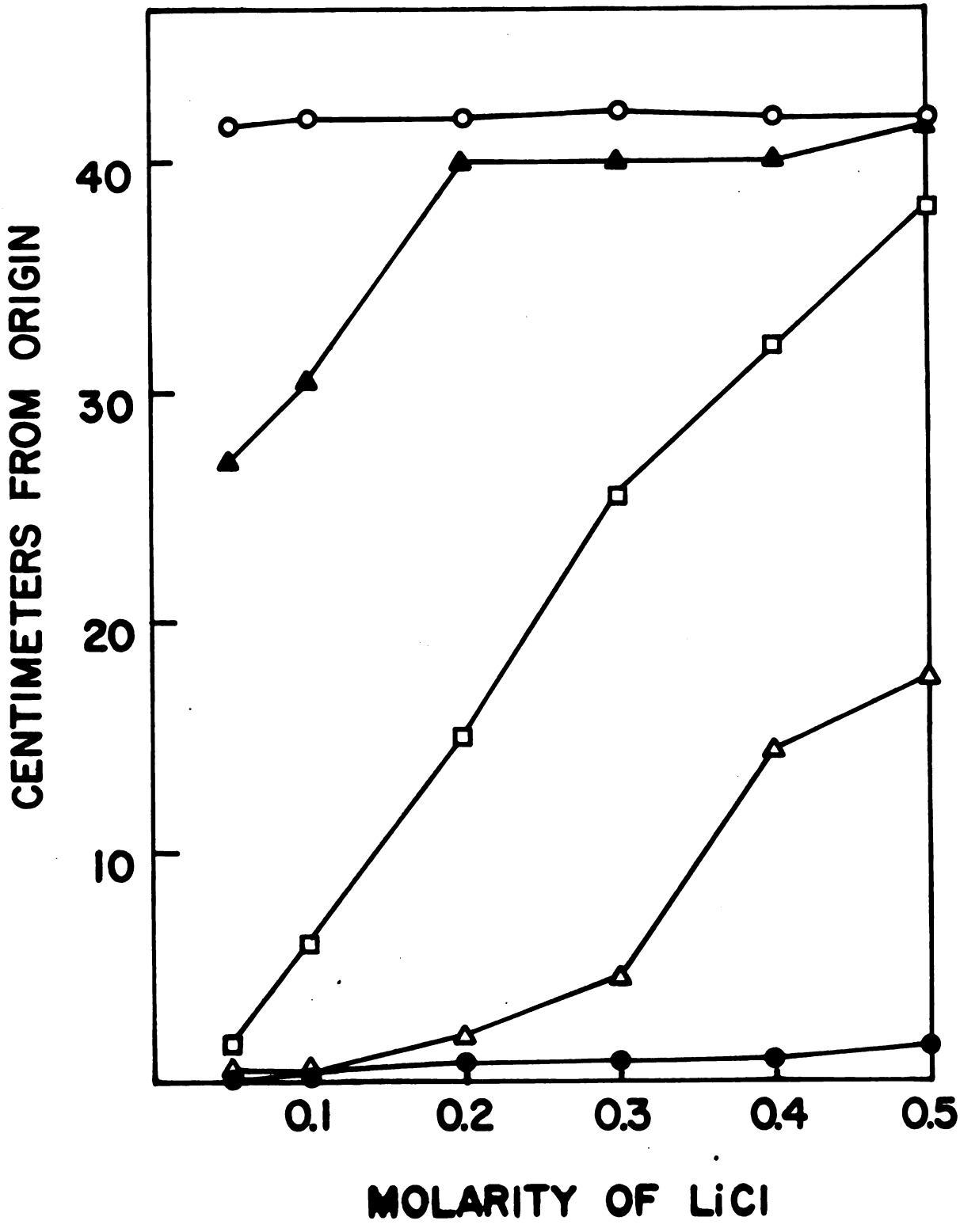
Protein was determined by the biuret method (58) before column chromatography of the enzyme. After passage through Sephadex G-100 it was determined by the procedure of Lowry, Rosebrough, Farr and Randall (59). After passage through the DEAE-Sephadex column protein was estimated by 280/260. Then the fractions containing apiin synthase were concentrated by ultrafiltration, dialyzed against buffer (2-500 ml volumes of 0.01 M sodium phosphate, pH 7.4, 1×10^{-4} M EDTA and 1×10^{-2} M β-mercaptoethanol for 1 hour) and the protein was measured by the procedure of Lowry, Rosebrough, Farr and Randall (59).

Standard Assay for Apiin Synthase. -- Apiin synthase was measured by quantitatively isolating [^{14}C]apiin formed from "7GA" and UDP-[U-^{14}C]apiose. The following standard assay was used. In a 12 ml conical centrifuge tube, 2.5 μmoles

of sodium phosphate, pH 8.0, containing 1.2 nmoles of EDTA, 80 nmoles NAD^+, 2.2 nmoles of UDP-[U-^{14}C]glucuronic acid (110,000 dpm, glucuronic acid portion uniformly labeled within ±15%) and 0.2 milliunits of duckweed UDPGA cyclase in a final volume of 25 µl. After incubation at 25° for 20 minutes, the amount of UDP-[U-^{14}C]apiose, UDP-[U-^{14}C]-xylose and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P present in the standard assays was determined by chromatographing and further treating a standard assay as described on page 28 of Part 2. More than 95% of the UDP-[U-^{14}C]glucuronic acid had been converted to UDP-[U-^{14}C]apiose and UDP-[U-^{14}C]-xylose in the standard assay. The amount of UDP-[U-^{14}C]apiose and UDP-[U-^{14}C]xylose formed was about the same since the purity of various preparations of UDPGA cyclase used was about the same with respect to UDPGA decarboxylase. As shown in Part 2 page 28 34% of the UDP-[U-^{14}C]glucuronic acid had been converted to UDP-[U-^{14}C]xylose and 63% was UDP-[U-^{14}C]-apiose. Sometimes the amount of UDP-[U-^{14}C]apiose formed was 3-5% more and the amount of UDP-[U-^{14}C]xylose formed was the same amount less when the UDPGA cyclase contained less contaminating UDPGA decarboxylase. To conserve apiin synthase, UDP-[U-^{14}C]glucuronic acid and duckweed UDPGA cyclase the standard assay contained only enough UDP-[U-^{14}C]glucuronic acid to yield a concentration of UDP-[U-^{14}C]apiose which was approximately five times the K_m that apiin synthase has for UDP-[U-^{14}C]apiose. After incubation of UDPGA cyclase with UDP-[U-^{14}C]glucuronic acid, 5.5 nmoles of "7GA" in water and apiin synthase were added to make a final volume of 50 µl.

Fig. 1. Chromatography of some of the compounds present in the standard assay mixture on polyethylenimine impregnated paper with LiCl solutions. Chromatography of apiin, α-D-[U-^{14}C]apio-D-furanosyl-1:2-P, UDP-xylose, D-[U-^{14}C]-apiose and UDP-glucuronic acid was performed on paper prepared as described for solvent A and E in the Materials and Methods. The solutions of LiCl were allowed to migrate 45 cm which in each case took about 4 hours. After chromatography at 22° the distance each compound had migrated was determined.




CENTIMETERS FROM ORIGIN
40
30
20
10
0.1
0.2
0.3
0.4
0.5
MOLARITY OF LiCl

Only 5.5 nmoles of "7GA" would dissolve or stay in solution at 25° without raising the temperature to dissolve it (see below). Therefore 20 µl of a solution of "7GA" that had been boiled (0.28 nmoles/µl) were added to give the maximum quantity of "7GA" which was soluble in 50 µl at 25°. The solution containing 0.28 nmoles of "7GA" per µl was prepared by briefly boiling "7GA" in water and then cooling to 25°. Apiin synthase was added to the standard assay mixture, it was incubated for 5 minutes at 25° and then heated at 100° for 2 minutes. Protein was removed by centrifugation and the supernatant was applied to polyethylenimine impregnated paper for chromatography in solvent E. The supernatant from each assay was streaked over a 2 cm portion of the paper. The protein precipitate from each assay was suspended in 100 µl of 70% ethanol and removed again by centrifugation. The supernatant from the ethanol wash was applied on the same 2 cm wide portion of the paper. The chromatography paper was 12.5 cm long. After 4 hours of chromatography the origin, which contained [^{14}C]apiin, was cut out and the radioactivity measured as described in the General Methods. All the other compounds present in the assay mixture containing ^{14}C had been removed from the origin (Fig. 1).

Apiin synthase catalyzed the formation of apiin from exogenously supplied UDP-[U-^{14}C]apiose. A modification of the standard assay is presented. The modified standard assay was used only to obtain the data given in Figs. 8 and 9. In a 12 ml conical centrifuge tube, 2.5 µmoles of sodium phosphate buffer, pH 8.0, containing 1.2 nmoles of EDTA,

5.5 nmoles of "7GA" and a mixture of 0.032 nmoles UDP-[U-^{14}C]-apiose (17,300 dpm) and 0.014 nmoles of UDP-[U-^{14}C]xylose (7,500 dpm) were added. The reaction was initiated by the addition of apiin synthase, the mixture was incubated for 3 minutes at 25° and then heated at 100° for 2 minutes. The assay mixture was further treated as described above for the standard assay mixture.

A unit of apiin synthase is defined as the amount required to form one μmole of apiin per minute at 25° from UDP-apiose and "7GA" under the conditions of the standard assay.

Absorbance and Solubility of "7GA". The absorbance of a solution of recrystallized "7GA" in water at 25° was determined at 335 mμ and at 268 mμ. The molar absorbancy index, a_m, at 335 mμ was 19,000 liters mole^{-1} cm^{-1}. The molar absorbancy index at 268 mμ was 17,400 liters mole^{-1} cm^{-1}.

At 25°, 1.1 x 10^{-4} moles of "7GA" was dissolved in 1 liter of water. However, after boiling briefly 2.8 x 10^{-4} moles of "7GA" remained in solution in 1 liter of water after it was cooled to 25°.

RESULTS

IDENTIFICATION OF ONE OF THE REACTION PRODUCTS FORMED BY APIIN SYNTHASE

In vitro Enzymatic Formation of Apiin (Compound IV). -- Incubation of NAD^+, parsley UDPGA cyclase, UDP-[U-^{14}C]glucuronic acid, "7GA" and apiin synthase resulted in the formation of two radioactive D-apiose-containing compounds. In Part 2 one of these compounds was identified as the product of intramolecular phosphorylation of UDP-[U-^{14}C]apiose. The other is identified below. An incubation mixture consisted of 0.276 nmoles of UDP-[U-^{14}C]glucuronic acid (105,600 dpm), 160 nmoles of NAD^+, 4.8 µmoles of sodium phosphate, pH 8.0, containing 2.4 nmoles of EDTA, 11.0 nmole of "7GA" and 0.145 mg of protein containing both parsley UDPGA cyclase and apiin synthase activity from the ammonium sulfate material which had been passed through a Sephadex G-25 column (40 cm in height and 2.2 cm in diameter) in a final volume of 100 µl. After prior treatment of the enzymes by passage through Sephadex G-25 or G-100 exogenous "7GA" was required for the biosynthesis of the compound with an R_F of apiin (Compound IV below). After incubation at 25° for 30 minutes, it was heated at 100° for 2 minutes. Following removal of the denatured protein by centrifugation, the supernatant liquid was applied to washed Whatman No. 3MM paper and chromatographed in solvent B. Scanning the chromatogram for radioactivity revealed that four distinct radioactive compounds

were present. Numbering them from the origin, they were designated Compounds I, II, III, and IV and had R_F values of 0.12, 0.28, 0.66, and 0.73, respectively. Compound I was chromatographically identified in solvent systems A and B as UDP-[U-^{14}C]glucuronic acid. Compounds II and III were found to have the same R_F values in solvent systems A, B and C as UDP-[U-^{14}C]xylose and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. Hydrolysis of Compounds I, II and III in 0.1 N H_2SO_4 revealed the presence of only one radioactive component in each compound: D-[U-^{14}C]glucuronic acid in Compound I, D-[U-^{14}C]xylose in Compound II and D-[U-^{14}C]apiose in Compound III. Only Compound IV is discussed in detail since its synthesis alone required "7GA". UDP-[U-^{14}C]xylose and UDP-[U-^{14}C]apiose have been more extensively characterized in Part 2. α-D-[U-^{14}C]Apio-D-furanosyl cyclic-1:2-P is a degradation product of UDP-[U-^{14}C]apiose (Part 2).

Identification of Compound IV as Apiin. -- Compound IV migrated as a single, radioactive area on paper chromatography in solvent systems A to C. In each of these solvent systems, it had the same R_F as apiin whose R_F is distinctly different from those of D-[U-^{14}C]apiose, UDP-[U-^{14}C]glucuronic acid, UDP-[U-^{14}C]xylose and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. A small amount of an impurity was observed in the crystalline "apiin" when chromatograms were developed in solvent C (8). It is known that besides apiin, P. crispum contains small amounts of at least one other D-apiose-containing flavonoid. Nordstrom, Swain and Hamblin rigorously identified one such flavonoid as 7-(3',4',5,7-tetrahydroxyflavonyl)

O-?-D-apio-?-furanosyl-(?)-β-D-glucopyranoside (see also Part 1). Acid hydrolysis of "apiin" yielded the crystalline "7GA" which was used as a substrate in the incubation mixture described above for the formation of Compound IV and which also contained a small amount of impurity (about 5%), based upon the weight of each compound after elution. The impurity in "apiin" was observed only on chromatograms developed in solvent C where the R_{apiin} for the impurity was 0.83. The R_{apiin} was 1.0 for the major component of "apiin" which was assumed to be pure apiin. This major component migrated 28 cm when chromatographed in solvent C for 15 hours. The R_{apiin} for the impurity in "7GA" was 0.94 and the R_{apiin} for the major component of "7GA" was 1.14. The major component in "7GA" was assumed to be pure 7GA. Grisebach and Bilhuber (60) have isolated crystalline "apiin" from P. hortense and found it contained a small amount of 7-(3'-methoxy, 4',5,7-trihydroxy-flavonyl) O-β-D-apio-D-furanosyl-(1→2)-β-D-glucopyranoside on the basis of its hydrolysis and spectral properties. Grisebach and Bilhuber (60) identified the principal product (isolated from P. hortense) and Gupta and Seshadri (45) identified the only product (isolated from P. crispum) which they found after hydrolysis of crystalline apiin as 7-(4',5,7-trihydroxyflavonyl) O-β-D-glucopyranoside. Compound IV migrated with an R_F of the major component of the apiin and not the minor component of "apiin." Both of the components of "7GA" were eluted from chromatograms developed in solvent C. Each component of "7GA" was incubated as described above for the formation of Compound IV. The major component of "7GA", when incubated with UDP-[U-^{14}C]apiose and

apiin synthase resulted in the formation of only Compounds II, III and IV. Co-chromatography of this Compound IV with "apiin" showed that it had the same R_F as the major component of "apiin" on chromatograms developed in solvent C. None of Compound IV migrated with an R_F of the minor component of "apiin". The minor component of "7GA" when incubated as described above for the formation of Compound IV, yielded a very small amount of a substance which had the same R_F as the minor component of "apiin" in solvent C. On the basis of these results and the observation that all of Compound IV migrated with an R_F of the major component of "apiin" in solvent C, Compound IV is considered homogeneous. None of Compound IV (1% or less) migrated with any other R_F than the R_F of the major component of "apiin" in solvent C.

Compound IV was hydrolyzed in 0.5 N H_2SO_4 for 1 hour at 100° in the presence of 0.15 μmole of authentic D-apiose. This mixture and authentic D-[U-^{14}C]apiose similarly treated were each spotted separately onto Whatman No. 3MM paper. Chromatography in solvent B showed that Compound IV was no longer present and only a single radioactive compound with the same R_F as the authentic D-[U-^{14}C]apiose was observed. This compound contained eighty-six and one-half percent (86.5%) of the starting radioactivity which was all the radioactivity found on the chromatogram. This is the average of 3 experiments whose values were 87.9, 87.2 and 84.3 percent. The recovery on the chromatogram of authentic D-[U-^{14}C]apiose identically treated was 87.1 percent. This shows that the amount of

D-[U-^{14}C]apiose recovered was the same as the amount of radioactive material obtained from Compound IV. This suggests that all of the radioactive material from Compound IV was a single compound with the characteristics of D-[U-^{14}C]apiose in solvent B. The radioactive material with an R_F of D-apiose obtained from Compound IV was eluted. Aliquots of this radioactive material were co-chromatographed with carrier D-apiose (0.2 μmole per aliquot) in solvent A, B, C, D, F, G and H. This radioactive material in each solvent system migrated with the same R_F as the carrier D-apiose which was visualized with aniline hydrogen phthalate (73). Apiin has a distinctly different R_F from D-apiose in solvent systems A, B, C and D. The radioactive product of acid hydrolyzed Compound IV has the same chromatographic properties as the compound Gustine and Kindel (39) characterized by periodate oxidation to be D-apiose.

Compound IV was further identified as apiin by recrystallization of a mixture of Compound IV and crystalline "apiin" to constant specific activity. Compound IV (41312 dpm) and 2.25 mmoles of "apiin" were dissolved in 144 ml of 95% ethanol by boiling. The hot solution was filtered through Whatman No. 1 filter paper and the crystals were washed with 7.5 ml of boiling 95% ethanol. After this solution stood at 22° for 21 hours and 3 hours at 4°, the crystals were collected and air dried. This process was then repeated by redissolving the dried crystals in 95% ethanol (Table I).

To further identify Compound IV as apiin the acetate derivative of a mixture of Compound IV and "apiin" was

TABLE I. Recrystallization of a mixture of "apiin" and [^{14}C]Compound IV and a mixture of "apiin" acetate and [^{14}C]Compound IV acetate.

Crystallization number	"Apiin" recovered*	[^{14}C]Compound IV recovered*	Specific activity** ([^{14}C]Compound IV/"apiin")
	%	%	dpm/mmole
1	62.7	32.8	9604.9
2	77.7	74.7	9227.0
3	75.3	73.4	8995.8
4	59.1	62.0	9435.7
5	65.2	63.3	9153.7

Crystallization number	"Apiin acetate recovered*	[^{14}C]Compound IV acetate recovered*	Specific activity ($\frac{[^{14}C]\text{Compound IV acetate}}{\text{"apiin" acetate}}$)
	%	%	dpm/mmole
1	69.5	69.1	8919.0
2	51.1	53.1	9211.0
3	60.3	60.3	9270.0
4	41.3	42.7	9576.0

*Percent recovered from the previous recrystallization step.

**The expected specific activity of [^{14}C]Compound IV and "apiin" was 18360 dpm/mmole and the expected specific activity of [^{14}C]Compound IV acetate and "apiin" acetate was 9154 dpm/mmole.

recrystallized to constant specific activity. Compound IV and "apiin" were previously recrystallized together five times from 95% ethanol. The acetate derivatives of these compounds were prepared by the procedures of Gupta and Seshadri (45). Then the mixture of "apiin" acetate and Compound IV acetate was recrystallized to constant specific activity. This was accomplished by dissolving 5405 dpm of acetylated Compound IV containing 0.66 mmoles of "apiin" acetate in 99 ml acetone (analytical grade). This solution was stirred with heating until the precipitate dissolved. Further heating resulted in solvent evaporation. Just as a precipitate began to form the solution was cooled 5 minutes at 22°, the precipitate collected and air dried.

A summary of the recrystallization data involving Compound IV and "apiin" acetate are presented in Table I. The data show that the ratio (dpm/mmole) of Compound IV to "apiin" does not change significantly. The ratio of the acetate derivative of Compound IV and the acetate derivative of "apiin" also does not change significantly during recrystallization.

DISTRIBUTION OF APIIN SYNTHASE IN THE LEAVES, STEMS AND ROOTS OF PARSLEY

The distribution of apiin synthase activity was measured in the leaves, roots and stems of several mature parsley plants which were examined for activity following the extraction procedure described below for leaves. A summary of the analyses for apiin synthase activity is presented in Table II. The leaves are the best source of

apiin synthase activity as they yield more total activity as measured in the assay used (Table II), although some activity is observed in the stems and less in the roots. The enzyme isolated from stems had 2.5 times higher specific activity than the enzyme isolated from the leaves and roots.

TABLE II. Distribution of apiin synthase in the leaves, stems and roots of parsley.

Tissue	Total protein / 100 gm tissue	Specific Activity*	Total activity / 100 gm tissue
	mg	milliunits/mg protein	milliunits
Leaf	1050	0.020	21.07
Stem	147	0.051	7.45
Root	398	0.014	5.60

*Apiin synthase was measured in the standard assay with 0.2 nmoles UDP-[U-^{14}C]glucuronic acid (110,000 dpm) as substrate.

PURIFICATION OF APIIN SYNTHASE

A typical purification of apiin synthase is detailed below and summarized in Table III. All procedures were carried out at 0-5° unless otherwise stated.

Extraction. -- Parsley leaves from mature parsley plants were diced and 300 gms of diced leaves were homogenized in a Waring Blendor for 2 minutes with 660 ml of 0.1 M sodium phosphate, pH 8.0, 1 x 10^{-4} M EDTA and 1 x 10^{-2} M β-mercaptoethanol. The resulting slurry was squeezed through four layers of cheesecloth and centrifuged for 20 minutes at 14,500 x g. Apiin synthase was measured in the standard assay.

Ammonium sulfate. -- Ammonium sulfate (105.1 gm) was added to the supernatant material from the extraction step (641 ml) to bring the saturation to 30%. The solution was stirred 5 minutes and centrifuged at 14,500 x g for 20 minutes. Ammonium sulfate (75.2 gm) was added to this supernatant to bring the saturation to 48%, stirred 5 minutes and centrifuged at 10,000 x g for 10 minutes. The precipitate was taken up in about 25 ml of buffer to give a final volume of 50 ml. Apiin synthase was measured in the standard assay mixture after dialysis of 1 ml of the dissolved precipitate against 250 ml of buffer for 1 hour. Then it was dialyzed 2 more times against 250 ml of buffer for one hour until the ammonium sulfate was removed from the dissolved precipitate.

Sephadex G-100. -- A column of defined Sephadex G-100, 65 cm in height x 5 cm in diameter, was prepared and equilibrated with 0.01 M sodium phosphate, pH 7.4 containing 1×10^{-4} M EDTA and 1×10^{-2} M β-mercaptoethanol. The dissolved precipitate from the previous step was applied to the column and eluted with the above buffer. The column was eluted at 1.0 ml/minute and fractions of 12.2 mls were collected. The void volume began with fraction number 29. Most of the apiin synthase eluted into 9 tubes, numbers 42 - 50, which were combined and used below. (Fig. 2). Apiin synthase was assayed in the standard assay.

DEAE-Sephadex. -- A column 10.0 cm in height and 2.2 cm in diameter was prepared from defined DEAE-Sephadex. The DEAE-Sephadex had been pretreated in a beaker by washing as

follows: (1) with 0.1 M sodium phosphate, pH 7.4 until the pH remained at 7.4, (2) 4 times with 200 ml of 0.01 M sodium phosphate, pH 7.4, containing 1×10^{-4} M EDTA and 1×10^{-2} M β-mercaptoethanol. After the column was equilibrated with 500 ml of the above 0.01 M buffer, the material from the Sephadex G-100 step was applied. After application of the eluant from the Sephadex G-100 step containing apiin synthase the column was washed with 60 ml of 0.1 M NaCl in buffer. Apiin synthase was then eluted by increasing linearly the NaCl concentration. The linear gradient was set up with 200 ml of 0.3 M NaCl in buffer being gradually added to 200 ml of 0.1 M NaCl in buffer in the mixing flask. Fraction of 9.2 ml were collected. Almost all of the parsley apiin synthase eluted into 3 tubes, numbers 13-15, which were combined and concentrated to about 1.0 ml of ultrafiltration in a Diaflo cell (Fig. 3.). After dialysis for 2 hours against buffer, 1.0 ml of glycerol was added. Enzyme prepared in this manner constitutes the enzyme characterized below. Apiin synthase was purified 45 fold by this procedure and was almost free of UDPGA cyclase. Table III shows that more than 99% of the UDPGA cyclase activity in the original cell-free extract had been removed. Apiin synthase was assayed with the standard assay in all four steps.

TABLE III. Purification of apiin synthase from parsley leaves.

Step	Specific activity	Total protein	Purification	Yield	UDPGA cyclase* / Apiin synthase
	milliunits / mg of protein	mg	fold	%	
Extraction	0.08	3778	1.0	100	1.0
Ammonium sulfate**	0.07	1397	0.9	33	0.97
Sephadex G-100	1.09	80	13.3	28	0.41
DEAE-Sephadex***	3.73	5	45.7	6	0.06

*The ratio of the specific activity of UDPGA cyclase to the specific activity of apiin synthase was each set equal to 1 in the extraction step. The subsequent ratio are relative to 1.

**Activity was measured on a small portion after it was dialyzed against buffer.

***Activity was measured after concentration by ultrafiltration and dialysis as described in the text.

Fig. 2. The elution pattern of UDPGA cyclase and apiin synthase from Sephadex G-100. The ammonium sulfate fraction containing apiin synthase (■·····■·····■) and parsley UDPGA cyclase (o——o——o) was applied to the Sephadex G-100 column as described for the Sephadex G-100 step. The enzymes were eluted with buffer at 1.0 ml/minute and 12.2 ml fractions were collected. The amount of protein in each fraction was measured (●——●——●).

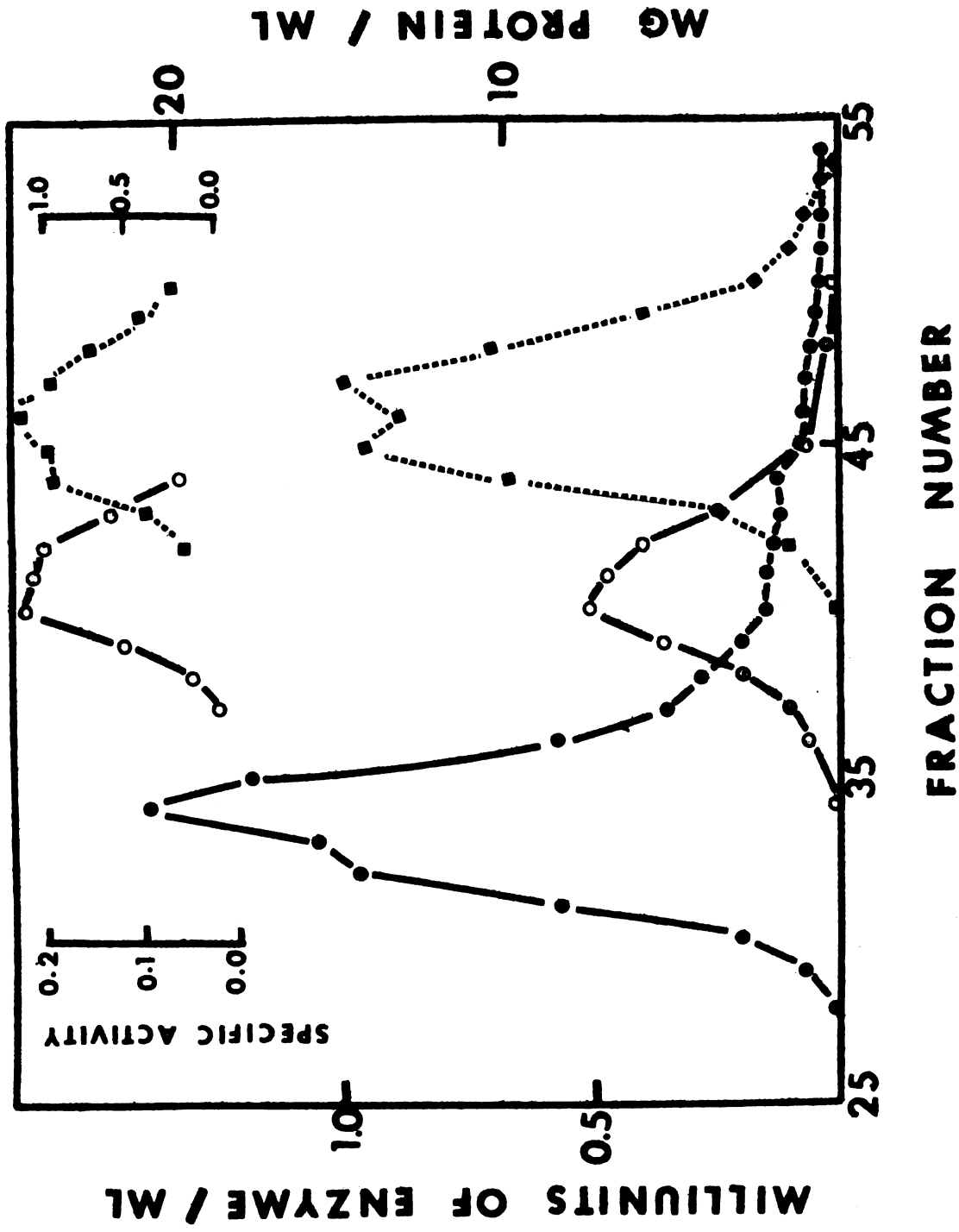
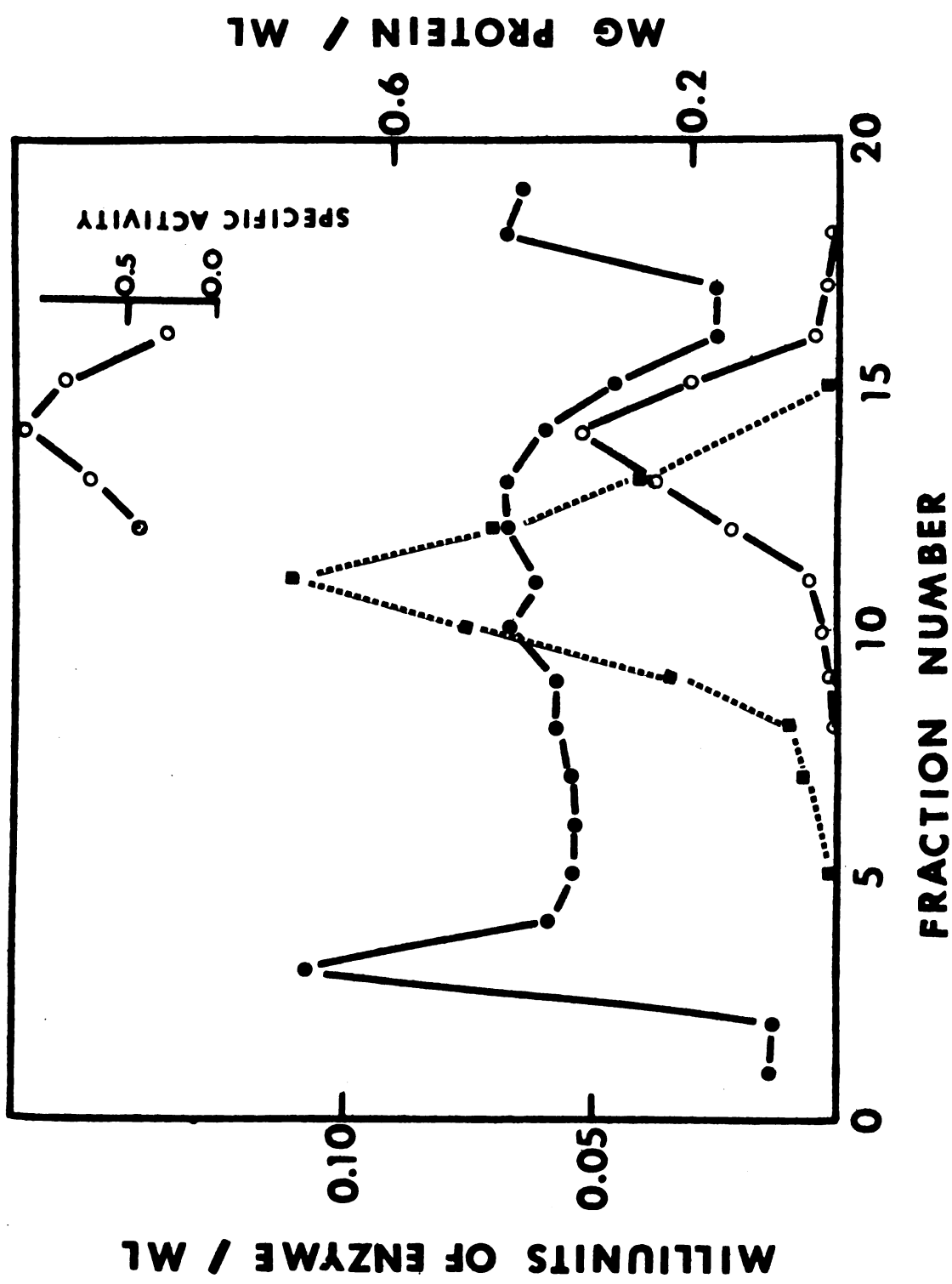

MG PROTEIN / ML
20
10
1.0
0.5
0.0
55
45
35
25
FRACTION NUMBER
0.2
0.1
0.0
SPECIFIC ACTIVITY
1.0
0.5
MILLIUNITS OF ENZYME / ML

Fig. 3. Chromatography of apiin synthase and UDPGA cyclase on DEAE-Sephadex. The Sephadex G-100 fractions containing apiin synthase (■·····■·····■) and some parsley UDPGA cyclase (o—o—o) were applied to the DEAE-Sephadex column as described for the DEAE-Sephadex step. Protein was measured (●———●———●) by the procedure of Lowry, Rosebrough, Farr and Randall (59) after concentration by ultrafiltration and dialysis. Fractions were collected starting with the application of the linear gradient described in the text. Fraction number 1 is the first one collected after application of the gradient. No UDPGA cyclase or apiin synthase was observed in the solutions collected before application of the gradient. Protein in fractions 1-5 and 18-20 was measured by absorbance at 280/260.


MG PROTEIN / ML
0.6
0.2
SPECIFIC ACTIVITY
0.5
0.0
20
15
10
5
0
FRACTION NUMBER
0.10
0.05
MILLIUNITS OF ENZYME / ML

PROPERTIES OF APIIN SYNTHASE

Linearity of the Apiin Synthase Reaction. -- The data in Fig. 4 (standard assay), Fig. 6 (standard assay) and Fig. 8 (modified standard assay) show that the transfer of D-apiose from UDP-apiose to "7GA" was linear with time for 5 minutes. The data in Fig. 5 (standard assay), Fig. 7 (standard assay) and Fig. 9 (modified standard assay) show that the reaction catalyzed by apiin synthase is linear with increasing concentrations of apiin synthase over the range of apiin synthase measured. The rate of apiin formation was directly proportional to the amount of apiin synthase present. Crude preparations of apiin synthase such as described for the extraction and ammonium sulfate step were almost linear with respect to enzyme concentration. However, some apparent inhibition of the reaction was noted particularly at higher concentrations of enzyme which was removed by passage through Sephadex G-25 or G-100. This treatment removes small molecular weight compounds which probably include some of the large quantity of the product, apiin, present in the cell-free extract described in the extraction step and the ammonium sulfate step.

Factors Affecting the Stability of Apiin Synthase. -- Stability of the enzyme to storage largely depended on the protein concentration, temperature and the addition of glycerol. When the enzyme was mixed with an equal volume of glycerol and stored at -20° good stability was achieved. Enzyme from the Sephadex G-100 step stored at 4° (4 mg/ml) for 12 hours lost almost all activity (> 95%). Addition of glycerol to make the solution 50% glycerol by volume preserved enzymatic activity

Fig. 4. Formation of apiin by apiin synthase as a function of time with low concentrations ($< K_m$) of "7GA". The formation of apiin by apiin synthase was followed using the standard assay. The assay mixture contained 0.6 nmoles of "7GA" in place of the 5.5 nmoles in the standard assay. The reaction was initiated by 0.032 milliunits (1.44 µg of protein) of apiin synthase purified through DEAE-Sephadex step. The standard assay mixture was incubated for the indicated times (1 to 5 minutes) at 25° before termination.

Fig. 5. Effect of apiin synthase concentration on reaction velocity with low concentrations ($< K_m$) of "7GA". The formation of apiin by apiin synthase was followed using the standard assay. The assay mixture contained 0.6 nmoles of "7GA" in place of the 5.5 nmoles in the standard assay. The reaction was initiated by the indicated amounts of apiin synthase purified through the DEAE-Sephadex step (0.0024 milliunits/1.0 µg of protein). The standard assay mixture was incubated for 3 minutes at 25° before termination.

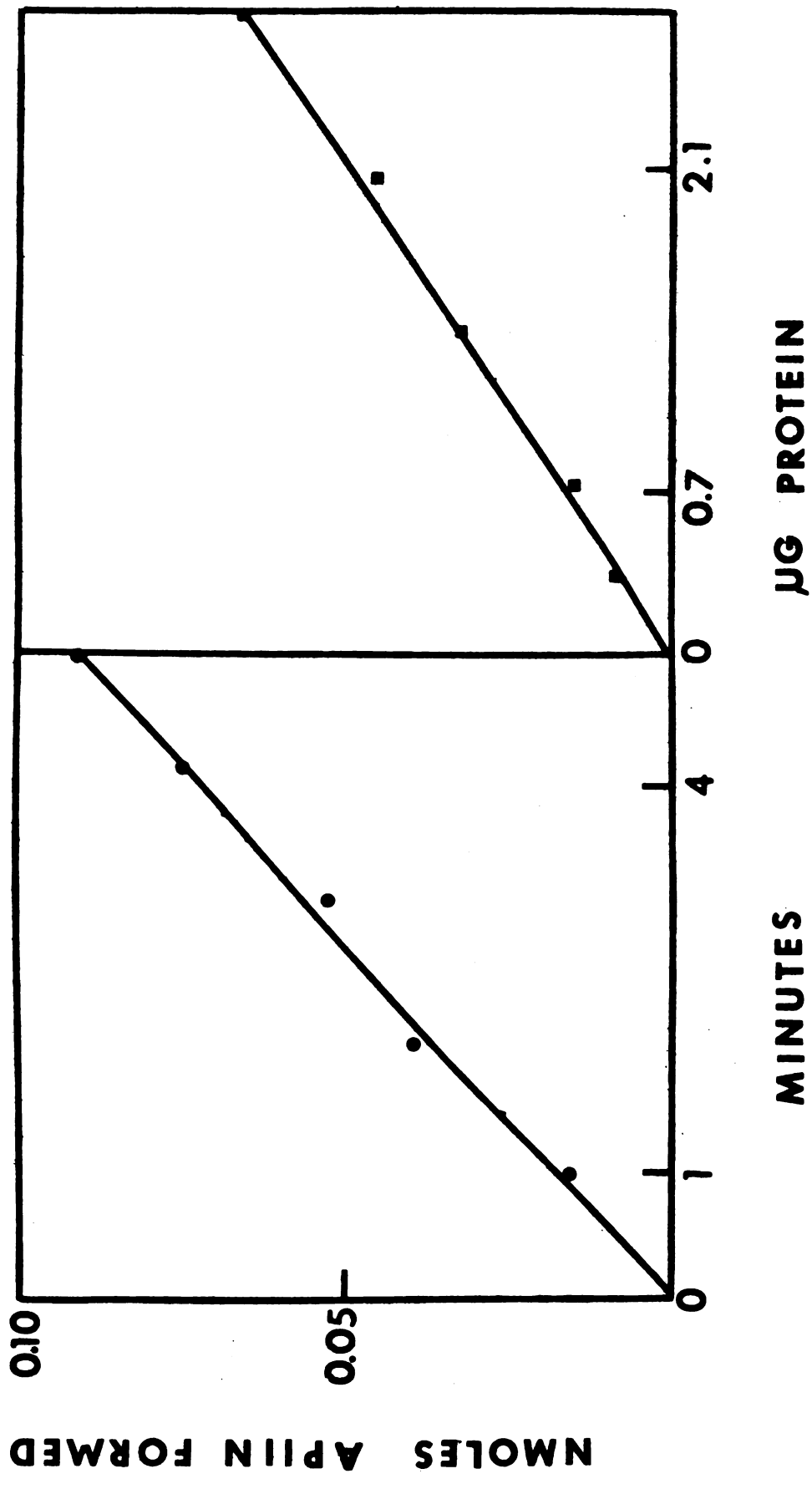

NMOLES APIIN FORMED
0.10
0.05
0
1
4
0
0.7
2.1
MINUTES
µG PROTEIN

Fig. 6. Formation of apiin by apiin synthase as a function of time with low concentrations ($< K_m$) of UDP-apiose. The formation of apiin by apiin synthase was followed using the standard assay except that it contained 0.2 nmoles of UDP-[U-^{14}C]glucuronic acid (100,000 dpm). The reaction initiated by the addition of 0.057 milliunits of apiin synthase (12.5 μg of protein) from the DEAE-Sephadex step. The assay mixtures were incubated for the indicated times at 25° and further treated as described in the Materials and Methods.

Fig. 7. Effect of the amount of apiin synthase on reaction velocity with low concentrations ($< K_m$) of UDP-apiose. The formation of apiin by apiin synthase was followed using the assay described in the legend of Fig. 6. However, the reactions were initiated by the addition of the indicated amounts of apiin synthase from the DEAE-Sephadex step (0.0046 milliunits/μg of protein) and were incubated for 3 minutes at 25° and further treated as described in the Materials and Methods.

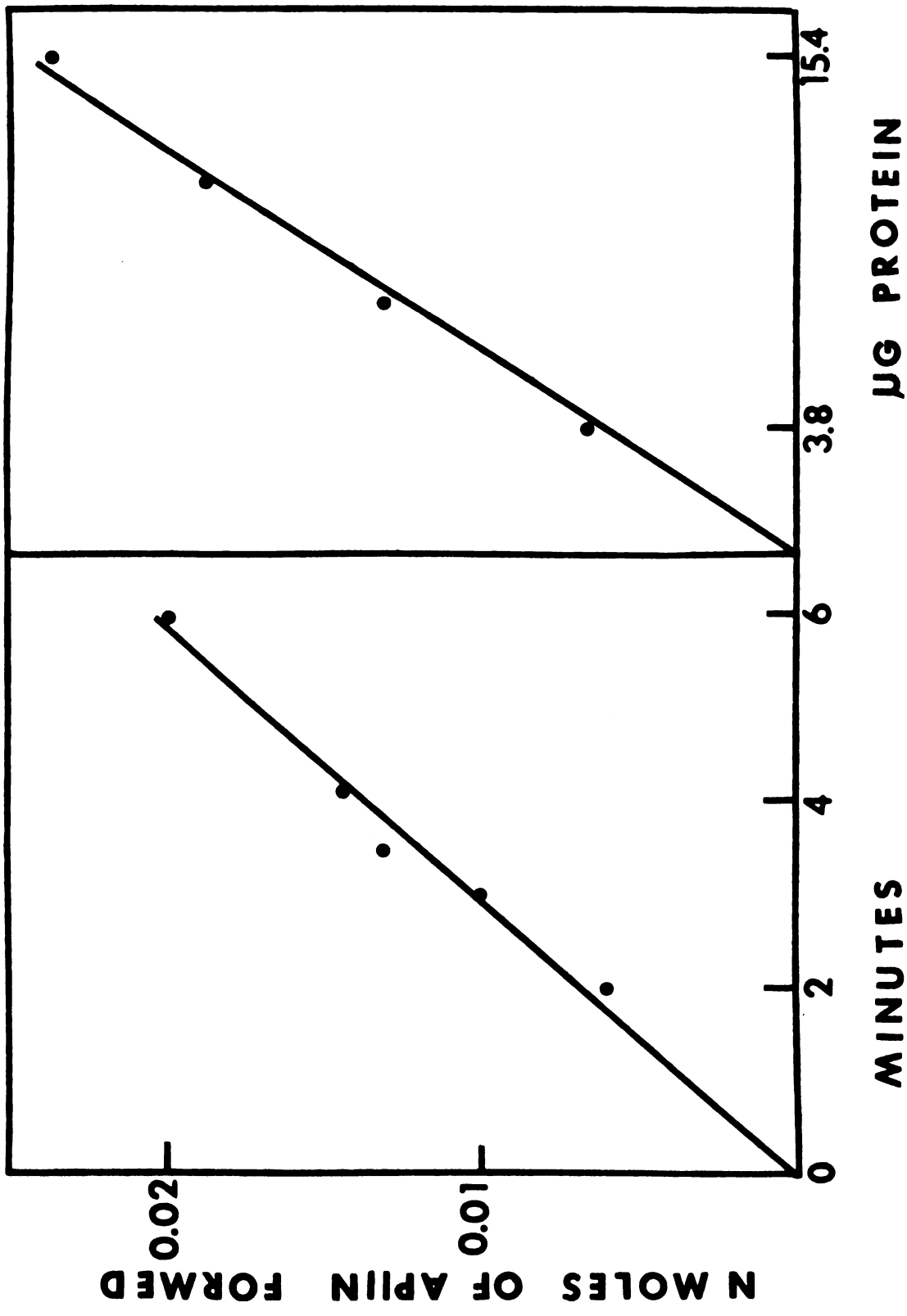
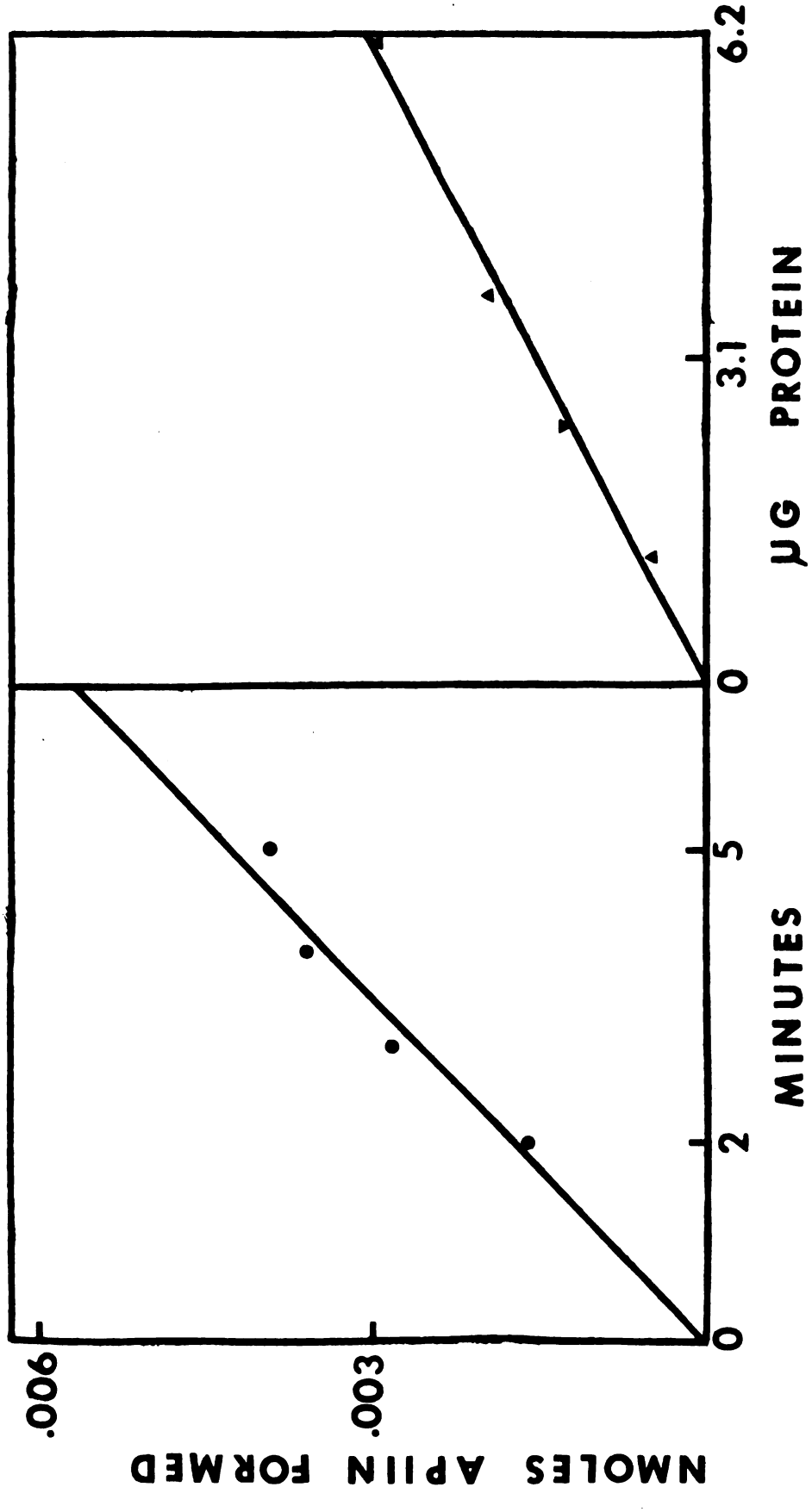

N MOLES OF APLIN FORMED
0.02
0.01
0
2
4
6
MINUTES
3.8
15.4
µG PROTEIN

Fig. 8. Formation of apiin by apiin synthase as a function of time with exogenous UDP-apiose. Apiin synthase was assayed by the modified standard assay described in the Materials and Methods. The reaction was initiated by 0.046 milliunits (6.2 µg of protein) of apiin synthase from the DEAE-Sephadex step. The reaction contained 0.032 nmoles of UDP-[U-^{14}C]apiose (17,300 dpm) and 0.014 nmoles of UDP-[U-^{14}C]xylose (7,500 dpm). The reaction was incubated for 3 minutes at 25° and then heated to 100° for 4 minutes.

Fig. 9. Effect of the concentration of apiin synthase on reaction velocity with exogenous UDP-apiose. Apiin synthase was assayed by the modified standard assay described in the Materials and Methods. The reaction was initiated by the indicated amounts of apiin synthase from the DEAE-Sephadex step (0.0148 milliunits/µg of protein) and contained 0.032 nmoles of UDP-[U-^{14}C]apiose (17,300 dpm) and 0.014 nmoles of UDP-[U-^{14}C]xylose (7,500 dpm). The reaction was incubated for 3 minutes at 25° and then heated to 100° for 4 minutes.


NMOLES APIIN FORMED
.006
.003
0
2
5
MINUTES
0
3.1
6.2
µG PROTEIN

at -20°. Apiin synthase after DEAE-Sephadex step (17.3 mg/ml) increased its apparent activity 30% with addition of an equal volume of glycerol. After 1 week at -20° it still had 112% of the activity observed before the addition of glycerol. Two weeks after adding glycerol only 80% of the original activity remained. The standard assay was used in the above assays.

pH Optimum of Apiin Synthase. -- Apiin synthase has optimum activity between pH 7.6 - 8.4 (Fig. 10). In this range the enzyme has almost the same activity with the two buffers tested. As indicated in Fig. 10 apiin synthase activity on either side of this range was near the optimum value and only gradually decreased in activity with a change in pH.

Affinity of Apiin Synthase for "7GA" and UDP-apiose. -- The K_m for "7GA" was calculated from the data presented in Fig. 11. It is 7.0×10^{-5} M. The K_m for UDP-apiose was calculated from the data presented in Fig. 12. It is 0.6×10^{-5} M.

Effect of Various Ions on the Activity of Apiin Synthase. -- Apiin formation by apiin synthase (from the DEAE-Sephadex step) was measured in the standard assay. The various ions tested were present in the standard assay at 1 mM final concentrations. The compounds tested were NH_4Cl, $FeCl_3$, $CaCl_2$, $MgCl_2$, NaCl, KCl, $NaMoO_3$, $NaBO_3$, $NiCl_2$, $CoCl_2$, $CuCl_2$ and $ZnCl_2$. None of these compounds increased apiin synthase activity. Only the latter four metal ions decreased the enzymatic activity with a decrease of 29% for $NiCl_2$, 40% for $CoCl_2$, 88% for $CuCl_2$ and 95% for $ZnCl_2$. The presence of EDTA, α,α'-dipyridyl, 8-hydroxyquinoline and KF at 1 mM final concentrations in the standard assay mixture had no effect on enzyme activity

Fig. 10. Effect of pH on the reaction velocity of apiin synthase. Incubation mixtures were prepared in a volume of 40 µl containing 2.25 nmoles of UDP-[U-^{14}C]glucuronic acid (88,000 dpm), 50 nmoles of NAD+, 0.8 moles of sodium phosphate, pH 8.0 and 0.2 milliunits of UDPGA cyclase. After incubation at 25° for 60 minutes, 11 nmoles of "7GA" and either 5.6 µmoles of sodium phosphate (▲——▲) or 5.6 µmoles of Tris-HCl (△·····△) buffer were added. The pH of these final solutions was measured in larger assay mixtures which did not contain the UDPGA cyclase, apiin synthase and UDP-[U-^{14}C]glucuronic acid. The reaction was initiated by the addition of 0.012 milliunits of apiin synthase (15 µg of protein) from the DEAE-Sephadex step making a final volume of 100 µl. The assay mixtures were further treated as described in Materials and Methods.

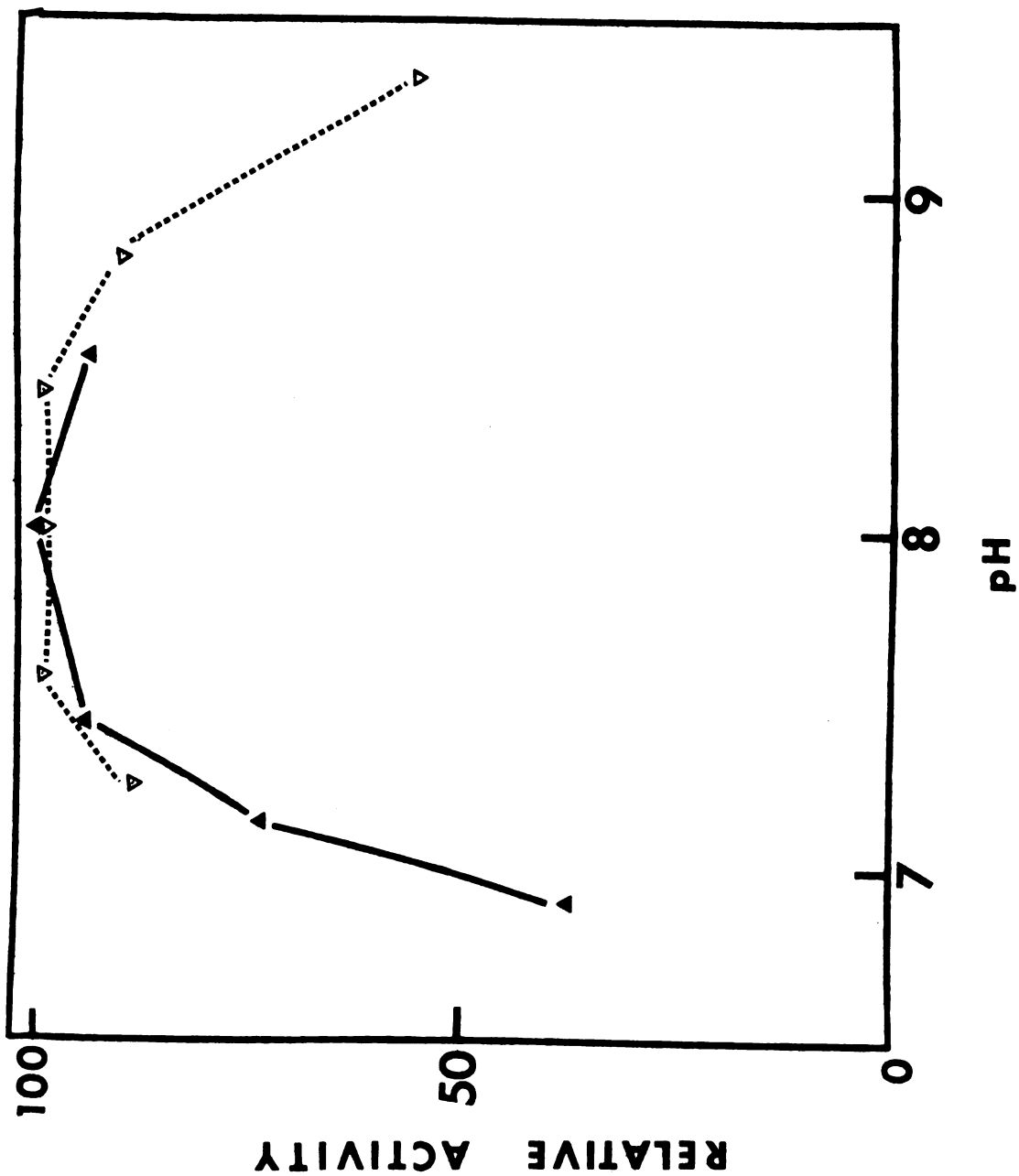

RELATIVE ACTIVITY
100
50
0
6
7
8
pH

Fig. 11. Effect of "7GA" concentration on the velocity of the apiin synthase reaction. The formation of apiin was measured in the standard assay with various concentrations of "7GA" ranging from 0.02 x 10^{-5} M to 9.07 x 10^{-5} M. Each assay contained 1.24 nmoles of UDP-[U-^{14}C]glucuronic acid (90,000 dpm) which was incubated with 0.12 milliunits of duckweed UDPGA cyclase (0.2 mg of protein) 2.5 µmoles of sodium phosphate, pH 8.0, containing 1.2 nmoles of EDTA and 100 nmoles NAD^+. After 15 minutes at 25° the 30 µl reaction mixture contained 0.84 nmoles of UDP-[U-^{14}C]apiose (63,000 dpm), 0.30 nmoles of UDP-[U-^{14}C]xylose (22,500 dpm), 0.1 nmoles of UMP and 0.1 nmoles of α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P (7,500 dpm) in place of the UDP-[U-^{14}C]glucuronic acid previously added to the mixtures. The various amounts of "7GA" were added to the assay mixtures and 0.032 milliunits of apiin synthase from the DEAE-Sephadex step (1.4 µg of protein) was used to initiate the reaction. The assay mixtures were further treated as described in the Materials and Methods. The insert in a Lineweaver-Burk plot of the same data. The K_m was determined by the least squares method using the data shown in the insert.

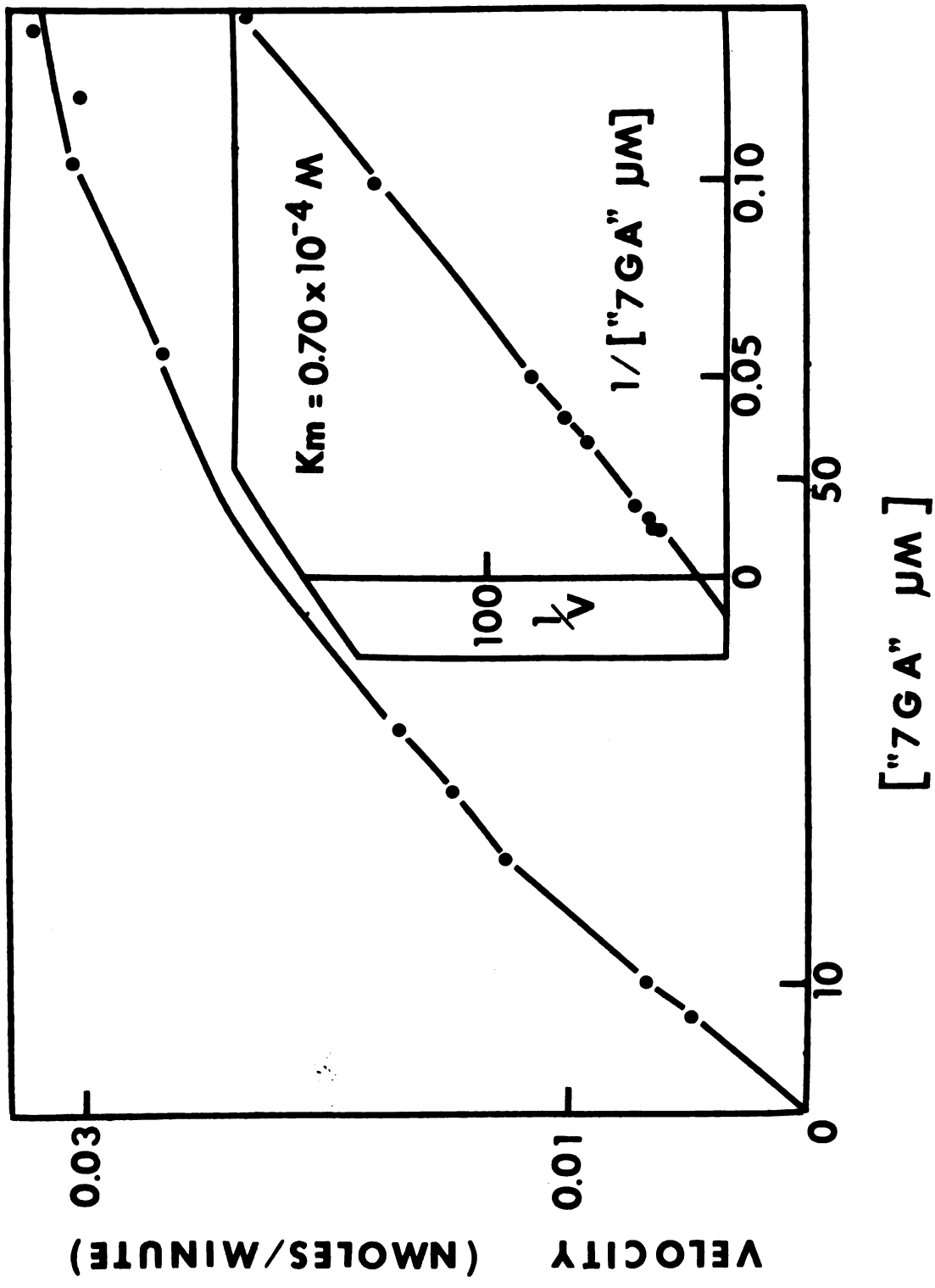
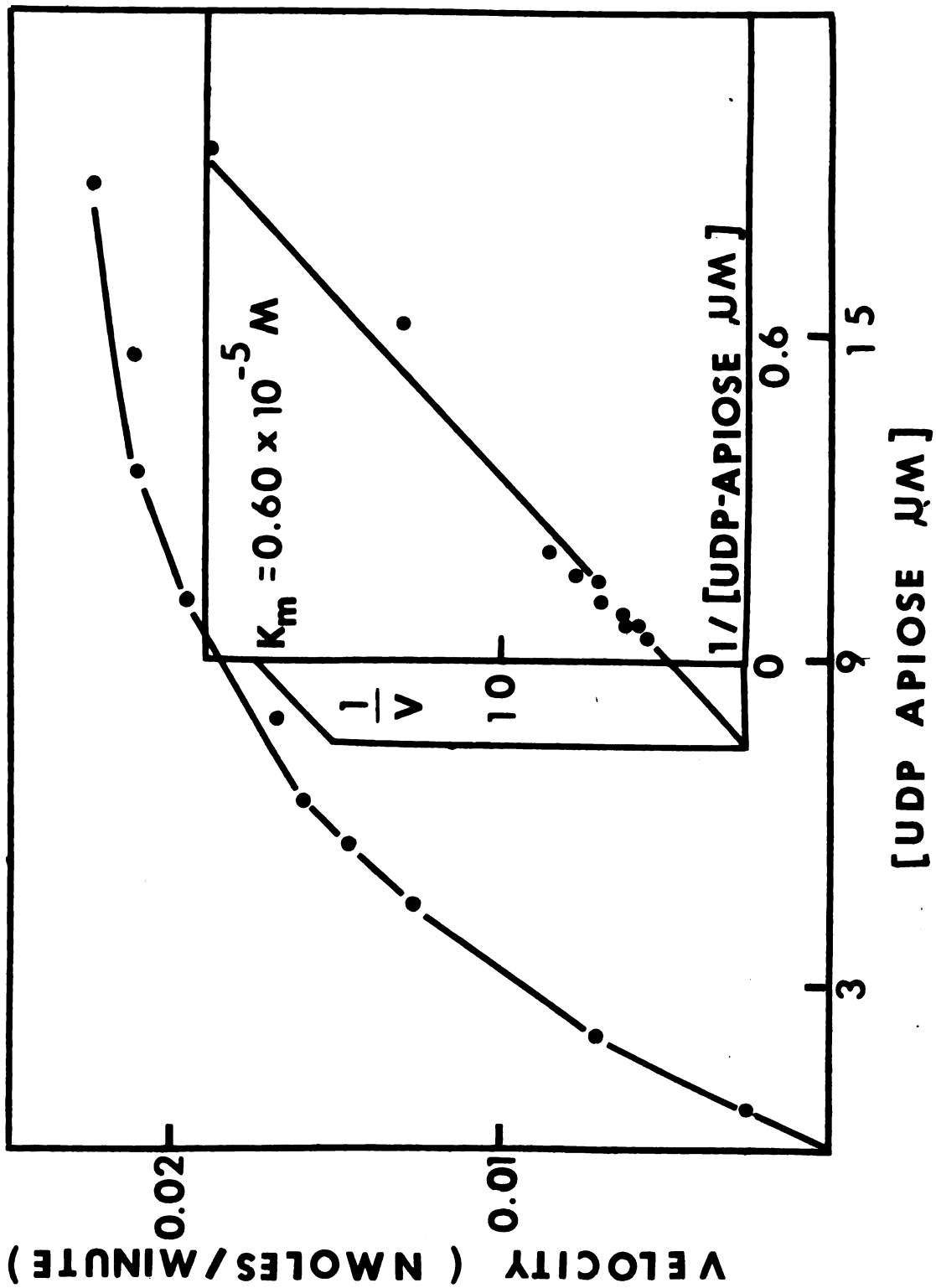

VELOCITY (NMOLES/MINUTE)
0.03
0.01
0
10
50
["7GA" μM]
Km = 0.70 x 10⁻⁴ M
1/["7GA" μM]
0.05
0.10
100
1/v
0

Fig. 12. Effect of UDP-apiose concentration on the velocity of the apiin synthase reaction. The formation of apiin was measured in the standard assay with various concentrations of UDP-apiose ranging from 0.48 x 10^{-6} M to 17.8 x 10^{-6} M. Assay mixtures were prepared as described in the standard assay and UDP-[U-^{14}C]glucuronic acid was added in varying amounts from 0.15 nmoles (66,000 dpm) to 1.58 nmoles (110,000 dpm). After incubation with duckweed UDPGA cyclase the amount of UDP-[U-^{14}C]apiose formed was determined. "7GA" (5.5 nmoles) was added and the reaction was initiated by the addition of 0.023 milliunits of apiin synthase (5.0 µg of protein) from the DEAE-Sephadex step. The reactions were incubated at 25° for 2 minutes. The assay mixtures were further treated as described in the Materials and Methods. The insert is a Lineweaver-Burk plot of the same data. The K_m was determined by the least squares method using the data shown in the insert.


VELOCITY (NMOLES/MINUTE)
0.02
0.01
3
9
15
[UDP APIOSE μM]
0
0.6
1/[UDP-APIOSE μM]
10
1/v
$K_m = 0.60 \times 10^{-5}$ M

except in the case of EDTA which slightly stimulated enzymatic activity (10%). The sodium phosphate buffer containing EDTA in the standard assay mixture was replaced by 2.5 μmole of Tris-HCl buffer, pH 8.0 in the above 17 assays.

Effect of Sulfhydryl Reagents on the Activity of Apiin Synthase. -- Apiin synthase after DEAE-Sephadex step was incubated with 1 mM final concentrations of p-chloromercuribenzoate, N-ethylmaleimide, iodoacetamide and oxidized glutathione in the standard assay mixture. All (100%) of the apiin synthase activity was lost with p-chloromercuribenzoate, 75% with iodoacetamide, 25% with oxidized glutathione and 18% N-ethylmaleimide.

Inhibition of Apiin Synthase Activity by Various Uracil Compounds. -- Enzyme from the DEAE-Sephadex step was assayed by the standard assay in the presence of varying concentrations of uridine, UMP, UDP, UTP, UDP-galactose, UDP-glucuronic acid and UDP-xylose (Table IV). All inhibited apiin synthase to some extent but UMP and UDP inhibited it most at the lower concentrations. UDP-glucuronic acid, UDP-xylose and UTP at 1×10^{-4} M concentrations only decreased the apiin synthase activity about 20%. UDP inhibited this much when present at 1/100th this concentration. UDP-glucuronic acid and UDP-xylose at 1×10^{-4} M concentrations only decreased the apiin synthase activity about 10% from that obtained with 1×10^{-5} M and 1×10^{-6} M concentrations of these compounds. Based upon these results it was concluded that UDP-xylose formed with the UDP-apiose in the standard assay (1.5×10^{-5} M) or added with exogenous UDP-apiose in the modified standard assay (0.6×10^{-6} M) probably does not affect significantly the

reaction of apiin synthase at the low concentrations present in the assays. The formation of UDP occurs as the apiin synthase reaction progresses resulting in the appearance in the standard assay of not more than a concentration of 0.5×10^{-6} M UDP at the end of a 5 minute assay. A small amount of UMP would also be formed from the intramolecular phosphorylation of UDP-apiose in the standard assay resulting in not more than 2×10^{-6} M UMP at the end of a 5 minute assay for apiin synthase. From the data presented in Table IV inhibition by the UDP formed in the assay should be less than 6% of the optimum rate. Inhibition of apiin synthase by the small amount of UMP formed as a breakdown product during the formation of UDP-apiose in the standard assay would be less than 10% of the optimum rate.

Fractionation of Apiin Synthase with Ammonium Sulfate. -- During the purification of apiin synthase, it was observed that most of the UDPGA cyclase isolated by the procedure described in the extraction step was removed during the purification of apiin synthase. The same extraction step was used to prepare UDPGA cyclase and most, but not all of the apiin synthase present, is removed during the subsequent purification step (Part 4). Since some UDPGA cyclase is not removed, apiin synthase was fractionated with ammonium sulfate to attempt to isolate apiin synthase without contaminating UDPGA cyclase. The fractions in which apiin synthase was found are reported below. The same fractions contained UDPGA cyclase and UDPGA decarboxylase activity and are reported in Part 4. Apiin synthase was isolated from parsley leaves as described

TABLE IV. Inhibition of apiin synthase by various uracil compounds.

Compound	Apiin synthase activity* obtained with the following concentrations of selected uracil compounds.			
	1×10^{-6} M	1×10^{-5} M	1×10^{-4} M	1×10^{-3} M
UTP	102	84	81	40
UDP	87	67	22	15
UMP	90	53	25	7
Uridine	61	68	69	35
UDP-glucuronic acid	92	87	80	57
UDP-xylose	91	91	82	42
UDP-galactose	87	87	57	39

*Activity is compared to the apiin synthase activity obtained when none of the above uracil compounds was added to the standard assay mixture which was given the value of 100.

TABLE V: Fractionation of apiin synthase with ammonium sulfate.

Step		Total protein	Specific activity	Total activity
molarity of ammonium sulfate	(% saturation of ammonium sulfate at 4°)	mg	milliunits / mg of protein	total milliunits
1.25→1.50	(29.5-34.5)	10	0.270	2.7
1.50→1.65	(34.5-39.0)	20	0.220	4.2
1.65→1.80	(39.0-42.5)	131	0.056	7.4
1.80→1.95	(42.5-46.1)	632	0.023	14.6
1.95→2.10	(46.1-49.7)	80	0.060	4.8
2.10→2.60	(49.7-61.5)	176	0.061	10.8

*Apiin synthase activity was measured in the standard assay with 1.05 nmoles of UDP-[U-^{14}C]glucuronic acid.

above in the extraction step. The cell-free extract was made 1.25 M in ammonium sulfate, stirred 5 minutes and centrifuged at 14,500 x g for 20 minutes. The supernatant was fractionated by the addition of 3.75 M ammonium sulfate in 0.1 M sodium phosphate buffer, pH 8.0, 1×10^{-4} M EDTA and 1×10^{-2} M β-mercaptoethanol. It was stirred 10 minutes and centrifuged at 10,000 x g for 10 minutes. The precipitate was collected and redissolved in 0.01 M sodium phosphate, pH 7.4, 1×10^{-4} M EDTA and 1×10^{-2} M β-mercaptoethanol. More ammonium sulfate was added to the supernatant and the process was repeated until 6 precipitates were collected. Each precipitate was dialyzed for 4 hours against buffer. After 4 hours dialysis all of the ammonium sulfate had been removed. The amount of ammonium entering the dialysis solution from the dissolved precipitate during dialysis was measured with the Nesseler's reagent (80). A summary of the analyses for apiin synthase activity in the various fractions is presented in Table V. Most of the protein fractions contained apiin synthase. They also contained UDPGA cyclase and UDPGA decarboxylase (Part 4). However, the last two fractions contained 35% of the total apiin synthase activity isolated in the extraction step and did not contain any UDPGA cyclase activity. These fractions contained only 4% of the UDPGA decarboxylase present in the extraction step (Part 4).

DISCUSSION

An important consideration in the purification of apiin synthase was the maintenance of the enzyme in dilute concentrations for minimal time periods. In the example given in Table I, little activity was lost on passage through a Sephadex G-100 column. Subsequent passage through a Sephadex G-200 column further purified apiin synthase by removing more UDPGA cyclase, however, some (64%) of the activity was lost. The Sephadex G-100 step has consistently given an appreciable purification with very little loss of activity. Most (98%) of the UDPGA cyclase isolated with apiin synthase in the cell-free extract was removed during purification of apiin synthase. Ammonium sulfate fractionation separated 35% of the apiin synthase activity isolated in the extraction step from the UDPGA cyclase activity (Table V).

The site of apiin synthesis has been a matter of some confusion and therefore was investigated. In 1965 Medicino and Picken (13) found in whole parsley plants that 16% of the apiin isolated per gm of tissue was from the roots and 84% was from the leaves. Their isotopic tracer experiments with [^{14}C]$NaHCO_3$, [3-^{14}C]serine and [2-^{14}C]acetate in parsley roots, leaves and whole plants indicated that apiin was exclusively synthesized in the roots. They concluded that apiin synthesis occurred exclusively in the roots and that apiin is transported from the roots to the leaves. Roberts, Shah and Loewus (35) in 1967 incubated [2-^{3}H]myo-inositol in parsley leaves which had been detached from the plant. They found incorporation of ^{3}H into D-apiose of apiin. I

isolated apiin synthase from the roots, stems and leaves of parsley. Only 16.5% of the apiin synthase isolated from equal quantities by weight of these three tissues was obtained from the roots. I also isolated UDPGA cyclase from equal quantities of roots, stems and leaves of parsley. Less than 1% of this enzyme was isolated from the roots of the total UDPGA cyclase isolated from the roots, stems and leaves (Part 4). The intracellular function of this enzyme is to form UDP-apiose, a substrate for apiin synthase. These results show that the enzymes catalyzing the final steps of apiin synthesis, the formation of UDP-apiose and the transfer of D-apiose to 7GA, occur primarily in the leaves and stems.

Data presented in this Part and Part 2 show that 7-(4',5',7-trihydroxyflavonyl) β-O-D-glucopyranoside and UDP-apiose are substrates of the reaction catalyzed by apiin synthase.

The minor component of "7GA" in P. crispum according to Nordstrom, Swain and Hamblin (18) is the glucoside of luteolin. The minor component of "7GA" was incubated for an hour with apiin synthase and UDP-[U-^{14}C]apiose. A very small transfer of D-[U-^{14}C]apiose (< 6% of the amount transferred in a similar experiment with the major component of "7GA") occurred, resulting in the formation of a compound with the R_F of the minor component of "apiin". Ortmann, Sandermann and Grisebach (57) tested the minor component of "7GA" isolated from P. hortense in an incubation mixture with UDP-[U-^{14}C]apiose and a cell-free extract isolated from P. hortense. They obtained 9% of the transfer of

D-[U-^{14}C]apiose obtained when 7GA was the substrate. The minor component of "7GA" in P. hortense is reported to be the glucoside of chrysoeriol (57).

In the glycosylation reactions of flavonoids reported by Barber (54) a requirement for ATP in the reactions was noted. He suggested the reaction required ATP in order to prevent the degradation of the sugar nucleotides involved in the transglycosylation reactions by enzymes present in his crude extract from mung bean. $MgCl_2$ was also included in his incubation mixtures although it did not stimulate his reactions and its removal did increase the formation of product slightly. The apiin synthase reaction was not stimulated by the addition of $MgCl_2$ or UTP.

The products of the reaction catalyzed by apiin synthase are apiin and UDP. The formation of apiin was shown by paper chromatography, recrystallization to a constant specific activity and other data presented in this study. [^{3}H]UDP was isolated and shown to be a product of the reaction when [^{3}H]UDP-[U-^{14}C]apiose was a substrate in the reaction catalyzed by apiin synthase (Part 2). The intracellular function of apiin synthase appears to be the transfer of D-apiose to form apiin. The low K_m for 7GA and UDP-apiose suggest that they are the substrates for apiin synthase. Ortmann, Sandermann and Grisebach recently reported that they would present data to show that 7GA had been formed from 4',5,7-trihydroxyflavone (apigenin) and UDP-glucose with a cell-free extract isolated from parsley (P. hortense) (57). The other substrate of apiin synthase, UDP-apiose, is formed in

parsley from UDP-glucuronic acid (Part 4).

Consistently 2 to 3 times as many total units of apiin synthase as total units of UDPGA cyclase (Part 4) were isolated from parsley in the extraction step.

It may be of physiological significance that UDP inhibits (13%) apiin synthase at concentrations of 1×10^{-6} M. For apiin formation to continue at high rate, UDP must be removed by resynthesis to UTP and UDP-sugars or in some other way at a sufficient rate to prevent accumulation of UDP. The UDP-sugars tested do not inhibit apiin synthase appreciably. It may also be of physiological significance that UDP inhibits both duckweed UDPGA cyclase (41%) and UDPGA decarboxylase (53%) even at concentrations of 1×10^{-6} M. This suggests that, for transglycosylation reactions using these sugar nucleotides as substrates to proceed at high rates, the UDP formed must be removed by resynthesis to UTP and UDP-sugars. This must occur at a sufficient rate to prevent accumulation of UDP and its inhibition of the formation of the sugar nucleotides like UDP-xylose and UDP-apiose (Part 4). These results agree with conclusions summarized by Horecker (83) who suggests that control of polysaccharide biosynthesis occurs in some organisms either through the regulation of the production of precursor nucleotides or through effects on the activity of transferase enzymes.

Paris, Paris and Fries (23) reported the isolation of a "glucoapiosylapigenin" from Digitalis purpurea (foxglove). I observed a compound which migrated with an R_F of apiin in solvent A was formed by a cell-free extract isolated

from D. purpurea by the procedure described in the extraction step. The protein was isolated as described in the ammonium sulfate step when ammonium sulfate was added to bring the saturation of ammonium sulfate from 0% to 65%. After the precipitate was dissolved in buffer some was passed through a Sephadex G-100 column. Protein which was retarded to the same extent as parsley apiin synthase was isolated and measured. Activity forming the compound with an R_F like apiin was observed in the cell-free extract, the dialyzed ammonium sulfate protein solution and the protein solution after passate through the Sephadex G-100 column. To the latter protein solution "7GA" was added for synthesis of the compound with the R_F of apiin. Although this compound was not further characterized D. purpurea enzyme, which had not been boiled, was required for its synthesis. Boiling the enzyme prevented the reaction from occurring. This experiment as well as others described in this part of the thesis suggest that apiin is formed from UDP-apiose and 7GA. Enzyme catalyzing this reaction has been isolated from 3 sources (P. hortense (57), P. crispum and D. purpurea) which indicates that this reaction represents an important pathway in plants for the biosynthesis of apiin.

In conclusion the data presented in this thesis shows that apiin and UDP are synthesized from 7GA and UDP-apiose by an enzyme partially purified from P. crispum.

PART 4

ISOLATION AND CHARACTERIZATION OF UDPGA CYCLASE FROM *P. crispum*

INTRODUCTION

In Part 2, the enzymatic formation of UDP-apiose and UDP-xylose from UDP-glucuronic acid by duckweed UDPGA cyclase and UDPGA decarboxylase was reported. Both UDP-apiose and UDP-xylose were characterized. The formation of UDP-xylose from UDP-glucuronic acid by enzymes isolated from both plants and animals has been reported several times (77). The enzyme catalyzing the formation of UDP-xylose from UDP-glucuronic acid has the systematic name of UDP-glucuronate carboxy-lyase (EC 4.1.1.35) and has the common name UDPGA decarboxylase (77). The enzyme catalyzing the formation of UDP-apiose from UDP-glucuronic acid has been given the name UDPGA cyclase[1]. The systematic name suggested for this enzyme is UDP-glucuronate carboxy-lyase (cleaving, cyclizing)[1].

Sandermann, Tisue and Grisebach (38) have reported the preparation of a cell-free extract from parsley which catalyzed the conversion of UDP-glucuronic acid to a D-apiose-containing compound. Although not fully characterized this compound was reported to be UDP-apiose. Gustine and Kindel (39) isolated cell-free extracts from both parsley and duckweed which formed UDP-xylose and a compound that was later identified as α-D-apio-D-furanosyl cyclic-1:2-P[2]. The latter compound has been shown to be the product of an intramolecular phosphorylation of UDP-apiose (Part 2). Therefore UDP-apiose was formed by

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[1]Gustine, D. L., Watson, R. R. and Kindel, P. K., manuscript in preparation

[2]Kindel, P. K. and Watson, R. R., manuscript in preparation

these cell-free extracts and non-enzymatically was converted to the cyclic phosphate. Duckweed UDPGA cyclase was partially purified and partially characterized by Gustine (41). A preliminary communication dealing with the characterization of duckweed UDPGA cyclase will appear shortly (29). Two preliminary reports have been presented which dealt primarily with the characterization of UDP-apiose and apiin synthase and also contained some of the information on parsley UDPGA cyclase (42,56). These three reports were taken in part or completely from Part 2, 3 and 4 of this thesis. The partial purification and characterization of parsley UDPGA cyclase is described in this part of the thesis. Some characterization data on parsley UDPGA decarboxylase, duckweed UDPGA cyclase and duckweed UDPGA decarboxylase are also presented. Evidence is presented that the UDPGA cyclase and UDPGA decarboxylase activities from parsley are associated with two different and separable proteins.

MATERIALS AND METHODS

Materials. -- NAD^+ was obtained from Sigma Chemical Co. UDP-[U-^{14}C]glucuronic acid was obtained from New England Nuclear Corp. Sephadex G-100, G-25 and DEAE-Sephadex A50 were obtained from Pharmacia Fine Chemicals, Inc. D-Apiose was isolated from "apiin" (8). Bovine serum albumin was purchased from Research Products Division, Miles Laboratories, Inc. α-D-[U-^{14}C]Apio-D-furanosyl cyclic-1:2-P^2 and UDP-[U-^{14}C]apiose (Part 2) were formed from UDP-[U-^{14}C]glucuronic acid.

Parsley seed (P. crispum, variety moss-curled) was purchased from Ferry-Morse Seed Co., Fulton, Ky. Parsley leaves, roots and stems were obtained from mature plants grown in a greenhouse. UMP, UDP, UTP, UDP-galactose and UDP-xylose were purchased from P-L Biochemicals, Inc. Duckweed UDPGA cyclase and duckweed UDPGA decarboxylase were prepared from L. minor by a modification of the isolation procedure described by Gustine (41). All other materials were from commercial sources. The Diaflo cell and UM-10 Diaflo membranes were purchased from the Amicon Corp.

General Methods. -- Paper chromatography was by the descending technique and was carried out with unwashed Whatman No. 3MM paper at 22° unless otherwise stated. The paper used with solvent D was pretreated with 2.5% neutralized polyethylenimine (46). The following solvents were employed: (A) 95% aqueous ethanol-1.0 M ammonium acetate, pH 7.5 (7:3 v/v), (B) ethyl acetate-H_2O-acetic acid-formic acid (18:4:3:1, v/v), (C) 1-butanol-acetic acid-H_2O (4:1:5, v/v, upper phase) and (D) 0.3 M LiCl. Radioactivity was detected on chromatograms with a Packard radiochromatogram scanner, Model 7201 (Packard Instrument Co.). All other radioactivity measurements were made with a Packard Tri-carb liquid scintillation counter, Model 3310, employing either (A) a scintillation solution made as described by Bray (84) or (B) 2,5-bis[2-(5-tert-butylbenzoxazolyl)-thiophene in reagent grade toluene (4 gm/l). When using solution B the portion of the chromatogram containing a radioactive compound was cut out and completely immersed in solution B in a scintillation vial and counted. The counting efficiencies

with solution A and B were 79 and 60%, respectively. Protein was determined by the biuret method (58) through the ammonium sulfate step. Thereafter it was determined by the Lowry Rosebrough, Farr and Randall procedure (59). Crystalline bovine serum albumin was used as a standard.

Definition of Units. -- A unit of parsley UDPGA cyclase or duckweed UDPGA cyclase is defined as the amount of enzyme required to catalyze the formation of 1 μmole of UDP-apiose per minute at 25° from UDP-glucuronic acid under the conditions of the standard assay. A unit of parsley UDPGA decarboxylase or duckweed UDPGA decarboxylase is defined as the amount of enzyme required to catalyze the formation of 1 μmole of UDP-xylose per minute at 25° from UDP-glucuronic acid under the conditions of the standard assay.

Standard Assays I and II for Parsley UDPGA Cyclase. -- Assay I for parsley UDPGA cyclase is based on the ability of UDP-apiose to undergo an intramolecular phosphorylation. After UDP-[U-^{14}C]apiose was formed it was hydrolyzed at a basic pH to UMP and α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P. The latter compound was then isolated and measured. The standard assay follows. To a 12 ml conical centrifuge tube were added 2.5 μmoles of sodium phosphate buffer pH 8.0, containing 2.5 nmoles of EDTA; 2.2 nmoles of UDP-[U-^{14}C]glucuronic acid (55,000 dpm, D-glucuronic acid portion uniformily labeled within ±15%); 45 nmoles NAD^+ and parsley UDPGA cyclase to make a final volume of 50 μl. The addition of UDPGA cyclase started the reaction. The mixture was incubated at 25° for 5 minutes and heated at 100° for 3 minutes. The protein was

removed by centrifugation and the supernatant was applied to Whatman No. 3MM paper. The protein precipitate was suspended in 100 µl of 70% ethanol and removed again by centrifugation. The supernatant from the ethanol wash was applied on the same 2 cm wide portion of Whatman No. 3MM paper and chromatography was carried out with solvent A. After chromatography the radioactive compounds, including α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P, were located and measured as described in the General Methods.

Assay II for parsley UDPGA cyclase is based upon the acid lability of UDP-apiose at pH 2.0. After UDPGA cyclase catalyzed the formation of UDP-[U-^{14}C]apiose, the sugar nucleotide was hydrolyzed at pH 2.0. The resultant D-[U-^{14}C]apiose was isolated and measured. An assay mixture was prepared as described in Assay I. After the addition of the enzyme to start the reaction, it was incubated at 25° for 5 minutes and the assay mixture was placed in an ice bath (see Fig. 8). Water, 0.1 µmole D-apiose, 0.1 µmole D-xylose and 9 µl of 1 N H_2SO_4 were added to make a final volume of 125 µl. The mixture was heated at 100° for 20 minutes and applied to Whatman No. 3MM paper. Even though no protein precipitate was observed, the conical centrifuge tube was washed with 100 µl of 70% ethanol which was also applied to the same portion of the Whatman No. 3MM paper. After chromatography with solvent B the radioactive portion containing D-[U-^{14}C]apiose was located and measured as described in the General Methods.

Assay I and II were compared by preparing identical assay mixtures and then measuring the parsley UDPGA cyclase activity

by both assays. Assay II measured an average of 94.5% of the parsley UDPGA cyclase activity measured by Assay I. The 94.5% is the average of six experiments whose values were 90.0, 90.1, 91.4, 94.4, 99.9 and 100.8% of the activity measured by Assay I. The 100.0% value of Assay I is the average of six experiments whose values were 94.4, 94.4, 95.7, 102.5, 105.6 and 107.0%. Some of the discrepancy between Assay I and Assay II is due to the small amount of UDP-[U-^{14}C]apiose which phosphorylates intramolecularly to form α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P during the incubation period. This compound is much more stable to acid hydrolysis under these conditions than is UDP-[U-^{14}C]apiose. More than half of the α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P is not hydrolyzed to free D-[U-^{14}C]apiose during the course of the incubation at 100° and pH 2. The amount of α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P which is not hydrolyzed to D-[U-^{14}C]apiose migrates with a different R_F than D-[U-^{14}C]apiose in solvent B. The stable product of partial hydrolysis of α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P, D-[U-^{14}C]apiose-2-phosphate, also migrates with a different R_F than D-[U-^{14}C]apiose in solvent B.[2] For this reason they are not measured and the amount of UDP-[U-^{14}C]apiose measured is less than the amount formed.

Standard Assays I and II for Duckweed UDPGA Cyclase. -- Duckweed UDPGA cyclase was measured as described for parsley UDPGA cyclase in Assays I and II.

Standard Assay for Parsley UDPGA Decarboxylase. -- Parsley UDPGA decarboxylase was measured with Assay II for parsley UDPGA cyclase. The radioactive compound migrating with the

R_F of D-[U-^{14}C]xylose was isolated and measured as described in the General Methods. The same assay was used for measuring radioactive D-[U-^{14}C]apiose and D-[U-^{14}C]xylose.

Standard Assay for Duckweed UDPGA Decarboxylase. -- Duckweed UDPGA decarboxylase was measured as described for parsley UDPGA decarboxylase.

Identification of the Products Formed by Parsley UDPGA Cyclase and Parsley UDPGA Decarboxylase. -- The identification of the two compounds formed by parsley UDPGA cyclase and parsley UDPGA decarboxylase is based upon the comparison of the characteristics of these compounds with the characteristics of UDP-apiose and UDP-xylose formed by duckweed UDPGA cyclase and duckweed UDPGA decarboxylase. The characterization of these later compounds has been described elsewhere (Part 2 and footnote 2).

The products of parsley UDPGA decarboxylase and duckweed UDPGA decarboxylase were both prepared from UDP-[U-^{14}C]glucuronic acid. The products both yielded D-[U-^{14}C]xylose after treatment at 100° for 15 minutes in 0.01 N hydrochloric acid. Degradation under these conditions which results in the liberation of a sugar is characteristic of a sugar nucleotide (62,68). The products of parsley and duckweed UDPGA decarboxylase migrated with the same R_F as UDP-xylose when chromatographed in solvents A, C and D (Part 2). Neither compound functions in the reaction catalyzed by apiin synthase (Part 2). Neither of these compounds phosphorylates intramolecularly after 5 minutes at pH 8.0 and 100°. On the basis of these data the product of the reaction catalyzed by parsley UDPGA

decarboxylase was concluded to be UDP-xylose.

The products of parsley UDPGA cyclase and duckweed UDPGA cyclase were both prepared from UDP-[U-^{14}C]glucuronic acid. The products both yielded D-[U-^{14}C]apiose after treatment at 100° for 15 minutes in 0.01 N hydrochloric acid. After heating at 100° and pH 8.0 the products of parsley and duckweed UDPGA cyclase migrated with the same R_F as authentic α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P when chromatographed in solvents A, C and D. Acid hydrolysis of the compound with an R_F of α-D-[U-^{14}C]apio-D-furanosyl cyclic-1:2-P yielded only D-[U-^{14}C]-apiose. Both products function in the reaction catalyzed by apiin synthase which results in the formation of a compound with an R_F of apiin. Acid hydrolysis of the compound with an R_F of apiin yielded only D-[U-^{14}C]apiose (Part 3). Based on these data UDP-apiose was identified as the product of the reaction catalyzed by parsley UDPGA cyclase.

RESULTS

DISTRIBUTION OF UDPGA CYCLASE IN THE LEAVES, STEMS AND ROOTS OF PARSLEY

The relative amounts of UDPGA cyclase in roots, stems and leaves of mature parsley plants were determined by extraction of protein from these tissues. The extractions were conducted as described in the extraction step of the purification of UDPGA cyclase from parsley leaves. After centrifugation for 20 minutes at 14,500 x g, the supernatant and the precipitate were collected and assayed. A summary of the analyses of the supernatant for UDPGA cyclase is presented in Table I. The

TABLE I. Distribution of UDPGA cyclase in the leaves, stems and roots of parsley.

Tissue	Total protein from 100 gm tissue	Specific activity	Total activity from 100 gm tissue
	mg	$\frac{\text{milliunits}}{\text{mg protein}}$	milliunits
Leaf	1050	0.0089	9.34
Stem	147	0.0102	1.50
Root			
supernatant	398	0.0003	0.11
precipitate*	450	0.0013	0.63

*Precipitate from centrifugation at 14,500 x g for 20 minutes. The precipitate was resuspended in buffer and UDPGA cyclase activity was measured. Most of the material which had precipitated was not protein since it did not react with biuret reagent (58). UDPGA cyclase was measured in Assay I with 0.2 nmoles UDP-[U-^{14}C]glucuronic acid (110,000 dpm) as substrate.

leaves are the best source of UDPGA cyclase activity although some UDPGA cyclase activity is observed in the stems and less in the roots. No UDPGA cyclase was observed in the resuspended precipitate from the stems or leaves.

PURIFICATION OF PARSLEY UDPGA CYCLASE

A typical purification of parsley UDPGA cyclase is detailed below and summarized in Table II. All procedures were carried out at 0-5° unless otherwise stated.

Extraction. -- The extraction step was carried out exactly as described in the purification of apiin synthase in Part 3.

Ammonium Sulfate. -- The ammonium sulfate step was carried out exactly as described in the purification of apiin synthase in Part 3.

Sephadex G-100. -- The Sephadex G-100 step was carried out exactly as described in the purification of apiin synthase in Part 3. Most of the parsley UDPGA cyclase was eluted into 6 tubes, numbers 38-43. Their contents were combined and used in the DEAE-Sephadex step.

DEAE-Sephadex. -- A column 10.0 cm in height and 2.2 cm in diameter was prepared from defined DEAE-Sephadex. The DEAE-Sephadex was treated as follows: (1) with 0.1 M sodium phosphate, pH 7.4 until the pH remained at 7.4 (2) 4 times with 400 ml of 0.01 M sodium phosphate, pH 7.4, containing 1×10^{-4} M EDTA and 1×10^{-2} M β-mercaptoethanol. Just before use the column was equilibrated with 500 ml of the above buffer. Half of the material from the Sephadex G-100 step was applied. After application the column was washed with 60 ml of 0.1 M

NaCl in buffer. UDPGA cyclase was then eluted by increasing the NaCl concentration linearly. The linear gradient was set up with 200 ml of 0.3 M NaCl in buffer in one beaker and 200 ml of 0.1 M NaCl in buffer in the mixing beaker. Fractions of 9.2 ml were collected. Almost all of the parsley UDPGA cyclase eluted into 8 tubes, numbers 23-30, which were combined and concentrated by ultrafiltration to about 1.0 ml. After 2 hours dialysis against buffer 1.0 ml of glycerol was added. Enzyme prepared in this manner constitutes the enzyme characterized below. UDPGA cyclase was purified 93 fold and was almost free from apiin synthase. More than 98% of the apiin synthase present in the cell-free extract (extraction step) was removed from the UDPGA cyclase after purification through the DEAE-Sephadex step. Much of the UDPGA decarboxylase activity present in the cell-free extract was not removed from UDPGA cyclase by purification through the DEAE-Sephadex step (Fig. 4, 10).

Linearity of the Parsley UDPGA Cyclase Reaction. -- The data in Fig. 1 show that the reaction catalyzed by parsley UDPGA cyclase is linear with two different concentrations of UDP-[U-^{14}C]glucuronic acid. It is linear for more than four minutes at the lower concentration and for a longer time at the higher concentration. The data in Fig. 2 show that the parsley UDPGA cyclase reaction velocity increases linearly with increasing concentrations of UDPGA cyclase. When parsley UDPGA cyclase from the ammonium sulfate step was assayed (after dialysis for 3 hours which removed the ammonium sulfate) the formation of UDP-apiose was not completely proportional to

TABLE II. Purification of UDPGA cyclase from parsley leaves.

Step	Specific activity	Total protein	Purification	Yield	Apiin Synthase* / UDPGA Cyclase
	milliunits/mg	mg	fold	%	
Extraction**	0.02	3735	1	100	1.0
Ammonium sulfate***	0.03	1233	1.8	58	0.5
Sephadex G-100	0.23	210	13.8	74	0.135
DEAE-Sephadex****	1.58	9.2	93	23	0.016

*Specific activity of UDPGA cyclase and of apiin synthase were each set equal to 1 at the extraction step.

**UDPGA cyclase was measured in the extraction step as described for Assay I except 0.2 nmoles UDP-glucuronic acid (11,000 dpm) was used because of the low amount of activity per ml. Activity for all four steps is expressed as initial velocity from the equation $V = v(1 + S/K_m)$ where v is the observed velocity and S is the amount of UDP-glucuronic acid. UDPGA cyclase activity in the ammonium sulfate step and the Sephadex G-100 step was measured as described for Assay I except 0.83 nmoles UDP-[U-^{14}C]glucuronic acid (45,000 dpm) was used as substrate. UDPGA cyclase activity in the DEAE-Sephadex step was measured in Assay I.

***UDPGA cyclase activity in the ammonium sulfate step was usually lower than the activity after the Sephadex G-100 step. During dialysis of the small portion of the ammonium sulfate step used for assay purposes some denaturation of protein often took place as evidenced by the appearance of a precipitate of protein.

****Activity was measured after concentration by ultrafiltration and dialysis. Numbers in the DEAE-Sephadex step are given as if all of the UDPGA cyclase in the Sephadex G-100 step had been used in the DEAE-Sephadex step instead of only one-half.

Fig. 1. Formation of UDP-apiose by parsley UDPGA cyclase as a function of time. The formation of UDP-apiose was followed using Assay I with either 1.71 nmoles (55,000 dpm) (●——●) or 0.528 nmoles (55,000 dpm) (o——o) of UDP-[U-^{14}C]glucuronic acid. The reactions were initiated by the addition of 0.08 milliunits of parsley UDPGA cyclase (40.5 μg of protein) from the DEAE-Sephadex step. The reactions were incubated for the indicated time (1 to 5 minutes) and further treated as described for Assay I.

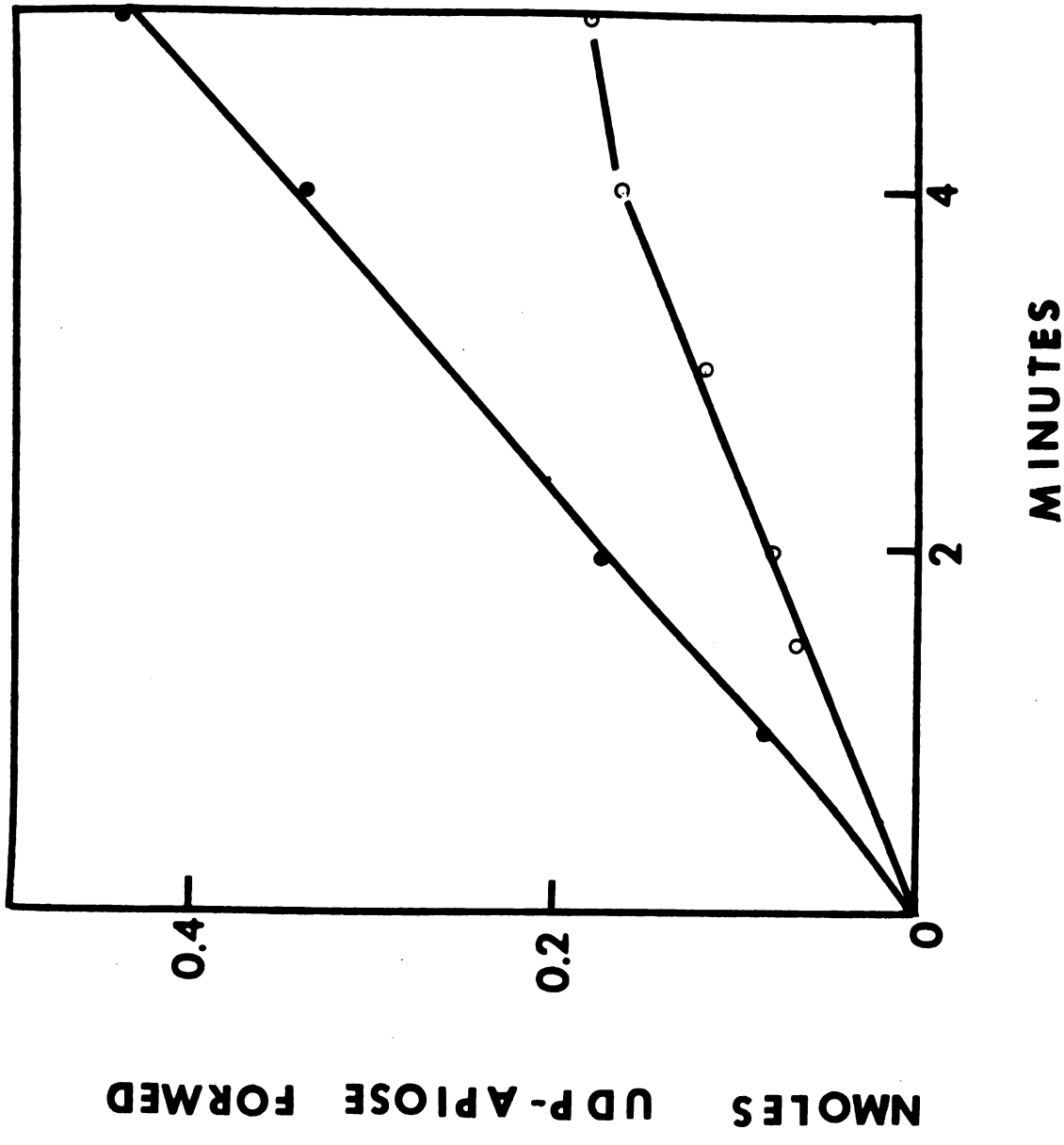
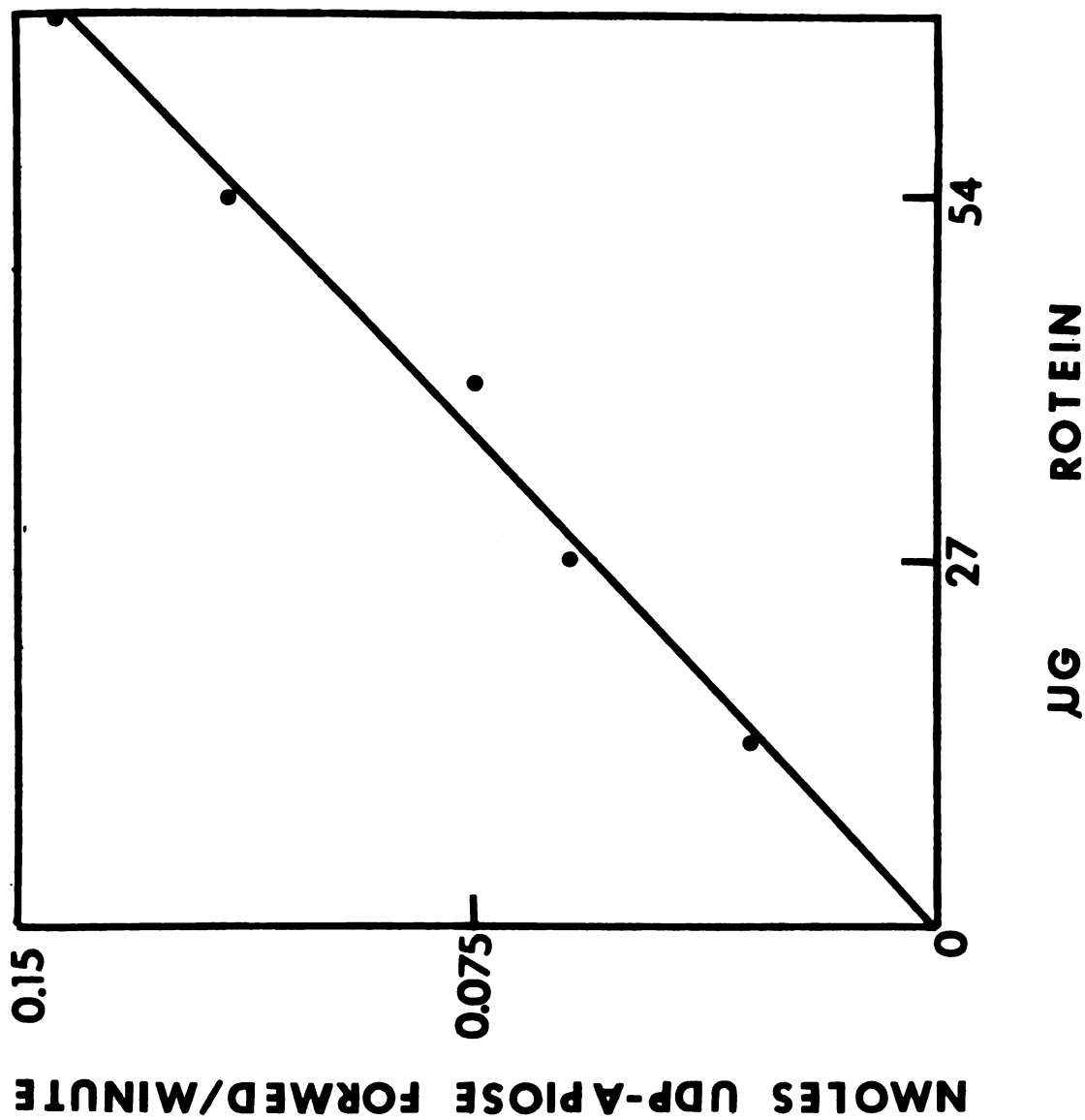

NMOLES UDP-APIOSE FORMED
0.4
0.2
0
2
4
MINUTES

Fig. 2. Effect of parsley UDPGA cyclase concentration on reaction velocity. The formation of UDP-[U-^{14}C]apiose was followed with Assay I. The assays were initiated by the indicated amounts of parsley UDPGA cyclase (2.1 milli-units/mg of protein) from the DEAE-Sephadex step.


NMOLES UDP-APIOSE FORMED/MINUTE
0.15
0.075
0
27
54
JUG ROTEIN

the amount of UDPGA cyclase present. However, after passage through a Sephadex G-25 or G-100 column there was a direct proportionality between the amount of UDPGA cyclase present in the assay and the amount of UDP-apiose formed in a specified time period.

Factors Affecting the Stability of Parsley UDPGA Cyclase. -- Stability of parsley UDPGA cyclase to storage and dialysis largely depended on protein concentration, temperature and addition of glycerol; the higher the protein concentration and the lower the temperature without freezing the extract, the better the stability. When UDPGA cyclase from the Sephadex G-100 step was stored at 4° (4-5 mg of protein/ml) for 12 hours it lost almost all activity. When UDPGA cyclase was concentrated by ultrafiltration and made 50% glycerol by volume enzyme activity was preserved during storage at -20° which did not freeze the solution. This was especially evident in more dilute solutions (0.1 to 10.0 mg of protein/ml). Storage of this material at -20° without glycerol resulted in its freezing. After 24 hours at -20° no enzymatic activity was observed. UDPGA cyclase from the DEAE-Sephadex step (13.4 mg of protein/ml) was made 50% glycerol by volume and stored at -20°. This treatment preserved 98% of the starting activity after 11 days as measured by Assay I and 91% after 19 days. However, when UDPGA cyclase from the DEAE-Sephadex step (10 mg of protein/ml and 30% glycerol by volume) was kept at 4° and 25°, enzymatic activity was rapidly lost. Storage of this material at 25° preserved about 15% more enzyme activity than at 4° throughout a 24 hour period as measured in Assay I. After

7.5 hours at 25° and 4°, 70 and 80%, respectively, of the activity had been lost.

pH Optimum of Parsley UDPGA Cyclase. -- Parsley UDPGA cyclase has optimum activity between pH 8.0 to 8.3 (Fig. 3). In this range the enzyme had the same activity with either of the buffers used. As indicated in Fig. 3 UDPGA cyclase activity decreased rapidly at pH values below pH 8.0 to 8.3.

Affinity of Parsley UDPGA Cyclase for UDP-glucuronic Acid. -- The K_m of parsley UDPGA cyclase for UDP-glucuronic acid was calculated from the data presented in the insert in Fig. 4 by the least squares method. It is 3.3×10^{-6} M.

Effect of Various Ions on the Activity of Parsley UDPGA Cyclase. -- UDPGA cyclase activity was measured in the presence of various ions. The final concentrations of these ions in Assay I was 1 mM. The compounds tested were $FeCl_2$, $FeCl_3$, $CaCl_2$, $MgCl_2$, $MnCl_2$, NaCl, KCl, $NaNoO_3$, $NaBO_3$, $CoCl_2$, NH_4Cl, $ZnCl_2$, $NiCl_2$ and $CuCl_2$. None of the above compounds stimulated enzyme activity and the latter four decreased the enzymatic activity by 30, 65, 70 and 85%, respectively. EDTA, α,α'-dipyridyl and KF at 1 mM final concentrations in Assay I had no effect on parsley UDPGA cyclase activity. The addition of o-phenanthroline and 8-hydroxyquinoline at the same concentration resulted in a slight (6%) decrease in enzyme activity. The effect of the above 19 compounds on the activity of UDPGA cyclase was determined in Assay I with the sodium phosphate buffer replaced by 2.5 μmoles of Tris-HCl, pH 8.0.

Effect of Sulfhydryl Reagents on the Activity of Parsley UDPGA Cyclase. -- UDPGA cyclase was incubated with 1 mM final

Fig. 3. Effect of pH on the reaction rate of parsley UDPGA cyclase. UDPGA cyclase was measured in Assay II with 2.4 μmoles of sodium phosphate buffer (●——●) pH 6.0 to 8.4 or 2.4 μmoles of Tris-HCl buffer (■····· ■) pH 6.0 to 9.0. The reaction in each assay was initiated by the addition of 0.0075 milliunits of parsley UDPGA cyclase (10 μg of protein) from the DEAE-Sephadex step.

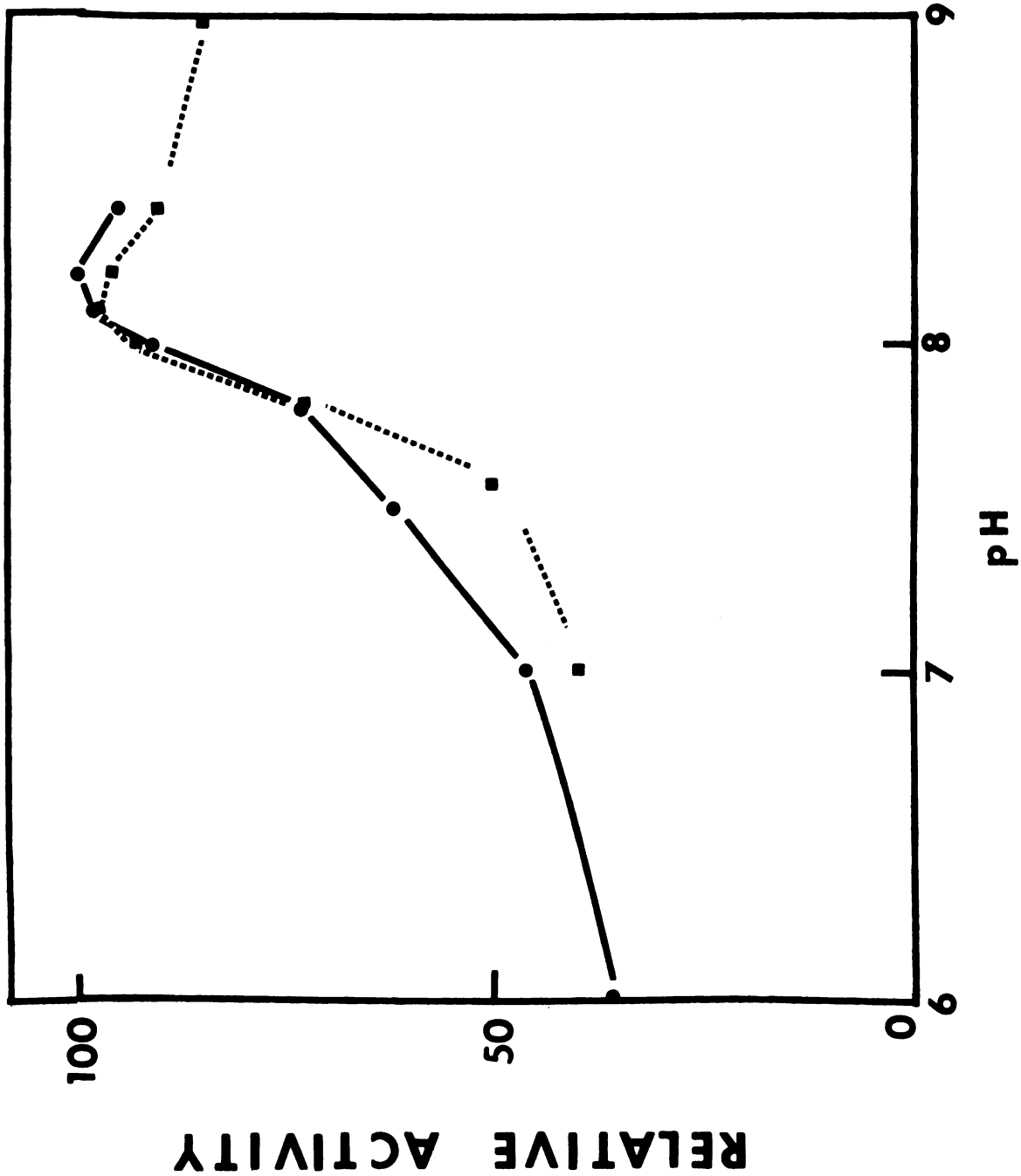
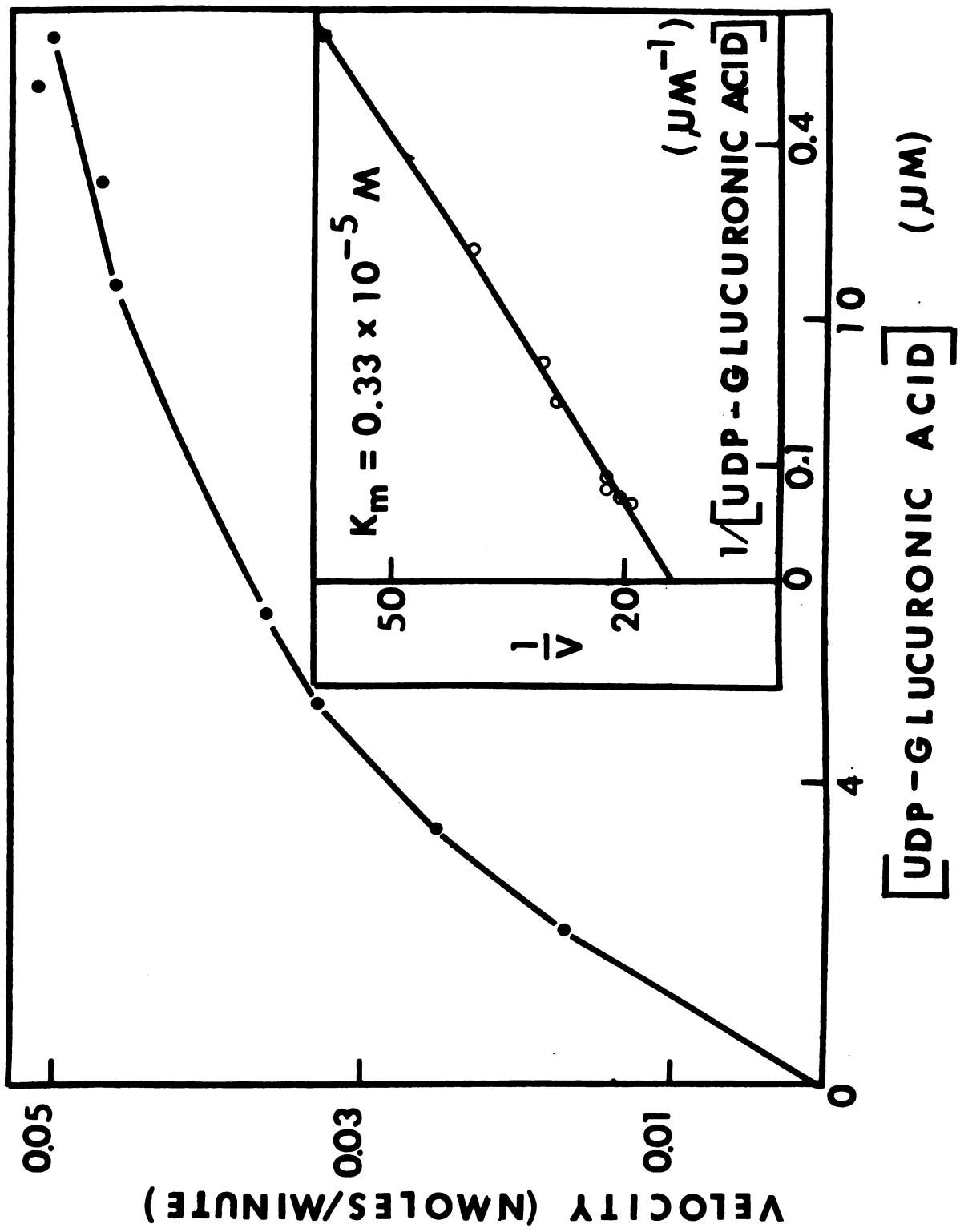

RELATIVE ACTIVITY
100
50
0
6
7
8
9
pH

Fig. 4. Effect of UDP-glucuronic acid concentration on the velocity of the parsley UDPGA cyclase reaction. The assay conditions were those described in Assay I except that the UDP-glucuronic acid concentration was varied as indicated. Each assay was initiated by the addition of 0.056 milliunits of parsley UDPGA cyclase (40.5 μg of protein) from the DEAE-Sephadex step. The insert is a Lineweaver-Burk plot of the same data.


VELOCITY (NMOLES/MINUTE)
0.05
0.03
0.01
0
4
10
[UDP-GLUCURONIC ACID] (μM)
$K_m = 0.33 \times 10^{-5}$ M
$\frac{1}{V}$
50
20
0
0.1
0.4
1/[UDP-GLUCURONIC ACID] (μM^{-1})

concentrations of p-chloromercuribenzoate, N-ethylmaleimide, iodoacetamide and oxidized glutathione in Assay I. More than 90% of the activity was lost with p-chloromercuribenzoate and N-ethylmaleimide (99 and 94%), 15% with iodoacetamide and 8% with oxidized glutathione.

Determination of Optimum NAD^+ Concentration for the Formation of UDP-Apiose. -- Present with the parsley UDPGA cyclase from the DEAE-Sephadex step was some of the parsley UDPGA decarboxylase present in the cell-free extract of the extraction step. The data in Fig. 5 show that parsley UDPGA cyclase has an absolute requirement for exogenously supplied NAD^+. No UDP-apiose is formed in the absence of exogenous NAD^+. The NAD^+ concentration for optimum UDP-apiose formation was 1.0 to 2.0 x 10^{-3} M.

Inhibition of Parsley UDPGA Decarboxylase and Parsley UDPGA Cyclase by UDP-Xylose. -- The activity of parsley UDPGA cyclase and parsley UDPGA decarboxylase were measured with Assay II in the presence of various concentrations of UDP-xylose (Table III). UDPGA decarboxylase activity was inhibited more at high concentrations of UDP-xylose than UDPGA cyclase activity. UDPGA cyclase activity was slightly stimulated at lower concentrations of UDP-xylose.

The Energy of Activation of Parsley UDPGA Cyclase. -- The energy of activation (E_a) was determined from the equation (85):

$$E = \text{slope} \times 2.303\ (R)$$

The value for the slope was obtained from Fig. 6. R equals 1.987 cal^{-1} (K^o) $(g\text{-mole})^{-1}$. E_a is 12.5 Kcal.

Fig. 5. Effect of NAD^+ concentration on parsley UDPGA cyclase reaction velocity. The formation of UDP-[U-^{14}C]-apiose was followed with Assay I. The reaction in each assay was initiated by the addition of 0.0085 milliunits of parsley UDPGA cyclase (12 μg of protein) from the DEAE-Sephadex step to each assay mixture containing the indicated amounts of NAD^+ (0.0 to 500 nmoles).

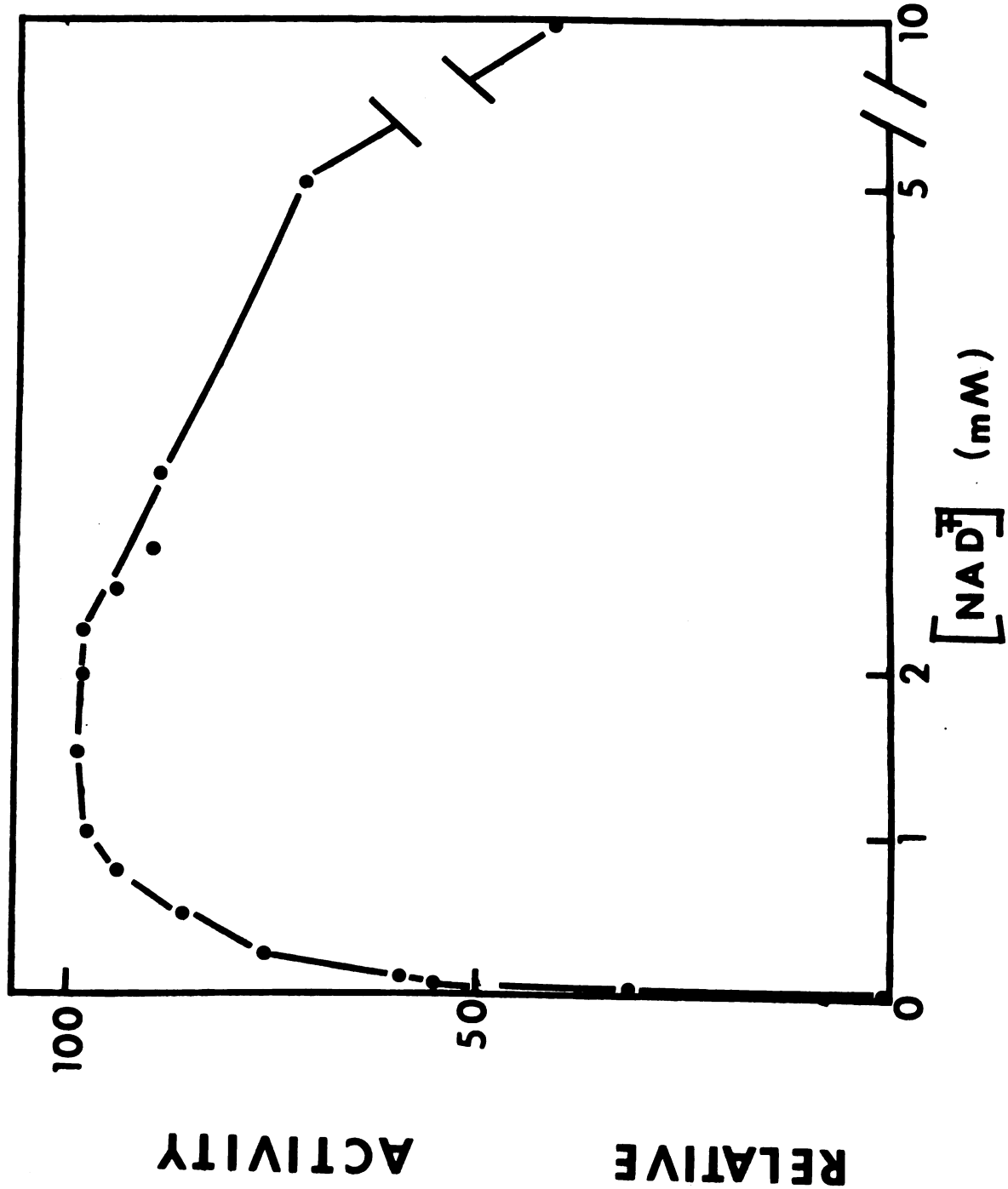

RELATIVE ACTIVITY
100
50
0
1
2
5
10
[NAD+] (mM)

Fig. 6. Effect of temperature on parsley UDPGA cyclase activity. Parsley UDPGA cyclase from the DEAE-Sephadex step contained 0.015 milliunits per 20 μg of protein. Each assay was initiated by this amount of UDPGA cyclase. The assays were incubated for 5 minutes at the various temperatures indicated. The reactions were heated at 100° for 3 minutes and further treated as described for Assay I. The initial velocity of the reaction, v_o, is defined as the milliunits of UDPGA cyclase activity measured in Assay I at the various incubation temperatures tested in this experiment.

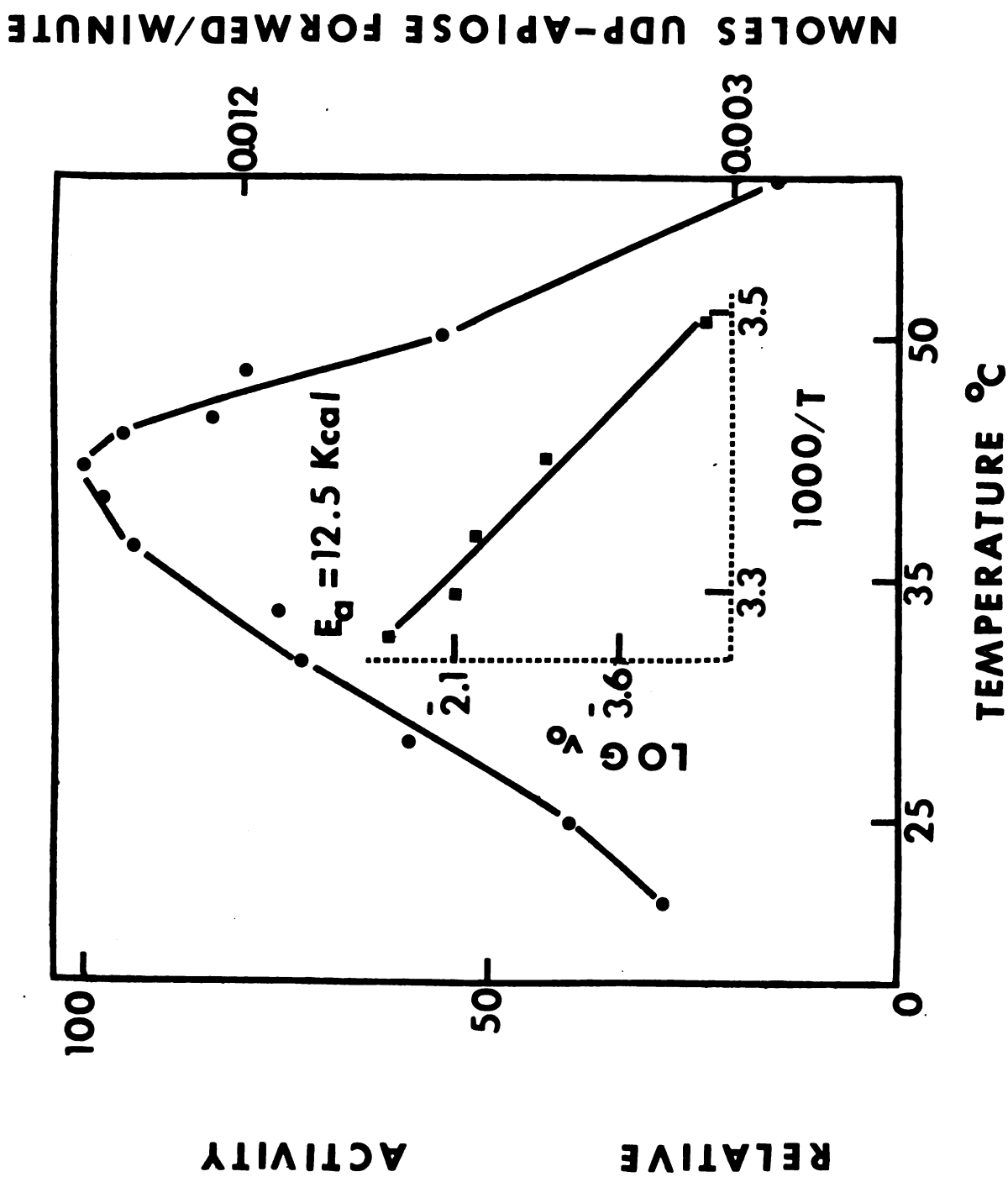

NMOLES UDP-APIOSE FORMED/MINUTE
0.012
0.003
E_a = 12.5 Kcal
1000/T
3.5
3.3
LOG %
-2.1
-3.6
TEMPERATURE °C
0
25
35
50
RELATIVE ACTIVITY
100
50

TABLE III. Inhibition of parsley UDPGA decarboxylase and parsley UDPGA cyclase by UDP-xylose

	Molar concentration of UDP-xylose in Assay			
	1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}
UDPGA decarboxylase activity*	100	93	76	29
UDPGA cyclase activity*	100	113	100	54

*Activity compared to that obtained when no UDP-xylose was added to the assay mixture and is equal to 100. UDPGA cyclase was assayed with Assay II and UDPGA decarboxylase was assayed with the standard assay in the presence of various concentrations of UDP-xylose.

Fractionation of Parsley UDPGA Decarboxylase and Parsley UDPGA Cyclase Activity with Ammonium Sulfate. -- To determine the concentrations of ammonium sulfate needed to precipitate each of the two enzymes, leaf tissue was prepared and treated as described in the extraction step. It was then fractionated with ammonium sulfate, as described in Part 3. A summary of the analyses for UDPGA decarboxylase and UDPGA cyclase activity in the various fractions is presented in Table IV. As it shows, 3 of the fractions contained both UDPGA decarboxylase and UDPGA cyclase. The ratios of the two activities did not vary significantly in each of these fractions. Two fractions, 1.25→1.50 and 1.95→2.10, contained 5% of the total UDPGA decarboxylase activity. They contained no UDPGA

TABLE IV: Fractionation of parsley UDPGA decarboxylase and parsley UDPGA cyclase with ammonium sulfate.

Step		Total protein	UDPGA decarboxylase*		UDPGA cyclase*	
molarity of ammonium sulfate	(% solution of ammonium sulfate at 4°)	mg	milliunits / mg of protein	total milliunits	milliunits / mg of protein	total milliunits
1.25→ 1.50	(29.5-34.5)	10	0.036	0.38	0.0000	0.00
1.50→ 1.65	(34.5-39.0)	20	0.080	1.60	0.0525	1.05
1.65→ 1.80	(39.0-42.5)	131	0.142	18.67	0.0565	7.20
1.80→ 1.95	(42.5-46.1)	632	0.0135	8.53	0.0050	3.16
1.95→ 2.10	(46.1-49.7)	80	0.006	0.96	0.0000	0.00
2.10→ 2.60	(49.7-61.5)	176	0.000	0.00	0.0000	0.00

*UDPGA decarboxylase was measured in the standard assay and UDPGA cyclase was measured in Assay II. Both assays contained 1.05 nmoles of UDP-[U-^{14}C]glucuronic acid as substrate.

cyclase activity. Kindel* observed that 13% of the UDPGA decarboxylase activity could be separated from the majority of the UDPGA cyclase activity with ammonium sulfate in a cell-free extract from duckweed.

PROPERTIES OF PARSLEY UDPGA DECARBOXYLASE

pH Optimum of Parsley UDPGA Decarboxylase. -- Parsley UDPGA decarboxylase has optimum activity at about pH 8.0 to 8.2 (Fig. 7). As indicated in Fig. 7, UDPGA decarboxylase activity decreased rapidly at pH values above or below pH 8.0 to 8.2.

Determination of Optimum NAD^+ Concentration for the Formation of UDP-xylose. -- Fig. 8 shows that parsley UDPGA decarboxylase has 65% of optimum activity without exogenous NAD^+. However, exogenous NAD^+ does stimulate the UDPGA decarboxylase showing that exogenous NAD^+ is required for optimum activity of this enzyme *in vitro*. The NAD^+ concentration for optimum UDP-xylose formation is about 2.0×10^{-3} M. More than optimum concentrations of NAD^+ suppressed enzymatic activity for both UDPGA cyclase (Fig. 5) and UDPGA decarboxylase, although the activity of UDPGA cyclase was suppressed much more than was the activity of UDPGA decarboxylase.

*Kindel, P. K., unpublished data

Fig. 7. Effect of pH on the reaction rate of parsley UDPGA decarboxylase. UDPGA decarboxylase was measured in the standard assay with 2.4 μmoles of sodium phosphate buffer (●——●) pH 7.0 to 9.0 or 2.4 μmoles of Tris-HCl buffer (o ..... o) pH 7.0 to 9.0. Each assay was initiated by the addition of 0.0075 milliunits of parsley UDPGA cyclase (10 μg of protein) from the DEAE-Sephadex step which contained 0.007 milliunits of parsley UDPGA decarboxylase.

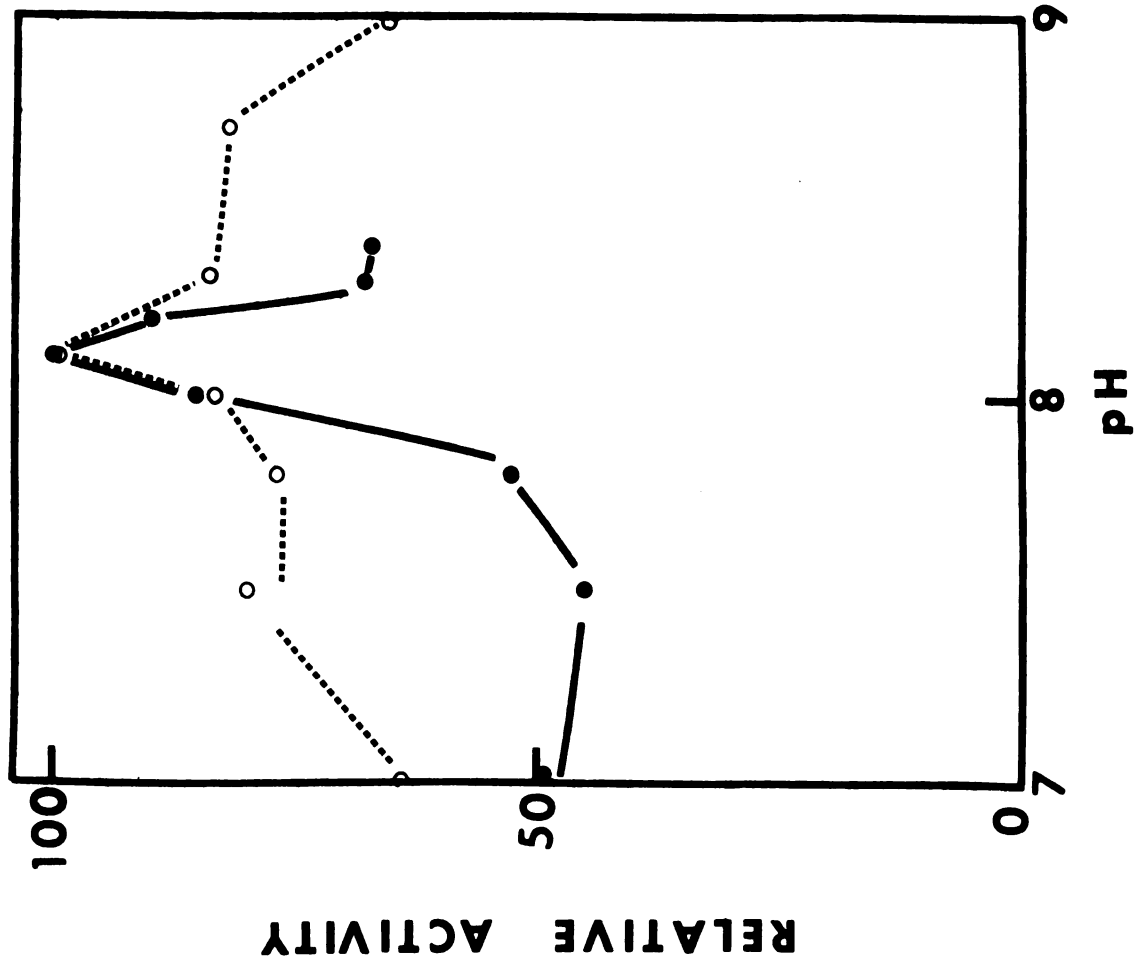
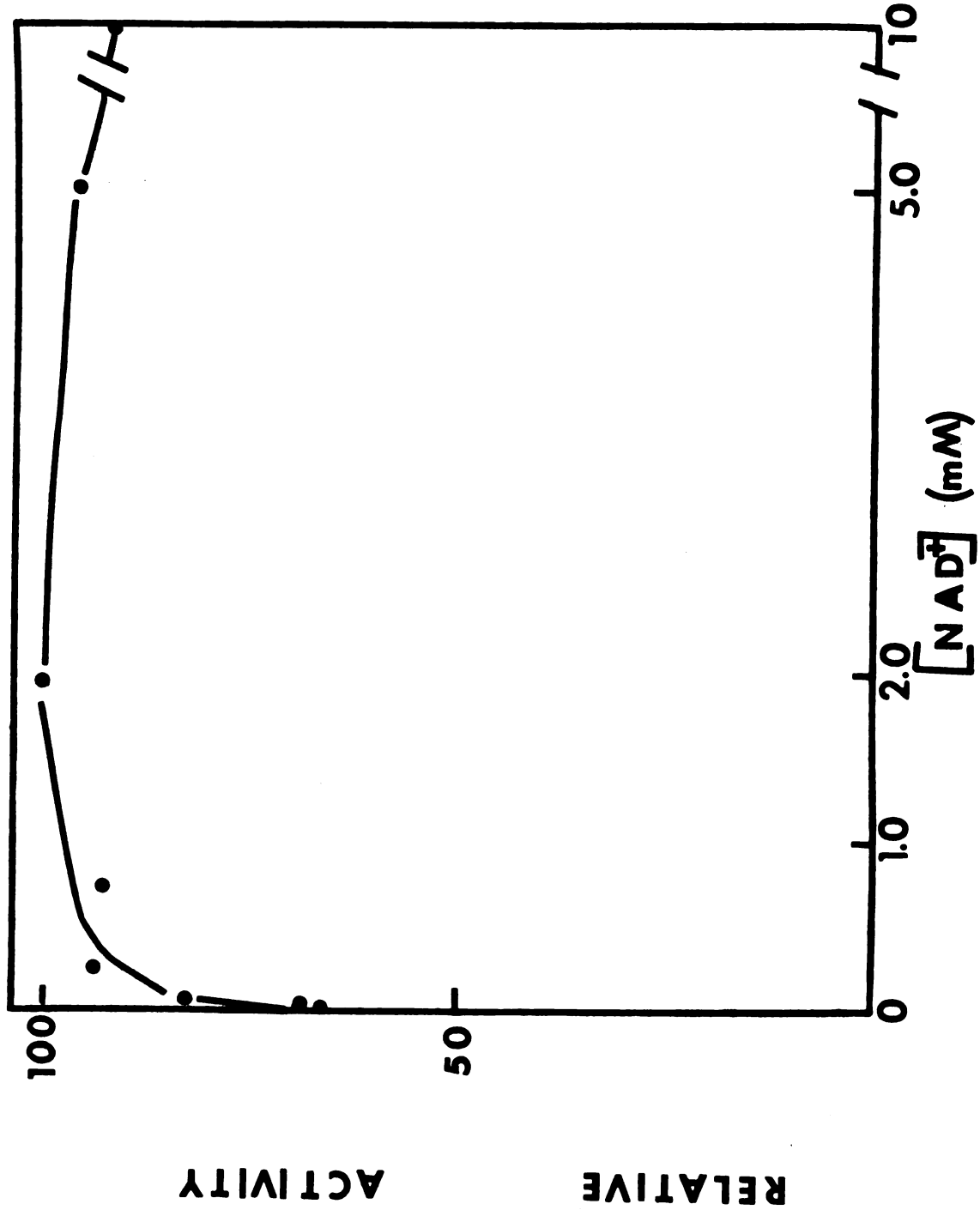

RELATIVE ACTIVITY
100
50
0
7
8
9
pH

Fig. 8. Effect of NAD^+ concentration on parsley UDPGA decarboxylase reaction velocity. The formation of UDP-[U-^{14}C]xylose was followed with the standard assay except that each assay mixture contained one of the concentrations of NAD^+ indicated in Fig. 5 (0.0 to 500 nmoles). The reaction was initiated by the addition to each assay mixture of 0.009 milliunits of parsley UDPGA decarboxylase (12 µg of protein) from the DEAE-Sephadex step which contained 0.01 milliunits of UDPGA cyclase.


RELATIVE
ACTIVITY
100
50
0
1.0
2.0
5.0
10
[NAD+] (mM)

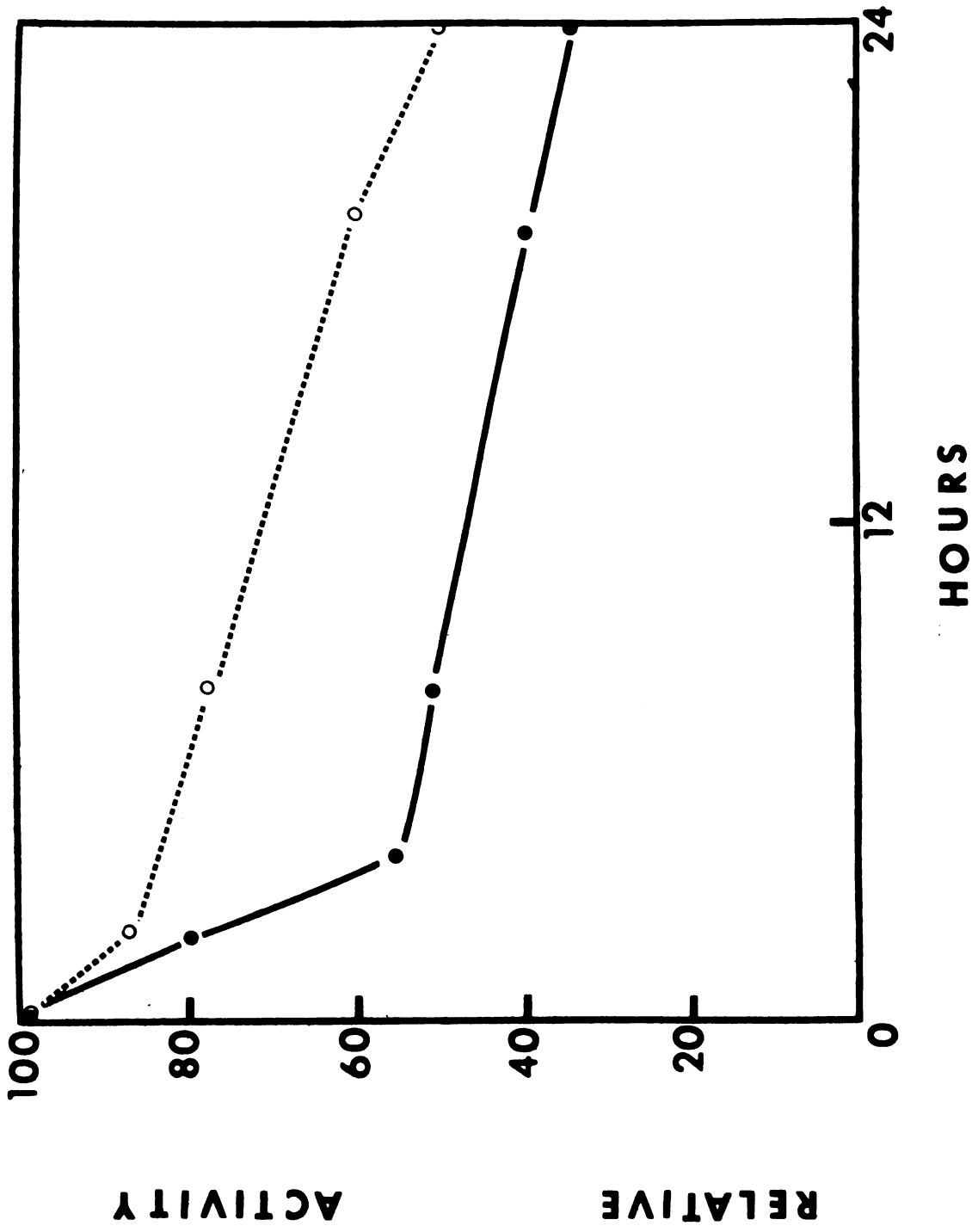
PROPERTIES OF DUCKWEED UDPGA CYCLASE AND DUCKWEED UDPGA DECARBOXYLASE

Factors Affecting the Stability of Duckweed UDPGA Cyclase. -- Gustine (41) observed using a modified Assay I which was incubated for 15 minutes that duckweed UDPGA cyclase lost activity very rapidly (24 hours) when stored in dilute solution at -20° or at 4°. The addition of glycerol to make a final concentration of 50% by volume stabilized the enzyme especially in dilute solutions at -20°. After 39 days of storage with these conditions Gustine (41) found that there was 3.7 times as much activity remaining in the solution which was 50% glycerol by volume (34% of the starting activity) compared to the solution without glycerol.

I investigated the stability of duckweed UDPGA cyclase from the DEAE-Sephadex step (10 mg protein/ml and 30% glycerol) that was kept at 4° and 25°. Storage at 25° preserved about 10% more enzyme activity than at 4° throughout a period of 24 hours. Storage at 4° with 10 mM NAD^+ both slowed the rapid loss of enzyme activity during the first 3 hours and slowed the loss of enzyme activity throughout the remainder of the 24 hour incubation period. After 24 hours only 35% of the original activity remained (Fig. 9) whereas 50% remained in the presence of 10 mM NAD^+.

Effect of Various Ions on the Activity of Duckweed UDPGA Cyclase. -- Duckweed UDPGA cyclase activity was measured in the presence of various ions. The final concentration of these ions in Assay I was 1 mM. The compounds tested were $FeCl_3$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, NaCl, KCl, $NaMoO_3$, $NaBO_3$,

Fig. 9. Effect of NAD^+ on the stability of duckweed UDPGA cyclase at 4°. Duckweed UDPGA cyclase from the DEAE-Sephadex step (10 mg of protein/ml) was stored at 4° in a solution 25% glycerol by volume. One portion was 10 mM with respect to NAD+ (O ····· O ····· O) and the other portion contained no NAD^+ (● —— ● —— ●). After incubation at 4° for the time intervals indicated (2,4,8,19 and 24 hours) 0.059 milliunits of UDPGA cyclase (60 μg of protein) was removed and measured with Assay I. NAD^+ was added to the assay mixture before enzyme was added from each portion so that the final concentration of NAD^+ in all assay mixtures was the same (2×10^{-3} M). The assays were begun by addition of enzyme and treated as described in Materials and Methods.


RELATIVE ACTIVITY
100
80
60
40
20
0
12
24
HOURS

NH_4Cl, $FeCl_2$, $ZnCl_2$, $CoCl_2$, and $CuCl_2$. None of the 14 compounds test increased duckweed UDPGA cyclase activity except $MgCl_2$ (21%). Gustine (41) tested duckweed UDPGA cyclase with only $MgCl_2$ and observed a 15% increase in enzymatic activity. Only the latter five compounds decreased the enzymatic activity by 9, 17, 76, 85, and 100% respectively. EDTA, α,α'-dipyridyl and KF at 1 mM final concentrations in Assay I had no effect on duckweed UDPGA cyclase activity. The addition of o-phenanthroline and 8-hydroxyquinoline at 1 mM levels resulted in a slight (10%) decrease in enzymatic activity. The effect of the above 19 compounds on the activity of UDPGA cyclase was determined in Assay I with the sodium phosphate buffer replaced by 2.5 μmoles of Tris-HCl, pH 8.0.

Effect of Sulfhydryl Reagents on the Activity of Duckweed UDPGA Cyclase. -- UDPGA cyclase was incubated with 1 mM concentrations of p-chloromercuribenzoate, N-ethylmaleimide, iodoacetamide and oxidized glutathione in Assay I. More than 95% of the activity was lost with p-chloromercuribenzoate and N-ethylmaleimide, 60% with iodoacetamide and 6% with oxidized glutathione.

The Energy of Activation of Duckweed UDPGA Cyclase. -- The energy of activation for duckweed UDPGA cyclase obtained from the equation above and from the data in Fig. 10 is 11.4 Kcal.

Inhibition of Duckweed UDPGA Cyclase and Duckweed UDPGA Decarboxylase Activities by Five Uracil Compounds. -- The two enzymes from the DEAE-Sephadex step were measured by Assay II in the presence of five uracil compounds (Table V). All five

Fig. 10. Effect of temperature on duckweed UDPGA cyclase activity. Duckweed UDPGA cyclase from the DEAE-Sephadex step contained 0.016 milliunits per 5 μg of protein. Each assay was initiated by this amount of UDPGA cyclase. The assays were incubated for 5 minutes at the various temperatures indicated. The reactions were heated at 100° for 3 minutes and further treated as described for Assay I. The initial velocity of the reaction, v_o, is defined as the milliunits of UDPGA cyclase activity measured in Assay I at the various incubation temperatures tested in this experiment.

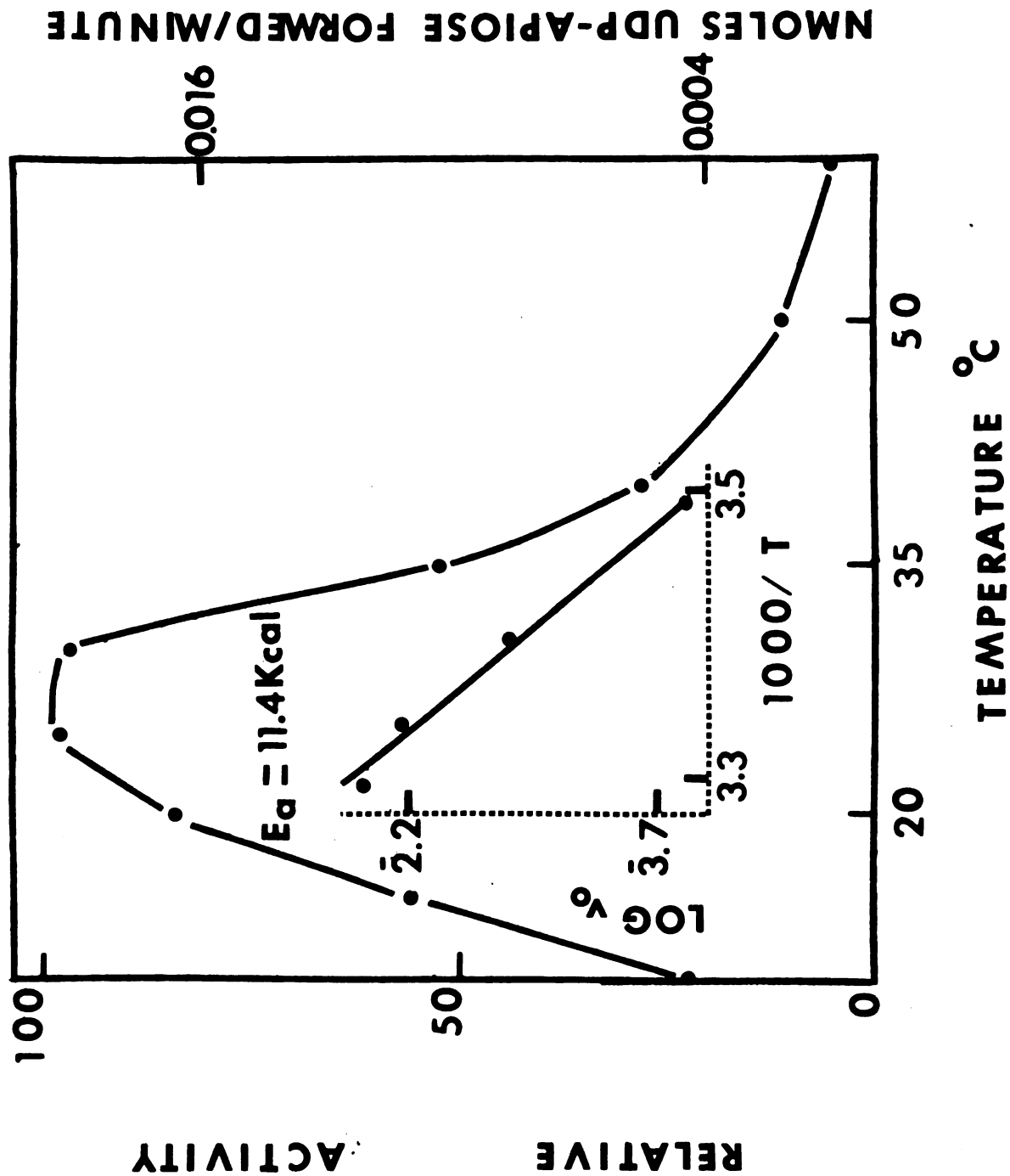

NMOLES UDP-APIOSE FORMED/MINUTE
0.016
0.004
TEMPERATURE °C
50
35
20
1000/ T
3.5
3.3
2.2
3.7
Ea = 11.4Kcal
LOG %
RELATIVE ACTIVITY
100
50
0

TABLE V: Inhibition of duckweed UDPGA decarboxylase and duckweed UDPGA cyclase by five uracil compounds.

Compound	Duckweed UDPGA decarboxylase activity* obtained with the following molar concentrations of selected uracil compounds			
	1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}
UTP	67	32	4	NT
UDP	47	32	9	0
UMP	86	97	88	60
UDP-galactose	90	53	49	52
UDP-xylose	99	91	57	12

Compound	Duckweed UDPGA cyclase* activity obtained with the following molar concentrations of selected uracil compounds			
	1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}
UTP	77	64	38	NT
UDP	59	50	47	45
UMP	87	88	94	98
UDP-galactose	95	82	92	66
UDP-xylose	117	98	70	57

*Activity is compared to the activity obtained when none of the above uracil compounds was added to the standard assay for UDPGA decarboxylase or to Assay II for UDPGA cyclase which was given the value of 100. NT = not tested.

compounds inhibited both activities. UTP and UDP inhibited both activities much more at lower concentrations than did the other three compounds. UDP-xylose inhibited UDPGA decarboxylase at high concentrations greatly, whereas UDP-galactose did not. Inhibition of UDPGA cyclase activity by these two sugar nucleotides even at high concentrations, was much less than the inhibition of UDPGA decarboxylase by UDP-xylose.

DISCUSSION

An important consideration in the purification of UDPGA cyclase was to maintain the enzyme in dilute concentrations for a minimal amount of time. Maintainence of the enzyme in dilute solutions (0.1-10.0 mg/ml) at 4^{o} and 25^{o} resulted in the rapid loss of activity.

Before UDPGA cyclase was isolated, Grisebach (84) suggested that only one enzyme would be necessary to form UDP-apiose and UDP-xylose from UDP-glucuronic acid because of the similarity of the intermediates he postulated in their formation. Subsequently Sandermann and Grisebach (40) and Sandermann, Tisue and Girsebach (38) described the isolation of cell-free extracts from parsley and duckweed forming UDP-apiose and UDP-xylose. The activities were not purified and no attempt was made to separate the two activities.

Partially purified duckweed UDPGA cyclase and parsley UDPGA cyclase were used in most of the experiments described above. Both enzymatic activities also contained UDPGA decarboxylase activity. The presence of two separate enzymes

one forming UDP-apiose and another forming UDP-xylose, was indicated by several experiments. In one of these experiments parsley protein was isolated and then fractionated by increasing the concentration of ammonium sulfate. After each increase in the concentration of ammonium sulfate the protein which precipitated was isolated and assayed. All of the parsley UDPGA cyclase was found in three of the six fractions isolated as was much of the parsley UDPGA decarboxylase. Some parsley UDPGA decarboxylase (5%) was found in two of the other fractions which did not contain any parsley UDPGA cyclase. Some separation of one of the enzyme activities from the other has been obtained by fractionating with ammonium sulfate protein isolated from duckweed. About 13% of the UDPGA decarboxylase was removed from the majority of the UDPGA cyclase activity*. Further evidence suggesting that two enzymes exist in parsley was found by measuring the NAD^+ requirement of the enzymes. Parsley UDPGA cyclase requires NAD^+ to form UDP-apiose. Parsley UDPGA decarboxylase activity does not need exogenously added NAD^+ to form UDP-xylose. NAD^+ (exogenously added) stimulates the formation of UDP-xylose and the concentration required for optimal UDP-xylose formation is similar to the optimal concentration needed for UDP-apiose formation.

The inhibitory effect of UDP-xylose is quite similar on both duckweed and parsley UDPGA decarboxylase. The inhibitory effect of UDP-xylose is quite similar on both duckweed and parsley UDPGA cyclase. The formation of UDP-xylose is inhibited more than the formation of UDP-apiose by various

concentrations of UDP-xylose. In fact, the UDP-xylose formation is inhibited as much as the UDP-apiose formation with 10 to 100 times lower concentrations of UDP-xylose. At concentrations of UDP-xylose where parsley UDPGA cyclase and parsley UDPGA decarboxylase activity are partly inhibited (30-88%), the parsley UDPGA decarboxylase activity is inhibited by up to twice as much. A similar observation was made with these enzymatic activities isolated from duckweed and purified through the DEAE-Sephadex step. All of the uracil compounds tested, UTP, UDP, UMP, UDP-xylose and UDP-galactose, inhibited duckweed UDPGA decarboxylase more than UDPGA cyclase.

The data do not show conclusively that the UDPGA decarboxylase activity precipitating with UDPGA cyclase in duckweed and parsley extracts represents either co-precipitation of UDPGA decarboxylase with other protein or one enzyme which forms both compounds. These data strongly indicate that separate enzymes are involved in the formation of UDP-apiose and UDP-xylose.

The inhibition by uracil compounds is greater on duckweed UDPGA decarboxylase that on duckweed UDPGA cyclase. This should allow the formation and subsequent isolation of UDP-apiose without contaminating UDP-xylose providing that the uracil compounds are added in the proper amounts. Subsequent removal of several of these uracil compounds could be accomplished using the isolation procedure for UDP-apiose described in Part 2. This is important as the preparation of UDP-apiose without contaminating UDP-xylose has not yet

been possible (Part 2).

It may be of physiological significance that UDP, a product of transglycosylation reactions such as those predicted for cell-wall polysaccharide formation (65,68), inhibits duckweed UDPGA cyclase 41% and inhibits duckweed UDPGA decarboxylase 57% at low concentrations (1×10^{-6} M). This suggests that for transglycosylation reactions to proceed UDP must be removed. This could occur by conversion of UDP to UTP and UDP-sugars at a sufficient rate to prevent accumulation of UDP and its inhibition of UDP-xylose and UTP-apiose formation. Besides duckweed UDPGA decarboxylase and duckweed UDPGA cyclase, parsley apiin synthase (Part 3) was inhibited significantly at low concentrations of UDP. UMP also had a similar effect on all three enzymes (Part 3).

The intracellular function of UDPGA cyclase and UDPGA decarboxylase is to catalyze the biosynthesis of UDP-apiose and UDP-xylose, respectively. The low K_m for UDP-glucuronic acid suggests that it is the substrate for parsley UDPGA cyclase. It would be of interest to determine the activity of parsley and duckweed UDPGA cyclase toward UDP-galacturonic acid as a possible substrate. Both UDP-glucuronic acid and UDP-galacturonic acid, a possible substrate, are probably present in parsley and duckweed (81). Nucleoside diphosphate galacturonic acid is very likely formed in duckweed, as large amounts of galacturonic acid have been found in the cell wall polysaccharides of duckweed (16). UDP-galacturonic acid has been formed and shown to be a precursor for polygalacturonans in Phaseolus aureus (43,44, 81).

Sandermann, Tisue and Grisebach (38) reported the isolation of particulate UDPGA cyclase from duckweed. They did not isolate any particulate UDPGA cyclase from parsley leaves. I also did not observe particulate UDPGA cyclase from parsley leaves and stems.

The requirement of parsley UDPGA cyclase for exogenously added NAD^+ has not been previously investigated. No synthesis of UDP-apiose with parsley UDPGA cyclase purified through the DEAE-Sephadex step was observed without exogenously added NAD^+. NAD^+ was not required to obtain 65% of the optimum parsley UDPGA decarboxylase activity observed in the presence of exogenously added NAD^+. Sandermann and Grisebach (40) reported that duckweed UDPGA cyclase, which had been purified only by passage through a Sephadex G-25 column, functioned without exogenously added NAD^+ at 3% of the rate obtained in the presence of NAD^+. Sandermann and Grisebach (40) observed that duckweed UDPGA decarboxylase functioned at 17% of the rate obtained in the presence of exogenously added NAD^+. They also found that NADH inhibited duckweed UDPGA cyclase and that $NADP^+$ was much less effective than NAD^+ as a cofactor in the UDPGA cyclase reaction.

The only significant differences observed between parsley and duckweed UDPGA cyclase were the energy of activation and the temperatures at which heat denaturation of the enzymes began to occur. The latter is the temperature for optimal activity of each enzyme. Parsley UDPGA cyclase begins to be denatured by heat at 37-42° and duckweed UDPGA cyclase begins to be denatured by heat at 24-27°. The

temperature for optimum activity of each enzyme is quite distinctive. It is about 27° for duckweed UDPGA cyclase and about 42° for parsley UDPGA cyclase. This shows that the parsley UDPGA cyclase was more stable to heat in the purified preparation tested. The only difference between the two experiments was the source of the protein and the protein concentration in the assay. These data also suggest that UDPGA cyclase should be assayed at 25° where heat denaturation should not affect either enzyme rather than at 30° which the enzyme commission suggests as a standard temperature for most enzyme assays (87). Because of the low stability of duckweed UDPGA cyclase and of UDP-apiose (Part 2) at temperatures above 25° it is suggested that all assays for any UDPGA cyclase be carried out at 25°. The energy of activation of duckweed UDPGA cyclase is lower than that of parsley UDPGA cyclase. This means that less energy is necessary to activate the substrate by formation of a substrate-enzyme complex (53) in the reaction catalyzed by duckweed UDPGA cyclase than the reaction catalyzed by parsley UDPGA cyclase. Based on the above observations the two enzymes are apparently not completely identical physically even though they both catalyze reactions forming UDP-apiose from UDP-glucuronic acid.

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