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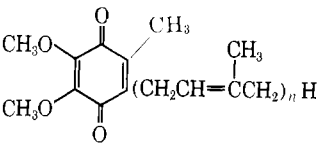
Discovery of Ubiquinones-1, -2, -3, and -4 and the Nature of Biosynthetic Isoprenylation*

G. Doyle Daves, Jr., R. F. Muraca, J. S. Whittick, Palle Friis,† and Karl Folkers

ABSTRACT: High-sensitivity mass spectrometry of paper chromatographically pure samples of the dominant ubiquinone from three diverse microorganisms has revealed the presence of trace amounts of ubiquinones-1, -2, -3, and -4. The presence of these ubiquinones in bacteria was confirmed by special chromatographic techniques using paraffin-impregnated thin layer plates. Ubiquinones-1, -2, -3, and -4 have previously been known by synthesis, but not by natural occurrence. Recently, 2-tetraprenylphenol, which is a precursor of ubiquinone-4, has been isolated from *Rhodospirillum rubrum*, and ubiquinone-5 has been isolated from *Escherichia coli*. The entire series, ubiquinones-1 through -10, is now known from nature. Pure ubiquinone-6 from *Saccharomyces cerevisiae* revealed the presence of ubiquinones-1 through -6. Similarly, pure ubiquinone-8 from *E. coli* revealed the presence of ubiquinones-1 through -8, and pure ubiquinone-10 from *R. rubrum* revealed ubiquinones-1 through -10. The difference in the concentrations of the ubiquinones at the extremes in molecular weight could be 500- to 1000-fold. There is no absolute structural specificity in certain species for the biosynthetic formation of a single multiprenyl pyrophosphate or in its reaction with *p*-hydroxybenzoic acid. Instead, biosynthetic isoprenylation takes place so that a series of ubiquinones are formed in which the number of isoprene units contained in the side chains varies from the number of units in the dominant ubiquinone for a given species (6, 8, or 10 for the species studied) to one. The relative concentrations of the ubiquinones diminish with diminishing chain length.

The occurrence in bacteria of ubiquinones-1, -2, -3, and -4 (Q_{1-4}, I, n = 1, 2, 3, and 4, respectively)[1] has been discovered. The existence in nature of ubiquinones-6 through -10 has been recognized (Lester and Crane, 1959) for a number of years. Recently, the isolation of a pure sample of Q_5 (I, n = 5) from *Escherichia coli* was reported (Friis *et al.*, 1966a), and 2-tetraprenylphenol, a precursor of ubiquinone-4, was characterized from *Rhodospirillum rubrum* (Olsen *et al.*, 1966). With the detection of ubiquinones-1 through -4 in nature, all of the ubiquinones with side chains of from one to ten isoprene units are now recognized as constituents of living systems, although these new lower isoprenylogs occur in very minor amounts as compared to the corresponding major ubiquinone of a given species.



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Mass Spectrometric Studies. The initial examination by high-sensitivity mass spectrometry of a paper chromatographically pure sample of Q_6 isolated from *Saccharomyces cerevisiae* revealed the presence of peaks which correspond to lower molecular weight ubiquinones. In addition to the peaks expected for Q_6 (Muraca *et al.*, 1967), peaks were observed (Figure 1a, top) which could be assigned to parent ions (M) of Q_5, Q_4, Q_3, Q_2, and Q_1. In each case, peaks of appropriate relative intensities due to the molecular ions of the corresponding hydroquinones were also observed. The observation of peaks due to hydroquinones is in accord with earlier data on the mass spectra of the ubiquinones (Muraca *et al.*, 1967) and the plastoquinones (Das *et al.*, 1965) in which peaks at M + 2 due

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[1] Abbreviations used: Q_{1-4}, ubiquinones-1, -2, -3, and -4; HBA, *p*-hydroxybenzoic acid.

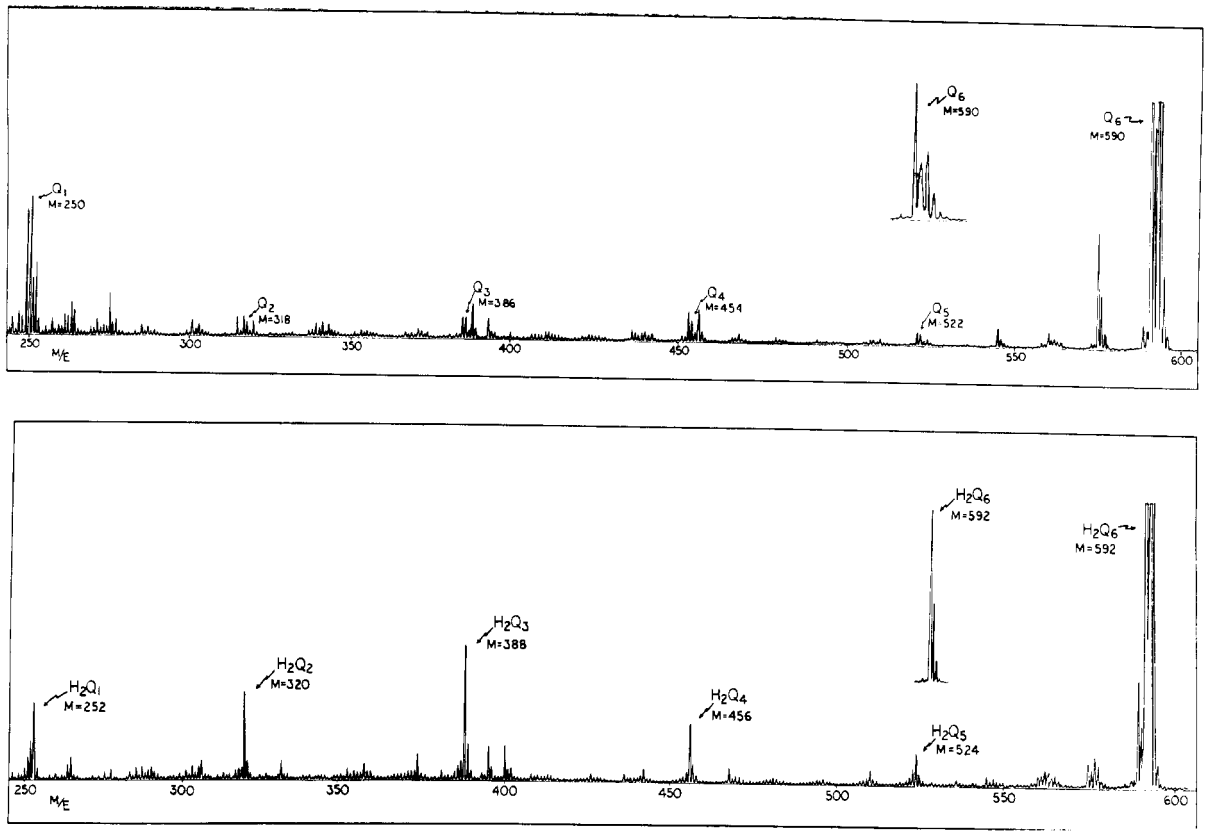


FIGURE 1: Partial mass spectra of ubiquinone-6 from *S. cerevisiae* (top) and of ubiquinol-6 obtained by reduction (bottom). Intensities are in arbitrary units and comparison of relative intensities are valid only for peaks of closely related *m/e* values. For accurate relative intensities see Muraca *et al.* (1967).

to the hydroquinones are always present. Following reduction of the sample of Q_6 to the hydroquinone, a spectrum was obtained (Figure 1b, bottom) which exhibited the expected shifts (Muraca *et al.*, 1967) in the relative intensities of the parent ions of the quinone and hydroquinone species.

Spectra obtained with low-voltage electrons supported the assignment of these peaks as molecular ions. The data in Table I show that the relative intensities of peaks due to parent ions of the various ubiquinones are essentially constant over a wide range of ionization potentials as expected for a homologous series of compounds. The only exception is the peak at *m/e* 250 (M^+ for Q_1) which suffered a 15- to 20-fold loss in intensity relative to the peak at *m/e* 590 in going from 70 to 8 (9) ev. The large decrease in the intensity of the peak at *m/e* 250 brought about by lowering the energy of the bombarding electrons is apparently due to a significant contribution by a fragment ion to the intensity of this peak at the 70-ev spectrum. It is noteworthy that while a cluster of four prominent peaks (*m/e* 249, 250, 251, and 252) is observed in the 70-ev spectrum (Figure 1a), only the peaks at *m/e* 250 (M^+ for Q_1) and 252 (for M^+ for H_2Q_1) are observed in the spectra obtained at low ionizing voltages.

Examination of paper chromatographically pure ubiquinones from *R. rubrum* and *E. coli* by similar mass spectrometric techniques also reveals the presence of the new Q_{1-4}. Actually, evidence was obtained for the presence in the sample of pure ubiquinone-8 from *E. coli* of ubiquinones-1 to -7, and in a sample of pure ubiquinone-10 from *R. rubrum* of ubiquinones-1 to -9.

TABLE I: Relative Intensities of Parent Ion Peaks of Ubiquinones from *S. cerevisiae* at High and Low Ionization Potentials.

Q	*m/e*	Ionizing Voltage 70 (ev)	9 (ev)	8 (ev)
Q_6	590	100[a]	100[a]	100[a]
Q_5	522	1.5	1.5	1.4
Q_4	454	2.5	2.4	2.6
Q_3	386	5.9	4.0	5.7
Q_2	318	10.0	10.2	11.0
Q_1	250	122	6.4	9.2

[a] Arbitrarily assigned.

A systematic investigation has not yet been made to determine whether isoprenylogs of molecular weights higher than that of the major Q component are also present in microorganisms. However, the presence of Q_7 in *S. cerevisiae* (major component, Q_6) has been noted; it seems reasonable to believe that most or all of the complete series of ubiquinones-1 to -10 may be present in many microorganisms. The possible existence in living systems of trace quantities of ubiquinones with more than ten units in the isoprenoid side chain, *i.e.*, Q_{11}, Q_{12}, etc., may be projected.

Since the mass spectrometric techniques used for these experiments do not yield quantitative data, it is not possible to know the relative concentrations of the various ubiquinones in a chromatographically pure sample. Relatively prominent peaks due to the parent ions of the lower molecular weight ubiquinones are observed. However, the lower isoprenylogs have higher volatilities and a greater tendency to form molecular ions (Muraca *et al.*, 1967); undoubtedly the actual concentrations of these isoprenylogs are very small.

The presence of Q_{1-4} in bacteria, first noted by mass spectrometric techniques, was confirmed by the following chromatographic experiment. A sample of ubiquinone obtained from *E. coli* pure Q_8 by reverse-phase paper chromatography (Friis *et al.*, 1966a) was subjected to reverse-phase thin layer chromatography using silica gel G plates impregnated with paraffin oil and developed in acetone-water (19:1). In this way, Q_5, Q_7, and Q_6 were readily detected using leucomethylene blue spray reagent. In order to concentrate the lower molecular weight ubiquinones preparative thin layer chromatography was carried out using paraffin plates developed in acetone–water (9:1). The area of the plates corresponding to Q_{1-5} was removed from the plates and eluted. The concentrated material obtained in this way was applied as a single spot to a paraffin-impregnated thin layer plate and developed using acetone-water (17:3). Leucomethylene blue positive spots corresponding to reference samples of ubiquinones 1–5 were observed. R_F values for the ubiquinones in this system are recorded in Table II.

An estimate of the relative abundance of the Q homologs in *E. coli* was obtained by fractionation of the ubiquinones using special chromatographic techniques (Friis *et al.*, 1966a). This study (Table III) shows a distribution of ubiquinones into three groups: the major component (Q_8), the minor components (Q_{5-7}), and the trace components (Q_{1-4}).

In the samples studied, ubiquinones-1 to -4 account for a very small percentage of the total ubiquinone content and have been characterized from three diverse microorganisms (*S. cerevisiae*, *E. coli*, and *R. rubrum*). The possible origin of Q_{1-4} from bacterial contaminants of these three microorganisms is recognized, but seems unlikely. It is pertinent that while fractionation of the ubiquinone obtained from *E. coli* produced Q_7, Q_6, and Q_5 in isolable quantities (Friis *et al.*, 1966a), the widely occurring ubiquinones-9 and -10 (Lester and Crane, 1959) were not detected. At present, it seems reasonable to assume that microorganisms actually produce several ubiquinones, and more than previously recognized, as do each of the microorganisms studied (*S. cerevisiae*, *R. rubrum*, and *E. coli*).

The significance of the multiplicity of ubiquinones in a single organism and their 500- to 1000-fold differences in concentrations (Table III) may be interpreted as reflecting a degree of nonspecificity in the biosynthesis of ubiquinone as it concerns the multiprenyl pyrophosphate (IV and V) requirement for the enzymatic isoprenylation of *p*-hydroxybenzoic acid (IV–VII and

TABLE II: Chromatography of Ubiquinones on Paraffin-Impregnated Thin Layer Plates.[a]

Q	R_F Values: Acetone–Water (19:1)	Acetone–Water (9:1)	Acetone–Water (17:3)
Q_1		0.90	0.78
Q_2		0.87	0.70
Q_3		0.83	0.60
Q_4		0.73	0.50
Q_5	0.70	0.61	0.40
Q_6	0.63	0.45	0.34
Q_7	0.57	0.35	0.24
Q_8	0.46	0.16	0.10
Q_9	0.39	0.06	0.05
Q_{10}	0.33	0.04	0.02

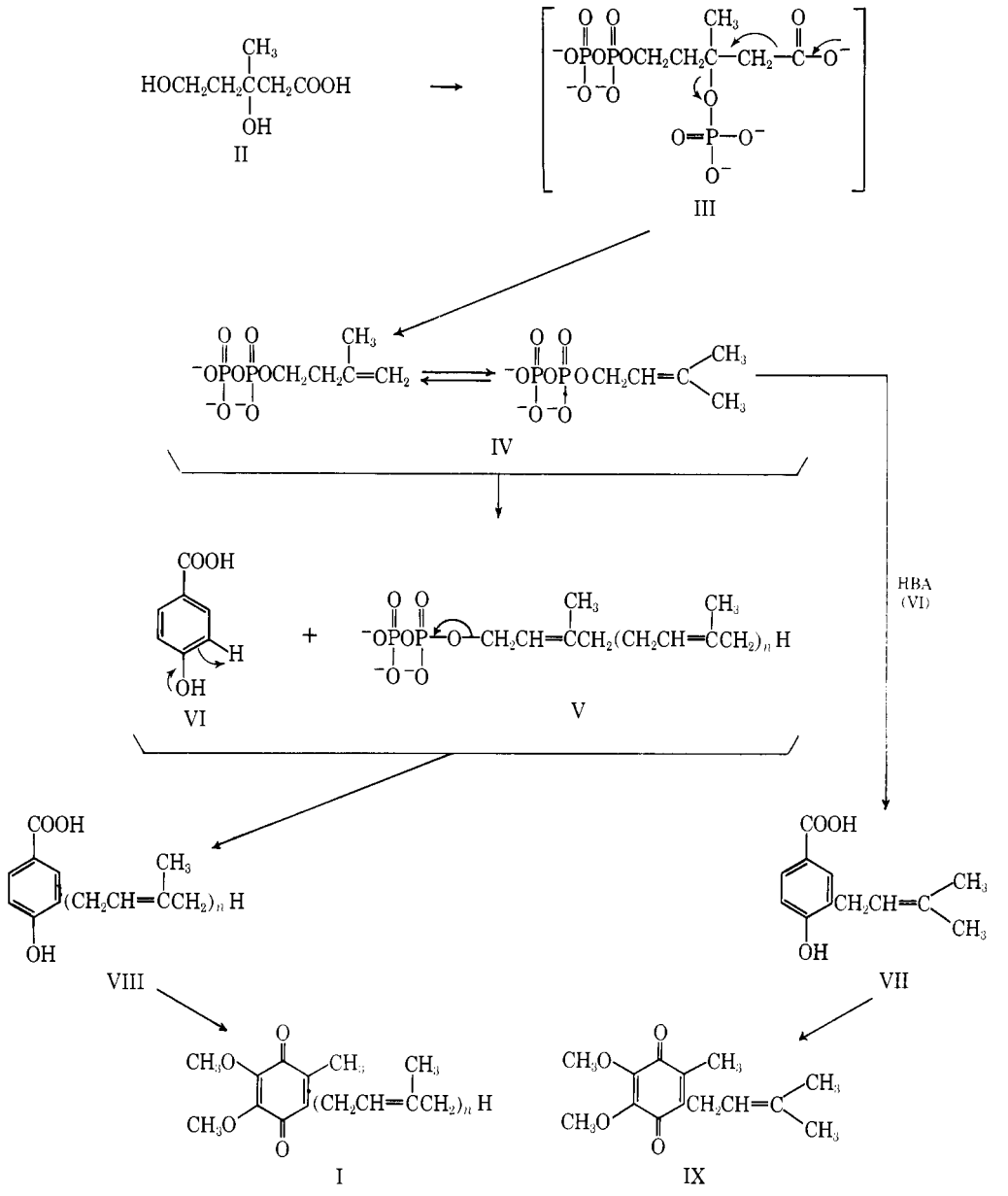
[a] Silica gel G plates (0.3 mm) impregnated with 5% Chevron White Oil No. 3 NF.

TABLE III: Ubiquinone Content of *E. coli*.

Q_n	Approx % Total Q[a]
10, 9	Not detected[b]
8	85
7	10
6	2
5	1
4, 3, 2, 1	<0.1[c]

[a] Per cent total Q estimated by summation of isolated samples of Q_{5-8} separated by extensive fractionation of Q_8 obtained from *E. coli*. [b] Reverse-phase paper chromatography. [c] Detected by mass spectrometry and by special chromatographic techniques using paraffin-impregnated thin layer plates; undetected by reverse-phase paper chromatography.

SCHEME I: Multiple Isoprenylation in the Biosynthesis of Ubiquinone.



V–VIII). The existence in these microorganisms of biosynthetic precursors of the ubiquinones such as 2-decaprenyl-, 2-nonaprenyl-, and 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone (Friis *et al.*, 1966a; Imamoto and Senoh, 1967) supports this view and is incompatible with a concept of stepwise build-up of the multiprenyl side chain in a late stage of ubiquinone biosynthesis.

The mechanism of the biosynthesis of the multiprenyl side chain from mevalonic acid (II), as presently summarized (Wagner and Folkers, 1964), and the condensation of side-chain components with *p*-hydroxybenzoic acid (VI), the ring precursor of ubiquinone (Olsen *et al.*, 1966; Friis *et al.*, 1966b), are formulated in Scheme I. The formation of ubiquinone-1 (IX) and higher isoprenylogs (I) with any number of isoprene units by such a mechanism is readily visualized.

The protein-lipid-containing enzyme systems which accommodate the build-up of the multiprenyl pyrophosphate (V) would be expected to have some structural specificity. The distribution and relative concentrations of so many ubiquinones in a single species indicate the prior formation of the corresponding multiprenyl pyrophosphates, and shows that the specificity of multiprenyl pyrophosphate biosynthesis (II–V) is not structurally absolute. It appears that the supporting enzyme complex and other conditions permit the biosynthesis of a multiprenyl pyrophosphate (V) of a

dominant chain length in the presence of multiprenyl pyrophosphates of subordinant chain lengths with variations between species (see also Whistance *et al.*, 1966).

The possibility that each of the ubiquinones (up to 10) observed in a single organism has separate and discrete metabolic functions seems unlikely. While there may be more than one site for functionality of ubiquinone it is reasonable that the most abundant ubiquinone in a given species would be dominant in functioning in each site. Clearly, the differences in the physical and chemical properties of the ubiquinones as influenced by the varying lengths of the multiprenyl side chains could also influence biochemical acceptability and functionality at various sites. It is apparent that those ubiquinones present in the greatest abundance are those which are the most physically and chemically similar which may reflect a common biosynthetic formation and a common capacity for functionality.

These views on the lack of absolute structural specificity for both the formation of the isoprenyl pyrophosphate and the isoprenylation of *p*-hydroxybenzoic acid would seem to be independent of any exchange reactions involving the multiprenyl side chain of the ubiquinones (Raman and Rudney, 1966; Billeter *et al.*, 1964; Martius and Fürer, 1963; Martius and Alvino, 1964).

Experimental Section

Preparation of Ubiquinone Samples for Mass Spectrometric Analysis. The hexane extracts (Friis *et al.*, 1966a; Olsen *et al.*, 1966) from collected cells of *S. cerevisiae*, *E. coli*, and *R. rubrum* (Olsen *et al.*, 1966) were fractionated by column chromatography over silica gel as previously described (Friis *et al.*, 1966a; Olsen *et al.*, 1966). The ubiquinones from these fractions were further purified by preparative thin layer chromatography on silica gel G plates developed in chloroform. These ubiquinone samples were then used for the mass spectrometric determinations. Ubiquinol samples for mass spectrometry were prepared as described (Muraca *et al.*, 1967).

These samples exhibited single spots (detected by leucomethylene blue spray reagent) upon chromatography on silicone-impregnated paper developed in 1-propanol–water (7:3) as described (Friis *et al.*, 1966a). Leucomethylene blue reagent is reported (Crane and Dilley, 1963) to have a lower limit of sensitivity of 1 $\mu g/cm^2$. In practice, samples of 10–30 μg are applied to a paper and the resulting spots as detected by leucomethylene blue are 3–5 cm^2 (or larger) in area. When larger samples (50–75 μg) are applied, significant streaking occurs. It is to be expected that quinones representing up to about 10% of the total sample can frequently be undetected by this general paper chromatographic procedure (Table III).

Detection of Ubiquinones-1 to -4 in E. coli by Reverse-Phase Thin Layer Chromatography. A 75-mg sample of ubiquinone-8 from *E. coli*, pure by paper chromatography, was streaked on silica gel G thin layer plates (0.3 mm) impregnated with 5% paraffin oil (Chevron White Oil No. 3 NF) and developed using acetone–water (9:1). The areas of the plates of R_F higher than a Q_6 reference spot were removed and eluted. In order to remove paraffin oil, the material was streaked on a thin layer plate (not paraffin impregnated) and the plate was developed using hexane–ether (9:1). A leucomethylene blue sensitive band just above the origin was removed and eluted. The material obtained by concentration of the eluate was applied as a single spot to a paraffin-impregnated thin layer plate on which ubiquinones-1 to -6 were also spotted as references. The plate was developed using acetone–water (17:3) and sprayed with leucomethylene blue spray reagent. The sample obtained from *E. coli* exhibited leucomethylene blue positive spots corresponding to each of ubiquinones-1 through -6 (Table II).

Estimation of the Distribution of Ubiquinones in E. coli (Table III). A 250-mg sample of ubiquinone-8 from *E. coli*, pure by paper chromatography, was extensively fractionated by a thin layer chromatographic technique utilized previously to isolate Q_5 (Friis *et al.*, 1966a). Using this technique, paper chromatographically pure samples of Q_8, Q_7, Q_6, and Q_5 were obtained. Summation of the quantities of the individual ubiquinones obtained and estimation of the distribution of ubiquinones in intermediate fractions produced the distribution data shown in Table III; the errors involved are such that the percentages obtained indicate only the relative abundance of the various ubiquinones.

Mass Spectral Determinations. The instrumentation and techniques used in these determinations have been described (Muraca *et al.*, 1967). All spectra were obtained using probe temperatures of 145–150°.

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Cyclic Hydroxamic Acids and Related Compounds from Maize. Isolation and Characterization*

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ABSTRACT: Two cyclic hydroxamic acids have been isolated from seedlings of the inbred strain of maize (CI31A). The major component, 2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one, is accompanied by small amounts of the analog lacking a methoxyl group. The related lactam 2-hydroxy-7-methoxy-1,4-(2H)-benzoxazin-3-one has been isolated from older plants. All three compounds occur in the intact plants as glucosides. This is the first report of the natural occurrence of this lactam or its glucoside.

Two cyclic hydroxamic acids which occur in cereal grasses have been described (Wahlroos and Virtanen, 1959; Virtanen and Hietala, 1960; Hietala and Virtanen, 1960). These compounds occur mainly as glucosides (Figure 1) from which the aglucones are rapidly released by enzymatic hydrolysis after the crushing or homogenization of the plants, although small amounts of the free aglucone V have been isolated from maize (Wahlroos and Virtanen, 1964). The aglucones, in turn, decompose in water to benzoxazolinones (Honkanen and Virtanen, 1961; Bredenberg *et al.*, 1962). Isolation of 2(3)-benzoxazolinone (III) from rye seedlings by Virtanen and Hietala (1955) led to the later isolation of the cyclic hydroxamic acids. Rye seedlings were reported to yield I and II (Virtanen and Hietala, 1960; Hietala and Virtanen, 1960) but the methoxy compounds IV and V were isolated from wheat and maize seedlings (Wahlroos and Virtanen, 1959). These appear to be the only examples now known of hydroxamic acids occurring in higher plants, although there are numerous reports of hydroxamic acids isolated from microorganisms (*cf.* Emery, 1965).

The cyclic hydroxamic acids have attracted attention because of their relationship to several phenomena of agronomic importance. Elnaghy and Linko (1962) have suggested a correlation between cyclic hydroxamic acid content and stem rust resistance in wheat, while BeMiller and Pappelis (1965) suggest a similar correlation with resistance of maize strains to stalk rot. Tolerance of certain plants to 2-chloro-*s*-triazine herbicides has also been related to the cyclic hydroxamic acids (Roth and Knusli, 1961; Hamilton, 1964). A linear relationship between the logarithm of the cyclic hydroxamic acid content of various inbred strains of maize and the resistance of these strains to attack by European corn borer (*Ostrinia nubilalis*) larvae has been inferred (Klun and Brindley, 1966). Purified V has been shown to be a feeding deterrent for corn borer larvae when added to a defined nutrient medium (Klun *et al.*, 1967).

Despite widespread interest in the properties of the cyclic hydroxamic acids, to our knowledge only a single investigation of the biosynthesis of these compounds has been reported (Reimann and Byerrum, 1964). These authors determined the derivation of the carbon atoms of V; however, the mechanism of synthesis of the hydroxamic acid functional group ha not been investigated. In the investigation reported here, compounds related to V have been isolated and identified. The structural relationships of these compounds suggest possible biosynthetic and functional relationships.

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