Properties of Graphical Representations of Multiple Classes of Binding Sites*

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ABSTRACT: Intercepts and slopes in various graphs of data for binding of small molecules by macromolecules with many classes of binding sites have been worked out for the following pairs of coordinates: $1/r$ *vs.* $1/A$, A/r *vs.* A, and r/A *vs.* r. These graphical parameters for multiple classes of sites are generally not what one might have expected from "intuitive" generalization from a single class.

The primary molecular step in many cellular interactions is the combination of a macromolecule (such as a protein) with one or several small molecules. The equilibrium and kinetic aspects, respectively, of such interactions have long been correlated quantitatively by the corresponding equations

$$r = \frac{nkA}{1 + kA} \tag{1}$$

$$v = \frac{v_{\max}(1/K_m)S}{1 + (1/K_m)S} \tag{2}$$

where r is the moles of bound small molecule per mole (total) protein, n the number of binding sites on the protein molecule, k the intrinsic binding constant (an *association* constant), and A the molar concentration of nonbound small molecule; and v is the initial velocity of the reaction, $v_{\max}$ the maximum initial velocity when substrate approaches infinite concentration, K_m the Michaelis constant (describing the *dissociation* reactions), and S the concentration of the substrate.

Equations 1 and 2 apply to a single site or to a single class of sites with the same intrinsic binding constant. For the evaluation of the parameters of these equations several linear transformations have been commonly used[1] (Klotz, 1946, 1953;

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nk}\frac{1}{A} \tag{3}$$

$$\frac{A}{r} = \frac{1}{nk} + \frac{1}{n}A \tag{4}$$

$$\frac{r}{A} = kn - kr \tag{5}$$

Scatchard, 1949). Their graphical equivalents are shown in Figure 1.

Where there are two or more independent classes of sites, the functions represented by the coordinates in Figure 1 will no longer give linear graphs. Nevertheless, it is still possible to determine limiting slopes and intercepts on the coordinate axes. The relation of these graphical parameters to *site* binding constants is not what seems to be commonly assumed on intuitive grounds. We have derived, therefore, the general expression for these graphical parameters. In addition we have considered in further detail the special case of a two-site system, which is often encountered in practice.

Many Independent Classes of Sites. If we have m classes of independent sites, each class, i, having n_i sites with an intrinsic binding constant, k_i, then we can generalize eq 1 and write

$$r = \sum_{i=1}^{m} \frac{n_i k_i A}{1 + k_i A} \tag{6}$$

The total number of sites, n_0, is given by

$$n_0 = \sum_{i=1}^{m} n_i \tag{7}$$

Anticipating certain sums that will be encountered in our analysis, we shall also define certain average binding constants by

$$\langle k \rangle_\gamma = \frac{\sum_{i=1}^{m} n_i k_i^{\gamma}}{\sum_{i=1}^{m} n_i k_i^{\gamma-1}} \tag{8}$$

where γ takes on values of 0, 1, or 2.

Figure 2 shows a schematic curve for a graph of variables $1/r$ and $1/A$. We wish to specify the values of intercepts 1 and 2 and of the limiting slopes 1 and 2.

Starting from eq 6 we may write

$$\frac{1}{r} = \left[\sum_{i=1}^{m} \frac{n_i k_i A}{1 + k_i A}\right]^{-1} = \left[\sum_{i=1}^{m} \frac{n_i k_i}{\left(\frac{1}{A} + k_i\right)}\right]^{-1} \tag{9}$$

From eq 9 we obtain the following results for the intercepts and limiting slopes in Figure 2.

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[1] Equations 3–5 have been expressed in terms of binding variables. The corresponding equations in terms of the variables of kinetics should be apparent from a comparison of symbols in eq 1 and 2.

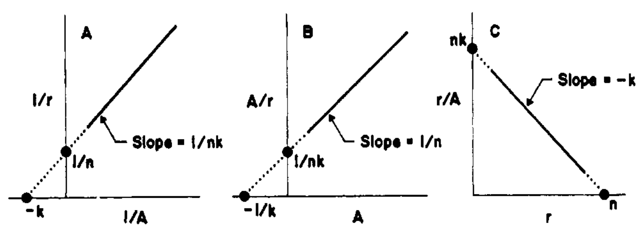

FIGURE 1: Graphical representation of the three commonly used linear transformations of the binding equation for a single site or a single class of sites. The slopes and intercepts of the three straight lines are indicated.

Intercept 1

$$\lim_{\frac{1}{A}\to 0}\left(\frac{1}{r}\right) = \left[\sum_{i=1}^{m}\frac{n_ik_i}{k_i}\right]^{-1} = \frac{1}{\sum_{i=1}^{m} n_i} = \frac{1}{n_0} \qquad (10)$$

Slope 1

$$\frac{d\left(\frac{1}{r}\right)}{d\left(\frac{1}{A}\right)} = (-1)\left[\sum_{i=1}^{m}\frac{n_ik_i}{\left(\frac{1}{A}+k_i\right)}\right]^{-2}(-1)\left[\sum_{i=1}^{m}\frac{n_ik_i}{\left(\frac{1}{A}+k_i\right)^2}\right] = \frac{\sum_{i=1}^{m}\frac{n_ik_i}{\left(\frac{1}{A}+k_i\right)^2}}{\left[\sum_{i=1}^{m}\frac{n_ik_i}{\left(\frac{1}{A}+k_i\right)}\right]^2} \qquad (11)$$

$$\lim_{\frac{1}{A}\to 0}\left[\frac{d\left(\frac{1}{r}\right)}{d\left(\frac{1}{A}\right)}\right] = \frac{\sum_{i=1}^{m}\left(\frac{n_i}{k_i}\right)}{\left[\sum_{i=1}^{m} n_i\right]^2} = \frac{1}{n_0\langle k\rangle_0} \qquad (12)$$

Intercept 2

$$\text{slope 1} = -\frac{\text{intercept 1}}{\text{intercept 2}} \qquad (13)$$

$$\text{intercept 2} = -\frac{\frac{1}{n_0}}{\frac{1}{n_0\langle k\rangle_0}} = -\langle k\rangle_0 \qquad (14)$$

Slope 2

$$\frac{d\left(\frac{1}{r}\right)}{d\left(\frac{1}{A}\right)} = \frac{\left[\sum_{i=1}^{m}\frac{n_ik_i}{\left(\frac{1}{A}+k_i\right)^2}\right]\frac{1}{A^2}}{\left[\sum_{i=1}^{m}\frac{n_ik_i}{\left(\frac{1}{A}+k_i\right)}\right]^2\frac{1}{A^2}} = \frac{\sum_{i=1}^{m}\frac{n_ik_i}{(1+k_iA)^2}}{\left[\sum_{i=1}^{m}\frac{n_ik_i}{1+k_iA}\right]^2} \qquad (15)$$

$$\frac{1}{A} \to \infty \text{ as } A \to 0$$

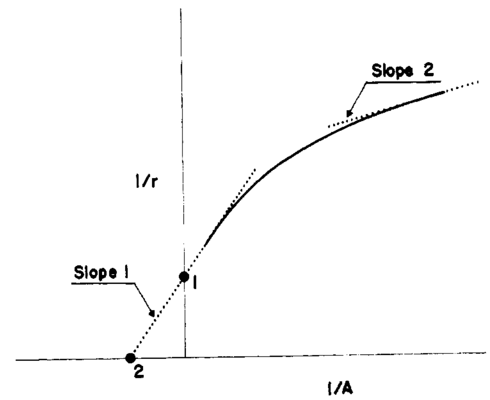

FIGURE 2: A schematic curve with intercepts and limiting slopes for a graph of variables $1/r$ and $1/A$ where m independent classes of sites are present.

$$\lim_{A\to 0}\left[\frac{d\left(\frac{1}{r}\right)}{d\left(\frac{1}{A}\right)}\right] = \frac{\sum_{i=1}^{m} n_ik_i}{\left[\sum_{i=1}^{m} n_ik_i\right]^2} = \frac{1}{\sum_{i=1}^{m} n_ik_i} = \frac{1}{n_0\langle k\rangle} \qquad (16)$$

Figure 3 shows a schematic curve for a graph with variables A/r and A. Starting from eq 6 we may write

$$\frac{A}{r} = \left[\sum_{i=1}^{m}\frac{n_ik_i}{1+k_iA}\right]^{-1} \qquad (17)$$

This leads to the following results for the intercepts and slopes in Figure 3.

Intercept 1

$$\lim_{A\to 0}\left(\frac{A}{r}\right) = \left[\sum_{i=1}^{m} n_ik_i\right]^{-1} = \frac{1}{n_0\langle k\rangle} \qquad (18)$$

Slope 1

$$\frac{d\left(\frac{A}{r}\right)}{dA} = (-1)\frac{(-1)\sum_{i=1}^{m}\frac{n_ik_i}{(1+k_iA)^2}k_i}{\left[\sum_{i=1}^{m}\frac{n_ik_i}{1+k_iA}\right]^2} = \frac{\sum_{i=1}^{m}\frac{n_ik_i^2}{(1+k_iA)^2}}{\left[\sum_{i=1}^{m}\frac{n_ik_i}{1+k_iA}\right]^2} \qquad (19)$$

$$\lim_{A\to 0}\left[\frac{d\left(\frac{A}{r}\right)}{dA}\right] = \frac{\sum_{i=1}^{m} n_ik_i^2}{\left[\sum_{i=1}^{m} n_ik_i\right]^2} = \frac{\langle k\rangle_2}{n_0\langle k\rangle} \qquad (20)$$

Intercept 2

$$\text{slope 1} = -\frac{\text{intercept 1}}{\text{intercept 2}} \qquad (21)$$

$$\text{intercept 2} = -\frac{\frac{1}{n_0\langle k\rangle}}{\frac{\langle k\rangle_2}{n_0\langle k\rangle}} = -\frac{1}{\langle k\rangle_2} \qquad (22)$$

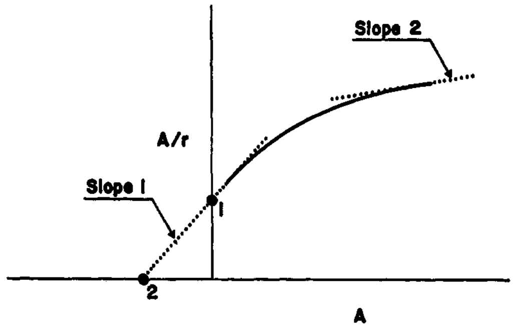

FIGURE 3: A schematic curve with intercepts and limiting slopes for a graph of variables A/r and A where m independent classes of sites are present.

Slope 2

$$\frac{\mathrm{d}\left(\frac{A}{r}\right)}{\mathrm{d}A} = \frac{\sum_{i=1}^{m} \frac{n_i k_i^2}{(1 + k_i A)^2} \frac{1}{A^{-2}}}{\left[\sum_{i=1}^{m} \frac{n_i k_i}{1 + k_i A}\right]^2 \frac{1}{A^{-2}}} = \frac{\sum_{i=1}^{m} \frac{n_i k_i^2}{\left(\frac{1}{A} + k_i\right)^2}}{\left[\sum_{i=1}^{m} \frac{n_i k_i}{\left(\frac{1}{A} + k_i\right)}\right]^2} \tag{23}$$

$$\lim_{A \to \infty} \left[\frac{\mathrm{d}\left(\frac{A}{r}\right)}{\mathrm{d}A}\right] = \frac{\sum_{i=1}^{m} n_i}{\left[\sum_{i=1}^{m} n_i\right]^2} = \frac{1}{\sum_{i=1}^{m} n_i} = \frac{1}{n_0} \tag{24}$$

Figure 4 uses r/A and r as coordinates. From eq 6 it follows that

$$\frac{r}{A} = \sum_{i=1}^{m} \frac{n_i k_i}{1 + k_i A} \tag{25}$$

From this we can obtain the following relations from graphical intercepts and slopes in Figure 4.

Intercept 1

$$\text{as } r \to 0,\ A \to 0$$

$$\lim_{A \to 0} \left(\frac{r}{A}\right) = \sum_{i=1}^{m} n_i k_i = n_0 \frac{\sum_{i=1}^{m} n_i k_i}{\sum_{i=1}^{m} n_i} = n_0 \langle k \rangle \tag{26}$$

Intercept 2

$$r = \sum_{i=1}^{m} \frac{\left(n_i k_i A\right)\left(\frac{1}{A}\right)}{\left(1 + k_i A\right)\left(\frac{1}{A}\right)} = \sum_{i=1}^{m} \frac{n_i k_i}{\left(\frac{1}{A} + k_i\right)}$$

$$\frac{r}{A} \to 0 \text{ as } A \to \infty$$

$$\lim_{A \to \infty} r = \sum_{i=1}^{m} \frac{n_i k_i}{k_i} = \sum_{i=1}^{m} n_i = n_0 \tag{27}$$

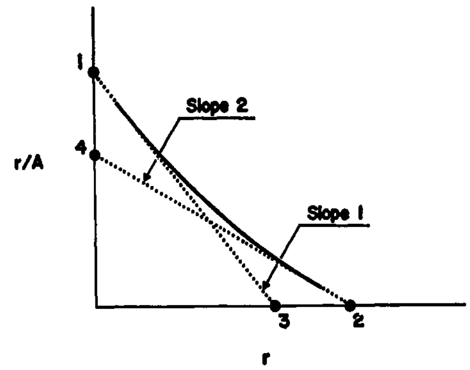

FIGURE 4: A schematic curve with intercepts and limiting slopes for a graph of variables r/A and r, where m independent classes of sites are present.

Slope 1

$$\frac{\mathrm{d}\left(\frac{r}{A}\right)}{\mathrm{d}r} = \frac{\frac{\mathrm{d}\left(\frac{r}{A}\right)}{\mathrm{d}A}}{\frac{\mathrm{d}r}{\mathrm{d}A}} \tag{28}$$

$$\frac{\mathrm{d}\left(\frac{r}{A}\right)}{\mathrm{d}A} = \sum_{i=1}^{m} (-1) \frac{n_i k_i}{(1 + k_i A)^2} k_i = -\sum_{i=1}^{m} \frac{n_i k_i^2}{(1 + k_i A)^2} \tag{29}$$

and

$$\frac{\mathrm{d}r}{\mathrm{d}A} = \sum_{i=1}^{m} \left[\frac{n_i k_i}{1 + k_i A} + (-1) \frac{n_i k_i A k_i}{(1 + k_i A)^2}\right] =$$

$$\sum_{i=1}^{m} \left[\frac{n_i k_i (1 + k_i A)}{(1 + k_i A)(1 + k_i A)} - \frac{n_i k_i^2 A}{(1 + k_i A)^2}\right] =$$

$$\sum_{i=1}^{m} \frac{n_i k_i}{(1 + k_i A)^2} \tag{30}$$

therefore

$$\frac{\mathrm{d}\left(\frac{r}{A}\right)}{\mathrm{d}r} = -\frac{\sum_{i=1}^{m} \frac{n_i k_i^2}{(1 + k_i A)^2}}{\sum_{i=1}^{m} \frac{n_i k_i}{(1 + k_i A)^2}} \tag{31}$$

$$\lim_{A \to 0} \left[\frac{\mathrm{d}\left(\frac{r}{A}\right)}{\mathrm{d}r}\right] = -\frac{\sum_{i=1}^{m} n_i k_i^2}{\sum_{i=1}^{m} n_i k_i} = -\langle k \rangle_2 \tag{32}$$

Intercept 3

$$\text{slope } 1 = -\frac{\text{intercept } 1}{\text{intercept } 3} \tag{33}$$

$$\text{intercept } 3 = \frac{-n_0 \langle k \rangle}{-\langle k \rangle_2} = \frac{n_0 \langle k \rangle}{\langle k \rangle_2} \tag{34}$$

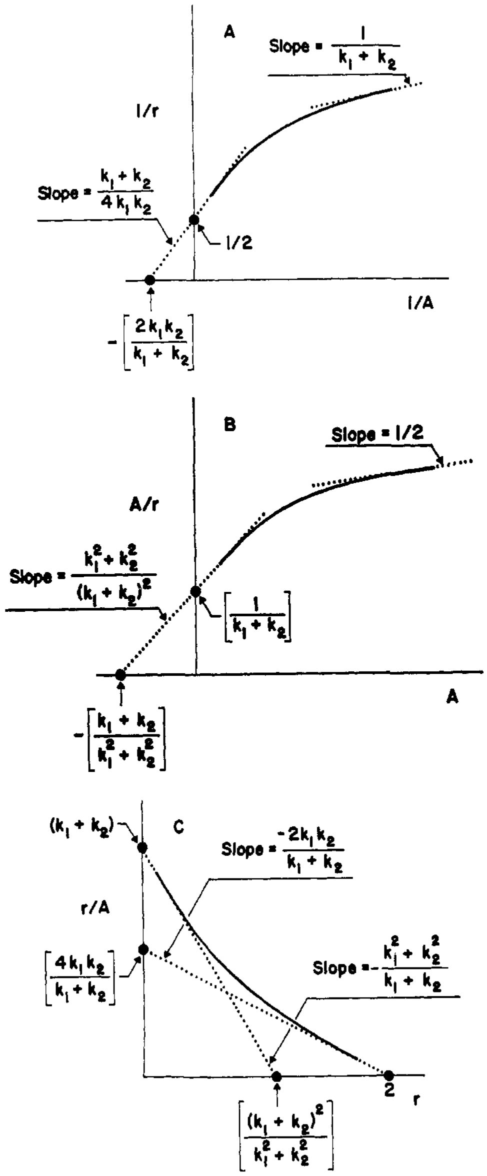

FIGURE 5: Schematic curves for each of the three pairs of coordinates for a system of only two independent binding sites. The intercepts and limiting slopes are indicated.

Slope 2

$$\frac{d\left(\frac{r}{A}\right)}{dr} = -\frac{\sum_{i=1}^{m} \frac{n_i k_i^2}{(1 + k_iA)^2} \frac{1}{A^{-2}}}{\sum_{i=1}^{m} \frac{n_i k_i}{(1 + k_iA)^2} \frac{1}{A^{-2}}} = -\frac{\sum_{i=1}^{m} \frac{n_i k_i^2}{\left(\frac{1}{A} + k_i\right)^2}}{\sum_{i=1}^{m} \frac{n_i k_i}{\left(\frac{1}{A} + k_i\right)^2}} \tag{35}$$

$$\lim_{A \to \infty} \left[\frac{d\left(\frac{r}{A}\right)}{dr}\right] = -\frac{\sum_{i=1}^{m} n_i}{\sum_{i=1}^{m} \frac{n_i}{k_i}} = -\langle k \rangle_0 \tag{36}$$

Intercept 4

$$\text{slope 2} = -\frac{\text{intercept 4}}{\text{intercept 2}} \tag{37}$$

TABLE I: Graphical Parameters (Figures 2–5) for Different Values of Site Binding Constants of Two-Site System.

	Relative Magnitudes of k_1 and k_2			
	$k_1 = k_2$	$k_1 = 2k_2$	$k_1 = 10k_2$	$k_1 = 100k_2$
	Figures 2 and 5A			
Intercept 1	0.5	0.5	0.5	0.5
Intercept 2	$-k_1$	$-0.67\ k_1$	$-0.18\ k_1$	$-0.02\ k_1$
Slope 1	$0.5/k_1$	$0.75/k_1$	$2.75/k_1$	$25.25/k_1$
Slope 2	$0.5/k_1$	$0.67/k_1$	$0.91/k_1$	$0.99/k_1$
	Figures 3 and 5B			
Intercept 1	$0.5/k_1$	$0.67/k_1$	$0.91/k_1$	$0.99/k_1$
Intercept 2	$-1/k_1$	$-1.2/k_1$	$-1.09/k_1$	$-1.01/k_1$
Slope 1	0.5	0.56	0.83	0.98
Slope 2	0.5	0.5	0.5	0.5
	Figures 4 and 5C			
Intercept 1	$2\ k_1$	$1.5\ k_1$	$1.1\ k_1$	$1.01\ k_1$
Intercept 2	2	2	2	2
Intercept 3	2	1.8	1.20	1.02
Intercept 4	$2\ k_1$	$1.33\ k_1$	$0.36\ k_1$	$0.04\ k_1$
Slope 1	$-k_1$	$-0.83\ k_1$	$-0.92\ k_1$	$-0.99\ k_1$
Slope 2	$-k_1$	$-0.67\ k_1$	$-0.18\ k_1$	$-0.02\ k_1$

therefore

$$\text{intercept 4} = -(-\langle k \rangle_0)n_0 = n_0\langle k \rangle_0 \tag{38}$$

It should be emphasized that these intercepts and slopes for multiple classes are not always the quantities one might have expected by "intuitive" generalization from a single class. For example, in Figure 4, intercept 3 is *not* the number of sites in the first of m classes but rather the parameter $n_0\langle k \rangle/\langle k \rangle_2$. Similarly intercept 4 is *not* the site binding constant for the mth class of sites but instead the quantity $n_0\langle k \rangle_0$.

Two Independent Sites. A concrete feeling for the significance of these graphical relations is perhaps best obtained by considering the special case of simply two independent sites

$$n_1 = 1; \qquad k_1 \tag{39}$$

$$n_2 = 1; \qquad k_2 \tag{40}$$

Examining Figure 5C, for example, we may substitute the above parameters into the appropriate equations, eq 26–38, for the intercepts and slopes. The following results are obtained.

$$\text{intercept 1:} \qquad k_1 + k_2 \tag{41}$$

$$\text{intercept 2:} \qquad 2 \tag{42}$$

$$\text{intercept 3:} \qquad \frac{(k_1 + k_2)^2}{k_1^2 + k_2^2} \tag{43}$$

$$\text{intercept 4:} \qquad \frac{4k_1k_2}{k_1 + k_2} \tag{44}$$

$$\text{slope 1:} \qquad -\frac{k_1^2 + k_2^2}{k_1 + k_2} \tag{45}$$

$$\text{slope 2:} \qquad -\frac{2k_1k_2}{k_1 + k_2} \qquad (46)$$

Although intercept 2 is equal to the number of binding sites (two in this example) it is apparent from eq 41 that intercept 1 is *not* equal to the binding constant for the first site. Similarly intercept 3 is not equal to the number of sites in class 1 (one in this example); on the contrary, intercept 3 must always be greater than unity (see eq 43). Furthermore, intercept 4 is *not* equal to k_2, the binding constant for the second site, as seems to be often believed.

Examining Figure 5A,B we find that here again the slopes and intercepts are not always what might be expected. Just as in Figure 5C the only parameter directly obtainable in these representations is the total number of binding sites; both intercept 1 in Figure 2 or 5A and slope 2 in Figure 3 or 5B are $1/n_0$. All the other slopes and intercepts represent various combinations of the different parameters.

If the magnitudes of k_1 and k_2 are far enough apart, some of the intercepts may approach the "intuitively" expected values. Table I illustrates this point, in conjunction with demonstrating the limits obtained for a range of relative magnitudes of the two-site binding constants.

Comparison of Site Binding Constants with Stoichiometric Binding Constants. The classical thermodynamic analysis of binding (Klotz, 1946) does not depend on any recognition of specific sites. The stoichiometric binding constants, K_i, so obtained, express the equilibria purely in stoichiometric terms. It has long been recognized, nevertheless, that the stoichiometric (or macroscopic or classical) binding constants must be related to the site (or microscopic) binding constants. For a two-site system, the relationships are analogous to those worked out for bifunctional proton-dissociating molecules (Adams, 1916; Edsall and Wyman, 1958).

$$K_1 = k_1 + k_2 \qquad (47)$$

$$K_2 = \frac{k_1k_2}{k_1 + k_2} \qquad (48)$$

For a multisite system, general relations between stoichiometric and site binding constants have been described by Fletcher *et al.* (1970).

Comparing the results of eq 47 and 48 with the intercepts of Figure 5C, we see that K_1 is indeed equal to intercept 1. On the other hand, K_2 is *not* equal to intercept 4, although they are related by a constant factor, 4.

Thus it is apparent that various site binding constants and stoichiometric binding constants can be deduced from graphical analyses for two-site (and multisite) systems.

However, the graphical intercepts and slopes are composites of contributions from both of the sites, and the individual values must be segregated out later.

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Biosynthesis of Bacterial Menaquinones (Vitamins K_2)*

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ABSTRACT: The biosynthesis of the menaquinones of *Escherichia coli*, *Mycobacterium phlei*, *Corynebacterium diphtheriae*, and *Streptomyces albus* has been studied using isotope tracer methods. To locate label derived from specifically labeled precursors, the menaquinones were chemically degraded by the following scheme: menaquinone → menaquinol diacetate → 1,4-diacetoxy-3-methyl-2-naphthaleneacetic acid → malonic plus phthalic acids. The latter acid was subsequently decarboxylated. The results demonstrate that menaquinones derive from shikimic acid and 2-ketoglutaric acid. 4-(2′-Carboxyphenyl)-4-oxobutyric acid can be incorporated into the menaquinone nucleus. No evidence could be obtained to suggest that preformed naphthalenoid compounds such as 1-naphthol, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, or 2-hydroxy-1,4-naphthoquinone, were involved as intermediates in the menaquinone biosynthetic pathway.

While all of the fat-soluble vitamins (A, D, E, and K) contain major structural features which identify them biosynthetically as isoprenoid compounds, those materials with vitamin E and vitamin K activity contain, in addition, an aromatic component. The benzene ring which constitutes the aromatic portion of the vitamin E series of compounds, *e.g.*, α-tocopherol, derives *in toto* from tyrosine (Whistance and Threlfall, 1968). The naphthoquinone ring system which figures in compounds with vitamin K activity, *e.g.*, phylloquinone and the menaquinones,[1] appears to have by contrast

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[1] In this paper, vitamins K_2 will be referred to as menaquinones following the recommended nomenclature (Folkers *et al.*, 1965). The abbreviations which are used are as follows: menaquinone with a 2-substituent of *n* prenyl units, MK-*n*; a menaquinone with a reduced double bond in the second prenyl unit, counting from the nucleus, MK-*n* (II-H_2).