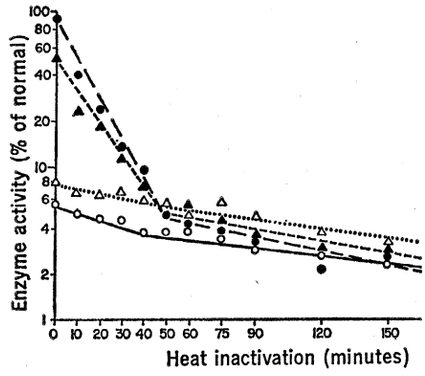

Fig. 2. Effect of heat inactivation and myoinositol on α-galactosidase activity of sonicated preparations of cultured fibroblasts from a normal subject and those from a patient with Fabry's disease. Heat inactivation of the enzyme in the cell preparation was carried out at 51°C for various periods of time. The heat-inactivated enzymes were then mixed with the substrate in 0.1*M* acetate buffer (*p*H 5.0), incubated as described in Table 1, with 5 m*M* substrate and 500 m*M* inhibitor (●—●, α-galactosidase activity of normal cells; ▲—▲, of normal cells, myoinositol added; ○—○, of cells from patient with Fabry's disease; and △—△, of cells from patient, myoinositol added).

to that found in the cell extracts from patients with Fabry's disease. Myoinositol had a mild stimulatory effect on patients' cell extracts as compared with the inhibitory effect on the normal cell extract (Fig. 2). Myoinositol had no inhibitory effect on the β-galactosidase activity in fibroblasts.

These observations are not compatible with the hypothesis of a regulator gene defect nor of a structural gene variation giving rise to an atypical enzyme. They do support the hypothesis, proposed on the basis of K_m data and electrophoretic separation of two different enzymes from leukocytes (*6*), that there is a separate heat-labile enzyme that is absent in the X-linked condition Fabry's disease. The present experiments demonstrate that the residual α-galactosidase enzyme found in fibroblast cells of patients with Fabry's disease is characterized by its higher K_m, its resistance to heat inactivation, and the lack of inhibition by myoinositol.

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29 February 1972

Flavonols: Pigments Responsible for Ultraviolet Absorption in Nectar Guide of Flower

Abstract. *The petals of the black-eyed susan (Compositae:* Rudbeckia hirta*) contain three flavonol glucosides (6,7-dimethoxy-3',4',5-trihydroxyflavone-3-O-glucoside, patulitrin, and quercetagetin). These compounds, which show intense spectral absorption at 340 to 380 nanometers, are restricted in distribution to the petal bases, which are ultraviolet absorbing as a result. Such ultraviolet-absorbing petal zones, known as "nectar guides," are invisible to us, but are visible and of orientation value to the pollinating insect that lands on the flower in search for food. This is the first time that ultraviolet absorption in a nectar guide has been interpreted in chemical terms. In view of the widespread occurrence of flavonols in flowers, it is suggested that these pigments serve specifically for demarcation of ultraviolet petal patterns visible and relevant to insects.*

The petals of flowers commonly show differential color markings at their base. Such markings have long been known as nectar guides (*1*). Located centrally on the flower, near the nectaries, the guides cue pollinating insects to the presence of adjacent food (*2, 3*). Nectar guides are frequently invisible to man. Consisting of ultraviolet-absorbing patches, they are discernible only to insects such as honey bees, whose visual sensitivity extends into the near-ultraviolet region of the solar spectrum (*3–5*). Although much is known about the floral pigments responsible for the visible colors of flowers, the chemical basis of ultraviolet absorption in nectar guides has remained unexplained. Evidence suggested that phenolic pigments were involved (*6*), but the pigments were not characterized. We have now demonstrated that ultraviolet absorption in the nectar guide of a composite flower, the familiar black-eyed susan (*Rudbeckia hirta*), is attributable to a mixture of flavonol glucosides.

To the human eye, the petals of *Rudbeckia* appear evenly yellow (Fig. 1A). Ultraviolet photography reveals the darkly absorbing nectar guides, which encompass the entire basal halves of the petals (see Fig. 1B). Reflection spectra of the base and apex of a petal (see Fig. 2A) show the two regions to be closely similar in the visible portion of the spectrum, but to differ sharply in the near ultraviolet (*7*).

Separate extraction, by prolonged soaking in methanol, of the severed bases and apices of a small quantity of petals, caused removal of all visible coloration from both samples, as well as of the ultraviolet-absorbing material from the bases. A comparison of the absorption spectra of the basal and apical extracts (Fig. 2B) shows both to have the carotenoid absorption responsible for yellow coloration (maxima at 420, 443, and 470 nm), but to differ in the near ultraviolet, where the basal extract has a maximum (350 nm), and the apical extract a minimum (360 nm). Thin-layer chromatography (TLC) (on polyamide TLC-11) of the basal extract showed three components, absent from the apical extract. It was these components that we set out to identify.

A sample of whole petals (6 g, dry weight) was extracted by soaking them for 3 weeks in methanol. Evaporation of the extract gave 1.4 g of a dark residue, from which 0.4 g of carotenoid material was removed by ether extraction. The ether-insoluble material was chromatographed on 37 g of polyamide CC-6 with aqueous butanone. Virtually all of the absorption in the 340- to 380-nm region was accounted for by three chromatographic fractions, which corresponded to those found by TLC of the basal extract. The least polar fraction yielded 21 mg of pale yellow crystals (compound **1**) on crystallization from aqueous methanol. The fraction of intermediate polarity yielded 10 mg of a pale green material (compound **2**) on crystallization from a mixture of acetonitrile and methanol, and the most polar component produced a yellow amorphous solid (compound **3**) on at-

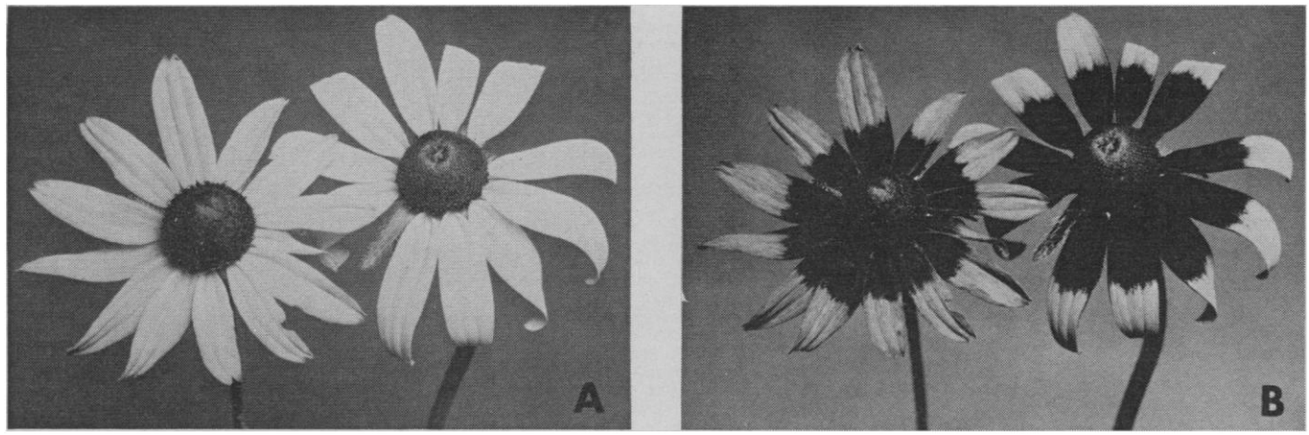
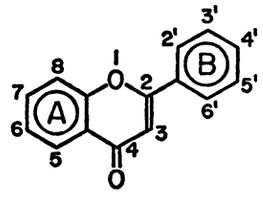


Fig. 1. (A) *Rudbeckia hirta*, photographed in visible light. (B) Same, photographed with ultraviolet transmitting lens and filter, in ultraviolet light. The absorbent basal portions of the petals are the "nectar guides."

tempted crystallization from aqueous methanol.

The methanolic ultraviolet spectrum of **1** (Fig. 2C) showed absorption maxima at 352 nm (molar extinction ε, 19,200) and 259 nm (ε, 20,600), with a shoulder at 268 nm. In methanolic sodium methoxide the long-wavelength absorption underwent a bathochromic shift of 58 nm, and the spectrum was stable for at least 1 hour. In methanolic aluminum chloride there was a bathochromic shift of 88 nm in the long-wavelength absorption, which was reduced to 28 nm on addition of hydrochloric acid. These spectra are characteristic of a flavone, with hydroxyl substituents at, inter alia, C-4′ and C-5; a pair of adjacent hydroxyl groups is also implied (*8*).



flavone

Acidic hydrolysis of **1** yielded glucose (*9*) and a water-insoluble crystalline aglycone whose high-resolution mass spectrum revealed an elemental composition of $C_{17}H_{14}O_8$, corresponding to a flavone bearing two methoxyl and four hydroxyl groups. In addition, an intense fragment with elemental composition $C_{15}H_{11}O_7$ indicated facile loss of CH_3CO from the molecular ion, a process characteristic of flavones bearing a methoxyl substituent at C-3, C-6, or C-8 (*10*).

Acidic hydrolysis of permethylated **1** (*11*) yielded another water-insoluble aglycone, the ultraviolet spectra of which (in the media discussed above) were in agreement with those described for 3-hydroxy-3′,4′,5,6,7-pentamethoxyflavone (*8*). This establishes the oxygenation pattern of **1**, and the attachment of the glucose moiety at C-3.

The 60-Mhz nuclear magnetic resonance (NMR) spectrum (CCl_4) of pertrimethylsilylated **1** showed: δ 7.57 (multiplet, 2), 6.87 (doublet, 1; $J = 9.0$ hz), 6.52 (singlet, 1; shifts to δ 6.38 on selective removal of the C-5 trimethylsilyl group), 5.91 (m, 1), 3.91 (s, 3), 3.73 (s, 3), 3.69 (m, 6), 0.0 to 0.3 (m, ~ 60). This is consistent with the presence of two methoxyl and three hydroxyl groups at positions 3′, 4′, 5, 6, and 7, and a glucose unit at C-3. The high-resolution mass spectrum discussed above implicated a methoxyl group at C-3, C-6, or C-8. Since C-3 bears the glucosyl moiety and C-8 a hydrogen, the methoxyl in question must be located at C-6. Basic hydrolysis of flavones provides a convenient method of characterizing substituents on ring B, which is converted into a benzoic acid derivative (*12*). When the water-insoluble aglycone from **1** was treated with 40 percent aqueous potassium hydroxide, the ring B fragment proved to be 3,4-dihydroxybenzoic acid by gas liquid chromatographic comparison of the trimethylsilylated product mixture with an authentic sample of this trimethylsilylated acid. This result established the presence of the pair of adjacent hydroxyl groups at C-3′ and C-4′ in ring B. Thus the remaining methoxyl substituent must be bound to C-7, and compound **1** must be 6,7-dimethoxy-3′,4′,5-trihydroxyflavone-3-*O*-glucoside, a previously unreported flavonol glucoside (Fig. 3).

Data from ultraviolet and NMR spectra obtained from compound **2** and its pertrimethylsilylated derivative were in agreement with those reported for patulitrin (*13*). The high-resolution mass spectrum of the aglycone of **2** yielded the expected elemental composition, and the sugar was shown to be glucose (*9*). Paper chromatographic comparison of **2** and authentic patulitrin in two solvent systems confirmed their identity (*14*) (Fig. 3). Similarly, the ultraviolet spectra of **3** and the NMR spectrum of its pertrimethylsilylated derivative indicated that **3** was quercetagetin (*13*) (Fig. 3). This assignment was substan-

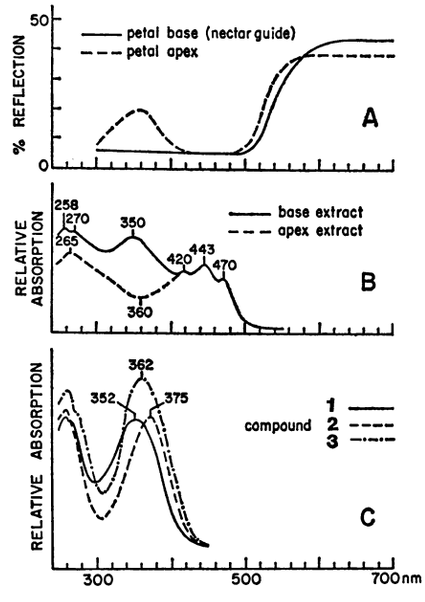
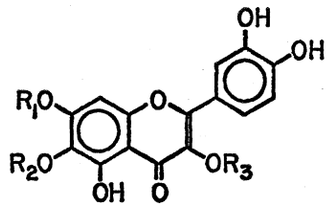


Fig. 2. (A) Reflection spectra of base and apex of living petal of *Rudbeckia hirta*. (B) Absorption spectra of methanol extracts of petal bases and apices. (C) Ultraviolet spectra of the three flavonols from the petal base (nectar guide).



Compound	R_1	R_2	R_3
1	CH_3	CH_3	glucose
2	glucose	CH_3	H
3	glucose	H	H

Fig. 3. Flavonols from nectar guide of *Rudbeckia hirta.*

tiated by the mass spectrum of the aglycone of **3**, sugar analysis (*9*), and paper chromatographic comparison of this aglycone with an authentic sample of quercetagetin (*13*).

Given the ultraviolet spectra of the three flavonols (Fig. 2C), their restriction to the petal bases, and the fact that they account for virtually all the absorption in the near-ultraviolet spectrum of a basal extract, this leaves no doubt that these are the compounds responsible for the absorbent quality of the nectar guide. Additional evidence was provided by the observation that artificial nectar guides, clearly visible by ultraviolet videoviewing (*5*), could be induced by delivery of droplets of methanolic solutions of the individual or mixed flavonols onto any part of the surface of a methanol-extracted *Rudbeckia* petal.

Flavonols are of widespread occurrence in flowers (*15*). Although visibly yellow like many flavonoids, they constitute the major group of floral pigments whose *chief* absorption matches the region in the near ultraviolet that the ultraviolet receptors of insects detect. No special function has hitherto been advanced for floral flavonols, and we propose that they serve primarily as ultraviolet-absorbing pigments, supplementing the other two major groups of floral pigments, the anthocyanins and the carotenoids, which account for most of the visible colors of flowers (*16*). Flavonols might thus be of considerable value in the study of floral speciation and evolution, and particularly of floral coevolution with ultraviolet-sensitive insects.

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16. Two other groups of flavonoids, the chalcones and the aurones, also include floral compounds with absorption maxima in the ultraviolet region of insect vision. They may therefore share the function of the flavonols, although they appear to be of more limited distribution than the latter.
17. Supported by grant AI-02908 and traineeship 5T01-GM00834 from the National Institutes of Health. We thank T. H. Goldsmith (Yale University) and Peter H. Raven (Missouri Botanical Gardens) for helpful discussions, T. J. Mabry (University of Texas) for samples of patulitrin and quercetagetin, and our colleague Peter A. Hyypio for identification of the flower.

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Evolutionary Clock: Nonconstancy of Rate in Different Species

Abstract. *By using various methods for comparing polypeptide sequences we find that the evolutionary divergence of rattlesnake cytochrome c from cytochromes c of species in other classes has been more rapid than that of cytochrome c of another reptile, the snapping turtle. This suggests that the evolutionary rate of change of cytochromes c is species-dependent as well as time-dependent.*

When two species of living organisms undergo divergent evolution from a common ancestor, their homologous proteins typically show time-related changes. Various proposals have been made for relating these changes to a time scale. Amino acid differences per 100 residues per unit time are sometimes employed (*1, 2*) as a measure. Other authors have used minimal mutation distances (minimum base differences between the codons of the replaced amino acids at corresponding loci in two polypeptides) for making quantitative comparisons (*3, 4*). In either case, certain anomalies appear in certain vertebrates with respect to the magnitude of these changes and their relationship to time. Such anomalies show up on "phylogenetic trees" as apparently negative rates of evolutionary divergence, or incorrect taxonomic placement of an organism in the wrong family (*3, 5*). In this report, we compare some vertebrate cytochromes c by the above methods and by a stochastic model of evolution (*6, 7*) and offer a possible explanation of these anomalies.

The term "evolutionary clock" embodies the concept of regularity, which Sarich (*8*) defined as the situation where "the probability of an amino acid sub-

Table 1. Differences in amino acid sites in various cytochromes c. The numbering of the sites is identical with that used by Bahl and Smith (*10*). The sequences are described in (*2, 10, 12, 13*).

Sites numbered	Total	Differences common to comparisons of
3, 35, 50, 89, 92	5	Birds-turtle and birds-snake
11, 12, 44, 46, 58, 61, 81, 83, 85, 86, 93, 100, 101, 103, 104	15	Birds-snake only
15, 33, 36, 62	4	Birds-turtle only
11, 12, 15, 33, 36, 44, 46, 50, 58, 61, 62, 81, 83, 85, 86, 89, 92, 93, 100, 101, 103, 104	22	Snake-turtle