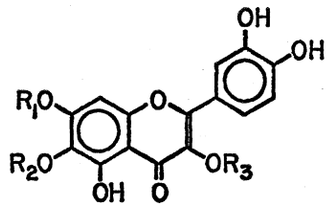

Compound	R_1	R_2	R_3
1	CH_3	CH_3	glucose
2	glucose	CH_3	H
3	glucose	H	H

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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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

stitution occurring in a given span of time is the same for each lineage in which that protein is found." We give an example based on comparisons of several vertebrate cytochromes c to show that the "evolutionary clock" does not run at a constant rate for all species.

The cytochromes c of four birds and two reptiles may be conveniently placed in a single taxon to include the adjoining classes of Reptilia and Aves (*9*). Rattlesnake cytochrome c has not been completely sequenced; the ordering of residues 11 to 26 was made by Bahl and Smith (*10*) on the basis of assumed homology with human cytochrome c. We have transposed residues 11 and 12 to increase the homology with the sequences of the other species. Arranging unsequenced peptides on the basis of "maximum homology" is not a good procedure in studies of molecular evolution. However, only three of the amino acid differences in the comparisons (Table 1) occur in the unsequenced region, and any differences in this region that are obscured by Bahl and Smith's alignment and our transposition of residues 11 and 12 would only increase the disparity which is the subject of the calculations below.

In the comparisons which follow, the birds have been placed in a single group, and are compared with each of the two reptiles, which are compared with each other.

In Table 1, we have noted the amino acid sites at which differences occur during the divergence of two reptilian cytochromes (snake and turtle) from each other and from the four avian species given in Table 2. Five sites differ in birds from both snake and turtle (Table 1). There are only four other sites in which turtle differs from birds, but there are 15 other sites in which snake and birds differ. We suggest that this shows that snake cytochrome c has evolved several times, perhaps three or four times, as rapidly as turtle cytochrome c during the time elapsed from the common ancestral origin of reptiles and birds.

So far, we have discussed the bird, turtle, and snake divergence on the basis of amino acid comparisons. The amino acid sequence comparisons are summarized in Table 2. From these data, it is possible to calculate (Table 3) the amino acid differences per codon, the minimum base differences per codon, and, by using the stochastic model (*6*, *7*), the number of random evolutionary hits per codon (REHC). Estimation of the first two measures was discussed adequately elsewhere (*1*–*5*). Random evolutionary hits (REH) is an estimate of the number of one-step nucleotide replacements that the structural DNA which codes for a protein has undergone, corrected for multiple-hit phenomena, the degeneracy and vagaries of the genetic code, spurious identities due to chance, and the fact that only a part of the structural gene is free to accept mutations at any one time. The experimental data from which the REH estimates are made are the number and type (in terms of minimum base changes per codon) of observed amino acid differences. The estimation assumes that the accepted point mutations in the DNA have occurred in a spatially random manner over the variable part of the structural gene. Details may be found in (*6*) and (*7*), where the stochastic model is discussed more fully. The REH calculation permits an estimate of the number of amino acid sites freely to vary during the divergence of any pair of homologous proteins, and the average number of nucleotide mutations that the DNA that codes for these sites has undergone.

Table 2. Differences in amino acid residues in various cytochromes c, by various methods of comparison (see text).

Comparison	Number of residues with indicated minimum base differences per codon			
	0	1	2	3
Rattlesnake versus				
King penguin	84	15	5	0
Duck	87	12	5	0
Pigeon	86	13	5	0
Chicken	85	13	6	0
Average	85.5	13.2	5.2	0
Snapping turtle versus				
King penguin	96	8	0	0
Duck	97	7	0	0
Pigeon	96	7	1	0
Chicken	96	8	0	0
Average	96.2	7.5	0.25	0
Rattlesnake versus				
Snapping turtle	82	17	5	0
Chicken versus				
King penguin	102	2	0	0
Duck	101	3	0	0
Pigeon	100	3	1	0
Average	101	2.7	0.3	0

In Table 2, the snake-bird comparison shows a much higher proportion of two-base changes (that is, differences per codon requiring a minimum of two-base changes) than the turtle-bird comparison. This produces a high calculated value (*6*) for REHC, but the number of calculated variable amino acid sites is the same, that is, 24, as the total found by direct comparison (Table 1). The more rapid evolution of snakes than turtles is thus further emphasized by the number of two-base changes. In addition, the number of variable sites in the snake-bird comparison is calculated as 25, in good agreement with the 24 differing amino acids. These 25 sites are calculated to have undergone 2.3 hits (accepted one-step nucleotide replacements) per site for a total of 58 per molecule, more than twice as great as the number of minimum base changes, which is 23.

Our viewpoint states that there should be about the same number of variable sites in the turtle-bird comparison, because we are again comparing birds and reptiles, but that these should have so far undergone far fewer changes because of the evidently slower rate (Table 3) of evolution in turtle cytochrome c. However, in this case, so few sites have been changed that

Table 3. Three per-codon measures for various comparisons.

Comparison	Amino acid difference per codon	Minimum base difference per codon	Random evolutionary hits per codon
Rattlesnake versus birds	0.18	0.23	0.58
Snapping turtle versus birds	.075	.077	.11
Rattlesnake versus snapping turtle	.21	.26	.55
Chicken versus other birds	.030	.033	.050

the calculation is in a range where one or two additional base differences affect it markedly. When calculated (*6*) for best fit, there is 0.22 hit per site on 54 variable sites, which is discordant with the snake-bird comparison, but one more two-base change would bring the computations into line for 0.55 hit per site on 25 variable sites, with one-fourth as many REHC for turtle as for snake (0.14 versus 0.58). The statistical variance in the estimate of the number of variable sites is thus large and very strongly dependent on the sample of sequences available. This large variance is not, however, a property of the estimate of the number of REHC. In fact, it can be shown (*11*) that the observed difference in the number of amino acid substitutions between the snapping turtle-chicken and rattlesnake-chicken exceeds chance fluctuations by a factor of more than 3.6.

A direct comparison of turtle and snake (Table 3) shows that the two reptiles have diverged widely, and we infer that most of this divergence is due to changes in snake. The four birds inter alia show very little divergence, even though the penguin is in a different superorder (*9*) from the other three. When snake, turtle, and chicken are compared for amino acid differences with the cytochromes c of seven nonreptilian and nonavian vertebrates [lamprey, dogfish, tuna, kangaroo, bovine, rabbit, and human (*2*)], in each case the snake differs more than either turtle or chicken. The average amino acid difference between snake and these seven species is 21.0 ± 4.5 (S.D.); the corresponding average for both turtle and chicken is 13.4 ± 4.2.

The Reptilia are a large and diverse class that includes both primitive and "modern" animals, so that it might be expected that turtle and rattlesnake would represent widely divergent lines. However, the difference between turtle and rattlesnake of 21 amino acid residues per 100 codons is notably larger than many differences between representatives of widely separated classes, for example, 17 between chicken and lamprey, or 16 between horse and dogfish, or even 15 between dog and screwworm fly in two different phyla.

The identification of the variable codons in rattlesnake cytochrome c is further supported by comparing it with bovine cytochrome c. All 20 of the sites differing in this comparison also differ in the snake-turtle comparison.

The differences in Tables 1, 2, and 3 suggest that the evolutionary rate of change of vertebrate cytochromes c is species-dependent as well as time-dependent.

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11. For the rattlesnake-bird comparison (Table 2), there is a total of 58 (2.3 × 25) evolutionary hits. For the snapping turtle-bird comparison, there are 14 (0.55 × 25) such hits. The standard deviation in observable number of base differences to be found between the two pairs of homologous structural genes which code for these cytochromes can be calculated from Eqs. 23 and 24 in (*7*) with $L = 25 \times 3 = 75$ and $X = 58$ and 14, respectively, and are S.D. rattlesnake-bird = 3.4 and S.D. snapping turtle-bird = 1.3. One can then expect an uncertainty in the number of amino acid differences of 2.6 (= 3.4 × 0.76) and 1.0 (= 1.3 × 0.76), respectively, because of codon degeneracy. If the difference in the number of amino acid differences is taken, the error in this difference is about $[(2.6)^2 + (1.0)^2]^{1/2} = 2.8$. The observed difference (Table 2) is 18.5 − 7.8 = 10.7, well over 3 standard deviations away, and significant.
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Rapid Light-Induced Decrease in Pineal Serotonin N-Acetyltransferase Activity

Abstract. *Light acting by way of the eye causes the dark-induced activity of serotonin* N-*acetyltransferase in the pineal gland of the rat to decrease with a halving time of about 3 minutes. This effect, which is one of the more rapid physiological changes known to occur in the activity of any enzyme that metabolizes biogenic amines, appears to explain the rapid increase in the concentration of pineal serotonin that is caused by light exposure at night.*

Serotonin *N*-acetyltransferase converts serotonin (5-hydroxytryptamine) to *N*-acetylserotonin (*N*-acetyl-5-hydroxytryptamine), the precursor of melatonin (5-methoxy-*N*-acetyltryptamine) (*1*). The activity of serotonin *N*-acetyltransferase in the pineal gland of the rat increases at night in the dark, when rats are active, to values that are 15 to 70 times greater than the day values (*2, 3*). It was not known if light could decrease the activity of this enzyme after it had been dark-induced. We now report that exposure to light at night causes a rapid decrease in the activity of pineal *N*-acetyltransferase.

Groups of male Osborne-Mendel (NIH strain) rats (200 to 225 g) were housed for 7 to 10 days in a room without windows, but with automatically regulated lighting that provided 14 hours of light and 10 hours of darkness. The lights were turned off at 7:00 p.m. At 11:30 p.m. the animals were removed from that dark room and transferred to a room where the lighting was about 100 lumen/m^2 (*4*). After being in the light for 0.25 to 10 minutes, the animals were stunned and immediately decapitated. The pineal glands were removed within 30 seconds of decapitation, were frozen on solid CO_2, and were stored for 12 hours at −20°C. Serotonin *N*-acetyltransferase activity in individual pineal glands was estimated as described (*2, 3*). When animals were placed in the lighted room the activity of pineal serotonin *N*-acetyltransferase was above 5000 units (Fig. 1). During a 10-minute exposure to light the activity fell to 400 units. The halving time ($t/2$) was slightly less than 3 minutes.

To determine whether we were actually observing an effect of light acting by way of the eye or if handling of the animals was causing the decrease in enzyme activity, we used blinded animals in a second study. The rats were blinded by bilateral enucleation while under ether anesthesia 36 hours prior to decapitation. They were housed in cages with control animals that had been