occurred when the RNA-driven C_0t (C_0t is the nucleotide concentration in moles per liter and t is time in seconds) of 0.1 to 200 mole liter^{-1} sec. Below a C_0t of 1.0, only a small amount of DNA was bound. Most of the DNA hybridized with mRNA at C_0t values between 1 and 200. Thus the hybridization reaction follows the course expected (*17*).

Messenger RNA's isolated from different sources (such as Ehrlich ascites carcinoma cells, mouse liver) give similar hybridization curves (Fig. 4) as they have similar complexity. An increase of the DNA molecular weight and an increase of the exonuclease digestion led to an increase of DNA binding (*18*). However, the background (nonspecific) DNA binding to poly(U)-Sepharose in the absence of mRNA also increased in these cases (*18*).

This background binding is due to the interaction between oligodeoxyadenylate (dA) regions of DNA with poly(U)-Sepharose. For this reason, it appears that a simple repetition of the hybridization and fractionation on poly(U)-Sepharose does not lead to a further purification of the material. Indeed, it was confirmed in rehybridization experiments. About 25 percent of the DNA that was absorbed on poly(U)-Sepharose after the first cycle of the hybridization with poly(A)$^+$ mRNA was involved in the hybridization during the second cycle. However, the background was also increased and reached about 3 to 4 percent (*18*). The use of mercuriated mRNA and purification of hybrids on SH-Sepharose resulted in significant purification because of the lower background binding (< 0.1 percent). The proportions of the hybridized material on SH-Sepharose and on the poly(U)-Sepharose were similar (Fig. 5). Thus, the SH-Sepharose technique may be used efficiently for the second cycle of purification or for both cycles.

This technique is being used for the isolation of fragments carrying the individual gene for mouse globin. Two-step hybridization-chromatography gives about 10,000-fold purification of the gene.

Another technique for the enrichment of native DNA with fragments containing some specific sequences, is the so-called "R-loop" method described by Thomas *et al.* (*19*). It depends on the formation of a hybrid between RNA and one strand of native DNA, under conditions favoring the stability of the RNA-DNA complex.

We checked the possibility of combining this hybridization procedure with the isolation of hybrids on poly(U)-Sepharose. Total mRNA was hybridized to fragments of native DNA with a molecular weight of $3 \times 10^{6\text{ to }6} \times 10^6$ under conditions for R-loop formation, and then the material was passed through a poly(U)-Sepharose column (Fig. 6). Some of the DNA was specifically retained on the column and could be eluted by heating at low ionic strength or by treatment with ribonuclease. Although it is difficult to calculate C_0t values accurately, the curve has essentially the same shape as in experiments described above.

Table 1. Competitive hybridization experiment with DNA of λgt-Dm 225. The mixture contained 0.4 μg of immobilized DNA; Dm poly(A)$^+$ mRNA, 1×10^6 count/min (5×10^5 count/min μg); or Dm cRNA, 5×10^6 count/min; and in some samples unlabeled Dm ploy(A)$^+$ mRNA, 20 μg (as competitor). The mixture was made up in 0.3 ml of double strength saline–sodium citrate and 0.1 percent sodium dodecyl sulfate; it was incubated for 20 hours at 65°C, and treated subsequently as described (*5*).

DNA	Incorporation (count/min)			
	^{3}H-labeled polyA$^+$ mRNA hybridized		^{3}H-labeled Dm cRNA hybridized	
	No competitor	Competitor	No competitor	Competitor
λgt-Dm225	483	82	848	820
λgt-λC	10	8	72	59
E. coli	5	5	32	52

The R-loop technique has some advantages in that it does not require any enzymatic treatment of DNA; the method does have some limitations, such as dependence of R-loop formation on the G · C (guanosine · cytosine) content of DNA and on the size of RNA.

Our exonuclease technique can also be used for obtaining the fragments with definite localization of structural gene. Now that several methods for the isolation of large pieces of native DNA containing structural genes are available, we believe that, after the containment problems are solved, such fragments of DNA can be studied in prokaryotic vectors.

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Nonnutritive Sweeteners: Taste-Structure Relationships for Some New Simple Dihydrochalcones

Abstract. *Six sweet and five nonsweet simple nonglycosidic dihydrochalcones were prepared, two of which have properties comparable to those of the intensely sweet neohesperidin dihydrochalcone.*

The discovery of a new class of intensely sweet compounds, obtained by a simple chemical modification of some naturally occurring flavonoids, was first reported by Horowitz and Gentili (*1*). For example, hydrogenation of the flavanones naringin (**1**) and neohesperidin (**2**), the predominant bitter constituents in grapefruit and Seville orange rind, respectively, gave the intensely sweet dihydrochalcones (DHC's) **3** and **4** in excellent yield (see Fig. 1). Dihydrochalcones seem an attractive class of sweeteners from a safety point of view since neohesperidin DHC (**4**) has not been reported to show any ill effects in either multi-

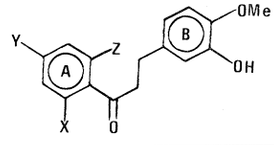
generation rat feeding studies or long-term dog feeding trials (*2*). However, the supply of glycosidic DHC's such as **3** and **4** appears to be limited to that available from natural sources since total synthesis of compounds of this complexity is likely to be economically unfeasible. A more serious problem is that the intense pleasant sweetness of DHC's **3** and **4** is notably slow in onset with considerable linger, which renders them unsuitable for many food uses.

The utility of DHC's **3** and **4** thus appears to be limited by both economics and taste properties. A recent report (*3*) indicating that simple DHC **5** (Fig. 1) exhibits a strong sweet taste greatly increased our interest in DHC's. It is clear from a comparison of **5** with **3** and **4** that the complex glycoside of **3** and **4** is not necessary for sweetness. Since simple DHC's such as **5** should be reasonably easily synthesized, we decided to prepare a series of structurally related compounds with the expectation that one or more of these materials might possess a sucroselike sweetness.

While numerous analogs varying in the substitution in the B ring of the DHC nucleus have been prepared, fewer varying in A-ring substitution have been reported (*4*). We therefore decided to concentrate our efforts on A-ring variants. A total of 11 compounds were prepared and submitted to sensory analysis (*5*). All new compounds were given a preliminary taste evaluation by sampling a saturated aqueous solution. Completely tasteless compounds were not further analyzed. Wherever allowed by solubility, the carboxylic acid DHC's were evaluated as free acids as well as alkali metal salt derivatives. All new compounds exhibiting any taste whatever were submitted for evaluation by a panel of six judges previously acquainted with the four primary tastes in the forms of dilute quinine hydrochloride (bitter), sucrose (sweet), sodium chloride (salty), and dilute hydrochloric acid (sour) solutions. This procedure for sensory analysis was essentially identical to that described earlier by Acton *et al.* (*6*). Judges were then asked to assign relative magnitudes of these four primary tastes to that of each tastant solution. In most cases, the relative amounts of these four tastes did not total 100 percent. Panelists frequently described the presence of other tastes such as medicinal, phenolic, and especially menthol-like. Total taste intensities were determined by comparison of the sample solution with a 8.55 percent (0.25*M*) sucrose standard. For comparison, sucrose, saccharin, cyclamate, and neohesperidin DHC (**4**) samples were included. Each sample was evaluated twice by each judge, once in the morning and once in the afternoon, and the results of two judgments for six judges, totaling 12 determinations, were averaged. A few of the most attractive compounds were evaluated in more than one session, resulting in more than 12 determinations.

The data thus obtained are summarized in Table 1. It should be clear that all our data have been determined relative to a 0.25*M* sucrose standard. The values obtained should not be construed as true on an absolute basis at all concentrations. We wish to emphasize that the data given are not intended to provide absolute values for either taste intensities or flavor percentages. Such precise information can be obtained only by analysis of each compound at several concentrations by eight to ten judges, with five to six evaluations by each judge at each concentration. Our data, however, are useful for illustrating the gross effects of

Table 1. Dihydrochalcone structure-taste relationships. Concentrations are in parts per million (ppm). Intensities are compared to sucrose on the basis of weight. Flavor judgments totaled 100 percent (see text). *N* = number of judgments.



Compound	*X*	*Y*	*Z*	*N*	Concentration of tastant solution (ppm)	Intensity relative to sucrose	Flavor judgment (%)			
							Sweet	Sour	Salty	Bitter
4	OH	*O*-β-neohesperidosyl	OH	12	250	663	88	3	2	5
5A	OH	OCH_2COOH	H	12	90	76	38	6	7	7
5B	OH	OCH_2COONa	H	12	95	63	42	0	8	32
6	OH	OH	H	12	90	57	1	0	1	4
7	OH	H	OH	12	90	247	21	5	9	23
8	OH	OH	OH	24	90	661	84	0	2	11
9A*	H	OCH_2COOH	H	1	1,800	0				
9B*	H	OCH_2COONa	H	1	1,680	0				
10A*	OH	OCH_2COOH	OCH_2COOH	1	2,300	0				
10B*	OH	OCH_2COONa	OCH_2COOH	1	2,200	0				
11A	OH	OCH_2COOH	OH	12	90	200	66	0	0	12
11B	OH	OCH_2COONa	OH	36	95	501	82	1	3	8
12A	OH	$OCH(CH_3)COOH$	OH	12	90	29	19	0	0	32
12B	OH	$OCH(CH_3)COONa$	OH	12	95	117	22	0	0	57
13*†	OH	$OCH(COONa)_2$	OH	1	495	0				
14	OH	$O(CH_2)_3COOK$	OH	12	250	308	74	9	5	11
15A*	OH	$OCH(COOH)$-CH_2CH_2COOH	OH	1	2,400	0				
15B*	OH	$OCH(COONa)$-CH_2CH_2COONa	OH	1	2,400	0				
Sucrose				24	85,500	1	98	0	1	1
Saccharin (sodium salt)				36	60	490	70	3	5	18
Cyclamate (sodium salt)				36	1,250	29	70	8	14	4

*The saturated aqueous solution was found tasteless and therefore was not analyzed by the taste panel. †The free acid form decarboxylates in aqueous solution to produce sweet DHC **11**.

various types of structure modification.

Our studies confirm the earlier report that **5** is a sweet compound (see **5A** and **5B** in Table 1) and that the glycoside portion of DHC **4** is unnecessary for sweetness (*3*). The taste intensity and flavor properties of DHC **5** appear to be much inferior to those of **4**, however (see **4**, **5A**, and **5B** in Table 1). Significantly better taste properties are encountered with DHC **11B**, which bears an additional hydroxyl group in the A ring, as is the case with **4**. In fact, carboxymethyl DHC **11B** compared quite favorably with glycosidic DHC **4**. Thus the relative intensity and sweetness seem to decline as the hydroxyl substitution on the A ring decreases, so that dihydroxy DHC **11** appears to be an excellent sweetener, while the monohydroxy compound **5** appears marginal at best and the norhydroxy compound **9** is tasteless. This trend is also observed in the noncarboxylic acid series **6**, **7**, and **8**, where dihydroxy DHC's **6** and **7** are very poor sweeteners and trihydroxy DHC **8** has an excellent sweet taste. It is also interesting to compare the data obtained for **8** with those for the glycoside **4**. It should be recognized that **8** is simply the aglycone portion of **4**. Approximately equal sweetness intensities were determined for **4** (663) and **8** (661), whereas Horowitz (*7*) reported "an enormous increase in sweetness" on proceeding from **8** to **4**. Our results suggest that the glycoside moiety of **4** is unimportant with regard to taste intensity. Aglycone **8** is somewhat less useful, however, because of its lower water solubility. Thus, the only useful function of the disaccharide portion of **4** appears to be to make the DHC molecule more soluble in water.

The results in Table 1 confirm that the carboxymethyl group can replace the bulky glycoside of **4** without significantly changing taste properties or decreasing water solubility (compare **4** and **11**). Surprisingly, the DHC's containing more than one carboxyl group are all tasteless (compare **10**, **13**, and **15**). The reasons for this phenomenon are not understood. However, as the number of attached carboxyl groups increases, the hydrophilic character of the molecule also increases, which may alter a critical hydrophobic-hydrophilic balance and result in tasteless compounds. If the molecule's hydrophilic character is too high, partitioning from saliva onto the receptor sites of the tongue may be depressed, with a concomitant loss of taste stimulation. This type of reasoning was used by Deutsch and Hansch (*8*) to explain taste-structure relationships in the *m*-nitroaniline class of sweeteners.

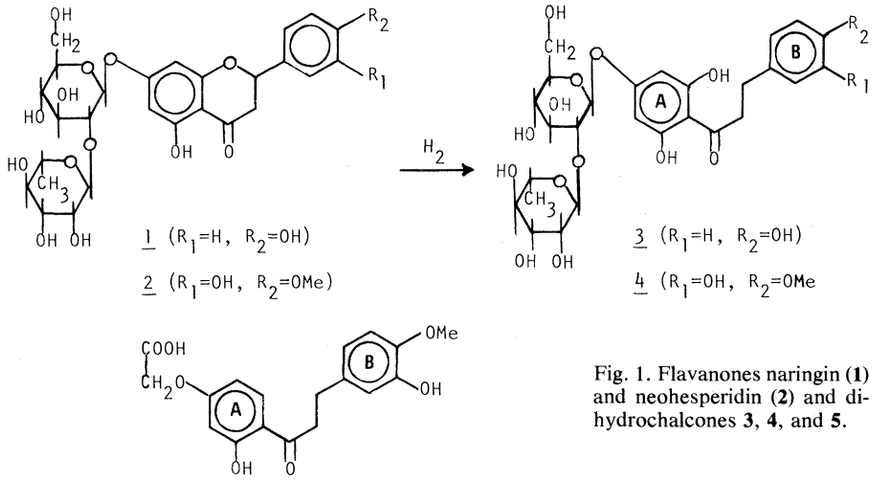


Fig. 1. Flavanones naringin (**1**) and neohesperidin (**2**) and dihydrochalcones **3**, **4**, and **5**.

Substitution on the carbon atom α to the carboxyl group significantly detracts from sweetness (compare **11** and **12**). The reasons for this effect are not clear. However, if the carboxyl moiety is involved in binding, such substitution may interfere. Alternatively, when the substituent is hydrophobic, as in **12**, the molecular hydrophobic-hydrophilic balance will be altered with a possible major effect on taste.

The distance of the carboxyl group from the DHC nucleus does not seem to have a major effect on taste properties. Only a moderate reduction in taste intensity is observed in going from carboxymethyl analog **11B** (501) to carboxypropyl derivative **14** (308), while only a slight decrease in taste quality is noted on proceeding from **11B** (82 percent sweet) to **14** (74 percent sweet).

It is curious that the alkali metal salts of the carboxylic acid DHC's seem to have better taste properties than the free acids (compare **11B** and **11A**). This is probably due to the greater water solubility of the salts.

The results shown in Table 1 seem to indicate that neohesperidin DHC (**4**) and the two carboxyalkyl analogs **11** and **14** are better sweeteners than both saccharin and cyclamate, but are somewhat inferior to sucrose when tasted in simple aqueous solution. Compound **4** is well known to have poor timing properties, showing an intense sweetness which is slow in onset followed by considerable linger. Both **11** and **14** also have these two difficulties, which makes them generally not useful for all areas of food and beverage use. Nevertheless, sweet DHC's seem to uniformly display a sweetness slow in onset followed by linger. This effect may be inherent in their structural nature. Most current thought concerning the mechanism of sweet taste perception assumes a primary event to be interaction of the tastant molecule with some proteinaceous component of a receptor cell membrane (*9*). Phenolic compounds are known to strongly hydrogen-bond to the amide groups of polyamides, in particular proteins. Therefore, the DHC's described above, which are phenolic compounds, might be expected to strongly bind to the receptor protein, resulting in a sweetness that lingers. Also if these phenolic DHC's indiscriminately bind to protein, immediate interaction with saliva protein may make rapid binding to the less readily available immobilized taste receptor proteins impossible. It may therefore take time for a useful concentration of free tastant molecules to accumulate in the vicinity of the receptor protein. Further investigation of the DHC class of compounds may yield an analog with a sucrose-like sweetness and the desired timing properties.

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