quantitation of the chromatograms. It was hypothesized that the quantitative differences were due to the retention of materials at numerous active sites on the column. This being true, the quantitative differences should be eliminated after the column active sites are saturated with the sample.

We therefore repeatedly injected the volatiles isolated from the fried cottonseed oil into the OV-101 column. Following each injection at 50°, the column was programmed from 50° to 190° at 5°/min and automatically cooled to 50°. After a minimum of seven consecutive injections of 5-μl samples of the ethyl ether solution of the isolated volatiles, excellent reproducible gas chromatograms were obtained, as shown in Figure 2. Equally reproducible gas chromatograms were obtained with the volatiles isolated from all the oil samples after 6–12 injections on either Porapak Q, OV-101, or methyl silicone SE-30 columns. Furthermore, closely reproducible gas chromatograms were obtained, with the use of the saturation method, from volatile flavor compounds isolated from canned beef stew.

It was further found that the active sorption sites would gradually become unsaturated if the column were left idle for some hours, even at room temperatures. Usually, a column would require saturation again when it was left idle overnight.

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Interaction of 5′-Nucleotides with Gelatin—a Model System for Flavor Potentiation

The association constants k (mM^{-1}) obtained with the equilibrium method in the system gelatin–5′-nucleotide–water (pH 6.5, 20°) are as follows: 5′-GMP 235, 5′-IMP 112, 5′-AMP 29, and 5′-CMP very small. They correspond in sequence and in relative magnitude exactly to the relative taste-potentiating activities of these compounds as reported by Yamaguchi *et al.* (1971). The described system, therefore, seems to be a valuable model for flavor potentiation. Possible mechanisms are stabilization of the helical structure and base stacking around the helices of proteins.

The theories of taste receptor stimulation are based upon the assumption that a chemical stimulus combines with some component of a receptor in such a way that the stimulus-receptor combination leads to neural activity and eventually to taste sensations. Several model systems are discussed in review papers (Beidler, 1961; Hofmann, 1969; Ohloff and Thomas, 1971; Unterhalt, 1970). For these interactions Beidler (1954) has proposed an equation which is derived from the mass action law. It is related to an equation described by Scatchard (1949), which is now widely used in binding studies with proteins (Steinhardt and Reynolds, 1969). Investigating the interactions of flavor compounds with food components (Solms *et al.*, 1973), we observed that 5′-nucleotides interact specifically with gelatin gel, and that the mode of action is a possible simulation of a taste-potentiating reaction. 5′-Nucleotides have no taste of their own, but have rather general well known taste-potentiating activities (Gutzeit-Walz and Solms, 1971; Kuninaka, 1967; Solms, 1967; Yamaguchi *et al.*, 1968). The relationships between taste-potentiating activity and structure have been extensively examined (Kasahara *et al.*, 1970; Mizuta *et al.*, 1972; Sato *et al.*, 1970). But nothing is known about the possible mechanism of taste potentiation; however, it can be assumed that it must be closely related to ordinary taste perception. In the present study, interactions in gelatin–nucleotide–water systems were investigated with the equilibrium method and the results were then compared with taste-potentiating activities reported in the literature.

EXPERIMENTAL SECTION

Materials. The nucleotides 5′-CMP, 5′-AMP, 5′-IMP and 5′-GMP were purchased from Zellstoff-Fabrik Waldhof, Mannheim, Germany. Gelatin R 220 was obtained from Ed. Geistlich Söhne, Wolhusen, Switzerland. In all calculations a value of 50,000 was used for the molecular weight of the gelatin.

Methods. A 10% gelatin gel was prepared by heating the dry gelatin in water to 50° with subsequent cooling. Samples of exactly 1 mg of gelatin gel were added to 25 ml of nucleotide solutions of different concentrations (5 to 90 mM) in 0.02 M sodium acetate buffer, pH 6.5. The systems were then equilibrated under shaking for 48 hr at 20°. The nucleotide concentrations in the supernatants were analyzed quantitatively by uv spectroscopy before (C_t) and after (C_f) equilibration. The bound nucleotides (C_b) were calculated as the difference (C_t) − (C_f). For the mathematical treatment of the data the Scatchard equation was used (Scatchard, 1949; Weder *et al.*, 1971)

$$\frac{\bar{r}}{n - \bar{r}} = kC_f$$

where $\bar{r}$ is the average number of moles of bound nucleotides per mole of gelatin, n is the maximum number of moles of nucleotides bound per mole of protein, k is an association constant (mM^{-1}), and C_f is the molar concentration of nonbound nucleotides at equilibrium. To determine the parameters k and n, one plots $\bar{r}/C_f$ against $\bar{r}$, as graphically presented in Figure 1. Each point shown in this figure averages six to eight measurements, with variations not exceeding ±6%. The drawing of the lines and the calculations of the binding parameters were done with a WANG 700 C computer, equipped with a writer plotter.

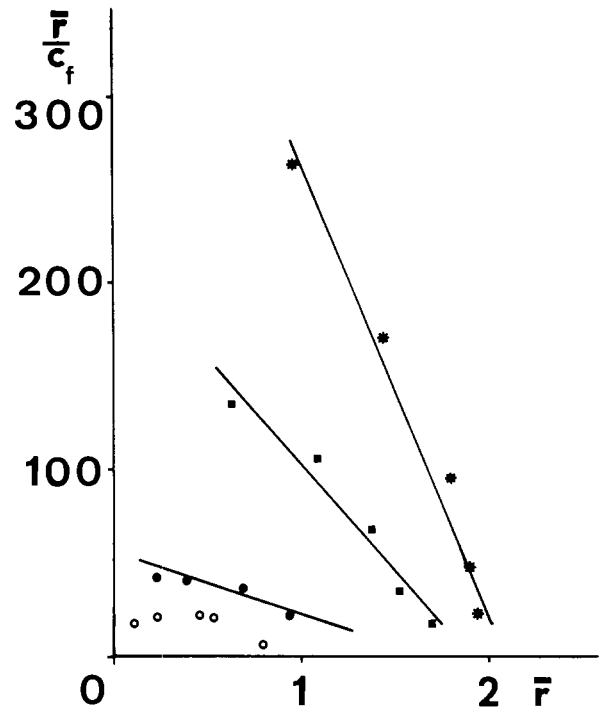

Figure 1. Scatchard plots for the interactions of 5′-GMP(*), 5′-IMP (■), 5′-AMP (●), and 5′-CMP (O) with gelatin.

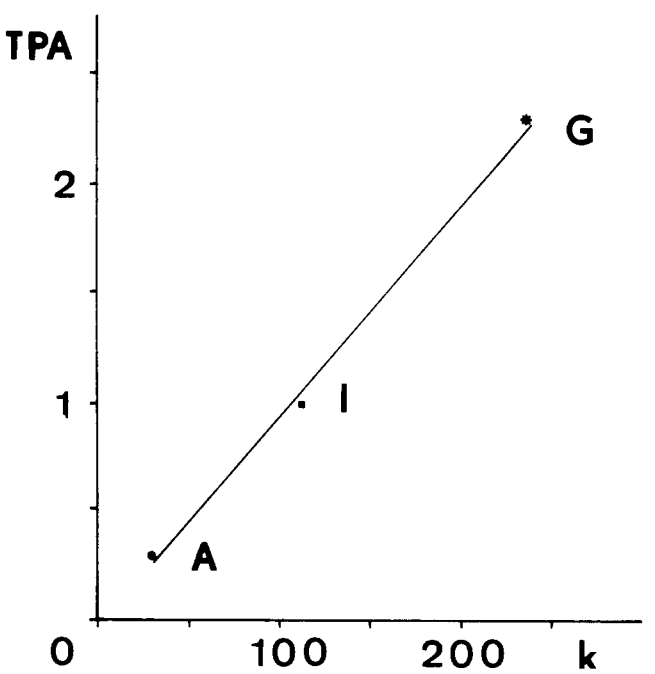

Figure 2. Relationship between relative taste-potentiating activities (TPA) and association constants with gelatin (k) for 5′-GMP (G), 5′-IMP (K), and 5′-AMP (A).

Table I. Binding Parameters of Nucleotides with Gelatin

Nucleotide	k (mM^{-1})	n
5′-GMP	235	2.1
5′-IMP	112	1.9
5′-AMP	29	1.8
5′-CMP	Very small	

RESULTS AND DISCUSSION

The Scatchard plots for the interactions are presented in Figure 1. It is obvious that the four tested nucleotides interact with gelatin in a quite different manner. 5′-GMP, 5′-IMP, and 5′-AMP show appreciable binding and give linear relationships on the plot. 5′-CMP shows a very low binding affinity and therefore a very large variation; a linear relationship could not be established in our experiments. The binding parameters k and n are shown in Table I. The association constants indicate that 5′-GMP interacts most strongly, followed by 5′-IMP, 5′-AMP, and 5′-CMP in decreasing order. Therefore, the association constants allow a net differentiation of the 5′-nucleotides in our system. The sequence is in accordance with the different taste potentiating effects found in taste panel experiments (Kuninaka, 1967; Kuninaka *et al.*, 1964), but also with data obtained from gustatory nerve fiber responses with rats (Sato and Akaike, 1965). It is interesting to note that the n values obtained are all in the same range; this indicates that the mechanisms of interaction might be similar. In order to compare the binding data of the nucleotides with their taste-potentiating effects, the k values, as obtained in our experiments, were plotted against the relative taste activities, as obtained in sensory tests and reported in the literature (Yamaguchi *et al.*, 1971). The plot is presented in Figure 2. It indicates that there is a very good relationship between the potentiating effects of the nucleotides 5′-GMP, 5′-IMP, and 5′-AMP and their respective association constants with gelatin. 5′-CMP has not been shown on the plot due to its very low binding properties; however, the reference point should be situated in a very low region on the reference line near 0. The data suggest that the presented model is a valuable simulation of a taste-potentiating reaction, since the taste activity of the 5′-nucleotides is directly related to the complex formation with gelatin gel. However, we were not able to find any data indicating that proteins related in structure to gelatin are possible essential components of receptor sites. Nucleotides are known to interact with protein-like molecules (Wagner and Arav, 1968) and thereby manifest a specificity in two ways: they stabilize the helical conformation of the molecules (Rifkind and Eichhorn, 1970) and undergo base-stacking around helical structures (Ts'o *et al.*, 1963). The nucleotides exert these actions in a specific order, namely GMP > IMP > AMP > CMP, which obviously parallels the taste-potentiating tendencies of these compounds. These observations have eventual biological implications. It is possible that the taste potentiating activity of 5′-nucleotides is due to the stabilization of the helical conformation of collagen-like proteins of receptor sides. This effect may then create a better environment for taste receptor stimulation by other chemical stimuli.

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Dibutyl- and Di-(2-ethylhexyl)phthalate in Fish

Low levels of dibutylphthalate (0–78 ppb) and di-(2-ethylhexyl)phthalate (0–160 ppb) were found in 21 samples of fish available to the Canadian consumer.

Dibutylphthalate (DBP) and di-(2-ethylhexyl)phthalate (DEHP) have recently been reported in inland waters (Corcoran, 1973; Hites, 1973) and in fish from North American sources (Mayer *et al.*, 1972; Zitko, 1972). Although these phthalate esters do not appear to present a serious short-term health hazard (Tepper, 1973) it was felt that a preliminary survey should be made to determine the levels of these phthalate esters in fish available to the Canadian consumer.

METHOD

Samples. Fish from Canadian lakes and rivers were supplied by Environment Canada; other samples were purchased at local markets. Canned fish were stored at room temperature; other samples were stored at −10° until analyzed.

Reagents. Solvents were distilled in glass and aliquots were concentrated, and the absence of DBP and DEHP was verified by glc. All glassware was rinsed before use with distilled ethyl ether. Silica gel, 0.2–0.5 mm (E. Merck) for column chromatography, was washed with ethyl ether, air dried, and activated for 16–20 hr at 120°.

Glc Conditions. A Varian Aerograph, Model 2100, gas–liquid chromatograph equipped with flame ionization detectors was used with nitrogen (40 ml/min) as the carrier gas. U-Shaped glass columns were used: (A) 6 ft × 3.5 mm i.d. packed with 3% XE-60 and (B) 7 ft × 2 mm i.d. packed with 5% OV-101, both on 100–120 mesh Chromosorb W (HP). The column temperature was 207°, the injection port 220°, and the detector block 270°. Electrometer setting was normally $10^{-11} \times 16$. The retention time for dibutylphthalate was 2.8 (Column A) and 3.2 min (Column B); for di-(2-ethylhexyl)phthalate it was 13.0 (Column A) and 22.4 min (Column B).

Glc–Mass Spectrometer. An Hitachi Perkin-Elmer (RMS-4) mass spectrometer coupled with a Perkin-Elmer Model 900 gas chromatograph fitted with flame ionization detectors was used. The 6 ft × ⅛ in. stainless steel column was packed with 3% silicone gum XE-60 on 100–120 mesh Chromosorb W (HP) and the carrier gas was helium at 40 ml/min. The operating conditions were detector 270°, injection port 240°, and oven 225°.

Sample Preparation. This was based on the method for the determination of DEHP in soy oil (Williams, 1973) with modifications to allow for the greater volatility and different chromatographic properties of DBP. The frozen or canned fish (100–200 g) was chopped into small pieces, macerated manually and heated, with occasional stirring, for 0.5 hr at 60–65° in an equal weight of hexane. The hexane was removed by decantation and the fish extracted two more times with a similar volume of hot hexane. The hexane extracts were combined and an aliquot was concentrated to dryness and weighed. An appropriate volume of hexane solution, containing up to 5 g of lipid, was then concentrated to 60 ml. This solution was extracted with acetonitrile saturated with hexane (6 × 60 ml) and the acetonitrile extracts were combined and concentrated to 300 ml. *m*-Chloroperbenzoic acid (0.5 g) was dissolved in aqueous sodium hydroxide (50 ml, 0.5 *N*), washed with ethyl ether (30 ml), and added to the acetonitrile solution. Sulfuric acid (50 ml, 0.5 *N*) was immediately added and the solution shaken and left overnight at room temperature. Water (200 ml) was added and the aqueous acetonitrile extracted with petroleum ether (3 × 100 ml). The petroleum ether extracts were combined, washed with sodium hydroxide (50 ml, 0.5 *N*) and water (2 × 100 ml), dried over sodium sulfate, concentrated to 20 ml, and transferred to a silica gel column (20 g) made up with 5% ethyl ether in petroleum ether. The column was eluted with 5% ethyl ether in petroleum ether (100 ml) and then with 15% ethyl ether in petroleum ether (250 ml). The 15% ethyl ether eluate was collected, concentrated to 0.3 ml using a micro-Snyder column, and examined by glc. Quantitation was carried out by comparison of peak height (DBP) or peak area (DEHP) with standard samples. Analysis of samples spiked at different stages in the method indicated that three extractions with hot hexane gave virtually complete extraction of the phthalate diesters. Overall recoveries of phthalate esters in spiked samples (0.1–0.5 ppm) were 60–65% for DBP and 65–70% for DEHP.

RESULTS AND DISCUSSION

Twenty-one samples of fish were analyzed for DEHP and DBP, and the results are given in Table I. Only very low levels of these phthalate esters could be detected in any of the fish. Analysis for low levels of phthalate esters is complicated by the presence of trace amounts of these compounds in solvents and chemicals used in the workup. Rigorous purification fails to remove all of the phthalates