DISTRIBUTION OF SODIUM BENZOATE IN AN ACIDIC FOOD SYSTEM AS AFFECTED BY LIPID AND PROTEIN CONTENT

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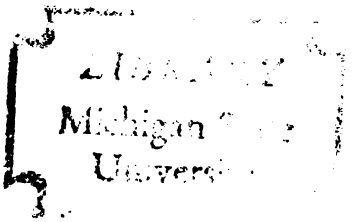
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ABSTRACT

DISTRIBUTION OF SODIUM BENZOATE IN AN ACIDIC FOOD SYSTEM AS AFFECTED BY LIPID AND PROTEIN CONTENT

By

Mary Catherine Ganzfried

The distribution of benzoic acid was determined spectrophotometrically as a function of corn oil and soy protein content of an agar model system. Both caused an exponential decrease in the benzoate concentration in the aqueous phase at concentrations from 0 to 15%, and a more gradual decrease at higher concentrations. A model system could be used to predict benzoate distribution in food products of known fat, protein, and moisture content. Commercial food products which declared 0.1% benzoate on the labels were analyzed for benzoate by a modified A.O.A.C. titrimetric method. The products showed variability in the benzoate content. The distribution of benzoate between the solid and liquid portions depended on the composition of the liquid phase. Products packed in simple brines which contained negligible amounts of fat and protein showed benzoate concentrations in the edible portion exceeding the maximum legal limit.

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Mary Catherine Ganzfried

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued . . . .)

TABLE OF CONTENTS (Continued . . . .)

LIST OF TABLES

LIST OF TABLES (Continued . . . .)

LIST OF FIGURES

INTRODUCTION

A Michigan processor had experienced difficulty in the measurement of benzoic acid in pepper products. Separate determinations of benzoic acid in the brine and fruit portions of these products showed a consistently higher preservative concentration in the fruit than in the brine. Two potential problems which could occur from this situation are (a) a benzoate concentration in the food as usually eaten in excess of the legally allowed level, and (b) a diminution of preservative capacity as a result of benzoic acid binding to food components which would make it unavailable to microorganisms.

This project was initiated to determine the ability of fat and protein in foods to bind benzoic acid. Measurements of benzoic acid partitioning in food products were made to evaluate the degree of concentration of benzoic acid which could occur in foods.

LITERATURE REVIEW

I. Use of Benzoic Acid and Sodium Benzoate in Foods

A. The Pure Food Act

At the turn of the century a controversy arose concerning the enactment of the Pure Food Act. Embedded in this amendment was the legality of the use of benzoic acid as a preservative in foods. The catsup manufacturers were the primary opponents of the passage of the bill. Three major points of debate were (1) justification of the use of benzoic acid due to the natural occurrence in cranberries and prunes at levels of 0.06% and 0.05%, respectively (Radin, 1914; Clague and Fellers, 1934), (2) the health hazard involved with the continuous consumption of foods containing the preservative, (3) the need for the preservative.

In a sense the battle was won on June 30, 1906 when President Roosevelt signed the Pure Food Bill into law. It was not a complete victory since nothing was settled with respect to the use of benzoic acid in foods. Political maneuvering prevented any definite action in creating laws or enforcing them once they were created (Wiley, 1929).

B. Detoxification Mechanisms and Toxicity Studies

In studying the toxicity of benzoic acid to animals, it became evident that mechanisms existed to dispose of the

chemical. Benzoic acid was readily excreted in conjugation with glycine, in the form of hippuric acid, and as benzoyl glucuronide. There was no apparent danger of its accumulation in the body (Banfield, 1952; Wiley, 1908; Williams, 1947). Radioisotope studies on humans using benzoic acid-C^{14} (Shatalova and Meerov, 1962) showed that 99% of the benzoic acid was excreted as hippuric acid within 12 hours and 100% within 24 hours. Wan and Riegelman (1972) determined a kinetic mechanism for the conversion of benzoic acid to hippuric acid by white rabbits and a Rhesus monkey. They established a steady-state concentration of hippuric acid such that the infusion of hippuric acid was equal to the excretion. Subsequent infusion of benzoic acid was used to determine that the conversion of benzoic acid to hippuric acid was described by Michaelis-Menten kinetics.

Toxicity studies with humans were carried out by Wiley and the Remsen Referee Board of Consulting Experts. Wiley's five-year study (Wiley, 1908) with healthy young men receiving up to 3.5 grams of benzoate/day showed injurious effects to digestion accompanied by indications of irritation and nausea, headache, and in a few cases vomiting. In the Remsen Referee Board study (Chittenden et al., 1909) the four-month experiment with 16 healthy young men showed no evidence of adverse effects at a low dosage of 0.3 grams of sodium benzoate per day. The large dose, increasing from 0.6 to 4.0 g/day, affected only four of the subjects in one

laboratory. The large dose was thought to have had an irritative action on the gastroenteric mucosa.

Acute toxicity tests with young rats have shown LD_{50} values of 2.1 g/Kg bodyweight (BW) when the dose was administered by stomach tube to fasted rats and 3.45 g/Kg BW when sodium benzoate was fed in a 10% aqueous solution (Deuel et al., 1954). Intravenous injection (Hager et al. 1942) resulted in an LD_{50} of 1.714 g/Kg BW. Symptoms following the I.V. injection were heightening of reflexes of the central nervous system, tremors, convulsions, and death occurring 1/2 to one hour after the injection.

Sub-acute feeding studies were in general agreement. Basal diets containing 5% or more sodium benzoate resulted in hyper-excitability, urinary incontinence, convulsive seizures, damage to livers and kidneys and death (Fanelli and Halliday, 1963; Deuel et al., 1954; Griffith, 1929). Diets with benzoate above 2% consistently retarded growth (Kowalewski, 1960; White, 1941; Fanelli and Halliday, 1963; Deuel et al., 1954; Griffith, 1929; Harshbarger, 1942). Below 2%, the rats grew normally in a 90-day feeding study (Deuel et al., 1954; Griffith, 1929).

Supplementation of glycine to benzoate-containing diets was found to overcome the adverse effects of the benzoate (White, 1941; Griffith, 1929; Harshbarger, 1942; Kowalewski, 1960). White (1941) put young male rats on a growth stunning diet (5g sodium benzoate/100 g basal diet)

for 3 to 6 weeks. Addition of 1% glycine to the diet resulted in a prompt resumption of growth and a steady growth rate as long as glycine feeding was continued. Survival and growth of the rats occurred only when the diets furnished a supply of glycine adequate for detoxification of benzoic acid and formation of new tissue proteins. Kowalewski (1960) observed a decrease in phospholipid content and carcass potassium with a 5% sodium benzoate diet. Addition of 1% glycine to the diet offset the decreases. Rittenberg and Schoenheimer (1939) used an excess of glycine containing N^{15} and found that only about one-third of the isotopic glycine fed with benzoic acid was excreted as hippuric acid. The other portion of the glycine came from endogenous sources.

Cats were found to be very sensitive to large doses of benzoic acid (Bedford and Clarke, 1972). They were found to have a defective glucuronic acid mechanism, therefore, all benzoic acid had to be excreted as hippuric acid. If the amount of benzoic acid was too great for glycine conjugation, the acid built up to toxic levels. A single dose in excess of 0.45 g/Kg BW resulted in clinical symptoms similar to the rats and probable death. The effects were stronger with rapid ingestion. A cumulative effect was observed within 48 to 72 hours with 0.03 g/Kg BW, while a 0.016 g/Kg BW level for 23 days had no effect.

C. Characteristics and Properties

Benzoic acid is manufactured chiefly by decarboxylation of phthalic acid. This is accomplished by the reaction of phthalic anhydride and a chromium-disodium phthalate catalyst followed by steam distillation. Yields are approximately 85%. Manufacture by chlorination of toluene is becoming less common due to the contamination by chlorine in the final product.

Benzoic acid is supplied as white crystalline needles or a powder. The molecular weight is 122.12. Two grades are available: technical (99%) and USP (99.5%). Containers for benzoic acid include 100# and 150# barrels, kegs, fiber drums and cartons, and bottles of various smaller sizes (Faith et al., 1950).

Sodium benzoate is supplied as dense and regular crystalline powder. The molecular weight is 144.11. It is packed in fiber drums with polyethylene liners (Annon., 1969).

Benzoic acid has a very low solubility in water (0.34 g/100ml) but is highly soluble in lipids. Sodium benzoate has a solubility in water of 50 g/100 ml, which makes it more adaptable to incorporation in food products (Chichester and Tanner, 1972).

Benzoic acid has a pKa of 4.2 and has been found to exhibit optimum microbial inhibition at a pH range of 2.5 to 4.0. It is generally considered to be most active against yeast and bacteria and less active against molds (Chichester and Tanner, 1972).

The US-FDA recognizes sodium benzoate and benzoic acid as safe for use in foods, but at a maximum level of 0.1% (Chichester and Tanner, 1972). This level was established empirically by the food industry taking into account a combination of factors: effective level and economic considerations, solubility of benzoic acid in acidic products, and a noticeable taste in fruit beverages around 0.1%. Toxicity studies failed to show injurious effects at this level.

The use of benzoate in foods has been advocated (Banfield, 1952) in the prevention of unnecessary waste of food which has limited availability and requires storage or transportation for optimal distribution. Its use is essential as a preservation safeguard in institutional packing where the containers are too large for adequate heat transfer during conventional cooking procedures.

II. Antimicrobial Activity

A. Effect of pH and Preservative Concentrations Required

Studies by Cruess (1932) were prompted when 0.1 to 0.2% sodium benzoate failed to prevent spoilage of commercially packed foods of low acidity. It was suspected that the hydrogen ion concentration of the medium might be the controlling factor. Evidence for this was (1) little difficulty had been encountered in processing juices, catsup, and fruit preserves, and (2) as much as three hundred times

the concentration was required at pH 7.0 to prevent growth of yeasts, molds and lactic acid bacteria as was required at pH 3.0 to 3.5. Another study (Cruess et al. 1931) with cubed melon preserves, maraschino style grapes, ripe olives, avocado pulp, and some vegetables showed that spoilage could be prevented with 0.1% or less sodium benzoate when the pH did not exceed 4.0. Near neutrality, 2% sodium benzoate failed to protect the products.

Winsley and Walters (1965) studied the growth and germination of Aspergillus niger, which had a growth optimum pH of 4.5 to 6.5. The concentration of benzoic acid needed to inhibit growth increased with pH, but the corresponding concentration of unionized molecules remained almost constant from pH 2.2 to 5.1. The investigators concluded that the unionized molecules were the predominantly active form. Penetration to or beyond the cell membrane of microorganisms would be more easily achieved by neutral molecules. Other investigators agreed with this theory (Entrekin, 1961; Aalto, 1953; Bosund, 1962;Patel and Foss, 1965).

Evans and Dunbar (1965) observed a reduction in the minimum inhibitory concentration of unionized benzoic acid (0.021% to 0.006%) when the pH was increased from 4 to 5. The possibility that less unionized acid was required at pH 5 to give the same biological response as at pH 4.0 due to a change in the resistance of the organisms seemed unlikely. The optimum pH for growth for Asp. niger was between pH 5.0

and 7.0. Based on similar results of other investigators, they concluded that less unionized acid was required because the benzoate anion was also exerting an effect.

B. Action on Sporulating Bacteria

Haggman and Nikkila (1962) found that 0.1% sodium benzoate at pH 7, corresponding to 10^{-5} M undissociated benzoic acid, did not prevent the multiplication and germination of the spores of Bacillus megaterium, but they did prevent generation of new spores. This was probably as a result of the inhibition of oxidative reactions. Gould (1964) studied three large-celled species of Bacillus cereus and three small-celled species: Bacillus subtilis CX, B.V. niger, and B. licheniformis NCTC 7589. At low concentrations (.01 to .03% sodium benzoate at pH 6.0) germination and outgrowth were permitted as far as the stage of emergence, but elongation and multiplication of the vegetative cells was prevented. More than 0.04% at pH 6.0 prevented rupture or lysis of the spore wall while still allowing germination to proceed.

C. Action on Yeasts

Oka (1960 a,b; 1964) has done much work in the elucidation of the mechanism of antimicrobial effects of various preservatives. He determined that for any antimicrobial compound to act in microbial cells, it was essential that the compound be transferred from the medium into the cells. The transfer process thus became an important first step for inhibition of microorganisms (Oka,

1960a). Benzoic acid and other similar inhibitors were shown to exist in the cell dissolved in the cell fluid and adsorbed on solid phases (Oka, 1960a). The antimicrobial effect was a function of the pH of the medium. However, at any pH, the same concentration of unionized molecules was found in the aqueous phase, so the adsorbed quantity, not the dissolved, determined the growth inhibiting effect (Oka, 1960a, 1964).

Spoilage was reported to occur in beverages and tomato sauce containing the maximum allowable benzoate concentrations and a low pH (Pitt and Richardson, 1973). The yeasts isolated, Sacc.bailii (S. acidifaciens), Pichia membranaefaciens, and Candida krusea, were all resistant to benzoic acid and acetic acid.

D. Action on the Metabolism of Microorganisms

Schwartz and Mandel (1972) determined that growth inhibition of Bacillus cereus by 1.0 mM salicylic acid was due to the inhibition of RNA synthesis. They measured the incorporation into the cells of several labeled compounds. No inhibition was observed for the incorporation of leucine and phenylalanine (protein precursors), diaminopimelic acid (an exclusive precursor of the B. cereus cell wall), thymidine (DNA precursor). Incorporation of uracil (RNA precursor) was inhibited immediately (50-75% less incorporation than controls). Guanine, adenosine and adenine were inhibited in a similar manner. Additional studies were

carried out to verify the selective inhibition of microbial RNA synthesis. Benzoic acid at a concentration of 4.0 mM produced incorporation patterns closely resembling those of salicylic acid.

Bosund studied the effect of benzoic acid on carbohydrate metabolism in an attempt to determine what reactions vital to the cell were susceptible to the inhibitor at concentrations suppressing the growth of the microorganisms (Bosund, 1962). The mode of action of salicylic acid on microorganisms was shown to be the uncoupling of oxidative phosphorylation (Bosund, 1959). Although benzoic acid resembled salicylic acid in the effects on bacterial cells, the mechanism for benzoic acid was not so well defined. Benzoic acid had only a weak and rather unspecific effect on oxidative phosphorylation (Bosund, 1962). Benzoic acid specifically blocked the oxidation of glucose and pyruvate at the acetate level (Bosund, 1959). No blocking was observed with Proteus vulgaris and E. coli cells precultured in a medium with acetate as the sole carbon source. The blocking occurred only if acetate was actually accumulating in the medium during oxidation (Bosund, 1960). The process principally inhibited during acetate oxidation was activation of the acetate molecule to acetyl-CoA or the subsequent condensation of acetyl-CoA and oxaloacetic acid (Bosund, 1959, 1962). The effect of benzoic acid on growth inhibition was believed to involve inhibition of the formation or

utilization of high-energy compounds (Bosund, 1959).

An earlier theory (Morse, 1951) postulated that antimetabolites functioned either by preventing incorporation of a metabolite into a more complex system or by themselves being incorporated into an analog of a vitamin coenzyme which then competed with the true coenzyme for the apoenzyme. The action of benzoates was either an effect upon the cell membrane or competition with a coenzyme for the enzyme protein.

III. Distribution in Oil-Water Systems

The solubility of benzoic acid in lipids is of importance in the preservation of systems containing both lipid and water. Experiments were done with eight creams (Hibbott and Monks, 1961) in which the preservative (methyl-p-hydroxybenzoate) was kept at a constant concentration of 0.15%. The amount of lipid in the creams remained constant, but partition coefficients of the preservative between the fat and the aqueous phases increased due to the choice of a variety of lipids. This resulted in a decreased preservative concentration in the aqueous phase. Although the total preservative was 0.15% for all creams, all those with 18-27.8 mg of preservative in 100 ml of the aqueous phase did not spoil, whereas creams with 11.6, 9.4, and 7.1 mg of preservative per 100 ml were unable to prevent growth of mold.

Hibbott and Monks (1961) and Anderson and Chow (1967) added propylene glycol or glycerol (2 to 20%) to oil:water

mixtures, replacing the same amount of water. The partition coefficients were reduced, increasing the preservative available in the aqueous phase, thus increasing preservative activity. At the higher concentrations, the glycerol itself had antimicrobial activity. Other investigators (von Schelhorn, 1964; Patel and Romanowski, 1970; Bean et al, 1965 and Bean, 1972) also observed that preservatives dissolved in fat were useless for preservation. The concentration in the aqueous phase determined the activity of the preservative in that system.

The distribution coefficient (the ratio of the concentration of the preservative in the lipid to the concentration in the aqueous phase) for a particular preservative was used to calculate the quantity of preservative required in a system to maintain an effective preservative level in the aqueous phase (von Schelhorn, 1964; Anderson and Chow, 1967; Patel and Romanowski, 1970). Von Schelhorn (1964) noted that the solubilities of preservatives in various kinds of oil did not differ very much, and for all practical purposes, it could be assumed that distribution coefficients between most kinds of oil and water were similar to those which he obtained for peanut oil. The effect of temperature on the partition coefficient of some preservatives was not consistent (Bean et al, 1965). Some increased while others decreased as the temperature was raised from 5^{o} to 45^{o}C. However, von Schelhorn (1964) showed that a change in

temperature from 20 to 46°C did not influence substantially the distribution coefficients, as long as the fat phase (oil or crystallized) was not changed.

Most preservatives studied were in the monomer form in the lipid phase, indicated by partition coefficients which were independent of the preservative concentration. Anderson and Chow (1967) reported the distribution of benzoic acid in arachis oil and isopropyl myristate as a monomer. However, in liquid paraffin the partition coefficient was not independent of the benzoic acid concentration, but the ratio of the square root of the concentration in the paraffin to the concentration in the aqueous phase was a constant. This was interpreted to indicate that benzoic acid was predominantly in the dimer form in the paraffin phase. The observation of the monomeric distribution of benzoic acid in arachis oil (Arachis is the genus of the common peanut) was in opposition to the report by von Schelhorn (1964) that benzoic acid dimerized in peanut oil.

IV. Interactions of Benzoic Acid with Food Components

A. Emulsifiers

An increasing number of failures of preservatives to protect creams from microbial spoilage were reported (Bean et al, 1965; Weddenburn, 1964). This period coincided with that during which, in general, there was a change from the use of anionic to nonionic emulsifiers. This reformulation modified the resistance of the products to microbial attack.

De Navarre and Bailey (1956) reported that 0.2% benzoic acid was effective against test organisms, an assortment of yeasts and molds. This preservative concentration no longer inhibited growth when 2% nonionic compounds (Tweens) were added to the media. These investigators suggested the formation of a Wurzschmitt cation-anion complex: condensation products of ethylene oxide with higher fatty alcohols formed cation-active polyoxonium hydroxides by dissociation in water which were capable of reacting with anions forming a stable cation-anion complex. This complex may have dissolved in the fats preferentially, leaving the media unprotected, thus favoring microbial growth. Bean et al (1965) and Kazmi and Mitchell (1971) reported that the mechanism of inactivation may be complex formation due to hydrogen bonding or solubilization within micelles. Patel and Kostenbauder (1958) suggested complex formation as opposed to solubilization into micelles based on a Langmuir-type plot of their data. Kazmi and Mitchell (1971) reported that a Langmuir plot heavily weighted those experimental points obtained at low concentrations of free drug which could lead to large errors on extrapolation to infinitely high preservative concentrations. An alternative rearrangement of the equation, known as the Scatchard equation, gave more even weighting to the different points on the curve. They concluded that their curve, which was not a straight line, indicated that binding sites within micelles did not behave independently of

each other. Possibly, the uptake of solute into micelles progressively altered the interaction between the binding sites and the solute.

B. Proteins

Research on the binding of organic acids by proteins was carried out at physiological pH where the protein, bovine serum albumin (BSA), was reactive and the organic acids were in the ionized form. Ionic interaction between groups of opposite charge occurred irrespective of the net charge on the protein (Goldstein, 1949).

Goldstein (1949) reported that, as a general rule, the affinity of a protein for an anion was increased by large anion molecular size, particularly by aromatic rings which contributed sizeable van der Waals forces to bond energy.

Benzoate was capable of binding with D-amino acid oxidase (Kotaki et al. 1966), where it protected the enzyme from inactivation by glyoxal. Benzoic acid was shown to be a poor competitive inhibitor of bacterial glutamate carboxylase. The Ki of benzoic acid was 74 mM compared to 2.2 mM for valeric acid, a better inhibitor (Fonda, 1972). Benzoic acid inhibited the binding of 8-nitrotheophylline to BSA. With the study focusing upon protein sites involved in binding of 8-nitrotheophylline (pH 2-9), the benzoate anion was a more effective inhibitor than benzoic acid (Meyer and Guttman, 1968).

Davison and Smith (1961) compared the binding to BSA of benzoic acid and hydroxy benzoates. As the hydroxyl

substituent was moved nearer to the acidic group, the molecules of drug bound per molecule of protein increased (ortho>meta>para≈benzoic acid). The presence of hydrogen donors in an ortho position conferred greater binding abilities than the same radicals in meta and para positions. The reduced binding in the latter positions was due to a hydration shell, as opposed to the internal hydrogen bonding possible only with ortho substitution. Two ortho hydroxyl groups resulted in extensive binding as did compounds with amino substitution for the hydroxyl. Phenol and cyclohexanol were not appreciably bound, confirming the primary importance of the carboxyl group (Lindenbaum and Schubert, 1956). Competition experiments (Davison and Smith, 1961) suggested that various cyclic acids probably were bound to the same site. Hexahydrosalicylic acid apparently did not compete with salicylic acid, indicating that the benzene ring may have been of some importance, or that the alcoholic hydroxyl group in the ortho position might have interfered.

Biological methods for the study of interactions have owed their usefulness to the diminution of drug activity caused by the interaction with proteins (Goldstein, 1949). Goldstein (1949) cited some of the applications. Bursck, in 1906, reported the inhibitory effect of serum on photodynamic action and other toxic effects of certain dyes, using paramecia as test organisms. He showed that serum

not only interfered with dye action but also altered diffusibility, fluorescence or light absorption, solubility and other properties. These phenomena he attributed to the formation of dye-albumin complexes. A quantitative analysis by Fawaz and Farah (1944) on cardiac glucosides, used systolic arrest of the frog heart for the assay of free drug concentration in the presence and absence of various serum protein fractions.

C. Starches

Goudah and Guth (1965) studied the interaction of benzoic acid and some derivatives with potato starch and arrowroot starch. No significant difference was observed between the binding of the two starches: 3.31 moles benzoic acid/potato starch equivalent and 3.41 moles benzoic acid/ arrowroot starch equivalent. Mansour and Guth (1968) reported that amylose was the main complexing component of starch with the drugs. The complex formation was due to the entrapment of the drug in the alpha-helical structure of amylose with supplementary stabilization by dipole-dipole interactions. The support of the inclusion theory (Goudah and Guth, 1965) was the decrease in degree of interaction with increasing molecular weight of the drug. Caffeine showed no interaction probably due to its large stereo-chemical configuration which would be difficult to fit in the voids of the helical starch molecule.

Hydrogen bonding was another factor to be considered as part of the complexation mechanism. The multiplicity of hydroxyl and carboxyl groups on the starch molecule enabled interaction with the polar drugs. The relative degree of interaction (meta and para hydroxybenzoic acids>>para-aminobenzoic acid>benzoic acid) could be explained by the electrophilic nature of the hydroxyl group contrasted with the weak electrophilic nature of the amino hydrogen and the lack of electrophilic nature of the benzene hydrogen (Goudah and Guth, 1965).

MATERIALS AND METHODS

I. Materials

The sodium benzoate used was a Pfizer U.S.P. dense powder with 100% activity. The soy protein was the Promine^{R}-D Isolated soy protein manufactured by Central Soya, Chicago, Ill. The corn oil used was Mazola 100% corn oil.

II. Methods

A. Preparation of the Model System

1. Brines

The brines were prepared following commercial specifications for a 50/50 pack out ratio (50% peppers, 50% brine by weight). The equilibrated brine must be 17-18 grains as acetic acid (1.7 to 1.8%) and 1.5 to 2.0% NaCl. The actual values for the brines prepared were 3.6% acetic acid and 3.0% NaCl to give the desired equilibrated concentration. A 10% sodium benzoate solution was used to prepare brines with benzoate concentrations of 0.0, 0.1, 0.2, and 0.3%. The benzoate solution had to be added to the water prior to the addition of the acid, particularly at the 0.3% level, to prevent the crystallization of benzoic acid. When crystallization did occur, the brine was filtered through Whatman #41 filter paper to remove the crystals. The pH of the solutions ranged from 2.7 for the 0% benzoate

brine to 3.0 for the 0.3% benzoate brine.

2. Agar Blocks

a. Fat

Agar blocks were prepared with 0.0, 0.5, 1.0, 2.0, 3.0, 5.0, 10, 15, 20, 30, and 40% corn oil by weight. All samples contained 2.0% agar. Deionized water was added as required. The agar and water were steamed until the agar dissolved. The hot solution was transferred to a tared blender jar and when necessary, water was added to replace any lost during steaming. The appropriate weight of oil was added and the sample was blended at high speed for 3 minutes in a Sorvall Omni-Mixer. The homogenized sample was poured into ice cube trays and put into a refrigerator to solidify. The cubes were approximately 30 ml in volume.

b. Protein

Agar blocks were prepared with 0.0, 0.5, 1.0, 2.0, 3.0, 5.0, 10.0 and 15.0% by weight Promine^{R}-D soy protein isolate and 2.0% agar. The procedure was the same as that used to prepare the fat samples. The agar solution was allowed to cool slightly as the protein tended to clump and gel when added to the hot solution. Blending was done at a slower speed and only long enough to dissolve the protein. This was to prevent excessive foaming.

c. Fat-Protein Combinations

Fat-protein combinations were prepared using food composition table values to simulate six food samples. The

combinations were (1) 0.4% fat, 5.4% protein (peas), (2) 0.5% fat, 7.8% protein (kidney beans), (3) 0.5% fat, 2.7% protein (mushrooms), (4) 1.0% fat, 3.3% protein (corn), (5) 2.1% fat, 16.4% protein (avocado), (6) 5.7% fat, 11.0% protein (soy beans). The procedure was the same as that used to prepare the protein blocks.

3. Packing of the Model System

Two or three cubes were weighed in tared laboratory sample containers. An equal weight of brine was added. Two or three replicates were made of each combination of benzoate concentration and fat or protein concentration. In one experiment, brine was added equivalent to two times the weight of the protein or fat containing agar cubes.

B. Preparation of Food Products

Six food products were purchased for this study: peas, corn, mushrooms, soy beans, kidney beans and avocado. Four replicates were made for each product in the 0% benzoate brine and six replicates for the three other brines.

Frozen peas and corn were allowed to thaw in the plastic bags. The peas were packed as they were, while the starchy liquid from the corn was discarded before packing.

Raw mushrooms were steam blanched for 2-2½ minutes depending on the size. No test was made for enzyme inactivation.

Soy beans and kidney beans were allowed to soak in warm distilled water overnight. The water was discarded and

replaced with fresh water. The beans were cooked over a steam table for 2½ hours.

Fresh avocados were cut into chunks and packed in the brine solutions.

C. Benzoate Analyses

1. Spectrophotometric Method

A Beckman DU spectrophotometer with a Gilford model 220 digital readout attachment was used for the determination of benzoate in the brines from the model system and food products. A scan from 250 to 285 nm showed a peak for the benzoate with minima at 267.5 and 277.5 nm and a maximum at 273 nm. Readings were taken at the three wavelengths. The height of the absorbance peak was used to measure the sodium benzoate concentration. A standard curve was made of benzoate dissolved in a ten-fold diluted equilibrated brine with no benzoate (dilution brine). The pH was near 3.0. The linear portion of the curve was at benzoate concentrations of 0.0 to 0.01% (Horwitz, 1965b).

a. Brines

The original brines added to the sample were analyzed spectrophotometrically. The 0% and 0.1% brines were diluted 20-fold in the dilution brine and the 0.2% and 0.3% brines were diluted 40-fold. The dilution allowed reading in the linear portion of the standard curve. The dilution brine was used as the blank.

b. Fat Samples

No interference was observed in the absorption of the brines from the fat samples in the 267.5 to 277.5 nm range. Samples with 0.0 and 0.1% benzoate brines were diluted 10-fold and 0.2% and 0.3% brines were diluted 20-fold.

c. Protein Samples

Protein dissolved in the brine was found to absorb in the region of the benzoate peak. Furthermore, the solutions became turbid upon dilution. Sodium benzoate standard curves were made for each protein concentration at each dilution (10 or 20 fold), using the appropriate 0% benzoate sample. The solutions were diluted in centrifuge tubes and centrifuged at 12,000 rpm (15,000 x g) in a Sorvall SS3 Automatic Superspeed centrifuge for 20 minutes prior to reading. The samples containing benzoate were analyzed by diluting, centrifuging and reading. The 0% benzoate sample of the appropriate protein concentration and dilution was used as the blank. Above 5.0% protein, all samples were diluted 20-fold.

d. Fat-protein Samples and Food Samples

The fat-protein samples and food samples were analyzed in the same manner as the protein samples. All samples were diluted 20-fold to reduce turbidity and decrease the number of standard curves.

2. A.O.A.C. Titrimetric Method

The A.O.A.C. method for benzoate determination was used for analysis of commercial food samples (Horwitz, 1965a).

The brine and tissue were analyzed separately (noting the proportions of the two phases) for determination of benzoate partitioning. Modification of the A.O.A.C. method was necessary to assure complete extraction of benzoate from all food samples.

Solid samples were chopped, if necessary, to facilitate blending. They were diluted 1:1 (w/w) with saturated NaCl and homogenized in a Sorvall Omni-Mixer for 2 to 3 minutes, or until the slurry appeared homogenous. Aliquots (150 grams) of the undiluted liquid samples or homogenates from solid samples were weighed into 400 ml beakers and 25-35 grams of solid NaCl were added. The solution was titrated to pH 8-9.5 with 10% NaOH. The alkaline sample was transferred to a 500 ml volumetric flask. Rinsing of the beaker was done with 10-15 ml of 0.02 N NaOH. Saturated NaCl solution was added to the mark. The solution was allowed to stand for at least two hours with periodic shaking.

The solution was then centrifuged for 20 minutes at 7500 rpm (6780 x g) in a Sorvall SS3 centrifuge and filtered through cheese cloth. Two hundred ml of the solution were pipetted into a 400 ml beaker and titrated to pH 1.0 with a 3.025 N HCl solution. To this solution 2 ml of methyl red indicator were added (200 mg methyl red/liter 0.02 N NaOH). Four chloroform extractions were done (70, 50, 40 and 30 ml chloroform) in a 500 ml separatory funnel. The chloroform was collected in a round-bottom flask. The methyl red was

extracted as an orange pigment in the chloroform. A clear chloroform phase resulting from the extraction of all the methyl red from the aqueous phase was an indication of the effectiveness of the extraction. Formation of an emulsion at the interface of the two phase system often occurred with food samples. The emulsion was easily broken by centrifugation of the emulsion layer for 15 sec. at 2000 x g in a clinical centrifuge. Except in cases where the emulsion problem was particularly severe, this procedure was only used after the final chloroform extraction.

Evaporation to dryness was accomplished in 20-25 minutes on a flash evaporator with the water bath kept at 40-44°C.

The residue was dissolved in 30 ml ethanol and titrated with 0.05 N NaOH to pH 8.2. One ml 0.05 N NaOH = 7.2 mg anhyd. sodium benzoate.

D. Protein Determination

The food samples were analyzed for protein content using the micro-Kjeldahl method described by Lillevik (1970) with some modifications. The digestion mixture consisted of 5.0 g $CuSO_4$: 5 H_2O and 5.0 g SeO_2 in 500 ml of concentrated H_2SO_4. Approximately 15 mg of protein for each sample were digested with 4 ml of the digestion mixture for one hour. After cooling, 1 ml of 30% H_2O_2 was added to each flask and digestion continued for an additional hour. The digested mixture was rinsed with 10 ml deionized water and

then neutralized with approximately 25 ml of a 40% NaOH solution. The released ammonia was trapped in 15 ml of a 4% boric acid solution containing 5 drops of the indicator. The distillation was continued until a final volume of 60 ml of the solution was collected in the receiving flask.

E. Fat Determination

The food samples and fat-agar samples were dried overnight at 100°C. The dry samples were extracted with ether in Soxhlett extractors at a rate of 4-5 drops/second for 8 hours. The extracted fat was collected in a tared flask and weighed following evaporation of the ether.

F. Calculations

1. Percent benzoate in the tissue of a sample separated into the tissue (solid) and brine (non-solid) phases.

B_t = % benzoate equilibrated in the tissue phase

B_i = % benzoate in the initial brine used for packing. Measured spectrophotometrically.

B_b = % benzoate in the brine phase of the sample after equilibration. Measured spectrophotometrically.

L = fraction of liquid in sample (non-solid fraction).

T = fraction of tissue in sample (solid fraction).

$$B_t = \frac{B_i - (B_b \times L)}{T}$$

2. Ratio of the percent benzoate in the tissue to the percent benzoate in the brine.

R = % benzoate in the tissue/% benzoate in the brine

$$R = B_t / B_b$$

3. The Partition Coefficient is the concentration of benzoate in the fat and/or protein divided by the concentration of benzoate in the aqueous phase.

P.C. = Partition Coefficient

$C_{f,p}$ = benzoate concentration in the fat and/or protein (in mg of benzoate in the fat and/or protein/grams of fat and/or protein in a 100 gram sample).

C_a = benzoate concentration in the aqueous phase (in mg of benzoate in the total aqueous phase/total grams of liquid in the brine and in the tissue in a 100 gram sample).

B_e = mg of benzoate/100 g of the initial brine after equilibration.

$B_{f,p}$ = benzoate (in mg) bound to the fat and/or protein in a 100 gram sample

A = grams of aqueous in the brine + grams of aqueous in the tissue in a 100 gram sample

F,P = grams of fat and/or protein in a 100 gram sample

a) $B_e = B_i \times 1000 \times L$

b) $B_{f,p} = B_e - (B_b \times 10 \times A)$

c) $C_{f,p} = B_{f,p}/F,P$

d) $$C_a = \frac{(B_b \times 1000)}{A}$$

e) $P.C. = C_{f,p}/C_a$

RESULTS

I. Model System

A preliminary equilibration study with a 2% agar-cube model system indicated that equilibration of the benzoate between the cubes and the brine was attained after six days storage with no significant change in readings after 12 days. All samples were stored for at least three weeks to assure equilibration.

Agar cubes which had been equilibrated in brine containing benzoate were transferred to fresh brine without benzoate to determine whether benzoate would transfer out of the blocks into brine as readily as it transferred in the other direction. The sets of samples used for this study were the protein-agar blocks, fat-protein agar blocks simulating food products (simulated foods), and laboratory-prepared food products (actual foods). With a knowledge of the benzoate concentration in the brine after the first equilibration, a prediction was made for the benzoate concentration in the brine following the second equilibration. The expected values were determined with equivalence equations:

$$\frac{\%\text{ benzoate in the brine (first equil.)}}{\%\text{ benzoate in the initial brine}} = \frac{X\ (\%\text{ benzoate in brine (second equil.)}}{\%\text{ benzoate in the cubes}}$$

A comparison of the expected values and actual values for the simulated foods and actual foods packed in an initial 0.2% benzoate brine is shown on Table 1.

Table 1. A comparison of expected and actual benzoate concentrations in the brines of samples after replacement of the equilibrated brine with a fresh brine and the establishment of a second equilibration. Values are given for the simulated food products and actual foods packed in an initial 0.2% benzoate brine.

PRODUCT	SIMULATED FOODS		ACTUAL FOODS	
	Expected % benzoate in brine	Actual % benzoate in brine	Expected % benzoate in brine	Actual % benzoate in brine
Kidney Beans [a,b]	0.047	0.049	0.044	0.043
Corn[a,b]	0.049	0.045	0.048	0.045
Peas	0.045	0.100	0.047	0.040
Mushrooms[a,b]	0.050	0.049	0.049	0.050
Soy Beans[a]	0.040	0.040	0.039	0.035
Avocado[a]	0.042	0.037	--	--

[a]No significant difference between expected and actual values of simulated foods at the 95% level of significance. Data used were for the three benzoate concentrations.

[b]No significant difference between expected and actual values of actual foods at the 95% level of significance. Data used were for the three benzoate concentrations.

A one-way analysis of variance was done for each product using values at all benzoate concentrations to test for significance between the expected and actual values. Notations were made for those products in which there was no

significant difference at the 95% level of significance. A comparison of expected and actual values for all simulated foods was significant, as was the same analysis for the food products. There was no significant difference when the simulated and actual foods were analyzed together.

Another study was done in which the fat and agar cubes were packed in brines containing 0.1, 0.2, and 0.3% benzoate, but using brine twice the weight of the cubes (33% cube, 67% brine). This increased the amount of benzoate which could be bound by the fat or protein. The equilibrated concentration of benzoate available in the fat or protein increased, as did the concentration in the aqueous phase. The net result was a ratio very close to the ratio for samples with equal weights of brine and cubes (50% cube, 50% brine). The ratios for the 0.2% benzoate data appear on Table 2.

To study the effect of the binding of benzoate by the agar, 1%, 2%, and 4% agar blocks were prepared. There was no significant difference among the three agar concentrations at the three benzoate levels. The percent benzoate in the equilibrated brine was approximately 0.054 for all three agar concentrations in the 0.1% benzoate brine and 0.105 and 0.114 for the 0.2 and 0.3% brines, respectively.

Table 2. Ratios of benzoate distribution for two pack-out ratios (50-50 and 33-67) of fat and protein agar cubes in a 0.2% benzoate brine.

% FAT OR PROTEIN	R=% Benzoate in tissue/% Benzoate in brine			
	FAT[a]		PROTEIN	
	50/50	33/67	50/50	33/67
0.0	1.09	1.30	1.29	1.36
0.5	1.09	1.04	1.36	1.58
1.0	1.33	1.25	1.36	1.52
2.0	1.24	1.30	1.47	1.52
3.0	1.37	1.22	1.52	1.76
5.0	1.62	1.54	2.00	2.07
10.0	2.11	2.84	2.26	2.49
15.0	2.57	2.49	2.52	2.88
20.0	2.75	2.95		
30.0	3.53	3.73		
40.0	3.73	4.91		

[a]No significant difference at the 95% level of significance between the two sets using values from the three benzoate concentrations.

II. Effect of Fat Concentration on Benzoate Distribution

Figure 1 is a plot of the concentration of benzoate in the aqueous phase vs. the percent fat (w/w) in agar blocks. Least squares exponential equations of the form $y=ae^{bx}$ fit the data, but did not show the curvature suggested by the points. The solid lines follow the equation, and the broken lines follow the data points.

The partitioning of a preservative between two or more phases was expressed in terms of a partition coefficient (von Schelhorn, 1964; Bean et al. 1965; Patel and Romanowski, 1970; Bean, 1972). This is the ratio of the concentration of benzoate in the fat to the concentration of benzoate in the aqueous phase. Figure 2 shows the data plotted as the partition coefficient vs. the percent fat. A power function equation ($y=ax^b$) was used. For the three benzoate concentrations, the partition coefficient at the lowest fat concentration (0.5%) was near 30. It decreased sharply to 12-13 for the 5% concentration and gradually decreased at higher fat concentrations. The final value at 40% fat was in the range of 6-8 for the three benzoate concentrations.

III. Effect of Protein Concentration on Benzoate Distribution

The concentration of benzoate in the aqueous phase vs. the protein concentration was plotted in Figure 3. The data points were quite scattered, but a least squares exponential equation of the form $y=ae^{bx}$ could be used though the correlation coefficients were -.802, -.948 and -.969 for the 0.1, 0.2, and 0.3% benzoate brines, respectively. There was no significant difference in the shape of the curves for the three benzoate concentrations. The percent decrease from equilibrated benzoate concentrations in the 0% samples to the equilibrated benzoate concentration in the 15% samples was similar for fat and protein. The values for protein were

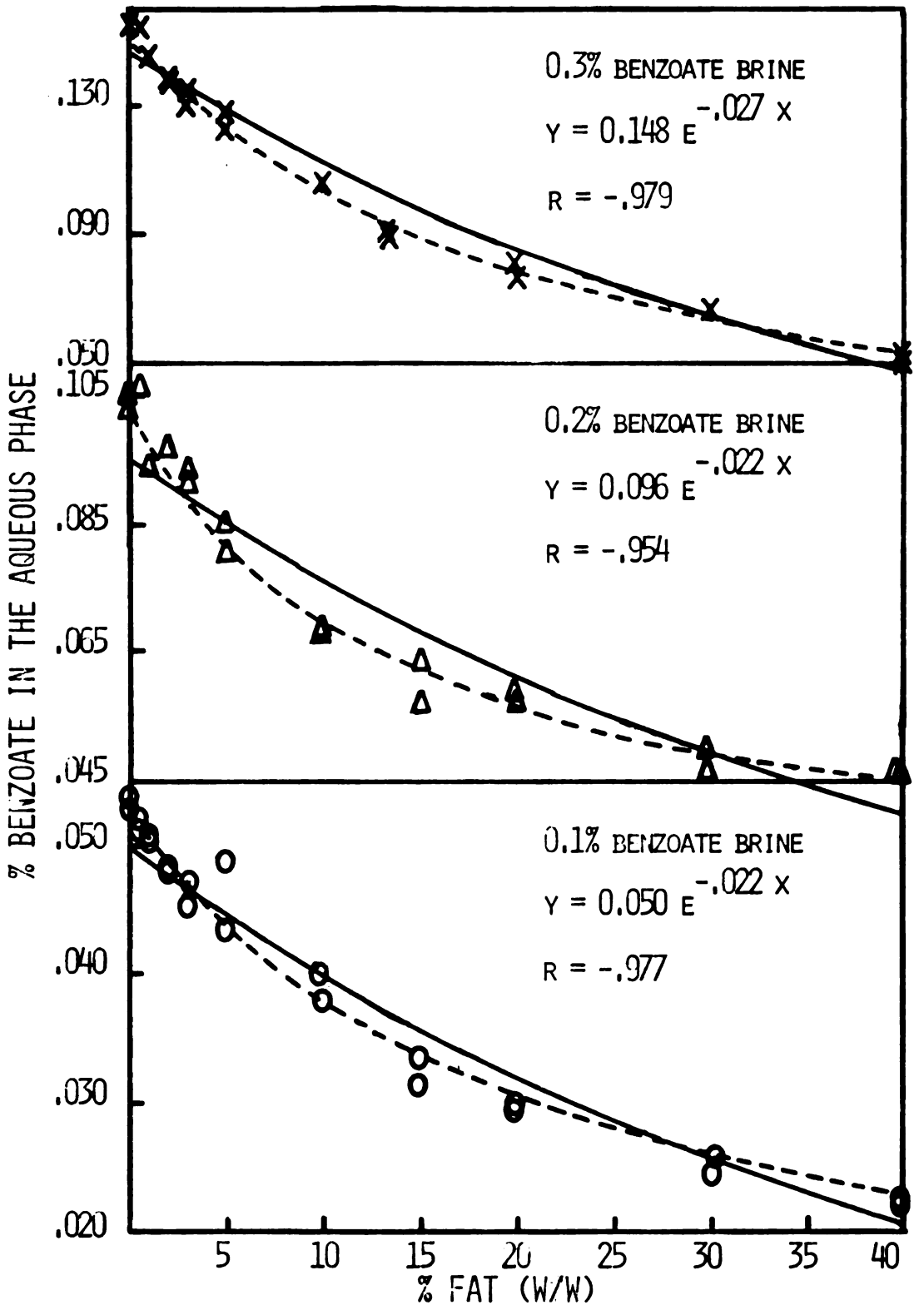

FIGURE 1. PERCENT BENZOATE IN THE EQUILIBRATED BRINE VS. % FAT IN AGAR CUBES FOR THREE INITIAL BENZOATE CONCENTRATIONS IN BRINE. THE SOLID LINES FOLLOW THE EQUATION, THE BROKEN LINES FOLLOW THE DATA POINTS.

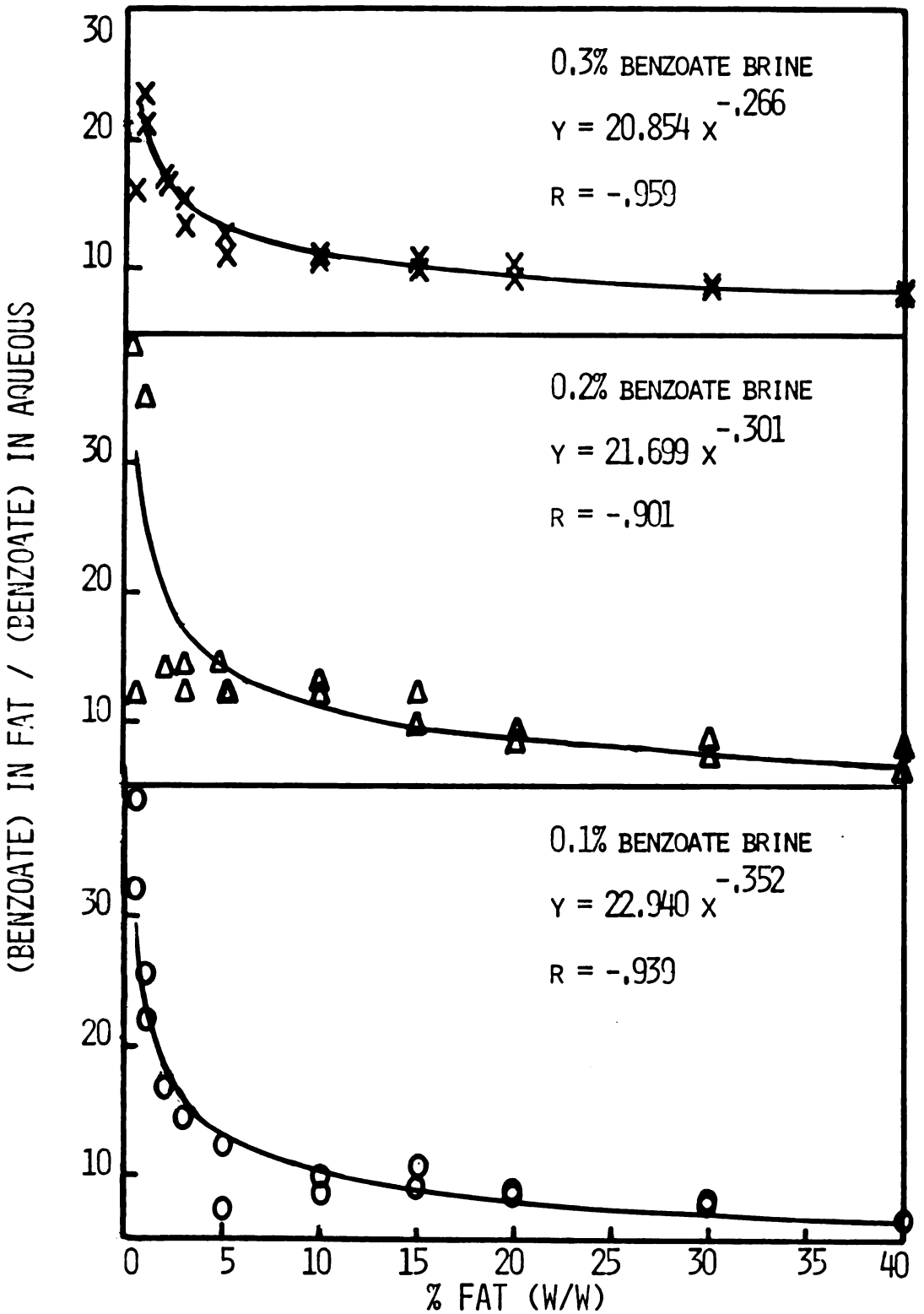

FIGURE 2. PARTITION COEFFICIENTS (BENZOATE CONCENTRATION IN FAT/BENZOATE CONCENTRATION IN AQUEOUS) VS. FAT CONCENTRATION IN FAT-AGAR BLOCKS IN BRINES OF THREE BENZOATE CONCENTRATIONS.

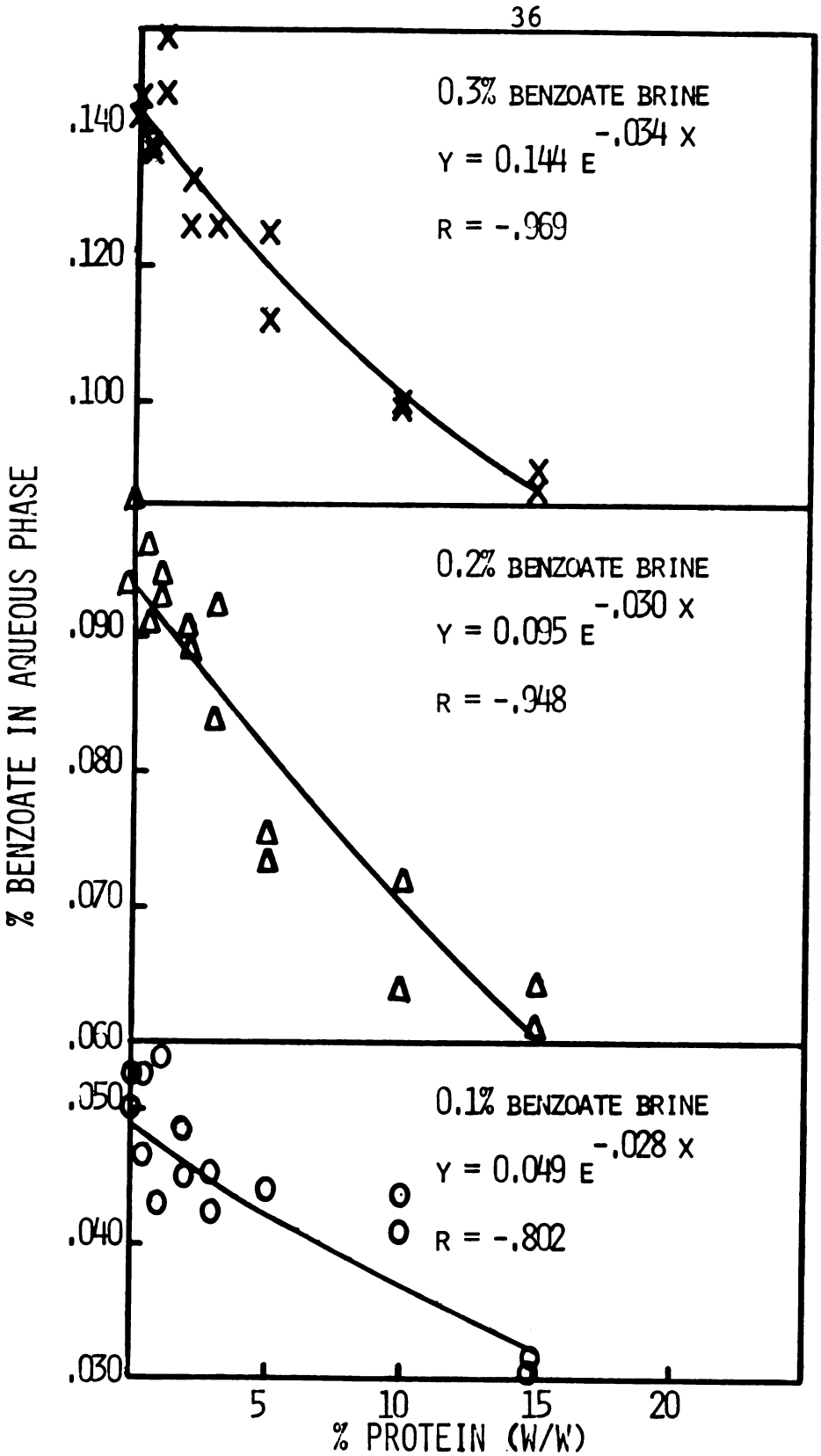

FIGURE 3. PERCENT BENZOATE IN EQUILIBRATED BRINES VS. % PROTEIN IN AGAR CUBES FOR THREE INITIAL BENZOATE CONCENTRATIONS IN THE BRINE.

35.0, 35.6, and 37.1% while those for fat were 37.7, 38.8, and 41.6% for 0.1, 0.2, and 0.3% benzoate brines, respectively.

Figure 4 shows the effect of protein concentration on the partitioning coefficient. The decrease in the coefficient values with an increase from 0.5% to 15% protein fit a power function equation ($y=ax^b$). There was some indication of an asymptotic decrease if the protein concentration was further increased.

IV. Effect of Binding of Benzoate on Antimicrobial Activity

An important aspect of the binding of benzoate by the fat is the effect which it has on the preservative activity of the benzoate. An attempt was made to determine the effective benzoate concentration in the aqueous phase of the model system. Malt extract (2%) was incorporated into the agar cubes. The equilibrated samples were inoculated with 10^8 cells/ml of Hansenula anomala or with brine from a spoiled jar of peppers known to contain yeast. Quantitation of growth was done by turbidimetric readings at 525 nm 0, 5, and 12 days after inoculation. A random sampling was done for plate counts. Agar block fragmentation interfered with turbidimetric measurements. The plate counts showed no evidence of any surviving cells in the pH 3 brine with 1.5% NaCl and no benzoate. The brine was replaced with water. A 30% sucrose solution was added to some replicates to give a final concentration of 1% sucrose. No growth was observed

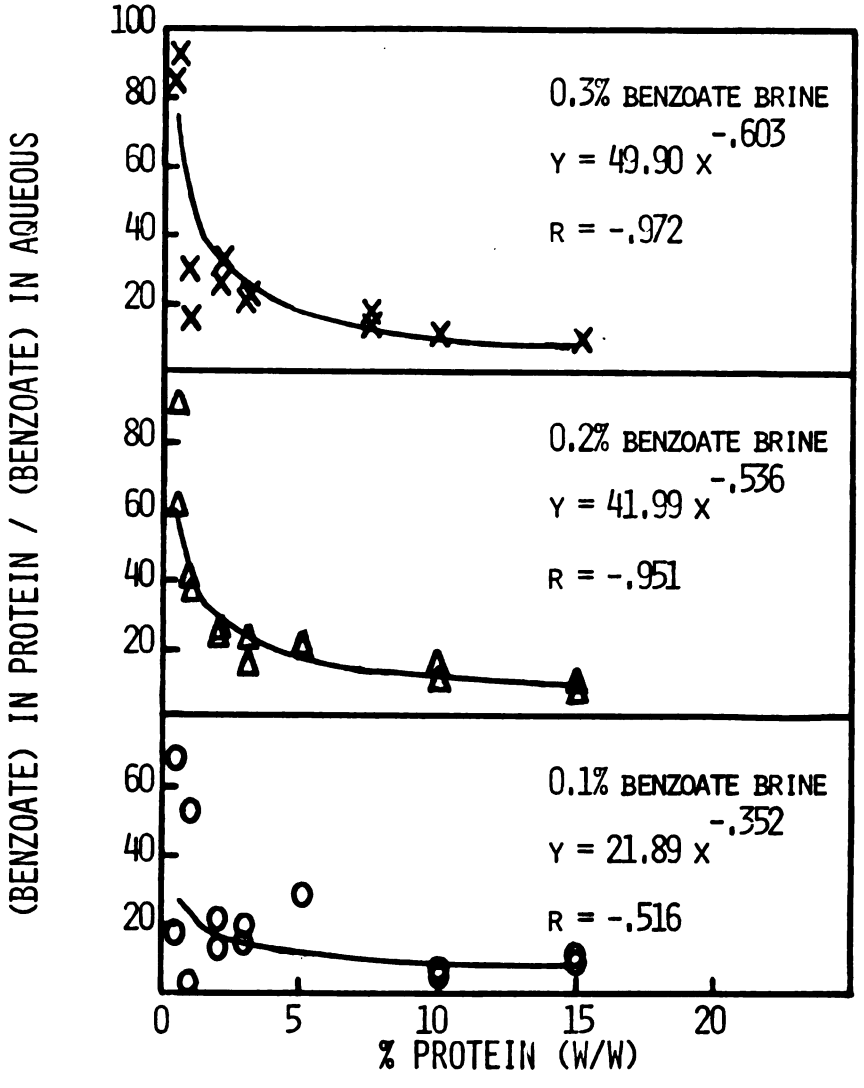

FIGURE 4. PARTITION COEFFICIENTS (BENZOATE CONCENTRATION IN PROTEIN/BENZOATE CONCENTRATION IN AQUEOUS) VS. PROTEIN CONCENTRATION IN PROTEIN AGAR BLOCKS IN BRINES OF THREE BENZOATE CONCENTRATIONS.

in any of the samples in two weeks. The inability to obtain growth of test organisms in the model system with no benzoate present made it impossible to show the decrease in preservative effectiveness which has been observed in other systems (Hibbott and Monks, 1961; Anderson and Chow, 1967).

V. Effect of Combinations of Fat and Protein on Benzoate Distribution

Figure 5 is a comparison of the partition coefficients of benzoate in fat and protein. The 0.2% benzoate data were used. Figure 6 shows the same data as Figure 5 but plotted as the ratio of the percent benzoate in the tissue to the percent benzoate in the liquid. This curve followed the equation of a parabolic function ($y=1.192 + 0.1097\ x - 0.0011\ x^2$).

Table 3 contains the ratios of the percent benzoate in the tissue to the percent benzoate in the liquid phase using the 0.2% benzoate brine (equilibrated ≈0.1%). The systems studied were a group of food products and protein-fat agar blocks to simulate these foods. For all ratios at the three benzoate concentrations, the paired analysis of variance showed no significant difference at the 95% level of significance between the simulated food products and the actual food products.

The parabolic curve of Figure 6 was used for prediction of the ratios for the simulated and actual food products packed in a 0.2% benzoate brine. These values appear in Table 4.

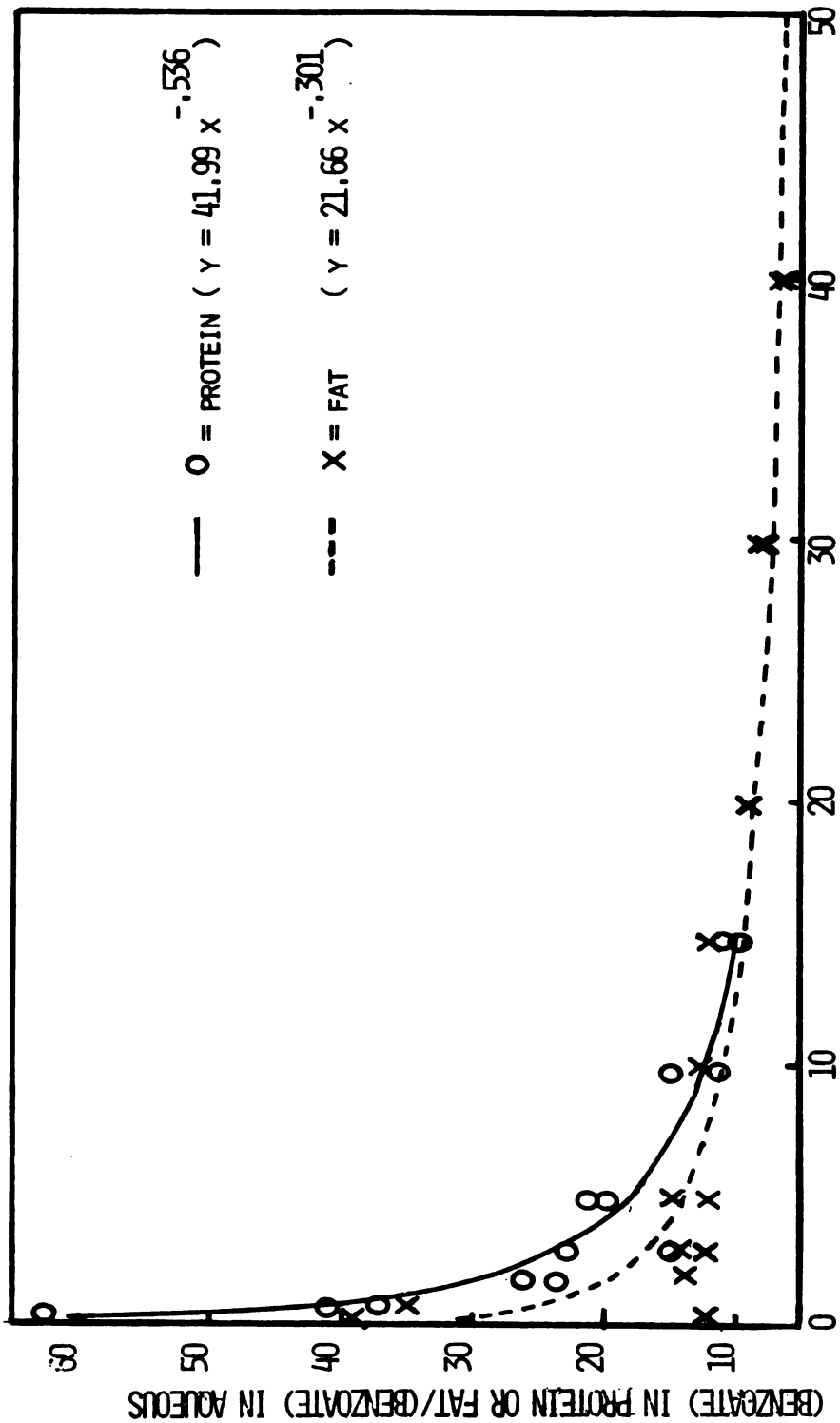

FIGURE 5. PARTITION COEFFICIENTS ((BENZOATE) IN FAT OR PROTEIN/(BENZOATE) IN AQUEOUS) VS. % FAT OR PROTEIN IN FAT OR PROTEIN AGAR BLOCKS PACKED IN 0.2% BENZOATE BRINE.

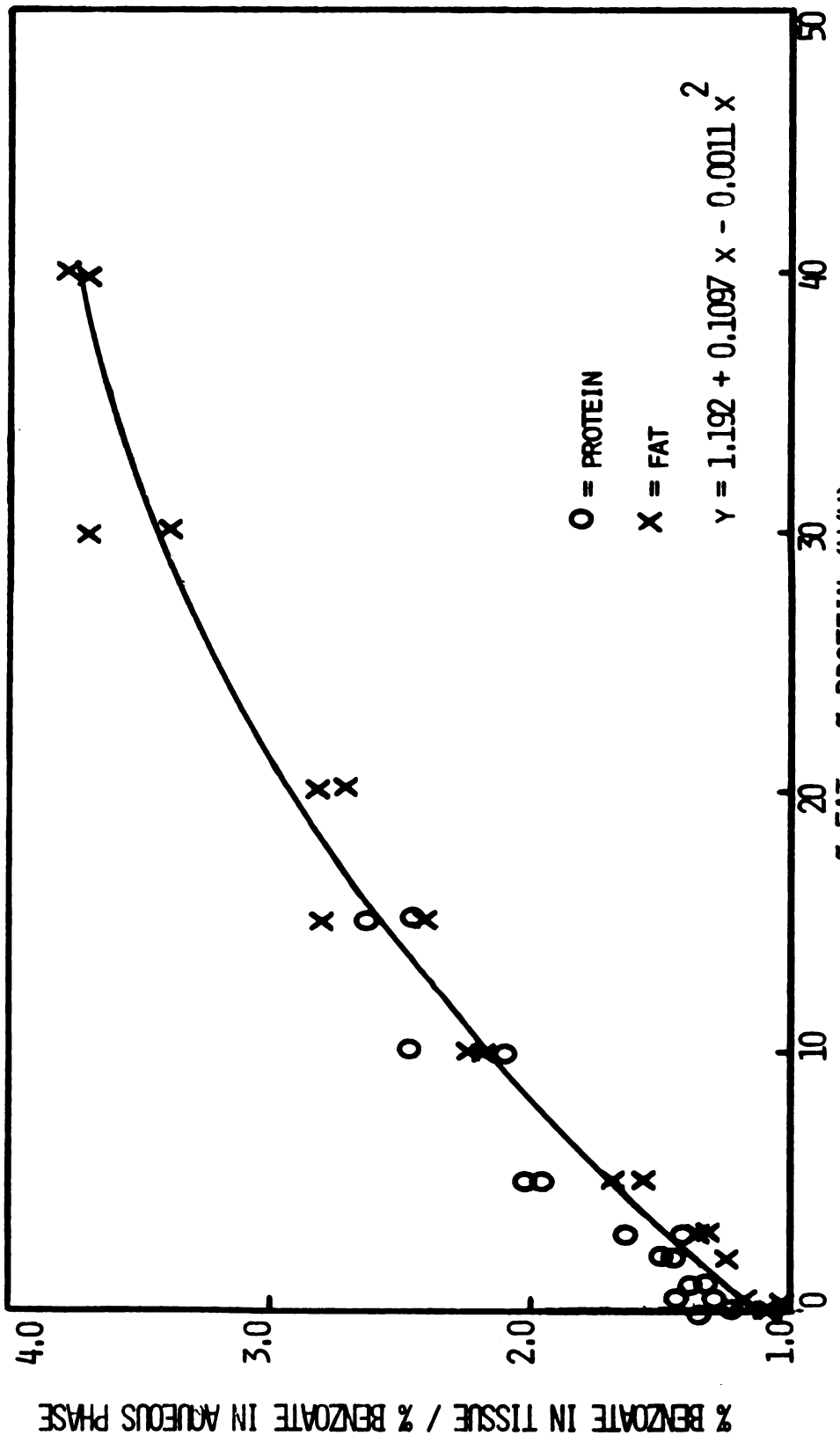

FIGURE 6. RATIO OF THE % BENZOATE IN THE TISSUE TO THE % BENZOATE IN THE AQUEOUS PHASE VS. % FAT + % PROTEIN. CURVE IS FOR 0.2% BENZOATE BRINE.

Table 3. Comparison of benzoate ratios (R:% benzoate in the tissue to the % benzoate in the liquid) of protein-fat agar blocks simulating food products and the actual food products.

PRODUCT	% BENZOATE IN TISSUE / % BENZOATE IN THE LIQUID	
	Agar Blocks	Food Products
Peas	2.31	2.24
Corn	1.94	2.14
Mushrooms	1.86	1.97
Soy beans	3.11	3.22
Kidney beans	2.15	2.62
Avocado	3.04	*

*Avocado food samples were not analyzed.

Table 4. Actual and predicted benzoate ratios (R) for simulated and actual foods based on equal and cumulative binding of benzoate by corn oil and soy protein (0.2% benzoate samples).

PRODUCT	AGAR BLOCKS			FOODS		
	% fat + % protein	Predicted ratio	Actual ratio	% fat + protein	Predicted ratio	Actual ratio
Peas	5.8	1.79	2.31	5.7	1.78	2.24
Kidney beans	8.3	2.02	2.15	9.9	2.17	2.62
Mushrooms	3.2	1.53	1.86	3.5	1.56	1.97
Corn	4.3	1.64	1.94	4.0	1.61	2.14
Soy beans	16.7	2.70	2.91	21.1	3.00	3.22
Avocado	18.5	2.83	3.04	---	---	---

VI. Commercial Food Products

All commercial food samples were separated into solid and liquid fractions and analyzed separately for benzoate content. The results were expressed as the ratio (R) of the percent benzoate in the tissue to the percent benzoate in the liquid. Table 5 contains data for different types of pepper packs.

Table 5. Benzoate distribution between tissue and aqueous in different types of paper products.

PRODUCT	Benzoate (%) in sample	Benzoate (%) in tissue	Benzoate (%) in brine	R
Mild peppers	0.082	0.103	0.074	1.39
Med.hot peppers	0.097	0.122	0.088	1.40
Hot pepper rings	0.152	0.178	0.135	1.32
Hot pepper rings	0.132	0.155	0.109	1.42
Mild pepperoncini	0.125	0.149	0.090	1.52
Sweet red peppers	0.080	0.099	0.059	1.69

The effect of pepper pigmentation on the benzoate distribution in hot mexi peppers and cherry peppers is shown in Table 6. Table 7 presents the benzoate distribution data for the components of a green cherry pepper.

Table 6. Effect of pepper pigmentation on the benzoate distribution between the tissue and brine

PRODUCT	Benzoate (%) in sample	Benzoate (%) in tissue	Benzoate (%) in brine	R
Hot mexi peppers				
a) red peppers	0.099	0.143	0.048	2.98
b) yellow peppers	0.093	0.130	0.048	2.72
Cherry peppers				
a) red peppers	0.073	0.086	0.050	1.71
b) green peppers	0.075	0.091	0.050	1.81

Table 7. Effect of cherry pepper components on the distribution of benzoate between the tissue and brine

FRACTION	% benzoate in fraction	% benzoate in brine	R
Green pepper (whole)	0.091	0.050	1.81
Green pepper w/o seeds	0.083	0.050	1.64
Seeds	0.171	0.050	3.40

Three jars of spoiled sweet banquet peppers and two jars of spoiled relish were analyzed for benzoate content and distribution. The results appear in Table 8.

Spoilage in the sweet peppers was evident by clouded brines and some sediment at the bottom of the jars. Gas

escape was observed upon opening one of the three jars. The peppers were turgid and did not appear damaged. Wet mounts of the brine showed the presence of yeast. The relish samples had gas pockets along the side of the jar. No wet mount was done with these samples.

Table 8. Benzoate partitioning between the solid and liquid phases in spoiled samples of peppers and relish

PRODUCT	Benzoate (%) in sample	Benzoate (%) in tissue	Benzoate (%) in brine	R
Spoiled sweet banquet peppers	0.088	0.106	0.069	1.55
"	0.092	0.105	0.078	1.35
"	0.071	0.082	0.062	1.32
Spoiled relish	0.065	0.070	0.055	1.27
"	0.128	0.142	0.105	1.35

Six refrigerated food products which listed 0.1% benzoate on their ingredient declaration were purchased and analyzed for benzoate. The results were tabulated in Table 9.

Italian salad dressing was analyzed in three phases: solid, aqueous and oil. The analysis results appear in Table 10.

A comparison of partitioning ratios between commercial food products and food products prepared in the laboratory (0.1% equilibrated benzoate brine) appears in Table 11.

Table 9. Benzoate distribution between solid and liquid phases of commercial products preserved with 0.1% sodium benzoate

PRODUCT	Benzoate (%) in sample	Benzoate (%) in tissue	Benzoate (%) in brine	R
Kosher pickles	0.127	0.132	0.122	1.086
Gelatin fruit cocktail salad	0.062	0.080	0.059	1.36
Mild Mexican sauce	0.090	0.101	0.086	1.17
Pineapple chunks	0.071	0.073	0.069	1.06
Baked beans	0.083	0.059	0.176	0.33
Cole slaw	0.070	0.043	0.155	0.27

Table 10. Distribution of benzoate among the solid, aqueous, and oil phases of Italian salad dressing

PRODUCT	Benzoate (%) in sample	Benzoate (%) in solid	Benzoate (%) in aqueous	Benzoate (%) in oil
Italian salad dressing	0.174	0.064	0.043	0.286
	$\frac{\text{(Benzoate) solid}}{\text{(Benzoate) aqueous}}$		$\frac{\text{(Benzoate) oil}}{\text{(Benzoate) aqueous}}$	
	1.488		6.65	

Table 11. Comparison of benzoate distribution ratios for food products prepared in the laboratory and commercial food products

LABORATORY PRODUCTS (0.1% Benz., equil.)	R	COMMERCIAL FOOD PRODUCTS	R
Peas	2.24	Hot mexi peppers	2.98
Corn	2.14	Gelatin fruit salad	1.36
Mushrooms	1.97	Mild Mexican sauce	1.17
Soy beans	3.22	Hot pepper rings	1.42
Kidney beans	2.62	Baked beans	0.33

DISCUSSION

I. Model System

The 2% agar-cube model system was satisfactory for the observation of benzoate partitioning as a function of lipid and protein content. It did not disintegrate in the pH 3 acetic acid brine, it attained equilibrium relatively rapidly (6 days), and the agar concentration had no effect on the benzoate which was bound. Two additional tests were conducted with the model system. A study was carried out to determine the ease of benzoate transfer out of agar cubes and food products into a brine containing no benzoate. The transfer out of the cubes and foods was proportional to the initial transfer from the brine into the cubes and foods. Refer to Table 1. In the second test, cubes were packed in benzoate-containing brines weighing twice as much as the cubes. These results were compared in Table 2 to cubes packed in an equal weight of brine. The ratios were close enough to conclude that an increased amount of brine containing benzoate did not change the distribution ratio of benzoate in the cube-brine system. This indicated that there were no limiting conditions in restricting the study to a 50/50 pack-out ratio.

Physical limitations of the agar blocks were (1) incorporation of more than 50% oil, which prevented

solidification and (2) incorporation of more than 15% protein, which was too viscous for mixing and cube formation.

II. Methods of Analysis

The spectrophotometric method of benzoate analysis, whenever it could be used, was preferred to the A.O.A.C. Official Titrimetric method. The spectrophotometric method required a knowledge of the components of the brine for the preparation of a standard curve and its use as a blank. It was not suitable for commercial food products because interference from food components was too great.

The modified A.O.A.C. method was satisfactory for all the food products tested. The addition of methyl red to the extraction mixture provided a visual method to determine when benzoate extraction was completed. Methyl red was chosen because its solubility in chloroform was approximately equal to the solubility of benzoic acid in chloroform. At the concentration at which the methyl red was added, it had a negligible effect on the final titration with NaOH. Its presence, however, obscured the phenolphthalein end point, requiring the titration to be carried out to pH 8.2. Methyl red changed from orange-red to yellow near pH 7.2, but the change was not sharp enough to be used as a titration end point.

III. Binding of Benzoate by Fat

A. Discussion of Binding Data

Benzoic acid, being more soluble in lipid than in

water, was expected to partition into the lipid over a range of lipid concentrations. Figure 1 indicated an exponential decrease in the benzoate concentration in the aqueous phase as the percent oil was increased. The greatest decrease was observed from 0 to 15% fat followed by a gradual decline at higher fat concentrations. This sharp decline followed by a slower decline at higher fat concentrations was observed by Bean (1972) with methyl paraben in soy oil (the partition coefficient = 80). His data were graphed on Figure 7. Figure 1 shows curves of the same shape for the three benzoate concentrations. The percent decreases in the benzoate present in the aqueous phase of 40% fat cubes compared to 0% fat cubes were 56.6%, 55.3%, and 64.1% for the 0.1, 0.2, and 0.3% benzoate brines, respectively. This was a decrease of over half the benzoate assumed to be active in a product.

The fat-binding data were plotted in Figure 2 as the partition coefficient (the concentration of benzoate in the fat divided by the concentration of benzoate in the total aqueous) vs. the fat concentration. The curves were similar to those in Figure 1, but they were better described by a power function equation than by an exponential equation. The partition coefficients at the various fat levels agreed reasonably well with those of von Schelhorn (1967). He reported a partition coefficient of 8.26 for mayonnaise containing 51.3% fat and 37.7% water. Extrapolation of the

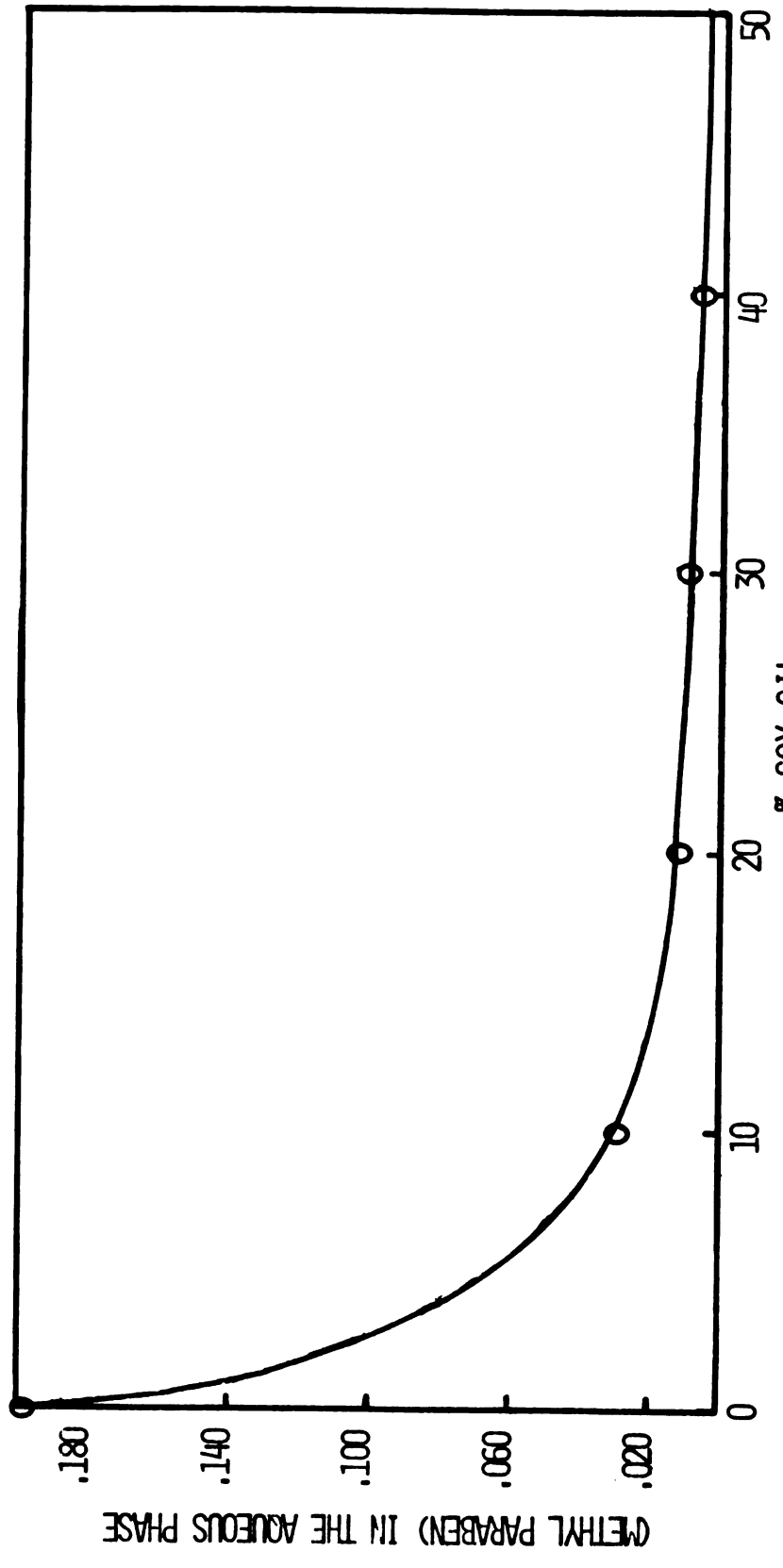

FIGURE 7. CONCENTRATION OF METHYL PARABEN IN THE AQUEOUS PHASE AS A FUNCTION OF SOY OIL CONCENTRATION (BEAN, L972).

curves in Figure 2 to 51.3% resulted in a range (the variability among benzoate concentrations) from 6-8, slightly lower than 8.26. The 7.90 value for marzipan at 25.5% fat and 12.3% water fell in the 7.75-8.75 range from the graph.

B. Preservative Activity

Many investigators have concluded that the preservative activity of benzoate is dependent upon the concentration of benzoate in the aqueous phase. Hibbott and Monks (1961) prepared eight creams, all containing 0.15% methyl-p-hydroxy benzoate. The amount of lipid was constant, but fats were chosen with a variety of partition coefficients, resulting in a range of preservative concentrations in the aqueous phase. All those creams with 18 to 27.8 mg of preservative in 100 ml of water did not spoil, whereas creams with 11.6, 9.4, and 7.1 mg of preservative per 100 ml were unable to prevent growth of mold. In addition, the inclusion in oil: water systems of propylene glycol or glycerol (2 to 20%), which reduce the partition coefficients, increased the preservative available in the aqueous phase, thus increasing preservative activity. Von Schelhorn (1964) reported a decrease in the effective preservation of ethyl and propyl esters of p-hydroxy benzoic acid with increasing fat concentration. The decreases in effective preservation of 70-90% with an increase from 0 to 10% fat, reflect the highly lipid-soluble nature of these preservatives.

Though it was not possible to study the preservative activity of benzoate as a function of fat and protein

composition with the model system developed for this study, it is reasonable to expect that similar declines in preservative activity related to bound preservative would be observed with this system if a suitable organism is found.

The total preservative required in a 2-phase system can be determined knowing the usual concentration required in the aqueous phase (C_{H_2O}), and knowing the volume in each phase (V_{oil}, V_{H_2O}) and the distribution coefficient (D) (Patel and Romanowski, 1970):

$$\text{Total preservative} = C_{H_2O}\ V_{H_2O} + D\ C_{H_2O}\ V_{oil}$$

This is valid in a system such as the model system which was near pH 3, where essentially all of the benzoate was in the undissociated form. Table 12 shows the proportion of undissociated benzoic acid at pH's from 2-6 (Kostenbauder, 1962).

Table 12. Proportion of benzoic acid undissociated at various pH levels (Kostenbauder, 1962)

pH	% Undissociated Benzoic Acid
2	99.4
3	94.3
4	82.5
5	13.7
6	1.6

pKa Benzoic Acid = 4.2

It is clear, that the undissociated benzoic acid in the aqueous phase must be considered in determining the activity of benzoate when it is used at pH 4 and above.

The total preservative required at any pH value can be determined by the following equations (Kostenbauder, 1962).

$$Ka = \frac{(H^+)\ (A^-)}{(HA)}$$ Ka = the dissociation constant of the acid

$$\text{The fraction undissociated} = \frac{(HA)}{(HA) + (A\text{-})}$$

$$\text{after substituting for HA} = \frac{(H^+)}{(H^+) + Ka}$$

$$\text{Undissociated acids} = (\text{Total})\ \frac{(H^+)}{(H^+) + Ka}$$

$$\text{Total required preservative} = \frac{\text{[Required conc. of undissoc.acid]}\ ((H^+) + Ka)}{(H^+)}$$

IV. Binding of Benzoate by Protein

Figures 3 and 4, showing plots of the binding of benzoate by protein were very similar to Figures 1 and 2 for fat. Again, there was no significant difference in the shape of the curves among the three benzoate concentrations. The shape of the curves for Figures 2 and 4 was similar to the shape of a curve obtained by Davison and Smith (1961) in studying the binding of benzoate at pH 5.4 to bovine serum albumin. They suggested that the curvilinear relationship

obtained was an indication that at least two binding sites existed. Kazmi and Mitchell (1971) used this relationship to interpret preservative-surfactant interaction. They suggested that the binding sites within micelles did not behave independently of one another. It was possible that uptake of solute into the micelles progressively altered the interaction between the binding sites and the solute leading to a change in both the number of sites available and the association constant. Perhaps the binding of benzoic acid by fat can also be explained this way.

Most of the protein-preservative interaction studies were carried out at pH 6-7. At this pH the benzoate ion was the form bound by the protein (Klotz, 1946; Meyer and Guttman, 1968). The present study showed that undissociated benzoic acid was bound by soy proteins at pH 3.0. It is probable that hydrophobic pockets exist in the protein molecules which are of the right size for binding of the hydrophobic benzoic acid molecules.

Figures 5 and 6 show similar relationships between the benzoate distribution coefficient and the concentration of fat or protein, but soy protein does appear to bind slightly more benzoate on a weight basis than corn oil. However, distribution coefficients in protein and fat were combined to determine if a reasonable prediction of benzoate distribution in food products could be made using data obtained with a model system.

The fact that the combination of fat plus protein simulating the food (colum 3 in Table 4) was a better prediction of the benzoate distribution in the food products (column 6 in Table 4) than the combined data which assume equal binding by protein and fat (column 5 in Table 4) suggests that protein does bind more benzoate on a weight basis than fat. However, the data indicate that addition of 0.5 to the predicted ratio (column 5 in Table 4) will provide a good estimate of the distribution between bound benzoate and soluble benzoate when the proximate composition of a food is known.

V. Commercial Samples

Commercial food products containing sodium benzoate were separated into the brine or liquid phase and the solid or tissue phase. The modified A.O.A.C. method was used to determine the benzoate in each phase. The results were expressed as the ratio of the percent benzoate in the tissue (or solid) to the percent benzoate in the brine (or liquid).

Different types of pepper products were analyzed (Table 5). The data showed accumulation of benzoate in the edible portion of food products for two reasons. First, the average benzoate concentration in the product was above the 0.1% limit. Secondly, in products, such as peppers, where benzoate accumulates in the tissue and the brine is usually not consumed, the benzoate in the food as eaten can be

greater than 0.1% even though the average concentration in the jar is 0.1% or less. The ratio of benzoate in the tissue to brine was the highest for red peppers (1.69). The ratio for green peppers (pepperoncini) was also quite high (1.52). The rest of the peppers with ratios from 1.32 to 1.42 were yellow. Table 6 showed higher ratios for the red peppers (2.98) than the yellow peppers (2.72) of hot mexi peppers, but also higher ratios for dark green cherry peppers (1.81) than red cherry peppers (1.71). The pigment had some effect, but it did not account for the difference between hot mexi peppers and cherry peppers. The data in Table 7 showed a high benzoate concentration in seeds. The large quantity of seeds in mexi peppers undoubtedly contributed to the high ratio observed with these peppers.

It would be of interest to know the spoilage rate in peppers with a high tissue benzoate concentration compared to similar products with a lower ratio. If previous studies of preservative effectiveness are correct, it is expected that more spoilage would occur with higher distribution ratios.

Table 8 contains the benzoate distribution data for three jars of spoiled sweet banquet peppers and two jars of spoiled relish. The results did not show any cause for spoilage on the basis of benzoate content. The benzoate concentration in the brines coupled with a low pH of 3.2 and a salt concentration of 1.5% should have been sufficient to

inhibit yeast growth. Pitt and Richardson (1973) reported spoilage in beverages and tomato sauce, where the benzoate levels were as high as legally allowed. They isolated strains of Saccharomyces bailii, Pichia membranaefaciens, and Candida krusei. The yeast were capable of growth in 0.02-0.07% benzoic acid and/or 1.5% acetic acid (pH 3.5). The spoilage was sporadic and believed to be due to post-processing contamination or contamination from improperly cleaned fillers.

Table 9 contains data for several refrigerated food products. The ratios for pickles, gelatin salad, Mexican sauce and pineapple chunks (close to one in most cases) were lower than those observed for peppers. The ratio of 0.274 for cole slaw was explained by the mayonnaise present in the liquid phase, which resulted in a low partitioning ratio. The ratio of the percent benzoate in the liquid/the percent benzoate in the solid was 3.6. The baked beans were diluted to aid in the separation of the beans and the sauce. The liquid was centrifuged and the supernatant and precipitate were analyzed separately. After accounting for the initial dilution, the benzoate concentration in the aqueous was 0.176%. Solubilization of proteins may account in part for this high benzoate concentration in the aqueous phase.

Table 8 has results for Italian salad dressing, analyzed as a three-phase system. The concentrations in the solid and aqueous were similar to those of other products

analyzed. The ratio of 6.65 for oil and aqueous was higher than would have been predicted from Figure 6. The value for the benzoate in the oil phase is not reliable due to problems with the final titration.

Table 11 compares the ratios for laboratory-prepared food products (0.1% benzoate after equilibration) and some of the commercial food products. The food products prepared in the laboratory had relatively high ratios (2-3), whereas the commercial products, on the average, stayed in the 1-1.5 range. The discrepancy may be attributed to the nature of the liquid phase. The laboratory products were packed in a simple brine containing only acetic acid, NaCl, and benzoate. The commercial brines, in addition, contained combinations of oils, spices, emulsifiers, and other ingredients. This would tend to favor more partitioning of benzoate into the liquid phase, thus reducing the partitioning ratio. The increased benzoate concentration in the liquid would not necessarily indicate a greater preservative capacity, since the benzoate would not be free, but bound to the ingredients in an inactive form.

CONCLUSIONS

In the present study of a model system and selected food products, previous observations of benzoate uptake by fat were confirmed. In addition, it was found that protein also binds benzoate. Von Schelhorn (1964) and Hibbott and Monks (1961) reported a decrease in antimicrobial activity of preservatives accompanying a decrease of the preservative in the aqueous phase of an aqueous-lipid system. In the present study, increased fat and protein concentrations decreased the amount of benzoic acid in the aqueous phase. The benzoate present in the aqueous phase of a product containing 15% fat and/or protein was only two-thirds of that present with no fat or protein. In a similar manner, the benzoate concentration in the aqueous was decreased by one-half by increasing the fat concentration from 0 to 40%. Equal weights of corn oil and soy protein bound benzoic acid in the same manner, but the binding of benzoic acid by protein was shown to be greater than the binding by corn oil.

The relationship between the benzoate distribution in a fat-protein model system and the quantity of fat and protein in that system can be used for the prediction of distributions in food products. With a relatively simple system, (peppers, cucumbers, or other fruits and vegetables

packed in a cover brine containing negligible amounts of fat or protein) the model system can be used to predict the ratio (R). In this type of system, the % benzoate in the liquid is equivalent to the effective benzoate concentration. The separation of the brine and tissue allows a measure of the benzoate concentration in the edible portion. In the case of a product such as cole slaw, (A product in which the fat and protein are in the nonsolid phase or in both the solid and nonsolid phases, and in which the phases are not normally separated for consumption), if the fat, protein and moisture contents are known, the model system can be used to predict the effective benzoate concentration in the entire system.

Analyses of commercially prepared peppers of different varieties, pickles, and Mexican sauce showed benzoate concentrations in the edible portion greater than 0.1%, which is the maximum legal limit. This was true even when the total benzoate concentration was below 0.1%.

All the products analyzed were labeled as containing 0.1% sodium benzoate, however, there existed variability in the actual amounts measured even between two jars of the same product. The range of benzoate concentration in the samples analyzed was 0.062 to 0.174%. It appears that often processors do not have sufficient control of benzoate addition to food products to assure that it will be within the legal limit.

The ratio of the percent benzoate in the solid/ percent benzoate in the liquid was decreased by the addition of ingredients such as oils, spices, emulsifiers in the liquid phase. This increase in the benzoate concentration in the aqueous phase, however, would not be expected to have an effect in increasing the preservative activity as the benzoate would be bound in an inactive form.

This study focused on the binding of benzoic acid by fat and protein. There is still too little known about the nature of this binding. Information concerning the mechanism of binding between benzoic acid and proteins at low pH, especially in food systems, is unavailable. It is expected that benzoate bound to protein will be unavailable as a preservative just as has been previously observed for benzoate in fat (Hibbott and Monks, 1961; von Schelhorn, 1964). This expectation needs to be tested.

The binding of benzoic acid by carbohydrates, particularly starches, and cell wall constituents should be studied to get a better picture of the binding of a preservative by these components of a food system.

Since ethyl p-hydroxybenzoic acid, propyl p-hydroxybenzoic acid and sorbate are also lipid soluble, it is expected that in food products similar distribution phenomena would be observed. This possibility should be investigated and the quantitative relationships developed.

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