of the C3 methyl group of this isomer should then have been shielded by the π electrons of the 6,7-double bond, while the methyl carbon should have been deshielded owing to the δ-substituent effect from C6 (Stothers et al., 1976; Grover et al., 1973; Mann et al., 1978). The respective ^{1}H NMR and ^{13}C NMR spectra of isomer **2a** showed an upfield shift for these methyl protons and a downfield shift for the methyl carbon relative to the positions of these signals in corresponding spectra of isomer **2b**. Therefore the diastereoisomeric ether **2a** was tentatively assigned 3*R**,5*R** configuration about its asymmetric centres and isomer **2b** assigned the 3*R**,5*S** configuration.

Finally, an extract of a commercial wine made from Muscat of Alexandria grapes was analyzed by GC and GC–MS. This extract contained the three unknowns. Cochromatography of the isolated and characterized monoterpene ethyl ethers **2a**, **2b**, and **3** with this extract confirmed the identity of the unknowns in the wine.

The ethers **2a**, **2b**, and **3** have been found to occur at higher concentrations in muscat wines than in wines prepared from less aromatic grape varieties. They could play a significant role in recent studies into the characterization of cultivars from the variety Riesling (Guentert, 1984; Rapp and Guentert, 1985). It has been demonstrated here that these ethers owe their origin in wines and spirits to the reactivity of diol **1** and to a lesser extent diol **4** in acidic ethanolic solution. An additional product was formed from diol **4** in acidified ethanol and this showed the following EIMS: m/z (relative intensity) 137 (3), 112 (4), 100 (7), 99 (100), 84 (5), 82 (8), 81 (7), 71 (90), 69 (10), 68 (9), 67 (19), 55 (17), 43 (42). This compound had a retention time longer than that of ether **3** and was tentatively identified from its MS as 3-ethoxy-3,7-dimethylocta-1,7-dien-6-ol.

It thus appears that ethyl ether formation is a significant reaction pathway in wines and spirits and could warrant further study.

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Registry No. **1**, 51276-34-7; (*R**,*R**)-**2a**, 95694-07-8; (*R**,*S**)-**2b**, 95694-08-9; **3**, 95694-06-7; **4**, 51276-33-6; ethanol, 64-17-5.

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The Nature of the Protein Constituent of Commercial Lemon Juice Cloud

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The cloud protein content of two commercial lemon juice concentrates was 29.8% of the original cloud. The protein can be completely solubilized in 10 M urea–6% citric acid, pH 2.5. The insolubility of the cloud protein could be attributed to at least three causes: heat denaturation, inherent insolubility, or a complex of protein with another constituent of the fruit. We present evidence that heat-denatured proteins do not contribute to the cloud, but that inherently insoluble and/or complexed protein are responsible for this constituent.

INTRODUCTION

A fine suspension of particulate material, known as cloud, contributes substantially to quality factors such as color, flavor, and texture of citrus juices. Most research on citrus cloud has dealt with the causes of its instability in orange juice (Baker and Bruemmer, 1970), a problem which is due mainly to soluble factors in the juice and which can usually be prevented by appropriate heat treatment during processing. Cloud instability may also be encountered when citrus-flavored beverages are formulated, and in such cases the problem resides in the nature of the cloud particles.

A better knowledge of the basic components and of the

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chemical and physical properties of cloud should lead to a better understanding of its stability as well as to other ways to improve its quality. Previous work on the properties and composition of cloud has produced useful information which opens the way for further, more detailed studies (Scott et al., 1965; Kanner et al., 1982; Venolia et al., 1974; Venolia and Peak, 1976; Mizrahi and Berk, 1970).

Since protein represents a substantial proportion of cloud and undoubtedly makes a significant contribution to its behavior, we have undertaken a detailed investigation of the properties of lemon juice cloud protein, and in particular, its sources in the intact fruit and its transition into cloud particles.

EXPERIMENTAL SECTION

Sources of Juice Samples. Two commercial lemon juice concentrates were used to prepare single strength samples, A and B. A is a pasteurized, 45.9° Brix concentrate (Ventura Coastal Corporation), produced by high temperature evaporation. It was stored frozen at −8 °C. B is a pasteurized, 61.9° Brix concentrate (Sunkist), produced and stored as A. C is 8.2° Brix single strength, fresh frozen juice, from the same production batch as B, just prior to concentration. It was stored frozen at −8 °C.

Preparation of Single Strength Juice Samples. Samples A and B were prepared by reconstitution of the concentrates to a final Brix of 8.2° (an arbitrary value for single strength juice) with deionized water. Sample C was used to determine the effect of heating on the soluble proteins of the juice serum and was thus used as provided by the manufacturer. Juice was obtained from the juice sacs of whole lemons with a 25 G 5/8 in. tuberculin syringe in an effort to minimize the high shear force the juice would experience during commercial processing or even by hand squeezing.

Samples A and B, after reconstitution, contained no visible particles and filtration through a 210-μm mesh resulted in no visible residue on the filter.

Isolation and Extraction of Cloud. Washing and solubilization of the cloud particles was carried out so as to avoid shear force, as it was found that preparation in which sonication or grinding were used tended to change the physical characteristics of the cloud.

The juice (10 mL) was centrifuged at 27000g for 15 min. These conditions produced a supernatant whose optical density (OD) at 600 nm was less than 0.05 (about 1% of the original turbidity of the juice). The supernatant was decanted, and the cloud pellet was redispersed in 10 mL of deionized water by vortexing. The suspension was centrifuged as before and the supernantant decanted. The washing process was repeated twice.

All reagents were of the highest purity obtainable. The term urea–citrate refers to 10 M urea–6% citric acid, pH 2.5. Samples of the washed cloud, as described above, were used for subsequent analysis as follows:

(a) Total cloud weight was obtained by freeze drying the cloud to constant weight.

(b) The cloud was prepared for protein analysis as follows: The washed cloud was extracted with 5 mL of acetone (to remove lipids and for dehydration) followed by centrifugation as done previously, and the acetone was decanted. The cloud was stirred for 15 min with 0.5 mL of dimethyl sulfoxide (Me_2SO) and 20 mL of isopropyl alcohol was then added. The suspension was mixed well and allowed to stand for 30 min in order for the proteins to reprecipitate. The suspension was centrifuged as before and decanted. The pellet was then dried under a stream of nitrogen. The Me_2SO extraction was necessary to remove the flavanone glycoside hesperidin, which tends to interfere with protein analysis.

(c) The cloud was prepared for solubilization in urea–citrate as follows: The washed cloud was extracted with 10 mL of methanol (in order to remove lipids), followed by centrifugation and decanting as done previously. The pellet was dried with a stream of nitrogen, and 10 mL of urea–citrate was added. The suspensions were stirred overnight at low speed with a small (1 cm) magnetic stir bar at 4 °C.

(d) Urea was removed from the cloud via extensive dialysis vs. 6% citric acid, pH 2.5, at 4 °C in Spectrapor membrane tubing of molecular weight cutoff 2000, obtained from Spectrum Medical Industries, Los Angeles, CA.

Model Systems of Heat-Denatured Proteins. A pectin esterase of low specific activity, obtained from orange peel (o.p.p.), was purchased from Sigma Chemical Co., St. Louis, MO. Bovine serum albumin (BSA) was purchased from Calbiochem-Behring Corp., La Jolla, CA.

The o.p.p. was used as a model protein system to approximate the types of proteins that are likely to be found in citrus cloud. BSA, a common mammalian protein, was also independently used as a heat-denatured model.

Solutions (1 mg/mL) of both o.p.p. and of BSA were prepared in both deionized water and in 6% citric acid, pH 2.5 (the pH of single strength lemon juice). The solutions were placed in an 85 °C water bath for 15 min.

Single strength, fresh frozen lemon juice (sample C) was also heated at 85 °C in a water bath for 15 min and compared to a nonheated control. Juice serum, from which the cloud had been removed first via centrifugation, was used similarly. After heating, the insoluble material (original cloud and/or heat denatured protein) was isolated and analyzed for protein.

Preparation of Whole Lemons. Fresh lemons were picked from a Eureka lemon tree growing at our laboratory in Pasadena. The lemons were thoroughly washed in tap water prior to use.

The peel (albedo and flavedo) was removed from a ripe but firm lemon. The remaining endocarp was squeezed by hand and the juice collected to establish the volume of single strength juice obtainable from the lemon. This value was used as the total volume in which the protein within the albedo would have been dispersed if the lemon had been processed commercially. The flavedo was removed from the peel with a scalpel and discarded. A portion of the albedo was first weighed and then homogeneized in 6% citric acid (pH 2.5) in a small laboratory model Waring blender. Further digestion was accomplished with a Brinkmann Polytron. The suspension was incubated at room temperature with occasional stirring for 2 h. The homogenate was centrifugated as with processed juices. The supernatant contained citric acid soluble protein, and the pellet contained citric acid insoluble protein.

To a portion of the citric acid soluble albedo protein was added 10 volumes of isopropyl alcohol. The suspension was agitated and the proteins allowed to precipitate for 30 min. The protein precipitate was isolated via centrifugation and prepared for protein analysis as described above. The citric acid-insoluble albedo protein was also prepared for protein analysis as described above.

The peel of a ripe but firm lemon was removed and discarded. The remaining endocarp was thoroughly squeezed by hand. The juice was filtered through a 210-μm filter, and the filtrate and retentate were saved. A portion of the filtrate was filtered through a 37-μm filter and again the filtrate and retentate were saved. The retentates from the 210- and 37-μm filtrations were homogenized in 0.05

N potassium hydroxide and prepared as described earlier. The filtrate from the 37-μm filtration was analyzed for protein directly.

Assay Methods. Kjeldahl nitrogen analysis was performed on five replicates of cloud from sample A, dried as described earlier, by Truesdail Laboratories, Inc., Los Angeles, CA.

Dried cloud samples and insoluble fresh lemon fruit material were solubilized in 0.05 N potassium hydroxide for 30 min, or until solubilization was complete.

Protein was determined from the Kjeldahl analysis on sample A as N × 6.25. Protein was also determined on sample A by the trichloroacetic acid precipitation procedure of Schaffner and Weissmann (1973) by using the sample, solubilized as described above, with bovine serum albumin as the standard. By comparison with the Kjeldahl results, a conversion factor was obtained. The trichloroacetic acid procedure was used for all subsequent protein determinations, and the conversion factor was applied to the results.

Molecular Weight Determinations. A molecular weight spectrum of cloud protein was obtained for samples A and B and for the soluble proteins in the juice serum of sample A. Cloud protein, solubilized in urea-citrate, was run on a 30 cm × 1.6 cm Sephacryl S-300 column by using urea-citrate for elution. The protein in the column fractions was precipitated with isopropyl alcohol, dried, and assayed, as described previously. Covalently linked bovine serum albumins of molecular weights 66000, 132000, 198000, and 264000, obtained from Sigma Chemical Co., St. Louis, MO, were used as molecular weight markers.

Amino Acid Analysis. Amino acid analysis on the cloud obtained from Sample A was performed by Woodson-Tennent Laboratories, Inc., Memphis, TN.

Isolation of Lemon Albedo Cells. A freshly cut piece of albedo tissue weighing 0.5 g was placed in a solution of 8 mg of Macerase (Calbiochem-Behring) in 4 mL of 0.7 M mannitol, buffered to pH 5.8 with 0.01 M ammonium citrate. After vacuum infiltration and standing at 25 °C for 1 h, the solution was removed and the albedo piece suspended in the same medium without Macerase. The cells were released by gentle vortexing and collected with a Pasteur pipette.

RESULTS AND DISCUSSION

Proteins are normally determined by the Kjeldahl procedure or by one of several colorimetric methods. The latter require the use of a pure protein, usually bovine serum albumin (BSA) as a standard, and their accuracy depends upon the similarity of the colorimetric response of the unknown and the standard. It seems unlikely that the mammalian protein BSA would be a good standard for the cloud proteins, so on these grounds the Kjeldahl procedure would be preferable. However, the Kjeldahl procedure is much more tedious for routine analysis but, more important, we have used urea, a compound rich in nitrogen, in high concentrations in much of our work. Traces of urea would introduce large errors in the Kjeldahl protein assay. Therefore, we have used the Kjeldahl procedure to analyze the cloud protein from a commercial concentrate and also analyzed the same sample by a colorimetric method. A conversion factor was derived by comparing the two results. All subsequent assays by the colorimetric method could then be corrected by using the conversion factor. In effect, the cloud proteins themselves serve as the standard for the colorimetric assay. The conversion factor, determined on sample A, was Kjeldahl protein (N × 6.25)/trichloroacetic acid precipitated protein = 1.72.

Table I. Total Cloud and Protein Weights[a]

sample	A	B
total cloud weight	245 ± 12	231 ± 8
total cloud protein	72.9 ± 2.6	69.1 ± 1.7
total cloud protein soluble in 10 M urea-6% citric acid, pH 2.5	75.7 ± 0.9	70.1 ± 2.1
soluble protein in 10 M urea, 6% citric acid, pH 2.5 that reprecipitates when dialyzed vs. 6% citric acid, pH 2.5	19.1 ± 1.7	15.2 ± 2.4

[a] Values given in milligrams per 100 mL of single strength juice ± SEM ($P < 0.05$).

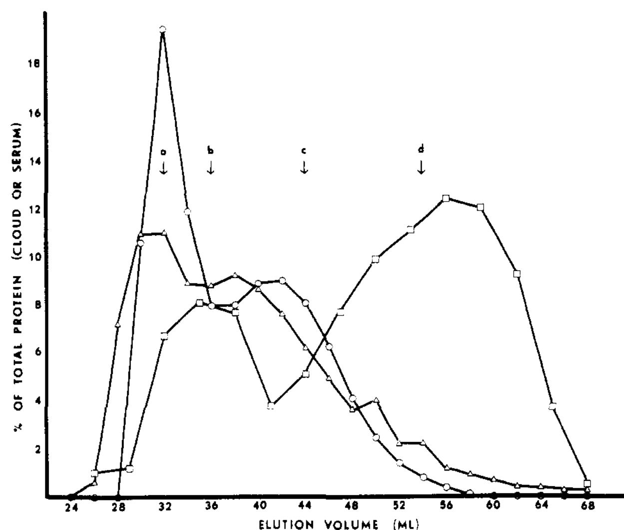


Figure 1. Fractionation of cloud and juice serum proteins obtained from commercial lemon juice concentrates on Sephacryl S-300: elution pattern of cloud proteins from sample A (○); elution pattern of juice serum proteins from sample A (□); elution pattern of cloud proteins from sample B (△). Covalently linked bovine serum albumin molecular weight standards are denoted by arrows: (a) 264000; (b) 198000; (c) 132000; (d) 66000.

The amount of cloud present in two commercial lemon juice concentrates, after adjustment to equal Brix values, was quite similar (Table I). Likewise, the protein concentrations in the two cloud samples were very similar, in both cases representing 29.8% of the total cloud (Table I).

In order to study the properties of the cloud proteins, it was first necessary to find a method for solubilizing them without producing chemical changes. No significant solubilization occured when the pH was raised from 2.5 to 7. At higher pH some of the protein dissolved, but this was probably due to base-catalyzed hydrolysis. The chaotropic reagent guanidine hydrochloride solubilized a large amount of the cloud protein, but even more effective was a combination of 10 M urea and 6% citric acid at pH 2.5, which dissolved all of the cloud protein (Table I). Urea alone could produce the same effect, but a much longer time was required, so we have routinely used urea-citrate for convenience.

To determine the molecular weight distribution of the cloud proteins, the solubilized material was chromatographed on a Sephacryl S-300 column which had been calibrated with standard proteins. The cloud proteins span a wide molecular weight range, from about 30000 to 300000 daltons. The soluble proteins in the juice serum span this same range but are heavily skewed toward the low molecular weight end (Figure 1).

Amino acid analysis was performed on sample A in the hope of being able to help explain the behavior of the cloud protein, such as a preponderance of a particular amino

Table II. Amino Acid Composition of Cloud (Sample A)

amino acid	% of cloud protein
tryptophan	13.2
methionine sulfoxide	0.1
aspartic acid	9.4
threonine	4.7
serine	4.8
hydroxyproline	0.5
glutamic acid	9.4
proline	3.9
glycine	3.9
alanine	5.1
cystine	2.3
valine	4.8
methionine	1.8
isoleucine	3.7
leucine	8.0
tyrosine	2.9
phenylalanine	6.2
hydroxylysine	0.1
histidine	3.2
lysine, total	6.4
arginine	5.2

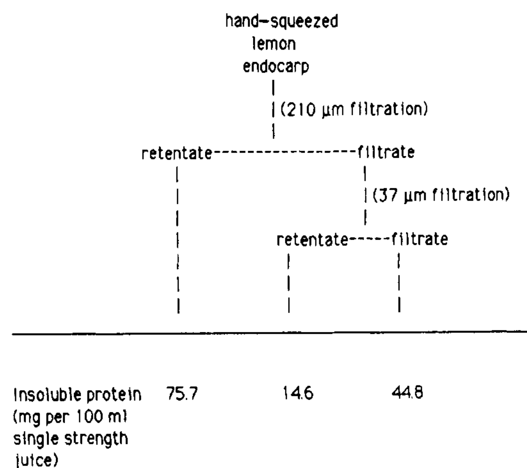
acid. No such anomalies were found however. This is not an unexpected result, as the total cloud protein represents a highly heterogeneous mixture. The amino acid composition of sample A is shown in Table II.

The proteins in lemon juice cloud are in an insoluble form. There are at least three plausible explanations for their insolubility: (1) Originally soluble protein is denatured and rendered insoluble during juice processing. (2) The protein is inherently insoluble in the juice serum. (3) Originally soluble protein forms an insoluble complex with another fruit constituent as a result of processing.

The possibility of denaturation was investigated by two approaches: studies of model proteins and simulating the juice concentration process on fresh, single strength juice. When BSA or o.p.p. were each dissolved in water and heated, all of the protein was denatured and precipitated. However, when the same proteins were each dissolved in 6% citric acid at pH 2.5 and subsequently heated, neither protein formed a precipitate. Although denaturation undoubtedly occurs under these conditions, the denatured protein remains soluble in the medium. In a confirming experiment, single strength, fresh frozen lemon juice (sample C) was heated and compared to an unheated control. Cloud isolated from both juices (heated and control) contained the same amount of protein. Juice serum isolated from sample C prior to heating and then subsequently heated also failed to show the formation of insoluble, heat-denatured protein. We concluded that juice processing does not contribute heat-denatured protein to the cloud.

Most of the lemon juice cloud particles are very small (2 μm or less). If it is assumed that at least some of the cloud protein is in an insoluble form in the intact fruit, the question arises as to whether this protein is present in existing particles of this small size in the fruit or is associated with larger material which is broken up into small fragments during processing. Juice withdrawn from intact juice sacs with a syringe was found to contain 46 mg of insoluble protein per 100 mL (Brix = 8.2°), which represents more than half of the protein content of cloud in commercial juices (Table I). Microscopic examination showed that most of these particles were in the range of 1–2 μm, suggesting that mitochondria (Bonner and Yarner, 1965) and/or other organelles could be involved. Little or no shear force was involved in extracting this juice, so these particles must have existed as such in the cells of the juice sacs. A similar result was obtained when fruit en-

Scheme I. Protein Concentrations from Hand-Squeezed Lemon Endocarp



docarp was hand-squeezed and filtered (Scheme I). The particles in the cells could be present as organelles, which are found in the cytoplasm, or particles suspended in the liquid in the vacuoles. When intact juice sacs were examined microscopically by using phase contrast optics, the cells inside the sacs could be seen. Particles could be observed in the cytoplasm of the cells but not in the vacuoles. Therefore, much of the cloud protein appears to be derived from organelles in the juice sac cells.

During commercial juice extraction much of the albedo of the fruit is disrupted, and it can be expected that some of the insoluble protein in the albedo will become part of the cloud. It was not possible to withdraw liquid from the intact albedo tissue with a syringe, but living cells were isolated by treatment of albedo tissue with a macerating enzyme. Microscopic examination of these cells showed small particles in the cytoplasm and none in the vacuoles, as was observed for fruit sac cells. It seems likely that these particles, like those in the juice sac cells, contain protein and contribute to the protein content of the cloud.

The total insoluble protein in the fruit endocarp, derived by adding the three values in Scheme I, represents almost twice the cloud protein of commercial lemon juice. The total insoluble protein in the albedo was found to be 127 mg/100 mL of single strength juice, also about twice the cloud protein. Thus, there is more than enough insoluble protein in either part of the fruit to supply all of the cloud protein. The 46 mg of insoluble protein per 100 mL derived by syringe extraction of juice sacs would represent a minimum value for the contribution of this part of the fruit to the cloud. Given the shear forces involved in commercial processing, perhaps most of the cloud protein is derived from the fruit endocarp. Much of this protein appears to be associated with organelles, and the rest is probably in the form of tissue fragments.

The cloud protein was completely solubilized in urea–citrate (Table I). When the solubilized cloud was dialyzed to remove the urea, approximately 25% of the cloud protein reprecipitated (Table I). This value represents a maximum percentage of the cloud protein that could be present as a complex with a high molecular weight substance, such as a polysaccharide, which would be expected to reform after removal of the urea (unless a low molecular weight substance, complexed with protein, was lost during dialysis). The failure of approximately 75% of the cloud protein to reprecipitate after dialysis may be due to an irreversible conformational change of the protein.

The pH of single strength lemon juice is approximately 2.5. This low pH is due mainly to citric acid, most of which

is stored in vacuoles of juice vesicle cells. The proteins, however, are synthesized at the site of ribosomes, which are located in the cell cytoplasm at a pH close to 5.5. The presence of many neutral amino acids (Table II) would tend to keep many of these proteins insoluble at this pH, due to isoelectric effects. When the cloud proteins are introduced to the pH 2.5 environment, after rupture of the citric acid vacuoles during processing, the proteins could remain insoluble due to a free energy barrier. The chaotropic effect of the urea, however, could allow the proteins to overcome this barrier and to become soluble. Considering the heterogeneity of the cloud proteins, when the urea is removed via dialysis it is unlikely that all of these proteins would revert to their insoluble state, due to many irreversible conformational changes that would be expected to occur.

We conclude that lemon cloud protein is a complex, heterogeneous material comprising approximately one third of the total cloud weight. It is probably derived primarily from the endocarp of the fruit, but it may also contain some material from the albedo. It appears to be composed, in part, of inherently insoluble protein which probably is derived from cellular organelles and lipid membranes. Some protein complexes with another fruit constituent could also play a role.

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Registry No. Pectin esterase, 9025-98-3.

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Bioavailability in Rats of Bound ^{14}C Residues from Corn Plants Treated with [^{14}C]Atrazine

Shahamat U. Khan,* Sam Kacew, and Stephen J. Molnar

Corn plants grown to the silage stage were treated with ^{14}C ring-labeled and unlabeled atrazine. The aerial portion of the plants was exhaustively extracted with solvents and the extracted material containing bound ^{14}C residues was fed to rats. For comparison extracted material from control plants fortified with [^{14}C]atrazine was also fed to rats. After 4 days, 88% of the dose was excreted in the feces and 10% in the urine from rats fed plant material containing bound ^{14}C residues. In contrast, only 32% of the dose was eliminated in the feces while 60% was voided via the urine when the corn material fortified with [^{14}C]atrazine was fed to rats. Most of the ^{14}C residues in feces from rats fed bound diet remained nonextractable and their amounts and nature were similar to those in the corn material. Atrazine added to the corn material before feeding was metabolized effectively when consumed by rats. The data demonstrated that bound residues in corn plant treated with atrazine have a low degree of bioavailability in rats.

Pesticide residues in plants may be present in three possible forms: (i) freely extractable residues; (ii) extractable conjugates bound to natural components of plants; (iii) nonextractable or "bound" residues incorporated into the plant constituents. Many studies using radioisotopes as tracers within pesticide molecules have revealed that a considerable portion of pesticide residues, as much as 19–75% of the total ^{14}C in various crops, may become bound in plants (Khan, 1982; Huber and Otto, 1983). Bound residues in plants are difficult to identify and are not generally detected in routine residue analysis. Thus, for a long time the possible plant burden of total pesticide residues may have been underestimated. These bound and usually chemically unidentified residues may however be important. For example, they might be released on digestion of the contaminated food, become available for absorption in body tissues, and cause toxicity.

In earlier studies it has been demonstrated that bound pesticide residues in plants have low bioavailability to animals (Paulson et al., 1975; Sutherland, 1976; Dorough, 1976; Marshall and Dorough, 1977). These observations are based on the assumption that urinary and/or biliary excretion of the radioactivity from a radiolabeled pesticide or its metabolites indicates that the material is bioavailable. However, if the radioactivity is excreted quantitatively in the feces, it is not considered bioavailable. Paulson et al. (1975) fed bound residues in alfalfa plants treated with [^{14}C]phenyl propham to rats and found 93–95% radioactivity was excreted in the feces with only

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