tissues as in separable lean of various cuts.

Lamb adipose tissue from leg and rib-loin cuts contained more iron, boron, and phosphorus than did similar tissue from pork. Calcium was higher and aluminum lower in fatty tissue of rib-loin than of leg of lamb. In pork, sodium was higher in adipose tissue of leg than in that of rib-loin.

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FOOD STABILIZERS AND PROTEIN DIGESTION

The Effect of Carrageenin on the Peptic Hydrolysis of Various Proteins

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Pepsin inhibition by carrageenin was studied to determine whether the addition of the polysaccharide to foods interferes with normal protein digestion. Different levels of carrageenin were added to solutions containing various concentrations of egg albumin, hemoglobin, casein, or soybean protein dissolved in HCl–citrate buffer, pH 1.6. Carrageenin at 0.085% level or lower did not affect pepsin activity in any of the solutions containing 1.0% or more protein, but there was marked inhibition of pepsin activity with higher concentrations of carrageenin or lower concentrations of protein. The protein level at which inhibition by a given concentration of carrageenin occurred, and the effect of pH on the degree of inhibition, varied from one protein substrate to another. Experiments in vivo further indicated that the levels of carrageenin used in foods do not interfere with normal protein digestion.

THE PROTEOLYTIC ACTION of pepsin may be inhibited by certain sulfated polysaccharides. In 1954, Levey and Sheinfeld (*1*) reported that heparin and chondroitin sulfate inhibit the pepsin-catalyzed hydrolysis of proteins, and more recently Houck *et al.* (*3*) reported that carrageenin, a sulfated polysaccharide from seaweed, is almost equally effective as an inhibitor of pepsin action. Data were obtained from studies both in vitro and in vivo suggesting that carrageenin affects pepsin activity. These authors reported that carrageenin ingested in drinking water (5 mg. per ml.) will inhibit ulcerogenesis in rats with pylorus ligation or subcutaneous injections of cortisone and in dogs subjected to large doses of histamine.

Since carrageenin is used as a stabilizer in many food products, the conditions under which pepsin inhibition by carrageenin occurs have been studied to determine whether addition of the polysaccharide to foods interferes with normal protein digestion. The results of these studies are presented.

Reagents and Methods

Casein, Hammerstein quality; soybean protein, 88% protein; and pepsin (3× Crystalline) were obtained from Nutritional Biochemical Corp., and commercial grade carrageenin was obtained from Marine Colloids, Inc. Egg albumin was prepared from fresh hen eggs, and hemoglobin from fresh beef blood according to the procedure of Anson and Mirsky (*1*).

For tests in vitro, different levels of carrageenin were added to solutions containing various concentrations of egg albumin, hemoglobin, casein, or soybean protein dissolved in hydrochloric acid–citrate buffer, pH 1.6. The quantity of pepsin which was then added depended on the substrate and the assay used for following protein hydrolysis. Three different assays were employed, since no single procedure was suitable for measuring protein hydrolysis when different proteins are used as substrates. The Riggs and Stadie assay (*6*) was used with egg albumin, the Anson and Mirsky assay (*1*) with hemoglobin, and the Volhard and Lohlein assay (*2*) with casein or soybean protein as the substrate.

For studies in vivo, male rats weighing approximately 150 grams were fasted 18 hours, and then given, by stomach tube, 2 ml. of a solution containing milk proteins with or without carrageenin. The dosing solutions were preparations of cow's milk modified to resemble human milk (carrageenin is used in most prepared infant formulas as a stabilizer). Exactly 1 hour after receiving the dosing solutions, the rats were killed with chloroform, the abdomen opened immediately, and the gastrointestinal tract ligated in two places—the esophagus directly above the stomach and the intestine 20 inches below the pylorus. The ligated segment was then removed, and the contents of the stomach and intestine were washed into a beaker with water. The

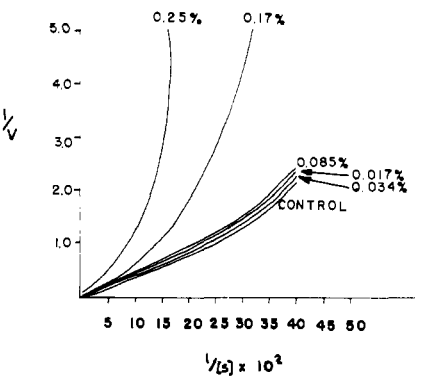

Figure 1. Effect of carrageenin on hydrolysis of egg albumin by pepsin

Egg albumin concentration [*s*], is expressed as mg. per ml. of incubation mixture, and velocity, *v*, is expressed in arbitrary units

Table I. Effect of Carrageenin on Digestion of Milk Proteins in vivo[a]

Rat Weight,[b] grams	Carrageenin Concn., %	Protein Digested,[c] mg.
144 ± 17	0.000	44.3 ± 8.6
145 ± 24	0.017	50.4 ± 5.4
151 ± 36	0.175	41.9 ± 5.4
147 ± 8	0.525	36.6 ± 5.8

[a] 2 ml. of a cow's milk formula modified to resemble human milk was given each rat by stomach tube. Formulas contained 1.72% protein. Carrageenin was added to formulas at levels shown in table.

[b] Av. weight (six rats per treatment) ± std. dev.

[c] Av. mg. of protein ± std. dev. digested during a 1-hour period.

mixture was brought to boiling temperature very rapidly. The protein in the boiled solution was precipitated with 4% trichloroacetic acid and the nitrogen content of the precipitate determined by the semimicro-Kjeldahl method. The difference between the protein—grams of nitrogen multiplied by 6.38—in the test solutions before ingestion, and the amount found in the stomach and upper intestine was considered to be the amount of protein digested in one hour.

Results

Egg albumin was incubated with pepsin (100 μg. per ml.) for four minutes at pH 1.6 and 30° C. Preliminary experiments showed that the rate of hydrolysis of egg albumin was proportional to the pepsin concentration in the range 0 to 100 μg. per ml. and that the reaction rate remained constant for at least 10 minutes. The effect of various concentrations of carrageenin on pepsin activity is shown in Figure 1 as a Lineweaver-Burk plot. Carrageenin at 0.25% or lower did not affect pepsin activity in any of the solutions containing 2.5% ($1/[s] \times 10^2 = 5$) or more of egg albumin, but there was inhibition in all solutions containing less than 0.5% protein and 0.17% or more carrageenin. When the level of carrageenin was below 0.085%, there was no inhibition of pepsin activity even at very low concentrations of protein (0.25%). The data obtained with hemoglobin as substrate were similar to the data with egg albumin shown in Figure 1.

When soybean protein or casein was the substrate, a concentration of 0.5 mg. of pepsin per ml. of solution was incubated with the substrate for 15 minutes at pH 1.6 and 30° C. The effect of carrageenin on pepsin activity with casein as substrate is shown in Figure 2. There was little inhibition by carrageenin at any concentration studied when 1.0% or more protein was present in the incubation mixture.

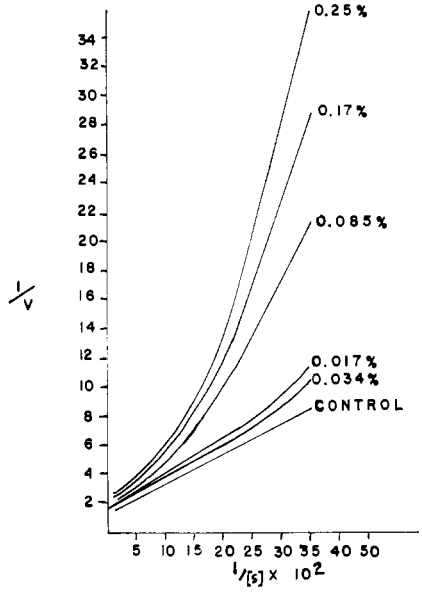

Figure 2. Effect of carrageenin on hydrolysis of casein by pepsin

Casein concentration [s], is expressed as mg. per ml. of incubation mixture, and velocity, v, is expressed in arbitrary units

Likewise, there was little or no inhibition by 0.034% or less of carrageenin at any concentration of protein studied (0.3% or more). The results obtained with soybean protein were almost identical to the results with casein shown in Figure 2.

The effect of carrageenin on the pepsin-catalyzed hydrolysis of casein was further studied by measuring pepsin activity in the presence or absence of carrageenin at pH values ranging from 1 through 7. Initially, the incubation solutions contained 1.329 mg. of casein, 0.5 mg. of pepsin, and 0.0 or 0.85 mg. of carrageenin per ml.; pepsin activity is inhibited at these concentrations of casein and at 0.85 mg. of carrageenin per ml. The solutions were incubated for 15 minutes at 40° C., and the desired pH was maintained in a given incubation mixture by continuous titration with dilute hydrochloric acid or sodium hydroxide solution. Pepsin activity measurements were not made in the pH range 4.1 to 5.9, since casein is only very slightly soluble in water at these concentrations of hydrogen ions.

Pepsin activity (mg. of amino nitrogen × 10) in the presence or absence of carrageenin with casein as substrate is shown in Figure 3 as a function of pH. Pepsin activity reached a maximum around pH 2.5 and rapidly decreased as the solutions were made more alkaline. The high (0.085%) concentration of carrageenin inhibited pepsin activity at all pH values from 1 through 7, but the degree of inhibition was less at values above 2.

The effect of pH on pepsin activity in the presence or absence of carrageenin was also studied using hemoglobin as a

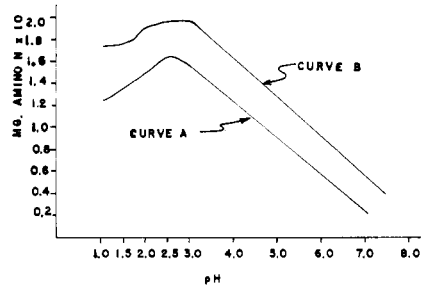

Figure 3. Effect of pH on hydrolysis of casein by pepsin in the presence (curve A) or absence (Curve B) or carrageenin

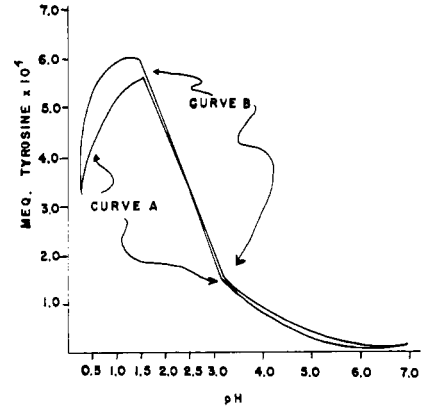

Figure 4. Effect of pH on hydrolysis of hemoglobin by pepsin in the presence (Curve A) or absence (Curve B) of carrageenin

substrate. In these experiments, the incubation solutions contained 2.24 mg. of hemoglobin, 0.01 mg. of pepsin, and 0.0 or 0.85 mg. of carrageenin per ml. The solutions were incubated for 15 minutes at 25° C. The results, expressed graphically in Figure 4, indicate that carrageenin does not inhibit the peptic hydrolysis of hemoglobin at pH values greater than 2.

The data in Table I indicate that protein digestion proceeded normally in rats fed 2 ml. of test formulas simulating human milk which contained 1.72% protein and from 0 to 0.175% carrageenin. None of these differences were statistically significant. The 36.6 mg. of protein digested by rats receiving a formula with 0.525% carrageenin was, however, significantly ($P = 0.05$) lower than the other groups. Formulas containing a carrageenin level greater than 0.525% were not tested, since such solutions are extremely viscous and will not flow through a stomach tube under the conditions of the procedures used with solutions containing less carrageenin.

Conclusions

It is concluded from the experiments in vitro that carrageenin is a competitive inhibitor of pepsin activity—the in-

hibiting ability depends on the concentration of protein substrate relative to the concentration of carrageenin—and that the degree and character of this inhibition depends on the particular substrate used in the assay for enzymatic activity. The data support the conclusion of Piper and Fenton (*5*) that carrageenin does not react with the active center of the enzyme but inhibits by combining with the substrate. The carrageenin in food products should not interfere with normal peptic digestion of protein, since the concentration of protein is higher and the concentration of carrageenin much lower in such products than in the experimental mixtures where inhibition was demonstrated. Although the exact concentration of protein necessary to prevent inhibition of pepsin by a given concentration of carrageenin depends on the kind of proteins in the mixture, a low concentration (0.25%) of any dietary protein would probably be sufficient at the carrageenin levels used in food products. The conclusions drawn from the experiments in vitro are supported by the data from animal feeding experiments summarized in Table I.

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PLANT ANALYSIS

Supplementary Chromatographic Method for Determining Saponins in Alfalfa

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A supplementary procedure is described for improving accuracy in the gravimetric determination of saponins through application of quantitative paper chromatography.

An earlier gravimetric method for approximate determination of saponins in alfalfa involved adsorption of saponins on carbon and their subsequent recovery by elution (*2*). Complete recovery of saponins was demonstrated; however, results were known to be somewhat higher than the true values. Despite this shortcoming, results obtained by the method could be used in studies of the significance of alfalfa saponins in chick diets. However, the desirability of a more accurate total saponin assay was recognized, and a supplementary chromatographic procedure has been developed for application to the analytical products eluted from carbon. By this supplementary treatment, inaccuracies due to incomplete separation of saponins and nonsaponins can be considerably reduced. Some of the techniques applied in these instances to saponins might be useful in estimating some other minor constituents of plants which present similar obstacles to analysis.

For brevity, the impure saponins eluted from carbon with ethyl alcohol and pyridine in the preceding procedure (*2*) are referred to here as CAP saponins.

Apparatus

Two chromatographic accessories were used, a streaking pipet (Figure 1), and a sample applicator. The sample applicator supports, illuminates, and dries chromatographic paper during sample application. Its novel feature is a traveling clamp that carries the pipet during streaking and automatically lowers and raises it at the ends of sample streaks. Both accessories were manufactured by Research Specialties Co., 200 Garrard Blvd., Richmond, Calif.

Procedure

CAP saponins, while still in the dish in which they had been weighed, were dissolved in 1 to 1.5 ml. of 60% ethyl alcohol. With the sample applicator and streaking pipet, this solution was applied to a sheet of Whatman 3MM paper in a streak parallel to and 2.5 cm. from an edge of the paper. Streak length was limited either by the weight of CAP saponins available or by paper and equipment dimensions. Maximum streak length was about 40 cm. The most suitable density of loading was about 1 mg. of CAP saponins per cm. of streak. To achieve satisfactory loading, it was necessary to draw the pipet along the streak repeatedly with intervals for drying between applications. Uniform loading throughout the streak and absence of sample spreading at the ends of the streak were necessary conditions. Saponin solutions have a strong tendency to form drops at the ends of pipet tips. To prevent this, with consequent spreading at the ends of the streaks, it was necessary to shrink the bore of the pipet tip to not more than 0.13 mm. and to limit its length to not more than 8 mm. The ends of the tips were squared and chamfered around the edges by grinding. Fire polishing instead of grinding was unsatisfactory.

When streaking was completed, the pipet was rinsed with 60% ethyl alcohol. Rinsings were delivered into the dish that initially contained the CAP saponins, the liquid was evaporated, and the dish and residue were vacuum dried at 65° C. and weighed to determine by difference the weight of CAP saponins applied to the paper.

After the length of the streak had been measured and recorded, the width of the paper was accurately trimmed to the ends of the streak, and an ascending chromatogram was developed on the paper. The developing solution used was the upper phase of a *n*-butyl alcohol–1*M* ammonium hydroxide–95% ethyl alcohol (60:30.5:13) solvent mixture. Development was stopped when the liquid front had advanced 15 cm. above the level of the sample streak. Development time at 30° C. was about 3 hours.

Saponins were located on the dried chromatogram by staining a 1-cm. wide test strip that was cut from the top to the bottom of the chromatogram at its middle. The strip was stained by drawing it through a mixture of sulfuric acid and acetic anhydride (*1*). Positions of saponins from alfalfa leaves and stems became visible on the test strip by the appearance of a blue band surmounted by a red- to plum-colored band in the region from about R_f 0.15 to about 0.55. The upper and lower limits of the