enzyme or browning reactions to occur at appreciable rates, while the loosely bound water provides a matrix (or solvent) in which diffusion is rapid enough for these reactions to occur. The increase in browning and enzyme reaction rates with increasing water activity is similar to the measured increase in water molecular mobility which occurs in the loosely bound water region (see Figure 5). The water activity below which most bacteria will not grow (~0.9) corresponds to the transition between nonfreezeable (loosely bound) water and free water. It is tempting to speculate from this result that free water may be necessary to support bacterial growth. Because the water requirements of bacteria differ to some extent, it is clear that growth/no growth is dependent upon more than simply the presence of free water. In any case, it is clear that water activity, particularly in reduced moisture content foods, will be a function not only of the solute content, but also the nature of the food–water binding.

These correlations between the measured hydration state of the soy protein and rates of food degradation processes are speculative, since we do not have data on the latter for this material. The hydration model presented here does provide a sound basis for further experiments to investigate the reasons for the water activity/content dependence of degradation processes such as microbial growth, lipid oxidation, enzyme activity, and nonenzymatic browning, and to use hydration state as a means of food stability control.

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A Simple Method for Determination of Tryptophan in Food Samples

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A very simple and sensitive method for determination of tryptophan in food samples is described. The food proteins are completely solubilized by partial hydrolysis with papain in the presence of 8 M urea. An aliquot is diluted with 8 M urea and the fluorescence of tryptophan is directly measured at an emission wavelength of 348 nm, during excitation at 288 nm. Tryptophan is used as an internal standard. The results are compared with values obtained from a conventional method involving basic hydrolysis.

Tryptophan is one of the essential amino acids for human beings. Determination of the amount of tryptophan in foodstuffs is therefore of considerable importance.

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However, most of the methods used today are either laborious or not generally applicable. For a review on tryptophan analysis see Friedman and Finley (1975).

The native fluorescence of tryptophan is sufficiently intense and specific to permit its use for quantitative assay purposes (Udenfriend, 1962). The quenching effect of microenvironment on tryptophan fluorescence observed

Table I. Tryptophan Content of Some Food Samples;
Duplicate Analysis

| | | g of Trp/16 g of N | |
Sample	% N	Fluoro-metric method	Conventional method
Mixed school lunch			
B I	4.20	1.57	1.61
T II	4.53	1.14	1.20
T III	3.40	1.40	1.30
T IV	5.95	1.49	1.44
T V	4.96	1.32	1.26
Canned baby foods			
Steak casserole	10.2	1.33	1.33
Haddock	7.1	1.38	1.25
Ham	10.5	1.52	1.30

in proteins can be reduced by denaturating agents (Shelton and Rogers, 1971; Ostashewsky et al., 1973) or by partial hydrolysis. Thus, Sasaki et al. (1975) did accurate determinations of the tryptophan content of some purified proteins after digestion with chymotrypsin and Pronase, followed by dilution in 6 M urea. Such a method, however, requires that the proteins are in solution. It has been possible in this laboratory to solubilize completely proteins in mixed diet samples with papain in 8 M urea in a single incubation step (Nair et al., 1976). The purpose of the present study was to evaluate whether a direct fluorometric measurement on this material would yield the tryptophan content.

MATERIALS AND METHODS

Purified Proteins. Trypsin from beef pancreas (2× crystallized) was purchased from Mann Research Laboratories, New York, N.Y. Bovine serum albumin (crystallized) was from BDH Chemicals LTD., Poole, England, and human serum albumin (lyophilized) was from AB Kabi, Stockholm, Sweden. Ribonuclease A from bovine pancreas (5× crystallized), lysozyme from eggwhite (3× crystallized), pepsin from stomach mucosa (2× crystallized), and hemoglobin from beef blood (2× crystallized) were purchased from Sigma Chemical Co., St. Louis, Mo. Cytochrome c from horse heart was purchased from Mann Research Laboratories, New York, N.Y.

Food Samples. The food samples (specified in Table I) were homogenized and fat extracted with chloroform. The fat-free residue was lyophilized and ground to a fine powder (Borgström et al., 1975).

Enzymatic Hydrolysis of the Samples. Samples containing about 10 mg of protein were incubated, rotating end over end, over a period of 24 h at 37 °C with 1 mg of papain (2× crystallized suspension from Sigma Chemical Co., St. Louis, Mo.) in 5 ml of freshly prepared incubation mixture. The incubation mixture contained 8 M urea, 0.005 M thioglycollate, 0.002 M EDTA, and 0.1 M sodium borate and was adjusted to pH 7.6. After incubation the urea solutions were separated from the insoluble residues by centrifugation. By analyzing the residues for amino acids the solubilities of the proteins were calculated and were found to be more than 99% in all the samples.

Fluorometric Measurements. The apparatus used was an Aminco Bowman spectrophotofluorometer, equipped with an Aminco photomultiplier photometer. The excitation wavelength was 288 nm and the emission wavelength was 348 nm. An 8 M urea solution (2 ml) containing 0.1 M sodium borate and adjusted to pH 7.6 was transferred to a quartz cell. The blank fluorescence was adjusted to zero. An aliquot of 100 μl from the sample previously incubated as above was added and mixed thoroughly. The fluorescence was measured and 10 μl of

Table II. Tryptophan Content of Some Pure Proteins Determined by Spectrofluorophotometry; Duplicate Analysis

| | Trp content, mol/mol of protein | |
Sample	Fluoro-metric determinations	Lit. value
Bovine serum albumin	1.8	2 (King and Spencer, 1970)
Cytochrome c	1.0	1 (Margoliash and Schejter, 1966)
Hemoglobin	6.0	6 (Walsh and Neurath, 1964)
Human serum albumin	1.0	1 (Kirschenbaum, 1972)
Lysozyme	4.0	6 (Dayhoff and Eck, 1967-1968)
Pepsin	5.1	5 (Dopheide and Jones, 1968; Kostka et al., 1970)
Ribonuclease A	0.0	0 (Dayhoff and Eck, 1967-1968)
Trypsin	4.0	4 (Dayhoff, 1970)

a tryptophan standard solution containing 0.392 nmol of tryptophan per μl was added. After mixing, the fluorescence was measured again and the tryptophan content was calculated by extrapolating the fluorometer readings to zero fluorescence. The results were corrected for fluorescence originating from papain by analyzing a sample containing only the enzyme. The tryptophan standards were prepared daily by dilution of a stock solution. The stock solution was prepared every week and stored in a refrigerator. The tryptophan (chromatographically homogeneous) was purchased from BDH Chemicals Ltd., Poole, England.

Analysis of Tryptophan by a Conventional Method. Tryptophan contents of the samples were also determined after basic hydrolysis with barium hydroxide. The method used was as described by Slump and Schreuder (1969).

Reagents. The reagents not specified above but used in the experiments were all of analytical grade.

RESULTS AND DISCUSSION

Analysis of Pure Proteins. To study whether the partial hydrolysis with papain in 8 M urea is sufficient to eliminate the quenching effect on tryptophan fluorescence observed in intact proteins, some well-defined proteins were analyzed. The results of the analysis are shown in Table II. For most of the protein samples the tryptophan content determined shows good agreement with the theoretical values. Lysozyme shows, however, less agreement with a value about 70% of the theoretical one. The reason for this exception is not clear.

These results are quite comparable with those obtained by Sasaki et al. (1975) who analyzed over 15 proteins with very good yield in most cases. It is obvious that papain incubation reduces the quenching effect of the intact protein molecule on the tryptophan fluorescence in most proteins and enables a quantitative determination of its content.

Analysis of Food Samples. The complex mixture of various compounds present in food samples makes it necessary to examine whether, under the conditions employed, tryptophan is the only component with measurable fluorescence. Figure 1 shows the excitation and emission spectra of pepsin and a mixed food diet after hydrolysis with papain in 8 M urea. The curves obtained from the food sample are qualitatively identical with those obtained from the pure protein. This indicates that in the food sample there is no extraneous fluorescence or absorbance in the region of measurement.

The fluorescence of a fluorophor can vary markedly with pH and one can expect a different behavior for different
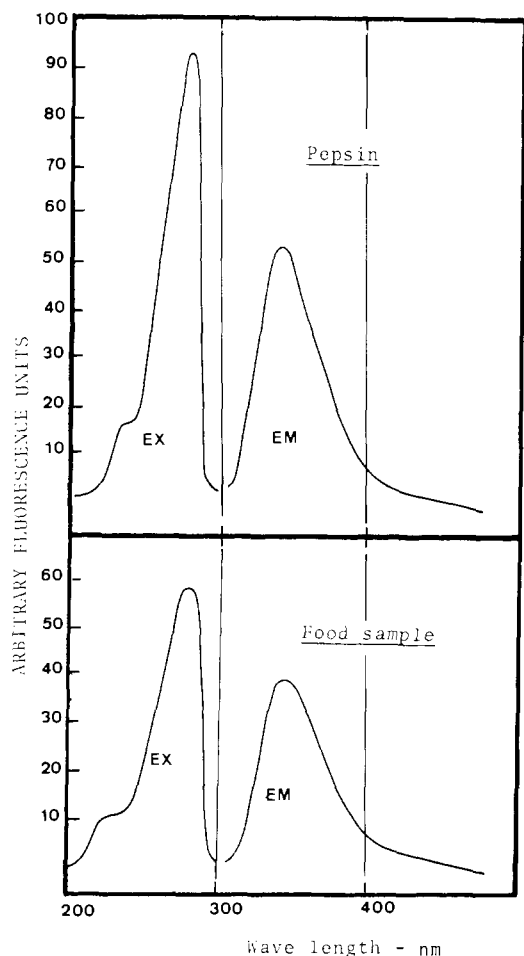
Figure 1. The fluorescence excitation spectra (EM 348 nm) and the fluorescence emission spectra (EX 288 nm) for a pure protein and a food sample after treatment with urea and papain for 24 h. The measurements were performed in 8 M urea containing 0.1 M sodium borate at pH 7.6. The spectra are only corrected for background fluorescence.
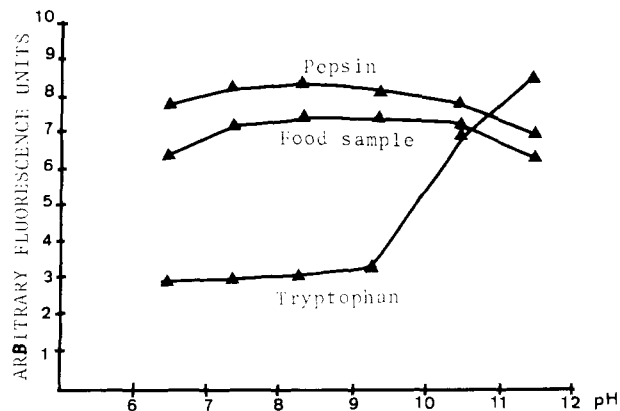

Figure 2. Effect of pH on the fluorescence of tryptophan, a pure protein (pepsin), and a food sample. The pure protein and the food sample were treated with papain in urea as described elsewhere. The concentration of tryptophan residues is not the same in the different samples.

substances. To evaluate the effect of pH on the fluorescence of the pepsin sample and the food sample after incubation the fluorescence was measured as described above but in urea solutions of different pH. The results as presented in graphs (Figure 2) show the same characteristic shape for the fluorescence of the food sample as for the fluorescence of the pure protein.

These findings suggest that the fluorescence detected under these conditions in the food sample is due to the tryptophan content of the food proteins. The fluorescence originating from peptide-bound tryptophan present in these samples is much less influenced by changing pH than the fluorescence of free tryptophan (Figure 2). It is necessary to perform measurements as described in this article in the pH region 7 to 9 to acquire correct values when free tryptophan is used as the internal standard.

A number of feedingstuffs were treated as described in the Materials and Methods section, and analyzed for tryptophan. The results obtained were compared to those from the conventional method of tryptophan analysis. As seen from Table I the values from the fluorometric analysis show general agreement with those from the conventional method of analysis. The latter method includes basic hydrolysis of the sample with barium hydroxide, gel filtration of the hydrolysate after precipitating the barium, and spectrophotometric measurement after ninhydrin reaction. The average recovery of added tryptophan by this method is estimated to be 70–97% in general and when the barium hydroxide was boiled to remove air, recovery was raised to 97–100% for duplicate analysis (Slump and Schreuder, 1969).

Accuracy and Precision of the Method. The results from the analysis of pure proteins by the present method (Table II) show that the tryptophan content can be determined with a fair degree of accuracy by virtue of its native fluorescence. To determine the precision of the method a number of analyses of a food sample were carried out. The standard deviation was 7% for the overall analysis and 3.5% for repeated fluorometric measurements on the same hydrolysate. The present method involves few steps and avoids difficulties normally met with the analysis of tryptophan in feedingstuffs due to its low concentration, losses during hydrolysis, and in some cases formation of colored by-products. Due to its speed (20 measurements/h) it should prove useful for screening large numbers of samples.

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Supplementation of Arabic and Indian Breads with Fish Protein Concentrate

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The utilization of different kinds of fish protein concentrates (FPC) in the supplementation of Arabic bread and Indian bread (puri) was studied. Taste panel tests showed that the supplemented breads were well accepted when 10% of bread flour was replaced by FPC. Such a supplementation with the best test FPC used elevated the protein efficiency ratio of the bread to equal that of casein. It was demonstrated that extremely high dry heat (650 °C) required for the Arabic bread or cooking in oil (190 °C) for puri did little to decrease the protein value of FPC when the heating time was about 1 min. Significant differences in nutritional value were observed between the different kinds of FPC used.

Bread, in all its various forms, is eaten in most countries and the quantitative consumption of bread is not easily surpassed by any other single food product. This is the reason why bread has been the main food item in several studies involving the improvement of the nutritional status of many people.

So far studies have been carried out where bread has been enriched with amino acids, especially lysine, oilseed protein concentrates, and fish protein concentrates. The so-called "modern bread" in India is an example of good commercial success of lysine-enriched products (Altschul, 1969). Both Stillings et al. (1971) and Hegsted (1968) gave similar reports that 0.4% lysine added to wheat flour results in the same nutritional protein value as wheat flour with 5% fish protein concentrate (FPC). Enrichment of 1 kg of wheat flour at these levels costs 0.8 cent using lysine and 2.5 cents using FPC (Beigler, 1969). However, Stillings et al. (1971) in a comparative study between FPC and lysine supplemented breads reported that maximum responses of weight gain, protein efficiency ratio (PER), and net protein utilization (NPU) obtained with FPC were greater than those obtained with lysine.

Among plant protein concentrates soy protein has been the most widely used, giving the best results. However, in comparative studies, the biological value of soy protein concentrates has been found to be inferior to FPC (Pond et al., 1971).

Several baking studies with FPC have been reported in the literature. Generally, flour samples with FPC levels of 3–9% have given acceptable results depending on the experimental product and on the quality of FPC used. In the U.S. the Bureau of Commercial Fisheries (now the National Marine Fisheries Service) had initiated several food experiments with FPC as reported by Sidwell et al. (1970) and Kwee et al. (1969). In bread the color was found to be affected by the kind of fish used for FPC. Incorporation of FPC into bread also decreased the loaf volume.

The main objective in the present study was to incorporate FPC in breads widely eaten in some malnourished areas. The Arab countries, with a population close to 250 million, derive 60–70% of the daily protein intake from grains (Abbott, 1969). One of the most important foods is the Arabic bread, commonly used in all Arab lands. The average daily bread consumption is 5 to 12 loaves, each weighing approximately 100 g. This bread has some very interesting characteristics. It is made of yeast leavened dough and baked in an extremely hot oven, 500–600 °C, for approximately 1 min. During baking the bread puffs up leaving a hollow pocket inside. During cooling the bread flattens out, but the bottom and top crust remain separated from each other. In this study FPC was incorporated in the Arabic bread in order to study the effect of this protein supplement on the acceptability and nutritional quality of the bread. Dalby (1969) has reported on studies where different vegetable protein sources, such as chick peas, horse beans, and cottonseed flour, were incorporated in Arabic bread. Only the acceptability of the bread was tested. Hallab and Khatchadourian (1974) enhanced the nutritive value of Arabic bread by addition of chickpea flour and soybean flour.

In India, nearly 60% of the daily protein intake is derived from cereals, and the second largest protein source consists of legumes and nuts (West, 1969). The dietary food staples vary widely among the Indian people. Chapati, a type of unleavened bread, is mostly used in India; however, another bread called puri, generally prepared from whole wheat flour and cooked in oil, is also common in many parts of India. Puri was chosen in order to study the effect of cooking in oil on the nutritional value of FPC supplemented bread.

Since the quality of FPC varies depending on the raw materials and processing methods (Makdani et al., 1971), three different FPC's were selected in the present study.

MATERIALS AND METHODS

Materials. Three different FPC preparations were used. FPC I was purchased from the Alpine Marine Protein Industries, Inc., New Bedford, Mass., and was prepared from red hake by extraction with 1,2-dichloro-

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