Acylation of Fish Protein: Effect of Reaction Conditions on Products

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Introduction

Functionality is the attribute of a food material that gives the desired properties to the food system in which it has been incorporated. For example, it is the emulsion-stabilizing functionality of egg yolk that makes possible the fat-in-water emulsion that is the basis of mayonnaise. It has been shown that the functional properties of fish muscle proteins can be improved by chemically modifying these proteins (Groninger and Miller, 1975; Miller and Groninger, 1976). Modification through the acylation of fish myofibrillar protein has been described by Groninger (1973) and Chen et al. (1975). Acylation involves the chemical bonding of a small molecule, in most instances acetyl or succinyl, to the protein which results in an alteration of the electrical charge on the protein.

Recent studies of the chemical and nutritional properties of acylated fish protein (Groninger and Miller, 1979) included determination of their nutrititive value and biological utilization relative to acylation reaction conditions. Rat feeding tests showed clearly that acylation resulted in decreased nutritional value of the modified protein as measured by the protein efficiency ratio and the feed efficiency. A modified protein with improved functional properties and reasonably good nutritional value was produced, however, at low to moderate levels of acylation. Such a product would be desirable for food applications in which the modified protein additives might be a significant part of the protein intake. Therefore commercial applications of the process will require selection and control of reaction conditions to provide modified protein with the needed functional properties as well as reasonably good nutritive value. For this purpose, detailed knowledge of acylation reaction conditions and the effects on products is important.

Although the major target of the acylation reaction is the amino group of the amino acid lysine (Grant-Green and Friedberg, 1970), minor reactions occur to form secondary products with cysteine (Habeeb et al., 1958), tyrosine (Riordan and Vallee, 1963), and with serine and/or threonine (Gounaris and Perlmann, 1967). In most reported work on the acylation of proteins for food purposes, little attention has been given to the formation of these secondary products. Since these secondary products may be important from the point of view of nutrition, food safety, and the functionality of the modified protein, work on secondary products was initiated.

The objective of this work was to determine the relative reactivity of the various sites on the fish protein that might involve the acylation reagent; this includes the epsilon-amino group of lysine, the sulfhydryl group of cysteine, the phenolic group of tyrosine, and the hydroxyl groups of serine and threonine when fish myofibrillar protein is acylated under various conditions. The inhibitory effect of sulfite on the acylation reaction was also evaluated.

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Methods

The various methods used in this work are described as follows.

Preparation of Myofibrillar Proteins

The procedure for the preparation of myofibrillar proteins was based on the method described by Groninger (1973). Fresh or defrosted rockfish (*Sebastes* spp.) fillets were comminuted by passing through a food grinder with a 0.2 cm orifice plate. The comminuted fish muscle was suspended three times in a large excess volume of cold 0.1 *M* NaCl to remove a large proportion of water-soluble proteins, minerals, and other extractives. The supernatant was removed by centrifugation for 10 minutes at 8,000 *g*, 0°C. To remove additional soluble protein, the muscle protein was blended for 20 seconds in a high-speed blender with about 3 volumes of 0.1 *M* NaCl at 0°C and centrifuged for 10 minutes at 8,000 *g*, 0°C. Blending and subsequent centrifugation was also repeated three times. The myofibrillar proteins were solubilized by blending for 30 seconds in 0.6 *M* NaCl at 0°C. Connective tissue and nonsolubilized myofibrillar protein were removed by centrifugation for 15 minutes at 13,000 *g*, 0°C. The typical protein preparation contained 30 to 32 mg protein/g.

Acylation of Fish Protein

Salt-solubilized myofibrillar protein, at a concentration of approximately 30 mg/g, was acetylated by reacting with acetic anhydride under a constant agitation in an ice bath at different pH's and with or without the addition of sodium sulfite. Acetic anhydride was added dropwise by a microburette in a constant rate of addition (0.5 g/minute). Simultaneous-

ly, 2 *N* NaOH was added to provide a pH stat-like condition during the reaction period, which normally took 40-60 minutes in a 600 ml of total reaction volume.

Succinylation was carried out using a procedure similar to the acetylation procedure except that incremental amounts of solid succinic anhydride were added.

Protein Content

Protein content was determined by the method of Lowry et al. (1951).

Extent of Acylation

The extent of acylation of protein was measured by the method of Kakade and Liener (1969) and calculated as percent acylation based on the amounts of free amino groups in the acylated and the native samples.

Acylhydroxamates

To estimate the amount of acylated secondary side groups in protein, acylhydroxamates were determined by Hestrin's method (1949). Acylhydroxamates are formed by deacetylation of acylated residues with alkaline hydroxylamine and subsequently the ferric acylhydroxamate complex is formed which has an absorption maximum of 540 mm.

In the preliminary work with acetylated compounds, it was found that deacetylation with hydroxylamine occurred selectively on protein residues under conditions of controlled pH. As shown in Table 1, all of the acetyl compounds were readily deacetylated at pH 12.5 while S-mixture and O-acetyltyrosine were reacted at pH 7.5, although the recovery was slightly reduced. O-acetyltyrosyl derivatives were readily deacetylated when acetylated proteins were tested as described by Riordan and Vallee (1972). Furthermore, an estimation of cysteine could be made in the presence of O-acetyltyrosine when deacylation was performed at pH 6.5 to 6.0. However, this was not satisfactory for a quantitative test because of variability.

Therefore, the deacylation values measured at pH 12.5 are the total amount of acyl groups on secondary residues including tyrosyl, sulfhydryl, and hydroxyl; and the values measured at pH 7.5 are the sum of tyrosyl and sulfhydryl residues. The deacylation values measured at pH 7.5 were very similar to those values of O-acyl tyrosine and S-acyl cysteine, which were measured individually by the methods of Riordan et al. (1965) and Habeeb (1972). The amounts of acylated O-hydroxyl residues as the total of O-serine and O-threonine were simply computed by subtracting the amount of O-acyl tyrosine plus S-acyl cysteine from the total deacylation value measured at pH 12.5.

Percent acylation given in the data was calculated as the percent ratio of each secondary residue in mg/100 g protein based on the contents of corresponding amino acids from the data of amino acid composition of rockfish protein by Groninger and Miller (1979).

The hydroxylamine deacylation reagent consisted of 10 ml of 2 *M* NH_2OH and 10 ml of 3.5 *N* NaOH for the test at pH 12.5; a mixture of 10 ml 2 *M* NH_2OH and an appropriate amount of 3.5 *N* NaOH for the test at pH 7.5. In general procedure for deacylation, 2 ml of 1 *M* hydroxylamine reagent was added to 1.0 ml aliquots of protein solution and reacted for 10 minutes at room temperature. The reaction was stopped by addition of 1.0 ml of concentrated HCl diluted at 1:2 (v/v) and subsequently 1.0 ml of 1 percent ferric chloride in 0.1 *N* HCl was added. The protein precipitate formed was removed by filtration, and the absorbance of the supernatant was measured by 540 nm 20 minutes after the addition of ferric chloride. Quantitation in all cases was based on standard curves using acetylcholine and monomethylsuccinate.

Table 1.—Deacetylation recovery of O- and S-acetyl compounds with hydroxylamine at pH 12.5 and 7.5.

Test mixture	Concentration (m*M*)	Recovery at pH 12.5 (m*M*)	12.5 %	7.5 (m*M*)	7.5 %
O-mixture 1 m*M* O-acetylhydroxyproline 0.25 m*M* O-acetyltyrosine 1 m*M* N,O-diacetylserine	2.25	2.21	98	0.25	11
S-mixture 1 m*M* S-acetylthiocholine 1 m*M* S-acetylmercaptosuccinate 1 m*M* N,S-diacetylcysteine	3.00	3.05	102	2.78	93
O- and S-mixture Same amount of O- and S-mixture	2.63	2.55	97	1.41	54

Analysis of O-acyl Tyrosine

A spectral determination method for tyrosine was adapted to estimate acyl O-tyrosine after deacylation with hydroxylamine. This test is based on the shift of absorption maximum from 263 to 278 nm and an increase in molar absorptivity when O-tyrosine residues of protein are deacylated.

In practice, solubilized fish myofibrillar protein was found to be too turbid for direct spectrophotometric analysis without clarification by proteolytic treatment prior to deacylation. This was accomplished by incubating 10 ml of solubilized protein suspension in barbital buffer (pH 7.5) with 1 ml of trypsin-bromelain mixture (50 + 100 mg/40 ml H_2O for 30 to 60 minutes at room temperature. If necessary, the protein solution was incubated for an additional 60 minutes at 30°C in a shaker bath. The mixture was clarified by centrifugation for 15 minutes at 18,000 *g*, 0°C. Deacylation of O-acyl tyrosine in 1.0 ml of enzyme-treated protein solution was accomplished with 2 ml of 1 *M* hydroxylamine reagent (pH 7.5) for 10 minutes at room temperature. The volume was adjusted to 5 ml with water, and the absorbance was measured at 278 nm. The increase in absorbance at 278 nm between the native and acylated proteins, E_{278} = 1,100 per mole, was used to determine the amount of O-acyl tyrosine in acylated fish protein.

Analysis of S-acyl Cysteine

Sulfhydryl was estimated using

Ellman's reagent and the general procedure of Habeeb (1972); however, it was adapted for use on modified fish protein. In this modified procedure, acylated sulfhydryl groups were deacylated with hydroxylamine prior to reaction with Ellman's reagent, and the difference in absorbance at 412 nm between the control and modified protein was measured. A result of the quantitive determination of N,S-diacetylcysteine through the reaction with 5,5-dithiobis-(nitrobenzoic acid) (DTNB) reagent is shown in Table 2. At least 2 ml of 1 *M* hydroxylamine for deacetylation and 1.0 ml of 2 m*M* DTNB reagent were required to develop a quantitive color reaction. The concentration of the sample was shown to be a critical factor, and the absorbance of the range of 0.4-0.7 was used for best recovery.

For deacylation, 1 ml of 2 percent sodium dodecyl sulfate phosphate buffer solution (pH 8.0) with 0.2 ml of 0.2 *M* EDTA and 2 ml of 1 *M* hydroxylamine (pH 7.5) were added to 1 ml of enzyme-treated protein solution. After deacylating for 10 minutes at ambient temperature, 1 ml of 2 m*M* DTNB was added, the volume was made up to 5 ml with water, and the absorbance was measured at 412 nm. A molar absorptivity of 13,000 was used for the calculation of the amount of S-acyl sulfhydryl. Because hydroxylamine appeared to cause fading of the color, the absorbance was read within several seconds after the addition of the DTNB reagent.

Table 2.—Recovery and optimum reaction conditions for deacylation of N,S-diacetylcysteine and subsequent quantitation with 5,5′-dithiobis-(2-nitrobenzoic acid).

N,S-diacetyl-cysteine (0.2 m*M*) (ml)	Deacylation with 1 *M* hydroxylamine (ml)	2 m*M* DTNB reagent (ml)	Recovery (percent)
1.0	2.0	1.0	99
1.0	2.0	0.5	55
1.0	2.0	0.2	38
1.0	1.0	1.0	48
1.0	1.0	0.5	34
1.0	1.0	0.2	27

Results

For the purpose of simplification, the results are presented in the following six sections.

Acetylation of Amino Group

Salt-solubilized fish myofibrillar proteins were acetylated with acetic anhydride at three different pH's (9.5, 7.5, and 6.5), and at various levels of acetylating agent at 0°C. The extent of acetylation was expressed in terms of percentage of reactive lysine residues with 2,4,6-trinitrobenzenesulfonic acid reagent. As shown in Figure 1, the rate of reaction was affected by both pH and concentration of reagent. The reaction proceeded much faster at pH 9.5 than at lower pH's. The reaction was almost complete even under relatively mild conditions with 30 to 50 m*M* anhydride (ratio to protein, 1:8 to 1:5) at each pH until the proteins were 80-90 percent acetylated. At 40 m*M* anhydride concentration, acetylation was about 90 percent complete at pH 9.5, whereas only 75 and 65 percent were complete at pH 7.5 and 6.5, respectively. This shows that different amounts of anhydride were required to provide the same degree of acetylation at different pH's. For example, approximately 30, 45, and 55 m*M* of anhydride were added to yield 80 percent acetylation at pH 9.5, 7.5, and 6.5, respectively. With amounts of acetic anhydride in excess of 80 m*M* (ratio to protein, 1:4), the reaction was completed rapidly, leading to over 90 percent acetylation at each pH.

The effect of the rate of anhydride addition was evaluated; however, significant differences in overall reaction rates were not observed when 0.5 to 1.0 g/minute anhydride was added. In all other experimental work, acylating agents were added in the rate of 0.5 g/minute.

Acetylation of Secondary Groups

The relative reactivities of secondary groups are shown in Figures 2 to 5. The general features of the acetylation of total secondary groups were

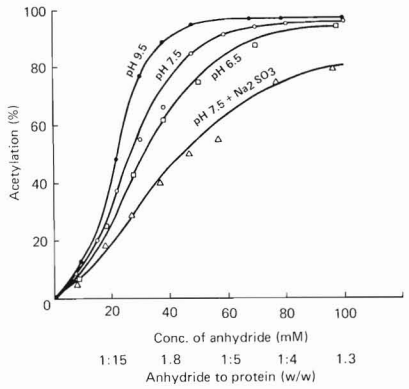

Figure 1.—The reactivity of amino groups of fish myofibrillar protein during acetylation with acetic anhydride (protein content, 30.2-31.6 mg/g).

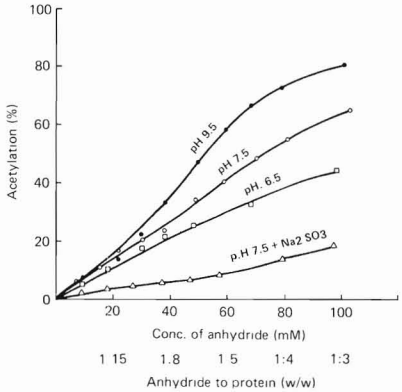

Figure 2.—Overall reactivity of the total secondary groups of fish myofibrillar protein with acetic anhydride (protein content, 30.2-31.6 mg/g).

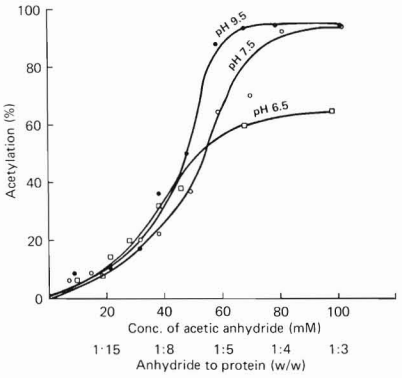

Figure 3.—The reactivity of tyrosyl group of fish myofibrillar protein with acetic anhydride (protein content, 30.2-31.6 mg/g).

similar to the acetylation of the amino group, except that the reaction rate was greatly reduced at lower pH's (Fig. 2). For example, at 40 m*M* anhydride, about 35, 25, and 20 percent of total secondary groups were reacted at pH 9.5, 7.5, and 6.5, respectively, while at least 65 to 90 percent of the amino groups were acylated at the same anhydride concentration.

The rates of acetylation of secondary groups at pH 6.5 and 7.5 appeared to be relatively linear; however, at pH 9.5, concentration of acetic anhydride between 40 to 70 m*M* increased the rate considerably. When Figures 1 and 2 are considered, it can be seen that total acylation at secondary residues of protein might be kept relatively low by controlled use of anhydride and somewhat minimized by acetylating at pH 9.5. On the other hand, relatively high levels of acetylation of the secondary groups can only be accomplished after the acetylation of the amino group is almost complete.

Acetylation of Tyrosyl Group

As shown in Figure 3, the phenolic group of tyrosine can be acetylated to a level of more than 90 percent by using a high concentration of anhydride and extended reaction time. The reactivity of the tyrosyl group was relatively low at lower levels of reagent. For example, at 40 m*M* anhydride concentration, approximately 30 percent of tyrosyl groups were reacted; and at above 50 m*M* of anhydride at pH 6.5, the reaction rate decreased considerably. Again, it is noticeable that the rate of the reaction tended to accelerate dramatically at pH 9.5 and 7.5 with 40 to 60 m*M* anhydride, at which point the acetylation of amino groups was nearly complete. This tendency was revealed more clearly on tyrosyl groups than in the overall reaction of total secondary groups because the acylation of tyrosyl groups is quantitatively much greater than acylation of sulfhydryl and hydroxyl groups, particularly at higher concentration of anhydride. The pH dependence of the reaction was not as great between pH 7.5 and 9.5 as between pH 7.5 and 6.5, and this effect was more evident at higher concentrations of anhydride than at lower levels.

Acetylation of Sulfhydryl Group

Unlike tyrosyl or hydroxyl residues, the sulfhydryl group of fish protein showed a high reactivity with acetic anhydride even at lower levels of anhydride concentration, yielding over 50 percent acetylation with 40 m*M* anhydride (ratio to protein, 1:8) at both pH 9.5 and 7.5 (Fig. 4). The pattern of the reaction was also different from those of the other residues in that the overall reaction proceeded similarly to the amino group reaction. The reaction rate of pH 6.5 was significantly less than at pH 7.5 and 9.5 with anhydride levels greater than 30 m*M* (ratio to protein, 1:10). A relatively high acylation reactivity of sulfhydryl in papain compared with more simple compounds has been reported by Finkle and Smith (1958), Wallenfels and Eisele (1968), and Chaiken and Smith (1969a). Chaiken and Smith (1969b) suggested that an explanation for the high rate of reaction of the sulfhydryl group with some acylating agents may be intraprotein interactions involving the sulfhydryl, imidazolium, and carboxylate groups of the protein which may cause increased nucleophility of the sulfur anion.

Acylation of Hydroxyl Group

It has been generally known that the hydroxyl groups of serine and threonine show the lowest reactivity with acetic anhydride compared with other secondary side groups. The reaction rates at pH 6.5 and 7.5 decrease at about 30 m*M* anhydride and lower concentrations and increase at high levels of anhydride concentration. The reaction rate at pH 9.5 is relatively linear up to 100 m*M* anhydride. No more than 28 percent was modified with 40 m*M* anhydride at pH 9.5 (Fig. 5).

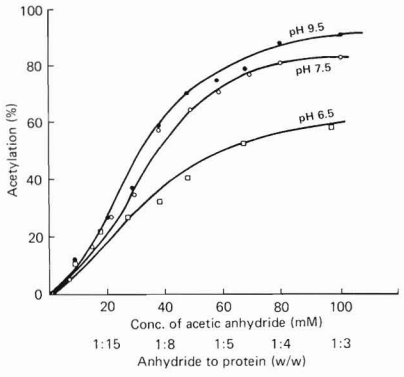

Figure 4.—The reactivity of sulfhydryl group of fish myofibrillar protein with acetic anhydride (protein content, 30.2-31.6 mg/g).

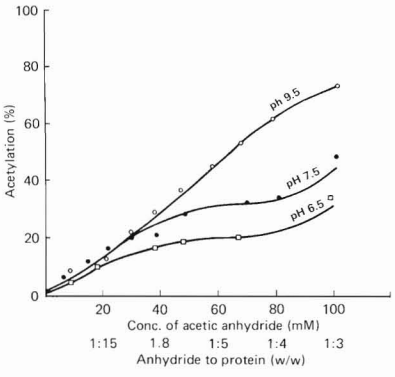

Figure 5.—The reactivity of hydroxyl group of fish myofibrillar protein with acetic anhydride (protein content, 30.2-31.6 mg/g).

Inhibitory Effect of Sodium Sulfite

It is important to know if the reaction of acylating agents with the secondary functional groups on protein can be modified by use of chemicals that might be acceptable food additives. Sulfite, a widely used additive, has been shown by Pfleiderer et al. (1968) to protect all of the sulfhydryl groups in lactate dehydrogenase during the acetylation with acetylimidazole.

To evaluate the inhibitory effect of sodium sulfite on acylation of secondary groups, fish myofibrillar protein was acylated with acetic and succinic anhydride in the presence of up to 20 m*M* sulfite. As the results of Figures 1

and 2 and Table 3 indicate, sodium sulfite was effective in retarding the reaction with sulfhydryl, tyrosyl, and the amino group. In contrast to the result of Pfleiderer et al. (1968), however, the sulfite was more effective in retarding the reaction of tyrosyl than the sulfhydryl group. There was decreased acylation of the amino group and protection of the hydroxyl and sulfhydryl groups. In general, the protection effect was increased with higher levels of sodium sulfite (Table 3). However, the effect differed depending on the kinds of secondary group. For example, acylation of tyrosyl groups was completely inhibited at a level of 15 m*M* of sodium sulfite while acylation of sulfhydryl and hydroxyl was reduced to approximately 50 percent at 5 m*M* of the sulfite and higher levels of sulfite did not retard the reaction further. The retardation effect was greater for the secondary groups than for the amino group. At a level of 15 m*M* sulfite, acetylation was reduced 20 percent on the amino group, 70 percent for total secondary groups, 60 percent on the sulfhydryl, 55 percent on the hydroxyl, and 100 percent on the tyrosyl groups.

Sulfite, as shown in Table 4, was also effective in reducing the succinylation of protein functional groups. A significant retardation of succinylation of the sulfhydryl group occured in the presence of sulfite, while the reaction of the hydroxyl group was not greatly affected. Succinylation of the tyrosyl group was not detected as other investigators have reported earlier (Riordan and Vallee, 1964; Gounaris and Perlmann, 1967). Although the extent of succinylation was lower than acetylation, the protective effect was revealed almost the same as in the case of acetylation. Fifteen m*M* sulfite reduced succinylation 37 percent on amino, 60 percent on sulfhydryl, and 28 percent on hydroxyl groups.

The pH dependence of the retardation effect of sodium sulfite is shown in Table 5. A major decrease in the acetylation of amino and tyrosyl was shown below pH 7.5; however, there were only minor changes for sulfhydryl and hydroxyl.

From these results it is concluded that sulfite can be used effectively to retard acylation of secondary groups; however, it may be less useful for succinylation reactions since only the sulfhydryl reaction is significantly retarded with the acylation agent. For example, acetylation with an anhydride to protein ratio of 1:10 at pH 9.5 results in 80 percent acetylation on amino and 25 percent on total secondary groups that include 18 percent on tyrosyl, 46 percent on sulfhydryl, and 22 percent on hydroxyl (Fig. 1). The same level of acetylation on amino group was obtained at an anhydride to protein ratio of 1:5 with 15 m*M* sulfite at pH 9.5 (Table 5); however, acetylation was reduced 24 percent on secondary groups that include 60 percent reduction on tyrosyl and 13 percent on sulfhydryl, but little or no change in hydroxyl.

Discussion

The results of acetylation with acetic anhydride show that the amino groups of fish myofibrillar proteins were readily acetylated to a level of 80-90 percent under relatively mild conditions. However, the overall reactivity of secondary groups, particularly hydroxyl, was much less than that of the amino group, a function of pH and reagent concentration. The effect of pH on the reaction rate of protein

Table 3.—Effect of sulfite on acetylation of functional groups in fish myofibrillar protein at pH 7.5.

Sodium sulfite (m*M*)	Acetic anhydride (m*M*)	Percent acetylation on: Amino group	Total secondary groups	Tyrosyl group	Sulfhydryl group	Hydroxyl group
0	100	94.8	78.2	95.1	95.1	69.7
5	97	88.4	28.7	26.5	46.3	28.0
10	106	83.8	26.2	13.2	40.2	30.7
15	107	78.3	22.2	0	40.2	30.3
20	107	75.0	20.8	0	39.0	28.3

Table 4.—Effect of sulfite on succinylation of functional groups in fish myofibrillar protein at pH 7.5.

Sodium sulfite (m*M*)	Acetic anhydride (m*M*)	Percent succinylation on: Amino group	Total secondary groups	Tyrosyl group	Sulfhydryl group	Hydroxyl group
0	95	80.8	6.6	0	36.6	6.7
5	86	70.9	5.6	0	28.0	6.0
10	86	66.3	5.4	0	15.9	6.2
15	86	51.2	4.1	0	15.9	4.8

Table 5.—The influence of pH on the retardation of the acetylation of fish myofibrillar protein in the presence of sulfite.

pH	Sodium sulfite (m*M*)	Acetic anhydride (m*M*)	Percent acetylation on: Amino group	Total secondary groups	Tyrosyl group	Sulfhydryl group	Hydroxyl group
6.5	15	60	33.2	9.2	0	36.8	11.7
7.5	15	60	75.0	12.9	5.2	37.8	14.0
9.5	15	60	79.6	19.2	7.0	41.5	22.6

residues tended to reflect, as generally recognized, that the acetylation reaction was more favorable at pH 9.5 than at pH 7.5 or 6.5. The pH effect on the reaction rate was greater on the secondary groups than on the amino group at higher levels of anhydride, whereas at lower levels of anhydride concentration, the differences were very small.

Tyrosyl showed a large increase in acetylation rate at higher levels of anhydride. Also, a large decrease in the acetylation of tyrosyl occurred in the presence of sulfite. This large increase in acetylation rate might be explained by exposure of buried tyrosyl groups as a result of acetylation-induced conformational changes in the protein (Habeeb, 1966). The decrease in the acetylation of tyrosyl in the presence of sulfite might be due to a sulfite-induced conformation change in protein as was suggested by Pfleiderer et al. (1968).

Conclusions

Protein amino groups were readily acylated under mild conditions at pH 9.5 to 6.5 with 30 to 50 m*M* anhydride (ratio of anhydride to protein, 1:10 to 1:5, weight:weight) added at the rate of 0.5 g/minute resulting in 80-90 percent acetylation. Under the same conditions, the reactivities of secondary groups were much less; only 20-30 percent as total secondary group, 20-35 percent of tyrosyl, 40 percent of sulfhydryl, and 20 percent of hydroxyl groups were acetylated. Sodium sulfite was effective in retarding the acylation of these secondary groups. Fifteen m*M* sulfite reduced acylation by 20 percent on amino, 60 percent on sulfhydryl, 100 percent on tyrosyl, and 55 percent on hydroxyl groups; and 15 m*M* sulfite reduced succinylation by 37 percent on amino, 60 percent on sulfhydryl, and 28 percent on the hydroxyl group.

The relative acylation reactivities of protein groups might be considered in terms of the modification of proteins for food use. In much of the reported work on food-protein modification, about 80 percent acylation of the amino group is indicated. If 80 percent acylation of the amino group is used as an example and if minimal amounts of secondary products are desired, it is recommended that the reaction conditions be pH 9.5 with an anhydride to protein ratio of 1:10. Under these conditions, acylation would result on 20-30 percent of the total secondary groups that include 20-30 percent tyrosyl, 40 percent sulfhydryl, and 20 percent hydroxyl groups. It is further recommended that when the need for the least secondary product is indicated, then the acylation should be performed in the presence of sulfite.

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