The Reaction of Iodoacetate with Methionine

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Iodoacetate acid and its amide have been widely used in protein chemistry as reagents for sulfhydryl groups. Although both can be expected to undergo other reactions with proteins, for example, with the e-amino groups of lysine residues, the imidazole groups of histidine residues, and the phenolic hydroxyls of tyrosine residues, these reactions are generally slower than is the one with sulfhydryl groups (1, 2), particularly at neutral or slightly acid pH.

The reaction of iodoacetate with the sulfur of methionine residues, however, although little mentioned in the literature that deals with the action of iodoacetate on proteins, might be expected, on the basis of experience with other alkylating agents, to take place readily at neutral pH. For example, experiments carried out in the laboratory of Dr. Max Bergmann in 1942 to 1944 (3) with bis(β chloroethyl)sulfide (mustard gas), showed that the formation of the sulfonium salt of methionine proceeded much more rapidly than did reaction of the alkylating agent with either the amino or the carboxyl groups of amino acids. Tocnies and Kolb (4) in their detailed studies of the formation of various sulfonium salts of methionine, demonstrated that both iodoacetate and bromoacetic acids reacted with methionine at a appreciable rate. The present investigation was prompted by the observation that when proteins that had been reduced with sodium borohydride were treated with iodoacetate to cover the SH groups formed (5), quantitative analyses for methionine gave results that were occasionally slightly low. The recoveries of methionine were also sometimes low upon analysis of standard ribonuclease that had been treated with iodoacetate under certain conditions (6). In order to ascertain whether sulfonium salt formation might have taken place, the chemistry of the reaction of iodoacetate with methionine has been examined in more detail. Ion exchange chromatography has been used as a means of following the reaction.

The ability to detect the alkylation of a methionine residue in a protein will depend in part upon the nature of the products that the resulting sulfonium salt yields when the protein is hydrolyzed in preparation for amino acid analysis. The mechanism of the decomposition of sulfonium salts of methionine has been studied frequently in the past 10 years, as the methyl donating role of sulfonium compounds in mammalian and microbial metabolism has become known (du Vigneaud (7); Chalkinger (8)). Cantoni’s (9) establishment of the significance of S-adenosyl-L-methionine in this connection has recently led to further study of its chemical and enzymatic decomposition by Parks and Schlenk (10), Shapiro and Mather (11), and Stekol et al. (12, 13). These studies, taken together with the earlier investigations of the methylsulfonium salts by Lavine and Floyd (14, 16) and Lavine et al. (15), and of the sulfonium salt formed from methionine and mustard gas (3), permit formulation of the scheme given in Fig. 1, which shows the compounds to be expected upon decomposition of methionine carboxymethylsulfonium salt. The occurrence of each of the three possible cleavages shown in Fig. 1 has been demonstrated by the chromatographic analyses described in the present report. It has been found that methionine itself is to some extent regenerated when the sulfonium salt is heated in strong acid, and this may account in part for the fact that examination of hydrolyzates of proteins that have been treated with iodoacetate has not heretofore permitted the detection of sulfonium salt formation.

**EXPERIMENTAL.**

**Analytical Studies of Reaction of Methionine with Iodoacetate—**

Stock solutions were prepared to contain per ml., 6 mg. of methionine and 22 mg. of iodoacetic acid, respectively. Before being made up to volume, each solution was brought to pH 4 with NaOH. For each analytical experiment, 0.5 ml. of the methionine solution, 1.0 ml. of the iodoacetic acid solution, and 1.0 ml. of water were mixed, brought to the desired pH with NaOH, and incubated at the indicated temperature in a pH-stat. After a predetermined time, 0.5 ml. of the reaction mixture was added to an equal volume of citrate buffer at pH 2.2 (cf. (17), Table I), and 0.5 ml. of the resulting mixture was immediately analyzed by ion exchange chromatography with the aid of automatic recording equipment, according to the procedure of Spackman et al. (18).

**Preparation of Methioninecarboxymethylsulfonium Iodide—** To 300 mg. of L-methionine in 25 ml. of water was added 1.0 gm. of iodoacetic acid. The mixture was kept at 40° for 24 hours, after which a 0.02 ml. sample was withdrawn for chromatographic analysis. The excess iodoacetic acid was removed by extraction with ether; samples were withdrawn for the experiments described below, and the remaining aqueous solution was lyophilized. Before elementary analysis, the slightly yellow powder, which is very hygroscopic, was dried over P₂O₅ in high vacuum for 3 hours at room temperature.

\[ \text{CH₃O₃NSI} \text{ H₂O (393.2)} \]

Calculated: C 23.80, H 4.57, N 3.97, S 9.08

Found: C 23.82, H 4.66, N 3.90, S 9.16

**Decomposition of Methioninecarboxymethylsulfonium Iodide—** To study the decomposition of the sulfonium salt, 0.05 ml. aliquots of the reaction mixture described above were added to 5 ml. of citrate buffer at pH 2.2 and to 5 ml. of 0.2 M phosphate buffer at pH 6.5, and the resulting mixtures were heated at 100°...
Acid and oxidized by the procedure of Schram et al. (19) except sulfonium salt (about 5 mg.) was dissolved in 10 ml. of performic acid and oxidized for 4 hours with performic acid at 40° (19) and excess performic acid was removed by two lyophilizations on the rotary evaporator instead of at a bath temperature of 40°.

**Diagram:**

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  (CH3-S-CH2COOH)
     |        | CH2
     |        | CH2
  S-Carboxymethyl-
  homocysteine  →  CHNH2
                         COOH
  Carboxymethyl-
  sulfonyl
  salt of methionine

   CH2OH
   CH2
   CHNH2
  Methionine
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**FIG. 1.** Products of the decomposition of the methionine-carboxymethylsulfonyl sulfonium salt.

For 1 hour. Aliquots (2 ml.) from each reaction mixture were analyzed chromatographically. A larger sample (0.25 ml.) of the sulfonium salt solution was also heated with 6 N HCl (1.0 ml. for 1 hour and for 20 hours) in order to determine the nature of the products that might be formed during hydrolysis of a protein that had been treated with iodoacetate.

**Oxidation of Methioninecarboxymethylsulfonium Iodide**—The sulfonium salt (about 5 mg.) was dissolved in 10 ml. of performic acid and oxidized by the procedure of Schram et al. (19), except that the performic acid was removed by two lyophilizations on the rotary evaporator instead of at a bath temperature of 40°. The residue was dissolved in citrate buffer at pH 2.2 and chromatographed.

**RESULTS**

**Formation of Methioninecarboxymethylsulfonium Iodide**—When the solution present after methionine and iodoacetate have been allowed to react at 40° for 24 hours is chromatographed on a sulfonated polystyrene resin, the effluent curve shown in Fig. 2a is obtained. Only the most intense peak of homoserine emerges. That the two major peaks are attributable to the stereoisomeric L-methionine-d- and L-carboxymethylsulfonium salts is proved by the preparation from the reaction mixture of material possessing the analytical composition of the pure sulfonium iodide, as given above, and by the behavior of the reaction product upon heating, which is described below. The third peak, representing about 2 per cent of the total ninhydrin positive material, is almost certainly homoserine, which has been identified as a decomposition product of the sulfonium salt (cf. next section). The recovery of the sulfonium salt from chromatograms of the type shown in Fig. 2 is usually about 75 to 80 per cent. Because of the lability of the sulfonium salt, discussed below, some decomposition during chromatography at 50° is to be expected.

The extent of the reaction of iodoacetate with methionine under various conditions has been followed by chromatograms of the type shown in Fig. 2. It has been found that the rate of the reaction is not pH-dependent. Thus, after 2 hours of reaction at 40°, between 70 and 75 per cent of the theoretical quantity of sulfonium salts is formed at pH 4, 5.5, 7.0, and 8.5. At 25°, as might be anticipated, the rate is slower, the extent of sulfonium salt formation being about 5 per cent in 20 minutes at pH 8.5, about 30 per cent in 120 minutes, and about 70 per cent in 340 minutes. At 10° and pH 8.5, only 10 per cent conversion to the sulfonium salt occurs in two hours.

**Decomposition of Methioninecarboxymethylsulfonium Iodide**—When the sulfonium salt is boiled in water for 1 hour, 0.87 equivalent of acid is liberated, which is characteristic of the behavior of other sulfonium salts (3). To investigate the nature of the products formed when the sulfonium iodide is heated, chromatography on IR-120 has been used. Effluent curves obtained after heating the salt at 100° for 1 hour at pH 6.5 and at pH 2.2 are shown in Figs. 2b and c. At both of these pH values cleavage leads almost quantitatively to the formation of homoserine and its lactone. The position of the peak at about 160 ml. on the long column, which is just ahead of the position of elution of glutamic acid, is the same as that of an authentic sample of homoserine. Homoserine is formed to a lesser extent at pH 2.2 than at pH 6.5, and along with this diminution in yield, there is an increase in the quantity of the compound possessing the expected chromatographic behavior of homoserine lactone. This basic compound emerges from the 15-cm column just after ammonia at exactly the position assumed by the substance formed when homoserine is heated at pH 2.2, conditions known to cause lactonization.

The sulfonium salt decomposes in a different manner, however, when heated in strong acid. The mixture of products formed upon hydrolysis for 24 hours under the conditions used for protein hydrolysis is shown in Fig. 2d. Only small quantities (about 5 per cent) of homoserine, or its lactone (about 6 per cent) are formed. Methionine is regenerated to the extent of 20 per cent by the acid treatment. There is also another major peak (in about 50 per cent yield) which appears just after the proline position on the long column. This peak, which is also seen in trace amounts in the chromatograms shown in Figs. 2b and c, emerges at exactly the same position as a synthetic (21) sample of S-carboxymethylhomocysteine. Moreover, a compound with exactly this chromatographic behavior is one of the two major products that can be detected after iodoacetate is allowed to react with homocysteine thiolactone in the presence of sodium borohydride (5) at pH 10. The other product is homocystine, a trace of which (2 per cent) may also be seen in Fig. 2d. The sulfonium salt is more stable in 6 N HCl than in buffers at pH 2.2 or 6.5, since after 1 hour of heating in strong acid, about 60 per cent of the sulfonium salt remains unchanged.

**Oxidation of Methioninecarboxymethylsulfonium Iodide**—When the sulfonium salt was oxidized for 4 hours with performic acid at 4° (19) and excess performic acid was removed by two lyophilizations on the rotary evaporator, chromatography showed that about 85 per cent of the sulfonium iodide had been recovered unchanged. When reaction was allowed to proceed for 20 hours, the recovery was 65 per cent.

**DISCUSSION**

From the evidence presented in this communication it can be concluded that whenever proteins are allowed to react with iodoacetate, formation of the carboxymethylsulfonium derivative of methionine is an ever present possibility. When exclusive
Fig. 2. Chromatographic analyses of preparations of methioninecarboxymethylsulfonium salts and the products formed upon heating the aqueous solutions at different pH values. The chromatography was performed with columns of Amberlite IR-120 (20) with automatic recording equipment (18). (a) The sulfonium salts obtained by treating L-methionine with iodoacetic acid for 24 hours at 40°. (b) Products formed when the sulfonium salt analyzed above was heated for 1 hour at 100° in 0.2 M phosphate buffer at pH 6.5. (c) Products formed when the sulfonium salt was heated for 1 hour at 100° in 0.2 N citrate buffer at pH 2.2. The ammonia is in part a contaminant picked up from the air by the acid solution during the heating period. (d) Products formed when the sulfonium salt was heated for 20 hours in 6 N HCl, at 110°, under the conditions used for the hydrolysis of proteins. The ammonia peak arises in part from the ammonia in the HCl.

Coverage of sulfhydryl groups is desired, therefore, it is important to limit the time in which the protein and iodoacetate are in contact to the minimum that measurement shows is necessary to effect complete coverage of the sulfhydryl groups. If this period is of the order of 20 minutes, as it is when borohydride-reduced proteins are allowed to react with iodoacetate (5), the extent of the reaction with methionine is usually insignificant. With the longer times necessary to cover sulfhydryl groups that are sluggish, side reactions with methionine, and possibly other groups as well, are inevitable. Since the extent of the reaction with methionine is independent of pH in the range pH 4 to 8, this side reaction, unlike the ones with amino, imidazole, and
phenolic groups, cannot be minimized by working at mildly acid pH values. This fact, which doubtless can be troublesome in some cases, may prove to be an advantage in others. It may be possible to use iodoacetate as a reagent to alter specifically the methionine residues in proteins by allowing the reaction to take place in the range pH 2 to 5, particularly if sulphydryl groups are absent. Studies are in progress (6) to determine whether ribonuclease can be inactivated by the use of this reaction to convert the methionine residues in the protein to sulfonium salts.

The instability of sulfonium salts makes it difficult to determine the extent to which the methionine residues in proteins have reacted. Hydrolysis with acid decomposes the sulfonium salt completely with the regeneration of some methionine and the formation of S-carboxymethylhomocysteine and homoserine (and its lactone). The presence of small peaks attributable to these latter substances in chromatograms of hydrolysates of iodoacetate-treated proteins may be taken as a sure indication that reaction with methionine residues has occurred. The size of these peaks, however, cannot be used to determine the extent of the reaction, because the sulfonium salt bound in peptide linkage may decompose differently on heating than does the sulfonium salt of free methionine. For example, when ribonuclease is allowed to react with iodoacetate at pH 2.8, under conditions promoting combination with methionine residues exclusively (6), and the product is heated at pH 3 for 1 hour, hydrolyzed, and analyzed, methionine, homoserine (and its lactone), and S-carboxymethylhomocysteine are all found in the hydrolysate. The sulfonium salt of free methionine treated in this manner would be desulfurized completely, yielding homoserine (and its lactone) and S-carboxymethylmethioninesulfonium salt have been synthesized, allowing to interact for any appreciable length of time. The carboxymethylmethionine-sulfonium salt has been synthesized, and the mechanism of its decomposition has been investigated. After heating the salt at 100° for 1 hour at pH 2.2 or 6.5, homoserine and its lactone are formed almost quantitatively. When the sulfonium salt is heated in 6 N HCl, however, methionine is regenerated, and a large amount of S-carboxymethylhomocysteine is also formed. The instability of the sulfonium salt makes it difficult to determine the extent to which the methionine residues in proteins have reacted. Because alkylation of methionine occurs at mildly acid pH values, it may be possible to use iodoacetate as a reagent to alter specifically the methionine residues in proteins, particularly if sulphydryl groups are absent.

REFERENCES


SUMMARY

In order to understand more fully the nature of the products that may be formed when iodoacetate reacts with a protein, the reaction of this alkylating reagent with methionine has been studied. Ion exchange chromatography has been used to follow the reaction. It has been found that the formation of the carboxymethylsulfonium salt of methionine proceeds at a rapid rate and with equal facility throughout the range pH 2 to pH 8.5. In fact, at neutral pH, only the familiar alkylation of sulphydryl groups is faster, and hence it is necessary to take into account the possibility of reaction with the sulfur atoms of a methionine residue whenever proteins and iodoacetate are allowed to interact for any appreciable length of time. The carboxymethylmethioninesulfonium salt has been synthesized, and the mechanism of its decomposition has been investigated. After heating the salt at 100° for 1 hour at pH 2.2 or 6.5, homoserine and its lactone are formed almost quantitatively. When the sulfonium salt is heated in 6 N HCl, however, methionine is regenerated, and a large amount of S-carboxymethylhomocysteine is also formed. The instability of the sulfonium salt makes it difficult to determine the extent to which the methionine residues in proteins have reacted. Because alkylation of methionine occurs at mildly acid pH values, it may be possible to use iodoacetate as a reagent to alter specifically the methionine residues in proteins, particularly if sulphydryl groups are absent.
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