Modification of an Arginyl Residue in Pepsin by 2,3-Butanediione*

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SUMMARY

2,3-Butanediione (biacetyl) was found to modify an arginyl residue in porcine pepsin at pH 6.0, 25°. In an inhibition study, half-inactivation occurred after 21 hours of reaction, and maximum inactivation of 80 to 85% was reached after 24 hours. Amino acid analysis of the modified pepsin revealed that 1 arginyl residue remained, indicating that 1 arginyl residue had been modified. The loss of activity and loss of arginine occurred concomitantly during the course of reaction. The presence of substrates retarded enzyme inactivation, as well as arginyl loss, at corresponding rates. Porcine pepsinogen treated with biacetyl in a pH 7.0 solution did not lose activity significantly, as shown by its proteolytic activity after subsequent activation by acid. Biacetyl-modified pepsin continued to be susceptible to specific esterification reagents: diazoacetyl-DL-norleucine methyl ester, p-bromophenacyl bromide, and 1,2-epoxy-3-(p-nitrophenoxy)propane.

Peptides from a chymotryptic digest of pepsin and of biacetyl-treated pepsin were separated by high voltage paper electrophoresis at pH 6.5. Two peptides were isolated, containing one each of the only 2 arginyl residues of pepsin. The sequences of these peptides were ascertained from amino acid compositions and amino-terminal determinations. One of the peptides (A₁) from biacetyl-pepsin contained almost no free arginine. An additional spot, apparently representing modified arginine, was observed for the digest of Peptide A₁ in high voltage electrophoresis. These results indicated that the arginyl residue in Peptide A₁, located 12 residues from the carboxyl terminus of pepsin, is the site of biacetyl modification. This arginyl residue apparently does not directly participate in the catalysis of the enzyme.

Biacetyl was also found to inactivate other gastric proteases in similar fashion, i.e. human gastricsin, human pepsin, and bovine rennin.

Several specific inactivators have been used successfully in studies of the structure of the active center of pepsin. They can be divided into three different types: diazo compounds, substrate-like epoxides, and phenacyl bromide derivatives. The ability of certain diazo compounds to inactivate pepsin was originally reported by Delbrie and Fruton (2). Since then, a number of other diazo inactivators have been reported (3-12). Of these, diazoacetyl-DL-norleucine methyl ester has been investigated in particular detail by Rajagopalan et al. (3) and by Lundblad and Stein (4). The data from these and other studies show that the diazo inactivators esterify a unique aspartyl residue contained in the sequence: Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu (11-13). Because the reaction of pepsin with diazo compounds results in complete loss of activity, this aspartyl residue is probably involved in catalysis of the enzyme. The second group of inactivators is made up of the substrate-like epoxides, such as 1,2-epoxy-3-(p-nitrophenoxy)propane reported by Tang et al. (14) and Tang (15). These compounds inactivate pepsin by esterifying a carboxyl group in the active center which differs from the carboxyl groups that react with other types of inactivators (16, 17). Since this reaction completely inactivates the enzyme, the esterified carboxyl group is probably essential for catalysis. Finally, the phenacyl bromide derivatives, reported by Erlanger et al. (18, 19), apparently esterify a carboxyl group not directly involving enzymic catalysis, since the modified enzyme remains partially active. All three types of inactivators described above, however, act upon carboxyl groups.

Porcine pepsin contains 2 arginyl residues located in the positions 12 and 20 residues from the COOH terminus (20-22). A comparison of amino acid sequence of this region in several homologous enzymes, including human pepsin and gastricsin (23) and bovine rennin (24), has revealed the relative positions of the arginyl residues to be identical. This suggests that the arginyl residues may be important in acidic protease function, and that they have thus been retained during the evolutionary process.

To elucidate the role of arginyl residues in peptic activity, we designed a study on the chemical modification of arginyl residues in pepsin. Although a number of reagents are available for arginine modification (25-28), the useful ones are necessarily reactive in acidic solutions, since pepsin inactivates spontaneously in alkaline solutions. 2,3-Butanediol (biacetyl) was chosen for testing since this reagent has been shown to react with arginyl residues under mild conditions in a near-neutral

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solution (29–31). Moreover, biacetyl has been successfully used to modify arginyl residues in antibodies (32) and carboxypeptidase A (33).

The results of this study indicated that biacetyl modifies the arginine residues from the COOH-terminal of pepsin, causing an 85% loss of enzyme activity.

**EXPERIMENTAL PROCEDURE**

**Materials**

Porcine pepsin (3 times crystalline) and bovine hemoglobin substrate were obtained from Pentex; aminopeptidase M was purchased from Henley and Co., and crystalline rennin from Nutritional Biochemicals Co. Human gastricsin and pepsin were prepared as described previously (34, 35). Diazocetyl-DL-norleucine methyl ester was a gift from Dr. John N. Mills, Oklahoma Baptist University, Shawnee, Okla. The synthetic DL-norleucine methyl ester was allowed to react with 0.3 ml of biacetyl in 30 ml of 0.2 M potassium phosphate buffer, pH 6.0. Biacetyl, 10 μl, was added to this solution, which was then incubated with occasional shaking at 25° for 24 hours. The product was lyophilized thoroughly, redissolved in small amounts of water, and lyophilization was repeated. The aminopeptidase M hydrolysate of the modified B chain of insulin contained 1 residue of lysine (the same as that in the unmodified B chain) and no arginine, indicating the complete modification of arginine by biacetyl.

**Methods**

**Proteolytic Activity**—The proteolytic activity was measured with bovine hemoglobin as substrate. The procedure was essentially that of Anson and Mirsky (37).

**Milk-clotting Activity**—The procedure of Berridge was followed for the determination of milk-clotting activity (38).

**Inactivation Studies**—A typical reaction mixture consisted of 1 ml of a 0.1% solution of pepsin in 0.2 M potassium phosphate buffer, pH 6.0. To this solution, about 10 μl of biacetyl were added. The reaction was carried out at 25 ± 1°. Aliquots of 10 μl were withdrawn at different time intervals for the assay of enzyme activity. An enzyme solution containing no biacetyl was incubated along with the reaction mixtures to serve as a control.

**Preparation of Biacetyl-Pepsin**—Crystalline pepsin (30 mg) was allowed to react with 0.3 ml of biacetyl in 30 ml of 0.2 M potassium phosphate buffer, pH 6.0. After standing for 24 hours at room temperature, the solution was dialyzed against distilled water at 4°. The resulting mixture was dialyzed against distilled water.

**Reaction of Biacetyl-treated Pepsin with p-Bromophenacyl Bromide**—The reaction of pepsin or biacetyl-treated pepsin with p-bromophenacyl bromide was carried out according to the method of Erlanger et al. (18). The reacted pepsin was dialyzed at 4° against distilled water.

**Biacetyl Modification of Oxidized B Chain of Bovine Insulin**—The oxidized B chain of bovine insulin (3 mg) was suspended in 1 ml of 0.02 M potassium phosphate buffer, pH 6.0. Biacetyl, 10 μl, was added to this solution, which was then incubated with occasional shaking at 25° for 24 hours. The product was lyophilized thoroughly, redissolved in small amounts of water, and lyophilization was repeated. The aminopeptidase M hydrolysate of the modified B chain of insulin contained 1 residue of lysine (the same as that in the unmodified B chain) and no arginine, indicating the complete modification of arginine by biacetyl.

**Biacetyl Modification of Dipptide L-Alanyl-L-arginine**—Dipeptide (8 mg) was dissolved in 1 ml of 0.02 M potassium phosphate buffer, pH 6.0, to which 10 μl of biacetyl were added. The solution was left standing at room temperature for 24 hours and then lyophilized. The resulting material was subjected to high voltage electrophoresis as a 20-cm band on Whatman 3MM paper at pH 3.5 (60 volts per cm for 1 hour). A guide strip was cut out and treated with cadmium-ninhydrin reagent to reveal the peptide bands. Only trace amounts of the original dipetide were present (relative mobility to free arginine was 0.95). A strong peptide band with a mobility of 0.80 (i.e. relative to free arginine, 1.0) was present, apparently representing the biacetyl-modified dipeptide. This peptide band was cut out, and the material was recovered by elution. The hydrolysate obtained with methanesulfonic acid contained free alanine, but no free arginine.

**Amino Acid Analysis**—Amino acids were quantitated with a Spinco model 120B amino acid analyzer having a modified “range card” in the recorder to permit analysis in the range of 0.001 to 0.1 mole; the procedure of Spackman (39) was followed.

For quantitation of biacyetyl-modified arginine, several hydrolytic methods were tried. Yankelev (31) reported that about 12% of modified arginine was regenerated to free arginine in hydrolysis with 6 N HCl. We found that the hydrolysis of biacyetyl-modified alanylarginine by 6 N HCl resulted in about 20% of the modified arginine being regenerated back to free arginine. (In some earlier experiments, when HCl hydrolysis was used, the analytical data for arginine were corrected for regeneration.) The digest with aminopeptidase M did not regenerate free arginine from either modified alanylarginine or the B chain of insulin. However, this enzymic digest of pepsin could not fully release the arginine content of 2 residues, and thus was unsuitable for amino acid analysis of the modified pepsin. A third method, hydrolysis with methanesulfonic acid, devised by Liu and Chang (40), was most successful. The hydrolysate of biacyetyl-modified alanylarginine produced no free arginine.

The amount of modified arginine could not be analyzed directly in the amino acid analyzer. We confirmed the report of Yankelev (31) that several overlapping peaks appearing in the regions were basic amino acids normally eluted. Therefore, the
amount of modification of arginine was determined by the difference in arginine content before and after modification.

The hydrolysis with methanesulfonic acid was carried out by the method of Liu and Chang (40). Protein or peptide (0.02 to 0.05 μmole) was hydrolyzed with 0.2 ml of 3 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole hydrochloride at 110° for 24 hours.

Amino-terminal Residue Determination—The NH₂-terminal residues of the peptides were determined after reaction with dansyl chloride according to the method of Gray and Hartley (42).

High Voltage Paper Electrophoresis—The commercial apparatus produced by Savant Instruments, Inc. was used with the following buffer systems: pyridine-acetic acid-water (25:1:225 by volume), pH 2.5; formic acid-acetic acid-water (1:4:45 by volume), pH 3.5; pyridine-acetic acid-water (25:1:225 by volume), pH 3.5. Whatman No. 1 paper was used except in the preparative electrophoresis where the peptide sample was applied as a band on Whatman No. 3MM paper before being subjected to high voltage electrophoresis.

Chymotryptic Digest of Porcine Pepsin and Biacetyl-treated Pepsin—The 0.25 and 1% protein solutions were incubated at 37° for 3 hours with α-chymotrypsin (substrate to enzyme ratio, 50:1). The buffer was 0.2 N N-ethylmorpholine acetate solution, pH 8.0.

Digestion with Amino-peptidase M—Samples containing 0.05 μmole of peptide were incubated with 100 μg of the enzyme in 500 μl of 0.2 M N-ethylmorpholine acetate, pH 8, at 25° for 24 hours. The digestion was at times sealed down to 0.01 μmole of peptide with 100 μl of buffer and 50 μg of enzyme. The samples were lyophilized at the end of incubation, redissolved in 0.2 M sodium citrate buffer, pH 2.2, and directly applied in the column of the amino acid analyzer for the determination of free amino acids.

RESULTS

Inactivation of Pepsin by Biacetyl—Fig. 1 shows the course of inactivation of pepsin by biacetyl in solutions with pH values of 3.0, 4.0, 5.0, and 6.0. In all cases, maximum inactivation was about 85%. Prolonging the incubation did not significantly increase the inactivation. Because the reaction apparently proceeded more rapidly at higher pH values, pH 6.0 was chosen for further experiments. Varying the biacetyl concentration from 5 to 20 μl per ml of incubation mixture failed to alter the course of inactivation appreciably. The use of citrate or phosphate buffer had no apparent effect. Another arginine-modifying reagent, benzil (27), incubated as a saturate solution in the same buffer, had no measurable inactivation ability. Phenylglyoxal at 10 mM inactivated about 50% of enzyme activity after 4 hours of reaction. At 10 mM, however, no inactivation was observed.

After 20 hours of reaction of biacetyl with pepsin, the mixture was dialyzed in the cold against distilled water. The dialyzed sample regained no activity, indicating that the inactivation was irreversible and probably due to the modification of arginyl residues in pepsin molecules. This was confirmed by amino acid analysis, which showed a loss of about 1 residue of arginine in biacetyl-treated pepsin (Table I). The same result was obtained whether the hydrolysis was carried out with HCl or methanesulfonic acid.

Porcine pepsinogen was incubated with biacetyl under the same conditions used in the inactivation of pepsin, except that the pH of the solution was 7.0. A considerably slower inactivation rate was observed, approximately one-tenth of that of pepsin in pH 6.0 solution.

Properties of Biacetyl-treated Pepsin—The activity of biacetyl-treated pepsin was measured with two synthetic substrates: N-carbobenzoxy-L-tyrosyl-L-phenylalanine and N-carbobenzyloxy-L-glutamyl-L-tyrosine. With these substrates, the specific activity of biacetyl-treated enzyme was only 15% of that of native enzyme, confirming the results obtained with the hemoglobin assay (Fig. 1). The activity of biacetyl-treated pepsin was also measured in solutions ranging from pH 1.0 to 5.0, with hemoglobin as substrate. Maximum activity was observed at pH 2.0, the same as for untreated pepsin, indicating that the loss of enzyme activity after modification was not due to a shift of pH

![Fig. 1. Rate of inactivation of porcine pepsin by biacetyl at 25° in solutions of different pH values. The solution contained 1 mg of pepsin, 10 μl of biacetyl in 1 ml of 0.2 M potassium phosphate buffer. Activity was measured with hemoglobin as substrate.](http://www.jbc.org/)

**Table I**

| Enzyme         | Treatment    | Remaining enzyme activity | Basic amino acid | Loss in arginine |
|----------------|--------------|---------------------------|------------------|------------------|
| Porcine pepsin | None         | 100                       | Lys 1.00         | 2.00             |
| Human pepsin   | Biacetyl     | 15                        | 0.84 0.81        | 1.19             |
| Human gastricsin| None         | 100                       | 0.00 1.00        | 3.00             |
|                | Biacetyl     | 20                        | 0.65 2.03        | 0.97             |
| Porcine pepsinogen | None       | 100                       | 0.84 1.00        | 1.70             |
| Bovine rennin  | Biacetyl     | 92                        | Not determined   |                  |
|                | Biacetyl     | 25                        | Not determined   |                  |

* The residue numbers of basic amino acids were taken from the following studies: porcine pepsin, Rajagopalan et al. (40); human pepsin and gastricsin, Mills and Tang (41) and Huang and Tang (42). The biacetyl-treated enzymes were hydrolyzed in methanesulfonic acid (see "Methods").

* Loss of arginine was due to biacetyl modification. The data were the difference of residues of arginine in the native and biacetyl-treated enzymes.
The ultraviolet absorption spectra of biacetyl, native pepsin, and biacetyl-treated pepsin. Spectra were measured at a protein concentration of 0.1 mg per ml in water. Biacetyl concentration was 0.05 mg per ml in water.

![Figure 2. Ultraviolet absorption spectra of biacetyl, native pepsin, and biacetyl-treated pepsin. Spectra were measured at a protein concentration of 0.1 mg per ml in water. Biacetyl concentration was 0.05 mg per ml in water.](http://www.jbc.org/)

**Table II**

| Inactivation | Percentage of activity in biacetyl-pepsin | Percentage of enzyme activity remaining | Percentage of inactivator | No. of inactivator incorporated |
|--------------|------------------------------------------|----------------------------------------|---------------------------|--------------------------------|
| Diazoacetyl-DL-norleucine methyl ester | 17% | 0% | 100% | 1 residue of norleucine incorporated |
| p-Bromophenacyl bromide | 17% | 8% | 92% | Not determined |
| EPNPb | 17% | 0% | 100% | 2 residues of DPNP incorporatedc |

a The norleucine incorporation was determined by amino acid analysis according to the method of Rajagopalan et al. (3).
b EPNP is the abbreviation of 1,2-epoxy-3-(p-nitrophenoxy)propane.
c The EPNP content in pepsin was determined spectrophotometrically according to the method of Tang (15).

The treated enzyme showed a marked increase in absorbance in the wave length region 235 to 280 nm over that of untreated pepsin (Fig. 2). Although Yankeelov reported that biacetyl-modified arginine can be partially regenerated by salt (30), we observed no recovery of lost enzyme activity upon incubation of biacetyl-treated pepsin in 1 M NaCl, pH 6.0.

Biacetyl-treated pepsin was further incubated with three other known inactivators of pepsin. The first, diazoacetyl norleucine methyl ester (3), completely inactivated the biacetyl-treated pepsin (Table II). Amino acid analysis showed that 1.3 residues of norleucine were incorporated. The second, 1,2-epoxy-3-(p-nitrophenoxy)propane (15), likewise completely abolished the remaining activity of biacetyl-treated pepsin. The third, p-bromophenacyl bromide (18), reduced the remaining enzymic activity of biacetyl-treated pepsin, but (as in the inactivation of native enzyme) failed to abolish it altogether (Table II).

**Table III**

| Additions | Concentrations | pH 6.0 | pH 1.0 |
|-----------|----------------|--------|--------|
| None | - | % | % | % | % |
| Biacetyl | 1.0 | 0 | 100 | 0 | 100 | 0 |
| Biacetyl + Ac-Tyr-Tyr | 6.16 x 10^{-4} | 66 | 34 | 66 | 34 |
| Biacetyl + Z-Glu-Phe | 1.16 x 10^{-5} | 53 | 47 | 53 | 47 |
| Biacetyl + APDTa | 5.50 x 10^{-5} | 54 | 46 | 54 | 46 |

a The abbreviations used are: Ac-Tyr-Tyr, N-acetyl-L-tyrosyl-L-tyrosine; Z-Glu-Phe, N-carbobenzoxy-L-glutamyl-L-phenylalanine; and APDT, N-acetyl-L-phenylalanyl-L-diiodotyrosine.

![Figure 3. The effect of synthetic substrate, N-acetyl-L-tyrosyl-L-tyrosine, on the rate of inactivation by biacetyl and on the rate of arginine loss. Keys: -P, and -P, inactivation of peptic activity in the presence and absence of substrate; and -Arg, and -Arg, the loss of arginine in the presence and absence of substrate.](http://www.jbc.org/)

Effect of Synthetic Substances on Inactivation of Pepsin by Biacetyl—To determine whether the biacetyl-modified arginyl residue is located in or near the active center of pepsin, inactivation was carried out in the presence of a number of known synthetic enzyme substrates. In consequence, the rate of inactivation was found to be significantly reduced by N-acetyl-L-phenylalanyl-L-diiodotyrosine and N-acetyl-L-tyrosyl-L-tyrosine (Table III). Experiments carried out in pH 6 and 3 had the similar results. N-Carbobenzyoxylglycyl-L-phenylalanine and its amide, two poor substrates, and glycylglycine had no protecting effect when present at 1 mM. The course of inactivation, as well as the loss of arginine by the reaction of biacetyl, was measured in both the presence and the absence of substrate (Fig. 3). In the presence of synthetic substrate, the rates of loss of enzyme activity and arginine residue were slower than in control experiments. When the data obtained at 3, 6, and 9 hours were averaged (Fig. 3), N-acetyl-L-tyrosyl-L-tyrosine...
Modification of an Arginine in Pepsin

Amino acid composition, amino-terminal residues, and identified peptides which moved toward the anode were omitted from the figure.

Identification of Modified Arginyl Residue—Since the amino acid sequence around the 2 arginyl residues in porcine pepsin is known (20–22), experiments were designed to determine which of the arginines was modified by biacetyl. Our approach was similar to that used to determine the arginine sequences in human pepsin (23). Pepsin and biacetyl-modified pepsin in 0.25% concentrations were digested separately by ø-chymotrypsin. The peptide mixtures were subjected to high voltage electrophoresis at pH 6.0. In the native pepsin digest, only two peptide spots, A1 and A2, migrated toward the cathode (Fig. 4). These two peptides were then recovered from preparative high voltage electrophoresis, and their amino acid compositions as well as their NH2-terminal residues were determined (Table IV). Peptide A1 was found to be derived from the sequence Asp–Arg–Ala–Asn–Asn–Ile–Arg–Glu–Tyr (17). The second peptide contained 9 residues from the carboxyl terminus of pepsin and had the sequence Asn–Lys–Val–Gly–Leu–Ala–Pro–Val–Ala. It contained no arginine.

The electrophoretic pattern of peptides derived from 9- and 24-hour digests of 0.25% biacetyl-treated pepsin showed a near-absence of Peptide A1 (Fig. 4), apparently due to the modification of arginine C-12 (12th residue from COOH terminus of pepsin) (20)). The electrophoretic pattern of Peptide A2 did not differ appreciably from that of biacetyl-modified pepsin (Fig. 4). However, Peptides A1 and A2 manifested the same color intensity either after prolonged digestion with ø-chymotrypsin or when the concentration of the modified pepsin was 1% instead of 0.25%. Thus, the hydrolysis by ø-chymotrypsin to form modified Peptide A1 was slower in modified pepsin, where arginine was 1 residue away from the bond hydrolyzed. Since the modified arginine is known to retain its positive charge (32)), modified Peptide A1 would migrate to the same position as the unmodified peptide.

Thus, the hydrolysis by ø-chymotrypsin to form modified Peptide A1 was slower in modified pepsin, where arginine was 1 residue away from the bond hydrolyzed. Since the modified arginine is known to retain its positive charge (32)), modified Peptide A1 would migrate to the same position as the unmodified peptide. These inferences led us to perform experiments to identify the biacetyl-modified arginine in Peptides A1 and A2 obtained from the digest of modified pepsin recovered from preparative high voltage electrophoresis.

Peptides A1 and A2 from biacetyl-pepsin were digested separately with aminopeptidase M. The resulting amino acid mixtures were subjected to high voltage paper electrophoresis at pH 3.5 (Fig. 5). Peptide A1 from either native or biacetyl-modified pepsin showed an arginine spot, with no additional spot near the region where the basic amino acids are located. The digest of Peptide A1 from native contained free arginine, as expected. However, the digest of Peptide A1 from biacetyl-treated pepsin contained lysine and only trace amounts of arginine. Additionally, this digest contained a spot with a relative mobility of 0.74 (the mobility of arginine being defined as 1.0); this was assumed to be the biacetyl-modified arginine. Since it is known that the.

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TABLE IV
Amino acid composition, amino-terminal residues, and identified sequences of peptides A1 and A2.

| Peptides | Amino acid composition | NH2-terminal | Sequencea |
|----------|------------------------|--------------|-----------|
| A-1      | Asp–Arg–Ala–Asn–Asn–Ile–Arg–Glu–Tyr | Asp         | Asp–Arg–Ala–Asn–Asn–Ile–Arg–Glu–Tyr |
| A-2      |                         | Ile          | Asn–Lys–Val–Gly–Leu–Ala–Pro–Val–Ala |

a Sequence was taken from the work of Dopheide et al. (20).

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Fig. 4. Pattern of high voltage paper electrophoresis of basic chymotryptic peptides from pepsin (P) and biacetyl-treated pepsin (BP). The Subscripts 9 and 20 indicate the hour of digestion of ø-chymotrypsin. The position of peptides are indicated in dark areas. The circled areas indicate that only faint spots were found for Peptide A1 from biacetyl-reacted pepsin. An amino acid mixture (A.A.) was also run to give the position of neutral amino acids (N) and arginine (Arg). The electrophoresis was carried out in pH 6.5 at 70 volts per cm for 60 min. The acidic peptides which moved toward the anode were unaltered from the figure.

Fig. 5. Amino acids from the aminopeptidase M hydrolysate of arginine peptides from pepsin (A1 and A2) and biacetyl-treated pepsin (B.A1 and B.A2). Hydrolysate of Peptide A1 from native pepsin (A1) and biacetyl-pepsin (B.A1) gave an arginine spot. Hydrolysate of Peptide A1 from native pepsin (A1) contained both arginine and lysine spots. The same hydrolysate from biacetyl-pepsin (B.A1) showed only traces of arginine (circled area) and an additional spot (B-Arg) is apparently biacetyl-modified arginine. The hydrolysate of biacetyl-modified B chain of insulin also gives only traces of arginine and a spot of B-Arg. Amino acid mixtures (A.A. and A.A.) were also run to give the positions of neutral amino acids (N), histidine (His), arginine (Arg), and lysine (Lys). The nature of three faint spots (circled area) near the neutral amino acids in Samples A1, B-A1, and B-Ins is not certain. The high voltage paper electrophoresis was performed in pH 3.5 at 60 volts per cm for 1 hour.
B chain of insulin can be completely hydrolyzed to free amino acid by aminopeptidase M (45), a digest of biacetyl-treated insulin B chain was also subjected to electrophoresis for comparison. This hydrolysis showed the presence of lysine, histidine, and trace amounts of arginine. However, the spot apparently representing the biacetyl-arginine was clearly present. From these results, we concluded that the biacetyl reacted with the native pepsin at the arginine residue which is in the sequence of Peptide A1: Asp—Arg—Ala—Asn—Asn—Lys—Val—Gly—Leu—Ala—Pro—Val—Ala. The COOH-terminal alanine is the COOH-terminal of pepsin. Therefore, the modified arginine must be located 12 residues from the carboxyl terminus of the enzyme.

Reaction of Biacetyl with Other Gastric Proteases—Several other gastric proteases, human gastricsin, human pepsin, and bovine rennin, were treated with biacetyl. As shown in Table I, enzymic inactivation of about 75 to 80% was observed in all three cases. The amino acid analysis of human enzymes showed a loss of about 1 residue of arginine.

**DISCUSSION**

The inactivation of pepsin by biacetyl is apparently due to the modification of arginine located 12 residues from the carboxyl terminus (C-12) in the enzyme molecule. Several pieces of evidence support this conclusion: (a) biacetyl-treated pepsin lost 1 residue of arginine, as determined by amino acid analysis, (b) the relationship between the loss of activity and loss of arginine remained about the same during the course of inactivation (Fig. 3), (c) the peptide containing arginine C-12 was nearly absent in the α-chymotryptic digest of biacetyl-treated pepsin (0.25% pepsin) while the peptide containing an arginine located 20 residues from the carboxyl terminus (C-20) remained essentially unchanged, (d) an enzymic hydrolysate of Peptide A1 containing arginine C-12, was almost devoid of free arginine and contained a ninhydrin-positive spot, apparently representing biacetyl-modified arginine (Fig. 5).

The modification of arginine C-12 caused a similar degree of decrease in the hydrolysis rate of both protein and dipeptide substrates. The dipeptide substrates contain two side chains which may participate in the binding to the active center of the enzyme. However, pepsin is known to contain not only two major side chain-binding sites, but also additional hydrophobic binding sites which interact with the side chains of amino acids that are 1 or 2 residues removed from the 2 major binding residues (46–48). The results of this study suggest that the loss of activity is due to an impairment of the primary binding or catalytic site of the enzyme.

The role of arginine C-12 in the catalysis of pepsin is uncertain. It is unlikely, however, that this arginine side chain participates directly in enzymic hydrolysis, since the enzyme remains partially active after modification. Besides, the biacetyl-modified pepsin remains reactive with other specific pepsin inactivators, i.e., diazocetyl-DL-norleucine methyl ester (3) and 1,2-epoxy-3-(p-nitrophenox)propane (15). Hartsuck and Tang (17) have postulated an arginyl residue of pepsin is involved in polarizing the carbonyl group of the peptide bond to be cleaved. Kitson and Knowles (49) have reported that treating pepsin with phenylglyoxal, an arginine-modifying reagent, produced 45% inactivation. We have confirmed this observation. They also reported a shift of pH-kat curve (on the low pH side) of about half-pH unit. They suggested that the arginine modified by phenylglyoxal (probably also arginine C-12) may form a salt linkage to a carboxyl group in the active center of the enzyme. Thus the modification would cause a shift in the pH-kat curve and in the pH-Amax curve. However, a change of pK in an active center residue mediated indirectly by the conformational change after arginine modification has not been ruled out. We feel that if arginyl C-12 plays a role as suggested by either Hartsuck and Tang (17) or Kitson and Knowles (49), a more complete inactivation would be expected after the modification.

The presence of substrates of pepsin slows the rate of inactivation. This protecting effect appears to be due to the binding of a substrate molecule in the active center of the enzyme. The fact that poor substrate and nonsubstrate peptides do not protect and that protection by substrates is observed at both pH 3 and 6 supports this view. However, the protection experiments do not establish the location of arginine C-12 in the three-dimensional structure of the enzyme. It is still possible, that the effect observed is due to a substrate-induced change of conformation in the pepsin molecule.

The absence of significant modification of arginine C-20 is probably due to a steric hindrance. Since biacetyl is not an "active center-directed reagent," the reactivity of two arginines should not differ significantly, if they are equally accessible. Therefore, it is possible that the side chain of arginine C-20 is relatively "buried" in the tertiary structure of the enzyme. Yankeelov (31) showed that biacetyl reacts with amino acid imidazole groups at a much slower rate. These minor reactions are probably not responsible for the inactivation of pepsin. No significant loss of lysine and histidine was evident after modification. Additionally, a number of studies on the modification of amino groups in pepsin have indicated that the loss of enzymic activity results from N-acetylation (50), from loss of amino groups (51), or from N-ethoxycarbonylation (52). All of these results argue against the modification of the amino terminus as the basis for inactivation.

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Modification of an Arginine in Pepsin

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Modification of an Arginyl Residue in Pepsin by 2,3-Butanedione
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