Modification of Arginine in Proteins by Oligomers of 2,3-Butanedione*

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ABSTRACT: A crystalline trimer and a crystalline dimer of 2,3-butanedione (biacetyl) have been prepared which selectively modify arginine at neutral pH and 25°. Free arginine reacts with the dimer to give an intermediate which is converted into three ninhydrin-positive products. The trimer reacts with arginine under the same conditions to give the same products, but also causes losses through α-amino group modification. The adducts resemble free arginine in amino group reactivity, but do not react with Sakaguchi reagent. These products are stable in neutral solution, arginine being released in only 4% yield after 50-hr incubation at pH 7.0, 37°. Acid hydrolysis of the derivatives mainly produces materials not seen during automatic amino acid chromatography, but also releases 12% arginine. Arginine recoveries from modified proteins may be corrected accordingly. Trimeric biacetyl modified 93% of the arginine and 38% of the lysine in bovine plasma albumin. Under identical conditions the dimer shows similar specificity and reactivity. Enzymatic activity of bovine pancreatic ribonuclease A was retained (45%) when more than three of the four arginine residues of that protein were modified by the trimer. By adjustment of conditions, side reactions with lysine in bovine plasma albumin and bovine pancreatic ribonuclease A were reduced to *ca.* 3% while arginine modification was maintained at 50–60%. The results suggest that these oligomers of biacetyl have potential for structure–function studies of proteins.

Reagents for the modification of guanidinum groups in proteins under mild conditions have been uncovered only very recently. An early report indicated that 2,3-butanedione (biacetyl) could be self-condensed to give a reagent capable of modifying the guanidinium group at pH 8 (Yankeelov *et al.*, 1966). Shortly thereafter, related procedures were utilized to modify arginine at active sites of antibodies (Grossberg and Pressman, 1968). In addition, biacetyl was used to implicate arginine in the function of carboxypeptidase A (Vallee and Riordan, 1968). A crystalline trimer of biacetyl was also reported to be an effective reagent for arginine in proteins at neutral pH (Yankeelov *et al.*, 1968). In other studies under mild conditions glyoxal (Nakaya *et al.*, 1967) and phenylglyoxal (Takahashi, 1968) have been used.

A need for mild arginine reagents was clear from the work of Koshland and Englberger (1963). This work stimulated our interest in the problem. From mechanistic considerations it appeared likely that simple α-diketones would react with the guanidinium group under mild conditions. Accordingly, biacetyl was selected for study as a modifying agent. Under conditions initially employed for this reagent, however, reaction appeared to occur after a preliminrary self-condensation (Yankeelov *et al.*, 1966).

The present communication, an outgrowth of our earlier work, describes (a) the preparation of a crystalline dimer and a crystalline trimer of biacetyl, (b) the reactions of these oligomers with free arginine, and (c) the actions of these reagents on bovine pancreatic ribonuclease A and bovine plasma albumin.

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Materials and Methods

This study employed the following materials listed with their corresponding sources: 2,3-butanedione (99.9% purity), Matheson Coleman and Bell; arginine (Grade A, free base), Calbiochem; powdered glass SC-12300, E. H. Sargent and Co.; RNA (sodium salt), Schwartz; bovine albumin, Mann Research Laboratories; bovine pancreatic ribonuclease A (Type III-A), Sigma Chemical Co.; trinitrobenzenesulfonic acid, Eastman. Other reagents were of analytical grade or the highest quality available. Sodium phosphate buffers, redistilled organic solvents, and distilled, deionized water were used throughout these studies.

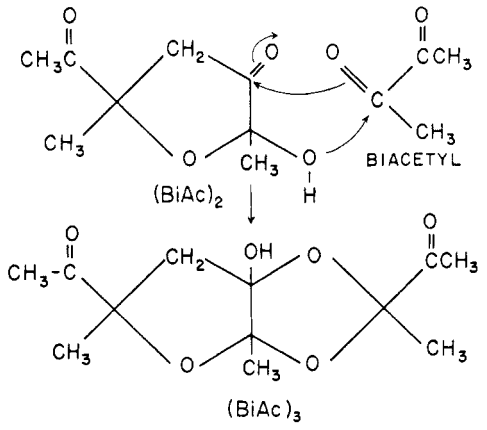
Melting points were taken on a Fisher–Johns apparatus with dealkalinized cover glasses (Shriner *et al.*, 1964). Elemental compositions and molecular weights were determined by Galbraith Laboratories, Inc., Knoxville, Tenn. Infrared, ultraviolet, and nuclear magnetic resonance spectra were obtained with Perkin-Elmer Infracord, Zeiss PMQ II and Varian A-60A spectrophotometers, respectively. Colorimetric measurements were made on a Bausch and Lomb Spectronic 20 spectrophotometer. Determinations of pH were made on a radiometer Type 4c meter.

Complete amino acid analyses (21-hr separations) were done with a Technicon Type NC-1 analyzer with Type B resin. Calculated recoveries of amino acid residues were based on the combined content of aspartic acid, valine, and phenylalanine unless otherwise specified. Separate analyses of the basic amino acids of protein hydrolysates or modified samples of free arginine were accomplished by special adaptation of short columns. Beckman Type AA-27 resin was used in a 15-cm column for analyses of protein hydrolysates while

Technicon Type B resin was employed in a 12-cm column to examine reactions of free arginine. The 15- and 12-cm columns were operated at 50° with pH 5.35 and 5.28 citrate buffers, respectively (Spackman *et al.*, 1958).

Nitrogen was determined as described by Lang (1958). Amino groups were analyzed by trinitrobenzenesulfonic acid in borate buffer according to Habeeb (1966, 1967). Both reagent and protein blanks were included. Manual ninhydrin analyses with dimethyl sulfoxide as solvent were performed according to Moore (1968) while Sakaguchi estimation of arginine was according to Izumi (1965). Protein samples were dialyzed at 4° unless otherwise indicated using aged size 8 dialysis tubing (Union Carbide Corp., Food Products Division). RNase A[1] was assayed with dialyzed RNA according to Kalnitsky *et al.* (1959) with activity losses estimated by reference to a standard curve for untreated enzyme.

Crystalline Trimeric Biacetyl. 2,5-Diacetyl-3a,5,6,6a-tetrahydro-6a-hydroxy-2,3a,5-trimethylfuro[2,3-d]-1,3-dioxole $(BiAc)_3$. This compound was first described by Cresswell *et al.* (1961). Powdered glass was cleaned as described by Carlson (1960) and alkalinized by washing with excess 0.1 N NaOH. The glass was washed free of base and oven dried at 110°. Biacetyl (196 g, 2.28 moles) containing 0.1% water (v/v) was mixed with 415 g of the prepared glass in a closed vessel. The suspension, which gradually turns to a paste, was mixed twice daily. After 5–7 days at room temperature the mixture usually hardens. The product was extracted with ether and the extract dried over $MgSO_4$. Following removal of solvent and overnight storage in the cold, 76 g (39%) of $(BiAc)_3$ was collected. Recrystallization from ether gave colorless needles: mp 112.5–114° (lit.[2] mp 73–75°); λ_{max}^{KCl} 2.86, 5.82 μ; nmr ($CDCl_3$) τ 5.52 (1 H), 7.66 (3 H), 7.75 (3 H), 8.54 (3 H), 8.60 (3 H), 8.66 (3 H), 6.74 (d, J = 13.5 cps, 1 H), 8.09 (d, J = 13.5 cps, 1 H); $\lambda_{max}^{1\ mM\ HCl}$ 286 mμ (ϵ 84). *Anal.* Calcd for $C_{12}H_{18}O_6$: C, 55.81; H, 7.02; mol wt 258. Found: C, 56.00; H, 7.04; mol wt (osmometer, acetone), 256. This product is stable at room temperature if kept free of moisture.



Crystalline Dimeric Biacetyl. 5-Acetyltetrahydro-2-hydroxy-2,5-dimethyl-3-oxofuran $(BiAc)_2$. The following procedure was developed from the brief descriptions of Diels *et al.* (1914) and of Birch and Moye (1957). A solution of 1.0 N KOH (350 ml) was added dropwise to a stirred solution of 100 ml of biacetyl (1.14 moles) in 400 ml of water chilled to 0°. The addition was performed over a 55-min period while maintaining the temperature at 0 ± 2° by ice bath cooling. Fifteen minutes after the KOH addition had been completed, the solution was brought to pH 2.3 by addition of concentrated H_2SO_4 while maintaining temperature control. The resulting solution was saturated with NaCl and extracted with ether. The extract was dried over $MgSO_4$. Removal of solvent produced a yellow oil which was distilled through a 10-cm Vigreux column at 0.2–0.3 mm. The fractions boiling at 95–102° (n_D^{20} 1.457–1.459) were collected to give 67.5 g of purified dimer. In the absence of seeds, crystallization occurred after 19 days of storage at −20° with periodic thawing. Vacuum filtration followed by pressing with filter paper gave 26 g (27%) of crystalline dimer. When distillates were seeded immediately the yield was raised to 45–48%. Recrystallization from ligroin (60–90°) produced colorless needles, mp 59–61° (lit. (Birch and Moye, 1957) mp 51–54°): λ_{max}^{KCl} 2.97, 5.68, 5.82 μ; nmr ($CDCl_3$) τ 5.60 (1 H), 7.68 (3 H), 8.53 (6 H), 6.66 (d, J = 19 cps, 1 H), 7.80 (d, J = 19 cps, 1 H); $\lambda_{max}^{1\ mM\ HCl}$ 279 mμ (ϵ 47). *Anal.* Calcd for $C_8H_{12}O_4$: C, 55.81; H, 7.02; mol wt 172. Found: C, 55.83; H, 7.10; mol wt (osmometer, acetone), 173. This compound was stored with desiccant at −20°.

Protein Modifications. Proteins were dissolved (6 mg/ml) in a freshly prepared solution of 9.3% (w/v, *ca.* 0.4 M) $(BiAc)_3$ in 0.5 M, pH 7.0, sodium phosphate buffer. The reaction mixtures were incubated at 25° in a constant temperature bath and protected from light.[3] Aliquots were removed at specified intervals and dialyzed against water (1000 volumes) for 24 to 48 hr with several changes. These conditions for modification will be referred to as standard conditions in the text. Retentates were lyophilized and hydrolyzed (6 N HCl, 110°, 24 hr) prior to automatic analysis.

To achieve highly specific modification of RNase A the standard conditions were adjusted as follows. (1) $(BiAc)_3$ and buffer concentrations were lowered to 0.2 M. (2) The pH was lowered to 6.0. (3) Reaction time was restricted to 6 hr. (4) Samples were dialyzed against pH 5.1, 0.01 M sodium acetate buffer instead of water.

Colorimetric Assays. Nitrogen was determined after protein solutions were dialyzed free of reagents. Readings were made at 420 mμ. Aliquots of retentates containing 0.6–1.0 mg of protein were assayed for amino groups with 2,4,6-trinitrobenzenesulfonic acid. Amino acids from protein hydrolysates were dissolved at 1 mg/ml with water. Subsequent dilutions were made in water and pH 5.5, 4 M sodium acetate buffer for Sakaguchi and ninhydrin determinations, respectively. The Sakaguchi color of each sample was normalized according to its ninhydrin or nitrogen value.

[1] Abbreviations used are: $(BiAc)_3$ or trimeric biacetyl, 2,5-diacetyl-3a,5,6,6a-tetrahydro-6a-hydroxy-2,3a,5-trimethylfuro[2,3-*d*]-1,3-dioxole; $(BiAc)_2$ or dimeric biacetyl, 5-acetyltetrahydro-2-hydroxy-2,5-dimethyl-3-oxofuran; RNase A, bovine pancreatic ribonuclease A.

[2] This compound exhibits a higher melting form of mp 121–122° not encountered in many of the earlier preparations. The melting points reported here were obtained with dealkalinized glass cover slips. It is likely that the mp of 73–75° reported by Cresswell *et al.* (1961) is related to alkaline glass surface effects (Shriner *et al.*, 1964).

[3] In earlier studies (Yankeelov *et al.*, 1968), when no precaution was taken to exclude light, histidine losses were detected in modified bovine plasma albumin.

TABLE I: Comparison of Arginine and Derivatives.

Property	0.1 M Sodium Acetate Buffer	
	Arginine Free Base	Arginine Derivatives
Weight of sample per volume of buffer (μg/ml)	5,220	12,960
Observed (μg/ml) nitrogen content	1,681	1,687
Molarity based on nitrogen determination[a]	0.0300	0.0301
Estimated molecular weight[b]	174 (Theory: 174)	430 (Theory: 432)
2,4,6-Trinitrobenzenesulfonic acid color[c] A_{335}	1.33	1.32
Ninhydrin color[d] A_{570}	0.550	0.550
Sakaguchi color[e] A_{520}	1.020	0.0052

[a] Expressed as arginine equivalents per liter. Theoretical nitrogen content of arginine: 32.16%. [b] Based on sample weights, nitrogen determination, and the assumption that no nitrogen loss occurs in the formation of derivatives. The theoretical weight of adducts of 3 moles of biacetyl/mole of arginine is 432. [c] 1 ml of $^1/_{60}$ dilution assayed, 1.00-cm path length. [d] 0.1 ml of $^1/_{15}$ dilution assayed, 1.00-cm path length. [e] 1 ml of a $^1/_{150}$ dilution of arginine assayed; derivatives assayed at 10-fold higher concentration and adjusted A_{520} recorded for direct comparison. Samples read in 0.5-in. matched test tubes.

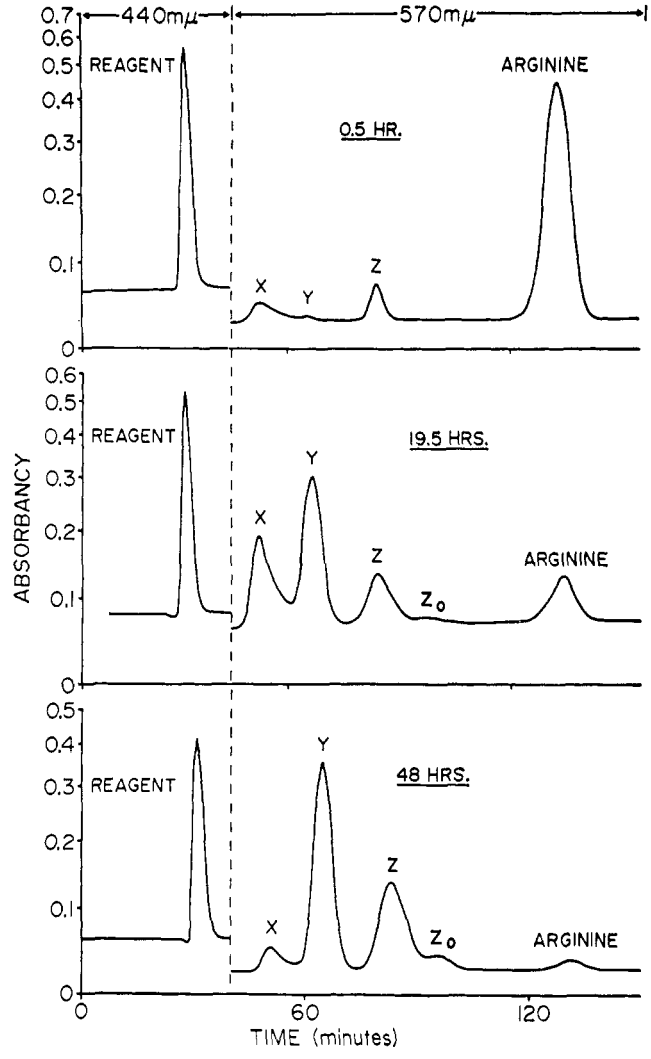


FIGURE 1: Chromatographic profiles of reaction mixtures of $(BiAc)_2$ with free arginine. Reaction was allowed to occur in 0.5 M, pH 7.0 phosphate buffer (25°). Arginine and $(BiAc)_2$ concentrations were 10 and 80 mM, respectively. At the times indicated samples were removed and treated with 3 volumes of 0.25 N HCl. The acid-quenched mixtures (0.08 ml) were analyzed on the 12-cm column of the amino acid analyzer. Excess reagent appears at column volume producing greater color at 440 than 570 mμ. Z contains background ammonia.

Results and Their Evaluation

Reactions of Free Arginine with $(BiAc)_2$ and $(BiAc)_3$. Figure 1 shows chromatographic profiles of a reaction mixture of arginine and $(BiAc)_2$ after increasing periods of reaction. These results suggest that modification occurs exclusively at the guanidinium group. The reaction proceeds through an intermediate designated X which is converted into products Y, Z, and Z_0, the latter being a minor component emerging after, but incompletely resolved from Z. Intermediate X is not detected on the 130-cm column. In contrast, Y, Z, and Z_0 appear (in that order) fully resolved by the 21-hr separation (not shown).

The time dependency of guanidinium group modification of arginine by $(BiAc)_3$ is less readily explored by amino acid chromatography since excess reagent causes substantial modification of the α-amino group. Reaction of arginine with stoichiometric amounts of $(BiAc)_3$ produces the same ninhydrin-positive products (Y, Z, and Z_0) as the $(BiAc)_2$–arginine reaction, although more than a single intermediate is detectable by amino acid chromatography. The final products from the two reactions are indistinguishable in four chromatographic systems.

Preparation of Products from Arginine–$(BiAc)_2$ Reaction. Arginine hydrochloride (2.11 g, 10 mmoles) was added to a solution of $(BiAc)_2$ (2.58 g, 15 mmoles) in 100 ml of 0.1 M, pH 7.0 phosphate buffer. The solution was allowed to stand in a dark vessel at room temperature (*ca.* 25°) for 5 days. The reaction mixture was filtered to remove a small amount of insoluble material. Short-column analysis revealed that both arginine and intermediate X had been reduced to 1% of the arginine treated. The filtrate was lyophilized and the resulting tan powder was triturated with ether and the extract discarded. The remaining solid was extracted with absolute ethanol (2 × 80 ml) and the extract was filtered. Removal of the ethanol at reduced pressure left a residue of products which was dissolved in 25 ml of water and treated with Norit. The solution was applied to a Sephadex G-10 column (5.2 × 100 cm) and eluted with 0.1 M, pH 5.0 pyridine–acetate buffer at 100 ml/hr. Fractions of 20 ml were collected. A single

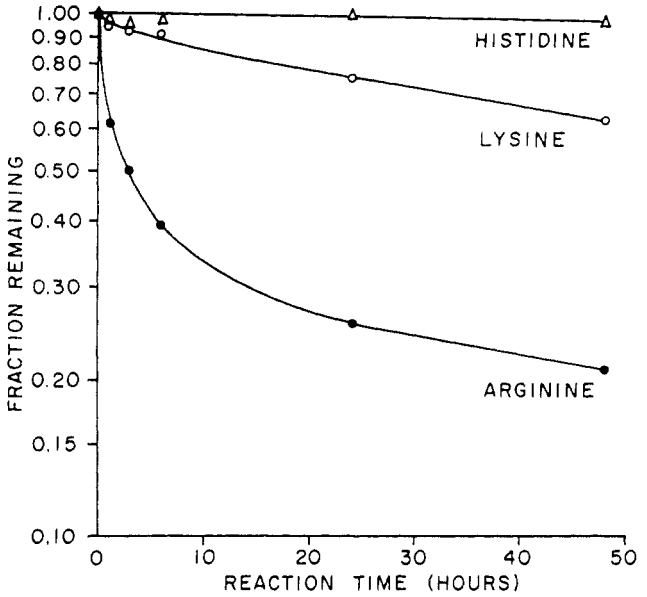


FIGURE 2: Semilogarithmic plot showing time dependence of amino acid recoveries from RNase A treated with $(BiAc)_3$ under standard conditions. Aliquots were removed at fixed intervals, dialyzed against water, and lyophilized prior to hydrolysis. The data have not been corrected for regeneration of arginine.

ninhydrin-positive peak emerged (fractions 44–50) which was collected and treated with Norit. The colorless solution was lyophilized and further dried at 0.040 mm (25°) to give 1.92 g of a white solid (44% yield based on adducts containing 3 moles of biacetyl per mole of arginine).

Properties of the Derivatives. The properties of arginine and derivatives Y, Z, and Z_0 prepared from arginine and

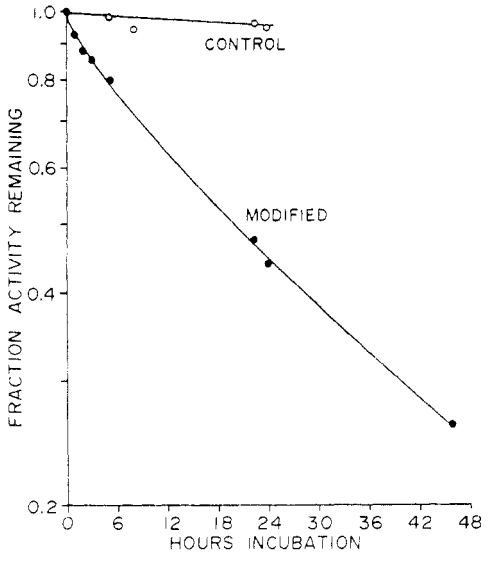


FIGURE 3: Semilogarithmic plot of fraction RNase activity remaining against time of incubation of RNase A with $(BiAc)_3$ under standard conditions. Aliquots were removed at fixed intervals, diluted directly in cold, pH 5.2 acetate–KCl buffer (ionic strength = 0.4), and assayed at this pH with RNA as substrate according to Kalnitsky *et al.* (1959). The control sample was treated identically with the exception that $(BiAc)_3$ was omitted.

TABLE II: Comparison of $(BiAc)_2$- and $(BiAc)_3$-Treated Proteins.[a]

Protein	Reagent	% Modified	
		Arginine[b]	Lysine
Bovine plasma albumin	$(BiAc)_2$	77	18
	$(BiAc)_3$	83	24
RNase	$(BiAc)_2$	70	22
	$(BiAc)_3$	85	25

[a] Proteins were treated with 0.4 M reagent for 24 hr under standard conditions. [b] Data for arginine are corrected for regeneration of arginine during acid hydrolysis by dividing the observed loss by 0.88.

$(BiAc)_2$ are compared in Table I. Solutions were prepared with the assumption of molecular weights of 432 and 174 for the derivatives and arginine, respectively. The equal nitrogen values show that the mixture of products contains 3 moles of biacetyl per mole of arginine. The ninhydrin color yields are identical while the nearly equal 2,4,6-trinitrobenzenesulfonic acid colors indicate that no 2,4,6-trinitrobenzenesulfonic acid reactive nitrogen is present in side chains of these products. The Sakaguchi assay gives a color yield for the derivatives that is less than 1% that obtained for free arginine. The latter result shows that arginine is not released under the strongly alkaline conditions of the assay. When these derivatives are incubated in 1 M NaCl–0.05 M, pH 7.0 phosphate buffer at 37° for 50 hr, the Sakaguchi color yield increased to only 5% that of arginine. The absorption spectrum of the products (not shown) has bands at 257 and 287 mμ.

The extent of hydrolytic release of arginine from the purified products was determined in the following way. A stock solution was prepared that was 12 mM in arginine derivatives and 2 mM in leucine added as an internal standard. This stock solution (1 ml) was diluted with an equal volume of 12 N HCl, hydrolyzed at 110° for 24 hr, and subjected to complete amino acid analysis. An identical aliquot of the original stock solution was diluted with pH 2.2 citrate buffer and also analyzed. The extent of arginine regeneration was 12% based either on absolute recoveries or the use of leucine as an internal standard. Only traces of other hydrolytic fragments were found on the 21-hr chromatogram. One of these components (*ca.* 2%) appears just prior to the usual position of lysine while a second (3–4%) is eluted before arginine.

Comparison of $(BiAc)_2$ and $(BiAc)_3$ as Protein Reagents. Table II compares the effects of the two oligomers of biacetyl on bovine plasma albumin and RNase A. The data show that while the reagents have similar specificity under standard conditions, $(BiAc)_3$ modifies both proteins more extensively. Since $(BiAc)_3$ is also more conveniently prepared and stored, it was chosen for more complete study.

Modification of RNase A at Neutral pH. Figures 2 and 3 reveal that arginine of RNase A treated with $(BiAc)_3$ under standard conditions is lost much more rapidly than enzymatic activity. For example, after 6-hr reaction, over 60%

TABLE III: Amino Acid Analysis of RNase A Modified with $(BiAc)_3$.[a]

Residue	Theory	Native	Modified
Aspartic acid[b]	15	15.0	15.0
Threonine[c]	10	9.64	9.94
Serine[c]	15	14.2	14.3
Glutamic acid	12	12.0	12.2
Proline	4	4.01	4.21
Glycine	3	3.06	3.10
Alanine	12	12.0	12.1
Cystine/2	8	6.16	6.54
Valine	9	8.83	8.98
Methionine	4	3.53	3.59
Isoleucine	3	2.34	2.43
Leucine	2	2.01	2.02
Tyrosine[c]	6	5.96	4.49[d]
Phenylalanine	3	2.88	2.96
Lysine	10	9.89	6.26
Histidine	4	3.76	3.70
Arginine	4	3.85	0.818

[a] Sample modified for 48 hr under standard conditions. [b] Aspartic acid taken as reference amino acid. [c] Correction factors for losses during hydrolysis: threonine, 1.05; serine, 1.12; tyrosine, 1.14 (Ooi *et al.*, 1963). [d] Recovery of tyrosine in modified protein is quantitatively restored by including 10- to 100-fold excess phenol in the hydrolysis mixture (see Discussion).

of the arginine was modified while only 20% of the enzymatic activity was eliminated. After 24-hr treatment, more than three of the four arginines present in the molecule were modified while 45% of the enzymatic activity was retained. Table III gives the complete amino acid composition of this protein after 48-hr treatment with $(BiAc)_3$ under standard conditions. The structure–function implications of these observations will be discussed later.

These results with RNase as well as results with bovine plasma albumin reported earlier (Yankeelov *et al.*, 1968) were obtained at neutral pH and high reagent concentration where significant losses of lysine also occur. It was, therefore, of interest to seek conditions that might permit increased specificity without decreased efficacy.

Concentration Dependence. The dependency of amino group and arginine loss from bovine plasma albumin on reagent excess is shown in Figure 4. The upper limit of $(BiAc)_3$ concentration was 9.3% (w/v, *ca.* 0.4 M) or 192-fold excess relative to arginine in bovine plasma albumin. The dependency of amino group loss (curve 1-A) with molar excess of reagent is not markedly different from the dependency of arginine loss (curve 1-B). Consequently, only a slight improvement in specificity can be achieved by working at reduced trimer concentration at neutral pH.

Curve 2 of the same figure indicates that 5–10% more arginine remains modified when reagent is removed by dialysis against water rather than neutral, high salt buffer. The difference is due to a small quantity of reversibly modified arginine remaining in the water dialyzed samples.

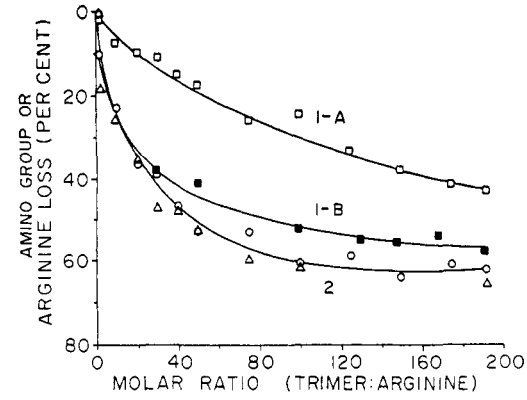


FIGURE 4: Dependence of amino group and arginine loss from bovine plasma albumin on molar ratio of $(BiAc)_3$ to protein arginine. Ratios were calculated from an assumed mol wt of 66,000 and an arginine content of 23 residues for bovine plasma albumin (Spahr and Edsall, 1964). Weighed quantities of $(BiAc)_3$ were added to bovine plasma albumin (6 mg/ml) in pH 7.0 phosphate buffer and the solutions were incubated at 25° for 24 hr. Curves 1-A (□) and 1-B (■) are data for amino groups (2,4,6-trinitrobenzenesulfonic acid) and arginine (amino acid analysis), respectively. Reaction in 0.2 M buffer was followed by dialysis against 1 M sodium chloride–0.05 M, pH 7.0 phosphate buffer with a terminal dialysis against 0.05 M phosphate buffer. Curve 2 shows arginine loss when reaction occurred in 0.1 M buffer and dialysis was restricted to water: (○) Sakaguchi assay; (△) amino acid analysis.

pH Dependence. Preliminary studies with $(BiAc)_3$ (J. A. Yankeelov, Jr., unpublished data, 1969) revealed that while both arginine and lysine modifications were enhanced at alkaline pH, there is an overall loss in specificity. Conversely, slightly acidic pH improves the specificity of the reagent. For example, when bovine plasma albumin was treated with $(BiAc)_3$ in pH 6.0 phosphate buffer under otherwise standard conditions, lysine loss was markedly reduced (Figure 5). After 6-hr exposure at the lower pH, lysine loss was 3% while arginine loss was 53%. After 48-hr reaction lysine loss was only 10% while arginine loss progressed to 70%. A more stringent test of this improved specificity

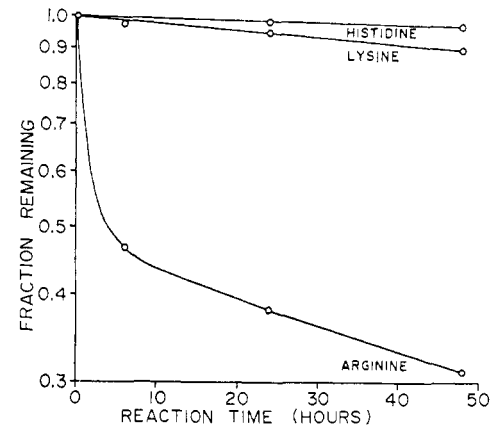


FIGURE 5: Semilogarithmic plot showing time dependence of amino acid recoveries from bovine plasma albumin treated with $(BiAc)_3$. The conditions were identical with those given for Figure 2 with the exception that the pH during modification was 6.0 instead of 7.0.

was performed with RNase A as described below. This enzyme is known to have an amino-terminal lysine, the α-amino group of which is accessible to other reagents (Takahashi, 1968).

Specific Modification of RNase A. RNase treated with 0.2 M $(BiAc)_3$ at pH 6.0 for 6 hr had 50–60% of its arginine modified as measured by either amino acid chromatography or Sakaguchi assay. Amino group loss was 2% by 2,4,6-trinitrobenzenesulfonic acid assay which was in good agreement with a 97% recovery of lysine found by automatic chromatography. The treated protein, with two of its four arginine residues modified, retained 80% of its activity toward RNA. The modified protein was found to have an $A_{277.5}$ only 7% greater than the native enzyme. The modification occurring at the lower pH is largely reversible since dialysis against 1 M NaCl–0.05 M, pH 7.0 phosphate buffer at room temperature for 48 hr resulted in 77% regeneration of modified arginine. This high level of reversibility is in sharp contrast to that found for the products obtained at pH 7.0 or 8.0 (see Discussion).

Discussion

Arginine in proteins can be modified with $(BiAc)_3$ or $(BiAc)_2$. The trimeric reagent can be simply prepared and is stable. Although chromatographic study of reaction of the two reagents with free arginine indicates that the $(BiAc)_3$ reaction occurs by the more complex path, $(BiAc)_3$ and $(BiAc)_2$ produce the same irreversible arginine derivatives. Three products (two major, one minor) are formed which contain an average of 3 moles of biacetyl/mole of arginine. At neutral or mildly alkaline pH, $(BiAc)_3$ extensively converts arginine in proteins into the irreversible products. Under these conditions a significant side reaction of amino groups also occurs.

The extent of hydrolytic regeneration of arginine from its derivatives under conditions routinely used for protein hydrolysis is 12%. This extent of regeneration is also consistent with residual arginine found in exhaustively modified, performic acid oxidzied RNase (J. A. Yankeelov, Jr., unpublished observations, 1969) where all arginines are presumably exposed. Thus, the corrected loss of arginine can be obtained by dividing the observed loss by 0.88.

The action of $(BiAc)_3$ on free amino acids other than arginine was studied earlier (Yankeelov *et al.*, 1968). While observation of a side reaction of amino groups of proteins was in accord with the amino acid study, a side reaction with tyrosine of proteins, suggested by low tyrosine recoveries from hydrolysates, was anomalous since free tyrosine was unreactive to $(BiAc)_3$. Accordingly, hydrolysis of modified proteins in the presence of excess phenol restored tyrosine recoveries, but was without effect on arginine or lysine. Subsequent experiments have verified that the tyrosine anomaly is linked to the presence of modified arginine during the hydrolytic step (J. A. Yankeelov, Jr., unpublished observations, 1969).

Although recoveries of the remaining amino acids from modified proteins have been very satisfactory, reactions of $(BiAc)_3$ or $(BiAc)_2$ with other protein side chains cannot be ruled out unequivocally. In any case, the study of free amino acids suggests such reactions in proteins would not exceed the lysine reaction in importance.[4]

If the standard reaction conditions for $(BiAc)_3$ are altered by lowering the pH to 6.0 and restricting the exposure of the protein, two marked changes in reaction character occur. (1) The modification becomes highly specific for arginine and (2) the residues blocked can be released by dialysis against neutral, high salt buffer. The most likely explanation for this dual nature of the reagent is that the lower pH restricts the reaction to formation of adducts which ordinarily serve as intermediates at higher pH. While proteins often become colored after extensive modification at neutral pH, they remain colorless at pH 6.0.

In the light of the above considerations it is appropriate to discuss implications of the results obtained with RNase A. Modification of RNase A with 0.2 M $(BiAc)_3$ at pH 6.0 resulted in a loss of two of its four arginine residues, but a loss of only 20% of its enzymatic activity. Under these conditions total lysine loss by a combination of deamination (if present) and adduct formation was near the level of experimental error (0.2–0.3 residue).

Modification of RNase A under standard conditions caused more rapid loss of arginine than enzymatic activity. The results obtained under both sets of conditions suggest no essential role for arginine. More specifically the data indicate that integrity of three of the four arginines present in the enzyme is not essential for binding or catalytic function.

Surprisingly, phenylglyoxal has recently been shown to rapidly inactivate RNase A while concomitantly modifying two to three residues of arginine (Takahashi, 1968). Takahashi suggested, as one of several possible interpretations, that the inactivation may have been due to the introduction of bulky phenyl groups into the protein. The observations of the present study clearly support this alternative.

The four arginines present in RNase A are located at positions 10, 33, 39, and 85. According to Wyckoff *et al.* (1967) arginine 33 in RNase S has a charge–charge interaction with aspartic acid 14 of the S peptide. Such an interaction is consistent with studies of Hofmann and coworkers (Hofmann *et al.*, 1966) on the role of aspartic acid 14 in binding of S peptide to S protein. It is likely that similar interactions occur in RNase A. For these reasons we suggest that arginine-33 is also the residue most resistant to chemical modification.

The possibility that phosphate ion is protecting either arginine-39 or -85 is unlikely since similar results were obtained in sodium pivalate buffer. Furthermore, treatment of RNase A with $(BiAc)_2$ in *N*-ethylmorpholine buffer at pH 8.0 produced only modest losses in enzymatic activity (J. A. Yankeelov, Jr., unpublished observations, 1969).

In summary, results with bovine plasma albumin and RNase A show that arginine in proteins reacts selectively with $(BiAc)_3$ or $(BiAc)_2$ at neutral pH to produce stable derivatives. At pH 6.0 these proteins were modified specifically and reversibly by $(BiAc)_3$. Reaction at the lower pH may be particularly useful for study of proteins such as pepsin which denature under mildly alkaline conditions. Although several derivatives are formed and the precise nature of the products is dependent on reaction conditions, $(BiAc)_3$ and

[4] Although thin-layer chromatographic examination of reduced glutathione before and after $(BiAc)_3$ treatment revealed a sulfhydryl reaction, its relative importance in protein studies is yet to be assessed.

$(BiAc)_2$ appear to be useful reagents for the investigation of arginine in proteins.

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