α-Amino Alcohols as Products of a Reductive Side Reaction of Denatured Collagen with Sodium Borohydride*

Mercedes A. Paz, Edward Henson, Robert Rombauer, Lorraine Abrash,†
Olga O. Blumenfeld,‡ and Paul M. Gallop§

ABSTRACT: Evidence is presented for the occurrence of a small level of a peptide bond reductive side reaction, during the treatment of denatured collagen with sodium borohydride. The resulting α-amino alcohols led to the earlier and erroneous conclusion that α-amino aldehydes were present in this protein.

In this paper this conclusion is corrected. Experimental approaches with tritiated sodium borodeuteride which were used to establish the origins of α-amino alcohols are described. The use of tritiated sodium borodeuteride practically eliminates the chromatographic isotopic effect prevalent in the purification of derivatives of the amino alcohols when prepared with tritiated sodium borohydride. Accordingly, specific activity measurements can now clearly determine if one or two nonexchangeable tritium atoms are incorporated during a reduction, and this can be confirmed by mass measurements which establish whether one or two nonexchangeable deuterium atoms are also present.

In recent years two structural proteins, collagen and elastin, have been shown to contain covalently bound carbonyl compounds which appear to play a role in the biosynthesis of intra- and intermolecular cross-links. The structure of several of these carbonyl compounds has been established and, in the main, they appear to be derived biosynthetically by modification of lysyl residues within the backbone of the proteins (Paz *et al.*, 1969; Gallop *et al.*, 1968; Blumenfeld and Gallop, 1966; Franzblau *et al.*, 1969; Lent *et al.*, 1969; Partridge *et al.*, 1966; Bornstein and Piez, 1966; Bailey and Peach, 1968; Tanzer and Mechanic, 1968).

In most of these investigations, reduction with tritiated sodium borohydride has been employed to reduce the carbonyl compound or Schiff base in order to facilitate its isolation and characterization.

We have recently observed that during the reduction of carbonyl compounds in collagen a significant amount of peptide bond reduction may accompany the desired sodium borohydride reduction of the carbonyl moiety, particularly if the collagen is denatured and the reduction is performed at pH values above neutrality. Crestfield *et al.* (1963) reported some peptide bond cleavage during sodium borohydride reduction of ribonuclease.

In our earlier investigations we isolated α-amino alcohols and erroneously concluded that they could not have arisen from peptide bond reduction since they were onefold rather than twofold reduced. (Carbonyl compounds or Schiff bases incorporate one tritium/mole—onefold reduced; compounds resulting from the reduction of peptide bonds incorporate two tritiums/mole—twofold reduced.) The direct isolation from collagen of certain 2,4 dinitrophenylosazones in small amounts appeared to support our conclusion that α-amino aldehydes were components of collagen (Gallop *et al.*, 1968; Paz *et al.*, 1969). Reinvestigation of this problem using tritiated sodium borodeuteride, as described below, showed that the α-amino alcohols are indeed twofold reduced and that our earlier results led us to this erroneous conclusion by virtue of an isotope effect during purification. However, the isolation of osazones still remains unexplained.

Since the use of more drastic conditions of reduction with sodium borohydride in the more cross-linked collagens, elastin, and other biological materials may be a necessity, it is important that new products which are isolated be evaluated as to the number of reductive stages to differentiate between reductions from the carbonyl or carboxylic acid oxidation levels to alcohols or amines. Accordingly, the use of tritiated sodium borodeuteride should be helpful in this respect.

Materials

All chemicals used were reagent grade unless otherwise specified.

Ichthyocol was prepared from the tunics of carp swim bladders (Gallop and Seifter, 1963) and denatured by suspending the dried tropocollagen in water and heating 10 min at 60°. The gelatin solution was clarified by centrifugation. Protein concentration was obtained from its nitrogen content (Kjeldahl determination).

Tritiated sodium borohydride, [^{3}H]$NaBH_4$, was purchased from New England Nuclear Corp., Boston, Mass., and sodium borodeuteride, $NaBD_4$, from Alpha Inorganics, Inc., Beverly, Mass.

Preparation of 4-p-Nitrobenzamidobutyraldehyde. *p*-Nitrobenzoyl chloride (5.5 g) dissolved in 25 ml of *p*-dioxane was

* From the Department of Biochemistry and the Unit for Research in Aging, Albert Einstein College of Medicine, Yeshiva University, New York, New York. *Received November 14, 1969.* This work was supported by grants from the National Institutes of Health, U. S. Public Health Service Grants AM-12791, AM-05821, AM-03172, AM-11913, and HD-00674.

† Postdoctoral Research Fellow, Helen Hay Whitney Foundation, 9-5-26-0128-140.

‡ Development awardee, July 1967, U. S. Public Health Service (1-K3-GM-5998).

§ Recipient of Research Career Award 5-KG-AM-19435 from the U. S. Public Health Service.

added dropwise, at 0–5°, to a mixture of 4.0 g of 4-aminobutyraldehyde diethyl acetal (Aldrich Chem. Co.) and 4.0 g of triethylamine in 25 ml of *p*-dioxane. The mixture was stirred for 1 hr, at 0–5°, and water was added. An oil deposited, which was extracted into ethyl acetate, dried over Na_2SO_4, and concentrated to give 6.5 g (80%) of crude 4-*p*-nitrobenzamidobutyraldehyde diethyl acetal, which was crystallized from cyclohexane–ethyl acetate, mp 62–63°. Mass spectra showed a P-1 ion, typical of acetals, at *m/e* 309 (1.5%), a base peak at *m/e* 103 (100%) and other ions at *m/e* 150 (80%), 219 (26%), and 265 (16%). To 3 g of the recrystallized diethyl acetal dissolved in 15 ml of *p*-dioxane was added 15 ml of 0.1 N HCl and the mixture was refluxed for 30 min. After concentrating to an oil, the product was dissolved in ethyl acetate, and washed with water. The ethyl acetate solution was dried over Na_2SO_4 and again concentrated to an oil, which crystallized on addition of petroleum ether. The crude aldehyde was recrystallized from cyclohexane–ethyl acetate, mp 121–122°. Mass spectra showed a parent ion at *m/e* 236 (11.3%), a base peak at *m/e* 150 (100%), and ions at *m/e* 218 (3.5%), 207 (5.5%), 193 (19.5%), and 179 (10%).

Preparation of 4-p-Nitrobenzamidobutanol. *p*-Nitrobenzoyl chloride (1.86 g) was added to a mixture of 0.89 g of 4-aminobutanol and 0.80 g of sodium hydroxide in 20 ml of water at 10–15°. The mixture was stirred, at 10–15°, for 2 hr and extracted with ethyl acetate. The ethyl acetate solution was dried with Na_2SO_4 and concentrated to an oil which crystallized on addition of petroleum ether to give 2.0 g of 4-*p*-nitrobenzamidobutanol, mp 81°. Mass spectra indicated a parent ion at *m/e* 238 (11%), a base peak at *m/e* 150 (100%), and ions at *m/e* 221 (6%), 207 (9%), 193 (11%), and 179 (15%); the molar extinction coefficient was 11.3 at λ_{max} of 267 mμ.

Methods

Standardization of Tritiated Sodium Borodeuteride, [^3H]-NaBD$_4$. [^{3}H]$NaBH_4$ (12.6 mg) was added to 650 mg of $NaBD_4$ and the mixture was thoroughly ground together in an agate mortar.

Two model carbonyl compounds were reduced to alcohols and checked for specific activity in terms of disintegrations per minute per micromole and for the masses of the parent ions and other ions. A medium-resolution mass spectrometer (Hitachi RMU6E, with direct inlet to the source) was used. The mass spectra were compared with those of the corresponding alcohols prepared by direct synthesis. One model compound employed, δ-amino-γ-ketovaleric acid, has been used before (Gallop *et al.*, 1968). The use of the second model compound, 4-*p*-nitrobenzamidobutyraldehyde, is preferred, as the isolation of the corresponding alcohol is more rapid and expedient. Its reduction was carried out as follows: 10 mg of 4-*p*-nitrobenzamidobutyraldehyde dissolved in 3 ml of 1:1 ethanol–water was reduced with 10 mg of [^{3}H]-$NaBD_4$ for 1 hr at 25°. Reduction was terminated with addition of a few drops of 6 N HCl and 10 ml of water. The product, 4-*p*-nitrobenzamidobutanol, was extracted into ethyl acetate, which was then washed with water, dried over anhydrous Na_2SO_4, reduced in volume *in vacuo*, and chromatographed on thin layers of silica gel with ethyl acetate–benzene (7:3). The reduced compound (R_F 0.3) was eluted with warm alcohol and its specific activity determined as previously described (Gallop *et al.*, 1968) using a molar extinction coefficient of 11.3 at a λ_{max} of 267 mμ. The specific activity was found to be 4.8×10^6 dpm/μmole and the identical value was found in the reduction of the other standard, δ-amino-γ-ketovaleric acid.

4-*p*-Nitrobenzamidobutanol was examined in the mass spectrometer and found to have a spectrum identical with that of the authentic compound, prepared by synthesis, with the exception of the molecular ion (*m/e* 239) and other ions which now contained the deuterium present on the C_1 carbon atom.

Reduction of Acid-Soluble Carp Swim Bladder Collagen with Tritiated Sodium Borodeuteride; Isolation of Reduced α-Amino Alcohols as Their 2,4-Dinitrophenylamino Derivatives, Their Specific Activity Determination, and Mass Spectrometry. Denatured ichthyocol (3 g) in 150 ml of water was reduced with 32 mg of previously calibrated tritiated sodium borodeuteride. The reduction was carried out at pH 8–9 in 0.006 M Versene, at room temperature, for 1 hr and 20 min. The reduction was terminated by adding 1 N HCl to pH 4 and the solution was lyophilized.

The steps of the procedure which follow were described in our previous publication (Gallop *et al.*, 1968); they are (1) acid hydrolysis of reduced collagen, (2) separation of radioactive α-amino alcohols into basic and neutral fractions by chromatography on Dowex 1 (OH^-), (3) dinitrophenylation of the α-amino alcohols in the basic and neutral fractions, (4) chromatography of dinitrophenylated derivatives on silicic acid columns and two-dimensional chromatography on thin layers of silica gels, (5) specific activity determinations, and (6) mass spectrometry of isolated 2,4-dinitrophenyl α-amino alcohols.

The following modification in procedure was adopted in step 4 to ensure a more complete purification of the dinitrophenylated compounds. Each radioactive peak obtained from chromatography on silicic acid columns was rechromatographed by two-dimensional chromatography on thin layers of silica gels using solvents previously described (Gallop *et al.*, 1968); it was then eluted and the latter step was repeated before specific activity determination was performed.

Results

Mass Spectrometry of Reduced Deuterated 2,4-Dinitrophenyl-α-amino Alcohols. From the mass spectral examinations of reduced standard compounds, 2,4-dinitrophenyl-δ-amino-γ-hydroxyvalerolactone and 4-*p*-nitrobenzamido-1-butanol it was possible to establish the respective contents of hydrogen and deuterium in the sodium borodeuteride used for reduction. The masses of the parent deuterated compounds are, respectively, 282 and 239; those of the nondeuterium-containing compounds are 281 and 238. After appropriate corrections of peak intensities for isotopic contributions from naturally occurring ^{13}C, ^{15}N, and for the M-1 contribution in the spectrum of the authentic nondeuterated standards it was calculated that 3 ± 1% of the $NaBD_4$ was hydrogen. Thus, in the nondeuterated, synthetic 4-*p*-nitrobenzamido-1-butanol the intensity of the M-1 peak (237) was 8% of that of the molecular ion *m/e* 238. In the deuterated, tritiated compound, 11 ± 1% of the intensity of the molecular ion *m/e* 239 was found at *m/e* 238. Assuming

TABLE I: Molecular Weights and Specific Activities of Reduced Compounds or Their Derivatives Isolated from Ichthyocol Reduced with Tritiated Sodium Borodeuteride.

Compound	*m/e* of Parent Ions Detected in the Mass Spectrometer			Specific Activity, Moles of Tritium Incorporated/ Mole of Compound
	% Naturally Occurring	% Monodeuterio	% Dideuterio	
DNP-glycinol	227 (<1%)	228 (<1%)	229 (~99%)	1.75
DNP-α-alaninol	0	0	243 (100%)	1.79
DNP-serinol	0	0	259 (100%)	2.00
DNP-threoninol	0	0	273 (100%)	1.92
DNP-aspartol	0	0	269 (100%)	
O,N-diDNP-glycinol	0	0	395 (100%)	1.79
O,N-diDNP-threoninol	0	0	439 (100%)	1.80

that 8% of the M-1 resulted by hydrogen loss from fragmentation, approximately 3 ± 1% of the M-1 resulted from the hydrogen content of [^{3}H]$NaBD_4$, which results mainly (2%) from the [^{3}H]$NaBH_4$ used to tritium label the $NaBD_4$. Reduction of other compounds, which gave no significant M-1 ion during fragmentations, indicated that [^{3}H]$NaBD_4$ was 98–99% deuterium. This value permits to calculate the expected heights of parent peaks of the isolated reduced compounds whether they derive from the reduction of a carbonyl compound to an alcohol whereby 1 mole of deuterium is utilized per mole, or from reduction of a carboxyl group to an alcohol where 2 moles of deuterium is incorporated per mole; in addition this value helps to decide whether parent ions of nondeuterated reduced compounds are inherently present. Accordingly, in an unknown tritiated deuterated compound derived from carbonyl reduction the presence of M-1 ions greater than about 3% of the parent ions, either signifies that the M-1 ion arose from fragmentation, or that the parent ion of the nondeuterated compound is present; in compounds derived from carboxyl reductions, the parent ions will appear at two mass units higher than in the nondeuterated compounds. Peaks at masses M-1, having intensities higher than 6% are due either to fragmentation, or arise by virtue of the presence of parent ions of onefold reduced compounds; those at M-2 of intensities greater than background may signify that nondeuterated reduced compounds are naturally present. Appropriate corrections for isotopic contributions of naturally occurring ^{13}C, ^{15}N, and ^{18}O should be included in these calculations.

These observations applied to the isolated 2,4-dinitrophenyl-α-amino alcohols indicate that they all contained two deuteriums per mole at the alcohol position and consequently originated from peptide bond reduction. These results are shown in Table I. Only in the case of *N*-2,4-dinitrophenylglycinol a small amount (less than 1%) of onefold reduced compound was observed. A small level of compound containing no deuterium was also found indicating the presence of a trace of glycinol in the hydrolysate which was either a component of the protein or arose from an amino acid during hydrolysis.

Specific Activity of Reduced Deuterated 2,4-Dinitrophenyl-α-amino Alcohols. In the reduction of collagen with tritiated sodium borohydride an isotope effect was observed in the chromatography of α-amino alcohols on columns of silicic acid (Gallop *et al.*, 1968). Upon chromatography to constant specific activity a correction factor needed to be applied. No significant chromatographic separation due to isotope effect was encountered in the separation of deuterated dinitrophenylated compounds. This greatly facilitated their purification and calculation of specific activity, as no assumptions were required. In Table I are listed the specific activities of the isolated compounds. They are close to the values expected for twofold reduced compounds and are consistent with the mass spectral data.

Discussion

The results obtained in this study demonstrate that most of the α-amino alcohols isolated from sodium borohydride reduced denatured collagen derive from a side reaction involving the reduction of peptide bonds and are not, as previously concluded, evidence for the presence of α-amino aldehydes in the protein. The earlier and erroneous conclusion resulted from an incomplete and selective purification of the isolated dinitrophenylated compounds of lower specific activity values due in part to an isotope effect which promoted the separation of tritiated product from their carrier compounds. With the tritiated sodium borodeuteride employed in this study, most of the isotopic chromatographic effects were minimized and this facilitated the purification of the reduced compounds for specific activity determination; in addition mass determinations of the isolated compounds helped determine their origins.

It should be noted that the results obtained in this communication point to the ease of reduction of peptide bonds in denatured collagen during relatively mild conditions of reduction with sodium borohydride. This was not observed in native calf skin tropocollagen (Paz *et al.*, 1969), nor during reduction of hemoglobin at pH 7.5 (Bookchin and Gallop, 1968) but was observed during reduction of trypsin, at pH 9,[1] and could occur in other proteins depending on

[1] Unpublished experiments.

TABLE II: Relative Susceptibility of Amino Acid Residues in a Polypeptide Backbone to Reduction with Sodium Borohydride.

Amino Acid Residue	Ratio of % Radioactivity Recovered in α-Amino Alcohols to Residues/100 Residues		
	Ichthyocol	Calf Skin Collagen	Trypsin
Aspartic acid	2.3	2.2	2.4
Glutamic acid	1.1	1.1	0.4
Serine + threonine	3.9	3.0	4.3
Glycine	1.0	1.2	0.3
Alanine	1.1	1.2	0.7
Lysine	3.1	4.0	Not determined
Other residues[a]	<0.1	<0.1	<0.1

[a] This includes residues whose amino alcohols would have been detected and accordingly, this excludes histidine, arginine, and tryptophan.

their inherent properties and the conditions used. Such selectivity in ease of reduction of peptide bonds in proteins was also pointed out by Crestfield *et al.* (1963). Even though the level of these reductions may be low (3–6 residues/1000 as found in collagen), it may greatly exceed the content of reduced carbonyl compounds under consideration in the protein. Thus, in collagen the contents of reduced carbonyl compounds, hydroxynorleucine, and the reduced aldol product of α-aminoadipic acid semialdehyde, together, are in the order of less than 1 residue/1000 (Paz *et al.*, 1969).

It was observed here that some selectivity exists in the reduction of peptide bonds. This is shown in Table II, where the relative levels of amino acids converted into α-amino alcohols are compared in collagen and trypsin. In both proteins there is an obvious tendency for reductions at seryl and threonyl positions. In collagen lysyl bonds also appear to be easily reduced, however, in reduced trypsin, the absence or presence of lysinol was not ascertained. Peptide bonds at aspartyl (or asparaginyl), residues are sensitive, but glycyl, alaninyl, and glutamyl (or glutaminyl) positions show a lesser susceptibility to reduction. Bonds involving most other residues do not appear to be affected. Obviously the nature of the residue which donates the amino group in the peptide bond will influence the ease of reduction. It is known, that $LiAlH_4$ under anhydrous conditions can promote selective reductions of peptide bonds in which proline is the imino donor (Ruttenberg *et al.*, 1964). In our study, it would appear, by comparing two such dissimilar proteins as collagen and trypsin, that the bonds involving the hydroxyamino acids are sensitive to reduction more or less independently of their adjacent residues. In trypsin, examination of the amino acid sequenced regions of the polypeptide chains reveals that seryl and threonyl residues are not followed by proline. Their sensitivity to reduction therefore probably resides in the intrinsic nature of these residues. It would be of interest to know if there is selectivity in the specific seryl or threonyl bonds which are cleaved and whether this is related to sequential factors or to accessibility due to conformation and/or configuration.

In view of the sensitivity of the seryl and threonyl bonds to sodium borohydride reduction, experiments in which this reagent has been used to demonstrate *o*-glycosidic attachments to seryl and threonyl residues in glycoproteins, should be viewed with caution. Disappearance of serine or threonine with nonquantitative conversion into alanine and α-aminobutyric acid might indicate a base promoted β elimination of carbohydrates from their *o*-glycosidic attachments to seryl and threonyl hydroxy groups, but it is also likely that some serine and threonine could be converted into serinol and threoninol by peptide bond reduction and be missed in the analysis. Indeed, treatment of glycopeptides or glycoproteins with alkaline sodium borohydride often leads to loss of serine and threonine without a corresponding increase in alanine and α-aminobutyric acid (Kathan and Adamany, 1967; Winzler *et al.*, 1967; Lisowska, 1969). Although this may be due to an incomplete reduction of dehydroalanine or dehydro-α-aminobutyric acid (Tanaka and Pigman, 1965), the presence in hydrolysates of glycopeptides treated with tritiated alkaline sodium borohydride of unidentified radioactive peaks in the regions of elution of serinol and threoninol (Winzler *et al.*, 1967), suggests that these α-amino alcohols may indeed be present. Accordingly in experiments involving tritiated sodium borohydride treatment of glycoproteins the level of α-amino alcohols should be ascertained before quantitative conclusions are made concerning the process under investigation.

We have noted previously that no significant amounts of α-amino alcohols are produced during reduction of native calf skin tropocollagen but that they arose when reduction was carried out on the denatured protein (Paz *et al.*, 1969). It is probable that the study of carbonyl compounds in more crosslinked and insoluble collagens or elastin will necessitate the use of sodium borohydride reductions on previously denatured proteins where the occurrence of α-amino alcohols will present problems of interference with the reduced compounds under consideration. In this and previous publications (Paz *et al.*, 1969; Gallop *et al.*, 1968; Blumenfeld and Gallop, 1966), we outline in detail the means of identification of α-amino alcohols by their chromatographic behavior on columns of amino acid analyzers (Spinco and Technicon), or as their 2,4-dinitrophenyl derivatives on silicic acid columns or thin layers of silica gels. Thus during reductions of proteins with sodium borohydride, even if peptide bond reduction occurs, the α-amino alcohols resulting from this side reduction should be recognized and their interference eliminated. In some instances where this interference involves chromatographic behavior and the compound under consideration is not periodate sensitive, treatment with periodate may eliminate the radioactive α-amino alcohols. Moreover, the use of tritiated sodium borodeuteride should be helpful in avoiding artifacts such as we had described in earlier publications (Schneider *et al.*, 1967; Gallop *et al.*, 1968; Paz *et al.*, 1969).

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Iron–Mercaptoethanol–Inorganic Sulfide Complex. Possible Model for the Chromophore of Nonheme Iron Proteins*

C. S. Yang† and F. M. Huennekens

ABSTRACT: An iron–mercaptoethanol–inorganic sulfide complex is formed upon mixing $FeCl_3$ with an equal concentration of Na_2S and an excess of 2-mercaptoethanol at pH 9. The absorption spectrum of this complex, characterized by maxima at 325, 412, and 450 mμ, is similar to that of the iron–sulfur chromophore of nonheme iron proteins. Iron and inorganic sulfide are present at a 1:1 ratio in the complex and the iron is in the trivalent state. Studies with analogs of mercaptoethanol suggest that both the sulfhydryl and hydroxyl groups of the parent compound are involved in iron binding, resulting in the formation of a 5-membered chelate ring. Mononuclear and binuclear structures are considered for the complex. The "model complex" is inherently unstable due to intramolecular electron transfer in which Fe^{3+} is reduced to Fe^{2+} by the mercaptoethanol. In the presence of oxygen, the resulting Fe^{2+} is readily oxidized to the ferric state, regenerating the model complex. The combination of these two reactions results in reduction of O_2 and oxidation of mercaptoethanol. The actual O_2 uptake of the system is much faster than would be expected from the above mechanism, and a more efficient process for reduction of O_2 must be involved. At 110°K, the complex system shows an electron paramagnetic resonance signal at $g = 4.1$, accompanied by weaker signals in the central field ($g_\perp = 2.01$ and $g_{||} = 1.96$). The latter signals are believed to be associated with a transient species containing an unpaired electron.

Nonheme iron proteins,[1] which are involved in various electron-transfer processes in biological systems, have received intensive study during the past few years (reviewed by Malkin and Rabinowitz, 1967, Kimura, 1968, and San Pietro, 1968). These proteins generally show characteristic absorption bands (or shoulders) in the regions of 315–335, 410–420, and 450–460 mμ and, upon reduction, a unique electron paramagnetic resonance signal is seen at $g = 1.94$ (reviewed by Beinert, 1966). These properties have been attributed to a complex in which the iron is bonded to sulfhydryl groups on the protein and to inorganic sulfur. In several cases, more detailed structures have been envisioned for these complexes (Blomstrom *et al.*, 1964; Tanaka *et al.*, 1965; Brintzinger *et al.*, 1966; Bayer *et al.*, 1969). However, the exact structures of the iron–sulfur chromophore of these nonheme iron proteins have not yet been established.

Searching for a possible model for the above proteins, Beinert *et al.* (1965) showed that pentacyanonitrosyl ferrate gives an electron paramagnetic resonance signal in which the g value is <2. On the other hand, the typical absorption spectrum of the nonheme iron protein is not duplicated in the above compound or in other simple iron complexes

* From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received August 12, 1969.* This work was supported by grants from the American Heart Association (66-796) and the National Cancer Institute, National Institutes of Health (CA 6522).

† Present address: Department of Chemistry, Yale University, New Haven, Conn.

[1] In this paper, "nonheme iron proteins" refer to those that also contain "labile sulfide." This category includes both smaller molecular weight ferredoxins and larger nonheme iron flavoproteins.