158–159 and 213°, respectively. Since imidazole picrate also melts at 212–213° it is possible that dealkylation occurred during pyrolysis, or alternatively, that the *N*-methyl group has migrated to the 2 position. From the above it appears highly likely that ophidine is identical with two other methylhistidine dipeptides obtained from whale muscle, cetasin (Müller, 1959), and balenine (Pocchiari *et al.*, 1962). Allowing for small differences due to sample preparation and scale representation (cm^{-1} *vs.* wavelength), the infrared spectrum of cetasin picrolonate (Müller, 1959) is apparently the same as that of ophidine (Tsunoo *et al.*, 1959). Balenine (also called isoanserine) has been synthesized from 3-methylhistidine (Rinderknecht *et al.*, 1964; Dennis and Lorkin, 1965).

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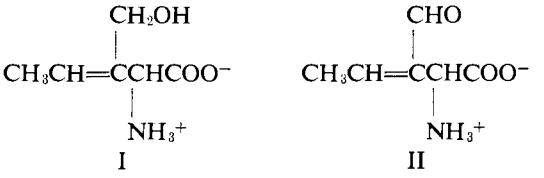
Identification of Two New β-Unsaturated Amino Acids in the Mushroom, *Bankera fuligineoalba**

Richard R. Doyle† and Bruce Levenberg‡

ABSTRACT: Two monounsaturated α-amino acids have been discovered in fruiting bodies of the mushroom, *Bankera fuligineoalba*. One of them, I, isolated as a pure, crystalline solid, has been characterized by chemical and spectral criteria as L-2-amino-3-hydroxymethyl-3-pentenoic acid. Nuclear magnetic resonance spectral data tentatively permit the assignment of the methyl and hydroxymethyl groups to the *cis* configuration. The other, II, was obtained in purified form, and, on the basis of its ultraviolet spectrum and its conversion into I by alkaline KBH_4 has been tentatively assigned the structure L-2-amino-3-formyl-3-pentenoic acid.

In the course of a search for new amino acids from higher fungi, we examined methanolic extracts of the mushroom, *Bankera fuligineoalba*. Using two-dimensional paper chromatographic techniques, the presence of two unusual compounds was observed, both of which afforded atypical colors after reaction on paper with ninhydrin, but only one absorbed in the ultraviolet region. The purpose of this report is to describe the isolation and properties of these compounds and to offer evidence in favor of their proposed formulation as L-2-amino-3-hydroxymethyl-3-pentenoic acid (I) and L-2-amino-3-formyl-3-pentenoic acid (II).



Experimental Work and Results

General. All concentration steps were done *in vacuo* (water aspirator) using rotary or test-tube-type flash evaporators operating at temperatures not over 35°. Infrared spectra were obtained from KBr disks using the Perkin-Elmer Model 237 spectrometer. Nuclear

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† Recipient of a postdoctoral fellowship (5-F2-CA-30,540) from the U. S. Public Health Service. Present address: Department of Chemistry, Denison University, Granville, Ohio 43023.

‡ Recipient of a career development award (K3-GM-3115) from the U. S. Public Health Service.

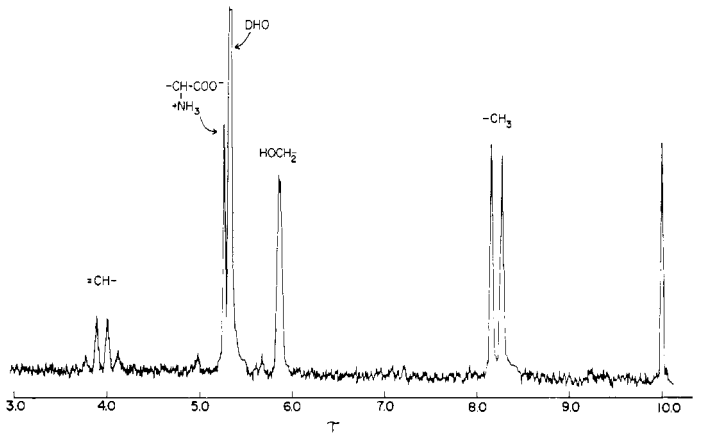


FIGURE 1: Nuclear magnetic resonance spectrum of L-2-amino-3-hydroxymethyl-3-pentenoic acid (I) in D_2O.

magnetic resonance spectra were taken with a Varian A-60 spectrometer on solutions prepared in D_2O containing the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate. Ultraviolet spectra were recorded on a Cary Model 15 spectrophotometer. The melting point was determined in a Mel-Temp apparatus and is corrected. Paper electrophoreses were carried out in the Savant Model FP-22A flat-plate instrument.

Paper Chromatography. Descending techniques were employed throughout. Preparative runs were carried out on Whatman No. 17 papers (18 × 22 in.) with wicks made from Whatman No. 3MM paper sewn onto the leading edge in order to retard the rate of solvent flow and thereby improve resolution. Except where noted, all other chromatograms were run on sheets (18 × 22 in.) of Whatman No. 3 paper. Solvent systems used were as follows: (A) 1-butanol–acetic acid–water (18:2:5), (B) 1-butanol saturated with 1.5 N HCl, (C) *t*-amyl alcohol–water (saturated), (D) pyridine–ethanol–water (10:7:3), (E) methanol–1-butanol–acetic acid–water (10:10:1:5), (F) methanol–1-butanol–acetic acid–water (15:10:1:5), (G) liquid phenol–water (10:2), atmosphere equilibrated with 0.3% aqueous NH_3, and (H) 1-butanol–methyl ethyl ketone–NH_4OH–water (5:3:1:1). Amino acids were detected by dipping dried chromatograms through an acetonic solution of ninhydrin (0.2%, w/v) containing about 5000 ppm of redistilled 2,4,6-collidine. Colors were allowed to develop at room temperature. R_F values of the new amino acids in the solvents employed for their detection and isolation are as follows: A (I) 0.22, (II) 0.14, B (I) 0.53, F (II) 0.39, and H (I) 0.20 and (II) 0.22 (streaking due to decomposition).

Source and Preparation of the Mushroom Extract. Through the cooperation of Professor Alexander H. Smith, Curator of Fungi, Department of Botany, The University of Michigan, mature fruiting bodies of *Bankera fuligineoalba* were obtained late in October of 1964 from local forest reserves in southeastern Michigan. After removal of adhering pine needles and other debris, the caps (4 lb) were rinsed thoroughly in distilled water, drained of excess liquid, suspended in 3 l. of absolute methanol containing 3.5 ml of concentrated H_2SO_4, and homogenized without delay in a large-capacity Waring Blendor for 4–5 min. The brei was filtered through several layers of cheesecloth and the remaining pulp was re-extracted in the blender with 1 l. of 80% methanol containing 1.1 ml of concentrated H_2SO_4. The combined filtrates (4.9 l.) were concentrated to 850 ml, centrifuged to remove particulate matter, and stored at −17°.

2-Amino-3-hydroxymethyl-3-pentenoic Acid (I). ISOLATION. In a typical isolation, 300 ml of freshly thawed concentrate (pH 3.5) was passed through a column (7.6 × 14 cm) of 15–50 mesh Amberlite IR-120 resin (H^+ form) over a 2-hr period. The column was washed rapidly with 3 l. of distilled water and the resin was then eluted with 1.5 N NH_4OH at a rate of 1 ml/min. The effluent was collected in 200-ml fractions which were monitored by paper chromatography in solvent A. Fractions containing appreciable quantities of I were combined, concentrated to 24 ml, and streaked on three sheets of Whatman No. 17 paper. Development was carried out for 48 hr in solvent A. Areas of the dried chromatograms containing I were located by the use of narrow test strips which were cut from the main sheet and dipped through acetonic ninhydrin. The compound was eluted with water from the remainder of the papers, concentrated to a few milliliters, and rechromatographed for 90 hr on three sheets of Whatman No. 3MM paper in solvent B. Compound I was located on the thoroughly dried chromatograms as before and eluted with water. The eluate was concentrated to 10 ml and decolorized with a small amount of activated charcoal. After removal of the charcoal by centrifugation, the solution was diluted to 70 ml with water, adjusted to pH 7.3 with NH_4OH, and passed through a column (2.9 × 10 cm) of Dowex 1 resin (acetate form) to remove chloride ions. The column was washed with five to six bed volumes of distilled water and the combined eluates containing I were concentrated to dryness. Ammonium acetate was removed by repeated evaporation to dryness *in vacuo* at 50–55° and finally by pumping under high vacuum for 2 days. The amino acid was then crystallized from a concentrated, aqueous solution by addition of methanol in the cold, and recrystallized twice in the same manner. A white, microcrystalline solid (85 mg) was obtained, representing approximately 0.07% of the dry weight of the mushroom cap.

PROPERTIES. The highly water-soluble substance (neutral to pH test paper) possessed mp 160–161° (with gas evolution), $[\alpha]_D^{25°}$ +182 (*c* 0.8, water, pH 6) and +201° (*c* 0.8, 0.3 N HCl). A positive result was obtained in a test of its ability to chelate Cu^{2+} (Woiwod, 1949). Quantitative determination with ninhydrin (Moore and Stein, 1948) gave an equivalent weight of 143 (L-leucine standard). The infrared spectrum showed absorptions at the following frequencies: 3257, 3155, 3058, 2959, 2915, 2874, 2809, 2717, 2591, 2525, 2463, 2398, 2062, 1661, 1621, 1595, 1527, 1397, 1351, 1224, 1130, 1111, 1076, 1040, 994, 953, 909, 853, 826, 818, 765, and 741 cm^{-1}. The nuclear magnetic resonance spectrum is presented in Figure 1.

Anal. Calcd for $C_6H_{11}NO_3$: C, 49.6; H, 7.6; N, 9.7; O, 33.1. Found:[1] C, 49.8; H, 7.6; N, 9.9; O, 32.8.

[1] Huffman Laboratories, Wheatridge, Colo.

CATALYTIC HYDROGENATION. An aqueous solution of I (16 μmoles in 10 ml) was acidified with acetic acid and hydrogenated for 1 hr at room temperature and atmospheric pressure using 70 mg of Adam's catalyst (PtO_2). After filtration, the clear, colorless solution was evaporated to dryness and redissolved in a small volume of water. Chromatography in solvent A revealed that I had completely disappeared and been replaced by a new spot (giving a typical blue color with ninhydrin) migrating in the isoleucine area (R_F 0.52) of the chromatogram. However, on chromatography in solvent C (a system able to resolve the two stereoisomers of L-isoleucine) for several days, the new material could readily be separated into two amino acids, identical in R_F and by cochromatography with authentic L-isoleucine and L-alloisoleucine, respectively. A determination on the amino acid analyzer confirmed the identification of the two reduction products of I, and, furthermore, showed them to be present in equal quantity. In another experiment, I was hydrogenated in the same manner, using, however, only 10 mg of catalyst. Paper chromatography of the reduced sample showed, in addition to the isoleucine isomers, some unreacted I and two new ninhydrin-positive compounds. The first of these produced with ninhydrin a yellow → olive-green → blue color sequence characteristic of I, and cochromatographed with a known sample of 2-amino-3-methyl-3-pentenoic acid[2] in solvents B–E. The second product afforded a normal blue reaction with ninhydrin and migrated as a neutral zwitterion on paper electrophoresis.[3] However, when dissolved in 2.5 N HCl, evaporated to near dryness, and taken up once again in water, it was converted into a substance behaving as a cation in the electric field. On treatment with 2.5 N NH_4OH, the cationic species could be reconverted into the original zwitterionic form and the process repeated.

PERIODATE–PERMANGANATE OXIDATION. An aqueous solution containing 145 μg (1.0 μmole) of I was oxidized at pH 7.5 for 90 min at 25° by the periodate–permanganate reagent (Lemieux and von Rudloff, 1955a). Analyses for both formaldehyde (Lemieux and von Rudloff, 1955b) (erythritol standard) and acetaldehyde (Bhattacharyya and Aminoff, 1966) (crotonic acid standard) showed yields of 58 and 74%, respectively, of that expected on the basis of a quantitative oxidation of the proposed structure.[4] (The acetaldehyde result was confirmed, qualitatively and quantitatively, by trapping the gaseous oxidation product in a buffered (pH 7.0) solution of DPNH[5] and yeast alcohol dehydrogenase and observing the extent of DPN^+ formation.) The values found for both aldehydes are well within the range of maximum yields obtainable by this method for a wide variety of compounds containing terminal methylene (Lemieux and von Rudloff, 1955b) and other olefinic groups (von Rudloff, 1965).

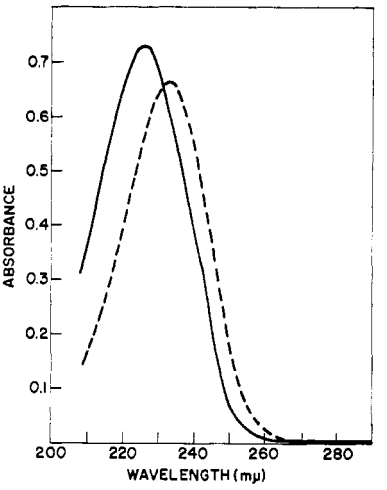


FIGURE 2: Ultraviolet spectra of L-2-amino-3-formyl-3-pentenoic acid (II) and tiglaldehyde. Solid line, II; dashed line, tiglaldehyde. Spectra were taken in 0.1 N HCl.

2-Amino-3-formyl-3-pentenoic Acid (II). PREPARATION OF A PURIFIED STOCK SOLUTION. In a manner identical with that used for the isolation of I, 300 ml of freshly thawed concentrate was passed over Amberlite IR-120 (hydrogen form). The resin was washed with water and subsequently eluted with 1.5 N NH_4OH. Eluates from the column were monitored in the ultraviolet region, and aliquots from those fractions that showed specific absorption in the region from 220 to 230 mμ were concentrated to a small volume and examined by paper chromatography in solvent A. Fractions that contained an absorbing spot coincident with one giving a sepia color with ninhydrin were pooled, concentrated, and streaked on four sheets of Whatman No. 17 paper. Chromatography was carried out in solvent A for 47 hr. Location of areas containing II was made by applying criteria of ultraviolet absorption and ninhydrin color reaction to narrow test strips. Water was used to elute the amino acid from the papers, after which the solution was concentrated and decolorized with activated charcoal. The filtrate was rechromatographed on a sheet of Schleicher & Schuell no. 589 paper for 30 hr in solvent F. Location of II on the dried chromatogram was made as before, and the compound was leached from the paper with water. The eluate was concentrated to a few milliliters, centrifuged to remove some dustlike paper fibers, and stored at 2°. This stock solution, which exhibited the ultraviolet spectrum shown in Figure 2 and, by chromatographic criteria, contained II as the sole ninhydrin-positive, ultraviolet-absorbing component, was used as such for further study.

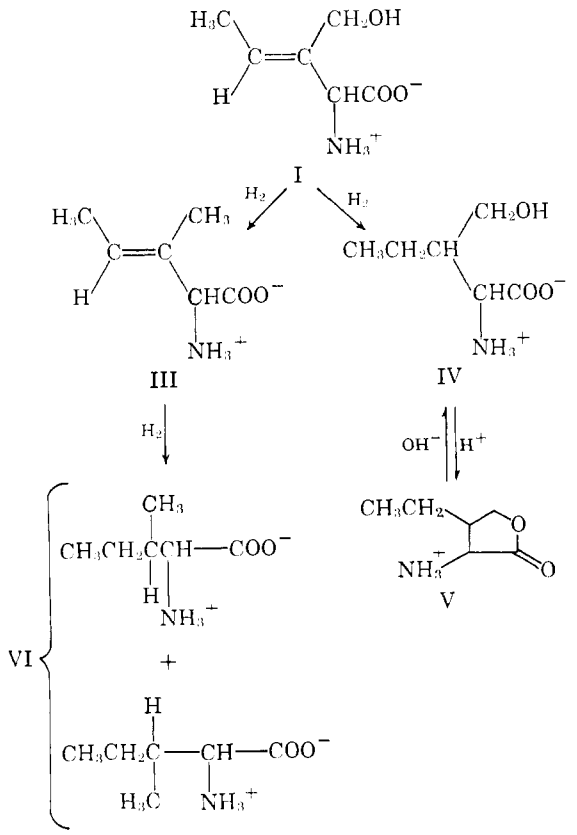
[2] This compound was synthesized in small yield by a Strecker reaction (Greenstein and Winitz, 1961d) between tiglaldehyde, $(NH_4)_2CO_3$, and NaCN, followed by alkaline hydrolysis of the intermediate hydantoin, and purification of the resulting unsaturated α-amino acid on cation-exchange resin columns and by preparative paper chromatography (B. Levenberg, in preparation).

[3] This same compound was also noted after hydrogenation of I with the larger amount of catalyst. It was present, however, in far smaller quantity than the mixture of isoleucine isomers obtained.

[4] At concentrations severalfold higher than those actually determined, neither aldehyde interfered in any way with the estimation of the other.

[5] Abbreviations are as listed in *Biochemistry 5*, 1445 (1966).

SCHEME I



ALKALINE BOROHYDRIDE REDUCTION. The above stock solution of II (1 ml) was made 0.2 N in KOH and added to 100 mg of KBH_4. The solution was stirred for 30 min at 25°, acidified with HCl to destroy excess borohydride, and finally centrifuged to remove precipitated boric acid. The supernatant, which no longer possessed ultraviolet absorption, could be shown by paper chromatography in solvents B–E and G to contain, in place of II, an amino acid indistinguishable from I in its color response toward ninhydrin, R_F values, and by cochromatography with pure material. Determinations with the quantitative ninhydrin reagent (leucine standard) indicated that the reduction of II to I was essentially quantitative.

Discussion and Conclusions

The rather unusual sequence of color changes accompanying the reaction on paper chromatograms of the two new *Bankera* amino acids with acetonic ninhydrin greatly facilitated their initial discovery and subsequent detection throughout the course of isolation. Both compounds were first noticed by their appearance as ninhydrin-positive spots of peculiar hue on two-dimensional paper chromatograms developed in solvents H (40 hr) and A (22 hr), respectively. Compound I appears initially with a pale yellow hue that changes after a short while to olive-green and finally to a stable blue color not unlike that given directly by most of the common amino acids. Coincident with its ultraviolet absorption, II develops at first a light yellow-brown color, changing in a very short time to a stable sepia tint. While of little value in predicting the specific nature of functional groups along the carbon chain, such atypical ninhydrin colors did suggest, by comparison with analogous data compiled for other amino acids (Fowden, 1960), the possible occurrence of double-bond character at a carbon atom one or two removed from that bearing the amino group.

Through the application of ion-exchange and preparative paper chromatography, I was isolated in the form of the free amino acid, a colorless, microcrystalline, highly water-soluble compound possessing the empirical formula $C_6H_{11}NO_3$. Aqueous solutions were neutral. The migration characteristics of I in paper electrophoresis at several values of pH were typical of a neutral amino acid. Its ability to chelate with Cu^{2+} showed it to be an α-amino acid. On the basis of its quantitative reaction with ninhydrin, I had an equivalent weight of 143, in excellent agreement with the above formula. The specific rotation of +182° in water is extremely high in comparison with the values given by other amino acids (Greenstein and Winitz, 1961a) with the exception of the β-unsaturated amino acids, α-phenyl-L-glycine ($[\alpha]_D^{25°}$ +172.4°) and β-methylene-L-norvaline (Doyle and Levenberg, 1967) ($[\alpha]_D^{25°}$ +197°).[6] In acid, the rotation of I shows a small though definite shift to a more positive value, indicating that it belongs to the L series (Greenstein and Winitz, 1961b). This assignment was confirmed independently by the observation that highly purified L- (but not D-) amino acid oxidase under anaerobic conditions catalyzed electron transfer between I and the flavin prosthetic group of the enzyme at a relatively slow, but measurable rate. The infrared spectrum showed absorption bands at 2062, 1595, 1527, and 1397 cm^{-1} characteristic for zwitterionic amino acids (Greenstein and Winitz, 1961c). In addition, it contained peaks at 1661 and 1351 cm^{-1} which could be assigned to C=C and OH bending vibrations, respectively (Dyer, 1965a). The nuclear magnetic resonance spectrum (Figure 1) showed absorption at τ 3.95 (q, J = 7 cps), 5.28 (s), 5.88 (s), and 8.21 (d, J = 7 cps) equivalent to one, one, two, and three protons, respectively. The quartet at τ 3.95 is assigned to a vinyl proton which is coupled with the methyl group at τ 8.21. The sharp singlet at τ 5.28 which shifts to lower field upon addition of CF_3COOH is assigned to the α-hydrogen. The absorption at τ 5.88 is assigned to the methylene group of an allylic alcohol function. The long-range coupling (J = 1 cps) between the methylene protons and the vinyl hydrogen is strongly indicative of a *trans* rather than a *cis* configuration between the two (Dyer, 1965b).[7] This would lead to the *cis* relationship between the methyl and hydroxymethyl groups about the double bond, as shown in structure I of Scheme I.

The analyses, spectral data, and properties of I suggest that the compound is 2-amino-3-hydroxymethyl-3-pen-

[6] B. Levenberg, unpublished data.

[7] The authors are indebted to Dr. John R. Dyer for calling this point to their attention during the process of editorial review of the manuscript.

tenoic acid. This structure receives strong support from the result of catalytic hydrogenation of I in aqueous acid (Scheme I). The principal product was an equimolar mixture of isoleucine and alloisoleucine (VI), which is probably formed by hydrogenolysis of the allylic hydroxyl group followed by reduction of the double bond (House, 1965). The appearance of both isoleucine isomers furnishes compelling evidence that the position of unsaturation in the molecule involves carbon atom 3. When the extent of catalytic hydrogenation was incomplete, there were detected two new ninhydrin-positive compounds, in addition to the isoleucine isomers and some unreacted I. One of these, III, was identified as 2-amino-3-methyl-3-pentenoic acid, formed presumably as an intermediate in the reduction process in which the double bond of I has not yet been hydrogenated. The other, IV, migrated in paper electrophoresis as a neutral zwitterion, but on mild acid treatment it was converted into a positively charged species (V), which was transformed once again to IV on exposure to alkali. The latter, presumably the saturated hydroxyamino acid, would form on acidification the aminolactone V (Wieland and Wieland, 1959), mild basic hydrolysis of which would reconvert it into IV. The hydroxyamino acid (IV) is thus considered to be formed as a side product in which the double bond of I has been reduced first, thereby preventing hydrogenolysis of the hydroxyl group. Further evidence favoring structure I for the new amino acid was provided by the result of periodate–permanganate oxidation. As products of the reaction, acetaldehyde and formaldehyde could both be obtained in reasonable yield only from the particular unsaturated hydroxyisoleucine isomer proposed.

Viewing I as the β-ethylidene analog of L-homoserine, a sample was tested as a possible inhibitor or substrate of partially purified homoserine dehydrogenase from *Rhodospirillum rubrum*. Over a wide range of concentrations and employing two different assay procedures, I was found to be inactive in either capacity.

Aqueous solutions of purified II exhibit an ultraviolet absorption spectrum closely resembling that of the α,β-unsaturated aldehyde, tiglaldehyde (Figure 2). Compound II was quite unstable (particularly at alkaline pH), although stock solutions could be maintained reasonably well for several weeks at 0° if they were acidified below pH 2. All attempts to obtain the compound or a derivative thereof[8] as a pure solid were unsuccessful. However, it was clearly possible to show that when treated with alkaline borohydride, II was converted into I. Formed in this way, the latter possessed the L-configuration, as evidenced by its reaction with L- (but not with D-) amino acid oxidase, in a manner completely analogous to that observed with naturally occurring I. On the basis of these observations, the proposal is made that II be tentatively assigned the α,β-unsaturated aldehyde structure corresponding to that of the alcohol I.

It is not yet known whether the new amino acids exist in the free state only, or if they may occur in the protein and/or peptide fraction of the mushroom as well. Their distribution in nature thus far appears to be confined exclusively to *B. fuligineoalba*, one of the few species known in this genus. Chromatographic analyses of extracts from mushrooms of close taxonomic relationship (*e.g.*, the *Hydnum* group) have failed to reveal the presence of either substance. Together with β-methylene-L-norvaline (Doyle and Levenberg, 1967) and a possibly related cyclic amino acid (Honkanen *et al.*, 1964) both from *Lactarius helvus*, these compounds represent the only reported examples of the natural occurrence of aliphatic β-unsaturated α-amino acids. The possibility of their metabolic interconversion in *B. fuligineoalba* through the activity of a specific alcohol dehydrogenase as well as their potential role as leucine or isoleucine antagonists in protein synthesis is presently being investigated.

[8] Although spectrophotometric evidence clearly suggested that II forms a semicarbazone, such a derivative was readily cleaved to the original reactants when attempts were made to isolate it.

Acknowledgment

The authors wish to thank Mrs. Linda Curl for her excellent technical assistance and Dr. A. H. Smith for invaluable services in the field. Dr. Prasanta K. Datta kindly carried out the tests with homoserine dehydrogenase. The expert help of Dr. Vincent Massey in performing the D- and L-amino acid oxidase assays and of Andrea M. Mills in carrying out determinations on the amino acid analyzer is gratefully acknowledged, as is that of Dr. Jack J. Distler in obtaining the polarimetric data.

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Equine Antihapten Antibody. VI. Subunits of Polyalanylated γG(T)-Immunoglobulin*

Robert J. Genco,† Fred Karush,‡ and Harriet S. Tenenhouse

ABSTRACT: After extensive alanylation both γG(T) antihapten and γG(T) diphtheria antitoxin were converted into their monomeric subunits. The proteins were mildly reduced and alkylated and underwent spontaneous dissociation into the subunits in neutral aqueous solution. The heavy and light chains were separated by gel filtration with Sephadex G-200 in buffered saline. Their identification was established by immunoelectrophoresis with rabbit antisera specific for equine heavy and light chains. The heavy chain derived from the polyalanylated antihapten antibody exhibited a reduction of at least 100-fold in its association constant for the homologous hapten compared with the parent antibody. The optical rotatory dispersion patterns of the polyalanylated γG(T) and subunits revealed no extensive conformational alteration resulting from their dissociation. The calculated curve for an equimolar mixture of heavy and light chains differed significantly from that observed for the parent protein only in the smaller Cotton effect at 224 mμ of the mixture. The interpretation of this finding, coupled with the earlier measurements of the optical rotatory dispersion pattern of the Fab fragment of rabbit γG (Steiner, L. A., and Lowey, S. (1966), *J. Biol. Chem. 241*, 281), is that the light-chain–heavy-chain interaction affects the conformation of the binding region of the antibody. Furthermore, in view of the optical rotatory dispersion behavior of reconstituted myeloma γG (Dorrington, K. J., Zarlengo, M. H., and Tanford, C. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 996), it is inferred that this structure is dependent upon the specific interaction of complementary heavy and light chains. The loss of binding activity following dissociation may then be attributed to a change in the conformation of the heavy chain associated with its combining region.

The polyalanylation of immunoglobulins has served as a useful chemical modification of these proteins primarily because of the enhanced aqueous solubility acquired by the subunits of the immunoglobulins (Fuchs and Sela, 1965; Freedman and Sela, 1966; Haber and Richards, 1966; Dorrington *et al.*, 1967). The availability of soluble light and heavy chains, particularly if prepared without exposure to denaturing solutes, would allow further clarification of their respective roles in determining the combining region of the antibody molecules. To exploit this possibility it was necessary to establish that polyalanylation of the antibody molecule did not alter its binding sites. The absence of extensive alteration of these sites was inferred from the results of an earlier study (Karush and Sela, 1967) in which it was shown that alanine enrichment of 200–600 residues/molecule of antihapten antibody did not reduce the average association constant for hapten binding by more than twofold.

In the course of this earlier study it was observed that mild reduction and alkylation of polyalanylated γG(T)[1] resulted in the spontaneous dissociation of the proteins into subunits in neutral aqueous solution, that is, in the absence of any additional dissociating solute. This observation was the basis for the further investigation whose results are described in this report. Extensively alanylated γG(T) anti-Lac antibody and γG(T) diph-

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† Present address: Department of Oral Biology, School of Dentistry, State University of New York at Buffalo, Buffalo, N. Y.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Lac, *p*-azophenyl β-lactoside; Hy, hemocyanin; PSS, 0.15 M NaCl–0.02 M phosphate buffer (pH 7.4); *S*-CmC, *S*-carboxymethylcysteine; Lac dye, *p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside; γG(T), T component of equine antiserum (Weir *et al.*, 1966).