carbonate while being cooled in an ice–salt-bath. It was then extracted with ether and the combined extracts were dried over magnesium sulfate. The aqueous layer which smelled strongly of ammonia was discarded. The ether solution was evaporated *in vacuo* and the residue was dissolved in 30 ml. of an 80% hexane–benzene mixture. This solution was poured on an alumina column (6″ × 1″). Elution with 100 ml. of 80% benzene–hexane yielded 0.0724 g. of a colorless oil which had a strong lemon-like odor (**base** A). Further elution with benzene and chloroform brought down only traces of substances which did not react with hydrogen chloride in ether. Washing with 95% ethanol (200 ml.) eluted 0.1973 g. of a white crystalline substance, **base** B.

Base A gave a hydrochloride which sublimed easily. The melting point of this salt taken in a sealed tube on the Kofler hot-stage was 213–215°. Found: C, 67.64; H, 8.34; N, 11.48. The analysis does not fit a C_{13}-compound; H and N would fit $C_{13}H_{20}N_2 \cdot HCl$. The substance also reacted with phenyl isothiocyanate in hexane to give a **phenylthiourea** derivative which after one sublimation melted at 144–145°. Found: C, 73.50; H, 7.03. The infrared spectrum of the hydrochloride in chloroform shows the following bands: 2.92 (very weak imino band), 3.71, 3.98 (ammonium region), 6.21^w, 6.32^s, 6.67^s, 6.75^m, 6.87^s, 7.31^m. The free liquid base A in chloroform showed: 2.95 (sharp imino band); 3.41, 3.51 (two different very characteristic C–H stretching frequencies); 6.23^s; 6.65^s, 6.83^s, 6.98^m, 7.57^s, 7.95^s, 8.50^m. A very strong band at 14.40 still indicated a monosubstituted intact benzene ring. We suspect that hydrogenolysis of the intermediate 1-benzyl-2-phenylhydrazine might have led to benzylamine and aniline; the latter might undergo further reduction analogous to the formation of dicyclohexylamine from aniline.[43]

Base B could be purified by vacuum sublimation to yield a colorless crystalline substance, recrystallized from ether, which sintered at 117° and melted at 119–120°. The mixed melting point with phenylbenzamidine (m.p. 117°) showed a large depression (87–110°).

Anal. Calcd. for $C_{26}H_{24}N_4O_3$: C, 70.88; H, 5.47; N, 12.73. Found: C, 70.66; H, 5.43; N, 12.64.

Hydrochloride.—The hydrochloride prepared in ether melted at 220–222°. An infrared spectrum obtained from solutions of varying concentration of base B in chloroform showed the following bands: 2.98, 3.05 (imino or amino bands; 5.92 (carbonyl of amid (?); 6.23^s, 6.36^s, 6.70^s, 6.88^s, 7.67^m. The presence of a monosubstituted benzene ring was shown by the typical aromatic fine structure between 5–6 μ and the strong band at 14.25 μ.

Picrate.—The picrate, prepared in ether, proved to be almost insoluble in any organic solvent. Recrystallized from much acetone, it formed a bright-yellow crystalline powder and melted at 239–241°.

Anal. Calcd. for $C_{17}H_{16}N_5O_9$: C, 47.01; H, 3.72; N, 16.13. Found: C, 47.00, 46.87; H, 3.66, 3.78; N, 16.14.

The parent base of this picrate does not correspond to $C_{26}H_{24}N_4O_3$ but to $C_{11}H_{13}N_2O_2$ or $C_{22}H_{26}N_4O_4$. The nature of this discrepancy has not been investigated yet.

Catalytic Hydrogenation of Anisalphenylhydrazine "Oxide."—Platinum oxide (0.222 g.) was prereduced in 20 ml. of glacial acetic acid with 271 ml. of hydrogen. To the suspension was then added 1.204 g. (approx. 0.005 mole) of anisalphenylhydrazine "oxide" and the mixture was stirred at room temperature with hydrogen at atmospheric pressure. The hydrogen uptake leveled off after some 20 hours; 707 ml. of hydrogen (approx. 7 moles) had been absorbed during that time. The mixture no longer contained undissolved starting material. It was filtered from catalyst and diluted with 10 ml. of 6 *N* hydrochloric acid and 30 ml. of water. Extraction of the solution with ether and evaporation of the ether extracts showed that practically no neutral or acidic substances had been formed during the reduction. The acid solution was cooled in ice and was then saturated with solid potassium hydroxide. The resulting mixture was extracted with three 50-ml. portions of ether and the ether solution was washed once with 5 ml. of water after which it was evaporated to a volume of 40 ml. It was then extracted with 12 consecutive 2-ml. portions of 0.1 *N* hydrochloric acid and one 2-ml. portion of water. Each fraction was allowed to dry in a vacuum desiccator over potassium hydroxide. The weight of the fractions varied between 30–40 mg. Melting points of the crude hydrochlorides varied from 165–180° to 178–197° between the 1st and the 12th fraction.

The ether solution which had remained after these extractions was dried over magnesium sulfate and was evaporated *in vacuo* to leave a liquid residue of 0.4 g. This material was still basic and gave a hydrochloride melting at 210–218° (dec.).

BETHESDA, MARYLAND

(43) *Cf.* R. Willstätter and D. Hatt, *Ber.*, **45**, 1476 (1912); G. S. Hiers and R. Adams, *ibid.*, **59**, 162 (1926).

[CONTRIBUTION FROM THE CHEMICAL LABORATORY, UNIVERSITY OF CALIFORNIA, LOS ANGELES]

The Free Amino Groups of Crystalline Bovine Plasma Albumin[1]

BY LAWRENCE E. MCCLURE, LEROY SCHIELER AND MAX S. DUNN

RECEIVED OCTOBER 30, 1952

Alanine, aspartic acid, histidine and methionine were identified by paper chromatography after freeing them from their thiohydantoins obtained from phenylisothiocyanated bovine plasma albumin. It may be concluded from this evidence that the α-amino groups of these amino acids are free in this protein. The percentages of these amino acids as well as arginine, lysine and tyrosine were significantly lower in deaminized than in the native protein. This finding is explained by assuming that nitrous acid acted on the exposed side chains of these amino acids to form derivatives which, though present in a hydrolysate of the protein, were without effect on the growth of organisms used to determine the parent amino acids. The number of amino acid residues per mole of protein was shown to range from 2 for tryptophan to 100 for glutamic acid based on 66,100 the derived molecular weight. The authors' data are consistent with the view that 2 or 3 terminal α-amino groups in bovine plasma albumin are contributed by aspartic acid, one by methionine, one by histidine and an undetermined number by alanine.

The authors' results on the free amino groups of bovine plasma albumin are presented at this time because they differ somewhat from the recent report of Van Vunakis and Brand[2] that there is only one terminal group, identified as that of aspartic acid, per mole of serum albumin. The latter workers determined aspartic acid by chromatographic analysis of its dinitrophenyl (DNP) derivative. In the present work, amino acids with free amino groups were identified by the phenyl isothiocyanate (PIC) method of Edman[3] and were determined by microbiological assay of the native and deaminized protein.

(1) Paper 72. For the preceding related paper (Paper 62) see M. S. Dunn and L. E. McClure, *J. Biol. Chem.*, **184**, 223 (1950). This work has been aided by grants from Swift and Company and the University of California.

(2) H. Van Vunakis and E. Brand, Abstracts of Papers, 119th Meeting, Amer. Chem. Soc., 28c, April, 1951.

(3) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

TABLE I

R_f VALUES OF AMINO ACIDS ISOLATED FROM PHENYLTHIOHYDANTOINS

Spot number	Water-satd. phenol		Pyridine–water (7:3), *p*H 7.0		Water-satd. *n*-butanol		Water-satd. benzyl alcohol		Amino acid
	A[a]	B[b]	A	B	A	B	A	B	
1	0.07	0.06	0.21	0.20					Aspartic acid
2	.37	.36	.27	.28					Histidine
3	.55	.54	.44	.47					Alanine
4	.78	.83	.62	.62	0.10	0.08	0.02	0.02	Methionine

[a] A, R_f value for an analytically-pure sample of the amino acid. [b] B, R_f value for the unknown spot.

Experimental

The Identification by the Phenyl Isothiocyanate Method of the Amino Acids Contributing Free α-Amino Groups to Bovine Plasma Albumin.[3]—A 10-g. sample of the crystalline plasma albumin was dissolved in 173 ml. of water, an equal volume of pyridine was added and the mixture was adjusted to *p*H 9.0 with *N* NaOH. Phenyl isothiocyanate (16.5 ml. in one portion) was added, the mixture was stirred continuously, the *p*H was maintained at 9.0 with *N* NaOH and the reaction was allowed to proceed for 3 hours.

The light-yellow solution of the phenyl thiocarbamate (PTC) albumin (upper layer) and the excess reagent (lower layer) were separated and the former was extracted twice with an equal volume of benzene to remove pyridine and phenyl isothiocyanate. The PTC albumin solution was acidified to *p*H 3.0 with *N* HCl giving immediately a light-yellow precipitate. The suspension was allowed to stand for 18 hours in the refrigerator. The solid was collected and washed successively with one 200-ml. portion each of water, absolute ethanol and ether. The yield of product, dried to constant weight at 37°, was 15.8 g.

The dry PTC albumin, ground to a fine powder, was suspended in 70 ml. of dry nitromethane in a 125-ml. glass-stoppered flask immersed in an ice–water-bath. The mixture was saturated with dry HCl gas, the flask was removed from the bath and the reaction was allowed to proceed (with stirring for the first 8 hours) at room temperature for 18 hours. The suspension was filtered, the precipitate (albumin cleaved from the PTC albumin) was discarded and the filtrate (containing the phenylthiohydantoins of amino acids with amino groups free in the protein) was evaporated to dryness on a steam-bath. A mixture of the residual material and 50 ml. of saturated barium hydroxide solution was heated for 12 hours at 120° in a sealed Pyrex tube.

The hydrolyzate was removed from the tube, heated to boiling during the addition of sulfuric acid to coagulate the $BaSO_4$ precipitate, and filtered hot to hold the amino acids in solution. The filtrate was evaporated to dryness and the residue was dissolved in 5 ml. of water.

Whatman No. 1 paper (20 cm. × 20 cm.) was spotted with this solution delivered from a capillary pipet and two-dimensional chromatograms were obtained with the solvent systems (a) water-saturated phenol and pyridine–water, 7:3, at *p*H 7.0 and (b) water-saturated *n*-butanol and water-saturated benzyl alcohol. The R_f values are listed in Table I.

Deamination of Bovine Plasma Albumin.—A 10% solution of $NaNO_2$ was added slowly with vigorous stirring to a suspension of 4 g. of crystalline bovine plasma albumin[4] in 50 ml. of a 50% acetic acid solution until gas evolution ceased. The volume of 10% $NaNO_2$ solution used was 21 ml. and the time was 2 hours. The light-yellow precipitate, which began to form almost immediately and continued to increase in amount during the addition of the $NaNO_2$, was collected after the suspension had stood at room temperature for 18 hours. The precipitate was washed with water until free of acid, with absolute ethanol four times and with ether twice.

Anal. Found: N, 14.58; moisture, 6.12; ash, 0.1.

The Determination of Amino Acids in Bovine Plasma Albumin and Its Deamination Product.—A 500-mg. sample (in duplicate) of each preparation and 5 ml. of 6 *N* HCl were heated for 18 hours at 120° in a Leiboff tube. Each hydrolyzate was transferred quantitatively to a 200-ml. volumetric flask and brought to *p*H 7.0 with NaOH. The mixture was diluted to volume and preserved with toluene. For the determination of tryptophan each duplicate sample was hydrolyzed as described with 2.5 *N* NaOH. Proline was determined microbiologically by the method of Dunn, *et al.*,[5] serine by the authors' unpublished method, and the other amino acids by the procedure of Dunn, *et al.*[6] Cystine was calculated from the sulfur obtained by subtracting methionine sulfur from total sulfur. The amino acid values are shown in Table II.

TABLE II

AMINO ACID COMPOSITION OF BOVINE PLASMA ALBUMIN AND ITS DEAMINATION PRODUCT

Amino acid	Whole protein,[a] % Authors	Whole protein,[a] % Literature[c]	Deaminized protein,[b] % Authors
Arginine	5.64	6.1 (5.9–6.2)	4.73
Aspartic acid	10.1	10.8 (10.5–11.1)	9.55
Cystine	6.7	6.3 (5.9–6.5)	[d]
Glutamic acid	22.4	16.9 (16.9–17.0)	22.8
Glycine	1.76	1.91 (1.82–2.0)	1.79
Histidine	3.78	4.3 (3.6–5.9)	3.62
Isoleucine	2.88	2.79 (2.6–3.0)	2.95
Leucine	11.4	12.8 (11.8–13.7)	11.3
Lysine	11.9	11.9 (10.3–12.8)	0.1
Methionine	0.92	0.83 (0.80–0.86)	0.76
Phenylalanine	6.31	6.3 (6.0–6.6)	6.17
Proline	4.68	5.2 (4.75–5.6)	4.60
Serine	3.80	4.4 (4.2–4.9)	[d]
Threonine	5.54	6.5 (5.8–7.1)	5.50
Tryptophan	0.61	0.55 (0.49–0.58)	0.60
Tyrosine	4.41	5.1 (4.3–5.5)	1.08
Valine	5.98	6.4 (5.9–6.6)	6.01

[a] Each value is the average of the assay values for two samples calculated on a moisture- and ash-free basis. The average deviation of the values from the average was 0.73 (0.0–1.4%). The mean deviations from the means of the assay values found at different levels of samples averaged 2.2 (0.56–6.4%). [b] Same as in (*a*) except that the first average was 1.4 (0.0–4.5%) and the second 1.7 (0.75–4.4%). [c] The average and range of values reported by Brand,[7] Henderson and Snell,[8] Velick and Ronzoni[9] and Stein and Moore.[10] The mean deviations from the means of Henderson and Snell's assay values found at different levels of samples averaged 5.6 (0.6–10.7)%. The precision of the values obtained by the other authors was not stated. The authors' values and those in the literature are in reasonable agreement except for glutamic acid. Although the value found for this amino acid in the present experiments is higher than the literature values, it was derived from apparently dependable assay data. Furthermore, a smaller value would account less satisfactorily for the total residue percentage shown in Table III. [d] Not determined.

Results and Discussion

As indicated in Table I alanine, aspartic acid, histidine and methionine were the only amino

(4) Armour and Company, Chicago, product same (Lot 46) as that employed previously.[1]

(5) M. S. Dunn, L. E. McClure and R. B. Merrifield, *J. Biol. Chem.*, **179**, 11 (1949).

(6) M. S. Dunn, M. N. Camien, R. B. Malin, E. A. Murphy and P. J. Reiner, *Univ. Calif. Publ. Physiol.*, **8**, 293 (1949).

(7) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

(8) L. M. Henderson and E. E. Snell, *J. Biol. Chem.*, **172**, 15 (1948).

(9) S. F. Velick and E. Ronzoni, *ibid.*, **173**, 627 (1948).

(10) W. H. Stein and S. Moore, *ibid.*, **178**, 79 (1949).

acids which could be isolated by paper chromatography from the phenylthiohydantoins formed by the reaction of the plasma albumin with phenyl isothiocyanate. From this evidence it would appear that the amino groups of these four amino acids are free in plasma albumin molecule.

Data on the amino acid composition of bovine plasma albumin and its deamination product are given in Table II. Of the amino acids determined only arginine, aspartic acid, histidine, lysine, methionine and tyrosine were significantly decreased in the deamination product. Alanine was not determined. Similar results have been reported for arginine,[11] histidine,[11] lysine[11] and tyrosine,[12,13] in other proteins by workers who employed chemical methods, but it appears that there is no evidence relating to aspartic acid and methionine.

As pointed out by Fox, *et al.*,[14] microbiological assays of deaminized proteins would be undependable if amino acid derivatives with stimulatory or inhibitory potentialities were formed by deamination. This uncertainty is emphasized by the observations that derivatives possibly formed by the action of nitrous acid on amino acids in proteins are inhibitory or vary from 0 to 100% activity in replacing the precursor amino acid in promoting growth of the assay organism. Such possible stimulatory derivatives which have been tested include citrulline,[15] malic acid,[16] α-ketoglutaric acid,[17] imidazolepyruvic acid,[18] α-hydroxy-β-methylvaleric acid,[19] α-keto-β-methylvaleric acid,[19] α-ketoisovaleric acid,[19] methionine sulfoxide,[20] phenyllactic acid,[20,21] phenylpyruvic acid,[21] indole[23] and α-hydroxyisovaleric acid.[19] Inert or inhibitory derivatives are pyruvic acid,[24] ornithine,[15,25] α-hydroxyglutaric acid,[17,22] pyrrolidine carboxylic acid,[17] imidazoleacetic acid,[18] imidazole aldehyde,[18] α-hydroxyisocaproic acid,[19] methionine sulfone,[21] phenylacrylic acid,[26] *p*-hydroxyphenylacrylic acid,[26] *p*-nitrophenylalanine,[27] and indoleacrylic acid.[27] Because of the absence of drifts and the close concordance of the values obtained at the different levels of samples in the microbiological assays, it is a reasonable assumption that microbiologically-active derivatives were absent, present in negligible amounts or inoperative under the assay conditions.

(11) Z. H. Skraup, *Biochem. Z.*, **10**, 245 (1908).
(12) Z. H. Skraup and P. Hoernes, *Monatsh.*, **27**, 631 (1906).
(13) H. B. Lewis and H. Updegraff, *J. Biol. Chem.*, **56**, 405 (1923).
(14) S. W. Fox, T. L. Hurst and K. F. Itschner, THIS JOURNAL, **73**, 3573 (1951).
(15) B. E. Volcani and E. E. Snell, *J. Biol. Chem.*, **174**, 823 (1948).
(16) S. Korkes and S. Ochoa, *ibid.*, **176**, 463 (1948).
(17) L. R. Hac, E. E. Snell and R. J. Williams, *ibid.*, **159**, 273 (1945).
(18) H. P. Broquist and E. E. Snell, *ibid.*, **180**, 59 (1949).
(19) D. M. Hegsted, *ibid.*, **187**, 365 (1950).
(20) M. N. Camien and M. S. Dunn, *ibid.*, **187**, 365 (1950).
(21) B. A. Prescott, E. Borek, A. Brecher and H. Waelsch, *ibid.*, **181**, 273 (1949).
(22) S. Eiduson, M. N. Camien and M. S. Dunn, *Archiv. Biochem.*, **29**, 302 (1950).
(23) E. E. Snell, *ibid.*, **2**, 389 (1943).
(24) E. E. Snell, *J. Biol. Chem.*, **158**, 497 (1945).
(25) R. J. Sirney, L. T. Cheng and C. A. Elvehjem, *ibid.*, **190**, 547 (1951).
(26) A. Furst, H. A. Harper, R. J. Seiwald, M. D. Morris and R. A. Nevé, *Archiv. Biochem. Biophys.*, **31**, 160 (1951).
(27) H. A. Harper, A. Furst and F. D. Morris, *Wasmann J. Biol.*, **8**, 299 (1950).

It seems probable that the α-amino groups of arginine, lysine, methionine and tyrosine are not free in the bovine plasma albumin molecule even though the content of these amino acids was significantly less in the deaminized than in the native protein. Presumably, nitrous acid acts on the ε-amino group of lysine, the guanidino radical of arginine and the hydroxyphenyl radical of tyrosine to form the hydroxy, nitrosated and possibly other types of derivative which, though present in a hydrolysate of the protein, were without effect on the growth of the organism employed to determine the parent amino acid.

In the estimation of the molecular weight of bovine plasma albumin from the data of Table III, the analyses for tryptophan and methionine are the most critical. These analyses are more consistent with a molecular weight of 66,000 than with the values 69,000 and 70,000, reported by Scatchard, *et al.*,[28] and Adair and Robinson,[29] respectively, who employed osmotic pressure methods. Brand's[7] analyses for these two amino acids lead to the values of 70,300 and 73,600, respectively, compared to the values of 66,400 and 64,800 reported here.

TABLE III

AMINO ACID RESIDUES PER CENT. AND PER MOLE OF BOVINE PLASMA ALBUMIN

Amino acid	Residue, %	Minimum mol. wt.	No. residues per mole[a]		Mol. wt.
Alanine	4.99	1,785	37[c]		66,045
Arginine	5.06	3,090	21 ± 0.1	(24)	64,900
Aspartic acid	8.74	1,330	50 ± 0.1	(53)	66,500
Cystine	5.7	3,580	18[d]	(18)	64,400
Glutamic acid	19.7	655	100 ± 4	(85)	65,500
Glycine	1.34	4,250	16 ± 0.3	(17)	68,000
Histidine	3.34	4,100	16 ± .4	(16)	65,600
Isoleucine	2.48	4,550	15 ± .2	(14)	68,300
Leucine	9.82	1,150	58 ± 1	(69)	66,700
Lysine	10.4	1,240	53 ± 3	(56)	65,800
Methionine	0.81	16,200	4 ± 0.08	(4)	64,800
Phenylalanine	5.62	2,650	25 ± .8	(24)	66,300
Proline	3.94	2,640	27 ± .8	(32)	66,400
Serine	3.14	2,770	24 ± .8	(28)	66,400
Threonine	4.95	2,040	32 ± .6	(36)	65,300
Tryptophan	0.56	33,200	2 ± .04	(2)	66,400
Tyrosine	3.97	4,110	16 ± .3	(20)	65,800
Valine	5.07	1,950	34 ± .7	(37)	66,300
Amide[b]	1.17				
Total	99.9		Average		66,100 ± 760

[a] The values shown in the parentheses are those of Brand[7] calculated on the basis of an assumed molecular weight of 66,100. [b] Determined as ammonia distilled from an acid hydrolysate of plasma albumin made slightly basic with *N* NaOH. [c] Taken from Stein and Moore.[10] [d] Calculated from difference between total sulfur determined by a chemical method and sulfur of methionine determined by microbiological assay.

The data in Tables II and III are consistent with the view that two or three terminal α-amino groups in bovine plasma albumin are contributed by aspartic acid, one by methionine, one by histidine and an undetermined number by alanine.

LOS ANGELES, CALIFORNIA

(28) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946).
(29) G. S. Adair and M. E. Robinson, *Biochem. J.*, **24**, 1864 (1930).