Biochem. J. (1976) **159**, 529–533
Printed in Great Britain

Determination of the Number and Relative Position of Tryptophan Residues in Various Albumins

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(*Received* 20 *April* 1976)

A technique is described by which both the number of tryptophan residues and their approximate locations in the peptide chain of a protein can be determined by cleavage with *N*-bromosuccinimide followed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The number of new peptide bands appearing in the gel is a function of the number of tryptophan residues, and the relative migration of the bands permits calculation of peptide molecular weights and an estimation of the positions of the tryptophan residues in the peptide chain. The technique uses a sample of about 0.5 mg and is suitable for any protein that contains a small number of tryptophan residues. These are the very specimens that are difficult to assay accurately for tryptophan by spectrophotometric or colorimetric methods. Tryptophan residues which are within about 20 residues of the ends of the peptide chain or of each other would not be detected. The specificity of the cleavage with *N*-bromosuccinimide was ascertained by utilizing human serum albumin, which is known to have a single tryptophan residue at position 214. The technique was then applied to a comparative study of the numbers and locations of tryptophans in the serum albumins of 16 species, namely 11 mammals, three birds and two amphibians. The numbers of tryptophan residues were confirmed by an independent colorimetric method. All of the mammalian albumins contained a tryptophan residue near position 213. The three avian albumins examined have no tryptophan. Frog and toad albumins contained two tryptophan residues, which appear to be situated at different positions from those in mammalian albumins.

The modification and cleavage of peptides by *N*-bromosuccinimide has been reviewed by Witkop (1961) and Ramachandran & Witkop (1967). In aqueous solutions, *N*-bromosuccinimide is an active reagent which is capable of the cleavage of peptide chains at tryptophan, tyrosine and histidine residues. The reagent can, however, be made relatively specific for tryptophan residues. Peters (1959) and Ramachandran & Witkop (1959) independently determined that optimum cleavage at the lone tryptophan residue in human serum albumin and the two tryptophan residues in bovine serum albumin was achieved in 8M-urea buffered with acetate at about pH4. The reaction required 10–20 mol of *N*-bromosuccinimide/mol of protein and was essentially complete within 15 min. Cleavage yields of 30–50% were determined by quantifying the new *N*-terminal residues with fluoro-2,4-dinitrobenzene.

A concentrated urea/acetate solution offers two advantages, in that (*a*) proteins are at least partially denatured such that 'buried' tryptophan residues are likely to be exposed and (*b*) the *N*-bromosuccinimide reacts with the urea to yield *N*-bromourea, a milder oxidizing agent, which under these conditions has increased specificity for tryptophan residues (Funatsu *et al.*, 1964).

We have expanded on the original studies with human and bovine serum albumin to develop a method which allows for the simultaneous determination of the number and relative positions of tryptophan residues. After cleavage, the reaction products are analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The number of new peptide bands appearing in the gel is a function of the number of tryptophan residues, and the relative migration of the bands permits calculation of peptide molecular weights and an estimation of the site(s) of cleavage.

The optimal conditions for the cleavage of the lone tryptophan bond at position 214 in the human albumin sequence (Meloun *et al.*, 1975) were determined, and the technique was then applied to a comparative study of the numbers and locations of tryptophan residues in the serum albumins of 16

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species, namely 11 mammals, three birds and two amphibians. The results have been compared with the known locations of the single tryptophan residue in human albumin and the two tryptophan residues in bovine albumin (Brown, 1975). The number of tryptophan residues was confirmed by an independent method.

The technique uses about 0.5mg of protein and should be generally useful for the determination of the number of tryptophan residues and their positions in proteins that contain a small number of tryptophan residues, since such proteins produce a limited number of new peptides when made to react with *N*-bromosuccinimide. These are the very specimens that are difficult to assay accurately for tryptophan by spectrophotometric or colorimetric methods.

Materials

Albumins

Chicken, horse, human, pig, sheep, goat, guinea-pig, rabbit and dog albumins were purchased as fraction V powders from Miles Laboratories (Elkhart, IN, U.S.A.). Crystallized bovine plasma albumin was purchased from Armour Pharmaceutical Co. (Chicago, IL, U.S.A.). Bullfrog (*Rana catesbeiana*) mouse, rat and toad (*Bufo marinus*) albumins were prepared by standard procedures for the purification of albumins, which included salt fractionation, gel filtration and ion-exchange chromatography, or the trichloroacetic acid-precipitation/ethanol-extraction method (reviewed by Peters, 1970). Duck and turkey albumins were preparations designated 'II' from an earlier publication (Peters *et al.*, 1958). All albumins were more than 95% homogeneous as judged by electrophoresis in cellulose acetate or polyacrylamide gel. Albumin preparations were stored at −20°C.

Chemicals

Reagent-grade *N*-bromosuccinimide (Fisher Scientific Co., Fairlawn, NJ, U.S.A.) was further purified by two recrystallizations from acetone. Acrylamide (Eastman Kodak, Rochester, NY, U.S.A.) was also recrystallized twice from acetone. Sephadex G-10 and Dextran Blue 2000 were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). L-Tryptophan was from Nutritional Biochemicals Corp. (Cleveland, OH, U.S.A.). Pronase was 'B' grade from Calbiochem (La Jolla, CA, U.S.A.). *p*-Dimethylaminobenzaldehyde was Eastman Kodak no. 95, recrystallized from dilute HCl by the addition of NaOH. A stock solution of 10M-urea was deionized on a mixed-bed ion-exchange column of Rexyn I-300 (Fisher Scientific Co.). Other chemicals were reagent grade.

Methods

The protocol was designed to accommodate a small amount of protein (0.5–0.6mg) in a 1-day procedure. Reaction with *N*-bromosuccinimide was conducted in 8M-urea/2M-acetic acid. The excess of reagent and the acetic acid were then separated from the protein on a column of Sephadex G-10, which had been previously equilibrated with 8M-urea; elution was with 8M-urea. The protein in 8M-urea was then incubated with sodium dodecyl sulphate/β-mercaptoethanol solution before electrophoresis.

Column procedures

The columns were prepared from disposable Pasteur pipettes [0.52cm (internal diam.)×14.6cm], which were plugged with a small amount of glass wool and then filled with hydrated Sephadex G-10 to 8.5cm above the plug (1.8ml bed volume). Individual rinsing reservoirs for each column were constructed from the barrel of a disposable 5ml syringe equipped with a 19-gauge needle which had been inserted through a number 000 cork. The void volume of each column was individually determined with a 50μl sample of a 0.2% (w/v) Dextran Blue 2000/8M-urea solution. The columns were rinsed with at least 5ml of 8M-urea before each experiment.

Reaction with N-bromosuccinimide

About 0.5mg of freeze-dried protein was dissolved in 90μl of 8M-urea/2M-acetic acid and allowed to react at 23°C for 20min with a 20:1 molar ratio of *N*-bromosuccinimide, added in 10μl of 8M-urea/2M-acetic acid. A 50μl portion of the reaction mixture was then applied to a Sephadex G-10 column and eluted with 8M-urea. The protein was collected as a 150μl fraction, which corresponded to the void volume of each column.

Polyacrylamide-gel electrophoresis

After elution from the Sephadex G-10 column, a 100μl portion of the *N*-bromosuccinimide-treated protein was mixed with 25μl of 5% (w/v) sodium dodecyl sulphate/10% (v/v) β-mercaptoethanol in a small screw-cap vial (1cm×3.5cm) and left at 23°C for 90–120min. A 10μl portion of each reduced sample was then subjected to electrophoresis on polyacrylamide gel [5mm (internal diam.)×55mm], prepared from 6% (w/v) acrylamide, at 8V/cm for 70min. Protein bands were stained with Coomassie Blue and destained by rinsing (Weber *et al.*, 1972).

Standard determination of tryptophan

The tryptophan content of albumins was determined chemically by a small-scale modification of the methods of Spies (1967). Portions (about 1mg) of various albumins were freeze-dried in test tubes (1cm×6cm). To each tube was added 30μl of 0.4M-

Tris/HCl buffer, pH 9.2, and 10 μl of Pronase (1 mg/ml in the same buffer). The specimens were allowed to digest for 16h at 37°C, covered tightly with Parafilm. To all samples, plus a series of L-tryptophan standards and blanks, were added 30 μl of water and 675 μl of a solution of *p*-dimethylaminobenzaldehyde (3 mg/ml in 10.5 M-H_2SO_4). Blanks were included with *p*-dimethylaminobenzaldehyde. The contents were mixed on a Vortex mixer and left for 3h at 23°C in the dark. A 10 μl portion of freshly prepared $NaNO_2$ (0.45 mg/ml) was then added, the contents were mixed again and left for 30min at 23°C in the dark. Absorbance was recorded over the range 530–670nm in a Hitachi–Perkin–Elmer model 124 spectrophotometer. The amount of tryptophan in the unknowns was determined from the net absorbance at the maximum at 590nm compared with that of the standards.

Determination of albumin

Albumin concentrations were determined by the biuret method (Gornall *et al.*, 1949) or the Lowry technique (Lowry *et al.*, 1951), referred to bovine albumin as standard. Concentration of bovine albumin was determined by using the factor $E_{280}^{mg/ml} = 0.667$ (Janatova *et al.*, 1968).

Results and Discussion

In Plate 1(*a*) electrophoresis in sodium dodecyl sulphate/polyacrylamide gels demonstrates the products of the reaction of *N*-bromosuccinimide with human serum albumin at various molar ratios. A 20min reaction time was based on the earlier studies of Peters (1959) and Ramachandran & Witkop (1959). As noted by the previous workers, cleavage products are present in only trace amounts at or below a 5:1 molar ratio of *N*-bromosuccinimide to albumin. Above a 20:1 ratio, cleavage at sites other than tryptophan becomes more apparent, such that at ratios of 80:1 or 100:1 there is general destruction of the protein. We therefore selected a 20:1 ratio of reagent to albumin.

Cleavage products at various reaction times are shown in Plate 1(*b*). The reactions were terminated by quenching with a 10-fold molar excess of L-tryptophan. Plate 1(*b*) shows that there is little difference between reaction times from 0.5 to 60min. In other experiments (not shown) no changes in products or yield were observed at reaction times up to 4h. A 20min reaction time was selected because it was in the region of optimum yield and allowed for the convenient handling of up to ten samples.

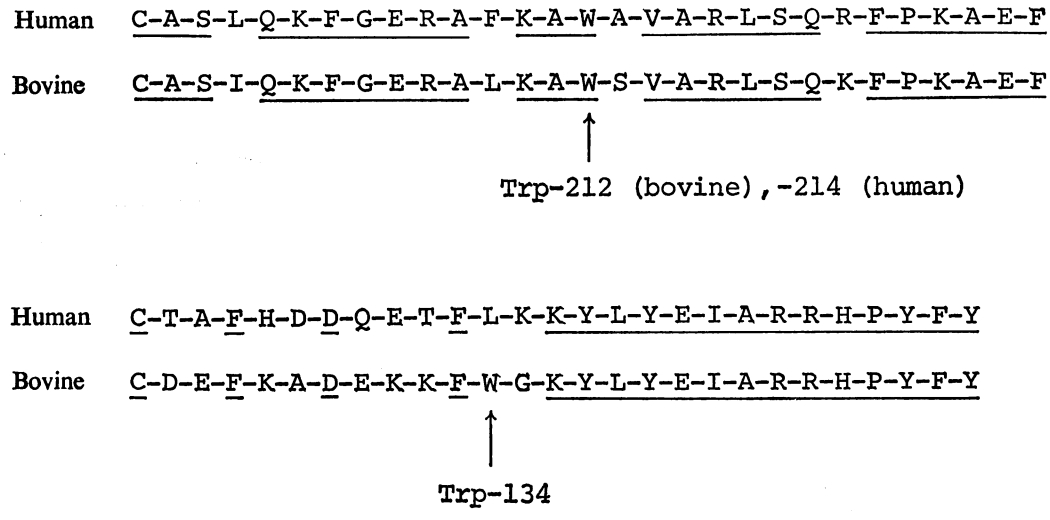
The serum albumins of 16 species and two known proteolytic fragments of bovine albumin were investigated for tryptophan content and tryptophan residue position, with the results shown in Plate 2. For human albumin or other proteins with a lone tryptophan residue, the reaction products should include the intact molecule and two fragments. The molecular weights of the two cleavage products from human albumin were calculated from their relative mobilities in the electrophoretic gels (Weber *et al.*, 1972) and compared with the values calculated from the known sequence of human albumin (Behrens *et al.*, 1975; Meloun *et al.*, 1975). The experimental values of 23800 and 42400 for the *N*- and *C*-terminal portions respectively agreed well with the theoretical values of 24610 and 41830, confirming the relative location of the tryptophan residue in the published sequence.

When two tryptophan residues are present, limited cleavage should yield the intact molecule plus five peptide fragments. For bovine albumin, the expected peptides would correspond to residues 1–134, 1–212, 135–581, 213–581 and 135–212 (Brown, 1975). As for human albumin, the theoretical and experimental molecular weights for these peptides (Plate 2*a*) confirmed the location of these tryptophan residues in the proposed sequence (Brown, 1975). The cleavage results for other albumins in Plate 2 can thus be compared with the known cleavage products of human and bovine albumins. The avian albumins, chicken, duck and turkey, and the peptic fragment P-A contain no tryptophan residues, whereas the 11 mammalian albumins contain either one or two. In each of these mammals one tryptophan residue is found near residue 213, as is the lone tryptophan residue in human albumin, and a second one, if present, is found near residue 134, as in bovine albumin. The fact that 11 species, representing six placental-mammalian orders, all possess a tryptophan residue near position 213 is significant. In evolutionary terms the implication is that this tryptophan residue probably first appeared in some pre-placental mammal more than 70 million years ago, since the various placental orders diverged between 50 and 70 million years ago (Romer, 1959). The conservative nature of amino acid substitutions in this area of the albumin chain (Table 1) suggests an important biological function, which is likely to include the binding of bilirubin and fatty acids (Reed *et al.*, 1975).

All of the mammals examined which have two tryptophan residues (cow, goat, sheep and pig) belong to the order Artiodactyla. Within that order the pig family (Suidae) diverged from the bovine family (Bovidae) about 25–30 million years ago (Romer, 1959). Since the horse (Perissodactyla) and cow have enjoyed distinct ancestry for 50 million years (Romer, 1959), it has been inferred that the tryptophan residue at position 134 appeared about 30 to 50 million years ago and has been conserved for at least 25 million years. In the sequences of human and bovine albumins, there is an area of substantial amino acid substitution preceding the tryptophan-134 residue and one of relatively strict conservation

Table 1. *Comparison of amino acid sequences near the tryptophan residues of human and bovine albumins*

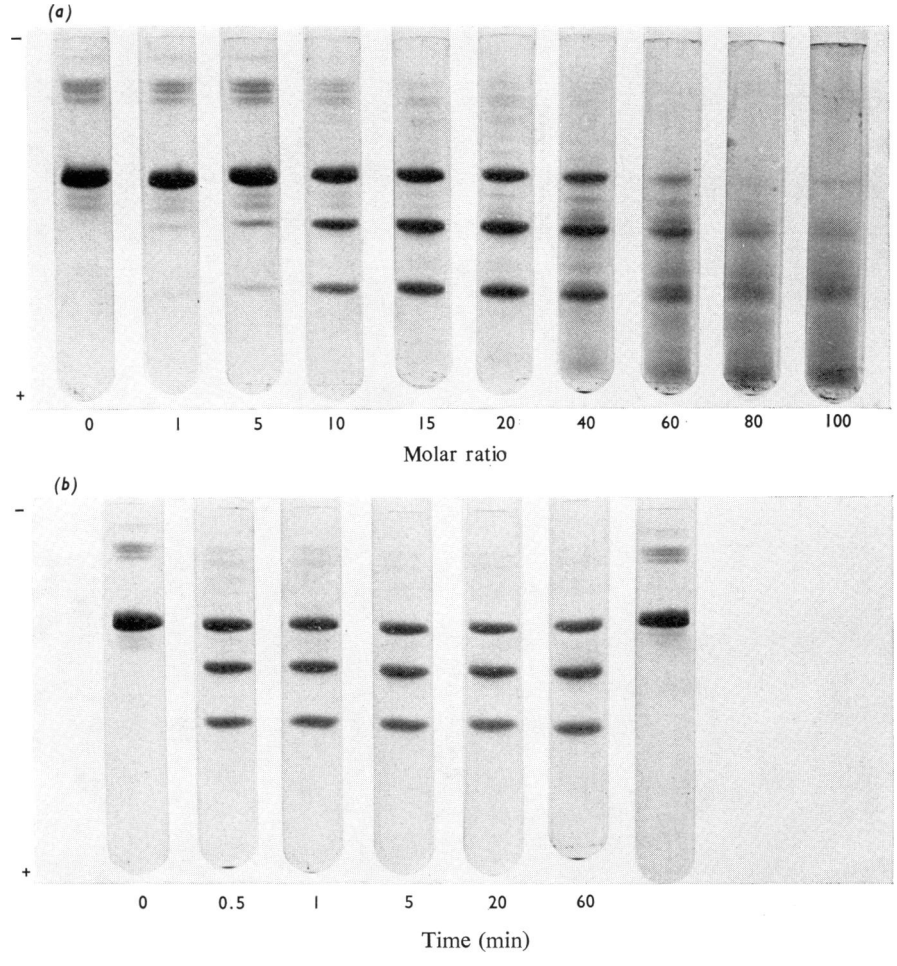
Data for human albumin are from Meloun *et al.* (1975) and those for bovine albumin are from Brown (1975). For convenience, the one-letter code for amino acids is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Residues common to the two albumins are underlined.



following it (Table 1). The biochemical function, if any, of this area of the molecule remains to be elucidated. In view of the apparently strict conservation of the position of tryptophan residues in the albumins derived from these placental mammals, it will be of future interest to investigate the albumins from some other mammals, for example, the marsupials and the very primitive platypus, as well as from reptiles. It might be pointed out that proteins with physicochemical properties typical of albumins are also found in the amphibians (Wallace & Wilson, 1972; Nagano *et al.*, 1973; Feldhoff, 1973) and hence albumin phenotypic properties have been maintained for at least 300 million years.

The tryptophan content of each of the albumins used in this study was also determined by an independent colorimetric assay (Table 2). The results obtained by both methods are in concurrence for all of the avian and mammalian albumins, but with the amphibian species the gel procedure does not give a clear-cut answer. From the gels it appears that the toad and the frog both have one tryptophan residue, which seems to be located about 100 residues from one end, whereas the colorimetric results indicate two tryptophan residues per molecule. Several explanations for the inability to locate a second tryptophan residue point out possible limitations of the methodology. First, a tryptophan residue may be 'buried' and unreactive, although this is unlikely in an 8M-urea solution at low pH. Second, the tryptophan residues may be so situated that the cleavage products could not be resolved on the gel, or would be too small to be stained effectively. For adequate resolution the tryptophan residues should be located more than 20 residues from each other and from either end of the peptide chain. An example is gel (ix) (Plate 2*b*), which shows the reaction products of the 35000-mol.wt. peptide, P-B. This peptide corresponds to the first 306 residues of bovine albumin and contains two tryptophan residues (Feldhoff & Peters, 1975). The expected mol.wts. of fragments derived from this peptide are 9000, 10500, 15500, 19500 and 24500, and in fact each can be seen on the gel.

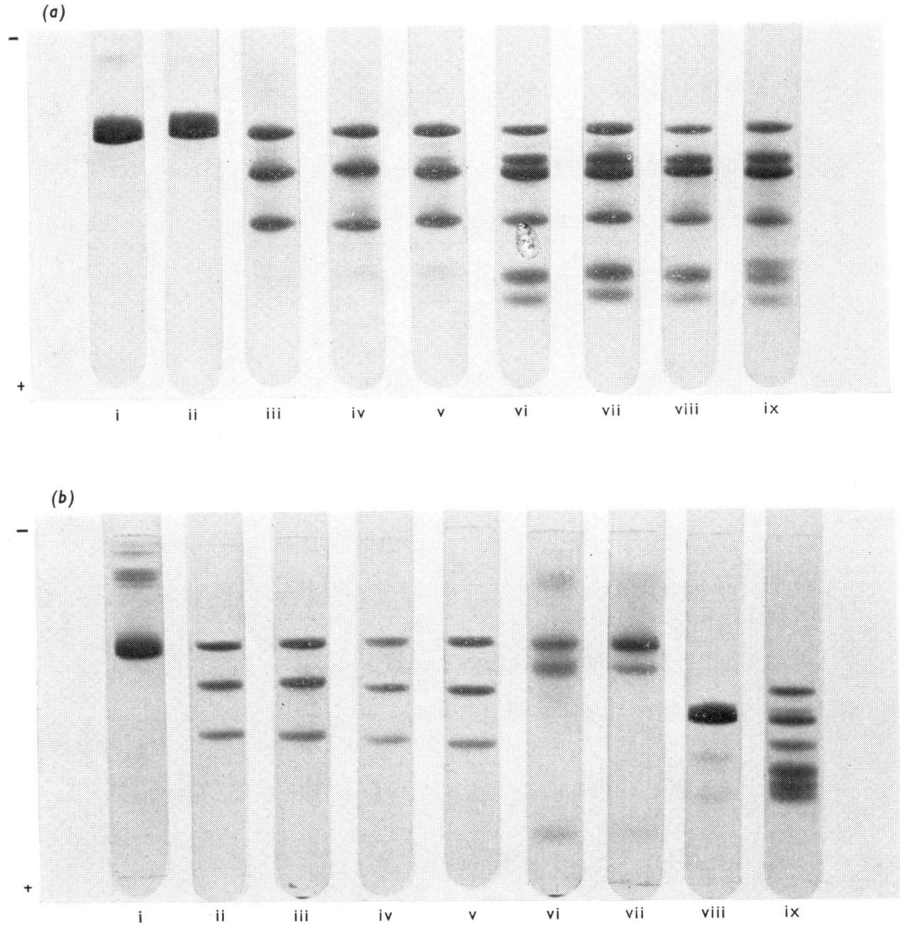
The limits of the methodology cannot be defined accurately until many different proteins are studied, but from a practical point of view it will be difficult to determine either the numbers or the relative position when there are more than three tryptophan residues, because of the large number of overlapping reaction products formed. For some proteins it may be useful to study the products with and without disulphide-bond reduction to determine whether or not the tryptophan residues are located within disulphide loops. In the serum albumins the tryptophan residues are found within disulphide loops, therefore gels run without reduction show only one band migrating in the region of the intact molecule. Any study using this methodology will be facilitated by knowing the sequence and tryptophan position in



EXPLANATION OF PLATE 1

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of reaction products of N-bromosuccinimide with human albumin (a) at various molar ratios and (b) after various periods of time

(*a*) Reaction was at 23°C for 20min in 8M-urea/2M-acetic acid. Gels are 6% (w/v) polyacrylamide. *N*-Bromosuccinimide/albumin molar ratios are listed below each gel. (*b*) Reaction was at a 20:1 molar ratio of *N*-bromosuccinimide to albumin and 23°C. Reaction times (min) are listed below each gel. In the right-hand specimen, marked (20), a 10-fold molar excess of L-tryptophan was added before the *N*-bromosuccinimide.



EXPLANATION OF PLATE 2

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of reaction products of N-bromosuccinimide with various albumins

Reaction was at a 20:1 molar ratio of *N*-bromosuccinimide to each albumin for 20min at 23°C. (*a*) (i), Chicken; (ii), turkey; (iii), man; (iv), rabbit; (v), horse; (vi), cow; (vii), goat; (viii), sheep; (ix), pig. (*b*): (i), duck; (ii), mouse; (iii), guinea pig; (iv), dog; (v), rat; (vi), frog; (vii), toad; (viii), cow fragment P-A; (ix), cow fragment P-B. Fragments P-A and P-B are from Feldhoff & Peters (1975).

Table 2. *Tryptophan content of albumins by two methods*

The values are mol of tryptophan/mol of albumin (mol.wt. 66000). Colorimetric analysis of each albumin was performed as part of this study. Samples are listed in the same order as they appear in Plate 2. Values are means ± S.D. with the number of analyses in parentheses.

Albumin species	Colorimetric	Cleavage with *N*-bromosuccinimide
Chicken	0.2* ±0.05 (2)	0
Turkey	0.1 ±0.05 (2)	0
Man	1.0*† ±0.1 (4)	1
Rabbit	1.1* ±0.1 (4)	1
Horse	1.2 ±0.1 (2)	1
Cow	2.0* ±0.1 (6)	2
Goat	1.8 ±0.05 (3)	2
Sheep	2.4 ±0.05 (3)	2
Pig	2.2 ±0.1 (3)	2
Duck	0.1 ±0.05 (2)	0
Mouse	1.0 ±0.05 (2)	1
Guinea pig	1.4 ±0.2 (2)	1
Dog	1.1* ±0.1 (4)	1
Rat	1.0* ±0.1 (4)	1
Frog	2.1 ±0.1 (2)	1
Toad	1.8 ±0.15 (4)	1
Cow, fragment 'P-A'	0‡	0
Cow, fragment 'P-B'	2‡	2

* Confirmatory data for these species are available (reviewed by Peters, 1970).

† The human albumin fraction V gave higher results (2.6 mol/mol) unless it was precipitated with trichloroacetic acid (8%, w/v) before analysis to remove a contaminant, probably acetyltryptophan.

‡ Fragments 'P-A' and 'P-B' from Feldhoff & Peters (1975).

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a similar protein derived from a different tissue or organism; however, this is not a prerequisite to obtaining useful information. For most studies it would be advisable to determine the optimum molar ratio of *N*-bromosuccinimide to protein as in Plate 1 and to include human and bovine albumins as experimental controls.

We are indebted to Dr. James R. Brown of the University of Texas, Austin, for furnishing the sequences of human and bovine albumins before publication. We are also grateful to James A. Robinson for several weeks of volunteer work on this project and to Carol MacKay for technical assistance. Dr. Allen G. Ramsay and his assistant, David L. Gallagher, kindly provided us with blood samples from the marine toad, *Bufo marinus*. This work was supported by USPHS grant HL-02751 and the Stephen C. Clark Research Fund of The Mary Imogene Bassett Hospital. The Mary Imogene Bassett Hospital is affiliated with Columbia University.

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