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Note

Separation of cyanogen bromide fragments from normal and abnormal human serum albumin by reversed-phase high-performance liquid chromatography

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Human serum albumin consists of a single polypeptide chain of 585 residues, containing 17 intra-chain disulphide bonds, a unique free cysteine and six methionine residues[1–3]. Isolation of cyanogen bromide (CNBr) fragments from normal albumin[2,4–7] as well as from some of its genetic variants[8–10] has so far been accomplished by polyacrylamide gel electrophoresis or by conventional chromatographic techniques.

The first procedure[10,11], which has also been used on a preparative scale[10], appears to be complicated by difficulties in isolating all the fragments in a pure form and in a suitable amount for structural studies. The second method consists of a multi-step procedure: CNBr cleavage, carried out on unreduced albumin, gives three large fragments (named B, C and A, according to McMenamy *et al.*[4], from the N-terminal end of the molecule), which have been separated by gel filtration. Fragment C consists of a single polypeptide chain while fragments B and A are made up, of two and four chains, respectively, held together by S–S bonds. The peptides contained in A and B can be obtained in a purified form only after reduction, carboxamidomethylation and ion-exchange chromatography. The separation of all the CNBr fragments from the reduced carboxymethylated albumin has been achieved, in this present study, by a simple reversed-phase high-performance liquid chromatographic (HPLC) method; the reported procedure has been employed to characterize a genetic variant of this protein, the fast-migrating Mi/Fg albumin[12].

Our results illustrate the well known advantages of HPLC for the separation of large fragments[13–20] and its specific suitability for the detection of amino acid replacements within the primary structure of the proteins[21–27].

EXPERIMENTAL

Materials

Trifluoroacetic acid (TFA) (sequanol grade) was obtained from LKB (Bromma, Sweden), acetonitrile (HPLC grade) was supplied by Merck (Darmstadt, F.R.G.) and filtered through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) prior to use. The distilled water that was used as the mobile phase for HPLC separations was filtered through a LiChroprep RP-8 column (Merck). Cyanogen bromide,

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iodoacetic acid and dithiothreithol were purchased from Eastman Kodak (Rochester, NY, U.S.A.). All the other chemicals were of analytical-reagent grade and were obtained from Carlo Erba (Milan, Italy).

Purification, carboxymethylation and cyanogen bromide digestion of albumin

Normal human serum albumin and Mi/Fg variant were purified according to Winter[28]. Carboxymethylation was performed as described by Swenson *et al.*[29]. Albumin at a concentration of 10 mg/ml was reduced under nitrogen at 25°C for 3 h in 1 *M* Tris–HCl (pH 8.0) containing 6 *M* guanidine and a 10-fold molar excess of dithiothreithol over the –SH concentration. Iodoacetic acid (1.3-fold molar excess over total thiols) was added, and the mixture was maintained in the dark for 60 min before the reaction was terminated by addition of 2-mercaptoethanol. Excess of reagents was removed by exhaustive dialysis against distilled water and the protein was freeze-dried. Cyanogen bromide cleavage was carried out on 0.5 μmol of carboxymethylated albumin at a concentration of 10 mg/ml in 70% formic acid; a 100-fold molar excess of cyanogen bromide over the methionine residues was added and the reaction was allowed to proceed for 24 h in the dark at 25°C under a nitrogen atmosphere. The mixture was then diluted 20-fold with distilled water and freeze-dried[30].

HPLC separation of cyanogen bromide fragments

The separation was performed essentially as reported by Tarr and Crabb[20]. A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with two M6000 pumps, a U5K sample injector, a Model 680 automated gradient controller and a variable-wavelength detector (Japan Spectroscopic Co., Tokyo, Japan) was employed.

A cyanogen bromide digest of serum albumin was dissolved in 0.05% aqueous trifluoroacetic acid (TFA) (solvent A) and 100 μl of the solution (corresponding to about 6 nmol of protein) were injected on to a Vydac C-18 column (10 μm, 25 cm × 4.6 mm I.D.) (Separations Group, CA, U.S.A.) equilibrated with 80% of solvent A and 20% of acetonitrile–2-propanol (2:1, v/v) containing 0.05% of TFA (solvent B). Elution of peptides was achieved by use of a 30-min linear gradient from 20% to 60% of solvent B at a flow-rate of 2 ml/min.

Two fragments, which were coeluted under the above conditions, were separated on a μ-Bondapak C_{18} column (10 μm, 30 cm × 3.9 mm I.D.) (Waters Assoc.), washed with 0.05% aqueous TFA (solvent A) and equilibrated with 80% solvent A and 20% of acetonitrile–0.05% TFA (solvent B). A linear gradient was run at 2 ml/min from 20% to 35% of solvent B over a period of 15 min; the concentration of solvent B was then linearly increased to 60% in an additional period of 15 min.

All runs were performed at room temperature and individual peaks were collected manually. Amino acid analysis was performed on a Beckman Model 120B automatic analyser, equipped with high-sensitivity cuvettes, according to Dévényi[31]. N-Terminal amino acids were identified as the dansyl derivatives according to Hartley[32].

RESULTS AND DISCUSSION

Seven fragments (ranging between 31 and 175 aminoacyl residues) were expected, from the known sequence of human serum albumin[1-3], by submitting the whole reduced carboxymethylated protein to CNBr cleavage. Several attempts to separate all these fragments in a single run, using both μBondapak C_{18} or Vydac C_{18} as reversed-phase supports, and various solvent conditions, were unsuccessful. However, their complete separation was achieved using a two-step method: in the first step the CNBr digest of the reduced carboxymethylated albumin was passed through

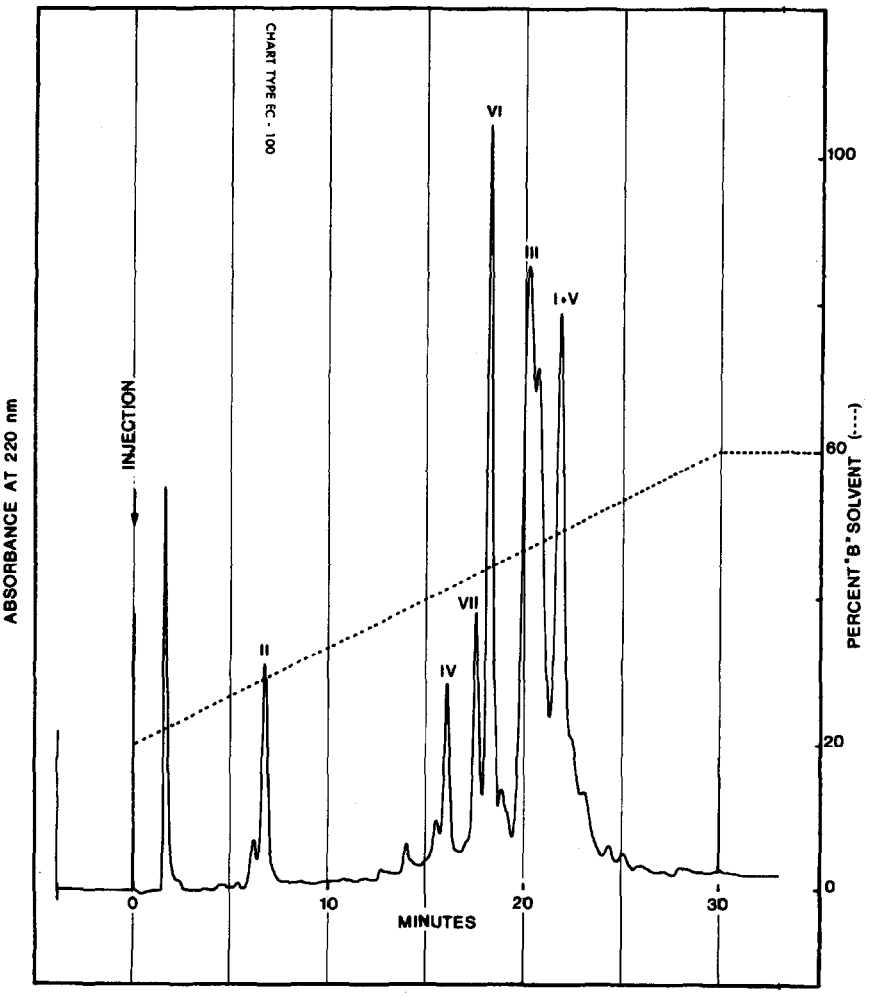

Fig. 1. Elution pattern of cyanogen bromide fragments of the whole reduced and carboxymethylated normal human serum albumin. The digest was dissolved in 0.05% aqueous TFA (pH 2.3) (solvent A) and 100 μl (corresponding to about 6 nmol of protein) were injected on to a Vydac C_{18} column (25 cm × 4.6 mm I.D.) equilibrated with 80% of solvent A and 20% of acetonitrile–2-propanol (2:1, v/v) containing 0.05% of TFA (solvent B). The elution was performed at room temperature, using a 30 min linear gradient from 20% to 60% of solvent B. Flow-rate, 2 ml/min; absorbance range, 1.28 full-scale.

a Vydac C_{18} column and in the second the mixture of two unresolved peptides was purified on a μBondapak C_{18} column.

As shown in Fig. 1, five fragments are eluted as separate peaks when the whole CNBr digest of human albumin is chromatographed on a Vydac C_{18} column. The fragments eluted under each peak were identified by amino acid and N-terminal analysis, and are designated by roman numerals starting from the N-terminus of the molecule; the results in Table I show that these peptides are obtained in homogeneous form. The fact that CNBr III gives a double peak may be due to the homoserine–homoserine lactone equilibrium[33] or, possibly, to modifications of some aminoacyl residues occurring on CNBr-acid treatment of the protein; such a conclusion is drawn from the observation that the same amino acid composition and N-terminus are found by analysing the separately collected material of the "two" peaks. As each of the separated fragments is recovered in a good and very similar yield (about 70% as

TABLE I

AMINO ACID COMPOSITION OF THE CNBr FRAGMENTS OF NORMAL SERUM ALBUMIN SEPARATED BY HPLC ON A VYDAC C_{18} COLUMN

Amino acid	*Amino acyl residues**									
	CNBr II		*CNBr III*		*CNBr IV*		*CNBr VI*		*CNBr VII*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Lys	2.00	2	18.72	19	2.92	3	10.79	11	4.95	5
His	1.02	1	5.02	5	–	–	2.99	3	–	–
Arg	2.94	3	8.93	9	–	–	3.84	4	–	–
Asp	6.89	7	14.06	14	4.86	5	7.10	7	4.02	4
Thr**	–	–	6.08	6	–	–	8.80	9	1.03	1
Ser**	–	–	8.75	9	2.03	2	5.01	5	1.05	1
Glu	5.07	5	24.15	24	2.08	2	15.06	15	5.01	5
Pro	3.98	4	5.10	5	2.07	2	5.05	5	–	–
Gly	0.92	1	3.10	3	1.07	1	–	–	2.05	2
Ala	2.03	2	22.79	23	5.00	5	7.03	7	7.81	8
Val***	2.86	3	5.00	5	2.79	3	10.88	11	2.03	2
Met	–	1	–	1	–	1	–	1	–	–
Ile***	–	–	3.88	4	–	–	1.89	2	–	–
Leu	2.91	3	18.91	19	3.06	3	9.94	10	3.03	3
Tyr	–	–	6.01	6	0.97	1	1.89	2	–	–
Phe	0.96	1	9.95	10	1.92	2	4.08	4	2.81	3
Cys§	2.87	3	11.75	12	0.78	1	5.87	6	2.79	3
Trp	–	–	n.d.§§	1	–	–	–	–	–	–
Homoserine	0.85	–	0.91	–	0.85	–	0.89	–	–	–
Total	–	36	–	175	–	31	–	102	–	37
NH_2 terminus	Ala		Cys		Pro		Pro		Asp	

* A = Found values (the values are the means of five independent determinations); B = values expected from the sequence[3].

** Corrected for destruction during acid hydrolysis.

*** Corrected for slow release during acid hydrolysis.

§ Determined as S-carboxymethylcysteine.

§§ Not determined.

calculated by amino acid analyses), any problem of limited solubility and/or irreversible interaction with reversed-phase support can be excluded. CNBr fragments V and I are coeluted in a peak at 22–23 min; modifications of the steepness and/or of the relative proportions of acetonitrile and 2-propanol in the elution gradient failed to improve the resolution. However, the separation of CNBr V and CNBr I was achieved by rechromatographing the mixture of the two fragments obtained from the first run on a μBondapak C_{18} column (Fig. 2).

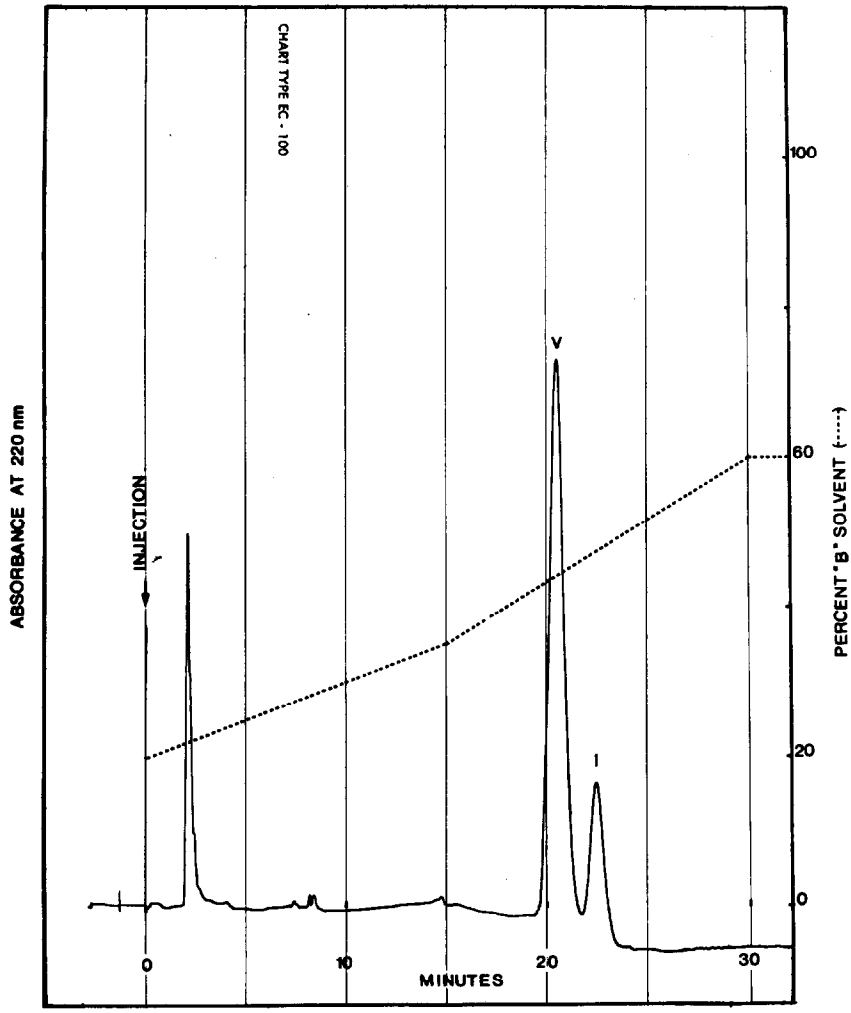

Fig. 2. Elution pattern of cyanogen bromide fragments V and I. The mixture of CNBr V and I, obtained from a Vydac C_{18} column (see Fig. 1) was dissolved in 0.05% aqueous TFA (solvent A) and 100 μl (corresponding to 5 nmol of peptides) were injected on to a μBondapak C_{18} column (30 cm × 3.9 mm I.D.) equilibrated with 80% of solvent A and 20% of acetonitrile–0.05% TFA (solvent B). The elution was performed at room temperature, using a linear gradient from 20% to 35% of solvent B over a period of 15 min; the concentration of solvent B was then linearly increased to 60% in an additional period of 15 min. Flow-rate, 2 ml/min; absorbance range, 0.64 full-scale.

As shown by comparing the elution profiles in Figs 1 and 2, the retention time of CNBr V on a μBondapak C_{18} column is slightly decreased, thus providing its complete resolution from CNBr I.

The two fragments are recovered with the same yield and the data reported in Table II indicate their homogeneity. As expected for a small-pore support, the μBondapak C_{18} column is not very effective in separating all the largest, most hydrophobic peptides from the whole CNBr digest of the albumin; under the conditions shown in Fig. 2, CNBr VI (102 aminoacyl residues) is incompletely resolved from CNBr III and CNBr V (175 and 117 aminoacyl residues, respectively), which are coeluted in a broad peak. No improvement in the resolution on this support can be obtained by replacing acetonitrile with a mixture of acetonitrile and 2-propanol as the eluent; in contrast, the second solvent, particularly in acetonitrile–2-propanol (2:1), gives the best resolution on Vydac C_{18}.

Characterization of Mi/Fg albumin

The above procedure was used for the characterization of Mi/Fg albumin, a

TABLE II

AMINO ACID COMPOSITION OF CNBr FRAGMENTS I AND V OF NORMAL SERUM ALBUMIN SEPARATED BY HPLC ON A μBONDAPAK C_{18} COLUMN

Amino acid	*Amino acyl residues**			
	CNBr I		*CNBr V*	
	A	*B*	*A*	*B*
Lys	6.83	7	11.72	12
His	3.81	4	3.06	3
Arg	2.04	2	5.87	6
Asp	8.92	9	7.06	7
Thr**	5.88	6	5.80	6
Ser**	3.00	3	4.05	4
Glu	13.06	13	17.12	17
Pro	1.02	1	6.80	7
Gly	2.92	3	3.04	3
Ala	8.94	9	8.06	8
Val***	6.84	7	9.72	10
Met	–	1	–	1
Ile***	1.05	1	1.07	1
Leu	9.02	9	13.79	14
Tyr	1.88	2	6.85	7
Phe	5.87	6	5.07	5
Cys§	4.02	4	5.86	6
Homoserine	0.89	–	0.92	–
Total		87		117
NH_2 terminus	Asp		Phe	

* A = Found values (the values are the means of two independent determinations); B = values expected from the sequence[3].

** Corrected for destruction during acid hydrolysis.

*** Corrected for slow release during acid hydrolysis.

§ Determined as S-carboxymethylcysteine.

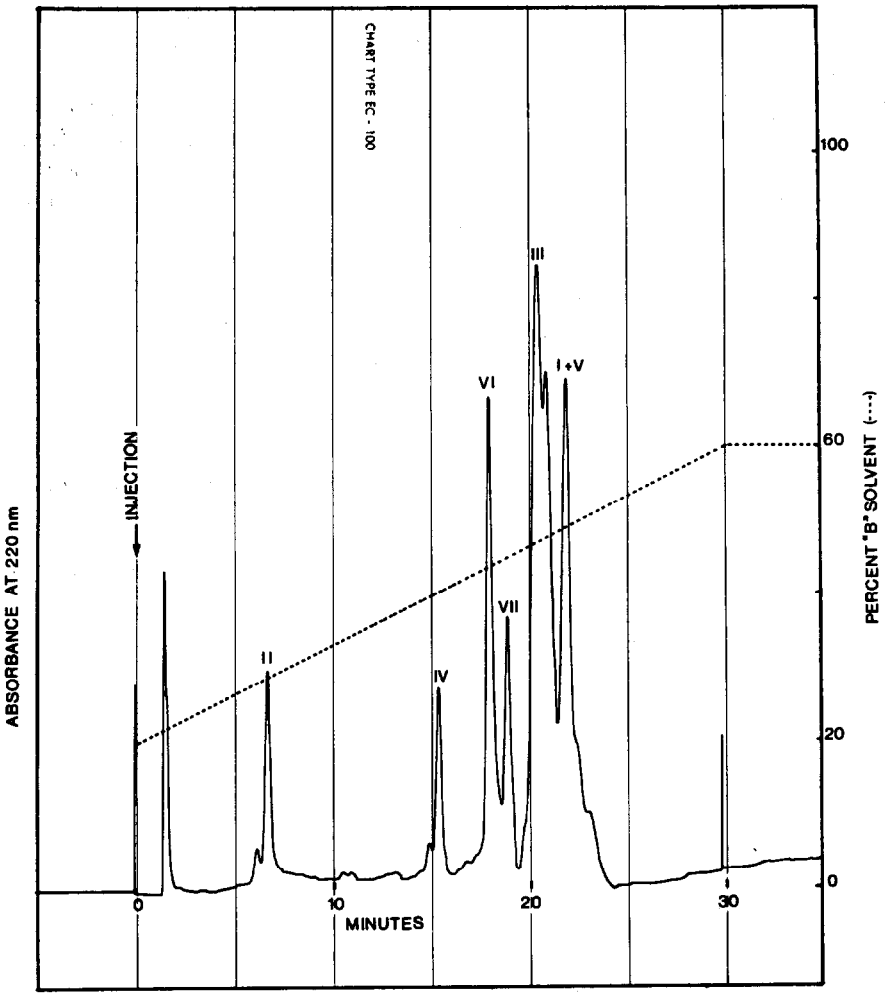

Fig. 3. Elution pattern of cyanogen bromide fragments of the whole reduced carboxymethylated Mi/Fg albumin. Experimental conditions as in Fig. 1.

fast-migrating genetic variant of this protein, which was obtained from an apparently healthy 15-year-old boy, homozygous for this variant. CNBr fragments were isolated from the reduced, carboxymethylated protein; the chromatographic pattern in Fig. 3 shows that all the peaks except one are eluted with the same retention time as the corresponding peaks from normal serum albumin. The abnormal peptide was identified by its amino acid composition and N-terminus (Table III) as the modified CNBr VII (residues 549–585 of the sequence), which contains a glutamyl instead of a lysine residue. Mi/Fg albumin can thus be added to the few[10,28] among the several reported genetic variants of albumin[34] that have been characterized at a molecular level.

Although no disease has been associated with genetically determined variations

TABLE III

AMINO ACID COMPOSITION OF CNBr VII FRAGMENTS ISOLATED FROM NORMAL AND Mi/Fg ALBUMINS

Amino acid	*Amino acyl residues*	
	Normal CNBr VII	*Mi/Fg CNBr VII**
Lys	4.95	3.95
Asp	4.02	4.06
Thr**	1.03	0.95
Ser**	1.05	1.09
Glu	5.01	6.03
Gly	2.05	2.08
Ala	7.81	7.79
Val***	2.03	1.97
Leu	3.03	2.99
Phe	2.81	2.88
Cys§	2.79	2.79
NH_2 terminus		Asp

* The values are the means of two independent determinations.
** Corrected for destruction during acid hydrolysis.
*** Corrected for slow release during acid hydrolysis.
§ Determined as S-carboxymethylcysteine.

of albumin, screening for variants could be of interest as albumin is the major carrier of several physiologically active substances and drugs. Thus it is possible that some variants exhibit different functional properties, as the decreased affinity of Naskapi and Mexico albumins for warfarin[35] seems to indicate.

Conventional methods of protein analysis are limited to the detection of charge differences; the use of HPLC, as in the case of substituted haemoglobin[27], could help in recognizing a new class of albumin variants.

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REFERENCES

1 T. Peters, Jr., in F. W. Putman (Editor), *The Plasma Proteins*, Vol. I, Academic Press, New York, 2nd ed., 1975, pp. 133–186.
2 B. Meloun, L. Moravek and V. Kostka, *FEBS Lett.*, 58 (1975) 134.
3 A. Dugaiczyk, S. W. Law and O. E. Dennison, *Proc. Nat. Acad. Sci. U.S.*, 79 (1982) 71.
4 R. H. McMenamy, H. M. Dintzis and F. Watson, *J. Biol Chem.*, 246 (1971) 4744.
5 B. Meloun and J. Kusnir, *FEBS Lett.*, 27 (1972) 121.
6 D. R. Babin and S. M. Goos, *Eur. J. Biochem.*, 34 (1973) 409.
7 B. Meloun, M. A. Saber and J. Kusnir, *Biochim. Biophys. Acta*, 393 (1975) 505.
8 D. Gitlin and J. D. Gitlin, in F. W. Putman (Editor), *The Plasma Proteins*, Vol. 2, Academic Press, New York, 2nd ed., 1975 pp. 321–374.
9 C. Lapresle, *FEBS Lett.*, 76 (1977) 204.

10 S. G. Franklin, I. S. Wolf, A. Zweidler and B. S. Blumberg, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 2505.
11 S. G. Franklin, S. I. Wolf, Y. Ozdemir, G. T. Yuregir, T. Isbir and B. S. Blumberg, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 5480.
12 G. Vanzetti, F. Porta, L. Prencipe, A. Scherini and M. Fraccaro, *Hum. Genet.*, 46 (1979) 5.
13 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11199.
14 C. Black, D. M. Douglas and M. L. Tanzer, *J. Chromatogr.*, 190 (1980) 393.
15 M. Van der Rest, H. P. J. Bennet, S. Salomon and F. H. Glorieux, *Biochem. J.*, 191 (1980) 253.
16 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
17 J. J. L'Italien and R. A. Laursen, *J. Biol. Chem.*, 256 (1981) 8092.
18 W. C. Mahoney, *Biochim. Biophys. Acta*, 704 (1982) 284.
19 Z. I. Randhawa, R. T. Jones and E. Lie Injol, *Anal. Biochem.*, 129 (1983) 184.
20 G. E. Tarr and J. W. Crabb, *Anal. Biochem.*, 131 (1983) 99.
21 T. A. Stoming, F. A. Garver, M. A. Gangarosa, J. M. Harrison and T. H. J. Huisman, *Anal. Biochem.*, 96 (1979) 113.
22 J. B. Wilson, H. Lam, P. Pravatmuang and T. H. J. Huisman, *J. Chromatogr.*, 179 (1979) 271.
23 W. A. Schroeder, J. B. Shelton, J. R. Shelton and D. Powars, *J. Chromatogr.*, 174 (1979) 385.
24 J. Sugihara, T. Imamura, T. Imoto and T. Yamase, *Biochim. Biophys. Acta*, 669 (1981) 105.
25 G. P. Boisell, H. Waycman, H. Fabritius, R. Cabannes and D. Labie, *Biochim. Biophys. Acta*, 670 (1981) 203.
26 H. Lam, B. B. Webber, J. B. Wilson and T. H. J. Huisman, *J. Chromatogr.*, 269 (1983) 119.
27 J. Rochette, P. G. Righetti, A. Bianchi Bosisio, F. Vertongen, G. Schneck, J. P. Boissel, D. Labie and H. Wajcman, *J. Chromatogr.*, 285 (1984) 143.
28 W. P. Winter, L. R. Weitkamp and D. L. Rucknagel, *Biochemistry*, 11 (1972) 889.
29 R. P. Swenson, C. H. Williams, Jr., V. Massey, S. Ronchi, L. Minchiotti, M. Galliano and B. Curti, *J. Biol. Chem.*, 257 (1982) 8817.
30 E. Gross, *Methods Enzymol.*, 11 (1967) 238.
31 T. Dévényi, *Acta Biochim. Biophys, Acad. Sci. Hung.*, 3 (1968) 429.
32 B. S. Hartley, *Biochem. J.*, 119 (1970) 805.
33 H. D. Armstrong, *J. Amer. Chem. Soc.*, 71 (1949) 3399.
34 A. L. Tarnoky, *Advan. Clin. Chem.*, 21 (1980) 102.
35 C. Wiling, B. S. Blumberg and E. S. Vesell, *Science*, 195 (1977) 991.