DECREASED TYROSINE/TRYPTOPHAN RATIO IN A 6.6S CRYOGLOBULIN, AS DETERMINED BY A SPECTROPHOTOMETRIC TECHNIQUE

Norman A. Cummings

Oral Medicine and Surgery Branch, National Institute of Dental Research, National Institutes of Health, U.S. Public Health Service, Department of Health, Education and Welfare, Bethesda, Maryland 20014

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A large number of studies have reported the occurence of cryoglobulins (globulins that reversibly precipitate from the serum on cooling) in a variety of clinical disease states, including multiple myeloma and many of the connective tissue disorders. Currently little is known about the physico-chemical behavior of cryoglobulins, and the mechanism for cryoprecipitation has not been established.

The work of Haber (1964) and of Whitney and Tanford (1965) concerning the circumstantial relationship of amino acid sequence to antibody activity further strengthens the concept that protein specificity arises from specificity in content and sequence of amino acids. Work done in this laboratory has shown that cryoprecipitation of a 6.6S cryoglobulin is inhibited by reduction with mercaptoethanol; reoxidation removes this inhibition, while reduction and alkylation irreversibly destroys cryoprecipitability (Cummings, 1964). These data stress the importance of amino acid content and sequence as determinants of cryoprecipitability in cryoglobulins.

Investigation of possible amino acid differences between cryoglobulin and normal IgG has been instituted by spectroscopic analyses of the respective proteins in 0.1 M NaOH. This paper reports the results of that data with regards to tyrosine and tryptophan content.

MATERIALS AND METHODS

The paraprotein (1) was isolated from the serum of a cryoglobulinemic patient with multiple myeloma by repeated cold precipitation at 4°C., washing with water, and re-solution in the warmth (37°C.).

Normal IgG was obtained from Pentex, Inc., as human gamma globulin, Lot 26. Anion-exchange chromatography of this preparation was carried out at 4°C. on diethylaminoethylcellulose (DEAE) (Peterson and Sober, 1956; Sober, et. al., 1956) in a Tris-Succinate gradient, pH 8.6 to 4.5, (Peterson and Chiazze, 1962). Only the first, or "unretarded" peak, which was eluted in starting buffer of .04M Tris- .005 M Succinic Acid, pH 8.6, K=0.50 mmho, was used for further study.

Analytical ultracentifugation was carried out in a Spinco Model E ultracentrifuge with double sector cells. Sedimentation coefficients were calculated directly from photographic plates with the use of a Gaertner comparator (Troutman, 1966).

Antisera were prepared in rabbits against human serum and IgG; immunoelectrophoretic analysis was performed on 7.6 x 2.5 cm. glass slides coated with 1 per cent agar in pH 8.6 barbital buffer, 0.05μ (Grabar and Williams, 1953).

Gel filtration chromatography (Porath and Flodin, 1959) was performed in glass columns (1.9 x 54 cm.) using Sephadex G-200 (Pharmacia, Inc., Lot No. 3380).

The proteins were dissolved in, and dialyzed against 0.10 M NaOH, and analyzed spectrophotometrically. (Bencze and Schmid, 1957). Optical densities were first read in a dual beam model DB spectrophotometer with automatic recordings of wavelength vs. absorption (Beckman Instruments, Inc.) at 24°C. The entire region between 255 mμ and 320 mμ was checked manually at 0.25 mμ (255 to 300 mμ) and 0.50 mμ (300 to 320 mμ) increments in a Zeiss Model PMQ II spectrophotometer at 24°C.

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(1) Kindly supplied by Dr. William Terry, Immunology Branch, National Cancer Institute.

Protein concentrations were determined spectrophotometrically by using $E^{1\%}_{1cm.,280m\mu}$ of 14.5 for IgG and 13.3 for the cryoglobulin. These extinction coefficients were determined by dry weights at 110°C. in vacuo performed on aliquots of the same solutions which were spectrophotometrically analyzed (Hunter, 1966).

Calculations

The absorbance readings for each protein were plotted against wavelength, and a line was drawn tangent to the two maxima (about 282 and 294 mμ). The slope a/b of this line was divided by A_{max}, the maximum absorbance of the absorption curve, and the value S obtained:

$$S = \frac{(a/b)\ 10^3}{A_{max}} = \frac{\Delta A \cdot 10^3 / \Delta m\mu}{A_{max}}$$

The S value was used to determine the molar ratio (tyrosine/tryptophan), R, and absorptivity, $E^{1\%}_{1cm.}$, by extrapolation from tables prepared from similar absorption curves of mixtures of the two free amino acids in known ratios (Bencze and Schmid, 1957). In all cases, appropriate correction for extraneous absorption was made. The total content of tyrosine and tryptophan is determined by:

$$C_{tyr+trp} = A_{max} / E^{1\%}_{1cm.}$$

Results:

With gel filtration chromatography (Sephadex G-200), both the IgG and the cryoglobulin were eluted at the same volume as single peaks. They each migrated as a single symetrical homodisperse peak in the analytical ultracentifuge; the $S_{20,w}$ of the IgG and cryoglobulin, extrapolated to infinite dilution, was 6.70 and 6.61 respectively. Each protein gave a single line against rabbit anti-human serum and anti-IgG antisera on immunoeletrophoresis.

Figures from representative spectrophotometric analyses are given in Table 1. The amount of tyrosine and tryptophan (gm. of amino acid/100 gm. of protein) was 9.1 in IgG and 8.375 in cryoglobulin, with tyrosine/tryptophan ratios of 2.06 and 1.74 respectively.

TABLE 1

Representative determinations of tyrosine and tryptophan in IgG and cryoglobulin.

	IgG	Cryoglobulin
A_{280}	0.638	0.320
A_{294}	0.594	0.305
a	0.10	0.05
b	30.0	50.0
S	5.22	3.125
$E^{1\%}_{1cm.}$	168.40	172.45
R(tyr/trp)	2.06	1.746
$C_{tyr+trp}$ (gm/100gm. protein)	9.1	8.375

TABLE 2

Tyrosine and tryptophan content of various human IgG's. The last line gives the results for the 6.6S cryoglobulin. Tyrosine and tryptophan are in gm. amino acid/100gm. protein. R=tyrosine.tryptophan.

Study	Tyrosine	Tryptophan	R
Cummings, 1968 (Present report)	6.13	2.97	2.06
Tristram and Smith, 1963	6.08	3.12	1.95
Crumpton and Wilkinson, 1963	5.76	2.63	2.19
Woods & Engle, 1960	5.88	2.58	2.28
Brand, 1946	6.08	2.61	2.33
Cryoglobulin (Present report)	5.32	3.05	1.74

Discussion:

Determination of amino acid content in intact proteins by spectrophotometric techniques offers certain advantages over chemical methods, particularly

since hydrolysis is not involved. Tryptophan is especially susceptible to both hydrolytic loss in 6N HCI, as well as a low recovery rate by standard ion-exchange analysis (Tristram and Smith, 1963).

Since phenylalanine absorbs only weakly, and the other amino acids show no absorption over the 250 to 320 mμ range, it was assumed that the absorption curve of proteins was the sum of the absorption due to tyrosine and tryptophan. Treatment of the protein solution in alkali as a two-component system for spectrophotometric analysis allowed estimation of these two amino acids (Goodwin and Morton, 1946). The method of Bencze and Schmid (1957), which is used here, further defined this technique by using the slope of the line tangent to the characteristic maxima (rather than measurement at fixed wavelengths), thus circumventing the effect of the bathochromic shift. (See Beaven and Holiday, 1952). This type of analysis of proteins has led to values for tryptophan and tyrosine in good agreement with those secured by chemical methods. (Greenstein and Winitz, 1961).

Various figures for the amino acid content of human IgG are available. Several such studies are summarized in Table 2. As seen, the tyrosine/tryptophan ratio for the normal human IgG is consistent with the figures reported from a variety of these proteins. The ratio is significantly lowered in the cryoglobulin, even with regard to the expected variation of amino acid content in an immunoglobulin. This lowered ratio is due primarily to a decrease in tyrosine content, and explains the lower $E^{1\%}_{1cm.}$ at 280 mμ for the globulin. The figures for tyrosine represent 50.6 and 44.0 residues per 150,000 gm. of protein for the normal IgG and for the cryoglobulin, respectively. The comparable figures for tryptophan are 22.8 and 22.4 residues/150,000 gm.

The chemical basis for cryoprecipitation is unknown, but the importance of amino acid content and sequence may be paramount. Amino acids in proteins may behave in part as ions or as organic molecules; which behavior predominates depends both on the inborn structure and on various environmental factors. Isolated (L)tyrosine per se is relatively insoluble in water, although its

solubility increases almost 100% as the temperature is raised from 20 to 40°C. (Greenstein and Winitz, 1961). On the other hand, there is evidence that the presence of non-polar side chains on the protein backbone induces a lattice or cagelike arrangement of water of hydration around the molecule, and helps stabilize the protein. Thus a decrease in tyrosine might be useful in interpreting the solubility characteristics of the cryoglobulin. However many other variables besides tyrosine content may be involved, and there is no evidence that this finding directly explains the difference in solubility behavior.

The position of the amino acids, particularly with respect to availability of charged, soluble moieties to the water and buffer milieu at different temperatures, is being further studied. These experiments are preliminary to a more complete investigation, and point out for the first time a definite decrease in the tyrosine/tryptophan ratio of this peculiar paraprotein.

Summary:

The tyrosine/tryptophan ratio in a human IgG and in a 6.6S cryoglobulin has been determined by the spectrophotometric technique of Bencze and Schmid. This ratio is significantly lower in the cryoglobulin, and may explain some of the abnormal solubility characteristics.

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