THE SPECIFICITY OF KERATINS*†

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An innate quality of proteins is, in general, species specificity. However, certain proteins may not exhibit this attribute. In fact, the serological species differences of keratins have been generally accepted as either poorly defined or not demonstrable.

Krusius (1), in an extensive study on the immunological nature of the keratins, found that keratins from different species were not immunologically distinct. This author, however, realized that the method he used for the preparation of his keratins may have led to hydrolysis and deep seated alterations of the proteins, which would account for the lack of specificity observed. It should be stated here that antiformin was used as the dissolving agent, and that very little attention was paid to the control of the hydrogen ion concentration or to the possible denaturation of the compounds. Data concerning the chemical analyses of these proteins were incomplete.

Recently, Goddard, and Michaelis (2), observed that on treatment of wool with alkaline thioglycolate, under proper conditions, a protein was obtained, in which no other reaction had become manifest than the splitting of the disulfide bonds. They further noted that keratin owes its peculiar resistance against dissolving agents to the disulfide bonds in their original position, which are mainly responsible for the pattern of the structure of keratin and its physical properties.

These observers were able to split these disulfide bonds by reducing agents in such a manner as to leave intact the chemical composition and to avoid hydrolytic splitting. The reduced protein obtained was

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called "kerateine," and the reoxidized product was designated as "metakeratin." These proteins behaved more like ordinary proteins than native keratin, both with respect to solubility and behavior toward proteolytic enzymes.

The rôle of sulfhydryl-dithiol groupings in serological specificity has been investigated. Blumenthal (3), in fact, found that the precipitability of serum albumin is irreversibly decreased after reduction of the disulfide groups, and as an explanation, the author suggests either the participation of the —S—S— and the —SH groups in the reaction, or a splitting of the antigenic molecule by opening of the —S—S— linkage. Pillemer, Ecker, Myers, and Muntwyler (4) further showed that sulfhydryl groupings enter into the serological specificity of crystalline urease.

Keratin, due to its very high percentage of disulfide sulfur (10 to 15 per cent), which can be easily reduced to a sulfhydryl protein, appeared to be a useful material for the study of a sulfhydryl protein in immunological reactions. Furthermore, it seemed of interest to reinvestigate the species specificity of these unusual proteins.

Methods

Chemical.—The keratins were prepared essentially by the method outlined by Goddard and Michaelis (2). Since a few modifications were introduced, the method of preparation as used in this laboratory is given.

"Parent" Proteins.—100 gm. of either wool, chicken feathers (White Rock), or human hair (mixed) previously defatted by continuous extraction with warm alcohol, followed by warm ether, was added to 2 liters of 0.5 M disodium thioglycolate, care being taken not to exceed a pH of 10. A smaller yield of the protein is obtained at this pH (Goddard and Michaelis advise a pH of 11 to 12) but a less drastic treatment of the protein occurs at this hydrogen ion concentration. The mixture was then shaken for 4 hours, after which time the undissolved residue was removed by centrifugation, followed by filtration through a Buchner filter. The filtrate was precipitated by the careful addition of acetic acid, and allowed to stand for 30 minutes in the ice chest, 3°C. The precipitate was then collected on the centrifuge, washed 5 times with acetone, and finally 5 times in ether. After freeing from ether by vacuum, the protein was redissolved in M/10 Na_2CO_3, and reprecipitated immediately with dilute acetic acid. The precipitate was again collected on the centrifuge, transferred to a mortar, ground 3 times with acetone,

and finally ground 3 times with ether. After freeing of the ether by vacuum, the protein was suspended in water and dialyzed for 96 hours against running tap water. The dialyzed protein was washed twice with acetone, ground up in a mortar 3 times with acetone, treated similarly with ether and finally dried by vacuum. A fine, grayish white powder was obtained in each case. The final products yielded a very faint nitroprusside test.

Reduced Keratin (Kerateine).—This product was prepared by reducing 10 gm. of the parent protein with 5.0 gm. of thioglycolic acid under a stream of O_2-free nitrogen at a pH between 8 to 9 for 3 hours. The protein was then precipitated with the careful addition of 10 per cent metaphosphoric acid, collected on the centrifuge, transferred to a mortar and ground 5 times with acetone and ether, respectively, and then dried in a vacuum desiccator.

Oxidized keratin (metakeratin) was prepared by dissolving 10 gm. of the parent protein in 200 cc. of water and sufficient M/10 NaOH was added with constant stirring to maintain the pH between 8 to 9. When the protein had completely dissolved, 50 mg. of Cu_2O was added and a stream of air led through the solution for a period of 24 hours. The protein was precipitated with dilute acetic acid and processed in a manner similar to the method used in the preparation of kerateine.

The various compounds were analyzed for nitrogen by the micro Kjeldahl method, for sulfur by Frear's (5) procedure, for cystine by the method of Folin and Marenzi (6). The isoelectric points were determined according to Michaelis and Rona (7), pH of the mixtures being determined with the glass electrode. The amino nitrogen was estimated in the constant pressure apparatus of Van Slyke (8).

Immunological.—White, male, rabbits were injected intraperitoneally at 3 day intervals with the various proteins. Initial doses of 50 mg. were employed, followed by an increase of 50 mg. at each subsequent injection, until a final quantity of 300 mg. was given. The proteins were prepared freshly in M/10 Na_2CO_3 and then carefully adjusted to a pH of 8 with dilute HCl. Due to the rapid autooxidation of the reduced keratin, the kerateines were prepared rapidly and immediately injected. When the animals showed a sufficiently high precipitin titer (1:25,000), they were bled out for serum.

The test antigens were prepared by dissolving the keratins in M/10 Na_2CO_3, the pH being adjusted to 8 with N/10 HCl and diluted to the desired concentrations with 0.9 per cent saline. Again, rapidity is necessary in the case of the kerateine compounds.

The precipitin (ring) test was used in the cross-reaction tests. One drop of the diluted antigens was layered on one drop of the antiserum in Hektoen tubes. The precipitations were read after 1 hour at room temperature, and again after overnight standing in the ice chest (± 3°C.).

RESULTS

The chemical analyses of the various keratins are given in Table I. It is evident from the data that the total nitrogen, sulfur, cysteine, isoelectric points are essentially identical in all of the preparations employed. The results obtained compare favorably with those procured by Goddard and Michaelis for wool proteins. The various compounds examined were soluble in M/10 NaOH or M/10 Na_2CO_3 and were digested by trypsin.

The results of the cross-precipitation reactions are summarized in Table II. The reactions definitely show that species specificity is an individual characteristic of the keratins. It will be noted that cross-

TABLE I

Analysis of the Keratin Compounds (Oxidized and Unoxidized)

Protein	Total S	Total N	Cystine	Amino N	Isoelectric point
	per cent	*per cent*	*per cent*	*per cent*	*pH*
Wool metakeratin	3.55	16.30	12.91	0.84	4.5–5.0
Wool kerateine	3.49	16.41	12.83 (88% cysteine)	0.85	4.5–5.0
Feather metakeratin	3.61	16.39	13.10	0.84	4.5–5.0
Feather kerateine	3.68	16.33	13.00 (90% cysteine)	0.84	4.4–4.8
Hair metakeratin	3.59	16.30	12.84	0.85	4.5–4.9
Hair kerateine	3.63	16.37	12.84 (88% cysteine)	0.86	4.5–5.0

reactions are often encountered. A possible explanation for this observation is given below. It is interesting to note that optimal species specificity is obtained only when the reduced keratin (kerateine) was allowed to react with the antiserum prepared by the injection of the homologous reduced keratin.

The same phenomenon but to a lesser degree is seen when the metakeratins are layered on their homologous antiserums.

These observations suggest that not only are the keratins species specific, but that immunological differences are detectable in a single keratin preparation, depending on the state of oxidation or reduction of the protein.

TABLE II

Cross-Reactions between the Various Keratin Compounds

Antisera from	Antigens from											
	Hair metakeratin		Hair kerateine		Wool metakeratin		Wool kerateine		Feather metakeratin		Feather kerateine	
	1 hr.	Overnight	1 hr.	Overnight	1 hr.	Overnight	1 hr.	Overnight	1 hr.	Overnight	1 hr.	Overnight
Hair metakeratin (oxidized)	+++	+++	++	++	+	+	±	±	±	±	±	+
Hair kerateine (reduced)	+±	++	++++	++++	±	+	+	+	±	±	±	±
Wool metakeratin (oxidized)	+±	±	±	±	+++	++++	+	+	±	+	−	−
Wool kerateine (reduced)	±	±	±	±	+±	++	++++	++++	±	±	±	±
Feather metakeratin (oxidized)	±	±	+±	±	±	±	−	−	+++	+++±	+±	++
Feather kerateine (reduced)	±	±	±	±	±	±	±	±	+	+	++++	++++

Concentration of antigen, 0.004 per cent.

DISCUSSION

It would appear, as data upon the basic amino acid contents of proteins accumulate, that recognition must be given to the view that there exists a central basic nucleus characteristic for any one biological type of protein around which the remaining amino acids are united. Recent work by Block (9) has revealed that the keratins fall into one such group of proteins with histidine:lysine:arginine ratios of 1:4:12.

If this view is accepted all keratins must possess an individuality in chemical structure that characterizes them as keratins, but among these chemically similar substances there must exist a special variant in each species to account for the specificity exhibited by each type of keratin. This may be ascribed to the nature and the spatial arrangements of the terminal amino acids, especially cystine and cysteine. From the recent experiments of Landsteiner and van der Scheer (10), it appears plausible that the immunological characters of proteins are determined by the arrangements of the amino acids on the surface of the molecule. It should also be mentioned here that Landsteiner, Longsworth, and van der Scheer in a recent publication (11) found that the pronounced serological differences encountered among various hemoglobins depend on structural features of the molecules other than those which determine the charge.

Wrinch (12) has recently postulated that proteins may be formed by series of amino acids which may be bent into sequences of hexagons. The simplest of these structures is formed by two amino acids and a regular structure contains six amino acid residues. She states that progressively larger molecules with a threefold symmetry can be built up and that these molecules may polymerize by virtue of CO— and —NH groups.

These structures are laminar; on one surface only the hydroxyl groups emerge, while on the other side, side chains of the amino acid and hydroxyls both become apparent. Compact bodies can be formed from laminae by piling laminae one on the other. Such piled laminae would be joined front to front in the case of a disulfide protein by the —S—S— bridge of cystine molecules, which have their —C (NH_2) COOH parts in both laminae.

Serious consideration must be given to this theory as it appears

congruous with the known facts of protein nature and behavior. Therefore, if this hypothesis is accepted, the differences observed between the reactions of oxidized and reduced keratins may depend on the fact that reductions of the —S—S— linkage would produce an inter- or intramolecular rearrangement, whereby the laminae would be rearranged in a different manner.

The other possibility remains that the —S—S— or —SH groups may operate as "determinants." Although Marrack (13) in his monograph states "that there is no evidence that naturally occurring proteins contain characteristic 'determinant groups,'" it is evident from the above that until further work with sulfhydryl proteins is performed, the issue remains controversial.

SUMMARY

Kerateine and metakeratin were prepared from wool, chicken feathers and human hair. Chemical analyses revealed that the compounds are closely related.

The results of serological studies disclose that species specificity is an individual characteristic of the keratins employed and that the specificity observed is dependent on the redox state of the sulfhydryl groupings in the protein molecule.

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