THE SPECIFICITY OF KERATEINE DERIVATIVES*

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In a previous communication, it was shown by Pillemer, Ecker and Wells (1) that keratins are species specific, and that their specificity depends on the redox state of the sulfhydryl groupings in their molecule.

Since keratins contain a high percentage of disulfide sulfur (10 to 15 per cent cystine) and are readily reduced to sulfhydryl proteins by alkaline thioglycolate, a large number of substituent groups may be introduced into their molecules by reaction with simple organic halogen compounds according to the following formula (2):

$$A—SH + R—X \rightarrow A—S—R + HX$$

The present report presents evidence showing that substitution of the SH group of kerateines by simple chemical compounds influences the serologic behavior as well as the chemical characteristics of the derived proteins.

Methods and Materials

The Preparation of the Kerateine Derivatives.—Kerateine and metakeratin were prepared from wool and chicken feathers (White Rock) by the method described in a previous communication (1). The derivatives were prepared from wool and feather kerateine by reaction with iodoacetic acid, α-bromopropionic acid, α-bromo-*n*-butyric, α-bromoisobutyric, α-bromo-*n*-valeric, α-bromoisovaleric, α-bromo-*n*-caproic, α-bromoethylbenzene, and benzylchloride. The nomenclature designated by Goddard and Michaelis (2) was adopted for the derived kerateines, *i.e.*, by reaction with iodoacetate, carboxymethylkerateine was obtained.

Preparation of Carboxymethylkerateine.—For the most part, the method outlined by Goddard and Michaelis (2) was followed. 10 gm. of kerateine were suspended in 100 cc. of double-distilled water, and M NaOH carefully added to give a faint pink color with phenolphthalein. 6.5 gm. of thioglycolic acid, neutralized to a pH of 8 with NaOH, were added, and a continuous stream of O_2-free nitrogen led through the mixture. After 4 hours, 15 gm. of recrystallized iodoacetic acid, neutralized to a pH of 8, were added. A pH of 8 was maintained by the addition of 0.5 M NaOH from time to time. After 3 hours at room temperature, the preparation was again reduced with thioglycolate in a similar manner, and again treated with iodoacetic acid for 2 hours. The protein

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was precipitated with acetic acid, collected in the centrifuge, transferred to a mortar, ground up three times with acetone, and the suspension dialyzed against running water for 4 days. The protein was next precipitated with 10 per cent metaphosphoric acid, collected in the centrifuge, and transferred to a mortar. It was then ground up three times with acetone and three times with ether, and finally dried *in vacuo* by a method described by Ecker and Pillemer (3).

The Preparation of α-Carboxyethyl-, α-Carboxy-n-Propyl-, α-Carboxyisopropyl-, α-Carboxy-n-Butyl-, α-Carboxyisobutyl-, α-Carboxy-n-Amylkerateine.—The preparation of these compounds was almost identical in manner. 10 gm. of kerateine were suspended in 100 cc. of double-distilled water for each preparation, and M NaOH added to give a faint pink color with phenolphthalein. 5 gm. of thioglycolic acid neutralized to a pH of 8 were added to each preparation, and O_2-free nitrogen led continuously through the mixture for 4 hours. Then either 10.7 gm. of α-bromopropionic acid, or 11.7 gm. of α-bromo-*n*-butyric or α-bromo-isobutyric, or 12.6 gm. of α-bromo-*n*-valeric or α-bromo-isovaleric, or 13.6 gm. of α-bromo-*n*-caproic acid were added. All the compounds were brought to a pH of 8 before addition to the reduced protein mixtures, and sufficient 0.5 M NaOH was added from time to time to maintain the pH at about 8. These preparations were allowed to stand for 12 hours, after which time they were reduced in the manner outlined above, and treated a second time with the organic halogen acids for 3 hours. The proteins were then dialyzed for 4 days against running water, and carefully precipitated with 10 per cent metaphosphoric acid. The precipitate was collected in the centrifuge, transferred to a mortar, ground up five times with acetone and five times with ether, and finally dried *in vacuo*.

Preparation of Benzyl- and β-Phenylethylkerateines.—10 gm. of kerateine were suspended in 125 cc. of double-distilled water for each preparation, and M NaOH added to give a pink color with phenolphthalein. 5 gm. of thioglycolic acid neutralized to phenolphthalein were added to each preparation, a stream of O_2-free nitrogen led through the mixture for 4 hours. Then either 8.8 gm. of benzyl chloride or 13 gm. of α-bromo-ethylbenzene dissolved in 125 cc. of absolute ethyl alcohol were slowly added to the mixtures under constant stirring. The preparations were allowed to stand 12 hours at room temperature. The suspensions were then dialyzed for 24 hours against running water, precipitated with 10 per cent trichloroacetic acid, collected in the centrifuge, transferred to a mortar, ground five times with acetone and five times with ether, and again dialyzed for 4 days and processed again as above.

Chemical Analysis.—The proteins were analyzed for nitrogen by the micro Kjeldahl method, for cystine by the method of Folin and Marenzi (4), for sulfur by the Frear (5) procedure. The amino nitrogen determinations were made by the method of Van Slyke (6) in the constant pressure apparatus. The isoelectric points were estimated by the method of Michaelis and Rona (7), the pH of the mixtures was determined with the glass electrode. The digestibility of the proteins with trypsin was determined by the method described by Myers, Free, and Beams (8).

Preparation of Antigens.—Fresh solutions were prepared from the derived compounds prior to injections. The modified proteins were dissolved in 0.1 M Na_2CO_3 and immediately neutralized to a pH of 7.8.

Animals.—White male rabbits were employed. All animals were kept on a mixed diet, especially high in vitamin C. All food was withheld for a period of 24 hours prior to the final bleeding of the animals. Four animals were injected with each antigen.

Method of Immunization.—50 mg. of kerateine, metakeratin, benzylkerateine, and β-phenylethylkerateine were injected intraperitoneally at 3 day intervals. The other proteins were given intravenously in quantities of 20 mg. daily, for a period of 6 days, followed by a rest period of 1 week, and then followed by two or more courses of injections. As soon as a sufficiently high titer (of at least ±1:10,000) was realized, the animals were anesthetized and bled to exsanguination from the femoral artery into sterile flasks. After clotting, the clear serum was decanted, centrifugalized, and placed in ampoules.

Precipitin Tests.—The ring test was performed in chemically clean Hektoen tubes. One drop of dilute antigen was layered over one drop of immune serum. The tubes were observed after standing at room temperature for 1 hour, and again after standing overnight in the refrigerator (±2°C.). It should be pointed out that feather kerateine derivatives were employed for immunization, while the wool kerateine derivatives were used as test antigens. This was done in order to reduce the possibilities of interaction between the parent proteins and the immune serums.

Inhibition Tests.—1 millimol of each substance to be tested was added to 5 cc. of water, and sufficient NaOH added to dissolve the compound completely. The solution was then neutralized to litmus with HCl and brought up to a volume of 10 cc. 0.05 cc. of each solution was then added to 0.2 cc. of the diluted antigen. The control tube contained only 0.05 cc. of saline, immune serum, and homologous antigen. Readings were taken after 1 hour at room temperature, and after standing overnight in the refrigerator (±2°C.).

Chemical Results

The analytical data for the derived kerateines are given in Table I. It is readily seen that the sulfur and nitrogen contents are similar to the values of the parent protein (kerateine) and that nearly all of the cystine has been reduced and substituted.

Although Michaelis and Schubert (9) have observed that organic halogens react with amino groups, it will be seen that no demonstrable substitution of the free amino groups occurred under the conditions employed in these experiments. As Michaelis (10) has pointed out, such a reaction does not occur, under controlled conditions, in the first place because this reaction requires a high degree of alkalinity, and secondly, because the free amino groups of the proteins are not α-amino groups, but are the ε-amino groups of lysine, and these, in general, are far less reactive.

It should also be pointed out here that none of the kerateine derivatives has been prepared in an absolutely pure state, nor should they be regarded as chemical entities. Furthermore, it is self-evident that the reduction of the —S—S— groups is a reversible reaction, and that the reactions with the organic halogen compounds can proceed only to the extent to which the —S—S— groups have been originally reduced.

As to the isoelectric points and solubilities of these kerateine derivatives,

it is to be noted that these proteins differ in their isoelectric points and solubilities depending on the nature of the substituent group. The proteins substituted with the aliphatic groups show a much lowered isoelectric point, as would be expected from the introduction of the carboxyl groups. On the other hand, benzylkerateine has an isoelectric point slightly higher than

TABLE I

Analysis of Kerateine Derivatives

Protein	Total S	Total N	Cystine	Amino N	Isoelectric point	Solubility	Digestibility by trypsin
	per cent	*per cent*	*per cent*	*per cent*			
Reduced keratin (kerateine)	3.14	16.43	13.1, 80% as cysteine	0.84	4.6–4.9	Not soluble in 0.1 N Na acetate. Soluble in 0.1 N NaOH or Na_2CO_3	+
Oxidized keratin (metakeratin)	3.21	16.21	13.3	0.82	4.6–4.9	" "	+
Carboxymethylkerateine	2.62	16.55	2.56	0.82	3.8–4.2	Soluble in 0.1 N sodium acetate and M/30 HCl	+
α-Carboxyethylkerateine	2.63	16.33	2.71	0.84	3.5–3.7	" "	+
α-Carboxy-*n*-propylkerateine	3.03	16.01	2.50	0.85	3.6–3.8	" "	+
α-Carboxyisopropylkerateine	2.91	15.70	4.10	0.84	3.6–4.0	" "	+
α-Carboxy-*n*-butylkerateine	2.74	15.00	3.20	0.81	3.6–4.2	" "	+
α-Carboxyisobutylkerateine	2.69	15.30	2.64	0.85	3.8–4.2	" "	+
α-Carboxyamylkerateine	2.83	15.72	2.86	0.82	3.8–4.2	Soluble in 0.1 N sodium acetate	+
Benzylkerateine	3.09	15.33	4.96	0.83	5.3–5.6	Soluble (very slowly) in 0.1 N NaOH	+
β-phenylethylkerateine	3.07	15.65	4.3	0.82	4.4–4.8	" "	+

the present protein kerateine, while β-phenylethylkerateine has practically the same isoelectric point as kerateine. These changes are what would be anticipated from the chemical nature of the substituted groups.

In contrast to wool and feather keratins, which are resistant to tryptic digestion, all of the kerateine derivatives were readily digested by duodenal contents (protease).

Immunological Results

In Table II are given the results of the cross-reactions between the various modified antigens and their immune serums. It is to be noted that the

most marked reactions occur at the interface of the proteins and their homologous immune serums, and the original species specificity of the keratins has been abolished to a great extent. However, all of the individuality of the original proteins was not destroyed, as some reaction occurred between the derivatives and the parent protein. For this reason, the immunizing antigens and the test antigens were prepared from feather and wool derivatives, respectively.

The derived proteins containing the shorter aliphatic groups, *i.e.*, carboxymethylkerateine and α-carboxyethylkerateine, yield the most specific reactions, while the range of activity broadens when the length of the side

TABLE II

Cross-Reactions of the Kerateine Derivatives

Immune serums for feather kerateine derivatives	Wool kerateine derivatives*									Feather kerateine	Wool kerateine
	Carboxymethyl K†	α-Carboxyethyl K	α-Carboxy-*n*-propyl K	α-Carboxy-isopropyl K	α-Carboxy-*n*-butyl K	α-Carboxy-isobutyl K	α-Carboxyamyl K	β-phenylethyl K	Benzyl K		
Carboxymethyl	++++	±	−	−	−	−	−	−	−	+	−
α-Carboxyethyl	±	++++	−	±	−	−	−	−	−	+	−
α-Carboxy-*n*-propyl	−	±	++++	++	±	−	−	−	−	±	−
α-Carboxyisobutyl	−	−	+±	+	+++	++++	++	−	−	+	−
α-Carboxyamyl	±	−	±	±	+±	+±	++++	±	−	+	−
Benzyl	−	−	−	−	−	−	±	+++	++++	+	−
Kerateine (wool)	+	+	±	+	±	+	+	+	+	±	++++
Kerateine (feather)	−	−	−	−	−	−	−	−	−	++++	±

* Concentration of antigen, 0.01 per cent. Readings after 2 hours at room temperature.

† K represents the kerateine molecule.

chain is increased in the determinant group. However, the strongest reactions always occur between homologous antigens and their immune serums. In general, it appears that the reaction observed with the proteins containing the longer chained substituents, may, in part, be ascribed to the general physicochemical properties of long aliphatic chains. The results compare favorably with those obtained by Landsteiner and van der Scheer (11) in their studies on antigens containing azo components with aliphatic side chains.

A striking specificity is exhibited in the case of the two compounds containing benzene rings in their side chains. Benzylkerateine and β-phenylethylkerateine could be easily differentiated from those compounds containing aliphatic side chains as determinants.

Inhibition Tests.—Landsteiner (12), applying his fundamental observation that ordinary precipitin reactions can be inhibited by the addition of

an excess of antigen, showed that the precipitin reactions of artificial antigens are inhibited specifically by the addition of an adequate quantity of the same chemical substance or of one similar to that used in building up the antigenic complex. These observations have been repeatedly confirmed. The advantage of such a test is at once realized, as a great variety of compounds can be examined, and the non-specific influence of the colloid

TABLE III

Immune serums for feather kerateine derivatives	Readings taken after	Substances used for inhibition tests											
		Control*	Acetic acid	Iodoacetic acid	Propionic acid	Bromopropionic acid	*n*-Butyric acid	Isobutyric acid	*n*-Valeric acid	Isovaleric acid	*n*-Caproic acid	*n*-Caprylic acid	Benzoic acid
Carboxy-methyl	1 hr.	+++	±	±	+	+	++	++	+	+	±+	+±	+++
	Night in ice chest	++++	±	+	++	++	+++	+++	+++	+++	+±	+±	++++
α-Carboxy-ethyl	1 hr.	++++	+	+	±	±	++	++	±	+	±	±	+++
	Night in ice chest	++++	++	++	±	+	+++	+++	+±	+±	+±	+	++++
α-Carboxy-*n*-propyl	1 hr.	+++	++	++	+	+	−	±	±+	±+	±	±	++
	Night in ice chest	+++	+++	+++	++	++	±	+	++	++	+±	+	++++
α-Carboxy-isobutyl	1 hr.	+++	++	+++	±	±	±	++	±	−	±	±	+++
	Night in ice chest	++++	+++	+++	++±	+++	++	++±	+	±	+	±	++++
α-Carboxy-amyl	1 hr.	++++	+++	+++	++	++	+±	+++	±	±	−	−	++
	Night in ice chest	++++	++++	+++	+++	+++	+±	+++	++	+	±	±	+++

* Control tube contained one drop of a mixture composed of 0.05 cc. saline and 0.2 cc. test antigen, which was layered over one drop of its homologous immune serum.

Concentration of antigen 1:1500.

portion of the molecule, as well as mode of attachment of the determinant group, are substantially excluded.

The inhibition test served to confirm the results obtained in the cross reaction of the modified kerateines, and also yielded additional significant information. The tests exhibited in Table III again demonstrate that the most marked inhibitions take place with homologous substances, and that upon increasing the length of the aliphatic chain employed, the specificity of the immune serum is lessened. Substitution of a hydrogen atom in the aliphatic chain by iodine or bromine did not materially affect the reaction. It is of interest to note that benzoic acid had little or no inhibiting effect on the reaction of the aliphatic derivatives and their immune serums.

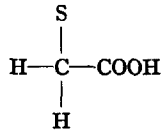
In Table IV are given the inhibition reactions for benzylkerateine and its immune serum. It is seen that substances having a similar chemical composition to the substituted group in the artificial antigen, give the strongest reactions. Benzoic acid, phenylacetic acid, phenylcarbinol, substances similar in structure to the introduced benzyl radical, give the most pronounced inhibitions, while the aliphatics, so prominent in the reactions in Table III, give little or no reaction.

In an attempt to localize the polar group or field responsible for the specific binding of these derived kerateines to their homologous immune serums, numerous compounds of varying composition have been employed as inhibiting agents. The results are given in Table V. It was found that

TABLE IV

Immune serums for feather kerateine derivatives	Reading taken after	Substances used for the inhibition tests										
		Control	Benzoic acid	Acetic acid	Phenylacetic acid	Phenol	Glutathione	Caproic acid	Caprylic acid	Phenylalanine	Phenylcarbinol	Valeric acid
Benzylkeratin	1 hr.	+++	±	+++	+	++	+++	++	++	+	±	+++
	Night in ice chest	++++	+±	++++	+±	++	++++	+++±	+++±	++	+	++++

thioglycolic acid gave the most pronounced inhibitions with the shorter chain aliphatic-containing kerateines. It therefore becomes apparent that the group



is of primary importance in the determination of the specificity of these compounds, and likewise brings out the fact that extreme caution must be taken in freeing the proteins from the thioglycolic acid used in their preparation. That the carboxyl group is necessary is evident from the observation that acetamide and methyl alcohol have little or no inhibitory action on these compounds. Likewise, substitution of a hydrogen atom in the aliphatic chain by $—NH_2$, —OH, or I_2 does not materially affect the reaction.

Again, on increasing the length of the aliphatic chain of the inhibiting agent, *i.e.*, sebacic and caprylic acids, the specificity of the immune serum is lessened. It is altogether probable that certain non-specific physicochemical factors enter into this phenomenon.

TABLE V

Immune serums for	Reading taken after	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Control*
Carboxy-methyl-kerateine	1 hr.	−	±	±	+	+++	+++	++++	+++	+++	+	+	+±+	+++	+++	+++	++	+++	±	+±	++++ (4)
	Night in ice chest	±	+±	+±	+±	++++	++++	++++	++++	++++	++±	+±	+++±	+++±	+++	++++	+++	++++	+	+±	++++ (4)
α-Carboxy-n-propyl-kerateine	1 hr.	+	++	++	++	+++	++	+++	+++	++	+±	++	++	++	+±	+++	+++	+++	±	±	+++ (3)
	Night in ice chest	+±	++	++	++±	++++	+++	++++	++++	+++	+++	++±	++++	+++	++±	++++	++++	++++	±	+	++++ (4)
α-Carboxy-n-amyl-kerateine	1 hr.	++	+++	+++	+++	++++	++++	++++	+++	+++	+++	+++	+++	++±	++	+++	+±	++++	−	−	++++ (4)
	Night in ice chest	++±	+++±	+++±	+++±	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	±	±	++++ (4)
Benzylkerateine	1 hr.	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	±	±	+++	+++	+++	+++	++	++	+++ (3)
	Night in ice chest	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+±	+	++++	++++	++++	++++	+++	++++	++++ (4)

The following substances were used for inhibition tests: (1) Thioglycolic acid, (2) glycolic acid, (3) glycocol, (4) acetic acid, (5) acetamide, (6) ethyl acetate, (7) acetone, (8) methyl alcohol, (9) propyl alcohol, (10) lactic acid, (11) succinic acid, (12) benzoic acid, (13) phenylacetic acid, (14) cysteine, (15) glutathione, (16) trichloroacetic acid, (17) taurine, (18) sebacic acid, (19) caprylic acid.

* Control tube contained one drop of mixture composed of 0.05 cc. saline and 0.2 cc. test antigen 1:500 which was layered over one drop of its homologous immune serum.

Concentration of antigen 1:500.

As is indicated in Table IV, the most marked inhibitions with benzylkerateine-immune serum occurred with substances containing an aromatic nucleus, while no or only slight reactions took place with the aliphatics.

COMMENT

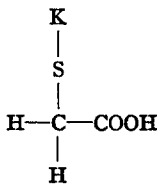
The ideal method of combining new groups into the protein molecule is one that results in the introduction of the substituent groups into a definite part of the molecule, unaccompanied by any hydrolytic or oxidative changes.

By methods described above several kerateine derivatives have been prepared. Each of these proteins shows a distinct chemical and immunological individuality different from that of the parent protein, while the chemical analysis of these compounds shows that the only reaction manifested in the process of modification was the substitution of the hydrogen in the —SH groups of the reduced keratin.

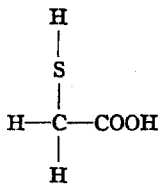
The serologic evidence presented here would warrant the conclusion that either the introduction of new groups into the kerateine molecule produces a new structural arrangement which is more prominent than the other polar groups in the molecule, or that the blocking of the polar —SH groups by the substituted group results in the formation of a new immunologic individuality. The weight of the experimental evidence is in favor of the former view, but it would be premature to draw conclusions at this time.

As to the inhibition reactions, these tests confirmed the view that the specificity of the new protein is due to the introduced determinant groups, which in all probability are substituted into the sulfhydryls of the parent protein (kerateine).

The observation that thioglycolic acid shows the most specific inhibition with the shorter chained aliphatics, *i.e.*, carboxymethylkerateine, is interesting, since the chemical structure of thioglycolic acid may in part be considered quite similar to that of the determinant group of carboxymethylkerateine, and also in part similar to the remainder of aliphatic acid derivatives of kerateine. In a comparison of the chemical composition of carboxymethylkerateine and thioglycolic acid, this observation becomes obvious:



carboxymethylkerateine



thioglycolic acid

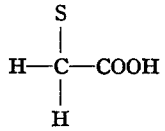
These studies, furthermore, substantiate the views that cystine and cysteine molecules are intimately connected with the species specificity exhibited by the keratins. This hypothesis is in line with the recent findings of Landsteiner, Longsworth, and van der Scheer (13), who noted that the serologic differences observed between the ovalbumins or the hemoglobins are due to structural features of the molecule other than those which determine the charge.

Since the most marked changes in the serologic behavior of modified proteins occur in those instances in which the benzene ring has been affected, tyrosine has been considered to play a major rôle in the determination of the specificity of proteins. The observations of Hopkins and Wormall (14), and Clutton and his associates (15), in which the free amino groups of proteins were used for the attachment of new determinant groups, indicate that a protein may be altered immunologically by a process not affecting the benzene ring. From the studies reported herein, it is also evident that substitution in the —SH groups of kerateines by simple chemical compounds influences the serologic behavior as well as the chemical characteristics of these derived proteins.

SUMMARY

Carboxymethyl, α-carboxyethyl-, α-carboxy-*n*-propyl-, α-carboxyisopropyl-, α-carboxy-*n*-butyl, α-carboxyisobutyl, α-carboxyamyl-, benzyl-, and β-phenylethylkerateines were prepared from the parent protein, reduced keratin or kerateine. Chemical analysis disclosed that the various compounds differed in their isoelectric points and solubilities depending on the nature of the substituent group introduced. In general, it was found that in so far as could be determined, nearly all of the available sulfhydryl groups were substituted, while no detectable substitution of the free amino groups of the proteins occurred.

The results of the serologic studies revealed that the kerateine derivatives acquired a new immunologic character dependent on the nature of the introduced determinant group. Inhibition tests confirmed the results obtained. Evidence was also produced to show that the grouping



may play a rôle in some of the reactions observed.

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