THE SPECIFICITY OF OXIDIZED AND REDUCED PROTEINS OF THE OCULAR LENS*

By E. E. ECKER, Ph.D., and L. PILLEMER, Ph.D.

(From the Institute of Pathology, Western Reserve University and the University Hospitals, Cleveland)

(Received for publication, December 18, 1939)

The organ specificity of the ocular lens was first reported by Uhlenhuth (1). He discovered that lens antiserums precipitate, even in high dilutions, the lens proteins of other animals. He studied the lens proteins of many species of animals including the fish. The fish lens, however, showed a different behavior, and did not readily flocculate in the presence of other lens antiserums. Only in strong serum concentrations and after hours of standing did flocculations occur. On the basis of these observations, Uhlenhuth reported that the lens proteins of mammals, birds and amphibians contain, in part, similar proteins, while only traces of these substances are found in the lens proteins of fish. Von Szily (2) and others have since demonstrated a limited species specificity in the protein of the embryonal lens.

In spite of a vast quantity of research into immunologic behavior of the protein of the lens, the methods that have been employed in its preparation are open to adverse criticism, because of failure to control the hydrogen ion concentration, or to prevent possible denaturation during preparation. In most instances, also no attempt was made to purify the compounds. It is noteworthy that in these studies no attention has been paid to the redox state of this interesting sulfhydryl protein in relation to its immunologic characteristics.

Recent work from this laboratory (3) revealed that species specificity is an individual peculiarity of the keratins, and that it is dependent on the oxidation-reduction state of the sulfhydryl groupings in the protein. These studies also have shown (4) that sulfhydryl groups apparently enter into the antigenic composition of urease. Indeed, marked immunologic differences were displayed by the oxidized and reduced forms of this enzyme. Since the lens is rich in sulfhydryl sulfur, it was considered that this would be a suitable material for the continuance of studies on the

* Aided by a grant from the Commonwealth Fund.

immunologic behavior of this group of proteins. In all these studies great care has been exercised to avoid denaturation, and to maintain the desired redox state of the protein.

Experimental Methods

Chemical.—The lens proteins were prepared in a manner similar to that described for the keratins (3).

Parent Proteins.—The lenses of swine, chicken, sheep and fish (pike) were used. After these had been freed of all ligaments and extraneous materials, 25 gm. of each were added to 1 liter of 0.2 M disodium thioglycolate, and care was taken to maintain a pH of 9 or less. The mixture was shaken at repeated intervals and allowed to stand at room temperature for about 12 hours, after which the undissolved residue (practically nil) was removed by passage through a Buchner filter. The filtrate was precipitated by the careful addition of acetic acid until complete precipitation was obtained. The precipitate was then collected in a Buchner funnel, transferred to a mortar, and washed three times with acetone and ether respectively. After removal of the ether by vacuum, the protein was suspended in water and dialyzed for 48 hours against cold running tap water. The dialyzed protein solution then was precipitated again with dilute acetic acid, collected in the centrifuge, washed three times with acetone and ether, and finally dried *in vacuo*. A fine white powder was obtained which yielded a faint nitroprusside reaction.

Oxidized Protein.—Approximately 10 gm. of the parent protein were dissolved in 200 cc. of distilled water, and sufficient M/10 sodium hydroxide was added under constant stirring to maintain a pH of 8 to 9 (colorimetrically). 50 mg. of cuprous oxide was then 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 the manner described for the parent protein

Reduced Protein.—10 gm. of the parent protein were reduced with 100 cc. of 5 per cent disodium thioglycolate solution (pH 9) for 3 hours, and precipitated carefully with 10 per cent metaphosphoric acid. The precipitate was collected in the centrifuge, transferred to a mortar and ground up five times with acetone and five times with dry ether. It was dried finally in a vacuum desiccator. A reduction was also accomplished similarly by the use of potassium cyanide.

All the protein preparations were then analyzed for nitrogen by the micro Kjeldahl method, for cysteine by colorimetric comparison to a known cysteine standard with nitroprusside, and their isoelectric points were determined according to the methods of Michaelis and Rona (5). The hydrogen ion concentrations were controlled by a glass electrode.

Immunologic.—White, male rabbits (2 to 3 kg.) received intravenous injections of 20 mg. of protein daily for several 6 day periods. All the proteins were prepared freshly in M/100 sodium hydroxide solution, and the pH was adjusted to 7.8 with 0.01 N hydrochloric acid. In order to avoid possible auto-oxidation of the reduced lens protein, it was prepared rapidly and injected immediately. All the test antigens were prepared in the same manner and diluted to the desired concentrations with 0.9 per cent sodium chloride solution. The precipitin (ring) test was used in all the cross reactions, employing Hektoen tubes.

RESULTS

The results of chemical analysis of the various proteins are shown in Table I. From the data secured, it is evident that although the total nitrogen content of the different proteins is almost identical, marked variations are displayed in the isoelectric points and also in the content of cysteine. The protein from fish lens has the highest isoelectric point, as well as the largest quantity of sulfhydryl-disulfide sulfur, while that of chicken lens has the lowest isoelectric point and also the smallest cysteine content. The proteins secured from the swine and sheep lenses are almost identical in their chemical characteristics, maintaining an intermediary position between those of the chicken and the fish.

TABLE I

The Chemical Composition and Physical Properties of Different Lens Proteins

Source of protein	Nitr^{v}gen	Isoelectric point	Cysteine
	per cent		*per cent*
Fish lens	16.28	4.90–5.00	Oxidized 0.00 Reduced 8.50
Sheep lens	16.16	4.25–4.50	Oxidized 0.00 Reduced 5.20
Swine lens	16.21	4.25–4.50	Oxidized 0.00 Reduced 5.00
Chicken lens	16.23	3.90–4.00	Oxidized 0.00 Reduced 4.30

The results of the cross precipitation reactions are summarized in Table II. They show that only in distantly related species, such as the fish and the chicken, is species specificity a prominent characteristic of the lens proteins. Indeed, in the immunologic tests of the proteins from swine and sheep lenses, almost identical results were obtained, while those of the fish and the chicken could be easily differentiated. This would indicate that lens proteins are only relatively species specific, and that the phenomenon is independent of the redox state of the proteins.

Of particular significance is the finding that the oxidized lens proteins precipitated their homologous antiserums in lesser degree than the reduced antigens, regardless of whether the homologous antiserums were prepared from oxidized or from reduced proteins.

These observations suggest that the spatial arrangement and recurrence

of sulfhydryl-disulfide groupings play a more important rôle in the specificity of the compounds than the state of oxidation or reduction, although immunologic differences may be encountered in a single lens preparation, depending on the redox state of the individual protein.

A comparison of the chemical analyses of these proteins and their serologic characteristics reveals a marked similarity between chemical composition and immunologic variations. The swine and the sheep, which are biologically more closely related, also show closer chemical and immunologic

TABLE II

The Cross Precipitin Reactions of Lens Proteins

Antiserums from	Fish lens*		Sheep lens*		Swine lens*		Chicken lens*	
	Reduced	Oxidized	Reduced	Oxidized	Reduced	Oxidized	Reduced	Oxidized
Fish lens reduced	++++	++	−	−	−	−	++	∓
Fish lens oxidized	++++	++	−	−	−	−	++	∓
Sheep lens reduced	−	−	+++	+∓	+++	++	+	∓
Sheep lens oxidized	−	−	+++	++	+++	++	+	∓
Swine lens reduced	−	−	+++	+∓	++++	+∓	+	+
Swine lens oxidized	−	−	+++	++	++++	+∓	+	+
Chicken lens reduced	−	−	+∓	+	++	+	++++	+∓
Chicken lens oxidized	−	−	++	∓	++	+	++++	+∓

* Concentration of antigen 1:4000.
Readings after 1 hour at room temperature.

relations in their lens proteins than do those of the fish and chicken, which are both chemically and serologically distinct.

Markin and Kyes (7) have just reported that the coincidence in structure obtaining between beef and dog lens proteins does not extend to pigeon lens proteins. Pigeons highly sensitized to beef lens proteins are not sensitive to pigeon lens proteins and are not desensitized to beef by the injection of pigeon lens proteins. From the results of their experiments with anaphylaxis they conclude that "the extreme specialization of the optic lens results in a tissue whose constituent proteins are the same in two mammalian species as widely separated as dog and beef and are therefore

in full accord with the results obtained with the precipitin tests by Uhlenhuth, Hektoen and others."

It must be emphasized that the ocular lens is a highly differentiated organ without specific blood proteins.

DISCUSSION

It is regrettable that no complete data exist on the basic amino acid contents of the various lens proteins, such as those of Block (6) on the keratins. Such data would have been of inestimable value in the interpretation of the results obtained in this study.

The marked relationship between the chemical properties and the immunologic characters of these proteins points to the fact that their serologic behavior is dependent upon the presence of definite chemical, structural features. It is altogether probable that spatial relationships instead of the redox state of the sulfhydryl-disulfide system, play a major rôle in this particular instance.

The differences encountered between the reactions of the oxidized and reduced proteins on their homologous antiserums may be interpreted as similar to the reactions noted in the studies of the urease-anti-urease systems (4), *i.e.*, both oxidized and reduced lens proteins, after injection, may be converted into a similar oxidation-reduction state. It is altogether likely that the reducing action of the tissues possesses a great enough potential to reduce the oxidized lens protein. At least, this was proved with respect to urease. If the above hypothesis is valid, each antigen would yield a similar antiserum, which would react maximally with the reduced lens protein, while the reaction with the oxidized lens antigen would be weaker because of intermolecular or intramolecular changes in the oxidized antigen molecule.

Finally, it should be pointed out that lens proteins are natural sulfhydryl proteins, while keratins are natural disulfide sulfur proteins. This chemical difference may account for the varying serologic reactions of the lens protein and of the keratins (3). Additional studies are now being carried out to investigate this point.

SUMMARY

Oxidized and reduced proteins were prepared from the ocular lenses of sheep, swine, chicken and fish (pike). The proteins were prepared under conditions designed to avoid denaturation and to produce relatively pure compounds.

Serologic studies revealed that species specificity is demonstrable in the

proteins from chicken and fish lenses, but in the more closely related species (swine and sheep) this characteristic is not so evident.

Serologic differences may be detected in the lens preparations from a single species, depending on the redox state of the protein.

BIBLIOGRAPHY

1. Uhlenhuth, P., Festschrift fur Robert Koch, Jena, Gustav Fischer, 1903.
2. Von Szily, *Klin. Monatsbl. Augenheilk*, **12,** 150; quoted by Dietzel, F. W. L., Onderzoekingen over de Specificiteit van het Lenseiwit, Thesis, Leiden, 1929.
3. Pillemer, L., Ecker, E. E., and Wells, J. R., *J. Exp. Med.*, 1939, **69,** 191.
4. Pillemer, L., Ecker, E. E., Myers, V. C., and Muntwyler, E., *J. Biol. Chem.*, 1938, **123,** 365.
5. Michaelis, L., and Rona, P., *Biochem. Z.*, 1910, **27,** 38.
6. Block, R., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32,** 1574.
7. Markin, L., and Kyes, P., *J. Infect. Dis.*, 1939, **65,** 156.