The Effect of Heat on Bovine Lens Proteins

It is well known that infrared and microwave rays can induce lens cataract, and it is believed that this phenomenon is due to a direct heating effect on the lens[1]. Since lens proteins are of fundamental importance in the development of lens cataract, the present study was directed at analyzing the effect of heat on their solubility, electrophoretic mobility and antigenic properties.

Methods. Bovine eyes were obtained from the slaughterhouse immediately after sacrifice of adult animals and the lenses with capsule intact cleanly dissected from surrounding tissues. The lenses were homogenized in distilled water and centrifuged at 27,000 *g* for 30 min at 4 °C. The supernatant was used for analysis.

α-Crystallin was prepared according to the method of FRANCOIS, RABAEY and WIEME[2]. The lens supernatant was adjusted to pH 5.0 by the addition of 0.1 *N* HCl. The precipitate was separated by centrifugation and dissolved in distilled water at pH 7.2 by adding a minimum quantity of 0.1 *N* sodium hydroxide. It was purified by 3 repeated precipitations at pH 5.0, and redissolved at pH 7.2 with 0.1 *N* NaOH.

The supernatant obtained after the separation of α-crystallin was treated with absolute alcohol to reach the final concentration of 13% (v/v). A small amount of α_2-crystallin which precipitated was discarded. The supernatant thus obtained was dialyzed in the cold against distilled water, lyophilized, and dissolved in distilled water at pH 7.2 using 0.1 *N* NaOH. This solution contains a mixture of β- and γ-crystallins.

Aliquots of lens supernatant, isolated α-crystallin and β- and γ-crystallins were heated in a constant temperature water bath at 50, 55, 60, 65, 70 and 100 °C for 30 min. After heating, the solutions were centrifuged and the clear supernatants subjected to cellulose acetate electrophoresis. The antigenic properties of the lens proteins were studied by double diffusion in agar and immunoelectrophoresis[3]. Antisera to adult bovine lens were produced in rabbits. The protein content of lens supernatant was determined by the method of LOWRY et al.[4].

For cellulose acetate electrophoresis 4 λ of lens supernatant were used. Electrophoresis was performed in 0.05 *M tris*-borate buffer pH 8.6, at 35 V/cm for 1 h. The strips were stained in ponceau xylidine and cleared in 5% acetic acid.

Results. The cellulose acetate electrophoretic pattern of freshly prepared bovine whole lens supernatant (86.8 mg protein/ml), after heating at different temperatures, is shown in Figure 1. The mobility of α-crystallin decreased at temperatures of 60 °C and higher while the band stained more intensely. β- and γ-crystallins, however, showed decreased staining intensity from 60 °C on. At 100 °C no staining reaction was noted for β- and γ-crystallins and a very faint band for α-crystallin. Immunoelectrophoretic analysis showed only an α-crystallin band at 100 °C, while the β- and γ-bands present at lower temperatures had disappeared. A decrease in mobility of electrophoretic bands 1–3 between 22 °C and 50 °C was also noted.

The changes in the soluble protein content after heating are shown in Table I. The solution was distinctly cloudy at 70 °C, and a heavy precipitate was present at 100 °C. The protein value decreased markedly above 70 °C. These observations suggest that the disappearance of the β- and γ-electrophoretic bands with increase in temperature is due to the precipitation of these proteins.

When isolated α-crystallin, 33.3 mg/ml, was heated, the solution remained clear except for slight cloudiness at

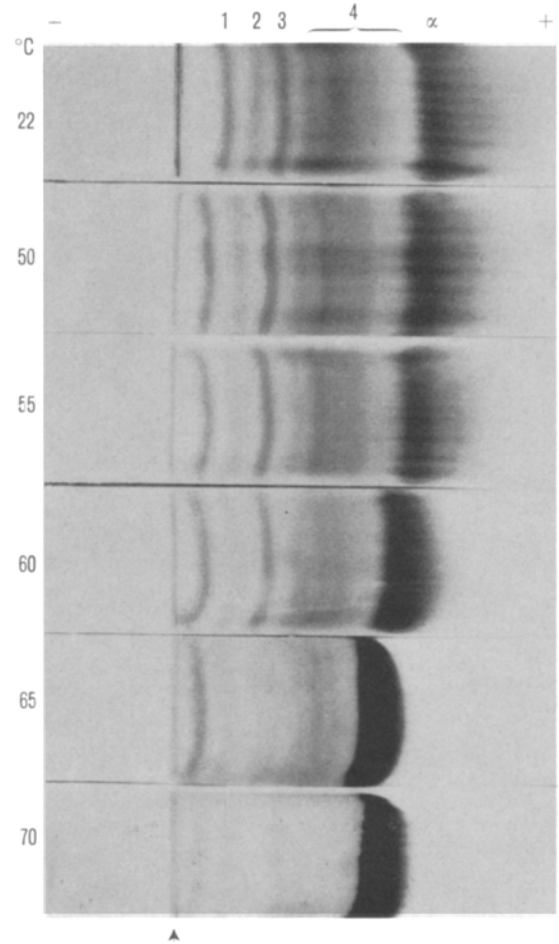

Fig. 1. Cellulose acetate electrophoretic pattern of whole lens soluble protein, aliquots of which had been heated for 30 min at different temperatures. The arrow indicates the application site. Note the decrease in mobility of α-crystallin and the decrease in staining intensity of the slower migrating proteins. Bands 1–3 probably represent γ-crystallins, and the reactions indicated by band 4 probably β-crystallins.

Table I. Change in appearance of solution and protein concentration as a result of heat treatment

Temperature °C	Appearance of solution of whole bovine lens proteins	Soluble protein mg/ml
22	Clear	86.8
50	Clear	72.0
60	Clear	70.0
70	Cloudy	61.0
100	Complete precipitate	4.17

[1] S. LERMAN, in *Cataracts, Chemistry, Mechanism and Therapy* (Charles C. Thomas, Springfield, Ill. 1964), p. 175.
[2] J. FRANCOIS, M. RABAEY, and R. J. WIEME, Arch. Ophthal. *53*, 481 (1955).
[3] H. MAISEL, Arch. Ophthal. *68*, 254 (1962).
[4] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).

100 °C. The electrophoretic mobility of the isolated protein decreased with increasing temperature (Figure 2). The immunologic specificity of α-crystallin did not change, even after heating at 100 °C (Figure 3). There was only a slight decrease in soluble protein concentration at 100 °C (Table II).

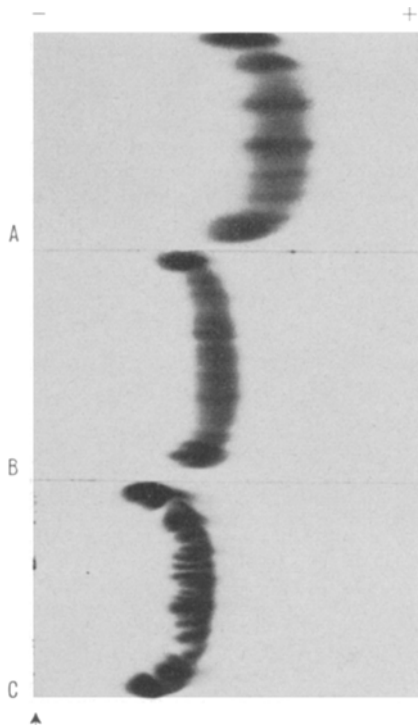

Fig. 2. Note the decrease in anodic electrophoretic mobility of α-crystallin when heated at different temperatures. A = unheated, B = 70 °C, and C = 100 °C.

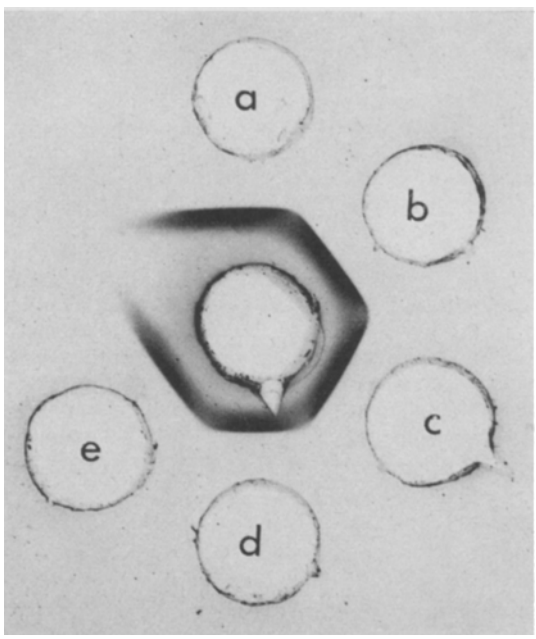

Fig. 3. Immunologic analysis of isolated α-crystallin heated at different temperatures. The precipitin bands merge in a reaction of identity; a–e indicate in sequence temperatures of 22, 50, 60, 70, and 100 °C.

When the mixture of β- and γ-crystallins was heated there was a decrease in staining intensity of all protein bands from 60 °C on, and at 100 °C no staining reaction was noted (Figures 1 and 4). This decreased staining is due to the precipitation of protein as indicated by the appearance of the solution and by the decrease in soluble protein concentration with increase in temperature (Table II). A slight decrease in anodic electrophoretic mobility of the slow migrating proteins was noted.

Discussion. Denaturation involves a rearrangement of the peptide chains in the protein molecule[5]. Closely folded chains are unfolded and/or refolded[6]. The particular mode of denaturation determines whether the disrupted peptide chains remain in the unfolded state, are

Table II. Change in appearance of solution and protein concentration as a result of heat treatment

Temperature in °C	Appearance of solution of isolated α-crystallin	Soluble protein in mg/ml	Appearance of solution of isolated β- and γ-crystallins	Soluble protein in mg/ml
22	Clear	33.3	Clear	83.3
50	Clear	33.0	Slightly cloudy	78.2
60	Clear	33.5	Moderately cloudy	45.0
70	Clear	33.3	Very cloudy	11.76
100	Slight cloudiness	30.0	Complete coagulate	3.78

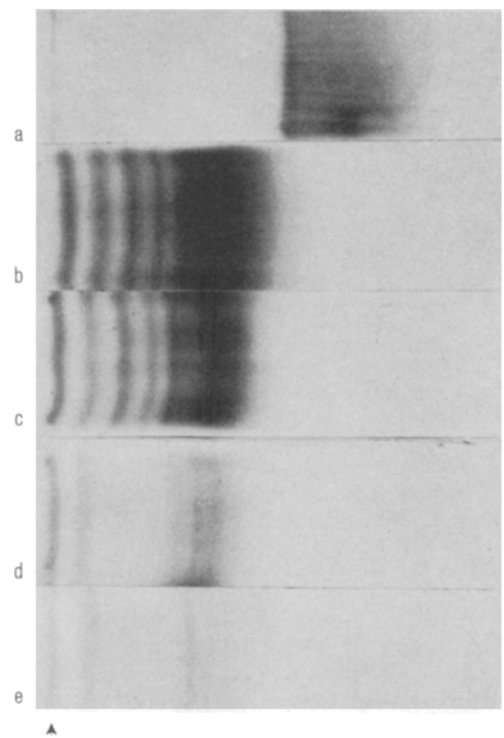

Fig. 4. Electrophoretic pattern of isolated β- and γ-crystallins after heat treatment. a = isolated unheated α-crystallin, b = β- and γ-crystallins at 22 °C, c–d = β- and γ-crystallins after heating to 50, 65, and 70 °C respectively.

[5] H. Wu, Chin. J. Physiol. *5*, 321 (1931).
[6] R. Lumrey and H. Eyring, J. phys. Chem., Wash. *58*, 110 (1954).

refolded to give the original pattern, or whether some other pattern from the original will result. The extent of denaturation can vary from slight structural changes to complete rearrangement of the peptide chains.

If denaturation is due to heat, the protein remains in the zwitterionic state. Hydrogen bonds between peptide chains are cleaved and bonds between hydrophobic groups may disappear. The insolubility of heat-treated proteins is probably caused by the S–S interchange reaction, and the resultant formation of new intermolecular S–S bonds[7].

The present experiment shows that heat has significantly different effects on α-crystallin compared to β- and γ-crystallins. α-Crystallin is not significantly precipitated even at 100 °C, in contrast to β- and γ-crystallins which precipitate increasingly with rise in temperature above 50 °C.

α-Crystallin is, however, distinctly modified by the rise in temperature, since its electrophoretic mobility is altered. The change in molecular conformation which must account for this, does not affect its antigenic sites, since the immunologic specificity of α-crystallin remains identical from 22–100 °C. Further evidence of a change in α-crystallin structure is offered by its more intense color reaction with ponceau xylidine after heating (Figures 1 and 2). This phenomenon has been found for other proteins, and ascribed to the unfolding of the peptide chains. The higher color reactivity shows that some of the reacting groups are buried inside in the native protein or screened off in another manner.

JANSEN et al.[8], studied the thermal coagulation of bovine and human serum albumin and reported that the physical properties depend upon the free sulfhydryl group and if this sulfhydryl group is destroyed by heating or other chemical reagents, clear clots result.

KINOSHITA and MEROLA[9] reported that there is a masking of sulfhydryl groups as the lens matures being strikingly absent in cataractous lenses[10]. This suggested that during cataract formation, sulfhydryl groups are oxidized to disulfide bonds.

β- and γ-crystallins contain free sulfhydryl groups while they are absent in α-crystallin as revealed by a negative nitroprusside test[11]. It may be concluded that during heating the free sulfhydryl groups of β- and γ-crystallins are converted into disulfide linkages with the formation of an insoluble precipitate, while the solubility of α-crystallin is unaffected by heat because it lacks free sulfhydryl groups[12].

Résumé. La chaleur est capable de précipiter les protéines du cristallin bovin β et γ, alors que l'alpha cristallin n'est pas précipité à des températures allant jusqu'à 100 °C. Cette constatation semble être en relation avec la formation de ponts disulfures moléculaires.

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[7] R. C. WARNER and M. LEVY, J. Am. chem. Soc. *80*, 5735 (1958).
[8] E. V. JENSEN, V. D. HOSPELHORN, D. F. TAPELY, and C. HUGGINS, J. biol. Chem. *185*, 411 (1950).
[9] J. H. KINOSHITA and L. O. MEROLA, Arch. Biochem. *81*, 395 (1959).
[10] Z. DISCHE, E. BORENFREUND, and G. ZELMENIS, Arch. Ophthal. *55*, 471 (1956).
[11] W. F. BON, J. biol. Chem. *236*, 88 (1961).
[12] Acknowledgment: The authors gratefully acknowledge the technical assistance of Mrs. SANGITA MEHTA.
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Chitin in Tunicata

The occurrence of cellulose as so-called tunicin in the mantle of tunicates is a well-established fact. Obviously this sort of cellulose is the same as that occurring in plants[1,2]. The analogy concerns not only the chemistry but also the structure, as the texture of the microfibrils exactly resembles the arrangement of fibrils found in the cell walls of certain plants[3,4]. The tunicates are the only group of animals known so far in which cellulose occurs; chitin, which is closely related to cellulose, has never been found in tunicates[5,6] or in other chordates[6]. Thus it was quite surprising when, in connection with investigations on the distribution of peritrophic membranes in animals not belonging to the Arthropoda, it was possible to find in the intestine of several ascidians membranes containing considerable amounts of chitin. Until now only some of the larger ascidians have been examined: *Phallusia mammillata, Ciona intestinalis, Halocynthia papillosa, Clavelina lepadiformis* and *Corella* sp. *Phallusia* and *Ciona* from the Mediterranean (Banyuls and Naples) were fixed in formalin; *Clavelina* from Heligoland was fixed in bouin; *Halocynthia* was the only living material, coming from the Adriatic; *Corella* was fixed in formalin and supplied by the Carolina Biological Supply Comp., Burlington N.C., USA.

All these species filter plankton in their branchial sacs, wrap it in mucus and transport it by means of their cilia into the intestine. Here the band of mucus is loosely wrapped in additional membranes, being secreted by the cells of the intestine. The secretion of these peritrophic membranes may easily be seen in cross sections of the intestine. Histochemical reactions, such as staining with alcian blue, toluidine blue, bromophenol blue, and the PAS reaction, indicate that these peritrophic membranes are built up of, or at least contain, glycoproteids. The band of mucus with plankton coming in from the branchial sac, is sometimes transported through the intestine as a rather straight band, and is sometimes coiled up between membranes which are wrapped loosely around these remarkably flat coils. It may be that it depends on the

[1] A. FREY-WYSSLING and R. FREY, Protoplasma *39*, 656 (1950).
[2] K. H. MEYER, L. HUBER, and E. KELLENBERGER, Experientia *7*, 216 (1951).
[3] A. FREY-WYSSLING, *Die pflanzliche Zellwand* (1–355, Berlin 1959).
[4] P. A. ROELOFSEN, *Handbuch der Pflanzenanatomie* (Berlin 1959), Bd. III, Teil 4, p. 1–339.
[5] K. M. RUDALL, Symp. Soc. exp. Biol. *9*, 49 (1955).
[6] CH. JEUNIAUX, *Chitine et Chitinolyse* (Paris 1963).