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Multiple changes in lens protein composition associated with the Cat^{Fr} gene in the mouse

By T. H. DAY* and R. M. CLAYTON†
**Institute of Animal Genetics, Edinburgh University, and Genetics Laboratory, University of Nottingham, and*
† Institute of Animal Genetics, Edinburgh University

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SUMMARY

The ocular cataracts produced by the dominant Cat^{Fr} gene in the mouse, *Mus musculus*, are associated with quantitative changes in the lens proteins (crystallins). The three classes of crystallin are affected differentially in homozygotes. Heterozygotes show a smaller effect. The quantitative levels of crystallin subunits are also affected and these changes are different for each subunit. The overall loss in protein is not readily explicable in terms of a generalised leakage or a general tendency to insolubilisation. Possible mechanisms for the action of the gene are suggested.

1. INTRODUCTION

It has long been accepted that mutants recognized by a morphological abnormality are likely to show pleiotropic patterns of manifestation (e.g. Grüneberg, 1952). Even at the biochemical level of recognition the quantities of several metabolites may be affected differentially as a result of some metabolic block (Harris, 1970). However, specific proteins are normally affected singularly and independently of changes in other proteins. Where several proteins are affected together it may be either because they are related heteropolymers containing one mutant subunit (L.D.H. – Markert & Whitt, 1968), or the same protein may possess varying numbers of some prosthetic group or carbohydrate moiety attached (Plapp & Cole, 1967; Stratil & Spooner, 1971), thus generating apparent multiplicity.

The lens of the vertebrate eye has a high concentration of proteins, the crystallins. In mammals these comprise three groups distinguishable by molecular weights, electrophoretic mobilities and antigenicities. The α and β crystallins are both series of heteropolymers and the γ's are monomers.

Numerous mutants, especially in the mouse but also in man and rat, have been described which affect the lens of the eye. Of these several cause cataracts, or opacity, of the lens. In all types of cataract, whether hereditary, senile or experimentally produced, there is an increase in the quantity of insoluble lens protein (albuminoid) (see Pirie & van Heyningen, 1956). In healthy bovine lenses the insoluble albuminoid fraction is largely derived from α-crystallin (Rao, Mehta &

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Cooper, 1965; Ruttenberg, 1965; Waley, 1965), and there is only weak immunological cross-reaction with the β- and γ-crystallins (Manski, Behrens & Martinez, 1968). The origin of albuminoid, however, appears to vary with the species, so that in the rat and dogfish it is more closely related to γ- rather than α-crystallins (Lerman, Zigman & Forbes, 1968).

Tapasztó (1962), using human cataractous lenses, has demonstrated a disproportionate loss of α-crystallin with a concommitant increase in albuminoid. Mach (1963), François, Rabaey & Stockmans (1965) and Charlton & van Heyningen (1968) have all found that there is a preferential decrease in the low molecular weight proteins, while α-crystallin remains unchanged or is increased. Further, the calculations of Mach indicate a net loss of protein from the lens. One possible explanation is that there is a preferential proteolysis of the γ-crystallins, but there is no evidence to support this. Alternatively, there could be leakage from the lens of only the low molecular weight material. This has been suggested by Sippel (1967) and tested by Charlton & van Heyningen (1968) whose results are not decisive. This whole problem of changes in lens proteins accompanying cataract has been recently reviewed by van Heyningen (1969).

The studies on hereditary cataracts in mice have not resulted in a coherent idea of the role of lens proteins in these defects. Smelser & van Sallman (1949), using a strain of mice which had not been characterized genetically with respect to the cataract trait, showed that there was a general reduction in the quantity of soluble protein, but that the fraction with the lowest electrophoretic mobility (presumably the γ-fraction) was reduced relative to the two faster fractions. Konyukhov & Wachtel (1963), using mice homozygous for the mutation 'recessive cataract' (cac), found a reduction in the α-components, loss of one of the β-bands and reduction in some of the γ-bands and an increase in others. The situation is further confused by the original nomenclature used by Konyukhov & Wachtel. Using chromatographic fractionation and electrophoresis in media other than polyacrylamide we have shown that the β-components have the highest mobilities in polyacrylamide gels, the γ's have intermediate mobilities and the α's are retarded by the gel and so remain at or near the origin (Day, 1971; T. H. Day & R. M. Clayton, in preparation).* It would therefore appear that it is the β-crystallins which are most altered in recessive cataract mice. However, Moser & Glueck-sohn-Waelsch (1967), using the same stocks as Konyukhov & Wachtel failed to find any difference between cataractous and isogenic non-cataractous animals. There is clearly no consistent pattern of changes which results in, or is the result of, cataract formation.

This communication is a report of some experiments performed on mice carrying the dominant cataract mutation (Cat) originally described by Paget (1953) who named it Cataracta Hereditaria Subcapsularis. A similar mutation was later described by Fraser & Schabtach (1962) who called it Shrivelled, and Verusio &

* Since this paper was written, Konyukhov & Platonov (1971) have made an identification of mouse crystallins similar to ours. (Konyukhov, B. V. and Platonov, E. S. (1971). Soluble lens proteins of mutant stock mice in cataract development. *Expl. Eye Res.* **11**, 230–238.)

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Fraser (1966) showed these two independent mutations to be allelic. More detailed descriptions of the morphological defects and ontogeny of the dominant Cataract Fraser (Shrivelled) mutant have been provided by Zwaan & Williams (1968, 1969).

Homozygous Cat^{Fr}/Cat^{Fr} animals show changes at 14 days of gestation and these are progressive. By birth an anterior polar cataract has developed. In normal animals epithelium secretes the collagen and glycoprotein of the capsule (Duke-Elder, 1969) and as the fibres develop from epithelium cells, capsule synthesis ceases and crystallin synthesis is undertaken. A characteristic of this mutant is the hyperplasia of the lens epithelium which secretes an excessively thick capsule. This is similar to another mouse mutant, Small eye (Clayton & Campbell, 1968; R. M. Clayton & J. C. Campbell, in preparation) which also shows an excessive production of capsule and extreme hyperplasia of the lens epithelium. These lenses are also cataractous.

This paper describes the protein composition of Cat/Cat, Cat/+ and +/+ mice. The findings have been: (1) that the heterozygote may be distinguished from both homozygotes, and (2) that there are apparently independent quantitative changes in the levels not only of the major protein classes but also of the subunits or peptide chains comprising these classes.

2. MATERIALS AND METHODS

The mice were bred from animals generously given by Professor F. Clarke-Fraser. The 'Cat^{Fr}' mutation was maintained in the homozygous condition on an A/J background by brother–sister matings and heterozygotes were obtained by crossing with an A/J/Fr stock that had been separated from the A/J line by about 30 generations of inbreeding. Animals from the A/J/Fr stock were used as the wild-type homozygotes.

Individual lenses were homogenized and electrophoresed in polyacrylamide gels as described by Konyukhov & Wachtel (1963) with minor modifications (T. H. Day & R. M. Clayton, in preparation).

Determination of the proportions that are water soluble, urea soluble and urea insoluble was as follows: lenses from 3 to 15 animals (13–14 months old) were weighed, homogenized in a known quantity of 0·05 M Tris/HCl buffer, pH 7·2, and then centrifuged at 3000g for 15 min; the absorbance of the supernatant was measured at 280 nm in a Beckman DB spectrophotometer; the pellet was washed three times and to it was added a known quantity of buffer containing 8M urea; after about 18 h at 2 °C the mixture was centrifuged again and the absorbance of the supernatant measured; the urea-insoluble pellet was washed three times, dried in an oven overnight and the residue weighed; samples of the supernatants after both centrifugations from each of the three genotypes were electrophoresed in polyacrylamide gels; the absorbances were translated into concentrations of protein by using a standard solution of bovine serum albumen.

Sephadex G75 (Pharmacia GB Ltd., London) was used for chromatography in glass columns 0·5 cm × 150 cm. The columns were packed according to the manufac-

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turer's instructions and well washed with the elution buffer. Approximately 50 mg. of protein was applied to each column and the material eluted with 0·05 M Tris/HCl buffer pH 7·2 containing 10 mM 2-mercaptoethanol. The flow rate was 2·0 ml/h maintained by gravity and 1·0 ml fractions were collected. Absorbances were measured at 280 nm. Fractions from the centre of the three peaks (α-, β- and γ-crystallins) were pooled and concentrated by the addition of dry Sephadex G 200, G 50 and G 25 respectively. Samples from each peak were electrophoresed in polyacrylamide gels to confirm that the chromatographic peaks were indeed α-, β- and γ-crystallins.

3. RESULTS

(a) *Gross quantitative differences between the genotypes*

Our results (Plate 1) consistently showed no differences in mobility of the β- or γ-bands between individual animals of the three genotypes. The high molecular weight α-crystallin fraction remains near the origin in these gels. While applying approximately the same volume of lens extract to each gel (15 μl ± approx. 1 μl) there was a repeatable reduction in the intensities of protein bands in the Cat^{Fr}/Cat^{Fr} animals. A similar but less extreme reduction is seen in the $Cat^{Fr}/+$ animals.

Some features of difference between the genotypes have been summarised in Table 1. It is evident that cataractous lenses are smaller, and incidentally, much softer, than normal ones, and that the heterozygote is intermediate between the two homozygotes. There is also a difference in the composition of the lenses. In cataractous lenses, protein constitutes only 10 % of the wet weight of the lens whereas in wild type animals this proportion is 30 %, and the heterozygotes are intermediate. The reduced size of the lenses and the reduced proportion of them that is protein means that there is a very drastic drop in the amount of protein in an individual lens.

This decrease in protein is seen to be proportionally distributed between the protein that is soluble in normal buffers, that which is only soluble in urea and that which remains insoluble even in urea. There is no evidence in these animals that cataracts are accompanied by a conversion of soluble into insoluble protein.

One may ask whether it is the low molecular weight proteins that are preferentially reduced as has been observed in other types of cataracts. The electrophoresis patterns already described suggest that some proteins are present in disproportionately reduced quantities. 20 to 30 lenses of each genotype were homogenized and chromatographed on Sephadex G 75 columns. The elution patterns, given in Plate 1D, indicate a disproportionate reduction in the amount of β- and γ-crystallins so that in homozygous cataractous lenses the proportion of the total soluble protein which is α-crystallin is over 80 % compared to under 30 % in normal wild-type lenses (see Table 1). The heterozygote pattern is again intermediate between the two homozygotes. It should be noted that the extracts were centrifuged at 3000g for 30 min immediately before being applied to the columns so that the

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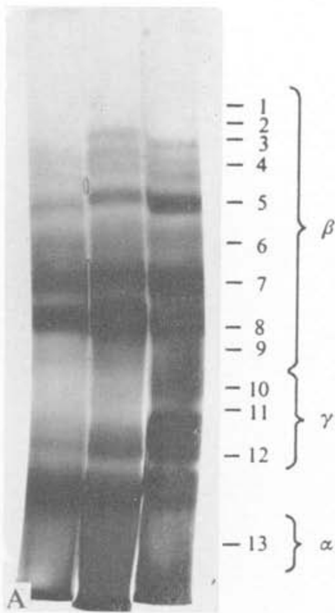

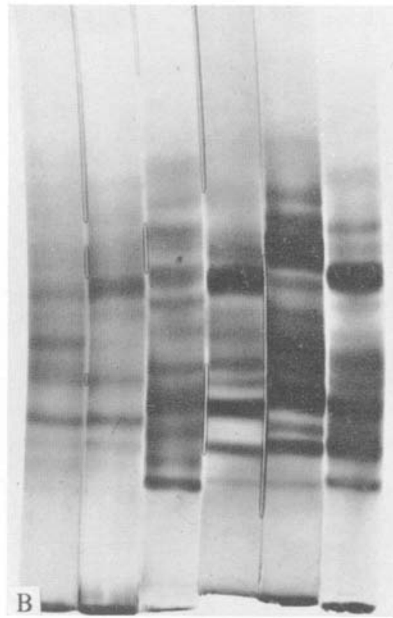

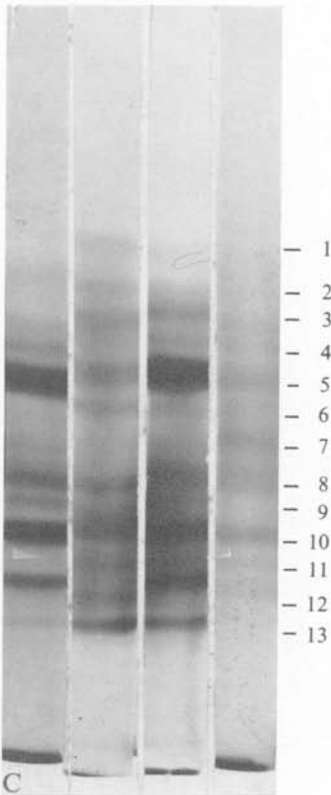

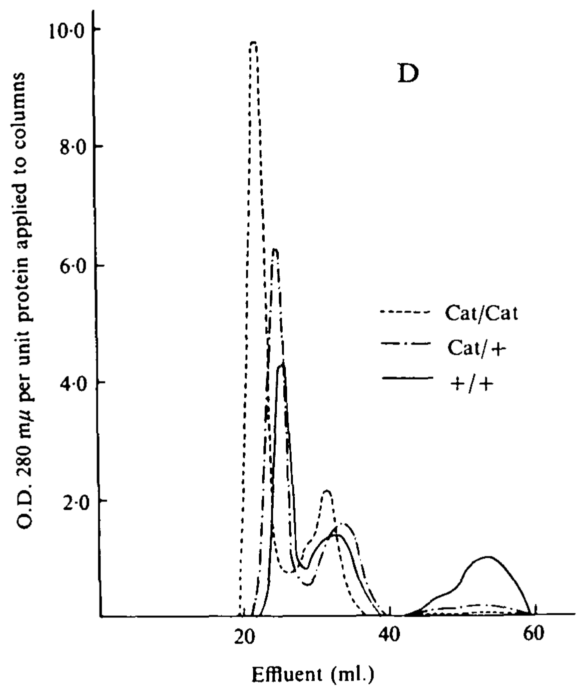

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Table 1. *Protein composition of Cat^{Fr}/Cat^{Fr}, Cat^{Fr}/+ and +/+ lenses as percentages of wet weight*

Genotype	+/+	Cat^{Fr}/+	Cat^{Fr}/Cat^{Fr}
Total no. of lenses examined	36	36	26
Mean wet weight of a single lens (mg)	6·1	5·2	3·4
Percentage of lens (w/w) which is protein	29·9	20·4	9·8
Percentage of lens (w/w) which is protein soluble in Tris HCl buffer pH 7·2 ('soluble protein')	18·2	10·8	6·3
Percentage of lens (w/w) which is protein insoluble in Tris buffer but soluble in Tris buffer + 8M urea ('urea soluble protein')	9·8	8·3	2·4
Percentage of lens (w/w) which is insoluble in Tris buffer + 8M urea ('insoluble residue')	1·9	1·3	1·1
Percentage of 'soluble protein' in Sephadex G75α peak	29	64	83
Percentage of 'soluble protein' in Sephadex G75β peak	40	30	17
Percentage of 'soluble protein' in Sephadex G75γ peak	31	6	0 (undetectable)

peaks eluted in the void volume of the column are essentially free of albuminoid except for that precipated during the course of filtration.

(*b*) *Soluble protein*

The soluble proteins from each genotype were compared both as intact aggregates in gels not containing urea, and as subunits in gels run in the presence of urea. A comparison of the electrophoresis of the intact proteins shows that the decrease in total protein does not result from the loss of all proteins equally. Thus of the β-crystallin bands 1–4, 8 and 9 (see Plate 1A), the loss is greatest in Cat^{Fr}/Cat^{Fr} and intermediate in Cat^{Fr}/+ compared with the wild-type animals. β 7 is affected differently, being more intensely stained than 6 in Cat^{Fr}/Cat^{Fr} and Cat^{Fr}/+ and less intensely stained in +/+. Indeed in the Cat^{Fr}/+ it is one of the two most heavily stained bands and appears disproportionately intense compared to the remaining bands. It is probably the major constituent of β's remaining in the Cat^{Fr}/Cat^{Fr}. The gamma crystallin bands 10, 11 and 12 are much reduced but again there is preferential loss, band 12 being relatively persistent, 10 being almost lost and 11 reduced in the heterozygote, while 10 is virtually absent and 11 remains only as a trace in the Cat^{Fr}/Cat^{Fr} homozygotes. The reason why γ-crystallins from Cat^{Fr}/Cat^{Fr} extracts are detectable in polyacrylamide gels, but not in the eluents from chromatographic columns, is probably the very much shorter time that the proteins are subjected to *in vitro* conditions during electrophoresis. It is likely that the γ-crystallins are rendered considerably less stable by their separation for several hours from the higher molecular weight α- and β-crystallins. The α-crystallin is not analysable in polyacrylamide gels in the absence of urea due to the large molecular size.

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If we turn to the patterns found after dissociation in urea we find that the dissociated soluble proteins also show differences between +/+, $Cat^{Fr}/+$ and Cat^{Fr}/Cat^{Fr}. Thus bands 1, 2, 3, and 6 are reduced, 4 and 5 less so, 8 is actually relatively increased, 9 relatively unchanged and 11 and 13 lost, in Cat^{Fr}/Cat^{Fr} as compared to +/+ extracts. (Plate 1B, C.)

(c) *The urea soluble protein*

The protein fraction which was insoluble in Tris/HCl buffer, but which could be solubilized by 8M urea produced essentially the same bands as the soluble protein, but there are yet again differences between genotypes. The relative intensities of each band in the 'soluble' and 'urea soluble' fractions appear much the same in the wild type lenses except for 1, 2 and 6 and possibly 3 and 13 which are relatively more marked in the insoluble fraction, while in Cat^{Fr}/Cat^{Fr} lenses, bands 7 and probably 3 and 4 have tended to move from the 'soluble' into the 'urea soluble' compartment (Plate 1B, C). While some individual proteins may have become insolubilized in the cataractous lenses, there is clearly not a gross conversion of 'soluble protein' into 'urea soluble protein' (see Table 1). This is shown by a comparison of the 'soluble' and 'urea soluble' categories in wild-type lenses (18·2 and 9·8% respectively) and in Cat^{Fr}/Cat^{Fr} lenses (6·3 and 2·4%).

(d) *The 'insoluble residue'*

No attempt was made to analyse the 'insoluble residue' qualitatively. This category was measured quantitatively by weighing the dried residue after extraction firstly with Tris buffer, and then with Tris buffer containing urea. It may well contain material that is not protein. However, one may conclude that the apparent deficit of protein in the other two categories ('soluble' and 'urea soluble') is not accompanied by an increase in the 'insoluble' category. This means that cataract is not accompanied by an irreversible insolubilization of protein.

Table 1 summarizes the differences between the genotypes, in overall protein production and in the percentage of protein that is first, soluble in Tris/HCl, secondly insoluble in Tris/HCl but soluble in urea, and thirdly insoluble even in urea. It will be seen that the heterozygote is intermediate between the two homozygotes for all measurements.

4. DISCUSSION

The analyses of the protein composition of the three genotypes have been made for total protein content, soluble protein content, insoluble but dissociable protein and insoluble/non-dissociable protein, and also in terms of the relative composition of the major crystallin classes. We have found no evidence that cataractous lenses contain any qualitatively changed proteins, and in consequence it seems unlikely that the Cat^{Fr} locus determines the sequence of a crystallin. However, it is plain not only that the overall content of protein is seriously reduced in the Cat^{Fr}/Cat^{Fr} mice and less so in heterozygotes, but also that the conversion of protein into the insoluble compartment does not differ much in the three geno-

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types. The overall changes in the protein composition demonstrated by column chromatography suggest that the serious diminution of γ-crystallins and the less extreme diminution of the β's does not necessarily involve insolubilization but is more likely to reflect a diminution in synthesis or a disproportionate loss from the lens. Nevertheless, when we turn to the subunit composition of the proteins as demonstrated by electrophoresis in the presence of urea, this tentative conclusion is contradicted by the finding that the relationship between changes in the 'soluble' and 'urea soluble' compartments differs between the individual sub-unit bands. This must mean that the regular changes in overall composition demonstrated by the previous technique evidently are mere summaries of the many specific changes affecting the subunits.

Zwann & Williams (1969) observed that the Cat^{Fr}/Cat^{Fr} lens shows breakdown of fibres. Analogy with the bovine lens (Papaconstantinou, 1967) and amphibian lens (McDevitt, Meza & Yamada, 1969) suggests that the fibres contain all of the γ-crystallins but only some of the α's and β's. The epithelium, on the other hand, probably contains only α- and β-crystallins. The epithelium of the Cat^{Fr}/Cat^{Fr} mutants shows local hyperplasia and secretion of excess capsular material, but it is not known whether the α- and β-crystallin content of such epithelial cells is normal. In 'Small-eye', another mouse mutant that shows similar but far more extreme epithelial changes than Cat^{Fr}/Cat^{Fr} homozygotes, there is also severe loss of crystallins. The extent of this protein loss is related to the degree of morphological abnormality, and in very cataractous lenses, in which the epithelium is severely affected, the amount of α-, β- and γ-crystallins detectable is negligible (R. M. Clayton & J. C. Campbell, in preparation). For many years it has been known that the composition in terms of crystallin content of the earliest fibres is different from that of fibres deposited later. Thus localized cataract damage causing a breakdown or failure in formation of restricted areas may come to have multiple effects on the protein composition of a lens. This follows because a restricted group of fibres laid down at a certain period of development should not be expected to have the same crystallin composition as that of the total lens.

It is clearly not possible to determine from the data presently available whether the primary lesion in hereditary cataracts affects cellular integrity and maintenance of lens cells laid down at particular and restricted periods of development. Another possibility is that the primary lesion affects one of the peptide chains of the crystallins, which, while being qualitatively identical to the wild-type peptide, is nevertheless synthesized in gradually changing proportions during development. Thus the integrity of cells which normally contain a high proportion of the subunit in question may break down as a result of the imbalance in their subunit composition.

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EXPLANATION OF PLATE

Plate 1A. Soluble protein from aliquots of lenses prepared as described in the text, electrophoresed in polyacrylamide gels. (Tris glycine buffer pH 8·9, 10 mM 2-mercaptoethanol.) Left to right, Cat^{Fr}/Cat^{Fr}, Cat^{Fr}/+, +/+. The crystallins are identified by class (Day, 1971) and numbered from anode to cathode.

Plate 1B. Saline soluble and saline insoluble proteins from the aliquots shown in 1A, dissociated by 8M urea containing 10 mM mercaptoethanol, electrophoresed in polyacrylamide gels (tris glycine buffer pH 8·9. 10 mM 2-mercaptoethanol, 6M urea). Left to right, saline insoluble proteins, Cat^{Fr}/Cat^{Fr}, Cat^{Fr}/+, +/+ ; saline soluble proteins, Cat^{Fr}/Cat^{Fr}, Cat^{Fr}/+, +/+.

Plate 1C. Urea polyacrylamide electrophoresis, as in plate 1B. Left to right Cat^{Fr}/Cat^{Fr} saline soluble, Cat^{Fr}/Cat^{Fr} saline insoluble, +/+, saline soluble; +/+, saline insoluble. Bands numbered from anode to cathode.

Plate 1D. Column chromatographic separation of 50 mg of lens protein from each of three genotypes +/+, Cat^{Fr}/+, Cat^{Fr}/Cat^{Fr}. Conditions as described in text.

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