Cataract as a Protein Condensation Disease
The Proctor Lecture
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The progression of cataractogenesis in the normal aging population can be characterized as a continual increase in the intensity of light scattered from the lens. This increase is an exponential function of age and has a time constant ($\Delta T$) that on average is approximately 35 years. An important molecular mechanism for such light scattering is the condensation of proteins into aggregates. Such aggregated states are also the basic pathogenic factor for a wide class of molecular condensation diseases, including sickle cell and Alzheimer's diseases. We present general strategies for the inhibition of such diseases. Assay methods to measure the efficacy of reagents that inhibit lens protein aggregation and phase separation in vitro are also presented, along with the chemical principles used in their selection. We also describe the results of in vivo studies that confirm the efficacy of such inhibitors in animal model systems. In the case of the human lens, a putative cataract inhibitor may be regarded as effective if, on average, it increases the time constant ($\Delta T$) for the exponential increase of scattering with age by $\sim$20% or more. A rough statistical estimation suggests that it may be feasible to detect such an increase in a human population during a 2-year period using modern optical methods.

INTRODUCTION AND BACKGROUND
Cataract and Molecular Condensation Diseases

The eye is that living instrument which conveys the light of the external world into the inner world of the mind, wherein we receive the miraculous gift of vision. So precious is this gift, that Science must search for ways to keep this portal clear for the flow of light, just as we strive to keep open the circulation of our blood, or the flow of neurotransmitters in our brain. Indeed, Science is called upon to “make war upon the bloody tyrant, Time.” For, in the course of age, our lenses grow cloudy and cataractous. The circulation of our blood is impeded by atherosclerotic plaques, and our neural processing is obstructed by amyloid plaques.

In the struggle between Science and Time, we are fortunate to live in a period in which Science is beginning to uncover the molecular basis for the various obstacles to vision, circulation, and to thought itself. Indeed, we shall argue that apparently unrelated diseases such as cataract, sickle cell disease, atherosclerosis, and Alzheimer’s disease, are all representatives of a broad class of pathologies that we shall designate as “molecular condensation diseases.” The basic element common to all members of this class is an attractive energy of interaction between specific biologic molecules, which produces condensation into dense, frequently insoluble phases. These dense condensate phases are responsible for compromising cellular and organ function.

Having recognized the elements common to all molecular condensation diseases, it becomes possible to enunciate a general strategy, described below, for the inhibition of each specific disease in this class. Indeed, as the 20th century draws to a close, we can glimpse a conceptual pathway that, if followed by interdisciplinary research teams, holds the promise of inhibition of molecular condensation diseases. Thus we may hope to stay the ruinous hand of time.

Turbidity of the Aging Normal Human Lens

If we are to understand cataractogenesis rightly, it is useful to characterize semiquantitatively the increase in turbidity and light scattering that occurs in the aging normal lens. Analysis of recent light scattering measurements shows that light scattered by a newborn human lens is 50 times greater than that of the liquid toluene that is used as a standard in the measurement of absolute light intensity. As the lens ages, the intensity of the light scattered backward, nearly opposite the direction of the incident light, increases approximately exponentially with age. Under conditions in which the ophthalmologist will detect significant turbidity, the backward scattered light intensity has been found to increase by a factor of approximately 6 to 8 as compared with the newborn lens. These findings suggest that even the “clear” infant
lens, a bag of interdigitated fiber cells each packed with cytoplasmic protein, is far from a perfect medium for light transmission. Furthermore, the optical properties of this jellylike bag are not stable. With age, on average, each lens shows a continual increase in turbidity until a threshold for "cataract" is crossed.

Healthy persons differ simply by virtue of variations in the rate of progression of turbidity. Thus, the common meaning of the term "disease" is really not relevant to the process of cataractogenesis. Having said all this, it is still quite relevant to ask: Can we hope to stay the ruinate hand of time? What are the molecular changes that are responsible for the increasing level of turbidity? How may we arrest these changes? To answer these questions, we must first examine the physical conditions needed for the transparency of any continuous medium.

**Physical Basis for the Transparency or the Turbidity of a Continuous Medium**

Attenuation of the power in a beam of electromagnetic radiation passing through a medium takes place by two processes: absorption and scattering. In the former process, part of the power in the field is dissipated into heat as the result of energy-absorbing electronic transitions. In the latter process, part of the radiation is redirected (scattered) away from the incident direction, thereby reducing the transmitted power. Furthermore, depending on the precise angular dependence of the scattered light intensity, the wave fronts of the transmitted light can be distorted and the image-forming capability of the medium can become degraded. In the case of cataract, the light scattering process is the primary factor responsible for the turbidity and wave front distortion by the lens.

From a fundamental physical point of view, it can be shown that a perfectly uniform medium having density \( \rho \) exhibits no scattering of light away from the forward direction. This is true even though the incident field stimulates each particle in the medium to produce spherically spreading scattered light waves. It should be borne in mind that the net field at each point in space is the sum of the wave fields produced by each particle. If the density is a constant, then the superposition of the phases of all the scattered waves produces complete cancellation and the net scattered field is zero. Thus, it follows that a continuous medium can scatter light only if it contains spatial fluctuations \( \delta \rho(\tau) \) in density about the mean density \( \bar{\rho} \), that is, \( \delta \rho(\tau) = [\rho(\tau) - \bar{\rho}] \).

If fluctuations in density are the origin of light scattering, it is of first importance to identify the specific fluctuations that occur in the aging cataractous lens. The theory of the scattering of light only gives a generic description of the nature of the scattering fluctuations, and it uses concepts unfamiliar to the biologist. Mathematically, the theory says that the amplitude of the field scattered into a particular direction is directly proportional to the Fourier amplitude of the density fluctuation having wave vector: \( \vec{K} = \vec{k} - \vec{k}_0 \). Here \( \vec{k} \) is the scattered wave vector, \( \vec{k}_0 \) is the incident wave vector, and \( \vec{K} \), the difference between \( \vec{k} \) and \( \vec{k}_0 \), is called the scattering vector. In fact, it was Albert Einstein\(^6\) in 1910 who used this connection between the scattered electric field and the \( \vec{K} \)th Fourier amplitude of the density fluctuations in his investigation of the extraordinary phenomenon of the opalescence of a fluid in the vicinity of its critical point. We now turn to a more pictorial description of the actual density fluctuations that have been found responsible for light scattering from the lens.

**MICROSCOPIC BASIS FOR LENS OPACIFICATION**

In 1971, the current author suggested\(^7\) that the aggregation of lens proteins into randomly distributed high molecular weight clusters could produce sufficient fluctuations in protein density so as to account for the turbidity of cataract. The molecular weight required for such aggregates was calculated theoretically to be \( \sim 50 \times 10^6 \) g/mol. Experimental studies conducted soon after this prediction confirmed the existence of high molecular weight aggregates in aging lenses\(^8\) and also showed their molecular weight to be in the range predicted theoretically.\(^7\) Since those early days, there have been a host of studies dedicated to identifying the precise chemical interactions responsible for the interprotein attraction and binding.\(^8\) Indeed, a wide variety of posttranslational chemical modifications of the lens proteins believed to be associated with these aggregates has been reported. Such modifications include intermolecular disulfide crosslinks, methionine oxidation, carbamoylation, glycosylations of amino groups, and racemization. In addition to these biochemical investigations, which require the actual removal of the aggregates as part of the insoluble fraction of entire lens homogenates, it has proven possible to obtain, under clinical conditions, evidence for those aggregates in the living human lens. Indeed, by using the noninvasive method of quasielastic light scattering spectroscopy (QLS),\(^3,9\) one may quantitatively observe the age-related increase in high molecular weight protein aggregates in the aging human lens by suitably modifying the traditional slit lamp microscope.\(^2\) All these lines of evidence clearly support the important role that such aggregation plays in cataractogenesis.

Having said this, it is well to reassert that a variety of studies\(^8\) show that multiple biochemical factors contribute to cataractogenesis. Nevertheless, these multiple factors have the net effect of producing "clumps"
or aggregates of proteins. These spatial fluctuations in protein density produce increased light scattering and ultimately can result in opacification of the lens.

From a purely biologic point of view, it might seem logical to block cataractogenesis by specifically inhibiting each of the possible types of posttranslational chemical modifications mentioned above. However, even a brief consideration of such a program makes it clear that it is impractical to control the intracellular biologic milieu with the delicacy and specificity needed to block each of the specific posttranslational modifications so far detected. On the other hand, if one steps back a bit, one sees that modifications that promote aggregation can be expected to increase the net attractive interactions between the cytoplasmic proteins. High molecular weight protein aggregation is but one of the effects of this attractive interprotein interaction. Another effect is the phenomenon called "cold cataract." An examination of this phenomenon will reveal an unexpected and more practical avenue for the inhibition of cataractogenesis.

We have seen above that the cytoplasmic solution of proteins within each lens cell is in fact not stable. Microscopic protein aggregation is one manifestation of this instability. A second instability, and a source of spatial inhomogeneity of the dense cytoplasmic protein solution, is the spontaneous separation of the solution into coexisting protein-rich and protein-poor domains. In "cold cataract," on lowering the temperature of say, a calf lens, below approximately 15°C, it is observed that the lens becomes cloudy and opaque. This opacification is reversible, and on raising the temperature, the lens becomes clear once again. The experiments of Tanaka and Benedek were the first to show that this reversible opacification was the result of a phase separation of the cytoplasmic proteins into coexisting protein-rich and protein-poor domains. The spatial fluctuation in the protein density from one domain to another produces sufficient light scattering so as to cause opacification of the lens. The actual temperature \( T_c \) below which such phase separation occurs depends very sensitively on the magnitude of the net attractive interprotein interaction energy. Indeed, as the interprotein attraction increases, the temperature for phase separation rises. If the temperature \( T_c \) increases above body temperature, the lens cytoplasm becomes opaque on the formation of protein-rich and protein-poor domains. In many animal model systems cataractogenic agents and genetic errors have been shown to produce lens opacification associated with this phase separation effect.

This qualitative discussion suggests that the phase separation phenomenon itself might be used to quantify the net attraction between proteins. Thereby, phase separation might well be used as a tool to discover protein modifications that can reduce the attraction between proteins—and hence inhibit the cataractogenic fluctuations such as aggregation and phase separation. In view of the importance of this possibility, we present a very brief summary of a few quantitative features of liquid-liquid phase separation.

Consider a simple binary mixture consisting of a single species of protein \((\phi)\) as solute molecule and water (aqueous) \( (\omega) \) as solvent. Let \( N_\omega \) and \( N_\phi \) be, respectively, the number of water and protein molecules in the solution. Also, let \( \Omega_\omega \) and \( \Omega_\phi \) be the effective volumes of a water and a protein molecule. We shall specify the protein concentration in terms of its volume fraction \( \phi \) in solution, where \( \phi = N_\phi / V \) and \( V = N_\omega \Omega_\omega + N_\phi \Omega_\phi \) is the total solution volume. The water volume fraction is \( \Omega_\omega / V = (1 - \phi) \). If the interaction between proteins is sufficiently strong so that binary liquid phase separation can occur below some critical temperature \( T_c \), then such separation will become manifest in the following way. Let the temperature of the solution be fixed at \( T < T_c \), and let the protein volume fraction \( \phi \) be increased steadily from \( \phi = 0 \). For small values of \( \phi \), all the protein added will be soluble. However, as \( \phi \) increases, a value \((\phi^1)\) will be reached, above which further protein will appear either in the form of dense droplets, each of which contains a density of protein corresponding to a volume fraction \( \phi^1 \), or dilute droplets corresponding to volume fraction \( \phi^i \). Indeed, for all values of the total protein concentration \( \phi_1 < \phi < \phi^i \), the total protein added will segregate into the two phases in the form of domains. Each domain will have protein densities given by \( \phi^1 \) and \( \phi^i \). It is possible to measure \( \phi^1 \) and \( \phi^i \) at each value of the temperature \( T < T_c \). A graph of \( \phi^1 \) and \( \phi^i \) versus \( T \) is called the liquid-liquid coexistence curve. In Figure 1 we show the actual coexistence curve for the calf lens protein \( \gamma_{\text{im}} \) (or \( \gamma_D \)) crystallin. It will be noted that as the maximum temperature \( T_c \) for liquid-liquid phase separation is approached, \( \phi^1 \) and \( \phi^i \) approach one another. In the case of \( \gamma_{\text{im}} \) crystallin protein, \( T_c = 5.2°C \). In Figure 1 we also show the coexistence curve for \( \gamma_{\text{im}} \) (or \( \gamma_C \)) crystallin. This member of the \( (\beta/\gamma) \) crystallin family has essentially the same molecular weight as does \( \gamma_{\text{im}} \) and has a high degree of homology in amino acid sequence with \( \gamma_{\text{im}} \). Nevertheless, the small difference in amino acid sequence produces sufficient difference in the interprotein interaction energy that the critical temperature \( T_c \) for a pure \( \gamma_{\text{im}} \) solution is 37°C.

Dr. Jayanti Pande has carried out a variety of investigations that show vividly that chemical modifications of the \( \gamma \) crystallin proteins can produce dramatic alterations in \( T_c \). Indeed, certain modifications can increase \( T_c \) and others decrease \( T_c \). Also, she has shown that dimerization produces a large increase in \( T_c \). Clearly, if the net interprotein interaction energy is such that \( T_c \) exceeds body temperature for the mixture...
of cytoplasmic proteins, lens opacification will result. In view of this, it is important to express quantitatively the connection between the critical temperature $T_c$ and the energy with which protein and water molecules interact with one another. This connection has long been well known for binary mixtures of small molecules. In the case of proteins, a simple model shows that if $\sigma$ represents the number of sites on each protein that can interact with either a water or another protein molecule, $k_b$ is Boltzmann's constant, and $\mu$ is a pure number approximately equal to 10, then for a binary mixture of protein and aqueous:

$$k_B T_c = \frac{(\sigma/\mu)(E_{\text{pw}} - (E_{\text{ww}} + E_{\text{pp}})/2)}{(1)}$$

In this equation, $E_{\text{pw}}$ is the mean attractive (negative) energy of interaction between a site on the protein and a water molecule, $E_{\text{ww}}$ is the attractive (negative) water-water interactive energy and $E_{\text{pp}}$ is the mean attractive (negative) energy of interaction between two sites on adjacent proteins. This equation is useful in expressing the fact that the value of the critical temperature $T_c$ for phase separation is determined as a difference between the mean (negative) like-like attractive energies $(E_{\text{ww}} + E_{\text{pp}})/2$ and the mean (negative) like-unlike attractive energy $E_{\text{pw}}$ between protein and water. Indeed, it is essential that the net like-like attraction between water molecules and protein molecules be numerically larger than the attractive like-unlike energy so that phase separation is possible at a positive temperature. The preponderance of like-like attraction above the like-unlike protein-water attraction is a driving force for the segregation of the solution into separate domains containing either water or protein. On the other hand, a complete separation of protein and water molecules is very disadvantageous from an entropic point of view. As a result, to an extent determined by the temperature, the protein phase must contain water molecules, whereas the aqueous phase also contains protein. This results in the coexistence of protein-rich and water-rich (or protein-poor) phases as observed experimentally. Droplets of these phases contained in the individual fiber cells produce the protein density inhomogeneities responsible for the opacification that occurs in “cold cataract.”

We shall see subsequently how equation 1 can serve as the conceptual basis of an assay method that can provide useful guidance in the search for cataract-inhibiting reagents. At this point, however, we conclude our description of the microscopic physicochemical bases for lens opacification by describing a third possible source of spatial fluctuation in protein density: a fluctuation associated with “osmotic cataract.” The cytoplasm of the lens fiber cells contains a high concentration of protein. Thus, the chemical potential for water in the lens interior is much lower than the water chemical potential in the aqueous chamber surrounding the lens. This chemical potential gradient would result in considerable flow of water into the lens were it not for active pumps in the lens epithelial cells. These pumps must function effectively so as to maintain the high protein concentration and hence, the refractive properties of the lens. If the epithelial pumps become compromised, then aqueous can flow into and accumulate within the lens. The water accumulation can take the form of “water clefts”: lakes of water whose dimensions easily can exceed the wavelength of light. Such clefts are powerful scatterers of light and can produce lens opacification.

In the current article, we do not consider problems connected with the viability of the system of epithelial lens pumps. Instead, we focus our attention on maintaining the stability of the cytoplasmic lens proteins against high molecular weight aggregate formation and phase separation. Both these phenomena are the result of attractive net interactions between the intracellular proteins. Such attractive interactions between proteins not only can produce cataract, but are responsible for the manifestations of a variety of other diseases. We now draw the reader's attention to some other members of the class of protein condensation diseases, of which cataract is but one example.

**MOLECULAR CONDENSATION DISEASES**

I believe that it is useful to introduce the term “molecular condensation diseases” to describe a class of dis-
A second protein condensation disease is Alzheimer’s Disease. The consequent death and absorption of neurons causes the devastating loss of brain function characteristic of Alzheimer’s disease. Similar neurodegeneration occurs in other amyloidogenic protein diseases, such as scrapie and bovine spongiform encephalopathy. These, too, are protein condensation diseases in which the proteins involved condense into characteristic fibrillar phases.

**Multiple Myeloma**

In the neoplastic disease known as multiple myeloma, patients often die from amyloid deposits in the kidneys. These nephrotic deposits are the result of condensation of so-called Bence Jones proteins, which have been shown to be the light chain components of human immunoglobulins that are overproduced in patients with multiple myeloma. Examples of molecular condensation diseases not involving proteins are presented next.

**Cholesterol Gallstone Disease**

Here, a crucial nucleating factor for the nucleation of gallstones is the formation of crystals of cholesterol monohydrate. Normally, cholesterol is solubilized in bile by molecules consisting of bile salts, lecithin, and cholesterol. Supersaturation of cholesterol in the bile leads to condensation of cholesterol into a solid phase, which serves as the nucleus for accretion of various bile components into stony aggregates. Here again, a phase transition produces a condensate that compromises organ function.

**Nephrolithiasis**

Similarly, nephrolithiasis is the result of condensation of calcium salts, uric acid, cystine, and MgNH₄PO₄ into kidney stones, which can block the ureter and severely affect urinary function. Other molecular condensation diseases are atherosclerosis, gout, and type II diabetes.

**General Strategies for the Inhibition of Molecular Condensation Diseases**

Having now described the basic intermolecular interactions that produce molecular condensation diseases, and having given a few specific examples of such diseases, we are now in a position to enunciate strategies for their inhibition. In general, two main lines of attack suggest themselves. The first strategic line consists of shifting the position of boundaries in the phase diagram so that under physiological conditions the thermodynamic equilibrium state corresponds to a single soluble phase. The required shifting of the phase boundaries can be carried out by chemically modifying the proteins in such a way as to reduce the magnitude of the net attractive energy of interaction between proteins. This will generally result in...
moving the phase boundaries to lower temperatures. If the relevant phase boundary falls below body temperature, the phase transition that produces the protein condensation will not occur under normal body conditions. As an example, if the phase transition involves liquid-liquid phase separation as in several animal model systems for cataract, then equation 1 shows how the maximum temperature \( T_c \) on the coexistence curve depends on the interaction energies \( E_{pp} \), \( E_{pp} \), and \( E_{ww} \). According to this equation, if \( E_{pp} \) can be increased relative to \( E_{ww} \) and \( E_{pp} \) by increasing the hydrophilicity of the protein, then the phase separation temperature can be shifted downward away from body temperature.

According to this general line of reasoning, one does not try to interfere with naturally occurring post-translational chemical modifications. Instead, the pharmaceutical strategy consists in purposefully introducing specific modifications that relocate the phase boundaries so that the condensed phase is not possible thermodynamically. Under these conditions, the proteins will remain in the soluble state and cellular and organ damage will not occur. This strategy for inhibiting cataract disease has already been enunciated in the literature. 25

The second general strategy for the inhibition of molecular condensation diseases consists in controlling the kinetic factors that govern the formation of the condensed phase. In principle, if the kinetic evolution can be slowed sufficiently, the effect of the disease process can be greatly postponed or even eliminated entirely. As an example of the latter prospect, consider the case of sickle cell disease. The sickling process in the erythrocyte begins with the deoxygenation of the hemoglobin. The formation of the hemoglobin S fibers, however, requires the formation of fiber nuclei and fiber growth. The time for such nucleation and growth is extremely sensitive to the concentrations of Hemoglobin S and normal hemoglobins in the erythrocyte. 21 If these concentrations can be changed even a little, the time required for gelation can be made to exceed the time of passage of the erythrocyte through the capillary beds. Indeed, Eaton and Hofrichter 26 have suggested that precisely this mechanism may be the mode of action of the drug hydroxyurea, which is now undergoing clinical trials for the amelioration of sickle cell disease.

In the case of cataract disease, suppose that the time constant for the exponential rate of increase of lens turbidity could be increased by 20%. Then, on average, the age at which the threshold for ‘cataract’ would be crossed in the normal population would be 20% greater and would exceed the average lifespan. Hence, the occurrence of cataract would become relatively rare. Indeed, this remarkable fact has already been pointed out by Kupfer in 1985. 27 Similarly, a reduction of the rates of nucleation and growth of fibers of \( \beta \) amyloid in the brain parenchyma could dramatically lower the incidence of Alzheimer’s disease.

In summary then, we envision two main lines of attack on molecular condensation diseases. In one approach, the interaction between the molecules involved are altered sufficiently so that in the physiological milieu, the thermodynamically stable state is a single soluble phase. In the second approach, the kinetic evolution of the condensed phase is slowed sufficiently so that the pathogenic condensation does not occur.

**CHEMICAL STRATEGIES FOR CATARACT INHIBITION**

**Protein Phase Separation and Aggregation**

On the basis of the discussion in the preceding section, it is clearly advantageous to discover reagents that, on reacting with the lens proteins, produce modifications that reduce the net interprotein energy of attraction. In searching for such reagents, it is important to recognize that the phase separation temperature \( T_c \) provides a measure of the noncovalent interprotein energy of attraction. Protein modifications that lower \( T_c \) will inhibit phase separation and will mitigate high molecular weight aggregate formation associated with noncovalent binding. Such modifications could also reduce the rate at which covalently linked aggregates are formed by increasing the reaction barrier height in the intermolecular potential. With these considerations in mind, we may regard protein modifiers that reduce \( T_c \) as cataract inhibiting and those that increase \( T_c \) as cataract inducing. 17

**Cataract-Inhibiting and Cataract-Inducing Protein Modifications: Role of Hydrophobic and Hydrophilic Effects**

Dr. Jayanti Pande has conducted thorough investigations of the effect of specific protein modifications on the phase separation temperature \( T_c \). She studied the lens protein \( \gamma IV \) crystallin and, in particular, made modifications of the sulfur-centered residues, cysteine and methionine, by alkylating with the highly polar reagent N-bromoacetylthanolaminephosphatase (NBAEP). 28 These studies showed a dramatic decrease of \( T_c \) amounting to \( \sim 22^\circ C \) when approximately 30% to 40% of these residues are so modified. This reagent also reduces the temperature for opacification of the intact calf lens ex vivo by 3° to 9°C. These findings are consistent with the view that the reduction in \( T_c \) is related to an increase in the hydrophilicity of the protein in the vicinity of the sulfur-containing residues. 26

In a subsequent investigation, 17 the reagent N-ethylmaleimide (NEM), a polar but uncharged modification...
that reacts only with the cysteine residues, was used. It was found that the reduction of \( T_c \) produced by this reagent was only half as large as that produced by the charged modifier NBAEP. By comparing the intrinsic hydrophilic character of these two modifiers, it was possible to conclude that the hydrophilic character of the doubly charged ionic phosphate group of NBAEP was responsible for its more potent effect on \( T_c \).

Dr. Pande has also identified specific modifications of lens proteins that are cataract inducing.\(^{19} \) By studying aqueous solutions of the lens protein \( \gamma II \) crystallin exposed to ambient oxygen in the absence of reducing agents, she discovered that protein thiol groups are oxidized and a new species is produced that appears to be intermolecular disulfide-linked dimers as well as noncovalently associated dimers. By separating these dimers and obtaining their coexistence curve, it was found that the phase separation temperature for a solution of such dimers is more than 40°C higher than that of the monomeric \( \gamma II \). These experiments\(^{18} \) show clearly the close connection between an increase in \( T_c \) and protein aggregation. Moreover, they demonstrate that whereas some sulfur-centered modifications (NBEAP and NEM) can decrease \( T_c \) and thereby are cataract inhibiting,\(^{25} \) other cysteine modifications, for example, thiol intermolecular crosslinking, dramatically increase \( T_c \) and are cataractogenic. Indeed, disulfide crosslinked \( \gamma \) crystallin aggregates are found in human nuclear cataract.

**In Vitro Assays for Putative Cataract Inhibitors**

The discussion above suggests a practical assay methodology that can be used in the evaluation of putative cataract inhibitors. Such inhibitors must decrease the net energy of attraction between protein molecules. Consequently, they must reduce the critical temperature \( T_c \) for phase separation in aqueous protein solutions. Thus, a simple in vitro assay method consists in measuring the decrease in phase separation temperature (\( T_c \)) in an aqueous protein solution as a function of the concentration \( C \) of the inhibitor molecule. The magnitude of the rate of decrease (\( dT_c/\, dC \)) can be used to measure the potential effectiveness of the proposed inhibitor. In practice, the aqueous-protein solution can be calf lens homogenate from which fiber cell membranes have been removed. Such test solutions generally have a protein homogenate concentration in the range of 50 to 100 mg/ml, and opacify at approximately 10°C in the absence of the added inhibitor. Alternatively, one may employ an aqueous solution of a single \( \gamma \) protein species such as \( \gamma II \). Or one may incubate the entire lens in a bathing medium containing the inhibitor to be assayed. In each case (\( dT_c/\, dC \)) can be measured for each specific inhibitor molecule.

This assay method has been used to measure (\( dT_c/\, dC \)) and evaluate the potential effectiveness of a wide variety of reagents. These reagents include glycols, aldehydes, and an acrylamide\(^{29} \), a variety of naturally occurring cellular constituents\(^{30} \), and several amine-modifying reagents.\(^{31} \)

**Pantethine as an Inhibitor of Protein Aggregation and Phase Separation**

A particularly interesting naturally occurring compound, pantethine, has been found to have a strong effect in reducing the phase separation temperature for calf lens homogenate. Indeed (\( dT_c/\, dC \)) for this reagent is \(-280°C/M.\) Pantethine is a disulfide form of pantethine, which is, in turn, a precursor of coenzyme A lacking the nucleotide segment.\(^{32} \) In a recently published paper\(^{28} \) J. Pande has demonstrated that pantethine also suppresses strongly the aggregation of \( \gamma II \) crystallin into the form of dimers. Pantethine inhibits dimer formation when it reacts with the protein at a pantethine to protein mole ratio of 2:1. The mechanism of action of pantethine appears to be as follows. The pantethine-protein complex takes place at two cysteine residues on each protein by a thiol-disulfide exchange mechanism, which produces a mixed disulfide structure as shown in Fig. 2.\(^{28,33} \) Each complexation reaction releases one molecule of pantetheine into the solution. The mixed disulfide complex has two important effects: it clearly decreases the net attraction between proteins, and it also inhibits the formation of intermolecular disulfide bridges between proteins. Thus, this reagent has the effect of suppressing both phase separation and protein aggregation. Further importance attaches itself to this reagent in view of the work of Clark and his coworkers,\(^{34-36} \) who have shown that this reagent is effective in suppressing protein aggregation and phase separation in animal model systems, as discussed below.

**IN VIVO TESTING OF PUTATIVE ANTICATARACT REAGENTS**

**Animal Model Systems**

The discussion of the previous sections clearly suggests that reagents that sufficiently reduce the magnitude of the attractive energy between proteins are expected...
to be inhibitors of cataractogenesis. To test the validity of this line of reasoning, Clark and coworkers\textsuperscript{34–36} have recently reported the results of systematic studies of the effect of a variety of putative anticataract reagents using different animal model systems.

In one of these studies,\textsuperscript{34} the selenite animal model was used, and the anticataract activities of 14 reagents were evaluated. Rats subjected to a single subcutaneous injection of sodium selenite at a dose of 19 μmole/kg body weight rapidly develop lens opacity, which evolves to mature cataracts by day 4 or 5. Cataract formation in the selenite model apparently involves multifactorial alterations in lenticular metabolism and in interprotein interactions.\textsuperscript{34} These authors studied the effect of the following classes of reagents: pantethine, three antioxidants, three thiophosphates, three disulfides, and two chelators. They also determined for each putative inhibitor the quantity (\(dT_c/\,dC\)) that measures the rate at which the phase separation temperature of calf homogenate decreases with the concentration (\(C\)) of the putative anticataract reagent. Their results demonstrated that the magnitude of \((dT_c/\,dC)\) may, in fact, be a good indicator of protective action on opacification in vivo. Indeed, the three most effective reagents in inhibiting the development of cataract were found to be pantethine, glutathione isopropyl ester, and glutathione for which \((dT_c/\,dC)\) was found to be \(-212, -216,\) and \(-248 °C/\,M\), respectively. Correspondingly, the reagents providing "some" protection had a moderate effect on \(T_c\), that is, \((dT_c/\,dC)\) approximately \(-50\) to \(-80°C/\,M\). The evident correlation between the degree of suppression of cataract and the magnitude of \((dT_c/\,dC)\) implies that the degree of suppression of the temperature for cytoplasmic phase separation is indeed a useful method for the identification of reagents potentially capable of inhibiting cataract.\textsuperscript{34}

In a parallel study, Clark et al.\textsuperscript{35} studied the effect of two strong phase separation inhibitors, pantethine and amino phosphorothioate (WR-77913), on increased light scattering and lens opacification for five different animal model systems of cataract: \(γ\) radiation, selenite, galactose, streptozocin, and Royal College of Surgeons. In the first two model systems, each test anticataract reagent was delivered by subcutaneous or intraperitoneal injection, 15 to 30 minutes before the cataractogenic insult. In the latter three model systems, in which the insult is continuous, repeated intraperitoneal administration of the reagents were necessary. The results of this investigation showed that systemic administration of the reagents pantethine or WR-77913 delayed or inhibited lens opacification in each of the model systems studied.\textsuperscript{35}

The authors found that these reagents were, in fact, effective only when administered before the administration of the insult or during the earliest stages of the opacification process.\textsuperscript{35,36} The precise biochemical mode of action of these two inhibitors is as yet not understood. These investigations themselves do not prove that the inhibiting effect of pantethine and WR-77913 is simply the result of reducing the net attraction between lens proteins. Nevertheless, it should be noted that these reagents have the overall effect of inhibiting or delaying the abnormal rise in phase separation temperature that is associated with opacification in each of these model systems.

**Human Age-Related Cataract Model**

In the final analysis, the efficacy of a putative cataract inhibiting reagent must be evaluated in a population of human beings. We have already discussed in the first section the fact that in the normal aging lens, the intensity \(I\) of the total backscattered light intensity increases exponentially with age according to:  

\[
I(t) = I(0)e^{t/\Delta T}
\]

Here, \(I(0)\) is the intensity of light backscattering for the newborn lens, \(t\) is the age, and \(\Delta T\) is the time constant for age-related opacification, which is \(~35\) years. The quantity \(\Delta T\) can be measured in a trial lasting \(τ\) years by determining the fractional increase in scattered light \([I(t+τ) - I(t)]/I(t) = ΔI/ΔT\). If \(τ\) is much smaller than \(ΔT\), then we see from the equation above that \(ΔI/ΔT = (τ/ΔT)\). We may envision a test of efficacy of a putative cataract inhibitor by essentially measuring the time constants \(ΔT\) and \(ΔT\) for both the treated and untreated populations respectively. For a reagent to be deemed effective, \(ΔT\) must be larger than \(ΔT\) by about 20% as mentioned in the previous section on general strategies for inhibiting molecular condensation diseases. One can estimate roughly, using this criterion, the feasibility of a 2-year human clinical trial. In the normal aging group, the fractional increase \((ΔI/ΔT)_{\text{normal}}\) in scattered light intensity will amount to \((2/35) ≈ 0.057\). In the treated group, an effective drug will reduce this fractional increase to \((ΔI/ΔT)_{\text{treated}} ≈ 0.047\). Thus, the trial methodology and population must be chosen such that this difference of 0.01 in \((ΔI/ΔT)\) will be clearly detectable. To do this \((ΔI/ΔT)\) should be measured on average with an accuracy of 0.003. Let us denote as \(±\varepsilon\) the uncertainty associated with a measurement of \((ΔI/ΔT)\) for a single person. Then, if \(n\) persons are in each trial, we may estimate that to obtain the needed accuracy, \(n\) and \(e\) must be related by \((e/\sqrt{n}) = 0.003\). If the methodology used permits a value of \(e ≈ 0.05\), the trial population is \(n ≈ 300\). Clearly this mode of estimation is primitive, and can be improved by more sophisticated statistical analyses. Nevertheless, such a trial of drug efficacy appears technically feasible.
Modern Optical Methods for the Measurement of Light Scattering From the Human Lens In Vivo

With these considerations in mind, it is useful to consider briefly the major methodologies now available for assessing the magnitude of light scattered from the living human lens. These methodologies can be divided into three groups: group 1—quasielastic light scattering spectroscopy; group 2—Scheimpflug slit lamp microscopy using photoelectric or photodensitometric detection; and group 3—comparative visual classification of photographic images of lens opacities. Of these methodologies, those in group 1 and group 2 are particularly well suited to the quantitative detection of light scattering in normal lenses or very early stages of cataract. The method of group 1 consists in detecting photoelectrically the mean intensity and the temporal fluctuations in the intensity of light scattered from the lens in vivo.2,3,37–41 From the time dependence of the fluctuations, it is possible to decompose the scattered light into contributions from rapidly diffusing, slowly diffusing, and stationary scattering elements in the lens. By calibrating such spectrometers against a standard fluid scatterer, it is possible to make a measure of the so-called Rayleigh ratio, which is an absolute measure of the light scattering power of the region of the lens under study. In this method, the region of the lens that is examined is typically a cylinder tens of micrometers in diameter and ~100 μm long. Since the light scattering from the lens is not spatially uniform, an accurate means of fixing the position of the illuminated region in the lens is necessary in this method. Well-engineered clinical instruments capable of reliable evaluation of the increase in scattered light intensity are now becoming commercially available.

The method of group 2 consists in using a camera with suitably designed optics to form an image of a cross-section of the lens in the film plane. A particular optical design for such a camera is the so-called Scheimpflug System.42 The lens image can be used to deduce the scattered light intensity either by using densitometric analysis of a photographic film or photoelectrically using a charged coupled device (CCD) in the image plane of the camera. This method has been used clinically in a number of in vivo studies.43–50

Finally, the method of group 3 represents a method of visual comparison by trained observers who compare the image of lens opacities as observed by eye in the slit lamp microscope or as recorded on photographic film with photographs of standards. This method is used primarily to study the temporal evolution of clearly evident lens opacities. These methods51–55 are most appropriately used to document the later course of development of mature cataract.

In the analyses given previously, we have made a rough theoretical estimate of the population size needed for a 2-year clinical trial of a putative cataract inhibitor. Clearly, it is very important to establish experimentally the trial conditions needed to determine ΔT – ΔT with the requisite accuracy using each of the methodologies described above. It appears that the method of quasielastic light scattering spectroscopy is well suited for such trials. The methods in group 2 should also be quite suitable. The method of group 3 would be particularly useful for studies in older populations in which frank cataractous changes are occurring.

CONCLUSIONS

We have reviewed a variety of experiments that demonstrate that the opacification associated with cataract is the result of loss of solubility of the lens proteins and their condensation into high molecular weight aggregates, or spatially separated domains containing coexisting protein-rich and protein-poor liquid phases. Moreover, we have perceived that cataract is but one entity in a broad class of diseases that may be designated as Molecular Condensation Diseases. The central element of pathogenesis in this class of diseases is an enhanced intermolecular attraction that results in a condensation of normally soluble molecules into insoluble phases. The explicit identification and designation of this class of Molecular Condensation Diseases is practically quite useful. By recognizing the attractive molecular interactions common to diseases in this class, it becomes possible to formulate a general strategy for the inhibition of each such disease. Indeed, one may envision inhibition by chemical modification of the constituent molecules so as to reduce the net intermolecular attractions. This can relocate the phase boundaries so that under physiological conditions the condensed phase will not be thermodynamically possible. On the other hand, one can alter the conditions that govern the kinetic evolution of the condensed phase so that its formation is effectively prohibited.

In the case of cataract disease, we have described a variety of reagents that inhibit protein aggregation and phase separation in vitro. Furthermore, we have described experiments on animal model systems in which these reagents also inhibit the development of cataract in vivo. We have pointed out that for a putative antcataract reagent to be deemed effective, it need only increase by 20% the time constant ΔT describing the exponential increase in the intensity of light scattered by the aging normal lens. To detect such an increase, it is necessary that quantitative optical methods be used to assess accurately age-related changes in the intensity of light scattered from the
lens. In fact, we describe well-documented technical advances well suited to this purpose. A crude statistical analysis suggests that a trial lasting 2 years involving ~300 subjects could be sufficient to determine efficacy.

Indeed, the scientific progress documented above has brought us to a threshold beyond which we can clearly see the path that leads to clinically effective pharmacologic inhibition of cataract. To move along this path, we must create a drug development effort that can address the crucial issues of effective drug delivery, including assays for drug penetration into the lens, and drug toxicity assessment. When this effort is then coupled to the modern optical methodologies for quantitative evaluation of light scattering from the lens, we can conduct significant human trials of the efficacy of cataract inhibiting drugs.

Acknowledgments

The research findings summarized here are a distillate of the work of many gifted colleagues and students. In 1969, a seminal discussion with Dr. Jiri Kinoshita resulted in my analysis of the important role of high molecular weight protein aggregates in cataracts. Early on, Dr. David Miller began to teach me the microscopic basis for many clinical problems in ophthalmology. Each of the postdoctoral research associates who worked with me have contributed vital new biochemical and biophysical knowledge and insights. These were: Drs. Judith Jedziniak (recently deceased), David Nicoli, Toyochi Tanaka, John I. Clark, Mireille Delaye (deceased), Phyllis Hammer, Roelant Siezen, John Thomson, Peter Schurtenberger, George Thurston, Victor Taret, Jayanti Pande, and Aleksey Lomakin. The many graduate and undergraduate students and technical staff who are coauthors of our research papers have been a constant source of vitality and originality. My colleagues, Drs. Peter Magante, Leo Chylack, and Teo Libondi helped demonstrate clinically that quasielastic light scattering could measure the development of protein aggregates in the living lens.

I wish also to acknowledge, with special gratitude, Dr. Carl Kupfer and the dedicated administrative staff of the National Eye Institute along with its Study Sections, whose guidance and financial support have made our work possible.

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