Biochemistry of Retinal Dystrophy

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The study of progressive hereditary degeneration of the retina in laboratory animals in conditions resembling human retinitis pigmentosa was one of Professor Arnold Sorsby's major research interests during the past three decades. It was in this field of work that I was privileged to be his collaborator for a number of years. The search for a biochemical lesion as the causative agent in such conditions was carried out in the M. R. C. Wernher Research Unit for Ophthalmological Genetics, in London, and encompassed detailed histopathological and biochemical investigations. In addition, the experimental production of similar lesions using specific retinotoxic substances was pursued with great vigour in the hope that knowledge of the pharmacological mode of action of such compounds would offer some means of counteracting their toxic effects. The ultimate aim of such an approach was the provision of therapeutic measures to combat or offset the retinal degenerative process in human retinitis pigmentosa (Sorsby, 1941; Noell, 1953; Edge et al., 1956; Sorsby and Nakajima, 1958a, b; Sorsby and Harding, 1962a, b; Sorsby and Reading, 1964; Reading and Sorsby, 1966; Reading, 1970).

It would be impracticable to review all the work done in all laboratories on this problem, so it is intended in the present paper to describe and review the biochemical and histochemical studies on retinal dystrophies in animals other than man. In addition, an attempt is made to correlate present knowledge to give a rational analysis of the sequential nature of the biochemical lesion which undoubtedly underlies the progress of these genetically determined retinal degenerations.

Histopathology

Keeler (1927, 1928a, b) was the first to record the finding of a strain of ‘rodless’ mice in which the visual cells were almost completely absent. Brückner (1951) presented the first ophthalmoscopic description of the fundus of albino and pigmented wild mice affected with retinal dystrophy. The incidence was as high as 50% in some of the strains examined.

Sorsby et al. (1954) carried out histological investigations on Brückner’s mice, and established that the degeneration process in the retina developed subsequent to cellular differentiation but before the final stages of development of the rods. Similar changes were observed in the rat by Bourne, Campbell, and Tansley (1938) and by Lucas, Attfield, and Davey (1955), in the Irish setter by Lucas (1954), and in other breeds of dog by Barnett (1966). In affected mice, retinal development is normal until around the 10th postnatal day, whereupon early degenerative changes are seen. In the rat and dog, retinal degeneration starts about 12 days after birth, though full differentiation of the rods does not occur until 21–28 days. The pattern of changes in the rat broadly resembles those occurring in human retinitis pigmentosa (Cogan, 1950).

The condition was shown to have a simple autosomal recessive inheritance in the three species mentioned. Sorsby et al. (1954) stressed the fact that affected retinæ were degenerate before the neuroepithelium had reached the stage of full differentiation. They suggested the term dystrophy rather than abiotrophy to describe such disorders.

Electron Microscopy

Lasansky and De Robertis (1960) examined the dystrophic mouse retina by electron microscopy, confirming the failure of the full development of the rod cells, and showed that the outer segment of the rod in the normal mouse proceeds from a primitive cilium to the formation of membranous material which is reoriented into sacs, finally building up into a definite regularly layered structure. In the dystrophic animals, changes occur in the reorientation stage and the regularly layered structure never appears; in its stead, degeneration sets in. Dowling and Sidman (1962), in a similar examination of the eyes of rats affected with retinal dystrophy, reported an overproduction of visual pigment associated with the appearance of swirling sheets of ‘extracellular

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lamellae' lying between the outer segments of the rods and the pigment epithelium. These authors also described the electroretinogram (ERG) pattern in the dystrophic rat and showed that it developed almost to the normal adult state but thereafter showed abnormalities.

Biochemical Investigations

Virtually all the biochemical investigations have been carried out on retinae or eyes of pink-eyed piebald agouti rats descended from the original strain of Bourne et al. (1938).

Carbohydrate metabolism. The dependence of the retina on carbohydrate as a fuel for the supply of energy prompted early investigators of retinal dystrophy to direct their attention towards studies of retinal glucose metabolism. Graymore, Tansley, and Kerly (1959) found anaerobic glycolysis in the affected retina to be virtually the same as in the normal retina until the third postnatal week. These workers were unable to establish a relation between anaerobic glycolysis and visual cell degeneration. Walters (1959) and Brotherton (1962) reported a decrease in the rate of anaerobic glycolysis in affected retinae, but this was not pronounced until 16–25 days of age.

Aerobic glucose metabolism was investigated in detail using radioisotopic methods by Reading and Sorsby (1962). The normal animals used for comparison purposes in these experiments were black-hooded (PVG) rats, a strain characterized by an absence of spontaneous retinal degeneration. The age range studied was from 10 to 60 days. No striking differences between normal and affected retinae were found in the over-all pattern of glucose metabolism from 10 to 21 days after birth. At later stages, dystrophic retinae showed obvious decreases in lactic acid, carbon dioxide, and amino acid production, together with respiratory depression and a fall in the rate of the utilization of glucose. Dystrophic retinae did not retain the amino acids formed from glucose intracellularly; in fact the amino acids 'leaked' into the fluid medium bathing the tissue. This occurred before the animals had reached the age of 21 days.

Protein and amino acid metabolism. Leakage of amino acids suggested possible changes in protein metabolism in the affected retina, so the uptake, transport, and incorporation of 14C glycine into total retinal protein as a measure of the over-all rate of protein synthesis was studied both in vitro and in vivo (Reading and Sorsby, 1964a, b). Experiments were carried out on litter mates of affected rats in comparison with normal animals at corresponding ages from 6 to 24 days after birth. In vitro, retinal tissue was incubated for 2-hour periods in a phosphate medium with added glucose and uniformly labelled 14C glycine. In vivo, each rat received a subcutaneous injection of 10 μCi of 14C glycine in saline. Retinal protein was fractionated by tricholoracetic acid precipitation followed by removal of nucleic acid and lipids. The isolated protein fractions were then assayed for radioactivity.

In vitro, glycine incorporation into retinal protein was lower in dystrophic than in normal retinae. At 6 to 8 days of age the differences were most pronounced (p = 0.04) and became less with increasing age, until at 24 days, incorporation rates were equal in both normal and dystrophic animals (Fig. 1).

![Graph](http://jmg.bmj.com/)

**Fig. 1.** Rate of retinal protein synthesis (in vitro). (From Biochemistry of the Retina, p. 78. Ed. by C. N. Graymore. Academic Press, London and New York. 1965).

No differences in amino acid uptake or in transport of amino acid into retinal tissue were found between normal and affected retinae at the early stages. Only at 24 days was the intracellular concentration of glycine less in dystrophic than in normal tissue. The in vitro results were confirmed by measuring the rate of retinal protein synthesis at various intervals after the subcutaneous injection of radioactive glycine into live 8-day-old litter mate rats. The dystrophic animals showed a conspicuous decrease in the rate of protein synthesis and a reduction in the rate of protein breakdown, indicating a slower turnover of retinal protein. The rates of protein synthesis in the livers of both normal and
affected rats were equal, so the differences observed in the retinae were unlikely to be due to differences between the animal strains. In addition, the rates of amino acid transport into the retinae were the same in normal and dystrophic animals, in vivo.

The depression in protein synthesis and turnover was apparent some 6 to 8 days before histological changes were detectable. This suggested a discrepancy in the production of an essential protein, possibly one with structural as well as functional properties. The anomaly could involve either a reduction in total protein or the production of a protein of slightly abnormal structure.

**Hexose monophosphate shunt (HMP) pathway.** These findings prompted an investigation of biochemical mechanisms associated with the visual cycle. The HMP pathway of direct glucose oxidation has been implicated in the normal functioning of the visual cycle in the mammalian retina by the elegant researches of Putterman (1963). After light falls on the retina causing bleaching and the release of the chromophore (all-trans retinal) from the visual pigment, the reduction of retinal to retinol (vitamin A alcohol), by the specific retinal alcohol dehydrogenase (RADH), is dependent on an adequate supply of the reduced pyridine nucleotide coenzyme, NADPH. This enzymatic reduction by RADH involves the oxidation of NADPH to NADP, so producing an increase in the cytoplasmic NADP/NADPH ratio. This results in a stimulation of glucose oxidation via the HMP shunt pathway which requires NADP for its two dehydrogenases. In this way, the two processes are 'metabolically coupled' by the common coenzyme. In the normal retina, the HMP shunt shows a low level of activity in dark adaptation, with short bursts of activity when light falls on the retina.

The activity of the HMP shunt pathway was investigated in developing normal and dystrophic rat retinae (Reading, 1964). This was done in vitro by measuring the incorporation of $^{14}$C into respiratory carbon dioxide produced by incubating excised retinal tissue with specifically $^{14}$carbon-1 or $^{14}$carbon-6 labelled glucose substrates. The results obtained showed an increase in HMP shunt activity in dystrophic retinae. In normal, undifferentiated retinae at 6 to 8 days of age, HMP shunt activity is relatively high, the activity of this pathway decreasing as development proceeds. In contrast, HMP shunt activity in dystrophic retinae is approximately twice that of the normal at corresponding ages, during the early stages of development of the lesion (Table I). Though the statistical significance of the results is of a low order, the trend towards an increased activity is obvious. Bonavita (1965) gave confirmatory evidence to the study of over-all HMP shunt activity described above. He determined the specific activities of the two primary enzymes of the HMP shunt pathway, viz. glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in normal and dystrophic rat retinae. Bonavita found decreases in the activities of both G6PD and 6PGD during development and differentiation in both normal and affected retinae. However, a significant difference was found between normal and affected animals in that G6PD specific activity reached a peak value in dystrophic retinae at 12 days of age (about 60% higher than normal), and this decreased to reach the values of normal retinae, both values then remaining about equal up to 120 days of age. A similar pattern was shown by 6PGD (i.e. a peak activity at about 12 days), though lower values were found in dystrophic retinae at all stages of development.

**Retinal alcohol dehydrogenase (RADH) activity.** The enzyme RADH is metabolically coupled to the HMP shunt in the retina, as previously explained. Since changes in the latter pathway had been found, it was thought that there might be a reflection of these manifested by early changes in RADH activity in dystrophic retinae. A study of RADH activity carried out by Reading and Sorsby (1966a) showed that the enzyme increased in activity in normal retinae until the animals were 1 month old, and then levelled off to reach a steady state of activity in the adult (Fig. 2). In dystrophic retinae, RADH enzyme activity developed similarly until the rats were about 2 weeks old, whereupon wide variations in activity were recorded. Subsequently an obvious decrease in activity was found from 3 weeks onwards, until retinae from 4-week-old dystrophic rats possessed only 40% RADH activity of corresponding normal retinae. Decrease in RADH activity in dystrophic retinae was therefore

<table>
<thead>
<tr>
<th>Age (dy.)</th>
<th>Normal</th>
<th>Dystrophic</th>
<th>Increase in Dystrophic</th>
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<tbody>
<tr>
<td>6-7</td>
<td>2.9 ± 0.6</td>
<td>4.1 ± 0.8</td>
<td>41.0 (p = 0.3 - 0.2)</td>
</tr>
<tr>
<td>14</td>
<td>1.7 ± 0.3</td>
<td>2.5 ± 0.5</td>
<td>49.0 (p = 0.2 - 0.1)</td>
</tr>
<tr>
<td>27-28</td>
<td>1.2 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>50.0 (p = 0.05 - 0.02)</td>
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(± SEM in parentheses.) Results expressed as ratios of specific yields of $^{14}$CO$_2$ from $^{14}$C-1 glucose, and $^{14}$C-6 glucose.

(From Reading, 1964.)
It is thought that the 'M' isoenzyme is concerned mainly with anaerobic glycolysis (lactate formation) and the 'H' isoenzyme associated with highly oxygenated tissues is inhibited by high concentrations of pyruvate. Tissues with predominantly 'H' type LDH tend to oxidize pyruvate via the Krebs cycle rather than convert it to lactate, so the retina is characterized by having a predominantly 'M' type pattern. Bonavita et al. (1963) and Graymore (1964a, b) both showed an anomalous isoenzyme pattern for LDH in dystrophic rat retinae. They found the 'M' type to be deficient from birth. Graymore suggested that the 'M' fraction played an important role in the differentiation process in the retina.

Anomalies in visual pigment formation. Dowling and Sidman (1962), in their extensive investigation of retinal dystrophy in the rat, demonstrated an overproduction of visual pigment in this condition. This was shown by electron microscopy and by rhodopsin analysis of the back portion of the eye which included retina, pigment epithelium plus choroid and sclera. As already stated, the excess rhodopsin was found in the form of swirling sheets or bundles of extracellular lamellae having a double membrane structure which, though being disorganized, resembled the general appearance of normal outer segments under the electron microscope. In fact, the authors described this as the first recognizable abnormality. They stated that by the age of 20 days, dystrophic animals possessed about twice as much rhodopsin per eye as corresponding controls. Rhodopsin content increased rapidly in both affected and normal eyes until about 30 days of age, when the dystrophic eye contained almost double the amount of rhodopsin as the control. Subsequently, rhodopsin content in the eyes of dystrophic rats fell rapidly and by 40 days the level was about half that of the normal. Evidence was also presented that decreases in rhodopsin content, the onset of histological changes, and changes in the ERG of the dystrophic eye could be delayed by keeping the animals in the dark from birth.

Vitamin A metabolism. The distribution of retinal (vitamin A aldehyde) and retinol (vitamin A alcohol) when compared in normal and dystrophic eyes under the conditions of dark and light adaptation were found to differ in the two strains (Reading, 1966). Litter mates 1 to 4 weeks old were light or dark adapted and killed by cervical fracture. After enucleation, retinae were separated from the tissues of the back of the eyes (referred to as 'pigment layers' for convenience). In the dark adaptation

 Isoenzyme changes in retinal dystrophy. Futterman and Kinoshita (1959) subjected extracts of bovine retinae to zone electrophoresis on starch paste and assayed isolated fractions for lactic dehydrogenase (LDH) activity. They found five discrete fractions, all with the capability of converting pyruvate to lactate, but having different affinities for the pyridine nucleotide coenzymes, NADH and NADPH, which depended on substrate (pyruvate) concentration.

Bonavita, Ponte, and Amore (1963) reported only four distinct fractions from the rat retina, but Graymore (1964a, b) using cellulose acetate strips as the supporting medium for electrophoresis, was able to show five fractions from rat retina. The five fractions consist of two major and discrete fractions, the 'M' (muscle) and 'H' (heart) isoenzymes, while the remaining three are hybrids of these. It

not obvious until after the third week of life. At 18 to 20 days of age, there was virtually no difference in enzymatic activity between normal and affected retinae. At this stage, the dystrophic animal shows substantial degenerative changes in the rod outer segments. It was concluded from these experiments that the decrease in RADH activity could be regarded as consequential to the primary biochemical lesion which causes cellular degeneration.
Biochemistry of Retinal Dystrophy

experiments manipulations were carried out under the illumination of a red darkroom safelight. The tissues were assayed for both retinol and retinal.

In normal animals, dark adaptation produced substantial increases in retinal content of retin a e, while light adaptation (bleaching) caused decreases in retinal content; changes that were to be expected. However, it was found that prolonged bleaching, even when the retina had reached a state of functional maturity at 4 weeks, never reduced the level of retinal to below 11-0-12 0 μg./g. (wet wt.) tissue. Conversely, in dystrophic animals, bleaching produced a conspicuous depletion of retinal in retinae. In addition, dark adaptation in dystrophic animals resulted in the regeneration of less visual pigment than normal, and this process decreased with increase in age (Fig. 3).

The most striking differences between normal and dystrophic animals were seen in the retinol content of the 'pigment layers' at 2, 3, and 4 weeks of age. In the eye of the dystrophic rat, concentrations of retinol after light adaptation were at least twice those in the normal rat at corresponding ages (Fig. 4). The figures obtained must have been subject to some dilution owing to the presence of excess tissue (choroid and sclera), so that the absolute differences could have been greater than those recorded. The wet weights of retinae and 'pigment layers' from normal and dystrophic animals showed a remarkable similarity over the age-group examined (Table II).

Changes in ATPase. The implication of ATPase in Na⁺ and K⁺ transfer in cell membranes and the changes reported by Dowling and Sidman (1962) in the ERG at 22 days, coinciding with degeneration of rod inner segments and nuclei in the dystrophic rat, prompted Bonavita, Guaneri, and Ponte (1966) to compare the development of activity of Na⁺ -K⁺ -activated ATPase in normal and dystrophic rats. They found that Mg⁺⁺ -activated and Mg⁺⁺ -Na⁺ -K⁺ -activated ATPase activities increased during postnatal development of the retina in both normal and affected rats. However, a decline in Na⁺ -K⁺ -ATPase activity could be measured much earlier than 22 days after birth. These authors expressed their results in the form of the ratio Mg⁺⁺ -Na⁺ K⁺ /Mg⁺⁺ stimulated ATPase activity. In this way they found a pronounced decrease in the ratio by 12 days of age in dystrophic retinae. In normal retinae, this ratio remained constant over the period 2 to 120 days of age.

Discussion of Present State of Knowledge

Primary biochemical lesion. The most important biochemical findings recorded up to the present are first, the apparent overproduction of visual pigment in the eyes of dystrophic rats compared with normal-sighted animals at corresponding ages (Dowling and Sidman, 1962), and secondly, the excessive local concentration of retinol (vitamin A alcohol) found in the 'pigment layers' of dystrophic rats, occurring before the onset of histological changes (Reading, 1966).
These two observations indicate that there is excessive production of visual pigment of an unusually labile nature, labile, that is, to the action of light, since affected rats reared in darkness develop visual cell degeneration much more slowly than those reared under normal lighting conditions.

Dowling and Sidman (1962) stated that the excess visual pigment was qualitatively normal, judged by the wavelengths of the peaks of its absorption spectrum and those of the products of bleaching. Caution must be attached to any interpretation based on this observation, however, since the spectral characteristics simply show that the 'dystrophic visual pigment' contains the normal chromophore (11-cis retinal) and that the protein moiety (opsin) has a structure similar in molecular size and amino acid composition to that present in the normal unaffected eye. The protein moiety of the 'dystrophic pigment' could possess a relatively minor anomaly in one of its constituent peptide chains, viz. one or more amino acids 'out of place'. Such a situation accords with the genetic nature of the condition, and suggests reasons why the visual pigment of the dystrophic animal is excessively labile to the action of light.

The most recent ideas concerning the breakdown of visual pigment (rhodopsin) and the propagation of the nerve impulse have been put forward by Bonting (1969). Bonting and his associates working at Nijmegen have obtained good experimental evidence to support their ideas which are based on the known constitution of rhodopsin, i.e. a complex of protein (opsin), phospholipids (e.g. phosphatidylethanolamine), and retinal. They regard the visual mechanism as a process in which the rhodopsin present in the outer segments of the rod cells absorbs a quantum of light energy and causes first a stereoisomeric change of the chromophore from 11-cis to all-trans retinal. The all-trans retinal then moves from its normal bonded position in the rhodopsin molecule (i.e. forming a Schiff base with the amino group of phosphatidylethanolamine) to the ε-amino group of lysine, a basic amino acid constituent of the protein opsin. This ε-amino group carries a positive charge and during its combination with retinal, this positive charge is lost. This opens up a channel for the passage of Na⁺ and K⁺ ions in the outer rod sac membrane, and so cationic fluxes occur that result in the propagation of the nerve impulse. In the regeneration process, the normal cationic gradient with high Na⁺ concentration on the outside and high K⁺ concentration on the inside of the membrane is re-established by the Na⁺ - K⁺ - ATPase present in the rod sac membrane, the substrate ATP being supplied from the inner segment of the rod cells. It is tempting to speculate that if the amino acid sequence of opsin produced under the influence of an abnormal allele is slightly altered from the protein normally produced, then the changes described above may occur with greater or less facility. Not only must the amino acid structure of the protein be important, but also the conformational nature or the tertiary structure of the protein must be maintained within very close limits, since the protonation of one particular group in one amino acid (lysine) situated in a specific position in the peptide chain is of paramount importance to the proper functioning of the over-all mechanism.

Sequential nature of lesion. It is obvious from the foregoing discussion that very little can be done to prevent the development of the primary biochemical lesion. However, the series of changes that occur and finally result in the breakdown and disappearance of the entire neuroepithelium of the retina must involve some sequential biochemical mechanisms. It appears that the excessive local concentration of retinol in the pigment epithelium (Reading, 1966) arises from the action of light on an unusually labile type of visual pigment; in fact, a 'dystrophic type rhodopsin' itself present in abnormally large amounts in the early stages of the condition. The concentration of vitamin A to maintain tissue integrity, especially that of the retina and its associated layers in the eye, is critical. Vitamin A deficiencies, produced in rats and rabbits by replacing vitamin A alcohol with vitamin A acid in the diet, show obvious types of retinal degeneration (Dowling, 1964; Sorsby, Reading, and Bunyan, 1966).

The build-up of retinol in the pigment epithelium will tend to cause breakdown of the membranes of the subcellular lysosomes in this tissue, thus releasing bound acid proteases and hydrolases. This accounts for the cellular digestion and subsequent disappearance of cellular debris. Preliminary evidence has been found for the release of acid cathepsins (proteases) into the cytoplasm of retinal cells from dystrophic rats. Such changes do not occur in corresponding normal rats (H. W. Reading and M. Burden, unpublished results). So far it has been impossible to estimate such changes in the pigment layers owing to the limits of sensitivity of the methods of estimation. Though the evidence for the appearance of the protease in the soluble cytoplasm of the retina cells is unequivocal, it is difficult to judge the stage at which such enzymatic release could have a pathological effect. Supporting evidence for the release of lysosomal enzymes has recently been provided by
Biochemistry of Retinal Dystrophy

283

elegant cytochemical studies on the eyes of dystrophic rats (Yates et al., 1970). In these studies acid phosphatase appears to be released in the pigment epithelium and probably permeates the outer segments of the rod cells. In addition, the early release of a lysosomal acid protease has also been demonstrated.

The evidence for the release of lysosomal enzymes offers some hope of alleviating or offsetting the development of retinal degeneration in animals, possibly including man. It may be possible to block some of the sequential stages in the neuro-pathological process. Pharmacologically active agents are known which stabilize lysosomal membranes, such as cortisol and cortisone (Allison, 1968), just as other substances, such as excess vitamin A, disrupt the membrane or render it permeable (Dingle and Lucy, 1965).

In this way, the future holds some hope for the direct application of the basic researches which have been carried out on inherited retinal dystrophies; researches that were instituted and pursued with great drive by Arnold Sorsby. It appears at the present moment that a cytochemical approach offers the best means of attacking the unanswered questions. This approach would eliminate the difficulties inherent in obtaining sufficient quantities of eye tissue to show significant changes, which has always been the major problem for biochemists and others working on retinal dystrophy. It need hardly be stated that the experimenter must always rely on tissues obtained from experimental animals.

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References


