GRANIT, R. (Stockholm, Sweden). **Absorption and nervous Transmission of the visible Spectrum.**

To-day retinology is a very live branch of sensory physiology and it advances rapidly along a broad front. It is impossible to do justice to the subject in any other way than by selecting a group of problems from this front line and try to give an indication of methods, results and prospects. Having recently (GRANIT, 1955a) discussed the advances in the neurology of the retina I do not now feel any inclination to present the same material again. There are, to be sure, new results on centrifugal control of the retina (GRANIT, 1955b; DODT, 1955) but in this field we will now have to wait for the histologists to do their share of the work by applying the new stains for unmyelinated or very thinly myelinated small fibres to the optic nerve and tract in order to study the course of these fibres from the brain downwards. Very convincing pictures of centrifugal fibres within the retina itself have been published by the past masters of histology, by CAJAL (1894) in dogs, by DOGIEL (1895) in birds, by POLYAK (1941) in monkeys — to mention higher species only — but the optic nerve and tract are missing links in our knowledge. Therefore the possibility of recurrent collaterals from the retinal portion of the optic nerve must be kept in mind. However, CAJAL who studied recurrent collaterals in so many places in the central nervous system could hardly have been unaware of this possibility and he, explicitly described the retinal efferent fibres as true centrifugal ones. Indeed, he went as far as to suggest a functional role for them. There is no doubt but that centrifugal effects on the retina can be demonstrated (GRANIT, DODT). Both excitatory and inhibitory effects on the retinal ganglion cells have been found. Further progress would be greatly facilitated by more precise knowledge about the histological aspects of the problem.

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Electroretinography is spreading rapidly into the ophthalmological hospitals all over the world and has also attracted several workers outside the clinic. Yet the advances made in this field, valuable though they be, tend to fall into a category of knowledge which is of greater interest to the specialist than to a physiological congress.

This discussion was announced as concerned with the primary mechanism of excitation. This, in the retina, undoubtedly is photochemical. Light quanta which have been absorbed in the receptors initiate chemical changes which in their turn set up generator potentials for excitation. The very great advantage of the retina studied as a chemical sense organ is that spectral absorption serves as a guide for identification of the photochemically active substances. Identification by spectroscopy is one of the major tools of the chemist. Retinal photochemistry begins with identification by spectroscopy as an introduction to the general problem of chemical excitation. For this reason, too, it is a theme of considerable physiological importance. An added stimulus to retinal photochemistry has been our natural interest in the beautiful world of colours.

Historically colour vision was the starting point. Deductions from psychophysical data, applied with due caution, still provide valuable hints, particularly when compared with photochemical or electrophysiological data. In this field we have been witnessing a gradual retreat from some excesses of psychophysical naivism in the past. It still lingers on in the curious notion that, unless an animal has been shown to possess colour vision, its retinal distribution of spectral sensitivity is hardly worth studying from any other point of view than that of visual purple, because the results would be wholly irrelevant to the problem of colour vision. We shall see below that the cones of cat, frog and snake dispatch a message on a spectral colour band, the so-called photopic dominator, which agrees with the human photopic distribution of spectral sensitivity and

chemically can be synthetized by coupling vitamin A aldehyde to cone protein.

The intense activity in the field of retinal photochemistry has now led to the identification of some twenty photopigments from different animals. It should certainly be of general interest to throw a glance at the methods which within the brief span of five years have carried us so far. Actually, at the moment, photochemical and chemical work stands in need of some support by electrophysiological experimentation because the existence of an absorption curve referring to retinal extracts or suspensions does not as such constitute evidence for its significance as a mediator of excitation. Absorption of light is a more common property than photic excitation.

My work at the optic nerve level (GRANIT, summaries, 1945, 1947, 1955a) had led to the identification of two types of messages concerned with the representation of the visible spectrum: the broad-band dominators and the narrow-band modulators. At the time there could be no doubt but that the broad-band rod or scotopic dominator of several mammals, frog etc. agreed reasonably well with the absorption curve of visual purple or rhodopsin and hence expressed the spectral distribution of a homogeneous rod substance. This we know is a chromoprotein. Its maximum is around 5000Å (green) : WALD's early work (see e. g. WALD, 1953) had led to the conclusion that the prosthetic group was a carotenoid that he called retinene. MORTON and GOODWIN (1944) opened the door to biochemical analysis and synthesis by proving that this retinene was the aldehyde of the alcohol vitamin A1. In the retina of certain fishes (e. g. tench) containing vitamin A2 (WALD) there appeared a scotopic dominator whose absorption was shifted to 5300 Å (GRANIT, 1941). KUEHNE had realized that this substance differed from visual purple and had called it visual violet, nowadays mostly called porphyropsin. Our measurements on the tench gave a scotopic dominator whose position

showed good agreement with the absorption in DARTNALL's (1953) extracts from the same animal. MORTON, SALAH and STUBBS (1947) showed that the chromophoric group in eyes containing vitamin A2 was the corresponding aldehyde. WALD had called it retinene 2.

In order to establish agreement between electrophysiological and photochemical data some process of averaging has so far been necessary on the electrophysiological side. If the work is done on single fibres they are often found to be connected to a slightly variable pattern of receptors. Minor differences disappear in the averages from many fibres. If electroretinography of some kind be used, this in itself is an averaging procedure because the retinogram requires a very large number of receptors. The photochemical methods also deal with averages. Before proceeding it is therefore necessary to consider the nature of the retinal extracts which the photochemists use. The retinal receptors are treated with a dispersing agent, commonly digitonin. Some photopigments may be destroyed by this procedure (DARTNALL, 1955), the ones dissolved may have undergone spectral shifts owing to destruction of the living material (ARDEN, 1954; DOBROWOLSKI, JOHNSON and TANSLEY, 1955) or to the impurities which nearly always contaminate the extracts.

What can then be done to improve this situation? The classical way of dealing with impurities is to bleach the pigment and remeasure the absorption of the solution afterwards. The difference spectrum obtained in this manner can be compared with the original distribution of absorption before bleaching but, without electrophysiological control, it may, at times, be difficult to know which curve is correct, the original absorption curve or the difference curve. HUBBARD and WALD (1952) have raised certain objections to the difference spectra obtained after "bleaching". DARTNALL (1955), on the other hand, has tried to refine this method and also introduced differential

bleaching by which is meant selective use of specific wavelengths instead of a general "white". By this "homogeneity test" he showed that the visual pigment in the bleak (*Alburnus*) with maximum in 5330 (pigment 533 in his terminology) could be split into three photopigments, 533, 510 and 550, a very revealing disclosure. It certainly demonstrates the value of homogeneity tests. What is required now is parallel work on the electrical responses in this fish.

The challenge that visual pigments *in situ* may have absorption spectra slightly differing from their spectra in digitonin solution has not been left unanswered. Methods for the study of pigments *in situ* have been developed. One method has been to study suspensions of rod outer limbs which contain the pigment (Arden, 1954a, b). Densitometric methods have been used by Denton and Wyllie (1955). They have photographed the frog retina floated in Ringer's solution before and after bleaching and applied densitometry to their photographic plates. In addition to visual purple-rods they found another type of receptor, the socalled green rods of Kühne, which were transparent in green light but showed good absorption in blue light (4400 Å) which also bleached them. Precise curves were not obtained for the blue-sensitive substance which they suggest is identical with the one described long ago by electroretinography by Granit and Munsterhjelm (1937), and since also obtained by myself with the microelectrode technique. In the course of this work they also raised the interesting question of concentration of visual in the rods. It is, of course, difficult to obtain a precise estimate of this factor from rhodopsin in solution, partly because one does not know the number of rods, partly because the rods are dichroic (Schmidt, 1938; Denton, 1954) and so, *in situ*, concentrate more light than a correct estimate of their number per unit surface would lead to. Actually they came out with a figure as high as 70% concentration or a

density of 0.50. This figure agrees well with HUBBARD's (1954) calculations. It is considerably higher than in mammals. HAGINS and RUSHTON (1953) in rabbits estimated the concentration to 35%.

DOBROWOLSKI, JOHNSON and TANSLEY (1955) applied photography followed by densitometry to single rods of the clawed toad (*Xenopus*) and obtained an absorption curve with maximum at 5300 Å which showed agreement with DARTNALL's (1954) extracts from the same eye, his pigment 519; however, it was shifted towards the long wavelengths by this difference (530 — 519). It is nevertheless reasonable to assume that the pigments are identical and that the shift towards the long wavelength, as compared with values in solution, is due to the combination into which the pigment enters with the receptor or to an unidentified substance. In favour of the former alternative is ARDEN's (1954a) work with suspensions of frog rods in sucrose. The visual purple maximum proved to be at 5110 Å instead of at 5020 Å, which is the value generally found for frog visual purple in solution (cf. also BARER and SIDMAN, 1955). We shall see below, in discussing WALD's work, that the same chromophore (or prosthetic group of the chromoprotein) will deliver different absorption maxima when combined with different receptor proteins. It is, in point of fact, one of the major riddles in retinal photochemistry how the nature of the protein alters the absorption spectrum of one and the same chromophore the way it seems to do.

In addition ARDEN (1954, b) by his method found a narrow absorption band with maximum at 5440 Å in receptor suspensions. This, indeed, showed remarkable similarity to the narrow-band green modulator obtained by the microelectrode technique from optic nerve fibres. This made him raise the question of whether the use of detergents to dissolve the photopigments might not involve selection of certain types of pigments.

Perhaps the most interesting method of approaching the

problem of spectral absorption *in situ* is the one developed over a number of years by RUSHTON and several collaborators of which the most complete account is given by CAMPBELL and RUSHTON (1955). A similar method has independently been worked out by WEALE (1954, 1955). The principle consists in focussing a patch of light onto the retina and measuring the loss of absorption of this light as it is reflected back into a measuring instrument. In RUSHTON's version this method has been carried to a high degree of sensitivity, partly because it has been developed as a O-point test and partly because of the use of sensitive photomultiplier tubes for detecting absorption losses. Like the previous methods mentioned it is based on difference spectra in that absorption is measured before and after bleaching and the difference plotted. So far RUSHTON's (1955) most interesting result seems to refer to colour blind people. Thus in protanopes, applying a homogeneity test by partial bleaching, he found a foveal photosensitive pigment which gave good agreement with WILLMER's (1949) measurements of the protanope luminosity curve, which therefore would represent a single pigment with maximum in 5400 Å. The maximum of normals is at 5500 Å. Singularly interesting is the fact that the density of this pigment was found to be 50% greater than in normals. It corresponded, in fact, to a rhodopsin density at about 15° parafoveal. Again, the deuteranope was found to possess so little pigment that it was not practicable to make any discriminating measurements. The amount was only 10% of the density found in the protanope. Since deuteranopes have reasonably good vision, this gives an indication of the limitation of RUSHTON's method of measuring pigments *in situ*. There may well be pigments, useful in the visual act, which are beyond its range of analysis.

In animal such as e. g. the frog in which rods and cones occur in about equal numbers the question of whether a

pigment found by RUSHTON's method is a rod or a cone pigment may raise difficulties. These would seem to have been overcome in DONNER and RUSHTON's (1956) recent work demonstrating the directional or STILES-CRAWFORD effect in frog cones but not in rods. This directional effect signifies that a pencil of light entering the cones along their axis causes a greater effect than it does when entering obliquely. For the human eye FLAMANT and STILES (1948) had previously shown that the directional effect similarly could be used for distinguishing rods from cones.

In WEALE's work on cats (1953) and guinea pigs (1955a) by the method of measuring absorption losses of the reflected beam a number of photopigments were obtained in addition to visual purple, and good agreement was found with my own microelectrode work (see summary, 1947, 1955) with the same animals. Of particular interest seems the narrow absorption band in the blue region of the spectrum which undoubtedly is transmitted to the cortex. LENNOX and MADSEN (1955; cf. also MADSEN and LENNOX, 1955) have found independently organized sensitivity to blue in the cortex of cats and INGVAR (1955), on a large number of cerveau isolé cats with peripheral electroretinographic control of the average spectral sensitivity by the resonance method (GRANIT and WIRTH, 1953), has shown that the striate area contains places which give narrow-bands of the modulator type in the blue region of the spectrum. The tops of these narrow blue-sensitive bands could be 1000% above the visual purple curve measured at the same wavelength (4600 Å). Here is a challenge to the photochemists to identify the corresponding blue-sensitive pigments, so easily found by electrophysiological methods in the eyes of guinea pigs and cats. In fact, DODT and ELENIUS (1956) and DODT (1956), by spike recording, demonstrated very large blue-sensitive modulator-bands also in the eye of the rabbit. Why are these so elusive when approached from the photochemical end with retinal

extracts? Is the explanation simply that the dispersing agents used (chiefly digitonin) destroy them? Or do they not go in "solution"? Such discrepancies show that the methods of extraction have serious deficiencies, less obvious in what they show than in what they leave out.

It is now nearly twenty years since we (GRANIT and MUNSTERHJELM, 1937) first found evidence of a special blue-sensitive substance in the frog's retina. If this meeting were the proper forum for it, I could mention a very large number of papers which by electrophysiological or psychophysical methods have demonstrated blue-sensitive substances in the eyes of many different animals including man. The spectral distributions obtained are mostly of the narrow modulator type. I, for one, suspect that there is something in these narrow bands that creates special difficulties. It is best to admit this to be the case rather than to insist — in the first instance — on their being products of neural interaction. Interaction is a meaningless notion unless two pigments show spectral overlap. On the view that visual purple is the only substance sensitive to short wavelengths no interaction can occur. The explanation of the narrowness of blue-sensitive modulators by interaction thus presupposes that the photo-chemical workers (i) produce a blue-sensitive substance overlapping with visual purple and (ii) explain why they have found nothing at all to correspond to the large retinal and occipital effects by electrophysiological methods.

A narrow-band pigment proved to be the only one obtainable in the cone retina of the squirrel (*Sciurus*) when ARDEN and TANSLEY (1955a) measured its spectral distribution of sensitivity by electroretinography. It corresponded fairly well to the green modulator of the cat's retina, and when WEALE (1955) applied his method to the squirrel eye, a very similar curve was obtained. The souslik (*Citellus*) is a related species with a cone retina (BORNSCHEIN, 1954; ARDEN and TANSLEY, 1955b).

Electroretinography gave a band located in the green, as in the squirrel, but broader (ARDEN and TANSLEY, 1955b). Since they had indications of a secondary hump in 4700—4900 Å, this may explain why the band was broad. The narrowest modulator bands ever obtained in any eye are the ones DONNER (1953) found by the microelectrode technique in the retina of the pigeon which also had a broad-band cone pigment, as found by DONNER (1953) in confirmation of earlier results by myself with the same technique.

KUHNE, at the end of last century, showed that visual purple (rhodopsin) could be made to regenerate in solution and that the process required some material from the pigment epithelium which he called rhodophylin. His results were confirmed by CHASE, HECHT, LYTHGOE and others. The yield, however, was small, of the order of 15% of the initial concentration. MORTON's work, mentioned above, brought into focus the need for studying the reaction : rhodopsin $\leftrightarrows$ vitamin A aldehyde $\leftrightarrows$ vitamin A. We recall that the aldehyde was the chromophore in WALD's retinene and that WALD had found vitamin A to be the final stage of bleaching of visual purple in the living eye. However, it had not been found possible to produce vitamin A by bleaching visual purple solutions (for references, see GRANIT, 1947).

The late A. F. BLISS (1948, 1949) took up this question and found that he could reduce the aldehyde to its vitamin with the aid of alcohol dehydrogenase. These problems were then studied by WALD and his collaborators Ruth HUBBARD, BROWN and SMITH who have laid down great deal of highly succesful labour on the analysis of the reaction chain, both as "bleaching" (rhodopsin to vitamin A) and as regeneration (vitamin A to photopigment). The results of their work on regeneration are of particular interest to the present question regarding the nature of the absorption spectra of photopigments. They cannot in this connexion be taken up from the chemical standpoint.

Suffice it to say that the yield is high enough to suggest that independently of whether the reaction is run down to vitamin A or up to the chromoprotein which serves as the photopigment, some of the most essential components engaged in it (alcohol dehydrogenase, DPN) have been detected. The two retinenes (vitamin A aldehydes 1 and 2) can be reduced to vitamin A and can also by an apparently simple procedure be made to synthetize both rod and cone pigments in combination with the appropriate rod and cone proteins. These proteins are, however, unknown and so there is no chemical understanding of how the chromoproteins are being formed. The synthetic chromoproteins are, as it were, empirical syntheses. The retinenes themselves absorb light in the short wavelengths outside the visible spectrum. When they combine with rod or cone protein the absorption curve shifts towards the long wavelengths and now emerges in the visible region. Indeed, such chromoproteins are responsible for making this region of the spectrum visible.

The most interesting products of synthesis are the cone pigments iodopsin (WALD, BROWN and SMITH, 1955) and cyanopsin (WALD, BROWN and SMITH, 1953). The former is the aldehyde of vitamin A1 combined with chicken cone protein, the latter the aldehyde of vitamin A2 in combination with the same cone protein. In looking for physiological comparisons the authors found that the two absorption spectra obtained agreed remarkably well with my two electrophysiologically (microelectrodes) determined photopic dominators. The one based on vitamin A1 is found in eyes of e. g. frog, snake and (psychophysically) in man, the other, based on vitamin A2, is found in the cone eye of the Greek tortoise *(Testudo)* and in the mixed retina of the tench *(Tinca)* after light adaptation of sufficient strength to remove its scotopic dominator. The two latter eyes are vitamin A2 eyes. When these two retinenes (the aldehydes 1 and 2) were combined with rod proteins, absorption

curves were obtained which corresponded to the *scotopic* dominators of the two systems (rhodopsin and porphyropsin)

From WALD's work one gains the impression that he holds the prosthetic group of the broad-band chromoproteins always to be the two aldehydes 1 and 2. Why then such an array of broad-band absorption curves in different animals, as discussed above? Apparently WALD holds that the protein is the significant factor, the reason being that the same chromophore, e. g. the aldehyde of vitamin A1, synthetizes a rod or scotopic substance (rhodopsin) with rod protein (WALD and HUBBARD, 1950), and a cone or photopic substance with cone protein (iodopsin). The decisive factor is thus the receptor protein. Its chemical structure, however, is unknown and much work remains to be done before these difficult questions are solved.

Summarizing this brief discussion of recent work on the photopigments, we must emphasize that great advances have been made by different methods of approach most of which have been of considerable value from some particular point of view, none, however, perfect and sufficient by itself. Extraction, densitometry, absorption analysis *in situ*, biochemical synthesis have added to our knowledge of the photopigments. Electrophysiological methods have raised problems, some of which have been solved; others still require a great deal of work for a final understanding. At the moment there is too little electrophysiological work done in parallel with methods such as the ones mentioned. The problem is of the kind which requires convergence of methods approaching it from different angles. Those who have been long enough interested in this field to realize what it looked like in the thirties, not to speak of the twenties, have reasons for looking forward with some optimism to its further development.

ARDEN, G. B. *J. Physiol.*, 1954a, *123*, 377.
ARDEN, G. B. *J. Physiol.*, 1954b, *123*, 396.
ARDEN, G. B. and TANSLEY, K. *J. Physiol.*, 1955a, *127*, 592.

ARDEN, G. B. and TANSLEY, K. *J. Physiol.*, 1955b, *130*, 225.
BARER, R. and SIDMAN, R. L. *J. Physiol.*, 1955, *129*, 60P.
BLISS, A. F. *J. biol. chem.*, 1948, *176*, 563.
BLISS, A. F. *Biol. Bull.*, 1949, *97*, 221.
BORNSCHEIN, H. *Naturwissenschaften*, 1954, *41*, 435.
CAMPBELL, F. W. and RUSHTON, W. A. H. *J. Physiol.*, 1955, *130*, 131.
DARTNALL, H. J. A. *Brit. med. Bull.*, 1953, *9*, 24.
DARTNALL, H. J. A. *J. Physiol.*, 1955, *128*, 131.
DENTON, E. J. *J. Physiol.*, 1954, *124*, 16P.
DENTON, E. J. & WYLLIE, J. H. *J. Physiol.*, 1955, *127*, 81.
DOBROWOLSKI, J. A., JOHNSON, B. K. and TANSLEY, K. *J. Physiol.*, 1955, *130*, 533.
DODT, E. *J. Physiol.*, 1955, *128*, 12P.
DODT, E. *Acta physiol. scand.*, 1956. In course of publication.
DODT, E. and ELENIUS, V. *Pflüg. Arch. ges. Physiol.*, 1956. In course of publication.
DOGIEL, A. S. *Arch. mikr. Anat.*, 1895, *44*, 622.
DONNER, K. O. *J. Physiol.*, 1953, *122*, 524.
DONNER, K. O. and RUSHTOH, W. A. H. *J. Physiol.*, 1956, *132*, 17P.
FLAMANT, F. and STILES, W. S. *J. Physiol.*, 1948, *107*, 187.
GRANIT, R. *Acta physiol. scand.*, 1941, *2*, 334.
GRANIT, R. *Proc. phys. Soc. Lond.*, 1945, *57*, 447.
GRANIT, R. *Sensory mechanisms of the retina.* London : Oxford Univ. Press, 1947.
GRANIT, R. *Receptors and sensory perception.* New Haven : Yale Univ. Press, 1955a.
GRANIT, R. *J. Neurophysiol.*, 1955b, *18*, 388.
GRANIT, R. and MUNSTERHJELM, A. *J. Physiol*, 1937, *88*, 436.
GRANIT, R. and WIRTH, A. *J. Physiol.*, 1953, *122*, 386.
HAGINS, W. A. and RUSHTON, W. A. H. *J. Physiol.*, 1953, *120*, 61P.
HUBBARD, R. *J. gen. Physiol.*, 1954, *37*, 381.
HUBûARD, R. and WALD, G. *J. gen. Physiol.*, 195â, *36*, 296.
INGVAR, D. H.*Proc. Nord. Fören. Neurol.*, 1955. In course of publication
LENNOX, M. A. and MADSEN, A. *J. Neurophysiol.*, 1955, *18*, 412.
MADSEN, A. and LENNOX, M. A. *J. Neurophysiol.*, 1955, *18*, 574.
MORTON, R. A. and GOODWIN, T. W. *Nature*, 1944, *153*, 405.
MORTON, R. A., SALAH, M. K. and STUBBS, A. L. *Nature*, 1947, *159*, 744.
POLYAK, S. L. *The retina.* Chicago : Chicago Univ. Press, 1941.
RAMON Y CAJAL, S. *Trav. Lab. Rech. biol. Madr.*, 1933, *28*, suppl.
RUSHTON, W. A. H. *J. Physiol.*, 1955, *129*, 41P.
SCHMIDT, W. J. *Kolloidzschr.*, 1938, *85*, 137.
WALD, G. *Ann. Rev. Biochem.*, 1953, *22*, 497.
WALD, G., BROWN, P. K. and SMITH, P. H. *Science*, 1953, *118*, 505.
WALD, G., BROWN, P. K. and SMITH, P. H. *J. gen. Physiol.*, 1955, *38*, 623.
WALD, G. and HUBBARD, R. *Proc. nat. Acad. Sci., Wash.*, 1950, *36*, 92.
WEALE, R. A. *J. Physiol.*, 1953, *122*, 322.
WEALE, R. A. *J. Physiol.*, 1955a, *127*, 572.
WEALE, R. A. *J. Physiol.*, 1955b, *127*, 587.
WILLMER, E. N. *J. Physiol.*, 1949, *110*, 422.

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