106. THE ROLE OF THE PHOSPHOLIPIN IN VISUAL PURPLE SOLUTIONS

By E. E. BRODA

*From the Sir W. Ramsay and R. Forster Laboratories of
Chemistry, University College, London*

(Received 21 August 1941)

FOR sixty years since its discovery by Boll [1876] and Kühne [1877-8] visual purple has been identified exclusively through the absorption spectrum and the photosensitivity of its solutions. Recently more properties of this chromoprotein have been studied, namely its diffusion coefficient [Hecht *et al.* 1937], molecular weight [Hecht & Pickels, 1938], chromophore carrier weight [Broda *et al.* 1940], cataphoretic mobility [Broda *et al.* 1939; Broda & Victor, 1940], and some properties of its chromophore [Wald, 1938]. All these determinations, except that of the cataphoretic mobility, depend, however, ultimately on a knowledge of light absorption. Up to the present the spectrum and the photosensitivity have been used as the only purity criteria of visual purple.

The determination of either the spectrum or the photosensitivity, however, even in the purest solutions is complicated by the presence of coloured (yellow) 'impurities' [Dartnall *et al.* 1936]. Obviously the term 'impurity' must not imply more than that the substance concerned is different from the protein carrier and from the chromophore of visual purple; its interaction with the chromoprotein may well be of physiological significance.

Lythgoe [1937] suggested that the impurities consist of lipins, while Krause [1937] proved the presence in visual purple solutions of phosphorus and choline, both constituents of lecithin. A phospholipin content of the solutions was indeed to be expected, since these lipins are contained in the retina and in other parts of the eye [cf. Krause, 1934, 1]. The outer limbs of the rods contain a phospholipin which is blackened by osmic acid; it has been called 'myeloid' by Kühne and is considered by Krause [1934, 2] to be lecithin. Schmidt [1938] assumes that there are alternating layers of protein and phospholipin in the outer limbs. Part of the phospholipins, although lipophil, will dissolve along with the chromoprotein during the preparation of the visual purple solution, their solubility in water being due to the formation of hybrid ions [Kuhn *et al.* 1935].

The object of the present work was to measure the concentration of the phospholipins, and to investigate whether they can account for the light absorption attributed to the 'yellow impurities'. This information should not only help to define visual purple more accurately, but also to interpret the sensitivity curve of the human eye.

The concentration of the phospholipin

When fresh rod tissue from dark-adapted frogs' eyes is treated with light petroleum before extraction of the visual purple with digitonin solution as described by Lythgoe [1937], a deep yellow extract is obtained. The dissolved substance contains only about 1% P showing that in addition to phospholipins, sterols or fats are present. Neither sterols nor fat, however, can be taken up by aqueous digitonin, and therefore they cannot be present in the visual purple solution.

To determine the phospholipin concentration in a visual purple solution, a quantity of a pure solution (optical density at 500 $m\mu$ 1.20, at 400 $m\mu$ 0.37 cm^{-1}) was bleached and evaporated to dryness in contact with $CaCO_3$ at room temperature. The residue was extracted with chloroform until practically free from P. The yellow extract was evaporated, the residue after incineration dissolved in HNO_3 , and the P determined with molybdate. In this way the original solution was found to contain 0.0232 mg. P/ml. The fact that the whole of the phosphorus compound is soluble in chloroform as well as in water, justifies the calculation of the amount of phospholipin from the total P. If a P content of the lipin of 4% is assumed, the phospholipin concentration becomes 0.58 mg./ml. This corresponds to 0.48 mg./ml. for a solution diluted to standard optical density (1 cm^{-1} at 500 $m\mu$).

The protein concentration in the standard solution is 1.2 mg./ml. [Broda *et al.* 1940]; hence the weight of the lipin present is comparable with that of the protein. It greatly exceeds that of the prosthetic group, as at least 17,000 g. of protein are required [Broda *et al.* 1940] to carry 1 mol. of the prosthetic group with a probable weight of the order of 500 g.

The spectrum of the retina phospholipin

The spectrum of the clear lipin solution obtained from rod tissue as described above was determined with a Hilger Spekker photometer (Table 1). For comparison, the spectrum of egg lecithin ('purissimum', Merck) was also taken. No

Table 1. *The light absorption of phospholipins*

Extinction coefficients, calc. on a g./litre basis; solutions in chloroform

	Wave-length in $m\mu$					
	440	420	400	380	360	340
Retina phospholipin	0.035	0.045	0.06	0.10	0.18	0.51
Egg lecithin	0.035	0.04	0.045	0.055	0.07	0.15
β -Carotene [Zechmeister, 1934]	250	160	80	40	16	13

importance can be attached to numerical agreement or disagreement owing to the lack of purity of the retina phospholipin and the poor stability of phospholipins exposed to air and light. It is, however, found that the two substances have in common both the shape of the absorption curve and the order of magnitude of the extinction coefficient. This at 400 $m\mu$ is about 10^3 times smaller than that of the yellow pigments of the carotene or flavin classes.

In a qualitative sense the phospholipin accounts for the steady increase with decreasing wave-length of the absorption due to the impurities in the visible [Lythgoe, 1937] and ultraviolet [Goodeve, Lythgoe & Wood, unpublished]. Its presence explains why the ratio of the densities at 400 and at 500 $m\mu$ can be used as a purity criterion of visual purple [Lythgoe, 1937; Wald, 1938]. Finally, the oxidation of the phospholipins is probably the reason why the absorption of visual purple solutions over the whole range increases with time unless air is excluded.

To determine quantitatively the influence of the phospholipins on the absorption of the visual purple solutions, the absorption of an aqueous phospholipin solution with a concentration similar to that deduced from the P content of the visual purple solution would have to be measured. Unfortunately, however, aqueous phospholipin solutions free from protein are opalescent, although the solutions of visual purple like certain other protein solutions con-

taining phospholipin (e.g. solutions of euglobulin) are perfectly clear. Scattering must have greatly falsified the measurements of lecithin and cephalin from beef brain in water by Wadsworth & Crowe [1936], which are apparently the only determinations of a phospholipin spectrum so far carried out.

The optical density of the phospholipin in chloroform may, however, give an idea of the order of magnitude of its contribution to the total absorption of visual purple solutions. This way of approach appears to be valid, since the colour of phospholipin solutions is not greatly influenced by the solvent. A 1% solution of egg lecithin was tested and showed practically identical colours in amyl acetate, in carbon tetrachloride and in chloroform. The colours in hexane and in ethyl alcohol were slightly weaker, in benzene slightly stronger. The yellow colour of a solution in acetic acid had a faintly reddish tinge, while that of a solution in amyl alcohol had a faint greenish tinge. In no case, however, was the deviation of the colour from that in chloroform substantial. Above all, addition of water to the solutions did not produce any colour change as long as its amount was too small to make the solution opalescent.

On this basis, the contribution of the phospholipin to the density of a standard solution of visual purple would be about 0.02 at 440 m μ , 0.03 at 400 m μ , and 0.09 at 360 m μ . Clearly these values must not be taken to be more than rough approximations.

The influence of the phospholipin on the scotopic sensitivity curve of the eye

Schneider *et al.* [1939; 1941] found that the apparent quantum efficiency of the decomposition of visual purple in solution is nearly constant for wave-lengths greater than 440 m μ , but drops in the blue, violet and particularly in the ultraviolet. Its value at 400 m μ is only about 80%, and at 365 m μ only about 70% of that at 500 m μ . A genuine decrease of the quantum yield with decreasing wave-length would be unlikely. The apparent decrease was therefore attributed to the presence of an internal yellow filter in the solutions. This would absorb light without participating in the photoreaction, thus reducing the number of visual purple molecules decomposed per quantum absorbed by the solution as a whole. That is precisely how the phospholipins must act, since they absorb short wave light without contributing to the photoreaction. From the rough data for the extinction of the phospholipin, given in the preceding section, and taking into account that the density of the standard visual purple solution at 400 m μ is 0.3 and at 365 m μ 0.55 [Goodeve, Lythgoe & Wood, unpublished], it would be predicted that at 400 m μ about 10%, and at 360 m μ about 17% of the absorbed light would go to the phospholipin.

In the same spectral range there is a still greater discrepancy between the absorption curve of visual purple *in vitro* and the scotopic sensitivity curve of the human eye. As before, the two curves fit well above 500 m μ [Dartnall & Goodeve, 1937], but the ratio sensitivity: absorption at 365 m μ is only 1/4000 of its value at 500 m μ [Schneider *et al.* 1941]. This can hardly be due to any difference between the spectra of frog and human visual purples, which indeed has never been suggested. Schneider *et al.* [1941] pointed out that a yellow filter in the eye may be the reason of the disagreement. This filter may well consist of phospholipins. Much more lipin would be required to explain the discrepancy between the scotopic sensitivity curve and the absorption curve than to explain the drop in the apparent quantum efficiency in solution. This is not surprising, since only a small part of the retina lipin escapes the pre-extraction with light petroleum, and thus dissolves along with the chromoprotein.

Extrapolation from the extinction of the lipin in chloroform solution to 100% concentration shows that a lipin layer of a thickness of a fraction of a millimetre would impart to the filter in the eye the density 3.6 at 365 m μ , which is required by the results of Schneider *et al.* The lens and the vitreous humour contain some lipins, but their absorption is not very great [Graham, 1922]. It is concluded therefore that the bulk of the filter effect occurs in the retina. Although the light must travel over a greater distance in the lens and in the vitreous humour, the lipin concentration is much greater in the retina [Krause, 1934, 1]. It would be premature, however, to calculate the absorption in the retina as long as the extinction coefficient of the lipin *in situ* and the path of the light in the rods are not known.

The decrease with age of the sensitivity of the human eye to short-wave light will have to be correlated with the parallel increase of the lipin content of the eye tissues [cf. Krause, 1934, 1].

The bond between the visual purple and the phospholipin

The visual purple protein is possibly a globulin, since it is precipitated by removal of salts (dialysis) or by saturation of the neutral solution with sodium or magnesium sulphate [Kühne, 1877-8; Lythgoe, 1937]. Globulins interact specifically with phospholipins [Frazer, 1941]. Experiments with visual purple showed that the phospholipin cannot be removed from (bleached or unbleached) solutions by shaking with light petroleum; this points to chemical linkage of some kind as assumed for other lipoproteins on similar evidence by Mâchebeuf [1927]. The idea that the whole of the phospholipin is linked with the visual purple protein is supported by the clarity of the solutions; Sørensen [1930] has emphasized that the clarity of euglobulin solutions, which contain phospholipin, is due to a secondary bond. The fact that the d_{400}/d_{500} ratio of the purest solutions, as found in different laboratories, is nearly constant at about 0.3, indicates a roughly stoichiometric relation between the chromoprotein and the lipin, as far as the light-absorbing part of the lipin is concerned.

Further experiments showed that precipitated visual purple yields its phospholipin on treatment with solvents such as chloroform; electrodialysis also seems very effective in breaking the bond and removing the phosphorus. Only 2.5 mg. lipin were extractable from 18.3 mg. protein precipitated (and bleached) by 12 hr. electrodialysis through a collodion membrane at 220 v. Most of the phosphorus had been carried away through the membrane. The spectrum of the chloroform extract showed a maximum at 370 m μ indicating that it was due to indicator yellow (or 'retinene' in the terms of G. Wald) rather than to lipins. These facts suggest that the bond between the chromoprotein and the phospholipin is not strong.

Possibly visual purple solutions could be freed from lipin without bleaching or precipitating by electrodialysis in a flow of buffer.

SUMMARY

Solutions of visual purple contain phospholipin from the retina. Even in the purest solutions so far obtained the quantity of the lipin is comparable with that of the protein, and is much greater than that of the prosthetic group.

The light filter effect due to the phospholipin accounts, at least to a large extent, for the apparent decrease of the quantum efficiency of the decomposition of visual purple in solution in the blue, violet and particularly ultra-violet. The phospholipin in the retina serves to explain the discrepancy in the

same range between the photosensitivity curve of visual purple solutions and the human scotopic sensitivity curve.

The phospholipin is connected with the visual purple protein by a secondary bond which may be similar to that between euglobulin and lecithin.

Phospholipin is effectively removed from visual purple solutions by electro-dialysis.

I am indebted to Dr C. F. Goodeve and the late Dr R. J. Lythgoe for enabling me to carry out this work and for their constant help, Miss S. J. Hooper for the preparation of the visual purple, and the Rockefeller Foundation for a financial grant.

REFERENCES

- Boll (1876). *Ber. Akad. Wiss. Berlin*, p. 783.
 Broda & Victor (1940). *Biochem. J.* **34**, 1501.
 — Goodeve, Lythgoe & Victor (1939). *Nature, Lond.*, **144**, 709.
 — — — (1940). *J. Physiol.* **98**, 397.
 Dartnall & Goodeve (1937). *Nature, Lond.*, **139**, 409.
 — — — & Lythgoe (1936). *Proc. roy. Soc. A*, **156**, 158.
 Frazer (1941). *Trans. Faraday Soc.* **37**, 125, 186.
 Graham (1922). *J. Opt. Soc. Amer.* **6**, 605.
 Hecht, Chase & Shlaer (1937). *Science*, **85**, 2215.
 — — & Pickels (1938). *Proc. nat. Acad. Sci., Wash.*, **24**, 172.
 Krause (1934, 1). *The Biochemistry of the Eye*. Baltimore.
 — — (1934, 2). *Acta Ophthalm.* **12**, 372.
 — — (1937). *Arch. Ophthalm.* **18**, 807.
 Kühne (1877–8). *Heidelberg Physiolog. Untersuch.* **1**, 1.
 Kuhn, Hausser & Brydówna (1935). *Ber. dtsh. chem. Ges.* **68**, 2386.
 Lythgoe (1937). *J. Physiol.* **89**, 331.
 Mâchebœuf (1927). Thesis, Paris.
 Schmidt (1938). *Kolloidzshr.* **85**, 137; *Naturwissenschaften*, **26**, 491, 509.
 Schneider, Goodeve & Lythgoe (1939). *Proc. roy. Soc. A*, **170**, 102.
 — — — (1941). *Proc. roy. Soc. A*, (in the Press).
 Sørensen (1930). *Kolloidzshr.* **53**, 307.
 Wadsworth & Crowe (1936). *J. phys. Chem.* **40**, 739.
 Wald (1938). *J. gen. Physiol.* **21**, 795.
 Zechmeister (1934). *Carotinoide*. Berlin.