Schiff Bases Formed from Retinal and Phosphatidylethanolamine, Phosphatidylserine, Ethanolamine or Serine

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1. Conditions were established for the reaction of retinal with phosphatidylethanolamine, phosphatidylserine, ethanolamine and serine in chloroform, ethanol or ethanol-water solutions to form retinylidene compounds, or Schiff bases. 2. The Schiff bases were reduced to retinyl compounds with sodium borohydride. 3. Absorption maxima and molar extinction coefficients were determined for the various retinylidene and retinyl compounds and for the corresponding coloured products formed by their reaction with antimony trichloride.

In an earlier study of vitamin A in the eggs of the herring and other marine teleosts, retinal (vitamin A₁ aldehyde) was found to be the predominant form of the vitamin (Plack, Kon & Thompson, 1959). Although the lipid extracted from herring eggs did not show the characteristic properties of retinal, free retinal could be isolated by chromatography and we suggested that the retinal might be loosely combined with another lipid. The retinal complex represents only about 0.02% of the total lipid extracted from herring eggs, and is unstable. For this reason we have investigated this problem by making model compounds of retinal with phosphatidylethanolamine and phosphatidylserine and comparing their properties with those of the lipid extracts. In this paper we give the conditions for the reaction of retinal with the two phospholipids, with ethanolamine and with serine, and the spectrophotometric properties of the compounds formed.

Ball, Collins, Dalvi & Morton (1949), Pitt, Collins, Morton & Stok (1955) and Morton & Pitt (1955) described Schiff bases formed from retinal and various amino compounds and their relationship to derivatives of the visual pigment rhodopsin. An earlier suggestion by Krinsky (1958) that in rhodopsin the retinal might be present as a Schiff base combined with phosphatidylethanolamine or phosphatidylserine has been confirmed by Poincelot, Millar, Kimbel & Abrahamson (1969), who suggested that during the bleaching process the retinylidene group is transferred to the ϵ -amino group of a lysine residue in the protein, as found by Bownds (1967) and Akhtar, Blosse & Dewhurst (1968). Bonting & Bangham (1967), de Pont, Daemen & Bonting (1968), Daemen & Bonting (1969) and R. G. Adams, quoted by Bonting & Daemen (1969), considered *N*-

retinylidenephosphatidylethanolamine in relation to the visual process.

The Schiff bases considered here are retinylidene compounds of the general formula $C_{19}H_{27}\cdot CH:N\cdot R$ in the basic form. In acid solution the nitrogen becomes protonated, $C_{19}H_{27}\cdot CH:\overset{+}{N}H\cdot R$, and results in the characteristic increase in wavelength of maximum absorption. Reduction of the Schiff bases with sodium borohydride at an alkaline pH gives the corresponding retinyl compounds, $C_{19}H_{27}\cdot CH_2\cdot NH\cdot R$.

MATERIALS AND METHODS

Phospholipids. Synthetic $\beta\gamma$ -dipalmitoyl-L- α -phosphatidylethanolamine, grade II, was obtained from Sigma Chemical Co. (London) Ltd., London S.W.6. Determinations of phosphorus (Allen, 1940) and of ethanolamine (Axelrod, Reichenthal & Brodie, 1953) gave an ethanolamine/P molar ratio 0.90. Phosphatidylserine and phosphatidylethanolamine were prepared from ox brain by the method of Folch (1942); the crude extracts were dissolved in $CHCl_3$ -methanol (2:1, v/v), washed with 0.1 M-NaCl (Folch, Lees & Sloane-Stanley, 1957) and purified by chromatography (Rathbone & Maroney, 1963), phosphatidylethanolamine being eluted with $CHCl_3$ -methanol (4:1, v/v) and phosphatidylserine with $CHCl_3$ -methanol (3:2, v/v). A small portion of each fraction was used to identify the components present with the t.l.c. system of Skipski, Peterson & Barclay (1964). Fractions giving single ninhydrin-positive spots of the appropriate phospholipid were combined. Determinations of phosphorus, ethanolamine and serine (Axelrod *et al.* 1953) on the phosphatidylserine preparation gave serine/P molar ratio 0.82 and ethanolamine/serine molar ratio 0.005. Two phosphatidylethanolamine preparations gave ethanolamine/P molar ratio 0.71 and serine/ethanolamine molar ratio 0.01, and ethanolamine/P molar ratio 0.97 and serine/ethanolamine molar ratio 0.03.

Concentrations quoted of phosphatidylserine and phosphatidylethanolamine are based on the contents of serine and ethanolamine respectively.

Other chemicals. Crystalline all-*trans*-retinal (Eastman) was obtained from Kodak Ltd., Kirkby, Liverpool, and crystalline all-*trans*-retinol from Roche Products Ltd., Welwyn Garden City, Herts., and L-serine, ethanolamine, triethylamine, SbCl_3 and CHCl_3 were from British Drug Houses Ltd., Poole, Dorset. The ethanolamine and triethylamine were redistilled before use, and the SbCl_3 was purified and a 14% (w/w) solution in CHCl_3 made as described by Plack (1961). Absolute ethanol, doubly rectified, was from B.P. Chemicals (U.K.) Ltd., London W.1.

Absorption spectra. An Optica CF4 or Unicam SP.800 recording spectrophotometer was used to obtain absorption spectra in the u.v. and visible regions. Measurements of extinction at the absorption maxima were made with a Unicam SP.500 spectrophotometer.

Carr-Price test. To 0.5 ml. of a CHCl_3 solution of the sample in a 1 cm. spectrophotometer cell 2 ml. of the SbCl_3 solution was added, and the absorption curve of the colour product was recorded. A second 0.5 ml. sample was used to measure E_{max} with the Unicam SP.500 spectrophotometer.

Determination of retinal in the reaction mixtures. Concentrations of retinal in CHCl_3 or CHCl_3 -ethanol solution were determined immediately after the addition of the concentrated retinal solution, by dilution with CHCl_3 and measurement of the extinction at 390 nm. In ethanol or ethanol-water the reaction between retinal and ethanolamine or serine proceeded rapidly and the concentration of retinal was measured before its addition, by dilution with ethanol and measurement of the extinction at 380 nm. The values ϵ 43 200 in CHCl_3 (Hubbard, Gregerman & Wald, 1952-53) and ϵ 43 400 in ethanol (Robeson, Blum, Dieterle, Cawley & Baxter, 1955) were confirmed and were used to calculate the concentration of the retinal present.

Preparation of Schiff bases. All reaction mixtures were maintained in the dark at 25°. With synthetic phosphatidylethanolamine, the reaction mixture contained 40 mm-phosphatidylethanolamine, 1 mm-retinal and 0.4 M-triethylamine in CHCl_3 -ethanol (2:1, v/v), the ethanol being necessary to keep the phospholipid in solution. After 5 hr. essentially all the retinal had reacted, and samples were taken and diluted 45-fold with CHCl_3 or with ethanol for the determination of the absorption curves of the basic form in these solvents. The basic form of the Schiff base was converted into the acidic form by passing dry HCl through the solution in the spectrophotometer cell. The basic form of the Schiff base was reduced to the secondary amine, *N*-retinylphosphatidylethanolamine, by shaking the CHCl_3 or ethanol solution in the spectrophotometer cell with a few milligrams of NaBH_4 . For the Carr-Price reaction of the Schiff base, a sample of the reaction mixture was diluted tenfold with CHCl_3 , a further fivefold dilution being produced on addition of the SbCl_3 reagent (see above). For the Carr-Price reaction of the secondary amine, 0.4 ml. of the reaction mixture, 5 ml. of methanol, 4 drops of water and a few milligrams of NaBH_4 were mixed and left for 10 min. in the dark. To the mixture, 10 ml. of CHCl_3 and 10 ml. of water were added, and the whole was shaken and then centrifuged at 1000 g to separate the two phases. The aqueous layer was extracted in the same way with another 10 ml. of CHCl_3 , the two CHCl_3 extracts were combined, the solvent was removed under reduced pressure, and any water

remaining was removed by the addition, and removal under reduced pressure, of ethanol. The residue was dissolved in 4 ml. of CHCl_3 and the concentration of *N*-retinylphosphatidylethanolamine determined from the absorption curve of the solution in the u.v. and ϵ previously obtained by direct reduction. A 0.5 ml. sample of the CHCl_3 solution was used for the Carr-Price test.

For the other compounds, the reaction mixtures contained: 20 mm-phosphatidylethanolamine (from ox brain), 1 mm-retinal in CHCl_3 , sampled after 5 hr.; 40 mm-phosphatidylserine (from ox brain), 1 mm-retinal in CHCl_3 , sampled after 7 hr.; 40 mm-ethanolamine, 1 mm-retinal in CHCl_3 , sampled after 1 hr.; subsequent procedures for all three were as described for synthetic phosphatidylethanolamine. The formation of *N*-retinylideneserine required a large excess of serine, and the reaction mixture contained 0.2 M-serine, 0.02 mm-retinal, and 0.2 M- NaOH in ethanol-water (1:1, v/v). Combination of retinal was maximal in 30 min. and the absorption curve of the basic form of the Schiff base was determined without dilution. Other samples of the mixture were acidified with two drops of conc. HCl or reduced with a few milligrams of NaBH_4 to give the acidic and reduced forms respectively. The Schiff base was extracted from 10 ml. of the reaction mixture with 10 ml. of CHCl_3 , the mixture was centrifuged to separate the phases, and the CHCl_3 was removed from the extract under reduced pressure, ethanol being used to remove the residual water as before. The residue was dissolved in 1.5 ml. of CHCl_3 and 0.5 ml. samples were taken for the Carr-Price test. In the absence of excess of serine, the Schiff base broke down very rapidly, and the above extraction and Carr-Price test had to be done in 10 min. or less. A solution of the reduced Schiff base, *N*-retinylserine, in CHCl_3 was produced by reducing 10 ml. of the reaction mixture with a few milligrams of NaBH_4 until the solution was colourless, and adding 10 ml. of CHCl_3 to extract the compound. The CHCl_3 extract was washed with 2×5 ml. of water, and the solvent was removed under reduced pressure, ethanol being used to remove residual water. The residue was dissolved in 3 ml. of CHCl_3 , and absorption curves in the u.v. and in the Carr-Price test were determined as for synthetic phosphatidylethanolamine. With ethanol as solvent, the ethanolamine reaction mixture contained 40 mm-ethanolamine and 1 mm-retinal. Samples were taken after 30 min. and diluted with ethanol, with ethanol acidified with dry HCl or with ethanol followed by reduction with NaBH_4 to obtain the absorption characteristics of the basic and acidic Schiff bases and of *N*-retinylethanolamine. In ethanol-water (1:1, v/v), the reaction mixture contained 0.5 M-ethanolamine and 0.02 mm-retinal and was left for 30 min. The absorption characteristics of the basic, acidic and reduced Schiff bases were then determined without dilution, by following the procedures described for serine.

Determination of molar extinction coefficients and equilibrium constants. The molar extinction coefficients (ϵ) were obtained under the conditions described above for preparation of Schiff bases; ϵ = extinction at λ_{max} . due to Schiff base $\times$ dilution/molar concn. of Schiff base in reaction mixture. If essentially all the retinal was combined as Schiff base at equilibrium and a bimolecular reaction is assumed, then ϵ (provisional value) = $E_{\text{max}} \times$ dilution/initial molar concn. of retinal in reaction mixture.

Equilibrium constants were determined at 25° with a number of reaction mixtures containing different concentra-

tions of each amino compound, or of retinal, chosen to give between 20 and 80% of the retinal combined as Schiff base at equilibrium. With synthetic phosphatidylethanolamine, triethylamine was added to the reaction mixture at a concentration 10 times that of the phospholipid. Samples from the mixtures were taken at appropriate times and diluted with acidified solvent, and the extinctions at the $\lambda_{\max}$ of the acid Schiff bases were measured. Sampling was continued until the extinctions reached a maximum, the total time required varying from 30 min. to 48 hr., depending on the amino compound used and the concentrations of the reactants.

The maximum extinction was corrected for the absorption of unchanged retinal at this wavelength, the correction being obtained from a curve of correction against measured extinction. Two points only are required to draw this curve, the extinction at the wavelength used of a retinal solution of the same initial concentration as that in the reaction mixture and diluted the same way, which gives a value for both the measured extinction and the correction when no Schiff base is present, and the extinction when all the retinal is present as Schiff base (so that the correction is zero), derived from the initial concentration of retinal, the dilution and the provisional value of ϵ for the acid Schiff base, determined previously. The concentration of Schiff base at equilibrium was then calculated from the corrected maximum extinction, the dilution and the provisional value for ϵ , and a mean provisional value for K' obtained from:

$$K' = \frac{\text{Equilibrium concn. of Schiff base}}{\text{Equilibrium concn. of amino compound} \times \text{equilibrium concn. of retinal}}$$

$$= \frac{\text{Equilibrium concn. of Schiff base}}{(\text{Initial concn. of amino compound} - \text{equilibrium concn. of Schiff base}) (\text{initial concn. of retinal} - \text{equilibrium concn. of Schiff base})}$$

This calculation assumes a bimolecular reaction and no significant change in the concentration of water present. With the approximate value for K' , the composition of the equilibrium mixture for the concentrations of reactants used to determine ϵ was calculated. If x is the proportion of retinal combined as Schiff base, then:

$$K' = \frac{x \times \text{initial concn. of retinal}}{(\text{Initial concn. of amino compound} - x \times \text{initial concn. of retinal}) (\text{initial concn. of retinal} - x \times \text{initial concn. of retinal})}$$

from which x may be calculated.

K' and ϵ are interdependent, and the value of x above, when significantly different from 1, was used to give a more accurate value for ϵ . New values for K' were then calculated and the process repeated until the changes in K' , x and ϵ were insignificant. At this stage the individual values of K' were close. Changes in the value of ϵ affect the corrections for free retinal of the measured extinctions, since the second point on the correction curve is dependent on ϵ .

Values of ϵ for the basic and reduced Schiff bases were obtained by correcting the extinction measured for the contribution of free retinal or retinol at this wavelength and dividing by the concentration of Schiff base derived from the factor x above and the initial concentration of retinal. In the Carr-Price test, similar corrections were applied in calculating ϵ , the relevant data being obtained from

standard curves of the Carr-Price colour product of retinal and retinol.

Determination of velocity constants. Initial rates of formation of the Schiff bases were measured at 25° with a fixed concentration of retinal, 1 mM, and concentrations of the amino compound between 1 and 40 mM. With synthetic phosphatidylethanolamine, triethylamine was present at 10 times the concentration of the phospholipid, and with this compound the initial rates were also determined with a fixed concentration of the phospholipid, 1 mM, and concentrations of retinal between 1 and 10 mM. Samples of the reaction mixture were taken over a period of 30 min., diluted with solvent containing sufficient dry HCl (or conc. HCl with ethanol-water mixtures) to give a final acid reaction, and the extinctions read at $\lambda_{\max}$ for the acid Schiff base. These values were corrected for the contribution of unchanged retinal with the aid of the curve of correction against measured extinction at $\lambda_{\max}$, described above. The corrected values of extinction were converted into concentrations of Schiff base, plotted against time of sampling and the initial rates of formation obtained from the curves. With high initial concentrations of amino compounds, the initial rates cannot be obtained accurately from such progress curves and, since the concentration of Schiff base was small compared with the concentration of amino compound, a pseudo-unimolecular plot was used.

The initial rates of formation were used with the initial concentrations of amino compound and of retinal to give

values of k_t . The velocity constant of the back reaction was derived from $k_b = k_t/K'$.

RESULTS

Conditions for formation. With retinal and synthetic phosphatidylethanolamine, triethylamine

was used to give the basic conditions necessary for the formation of a Schiff base. A triethylamine concentration 10 times that of the phospholipid was found to give the maximum rate of formation, higher concentrations being inhibitory, and in the absence of triethylamine the rate was about one-tenth of the maximal rate. Ox brain phosphatidylethanolamine reacted with retinal at the maximal rate in the absence of triethylamine, and addition of the base decreased the rate of formation of *N*-retinylidene-phosphatidylethanolamine. Addition of triethylamine to mixtures of ox brain phosphatidylserine and retinal had no effect on the rate of reaction. Ethanolamine is itself a base, and triethylamine was

Table 1. Mean values ($\pm$ s.e.m.) of the equilibrium and velocity constants at 25° in the reactions between phospholipids, ethanolamine or serine and retinal to give Schiff bases

Water produced in the reactions (at most 1 mM) is assumed to be negligible in comparison with the concentrations present in the solvents. Numbers of determinations are given in parentheses.

$$K' = \frac{[\text{Schiff base}]}{[\text{amino compound}][\text{retinal}]} = \frac{k_f}{k_b}$$

Rate of formation of Schiff base = k_f [amino compound] [retinal]

Amino compound	Solvent	K' (mM ⁻¹)	k_f (mM ⁻¹ hr. ⁻¹)	k_b (hr. ⁻¹)
Phosphatidylethanolamine (synthetic)	CHCl ₃	2.99 $\pm$ 0.076 (5)	0.107 $\pm$ 0.0028 (5)	0.0358
	CHCl ₃ -ethanol (2:1, v/v)	7.12 $\pm$ 0.755 (5)	0.161 $\pm$ 0.0036 (7)	0.0226
Phosphatidylethanolamine (ox brain)	CHCl ₃	0.688 $\pm$ 0.0105 (2)	0.147 $\pm$ 0.0046 (3)	0.214
Phosphatidylserine (ox brain)	CHCl ₃	0.129 $\pm$ 0.0077 (4)	0.0253 $\pm$ 0.00269 (9)	0.196
Ethanolamine	CHCl ₃	2.42 $\pm$ 0.052 (3)	0.132 $\pm$ 0.0078 (13)	0.0546
	Ethanol	67.6 $\pm$ 9.94 (5)	1.26 $\pm$ 0.050 (5)	0.0186
	Ethanol-water (1:1, v/v)	0.378 $\pm$ 0.0192 (3)	1.78 $\pm$ 0.128 (4)	4.71
Serine	Ethanol-water (1:1, v/v)	0.0542 $\pm$ 0.00030 (4)	0.429 $\pm$ 0.0290 (4)	7.92

inhibitory to its reaction with retinal in chloroform solution. In ethanol-water, sodium hydroxide was used to produce the basic conditions necessary for the formation of *N*-retinylideneserine. Determination of the equilibrium constants at different pH values gave widely different results when total serine concentration was used but constant values when the concentration of the serine anion $-\text{O}_2\text{C}\cdot\text{CH}(\text{CH}_2\cdot\text{OH})\cdot\text{NH}_2$ was used, so it seems that $-\text{NH}_2$ groups reacted with retinal but not the protonated groups $-\text{NH}_3^+$ present in the zwitterionic form. In ethanol-water (1:1, v/v) the $\text{p}K_a$ of the amino group was found to be 9.15 by titration on a pH-meter standardized with aqueous buffer. A pH of 11.0 gave the maximum yield of *N*-retinylideneserine; this pH corresponds to 98% of the amino groups in the $-\text{NH}_2$ form.

Values for the equilibrium and velocity constants of the reactions are given in Table 1. The equilibrium constants K' incorporate a value for the concentration of water in the solvent, because the true equilibrium constant

$$K = \frac{[\text{Schiff base}][\text{water}]}{[\text{amino compound}][\text{retinal}]} = K'[\text{water}]$$

and the concentration of water was not measured. The values of K' have been used to give the composition of the reaction mixtures (Tables 2 and 3). Plots of the initial rates of formation against the concentrations of amino compound showed approximately linear relationships, indicating that the reactions were bimolecular.

Spectrophotometric properties. Tables 2 and 3 give the absorption maxima and molar extinction coefficients of the various compounds studied. With the Schiff base *N*-retinylidenephosphatidylethanolamine, prepared from synthetic phosphatidyl-

ethanolamine, in chloroform solution, the basic form had λ_{max} . 367nm. and the acidic form λ_{max} . 466nm. compared with λ_{max} . 390nm. for retinal; the displacement of λ_{max} . from the basic to the acidic form was thus 99nm. to longer wavelength. The corresponding changes in ϵ (Tables 2 and 3) may be compared with ϵ 43200 for retinal at λ_{max} . 390nm. Reduction of the Schiff base to *N*-retinylphosphatidylethanolamine removes one of the conjugated double bonds, and this compound had λ_{max} . 331nm. In the Carr-Price test, *N*-retinylidenephosphatidylethanolamine gave a violet product, stable for several minutes, with λ_{max} . 514nm. and a broad absorption curve, half-band width 118nm. *N*-Retinylphosphatidylethanolamine in the same test gave a mauve product changing rapidly to a yellow-brown colour. Absorption curves (Fig. 1) showed initially a main peak at 567nm. and a subsidiary peak at 463nm., the maximum extinction at 567nm. being reached about 50sec. after addition of the reagent. Successive scans showed that the 567nm. peak decreased in extinction and the 463nm. peak increased during a period of about 4min., and a minor peak or inflexion appeared at 520nm.

Both *N*-retinylidenephosphatidylserine and *N*-retinylserine had higher λ_{max} . than *N*-retinylidenephosphatidylethanolamine and *N*-retinylidenephosphatidylethanolamine in chloroform solutions of the acid form and in the Carr-Price test. With these exceptions the spectrophotometric properties of the compounds were similar.

DISCUSSION

The maximum water content of A.R. chloroform is quoted as 0.05%. Assuming this value to be w/v, the maximum concentration of water would be

Table 2. *Spectrophotometric properties of chloroform solutions of the retinylidene compounds (Schiff bases) formed from phospholipids, ethanolamine or serine and retinal, and of the corresponding retinyl compounds*

The theoretical fraction of retinal present as Schiff base at equilibrium was calculated with the equilibrium constants given in Table 1. —, Not measured.

Amino compound	Concn. in reaction mixture		Fraction of retinal present as Schiff base at equilibrium	Retinylidene compound (Schiff base)				Retinyl compound			
				Basic form		Acidic form		Colour product in Carr-Price test		u.v.	
	Amino compound (mm)	Retinal (mm)		$\lambda_{\text{max.}}$ (nm.)	ϵ	$\lambda_{\text{max.}}$ (nm.)	ϵ	$\lambda_{\text{max.}}$ (nm.)	ϵ	$\lambda_{\text{max.}}$ (nm.)	ϵ at higher $\lambda_{\text{max.}}$
Phosphatidylethanolamine (synthetic)	40*	1*	0.995	367	51900	466	47200	514	51900	331	37300
Phosphatidylethanolamine (ox brain)	20	1	0.93	372	48600†	468	50000†	518	48500†	331	36600†
Phosphatidylserine (ox brain)	40	1	0.83	372	56100†	475	50700†	543	57800†	—	—
Ethanolamine	40	1	0.99	371	51700	462	51600	512	50600	335	42700
Serine	200‡	0.02‡	0.92	370	—	475	—	535	—	—	—

* Solvent CHCl_3 -ethanol (2:1, v/v) containing 0.4 M-triethylamine; diluted 1 vol. to 44 vol. of CHCl_3 .

† Corrected for free retinal or retinol.

‡ Solvent ethanol-water (1:1, v/v); compounds extracted with CHCl_3 .

Table 3. *Spectrophotometric properties of ethanol and ethanol-water solutions of the retinylidene compounds (Schiff bases) formed from phosphatidylethanolamine, ethanolamine or serine and retinal, and of the corresponding retinyl compounds*

The theoretical fraction of retinal present as Schiff base at equilibrium was calculated with the equilibrium constants given in Table 1.

Amino compound	Concn. in reaction mixture		Fraction of retinal present as Schiff base at equilibrium	Retinylidene compound (Schiff base)				Retinyl compound	
				Basic form		Acidic form			
	Amino compound (mM)	Retinal (mM)		$\lambda_{\max.}$ (nm.)	ϵ	$\lambda_{\max.}$ (nm.)	ϵ	$\lambda_{\max.}$ (nm.)	ϵ
Ethanol									
Phosphatidylethanolamine (synthetic)	40*	1*	0.995	363	54 400	451	48 400	325	52 800
Ethanolamine	40	1	1.00	365	56 800	443	51 900	325	53 200
Ethanol-water (1:1, v/v)									
Ethanolamine	500	0.02	0.995	365	56 400	442	51 100	325	49 200
Serine	200	0.02	0.92	366	62 500†	452	50 300†	326	56 200†

* Solvent CHCl_3 -ethanol (2:1, v/v) containing 0.4M-triethylamine; diluted 1 vol. to 44 vol. of ethanol.

† Corrected for free retinal.

Fig. 1. Absorption curves obtained by successive scans of the colour product in CHCl_3 solution formed from *N*-retinylphosphatidylethanolamine (final concn. 0.02 mm, derived from synthetic phosphatidylethanolamine) and antimony trichloride. The scanning speed was 3.5 nm./sec. The 567 nm. peaks were passed in curve A at 44 sec., in curve B at 126 sec. and in curve C at 210 sec. after the addition of the reagent.

Table 4. *Calculated values for the equilibrium constants at 25° in the reactions between phospholipids, ethanolamine or serine and retinal to give Schiff bases*

The water content of CHCl_3 is taken as 28 mm, that of ethanol as 167 mm, that of CHCl_3 -ethanol (2:1, v/v) as 74 mm, and that of ethanol-water (1:1, v/v) 27.75 mm. These values are used with the values of K' (Table 1) to give

$$K = \frac{[\text{Schiff base}][\text{water}]}{[\text{amino compound}][\text{retinal}]} = K'[\text{water}]$$

Amino compound	Solvent	K
Phosphatidylethanolamine (synthetic)	CHCl_3	84
	CHCl_3 -ethanol (2:1, v/v)	530
Phosphatidylethanolamine (ox brain)	CHCl_3	19.3
Phosphatidylserine (ox brain)	CHCl_3	3.6
Ethanolamine	CHCl_3	68
	Ethanol	11 300
	Ethanol-water (1:1, v/v)	10 500
Serine	Ethanol-water (1:1, v/v)	1 500

28 mm. For the absolute ethanol used, containing up to 0.3% of water, the maximum water concentration would be 167 mm. In the reactions described here to form Schiff bases, the maximum concentration of water produced was 1 mm, and probably negligible compared with that present in the solvents. Values for the equilibrium constants, K' , given in Table 1 incorporate a value for the water content of

the solvent, and therefore differ with different batches of solvent. They are used here to predict the equilibrium composition of the reaction mixtures.

In Table 4, approximate values for the true equilibrium constants are given. These show that the effect of ethanol on the equilibrium constant for phosphatidylethanolamine in chloroform is marked, and that the widely different values of K' with

ethanolamine in ethanol and ethanol-water (Table 1) become close values of K . The results with synthetic and ox brain phosphatidylethanolamine do not show good agreement, but the higher value of the equilibrium constant for ox brain phosphatidylethanolamine in chloroform compared with ox brain phosphatidylserine is also reflected in a similar difference between ethanolamine and serine in ethanol-water. With equal concentrations of the two phospholipids, in the presence of retinal, a five-fold excess of *N*-retinylidenephosphatidylethanolamine over *N*-retinylidenephosphatidylserine would be predicted.

The equilibrium constant for ox brain phosphatidylethanolamine can be used to predict that, with a phosphatidylethanolamine concentration of 1–10 mM (about 0.7–7 mg./ml.) in the presence of 0.01 mM-retinal (2.8 μ g./ml.), 41–87% of the retinal will combine with the phospholipid as a Schiff base without the addition of a base. This is the type of mixture that can arise during the extraction of retinal, together with other lipids, from tissue samples and its concentration before further purification. The reaction rate would be relatively low at these concentrations, but overnight storage of the lipid extract containing retinal would give rise to the Schiff base, and this situation apparently arose during investigations of retinal in fish eggs (Plack *et al.* 1959). In the determination of retinal, subsequent procedures should be introduced to decompose the Schiff base.

With the constant concentration of water in the solvent, the equilibrium position of the mixtures will depend on the concentrations of the reactants and not merely on their molar ratios. Dilution of an equilibrium mixture containing the Schiff base in the basic form with solvent therefore produces a new equilibrium. In most instances the new equilibrium takes some time to be reached and it is possible to make dilutions for rapid spectrophotometric determinations, but with serine and ethanolamine in ethanol-water the velocity constants for the backward reaction are large (Table 1) and the new equilibrium is rapidly established on dilution.

The concentrations of ox brain phospholipids and of serine used did not give effectively all the retinal in the Schiff base form at equilibrium. Calculations with the equilibrium constants found show that for 99% of the retinal to be present as the Schiff base at equilibrium, the concentrations required would be: ox brain phosphatidylethanolamine, 144 mM; ox brain phosphatidylserine, 825 mM; serine, 1830 mM. Such concentrations were not attainable, both from lack of sufficient phospholipids and from limitations of solubility. The situation with serine cannot be improved by lowering the water content of the mixture. Thus in ethanol-water (9:1 v/v) the concentration of serine required would be 367 mM,

again not attainable on solubility grounds. The effect of the residual free retinal, or retinol after reduction, on values of $\lambda_{\max}$ is likely to be small, but corrections have been introduced into values of the molar extinction coefficient.

The values for $\lambda_{\max}$ and ϵ (Tables 2 and 3) are similar to those reported by Pitt *et al.* (1955) for retinylidenemethylamine. For the Carr–Price colour product of the Schiff bases, $\lambda_{\max}$ is higher in the present experiments and this may be due to antimony trichloride being used without acetic anhydride. Plack (1961) found a similar effect with retinal. With *N*-retinylidenephosphatidylserine and *N*-retinylideneserine, the Carr–Price $\lambda_{\max}$ value was markedly higher than with the compounds containing ethanolamine. The half-band width for the Carr–Price colour product of *N*-retinylidenephosphatidylethanolamine was 118 nm. compared with 110 nm. for retinylidenemethylamine (Pitt *et al.* 1955) and ϵ 51 900 compared with 118 000 for retinal (Plack & Kon, 1961) and 47 000 for retinylidenemethylamine (Pitt *et al.* 1955).

The behaviour of the retinyl compounds in the Carr–Price test (Fig. 1) was thought to be characteristic of these compounds, but Jurkowitz (1962) has reported a similar result with retinoic acid, a peak at 572 nm. decreasing in intensity with time and one at 470 nm. increasing in intensity with time. From Jurkowitz's (1962) data, the maximum value for ϵ at 572 nm. was 26 000 compared with about 45 000 for the retinyl compounds described here.

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