SUMMARY

1. A study has been made of the fate of salicylohydrazide and its *N*-acetyl derivative, and of salicylohydroxamic acid and its 5-bromo derivative, in the rabbit. The last two compounds have also been studied in man, rat and mouse.

2. Salicylohydrazide is metabolized mainly by conjugation with glucuronic acid (55 % of dose), and partly by hydrolysis to salicylic acid, which is excreted largely as salicyluric acid.

3. *N*¹-Acetyl-*N*²-salicyloylhydrazine is not deacetylated and is excreted mainly as its glucuronide (70 % of the dose). These hydrazides do not form ethereal sulphates. Their glucuronides have been isolated.

4. Salicylohydroxamic acid is metabolized by direct conjugation with glucuronic and sulphuric acids in the rabbit, and there is practically no conversion into salicylamide. The glucuronide was isolated. In man, mouse and the rat, it forms considerable amounts of salicylamide, which is excreted conjugated.

5. 5-Bromosalicylohydroxamic acid is excreted by man, mouse, rabbit and rat mainly as conjugates of 5-bromosalicylamide, and to a lesser extent as the glucuronide of 5-bromosalicylohydroxamic acid. The glucuronides of the amide and the hydroxamic acid were isolated from rabbit urine.

6. The *in vitro* tuberculostatic activity of 5-bromosalicylamide is about half that of 5-bromosalicylohydroxamic acid, its precursor *in vivo*.

7. The toxicity of aroyl hydrazides and hydroxamic acids in relation to their metabolism has been discussed.

We wish to thank Professor R. Cruickshank for making available the facilities for bacteriological investigations. One of us (W.M.M.) is grateful to St Mary's Hospital Medical School and to the Leverhulme Trust for Research Fellowships.

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Cationic Extracting Agents for Rhodopsin and their Mode of Action

By C. D. B. BRIDGES

*Medical Research Council, Group for Research in the Physiology of Vision,
Institute of Ophthalmology, Judd Street, London, W.C. 1*

(Received 13 November 1956)

Many different substances have been found to be capable of extracting rhodopsin from the dark-adapted frog retina. Kühne (1877) used a crude preparation of bile salts, and Tansley (1931) introduced the now widely used extracting agent digitonin, a steroid saponin found in foxglove leaves. Other workers have used sodium cholate (Sugita, 1926), sodium oleate, panaxtoxin and sodium salicylate (Hosoya, 1932).

With few exceptions, all substances capable of extracting rhodopsin dissolve in water yielding foaming solutions with a low air-solution interfacial tension. That is to say, they are surface-active agents.

The extraction of rhodopsin by cationic surface-active agents has not been previously described. Lythgoe (1938), using the cationic hexadecylpyridinium bromide, failed to extract rhodopsin

from the frog retina, an observation which led him to suggest that cationic surface-active agents might be inactive.

The present work describes the extraction of frog rhodopsin by a series of cationic surface-active agents. These form an homologous series of alkylammonium chlorides and two quaternary salts. The quantitative aspect of the extraction process is examined by allowing solutions of each extracting agent to equilibrate with an excess of rhodopsin present in the retinae of dark-adapted frogs.

As a result of this work, it has been possible to suggest a mechanism for the extraction process.

METHODS

Preparation of extracting agents

Free amines were obtained from L. Light and Co. Ltd. In order to prepare the chlorides, amylamine, octylamine, decylamine, dodecylamine and tetradecylamine were dissolved in light petroleum (b.p. 40–60°). Dry HCl gas was then passed through the solution, and the precipitated amine salt was filtered off, washed and recrystallized from acetone (for amylammonium chloride), chloroform and diethyl ether (for octylammonium chloride), ethanol and diethyl ether (for decylammonium chloride) or water (for tetradecylammonium chloride). Hexadecylammonium chloride and dodecamethylene-1:12-diammonium dichloride were prepared by dissolving the corresponding amines in warm ethanol, then adding an excess of conc. HCl. The bulk of the ethanol and water was removed by adding successive small quantities of benzene, and distilling off the ternary benzene-ethanol-water mixture.

Hexadecyltrimethylammonium chloride, hexadecylpyridinium chloride and digitonin were obtained from L. Light and Co. Ltd.

Extraction technique

The frogs were dark-adapted for about 16 hr., then in the illumination of a dim red Kodak safelight they were killed by decapitation. The retinae were then removed according to the method of Lythgoe (1937) and washed three times with McIlvaine's buffer, pH 4.6.

When the final washing fluid had been removed, the retinae were gently pressed dry between strips of Whatman no. 1 filter paper. A volume (1 ml.) of extracting agent of given concentration, buffered to pH 4.6, was added to the washed dried retinae. The mixture was stirred thoroughly and allowed to stand for 0.5 hr. It was then centrifuged for 0.5 hr. at 4000 rev./min., and the supernatant extract (usually containing some rhodopsin) pipetted off and retained. This process was repeated at least three times, the number of retinae being initially selected to ensure that their rhodopsin content was not exhausted. With dilute solutions of extracting agent it was occasionally necessary to repeat the extraction process five or six times in order to extract rhodopsin from the retinae.

Measurement of rhodopsin concentration

Each extract prepared as described was again centrifuged for 20 min. at 4000 rev./min., then treated with sufficient sodium borate to adjust the pH to about 7. The optical

densities at 490, 500 and 510 m μ were then measured in a spectrophotometer similar to that described by Dartnall (1952). All optical-density measurements were for a 0.5 cm. light path, versus a corresponding 'blank' of unused extracting-agent solution, at $20 \pm 0.5^\circ$. Measurements of optical density were repeated at least three times in such a way that 10 min. intervals separated each determination at a given wavelength. This procedure provided a check of the thermal stability of the extracts. The solutions were then bleached by exposure to a 6 v 24 w lamp at a distance of 2 ft. for a period of 10 min. Optical-density readings were taken at 490, 500 and 510 m μ as before. The solutions were then bleached for a further 10 min., and readings again taken at the three wavelengths. The 500 m μ density readings for the unbleached solution were averaged, as were those for the bleached solution. Subtraction of the bleached solution E_{500} from the unbleached solution E_{500} gave the ΔE_{500} or, since the λ_{max} of rhodopsin is 500 ± 2 m μ , the ΔE_{max} .

If the solution bleached to colourless photoproducts, the ΔE_{max} would be equal to the E_{max} of the unbleached rhodopsin. In actual fact, rhodopsin bleaches to yield indicator yellow. At pH 7, this substance has an absorption at 500 m μ , which is about 15% of that of the parent pigment at that wavelength (Lythgoe, 1937). Therefore,

$$\Delta E_{500} = 0.85 E_{500}.$$

Since

$$E_{500} = \epsilon_{500} cl$$

(where ϵ_{500} = molar extinction coefficient of rhodopsin at 500 m μ ; l = length of optical path over which the optical density has been measured; c = molar concentration of rhodopsin)

$$\Delta E_{500} = 0.85 \epsilon_{500} cl.$$

For a 0.5 cm. optical path and a molar extinction coefficient of 4.8×10^4 cm.² at 500 m μ (Collins & Morton, 1950b), we have $c = \Delta E_{500}/20$ 000.

Some solutions became faintly opalescent when bleached; results from these were discarded. It was always necessary to ensure that no rods remained in suspension after pipetting off the extracts from residual retinal material. The ability of these solutions to stabilize suspensions made this problem difficult to overcome. A small quantity of reddish photosensitive sediment was frequently observed after centrifuging these extracts prior to the optical measurements.

Measurement of absorption spectra

Absorption spectra were measured in the same apparatus and under the same conditions as described in the foregoing section. The precautions for ensuring that the solutions were thermally stable were as described by Dartnall (1952). To obtain solutions of suitable purity, the rod outer segments were separated from the rest of the retinae by the method of Saito (1938), and were then extracted with the given extracting agent.

RESULTS

General properties of extracts

In most cases, if the extracts were cooled to 3° a crystalline precipitate was formed. Visual examination of this precipitate revealed that most of the rhodopsin was associated with it. The precipitate dissolved slowly if warmed to 20°. Exposure of the

reddish crystals to daylight caused a slow change of colour, passing from red to orange, then bright yellow fading to pale yellow.

Similar precipitates were obtained if the pH of extracts prepared with the alkylammonium chlorides was raised to between 8 and 9. These precipitates dissolved slowly if they were suspended in a fluid of pH 6. Again, most of the rhodopsin was associated with the precipitated material (the bulk of which was presumably free amine).

Although no precipitation occurred when pure solutions of the quaternary salts were adjusted to pH 8–9, precipitates were occasionally observed in rhodopsin extracts made with these reagents. The precipitates did not appear to redissolve easily, even when suspended in fluids as acid as pH 4.6.

Loss of rhodopsin during storage

With the exception of the digitonin, hexadecyltrimethylammonium chloride and hexadecylammonium chloride extracts, loss of rhodopsin was appreciable after storage in darkness at 3° for a period of several weeks. The largest losses occurred in extracts prepared with high concentrations of the lower homologues. For example, an extract prepared with 1.25×10^{-2} M-octylammonium chloride lost about 73 % of its rhodopsin when stored for 60 days, compared with 35 % for a 2.58×10^{-2} M-decylammonium chloride extract and only 0.3 % for a 2.75×10^{-2} M-hexadecyltrimethylammonium chloride extract stored over the same period.

Often the solutions became somewhat opalescent and photometrically unstable, even after adequate time had been allowed for solution of the 'cold' precipitate. Generally speaking, the trend was towards improved keeping properties of the extracts as the hydrocarbon chain of the extracting agent was lengthened. Hexadecylpyridinium chloride was an exception to this tendency; there was 80 % loss of rhodopsin in an extract prepared with a 1.76×10^{-2} M solution of that substance.

Absorption and difference spectra

The $\lambda_{\max}$ of extracts prepared from the isolated rod outer segments ranged from 498 to 501 m μ . The true $\lambda_{\max}$ of the rhodopsin was obtained by calculating the $E_{\min}/E_{\max}$ values and correcting the observed maxima accordingly (cf. Collins & Morton, 1950a; Crescitelli & Dartnall, 1954). For extracts prepared with the alkylammonium chlorides, quaternary salts and digitonin, the true $\lambda_{\max}$ was found to be 502 ± 2 m μ .

Difference spectra, obtained by exposure of the extracts to white light, were identical for all extracting agents. The $\lambda_{\max}$ of the difference spectra was at 504 ± 1 m μ (at pH 7.0), confirming the value of 502 ± 2 m μ for the $\lambda_{\max}$ of the rhodopsin absorption spectrum.

It was found that the purity of the rhodopsin extracts (assessed by $E_{\min}/E_{\max}$) diminished as the hydrocarbon chain length of the extracting agent was decreased. Thus an extract prepared from isolated rod outer segments with 0.13 M-amyllumonium chloride had an $E_{\min}/E_{\max}$ of 0.62, whereas one prepared with 2.8×10^{-3} M-tetradecylammonium chloride had an $E_{\min}/E_{\max}$ of 0.44. The $E_{\min}/E_{\max}$ of extracts prepared from whole retinae with concentrations of extracting agent above the critical concentration for micelles (CCM) were higher than those prepared with concentrations below the CCM. Solutions at concentrations above the CCM contain micelles which are capable of solubilizing yellow lipid substances of retinal origin. This might account for the decrease in extract purity when the extracting agent concentration exceeds the CCM.

Quantitative aspects of the extraction process

When a batch of whole retinae was treated with successive volumes of extracting-agent solution, it was usually found that the first extract contained less rhodopsin than its successors. Thus when a batch of twelve retinae was extracted with 1 ml. volumes of 5.2×10^{-3} M-decylammonium chloride solution, the first extract had ΔE_{500} of 0.0364, whilst the second and third extracts had ΔE_{500} of 0.0520 and 0.0531 respectively. Similar results were obtained at other concentrations and with other extracting agents.

In Table 1 are listed some typical results obtained with amyllumonium, decylammonium and tetradecylammonium chlorides. The second and third extracts in each case contained about the same quantity of rhodopsin. Continued extraction of a retinal batch then yielded the same amounts of rhodopsin in each extract until the retinae were exhausted.

With low concentrations of extracting agent it was often necessary to extract the retinae five or six times before rhodopsin appeared in an extract.

Once a given retinal batch yielded an extract containing rhodopsin (extract R_1), albeit only in a very small quantity, the next extract (R_2) contained a greater quantity, which then remained constant for all succeeding extracts (R_3 , R_4 , etc.).

Whereas in the majority of cases the quantity of rhodopsin in R_1 was dependent upon the number of retinae extracted and the concentration of extracting agent, the rhodopsin content of succeeding extracts was dependent only on the latter. It was considered that extracts R_2 , R_3 , etc. contained the maximum amount of rhodopsin which could be extracted by that concentration of extracting agent. This amount of rhodopsin was, in fact, proportional to the extracting-agent concentration. If extracting-agent concentration is plotted against

Table 1. *Rhodopsin content of retinal extracts*

Rhodopsin content (as estimated by the ΔE_{500} at about pH 7) of successive extractions of batches of retinae by 1 ml. volumes of solutions prepared with three alkylammonium chlorides.

Concn. of extracting soln. (M)	No. of retinae	ΔE_{500} of first extract	ΔE_{500} of second extract	ΔE_{500} of third extract
1. Amylammonium chloride				
8.48×10^{-2}	12	0.0136	0.0368	0.0362
1.06×10^{-1}	12	0.0218	0.0433	0.0441
1.28×10^{-1}	12	0.0296	0.0501	0.0522
2. Decylammonium chloride				
5.20×10^{-2}	12	0.0364	0.0520	0.0531
1.29×10^{-1}	24	0.1066	0.1446	0.1438
2.08×10^{-1}	36	0.1735	0.2247	0.2239
2.58×10^{-1}	48	0.2190	0.2929	0.2942
3.87×10^{-1}	60	0.3319	0.4236	0.4248
5.15×10^{-1}	84	0.3349	0.5444	0.5425
3. Tetradecylammonium chloride				
2.00×10^{-2}	12	0.0655	0.0858	0.0870
2.80×10^{-2}	18	0.0934	0.1162	0.1169

Fig. 1. Extraction curves for all the extracting agents investigated. Ordinate: mean rhodopsin content (expressed as ΔE_{500}) of extracts R_2 and R_3 . Abscissa: molar concentration of extracting agent. Curve 1: amyllammonium chloride; curve 2: octylammonium chloride; curve 3: decylammonium chloride; curve 4: dodecylammonium chloride; curve 5: tetradecylammonium chloride; curve 6: hexadecylammonium chloride (●), hexadecyltrimethylammonium chloride (○), hexadecylpyridinium chloride (◐); curve 7: digitonin. The dotted contour cuts each extraction curve at the critical concentration for micelles (CCM). The inset shows area A at the origin magnified 10 times.

the corresponding rhodopsin content of extract R_2 one obtains, for a given extracting agent, a straight line passing through the origin (see Fig. 1).

The verification that ΔE_{500} for R_2 was equal to ΔE_{500} for R_3 was always carried out, in order to ensure that the extracting agent had been equilibrated with an excess of rhodopsin.

In general, the following expression was found to describe the relationship between the rhodopsin content of extract R_1 and that of extract R_2 :

$$\frac{N_1}{N_2} = \frac{xN_D - yn_a}{N_D}$$

(where N_1 = moles of rhodopsin in the first rhodopsin-containing extract (R_1); N_2 = moles of rhodopsin in the second rhodopsin-containing extract (R_2); N_D = moles of extracting agent/volume of solution used in each extraction; x = total number of times the retinae have been treated with extracting agent before R_2 . This figure includes any initial extracts which did not yield rhodopsin; y = number of retinae; n_a = constant for a given extracting agent).

The values of n_a for different extracting agents are given in Table 2. For the 16 carbon compounds, the value of n_a is about 2.8×10^{-8} moles, increasing with decreasing chain length to 4.5×10^{-6} moles for amylammonium chloride. The interpretation of the quantity n_a will be considered below.

Amount of rhodopsin extracted by different extracting agents

The amount of rhodopsin extracted by a given concentration of extracting agent is taken as the mean of the amounts in extracts R_2 and R_3 . The rhodopsin contents of these extracts depend only on the type and concentration of extracting agent.

(1) *Extraction of rhodopsin by the alkylammonium chlorides.* The general formula of the alkylammonium chlorides is given by (I). The homologues investigated had $n = 4, 7, 9, 11, 13$ and 15.

The slopes of the extraction curves shown in Fig. 1 may be used as a measure of the 'extracting efficiency' of each alkylammonium chloride. The 16-carbon hexadecylammonium chloride has an extracting efficiency maximal for the series; 3.0×10^{-3} moles of rhodopsin are extracted/mole of that extracting agent. This value compares with only 2.1×10^{-5} moles of rhodopsin/mole of the 5-carbon amylammonium chloride. The extracting efficiencies for these and other extracting agents are summarized in Table 2.

The lengthening of a 5-carbon chain to 8 carbons causes a 20-fold increase in extracting efficiency. This increase in extracting efficiency becomes progressively less for further increases in chain length, being 2.4 from 10 to 12 carbons and only 1.4 from 14 to 16 carbons.

The inverse value of the extracting-curve slope gives the number of moles of extracting agent required to extract 1 mole of rhodopsin: this represents the ratio of the numbers of extracting-agent molecules and rhodopsin molecules in solution. The ratio varies from 48 000 for amylammonium

Fig. 2. Change in the molar ratio of extracting agent: rhodopsin as the alkyl chain length is increased from 5 carbons (amylammonium chloride) to 16 carbons (hexadecylammonium chloride).

Table 2. *Extracting of rhodopsin by different cationic reagents and by digitonin*

Extraction efficiency is the no. of moles of rhodopsin extracted/mole of extracting agent. The figures given in the last column are the inverse values of the extracting efficiency. n_a is defined in the text.

Extracting agent	n_a (moles)	Extracting efficiency	Molecular ratio of extracting agent to rhodopsin
Digitonin	1.12×10^{-8}	4.1×10^{-3}	250
Hexadecyltrimethylammonium chloride	2.79×10^{-8}	3.0×10^{-3}	330
Hexadecylpyridinium chloride	2.83×10^{-8}	3.0×10^{-3}	330
Hexadecylammonium chloride	2.78×10^{-8}	3.0×10^{-3}	330
Tetradecylammonium chloride	4.06×10^{-8}	2.1×10^{-3}	470
Dodecylammonium chloride	7.30×10^{-8}	1.3×10^{-3}	800
Decylammonium chloride	1.25×10^{-7}	5.4×10^{-4}	1 800
Octylammonium chloride	1.19×10^{-6}	1.1×10^{-4}	8 800
Amylammonium chloride	4.47×10^{-6}	2.1×10^{-5}	48 000
Dodecamethylene-1:12-diammonium dichloride	4.80×10^{-8}	2.3×10^{-3}	430

chloride to 330 for hexadecylammonium chloride (see Table 2); its variation with length of alkyl chain is plotted in Fig. 2.

(2) *Extraction of rhodopsin by quaternary compounds.* Two 16-carbon quaternary compounds were investigated: these were hexadecyltrimethylammonium chloride (II) and hexadecylpyridinium chloride (III).

The ability of these two substances to extract rhodopsin is identical with that of hexadecylammonium chloride, namely 3.0×10^{-3} moles of rhodopsin/mole of extracting agent (see Table 2). In the region of the CCM (shown in Fig. 1 by the dotted contour which cuts each extraction curve at the CCM) there is a change in the slope of the extraction curve, the extracting efficiency above the CCM being only one-third of that below it. This is not shown clearly in Fig. 1, owing to the steepness of the curve.

(3) *Extraction of rhodopsin by dodecamethylene-1:12-diammonium dichloride.* This substance differs from dodecylammonium chloride in that it has an extra terminal $\overset{+}{\text{N}}\text{H}_3$ group (IV).

The extracting efficiency of dodecamethylene-1:12-diammonium dichloride is 2.3×10^{-3} moles of rhodopsin/mole of extracting agent. The corresponding value for dodecylammonium chloride is 1.3×10^{-3} . The diammonium derivative is therefore about 1.8 times as effective as its related monoammonium compound.

(4) *Extraction of rhodopsin by digitonin.* Digitonin has a structure quite different from that of the foregoing substances. It consists of a bulky polycyclic hydrocarbon structure united with 5 sugar molecules (4 hexose molecules and 1 of pentose). Since this substance does not ionize when dissolved in water, it is classed as non-ionic.

Its extracting curve is a straight line passing through the origin, except at the CCM, where there is an inflexion in the curve representing a reduction in the extracting efficiency of about one-half. The extracting efficiency of digitonin below its CCM is 4.1×10^{-3} moles of rhodopsin/mole of digitonin, a value somewhat larger than that of the C_{16} cationic compounds.

Effect of altering the duration of equilibration

Tests were carried out in order to ensure that the extracting agent solution had reached equilibrium

with the washed retinae. Several concentrations of decylammonium chloride were taken, and extraction curves were plotted as before, only this time the extracting agent solution was allowed to remain in contact with the retinae for 1.5 hr. instead of 0.5 hr. Within experimental error, there was no difference between the extraction curves for these different durations. Consequently, it was inferred that the extracting agent-retinae system had reached equilibrium after 0.5 hr.

Effect of altering the extraction pH

The previously described extractions were carried out on retinae which had been thoroughly washed in pH 4.6 buffer. In addition, the extracting solutions themselves were at pH 4.6. A series of extractions was carried out with solutions of decylammonium chloride at pH 6.8. The retinae were washed with pH 6.8 buffer before the extraction. The quantities of rhodopsin extracted at this pH were the same as those extracted at pH 4.6.

DISCUSSION

When a solution of an extracting agent is equilibrated with an excess of rhodopsin contained in frog's retinae, the amount of rhodopsin extracted is proportional to the extracting-agent concentration (provided this is below the CCM). In extracts prepared with a given extracting agent, the number of extracting-agent molecules is a constant multiple of the number of molecules of rhodopsin. For non-ionic digitonin this multiple is 246, a value compatible with Hubbard's (1954) finding that rhodopsin extracts prepared with digitonin contain complex micelles, each composed of 180–200 molecules of digitonin and 1 molecule of rhodopsin.

Digitonin not only extracts rhodopsin, it is also responsible for keeping the latter in solution. This is demonstrated by the fact that removal of digitonin from extracts by dialysis causes precipitation of the previously 'solubilized' rhodopsin (Chase & Haig, 1938; Broda, Goodeve & Lythgoe, 1940; Hubbard, 1954). There seems no doubt that digitonin forms a water-soluble complex with rhodopsin, and the similarity between the extraction curves of digitonin and those of the alkylammonium chlorides suggests that in these substances complex formation may also occur. Further support for this suggestion is provided by the fact that, in common with digitonin, the alkylammonium chlorides appear to act as solubilizing agents for rhodopsin, since precipitation of the extracting agent by methods which do not destroy rhodopsin also causes precipitation of that substance.

The majority of concentrations used in the present work were below the CCM, i.e. the extracting agents were present in the pure solutions as free ions

or molecules. It is therefore extremely unlikely that rhodopsin is solubilized in the same way as water-insoluble dyestuffs and hydrocarbons, namely by 'solution' in the hydrocarbon interior of the solubilizing agent's micelles. Consequently, the extracting agent must form some chemical complex with rhodopsin.

It is known that many cationic and anionic surface-active agents can form complexes with proteins (Putnam, 1948), and the reactions involved are in many ways similar to those of the present work. Thus the protein-precipitating activity of the sodium alkyl sulphates is found to increase with increasing chain length (McMeekin, 1942; Putnam & Neurath, 1945). McMeekin showed that successive additions of CH_2 to sodium octyl sulphate caused protein-precipitating activity to increase until a nearly maximum value was obtained, when further addition of CH_2 had little effect.

Like observations have been made in the present work. As the chain length of an alkylammonium chloride is increased, its rhodopsin-extracting efficiency becomes greater. The extracting agent: rhodopsin ratio decreases considerably as the chain length of the lower homologues is increased, but this effect becomes much less marked as the series is ascended.

Lundgren (1945) suggested that anionic surface-active agents combined with proteins by means of the electrostatic attraction between the negatively charged polar groups of the surface-active agent and oppositely charged groups of the peptide chain. Similarly, it may be suggested that the positively charged NH_3 groups of the alkylammonium ions become attached to corresponding negatively charged carboxyl groups on the peptide chain of the rhodopsin molecule. One would not expect that a complex of this type would be water-soluble, owing to the orientation of a large number of hydrophobic alkyl chains towards the aqueous phase. However, if then a second layer of alkylammonium ions is amphipathically adsorbed on to these alkyl chains in such a way that the hydrophilic polar groups point outwards, the complex becomes water soluble.

The nearly doubled extracting efficiency of dodecamethylene-1:12-diammonium dichloride may be attributed to the fact that the ion is hydrophilic at both ends, and therefore attachment of one end to the peptide chain always leaves a hydrophilic end in the aqueous phase. The complex so formed is water-soluble without the need for double-layer formation.

The greater extracting efficiency of the long-chain alkylammonium chlorides is due to their greater tendency for double-layer formation. In addition, the electrostatic combination of rhodopsin with alkylammonium ions is stabilized by the mutual affinity of adjacently bound ions, an effect which

becomes greater as the length of the alkyl chain is increased.

The surprising absence of any pH effect on the extraction curves of the present work is comparable with Putnam & Neurath's (1945) finding that the combination of sodium dodecyl sulphate with serum albumin was independent of pH over the range 4.0–6.8.

It is not clear how digitonin can react with rhodopsin. The binding of digitonin and rhodopsin molecules could take place by means of weak van der Waals forces between the non-polar regions of the peptide chain and the polycyclic hydrocarbon portion of the digitonin molecule. On the other hand, hydrogen-bond formation could occur between the sugar hydroxyl groups of digitonin and the carbonyl groups of the peptide chain. It seems probable that some factor of specific affinity is involved, since digitonin does not appear to form a complex with ovalbumin (Hubbard, 1954).

Number of anionic groups in rhodopsin

The extracting agent:rhodopsin ratio tends towards a minimum value as the length of the alkyl chain is increased. For hexadecylammonium chloride this ratio has a value of 330. The higher values obtained for the lower homologues might be due to the fact that only a proportion of the extracting agent molecules is combined with the extracted rhodopsin. Putnam and Neurath's observations on serum albumin tend to support this view, since these authors found that after complete precipitation with sodium octyl and decyl sulphates uncombined surface-active agent was always left in solution. This did not occur with sodium dodecyl sulphate.

If all the 330 hexadecylammonium ions are combined with one rhodopsin molecule, and if these are arranged in a bimolecular layer, then the rhodopsin molecule would possess a maximum number of 165 anionic groups. The combination of extracting agent molecules with other proteins of retinal origin would, however, reduce this figure.

Mechanism of the extraction process

Until a certain amount of extracting agent has been added to the retina, no rhodopsin can be extracted. For each extracting agent, this amount (n_a) is a constant (per retina) and becomes larger as the length of the alkyl chain decreases. In the 16-carbon compounds, n_a has a value of about 2.8×10^{-8} moles. If about 330 molecules of the 16-carbon compounds are required to extract one molecule of rhodopsin, and if the frog retina contains 2×10^{-9} moles of rhodopsin (Hubbard, 1954), then 2.8×10^{-8} moles of a 16-carbon compound should be sufficient to extract one-twentieth of the available rhodopsin.

The failure of these substances to extract this amount could be attributed to: (a) the formation of complexes with proteins situated in a position topographically more favourable than that of rhodopsin, e.g. proteins of connective tissue, nerve fibres, etc.; (b) the formation of an insoluble complex with rhodopsin by deposition of a monomolecular layer around the molecule.

In its 'native' state, the rhodopsin molecule may not necessarily be intrinsically insoluble. The failure of water to extract this protein may be due to the incorporation of the molecule within the structure of the rod outer limb. The rod could be considered as a macromolecule of proteins and lipid; the rhodopsin molecule is bound within this framework by means of polar and hydrogen bonds.

The penetration of a bulky positively charged alkylammonium ion into such a structure would profoundly disturb these electrostatic forces and cause considerable steric hindrance to hydrogen-bond formation. Thus the rod structure would disintegrate and, as outlined above, by suitable combination with the extracting-agent's ions, the rhodopsin would be brought into solution.

Structure, surface activity and the ability to extract rhodopsin

Almost all substances capable of extracting rhodopsin are surface active. Reference to Table 3 shows that the ability to extract rhodopsin is not confined to any particular group of surface-active agents; in fact, a surprisingly large number of substances with different structures appear to have extracting properties.

What type of chemical structure is required in an efficient extracting agent? From the foregoing discussion it is clear that two requirements must be

fulfilled. First, the extracting agent molecule must have a grouping capable of reacting with the peptide chain; this grouping must also be hydrophilic (the 'reacting group'). Secondly, the molecule must have a grouping conferring stability upon the bimolecular layer surrounding the 'solubilized' peptide chain (the 'stabilizing' group).

The reacting group may be positively charged (in the cationic extracting agents) or negatively charged (in the anionic extracting agents). With non-ionic digitonin it has been suggested that the reacting group may be the glycone moiety of the molecule; the stabilizing group would then be the remaining hydrocarbon aglycone.

A consequence of this structural duality (reacting group-stabilizing group) is that the molecule will have the structural characteristics of a surface-active agent. The hydrophilic group and the hydrophobic group of the surface-active agent are respectively the reacting group and the stabilizing group of the extracting agent.

SUMMARY

1. A series of cationic surface-active substances have been shown to be capable of extracting rhodopsin from the dark-adapted frog retina. These substances are: (a) The alkylammonium chlorides, of general formula $\text{CH}_3 \cdot [\text{CH}_2]_n \cdot \text{NH}_3^+ \text{Cl}^-$. In the present investigation n was 4, 7, 9, 11, 13 and 15. (b) The quaternary 16-carbon compounds hexadecyltrimethylammonium chloride and hexadecylpyridinium chloride. (c) The 12-carbon dodecylmethylene-1:12-diammonium dichloride.

2. When extracts were stored in darkness at 3°, loss of rhodopsin was appreciable in those extracts prepared with the lower homologues. Rhodopsin

Table 3. Scheme showing the classification of surface-active agents and groups containing substances capable of extracting rhodopsin

* Groups containing extracting agents for rhodopsin: (1) Present work. (2) Digitonin, saponin (Tansley, 1931). (3) Kühne (1877); Sugita (1926). (4) Sodium oleate (Hosoya, 1932). (5) Sodium palmityl 'sulphonate' (Lythgoe, 1938). (6) Panaxtoxin, Kalotoxin (Hosoya, 1932). (7) Sodium dodecyl sulphate destroys rhodopsin (Smith, 1941; confirmed by author).

losses were negligible in extracts prepared with the 16-carbon compounds (with the exception of hexadecylpyridinium derivative). Generally, the keeping properties of extracts improved as the length of the alkyl chain was increased.

3. When a batch of retinae was repeatedly extracted with small volumes of a given concentration of an extracting-agent solution, the first extract usually contained much less rhodopsin than its successors. These extracts all had the same rhodopsin content (provided the retinae did not become exhausted). This suggests that initially some of the extracting agent is 'inactivated' owing to its combination with non-photosensitive retinal proteins. The calculated amount of extracting agent so inactivated was found to be constant for a given extracting agent. As soon as this combination has been completed, rhodopsin is extracted in quantities proportional to the extracting-agent concentration.

4. The slope of the straight line obtained by plotting extracting agent concentration against rhodopsin concentration varies for different extracting agents. The molar ratio of extracting agent:rhodopsin increases from 330 for the 16-carbon hexadecylammonium chloride to 48358 for the 5-carbon amylammonium chloride. The extraction curves for the 16-carbon quaternary compounds are identical with that of hexadecylammonium chloride. The slope of the dodecamethylene-1:12-diammonium dichloride extraction curve is nearly half that of the corresponding monoammonium compound, dodecylammonium chloride.

5. It is suggested that the cationic extracting agent forms a water-soluble complex with rhodopsin. The reactions involved are as follows: (a) The extracting-agent cations form a single layer over the rhodopsin peptide chain. This is due to the electrostatic attraction between the polar head group of the cation, and oppositely charged groups on the peptide chain. The alkyl chains of this layer point towards the aqueous phase. (b) A second layer of cations

amphipathically adsorbed on to the first produces a water-soluble complex. The addition of an extra terminal NH_3 to dodecylammonium chloride (thus forming dodecamethylene-1:12-diammonium chloride) reduces the molar ratio of extracting agent:rhodopsin by nearly half. This is attributed to the fact that the ion has become hydrophilic at both ends of the alkyl chain, and hence double-layer formation is not necessary for the formation of a water-soluble complex.

I should like to record my thanks to Dr H. J. A. Dartnall for his encouragement throughout this work, and for his helpful criticisms of the manuscript; also to Miss Anne Ley for her invaluable assistance in the laboratory and for drawing the figures; also to the Medical Research Council for a grant, without which this work could not have been carried out.

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The Hydrolysis of Indoxyl Esters by Esterases of Human Blood

By ELUNED E. UNDERHAY

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

(Received 20 November 1956)

A new method for the localization of esterases in animal tissues by a histochemical staining process, using indoxyl esters as substrates, has recently been described by Barnett & Seligman (1951) and independently by Holt (1952). The indoxyl esters are hydrolysed by tissue esterases to indoxyl, which is subsequently oxidized to indigo, forming an in-

tensely coloured visual indicator of the sites of enzymic activity.

Detailed and characteristic histological pictures are obtained by a modification of this procedure, developed in these laboratories, which employs certain ring-substituted indoxyl esters as substrates (Holt & Withers, 1952; Holt, 1954). Recently, the