Summary

The nitration product of phenazine, which was previously described as 1,3-dinitrophenazine, was proved to be a mixture of 1,6- and 1,9-dinitrophenazines. These structures were determined by their conversion into dihydroxyphenazines by way of diaminophenazines.

From the nitration of phenazine N-oxide under high temperature, two isomers, 1,7-dinitrophenazine 5-oxide and 3,7-dinitrophenazine-5-oxide were obtained.

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19. Takeo Tsukamoto,* Tetsuya Komori,* Nadao Kinoshita,* Naoki Toida,** and Hirosi A. Kuriyama**: Chemical Studies on Visual Function. V.*** On the Chemical Nature of Rhodopsin. (1). Purification of Scotopsin and Its Amino Acid Component.

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Rhodopsin, the photo-sensitive pigment in the outer segment of the rods, has been studied in detail by Wald,[1] Robeson,[2] Oroshnik,[3] Hubbard,[4] and others so far as the structure of each isomer of its prosthetic group, retinene, is concerned. However, the protein fraction of rhodopsin has never been clarified except in a few details since Mirsky[5] pointed out that the bleaching and regeneration of rhodopsin should be considered as a result of reversible denaturation of proteins. Wald's theory[6] of thioacetal binding and Morton's theory[7] of Schiff-base binding have been advanced for the study of the active radical with which retinene may combine. Yet it remains unexplained what kind of amino acid of its protein portion combines with retinene, or whether it is combined with some non-protein substance. According to the result of our investigation[8] on the influence of arecoline compounds on the toad E.R.G., it is inferable that the substances related to arecoline which accelerate the rhodopsin regeneration also augment the height of b-wave in the retinal action potential with a few exception and such substances have one double bond in their molecular structure. However, it is necessary all the same that the relationship of various chemicals to rhodopsin and the chemical nature of rhodopsin should be examined for elucidation of the first stage in the agitation of the sense of sight in the retina. We are chiefly making a series of studies in scotopsin, the protein portion of rhodopsin kept in the dark.

Purification of Scotopsin from Cattle Retina—For the purification of scotopsin a modification of the method of Hubbard[9] is used. The retina extracted from fresh cattle eyeball and kept

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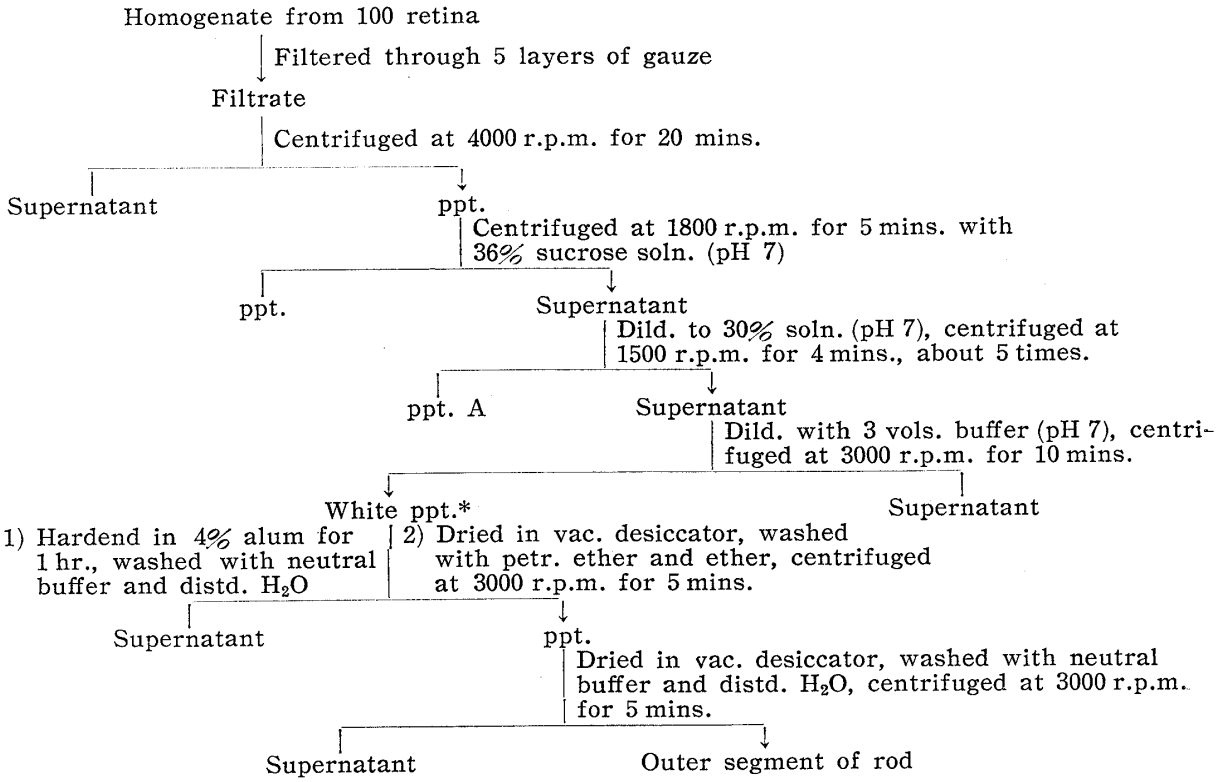
1) G. Wald, *et al.*: J. Gen. Physiol., **36**, 269, 415(1953); J. Biol. Chem., **222**, 865(1956).
2) C. D. Robeson, *et al.*: J. Am. Chem. Soc., **77**, 4120(1955).
3) W. Oroshnik: *Ibid.*, **78**, 2651(1956).
4) R. Hubbard: *Ibid.*, **78**, 4662(1956).
5) A. E. Mirsky: Proc. Natl. Acad. Sci., **22**, 147(1936).
6) G. Wald, P. K. Brown: J. Gen. Physiol., **35**, 797(1951).
7) R. A. Morton, F. D. Collins: Biochem. J. **47**, 18(1950).
8) Part IV. T. Tsukamoto, *et al.*: Kyushu J. Med. Sci., 8, 251(1957).
9) R. Hubbard: J. Gen. Physiol., **37**, 381(1953).

below −10° is left exposed to light for 1 hr. One hundred retinas are ground for homogenization in a mortar with 15 cc. of *M*/15 phosphate buffer (pH 7) and filtered through a gauze specified in the Japanese Pharmacopoeia. The unfiltered residue on the gauze is homogenized once more and filtered several times with the buffer, the buffer totalling 50 cc. This experiment is illustrated in Chart 1. The outer segments of the rods, which are isopicnotic with 30% sucrose solution, are treated with *M*/15 phosphate buffer (pH 7) and washed with ether, the final washing completing the purification of the segments. The purified outer segments of the rods are extracted within 24 hrs. with 10 cc. of 2% aq. solution of digitonin at 5~10°, centrifuged at 4000 r.p.m. for 30 mins., and scotopsin transferred into the supernatant is used immediately or is prepared into a dry powder at 2 mm. Hg at −65° for storage. The water for use in this purifying manipulation is distilled twice.

Electrophoresis and Ultraviolet Spectrometry—As shown in Fig. 1, the ultraviolet spectrum of scotopsin, measured by the Shimadzu Spectrophotometer Type QB 50, has an absorption maximum at 277 mμ. Scotopsin is dialyzed against Veronal buffer (pH 8.6, $I=0.1$) for 48 hrs. in a cellophane bag. The electrophoretic pattern is measured in 1.5 hrs. at 4°, 5 mA, 130 V, by means of the Tiselius-Svenson apparatus. Fig. 2 shows the result obtained.

Chart 1. Preparation of Outer Segments



* At this stage, if a minute quantity of ppt. A is present, this fraction is poured slowly into a tube containing 40% sucrose solution (dissolved in *M*/15 phosphate buffer, pH 7) and pH 7 phosphate buffer is further added. Then this tube is centrifuged at 3200 r.p.m. for 15 mins. A precipitates to the bottom and pure outer segment fraction collects between sucrose and phosphate buffer layer.

Molecular Weight Measurement by Light Scattering Method—The apparatus consists of a Brice Phoenix Photometer, Type 1000 and a Phoenix Differential Refractometer. The dry powder of scotopsin digitonide is dialyzed against distilled water within 48 hrs. and filtered through a sintered glass filter No. 4. The filtrate is centrifuged by an ultracentrifugal machine (Spinco Type) at 22500 r.p.m., 38000*g* for 40 mins. and filtered through a sintered glass filter No. 4 again. At this step, the intensity of light scattering at 0° and 90° is measured in 4 kinds of solution of different concentrations for determination of turbidity. The molecular weight of scotopsin digitonide was 286,000, calculated by the equation of Debye.[10]

Qualitative Analysis of Amino Acid by Hydrolysis—23.334 mg. of scotopsin digitonide (Kieldahl, N=1.31%) is dissolved in 1 cc. of distilled water and 0.5 cc. of this solution is used as a solution for acid and alkaline hydrolysis. In the acid hydrolysis, 0.75 cc. of HCl (Wako Junyaku,

10) P. Debye : J. Phys. & Colloid Chem., **51**, 18(1947); J. Applied. Phys., **15**, 338(1944).

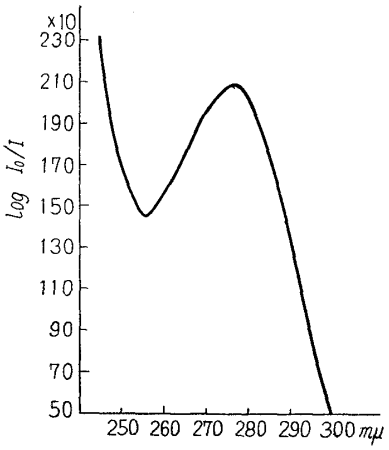


Fig. 1. Absorption Spectra of Cattle Scotopsin

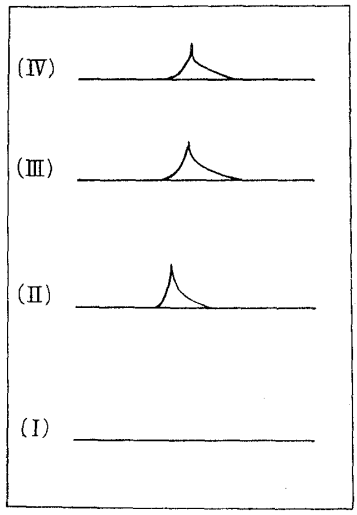


Fig. 2.
Electrophoretic Pattern of Cattle Scotopsin by Tiselius-Svenson Apparatus
Veronal buffer (pH 8.6, I=0.1) dialysed for 48 hrs.
Current, 5 mA; temp., 4°C, potential, 130 V.

	Slit angle (deg.)	Slit range	Exposed time (sec.)	Time (mins.)
(I)	20	0.3	10	0
(II)	50	0.2	10	60
(III)	50	0.2	10	80
(IV)	50	0.2	10	90

Original scotopsin digitoninide solution (Kjeldahl N, 0.34238 mg./cc.) was measured after 28-fold dilution with a supernatant layer of aqueous solution (2%) of digitonin. This supernatant layer was used after being centrifuged at 4,000 r.p.m. for 30 mins.

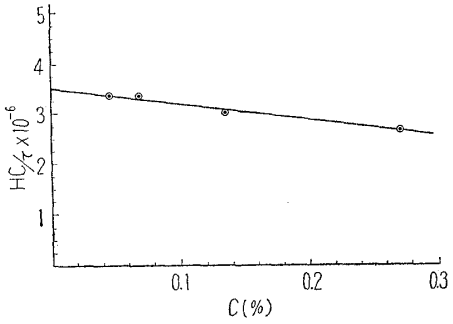


Fig. 3.
Molecular Weight Determination of Scotopsin Digitonide by Light Scattering Method
$(HC/\tau)_{C\to 0}=3.5\times 10^{-6}$
$H=2.287\times 10^{-6}$, $n_0=1.33700$, $n=1.33738$
$F=0.1215$, $R_W/R_C=1.066$
Temp.=10°, Wave length=5460 Å

Special Quality Reagent, 35%) is added to 0.5 cc. of aqueous solution of scotopsin digitonide, heated in a sealed tube at 105~110° for 24 hrs., and cooled. The brown precipitate is filtered off, the filtrate repeatedly concentrated under reduced pressure about 5 times, and dissolved in distilled water for use as a test solution of HCl-hydrolysis. The hydrolysate is dried over H_2SO_4, brought exactly to 0.5 cc. by the addition of distilled water, and 0.1 cc. of it is spotted for paper partition chromatography. In the alkaline hydrolysis, 1 cc. of an aqueous solution of $Ba(OH)_2$ (3.8%) is added to 0.5 cc. of aqueous solution of scotopsin-digitonide and this mixture is heated at 100° for 7 hrs. in an oil bath. The mixture is cooled and correctly neutralized with 1*N* H_2SO_4, and the white precipitate ($BaSO_4$) is washed with hot water. The filtrate is concentrated on a water bath, dried over H_2SO_4, and dissolved in water (0.5 cc.) to be used as a test solution for paper chromatography for which a round glass cylinder of 15 cm. inside diameter and 50 cm. in height is used and kept at a constant temperature (16°). On the bottom of this cylinder is placed a small vessel containing a quantity of the developing solvent, the upper layer of BuOH-AcOH-H_2O (4 : 1 : 5) which has been left standing for 3 days, with the lower layer left around the vessel in the cylinder. The filter paper (Toyo Roshi No. 131) is saturated with the vapor of the solvent for 5 hrs. Development is continued for 21 hrs. by the ascending method. The chromatogram is colored with 0.1% ninhydrin-EtOH solution, as shown in Fig. 4. The amount of alkaline hydrolysate produced was about 20 cc. before concentration. This solution is poured into a column of 1 g. of activated carbon which has been boiled for about 1 hr. in 20% AcOH solution at 100°, and filtered through No. 3 sintered glass filter of 1 cm. inside diameter. The aromatic amino acids remaining adsorbed are eluted with AcOH-PhOH mixture (2.5 g. of phenol dissolved in 50 cc. of 20% AcOH) The phenol layer is then removed by extraction with ether and the aqueous layer is concentrated for confirmation of the presence of aromatic amino acids by paper chromatography as described above. After the development, the paper was washed with ether, sprayed with about 0.5 cc. of a mixture of lutidine-AcOH-EtOH (2 : 15 : 50 by volume) and colored with 0.1% ninhydrin-EtOH solution. The

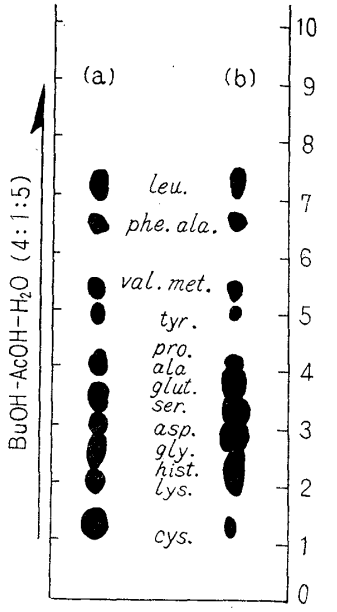


Fig. 4. One-dimensional Paper Chromatography of Scotopsin
(a) Acid hydrolysate (b) Alkaline hydrolysate

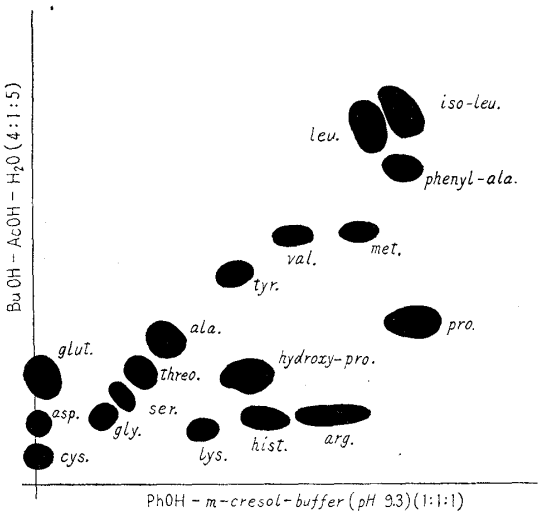


Fig. 5. Two-dimensional Paper Chromatography of Acid Hydrolysate of Cattle Scotopsin

control, i.e. the standard amino acids, phenylalanine, tryptophan, and tyrosine, were confirmed to have their spots at Rf 0.64, 0.58, and 0.50, respectively, coloring purple, reddish purple, and deep purple. From the alkaline hydrolysates of scotopsin only phenylalanine and tyrosine were found on the filter paper. Two-dimensional paper chromatography was carried out by a similar procedure to that described above with (a) BuOH•AcOH•H_2O mixture as described above and (b) the lower layer of a mixture of phenol-*m*-cresol-borate buffer (pH 9.3) (1 g. : 1 g. : 1 cc.) stood for one day. The filter paper (Toyo Roshi No. 131) is first saturated with the solvent (a) for 5 hrs. and developed as high as 30 cm. by the ascending method. The paper is next washed with ether and cut off along a line about 4 cm. below the top of the solvent front. The amino acid portion is coverd with a glass plate so as to bufferize the other part of this filter paper by spraying borate buffer (pH 9.3). When the paper is dry, the development is continued with the solvent (b) until it ascends 25 cm. The paper chromatogram produced is washed with ether and sprayed with lutidine-AcOH-H_2O (2 : 15 : 50) and with 0.1% ninhydrin-EtOH solution for coloration. The amino acids obtained from scotopsin digitonide were as follows: Aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, methionine, valine, phenylalanine, leucine, isoleucine, cystine,

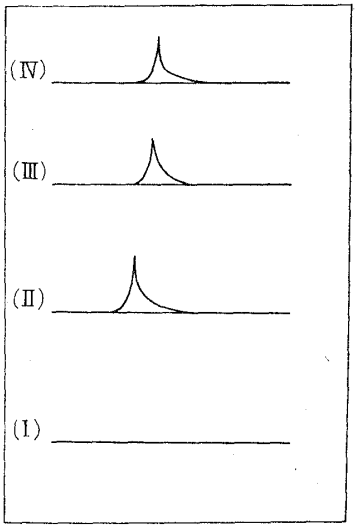


Fig. 6.
Electrophoretic Pattern of Frog Scotopsin by Tiselius-Svenson Apparatus

Veronal buffer (pH 8.6, I=0.1)
Dialysed for 48 hrs.
current···5 mA
potential···130 V
time···1.5 hrs.
temp.···15°

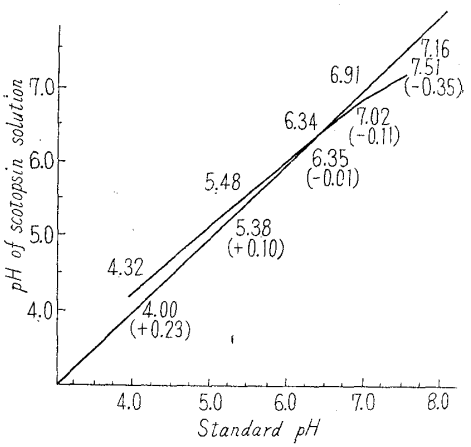


Fig. 7. Isoelectric Point Measurement Diagram

11) E. Kimura : Japan. J. Physiol., **3**, 25(1953).

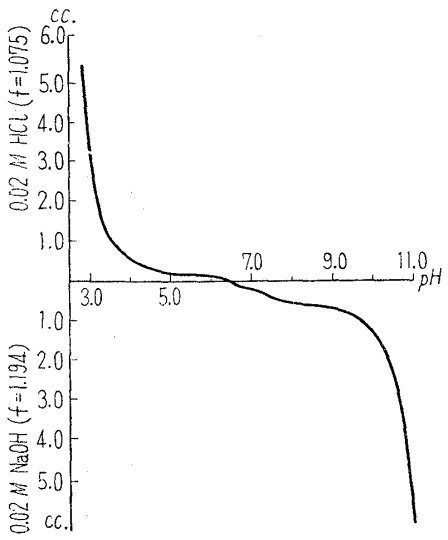


Fig. 8. Titration Curve for Scotopsin Digitonide Solution

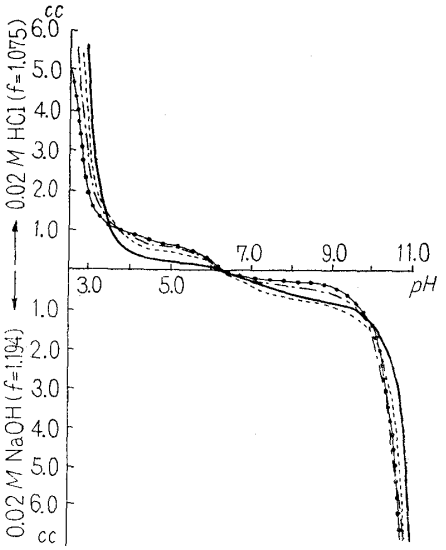


Fig. 9. Effect of Inorganic Salts

——— Aqueous solution of scotopsin digitonide
- - - - - - 0.1*M* KCl // // //
—·—·— 1*M* KCl // // //
—·—·— Satd. KCl // // //

proline, hydroxyproline, lysine, histidine, and arginine.

Titration of Scotopsin Solution—1200 bodies of a frog (*Rana nigromaculata japonica*) were used as experimental animals. The retinas were cut out from their eyeballs after being kept in the dark for 24 hrs.; the outer segments of these retinas were next separated by Kimura's method[11] and purified. These were extracted with digitonin solution (10 cc., 0.18 g. digitonin dissolved in *M*/15 phosphate buffer of pH 6.3). The extract, the original solution, was diluted nine-fold for spectrophotometry of rhodopsin with the Shimadzu Spectrophotometer Type QB-50. In the absorption spectrum of rhodopsin, the optical density ratio D_{400}/D_{500} mμ was 0.3. The test solution, a 100-fold dilution of the original, was bleached in the light for 1 hr. for titration of scotopsin; it was kept under a red light (20 V, 2 candle power) in a dark room during the titration of rhodopsin. The pH value of a dilute solution of scotopsin was measured with a glass electrode pH-meter

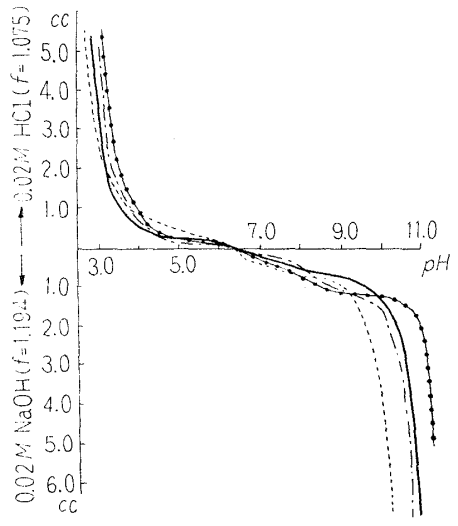


Fig. 10. Effect of Organic Solvents

—·—·— 1*M* KCl-80% EtOH soln. of scotopsin digitonide
—·—·—·— 1*M* KCl-80% MeOH soln. of // //
- - - - - - - - 1*M* KCl-1% HCHO soln. of // //
——— Aqueous soln. of // //

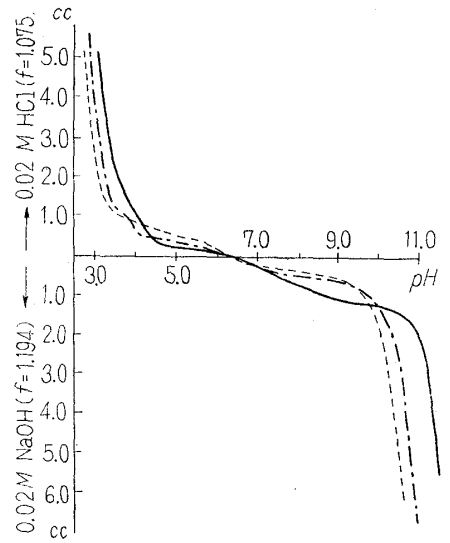


Fig. 11. Effect of Ethanol

——— 1*M* HCl-80% EtOH soln. of scotopsin digitonide
—·—·—·— 1*M* KCl-10% EtOH soln. of // //
- - - - - - 1*M* KCl soln. of // //

Model 40 A. The titration was carried out by using a solution of 0.02*M* HCl (f=1.075) and of 0.02*M* NaOH (f=1.194). The electrophoretic pattern of the original solution is illustrated in Fig. 6. The isoelectric point of scotopsin digitonide was determined as 6.34 by using Miyake's methods, devised for the determination of protamine (cf. Fig. 7). All these experiments were carried out at 16° except when the effect of temperatures was measured.

a) Aqueous Solution of Scotopsin—In the aqueous solution of scotopsin, the pK value of the dissociated radical of amino acid was estimated at 3.8~4.0, 5.6~6.0, 6.5~7.0, and 9.5~10.0 as arranged from the acidic to the alkaline side. These different values are regarded as those for aspartic acid, glutamic acid, histidine, cysteine, lysine, and tyrosine in Cohn and others' report.[12]

b) Effect of Inorganic Salts—The effect of inorganic salts on the titrated value of scotopsin varied according to dilution of 100 times with water, with 0.1*M* or 1*M* of KCl, or with saturated KCl. It was evident in the range of pH 3.5~10, from the isoelectric point to the acid side, the higher the ionic intensity of the KCl solution, the greater was the volume required for titration, and on the alkaline side quite the reverse was the case, and this variable tendency becomes marked in accordance with the variation of the pH from the isoelectric point. Results of this experiment agree with the experiment of Cohn[13] and Cannan[14] for clarification of the relation of certain proteins and the ionic intensity of inorganic salts.

c) Effect of Organic Solvents (Fig. 10)—i) Effect of HCHO : It has been generally conjectured that each amino group loses its basic nature when HCHO solution is added to the solution of proteins.[15] Our experiment has also shown that a dissociated basic radical is actively exchanged in nature in its three fractions, presumably histidine (pH 5.6~7.0), lysine (pH 9.4~10.6), and arginine (pH 11.6~12.6), due to the action of HCHO solution and that HCHO has no action on the carboxyl radical, the pH of which remaining unchanged in value.

ii) Effect of EtOH (Fig. 11) : Comparative examinations showed that the pH value on the acidic side for scotopsin moves towards the alkaline side as the concentration of EtOH increases from 0 to 10% and to 80%. On the alkaline side from pH 10, it shifts to the alkaline side, indicating that EtOH has an effect on the dissociation of both alkaline and acid radicals. The fact was recognized by Lichtenstein[16] in his experiments on gelatin.

iii) Effect of MeOH : The volume of NaOH needed for titration was small when the pH value of scotopsin was 8 or less, and in the alkaline side it was large when the pH value of scotopsin

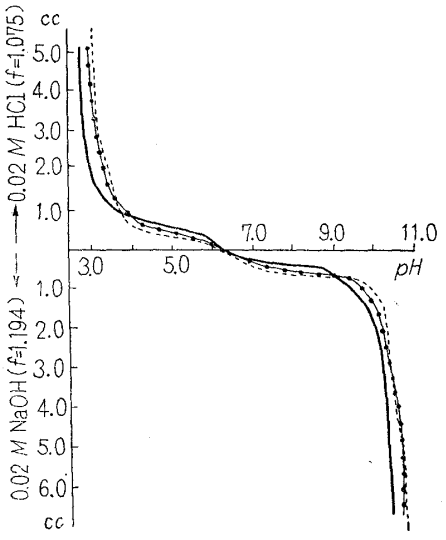


Fig. 12. Effect of Temperature

——— 27° 1*M* KCl soln. of scotopsin digitonide
—•—•— 17° 〃 〃 〃 〃
------ 7° 〃 〃 〃 〃

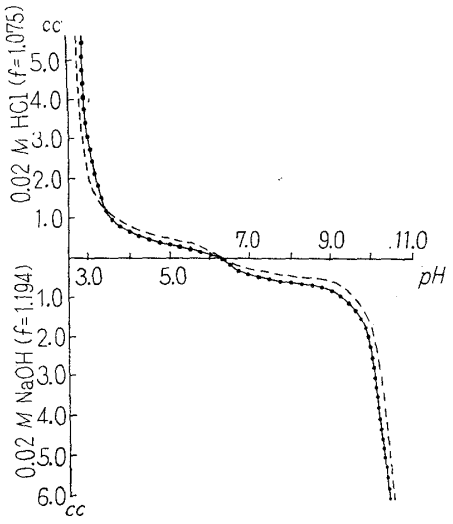


Fig. 13. Difference in Scotopsin and Rhodopsin

------ 1*M* KCl soln. of scotopsin digitonide
—•—•— 1*M* KCl soln. of rhodopsin digitonide

12) E. J. Cohn, J. T. Edsall : "Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions," Reinhold Publ. Corp., New York, 84, 445.
13) E. J. Cohn, A. A. Green, M. H. Branchard : J. Am. Chem. Soc., **59**, 1509(1937).
14) R. K. Cannan, A. H. Palmer, A. Kibrick : J. Biol. Chem., **142**, 803(1942).
15) D. Frenck, J. T. Edsall : Advances in Protein Chem., **2**, 278(1945).
16) I. Lichtenstein : Biochem. Z., **203**, 20(1940).

was over 8. The titration curve for scotopsin in MeOH solution shifted further to the alkaline side, finally to agree with that for the aqueous solution. On the acidic side it was recorded between the corresponding curves for EtOH and aqueous solution.

d) Effect of Temperature (Fig. 12)—At pH 4~9, the volume of HCl needed in titration was greater in proportion to a rise in temperature on the acidic side from the isoelectric point and it was smaller in proportion to a rise in temperature on the alkaline side from the isoelectric point. At pH 9~10 the titration curve for scotopsin shifted towards the acidic side with a rise in temperature. The fact is of great interest when considered in relation to other experiments indicating that a rise in temperature promotes the decomposition of rhodopsin.

e) Difference in Titration Curve between Scotopsin and Rhodopsin—When measured under the same conditions, the titration curve for scotopsin and that for rhodopsin differ from each other most markedly when located at pH 5.6 or so on the acidic side and at pH 8 to 9.5 on the alkaline. A general survey of the experimental results descrived above, concerning the titration curves for several kinds of solvents and the effect of temperature on a scotopsin solution, and of the pK value of the SH and imidazole radical reported by Cohn, *et al.*,[12] leads to the assumption that histidine and cysteine in its protein fraction participate in the fading process of rhodopsin.

Conclusion

1) A method was established for use in the purification of cattle scotopsin which was confirmed to be practically pure by absorption spectrometry and electrophoresis.

2) The molecular weight of scotopsin digitonide determined by the light-scattaring method was 286,000.

3) Amino acids in scotopsin digitonide was found to be composed of aspartic acid, glutamic acid, cystine, glycine, serine, threonine, alanine, tyrosine, valine, methionine, phenylalanine, leucine, isoleucine, hydroxyproline, proline, lysine, histidine, and arginine.[17]

4) The isoelectric point of scotopsin digitonide was 6.34.

5) A series of examination was made for the agents affecting the titration curves for rhodopsin and scotopsin extracted from a frog (*Rana nigromaculata japonica*). It was found that histidine and cysteine are two probable agencies concerned in the fading process of rhodopsin.

The authors are indebted to Mr. K. Hayashi, Faculty of Agriculture, University of Kyushu, for his kind advice in light scattering mesurement. The preparation of scotopsin and paper chromatography were partly carried out with the cooperation of Mr. S. Nishikawa of this Institute to whom acknowledgement is expressed.

Summary

The physical properties of scotopsin, the amino acid component of the substance, and various properties of scotopsin and rhodopsin shown on their titration curves were examined with the object of getting at the clue to clarifying the first process of visual excitement.

(Received July 22, 1957)

17) The quantitative examination has disclosed that glutamic acid is contained in a larger quantity than any other amino acids in cattle scotopsin. These quantitative results will be described in another paper.