[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

THE MOLECULAR WEIGHTS OF PHYCOCYAN AND OF PHYCOERYTHRIN FROM PORPHYRA TENERA AND OF PHYCOCYAN FROM APHANIZOMENON FLOS AQUAE

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In a previous paper a report has been given of the determination of the molecular weights of phycocyan and phycoerythrin from *Ceramium rubrum* by means of the centrifugal methods developed in this Laboratory.[1] For phycocyan the value 106,000 ± 5000 was found within the P_H region 6.8–7.9 and for phycoerythrin the value 208,000 ± 8000 within the P_H region 5.0–6.8. It was pointed out that within the limits of experimental error these values are equal to the molecular weight of egg albumin, 34,500, multiplied by the integers *three* and *six*, respectively. It seemed desirable to extend this study to proteins from other algae and to make determinations within a wider P_H range in order to state the stability regions of these proteins. The alga *Porphyra tenera* was chosen as a representative of the *Rhodophyceae* group not too closely related to *Ceramium*. As a representative of the *Cyanophyceae*, the blue-green algae, *Aphanizomenon flos aquae* was chosen.

Experimental

Preparation of Material

Preparation of Phycocyan and Phycoerythrin from Porphyra tenera.—The Japanese product, "Nori," which consists of dried *Porphyra tenera* was extracted with water as described by Kitasato;[2] 2800 g. was used for this work. Four hundred cc. of water was added for each 10 g. of the material, which was kept in the dark. After some days 1% of toluene was added as a preservative and the extraction was carried on for three weeks. In this way a reddish-violet colored solution was obtained. This solution was treated with about 20% of its weight of ammonium sulfate. The proteins thus precipitated were separated by centrifuging in a bucket centrifuge and brought into solution by washing with distilled water. This solution was then treated with increasing amounts of ammonium sulfate up to 25% of its weight. After each addition it was allowed to stand for a day. The precipitate formed was separated by centrifuging. In this way several mixtures containing different relative amounts of the two substances were formed.

The mixed precipitate was washed with cold water, under which conditions the phycocyan dissolved much quicker than phycoerythrin. By repeating the precipitation with ammonium sulfate and washing the precipitate with cold water, the separation of these substances was carried out completely.

Preparation of Phycocyan from Aphanizomenon flos aquae.[3]—The weed was collected from the surface of a lake near Upsala. The fresh material (30 kg.) was freed

[1] Svedberg and Lewis, THIS JOURNAL, **50**, 525 (1928).

[2] Kitasato, "Acta Phytochimica," **2**, 75 (1925).

[3] The preparation of this protein was carried out by Mr. Arne Tiselius.

from excess of water on a cloth filter and the residue was left to stand for three days, allowing autolysis to set in. Some gas was evolved and the phycocyan began to penetrate the cell membranes.

At this time the putrefaction was stopped by the addition of toluene. After five days the material was centrifuged and the residue was discarded. The supernatant liquid has a blue-green color and is fluorescent. To this a saturated ammonium sulfate solution was added in the proportion of 700 cc. of the ammonium sulfate to 1000 cc. of the liquid. The precipitate thus formed was separated by centrifuging and dissolved in distilled water. Recrystallization was repeated three times with the addition of one volume of saturated ammonium sulfate to five volumes of the solution. The first mother liquor formed by the addition of ammonium sulfate was green. As the process of recrystallization proceeded it became deep blue and strongly fluorescent. Well-defined crystals were formed when the precipitate was kept in an ice box for some days. A photomicrograph of the crystals is shown in Fig. 1. The crystals were dissolved in water and dialyzed against phosphate buffer (P_H 6.8) and against acetate buffer (P_H 4.6). The mother liquor was also dialyzed in the same way.

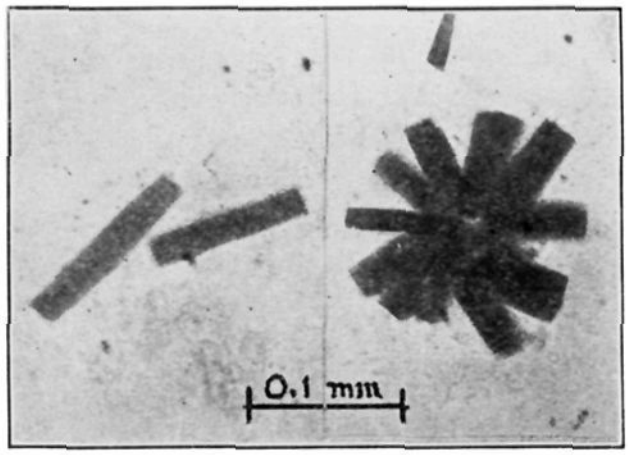

Fig. 1.

Specific Volume.—The partial specific volume was determined pycnometrically at 19.8°. The amount of protein in solution was determined by drying a certain amount of the solution at 105° and subtracting the amount of salt in the sample as known from the composition of the buffer.

The value 0.759 was obtained for phycocyan from *Porphyra tenera* at P_H 6.8 (solvent 0.0167 *M* in KH_2PO_4 and in Na_2HPO_4). The determination by A. Tiselius in this Laboratory gave 0.755 for phycocyan from *Ceramium rubrum*. The average value 0.746 was obtained for phycocyan from *Aphanizomenon flos aquae* at various hydrogen-ion concentrations between P_H 4.6 and 12.0 (solvent: for P_H 4.6, 0.01 *M* in acetic acid and in sodium acetate; for P_H 12.0, 0.01 *M* in Na_2HPO_4 and 0.00864 *M* in NaOH). No appreciable change with P_H was observed. The average value was further found to be almost identical with the values obtained by previous determinations for egg albumin,[4] hemoglobin,[5] serum albumin and serum globulin,[6] phycocyan and phycoerythrin (from *Ceramium rubrum*).[1]

Light Absorption.—The absorption in the visible spectrum was measured with a König–Martens spectrophotometer, and in the ultraviolet with a Judd–Lewis spectrophotometer. Phycocyan and phycoerythrin from *Porphyra tenera* and *Ceramium rubrum* were studied at P_H 6.8. The ultraviolet absorption of the phycocyan from *Aphanizomenon flos aquae* was measured at different hydrogen-ion concentrations. No change was observed between P_H 4.6 to 6.8, but an appreciable change took place

[4] Svedberg and Nichols, THIS JOURNAL, **48**, 3081 (1926).

[5] Svedberg and Fåhraeus, *ibid.*, **48**, 430 (1926); Svedberg and Nichols, *ibid.*, **49**, 2920 (1927).

[6] Svedberg and Sjögren, *ibid.*, **50**, 3321 (1928).

at P_H 12.0, where the protein begins to decompose. The chief results are given in Figs. 2, 3 and 4. The specific extinction coefficient, $\epsilon/c = 1/cd \log (I_0/I)$ (where c is the concentration in per cent., d the thickness of

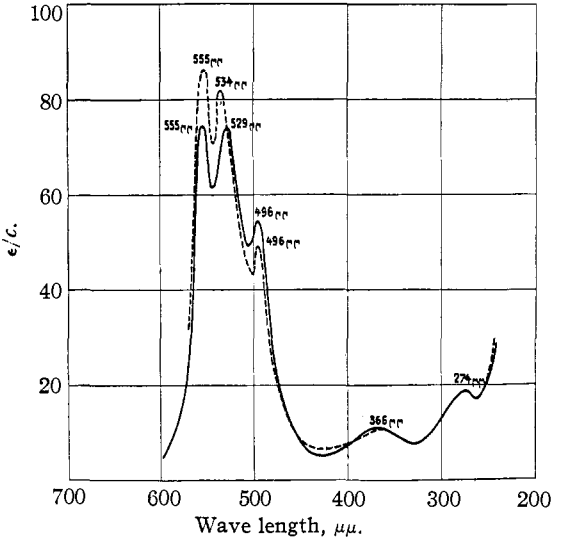

Fig. 2.

the solution, I_0 the intensity of the light after passing through the solvent and I the intensity after passing through the same thickness of solution)

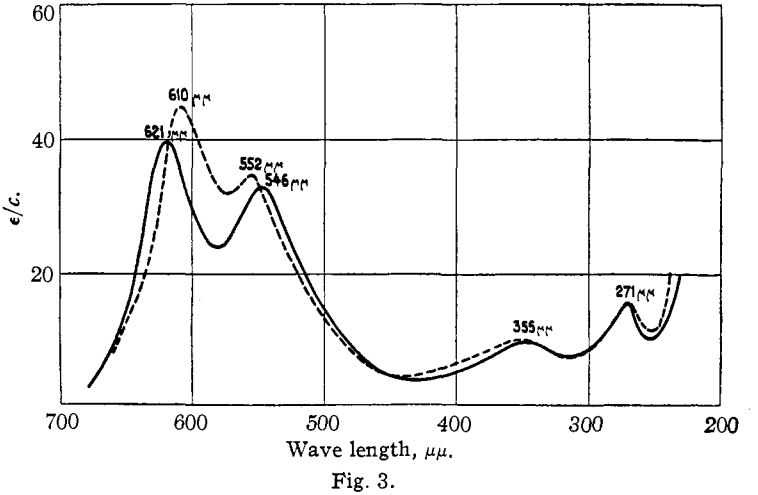

Fig. 3.

is plotted against the wave length. Figure 2, which contains the curves for phycoerythrin from *Ceramium* (solid line) and from *Porphyra* (dotted

line), shows clearly the resemblance of the light absorption of these proteins, as does Fig. 3 in the case of phycocyan from the same two algae. Figure 4 shows that the visible absorption of phycocyan from *Aphanizomenon* is quite different from the absorption of phycocyan from *Ceramium* and *Porphyra*. In the ultraviolet, however, the absorption of phycocyan from *Aphanizomenon* is almost identical with that of phycocyan from

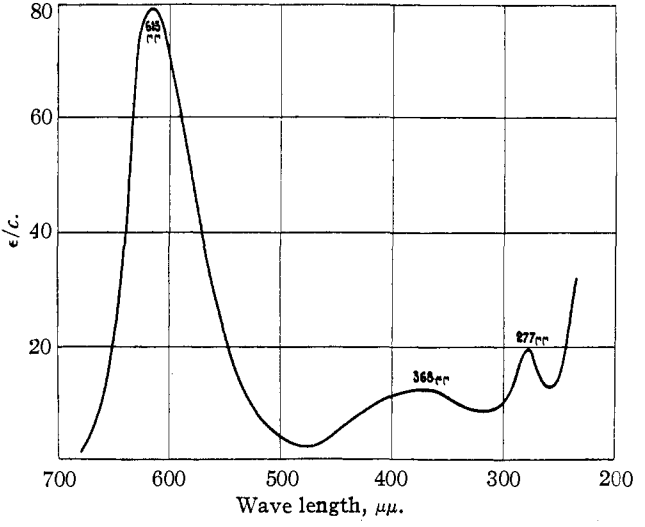

Fig. 4.

Ceramium and *Porphyra*. For the purpose of comparing the results obtained with the values obtained previously by different authors, the positions of the maxima are shown in Table I.

TABLE I

POSITION OF LIGHT ABSORPTION MAXIMA

	Phycocyan from *Ceramium rubrum*		Phycocyan from *Porphyra tenera*	
Prepared by	Kylin[7]	Tiselius	Kitasato	Katsurai
Observed by	Kylin	Katsurai	Kitasato[2]	Katsurai
Orange band	618–613	621	614	610
Yellow band	553–549	546	546	552
Long wave ultraviolet band	.....	350	330	355
Short wave ultraviolet band	.....	271	270	271

The values of the extinction coefficients obtained by Lewis in the ultraviolet region were found to be in error, probably owing to some toluene present. To secure freedom from toluene a current of wet nitrogen gas was blown through the protein solution just before the measurements were made.

[7] Kylin, *Z. physiol. Chem.*, **69**, 169 (1910).

TABLE II

POSITION OF LIGHT ABSORPTION MAXIMA

	Phycoerythrin from *Ceramium rubrum*					Phycoerythrin from *Porphyra tenera*	
Prepared by	Lewis	Lewis	Kylin	Kylin	Tiselius	Kitasato	Katsurai
Observed by	Lewis	Katsurai	Kylin	Katsurai[8]	Katsurai	Kitasato	Katsurai
Yellow band	566	554	569–565	554	560	562	555
Green band	540	528	541–537	536	533	526	534
Blue - green band	497.5	496	498–492	498	496	495	496
Long wave uv. band	360	368	.....	366	366	330	366
Short wave uv. band	262	275	.....	275	275	270	272

TABLE III

POSITION OF LIGHT ABSORPTION MAXIMA

	Phycocyan from *Aphanizomenon flos aquae*	
Prepared by	Tiselius	Boresch[9]
Observed by	Katsurai	Boresch
Orange band	615	620
Long wave ultraviolet band	368	...
Short wave ultraviolet band	277	...

Determination of the Molecular Weight

The molecular weights were determined by the sedimentation velocity and the sedimentation equilibrium methods. The apparatus and procedure have already been described.[10,4,5] The photographs were taken by using the mercury line $\lambda = 366\mu\mu$. In order to cut off the other lines, Wratten nickel-glass was used as a light filter. In the case of the sedimentation velocity method the molecular weight is given by the relation

$$M = \frac{RTs}{(1 - V\rho)\,D}$$

where R is the gas constant, T the absolute temperature, s the sedimentation constant[11] or $1/\omega^2 x \cdot dx/dt$, D the diffusion constant, V the partial specific volume of the protein, ρ the density of the solvent, x the distance from the axis of rotation, ω the angular velocity and t the time. The values of the specific sedimentation velocity and of the diffusion constant were all reduced to 20° by means of the relations $s_1/s_2 = \eta_2/\eta_1$ and $D_1/D_2 = T_1/T_2 \cdot \eta_2/\eta_1$, where η is the viscosity of the solution.

[8] The light absorption of phycoerythrin seventeen years old was measured; ref. 1, p. 532.

[9] Boresch, *Biochem. Z.*, **119**, 167 (1921).

[10] Svedberg, *Z. physik. Chem.*, **121**, 65 (1926); **127**, 51 (1927).

[11] The term "Sedimentation Constant" has been adopted as a simplified means of expressing the old term "Specific Sedimentation Velocity."

In the case of the sedimentation equilibrium method, the molecular weight is given by

$$M = \frac{2RT \ln (c_2/c_1)}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)}$$

where R, T, V, ρ and ω have their usual meaning and c_1 and c_2 are the concentrations at the distances x_1 and x_2 from the center of rotation. In all, 51 runs were carried out. The results of all of the determinations are given.

Phycocyan from Porphyra tenera.—The values obtained for the sedimentation constant were independent of the time of centrifuging, but the diffusion constant showed a drift with the time, thus indicating slight heterogeneity of the material. The presence of a non-centrifugible substance was also observed. The value of the diffusion constant used for calculating the molecular weight was computed by an extrapolation procedure, as in the case of edestin.[12] The results of each of the runs are given in Table IV. For P_H 4.6 and 6.8 the same buffers were used as previously mentioned; for P_H 11.0 the buffer was 0.0172 M in Na_2HPO_4 and 0.00282 M in NaOH.

TABLE IV

SEDIMENTATION VELOCITY MEASUREMENTS ON PHYCOCYAN FROM *Porphyra tenera*

Concn., %	P_H	R.p.m., mean	s, cm./sec. $\times 10^{13}$	$D \times 10^7$, cm.^{2}/sec.	M	Concn. of non-centrifugible matter, %
0.17	4.6	24,000	11.11	4.80	206.000	0
.37	6.8	43,000	5.69	5.30	109.000	10
.12	6.8	24,000	6.27	6.10	105.000	35
.10	11.0	41,000	2.06			10

As may be seen from the above table, the protein consists chiefly of molecules of the molecular weight of about 107,000 at P_H 6.8. It is extremely interesting that the molecular weight is about doubled at P_H 4.6 near the isoelectric point. At a P_H of 9.7 the molecules seem to be breaking up, as the s is slightly lower than at P_H 6.8. At higher P_H (11.0) the protein breaks up into small molecules of various sizes.

Determinations of the molecular weight by the sedimentation equilibrium method were carried out at P_H 6.8.

Values from 32,000 to 104,000 were obtained in one of the runs, and values ranging from 42,000 to 124,000 in the other. These results show that the molecules are not uniform. This protein contains to some extent molecules of a molecular weight smaller than 107,000.

In the case of Lewis's measurement of the molecular weight of phycocyan from *Ceramium rubrum*, the protein was prepared from the wet algae which was not dried by the sunlight, and the results of the determination of molecular weight have shown that the molecules are quite uniform. In

[12] Svedberg and Stamm, THIS JOURNAL, **51**, 2170 (1929).

the present work the protein was prepared from the sun-dried material, and the results of the determination show that the molecules are not uniform and that the material contains some smaller molecule.

In order to see the effect of sun drying, the protein was prepared from sun-dried *Ceramium rubrum* and the molecular weight was determined at the same P_H. As the yield of the protein was very small, only one run could be made but the result showed that part of the molecules were decomposed into smaller ones. The chief part of the phycocyan apparently had been completely destroyed in the drying process. This seems to explain the fact that the phycocyan used in the above experiments which had been prepared from sun-dried material did contain to some extent molecules of various sizes.

Phycoerythrin from Porphyra tenera.—In this case both the sedimentation and the diffusion constants were independent of time of centrifuging, thus indicating that the molecules had a uniform mass. Details of a typical run are given in Table V, and the results of all the runs are summarized in Table VI.

TABLE V

TYPICAL SEDIMENTATION VELOCITY RUN WITH PHYCOERYTHRIN FROM *Porphyra tenera*

Concentration, 0.12%; phosphate buffer ($M/60$ in KH_2PO_4 and in Na_2HPO_4) P_H, 6.8; V, 0.747 at 19.8; ρ, 1000; T, 293; length of column, 1.22 cm.; thickness of column, 0.60 cm.; exposure, 30 sec.; mean speed 22,630 r.p.m. ($\omega = 2370$).

Time interval, min.	Δx per 30 min., cm.	Mean x, cm.	$\omega^2 x \times 10^{-7}$	s, cm./sec. $\times 10^{13}$	D, cm.^{2}/sec. $\times 10^7$
45– 75	0.060	4.950	2.77	12.14	6.06
75–105	.070	5.020	2.82	13.84	6.25
105–135	.065	5.085	2.85	12.33	6.12
135–165	.065	5.150	2.88	12.28	6.60
165–195	.065	5.215	2.92	11.83	5.54
195–225	.065	5.280	2.96	11.11	5.13
			Mean	12.25×10^{-13}	5.95×10^{-7}

Molecular weight, 199,000.

TABLE VI

SEDIMENTATION VELOCITY MEASUREMENTS ON PHYCOERYTHRIN FROM *Porphyra tenera*

Concn., %	P_H	Mean speed, r.p.m.	s, cm./sec. $\times 10^{13}$	D, cm.^{2}/sec. $\times 10^7$
0.17	4.6	24,000	11.90	5.00
.65	6.8	43,000	10.90	5.00
.32	6.8	43,000	11.82	5.48
.16	6.8	43,000	11.11	4.55
.16	6.8	22,000	11.71	5.94
.12	6.8	22,630	12.25	5.95
.08	6.8	23,000	11.86	4.77
			Mean 11.65×10^{-13}	5.27×10^{-7}

Mean molecular weight, 214,000.

The values of the sedimentation constant and of the diffusion constant found by Lewis for phycoerythrin from *Ceramium* were 11.30×10^{-13} and 5.22×10^{-7}, respectively.

The results of the determination of the molecular weight by the sedimentation equilibrium method (using a wave length of $366\mu\mu$ for illumination) showed that the molecules are of uniform size with a molecular weight of 213,000. The run made with a shorter wave length ($\lambda = 270\mu\mu$) showed that the molecules are not uniform, indicating that the protein has decomposed and that the decomposition products contain no chromophore group.

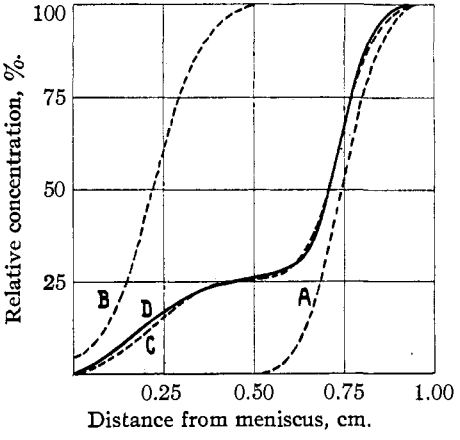

Fig. 5.

A run was made at P_H 11.0 (phosphate buffer 0.0172 *M* in Na_2HPO_4 and 0.0028 *M* in NaOH). The speed was 42,000 r.p.m. and the temperature in the cell was 21.4°. In this case there was a large drift in the diffusion constant with time. From the diffusion curve it was calculated that the protein consisted of 75% of molecular weight 208,000 and 25% of molecular weight 34,600 ($^1/_6 \times 208{,}000$). Figure 5 shows the calculated (C) and the experimental (D) relation between concentration and distance from meniscus after two hours of centrifuging, as well as the curves calculated for the cases in which the solutions were composed only of molecules of mass 208,000 (A) or only of molecules of mass $^1/_6 \times 208{,}000$ (B).

Phycocyan from Aphanizomenon flos aquae.—Throughout the low-speed runs (mean r.p.m. 24,000) the values obtained for the sedimentation constant were independent of the time of centrifuging, but a drift of the diffusion constant was observed, showing the heterogeneity of the molecules. Formation of a non-centrifugible substance was observed. From the diffusion curves of the runs made at high speed (r.p.m. 44,000) the proportion of the molecules of the different molecular weight was calculated. The results of the determination of the sedimentation constant in a low-speed run are summarized in Table VII.

The high-speed run (r.p.m. 44,000) at the same P_H showed that the protein molecules consist of 65% of molecular weight 208,000 and 35% of 104,000. For the purpose of seeing whether any separation can be effected by recrystallization, 10% of ammonium sulfate was added to the solution and the supernatant liquid was dialyzed against the same phos-

TABLE VII

SEDIMENTATION VELOCITY MEASUREMENTS ON PHYCOCYAN FROM *Aphanizomenon flos aquae* AT P_H 6.8

Concn., %	Mean speed, r.p.m.	s, cm./sec. $\times 10^{13}$	Non-centrifugible subs., %
0.11	24,000	11.66	6
.11	24,000	11.41	0
.11	23,000	10.87	3
.12	24,000	11.75	1
.24	23,000	10.82	0
.40	24,000	11.98	0
	Mean	11.41×10^{-13}	

phate buffer solution. The solution thus obtained was studied with a high-speed run. As the result of experiment, 63% of 208,000 and 37% of 104,000 was obtained, showing that practically no separation takes place by fractional crystallization. It is quite interesting to notice that a run carried out at P_H 4.6 showed that the protein molecules in this region are uniform, and have a molecular weight of 223,000. Figure 6 gives the calculated (C) and the experimental (D) relations between the concentration and distance from meniscus after 105 minutes for a solution of P_H 6.8, at 44,000 r.p.m. and 20°. The curves A and B represent the distribution of concentration in solutions containing only molecules of mass 208,000 and $^1/_2 \times 208{,}000$, respectively. From the experimental curve the sedimentation constant of the heavier component was found to be 11.15×10^{-13} and for the other component 5.77×10^{-13}. These values check very well with those found by Lewis for phycoerythrin and phycocyan from *Ceramium* at P_H of 6.8 (reduced to 20°), which are 11.30×10^{-13} and 5.59×10^{-13}, respectively. From a run carried out at very low hydrogen-ion concentration (P_H 12.0) the value 3.24×10^{-13} cm./sec. was obtained for the sedimentation constant, which is about the same as that of egg albumin, *viz.*, 3.32×10^{-13}, found by Nichols.

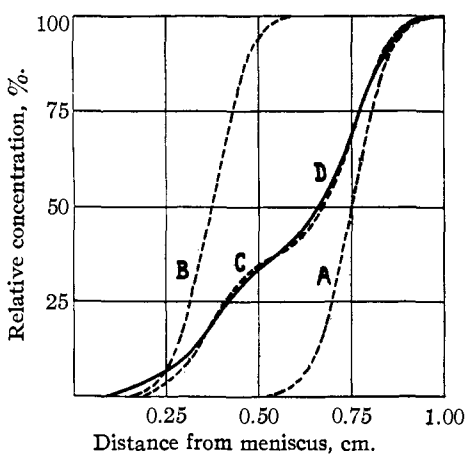

Fig. 6.

For the purpose of seeing whether the change of molecular weight by P_H is reversible, a solution of P_H 6.8 was dialyzed against acetate buffer of P_H 4.6 and the solution thus obtained was studied. As the result of experiment the uniformity of molecules was observed and the value

252,000 was obtained for the molecular weight, showing that the change of molecular weight is reversible.

Determinations of the molecular weight by the sedimentation equilibrium method were also carried out. The protein solution used for the experiment was that obtained by dialyzing the dissolved protein crystals. Two runs made at P_H 6.8 showed that the molecules are of different molecular weight. In one run it was observed that the molecules have a molecular weight from 106,400 to 184,000 and from 117,000 to 200,000 in the other. The run carried out at P_H 4.6 showed the existence of molecules of molecular weight from 115,000 to 207,000.

Discussion of Results

From the results obtained in these experiments, it seems probable that the phycoerythrin from *Porphyra tenera* and from *Ceramium* are identical, and the same is true regarding phycocyan from those algae. The molecular weights and stability regions are probably identical, but further determination is necessary to decide this point completely. Tiselius' determination of the isoelectric points of phycoerythrin and phycocyan from *Ceramium rubrum* showed that these proteins have different isoelectric points, *viz.*, P_H 4.5 for phycocyan and P_H 4.3 for phycoerythrin.

The phycocyan from *Aphanizomenon flos aquae* is different from the above-mentioned proteins. It has a different light absorption and a different isoelectric point (P_H 4.7 as measured by Tiselius) and it seems that the stability region of the molecules is also different. If we consider 34,500 as the unit of the protein molecule and call it A, these proteins consist of molecules of molecular weight A, 3A and 6A, and mixtures of these molecules. It is worth noticing that the existence of the intermediate molecular weights 2A, 4A and 5A was not observed. More determinations are necessary to make up the complete chart of the molecular weights of these proteins as a function of P_H. For the sake of convenience, the names R-Phycoerythrin and R-Phycocyan are proposed for the proteins from *Porphyra tenera* and *Ceramium rubrum* from the name of the family *Rhodophyceae* to which they belong and C-Phycocyan for that from *Aphanizomenon flos aquae* from *Cyanophyceae*.

The expenses connected with these experiments have been defrayed by grants from the Nobel Fund of Chemistry and from the foundation "Therese och Johan Anderssons Minne."

Summary

1. The specific volume, light absorption and molecular weights of phycoerythrin and phycocyan from *Porphyra tenera* and of phycocyan from *Aphanizomenon flos aquae* have been determined at different hydrogen-ion concentrations.

2. The specific volumes of these proteins are identical and independent of P_H and, within the limits of error, also identical with the specific volumes of all other proteins studied in this Laboratory, except the hemocyanins. This fact is in line with the result of the molecular weight determinations, according to which all the proteins in question are loosely built up of units of approximately the same mass, *viz.*, 34,500. It is also probable that they have the same general constitution.

3. The light absorption of the phycoerythrins from *Porphyra* and *Ceramium* are identical and the same is the case for the corresponding phycocyans. The light absorption of phycocyan from *Aphanizomenon* is different from that of the *Rhodophyceae* proteins.

4. Near the isoelectric points the molecular weights of these three proteins are identical and approximately 208,000 ± 5000. With increasing P_H decomposition of the molecules takes place. The phycoerythrin is the most stable one. At P_H 6.8 it is still undecomposed but at P_H 11.0 it consists of a mixture of 75% 208,000 and 25% $1/6 \times 208{,}000$. Phycocyan from *Porphyra* shows indications of decomposition at P_H 6.8 but the bulk of it consists of $1/2 \times 208{,}000$ at that P_H; at P_H 11.0 it is decomposed into small units of varying size. Phycocyan from *Aphanizomenon* is decomposed into a mixture of 65% of 208,000 and 35% of 100,000 at P_H 6.8 and is completely decomposed into the protein unit $1/6 \times 208{,}000$ at P_H 12.0.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF THE WASHINGTON SQUARE COLLEGE OF NEW YORK UNIVERSITY]

THE PYROCHEMICAL DECOMPOSITION OF AZIBENZIL

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Introduction

Schroeter's[1] method for the preparation of diphenylketene has been recommended by Staudinger[2] in a slightly modified form as the most convenient source of that substance. The operations involved are (1) the preparation of benzil monohydrazone, (2) oxidation of this in benzene with mercuric oxide to azibenzil and (3) decomposition of azibenzil at 110–120° to nitrogen and diphenylketene in an atmosphere of carbon dioxide. During the preparation of diphenylketene in this Laboratory by this procedure certain hitherto unreported observations were made. The purpose of the present communication is to report on these, and also to describe a convenient and somewhat modified procedure used successfully by us in the synthesis of diphenylketene.

[1] Schroeter, *Ber.*, **42**, 2336 (1909).

[2] Staudinger, *ibid.*, **44**, 1619 (1911).