*Studies on DNP-Protamines by Means of the Absorption Spectra**

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Studies on chemical structures of protamine molecules, such as clupeine and salmine obtained from sperms of *Clupea pallasii* and *Oncorhynchus keta* respectively, which were purified as now possible, have been carried out from various quarters by many investigators in our laboratory and the summarizing results have been briefly reported[1-5]. From the results of such various methods, viz. qualitative analyses of the constituent amino acids, methoxyl determinations of the protamine methyl esters[5,6] and electrometric titrations of protamines[7],* it was found that both protamine molecules are probably constituted of one simple polypeptide chain, containing neither diamino acids except arginine, nor dicarboxylic acids and sulfur containing amino acids. The DNP-method by F. Sanger[8] for the identification of N-terminal amino acids in proteins in which the α-amino groups are free, has already been applied to many proteins and peptides. The resulting yellow DNP-derivative either of protein or peptide has always a characteristic absorption spectrum which is associated with the presence of N-2,4-dinitrophenyl amino group. It is, therefore, likely that the determination of the wave lengths ($\lambda_{max.}$) and the absorption coefficients ($\epsilon_{\lambda max.}$) at their maxima, will lead in some cases to identify and estimate directly the N-terminal residue of the DNP-derivative, without laborious treatments of isolating DNP-amino acids after hydrolysis and fractionation.

In the present paper, the absorption spectra of DNP-derivatives of several amino acids and a few peptides were first determined and the differences among them were carefully investigated. Then, the absorption spectra of both DNP-protamines were observed, in order to compare them with those of the individual amino acids and peptides. Thus, such a method was found to be useful for the direct indentification of the N-terminal residue of clupeine or salmine. In fact, it was shown that the N-terminal residue of clupeine should be a mono amino acid while that of salmine should be proline, and moreover the calculation of the approximate values of the molecular weights of the protamines was enabled from their absorption coefficients.

Experimental

DNP-Amino Acids and -Peptides.—These were prepared according to the methods of F. Sanger and R. R. Porter[8,9] (cf. Table I). Purifications and crystallizations of DNP-derivatives were repeated until they gave the constant absorption coefficients at their $\lambda_{max.}$ respectively. In the case of DNP-arginine derived from arginine hydrochloride, it was ascertained by means of electrometric titration and paper chromatographic methods that only the α-amino group was replaced by DNP-group. Diglycylglycine used in this experiment was kindly supplied by the laboratory of Prof. Mizushima of the Chemical Institute in this University.

2,4-Dinitrophenol.—A material of commercial grade was purified by repeated recrystallizations from methanol using active charcoal, m.p. 114°C.

2,4-Dinitroaniline.—It was prepared by the method of O. Kym[10] by heating 2,4-dinitrochlorobenzene with acetamide at 200°C for ten hrs. It was then recrystallized from aqueous alcohol, m.p. 180°C.

Protamine Sulfates.—Each sample of protamines used, clupeine and salmine, was prepared and purified respectively by the method described in the previous papers,[1,2] and they were both the sulfate of the less soluble protamine of two fractions which were obtained using the solubility difference of their picrates in 67% aqueous acetone at

* The present work was presented to the 5th Annual Meeting of the Chemical Society of Japan held on April 3rd, 1952 in Tokyo Univ. and a part of the result of this work was briefly reported in this Bulletin (cf. reference 3).

1) T. Ando and K. Iwai, *Repts. Radiation Chem. Res. Inst.*, Tokyo Univ., No. 3, 14 (1948).

2) T. Ando and C. Hashimoto, *Repts. Radiation Chem. Res. Inst.*, Tokyo Univ., No. 4, 31 (1949).

3) T. Ando, S. Ishii, C. Hashimoto, M. Yamasaki and K. Iwai, This Bulletin, 25, 132 (1952).

4) T. Ando, K. Iwai, M. Yamasaki, C. Hashimoto, M. Kimura, S. Ishii and T. Tamura, This Bulletin, 26, 406 (1953).

5) T. Ando and C. Hashimoto, *Repts. Radiation Chem. Res. Inst.*, Tokyo Univ., No. 5, 56 (1950).

6) K. Iwai, The 5th Ann. Meeting of the Chemical Society, Japan (Tokyo, April, 1952).

7) C. Hashimoto, The 5th Ann. Meeting of the Chemical Society, Japan (Tokyo, April, 1952).

* The experimental results will be reported in the following paper.

8) F. Sanger, *Biochem. J.*, 39, 507 (1945).

9) F. Sanger and R. R. Porter, *Biochem. J.*, 42, 287 (1948).

10) O. Kym, *Ber.*, 32, 3539 (1899).

TABLE I
WAVELENGTHS AT ABSORPTION MAXIMA AND MOLECULAR ABSORPTION COEFFICIENTS OF DNP-DERIVATIVES§

DNP-Derivative	Absorption Maxima $\lambda_{1max.}$ mμ	($\lambda_{2max.}$) mμ	Molecular Absorption Coefficient at $\lambda_{1max.}$
DNP-Glycine	361	(265)	1.66×10^4
〃-Alanine	361	(265)	$1.63\times$ 〃
〃-Valine	363	(265)	$1.69\times$ 〃
〃-Isoleucine	362	(265)	$1.77\times$ 〃
〃-Arginine	357	(275)	$1.65\times$ 〃
〃-Serine	359	(265)	$1.72\times$ 〃
〃-Proline	387	(none)	$1.85\times$ 〃
〃-Glycylglycine	354	(265)	$1.52\times$ 〃
〃-Diglycylglycine	352	(265)	$1.52\times$ 〃
2,4-Dinitrophenol§§	361	(257)	$1.48\times$ 〃
2,4-Dinitroaniline	347	(262)	$1.28\times$ 〃

§ Controlled in pH ca. 9.1 by using the 1% aq $NaHCO_3$.
§§ This absorption band is probably due to 2,4-dinitrophenolate ion.

0°C. These samples* were shown to be tentatively homogeneous by the diffusion measurement[11].

Preparation and Purification of DNP-Protamine Sulfates.—DNP-Protamine sulfates were prepared principally according to the method of F. Sanger[8]. Protamine sulfate (0.104 g.) and sodium bicarbonate (0.104 g.) were dissolved in water (5 ml.), a solution of 2,4-dinitrofluorobenzene (0.12 g.) in ethanol (3 ml.) was then added and the mixture was mechanically shaken for about 4 hr. at the room temperature (20—25°C). The glutinous yellow products obtained were cooled, a few drops of 2 N sulfuric acid were carefully added to them, and then alcohol was added. The mixture was then centrifuged and the precipitates were washed with absolute alcohol and ether. Since the crude DNP-protamine sulfate thus obtained still contained a small quantity of dinitrophenol, some inorganic salts and unsubstituted protamine, further purification of it through activated alumina was tried to eliminate them. The crude DNP-product (0.100 g.) was completely dissolved in water (80 ml.), and the solution was carefully passed through an activated alumina column (Wako Pure Chemical Industries, Ltd., especially prepared for chromatographic purpose, 13 g., 1×15 cm.). DNP-Protamine remained at the top of the column giving a sharp yellow band and the unsubstituted protamine seemed to stay in the lower part, while dinitrophenol and inorganic salts passed through rapidly. After the column was washed with water, the colorless part of the protamine was first eluted with 0.1 N hydrochloric acid, and then the yellow substance at the top was eluted. The collected eluate of the yellow band was once filtered, and one or two drops of 2 N sulfuric acid were added, followed by several volumes of alcohol. Precipitates of yellow crystal-like sulfate were thus obtained. By repeating the above treatment, they were purified until their absorption coefficient at $\lambda_{max.}$ gave constant values. Both DNP-clupeine and -salmine sulfates obtained were yellow crystal-like substances.

Determination of Absorption Spectra.—Absorption spectra of various DNP-derivatives above obtained were determined with a Beckman quartz spectrophotometer, model DU. In the case of protamine sulfates, their 0.5% aqueous solution was used (pH 3.3). In order to get rid of the pH effect, DNP-derivatives were dissolved in 1% aqueous solution of sodium bicarbonate to keep pH at 9.1±0.2. All measurements were made at 18—20°C. The concentration of DNP-derivative solution used in these absorption measurements was always below 50 μM, so that Beer's law held good in all cases.

Results and Discussion

The results of measurements of absorption spectra for 2,4-dinitrophenol, 2,4-dinitroaniline and N-2,4-DNP-derivatives of several amino acids and peptides are shown in Fig. 1 and in Table I. As shown in Fig. 1, dinitroaniline

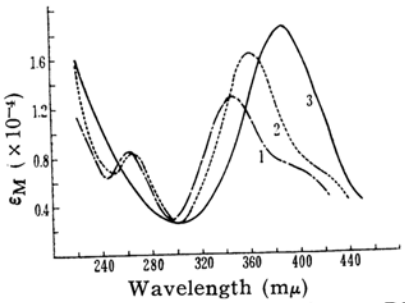

Fig. 1. Absorption spectra of some DNP-compounds in 1% aq.$NaHCO_3$.
Curve 1: Dinitroaniline; Curve 2: DNP-Alanine; Curve 3: DNP-Proline.

gave a characteristic absorption curve with maxima at the regions of 347 mμ and 262 mμ. But the absorption maxima of DNP-derivatives of monoamino acids, i. e. DNP-glycine, DNP-alanine etc., were observed at about 360 mμ and 265 mμ, being in longer wavelengths than those of dinitroaniline. α-DNP-Arginine also showed the absorption maxima at 357 mμ and 275 mμ, which were involved in nearly the similar regions as in the case of DNP-monoamino acids. In contrast with them, DNP-proline gave a characteristic absorption curve which had only a maximum at 387 mμ, a longer wavelength than DNP-derivatives of the above mentioned monoamino acids, and its absorption coefficient was higher than the latter, and no more absorption band could be seen near 265 mμ. The absorp-

11) K. Iso, T. Kitamura and I. Watanabe, *J. Chem. Soc. Japan*, 75, 342 (1954).
* Further experiments are being made on the problem whether these samples are homogeneous or heterogeneous.

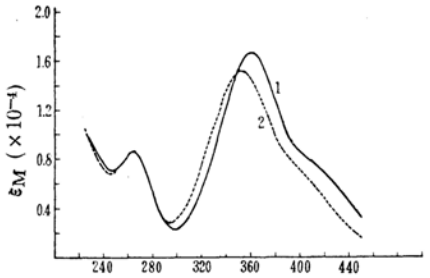

Fig. 2. Absorption spectra of DNP-glycine and DNP-diglycylglycine in 1% aq. $NaHCO_3$.
Curve 1: DNP-Glycine; Curve 2: DNP-Diglycylglycine.

tion spectra of DNP-glycylglycine and DNP-diglycylglycine showed the fairly similar shape to that of DNP-glycine, but both their maxima slightly shifted to the shorter wave-length side, and moreover their absorption coefficients were a little lower than that of DNP-glycine (cf. Fig. 2 and Table I). Sanger has also indicated that the DNP-glycyl peptides separated from insulin gave the same curve as DNP-glycine and DNP-glycylglycine at wave-lengths longer than 330 mμ[12].

From the measurements of these absorption spectra, it will be inferred that the difference among the curves of DNP-derivatives may mostly be attributed to the states of an amino group combining with the dinitrophenyl group, thus: in primary (dinitroaniline), secondary (DNP-monoamino acids or DNP-arginine), or tertiary amine (DNP-proline). The absorption maximum appearing in the longest wave-length region was found to shift to the longer wave-length side, in the order of primary<secondary<tertiary amine, and the absorption coefficient also was found to be higher in the same order. So far as the nitrogen combined with the DNP-group behaves as a secondary amine, the absorption curve of such a derivative seemed to show little difference whichever amino acid it may belong to. It was found also that the DNP-derivatives of glycylglycine and glycylpeptide gave absorption spectra essentially similar to that of the corresponding DNP-amino acid like DNP-glycine. Accordingly, from careful observations of the absorption spectra of the DNP-derivatives, it should at least be possible to determine whether proline or any other kind of monoamino acids occupies the α-N-terminal residue in the protein molecule, and further to estimate the number of N-terminal residues per 1 g. protein.

The absorption spectra of DNP-derivatives of clupeine and salmine are given in Fig. 3 and Table II.

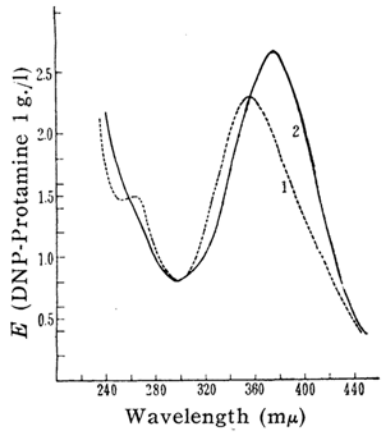

Fig. 3. Absorption spectra of DNP-Protamines in 1% aq. $NaHCO_3$.
Curve 1: DNP-Clupeine; Curve 2: DNP-Salmine.

As shown in the figure, DNP-clupeine gave two absorption maxima at about 357 mμ and 262 mμ, while DNP-salmine only one maximum at 375 mμ. Since neither clupeine nor salmine has absorption band with wave-length longer than 240 mμ, the absorption spectra

TABLE II

WAVELENGTHS AT ABSORPTION MAXIMA OF SOME DNP-PROTAMINES AND MOLECULAR WEIGHTS OF THE PROTAMINES

Protamine	Absorption Maxima of DNP-Derivative $\lambda_{1max.}$	($\lambda_{2max.}$)	Extinction†† $E\lambda_1$(1 g./l)	Molecular weight††	N-terminal residue
	mμ	mμ			
Clupeine (1951)†	357	(262)	2.23_3	7,100	Ala
Clupeine (1947)†	357	(262)	2.79_2	5,600	Ala
Salmine (1951)†	375	(None)	2.64_4	7,000	Pro
Salmine††† (1951)†	375	(None)	2.72_7	6,600	Pro

† The number in parenthesis shows the year the material was caught.
†† These values are the averages of two or three experiments.
††† This sample was kindly supplied by Dr. Watanabe's Laboratory.

12) F. Sanger, *Biochem. J.*, 45, 563 (1949).

above observed for both DNP-protamines are surely associated with the presence of a DNP-group. Comparing the results with those for DNP-amino acids, therefore, it can be concluded with a high probability that the N-terminal residue of clupeine is a mono-amino acid other than proline while that of salmine is proline. These results were consistent with the facts obtained in our laboratory; namely the N-terminal residue of our clupeine was shown to be alanine while that of salmine was proline by means of paper chromatography of DNP-protamine hydrolysates[3)4)], and partly by means of electrometric titration of both protamines.

Now, the number of DNP-groups per 1 g. of DNP-protamine* should be estimated from the absorption coefficient for the maxima at either 357 mμ or 375mμ. An assumption was made that the molar absorption coefficient of DNP-clupeine at 357 mμ is equal to that of DNP-alanine at its absorption maximum 361 mμ. As shown in Table II, the absorption coefficient of DNP-clupeine (1g./l) measured with an optical path-length 1 cm., at $\lambda_{max.}=$ 357 mμ was 2.233, while that of DNP-alanine (1 mol/l) with the same path-length, at $\lambda_{max.}=361$ mμ was 1.63×10^4 (Table I). Then it follows that there are $2.233/1.63\times10^4=1.37\times10^{-4}$ mol. DNP-groups in 1 g. of DNP-clupeine. As has already been shown, a clupeine molecule is a simple peptide chain consisting of arginine and several kinds of monoamino acids. The molecular weight for DNP-clupeine is thus $1/1.37\times10^{-4}=7{,}299$; accordingly that of free clupeine is 7,133. The molecular weight of clupeine is also calculated to 7,080 and 6,637, from the molecular absorption coefficients at 357 mμ of DNP-alanine (1.62×10^4) and at $\lambda_{max.}=352$ mμ of DNP-diglycylglycine (1.52×10^4) respectively.

Under the similar assumption that the molar absorption coefficient per DNP-group of DNP-salmine at $\lambda_{max.}=375$ mμ, is equal to that of DNP-proline at $\lambda_{max.}=387$ mμ, the following calculation may be made. The number of DNP-group in 1 g. of DNP-salmine is $2.644/1.85\times10^4=1.43\times10^{-4}$. It was already known that the salmine molecule also was a simple peptide chain consisting of arginines and other monoamino acids; consequently the molecular weight of DNP-salmine is calculated to $1/1.43\times10^{-4}=6{,}993$ and that of free salmine to 6,827. It also gives a value 6545, when calculated from the molar absorption coefficient of DNP-proline at 375 mμ (1.78×10^4) instead of using the coefficient for $\lambda_{max.}=387$ mμ.

Another sample of clupeine prepared from the ripe sperms of herrings caught in 1947 and that of salmine prepared in another laboratory*, also gave molecular weight 5,600 and 6,600 respectively, as shown in Table II.

From these results, it appears that both protamines, clupeine and salmine, probably have the molecular weights of the same order of 6,000–7,000. These values are also in fairly good agreement with those obtained by methoxyl determinations of the protamine methylesters, by electrometric titrations of the protamines, and by manometric determination of amino nitrogen in clupeine[4)] (cf. Table III).

TABLE III
MOLECULAR WEIGHTS OF CLUPEINE AND SALMINE

Method	Clupeine§	Salmine§
Methoxyl determination of the esterified product.		
Once esterification.	10,800	7,300
More repeated esterification.	6,100	6,100
Electrometric titration	4,600	4,400
Manometric van Slyke's method	6,400	None
Present method	5,600–7,100	6,100–7,000

§ The less soluble specimens of protamines were used.

Summary

1) DNP-Derivatives of amino compounds gave the characteristic spectral absorption curves, according to the states of the amino groups combined with the DNP-group. It was found, that 2,4-dinitroaniline as a primary amine, DNP-monoamino acids in the case of secondary amine and DNP-proline in that of tertiary amine gave respectively the absorption spectra with different absorption maxima and absorption coefficients (cf. Table I and Fig. 1).

2) The absorption curve of DNP-clupeine was of the similar shape to DNP-derivatives in the states of secondary amines, while that of DNP-salmine in those of tertiary amines. From this fact the difference between the

13) K. E. Rasmussen, *Z. physiol. Chem.*, 224, 97 (1934).

14) R.J. Block and D. Bolling, *Arch. Biochem.*, 6, 419 (1945).

* In fact, sulfate was always used in these experiments. The sulfuric acid content in our protamine sulfate was tentatively supposed to be 20% according to the analyses by K.E. Rasmussen for his clupeine sulfate,[13)] and R. J. Block and D. Bolling for their salmine sulfate.[14)]

* This sample used for comparing with our sample was kindly supplied by Dr. Watanabe's Laboratory in this Institute, and was not fractionated by using the solubility difference of the picrate in 67% aqueous acetone.

N-terminal residues of clupeine and salmine molecules was evidently recognized, that of the former being monoamino acid while that of the latter proline.

3) By comparing the absorption of DNP-protamine with the molecular absorption coefficient of the corresponding DNP-amino acid which occupies the N-terminal residue in the molecule, the molecular weights of DNP-derivatives of clupeine and salmine were estimated. Both clupeine and salmine were shown to have the molecular weights of the same order, 6,000–7,000.

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