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Microanalysis of Proteins and Peptides. II.[1] Fluorescent Labeling of Proteins and a Plasma Membrane Using Cycloheptaamylose-Dansyl Chloride Complex[2]

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A complex between cycloheptaamylose and dansyl chloride was prepared and the product (CDC) was applied to the fluorescent labeling of proteins in aqueous media. Excellent degree of labeling was achieved without using any organic solvent. CDC was found to be moderately soluble in the presence of excess cycloheptaamylose and highly soluble in the presence of urea in aqueous solution. CDC-cycloheptaamylose system was successfully applied to the fluorescent labeling of rat liver plasma membrane. CDC in urea solution provided dansylation method for proteins in a homogeneous system. Rapid dansylation was observed in all the methods described.

Dansyl chloride (5-dimethylaminonaphthalenesulfonyl chloride, DNS-Cl)[4] has widely been used for fluorescent labeling[5] and microanalysis[6] of proteins. However, DNS-Cl is scarcely soluble in aqueous media and consequently used as a suspension in organic solvent-water mixture. This procedure requires vigorous stirring throughout the reaction and prolonged reaction time. Addition of large amount of the organic solvent in order to improve the solubility of the reagent is liable to cause protein denaturation or precipitation. This procedure is not well suited for the microdetermination of proteins and almost inapplicable to the labeling of biomembranes which is susceptible to organic solvent. Recently, Rinderknecht[7] proposed the use of DNS-Cl adsorbed on Celite as a suspension in aqueous solution of proteins. Use of organic solvent was avoided and the procedure was much simplified in this method. However, the reaction was still homogeneous and the degree of labeling (D.L.) was often poor.[5d]

This paper deals with the preparation of the complex of DNS-Cl with cycloheptaamylose (C7A)[8] and the application of the product, C7A-DNS-Cl complex (CDC), to the dansylation of proteins and a plasma membrane. Satisfactory labeling was observed without using any organic solvent.

Materials and Methods

DNS-Cl was the gift of Dr. Y. Fujita, Seikagaku Kogyo Co. C7A was purchased from Hayashibara Biochemical Laboratories, Inc. and once recrystallized from water before use. Bovine serum albumin

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2) Preliminary accounts of this work has been presented: T. Kinoshita, F. Iinuma, and A. Tsuji, *Anal. Biochem.* (1974), in press.
3) Location: *Hatanodai-1, Shinagawa-ku, Tokyo.*
4) N. Seiler, "Methods of Biochemical Analysis," Vol. 18, ed. by D. Glick, Interscience Publishers, Inc., New York, 1970, pp. 259—337.
5) *a*) G. Weber, *Biochem. J.*, **41**, 145 (1952); *b*) R.F. Chen, *Arch. Biochem. Biophys.*, **128**, 163 (1968); *c*) F. Kierszenbaum, J. Dandliker, and B. Dandliker, *Immunochemistry*, **6**, 125 (1969); *d*) D.A. Deranleu and H. Neurath, *Biochemistry*, **5**, 1413 (1966); *e*) W.R. Gray, "Methods in Enzymology," Vol. 11, ed. by C.H.W. Hirs, Academic Press, Inc., New York, 1967, pp. 139—151.
6) *a*) K.R. Shelton, *Biochem. Biophys. Res. Commun.*, **43**, 367 (1971); *b*) T. Nakajima, H. Endou, F. Sakai, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **18**, 1935 (1970).
7) H. Rinderknecht, *Nature*, **193**, 167 (1962).
8) F. Cramer and H. Hettler, *Naturwissenschaften*, **24**, 625 (1967).

(BSA, fraction V), bovine α-chymotrypsin, and egg white lysozyme (6-times recrystallized) were obtained as described in the preceding paper.[1] Ovalbumin was purchased from Nutritional Biochemicals Corporation. Absorption and fluorescence spectra were taken on a Hitachi Model EPS-3T recording spectrophotometer and a Hitachi Model MPF-2A fluorescence spectrophotometer. Fluorescence intensity was measured with a Hitachi Model 103 fluorescence spectrophotometer.

Preparation of CDC——Six gram of C7A was dissolved in 300 ml of water on a boiling waterbath and the solution was cooled below 25° in a tap water. To this solution was added a solution containing 1.2 g of DNS-Cl in 5 ml of acetone dropwise under stirring in the course of 15 min. Yellow precipitate deposited on addition of the chloride solution. The mixture was then left standing in an ice bath for 30 min. The precipitate was collected by centrifugation and dried over P_2O_5 under reduced pressure giving 5.6 g of CDC as a yellow powder. IR in KBr disc showed absorption maxima at 3340 (O–H), 2890 (C–H) and 1610 (C=C) cm^{-1}. This powder was extracted with methanol and DNS-Cl was estimated by measuring the absorbance at 370 nm.

Dansylation of Proteins by CDC Procedure——To a solution containing 10 mg of protein in 1 ml of 0.1M phosphate buffer, pH 7.7 was added 10 mg of CDC and the mixture was stirred in an ice bath or at room temperature. After an appropriate period (5—60 min) undissolved material was removed by centrifugation if necessary, and the resultant solution was then passed through a column of Sephadex G-25 (1.2×18 cm) to remove residual DNS-Cl, dansyl amide, dansyl sulfonic acid and C7A. Dansylated protein was eluted from the column using 0.1M phosphate buffer, pH 7.7, as an eluant. D.L. for the protein was estimated by measuring the absorbances at 280 nm and 335 nm according to Mihalyi and Albert.[9] For the calculation of D.L. were used molar absorptivity 3370 at 335 nm[5b] and the ratio 0.34[9] of absorbances at 280 nm and 335 nm for protein bound dansyl group. Values of molecular weights (m.w.) and molar absorptivities (ε) for the proteins employed were as follows:[10] BSA, m.w.=69,000, $\varepsilon=4.55\times10^4$; ovalbumin, m.w.=45,000, $\varepsilon=3.3\times10^4$; chymotrypsin, m.w.=25,000, $\varepsilon=5\times10^4$; lysozyme, m.w.=14,000, $\varepsilon=3.8\times10^4$.

Dansylation of Proteins by CDC-Urea Procedure——To a solution containing 10 mg of a protein in 0.2 ml of 0.4M phosphate buffer, pH 7.7, was added a solution containing 50 mg of CDC in 0.8 ml of 8M urea, and the resultant clear solution was left standing for an appropriate period (5—60 min) in an ice bath or at room temperature. The labeled protein was purified and D.L. was estimated as described above.

Dansylation of a Plasma Membrane——A suspension of rat liver plasma membrane in water containing 1 mM $NaHCO_3$ and 1 mM $CaCl_2$ was prepared by the method of Emmelot[11] with slight modifications.[12] Protein concentration of this suspension was 1.33 mg/ml as estimated according to Lowry.[13] CDC-C7A solution for dansylation was prepared by stirring a mixture of 30 mg of CDC and 0.3 g of C7A in 15 ml of water for 5 min and filtering the mixture. To 3.8 ml of the membrane suspension in an ice bath was added 8.2 ml of the chilled CDC-C7A solution and the resultant mixture was kept at 1—3°. At intervals a 3 ml aliquot of the reaction mixture was taken out and centrifuged at 13000 rpm for 10 min. The precipitate was washed 3 times with each 3 ml of 16 mM C7A containing 1 mM $NaHCO_3$ and 1 mM $CaCl_2$, and then twice with each 3 ml of the same solution from which C7A was omitted, giving labeled membrane. On the other hand, to the combined supernatant and washings was added equivolume of 1 mM NaOH to convert the remaining DNS-Cl to dansyl sulfonic acid and the absorbance of the latter was measured at 320 nm after 30 min. The amount of dansyl group introduced into the membrane was calculated from decrease in the absorbance.

Results

Preparation of CDC

CDC was readily prepared by addition of DNS-Cl to aqueous solution of C7A. This compound is sparingly soluble in most organic solvents but more soluble in water than DNS-Cl itself. DNS-Cl was readily extracted from CDC with organic solvents such as methanol or acetone. Formation of 1:1 complex between C7A and DNS-Cl was suggested by the spectrophotometric estimation of the chloride after extraction of CDC with methanol.

9) E. Mihalyi and A. Albert, *Biochemistry*, **10**, 237 (1971).
10) "Handbook for Experiment of Biophysics," ed. by Biophysical Association of Japan, Yoshioka-shoten, Tokyo, 1970, pp. 362—372 (in Japanese).
11) P. Emmelot, C.J. Bos, E.L. Benedetti, and R. Randall, *J. Biol. Chem.*, **193**, 265 (1961).
12) S. Iwata, S. Omura, and T. Oda, "Biochemistry of Biomembranes," ed. by M. Nakao, *et al.*, Asaoka-shoten, 1969, pp. 107—117 (in Japanese).
13) O.H. Lowry, N.J. Rowebrough, A.L. Farr, and R. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Dansylation of Proteins by CDC Procedure

When a suspension of CDC in a protein solution is stirred, CDC dissolves with concomitant fluorescent labeling of the protein. In the dansylation of BSA (10 mg/ml) at room temperature and pH 7.7, CDC added (10 mg/ml) dissolved almost completely in the course of reaction for 30 min. Effect of pH on D.L. for BSA is shown in Fig. 1. Marked increase in D.L. with increase in pH value was observed from pH 6.7 to 7.7. Above pH 7.7, increment of D.L. was relatively small. Although CDC is more soluble in alkaline media, high pH may cause denaturation and degradation of proteins. Accordingly, CDC concentration of 10 mg/ml and pH 7.7 were chosen for the standard procedure.

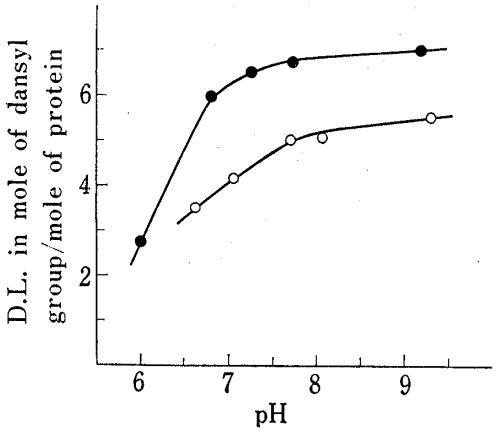

Fig. 1. Effect of pH on D.L. for BSA by CDC Procedure (○) and CDC–Urea Procedure (●)

Dansylation was carried out at 27° for 60 min.

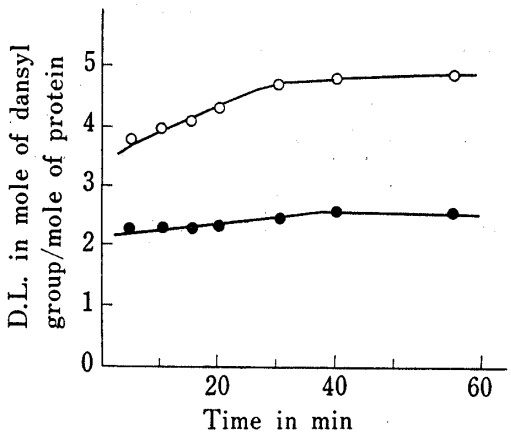

Fig. 2. D.L. for BSA plotted against Time of Dansylation by CDC Procedure at 2° (●) and at 27° (○)

TABLE I. D.L. for Proteins by CDC Procedure and CDC-Urea Procedure

Protein	Reaction conditions[a)]	D.L. in mole of dansyl group per mole of protein	
		by CDC procedure	by CDC-urea procedure
BSA	2°, 15 min	2.19	3.97
	2°, 60 min	2.40	3.93
	27°, 5 min	3.79	4.14
	27°, 30 min	4.80	5.91
Ovalbumin	2°, 15 min	0.58	3.06
	2°, 60 min	0.57	3.36
	27°, 5 min	0.52	3.25
	27°, 30 min	1.05	4.87
Chymotrypsin	2°, 15 min	0.31	0.60
	2°, 60 min	0.45	0.80
	27°, 5 min	1.10	1.34
	27°, 30 min	1.29	3.65
Lysozyme	27°, 60 min		0.54

a) All proteins were dansylated at pH 7.7 except for lysozyme which was labeled in 0.1M sodium borate containing 50 mg/ml CDC in 6.4M urea.

Fig. 2 depicts the D.L. for BSA plotted against varied reaction time in CDC procedure. Plateaus are reached at 40 min and 30 min by reaction at 2° and 27°, respectively. Fig. 2. also demonstrates that reaction of BSA with CDC is very rapid. D.L. at 5 min reaction reaches 80 to 90% of those at the plateaus. Table I shows D.L. for some proteins at pH 7.7 by CDC

procedure. These values are comparable to those by organic solvent method so far reported. For example, D.L. for BSA was reported to be 2.3 (0—3°, pH 7.5—8.2 for 5—12 hr),[5a] and 2.7 (0°, pH 9.2 for 45 min)[5c]; for ovalbumin, 1.7—2.4 (0—3°, pH 7.5—8.2 for 5—12 hr)[5a]; for chymotrypsin, 0.64 (at room temperature, in 0.1 M disodium hydrogen phosphate, for 30 min).[5d] By CDC procedure, reaction for only 5 min at room temperature gave reasonable D.L. of 3.8, 0.5 and 1.1 mole/mole for BSA, ovalbumin, and chymotrypsin, respectively. D.L. was considerably high in the reaction at 27° compared with those at 2°.

Dansylation of Proteins by CDC-Urea Procedure

In this procedure, highly concentrated CDC can be employed owing to its great solubility in the presence of urea. Fig. 3 displays the D.L. at pH 7.7, 27° for ovalbumin and BSA plotted against CDC concentration up to 70 mg/ml which is approximately the saturated concentration. D.L. reaches a plateau for ovalbumin at 20 mg/ml, but no plateau is observed for BSA in the concentration range examined. However, CDC concentration above 50 mg/ml much increases viscosity of the reaction mixture. Effect of pH on D.L. in CDC-urea procedure is exhibited in Fig. 1. The increase in D.L. with pH is diminished above pH 7.7. In the standard CDC-urea procedure was adopted CDC concentration of 50 mg/ml and pH 7.7.

D.L. for some proteins is summarized in Table I. D.L. is improved in CDC-urea procedure compared with CDC procedure.

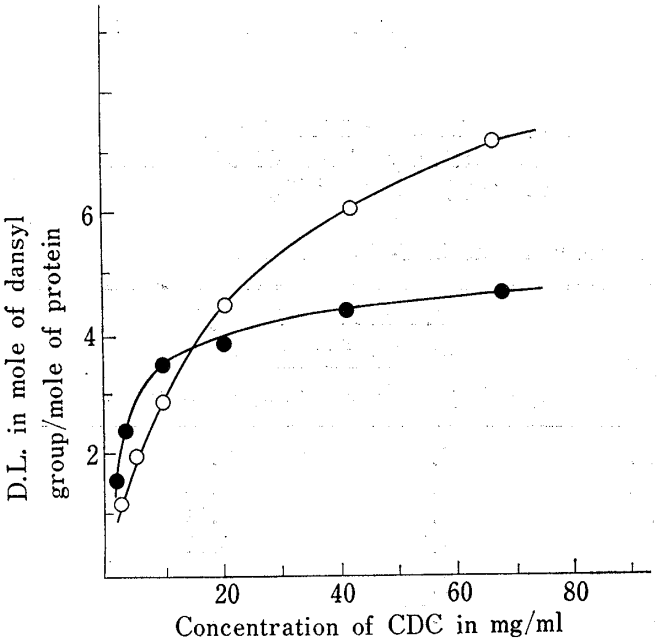

Fig. 3. Effect of CDC Concentration on D.L. for BSA (○) and Ovalbumin (●) in CDC-Urea Procedure

Dansylation was carried out at 27° for 60 min.

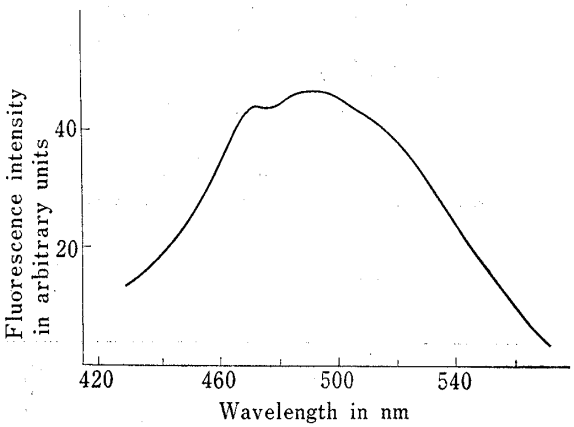

Fig. 4. Fluorescence Spectrum of Dansylated Rat Liver Plasma Membrane Excited at 365 nm

Protein concentration is 250 μg/ml.

Table II. Dansylation of Rat Liver Plasma Membrane by CDC-C7A Reagent at 2°

Reaction time (min)	Dansyl group introduced in the protein (nmole of DNS/mg of protein)
5	100
30	283
60	352

Dansylation of a Plasma Membrane

CDC was proved to be moderately soluble in aqueous solution containing excess C7A and this finding was applied to dansylation of a biological membrane. Table II summarizes the result of dansylation of rat liver plasma membrane in CDC-C7A solution. One hundred nanomoles (24 μg) of dansyl group was introduced per mg of protein in 5 min. Fig. 4 depicts the fluorescence spectrum of labeled rat liver plasma membrane isolated after reaction for 5 min. The dansylated membrane exhibited emission maximum at 490 nm when excited at 365 nm.

Discussion

DNS-Cl was mixed with C7A in aqueous media to give CDC in excellent yield whereas this chloride is extremely susceptible to hydrolysis.[14] In addition, CDC was found to be stable even when exposed to moist air for several days while DNS-Cl alone often decomposes on storage in an imperfectly sealed container. On the other hand, the molar ratio of DNS-Cl and C7A in CDC was indicated to be approximately 1: 1. These results suggest the formation of an inclusion compound between DNS-Cl and C7A. The preceding paper[1] demonstrated the inclusion of dansyl amino acids by C7A. DNS-Cl is assumed to be included in the hydrophobic cavity of C7A. Although the solubility of CDC in water is low, this reagent is moderately soluble in buffered protein solution. This may be partly due to the consumption of DNS-Cl during the reaction and partly due to some interaction of CDC with protein molecule. Satisfactory labeling was observed by reaction for 5 min at room temperature (Table I, Fig. 2).

Although effectiveness of CDC procedure was thus demonstrated, the reaction was still heterogeneous. CDC concentration in reaction mixture was therefore limited. Moreover, the procedure was inadequate for the quantitative analysis[6b] of proteins and for the dansylation of biomembranes which are best purified by centrifugation after the labeling. Interestingly, urea was found to greatly enhance the solubility of CDC and completely homogeneous reaction system was facilitated. Since urea alone does not solubilize DNS-Cl, this effect may be due to interaction between C7A and urea. This problem will be discussed elsewhere. CDC-urea procedure much improved D.L. for ovalbumin and chymotrypsin compared with CDC-procedure (Table I). Reaction for 5 min at room temperature was again indicated to be of practical value. Lysozyme is known to be a protein which is dansylated with poor D.L. Wahl and Lami[15] chromatographed lysozyme dansylated by organic solvent method and obtained a fraction of highest dansylation of 0.35. By means of CDC-urea reagent was obtained an overall D.L. of 0.54 by reaction for 60 min at room temperature.

A preliminary examination for the application of CDC-urea reagent to determination of protein was carried out using a small column of Sephadex G-25 (2×12 mm). Standard curve for BSA was linear in the range of 10 to 40 μg of the protein. CDC-urea reagent seems to be promising for the assay of proteins, although more elegant technique for separation of fluorescent byproducts of low molecular weight is required for better sensitivity.

Susceptibility of biomembranes to organic solvents has hitherto prevented their fluorescent labeling with DNS-Cl. CDC-C7A reagent was found to aid in overcoming this problem and, moreover, to give a highly dansylated plasma membrane in a short period of reaction. Satisfactory labeling of 100 nmole/mg was achieved in 5 min at 2° (Table II). The emission maximum of the labeled membrane was largely shifted to shorter wavelength of 490 nm (Fig. 4) compared with those of dansyl amino acids which fluoresce around 570 nm in aqueous media.[16] Chen[16] reported that dansyl DL-tryptophan fluoresces at 500 nm in dioxane and 498 nm in

14) Z. Tamura and T. Nakajima, *Protein, Nucleic Acid and Enzyme*, **12**, 729 (1967).
15) P. Wahl and H. Lami, *Biochim. Biophys. Acta*, **1969**, 233, 133.
16) R.F. Chen, *Arch. Biochem. Biophys.*, **120**, 609 (1967).

the presence of BSA. The marked blue shift shown by the dansyl group on the rat liver plasma membrane suggests its interaction with a highly hydrophobic environment similar to the hydrophobic region of BSA.

Recently, Schmidt-Ullrich and co-workers[17] reported a dansylation method for erythrocyte membranes. In this procedure, the fluorochrome was dispersed ultrasonically into lecithin-cholesterol micells and this preparation was then added to the membrane to carry out dansylation. However, the present method appears to be more simple and rapid than the lecithin method because of complete homogeneity of the reaction system.

For the fluorescent labeling of proteins and biomembranes, rapid reaction is preferred in order to minimize denaturation. CDC-procedure and CDC-C7A procedure provided rapid dansylation methods for these biopolymers. On the other hand, CDC-urea procedure enabled dansylation of proteins in homogeneous media. The methods herein described are expected to extend the applicability of DNS-Cl in the field of biopolymer research.

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17) R. Schmidt-Ullrich, H. Knüffermann, and D.F.H. Wallach, *Biochim. Biophys. Acta*, **307**, 353 (1973).