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# Microanalysis of Proteins and Peptides. I. Enhancement of the Fluorescence Intensity of Dansyl Amino Acids and Dansyl Proteins in Aqueous Media and Its Application to Assay of Amino Acids and Proteins[1]

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The fluorescence of dansyl amino acids and dansyl proteins in aqueous media was found to be greatly enhanced by addition of cycloheptaamylose. This finding was successfully applied for improvement of the assay procedure of amino acids and proteins. Dansyl amino acids of 50 picomoles per ml in final concentration was estimated. Interaction of cycloheptaamylose with dansyl amino acids was thermodynamically studied. The entropies and the enthalpies were found to vary with polarities of the dansyl amino acid.

Microquantities of biologically active amino compounds such as amino acids,[3] peptides,[3] proteins,[4] and catecholamines[5] are currently determined by the use of dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride, DNS-Cl)[3,6] because of the excellent reactivity and sensitivity of this reagent. Since the rate of dansylation in aqueous media is faster than that in non-polar solvent,[6] dansylation is carried out in aqueous solution. However, dansyl derivatives give poor quantum yield in polar solvent.[3,7] Therefore the compound in the aqueous solution must be transferred to non-polar media prior to measurement of the fluorescence.[3b] This procedure is time-consuming and inapplicable to dansyl derivatives insoluble in organic solvents such as dansyl proteins. In the present study, cycloheptaamylose (C7A)[8] was found to greatly enhance the fluorescence intensity of various dansyl derivatives in aqueous media. An application of this finding to the determination of amino acids and proteins, and thermodynamic studies on the interaction of C7A and dansyl amino acids are also described below.

## Materials and Methods

Dansyl L-amino acids were purchased from Seikagaku Kogyo Co. Stock solutions of dansyl amino acids were prepared by firstly dissolving 5 to 10 mg of the samples in 2 ml of methanol and then diluting to 50 ml with redistilled water. The stock solutions were further diluted as indicated in each experimental regend. Bovine serum albumin (BSA, fraction V), bovine chymotrypsin, egg white lysozyme (6-times recrystallized)

1) Preliminary accounts of this work has been presented: T. Kinoshita, F. Iinuma, and A. Tsuji, *Biochem. Biophys. Res. Commun.*, **51**, 666 (1973).
2) Location: *Hatanodai-1, Shinagawa-ku, Tokyo.*
3) *a*) N. Seiler, "Methods of Biochemical Analysis," Vol. 18, ed. by D. Glick, Interscience Publishers, Inc., New York, 1970, pp. 259—337; *b*) Z. Tamura, *Japan Analyst*, **17**, 908 (1968).
4) T. Nakajima, H. Endou, F. Sakai, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **18**, 1935 (1970).
5) H. Tsuzuki, K. Kitani, K. Imai, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **20**, 1931 (1972).
6) W.R. Gray "Methods in Enzymology," Vol. 11, ed. by C.H.W. Hirs, Academic Press, Inc., New York, 1967, pp. 139—151.
7) *a*) R.F. Chen, *Arch. Biochem. Biophys.*, **120**, 609 (1967); *b*) R.F. Chen, H. Edelhoch, and R.F. Steiner, "Physical principles and Techniques of Protein Chemistry," ed. by S.J. Leach, Academic Press, Inc., New York, 1969, pp. 214—240.
8) *a*) F. Cramer and H. Hettler, *Naturwissenschaften*, **54**, 625 (1967); *b*) F. Cramer, W. Saenger, and H-Ch Spatz, *J. Am. Chem. Soc.*, **89**, 14 (1967).

and protamine sulfate (salmine) were purchased from Wako Pure Chemical Co., Miles Laboratories, Ltd., Seikagaku Kogyo Co. and Nakarai Chemicals, Ltd., respectively. The proteins were dansylated using C7A-DNS-Cl complex (CDC) in urea solution as described in the subsequent paper of this series.[9] Degrees of labeling in mole of dansyl group per mole of protein estimated by the measurement of absorbances[9] were as follows: BSA, 4.03; chymotrypsin, 0.63; lysozyme, 0.54; protamine, 0.47. C7A was the gift of Mr. K. Tsukashima, Nikken Kagaku Kogyo Co., and once recrystallized from water before use. The measurement of fluorescence was performed with a Hitachi Model 103 fluorescence spectrophotometer, thermostated within an accuracy of 0.1°. The absorption spectra were taken on a Hitachi Model EPS-3T recording spectrophotometer.

**Assay Procedures**——For assay of dansyl amino acids, 2 ml of the sample solution containing 100 to 800 pmole/ml of dansyl amino acid was mixed with 2 ml of 16.0 mM C7A solution in 0.2M phosphate buffer, pH 7.4, and the resultant fluorescence was measured at 22.5°. Excitation and emission wavelengths were 365 and 515 nm, respectively. The assay procedure for dansyl proteins was essentially the same as that for dansyl amino acids except that 180 mM C7A solution in 0.2M phosphate buffer, pH 7.4, containing 8M urea was used in place of 16.0 mM C7A.

**Spectral Titrations**——To 2 ml of 13 μM dansyl amino acid solution was added 2 ml of C7A solution in 0.2M phosphate buffer, pH 7.4. The concentration of the C7A solution was varid from 2.6 to 16.0 mM. The fluorescence intensity was evaluated at three different temperatures of 4.0, 22.5 and 40.0° at the same wavelengths as above.

## Results and Discussion

### Establishment of the Assay

Addition of C7A to dansyl amino acid solutions brought shift of absorption spectra to longer wavelength, and of fluorescence spectra to shorter wavelength in the range of 5 to 18 nm with enhancement of fluorescence intensity by a factor of 10 to 20. Fig. 1 shows the absorption spectra of dansyl phenylalanine in the presence of various concentration of C7A. Cramer and co-workers[8b] reported the shift of absorption maxima of various dyes on the formation of inclusion compound with cyclohexaamylose. Fig. 2 demonstrates the fluorescence spectra of dansyl valine in the presence and absence of 8.0 mM C7A. Table I lists the emission spectral maxima (Em) of dansyl amino acids in the presence of 8.0 mM C7A. Em were observed within the range of 510 to 525 nm. Fig. 3 indicates the fluorescence intensity at 515 nm of dansyl valine plotted against varied C7A concentrations. The intensity increased with the concentration of C7A and reached a plateau at 6.7 to 8.0 mM C7A. The C7A concentration was

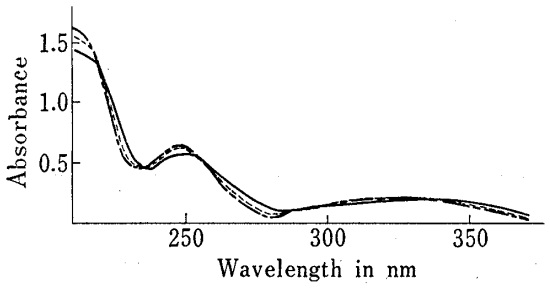


Fig. 1. Absorption Spectra of Dansyl Phenylalanine (50 μM) in the Presence of 0 mM (———), 1.3 mM (------) and 3.8 mM (——) of C7A

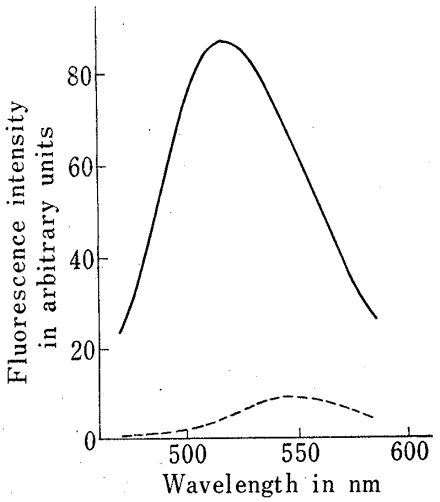


Fig. 2. Fluorescence Spectra of 26 μM Dansyl Valine in the Presence (——) and Absence (------) of 8.0 mM C7A Excited at 365 nm

9) T. Kinoshita, F. Iinuma, and A. Tsuji, *Chem. Pharm. Bull.* (Tokyo), **22**, 2421 (1974).

Table I. Emission Spectral Maxima in nm of Dansyl Amino Acids in the Presence of 8.0 M Urea

| Dansyl amino acid[a] | Em[b] | Dansyl amino acid[a] | Em[b] |
|---|---|---|---|
| DNS–Phe | 510 | DNS–Pro | 520 |
| Bis–DNS–Lys | 520 | DNS–Thr | 520 |
| DNS–Asp | 520 | DNS–His | 512 |
| DNS–Val | 520 | DNS–Gln | 520 |
| DNS–Trp | 520 | DNS–Asn | 520 |
| DNS–Hyp | 525 | DNS–Ala | 520 |
| DNS–Leu | 512 | O–DNS–Tyr | 520 |
| ε-DNS–Lys | 520 | DNS–Glu | 515 |
| DNS–Ser | 520 | | |

*a*) Concentration of dansyl amino acids was 9.0 μg/ml. For abbreviations, refer to Table III.
*b*) excited at 365 nm

fixed to an excess concentration of 8.0 mM and fluorescence intensity was measured at 515 nm unless otherwise indicated.

The above mentioned blue shift of Em and enhancement of fluorescence intensity may be attributable to the interaction of dansyl group with the hydrophobic cavity of C7A.[8] Chen[7a] pointed out that dansyl amino acids adsorbed onto the hydrophobic binding site of serum albumin show marked blue shift and enhancement of the fluorescence. He reported that dansyl DL-tryptophan in aqueous solution shows Em at 498 nm with a quantum yield (Q) of 0.61 in the presence of BSA whereas the same compound shows Em at 578 nm with Q of 0.068 in the absence of the protein. The cavity of

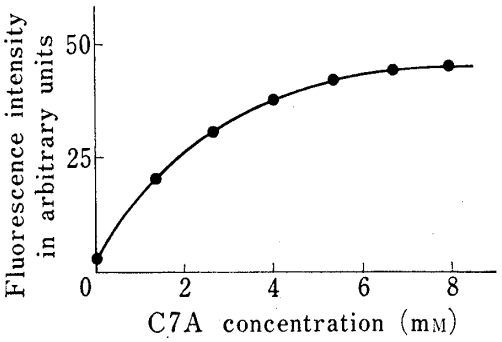


Fig. 3. Fluorescence Intensities of 13 μM Dansyl Valine in the Presence of Varied Concentrations of C7A

excitation: 365 nm, emission: 515 nm

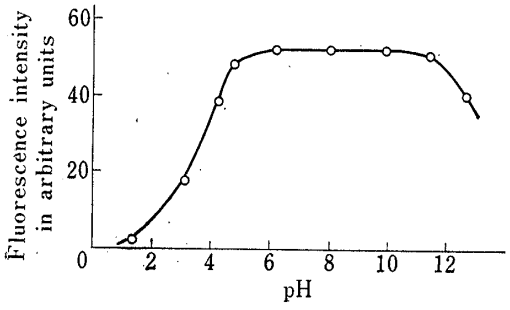


Fig. 4. Effect of pH on the Fluorescence Intensity of Dansyl Valine in the Presence of 8.0 mM C7A

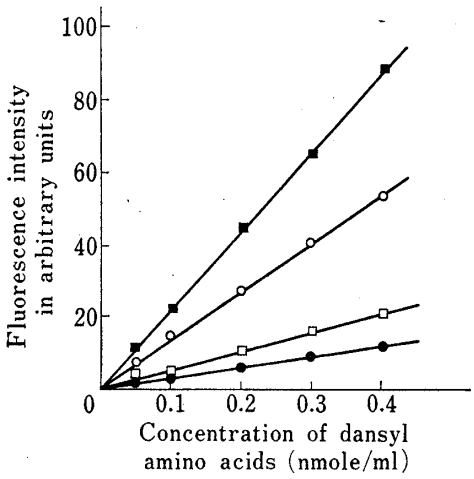


Fig. 5. Standard Curves for Dansyl Amino Acids: DNS-Leu (■), bis-DNS-His (○), O-DNS-Tyr (□), and DNS-Asp (●).

Standard curves for DNS-Ile, DNS-Val, bis-DNS-Lys, DNS-Ala, δ-DNS-Lys, DNS-Phe, DNS-Thr, DNS-Glu, DNS-Arg, DNS-Pro, DNS-Asn, DNS-Gln, DNS-Hyp, DNS-Ser, DNS-Cys-$SO_3H$, and DNS-Trp lie between those for bis-DNS-His and O-DNS-Tyr.

C7A appears to play a role similar to the hydrophobic region of BSA. Further discussions on the interaction will be given later.

Fig. 4 exhibits the influence of pH on the fluorescence intensity of dansyl valine in the presence of 8.0 mM C7A. Maximum intensity of the fluorescence was observed in a pH range of 6.0 to 10.0. The fluorescence was stable for two days in the dark at pH 7.4 at room temperature.

Standard curves for determination of dansyl amino acids are illustrated in Fig. 5. All standard curves are linear in the range in final concentration of 50 to 400 pmole per ml and passes through the origin. Although the fluorescence intensities were only about a half the intensities measured in methanol, dansyl amino acids were found to be determined up to 50 pmole per ml because of the high reproducibility. Coefficient of variation for dansyl leucine (50 pmole/ml) in 6 times measurement was 1.8%. This procedure may be followed more simply than that using organic medium for the fluorescence measurement.

Interferences of several compounds with fluorescence were examined by measuring fluorescence intensities in the presence of these compounds as listed in Table II. Urea, sodium chloride, sodium bicarbonate, L-lysine and D-glucose had practically no effect on the intensity at a concentration level of 25 mM. Perchlorate and benzoate markedly quenched the fluorescence, which is presumably due to competition of these materials with dansyl amino acids for the binding site of C7A molecule. Cramer and co-workers[8b] have demonstrated that perchlorate anion forms a complex with cyclohexaamylose. Occurence of perchlorate and benzoate at such level of concentration as used above may be less expected in biological samples.

Table II. Relative Fluorescence Intensity of DNS-Phe and DNS-Val in the Presence of Several Conpounds in Addition to 8.0 mM C7A

| | 50.2 μM DNS-Phe | 37.4 μM DNS-Val |
|---|---|---|
| None | 100.0 | 100.0 |
| Urea, 250 mM | 100.0 | 99.5 |
| NaCl, 250 mM | 103.0 | 103.8 |
| $NaHCO_3$, 25 mM | 101.0 | 101.0 |
| 250 mM | 107.3 | 108.0 |
| L-Lysine, 250 mM | 103.8 | 105.8 |
| D-Glucose, 250 mM | 105.0 | 104.6 |
| $NaClO_4$, 25 mM | 88.0 | 86.3 |
| 250 mM | 46.5 | 45.0 |
| Sodium benzoate, 25 mM | 83.3 | 81.0 |
| 250 mM | 28.3 | 25.4 |

The assay procedure for dansyl derivatives so far reported were sometimes much complicated and time-consuming because of poor fluorescence intensity of these substances in aqueous media.[3,7] Recently, Udenfriend and co-workers[10] reported methods for determination of amino acids,[10a,b] peptides[10a] and proteins[10c] by the use of fluorescamine. These methods are rapid and the reaction product between this reagent and amino compound gives intense fluorescence in aqueous solution. However, this reagent does not directly react with proline and hydroxyproline.[11] Although Weigele and co-workers[11a] have demonstrated

10) *a*) S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, and M. Weigele, *Science*, **178**, 871 (1972); *b*) S. Stein, P. Böhlen, W. Dairman, and S. Udenfriend, *Arch. Biochem. Biophys.*, **155**, 202 (1973); *c*) *Idem*, *ibid.*, **155**, 213 (1973).

11) *a*) M. Weigele, S. DeBernardo, and W. Leimgruber, *Biochem. Biophys. Res. Commun.*, **50**, 352 (1973); *b*) A.M. Felix and G. Terkelsen, *Anal. Biochem.*, **56**, 610 (1973).

that these amino acids can be assayed with fluorescamine after treatment with N-chlorosuccinimide, the sensitivity for hydroxyproline is lower than that of the present method.[11b] In addition, dansyl chloride has widely been empolyed in N-terminal determination,[3a,12] sequence analysis[3a,12,13] and peptide map determination[14] of proteins and ultramicroanalysis of amino acids in biological samples.[3a,15] For example, dansyl-Edman method[3a,12b] is extensively used in recent years for the sequence analysis. Accordingly, procedures for separation and identification of dansyl amino acids and dansyl peptides are, at present, well established and numerous standard samples of dansyl derivatives are available.[3a,12,15] Improvement of dansyl procedure, therefore, appears to be of significant value in the field of biochemical[7,12] and clinical[4] analysis.

In the present study, excellent sensitivity for dansyl amino acids (50 pmole/ml) was observed in aqueous media. This finding is expected to further extend the applicability of dansyl reaction. For example, dansyl amino acids and peptides can be directly estimated in aqueous solvent, which is known to nicely extract these substances from thin-layer plates after chromatography.[3] The present method seems to be especially useful for dansyl peptides and proteins insoluble in organic solvents. It may be effective for the assay of enzymes[16] or antibodies[17] dansylated for the purpose of biophysical investigation, since these proteins are often much precious and only little amount can be spared for assay. The possibility of application of the present method to the determination of proteins was hence examined as described below.

**Assay of Dansyl Proteins**

The fluorescence intensity of dansyl groups covalently bonded to protein molecule differs from protein to protein and even from amino acid to amino acid to which they are attached, and often give poor fluorescence intensity in aqueous media. C7A was expected to enhance the fluorescence of such dansyl groups. However, a preliminary examination using 16.0 mM C7A, which was much effective for dansyl amino acids, was unsatisfactory indicating that the use of more concentrated solution of C7A might be required. Although the solubility limit of C7A is 16 mM, it was found to be highly soluble in urea solution as described in detail in the subsequent paper.[9] C7A in urea solution was therefore tested for the assay of dansyl proteins.

Fig. 6 displays the effect of C7A dissolved in urea solution on the fluorescence intensity of dansylated proteins. Relative fluorescence intensity per mol of dansyl group is plotted against the concentration of C7A. At C7A concentration of 90 mM, the fluorescence intensity of a dansyl group on protamine, lysozyme, chymotrypsin, and BSA increased by a factor of 12.7, 5.6, 4.8, and 1.3, respectively. The considerably great fluorescence intensity of dansyl BSA in the absence of C7A may be due to the contact of the dansyl group with the hydrophobic region of the protein. McClure and Edelman[18] evaluated the sizes of the hydrophobic region of proteins, which decreased in the order BSA, chymotrypsin, and lysozyme using 2-*p*-toluidinylnaphthalene-6-sulfonate as a reporter. In the present study, the reverse order, lysozyme, chymotrypsin, and BSA was observed for the increment of the fluorescence intensity of dansyl groups on these proteins. This finding indicates that C7A is more effective for the dansyl groups, which are attached to less hydrophobic proteins and hence more quen-

12) *a*) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 379 (1963); *b*) S. Kimura, *Japan Analyst*, **23**, 563 (1974).
13) D.M. Fambrough and J. Bonner, *Biochemistry*, **5**, 2563 (1966).
14) V.A. Spivak, M.I. Levjant, S.P. Katrukha, and J.A.M. Varshavsky, *Anal. Biochem.*, **44**, 503 (1971).
15) L. Casola and G.D. Matteo, *Anal. Biochem.*, **49**, 416 (1972); J.P. Brown and R.N. Perham, *Eur. J. Biochem.*, **39**, 69 (1973).
16) D.A. Deranleau and H. Neurath, *Biochemistry*, **5**, 1413 (1966).
17) F. Kierszenbaum, J. Dandliker, and W.B. Dandliker, *Immunochemistry*, **6**, 125 (1969).
18) W.O. McClure and G.M. Edelman, *Biochemistry*, **5**, 1908 (1966).

ched. Alternatively, addition of C7A closes the gap of the fluorescence intensity among dansyl proteins.

Fig. 7 shows the standard curves for dansyl BSA and dansyl protamine in the presence of 90 mM C7A and 4M urea. Both standard curves are linear in the range of 0.75 to 6.0 μg of protein per ml and passed through the origin.

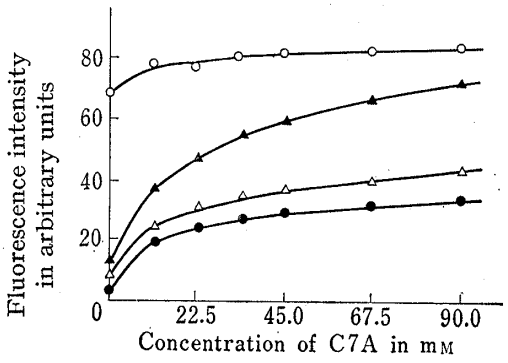


Fig. 6. Effect of C7A Concentration on the Fluorescence Intensity of Dansyl BSA (○), Dansyl Lysozyme (▲), Dansyl Chymotrypsin (△), and Dansyl Protamine (●)

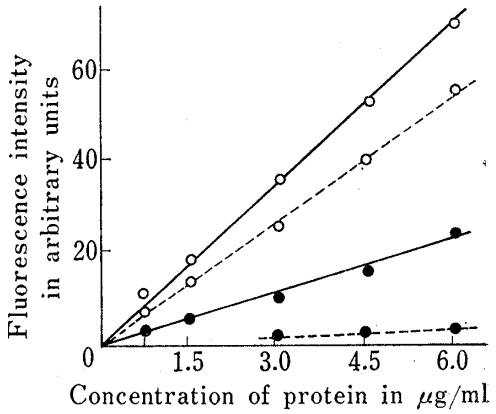


Fig. 7. Standard Curves for Dansyl BSA (○), and Dansyl Protamine (●) in the Presence (——) and Absence (------) of 90 mM C7A and 6 Urea

## Thermodynamic Study

The interaction of C7A with dansyl amino acids were further studied thermodynamically, assuming that the enhancement of fluorescence is resultant from formation of inclusion complex[19] between them. Fluorescence titration data of dansyl amino acids with C7A were analysed by the use of Benesi and Hildebrand equation.[20]

$$\frac{C_a}{\Delta I} = \frac{1}{K\Delta i}\cdot\frac{1}{C_d} + \frac{1}{\Delta i}$$

where $C_a$ and $C_d$ indicates total concentrations of dansyl amino acid and C7A, respectively. $\Delta I$ is the increment of the fluorescence of dansyl amino acid on addition of C7A, and $\Delta i$ denotes $\Delta I$/(concentration of dansyl amino acid-C7A complex). A plot of $C_a/\Delta I$ against $1/C_d$ for each dansyl amino acid was found to be a straight line which is indicative of the formation of 1:1 complex, as shown in Fig. 8. This figure also indicates that the association constants are temperature dependent.

Table III shows the summary of thermodynamic study. There was no remarkable difference among the free energy changes, while the entropies and enthalpies varied with the amino acids. The values of entropy changes for relatively hydrophobic dansyl amino acids such as dansyl phenylalanine, didansyl lysine or dansyl valine were negative or approximately zero. This may be explicable according to the concept by Bender and co-workers[21] that water molecules in the cavity of C7A cannot form their full complement of hydrogen bonds because of steric restrictions. Accordingly, increase in entropy due to the release of iceberg water, which brings about positive entropy in case of protein binding, may be minimized in this instance. Straub and Bender[22] observed negative entropy changes for the association of C7A with benzoyl acetic acid derivatives. On the other hand, relatively hydrophilic dansyl

19) P.V. Demarco and A.L. Thakker, *Chem. Commun.*, **1970**, 2.
20) H.A. Benesi and J.H. Hildebrand, *J. Am. Chem. Soc.*, **71**, 2703 (1970).
21) R.L. Vanetten, J.F. Sebastian, G.A. Clowers, and M.L. Bender, *J. Am. Chem. Soc.*, **89**, 3242 (1967).
22) T.S. Straub and M.L. Bender, *J. Am. Chem. Soc.*, **94**, 8881 (1972).

amino acids such as dansyl serine, dansyl glutamic acid or dansyl alanine showed positive entropy changes. This fact is hard to be interpreted by the above mentioned concept and may be explained as follows. Hydrophilic amino acid moieties carry more water molecules hydrogen-bonded to its polar sites than hydrophobic ones, and release of the water molecules in the interaction results in increase of entropy.

The low entropy and enthalpy values given by didansyl lysine may be attributed to its highly hydrophobic character. On the other hand, ε-dansyl lysine exhibits moderate entropy and enthalpy values presumably because its α-amino group is unprotected. Exceptionally, dansyl aspartic acid and dansyl hydroxyproline show considerably small entropy change despite their hydrophilic nature. γ-Hydroxyl groups of these compounds might be effective, in forming complexes of highly ordered structure, but further investigation will be required to obtain definite evidence.

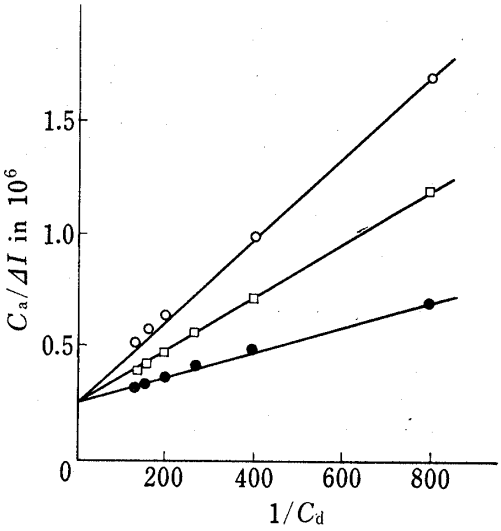


Fig. 8. Determination of the Association Constants for the Interaction of C7A with DANS-Val According to Benesi and Hildebrand at 40.0° (○), 22.5° (□), and 4.0° (●)

Table III. Thermodynamic Data in Interaction of C7A with Dansyl Amino Acids

| Dansyl amino acid[a)] | $k \times 10^{-2}$ (mole$^{-1}$) | $\Delta G$ (kcal·mole$^{-1}$) | $\Delta H$ (kcal·mole$^{-1}$) | $\Delta S$ (cal·mole$^{-1}$·deg$^{-1}$) |
|---|---|---|---|---|
| DNS–Phe | 0.9 | −2.6 | −5.6 | −10.0 |
| Bis–DNS–Lys | 1.9 | −3.1 | −4.2 | −3.7 |
| DNS–Asp | 1.4 | −2.9 | −3.1 | −0.7 |
| DNS–Val | 2.0 | −3.0 | −3.1 | 0.2 |
| DNS–Trp | 4.2 | −3.5 | −3.4 | 0.5 |
| DNS–Hyp | 1.3 | −2.8 | −2.4 | 1.5 |
| DNS–Cys–$SO_3H$ | 1.5 | −2.9 | −2.4 | 1.9 |
| DNS–Leu | 2.0 | −3.1 | −3.7 | 2.0 |
| ε-DNS–Lys | 2.5 | −3.2 | −2.6 | 2.1 |
| DNS–Ile | 1.9 | −3.1 | −2.4 | 2.2 |
| DNS–Arg | 1.1 | −2.7 | −2.1 | 2.2 |
| DNS–Pro | 2.2 | −3.2 | −2.2 | 3.2 |
| DNS–Thr | 1.8 | −3.0 | −2.0 | 3.5 |
| DNS–His | 1.6 | −3.0 | −1.8 | 3.8 |
| DNS–Gln | 1.3 | −2.9 | −1.6 | 4.4 |
| DNS–Asn | 1.1 | −2.8 | −1.4 | 4.6 |
| O–DNS–Tyr | 1.1 | −2.7 | −1.3 | 4.8 |
| DNS–Ala | 1.9 | −3.1 | −1.7 | 5.4 |
| DNS–Glu | 1.0 | −2.7 | −1.0 | 6.7 |
| DNS–Ser | 1.6 | −3.0 | −0.9 | 7.0 |
| DNS–OH | 1.4 | −2.9 | −0.8 | 7.2 |

a) abbreviations: DNS-Hyp, dansyl hydroxyproline; ε-DNS-Lys, ε-dansyl lysine; bis-DNS-lys, didansyl lysine; O-DNS-Tyr, O-dansyltyrosine; DNS-OH, dansylsulfonic acid; *etc.*

Thermodynamic study of cycloamylose-substrate complex is of interest because it can serve as a model for investigating the primary step of enzyme[22,23] or antigen-antibody[24]

23) F. Cramer and W. Kampfe, *J. Am. Chem. Soc.*, **87**, 1115 (1965); T.S. Straub and M.L. Bender, *J. Am. Chem. Soc.*, **94**, 8875 (1972).
24) F. Cramer, "Einschlussverbindungen," Springer-Verlag, Heidelberg, 1945, p. 65.

reactions. Although Cramer and co-workers[8b] have reported on the thermodynamics of conjugation of various azo dyes with cyclohexaamylose, the values of enthalpies were all positive and fell within the narrow range of 4.2 to 7.7 kcal/mole, presumably because there were little differences in polarity among the substrates employed. They have not described about entropies. The present study demonstrated that the entropies and enthalpies of the inclusion vary with the change in local polarity of the substrate molecules. These phenomena may be due to the amphiphilic nature of C7A and support the suggestion[8b] that hydrogen bonding, Van der Waales forces and hydrophobic interactions are concerned to the inclusion by cycloamyloses.

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