DANSYLPROTAMINE, A FLUOROMETRIC REAGENT FOR DETERMINATION OF NUCLEIC ACIDS

Noriko NAKAMURA, Toshio KINOSHITA^{®*}, Kazuyo MURAYAMA*,
and Fumio IINUMA**

Kanagawa Prefectural College of Medical Technology, Nakao-cho, Asahi-ku,
Yokohama 241, *School of Pharmaceutical Sciences, Kitasato University, Shirokane,
Minato-ku, Tokyo 108, and **Faculty of Pharmaceutical Sciences, Teikyo University,
Sagamiko, Kanagawa 199-01

DNA and RNA were found to enhance the fluorescence of dansylprotamine. This finding was applied to microdetermination of nucleic acids. This method is valid for samples containing 0.1 - 5.0 µg/ml of nucleic acids. Nucleic acids and dansylprotamine were found to bind each other through both electrostatic and hydrophobic interaction.

Nucleic acids have currently been determined using 3,5-diaminobenzoic acid¹⁾ or ethidium bromide²⁾³⁾. However, the former reagent requires heating for a long period in the presence of strong acid, and the latter reagent gives excitation and emission maxima at too close wavelengths (546 and 590 nm). Moreover, ethidium bromide has been found to be highly carcinogenic. Recently, new reagents for the fluorescent staining of DNA, 4',6'-diamino-2-phenylindole⁴⁾⁵⁾ and 2-[2-(4-hydroxyphenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)-benzimidazole trihydrochloride⁶⁾⁷⁾ were reported but these compounds do not stain RNA. In the present study, DNA and RNA were found to enhance the fluorescence of dansylprotamine (DNSP) whose fluorescence is quenched in aqueous media, and this finding was applied to the determination of DNA and RNA.

MATERIALS AND METHODS

Dansylchloride (DNS-Cl) and 8-anilino-1-naphthalene-sulfonate (ANS) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo). Cycloheptaamylose (C7A) was obtained from Hayashibara Biochemical Laboratories, Inc. (Tokyo), and once recrystallized from water before use. Protamine and DNA from salmon testis were the gift of Dr. M. Wakaomi, Yuki Gosei Yakuhin Kogyo Co. Ltd. (Tokyo). RNA from yeast was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo). A Hitachi Model 204-R fluorescence spectrophotometer was used for the fluorometric measurements, and a Hitachi Model 200-10 spectrophotometer was used for measuring absorbances.

Preparation of DNSP — Protamine was dansylated with cycloheptaamylose-dansylchloride complex (CDC)⁸⁾⁹⁾ in the manner described previously¹⁰⁾ with some modifications. To 10 ml of 16% C7A solution in aqueous 8 M guanidine hydrochloride was added 100mg of protamine, 250 mg of CDC and 1 ml of 40% triethanolamine in isopropanol. The resulting mixture was magnetically stirred for 10 min at 37°C. The mixture was then applied on a Sephadex column (2 cm x 20 cm) and DNSP was eluted from the column

with 0.1 M acetate buffer containing 1 M KCl, pH 4.0. The effluent was dialysed overnight against 0.1 M phosphate buffer, pH 7.4. Concentration of DNSP in the dialysed solution was determined by measuring its absorbance of DNS group at 335 nm¹⁰⁾ and the solution was properly diluted to give the DNSP reagent.

Determination of nucleic acids — To 50 μ l of a sample solution was added 3 ml of DNSP reagent (6 μ M DNSP in 25 mM phosphate buffer, pH 7.0) at room temperature and the fluorescence intensity was measured at excitation and emission wavelengths of 360 and 515 nm.

Fluorescence enhancement of DNSP with DNA-protamine complex — To 2 ml of 11.7 μ M DNS solution in glycine buffer was added 1 ml of an aqueous solution containing 50 μ g protamine and 20 μ g DNA. The fluorescence intensity was measured at excitation and emission wavelengths of 395 and 500 nm.

RESULTS AND DISCUSSION

Figure 1 shows the excitation and fluorescence spectra of the mixture of DNA and DNSP and that of DNSP alone. Although the fluorescence intensity of DNSP in aqueous media was low, it was markedly enhanced on addition of DNA. The increment of fluorescence was proportional to the concentration of DNA. Nucleic acids showed no significant fluorescence within the concentration range used for the determination.

Fig. 1 (a) Excitation and (b) emission spectra of the mixture of 50 μ l of aqueous 0.02 w/v% DNA solution and 3 ml of DNSP reagent (a_1, b_1) and reagent blank (a_2, b_2).

Fig. 2 Effect of DNSP concentration in the DNSP reagent on the fluorescence intensity.

Figure 2 displays the fluorescence intensity of DNA-DNSP mixture plotted against the concentration of DNSP. The fluorescence intensity showed a plateau at the concentration of DNSP between 1 and 7.5 μ M, whereas the reagent blank increased with increasing concentration of DNSP. The effect of pH was examined by measuring the fluorescence in the presence of DNA at different pH values in 25 mM phosphate buffer. The fluorescence intensity showed its maximum in the pH range between 6.4 and 8.0. Accordingly, 6 μ M DNSP in 25 mM phosphate buffer, pH 7.0, was used in the standard procedure. Linear

relations were found between increment of fluorescence intensity and the concentration of DNA and RNA in the range of final concentration from 0.1 to 5.0 $\mu\text{g/ml}$ as demonstrated in Fig. 3.

Table 1 lists the effect of several compounds on the fluorescence intensity of DNA-DNSP mixture. The fluorescence was not affected by glucose, leucine, benzoate and Tween 20. Although 10 $\mu\text{g/sample}$ of bovine serum albumin (BSA) did not affect the fluorescence, 100 $\mu\text{g/sample}$ of BSA increased the fluorescence of 10 $\mu\text{g/sample}$ of DNA by 9%. Mucopolysaccharides tested (chondroitin sulfate A, C, and heparin) greatly affected the fluorescence. Therefore, these compounds should be removed prior to the determination.

Fig. 3 Standard curves for DNA (●) and RNA (▲).

Table 1 Relative fluorescence intensity of DNA (10 $\mu\text{g/sample}$)-DNSP complex in the presence of several compounds.

To 50 μl of aqueous 0.02 w/v% DNA solution were added 1 ml of 18 nmol DNSP in 25 mM phosphate buffer, pH 7.0 and 2 ml of various compounds dissolved in 25 mM phosphate buffer, pH 7.0. Fluorescence intensity was measured at ex. 360 nm and em. 515 nm.

Compounds	Amount ($\mu\text{g/sample}$)	Recovery (%)	Compounds	Amount ($\mu\text{g/sample}$)	Recovery (%)
none	-	100			
glucose	10	100	bovine serum albumin	10	100
	100	100		100	109
sodium benzoate	10	100	chondroitin sulfate A	10	135
	100	100		100	357
Tween 20	10	100	chondroitin sulfate C	10	130
	100	100		100	328
L-leucine	10	100	heparin	10	242
	100	100		100	742

This method is very simple because stable fluorescence is obtained by only mixing DNSP solution with samples at room temperature. Furthermore, DNSP itself is stable at least for two months when stored in a refrigerator. DNSP method utilizes the fluorescence enhancement of DNSP on its interaction with nucleic acids and therefore is superior to the methods utilizing fluorescence quenching of dyes such as acridine orange.

DNSP is a fluorescence reagent having polyfunctional binding sites (polyarginine moiety) which bind tightly with nucleic acids. Several factors which may affect the binding were investigated. First, the

effect of ionic strength on the fluorescence intensity was examined using various concentration of KCl solution and the results are displayed in Fig. 4. The fluorescence decreased with the increasing concentration of KCl. The fluorescence continued to decrease up to the ionic strength of $\mu = 0.5$. The 100 mM phosphate buffer used in the determination did not affect the fluorescence. On the other hand, addition of urea reduced the fluorescence intensity as shown in Fig. 5. These facts suggest that both electrostatic and hydrophobic interactions between nucleic acids and DNS-group are involved in the enhancement of the fluorescence.

Fig. 4 Relative fluorescence intensity of DNA-DNSP complex under various ionic strength.

Fig. 5 Relative fluorescence intensity of DNA-DNSP complex in the presence of various concentration of urea.

To 50 μ l of aqueous 0.02 w/v% DNA solution were added 1 ml of 18nmol DNSP in 25mM phosphate buffer, pH 7.0 and 2 ml of various concentration of KCl or urea solution in 25 mM phosphate buffer, pH 7.0. Fluorescence intensity was measured at ex. 360 nm and em. 515 nm.

Fig. 6 Fluorescence spectra of 1.17×10^{-5} M ANS in the presence of protamine and DNA (final volume: 3 ml).

— ANS + 50 μ g protamine
 + 20 μ g DNA
 - - - ANS + 50 μ g protamine
 ANS alone

The induction of hydrophobic region on the interaction between protamine and DNA was further explored using ANS, which is an effective hydrophobic probe for proteins. ANS fluoresced very little in the presence of either intact (not dansylated) protamine or DNA alone, but its fluorescence was markedly enhanced on simultaneous addition of both protamine and DNA.

Figure 6 demonstrates the fluorescence intensity of ANS in the presence and absence of DNA-protamine complex. It has already been reported that protamine forms the random coil in aqueous solution but it is converted rapidly into the α -helix form on interaction with DNA and it twins itself round the cruck of double helical DNA¹¹⁾. The enhancement of fluorescence intensity of ANS in the presence of DNA-protamine complex indicates the induction of hydrophobic regions by the interaction between protamine and DNA presumably due to the increase of the α -helix form. The interaction of DNSP and nucleic acids may also result in the induction of hydrophobic regions which may contribute to the enhancement of the fluorescence intensity of the dansyl group. Kinoshita and Iinuma¹⁰⁾ have previously reported that heparin enhances the fluorescence of DNSP to a great extent. As heparin possesses little hydrophobic group in its molecule, this fact further supports the assumption that the fluorescence enhancement of DNSP is caused mainly by the induction of hydrophobic region in the protamine molecule.

Acknowledgements

The authors are grateful to Dr. M. Wakaomi, Yuki Gosei Yakuhin Kogyo Co. Ltd., for his generous gift of specimens of protamine and DNA.

REFERENCES

- 1) J. M. Kissane, E. Robins : J. Biol. Chem., 233, 184 (1958).
- 2) J. B. LePecq, P. Yot, C. Paoletti : Compt. Rend., 259, 1786 (1964).
- 3) A. S. Prasad, E. DuMocuhelle, D. Koniuch, D. Oberleas : J. Lab. Clin. Med., 80, 598 (1972).
- 4) W. C. Russel, C. Newman, D. H. Williamson : Nature(London), 253, 461 (1975).
- 5) J. Kapuscinski, B. Skoczylas : Anal. Biochem., 83, 252 (1977).
- 6) C. F. Cesarone, C. Bolgnesi, L. Santi : Anal. Biochem., 100, 188 (1979).
- 7) C. Labarca, K. Paigen : Anal. Biochem., 102, 344 (1980).
- 8) T. Kinoshita, F. Iinuma, A. Tsuji : Chem. Pharm. Bull., 22, 2421 (1974).
- 9) T. Kinoshita, F. Iinuma, A. Tsuji : Anal. Biochem., 61, 632 (1974).
- 10) T. Kinoshita, F. Iinuma, A. Tsuji : Chem. Pharm. Bull., 22, 2769 (1974).
- 11) R. W. Warrant, S. H. Kim : Nature, 271, 130 (1978).

Keyword phrases

nucleic acid determination ; fluorometry of nucleic acid ; DNA fluorometry; RNA fluorometry; dansylprotamine as fluorescence reagent; protamine interaction with nucleic acid.

(Received October 12, 1983)