FLUORMETRIC ASSAY FOR NUCLEASE ACTIVITY

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ABSTRACT

This project is aimed at the preparation of substrates for deoxyribonuclease (DNASE) assay, which permits the use of sensitive fluorescent techniques. Such an assay, if sufficiently sensitive, could then be adapted for inclusion as an experiment in multivator. The proposed procedure is to label the heterocyclic portion of DNA (the preferred substrate of DNASE) with a fluorescent species. As a result of enzyme action low molecular weight fluorescent products are formed. These, can be made to pass through a semi-permeable membrane and are then readily detected by fluorometry. Because of its high quantum yield of fluorescence, derivatives of fluorescein and related compounds were selected as suitable reagents to label DNA. A number of fluorescein derivatives are commercially available; in particular, fluorescein isothiocyanate is the reagent of choice for the preparation of fluorescent protein derivatives. Preliminary experiments with this compound, however, quickly showed that the amino and hydroxyl groups of DNA are very hard to label with this reagent and yields were found to be extremely low. Sulfonyl chlorides do, however, react quite readily with weakly basic amino groups to form sulfonamides, and these compounds show great stability to both acid and alkali. With this in view, model condensations of napthalene-sulphonyl chloride with adenine were carried out, and yields were shown to be excellent. A literature search suggested the use of the sulfonyl chloride of sulfonfluorescein as a likely reagent for labelling DNA. Preparation of the intermediate sulfone fluorescein from o-sulfo benzoic anhydride and resorcinol was readily accomplished and this after chromatographic purification could be transformed to the corresponding acid chloride with PCl_5. No attempt was made to purify the crude sulfonchloride, but it was immediately reacted with DNA. The condensation product was fluorescent and it could be purified by dialysis. A study of the spectral and the fluorescence properties of this compound are indicated before enzyme assays are undertaken.

EXPERIMENTAL

I. Reactions with Fluorescein Isothiocyanate

The reaction of isocyanates and isothiocyanates with amino and hydroxyl groups is well known. The products of reaction of isothiocyanates with amino groups are thioureas, with hydroxyl groups they are thiourethanes. Fluorescein isothiocyanate reacts with amino groups of proteins to give fluorescent protein derivatives. It was felt that perhaps similar derivatives could be made by reacting fluorescein isothiocyanate with nucleic acids. The difficulty is that the amino and hydroxyl groups are so deactivated by the purine and pyrimidine rings that they are largely present as imino and keto groups. This strong delocalization of electrons would not help attack by an electrophilic group such as isothiocyanate. The reaction should be alkali catalysed but it soon became clear that the reagent was hydrolysed by alkali. The fluorescein isothiocyanate used was adsorbed on celite (ex. Cal. Biochem). A variety of reaction conditions was used, but the reaction scheme generally followed this pattern; the nucleic acid was dissolved (or dispersed), the reagent was added, the mixture was shaken or stored for a period of time, centrifuged and then dialysed exhaustively or allowed to run through a Sephadex 25 column. The dialysed solutions or the column fractions were assayed spectrophotometrically as follows: the typical nucleic acid spectrum (minimum at 230$m\mu$, maximum at 260$m\mu$, no absorption at 490$m\mu$) is quite different from the isothiocyanate spectrum (maximum at 240$m\mu$, shoulder 250$m\mu$-280$m\mu$, maximum at 495$m\mu$). Thus one could look for a modified nucleic acid spectrum in the ultraviolet, and strong absorption near 490$m\mu$. It is possible that the fluorescein spectrum could mask out the nucleic acid spectrum almost completely, consequently there is some question about the composition of these Sephadex fractions which showed only the fluorescein spectrum; however, they were always very slow in coming off the column, and may be expected to be low molecular weight compounds. The dialysed material always showed considerable absorbance at 493$m\mu$ and fluorescence. Because of greater availability of material, RNA was studied much more thoroughly than DNA. The RNA is

a sample [OD_{260} ca 4, dissolved in saline] (supplied by Dr. Bodmer, Genetics Department, Stanford University). The reactions with DNA was attempted in aqueous solution at 95-98°C. No discernable absorption at 490mμ was seen in the nucleic acid fractions from a Sephadex column. Next the reaction was run at room temperature for 24 hours, with similar lack of success. The ratio of reagent to DNA (on a weight basis) was about 10 to 1. The room temperature reaction was run on DNA which had been incubated at pH 12 for 10 minutes to expose "buried" reactive groups. Alkali treated DNA was again incubated with fluorescein isothiocyanate, this time with ca 15/1 ratio of reagent to DNA for 3 hours. The resulting mix was treated in the usual way, one Sephadex fraction seemed to be interesting, but its fluorescence was low enough to raise some question about mixing of peaks in the column. Consequently it was dialysed vs. 10ml water. Both the DNA and the fluorescence seemed dialysable--but much more fluorescence than DNA passed through the membrane. If this reaction is to be run on DNA, it is suggested that a higher yield might be obtained if a much higher concentration of DNA is used, and that the reaction be run in the cold for several days--these suggestions result from experiments run on RNA. Thus, a number of preparations of yeast nucleic acid with fluorescein isothiocyanate have been made. The best yields were of the order of one fluorescein per 30 nucleotides. A typical preparation follows: 7.9mg of yeast nucleic acid is added to 1 ml water, 0.2ml 0.1M NaOH were added to dissolve the nucleic acid, as soon as possible, 2.0ml 0.2M Tris buffer pH 7.6 were added. The solution was chilled in an ice bath and 81.0mg of fluorescein isothiocyanate on celite (10 per cent were added). The solution was maintained cold for 5 hours. It was centrifuged briefly and the supernate was dialysed exhaustively vs. running tap water for several days. The ultraviolet absorption spectrum is the expected one for nucleic acid, the visible absorption spectrum is that expected for fluorescein. If the following assumptions are made it is possible to calculate the proportion of fluorescein to nucleotide: that the extinction coefficients for fluorescein thioureas are the same as for fluorescein, and that the formation of these derivatives does not affect the extinction of the nucleic acid in the ultraviolet. In spite of the exhaustive

dialysis, it is entirely possible that the derivative has absorbed fluor which is slowly released in a nonspecific fashion. The next step is to hydrolyse some fluorescein isothiocyanate and mix it with nucleic acid. Dialysis will show whether the dye has strongly adsorbed to the polymer. Adsorbed dye can perhaps be removed by dialysis at a variety of pH's, by solvent extraction at acid pH, or perhaps by acid precipitation of the nucleic acid. Once the material has been cleaned up, its efficiency as a substrate should be measured in a system such as the following: various dilutions of enzyme are mixed with buffer and substrate and dialysed vs. a known volume of buffer. The material on the outside of the membrane is assayed both for fluorescence and for ultraviolet absorbance. It is of interest to prepare a fluorescent DNA derivative, and the isothiocyanate reaction may well work when higher concentrations of DNA are employed.

II. Preparation and Characterization of the Naphthaline-2-Sulfonamide of Adenine.

Preparation of Naphthalene Sulfonyl Adenine:

Approximately 1mM (.1392gm.) adenine is dissolved in 1.5ml. 4N-NaOH, and to this is added naphthalene sulfonyl chloride (.2727gm., 1.2mM) dissolved in acetone (1ml). After filtration the filtrate is acidified to pH 3. It is allowed to stand for 1 hour and the product is then filtered off. It is recrystallized from ETOH (m.p. 196-7°). The product is soluble in 5% NaOH, 5% $NaHCO_3$; soluble in 5% HCl, and ether. Element tests (sodium fusion) showed presence of S and N and the ultra violet absorption spectrum shows the characteristics spectrum of a naphthalene derivative.

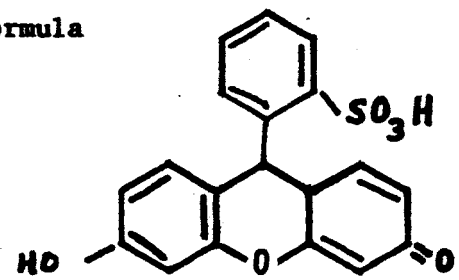
III. Preparation of Sulfon Fluorescein.

[Sulfon fluorescein is described in Beilstein and mentioned in Heterocyclic Compounds, (Vol. II, pp. 505 and 531) by Elderfield.]

The method of preparation is to grind together in a mortar 15gm O-sulfobenzoic anhydride and 22gm resorcinol and transfer it to 500ml round bottom flask which is then heated 180° internal temperature.

Anhydrous zinc chloride (7gm) is added with stirring, and heating is continued until the solution becomes very viscous. The resulting dark red mass consists largely of a mixture of sulfofluorescein and $ZnCl_2$ with basic zinc salts. The oil bath is allowed to cool to 90° and 200 ml. H_2O and 10 ml concentrated HCl are added to the mixture, and the temperature is raised until the water boils. Boiling is continued until the reaction mixture disintegrates and all zinc salts dissolve. The insoluble residue of sulfonfluorescein is filtered, ground with water in a mortar and refiltered. It is dried in an oven at 80°-100°. The precipitate is purified by dissolving in dilute NaOH, filtering and precipitating with 6 M HCl and this procedure is repeated four times. Yield: 22 grams, M.P. > 310°C.

Characterization of the Sulfon Fluorescein Preparation:

a) Proposed formula  molecular weight: 368.37

b) Elemental analysis: Calculated for $C_{19}H_{12}O_6S$: C,61.9; H,3.2; O,26.3; S,8.7. Found: $C_{19}H_{12})_6S$: C,58.2; H,5.54; O,28.96 (by difference); S,7.30.

c) Spectra: Ultra violet and visible absorption spectra of this compound were determined. The solutions were prepared as follows: 37.7mg of the preparation (0.1025 millimoles based on a molecular weight of 368) were dissolved in water made slightly alkaline with NaOH and diluted to approximately 50 ml., the pH adjusted with acid or alkali and finally the solution was quantitatively diluted to 100 ml. As 1.03×10^{-5} molar is somewhat dilute for a good ultra violet spectrum; a further spectrum (at pH 11) at 4×10^{-5} molar concentration was also run. The extinction coefficient at 495 mμ was found to be 57,300, and spectrophotometric titration shows a single pK at 6.3.

(d) Fluorescence Properties: The compound is quite fluorescent. All measurements were made using a Turner Model III Fluorometer. The fluorescence filter was a sharp cut-off filter at 510mμ. Solutions of the compound of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} molar concentrations were prepared at a pH of 4 and 9 (buffers 0.1M acetate and 0.1M phosphate), and fluorescence was measured at various wavelengths of activation. (The filters used were narrow pass at 360, 405, 436, 460, 490, 495mμ).

(e) Chromatographic Analysis of the Preparation of Sulfon Fluorescein: showed the preparation to be homogeneous. The best solvent system for thin layer chromatography was butanol (40), glacial acetic acid (10) and water (50). In this system the product had an Rf of 0.9.

Table I

Relative Fluorescence

Wavelength mμ	Molar Concentration			
	1.71 10^{-6}	1.71X 10^{-7}	1.71X 10^{-8}	1.71X 10^{-9}
360mμ				
pH4		1788	118	58
pH8		1350	180	15
405mμ				
pH4	979	79	3	1
pH8	583	50	6	1
436mμ				
pH4		969	39	27
pH8		897	81	17
460mμ				
pH4		384	17	6
pH8		498	42	9
490mμ				
pH4	120	14	4	[-2]
pH8	1183	109	18	7
495mμ				
pH4	230	[-40]	[-150]	[-80]
pH8		400	20	[-250]

IV. Preparation of the Sulfonyl Chloride of Sulfon Fluorescein:[1][2][3]

About 300mg of sulfonylfluorescein and 250 mg PCl_5 are combined in a plastic test tube. Five to six small metal ball bearings are placed in the tube and the mixture is agitated in a Vortex Jr. mixer for five minutes. Five ml cold acetone is added and the mixture immediately filtered. The acetone solution contains the sulfonyl chloride, and this is used to tag the DNA by several different procedures. These are:

(1) To ten ml 0.2 M NaCl containing in 20 mg chicken erythrocyte DNA is added 5 ml pH 10 phosphate buffer. With stirring, dropwise add the sulfonyl chloride up to 5 ml. A check was kept on the pH. It went down to ~7. The reaction mixture is left overnight. In the morning the pH was 6.5.

The mixture was then exhaustively dialyzed for 20-24 hours. Then equilibrium dialyzed for 3 hours. Both dialyses were done against .2 M NaCl. The solution outside the bag, after equilibrium dialysis was found to have a fluorescence of 9 RFU on the 30X scale. (Activation: 460, Fl >510.)

The inner portion of the bag was found to have a fluorescence of 350 RFU. A 1/50 dilution produced a fluorescence of 49 RFU.

Upon the preparation of the apurinic acid of the tagged DNA, the fluorescence was raised considerably, indicating intramolecular quenching. One ml of the tagged DNA was treated with 0.1 N HCl until a pH of 1.6 was reached. The mixture 1.0 ml was tranferred to dialysis tubing and dialyzed against 10 cc of dilute HCl of pH 1.6 for 20 hours. The fluorescence reading of the outer solution (12 ml) was 1020 RFU. This sharp increase in dialysable fluorescence may be attributed to the release of tagged purines (adenine and guanine) from the DNA inside the dialysis bag.

(2) The preparation of the tagged DNA was modified slightly. The

DNA was first treated with .1 M NaOH in the cold. (This step was introducted to unfold the greatly hydrogen-bonded DNA structure, thus increasing the number of amino and hydroxyl groups available for reaction with the sulfonyl chloride. It is well known that dilute alkali does not affect the convalent structure of DNA.) Concentrated H_3PO_4 was added to pH 10 (1 to 2 drops). The pH was then adjusted to pH 9 with a phosphate buffer solution. The sulfonyl chloride, freshly prepared, is then added in the cold in the same manner as above and the reaction allowed to proceed overnight in the cold with constant stirring.

After equilibrium and exhaustive dialysis of the above mixture the fluorescence of the reaction mixture was 730 RFU on the 30X scale. A 1/10 dilution had a reading of 7500 RFU. (Thus, there is apparent concentration quenching of the tagged DNA.) Upon treatment with concentrated HCl and heat, then return to alkalinity. The resulting concentration was about 1/25 of the original concentration. This 1/25 dilution mixture had a fluorescence of 1020 RFU.

Both of these experiments are preliminary; more recently, it has been found that precipitation of the reaction mixture with isopropanol is a good first step in purification of the tagged DNA. Absorption spectra of the purified tagged DNA should be performed after a sample has been completely freed of dialysable material. In addition, the fluorescein sulfonamide of adenine should be prepared to give a well-characterized spectral and fluorescence standard.

Conclusions

A) The naphthalene sulfonamide of adenine has been formed. This suggests that the sulfonamide of the adenine (and perhaps guanine and cytosine) when it is bound to the DNA polymer, is possible.

B) A fluorescein analogue, having a sulfonic acid replacing the fluorescein carboxyl group has been synthesized and found to be reasonably pure chromatographically. Absorption spectra of this compound are much like those of fluorescein. Spectrophotometric titration shows a single apparent pK at 6.3 - again much like fluorescein.

C) The acid chloride of sulfon fluorescein has been found to react with DNA leading to a non-dialyzable product which is fluorescent. The final purification, spectral and fluorescence properties of this compound should be investigated in detail.

Reference:

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3. Uehleke, H.: Naturwissenschaften 45, 87 (1958).