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# BIFUNCTIONAL REAGENT FOR STUDY OF BIOLOGICAL SYSTEMS[1]

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The detailed preparation, identification and importance of 4-fluoro-3-nitrophenylazide is described. This reagent has wide importance in biological systems.

## INTRODUCTION

Many bifunctional reagents have been prepared recently for the study of biological systems (Fasold, *et al* 1971). This paper describes in detail the preparation, identification and importance of 4-fluoro-3- nitrophenylazide (Fleet, *et al.* 1969) (FNPA) and its derivatives.

## MATERIALS AND METHODS

FNPA was prepared by adding 5 gm of 4-fluoro-3-nitroaniline (Aldrich Chemical Company Lot 021807) in a mixture of concentrated hydrochloric acid (30 ml) and water (5 ml). The mixture was stirred magnetically at about 40°C for 1 hr and cooled to -10°C (using an external bath of methanol–solid $C0_2$). An aqueous solution of sodium nitrite (2.8 gm in 3 ml water) was added dropwise over 20 min. and the mixture was stirred for 10 min. at –10°C. It was then filtered rapidly into a flask at -10°C. This filtrate was stirred between -20°3 and –10°C, and an aqueous solution of sodium azide (2.8 gm in 5 ml in 5 ml $H_2O$) was added dropwise (using red light). If there is frothing at this stage, a few drops of other may be added. After the addition of sodium azide was finished, the resulting precipitate was collected on a Buchner funnel, washed thoroughly with water, and dried at room temperature in a vaccum dessicator wrapped in aluminum foil (the product is light sensitive). The reaction is shown in Fig 1.

## RESULTS AND DISCUSSION

Elemental analysis data are given in Table 1. The mass spectra (Table 2) showed the molecular ion peak at mass = 182. A number of

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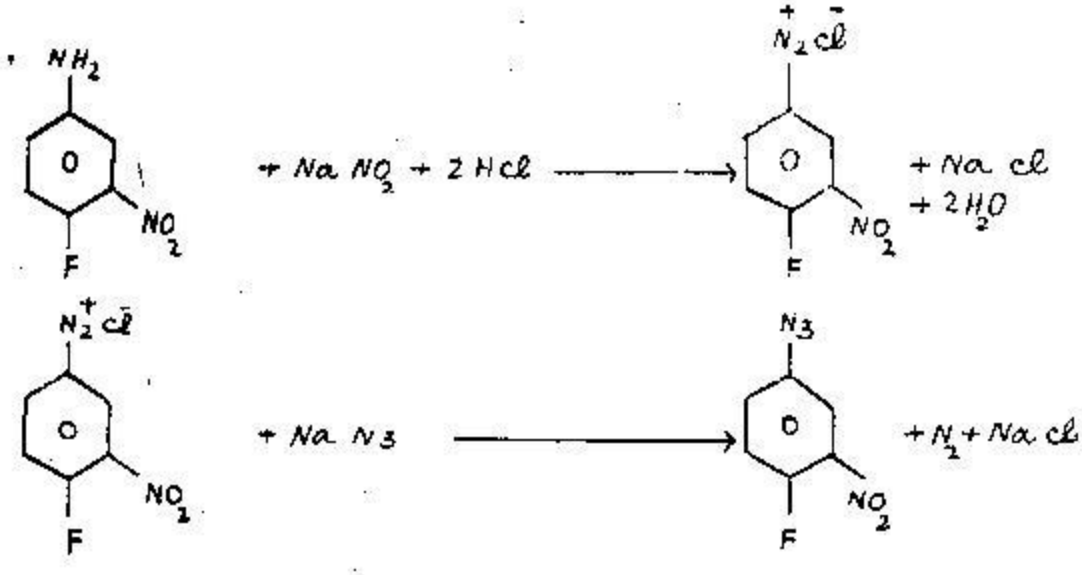
Fig. 1




broad metastable peaks were observed. The NMR spectrum showed a hydroxynitrophenylazide content to be 3 per cent of FNPA. The infrared spectrum gave the characteristic band at 2115 cm $^{-1}$ corresponding to azide. The $No_2$ group was indicated by absorption bands at 1365 and 1525 cm$^{-1}$. Thin-layer chromatography on cellulose silica gel plates using three solvent systems (acetone, chloroform, and 70 per cent ethanol) gave only one spot. Attempt to further purify the product by sublimation did not change mass and NMR spectra.

FNPA is insoluble in water but soluble in most organic solvents like acetone, chloroform, ether, ethanol, etc. At alkaline pH it hydrolyses to hydroxynitrophenylazide which is soluble in water. This product showed a molecular ion peak at 180

TABLE 1 *Elemental analysis data of fluoronitrophenylazide*

| Element | Calculated | Found |
|---|---|---|
| C | 39.6% | 39.64% |
| H | 1.65% | 1.85% |
| N | 30.8% | 31.0% |
| F | 10.5% | 10.9% |

TABLE 2 *Mass Specirum* [a] *of Fluoronitrophenylazide*

| Major Peaks [b] (m/e) | % of Base Peak | Major Metastable Peaks |
|---|---|---|
| 182 | 22.95 | 130.6 (182-154) |
| 154 | 33.13 | 75.6 (154-108) |
| 109 | 10.95 | 60.7 (108-81) |
| 108 | 100 | |
| 12 | 16.07 | |
| 81 | 23.00 | |
| 57 | 56.87 | |
| 50 | 30.73 | |
| 63 | 14.24 | |
| 31 | 23.76 | |
| 30 | 19.14 | |
| 28 | 66.65 | |

a The mass spectrum was taken by direct inlet system at room temperature. The source temperanure was 300°C, probe temperature 50-60°C and electron voltage 70 ev.
b All other peaks were less than 10 per cent of the base peak.

**Importance in Biological Systems :**

While the reactivity (reaction rate under mild conditions of pH and temperature) of the fluorine (the dark reaction group) of FNPA is unsatisfactorily low for use with most enzymes, this reagent may be used as such to prepare reactive derivatives of (1) matrix materials and (2) substrate analogs and enzyme cofactors for subsequent covalent linkage to enzymes or to one another by means of the photochemical reaction under conditions of optimal interaction of the two chemical moieties to be linked. In addition, derivatives of FNPA useful for reacting with the enzyme through the dark reaction, may be prepared by substituting the fluorine group with a reagent containing a nucleophilic group and one of the large number of reactive groups already

found to be useful in forming covalent bonds with enzymes through dark reactions under mild conditions. The following are the descriptions of chosen example of these reaction procedures.

A major advantage of this type of bifunctional reagent, in addition to the general reactivity of the photochemical group, is the high level of independence of the reactivity of the two groups (i.e., each of the two reactions in the covalent crosslinking can be done essentially completely independently of each other). Since the photochemical reaction is much less dependent upon the reaction conditions (pH, temperature, etc.) which influence molecular interactions, in most instances the preferred reaction sequence is expected to be the use of the dark reaction as the first step in formation of stable intermolecular crosslinks. The "activated" species containing the photochemical group can then be combined with the other species under optimal conditions of intermolecular association for the terminating photochemical step of the intermolecular crosslinking.

For example, FNPA may be used to prepare "activated" matrix materials such as derivatives of glass containing nucleophilic groups by a dark reaction at slightly elevated temperatures in slightly alkaline solution (the azide group is relatively stable on this reagent). This dark stable, photochemically active matrix material could then be combined with a wide variety of enzymes and or cofactors or substrate analogs for covalent linking to the matrix upon illumination with visible light. With this "activated" matrix matrix material, one may thereby produce immobilized enzymes under conditions of improved protection of the active site (favouring an improved enzyme activity yield) and may in similar manner produce column packing materials for purification of enzymes by the mild and highly specific procedure called "affinity chromatography"

This procedure may also prove useful for producing stable functional cofactar-enzyme linkages. For example, we can substitute for the fluorine on FNPA a flexible several-carbon chain containing on the distal end a group which will covalently link to the cofactor in a dark reaction. The production of a functional derivative of a cofactor in this manner would allow mixing with the enzyme under conditions for active complex formation. Irradiation of the enzyme-cofactor complex with visible light would be expected to covalently link the cofactor through the nitrene so produced to a site on the enzyme molecule near the "filled" active site. A stable enzyme-cofactor complex produced in this manner would seem more likely to be functional.

If the chemical characteristics of both members of the desired intermolecularly crosslinked complex were such that the relatively unspecific photochemical nitrene reaction is preferred for both ends of the bifunctional reagent, the following 4-step reaction program should accomplish this goal with relative ease. For example, the insoluble matrix material containing -C-C- or C-H bonds (organic polymers or derivatives of glass, etc.) can be reacted photochemically with FNPA. The resulting insolubilized fluorine group can be displaced by displaced by diamines (e.g., ethylene diamine, putrescine, etc.) thermochemically. The other amino group of this matrix derivative can then be reacted in the dark with more FNPA, linking the fresh nitrophenylazide group to the matrix through the second amino group dark reaction. This matrix-nitrophenyl-diamine-nitrophenylazide derivative will now be ready for photochemical linkage to the second member of the desired intermolecularly linked complex (i.e., cofactor, enzyme, whole cell or other enzyme system etc.).

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