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# Quantitative cytochemistry of nuclear and cytoplasmic proteins using the Naphthol Yellow S and dinitrofluorobenzene staining methods

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**Summary**

The 'total protein staining' of biological specimens with the electrostatically binding Naphthol Yellow S or the covalently binding dinitrofluorobenzene must be interpreted as methods which yield data on the specific amino acid pool of the proteins concerned. Both dyes bind to certain free amino-acid side-chains, giving different dye–protein ratios for various proteins. In the presence of DNA, dinitrofluorobenzene stains all proteins present in cell nuclei, whereas Naphthol Yellow S only stains the majority of the non-histone proteins. When protein staining methods are combined with the Feulgen–Pararosaniline ($SO_2$) procedure for DNA, decreased Feulgen–DNA contents were measured in dinitrofluorobenzene-stained isolated nuclei and lymphocytes.

## Introduction

The detection of specific proteins in cells or cell organelles can be achieved by a variety of staining methods. Most of these methods are based on the electrostatic or covalent binding of dye molecules to particular chemical groups (for example, amine, carboxyl) present in the amino acids of the protein. These dye binding sites, however, may be present in any protein and so all types of proteins become stained without exception. Therefore, specificity for one particular protein can only be achieved by immunocytochemical or enzyme cytochemical methods, which make use of the specific biochemical properties of the protein concerned. These areas of histochemistry, however, are beyond the scope of this paper, which will focus on the so-called total protein staining methods.

In cytochemistry, the main interest is no longer concentrated on specificity alone but is now more concerned with the possibilities of the quantitative application of the

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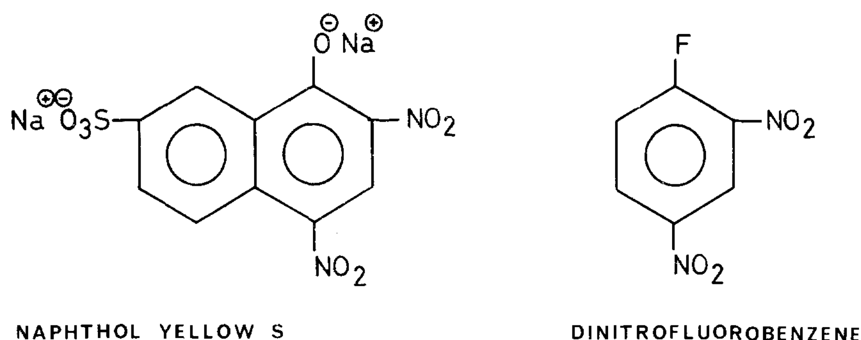


**Fig. 1.** Chemical structures of Naphthol Yellow S and dinitrofluorobenzene.

staining methods concerned. For this reason, we will discuss the application of two protein stains, Naphthol Yellow S (NYS) and dinitrofluorobenzene (DNFB) (Fig. 1). Several authors have shown in principle that these two reagents can be used quantitatively (Deitch, 1955, 1966; Tas *et al.*, 1974, 1978; Gaub *et al.*, 1975).

## Materials and methods

Preparations of isolated rat liver cells and isolated rat liver cell nuclei were obtained as described by Tas *et al.* (1978) and preparations of chicken erythrocytes as described by Van der Ploeg *et al.* (1979). Using fresh EDTA-treated venous blood, preparations of human lymphocytes were made according to Ornstein *et al.* (1976). Smears of the various preparations were air-dried and fixed with methanol–40% formaldehyde–acetic acid (85:10:5 by vol.) for 1 h at room temperature. After repeated washing in distilled water, the preparations were treated according to one of the following procedures.

(1) Staining with 0.1% (w/v) NYS dissolved in 1% acetic acid (pH 2.8) for 30 min, followed by rinsing in 1% acetic acid for 30 min (Tas *et al.*, 1974). For rat liver cells, the pH of the staining solution was adjusted to 4.0 with NaOH (Tas *et al.*, 1978).

(2) Staining with 0.7% DNFB dissolved in 50% ethanol containing 0.08 N sodium bicarbonate for 3 h at 65° C, followed by rinsing in 50% ethanol for 2 min at room temperature (Mitchell, 1967; Van der Ploeg *et al.*, 1979).

(3) Staining by the Feulgen–Pararosaniline ($SO_2$) procedure (Feulgen-Schiff method), as described previously (James, 1973; Tas *et al.*, 1974, 1978), followed by staining with NYS (Procedure 1). The Feulgen hydrolysis was carried out with 5 N HCl at 22° C for 60 min.

(4) Staining with DNFB (Procedure 2), followed by the Feulgen procedure (see Procedure 3).

For some experiments, the NYS and DNFB procedures were preceded by extraction of DNA with 5% (w/v) trichloroacetic acid (3 h, 60° C) and occasionally followed by extraction of basic proteins with 0.25 N HCl (1 h, 4° C) (Wang, 1967). After dehydration, the stained preparations were mounted in methyl salicylate ($n_D^{20} = 1.535$).

Cytophotometric measurements were performed with a Zeiss UMSP I as described by Tas *et al.* (1978, 1980). The Feulgen–DNA, NYS–protein and DNFB–protein contents (absorbances) of the different specimens were measured at 560, 430 and 420 nm respectively.

NYS–protein staining ratios were obtained with the aid of polyacrylamide model films possessing a periodic acid-soluble polyacrylamide gel matrix (Tas *et al.*, 1979). Bovine serum albumin or calf thymus histone was incorporated into the films (Tas *et al.*, 1974).

**Table 1.** Binding sites involved in the staining of proteins with Naphthol Yellow S and dinitrofluorobenzene.

| *Dye* | *Binding site* | $pk_a$ | *Source* | *Type of binding* |
|---|---|---|---|---|
| NYS | α-Amino | 9–11 | Terminal | Electrostatic |
| | ε-Amino | 10.5 | Lysine | |
| | Guanidino | 12.5 | Arginine | |
| | Imidazole | 6.0 | Histidine | |
| DNFB | α-Amino | – | Terminal | Covalent |
| | ε-Amino | – | Lysine | |
| | Sulphydryl | – | Cysteine | |
| | Phenolic hydroxyl* | – | Tyrosine | |
| | Imidazole* | – | Histidine | |

*Only a slight absorbance in the 400 nm region.

## Results and discussion

NYS, an acid dye, binds electrostatically at its sulphonic acid group to ionized basic groups present in the protein molecule (Deitch, 1955, 1966), while DNFB covalently binds to certain protein residues (Mitchell, 1967). Table 1 summarizes the binding sites involved in both cases. Of these sites, the α-amino groups probably do not contribute significantly to NYS staining in practice. From this fundamental knowledge about dye binding mechanisms, it can be concluded that these staining methods, which enable the cytophotometric measurement of the so-called 'total protein content' of cells or cell organelles to be obtained, must primarily be interpreted as methods which give a measure of the total amount of certain amino acid residues present. As a consequence, the dye–protein ratio will vary with different proteins. This is demonstrated by the clearly different NYS staining ratios obtainable for albumin (an acid protein) and histone (a basic protein) as shown in Table 2.

This phenomenon of protein dependent dye binding ratios has also been observed in 'biochemical' protein determinations using the well-known Lowry method (Lowry *et al.*, 1951; Pierce & Suelter, 1977) and the less common Coomassie Brilliant Blue method

**Table 2.** Naphthol Yellow S–protein dye binding ratios for albumin and histone. For method see Materials and methods.

| *Protein* (mol. wt) | *pI* | *nmol NYS/ μg protein* |
|---|---|---|
| Albumin (60 000) | 4.7 | 1.199 |
| Histone (14 700) | 10.8 | 2.893 |

**Table 3.** The Naphthol Yellow S- and dinitrofluorobenzene-protein content (in arbitrary units) of isolated tetraploid (T) and diploid (D) rat liver cell nuclei (RLN), isolated mononuclear tetraploid (MT) rat liver cells (RLC), human peripheral blood lymphocytes (HPL) and chicken erythrocytes (CE)*.

| | *NYS- and DNFB-protein content* (± S.D.; n = 20) | | | | |
|---|---|---|---|---|---|
| *Staining procedure* | *RLN-T* | *RLN-D* | *RLC-MT* | *HPL* | *CE* |
| NYS | 1120 ± 96 | 654 ± 74 | 30 075 ± 2730 | 757 ± 87 | – |
| TCA-NYS | 2397 ± 132 | 1230 ± 93 | 31 931 ± 2315 | 2101 ± 177 | – |
| TCA-HCl-NYS | 1632 ± 92 | 834 ± 84 | 31 012 ± 2569 | – | – |
| DNFB | 1592 ± 68 | – | 32 108 ± 2456 | 696 ± 103 | 1727 ± 133 |
| TCA-DNFB | 1324 ± 65 | – | 27 840 ± 2376 | 694 ± 108 | 1790 ± 114 |

*The nuclear and cellular preparations were stained according to different procedures, all including either staining with NYS at pH 2.8 (except RLC, which were stained at pH 4.0 in order to obtain accurate measurable absorbance values), or staining with DNFB.

(Pierce & Suelter, 1977; Van Kley & Hale, 1977). In the field of histochemistry, Cohn (1973) has reported different dye binding ratios for different proteins stained with alkaline Fast Green. Thus, it is clear that total protein staining methods are better described as staining methods for a specific amino-acid pool.

Apart from these fundamental aspects, a more practical but still important matter is the influence of DNA on the dye binding capacity of nuclear proteins. As shown in Table 3, the removal of DNA with hot trichloroacetic acid results in a two-fold increase in the NYS-protein content of isolated rat liver cell nuclei. On the other hand, DNFB staining appears to be uninfluenced by the presence of DNA. In order to check whether all basic proteins are blocked by DNA molecules, the trichloroacetic acid extraction was followed by a hydrochloric acid extraction procedure which removes the histones. The NYS-protein content measured under these circumstances was found to be significantly higher than the value measured from nuclei stained with NYS without any pretreatment. It is not easy to interpret this result, because it is not certain that the histones are removed completely by the hydrochloric acid treatment. It is possible, however, that the result indicates that ionic complexes formed between histones and acid nuclear proteins are important and must be considered.

For isolated rat liver cells, a significant increase in the NYS- or DNFB-protein content could not be observed after removing DNA (Table 3). On the other hand, lymphocytes and chicken erythrocytes showed staining characteristics similar to isolated nuclei, both with the NYS and DNFB methods. For mouse fibroblasts, Gaub *et al.* (1975) reported a 45% increase in the capacity of the cells to bind NYS after trichloroacetic acid extraction and Deitch (1955, 1966) reported a 30% increase in NYS binding by salamander liver nuclei after a similar treatment. It can be concluded, therefore, that NYS does *not* stain basic nuclear proteins and possibly may *not* stain a small part of the acid nuclear proteins

either when DNA is present. Nuclear protein staining with DNFB, on the other hand, is not influenced by the presence of DNA and, therefore, DNFB can be assumed to stain *all* nuclear proteins, although with the restrictions just discussed.

As regards the combined Feulgen–NYS and DNFB–Feulgen procedures, which enable DNA and protein to be measured simultaneously, it has been shown by several authors (Deitch, 1955, 1966; Gaub *et al.*, 1975; Mitchell, 1967) that in both cases the protein staining procedure and the DNA staining procedure had no chemical influence on each other. As mentioned in a previous paper (Tas *et al.*, 1980), this statement does not hold for *all* possible biological specimens. With isolated rat liver cells and chicken erythrocytes, similar Feulgen–DNA values have been found after simple DNA-staining and also when the Feulgen procedure was preceded by DNFB. In the case of isolated rat liver nuclei and human peripheral lymphocytes, however, the Feulgen–DNA readings decreased by about 20% after DNFB staining. This phenomenon was not observed when these preparations were stained by the combined Feulgen–NYS method. On the other hand, the DNFB–protein content remained unchanged after a subsequent Feulgen procedure in these examples. The NYS values were also found to remain unaltered.

Considering the findings presented in this paper as a whole, it can be concluded that NYS and DNFB each have their own specific advantages and limitations when these dyes are applied in quantitative (nuclear) cytochemistry. Both dyes bind to specific free amino-acid side chains, NYS by electrostatic interaction and DNFB covalently. There is no such thing as a 'general protein stain'.

In the case of intact untreated cell nuclei, NYS has been shown to stain *only* acid nuclear proteins, whereas DNFB stains *all* nuclear proteins. When each of the dyes are combined with Feulgen–DNA staining, attention should be paid to *possible* mutual influences of the staining procedures.

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