Fluorescamine: A Reagent for Assay of Amino Acids, Peptides, Proteins, and Primary Amines in the Picomole Range

Abstract. *Fluorescamine is a new reagent for the detection of primary amines in the picomole range. Its reaction with amines is almost instantaneous at room temperature in aqueous media. The products are highly fluorescent, whereas the reagent and its degradation products are nonfluorescent. Applications are discussed.*

McCaman and Robins (*1*) introduced a fluorometric method, now widely used, for assay of serum phenylalanine which is based on interaction with ninhydrin and peptides. Samejima *et al.* (*2*, *3*) found that it was the phenylacetaldehyde formed on interaction with ninhydrin, which combined with additional ninhydrin and peptide or any other primary amine to yield highly fluorescent ternary products. The structure of these products was subsequently elucidated by Weigele *et al.* (*4*), who then synthesized a novel reagent (*5*) that now replaces the fluorogenic ninhydrin reaction. This reagent, 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione (fluorescamine) (*6*) reacts directly with primary amines to form the same fluorophors (390 nm excitation, 475 nm emission) as are generated in the ninhydrin-phenylacetaldehyde reaction.

Several factors make fluorescamine suitable for assaying primary amines, including amino acids, peptides, and proteins. At *p*H 9, reaction with primary amines proceeds at room temperature, with a half time (*7*) of a fraction of a second. Excess reagent is concomitantly destroyed with a half-time of several seconds (*8*). The competing reactions are shown in Fig. 1. Fluorescamine, as well as its hydrolysis products, is nonfluorescent. Studies with small peptides have shown that the reaction goes to near completion (about 80 to 95 percent of theoretical yield) even when fluorescamine is not present in large excess.

For assay, primary amines are first buffered to an appropriate *p*H (≧ 7) and then fluorescamine, dissolved in a water miscible, nonhydroxylic solvent such as acetone is added. In a fraction of a second at room temperature, the reaction is complete and in less than a minute excess reagent is destroyed. The resulting fluorescence is proportional to the amine concentration and the fluorophors are stable over several hours. The above properties lend themselves well to automation. A relatively simple apparatus was developed for automated amino acid and peptide assay (*9*). Amino acid analysis of a solution representing 1 μg of hydrolyzed ribonuclease (Fig. 2) was compared with analysis of a 100-μg sample on a commercial analyzer. As little as 50 pmole of each amino acid can be determined with our apparatus. It should be noted that fluorescamine does not react with proline or hydroxyproline, which are not primary amines. This disadvantage may be overcome by introducing an appropriate intermediate step to convert these amino acids to primary amines. An advantage of the fluorescamine assay is that comparatively little fluorescence is obtained with ammonia. Therefore, ammonia does not interfere with analysis to the extent that it does in the colorimetric ninhydrin procedure.

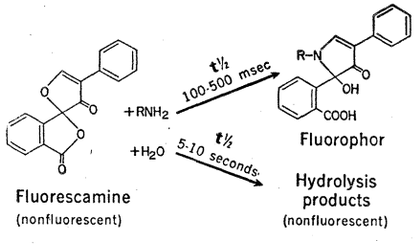

Fig. 1. Reaction of fluorescamine with primary amines and hydrolysis of the reagent at *p*H 9. Half-times are indicated.

Peptides generally yield greater fluorescence with fluorescamine than their component amino acids. Furthermore, peptides yield maximum fluorescence near *p*H 7, whereas amino acid fluorescence is generally maximum at *p*H 9, with little fluorescence appearing at *p*H 7 (Table 1). With the proper choice of buffers and reaction times, differences in fluorescence between peptides and amino acids can be made even greater. Since the relative fluorescence of individual fluorescamine derivatives is constant from *p*H 4 to 10, the variations depend on the reactivity of each amine at different *p*H values and on differences in quantum yield (*10*) for various types of fluorophors.

Many biologically active peptides, including oxytocin, vasopressin, bradykinin, angiotensin, substance P, insulin, and glucagon, yield highly fluorescent derivatives with fluorescamine. The oxytocin and vasopressin eluted from a chromatogram of an extract of a single rat pituitary can be determined (*11*). It has also been possible to detect peptides in column eluates of biological mixtures in the presence of amino acids by the persistence of fluorescence at low *p*H. The reagent may be used to determine the peptides in a tryptic digest of a protein. Even with the less sensitive ninhydrin-phenylacetaldehyde procedure it was possible to demonstrate over 20 peptide peaks in a chromatogram of 75 μg of a tryptic digest of hemoglobin (*3*).

Fluorescamine interacts with the primary amino groups of proteins to yield highly fluorescent derivatives. This can, of course, be used for protein labeling for conformational studies or for immunologic purposes. Fluorescamine-labeled antibodies have been prepared.

An important application has been the assay of proteins during protein purification procedures. Assays carried out manually can be used to determine 0.5 μg of protein. With a semiautomated procedure (similar to the one for amino acid assay) and a Bio-Gel column to separate nonprotein material, as little as 0.05 μg of protein can be assayed. Figure 3 shows a comparison of the fluorescamine technique with the standard Lowry procedure (*12*) for the monitoring of protein in a column effluent. The volumes used for fluorescamine assay were 10 to 20 percent of those used in the Lowry method, and smaller amounts could have been used. Background or noise was negligible with the automated fluorescence method, and two significant peaks not discernible by the Lowry procedure were observed.

Table 1. Influence of *p*H on the reaction of amino acids and peptides with fluorescamine. One volume of fluorescamine in acetone (10 mg per 100 ml) was added to three volumes of amine buffered with sodium borate (0.20*M* boric acid adjusted to the appropriate *p*H with sodium hydroxide).

Compound	Relative fluorescence	
	*p*H 7	*p*H 9
Alanine	0.10	0.73
Glycine	0.43	1.46
Leucine	0.30	1.35
Glycylglycylglycylglycine	1.60	1.40
Leucylalanine	2.80	1.43

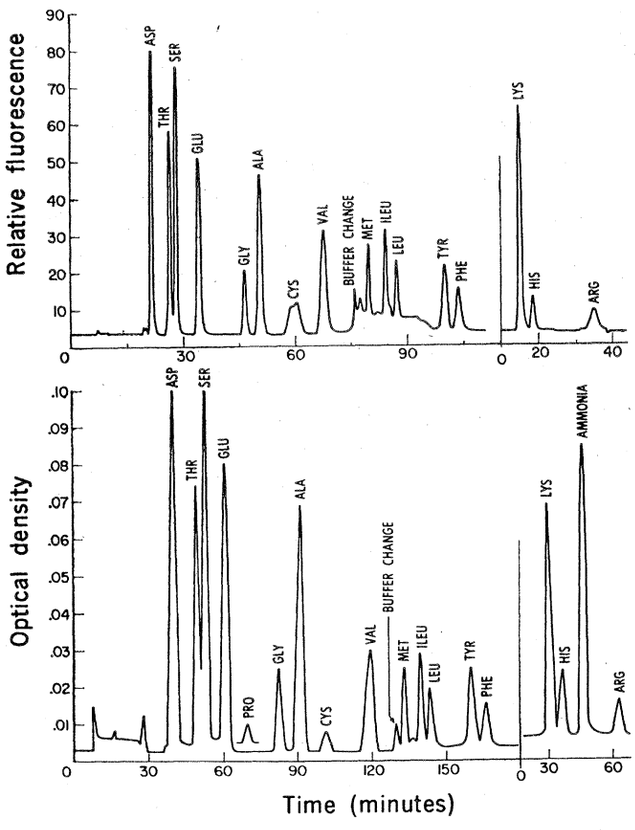

Fig. 2. Chromatography of hydrolyzed ribonuclease on the Beckman 120C analyzer (bottom) and the automated fluorescamine procedure (top). For the latter, Durrum DC-4A resin and Beckman concentrated buffers were used. The fluorescamine analyzer was assembled from an American Instrument Company filter fluorometer with a microflow cell, a strip chart recorder, high pressure metering pumps and various valves, columns, and other accessories from Chromatronix. The column effluent was first mixed with two volumes of sodium borate buffer to give a final *p*H of about 9.0 and then mixed with one volume of fluorescamine in acetone (15 mg per 100 ml). Flow rates were 7 ml/hour for the long column (30 by 0.28 cm) and 12 ml/hour for the short column (7.5 by 0.28 cm). Ala, alanine; Arg, arginine; Asp, aspartic acid; Cys, cystine; Glu, glutamic acid; Gly, glycine; His, histidine; Ileu, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

Fluorescamine can be used as an amine reagent not only in aqueous solution but also in organic solvents and on solids. It has been used as a spray to detect amino acids and peptides on thin layer chromatograms. As little as 20 pmole of each can be detected. Felix and Jimenez (*13*) have applied fluorescamine to determine completion of coupling reactions in solid-phase peptide synthesis. They can readily detect 0.1 percent of uncoupled material with greater simplicity than has previously been possible.

Fluorescamine yields intense fluorescence with other primary amines of biological importance. Thus, γ-aminobutyric acid, β-alanine, histamine, catecholamines, amphetamine, amino sugars, spermine, and spermidine also yield the characteristic fluorophor. Procedures for extracting and assaying the two polyamines in milligram quantities of tissue have been found feasible (*11*).

The introduction of ninhydrin more than 60 years ago proved most important to the developing field of protein chemistry. It is hoped that fluorescamine will be of similar value to future developments in biochemistry (*14*).

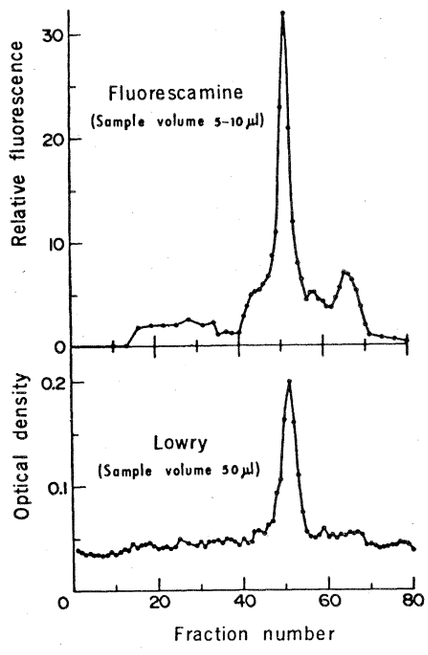

Fig. 3. Chromatography (on DEAE-Sephadex A-50, with a pyridine acetate gradient) of a partially purified preparation of guinea pig neurophysin monitored by the semiautomatic fluorescamine technique (top) and the Lowry procedure (bottom).

Note added in proof: M. Weigele, S. DeBernardo, and W. Leimgruber have found that proline, as well as other secondary amino acids, can be converted in simple fashion to primary amines which are detectable with fluorescamine.

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References and Notes

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3. K. Samejima, W. Dairman, J. Stone, S. Udenfriend, *ibid.* p. 237.
4. M. Weigele, J. F. Blount, J. P. Tengi, R. C. Czaijkowski, W. Leimgruber, *J. Amer. Chem. Soc.* **94**, 4052 (1972).
5. M. Weigele, S. L. DeBernardo, J. P. Tengi, W. Leimgruber, *ibid.*, p. 5927.
6. Fluorescamine is the trivial name for the compound. It can be obtained from Hoffmann-La Roche Inc., Nutley, N.J. 07110.
7. The reaction of fluorescamine with several primary amines, including aliphatic amines, amino acids, peptides, and a protein was followed in an Aminco-Morrow stopped-flow fluorometer (American Instrument Co.). The half-times of reaction at *p*H 9 ranged from 200 to 1000 msec, depending on the nature of the amine.
8. Inactivation of fluorescamine was followed by addition of one volume of fluorescamine in acetone to three volumes of aqueous buffer. At intervals, the remaining fluorescamine was treated with an excess of leucylalanine to form the corresponding fluorophor. At *p*H 9 the half time of inactivation was 5 to 10 seconds, depending on the buffer used.
9. S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, *Third American Peptide Symposium, Boston, June 1972*, J. Meienhofer, Ed. (Ann Arbor–Humphrey Science, Ann Arbor, Mich., in press).
10. Fluorescamine derivatives of several primary amines were prepared in crystalline form. Quantum yields were determined by the comparative method with quinine sulfate as a standard [R. F. Chen, *Science* **150**, 1593 (1965)]. The values ranged from 0.09 for the glycine methyl ester derivative to 0.34 for the leucylalanine derivative and were constant from *p*H 4 to 10.
11. K. Samejima, S. Stein, S. Udenfriend, unpublished observations.
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
13. A. M. Felix and M. Jimenez, *Anal. Biochem.*, in press.
14. Details of the above studies and several applications will appear in a number of subsequent reports.
15. We thank J. Stone for technical assistance; Dr. P. A. St. John, American Instrument Co., Silver Spring, Md., for permitting us to use special instrumentation for measurement of reaction rates and quantum yield; and Dr. H. Sachs for providing us with the column fractions shown in Fig. 3.

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