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DESIGN OF FLUOROMETRIC ANALYTICAL METHODS

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Abstract—The design is discussed of sensitive and specific fluorometric methods, which involves direct reactions (for cyanide, methyl ketones, and electrophiles). A non-stoichiometric fluorometric assay for cyanide is described and a discussion is given of the assay of atropine using induced coupled enzymes along with a cycling procedure to achieve extreme sensitivity and selectivity.

Fluorometry has been firmly established as method for the sensitive, specific and economic detection of trace quantities of substances. Generally, a fluorometric method has the potential of being 10–100 fold more sensitive than a colorimetric procedure, since dyes have a molar absorptivity of 10^5.[1] By coupling a fluorometric read-out with a catalytic or a non-stoichiometric chemical process even greater enhanced sensitivity may be achieved. The greatly improved fluorometric instrumentation becoming commercially available for automated analyses has further contributed to the increasing utility of fluorometry in such diverse applications as environmental pollution, clinical chemistry, biology and metallurgy.

Criteria for the choice of a set of reaction conditions for use in fluorometry include: rapid rates, fluorescent product stability, lack of internal quenching, and a highly fluorescent product. The total fluorescence intensity of the product, F (quanta/sec-4π geometry), is given by expression (1):

$$F = 2.3\Phi \cdot \epsilon \cdot c_x \cdot 1 \cdot \phi$$

where φ = radiant power of exciting light (quanta/sec);
ϵ = molar absorption coefficient;
c_x = concentration of solute x;
1 = optical path;
ϕ = quantum efficiency.[2]

This paper will describe the applications of a number of avenues of approach taken in the development of fluorometric assays.

ASSAY FOR CYANIDE

Benzoquinone has been shown to react with cyanide to yield 2,3-dicyano-1,4-dihydroxybenzene.[3]

The product had a λ_{ex} 400–420; λ_{em} 480–490, and could be assayed in the range of 0.2 μg/ml. Variously substituted benzoquinone and benzoquinonimine derivatives were investigated with respect to reaction rates, pH-buffers, and interferences. Hammett plots were made correlating reaction rates with the variously substituted benzoquinones. The parent benzoquinone and its 2,6-dichloro derivative possessed the optimum properties with respect to the assay of cyanide.

ASSAY FOR METHYL KETONES

It was known that *o*-nitrobenzaldehyde reacted with methyl ketone anions to yield indigo.[4] An intermediate in the formation of indigo was the highly fluorescent indoxyl (Scheme 1).

By proper choice of reaction conditions, a kinetic assay procedure was established for methyl ketones in the 10^{-5}–10^{-4} molar range. The pH (12.5) was found to be crucial in the assay since it was necessary to establish a high concentration of the methyl ketone anion to effect condensation with the *o*-nitrobenzaldehyde and allow the subsequent steps in the reaction leading to indoxylate ion and simultaneously minimize the rate of conversion of indoxyl to indigo.

ASSAY OF ELECTROPHILES

To meet the need for quantitative assays of electrophilic agents, such as acylating, sulfonylating and phosphorylating compounds, two new reagents were investigated: *o*-carboxyisonitrosoacetanilide and *N*-hydroxy-phthalimide. Scheme 2 illustrates the reaction of an electrophile with *o*-carboxyisonitrosoacetanilide leading to the highly fluorescent isatoic anhydride.[5]

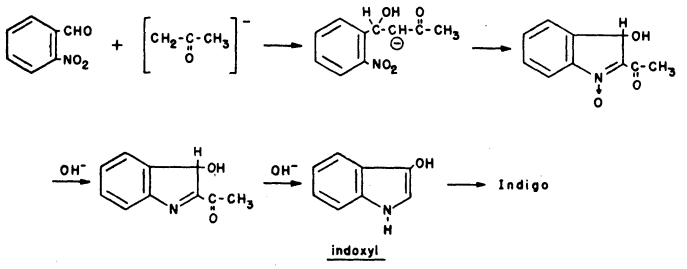
The problems encountered in this assay include determination of the concentration of the reactant to avoid quenching of the fluorescence of the isatoic anhydride anion but also allowing the rapid rate of the electrophilic reaction in the micromolar range. At the same time, it was necessary to avoid the side reactions of decomposition of the electrophile and the isatoic anhydride. Quantitative determinations were accomplished for solutions of electrophiles (benzene sulfonyl chloride, methyl-methoxyphosphonofluoridate, Parathion, Systox) in the 10^{-8} molar range using acetonitrile solvent and tetrabutylammoniumhydroxide. The reaction was complete in 10 min in the ng/ml range.

Similar results were obtained with *N*-hydroxyphthalimide following the reactions given in Scheme 3.

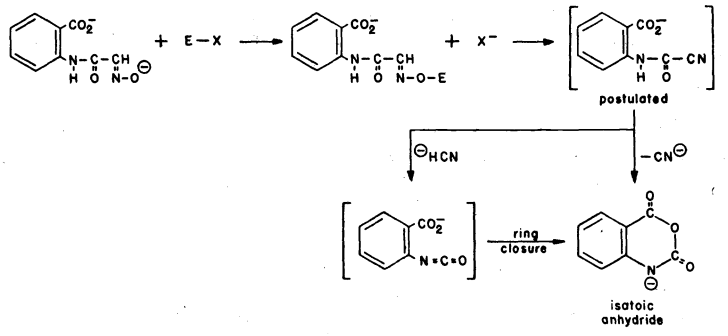
CATALYTIC-FLUOROMETRIC ASSAY OF CYANIDE

It was shown that cyanide ion reacts rapidly and uniquely with *p*-nitrobenzaldehyde, according to Scheme 4, to form a highly reducing anion adduct II capable of transferring electrons to resazurin (non-fluorescent) to produce resorufin (fluorescent), *p*-nitrobenzoate and cyanide. The regenerated cyanide then reacts again with the excess *p*-nitrobenzaldehyde to undergo the same series of reactions. By appropriate choice of reaction conditions the rate of product formation is dependent on cyanide concentration.[6] Thus, a fluorometric procedure becomes available wherein the resazurin is cyclically reduced to the fluorescent resorufin.

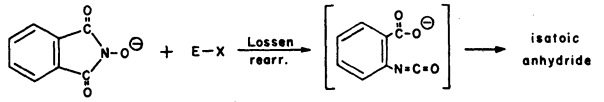
By monitoring the rate of fluorescence production, concentrations of cyanide in the 10^{-8}–10^{-9} molar range can be readily determined.



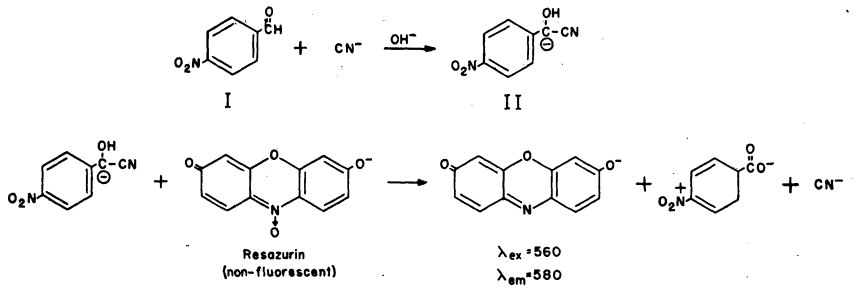
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

ENZYMATIC FLUOROMETRIC ASSAYS

The utilization of enzymes in assay procedures is predicated on the availablity of enzymes of known characteristics with respect to substrate association constants (K_m), inhibition constants (K_I), pH-activity relationships, etc. Commercially available enzymes are limited. However, microorganisms offer an untapped enzyme source. By a process of enzyme induction in adaptable microorganisms, the required enzymes may be obtained. The inducible microorganisms are propagated obligatorily in a synthetic growth medium containing the inducing substate as a sole source of carbon or nitrogen. The surviving cells are harvested and propagated further. The cells are processed to isolate the enzymes responsible for the metabolism of the inducing substrate. A variety of enzymes are thus obtained, including esterases, dehydrogenases, oxygenases—specific for the inducing substrate.[7] With this background, a fluorometric enzyme assay was devised for atropine, a compound of considerable medicinal interest.

Pseudomonas putida, indigenously growing about Atropa belladonna rhizomes were isolated and propagated in synthetic media containing atropine or tropic acid as the sole carbon source. The induced enzymes in the surviving organisms were isolated and found to display a potent atropinase (an esterase that splits atropine to tropic acid and tropine), tropic acid dehydrogenase (an enzyme that dehydrogenates tropic acid in the presence of DPN^+ to yield phenylmalonic and phenylacetic acids) and uncharacterized oxygenases. By coupling the atropinase and tropic acid dehydrogenase, atropine, and tropic acid could be determined spectrofluorometrically by the following sequence

a. Atropine $\xrightarrow{\text{atropinase}}$ tropic acid and tropine.

b. Tropic acid + DPN^+ $\xrightarrow[\text{phenylmalonic acid + 2 DPNH.}]{\text{tropic acid dehydrogenase}}$.

c. DPNH + resazurin $\xrightarrow{\text{diaphorase}}$ DPN^+ + resorufin.

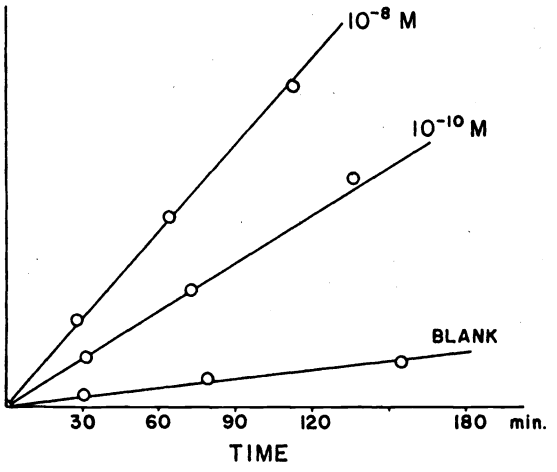


Fig. 1. The results of the cycling experiment in which 10^{-10} M atropine was determined.

d. lactate + DPN^+ $\xrightarrow[\text{pyruvate + DPNH}]{\text{lactic acid dehydrogenase}}$.

e. DPNH + resazurin $\xrightarrow{\text{diaphorase}}$ DPN^+ + resorufin.

(non-fluorescent) (fluorescent λ_{ex} 560, λ_{em} 580)

Atropine in the 10^{-7} M range can be readily assayed.

The next step was to apply a cycling procedure to the assay.[8]

This was carried out as follows:

Step 1. React 10^{-10} M solution of atropine with atropinase, producing tropic acid. Run a reagent blank concurrently.

Step 2. Treat tropic acid with 10^{-4} M DPN^+ and tropic acid dehydrogenase producing approximately 10^{-10} M DPNH.

Step 3. Treat with 0.1 N NaOH, preferentially destroying excess DPN^+, without affecting DPNH.

Step 4. Cycle DPNH as in Reaction *d*, *e*, using lactate, 10^{-3} M and resazurin, 10^{-5} M and diaphorase.

Step 5. Measure fluorescence at the end of 30, 60, 90, and 180 min.

Not only are the methods described above highly sensitive, but they are marked by specificity. Of the many esters tested with atropinase, only a cognate ester, phenylmalonic acid diethyl ester, showed a slight activity. Moreover, with tropic acid dehydrogenase, no other substrate has been found to possess activity other than tropic acid.

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