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Keyphrases

Alkaloids—*Clitocybe dealbata*
Muscarine—isolation
Biological analysis—muscarine
Chemical analysis—muscarine
Clitocybe species—muscarine, aging effect

Fluorometric Determinations Comparable to Eye Response

By JOSEPH E. SINSHEIMER, JAMES T. STEWART*, and JOSEPH H. BURCKHALTER

A filter-photomultiplier tube system approaching the spectral response of the human eye has been selected, its spectral response recorded, and the system evaluated with a series of model fluorescent compounds.

FLUORESCENT tagging agents are being synthesized in these laboratories for potential use in fluorescent antibody-antigen techniques (1). To determine more rationally structures to be synthesized, it was desirable to direct these studies by an objective and sensitive comparison of fluorescence. Cognizant that the antibody-antigen technique ultimately depended upon visual observation of fluorescence, a filter-photomultiplier tube detection system that approached the spectral response of the human eye (2, 3) was developed for use with commercially available fluorometers. As this system has general application for the comparison of materials to be employed for their visible fluorescence, the selection and evaluation of this system is reported.

EXPERIMENTAL

Apparatus—The Aminco-Bowman spectrophotofluorometer No. 4-8106, equipped with an Electro Instruments X-Y recorder No. 1620-809, and a detection system consisting of a Kodak Wratten filter No. 106 and a RCA 1P28 phototube were employed in this procedure.

Spectral Response of the Detection System—In order to describe the spectral response of a 106 filter–1P28 photomultiplier detection system, the following comparison of experimental to reference corrected spectra was employed. Relative quantum intensity data were obtained from a corrected fluorescent spectrum of quinine sulfate[1] by dividing the intensity at 10-mμ increments by the maximum intensity. The relative quantum intensity for the aluminum chelate of an azonaphthalene sulfonic acid dye (aluminum-PBBR chelate) has been described by White (4) and served as the second reference corrected spectrum.

Solutions of quinine sulfate (10×10^{-6} Gm./ml. in 0.1 N H_2SO_4) and the aluminum-PBBR chelate were placed in the fluorometer equipped with the 106 filter and their fluorescence emission spectra were taken in the usual manner at the appropriate excitation wavelengths (365 and 540 mμ, respectively).

Based upon these experimental spectra, values at 450 mμ for quinine sulfate and 630 mμ for aluminum-PBBR chelate were each set equal to 100% quantum intensity and values at the other wavelengths for each compound were made relative to it. Dividing these percentages by the relative quantum intensity from the corrected spectra gave quantum values which were then divided by their respective wavelengths to give energy per wavelength data. The energy data were plotted so that the values for both compounds were made equal at 580 mμ (a common wavelength nearest to that of 560 mμ, the peak for the average human eye). Values at the other wavelengths for both compounds were made relative to 100% for the maximum at 575 mμ of the combined curves. (See Fig. 1.)

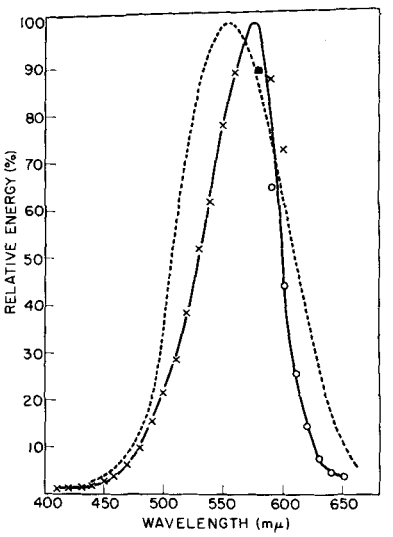

Fig. 1—Relative energy response of the 106 filter-1P28 photomultiplier system as compared to the average human eye. Key: ------, human eye responses; ——, 106-1P28 response; X, quinine sulfate data; O, Al-PBBR data.

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[1] Spectrum supplied by Dr. R. E. Phillips, G. K. Turner Associates, Palo Alto, Calif.

Visual Detection Measurements—A fluorescent compound was dissolved in an appropriate solvent and its emission spectrum determined in the Aminco-Bowman spectrophotofluorometer equipped with the 106 filter. Concentrations were chosen to be in a range linear with fluorescence and the proper excitation wavelengths were employed for each compound. The approximate area under an emission peak for a particular substance (peak height by width at half height in cm.) was divided by the concentration of that compound in moles per milliliter to provide intensity in terms of cm.^{2} mole^{-1} ml. data for each compound.

DISCUSSION AND RESULTS

A comparison of the blue fluorescence of a series of compounds synthesized in these laboratories to that of quinine sulfate by the quinine reference unit procedure (5) proved successful for estimating relative fluorescence of these compounds. However, this same comparison was not satisfactory for predicting success in the fluorescent antibody technique. That is, compounds found by this procedure to possess fluorescence of medium intensity compared to fluorescein isothiocyanate, a widely used antibody tagging reagent (6), did not provide observable fluorescent conjugates in the fluorescent antibody technique. Thus, a need for a detection system approaching that of the human eye was indicated.

The nine-stage photomultiplier tubes (RCA 1P21, 1P28, and 931A) commonly employed with commercially available fluorometers were investigated. As the relative spectral response of these tubes approach that of the eye at wavelengths above 500 mμ, there was only the need to modify the response at the lower wavelengths. Both a Kodak Wratten filter No. 106 and a Corning filter No. CS 3-124 were evaluated for this purpose with RCA 1P21, 1P28, and 931A tubes. A comparison of these systems for the spectral response of fluorescence obtained from quinine sulfate to the corrected relative response of quinine sulfate as reported by Lippert (7) was made. For the individual tubes used in the present study, the 106 filter was the most satisfactory and gave approximately the same relative response curves for each of the three types of tubes. However, as the 106–1P28 combination was two times more sensitive than the other system, it was used for more extensive study.

In a further description of the 106–1P28 detection system, quinine sulfate and the aluminum–PBBR chelate, suggested by White (4), were used as reference sources. These compounds covered the range of fluorescence emission that was of interest in approximating the eye response, and their corrected emission spectra have been established (4, 7). Emission spectra of these compounds were determined in the Aminco-Bowman spectrophotofluorometer with the 106 filter in front of a 1P28 photomultiplier tube. In this manner, the standard materials serve as a defined source so that the ratio of the experimental curves to their corresponding standard corrected curves serve to describe the effect of filter, photomultiplier tube, and emission monochromator. This procedure is analogous to the use of such standard compounds for the calibration of the emission portion of a fluorometer (4).

The relative quantum intensity data obtained in this manner were expressed in energy units by dividing by wavelength to give a direct comparison to the relative energy data available for the human eye. Finally, in order to form a continuous curve, the intensity values obtained for each reference compound were made relative to their values at 580 mμ as an equal value. This wavelength was chosen as being common to both reference spectra and the wavelength nearest to the 560 mμ maximum for the average human eye.

TABLE I—FLUORESCENCE RELATIONSHIPS TO RHODAMINE B

Compd.	Excitation λ (mμ)	Normal Emission λ (mμ)	Relative Fluorescence Without Filter	Relative Fluorescence With Filter[g]
Rhodamine B[a]	540	580	1.000	1.000
Rhodamine B isothiocyanate[b]	540	580	0.300	0.260
Disodium fluorescein[c]	490	520	12.500	5.000
Fluorescein isothiocyanate[b]	490	520	10.000	3.330
4-Naphtho-(1,3;4,5)-triazolylstilbene-2-sulfonic acid, sodium salt[d]	365	460	6.330	0.510
4,4′-Bis(4-anilino-6-di-ethanolamine-*s*-triazin-2-ylamino)-2,2′-stilbene-disulfonic acid disodium salt[e]	365	435	1.800	0.024
Quinine sulfate[f]	365	455	4.900	0.051

[a] Allied Chemical Co. [b] Baltimore Biological Laboratories. [c] Eastman Kodak. [d] Geigy Chemicals. [e] American Cyanamid Co. [f] Mallinckrodt Chemical Works. [g] All values in this column could be multiplied by a factor of 0.35 to compare fluorescence relative to rhodamine B determined without filter.

The relative energy responses of the 106–1P28 detection system used in these studies and derived as just described is given in Fig. 1. For purpose of comparison, the relative energy response of the human eye is also given in this figure.

An application of the 106–1P28 system for the evaluation of compounds whose fluorescence is of interest in the visible range is given in Table I. All compounds were compared at concentrations in the linear portion of their concentration-fluorescence curves. Two series of fluorescent intensities were compared to those of rhodamine B with and without the presence of the 106 filter. Of the compounds evaluated, rhodamine B with an emission maximum at 580 mμ nearest to the 575 mμ maximum of the system showed the least effect (35% reduction) with the presence of a 106 filter. While fluorescein shows a significant change in relative fluorescence in the two systems, this change is even more pronounced with compounds of blue fluorescence at the lower wavelengths.

Compounds exhibiting fluorescence at these lower wavelengths are of particular interest in our studies, so that the 106 filter–1P28 photomultiplier system has proven of value in our evaluation of the fluorescence of potential antibody tagging agents. This same system should have general application where a fluorescence detection system approaching that of the human eye is required.

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Keyphrases

Fluorometry—analysis
Eye response—fluorometry, comparable
Quinine—azonaphthalene sulfonic acid dye aluminum chelate, reference source
Rhodamine B—fluorescence standard

Quantitative Analysis and Alkaline Stability Studies of Allopurinol

By PHILIP D. GRESSEL and JOSEPH F. GALLELLI

Methods for quantitative chromatographic separation and spectrophotometric analysis are reported for allopurinol [4-hydroxypyrazolo(3,4-*d*) pyrimidine] in the presence of its alkaline decomposition products. Stability data are presented using rate constants obtained from Arrhenius-type plots; $t_{90\%}$ for an unbuffered sodium allopurinol solution is approximately 150 days at 25°. The formate salt of 3-amino-4-pyrazolecarboxamide accounts for essentially all the degradation under pharmaceutically usable conditions.

ALLOPURINOL, 4-hydroxypyrazolo(3,4-*d*)pyrimidine, is a new, potent inhibitor of xanthine oxidase used in the treatment of hyperuricemia associated with gout. Extensive studies with the parenteral form of this drug have been performed by the National Cancer Institute to evaluate its effectiveness in the prevention of hyperuricemia and uric acid nephropathy in patients treated with X-ray and/or chemotherapy. The chemical stability studies in the present work were oriented toward conditions likely to be encountered in the clinical studies.

Some preliminary work (1) had been done previously on the assay of allopurinol solutions, using the ratio of optical absorbance at 262 mμ:255 mμ. It is found, however, that the UV spectrum of the major decomposition product does not differ enough from that of allopurinol, at any pH, to enable one to perform accurate determinations (see Fig. 1). The preliminary work (1) utilizing simple spectrophotometric analysis had not resulted in the detection of any decomposition of a refluxing solution of sodium allopurinol. However, thin-layer chromatography conducted in this laboratory on similar samples clearly showed the presence of two compounds in significant amounts. This led to the search for a larger scale quantitative method of separation of the components in aqueous solutions.

A specific method of assay was developed for allopurinol in the presence of its decomposition products, utilizing ion-exchange chromatographic separation and spectrophotometric analysis. Kinetic studies employing the assay method were done for some alkaline conditions.

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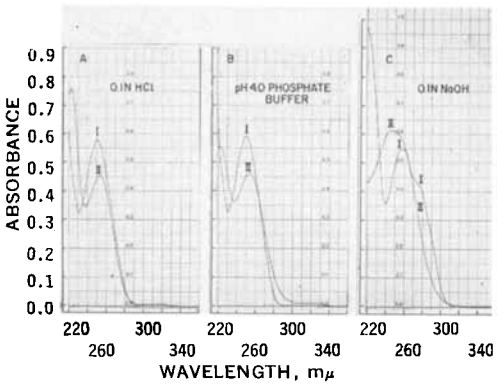

Fig. 1—Ultraviolet spectra of allopurinol (I) *and 3-amino-4-pyrazolecarboxamide* (II), *1.04 × 10⁻² M, in 0.1 N HCl, pH 4.0 phosphate buffer (0.1 M phosphate, 0.2 M NaCl), and 0.1 N NaOH.*

EXPERIMENTAL

Reagents—Allopurinol,[1] m.p. greater than 320°, was confirmed by IR spectrum (no official standards exist).

A strongly basic ion-exchange resin,[2] 200–400 mesh, was used; it was washed alternately with large volumes of 2 *N* HCl, water, and 2 *N* NaOH, (2) until the effluent was clear both by visual inspection and by ultraviolet spectrum. Then the procedure was repeated with the phosphate buffers, visual inspection. The pH 10 washings were continued until there was no change in pH. Stock solutions of phosphate buffers were prepared as follows: (*a*) pH 10 buffer, 0.100 *M* Na_2HPO_4, 0.200 *M* NaCl, adjusted to pH 10.00 with NaOH; (*b*) pH 4 buffer, 0.100 *M* $NaH_2PO_4 \cdot H_2O$, 0.200 *M* NaCl, adjusted to pH 4.00 with HCl.

Silica gel containing a fluorescent material[3] was

[1] Obtained from Burroughs Wellcome & Co., Tuckahoe, N. Y.

[2] Marketed as Dowex-1X8 (chloride form) by the Dow Chemical Co., Midland, Mich.

[3] Silica gel GF_{254} according to Stahl for thin-layer chromatography, manufactured by E. Merck Ag., Darmstadt, Germany; distributed by Brinkmann Instruments Inc., Westbury, L. I., N. Y.