Fluorescent Lipid Autoxidation Products

Potassium β-oxyacrolein, the enolic salt of malondialdehyde, underwent self-condensation reactions and polymerized at pH 7–8 to form fluorescing compounds which have been characterized by chromatography on LH-20 Sephadex columns and on silica gel G thin-layer plates as well as by their fluorescence spectra. Excitation maxima (Ex) were located at 390 and 468 nm while their emission maximum (Em) was at 550 nm. The fluorescing characteristics of the β-oxyacrolein condensation products (OACP) changed in the presence of amines and their Ex and Em shifted to 385 and 460 nm, respectively, with an increase in fluorescence intensity to values similar to those already reported for malonaldehyde–amine reaction products. Butanol extracts from the area of the lateral muscle of rancid herring showed Ex and Em corresponding to the nitrogen free fluorescing OACP as well as to the malonaldehyde–amine products.

In aqueous solution, concentrations of malonaldehyde up to 10^{-3} *M* were colorless and turned yellow, with a new absorption maximum at 477 nm only when heated at 100° or when left standing for extended periods of time at room temperature (Kwon and Watts, 1964). These authors assumed that a new reaction product must be responsible for the appearance of the light absorption in the visible spectrum and rationalized a hydrogen-bonded dimer structure of enolic malonaldehyde tautomers for it. Later it was shown that the infrared spectra of malonaldehyde differed depending on the concentrations of the solutions from which the samples had been prepared, i.e. in concentrated solutions the carbonyl absorption or concentrations decreased and new unexplained peaks appeared (Kwon and Van der Veen, 1968). Recent interest in the fluorescent properties of malonaldehyde derivatives as potential indicators for the autoxidation of polyunsaturated lipids (Chio and Tappel, 1969; Buttkus and Bose, 1972; Malshet and Tappel, 1973) made it desirable to further investigate the reagents, particularly malonaldehyde, involved in the formation of the fluorescing compounds.

EXPERIMENTAL SECTION

The fluorescing β-oxyacrolein condensation product (OACP) was prepared by mixing 3.10 ml of tetramethoxypropane (bp 178–179°, J. T. Baker Chemical Co.) with 0.75 ml of 2 *N* HCl to hydrolyze the acetal at 50° for 30–60 min. The reaction mixture was then cooled in ice, neutralized with about 5 ml of 1 *M* KOH, and adjusted to between pH 7.0 and 8.0. The resulting solution of the potassium salt of β-oxyacrolein was then reduced in volume in a rotary flash-evaporator (water pump) at 40–50°. To the remaining viscous, yellow-brown film was added anhydrous ethanol or benzene and the water and solvent were further removed by flash evaporation at the above temperature. The latter step was repeated one or more times. The flasks were immediately stoppered, wrapped in aluminum foil, and left at room temperature to be examined daily for increasing yellow fluorescence (excitation maxima (Ex) 360 nm). After 3 to 4 days the reaction mixture fluoresced in a bright yellow color and the reaction vessel was evacuated with an oil pump. The condensate in the Dry Ice trap, inserted between the pump and the reaction vessel, contained only water. The fluorescing product was extracted into redistilled 1-butanol and removed from an insoluble KCl precipitate. Most of the 1-butanol could then be distilled off from the salt-free OACP under reduced pressure and the remaining solvent was evaporated without the application of heat until a dry yellow-brown to red amorphous solid was obtained. Storage experiments of OACP dissolved in ethanol, dimethyl sulfoxide, or 1-butanol over a period of 0 to 4 days at room temperature showed that the fluorescence was most stable in 1-butanol while the solid appeared to be stable indefinitely if kept dry. Diluted 1-butanol extracts of the desalted, dried OACP were used for application to thin-layer plates, chromatography columns, and in spectroscopic measurements.

A solid, brown product could also be produced when an acid hydrolysate of malonaldehyde bis(dimethyl acetal) was adjusted to pH 3.0 and left standing at room temperature for several days. At more acidic pH values the color of the condensation product would change to dark brown and on to black and would not always be fluorescent.

Thin-layer chromatography (TLC) was carried out on 8 × 2 in. silica gel G plates using 1-butanol–methylene dichloride–water (20:4:1) as ascending solvent. The brightly yellow fluorescing spots of the OACP were detected in a Chromato-Vue box at 360 nm. The OACP product extracted with 1-butanol from the dried reaction mixture of potassium β-oxyacrolein was also separated into different components by chromatography on a Sephadex LH-20 column (16 × 1.5 cm). A volume of 0.5 ml of the saturated 1-butanol extract was applied to the column and eluted with ethanol.

Spectra of fluorescence were obtained with a Fluorispec Model SF1 (Baird-Atomic Inc.). The instrument was adjusted at Ex 350 and Em 450 nm to 100% with a solution of quinine (1.0 μg/ml of 0.1 *N* H_2SO_4) and the relative fluorescence of the dried OACP dissolved in 1-butanol was measured at Ex 390 and Em 550 nm. Visible and ultraviolet spectra were obtained with a Beckman DB-G grating spectrophotometer and infrared analysis was performed with a Unicam SP 200 and a Beckman 20A instrument. The samples were prepared as KBr pellets or in the form of Nujol mulls between KBr plates.

RESULTS AND DISCUSSION

Aldehydes readily undergo aldol condensation reactions which are catalyzed by acids and bases with a pronounced catalytic effect by amines (Nielsen and Houlihan, 1968). In the present experiments, potassium β-oxyacrolein was allowed to react in a concentrated semisolid film. When the reaction flask was removed from the flash evaporator it was immediately stoppered to prevent the aldehyde mixture from coming in contact with ammonia or other nitrogenous compounds from the air. At this point the reaction mixture was of yellow to brown color and displayed only a faint fluorescence or none at all when viewed under ultraviolet light (360 nm). However, after the flask had been left at room temperature for 3 days, a bright yellow fluorescence had developed which could be extracted from the reaction mixture into 1-butanol in which it appeared most stable and showed fluorescent Ex and Em maxima at 390 and 550 nm. On the addition of an excess of leucine ethyl ester to the solution of the OACP the Ex and Em maxima shifted to 385 and 460, respectively, and the fluorescence intensity gradually increased 25-fold.

TLC of the OACP on silica gel G using 1-butanol–methylene chloride–water (20:4:1) as ascending solvent separated the reaction products into three closely migrating spots

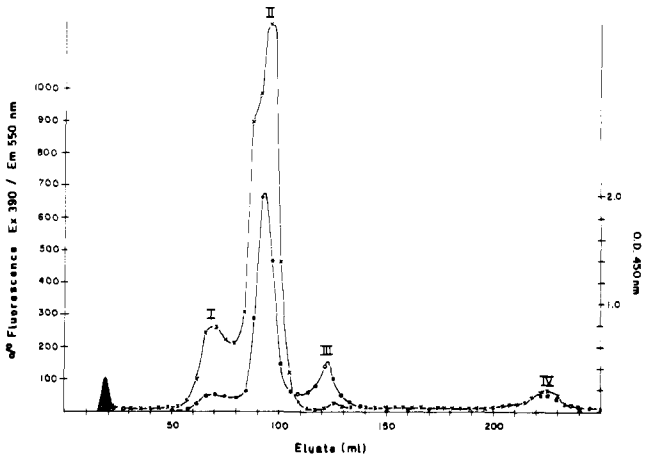

Figure 1. Chromatography of the aldol addition products from potassium β-oxyacrolein on a Sephadex LH-20 column (16 × 1.5 cm). Elution was carried out with absolute ethanol; flow rate ~0.4 ml/min; fractions of about 5 ml were collected. After the fluorimeter had been adjusted to 100% with a solution containing 1.0 μg of quinine per ml of 0.1 N H_2SO_4 at Ex 350 and Em 450, the percent fluorescence of the eluate was measured at an excitation setting of 390 and emission setting at 550 nm (X–X). The light absorption was measured with a spectrophotometer at 450 nm (O–O). The specific fluorescence maxima Ex/Em for the pooled fractions and their relative percent fluorescence values were: (I) 468/550 = 500%, 368/550 = 500%; (II) 468/550 = 6400%, 382/550 = 2800%; (III) 468/550 = 500%; (IV) 368/550 = 400%, 468/550 = 337%.

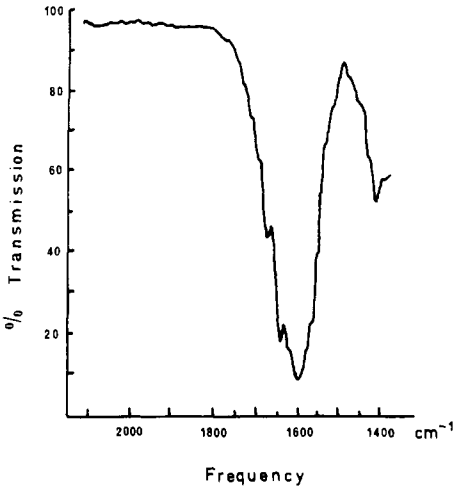

Figure 2. The predominant infrared absorption peaks of the β-oxyacrolein polymerization product (KBr pellet, 24-min scan) with the characteristic strong absorptions for the C=C stretching vibrations of aliphatic conjugated polyene compounds at 1645 and 1595 cm^{-1} and for the conjugated carbonyl stretching vibration at 1670 cm^{-1}.

with R_f values of 0.27, 0.35, and 0.42. The slower two spots showed the brightest yellow fluorescence. When the solvent evaporated from the TLC plates the fluorescence disappeared gradually.

Chromatography of a butanol extract of the OACP on a Sephadex LH-20 column with absolute ethanol separated the nitrogen free product into five fluorescing components (Figure 1). Although the fluorescence did coincide with yellow color, the relative intensities varied considerably. When TLC was carried out on the major peak (eluate fraction 80–105 ml) two major fluorescing spots with R_f values of 0.22 and 0.29 and one faintly noticeable spot at R_f 0.14 were observed.

While a crude butanol extract of the potassium OACP had an absorption maximum in the visible region of the spectrum at 445 and a minimum at 405 nm with an optical density ratio at 445 nm/405 nm of 1.15, the absorption maximum of the major fraction obtained from the LH-20 column was 460 nm with an optical density ratio 460 nm/405 nm = 1.85. In the uv region, one absorption peak of the nitrogen-free OACP was at 360 nm and another prominent peak was at 254 with a shoulder at 275 nm. The extinction coefficients of the absorption maxima decreased toward the visible end of the spectrum, i.e. 254 > 275 > 360 > 460 nm. The overall spectrum of the OACP, therefore, seems to bear a striking resemblance to that of 1-amino-3-iminopropene (Chio and Tappel, 1969) after standing for more than 1 hr in water or ethanol. Dissolving the reddish OACP in 1-butanol and heating it at 100° for 4 min the absorption maximum shifted to 470 nm and on prolonged heating finally turned a dark brown color and finally black. Excitation of these compounds with 360-nm light produced fluorescence emissions from bright yellow to red; however, the dark brown to black polymers did not emit any fluorescence. The color contribution of the OACP in the 2-thiobarbituric acid test during a 10-min heating period (Buttkus and Bose, 1972) was only 22% of the yield obtained from an equal weight of malonaldehyde and increased to 27% after standing for 48 hr in a 1:1 water–ethanol solution. Upon reduction of the fluorescing OACP with $LiAlH_4$ or $NaBH_4$ the absorption peaks at 460 and 360 nm disappeared and a pale yellow liquid remained.

From the above data it would appear that the products obtained from potassium β-oxyacrolein under the stated experimental reaction conditions have been formed by aldol type condensation mechanisms and contain predominantly open chain, conjugated double bond systems.

$$x\text{-OCH=CHCHO} \overset{2H^+}{\rightleftharpoons}$$

$$\text{HOCH=CHCH(OH)CH(CHO)CHO} \overset{-H_2O}{\rightleftharpoons}$$

$$\text{HOCH=CHCH=C(CHO)CHO} \overset{-H_2O}{\rightleftharpoons}$$

$$\text{HOCH=CHCH[=C(CHO)CH]}_{x-2}\text{=C(CHO)CHO}$$

The aldol condensation can presumably also proceed by the reaction of monomer, dimer, and trimer with their own kind or among each other to produce long conjugated polyene systems containing from 6 to 10 or more conjugated double bonds as in vitamin A aldehyde and the carotenes which have light absorption maxima around 400 to 480 nm and Ex and Em maxima of fluorescence at 327 and 510 nm (Noller, 1957; Rao, 1961; Udenfriend, 1962; Karrer and Jucker, 1948).

Infrared spectra also provide evidence in support of the general structural characteristics represented by the above formula for the β-oxyacrolein condensation product. For a conjugated polyene aldehyde absorption bands due to C=O stretching vibrations are assigned to the 1660–1680-cm^{-1} region and for multiple C=C stretching vibrations of aliphatic conjugated polyenes very strong absorptions in the 1580–1660-cm^{-1} region have been established (Cross, 1960). According to Fleming and Williams (1966) aliphatic conjugated polyenes are represented by a composite peak, made up primarily of two characteristic absorption bands at about 1650 and 1600 cm^{-1}, of which the lower frequency absorption usually is the more intense and overlaps with the one at the high frequency. These frequency assignments correspond very well with the ir spectrum of the OACP in a KBr pellet (Figure 2). In the Nujol mull the separation of these two peaks was even more pronounced, with the high frequency absorption at 1650 and the lower at 1580 cm^{-1}. For the C—H stretching vibration of the C=C—H grouping a medium intensity (50% transmittance) peak was at 3050 cm^{-1}. C–O stretching and O–H deformation absorptions were predominant in the 1050–1150- and 1260–1350-cm^{-1} regions, and a broad peak centered at about 3400 cm^{-1} in the KBr pellet and at 3200 cm^{-1} in the

Nujol mull spectrum indicated O–H stretching vibrations of intermolecular polymeric associations via hydrogen bonds.

The infrared spectrum of the OACP was, therefore, very similar to that obtained from the dichloromethane extract of a concentrated (0.5 *M*) malonaldehyde solution (Kwon and Van der Veen, 1968). Relative to their malondialdehyde spectrum prepared from a dilute solution, the spectrum of the concentrated reagent showed a decrease in carbonyl absorption and the appearance of O–H stretching vibrations.

In the presence of amines the proposed AOCP would be able to form enamine–imine type structures –N=CH—CH=CH—NH– and since enamine intermediates are operative in amine-catalyzed aldol condensations (Nielsen and Houlihan, 1968), further polymerization, shifts toward longer wavelength in light absorption, and higher fluorescent intensities might be expected. The slow loss of fluorescence of the OACP after removal of solvent from the TLC plates, i.e. direct contact with silicic acid or the gradual loss of fluorescence in dilute solutions of polar solvents such as water, ethanol, or dimethyl sulfoxide, at room temperature, are indications of the ease with which the equilibria of these condensation reactions can be altered.

These data also appear to be in agreement with the earlier work by Sawicki et al. (1963) who found that polar solvents inhibited the formation of fluorescence products between ethyl *p*-aminobenzoate and malonaldehyde while concentrated acid treatment and heating or the addition of amines enhanced it.

Herring muscle, containing at times up to 30% of lipids, generally showed a faint blue-white background fluorescence when fresh. However, with the onset of rancidity, storage for about 1 year at −12°, blotches of intensely yellow fluorescence were detectable particularly around the fins, the lateral line area, and in the regions of red muscle, metabolically active tissues which are high in lipids. At this stage it appeared as if the oxidized fluorescing lipids had permeated through the skin and butanol extracts of these muscle areas showed a distinct yellow fluorescence when examined under long-wavelength uv light. Marinaded products prepared from poorly stored herring also showed the bright yellow fluorescence. Applied to a Sephadex LH-20 column and eluted with absolute ethanol, the extracted fluorescing material from fish tissue appeared immediately after the void volume (Figure 1, cross hatched peak) and displayed two fluorescent Ex at 398 and 468 nm and respective Em at 470 and 550 nm, indicating the presence of two classes of fluorescing products with relatively large molecular weights up to 800. Due to some adsorption of the OACP to Sephadex LH-20, molecular weight estimates are uncertain; however, preliminary values for the OACP fractions I and II range from 190 to 310 mol wt units. Vapor pressure osmometer readings of the crude product in ethanol gave a mol wt of 200.

In conclusion, it would appear that the formation of nitrogen-free malonaldehyde polymers in biological tissue is possible under certain conditions; however, due to the omnipresence of amines, these polymers will probably exist predominantly in association with them.

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Cucurbit Root Starches: Isolation and Some Properties of Starches from *Cucurbita foetidissima* HBK and *Cucurbita digitata* Gray

Starches in potentially commercial amounts have been found in the roots of two species of feral xerophytic gourds, *Cucurbita foetidissima* and *Cucurbita digitata*. Commonly known as Buffalo gourd and Digitata gourd, these perennials are well adapted to marginal agricultural lands of semi-arid and arid environments. Starch can be readily isolated from the large storage roots. In each starch the granules resemble those of tapioca starch. Their average diameters are approximately 6 and 9 μ, respectively. Iodine affinity values of 4.07 and 4.42 suggest typical composition with respect to amylose and amylopectin content.

Curtis (1946) originally proposed that the wild perennial gourds of western and southwestern United States be investigated as possible seed oil and protein crops. Jacks et al. (1972) have reviewed subsequent work and concluded that certain xerophytic species do have such potential value. The feral xerophytic Buffalo gourd, *Cucurbita foetidissima*, has evolved in the semi-arid regions of western North America. A group of four closely related species