Stability of Patulin and Penicillic Acid in Fruit Juices and Flour

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Patulin and penicillic acid are fungal toxins which could be present in contaminated foods, particularly fruit juices and flours. A semiquantitative assay for these compounds, based on thin-layer chromatography, has been developed and applied to the determination of their stability in apple, grape, and orange juices, and in whole wheat and bleached flours. Both compounds were appreciably stable in grape and apple juice but not in orange juice or the flours, partially at least because of reaction with thiols. Heating the juices at 80° C. for short time periods did not completely destroy the toxins. Patulin was decomposed by the model thiol glutathione at pH's as low as 2.3 and 3.0. If high concentrations of patulin and penicillic acid are initially present in fruit juices of low SH content appreciable concentrations may well remain in the processed juice.

The antibiotics patulin {4-hydroxy-4H-furo[3,2-c]-pyran-2(6H)-one} and penicillic acid (3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid) are produced by a variety of Aspergillus and Penicillium species (Abraham and Florey, 1949). Some of these fungal species are likely contaminants of foods. Thus P. expansum, the common storage rot of fruit; A. clavatus, A. terreus, P. cyclopium, and P. urticae isolated from flour by Graves and Hesseltine (1966); and Byssochlamys nivea, the heat-resistant fruit juice contaminant identified by Kuehn (1958) as the Gymnoascus sp. of Karow and Foster (1944) have all been shown to synthesize patulin (Abraham and Florey, 1949; Efimenko and Yakimov, 1960). Similarly, penicillic acid is produced by fungi that include A. ochraceous, a contaminant of wheat and corn (Christensen, 1962), and P. cyclopium (Abraham and Florey, 1949; Gill-Carey, 1949).

Both patulin and penicillic acid are appreciably toxic to animals (Abraham and Florey, 1949). Dickens and Jones (1961) found them to be sarcomagenic to rats when administered by subcutaneous injection, and penicillic acid was similarly carcinogenic to mice (Dickens and Jones, 1965). Patulin is known to be a mitotic poison (Dustin, 1963), and Kraybill and Shimkin (1964) point out the possible health hazard if it is present in foods or animal feeds. In fact, patulin has been isolated from a Penicillium which infected a malt feed responsible for the death of cows (Ukai et al., 1954).

Although many workers have studied the chemical and biological reactivity of patulin and penicillic acid no data are available on their stability in foods, except for a report by Timonin (1946) that a dilute patulin solution lost its antibiotic activity in the presence of whole wheat flour or vitamin B1. Jefferys (1952) found patulin to be stable for several weeks, as an antibiotic, over the pH range 3.3 to 6.3 but it was slowly inactivated at pH 6.8. Patulin and penicillic acid retain their activity after 15 minutes at 100° C. (pH 2); patulin, however, does not survive this treatment at pH 9.5 (Heatley and Philpot, 1947). Both compounds react readily with thiols even at a pH as low as 4.5 (Dickens and Cooke, 1965; Geiger and Conn, 1945; Rinderknecht et al., 1947). Oxford (1942) reported that penicillic acid was unstable in the presence of amines and amino acids.

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In view of the hazardous nature of patulin and penicillic acid and their possible occurrence in fungus-infected foods we have studied their stability in fruit juices and flour—foods which could conceivably be so contaminated. The reaction between patulin and a model thiol, glutathione, at low pH has also been investigated.

MATERIALS

Foods. Canned grape drink was stated to contain water, Concord grape juice, citric acid, coloring, and vitamin C (18 mg. per 100 ml.). The pH was 3.0.

Canned apple juice (pH 3.5) had a vitamin C content of not less than 35 mg. per 100 ml. as stated on the label.

Canned orange juice (pH 3.5) contained 5% added sugar and had been reconstituted.

Whole wheat flour contained added maturing agents.

Bleached flour contained added maturing agents and was enriched with thiamine (0.44 mg. per 100 grams), riboflavin (0.26 mg. per 100 grams), niacin (3.5 mg. per 100 grams), and iron (2.9 mg. per 100 grams).

Fresh grape juice was prepared from Concord grapes, which were blended for a few seconds, then pressed by hand through a cloth towel. The juice was centrifuged, treated with pectinase (General Biochemicals) at 60° C., and after cooling centrifuged again and filtered; the pH was 3.1. Sodium benzoate (0.1%) was added to retard mold development.

Fresh apple juice was made from Joyce apples. These were washed, cut into quarters, and again pressed through cloth. The juice was treated with Takamine pectinase concentrate (Miles Chemical Co.) overnight and centrifuged. The juice (pH 3.5) was filtered through a Nalgene sterile filter unit.

Reagents. McIlvaine's buffers were prepared from 0.1M citric acid and 0.2M disodium hydrogen phosphate.

Standard solutions of crystalline patulin and penicillic acid in chloroform, acetone, or buffer (pH 3.4) were kept at 5° C. or in a freezer.

METHODS

All experiments to measure recoveries of patulin and penicillic acid from foods were performed in duplicate. Blanks were also done on each untreated food.

Fruit Juices. To each 50-ml. portion of juice was added 200 μg. each of patulin and penicillic acid as standard solutions (1 mg. per ml.). Samples were analyzed immediately and after periods of up to five weeks' storage in the dark

at 22° C. Analyses were also carried out on 50-ml. samples which were maintained at 80° C. for 10 and 20 minutes after the addition of the toxins then cooled rapidly in ice water.

Fifty milliliters of juice was shaken three times with 50 ml. of ethyl acetate; if emulsification occurred (with fresh grape juice) the layers were separated by centrifugation. The combined extracts were dried for at least 20 minutes with anhydrous calcium sulfate (Drierite), decanted, and passed through a chromatographic column (25 × 300 mm.) containing 15 grams of silica gel (Merck, 0.05 to 0.2 mm.) in ethyl acetate. The Drierite was washed with two portions of 25 ml. of ethyl acetate, and the washings were added to the column and eluted. The column was washed with a further 75 ml. of ethyl acetate. The combined eluate was evaporated to dryness on a steam bath under a gentle stream of nitrogen and 0.25 ml. of chloroform added to the residue.

The sulfhydryl (SH) content of fruit juices was determined by amperometric titration with silver nitrate in ammonia–ammonium nitrate–ethanol (Richmond and Somers, 1966).

Flour. Twenty-five grams of whole wheat flour or bleached enriched flour were intentionally contaminated with 125 μg. of patulin and 125 μg. of penicillic acid. Samples were analyzed immediately and after periods of time up to 2 weeks at 22° C. The flour was mixed with 12.5 ml. of distilled water and shaken with 125 ml. of ethyl acetate on a mechanical shaker for 30 minutes. The mixture was centrifuged and 50 ml. of the ethyl acetate layer withdrawn, dried with Drierite, and added to the silica gel column described above. The Drierite was washed with two portions of 25 ml. of ethyl acetate, which were passed through the column together with a further 50 ml. of ethyl acetate. The combined eluate was evaporated and the residue taken up in 0.25 ml. of chloroform.

Thin-Layer Chromatography. Thin layers (0.25 mm.) of Adsorbosil 5 (Applied Science Laboratories, Inc.) were activated at 80° C. for 2 hours, and spotted with the food extract and standards of patulin and penicillic acid (1 mg. per 5 ml. of chloroform). Plates were developed with anhydrous ether in an equilibrated tank, lightly sprayed with 0.3% aqueous ammonia, then with 4% aqueous phenylhydrazine hydrochloride (Yamamoto, 1956), and heated at 100° C. for 2 to 3 minutes. Amounts of patulin were estimated visually by comparing intensities of the yellow spots with standards at the same $R \cdot$. Penicillic acid was estimated similarly by yellow fluorescence under long wave ultraviolet light.

Reaction of Patulin with Glutathione. Accurately weighed quantities of patulin were dissolved in 100-ml. buffer solutions of pH 2.3, 3.0, and 6.9; ultraviolet spectra were recorded on a Cary 14 spectrophotometer. To 50 ml. of solution was added a weighed amount of reduced glutathione; the remaining 50 ml. of solution served as control. Solutions were kept at room temperature in the dark and analyzed for patulin after marked loss in the solutions containing glutathione was apparent from the ultraviolet spectra; 25-ml. portions were extracted with three equal volumes of ethyl acetate, the extracts were evaporated, and the patulin estimated by thin-layer chromatography.

RESULTS AND DISCUSSION

Methods of Analysis. Ethyl acetate was found to be an efficient solvent for the extraction of both patulin and penicillic acid from aqueous solution; ether was not suitable. Similar observations were made for patulin by Lochhead *et al.* (1946). All the patulin and penicillic acid in the food extracts was eluted from the silica gel column by ethyl acetate. Good recoveries of each compound added to fruit juices at levels of 4 p.p.m. and to flour at 5 p.p.m. were obtained as shown by the analyses at zero time (Table I). Thin-layer chromatography readily separated patulin and penicillic acid from each other and from interfering substances. Typical R_f values for patulin and penicillic acid were 0.77 and 0.50, respectively. It was possible to detect 0.2 μg. of each compound on the thin-layer chromatogram by the phenylhydrazine color reaction; estimation was carried out without delay since the background slowly turned yellow. Limits of detection of patulin or penicillic acid in the foods were of the order 0.1 to 0.3 p.p.m. Neither compound was found in the food samples used.

Stability Studies. The amounts of patulin and penicillic acid recovered from foods after different time periods at 22° and 80° C. are given in Tables I and II. The compounds were most rapidly destroyed in flour, both whole wheat and bleached. These results may be compared with those of Timonin (1946) that patulin is readily inactivated by flour in the presence of added water. He suggested that sulfhydryl compounds and vitamin B_1 present in the flour could react with patulin. A concentration of thiols in untreated wheat flour, of 1 μequivalent of SH per gram, reported by Tsen and Dempster (1963), would account for Timonin's result. The instability of patulin and penicillic acid in treated flour shows that they will not be found in appreciable amounts in commercial samples.

Table I. Percentage[a] of Patulin and Penicillic Acid Remaining in Foods at 22° C.

	Time, Weeks					Time, Weeks				
	0	1	2	3	5	0	1	2	3	5
			PATULIN					PENICILLIC ACID		
Canned grape juice	90, 85	85, 80	...	65, 65	50, 40	90, 90	90, 90	...	75, 75	60, 60
Fresh grape juice	85, 85	50, 50	60, 50	...	...	90, 90	70, 70	65, 70	...	...
Canned apple juice	90, 85	...	85, 85	75, 80	40, 50	85, 85	...	90, 85	90, 90	75, 50
Fresh apple juice	80, 75	85, 85	85, 75	85, 85	...	100, 100	95, 95	95, 95	90, 90	...
Canned orange juice	90, 90	50, 60	30, 30	25, 25	...	75, 75	40, 40	30, 30	25, 30	...
Whole wheat flour	80, 80	10, 5	5, 5	...	...	80, 70	10, 10	5, 5	...	...
Bleached flour	80, 80	20, 10	10, 10	...	...	75, 80	15, 15	10, 10	...	...

[a] Expressed as percentage of amount added—200 μg. of each compound in 50 ml. of juice, 125 μg. of each compound in 25 grams of flour.

Table II. Percentage[a] of Patulin and Penicillic Acid Remaining in Fruit Juices Kept at 80° C.

JUICE	Time, Minutes 10	20	Time, Minutes 10	20
	PATULIN		PENICILLIC ACID	
Canned grape	40, 30	20, 25	40, 50	40, 40
Fresh grape	85, 85	. . .	50, 50	. . .
Canned apple	50, 60	50, 40	70, 60	40, 50
Canned orange	25, 25	20, 20	30, 40	40, 30

[a] Expressed as percentage of amount added—200 μg. of each compound in 50 ml. of juice.

Table III. Reaction of Patulin with Reduced Glutathione

Initial Concentration, mM Patulin	Glutathione	Buffer pH	Time, Days	% Patulin Remaining
0.081	0.343	6.9	0.8	0
0.081	0	6.9	0.8	90
0.081	0.354	3.0	6.9	0
0.081	0	3.0	6.9	73
0.079	0.340	2.3	6.9	6
0.079	0	2.3	6.9	55

Of the fruit juices tested, orange juice caused the most rapid destruction of patulin and penicillic acid at room temperature. This was not a pH effect, and could be explained by the high level of sulfhydryl compounds in orange juice. Jansen and Jang (1952), and Miller and Rockland (1952), give levels of 0.02 to 0.03 mmole of SH per 100 ml. of juice, nearly all present as glutathione and cysteine. The orange juice used in our work contained 0.02 mmole of SH per 100 ml. This concentration of soluble sulfhydryl compounds would be sufficient to destroy patulin (and penicillic acid) at the observed rate, judging from the results shown in Table III for the model reaction of patulin and glutathione at low pH.

The moderate stability of patulin and penicillic acid in apple and grape juices can be attributed to very low levels of sulfhydryl compounds. The canned apple and grape juices used in this study in fact contained less than 0.003 mmole of SH per 100 ml., and Sedlák and Kaločai (1957) found that, in general, apples and black grapes contained negligible amounts of thiols. The levels of sulfhydryl compounds in fruits and vegetables reported by Sedlák and Kaločai (1957), and Zuman (1951), are a guide to the stability of patulin and penicillic acid in juices. Black currants, for example, contain high levels of sulfhydryl compounds (0.1 to 0.4 mmole per 100 grams) and would not be a good medium for stability of such thiol-reactive compounds as patulin and penicillic acid.

Tressler and Joslyn (1961) discuss heat treatment of juices. Since conditions vary from flash-pasteurization (90° C. for 30 seconds) to pasteurization at 70° C. for over 20 minutes, we used a temperature of 80° C., held for 10 or 20 minutes, in our measurements of the heat stability of patulin and penicillic acid in fruit juices. Table II shows that appreciable fractions of these compounds remain after heating. Since patulin and penicillic acid are reported to be stable at pH 2 when heated at 100° C. for 15 minutes (Heatley and Philpot, 1947), the destruction observed was probably due to other factors.

When patulin and excess glutathione were allowed to react at room temperature, shifts in the absorption maximum of patulin occurred. At pH 6.9, the maximum moved from 276 mμ to 285 mμ after 10 minutes and 294 mμ after 19 hours with marked decrease in absorbance. At pH 2.3 and 3.0 the absorption maximum moved to slightly shorter wavelength, with decrease in absorbance; after one week no maximum remained. No shift in position of the maximum occurred in control buffer solutions containing patulin. In view of the uncertainties of ultraviolet quantitation, patulin was determined after direct

extraction. The results are summarized in Table III, and the destruction of patulin by glutathione is demonstrated even at pH 2.3 and 3.0.

Brian et al. (1956) have shown that patulin can reach concentrations as high as 1000 p.p.m. in the sap from apples rotted by P. expansum. Our work has shown that if high concentrations of patulin and penicillic acid are initially present in fruit juices of low SH content it is probable that appreciable concentrations of these toxins will remain in the processed juice. This could be hazardous and the possible occurrence of these toxins in fruit juices should be the concern of food safety authorities.

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