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Antiviral Effect of Apple Beverages

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A variety of apple beverages were tested for antiviral activity against poliovirus 1 or coxsackievirus B5. Freshly prepared apple juice was particularly antiviral, but its activity declined more readily than that of commercial juice in response to heat and storage. The component responsible for activity was located both in the pulp and skin; after ultrafiltration, activity was present in fractions greater and less than molecular weight 10,000. Virus infectivity was not restored from virus-apple juice complexes with gelatin, serum, Tween 80, or polyethylene glycol.

Apples are one of the most important commercial fruits in North America; unfermented or fermented apple juice (cider) is a popular product of the apple industry. Moyer and Aitken (7) define sweet cider as fresh apple juice without preservation and apple juice as sweet cider that is treated to prevent spoilage. The introduction of flash pasteurization and its application to fruit juices provided impetus for the start of commercial production of apple juice in North America about 1937.

Recent studies in our laboratory compared the inactivation of poliovirus 1 by a number of commercial juices and beverages (6). Grape juice, apple juice, and tea were the most effective; grape products were shown earlier to inactivate a number of enteric viruses (5). Previously, Green (2) showed that extracts of tea inhibited influenza virus multiplication in embryonated eggs. In this report, the antiviral properties of apple beverages are examined and compared.

MATERIALS AND METHODS

Viruses and cells. Poliovirus type 1 (Sabin) and coxsackievirus type B5 were grown and assayed in HEp-2 cells. The sources of viruses and cells have been described (4). Virus stocks were stored in sealed ampoules at −70°C. Cells were grown in medium 199 containing 10% fetal bovine serum. Stock cultures were grown in Roux bottles as monolayers at 36°C. For virus assay, monolayers in plastic dishes (60 by 15 mm) were prepared from stock cultures 24 h before use.

Source and preparation of apples and apple beverages. Apple juices and drinks were purchased from local grocery stores; fermented apple beverages (ciders and wine) were purchased from liquor or wine stores. Information regarding contents of the products, provided on cans or bottles, was as follows: apple juices were vitamin supplemented, containing not less that 35 mg of ascorbic acid per 100 ml; apple juice from concentrate contained water, apple concentrate, and ascorbic acid, not less than 35 mg/100 ml; apple drinks varied with the brand, but all contained water, sugar, concentrated apple juice, apple flavor (natural and/or artificial), color, and ascorbic acid (not less than 18 to 20 mg/100 ml), and one or more of malic acid, citric acid, sodium citrate, corn syrup, or sodium benzoate; juice from fermented apples had an alcohol content of 6.6 to 11%.

Apple beverages are generally prepared from a mixture of not less than three apple varieties; McIntosh is one variety commonly used in Quebec and western North America (7). Commercial juice preparation comprises the following steps: washing, sorting, grinding, juice extracting by a press or centrifuge, screening, and filtering for clarification and pasteurization. (Ascorbic acid may be added as an antioxidant at some stage in processing; in North America, the final addition of ascorbic acid [35 mg/100 ml in Canada] provides a good source of vitamin C in the diet.) To obtain a clear apple extract, free of additives, for experimental or comparative purposes, juice was made from McIntosh apples in the laboratory. Apples in 2- to 5-pound (ca. 0.9- to 2.3-kg) lots were washed, cored, and homogenized with distilled water 1:1 (wt/vol). The homogenate was centrifuged at low speed, and the supernatant fluid was recentrifuged at 20,000 *g* for 1 h. The clear liquid was called laboratory-prepared apple juice. Juice from pulp or skin was prepared by separating pulp and skin and then proceeding as with the whole apples. Adjustments in pH were made with 1 N NaOH. Fourfold dilutions were made in water.

Virus inactivation. A virus inoculum of about 8,000 plaque-forming units in 0.05 ml of water was added to single, duplicate, or triplicate screw-capped bottles containing 2 ml of water (control), 2 ml of apple beverage, or dilutions at the natural pH or pH 7.0. Suspensions were incubated at ambient temperature (about 22°C) for 2 h. Virus was assayed by diluting each suspension 1:100 in medium 199 containing 10% fetal bovine serum. Triplicate aliquots of 2.5 ml were pipetted to monolayers. Cultures were shaken for 2 h at ambient temperature to allow adsorption of virus. The samples were poured off; an agar overlay was applied (3); and the cultures were incubated at 36°C

in a humidified 5% CO_2 atmosphere. Plaques were counted after 3 days; counts for each sample (plaques on three monolayers) were averaged. The counts obtained for duplicate or triplicate samples were averaged and expressed as percentage of the value obtained for the controls. The percent error (standard deviation divided by the mean and multiplied by 100) of the assay system varied from 2 to 25%, with a mean of 9%. Differences of 25% (the maximum experimental error) were considered significant.

Complex dissociation. One-milliliter volumes of aqueous solutions of 2% gelatin (Difco Laboratories, Detroit, Mich.), 2 or 0.2% Tween 80 (Sigma Chemical Co., St. Louis, Mo.), 1% polyethylene glycol 20,000 (Fisher Scientific Co., Pittsburgh, Pa.), or whole fetal bovine serum were mixed for 1 h with 1-ml portions of virus-apple juice (laboratory prepared) complexes (5).

Fractionation of apple juice. A 30-ml amount of commercial apple juice was adjusted to pH 7.0 and fractionated by filtration through a Diaflo membrane UM10 (Amicon Corp., Lexington, Mass.), designed to restrict passage of macromolecules in excess of molecular weight 10,000. The retentate was made up to 30 ml.

RESULTS

Apple juices, ciders, and a wine showed various degrees of antiviral activity against poliovirus 1, whereas apple drinks had little or no effect (Table 1). In general, the antiviral activity was as great or greater at pH 7.0 than at the natural pH.

Fresh laboratory-produced apple juice was compared with one of the more potent commercial apple juices for antiviral activity against poliovirus 1 and coxsackievirus B5 in three separate experiments (Table 2). The former juice was more potent for both viruses. With commercial juice, activity was as great or four times greater against poliovirus at pH 7.0 than at the natural pH; there was no antiviral effect against coxsackievirus. With laboratory-prepared juice, in comparison with pH 7.0, activity at the natural pH was as great or four times greater against poliovirus and at least 16 times greater against coxsackievirus.

The active agent in laboratory-prepared juice, in contrast to that in commercial juice, was unstable to heat and storage (Table 3). Activity was reduced by a factor of 16 after heating at 65°C for 1 min; storage at 4°C showed a 64-fold loss after 2 weeks and a 256-fold loss after 4 weeks; there was no reduction of activity after storage at −70°C for at least 1 month.

The antiviral activity of apples resided in both the pulp and the skin (Table 4). No reversal of activity was achieved with gelatin, fetal bovine serum, Tween 80, or polyethylene glycol (Table 5).

Filtration of commercial juice through a UM10 membrane indicated that activity against poliovirus 1 resided in both the filtrate and the retentate (Table 6).

TABLE 1. *Percent survival of poliovirus 1 in various commercial apple products after 2 h at ambient temperature*

Apple product	Natural pH (3.0-3.7)	pH 7.0
Juice		
1[a]	<1[b]	<1
2	2 (1-2)[c]	1 (1-1)
3	9 (6-11)	<1
4	37 (34-40)	2 (1-2)
5	21 (20-22)	13 (12-14)
Juice from concentrate	50 (49-51)	1 (1-2)
Drink		
1	102 (99-103)	49 (47-51)
2	90 (88-92)	89 (87-91)
3	95 (92-97)	1 (1-1)
Cider		
1	7 (6-7)	7 (5-7)
2	23 (22-24)	54 (52-56)
3	48 (45-51)	<1
Wine	4 (2-5)	<1

[a] Each a different brand.
[b] No plaques in three samples.
[c] Average of three samples, range in parentheses.

TABLE 2. *Titration of antiviral activity in commercial or laboratory-prepared apple juice after incubation at ambient temperature for 2 h*

Virus	Expt	Reciprocal of the dilution resulting in approx a 50% reduction in no. of plaques in:			
		Commercial juice[a]		Laboratory-prepared juice	
		pH 3.4[b]	pH 7.0	pH 3.3[b]	pH 7.0
Polio	1	16	64	256	256
	2	16	16	256	64
	3	16	64	256	64
Coxsackie	1	0	0	16	1
	2	0	0	64	1
	3	0	0	16	1

[a] Brand 1, see Table 1.
[b] The natural pH.

DISCUSSION

Apple juice, like many other fruit juices or extracts investigated earlier (4-6), contains compounds which inactivate viruses. Apple drinks are less effective as viral inactivators, probably because they are prepared with diluted apple juice. Although freshly prepared juice is a more potent antiviral agent than the commercial product, it loses potency more rapidly during storage. This instability may be due to oxidation of the tannins, which reduces their effectiveness

TABLE 3. *Effect of storage and heating of commercial and laboratory-prepared apple juice on the inactivation of poliovirus 1 after incubation at ambient temperature for 2 h*

Type of juice[a]	Treatment	Reciprocal of the dilution resulting in approx a 50% reduction in no. of plaques
Commercial[b]	Fresh	16
	4°C for 2 weeks	16
	65°C for 1 min	16
Laboratory	Fresh	256
	4°C for 2 weeks	4
	4°C for 4 weeks	1
	65°C for 1 min	16

[a] At the natural pH.
[b] Brand 1, see Table 1.

TABLE 4. *Titration of antiviral activity of juice prepared from apple skin or pulp after incubation at ambient temperature for 2 h*

Virus	Reciprocal of the dilution resulting in approx a 50% reduction in no. of plaques in:			
	Skin		Pulp	
	pH 3.3	pH 7.0	pH 3.3	pH 7.0
Polio	256	64	256	64
Coxsackie	16	1	16	1

TABLE 5. *Percent recovery of poliovirus 1 from laboratory-prepared apple juice[a] after 2 h at ambient temperature followed by a 1-h incubation with complex-dissociating agents*

Agent	pH of diluted apple juice	
	Natural	7.0
None	12 (10–14)[b]	48 (46–50)
1% gelatin	14 (10–18)	46 (45–46)
50% serum	11 (10–12)	40 (39–40)
1% Tween 80	8 (7–9)	36 (35–37)
0.1% Tween 80	13 (11–14)	30 (29–31)
0.5% polyethylene glycol	8 (8–8)	42 (40–43)

[a] Diluted 1/64 in water.
[b] Average of two samples, range in parentheses.

for reacting with viruses. Commercial production employs methods to reduce the enzymatic oxidative changes of tannins because they cause browning and decreased flavor (7). These include the addition of ascorbic acid and heating to inactivate the oxidases. However, methods used for clarifying juice undoubtedly remove many of the natural tannins and therefore may account for reduced antiviral activity of the commercial juice; our experiments with fresh juice indicated that flash pasteurization would reduce

TABLE 6. *Percent recovery of poliovirus 1 from filtration fractions of commercial apple juice adjusted to pH 7.0 after 2 h at ambient temperature*

Juice sample	Dilution in water	
	Undiluted	1/10
Unfiltered	<1[a]	15 (13–16)
UM10 retentate	3 (2–3)[b]	57 (56–58)
UM10 filtrate	1 (0–2)	40 (38–42)

[a] No plaques in three samples.
[b] Average of three samples, range in parentheses.

antiviral activity. Fortification with ascorbic acid would not likely increase the antiviral activity of apple juice; previously we found that ascorbic acid, though antiviral in aqueous solution, was ineffective after addition to several juices, including an apple drink.

Unlike grapes, where antiviral activity was in the skin but not the pulp (5), apple pulp was as active as the skin. This finding corresponds to the distribution of tannins in these two fruits (8, 9). When commercial apple juice was sieved by ultrafiltration, both filtrate and retentate showed antiviral activity, indicating that the size of the active component may vary, or that larger aggregates may be formed which retain antiviral activity. In earlier experiments we found that this was the case with commercial grape juice (5). As with grape juice, the effect of apple juice on poliovirus was greater than that on coxsackievirus; the natural pH and pH 7.0 showed little difference in activity against poliovirus, but the natural pH of grape juice and laboratory-prepared apple juice was markedly more effective against coxsackievirus. The latter phenomenon was apparently not due to an adverse effect of the acidic pH since commercial apple juice, pH 3.4, was not antiviral. An important difference between the apple and grape substances is in the stability of the association with virus. We were unable to restore virus infectivity from virus-apple juice complexes with a number of complex-dissociating compounds that have proved successful with enzyme-tannin (1) and virus-grape juice complexes (5). If apple components combine with virus during transit through the digestive tract, they may be useful as antiviral agents. Experiments to establish this potential are currently underway.

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