melissa extract by dilution(2) or by addition of gelatin.

Discussion. The condensed tannin of *Melissa officinalis* has some rather specific reactions with biologic materials. It reacts with gelatin, hide powder, and myxoviruses of subgroup 2 and, like all tannins, it also reacts with egg albumin and alkaloids(2). It does not seem to react with bovine serum, bovine serum albumin(1), influenza viruses, or certain erythrocytes. However, it may react to some degree with chick embryo cells(2). Condensed tannins of this type represent a challenge to the chemist; so far as is known, none has been purified as yet(8). Regeneration of the tannin by trypsin digestion of gelatin precipitates may be a unique method of tannin preparation.

It is tempting to postulate that melissa tannin reacts rapidly but reversibly with certain surface proteins of myxoviruses of subgroup 2, proteins that would have to be distinctly different from the surface proteins of influenza viruses.

As will be shown in subsequent publications, the tannin of melissa is not the only antiviral agent present in the aqueous extracts nor is melissa the only member of the mint family with such antiviral components.

Summary. Tannin-containing fractions from aqueous extracts of *Melissa officinalis* were prepared by gelatin precipitation, hide-powder adsorption, and lead acetate precipitation and it was found that the tannin recovered from these preparations was the hemagglutination inhibitor for Newcastle disease virus. Tannin prepared by gelatin precipitation showed antiviral activity in tests in eggs and in plaque-inhibition tests with Newcastle disease virus and hemagglutination tests with mumps virus. Aqueous extracts of the melissa plant blocked hemadsorption by parainfluenza viruses 1, 2, and 3. The tannin of this plant appears to have an affinity for myxoviruses of subgroup 2 but has no effect on influenza A and B viruses in hemagglutination and hemadsorption tests. The tannin is not primarily virucidal for Newcastle disease virus; its effect is a neutralization which can be reversed by dilution or by addition of gelatin.

We wish to acknowledge the excellent technical assistance of Donald S. Fox, Jr.

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Antiviral Substances in Plants of the Mint Family (Labiatae). II. Nontannin Polyphenol of *Melissa officinalis*.* (31873)

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Aqueous extracts of *Melissa officinalis* (lemon balm), a medicinal plant known in antiquity, have been shown to produce a variety of antiviral effects(1,2). It has now been established that a condensed tannin is responsible for the ability of these extracts (1) to inhibit hemagglutination by Newcastle disease virus (NDV) and mumps virus, (2) to protect eggs and chick cell cultures from

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TABLE I. Effect of Gelatin on Plaque Suppression by Aqueous Extracts of *Melissa officinalis.*

Virus	PFU*	No. of prep. tested; dilution†	Avg plaque-free zone,‡ mm	
			Gelatin	No gelatin
Herpes simplex (HF)	6×10^2-7×10^3	6; UD	16 (10)	25 (10)
Vaccinia (WR)	2×10^3	1; UD	16 (1)	23 (1)
		1; ½	12 (1)	21 (1)
		1; ¼	8 (1)	20 (1)
		1; ⅛	Trace (1)	14 (1)

* Plaque-forming units.
† UD = undiluted.
‡ Zone surrounding ¼-inch antibiotic disc impregnated with preparation. Numbers in parentheses indicate total number of different determinations averaged.

NDV, and (3) to prevent hemadsorption by NDV, mumps, and parainfluenza 1, 2, and 3 viruses but not by influenza A and B viruses (3). It is the purpose of this report to establish that melissa extracts also contain a second, nontannin component that is antiviral against herpes simplex and vaccinia viruses. This second antiviral substance will be shown to have properties of a nontannin polyphenol, much like caffeic acid.

Materials and methods. The preparation of chick embryo fibroblast cultures, their use in the disc-plate plaque-suppression test, and the infection and treatment of embryonated eggs have been described(1,2,4). The source, strain, and method of maintenance of the viruses used in this study have also been described(1-3). The methods of preparing tannin and tannin-free fractions of melissa extracts by gelatin precipitation and hide-powder adsorption are presented in a companion report(3). The procedure for precipitating plant polyphenols with lead acetate with subsequent regeneration of the polyphenols by the removal of Pb^{++} with resin AG-50W-X (H-form, Calbiochem) is a relatively standard method presented in prior reports(2,5).

Alternative methods for extracting leaves of *M. officinalis* were investigated. It was found that ethyl alcohol, *n*-butyl alcohol, ethyl acetate, and chloroform extracts had no antiviral effect against NDV or herpes simplex virus in the plaque-suppression test.

For the studies of caffeic acid (Aldrich Chem., Milwaukee), solutions of the compound were prepared in 0.1N NaOH at 56°C with stirring for 45 minutes. The pH was then adjusted to between 6.8 and 7.0. Further dilutions of this solution were made in sterile distilled water. All preparations used in egg and cell-culture experiments contained 100 units of penicillin and 100 μg of dihydrostreptomycin per milliliter.

Results and discussion. Early in these investigations the medium of Simpson and Hirst (6) was used for disc plaque-suppression tests with chick embryo fibroblast cultures. Although the phenol red called for in the original formulation was left out, the medium did contain 0.48% gelatin which should have neutralized any effect of the antiviral tannin of melissa. On numerous occasions, however, it was observed that, even in the presence of gelatin, significant zones of plaque suppression occurred in cultures infected with herpes simplex virus(1). In experiments to compare the antiviral effect of the aqueous extracts in cell-culture systems with and without gelatin, the presence of gelatin decreased the size of plaque-free zones but did not eliminate them (Table I). It was believed this result could most readily be explained by the presence of a second, nontannin, antiviral substance. However, it is significant that the plaque-free zones were much larger in the absence of gelatin than when gelatin was present, further suggesting that the tannin also has some antiviral effect on herpes simplex and vaccinia viruses. Also, in the presence of gelatin the plaques of vaccinia virus were larger and clearer while there was no detectable effect on plaques of herpes simplex virus.

To confirm the presence of this second antiviral component, tannin-free preparations were investigated and were found to have an antiviral effect in eggs and cell cultures (Table II). The tannin fraction was equally

TABLE II. Antiviral Activity of Tannin and Tannin-Free Fractions of *Melissa officinalis* Against Herpes Simplex and Vaccinia Viruses.

	Egg experiments								
			Survivors/total eggs				Disc-plaque suppression		
			Dilution						
Preparation	Virus and LD_{50}/0.3 ml/egg*	No. of prep. tested	UD	½	¼	UD, no virus	No. of prep. tested	PFU	Avg plaque-free zone, mm†
Tannin (gelatin ppt.)	Herpes 32	1	7/10	4/10	4/10	10/10	3	1×10^3 to 6×10^3	22 (5)
	Vaccinia 10	1	6/6	1/4	0/5	5/5	3	6×10^2 to 2×10^3	25 (3)
Tannin-free (supernate after gelatin ppt.)‡	Herpes 10-80	2	24/37	14/30	13/27	24/24	4	6×10^2 to 2×10^3	19 (10)§
	Vaccinia 10	1	3/5	1/5	0/5	5/5	1	2×10^3	26 (3) §
Tannin-free (supernate from gelatin and Pb^{++} ppt.)	Herpes 16	1	7/10	—	—	6/6	1	3×10^3	22 (1)
	Vaccinia 25	1	2/3 ‖	—	—	3/4	1	1×10^3	22 (2)
Tannin-free (after hide-powder adsorption)	Herpes —	—	—	—	—	—	1	3×10^3	20 (3)

* Eggs incubated at 36°C for 10 days postinfection. No untreated virus controls are included because no eggs survived these doses.

† Numbers in parentheses indicate total tests averaged.

‡ When tested in eggs, preparations were diluted to a volume equivalent to that of the original aqueous extract from which they were derived. However, the tannin-free fraction, produced by adding an equal amount of 2% gelatin, was in effect a two-fold dilution; hence, 0.6 ml was injected into eggs via the allantoic sac rather than the usual 0.3 ml.

§ These results are essentially for a ½ dilution of this preparation, all others are for ¼-inch antibiotic discs impregnated with preparations in a volume equivalent to the original aqueous extract.

‖ This experiment involved treating eggs 2 hr prior to addition of virus; in all others, eggs were treated 24 hr prior to addition of virus.

TABLE III. Antiviral Activity of Caffeic Acid.

Virus	Egg experiments		Disc-plaque suppression	
	LD_{50}/0.3 ml/egg	Survivors/ total eggs*	PFU	Plaque-free zone, mm
Herpes simplex	16-63	24/41	2×10^3	20 46, 48†
Vaccinia	20-80	7/35	4×10^2	17, 23, 25, 22 17, 19‡
Influenza A (PR8)	25	0/9	—	—
" B (GL)	12	0/10	—	—
Newcastle disease (11914)	32-100	1/16	—	—
None	—	42/50	—	—

* Eggs received 3.6 mg caffeic acid in 0.3 ml distilled water via the allantoic sac 3 hr prior to injection of virus by the same route. No untreated controls are included because no eggs survived these virus doses.

† These are results of tests with ½-inch antibiotic discs containing 960 μg of caffeic acid; all other determinations used ¼-inch discs with 240 μg.

‡ These results were from a caffeic acid preparation precipitated with lead acetate and recovered by resin treatment.

active and no doubt was contaminated by some of the second antiviral material. Even if this is true it still seems reasonable to conclude, based on the results in Table I, that the tannin itself also is active against herpes simplex and vaccinia viruses. There is little likelihood that the tannin-free fractions were contaminated with tannin because, by definition, tannins react with hide powder and are precipitated by gelatin(7). The tannin-free fractions have given no reactions typical of tannins nor do they interact with NDV, a property of this particular tannin(3).

To determine the nature of this nontannin antiviral substance, preparations freed of tannin by gelatin treatment were further treated with lead acetate. Such a procedure produces water-insoluble lead polyphenolates(5). When Pb^{++} was removed by ion exchange, the water-soluble plant polyphenol fraction exhibited significant antiviral activity (Table II). This strongly suggests that the second antiviral substance is a nontannin polyphenol or a mixture of polyphenols.

Aqueous extracts of melissa leaves have been reported to contain caffeic acid (3,4-dihydroxycinnamic acid) which is the basic unit of the condensed tannin of this plant(8). It has also been suggested that caffeic acid might be the substance responsible for the activity of melissa extracts(9). At best this could be only partially true because it is now known that a portion of the antiviral effect is due to a tannin(3). Caffeic acid was investigated and found to have antiviral activity very similar to that of tannin-free fractions of melissa (Table III). Caffeic acid also precipitated readily with lead acetate and, when freed of Pb^{++}, was active against vaccinia virus. Furthermore, both caffeic acid and the polyphenol fraction of melissa did not seem to be highly active in eggs infected with vaccinia virus, yet both had activity against this virus in cell cultures. Caffeic acid was inactive against NDV and influenza A and B viruses, which was equally true of the tannin-free fractions of melissa(3).

It has been reported that caffeic acid had some antiviral effect in mice and ferrets infected with influenza A (PR8) virus(9). The same report stated that, at 10 μg/ml, caffeic acid in contact with herpes simplex virus for 1 hour at 37°C inactivated "10^3 plaque forming units per ml." Since there was no indication that the acidity of the compound had been neutralized, the pH of the medium might readily account for the inactivation of this acid-labile virus. Neutralized caffeic acid at very substantial concentrations did not seem to be highly virucidal in contact with herpes simplex virus for 2 hours at 37°C (Table IV). There was a significant decrease in the number of viable virus particles but it is not clear how this compares with inactivation of 10^3 plaque forming units previously reported. The tannin fraction was somewhat

TABLE IV. Virucidal Effect of Caffeic Acid, Melissa Tannin, and Tannin-Free Fractions on Herpes Simplex Virus.

Preparation*	Reciprocal of $\log_{10}$ of LD_{50} in eggs
Caffeic acid (12 mg/ml)	3.4
Tannin (4× concentrated)	2.6
Tannin-free fraction (2× diluted)†	4.2
Virus control	4.7

* Virus diluted 1/10 in each preparation; control diluted in water. After 2 hr at room temperature, mixtures were further diluted in 10-fold increments in tryptose phosphate broth (Difco); 0.3 ml of each dilution was injected, via the allantoic sac, per egg, 6 eggs per dilution. Eggs were incubated 10 days at 36°C. All preparations were adjusted to pH 7.0 before they were mixed with virus.

† See text.

more virucidal, but this preparation was concentrated 4-fold compared to the usual aqueous extracts of melissa. On the other hand, the tannin-free fraction of melissa had little if any virucidal effect. However, this fraction is in effect a 2-fold dilution as a result of the addition of an equal volume of 2% gelatin solution to precipitate the tannin. Such a preparation is quite capable of producing an antiviral effect in herpes simplex-infected eggs and cell cultures, yet it seems to have little virucidal activity.

Preliminary chromatographic studies have been unable to establish firmly that caffeic acid is present in melissa preparations in significant quantities. Thin layer chromatographic plates prepared from hydrated silica gel (AR TLC-7GF, Mallinckrodt Chemical Works, St. Louis) were used with a developing solvent consisting of a mixture of benzene, acetic acid, and methanol (13.5:2.4:1.2). Caffeic and ferulic (not antiviral but reported to be a constituent of melissa leaves)(8) acids were used as controls and were detected by spraying the chromatogram with 1% ferric sulfate in distilled water. All preparations and controls received 1 μg of $K_2S_2O_5$ to retard oxidation of the polyphenols(5). Caffeic acid produced a brilliant greenish blue area with an R_f value of 0.42 when added to the plates in amounts that were comparable in antiviral activity to a variety of melissa preparations. A weak greenish blue area with an R_f value of 0.43 was observed with some concentrated preparations of melissa extracts, but the color soon faded while the color in the known caffeic acid area persisted for long periods.

There is little doubt that caffeic acid is present in melissa extracts, but at just detectable levels. It is thought that other substances in melissa extracts may have inhibited the caffeic acid migration. However, when caffeic acid was added to melissa preparations, there was no indication that its migration on the chromatogram was retarded. Ferulic acid also could be detected in certain melissa preparations (yellow; R_f, 0.68), but again at barely detectable levels. A sizable orange-yellow area with an R_f value of 0.16 was frequently produced by melissa preparations. The nature of this substance is unknown.

Summary. The presence of gelatin did not eliminate the antiviral activity of aqueous extracts of *Melissa officinalis* against herpes simplex and vaccinia viruses in disc plaque-suppression tests, suggesting that a second, nontannin, antiviral substance was present. By using tannin-free preparations it was found that the nontannin polyphenol fraction produced an antiviral effect against herpes simplex and vaccinia viruses in egg and cell-culture systems. This activity was very similar to that of caffeic acid. It was not clear whether the antiviral effect of either of these materials was exclusively due to a virucidal effect. Preliminary thin layer chromatography revealed the presence of caffeic acid in melissa preparations but at levels that seemed far too low to account for the antiviral effect.

We wish to acknowledge the excellent technical assistance of Donald S. Fox, Jr.

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Antiviral Substances in Plants of the Mint Family (Labiatae). III. Peppermint *(Mentha piperita)* and other Mint Plants.* (31874)

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One member of the mint family, *Melissa officinalis* (lemon balm) has been studied in detail and found to contain both tannin and nontannin polyphenol antiviral fractions(1-4). It has been reported that two other mint plants, peppermint and sage, contain the same condensed tannin, consisting of trimers of caffeic acid (3,4-dihydroxycinnamic acid), found in melissa(5). Because this tannin was demonstrated to be a potent inhibitor of Newcastle disease virus (NDV), studies were undertaken to determine if extracts of other plants of the mint family (Labiatae) also had antiviral activity. The following report will show that peppermint contains antiviral substances quite similar to those of melissa and, further, that a number of other mint plants also contain substances with antiviral activity.

Materials and methods. All plants used in this investigation were kindly supplied by S. B. Penick and Co., New York, as dried leaf preparations, with the exception of hyssop which was supplied as branches and seeds. Hot-water extracts of the plant materials were prepared exactly as described for melissa(2). The preparation of tannin and tannin-free fractions by gelatin precipitation or hide-powder adsorption has been reported previously(2,3). The antiviral test procedures have been fully described in previous reports, including methods for infecting, treating, and incubating embryonated chicken eggs(1,2) and the preparation of and medium for chick embryo fibroblast cell cultures and their use in the disc-plate plaque-suppression test(1,2, 6) and hemagglutination tests(2). Source, strain, and maintenance of viruses used in this study have also been previously described(1-3).

Results and discussion. Aqueous extracts of peppermint leaves produced virtually the same antiviral effects observed with extracts of melissa leaves(1) (Table I). The effects were noted in eggs only when the preparation was injected into the allantoic sac 3 to 24 hours prior to virus given by the same route, but there was no effect in influenza virus-infected eggs. However, peppermint extracts exhibited some activity in suppressing plaques of influenza A virus. This borderline activity was not confirmed in egg tests, was not observed with melissa extracts, and is unexplained at present. It is possible that this activity is the result of still another antiviral component at low concentration or of very low potency.

The antiviral effects of peppermint extracts were so similar to those of melissa that experiments were undertaken to determine if both tannin and nontannin antiviral substances were present, as has been found with melissa(4). The antiviral effect against NDV was concentrated in the tannin fraction while the tannin-free fraction showed activity against herpes simplex virus (Table II). Although the nontannin fraction of peppermint is not as potent as that of melissa, there clearly is a second antiviral substance inactive against NDV but active against herpes simplex. Investigations with melissa established that this second antiviral material

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