e.g., incidence of mammary tumors in virgin females, presence or absence of dual viral factors, has yet to be established(7).

Summary. Growth hormone preparations from several mammalian species were investigated for their ability to initiate secretion in organ cultures of prelactating mammary tissue from strain C3H and A mice. All the growth hormones studies showed some degree of activity in C3H mammary tissues. Bovine, ovine and porcine GH were 20-50% as active as ovine prolactin, although the prolactin content of the preparations was shown to be low. Simian and human GH, whose prolactin-like activity in other systems is well-known, showed activity equal to or greater than ovine prolactin in C3H mammary tissues. On the other hand, a single human GH preparation (one of three studied) was the only GH to show significant activity in strain A mammary tissues. These results suggest an intrinsic lactogenic activity of GH, particularly well-marked in the case of human GH, whose expression in mouse mammary gland cultures is in part determined by the genetic background of the tissues.

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Antiviral Substances in Plants of the Mint Family (Labiatae). I. Tannin of *Melissa officinalis*.* (31872)

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Aqueous extracts of the plant *Melissa officinalis* (lemon balm), used medicinally in antiquity, have a variety of antiviral effects including prevention of the plaque formation, hemagglutination, and death of embryonated eggs induced by Newcastle disease virus (NDV). Because gelatin blocked certain of

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these antiviral activities, it was suggested that a tannin might be involved(1,2). Furthermore, it is known that the leaves of this plant contain a unique tannin (comprising 5% of the total solids) constructed of units of caffeic acid (3,4-dihydroxycinnamic acid)(3). It is the purpose of this report to establish that the antiviral activity against NDV is exclusively related to this tannin, herein referred to as melissa tannin. Also, there are indications that melissa tannin has rather specific reactions, interacting with myxoviruses of subgroup 2 but not with the true influenza viruses.

Materials and methods. Tannin preparations. The source of melissa leaves and the preparation of aqueous extracts have been described(1,2). Tannin was precipitated from aqueous extracts by adding an equal volume of 2% solution of gelatin (Difco) dropwise, which produces a fluffy precipitate. (The gelatin was in excess of the amount required to precipitate all the available tannin.) The preparation was centrifuged at 1,200 rpm for 15 minutes, and the supernate was removed and stored at 4°C. The precipitate was then resuspended in distilled water and recentrifuged 3 times. After concentration under vacuum, these distilled water washes contained no detectable antiviral activity.

The tannin-gelatin precipitate was resolubilized by digestion with a mixture of 0.1% trypsin (Difco) and 0.1% disodium ethylenediaminetetraacetate (Versene, Fisher) in magnesium- and calcium-free Earle's balanced salt solution, usually in a volume one-fourth to one-half that of the original plant extract. After 2 to 3 hours at 36°C, the precipitate was completely digested. After the trypsin was destroyed by heating for 1 hour at 56°C, the solution was stored at 4°C. All solutions used for egg or cell-culture tests contained 100 U of penicillin and 100 μg of dihydrostreptomycin per milliliter.

A second method of concentrating the tannins involved adding 1 g of hide powder (slightly chromated, Merck, Darmstadt) to 40 ml of aqueous melissa extract. This hide-powder preparation was centrifuged at 1,200 rpm for 35 minutes, and the supernate was removed and stored at 4°C. Hide powder-tannin complex was then digested by the same technique used for tannin-gelatin precipitates. Large amounts of hide powder were required to remove all of the tannin, producing viscous solutions which were difficult to handle and somewhat difficult to trypsinize. The gelatin precipitation method was preferred.

A third procedure for precipitation of tannins used addition of lead acetate. (Because lead ions form water-insoluble compounds with many plant polyphenols, this type of preparation contained more low-molecular-weight polyphenols than did the other two preparations.) The lead can be removed by treating the preparation with AG-50W-X resin (Calbiochem). The entire procedure has been described elsewhere(2,4).

All preparations were adjusted to pH 6.9 to 7.2 before testing.

Antiviral tests. Newcastle disease (11914), influenza A (PR8), influenza B (GL), mumps (Enders), and parainfluenza 1 (C-35), 2 (Greer), and 3 (C-243) viruses were obtained from the American Type Culture Collection. Influenza A (WSN) virus was kindly supplied by Dr. E. M. Neumeyer, Du Pont Co., Newark, Del. The parainfluenza viruses were maintained by passage in rhesus monkey kidney cell cultures while the rest were maintained by passage in embryonated chicken eggs. Antiviral tests in eggs as well as hemagglutination-inhibition tests have been previously described(2). The plaque-inhibition test using antibiotic discs has also been described(1,5); however, for these studies the medium used was that described by Simpson and Hirst(6) but without gelatin and phenol red. Plaque counts in chick embryo fibroblast cultures (CEF) also were done in this same medium.

Hemadsorption-inhibition tests were performed on rhesus monkey kidney cell cultures infected with various myxoviruses(7) so that at least half the cells showed hemadsorption after 2 to 5 days' incubation at 36°C. When control cultures exhibited this degree of hemadsorption, the medium was removed from similar cultures and replaced with 1 ml of melissa extract diluted 4-fold in either Alse-

ver's solution (pH 6.1) or 0.02M phosphate-buffered saline (pH 7.1). Two tubes were used for each determination with 2 additional tubes receiving 1 ml of diluent only. After 30 minutes at room temperature, all tubes received 0.3 ml of guinea pig erythrocytes (0.4% in either Alsever's solution or phosphate-buffered saline). Erythrocytes were permitted to settle onto the infected cell sheets at 4°C for 30 minutes and then the tubes were examined for hemadsorption. Cultures were incubated 1 additional hour at room temperature and reexamined. Since there was no essential difference between these readings, only the latter ones are presented here.

Results. Fractions of the plant extract rich in tannin showed the same antiviral activity as the original aqueous extract, both in eggs and in cell cultures infected with NDV (Table I). For this study the tannin fraction was diluted to the original volume of the aqueous extract from which it was derived. The tannin-free supernate, however, was already diluted 2-fold by addition of gelatin solution so it was necessary to inject 0.6 ml into eggs rather than the usual 0.3 ml to equate it to the aqueous extract. Because there was residual gelatin present in the tannin-free supernate, it is not surprising that no protection against NDV was observed. Therefore, an additional preparation was made by precipitating the remaining polyphenols from the supernate with lead acetate, which does not precipitate gelatin. When freed of lead, this 4-fold concentrated preparation, now free of gelatin, also was inactive in suppressing plaques of NDV. From time to time, trypsinized gelatin was tested for an antiviral effect and none was observed.

With the more convenient hemagglutination-inhibition method, it was shown that all 3 methods of tannin preparation yielded fractions with good potency against NDV (Table II). Supernates from gelatin and hide-powder treatments had no detectable activity. Hemagglutination by mumps virus was also blocked by this tannin, and, in both NDV and mumps virus systems, this inhibition was removed by the addition of 1% gelatin just prior to the addition of the chicken erythrocytes.

TABLE I. Activity of Aqueous Extracts and Tannin and Tannin-Free Fractions of *Melissa officinalis* Against Newcastle Disease Virus.

Preparation	Egg experiments							Disc-plaque suppression			
	Virus dose (LD_{50}/0.3 ml)	Hr injected prior to virus	No. prep. tested	Survivors/Total eggs* Dilution Undil.	½	¼	Undil. (no virus)	No. prep. tested	Range of PFU	Avg plaque-free zone, mm (No. of tests)
Aqueous extract	32 to 160†	24	3	17/19	15/19	8/22	10/10	4	7×10^2–5×10^3	22 (10)
	100 to 200	3	3	17/18	15/19	8/19	16/17			
Tannin (gelatin ppt.)	32	24	1	8/10	3/9	—	4/4	2	4×10^2–7×10^2	20 (5)
	100	3	3	**17/22**	**14/24**	**5/18**	**5/5**			
Tannin-free (supernate from gelatin ppt.)	32	24	1	0/10	0/8	0/10	10/10	2‡	4×10^2–7×10^2	0 (6)
	100	3	1	—	0/8	0/8	4/4			

* Incubated at 36°C for 8 days. No untreated controls are included since at no time did any eggs survive these doses of virus.
† This is a summary of a number of experiments, hence the range of LD_{50}'s or PFU's.
‡ One of these preparations was concentrated 4 times by lead precipitation techniques to detect any possible remaining tannin.

TABLE II. Hemagglutination-Inhibition Tests with Aqueous Extracts and Tannin and Tannin-Free Fractions of *Melissa officinalis.*

	Means of reciprocal of highest dilution inhibiting hemagglutination*							
		Gelatin prep.		Hide-powder prep.			Control	
Virus	Aqueous extract	Tannin	Supernate	Tannin	Supernate	Pb prep.	2% gelatin	H_2O extract hide powder
Newcastle, 8 HA units†	64 (7) *<2 (1)*	64 (2) *<2 (2)*	<2 (2)	32 (1)	<2 to 4 (2)	64 (2)	<2 (2)	<2 (1)
Mumps, 8 HA units	64 (3) *<2 (1)*	16 (1)	<2 (2)	—	—	—	—	—
Influenza A (PR8), 2 HA units	<2 (1)	—	—	—	—	<2 (1)	—	—
Influenza B (GL), 2 HA units	<2 to 4 (1)	—	—	—	—	<2 (1)	—	—

* Number in parentheses indicates number of preparations tested (frequently more than one test was done on each preparation). Data in italics are results when 1% gelatin was added prior to addition of erythrocytes.

† HA units = hemagglutination units.

Neither the aqueous extract of melissa nor the lead acetate-precipitated tannin had any significant effect on influenza A and B viruses in the hemagglutination test (Table II). This agrees with the observed absence of antiviral activity against influenza viruses in eggs and in cell cultures. Also, this was confirmed in hemadsorption-inhibition tests in which influenza viruses produced normal hemadsorption in cultures containing melissa extracts (Table III). Hemadsorption was blocked in NDV and mumps virus as well as with the parainfluenza viruses, all classified in subgroup 2 of the myxoviruses. It is presumed that the tannin was responsible for this activity because, if the melissa extract was removed from these infected cell sheets and the cells washed with balanced salt solution, then hemadsorption could again be demonstrated.

Since the tanning of erythrocytes is a well-established reaction, chicken erythrocytes were treated with melissa extracts for 1 hour and then were centrifuged at 1,200 rpm and washed 3 times with phosphate-buffered saline. Such cells were readily agglutinated by NDV. Furthermore, in the presence of melissa extract, influenza viruses readily showed agglutination of chicken erythrocytes or hemadsorption of guinea pig erythrocytes. Thus, the tannin apparently does not alter the erythrocytes but rather reacts with certain viruses. This interaction with the virus particle, however, appears to be weak in that virus can be recovered from mixtures with

TABLE III. Hemadsorption-Inhibition Tests with Aqueous Extracts of *Melissa officinalis.*

		Degree of hemadsorption†			
	Virus dose	Melissa		Control	
Virus	($TCID_{50}$)*	Exp 1	Exp 2	Exp 1	Exp 2
Influenza A (PR8)	10^5	—	3+	—	3+
" A (WSN)	10^6	3+	2+	4+	3+
" B	10^6	—	2+	—	2+
Parainfluenza 1	10^3	—	±	—	4+
" 2	10^3	0	0	3+	3+
" 3	10^2	0	—	2+‡	—
Newcastle	10^7	0§	0	4+§	4+
Mumps	10^5	0‡	0	2+‡	3+

* 50% infectious dose for rhesus monkey kidney cells.

† Cultures incubated at 36°C for 3 days unless otherwise indicated. Exp 1 involved ¼ dilution of melissa in Alsever's solution (pH 6.1); Exp 2 involved 0.02M phosphate-buffered saline (pH 7.1).

‡ Incubated for 5 days.

§ Incubated for 2 days.

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.

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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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