THE INHIBITION OF THE INFLUENZA VIRUS BY TANNIC ACID AND RELATED COMPOUNDS

by

Robert S. Carson

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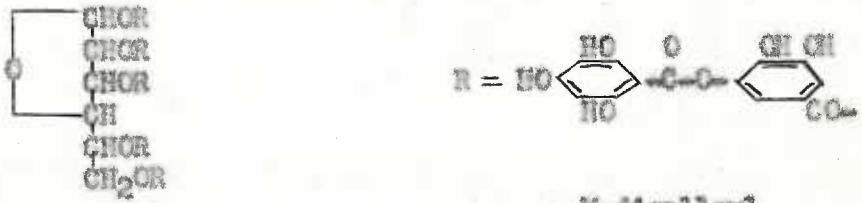
INTRODUCTION

The success of chemotherapeutic agents in the treatment of bacterial diseases has stimulated an intensive search for drugs to control viral infections. Although the majority of such investigations have resulted in negative findings, a number of compounds have either shown therapeutic promise, or have increased our understanding of mechanisms of viral multiplication.

In a thesis completed in 1950, Hosty[1] undertook an investigation of the effects of various metabolic inhibitors on the multiplication of the influenza virus in the chick embryo. His technique consisted of injecting a 10^{-6} to 10^{-7} dilution of virus, and a sublethal dose of inhibitor into the allantoic cavity of embryonated eggs. After the eggs were incubated for 24 to 48 hours, the allantoic fluids were harvested, and the amount of virus contained was determined by hemagglutination and infectivity titers. In order to test the validity of his experimental method, Hosty utilized tannic acid, which had previously been demonstrated by Green[2] to inhibit viral multiplication. The degree of inhibition proved to be so striking that it seemed desirable that tannic acid be investigated in more detail. The results of the study constitute the basis for this thesis.

CHEMISTRY OF TANNIC ACID

Tannic acid is a gallotannin obtained from the nutgall. In the crude form, it is a complex molecule, consisting of multiple digalloyl radicals in combination with glucose, along with impurities and various degradation products[4]. Fischer[5] considered the crude tannin to be a pentadigalloyl-glucoside (I). More recently, Nierenstein[6] has regarded the structure as a polydigalloyl-leucodigallic acid anhydride.

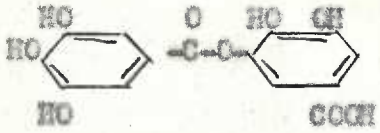


M-digalloyl

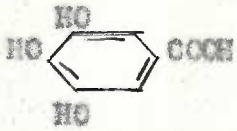
I. Fischer's formula for tannic acid.

Glucose is lost during the purification of tannic acid, so that the purer the specimen, the higher the content of digallic acid. Because of structural instability, even the pure form contains a complex mixture of organic compounds. It should be noted that tannic acid is readily oxidized in alkaline solution. In the present study, tannic acid solutions were alkalinized with NaOH, and it can be assumed that additional products were injected into the eggs. This assumption was supported in part by the observation that these neutralized solutions turned dark upon standing.

Attention was turned, therefore, to simpler compounds structurally related to tannic acid. These included digallic acid (II), gallic acid (III), pyrogallol (IV), and gentisic acid (V).

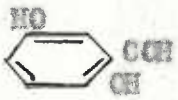


II. Digallic acid



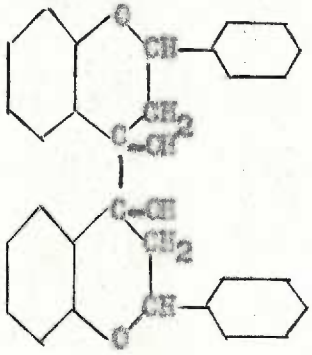
III. Gallic acid

IV. Pyrogallol



V. Centisic acid.

In the present study, a variety of crude tannins were tested for ability to inhibit viral multiplication. The majority of these tannins are classified as phlobotannins, structurally distinct from tannic acid, but possessing a similar ability to precipitate proteins. They are thought to be phenolic hydroxy derivatives of flavpinacol (IV)[4].



VI. Flavpinacol

In Table 1 is recorded the predominant type of tannin contained in the specimens utilized in the present study[7,8].

TABLE I

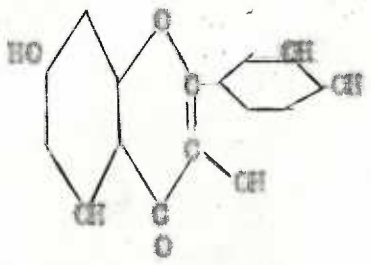
Predominant Tannins of Specimens

Studied in Present Investigation

Gallotannin	Phlobotannin
Tannic acid Merck	Mangrove extract
Nutgall tannin	Valonia powder
Chestnut tannin*	Wattle tannin
Myrabolan tannin	Hemlock tannin
Powdered sumac leaf	Quebracho tannin

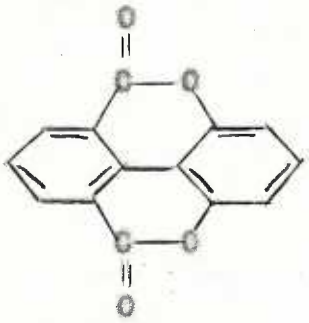
* Small amount of Phlobotannin also present.

Studies of the viral inhibitory properties of quercetin (VII) have been made[9,10,11]. This compound is structurally related to the flavpinacols.



VII. Quercetin

One other group of tannins, the ellagitannins, should be mentioned. The composition of this group has not yet been well established[4], but they are believed to be derived from a glucoside of ellagic acid (VIII).



VIII. Ellagic acid.

Of the tannins studied, valonia powder, myrabolan, and chestnut tannin are considered to contain some ellagitannin[7,8].

The caffetannins are not protein precipitants[4], and their effects on viral multiplication have not been investigated.

VIRAL INHIBITORY EXPERIMENTS WITH TANNINS AND RELATED COMPOUNDS

In 1934, Olitsky and Cox[12] demonstrated that tannic acid instilled intranasally in mice was able to prevent infection with the viruses of poliomyelitis and equine encephalomyelitis subsequently administered by the same route. They believed the effect of the tannic acid was to alter the susceptibility of the nasal mucosa to infection rather than directly on the virus itself. This observation aroused considerable interest in the possible intranasal use of tannic acid and other astringents such as alum[13], as prophylactics against the poliomyelitis virus. It is now realised, however, that the portal of entry for the poliomyelitis virus is not through the respiratory tract, but rather through the gastro-intestinal tract[14]. It is of interest to note that multiplication of Theiler's virus in the gastro-intestinal tract of mice is suppressed by oral administration of mercurial compounds[15].

In 1948, Green[2] observed that neutralised tannic acid in concentrations of 5 to 20 gamma per ml would inhibit the haemagglutinating properties of the PR8 influenza virus. He further found that when 1 mg of neutralised tannic acid was injected allantoically up to 6 hours preceding, or 1 hour following inoculation of from 10 to 100 ID/50 doses of PR8 virus, haemagglutinins were absent from fluids harvested after 48 hours of incubation. In the same paper, Green stated that if tannic acid and concentrated virus were mixed for 15 minutes, a reduction of viral infectivity titers by at least 3 logs would result.

In a later paper, Green[3] described the inhibition of the multiplication of the PR8 virus in chick embryos by a variety of alcoholic

extracts from tea. No attempts were made to determine the chemical composition of these extracts, but he assumed them to be tannins. These extracts were ineffective if given into the yolk sac.

More recently, Chantrill et al[36] reported that a number of plant extracts would inhibit the multiplication of influenza A virus and the bacteriophage of Pseudomonas pyocyanum. In both instances the inhibition by these extracts was lost when viral suspensions were prepared in nutrient broth or in a lactate medium containing 10% serum. When effective extracts were precipitated by lead acetate or phenazone, the supernatants did not inhibit viral multiplication. Crude tannins tested in a similar manner yielded the same results. From this data, Chantrill et al concluded that the effective plant extracts were tannins.

Cutting et al[9], studied the antiviral activities of a wide variety of flavonoid and related compounds in mice. They found that the oral administration of both quercetin and quercitrin was prophylactic against rabies virus inoculated intracerebrally 4 days later. When the action of these drugs on other viruses was studied[10], it was found that quercitrin not only prevented infection with the ectromelia virus, but that it also depressed multiplication when given after injection of the virus. The compounds studied were ineffective against the influenza A and the SK Columbia viruses. In preliminary studies made in this laboratory, quercetin was not demonstrated to inhibit influenza virus multiplication in the chick embryo[11].

EXPERIMENTAL

Introduction

The multiplication of the influenza A virus in the embryonated egg has been intensively investigated[17,18,19]. These studies indicated that, when virus is introduced into the allantoic cavity, the greater part is adsorbed by the cells of the chorioallantoic membrane. The union is rapid, most of the virus being taken up by the cells within a few minutes. Actual multiplication occurs within these cells, and after a period of 6 to 10 hours, during which time virus is not detectable either in or outside the membrane, large amounts are liberated into the allantoic fluid. From this site, additional cells in the membrane are infected, and the cycle is repeated.

On the basis of this knowledge, the observation by Green[2] that tannic acid exerted a dramatic inhibition of multiplication *in vivo*, as well as destroying virus *in vitro*, raised the question of the relationship of these two functions.

Several possibilities presented themselves as explanations. Because of the wide variety of impurities contained in tannic acid specimens, it seemed quite possible that *in vivo* inhibition resulted from these compounds, independent of the *in vitro* inactivation by tannic acid. A second possibility was that the membranes were altered by tannic acid in such a manner that viral multiplication was prevented. A final explanation was that tannic acid destroyed virus liberated into the allantoic cavity during the course of infection.

The present study was directed towards an investigation of these

considerations. In general, the technique described by Hosty[1] was followed and modifications were introduced when necessary. The usual procedure consisted of inoculating tannic acid allantoically at varying intervals before and after administration of influenza virus by the same route. After incubation at 37°C for 24 to 48 hours, samples of allantoic fluid were removed, and viral content determined by hemagglutination or infectivity titers. Titrations on each variable were determined on pooled samples from 6 - 8 eggs.

In the *in vitro* experiments, compounds were mixed with equal volumes of appropriate viral dilutions, and 0.2 ml amounts of these mixtures were injected immediately into eggs. In the *in vivo* experiments, inhibitors were administered 30 minutes after infection, in order to allow sufficient time for virus to penetrate the chorioallantoic membrane.

In the early experiments, viral inhibition by a variety of compounds related to tannic acid was studied. The final experiments undertook the investigation of factors influencing viral inhibition by tannic acid.

Materials

Eggs

Fertile hen eggs, 8 to 12 days old, were obtained from a local hatchery and the same source of supply was used throughout the course of the investigation. Eggs were candled immediately before use, and dead and doubtful eggs were discarded. Eggs were kept in an incubator at 37°C except when removed for experimental purposes.

Virus

Influenza A (PR8) virus was obtained originally from Dr. T. Francis Jr., Ann Arbor, Michigan. It was maintained at a high infectivity by frequent allantoic passage of 0.1 ml volumes of 10^{-6} dilution. Eggs containing seed virus were incubated 48 hours before harvesting. Between passage, virus was stored for not over 24 hours at 7°C. Stock virus was placed in sealed glass ampules and was stored in a CO_2 chest at -73°C.

Tannic acid

For the majority of experiments, Merck reagent grade tannic acid was used. Other varieties included, Merck tannic acid fluffy, and Mallinckrodt tannic acid U.S.P. XIII. The specimens of crude tannin were donated by Parke-Davis Company. Solutions were prepared by weighing appropriate amounts in 50 ml Erlenmeyer flasks and dissolving in not more than 25 ml tap water. After standing for 2 to 4 hours at room temperature, preparations were neutralised to pH 7.2 to 7.4 with 1N NaOH.

The volume was then brought to 50 ml. In Hosty's experiments, these solutions were sterilized by autoclaving for 10 minutes at 15 lbs. pressure. Because this resulted in a drop of pH to 4 to 5, the formation of a brownish precipitate, and a darkening of the solution, preparations used in the present study were sterilized by filtration through Seitz pads.

Special aspirating needles

Especially designed aspirating needles, modified from a description by Green and Freymann[20], were utilized in some experiments for serially removing samples from individual groups of eggs. The instruments were prepared from 1 ½ inch 20 gauge hypodermic needles by cutting off the tips and cutting 4 holes along the sides with an electrically rotated cutting disc. The holes were spaced alternately on opposite sides, 2 mm apart; the first hole being 2 mm from the tip. After holes were inserted, a short bevel was ground on to the end of each needle.

Red cell agglutination materials

Two types of cells were used. Human type O blood was removed aseptically from a volunteer and stored in a sterile tube at 7°C prior to use. Blood for chicken cells was withdrawn from the wing vein of a chicken, with a 10 ml syringe and 22 gauge needle that had first been rinsed in 10% heparin. Chicken blood was stored at 7°C in sterile 30 ml tubes which contained 5 ml of Alsever's solution. Cells could be satisfactorily preserved by this method for 2 to 4 weeks.

Both chicken and human cells were washed 3 times in 0.85% saline prior to use. At each washing, cell suspensions were centrifuged for 3 minutes at 2000 R.P.M. The type O cells were diluted to a 0.75% suspension and in the case of the chicken cells, a 0.25% suspension was used.

Saline was prepared with distilled water, because it was noted that Portland tap water contained non-specific hemagglutinins. Fresh saline was prepared each week. For the type O cells, a 1:100 dilution of rabbit serum inactivated at 56°C was employed. It was stored at -7°C and was thawed just prior to use.

Methods

All egg manipulations were performed under a hood, in a field which had first been sterilised by ultraviolet light. Aseptic technique was used for inoculations and removal of specimens.

Egg inoculation

Six to eight eggs were utilised for each variable studied. Before injection, the tops of the eggs were sterilised by wiping with cotton soaked in 70% alcohol. A hole was then drilled in the shell of each egg over the air sac. Inoculations were made by inserting needles to the hub with the point directed toward the center of the egg. Separate tuberculin syringes and 1 ½ inch 23 gauge needles were employed for virus and tannic acid administration. The inocula consisted of 0.1 ml amounts containing appropriate concentrations. In most instances tannic acid was given 30 minutes after virus. Following or between injections, the eggs were sealed with melted paraffin and were replaced in the incubator.

Harvesting

In the early experiments, the eggs were chilled from 1 ½ to 2 hours at 7°C prior to harvesting. They were then sterilised in the hood, and the tops removed with pointed curved forceps. 2 ml samples of allantoic fluid were removed from each egg with 10 ml syringes, using 20 gauge 1 inch needles. Individual samples of each variable were pooled for titrations. In later experiments the special aspirating needles already described were used, and samples removed without prior

chilling. After removing the seals from the eggs, the shells were sterilized with alcohol, and the needle was gently inserted with the point directed toward the center of the egg. By slow aspiration with a 10 ml syringe, 0.2 ml aliquots were removed from each egg and the individual samples pooled. The shells were then resealed with melted paraffin and eggs replaced in the incubator. By this method, 5 to 6 serial samples could be removed from the same groups of eggs.

10% membrane extracts were prepared by a technique described by Henle[18]. The membranes were removed from the eggs, and were washed 3 times in buffered saline.* After the final washing, the tissues were blotted dry, weighed, and placed in chilled Waring Blendors. Sufficient buffered saline was added to make a 10% suspension. Tissues were then emulsified for 3 minutes. Before hemagglutination titrations, suspensions were clarified by centrifugation at 2000 R.P.M. for 20 minutes.

Red blood cell agglutination

Two methods were employed. When human type O cells were used, 0.2 ml of undiluted allantoic fluid was placed in 0.8 ml of 0.85% saline in a Kahn tube. Serial two-fold dilutions were then made in tubes containing 0.5 ml of saline, and 0.5 ml was discarded from the last tube. A control tube containing saline but no virus was included with each titration. To each tube was added 0.5 ml of 1% inactivated rabbit serum, followed by 0.1 ml of 0.75% human type O cells.

Human type O cells were discarded in favor of chicken cells for two reasons: 1) end point was not clearly defined by this method and

* Equal parts 0.85% saline and phosphate buffer at pH 7.2.

often consisted of a gradient of 2 to 3 tubes 2) time for complete settling of the cells varied by 2 to 3 hours.

The chicken cell technique was similar to the human type O cell method except that no rabbit serum was used and 0.5 ml of a 0.25% suspension was used. After titrations were performed, the tubes were shaken, and after standing at room temperature for 2 ½ hours (or until button in control tube was clearly defined), the results were read.

Reading results

The control tubes without virus showed a small packed mass of cells with sharply defined edges in the bottom of the tube. In tubes considered positive to presence of virus, the red blood cells appeared to be evenly spread over the bottom of the tube. The endpoint was considered to be the highest dilution at which no tendency to button or ring formation occurred(22).

Infectivity titers

These were made by serially diluting allantoic fluids in sterile saline in units of 10. A separate pipette was used for each transfer, and the contents were thoroughly shaken before passage to the next tube. 0.1 ml amounts of dilutions were injected into 3 to 4 eggs. After incubation for 48 hours, the allantoic fluid was harvested and a 3 tube hemagglutination test was run on pooled samples from each dilution. Titers were expressed as the highest dilution of the original allantoic fluid which formed hemagglutinins upon passage. When it was desired to express the infectivity more accurately, eggs from each

dilution were individually tested for hemagglutination, and the 50% infectious dose (ID/50) was calculated according to the method of Reed and Muench[21].

VIRAL INHIBITION BY COMPOUNDS RELATED TO TANNIC ACID

Hoaty[1] demonstrated that 1 mg of neutralized and autoclaved tannic acid would inhibit the multiplication of a 10^{-7} dilution of the PR8 strain of influenza A virus. He found, as Green[2] did, that regardless of whether tannate was given to eggs first, mixed with virus and injected, or introduced following inoculation of virus, no virus could be demonstrated in treated eggs by either hemagglutination or infectivity tests after 24 hours of incubation. He also demonstrated a similar inhibition for the Lee strain of influenza B virus.

The first part of this present investigation undertook to study the inhibition of the PR8 virus by compounds related to tannic acid. It was early observed that autoclaving, as performed by Green, resulted in precipitate formation and a drop in pH of the tannic acid. Because of this undesirable chemical change, preparations were sterilized by filtration through Seitz pads. In order to compare the in vivo inhibition of tannate sterilized by these 2 methods, the experiment presented in Table 2 was designed. Duplicate solutions were prepared from 3 grades of tannic acid and after neutralization with 1 N NaOH, half of the preparations were sterilized by filtering and the other half by autoclaving. 0.1 ml amounts containing varying dilutions of these preparations, were then injected allantoically 30 minutes after the administration of 0.1 ml of a 10^{-6} dilution of PR8 virus. Allantoic fluid was harvested after 48 hours of incubation, and the presence or absence of virus was determined by 5 tube hemagglutination tests. As can be seen from the table, autoclaved and filtered tannate possess a similar degree of ability to

TABLE 2

Comparison of *In Vivo* Viral Inhibition

by Autoclaved and Filtered Tannates

Tannate	Dose mg	Red Cell Agglutination Autoclaved	Filtered
Reagent Grade Merck	1.0	0	0
	0.75	+	+
	0.5	+	+
Fluffy Merck	1.0	0	0
	0.75	0	0
	0.5	+	0
USP XXII Mallinckrodt	1.0	0	0
	0.75	+	0
	0.5	+	+
Control	--	+	

6 embryos used for each variable.
Virus dilution 10^{-6}.
Incubation time 48 hours.

prevent viral multiplication. Therefore, compounds tested during the course of the present investigation were sterilized by filtration.

In order to determine which fraction of tannic acid inhibited the influenza virus *in vivo*, related compounds, including digallic, gallic, pyrogallic, and gentisic acids, were tested. In these experiments, varying doses of the compounds were injected 30 minutes after administration of 10^{-6} to 10^{-7} virus. Eggs were candled after 24 and 48 hours incubation, and fluids were removed from the living embryos by the aspiration technique described earlier. Hemagglutination titers were then determined on pooled samples.

Table 3 is a summary of 2 experiments in which the *in vivo* inhibition by neutralized digallic and tannic acid was compared. Inhibition by the 2 compounds was similar in each experiment. In both experiments dosages as low as 0.25 mg prevented or markedly reduced hemagglutination titers determined after 24 hours of incubation. In the second experiment, inhibition was lost after 48 hours incubation with doses of the compounds below 0.75 mg.

The *in vivo* inhibition by pyrogallic acid was compared with that of tannic acid in the experiment shown in Table 4. As may be seen from the table, pyrogallic acid in a dosage of 2.5 mg, the approximate 50% lethal dose for embryos, prevented the appearance of virus, even after 48 hours of incubation. With a dose of 1 mg, a marked suppression of hemagglutination titer was obtained after 24 hours. This depression was less evident after 48 hours. Titers from embryos treated with 0.5 mg did not vary from the controls. It may be noted, that tannic acid in dosages of 1.0 and 0.5 mg, completely prevented appearance of hem-

TABLE 3

Comparison of *In Vivo* Viral Inhibition

by Digallic and Tannic Acid

Compound	Dose mg	Red Cell Agglutination (HCA) Titer* Experiment 1 24 Hrs	48 Hrs	Experiment 2 24 Hrs	48 Hrs
Digallic acid	1.0	0	0	0	0
	0.75	0	0	0	0
	0.50	0	0	0	2560
	0.25	-	-	0	1280
Tannic acid	1.0	0	0	0	0
	0.75	0	0	0	0
	0.50	0	0	0	5120
	0.25	0	80	40	2560
Control	-	80	10240	640	5120

6 embryos used for each variable.
Virus dilution 10^{-6}.

*Reciprocal of the highest dilution giving maximal hemagglutination

TABLE 4

Comparison of In Vivo Viral Inhibition

by Pyrogallic and Tannic Acid

Compound	Dose mg	Deaths 48 Hrs	HA Titer 24 Hrs	HA Titer 48 Hrs
Pyrogallic acid	10	6	nt	nt
	5	6	nt	nt
	2.5	3	0	0
	1.0	0	40	1280
	0.5	0	10240	10240
Tannic acid	1.0	0	0	1280
	0.5	0	0	2560
Control	-	0	10240	10240

6 embryos used for each variable.
Virus dilution 10^{-6}.

nt = not tested.

agglutinins after 24 hours of incubation. By the end of 48 hours, however, titers were only slightly lower than the controls. Because titers from control eggs had reached a maximum elevation by 24 hours, it was believed that the initial inoculum of virus may have been higher than in the previous experiment and that the increased dose was responsible for the loss of suppression. Experiments having a more direct bearing on this point will be presented later.

Table 5 shows the results of an experiment in which viral inhibition by gallic and tannic acid was compared. In this experiment, the gallic acid did not completely dissolve, and for purpose of comparison, dilutions were expressed on the basis of the original weight. As can be seen from the deaths recorded, toxic levels of gallic acid were obtained, and with 2.5 mgs, the approximate 50% lethal dose, a moderate inhibition of multiplication was noted. With a 1 mg dose, titers did not differ appreciably from the controls. Complete suppression of virus was obtained with 2 mg of tannic acid.

In Table 6 is shown a comparison of the *in vivo* inhibition by tannic and gentisic acids. No inhibition of virus was noted for gentisic acid even with doses as high as 10 mgs. In 2 eggs in which a lethal dose of 20 mgs was administered, only partial suppression of virus was obtained.

These experiments demonstrated that the digallic acid component was the most effective *in vivo* viral inhibitor. Because of the well known protein precipitating action of this moiety[4], it might well be expected that *in vivo* inhibition corresponded with ability to inactivate virus *in vitro*. In order to determine the *in vitro* inactivation

TABLE 5

Comparison of In Vivo Viral Inhibition

by Gallic and Tannic Acid

Compound	Dose mg	Deaths 48 Hrs	HCA Titer 24 Hrs	HCA Titer 48 Hrs
Gallic acid*	5	6	nt	nt
	2.5	3	40	320
	1.0	0	160	2560
Tannic acid	1.0	0	0	0
Control	-	0	320	10240

6 embryos used for each variable.
Virus dilution 10^{-7}.

* Incompletely dissolved. Calculation of dilutions on basis of original weight.

nt = not tested

TABLE 6

Comparison of In Vivo Viral Inhibition

by Gentisic and Tannic Acid

Compound	Dose mg	Deaths 48 Hrs	HCA Titer 24 Hrs	HCA Titer 48 Hrs
Gentisic acid	20*	2	40	640
	10	0	1280	10240
	5	0	1280	10240
	2.5	0	1280	10240
Tannic acid	1.0	0	0	640
	0.5	0	0	5120
	0.25	0	0	10240
Control	-	0	2560	10240

6 embryos used for each variable.
Virus dilution 10^{-6}.

* 2 embryos tested.

of virus by these compounds, they were compared in a single experiment which is tabulated in Table 7. In this experiment, dilutions of the compounds were mixed with equal volumes of 10^{-6} dilution of virus, and 0.2 ml of the mixture was injected immediately thereafter into the eggs. Incubation was extended to 96 hours in order to detect small amounts of virus. Dosage was expressed as the amount of the compound actually injected into the eggs. Hemagglutination tests indicated that tannic acid prevented multiplication with a dosage as low as 0.01 mg. Digallic acid prevented multiplication with a similar dose and in subsequent experiments the minimal effective dose was demonstrated to be under 0.001 mg. Pyrogallic acid completely prevented appearance of hemagglutinins with a dose of 0.1 mg and markedly suppressed the titer with a dose of 0.01 mg. The minimal effective dose of gallic acid was 1 mg and gentisic acid was completely inactive in the dosage used.

This experiment indicated that the *in vivo* inhibition of multiplication coincided with the rapid potent inactivation of virus *in vitro* by tannic and digallic acids. To a lesser extent, pyrogallic acid exhibited the same phenomenon. Because more striking inhibition was demonstrated for the portion of tannic acid capable of precipitating protein, the possibility was considered that crude tannins chemically distinct, but possessing this same ability, might also inhibit viral multiplication. Green's work with tea extracts[3] did not contribute to this consideration, because tea contains both phlobotannins and gallotannins.

A variety of crude tannins, obtained from Parke-Davis Company, were tested for ability to inhibit viral multiplication. For this

TABLE 7

Comparison of *In Vitro* Viral Inactivation by Tannic Acid and Related Compounds

Compound	Dose mg	RCA Titer
Tannic acid	1.0	0
	0.1	0
	0.01	0
	0.001	5120
	0.0001	10240
Digallic acid	1.0	0
	0.1	0
	0.01	0
Pyrogallic acid	1.0	0
	0.1	0
	0.01	80
Gallic acid	1.0	0
	0.1	10240
	0.01	10240
Gentisic acid	1.0	10240
	0.1	10240
	0.01	10240
Control	-	10240

6 embryos used for each variable.
Incubation time 96 hours.

purpose, a series of individual experiments were employed, in which tannins were tested for embryo toxicity and *in vivo* viral inhibition. Varying dilutions of the tannins were injected 30 minutes after administration of 10^{-7} virus. After 48 hours of incubation the eggs were candled, and dead embryos counted and discarded. The presence or absence of virus was determined in pools from living embryos, by 5 tube hemagglutination tests. In these experiments, titers from control embryos varied between 1:1280 and 1:5120.

The experiments on viral inhibition by gallotannins are summarized in Table 8. It may be seen from the table, that in addition to tannic acid, chestnut and myrabolan tannins inhibited multiplication. Nutgall tannin was toxic to embryos in the dosage used. Table 9 summarizes the experiments on inhibition by phlobotannins. The effective specimens included valonia, wattle, and hemlock tannins.

Because of the demonstration that viral inhibition was not limited to tannic acid, but was found in chemically unrelated tannins as well, a number of inorganic protein precipitants were tested for inhibitory ability. These compounds included aluminum potassium sulfate, mercuric bichloride, sulfosalicylic acid, and phosphotungstic acid. In these experiments, summarized in Table 10, the compounds were sterilized through Mandler filters without previous neutralization, and varying concentrations were injected 30 minutes after 10^{-6} virus. After 24 and 48 hours of incubation, eggs were candled, and fluids removed from living embryos, pooled, and hemagglutination titers determined. As can be seen from the table, both aluminum potassium sulfate and mercuric bichloride markedly inhibited viral multiplication with dosages below

TABLE 8

Summary of Experiments on Embryo Toxicity and

In Vivo Viral Inhibition by Crude Gallotannins.

Tannin	Dose mg	No. Eggs Surviving	Hemagglutination
Tannic acid Merck	10.0	0	nt
	7.5	0	nt
	5.0	1	0
	2.5	5	0
Nutgall tannin	10.0	0	nt
	7.5	0	nt
	5.0	0	nt
	2.5	0	nt
Chestnut tannin	10.0	0	nt
	7.5	3	0
	5.0	2	0
	2.5	3	0
Myrabolan tannin	10.0	0	nt
	7.5	1	0
	5.0	2	+
	2.5	6	+
Powdered sumac leaf*	10.0	3	+
	7.5	6	+
	5.0	5	+
	2.5	6	+

6 embryos used for each variable.
Virus dilution 10^{-7}.
Incubation time 48 hours.
nt = not tested.

* Incompletely dissolved. Calculation of dilution on basis of original weight.

TABLE 9

Summary of Experiments on Embryo Toxicity and

In Vivo Viral Inhibition by Crude Phlobotannins

Tannin	Dose mg	No. Eggs Surviving	Haemagglutination
Mangrove extract*	10.0	4	0
	7.5	6	0
	5.0	6	0
	2.5	6	0
Valonia powder	10.0	0	nt
	7.5	3	0
	5.0	6	0
	2.5	5	0
Wattle tannin	10.0	0	nt
	7.5	3	0
	5.0	5	0
	2.5	5	+
Hemlock tannin	10.0	0	nt
	7.5	0	nt
	5.0	2	0
	2.5	6	+
Quebracho tannin*	10.0	0	nt
	7.5	1	+
	5.0	1	+
	2.5	6	+

6 embryos used for each variable.
Virus dilution 10^{-7}.
Incubation time 48 hours.
nt = not tested.

* Incompletely dissolved. Calculation of dilution on basis of original weight.

TABLE 10

In Vivo Viral Inhibition by Inorganic Protein Precipitants

Compound	Dose mg	Deaths All Eggs	HCA Titer 24 Hrs	HCA Titer All Eggs
Phosphotungstic acid	20	6	nt	nt
	10	3	160	10240
	5	2	640	10240
Sulfosalicylic acid	20	6	nt	nt
	10	2	2560	5120
	5	0	2560	5120
Controls	-	0	1280	10240
Aluminum potassium sulfate	10	2	0	0
	5	1	0	0
	2.5	0	80	5120
Mercuric bichloride	0.1	6	nt	nt
	0.05	1	0	640
	0.025	0	0	2560
	0.0125	0	160	10240
Controls	-	0	160	10240

6 embryos used for each variable.
Virus dilution 10^{-5}.
nt = not tested.

the toxic levels. A moderate depression of titers for phosphotungstic acid was observed. The titers from sulfosalicylic acid treated eggs did not differ from the controls.

FACTORS INFLUENCING VIRAL INHIBITION BY TANNIC ACID

In the first part of this investigation, the *in vivo* inhibition of the PR8 virus by tannic acid, chemically related compounds, crude tannins, and other protein precipitating agents was studied. It was found that mono-phenolic derivatives of tannic acid were much weaker inhibitory agents than digallic acid or tannic acid itself. It was further noted that *in vivo* inhibition by these two compounds corresponded with their potent *in vitro* inactivation of virus. Finally, *in vivo* viral suppression was demonstrated for crude phlobotannins and for other protein precipitating agents.

These observations indicated that the in vivo inhibition by tannic acid was related to its protein precipitating capacity, and suggested that inhibition resulted from inactivation of virus liberated into the allantoic fluid. The second part of this study is concerned with an attempt to investigate this hypothesis more completely.

Green[2] observed that tannic acid inhibited viral multiplication when given up to 6 hours preceding, or 1 hour following injection of virus. He made no statement as to whether or not the intervals between injection of virus and tannic acid were extended. This relationship to inhibition was investigated in an experiment in which 2 mg of tannic acid were injected into eggs at varying intervals before and after infection with 10^{-7} virus. As can be seen from Table 11, tannic acid prevented multiplication, whether given 12 hours before, or 3 hours after viral injection. When the experiment was repeated, inhibition was demonstrated, even when 1 mg of tannic acid was given up to 96 hours

before the virus. On the other hand, 1 mg of tannic acid given after virus, was inhibitory only if a 1 hour interval was not exceeded.

In the experiment shown in Table 12, 2 mg of tannic acid were inoculated at 15 minute intervals up to 1 hour after the injection of 10^{-6} dilution of virus, and eggs were harvested after 30 and again after 72 hours of incubation. In the treated embryos, virus could not be demonstrated by either hemagglutination or infectivity tests at the end of 30 hours. By the end of 72 hours, hemagglutination had reached levels only slightly below the controls, and a single infectivity test had reached 10^{-7} or maximum elevation.

In order to determine more exactly the time of viral appearance, a similar experiment was undertaken in which 2 mg of tannic acid were injected 30 minutes after 10^{-6} dilution of virus, and eggs were harvested at more frequent intervals. As can be seen from Table 13, virus could be demonstrated in control eggs by infectivity tests determined after 12 hours incubation. By the end of 36 hours, hemagglutination was first apparent, and infectivity had reached maximum elevation. In the treated eggs, virus could not be demonstrated until after 60 hours of incubation, at which time both hemagglutination and infectivity had reached maximal proportions.

The relation of viral dose to duration of inhibition by tannic acid was studied in the experiment presented in Table 14. Growth curves of selected doses are illustrated in Figure 1. In order to obtain a standard and uniform dose, virus suspensions were prepared from frozen

TABLE 12

The Effect of Prolonging Incubation on the Inhibition of Viral Multiplication by Tannic Acid

Tannate Injected Minutes After Virus	30 Hours Incubation HCA Titer	30 Hours Incubation Infectivity*	72 Hours Incubation HCA Titer	72 Hours Incubation Infectivity*
15	0	0	640	nt
30	0	0	1280	nt
45	0	0	2560	nt
60	0	0	1280	7
Control	5120	7	5120	7

9 embryos used for each variable.
Virus dose 10^{-7}.
Tannate dose 2 mgs.

* Expressed as negative log of dilution.

nt = not tested

TABLE 13

The Relation of Duration of Incubation to Viral Multiplication in Treated and Non Treated Eggs

Incubation Period Hours	Tannate Treated RCA	Tannate Treated ET	Untreated Controls RCA	Untreated Controls ET
12	0	0	0	1
18	0	0	0	2
24	0	0	0	4
36	0	0	1280	7
48	0	0	5120	6
60	1280	6	5120	7

7 embryos used for each variable.
Virus dose 10^{-6}.
Tannate dose 2 mgs.

TABLE 14

Relation of Viral Dose to Inhibition by Tannic Acid

Red Cell Agglutination Titer After Hours Incubation	ID/50 doses of virus injected.													
	1		10		100		1000		10,000		100,000		1,000,000	
	T	C	T	C	T	C	T	C	T	C	T	C	T	C
6	0	0	0	0	0	0	0	0	0	20	0	640	0	1280
12	0	0	0	0	0	0	0	160	0	1280	10	5120	40	5120
18	0	0	0	320	0	1280	20	5120	320	5120	640	5120	640	10240
24	0	640	0	640	80	2560	320	5120	1280	5120	1280	5120	1280	10240
36	0	5120	320	10240	320	5120	640	5120	1280	5120	5120	5120	1280	10240
48	0	10240	5120	10240	5120	10240	10240	10240	10240	10240	5120	5120	1280	10240
72	0	10240	-	-	-	-	-	-	-	-	-	-	-	-

Serial samples of pools from 6 embryos used for each viral dose.
Dose tannate 2 mgs.

T = Tannate treated embryos
C = Control embryos.

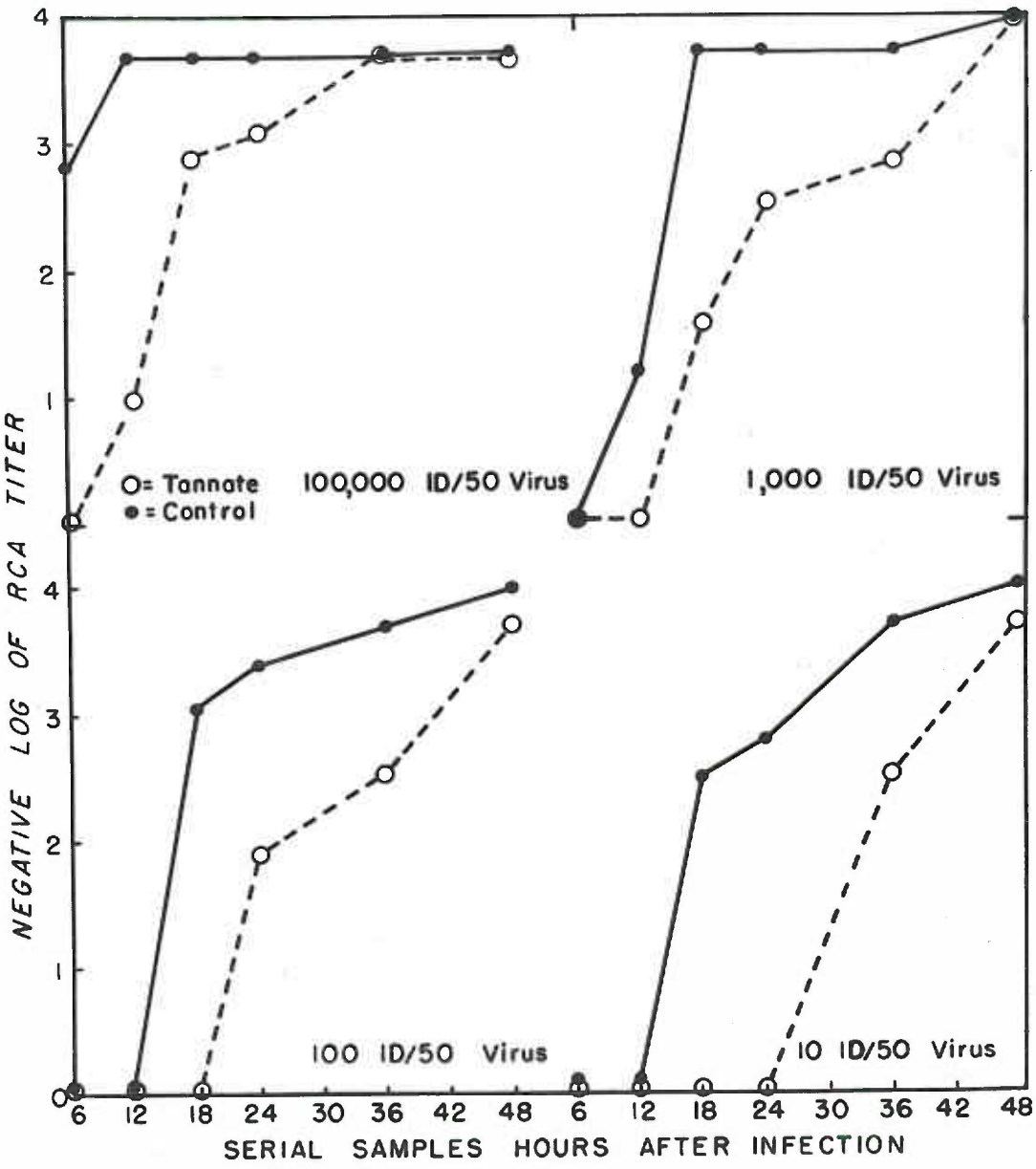


Figure 1. Selected growth curves from an experiment in which 2 mg of tannic acid was administered 30 minutes after varied ID/50 doses of virus.

allantoic fluid*, and the 50% infectious dose (ID/50) was determined at the time of inoculation. Ten fold dilutions of virus were injected into eggs and these were followed in 30 minutes by 2 mg of tannic acid. Aliquots of allantoic fluid were then serially removed from the same groups of embryos at 6 to 12 hour intervals, by the technique described in the section on methods.

The results of this experiment indicated, that with the exception of the ID/50 dose, inhibition by tannic acid proved to be only temporary. In general, the time of appearance of hemagglutinins was directly related to viral dosage in both treated and untreated embryos. A delay of approximately 6 hours occurred between the time that the titers of control eggs reached close to maximum elevation (1:1280 or higher), and the appearance of hemagglutinins in tannate treated eggs. When the experiment was repeated (Table 15), similar results were obtained.

The observation that inhibition of multiplication was only temporary when tannic acid was injected 30 minutes after infection, raised the question as to whether a similar loss of inhibition would occur, if tannic acid was inoculated before or mixed with virus. If the hypothesis is correct, that inhibition by tannate results from inactivation of virus in the allantoic cavity, then tannic acid given in the above manner would be expected to prevent virus from entering susceptible cells. This prevention of cell entry should result in permanent

*The technique for preparation of virus consisted of placing 1 ml amounts of freshly harvested virus in glass tubes. The tubes were placed in an ice bath while the ends were sealed with an oxygen flame. Tubes were then rapidly frozen in a dry ice-acetone mixture and were stored at -70°C.

TABLE 15

Relation of Viral Dose to Inhibition by Tannic Acid

Red Cell Agglutination Titer After Hours Incubation	ID/50 doses of virus injected.											
	3.2		32		320		3200		32,000		320,000	
	T	C	T	C	T	C	T	C	T	C	T	C
6	0	0	0	0	0	0	0	0	0	320	0	1280
12	0	0	0	40	0	1280	0	1280	0	2560	0	2560
18	0	640	0	5120	80	102400	160	102400	640	102400	1280	102400
24	160	5120	640	5120	640	5120	2560	5120	5120	102400	5120	102400
36	320	102400	5120	102400	102400	102400	102400	102400	102400	102400	-	-
48	320	102400	5120	102400	102400	102400	5120	102400	5120	102400	-	-

Serial Samples of pools from 6 embryos used for each viral dose.
Dose tannate 2 mgs.

T = Tannate treated embryos
C = Control embryos

inhibition of multiplication.

Table 16 shows the result of an experiment in which 2 mg of tannic acid were injected either 12 hours before, mixed with equal parts of virus and injected, or introduced 30 minutes after inoculation of a 10^{-5} dilution. Control eggs were injected with 0.1 ml of normal saline in the same manner. After 24 and 48 hours of incubation, hemagglutination titers were determined for the allantoic and amniotic fluids, and for 10% extracts of the various embryonic tissues. In the untreated eggs, hemagglutinins were demonstrable in both allantoic and amniotic fluids and membranes at 24 and 48 hours. In treated eggs, virus could be demonstrated in these areas, only when tannic acid was introduced 30 minutes after infection. No virus was demonstrable in the yolk sacs or embryos of either the treated or untreated eggs. The supposition that inhibition by tannic acid was due to viral inactivation in the allantoic cavity was further tested in the experiment shown in Table 17. In this experiment, a 10^{-6} dilution of virus was injected into the embryos 24 hours before the inoculation of varying doses of tannic acid. Samples of allantoic fluid were removed 1 and 24 hours after tannate injection. Chorioallantoic membranes were also removed 1 hour after tannate administration, and 10% extracts were tested for presence of virus. The inactivation of virus in the allantoic fluid was from the marked drop in hemagglutination titer that occurred 1 hour after tannate injection. On the other hand, with the exception of the 2 mg dose which showed a slight depression, the membranes from treated embryos had titers similar to the controls. The ability of the virus to multiply within the membranes is demonstrated by the return of hemagglutinins into the allan-

TABLE 16

Relation of Order of Tannic Acid Injection to Appearance of Virus in Embryonic Components

Source of RCA Determinations*	Time of Harvest Hrs Incubation	Relation of Tannate to Viral Injection: 12 Hrs. before		Mixed and injected		30 minutes after virus	
		Treated	Control	Treated	Control	Treated	Control
Allantoic fluid		0	10240	0	10240	80	2560
Amniotic fluid		0	80	0	160	10	640
Allantoic Memb.	24	0	20	0	10	0	20
Amniotic Memb.		0	0	0	10	0	20
Yolk Sac		0	0	0	0	0	0
Embryo		0	0	0	0	0	0
Allantoic fluid		0	10240	0	10240	5120	10240
Amniotic fluid		0	10240	0	640	160	2560
Allantoic Memb.	48	0	80	0	80	40	160
Amniotic Memb.		0	10	0	20	0	20
Yolk Sac		0	0	0	0	0	0
Embryo		0	0	0	0	0	0

6 embryos used for each variable.
Virus dose 10^{-5}
Tannate dose 2 mgs.

*Titers from tissues determined on 10% extracts.

TABLE 17

Hemagglutination Titers of Chorioallantoic Fluids and Membranes Before and After Injection of Tannate When Virus Was Inoculated 24 Hours Previously.

HCA Titer Before Tannate Al. Fluid	Tannate mg	HCA Titer After Tannate 1 Hour Al. Fluid	24 Hours Al. Fluid	1 Hour Al. Membranes
1280	2.0	0	5120	160
1280	1.0	0	5120	640
1280	0.5	10	5120	640
1280	Control	5120	5120	1280

6 embryos used for each variable.
Virus dilution 10^{-6}.

toic fluids 24 hours after inoculation of tannic acid.

A possible explanation for the return of virus in treated eggs, would be that strains resistant to tannic acid might develop, similar to those of the mumps virus for Friedlander polysaccharide(23). In order to exclude this possibility, two additional experiments were performed. In the first experiment, virus containing allantoic fluid from tannate treated eggs was re-injected at a 10^{-5} dilution into additional eggs and was again suppressed by tannic acid given 30 minutes later. After sufficient incubation to permit return of virus, the procedure was repeated. A total of 4 such passages did not demonstrate an increased resistance to tannic acid.

Figure 2 shows the hemagglutination titers from the second experiment in which undiluted virus was injected into eggs. After 11 hours of incubation, samples of allantoic fluid were removed and 0.5 mg of tannic acid was injected. Within 30 minutes, a drop in hemagglutination titer was observed, and 12 hours later a distinct rise occurred. A second injection of 2 mg of tannate after 27 hours of incubation, was followed by another fall in titer, and an increase 21 hours later. Titers for samples from control eggs given normal saline did not vary.

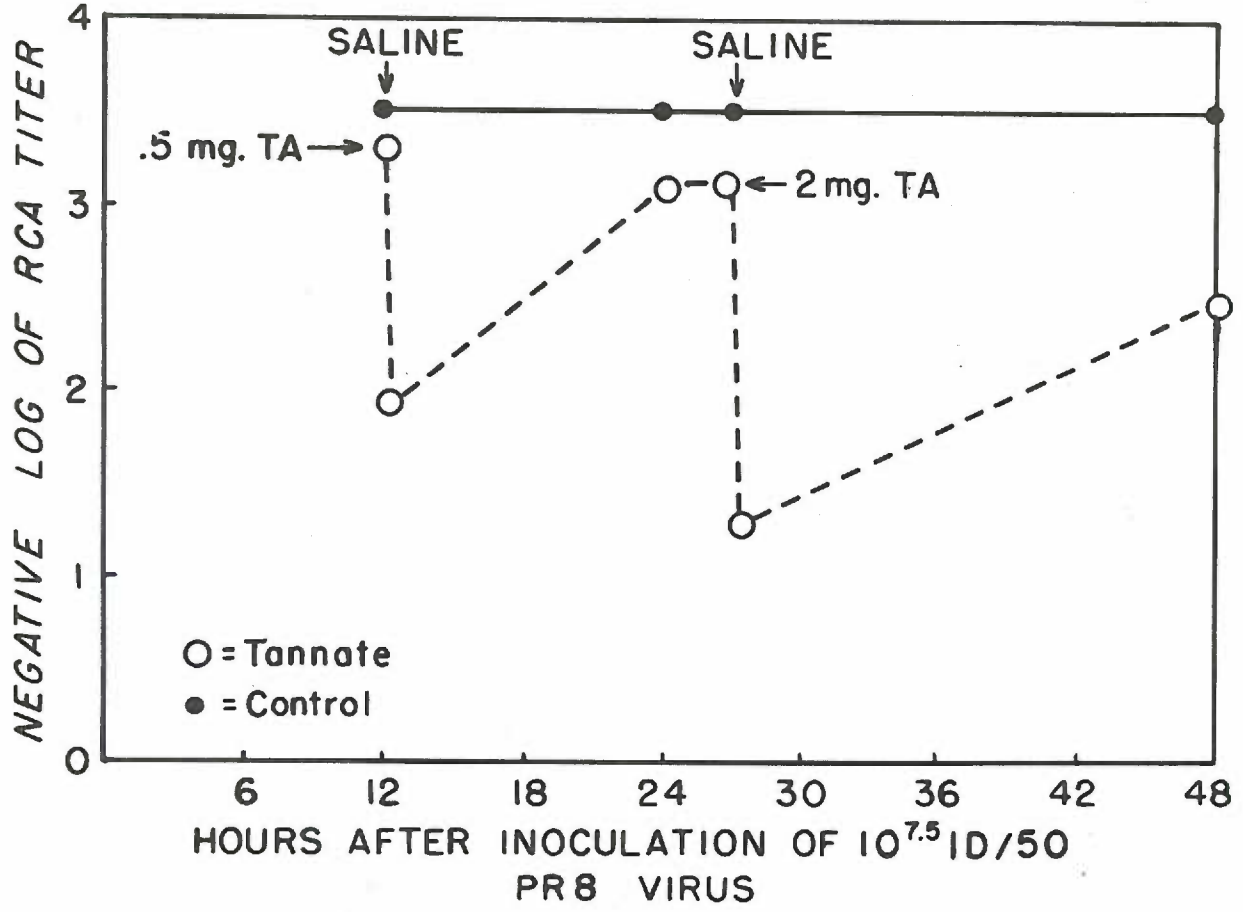


Figure 2. Effect of repeated injections of tannic acid on hemagglutination titers of allantoic fluid containing large amounts of virus.

DISCUSSION

Attempts to inhibit the multiplication of the animal viruses have been largely empirical. A wide variety of chemical and biological agents have been tried[24], but when this study was instigated, tannic acid was the most potent inhibitor demonstrable.

Although the most logical mode of action of tannic acid would be through its protein precipitating ability, this fact was by no means proven. One could not exclude the possibility that in such an impure substance, other compounds present might also exert an effect on the virus within the cell. It was also possible that tannic acid could alter the cell surface in such a way as to prevent viral multiplication. This last point has not yet been subjected to experimental determination, but such an alteration seems unlikely in view of the data thus far obtained. For the above reasons, viral inhibition by tannic acid was studied in considerable detail. This study constitutes the thesis herein presented.

In the early experiments, the multiplication of the PR8 virus was prevented by the introduction of crude tannins, inorganic protein precipitants, and tannic and digallic acids into the allantoic cavity 30 minutes after infection. If tannic or digallic acids were mixed with virus, complete inactivation of a 10^{-7} dilution occurred with as little as 0.01 mg even if the embryos are incubated for 96 hours. The later experiments indicated that the *in vivo* inactivation by tannic acid is temporary and that the virus invariably reappears in the allantoic fluid if sufficient incubation time is permitted.

The evidence now available suggests that both the in vivo and in vitro inactivation of influenza virus are probably related to the potent protein denaturing action of tannic acid. Similarly Chantrill et al[16] were unable to differentiate between the protein precipitating properties of tannins and viral inactivation. They observed that virus was protected in the presence of excess protein, and that the supernatants of tannin containing solutions which had previously been precipitated by protein, did not destroy virus. Talman[25] has demonstrated a quantitative relationship between loss of hemagglutination and the increase of optical density of virus containing allantoic fluids which were treated with varying quantities of tannic acid. She found that when the log of the hemagglutinating units per ml of fluid was plotted either against the log of the optical density, or milligrams of tannic acid, a straight line was obtained.

It has been demonstrated by Kilbourne and Horsfall[26] that during the course of infection, large amounts of viral, as well as non viral protein are liberated into the allantoic cavity. It would be reasonable, therefore, to assume that the appearance of virus after temporary suppression is due to the exhaustion of tannate by these proteins. Attempts to prove this point directly were hampered by the lack of a sensitive quantitative test for tannic acid. For this reason, indirect evidence and exclusion of alternative explanations for the reappearance of virus were utilized. These included the demonstration of the quantitative destruction of hemagglutinins in the fluids, the absence of resistance to tannate, and the presence of virus in the membrane. The latter is illustrated in Table 17 where a significant

amount of virus was found in the chorioallantoic membrane and was subsequently released into the allantoic fluid with continued incubation. Additional evidence for the presence of virus in the membrane is available from the experiment shown in Table 16.

An additional argument in favor of the extracellular site of action of tannic acid may be found in Figure 1. The temporary inhibition of virus was followed by multiplication at a rate comparable to the controls. If the tannic acid had blocked the intracellular production of virus, the gradual release from its inhibitory action would have changed the slope of the growth curve in a manner similar to that described for the fluoroacetate-poliomyelitis(27) and for the nitrocridin-influenza B(28) systems. From Figure 1, there also appears to be a direct relationship between the dosage of virus and the duration of inactivation by tannic acid. In the case of the interference phenomenon, largely an intracellular blocking mechanism, the challenging dose of infective virus does not influence the amount of inhibition(29, 30). To a lesser extent, the same criterion applies to the mumps-Friedländer polysaccharide system(31).

If one assumes that the action of tannic acid is due to inactivation of extracellular influenza virus, then two explanations are available for the increase which occurs in the membranes. 1) Virus need not necessarily be released into the extracellular environment but is able, through intracellular communication to spread within the tissue itself. 2) The second and more likely alternative is that the chorioallantoic membrane is reinfected from embryonal sources other than the allantoic fluid(32). Neither of these possibilities can be excluded by

the type of experiment herein described, and should be considered in all studies dealing with the quantity of virus in the allantoic fluid as compared with the membrane itself.

SUMMARY

1. Tannic acid, related compounds, crude tannins, and inorganic protein precipitants were tested for ability to inhibit the multiplication of the PR8 strain of influenza A virus in the embryonated egg.

2. A variety of compounds were found to possess viral inhibitory properties when injected 30 minutes after infection. These included tannic acid, digallic acid, chestnut tannin, myrabolan tannin, mangrove extract, valonia powder, wattle tannin, hemlock tannin, phosphotungstic acid, aluminum potassium sulfate, and mercuric bichloride.

3. The correlation of *in vivo* viral inhibition to potent *in vitro* inactivation was established for tannic and digallic acid.

4. The *in vivo* inhibition of virus by tannic acid was demonstrated to be temporary provided incubation was sufficiently prolonged. Inhibition was further shown to be a function of dose of virus injected. If 30 minutes was allowed for penetration of as little as 10 ID/50 of virus into the chorioallantoic membrane, hemagglutinins invariably appeared in the allantoic fluid. Once the effect of tannic acid was overcome, the virus was released into the fluid at a rate comparable with controls.

5. Further evidence is presented to show that the return of virus was not due to the development of resistant strains, and that virus within the chorioallantoic membrane was not affected by tannic acid.

6. It was concluded that tannic acid inhibited viral multiplication by inactivating virus in the allantoic cavity.

7. Reasons for this conclusion are discussed.

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