Antiviral and Antibacterial Activity of Thymus Extracts. (28715)

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Antibacterial and antiviral substances previously isolated by acetic acid extraction from the abalone, the oyster and certain other shellfish have been designated as paolins 1 and 2 respectively(1). Substances with similar biologic activities have now been obtained by the same general methods from the calf thymus; the present report describes this recent work.

Early investigators isolated an antibacterial fraction, histone, from calf thymus(2-4), but similar substances have also been isolated from other organs(2). About the time we initially reported the antiviral and antibacterial activity of extracts from calf thymus gland(5), Limasset and Truffaut also demonstrated inhibitory action of calf thymus material on tobacco mosaic virus(6).

Materials and methods. Preparation of thymus extracts. Extracts of calf thymus gland were made in (a) water, and (b) dilute acetic acid. Fresh calf thymus glands bought from slaughter houses were frozen and stored at −20°C for periods as long as 4 months before use. For water extraction, 1 part (by weight) of thymus, freed from fat, was ground in a Waring blendor with 2 parts (by volume) of distilled water; the mixture was then placed at 4°C for 72 hours after which it was partially clarified by centrifugation at 700 g for 2 to 3 hours. The supernate was dialyzed against distilled water at 4°C for 48 hours. After a small amount of insoluble material was removed by centrifugation the fluid was filtered through a Seitz E.K. Pad (size 14), and the filtrate was lyophilized. For acid extraction the same procedure was followed except that a 1:4 dilution of reagent grade acetic acid in distilled water was used in the extraction. The yield of dry material following either procedure was usually 0.5±% of the wet weight of the thymus.

Properties of the extracts. Both the acid and water extracts were white or brownish powders after lyophilization, were soluble in water, nondialyzable and gave positive reactions with all the well known protein tests. The antibacterial factor in the acid extract was stable at 90°C for 30 minutes in saline (pH 7.0) and its activity was somewhat enhanced by heating at 80°C. However, the antiviral factor in the water extract was destroyed at 80°C for 30 minutes under the same conditions and its activity was reduced after several hours at room temperature. Further chemical studies of these products are in progress.

Antibacterial assay in vitro. The following organisms were used for assay: a penicillin sensitive strain, FDA 209 and a penicillin resistant strain of *Staphlococcus aureus*; *Streptococcus pyogenes,* beta-hemolytic strain, type 12; *Bacillus subtilis*; *Salmonella typhosa* and *Paratyphi* A and B; *Shigella dysenteria* and *Paradysenteria flexneri;* and organisms belonging to the *Proteus* and *Pseudomonas* groups, 5 strains in each group (NIH No. 1 to 5), isolated from urine or wounds of patients.

Antibacterial assay was made in Difco nutrient broth medium containing various concentrations of the extract. The extract was dissolved in buffered saline pH 7.0 and heated to 70°C for 30-40 minutes (this was found adequate to kill viable bacterial contaminants) before being incorporated into the broth medium. Ten-fold dilutions of an 18-hour culture of the test organisms were made in saline and 0.1 ml of each dilution was inoculated into 2 ml of Difco nutrient medium with and without the test substance. A single dilution containing approximately 10^5 organisms as determined by plate counting was used for study of antibacterial spectrum of the acid extract. Growth was recorded 24 hours or 7 days afterwards. For assay with *S. pyogenes,* 1% fresh rabbit

blood was added to Difco nutrient broth which immediately hemolyzed the blood giving a deep red color; the growth of *S. pyogenes* resulted in decolorization of the medium.

Antiviral assay in tissue culture. The strain of Coxsackie A9 virus used in previous experiments on Coxsackie A9 virus multiplication(7) was employed for assaying antiviral activity in tissue culture. The thymus extracts to be assayed in the infected tissue cultures were dissolved in medium 199 containing 2% inactivated calf serum, in amounts sufficient to provide the following concentrations: 4, 20, 100 and 500 μg of dry extract per ml of solution. The presence of penicillin and streptomycin in the medium, 200 units and 100 μg per ml, respectively, was found adequate to inhibit viable bacterial contaminants in the extracts. None of these concentrations of either type of extract was found toxic for primary monkey kidney tissue culture cells observed for 2 weeks. In the assay a group of 6 primary monkey monolayer culture tubes were used for each concentration of the extract. After treatment for 48 hours with 1 ml of the medium containing extract under test each tube was inoculated with approximately 2 $TCID_{50}$ of Coxsackie A9 virus. These tubes along with a group of 6 infected but untreated control tubes were incubated for 2 or 3 days. Cytopathic effect was recorded daily. Fluid pooled from each group of tubes was then titered in primary monkey kidney monolayer cultures for viral content. The results were expressed as $TCID_{50}$ of virus per 1 ml of treated or control culture fluids.

Antiviral assay in vivo. *Poliovirus.* Swiss white mice, C.F.W. strain obtained from the NIH Animal Production Unit and weighing approximately 20 g were used for antiviral assay. Poliovirus type I, LSa strain, grown in primary monkey kidney tissue culture was inoculated intracerebrally, 10^5 $TCID_{50}$ in a volume of 0.03 ml for each mouse. Treatment consisted of a single dose of varying amounts of thymus extract dissolved in 0.5 ml volume of Hanks' salt solution, or buffered saline, pH 7.0, administered intraperitoneally 5 hours before or after inoculation. Equal amounts of Hanks' solution, or saline, were injected into the control mice. All mice were observed for 2 or 3 weeks after infection.

Coxsackie virus. The procedures were the same as for studies on poliovirus described above except for the following modifications. Newborn NIH Swiss white general purpose mice weighing about 2 g were used. Coxsackie A9 virus (obtained from Microbiological Associates, Bethesda, Md., Lot 20766) was grown in primary monkey kidney tissue culture. Approximately 10 $TCID_{50}$ of the virus contained in 0.04 ml of Hanks' salt solution or saline was administered intramuscularly. The thymus extract in 0.02 ml volume was administered intraperitoneally 5 hours before or after infection.

Toxicity tests in mice. Three groups of 12-15 newborn Swiss white mice were treated intraperitoneally with a single dose of 1 g/kg, 0.1 g/kg, or 0.01 g/kg body weight respectively of dry material from Lot 1 aqueous extract of thymus. The extract was dissolved in saline before use. The mice showed no toxic symptoms during a period of 2 weeks. They grew up normally and gained as much weight as the controls.

Results. Antimicrobial activity of acid and water extracts. Four lots of acetic extract and 3 lots of water extract were prepared from calf thymus tissue. In our first experiment the water extract (Lot 1) showed no antibacterial activity *in vitro* against *S. aureus* at a concentration of 500 μg/ml but only a few micrograms of the material inhibited Coxsackie A9 virus in tissue culture. On the other hand, low concentrations of the acid extract possessed *in vitro* antibacterial action but had no significant antiviral effect in tissue culture even at 500 μg/ml concentration. Subsequent preparations of extracts gave similar results although they appeared to be somewhat less potent (Table I).

Antibacterial spectrum of the acid extract. The presence of 4 to 20 μg/ml of the acid extract (Lot 4) prevented the growth of about 10^5 organisms of *S. aureus* FDA 209 strain, in 2 ml volumes of Difco nutrient broth cul-

TABLE I. Antibacterial and Antiviral Activity of Different Lots of Calf Thymus Extracts.

Lot No.	Bacterial (*S. aureus*) inhibiting dose, μg/ml	Viral (Coxsackie A9) inhibiting dose, μg/ml
	Water extracts	
1	>500	4
2	>500	ND
3	>500	100
	Acid extracts	
4	4	>500
5	20	>500
6	20	>500
7	20	ND

ND = not done.

ture. Other test organisms including all 5 strains of the *Pseudomonas* group and 3 of 5 strains of the *Proteus* group examined were inhibited by concentration of 20 to 500 μg/ml (Table II). It is evident from the data that gram positive as well as gram negative organisms were inhibited although a number of the latter were relatively insensitive.

The pattern of inhibition of a strain of *Pseudomonas* is illustrated by a typical experiment summarized in Table III. In this experiment bacterial growth was recorded 24 hours and 7 days after inoculation and there was no difference between the two readings. In the presence of 500 or 100 μg/ml of the acid extract (Lot 4), growth of an inoculum of 10^5 organisms was completely inhibited, and even the concentration of 20 μg/ml inhibited the inoculum containing 10^3 organisms.

Bactericidal action of acid extract. Varying numbers of *S. aureus* in Difco nutrient broth were incubated at 37°C for 24 hours with acid extract (Lot 4) at 500 μg/ml concentration. Bacterial growth was inhibited. At this time 0.5 ml amounts of each of the bacterial cultures were transferred and diluted 200-fold in fresh medium and incubated again for 7 days. At this dilution the concentration of thymus extract was below that required for inhibition. Transfers from tubes originally inoculated with 10^2 organisms or less yielded no growth, whereas transfers from tubes inoculated with the more concentrated bacterial suspension yielded organisms (Table IV). It thus seems that under these conditions the acid extract had an appreciable bactericidal effect.

TABLE II. Antibacterial Spectrum of Acid Extract of Thymus.

Organism	Inhibiting dose (μg/ml of broth)
S. aureus, FDA-209 pen sensitive	4
S. aureus, pen resistant	20
S. pyogenes, beta-hemolytic type 12	20
B. subtilis	100
S. paratyphi A	100
S. paratyphi B	100
S. typhosa	500
Shig. dysenteria	20
Pseudomonas group, 5 strains	20-100
Proteus group, 3 strains	100-500
Proteus group, 2 strains	>1000

Pen = penicillin.

TABLE III. Inhibition of *Pseudomonas* sp., NIH No. 5 by Acid Extract of Thymus.

Acid extract conc (μg/ml)	No. of organisms in inoculum					
	10^5	10^4	10^3	10^2	10^1	$<10^1$
500	—	—	—	—	—	—
100	—	—	—	—	—	—
20	+	+	—	—	—	—
0*	+	+	+	+	+	+

+ = growth; — = no growth. * Control.

Antiviral activity in tissue culture. The water extract of thymus did not inactivate Coxsackie A9 virus *in vitro* in the absence of living cells but it interfered with multiplication of Coxsackie A9 virus in monkey kidney monolayer cultures. In 3 typical experiments illustrated in Table V the presence of 4-100 μg/ml of the water extract reduced the yield of Coxsackie A9 virus by 2 to 4 logarithmic units. In a similar type of test, type 1 poliovirus, LSa strain, was also inhibited but to a lesser degree, since the yield of virus was reduced by approximately one logarithmic unit when concentration of the water extract was 100 μg/ml.

Antiviral activity against poliovirus in mice. Although the water extract of the calf thymus showed only slight inhibition of type 1 poliovirus in tissue culture, it exhibited a considerable protective effect in mice. Four assay experiments were carried out in mice at 4 different dates with the same lot of thymus extract (Lot 1). Since the results were com-

TABLE IV. Bactericidal Action of Acid Extract of Calf Thymus on *Staphylococcus aureus*.

	Conc (μg/ml)	Culture	No. of organisms in inoculum 10^5	10^4	10^3	10^2	10^1	$<10^1$
A	0*	Original	+	+	+	+	+	+
B	500	"	—	—	—	—	—	—
C	0	Subculture from B after 24 hr	+	+	+	—	—	—

+ = growth; — = no growth. * Control.

Note. Original Difco nutrient broth tubes were inoculated with indicated numbers of *Staph. aureus* and incubated for 24 hr. Transfers of 0.5 ml from each of these tubes were then made into 100 ml of fresh medium in flasks which were further incubated for a week.

parable they are summarized together in Table VI. In one assay the treatment was given 5 hours after infection, while in the other 3 experiments it was given 5 hours before challenge; no difference in the results was noted. The paralysis rate (all paralyzed mice died) of the 4 control groups was 68, 63, 65 and 62% respectively. The average paralysis rate in the controls was 63.8% while that in the groups treated with 2.5 mg/kg was 40.8% (Table VI). When the dose was gradually decreased, the effect became less and less evident. The results are statistically significant, P value being less than 0.01 at the 2.5 mg/kg dose level.

Similar experiments with poliovirus infection in mice were then carried out with a freshly prepared lot of water extract of thymus (Lot 2) and the data from one of them are presented in Fig. 1. In this assay, the paralysis rates were reduced from 60% in the control mice to 26% in the mice treated with 50 μg/mouse (2.5 mg/kg) of the extract. In this instance the 10 μg dose had little effect.

TABLE V. Inhibition of Coxsackie A9 Virus by Water Extract of Calf Thymus in Monkey Kidney Tissue Culture.

Trial	Conc of water extract (μg/ml)	Virus produced ($\log_{10}$ $TCID_{50}$)
1	100	2.1
	20	4.1
	0	6.1
2	100	3.3
	20	4.1
	0	6.1
3	20	1.9
	4	2.3
	0	4.3

Tissue culture tubes were inoculated with about 2 $TCID_{50}$ of virus and harvested for titration on third day.

Antiviral activity against Coxsackie A9 virus in suckling mice. Two replicate assay experiments were carried out in newborn mice at different dates with the water extract of thymus (Lot 1). A total of 28 litters or 204 mice were used. The treatment was given 5 hours before virus infection. The average death rate was reduced from 91% in the controls to 60% in those treated with 3.7-5.0

TABLE VI. Effect of Water Extract of Calf Thymus on Type I Poliovirus Infection in Mice (Summary of 4 Exp).

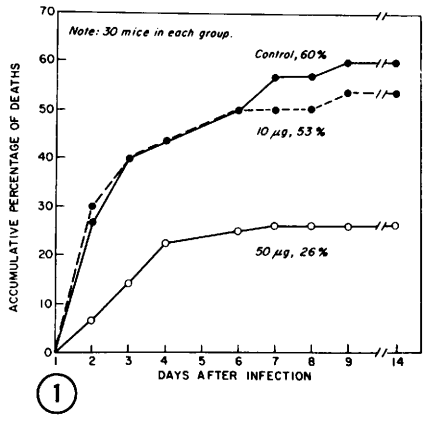
Mice	Treated, dose: mg/kg 2.5	1.0	.5	.25	Control 0
No. inoculated	115	90	155	65	155
No. paralyzed	47	41	79	34	99
% paralyzed	40.8	45.5	50.9	53.8	63.8

TABLE VII. Effect of Water Extract of Calf Thymus on Coxsackie A9 Virus Infection in Mice (Summary of 2 Exp).

Mice	Treated, dose: mg/kg 3.7-5.0	1.2-2.5	.2-.5	Control 0
No. inoculated	53	48	45	58
No. died	32	33	38	53
% death	60	68	85	91

mg/kg of the extract (Table VII). A third experiment was performed in a similar way except that the treatment consisting of a single dose of 2.5 μg/per mouse or 1.2 mg/kg was given 5 hours after infection. The results of the last experiment are illustrated in Fig. 2, showing that the death rate in control mice was 100% on the 10th day after infection, while that in treated ones was 60% on the 12th day. No deaths occurred thereafter. In the 3 experiments the dried extract was dissolved in saline and injected into mice within an hour or so.

Discussion. In searching for antiviral agents other than antimetabolites, mold products or synthetic compounds, we have studied extracts from certain shellfish(1) and from calf thymus. There are certain similarities as well as differences between these two groups of materials. The shellfish and thymus materials contained both antibacterial and antiviral activities which could be separated. In the shellfish material, these 2 antimicrobial substances were both present in the acid extract. In the thymus tissue the acid extract was mainly antibacterial while the water soluble one was chiefly antiviral. Generally speaking, the shellfish materials are more similar to each other than they are to the thymus substances. The extent of the differences among these substances may be comparable to that among different vitamins. This whole group of antimicrobial agents which are probably stored or manufactured by certain types of cells in higher organisms may be concerned with the defensive mechanism of the host, although their existence so far has been only detected by antimicrobial assay *in vitro* or in other living organisms.



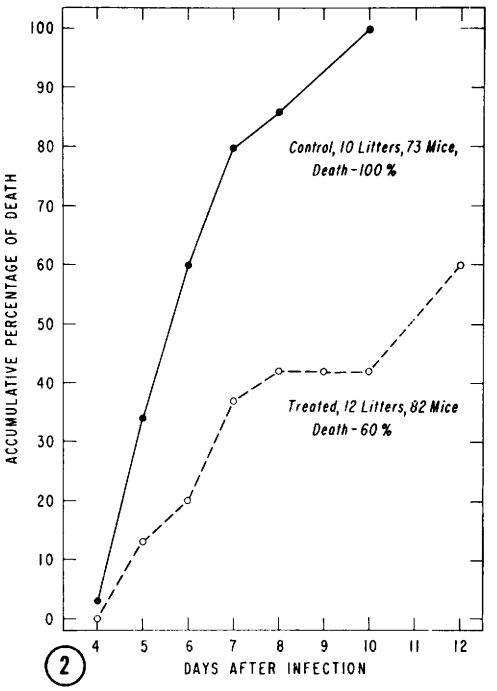


FIG. 1. Effect of water extract of calf thymus on type I poliovirus infection in mice. Prophylactic treatment consisted of a single interperitoneal injection of indicated doses (μg/mouse) of thymus extract given 5 hr before intracerebral infection.

FIG. 2. Effect of water extract of calf thymus on Coxsackie A9 infection in newborn mice. Treatment consisted of a single intraperitoneal injection of 2.5 μg/mouse of thymus extract given 5 hr after intramuscular infection.

Szent-Györgyi and his associates(8,9) reported the isolation of 3 substances from calf thymus, one a growth-promoting factor capable of accelerating neoplasia; the second, a growth inhibitor which greatly retards experimental tumors, and the third, a substance which interferes with fertility; they are not proteins and have low molecular weight. Our extracts which behave like protein and have been thoroughly dialyzed against water are most probably free from such substances. Since our extracts from calf thymus are not toxic and are active in small dosage, further work is in progress to see whether they have any practical value.

Summary. Two kinds of extracts were made from calf thymus gland: (a) in water, and (b) in dilute acetic acid. The water extract inhibited Coxsackie A9 virus and type I poliovirus in tissue culture and in mice. The acid extract showed *in vitro* antibacterial activity against *S. aureus, S. pyogenes,* organisms of the *Pseudomonas* group, and certain other bacteria.

The authors are indebted to Dr. C. J. Maloney for his advice in statistics. Thanks are also due to Mr. G. Caldes, for his technical assistance.

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Received September 3, 1963. P.S.E.B.M., 1963, v114.

Comparison of Acute Toxicity of Anticholinesterase Insecticides to Weanling and Adult Male Rats.* (28716)

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The acute toxicity of insecticides is usually studied in adult animals. Little information is therefore available on the susceptibility of young animals to these compounds. Previous studies(1,2) have demonstrated that young animals are much more susceptible than adults to some organophosphates. Thus the acute toxicity of O-ethyl O-(4-nitrophenyl) benzene thionophosphonate (EPN)(1) and O,O-diethyl O-(4-methylthio-m-tolyl) phosphorothioate (DMP, Bayer 29492)(2) was found to be much higher in 23-day-old male rats than in the adult rats of the same sex. The acute toxicity of the various insecticides now approved for use on food crops has not been studied systematically in the young rat. The present study was undertaken to obtain a comparison of the acute toxicity of several commonly used insecticides of the anticholinesterase series to weanling and adult male rats under similar experimental conditions.

Materials and methods. Weanling (23-day-old, 50 to 60 g) and adult (200 to 300 g) male Holtzman rats were used for these experiments. The toxicity of the following insecticides was measured: O,O-diethyl O-(4-nitrophenyl) phosphorothioate (parathion), O,O-dimethyl O-(4-nitrophenyl) phosphorothioate (methyl parathion), O-ethyl O-(4-nitrophenyl) benzene thionophosphonate (EPN), mixture of O,O-diethyl O-(2-ethylthio)ethyl phosphorothioate and O,O-diethyl S-2-(ethylthio)ethyl phosphorothioate (Systox), O,O-diethyl O-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl phosphorothioate (Co-Ral), O,O-diethyl S-2-(ethylthio)ethyl phosphorodithioate (Di-Syston), O,O-diethyl S-(p-chlorophenylthio)-methyl phosphorodithioate (Trithion), O,O,O,O-tetraethyl S,S′-methylene *bis*-phosphorodithioate (Ethion), O,O-dimethyl S-4-oxo-1,2,3-benzotriazin-3(4H)-ylmethyl phosphorodithioate (Guthion), O,O-dimethyl S-[1,2-*bis*-(ethoxycarbonyl)ethyl] phosphorodithioate (Malathion), 2,3-p-dioxanedithiol-S,S-*bis*-(O,O-diethyl phosphorodithioate) (Delnav), O,O-dimethyl 1-methyl-2-carbomethoxyvinyl phosphate (Phosdrin), tributyl phosphorotrithioite (Folex), dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate (Dipterex), octamethyl pyrophosphoramide (OMPA), and 1-naphthyl-N-methylcarbamate (Sevin). With the exceptions indicated below, the compounds were dissolved in ethanol (20%) and propylene glycol (80%) and the amount of toxicant in solution was adjusted so that an amount equivalent to 0.2% of the body weight was given intraperitoneally to weanlings and an amount equivalent to 0.1% was administered by the same route to the adults. Undiluted Malathion was given to adults. Dipterex was dissolved in distilled water. Co-Ral was used

* This work was supported by a grant from U.S.P.H.S.

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