the experimental disease. Although we were able to induce nephritis in experimental Groups C and E with a total dose of BSA half that previously used by other workers(4,5,7) it is necessary to emphasize that following the administration of unmodified BSA on day 7, the amount of BSA present in the circulation is either the same or more than that present at this time in the experiments of the other investigators. Hence, the amount of soluble antigen-antibody complexes formed in previous experiments may well be the same as those involved in this study. The total dose of 125 mg BSA/kg was arbitrarily chosen and conceivably much smaller doses of aggregated and unmodified BSA, given at the appropriate times would produce the same incidence of nephritis. It would then be possible to lower the dose of antigen to a level more comparable to the situation in poststreptococcal glomerulonephritis in man.

Summary. 1. A working hypothesis concerning the nature of serum sickness involving a dissection of the antigen into "antigenic" component and "combining" component of the antigen is presented. 2. Evidence is presented that injection of aggregated BSA in amounts capable of inducing an immune response does not lead to the renal lesions of serum sickness in the rabbit. If, however, this stimulus was combined with subsequent administration of soluble BSA serum sickness nephritis occurred. 3. BSA centrifuged for 5 hours at 105,000 $\times$ *g* had lesser capacity to induce serum sickness than did an unmodified BSA. 4. These observations are consistent with the view that the production of serum sickness nephritis can be dissected into two separate components which can be separately manipulated.

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Antiviral Activity of Higher Plants and Propionin on Lymphocytic Choriomeningitis Infection. (32057)

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In a previous paper(1) it was shown that two higher plants emerged as antiviral drugs from a screening program of Chinese medicinal agents. They were active at the animal level against lymphocytic choriomeningitis (LCM) infection. In this paper, we report further studies of the two plants, and of propionin, an antibiotic, which has newly been found active against LCM virus in mice. These natural products have also been compared with methotrexate which was reported to have antiviral action by Hass and Stewart (2) and Sidwell *et al*(3).

Materials and methods. 1. *Higher plants.*

The bulbs of *Narcissus tazetta* L. (H6727) and the buds of *Magnolia kobus* DC (H6512) were extracted by boiling in water for 30 minutes. The supernatants after centrifugation were concentrated at 60°C to represent a 40% solution of original weight of the plants, and then kept at 4°C as crude materials until use. It was found that *Narcissus tazetta* bulbs imported from Japan and San Francisco were more active than those from Hong Kong which had been used in previous experiments (1). Therefore, we used bulbs from San Francisco in this study.

2. *Propionin.* An unidentified substance in the cellular extracts of *Propionibacterium freudenreichii,* which was established as having antiviral activity against Columbia SK infection in mice(4,5,6), has been partially purified chromatographically in this laboratory(7,8). In this study, we used one of the fractions (propionin B1) which had been ascertained to have antiviral activity against Columbia SK virus in mice. Each 0.1 ml of water solution contained 2 mg of solid, which was the maximum non-toxic dose (MNTD), in mice.

3. *Methotrexate (Amethopterin).* A dose of 1 μg in 0.1 ml of water was the MNTD in mice by our schedule of treatment. The 2 μg dose showed definite toxicity, with 5 out of 10 uninfected control mice dying within 5 days (LD_{50}). The 5 μg dose showed LD_{100} toxicity.

4. In vivo *test.* Drugs were injected subcutaneously to 9 to 12 g mice in a dose of one MNTD or less 2 hours after intracerebral inoculation of $LD_{80\text{-}90}$ of LCM virus (strain NY621); injections were continued twice daily for 5 days. Observation was continued for an additional 9 days. At MNTD, mice showed no toxic signs except some slowing of growth (body weight) in comparison with control mice. The CNS symptoms (spastic paralysis) of the disease appeared during the second week after infection, never during in the first week. We considered that death of treated mice occurring within 6 days was due to drug-toxicity.

5. In vitro *test.* A strain of LCM virus adapted from original NY621 strain to KB cells in stationary culture maintained in Medium 199 with 2% calf serum was used as described in detail previously(1).

Results. 1. *Fractionation of H6727 with organic solvents.* The sticky, light brown, supernatant of the hot water extract of H6727, after centrifugation at 6000 rpm for 30 minutes, contained 5 mg solid (H6727-sup) per 0.1 ml, the MNTD against mice. The sticky substance (mostly starch) was precipitated and removed from the supernatant by adding 9 volumes of 95% ethanol. The alcohol-soluble fraction was then dried in a flash evaporator. The dried fraction was washed with 5 volumes of petroleum-ether (benzin), washed with ether, and then dissolved in the original volume of water (H6727-H_2O). Each 0.1 ml of H6727-H_2O contained 2 mg solid and showed one MNTD. The solid substance is a yellow, amorphus, deliquescent, powder which partly crystallized when it was kept in a desicator. Antiviral activity both *in vivo* and *in vitro* was demonstrated in H6727-H_2O without any quantitative loss of activity from the original material (H6727-sup).

2. *Fractionation of H6512 with organic solvents.* Preliminary experiments showed that the active principle was not soluble in petroleum-ether, ether, methanol or ethanol, but was soluble in water. The water fraction (H6512-H_2O) after washing with petroleum-ether, ether, and ethanol, contained 3 mg of a dark brown amorphous substance per 0.1 ml of the original volume; this constituted one MNTD against mice.

3. *Antiviral activity of H6727-H_2O, H6512-H_2O and propionin B1 on LCM infection in mice with comparison of amethopterin.* Table I showed the activities of these drugs. It was found that propionin showed definite activity, and that the partially purified samples of the two herbs possessed the same degrees of activity as the original crude extracts reported in the previous paper(1). Methotrexate showed severe toxicity as previously reported (2,3), and dosage was therefore limited to 1 μg per injection (twice a day for 5 days), as the MNTD. As shown in Table 1, methotrexate showed slight reduction of mortality of LCM infection. At the 2 μg dose, out of 20 mice used, 10 mice died within 5 days due

to drug-toxicity, and 5 mice died with paralysis between 8 to 14 days after inoculation of virus. At the 5 μg dose, all mice (20 out of 20) died within 5 days (LD_{100} of drug-toxicity). Thus amethopterin showed definite activity only at the toxic level and slight activity at MNTD, while 3 natural products demonstrated definite activity below the toxic level.

4. *Dose response and therapeutic activity of the drugs.* The upper part of Table II shows a clear dose response of the drugs on anti-LCM activity. H6727 and propionin were effective with as little as one-fourth MNTD

TABLE I. Antiviral Activity of *Narcissus tazetta* L (H6727), *Magnolia kobus* DC (H6512), Propionin and Methotrexate on LCM Infection in Mice.

Drug	Dose solid	Treated, died/total	Control, died/total	Treated/Control (%)
H6727-H_2O	2 mg*	12/40	38/40	32
H6512-H_2O	3 " *	16/40	38/40	42
Propionin-B1	2 " *	15/69	90/106	26
Methotrexate	2 μg	5/10†	18/20	56
"	1 " *	28/40	36/40	78
"	.5 "	17/20	19/20	90

* MNTD, maximum non-toxic dose.
† Of 20 mice used, 10 died within 5 days due to drug-toxicity. The denominator (10) is the number of mice that escaped lethal toxicity; the numerator is mice dead of infection.

TABLE II. Dose Response and Therapeutic Activity of H6727, H6512 and Propionin.

	No. of injections	Dose solid	Treated, died/total	Control, died/total	Treated/Control (%)
H6727-sup*		4 mg	11/40	38/40	29
		2	19/40	36/40	53
		1	14/20	19/20	74
		.5	9/10	10/10	90
H6512-H_2O*		3 mg	8/20	18/20	44
		1.5	12/20	18/20	67
		.8	17/20	18/20	94
Propionin-B1*		2 mg	4/20	17/20	24
		1	8/20	20/20	40
		.5	12/20	20/20	60
		.3	9/10	9/10	100
H6727-H_2O		1 mg (½ MNTD)			
2 hr later*	9		5/10	10/10	50
2 days " †	6		6/10	10/10	60
3 " " †	6		6/10	10/10	60
4 " " ‡	8		10/10	10/10	100
H6512-H_2O		3 mg (1 MNTD)			
2 hr later*	9		7/20	19/20	37
2 days " †	6		7/20	19/20	37
8 " " †	6		10/20	19/20	53
4 " " ‡	8		10/20	19/20	95
Propionin-B1		2 mg (1 MNTD)			
2 hr later*	9		3/10	9/10	33
2 days " †	6		3/10	9/10	33
3 " " †	6		5/10	9/10	56
4 " " ‡	8		10/10	9/10	100

* Drug administration was started 2 hr after infection, continued twice daily for 5 days (total 9 injections).
† Started 2 or 3 days after infection, continued twice daily for 3 days (total 6 injections).
‡ Started 4 days after infection, continued twice daily for 4 days (total 8 injections).

TABLE III. Effect of Drugs on Mortality of Paralyzed Mice.

	Mortality, died/paralyzed	Recovery,* survived/ paralyzed (%)
No drug	260/269 (96.7%)	3.3
H6727-sup and -H_2O	150/192 (78.1%)	21.9
H5512-H_2O	40/52 (76.9%)	23.1
Propionin-B1	60/74 (81.1%)	18.9

* Percentage of survivors of the paralyzed mice which had been given the drugs during the latent period of LCM disease.

(1 mg and 0.5 mg), and H6512 with one-half MNTD (1.5 mg). The lower part of Table II also shows therapeutic activity of the drugs, *i.e.*, they were effective when administration was started as late as 3 days post-inoculation and continued for at least 3 days (total 6 injections). Activity was not demonstrated even by 8 injections (4 days treatment) when administration was delayed for 4 days from infection.

5. *Effect of the drugs on mortality of paralyzed mice.* The NY621 strain of LCM virus caused almost uniform fatal paralysis in mice when given by intracerebral inoculation, with only about 3% of the paralyzed mice recovering and surviving. As shown in Table III, it was found that the percentage of survivors in the paralyzed mice was increased to about 20% in the treated groups which had been given the drugs during the incubation period. That is, the mortality of paralyzed mice in the treated group was lower than that of the control.

6. *Effect of H6727 on a single cycle of virus growth.* By using a tissue culture system (KB cells and the adapted strain of LCM virus), it was found that the antiviral activity of H6727 was strong enough to suppress the virus reproduction in a single cycle growth experiment that needed high multiplicity of infection. On the contrary, the activity of H6512 in tissue culture was weak and could be shown only when 10 TCID of virus was inoculated to KB cells. Also, propionin and methotrexate were found to be not active in the tissue culture system. Therefore, the single cycle experiment for analyzing the action mechanism was available only for H6727. As reported previously(1), preliminary experiments showed that the action of H6727 was probably at the intracellular level because it did not inactivate the virus by direct contact for 2 hours at 37°C, or overnight at 4°C. A dose of 0.1 ml of undiluted virus (10^7 $TCID_{100}$) was inoculated onto KB cell monolayers (10^5 cells in 1 ml of medium), one hour later the cells were washed 5 times with Hanks solution, then 25 μg of H6727-H_2O (one MNTD for KB cells) contained in 1 ml of medium was added immediately (1 hour), and 2,3 and 4 hours post infection and incubated for 20 hours. Infectivity of virus in the cells (cell-associated virus) and the cytopathic effect (CPE) at various incubation times were recorded. Table 4 shows the results. Administration at the time of appearance of progeny virus (4 hours) was still effective. However, although drug administration up to 3 hours after inoculation showed definite inhibitory effects on CPE, no inhibition was demonstrated after 4 hours. From these results, it appeared that the drug continued to inhibit virus reproduction after it failed to suppress CPE.

7. *Reversible inhibition of H6727 on reproduction of virus.* The suppressing mechanism of H6727 seems to be reversible. During the course of the single cycle growth experiment mentioned above, the processes of virus reproduction and CPE were found to be quickly (with 2 hours lag period) returned to a normal rate when the drug was removed from the culture medium by simple washing at various incubation periods. Table V shows the results.

Discussion. In spite of the weak activity of H6512, and the lack of activity of propionin, against an adapted strain of LCM virus in KB cells, each showed definite activity against the original strain of LCM virus in mice. The activity of the drugs in mice may be due to suppression of a specific lethal immune response; this response produces lymphocytic infiltration in the meninges and chorioid plexus with convulsions and death, similar to the effects of total body x-irradiation, cortisone or neonatal thymectomy(9). Or, the activity of the drugs may be related to the tolerance-inducing mechanism (dif-

TABLE IV. Effect of H6727 on a Single Cycle of Virus Growth.

Time H6727 added (hr post infection)	Incubation time after infection									
	1	2	3	4	5	6	7	9	13	20 hr
					Infectivity (TCID)					
1		3	3				3		3	3*
2			3				4		4	5
3							4		4	5
4									5	5
Control	3	3	3	5		6	7		7	7
					CPE†					
1		0	0	0	0	0	0	0	0	
2			0	0	0	0	1+	1+	1+	
3				0	0	1+	2+	2+	2+	
4					0	1+	2+	3+	4+	
Control	0	0	0	0	0	1+	2+	3+	4+	

* Indicates 10^3 $TCID_{100}$ of infectious virus associated to the cells.
† Drug was designated as effective when the viral cytopathic effect (CPE) showed only 0, 1+ or 2+ damage, in comparison with 4+ (maximal) in the control.

TABLE V. Reversible Inhibition of Virus Reproduction by H6727.

Period H6727 present in culture medium (hr post infection)	Incubation time after infection									
	1	2	3	4	5	6	7	9	12	20 hr
2 to 3*			3		3		5	7	7†	
2 to 4				3		3		5	7	7
2 to 5					3		4	5	6	7
2 to 7							4	4	6	7
2 to 12								4	4	7
2 to 20								4	4	5
Control (no drug)	3	3	3		5		7	7	7	7

* H6727 was added 2 hr post infection and removed 3 hr post infection by simple washing; the cells were incubated until 20 hr post infection in fresh medium.
† Indicates 10^7 $TCID_{100}$ of infectious virus (cell-associated) 12 hr post infection.

ferent from the antihypersensitivity reaction) of methotrexate, or of a derivative of nitrosourea, which was reported by Sidwell *et al*(3). Unexpectedly, methotrexate was found to be inactive on LCM virus in KB cells. Even at the concentration of 50 μg in 1 ml of medium which completely inhibited vaccinia (IHD strain) propagation without apparent toxicity when incubated for 5 days, methotrexate did not suppress the multiplication of LCM virus in the cells. Therefore, H6512, propionin and methotrexate are in a category of antiviral disease agents which show activity only *in vivo* and not *in vitro* and may act by other mechanisms than direct inhibition of virus multiplication. It should be noticed that such drugs would not have emerged from a regular screening test in which the first selection is done by using a tissue culture system, with only the active compounds carried on to a second selection at animal level.

Because the suppression of intracellular virus multiplication in KB cells by H6727 is reversible, effective treatment requires that administration in animals be continued until such time as other defenses are present, such as interferon or antibody. Further, more than one or two injections of the drug are required to reduce the mortality of LCM infection. Concerning the chemistry of H6727, 3 different alkaloids have previously been isolated from *Narcissus tazetta* L(10). The names are lycorine ($C_{16}H_{17}O_4N$), tazettine $C_{18}H_{21}O_5N$) and Suisenine ($C_{17}H_{19}O_5N$). We will attempt to determine whether the active principle is one of these alkaloids.

Summary. Further studies of 2 higher plants (*Narcissus tazetta* L and *Magnolia kobus* DC) with antiviral action against

lymphocytic choriomeningitis infection in mice have been reported, and the activity of a third substance, propionin, added. The anti-LCM activities of the 3 natural products have been compared with methotrexate.

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2-Amino-1,4-Naphthoquinone, an Oxidation Product of 2-Amino-1-Naphthol.* (32058)

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The carcinogenicity of aromatic amines is believed to result from their hydroxylated metabolites(1), which are unstable compounds that are easily oxidized. 2-amino-1-naphthol, a metabolite of the bladder carcinogen 2-naphthylamine, produces bladder tumors in about one year when implanted as a wax pellet in bladders of mice(2). Bryan *et al*(3) have shown that within a few hours this compound is largely oxidized in the bladder.

We are studying the interaction of the hydroxylated metabolites with DNA in order to determine their mode of action. The alteration of the melting profile(4) and the ability to prime for RNA synthesis(5) were the induced DNA changes produced by these metabolities. The DNA modifications were obtained only under conditions which allowed oxidation of the metabolites(4); in addition the oxidation products of 2-amino-1-naphthol also altered the melting profile of DNA.

These oxidation products are being examined by us because of the possibility that they are involved in the carcinogenicity of aromatic amines.

Results. The oxidation of 2-amino-1-naphthol (prepared as described in(6) was accomplished by stirring 4 g of the hydrochloride in 2 liters 0.1 M phosphate pH 7.2 for 24 hours at room temperature. The purple precipitate was removed and the yellow-brown supernatant, which exhibited blue-green fluorescence, was extracted several times with ether to give a deep orange extract. The residue obtained after evaporation of the ether layer to dryness was dissolved in methanol and chromatographed on a silica gel thin layer plate in ether/heptane = 2/1 to give 7 bands of various colors. The major orange band was eluted with methanol and evaporated to orange crystals of M.P. = 190-200°. The crystals were extracted with hot benzene

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