

## CHEMOTHERAPEUTIC STUDIES ON MOUSE HEPATITIS VIRUS (2)

Effect of Methanol on Mouse Hepatitis Virus *in vitro*

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## Introduction

Purification of mouse hepatitis virus (MHV) has not been succeeded until recently. It has been stated that mice infected with MHV produced the interferon-like substances within the host cells, and it produced remarkable disturbance of typical infectious cycles and symptoms (ALTUCCI 1959). FANTONI (1964) reported that he could be able to purify the MHV partially by the use of fluorocarbene to purify the MHV about one log of infectious titer.

It has been well known that some organic solvents could be able to purify some virus from the virus infected organ, as they precipitate the proteins. Methanol has been used for this purpose; COX (1947) has first reported the purification of influenza virus from infected colloid-allantoic fluid by 13% of methanol, and POLLARD (1949) has described on the purification of EEE virus, and MEF virus from the emulsion of mouse brain or spinal cord; later SCHWERD & SCHAFFER (1956) have reported on the purification of polio virus by 15% of methanol.

Present report describes on the effect of methanol on MHV and some trials of purification of MHV.

## Materials and Methods

## 1) Animals

Swiss albino mice weighing 9~10 g were used in the experiments. The mice were housed in air conditioned room at 21°C in 50~60% of humidity.

## 2) Virus

Mouse hepatitis virus (MHV) EHF 120 strain was used in all experiments. This strain has been isolated by BUECHER (1952) in Japan. Every 10 folds dilutions were inoculated to 6 or 7 mice 0.2 ml/mouse intraperitoneally. After 7 days observation, dead mice were confirmed by autopsy to be the infection of MHV. LD<sub>50</sub> was calculated by LEED & MUENCH (1938).

## 3) Purification by methanol

Purification of virus by methanol precipitation

was carried out by the method of POLLARD (1949). Ten percent of infected liver homogenate of mice was incubated at 0°C in ice box and the chilled methanol is slowly added into the homogenate. The mixture is kept in the ice box for about 3 hours, then the precipitate is centrifuged at 4,000 rpm for 30 minutes. The supernatant is tested for viral activity to mice. The supernatant is then resuspended to any volume desired in 0.2 mol (pH 7.2) phosphate buffer, and the virus is permitted to elute at room temperature for 1 hour, the suspension is then recentrifuged at 3,000 rpm for 20 minutes in the cold. The supernatant fluid contains most of virus.

## 4) Estimation of nitrogen

Nitrogen is estimated by the Folim-Chiucalet and egg albumin is used as the standard.

## Results

## 1) Fractionation of cellular components

It is generally confirmed that MHV multiplies in cytoplasm of liver cells, and its mean diameter is about 90 mμ (STARR 1960). On the other hand, the homogenate of MHV does not show the clear cut dose-response curve of mortality. The reason seemed to the homogenate contained many subcellular components. The first trial of our experiment is to determine whether these components influence the mortality at various infective doses or not.

So that these cellular components were divided by centrifugation in isotonic sucrose solution by the method of HOGEBOM (1955). These fractions were divided into 4 parts; P-O (original homogenate), F-1 (without nuclear and cell debris), F-2 (suspension of mitochondria), and F-3 (without nuclear, mitochondria, and microsome). These fractions were estimated for their nitrogen contents and infectivity of mice. The results are shown in Table 1.

As shown in the table, the virus titer did not increase markedly and specific activity per nitrogen content also did not increase. The mortality of

Table 1. Intracellular distribution of MHV in liver cells.

Note	Fractions	Nitrogen (mg/ml)	Volume (ml)	LD <sub>50</sub> (/0.2 ml)	Specific activity (LD <sub>50</sub> /r of N)
F-0	Original homogenate	11.0	15.0	10 <sup>-2.5</sup>	5.5 × 10 <sup>-6.5</sup>
F-1	Supernatant 700 g × 10 min.	8.8	15.0	10 <sup>-2.6</sup>	5.7 × 10 <sup>-6.6</sup>
F-2	Mitochondria 5100 g × 10 min.	2.2	10.0	10 <sup>-2.4</sup>	3.3 × 10 <sup>-5.4</sup>
F-3	Supernatant 25300 g × 10 min.	6.5	15.0	10 <sup>-2.6</sup>	8.0 × 10 <sup>-6.6</sup>

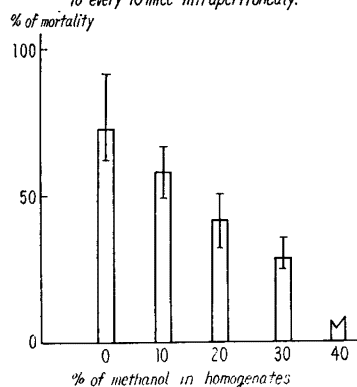
every dilutions did not show the dose response. These results suggested that the cell debris, nuclear, and mitochondria were not the main factors affecting the irregular mortality to every dilutions and it should be needed for the further purification.

## 2) Inactivation of MHV by methanol *in vitro*.

To study the inactivation effect of methanol, concentration of methanol were adjusted to 10%, 20%, 30% and 40% to the original homogenate.

After the addition of methanol, they were kept in the ice box at 0°C for 3 hours and centrifuged 4,500 rpm for 20 minutes. The supernatants were injected to every 10 mice intraperitoneally 0.2 ml per mouse and the mortalities were estimated. The results are shown in Figure 1.

Fig.1 Inactivation of MHV by methanol. After addition of methanol into the homogenates, they were kept in ice box at 0°C for 3 hours and centrifuged 4500rpm for 20 minutes. The supernatants were injected to every 10 mice intraperitoneally.



As shown in the figure inactivation was increased by the concentration of methanol. In 40% of methanol MHV was completely inactivated, but in 10% of methanol and 20% of methanol, they were not completely inactivated.

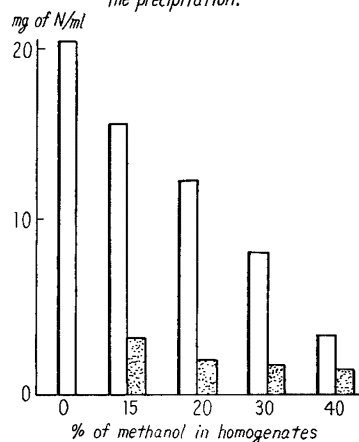
## 3) Nitrogen contents of every homogenates after addition of methanol

Nitrogen contents of supernatants after precipi-

tation of the proteins was estimated by Folin method (FOLIN 1955), and the elutions from the precipitates were also estimated by the same method. The results are shown in Figure 2.

As shown in the figure, nitrogen contents of the supernatants are gradually decreased by the increasing concentration of methanol. In addition, the same results were also obtained in the elutions from the precipitates.

Fig.2 Nitrogen loss by precipitation of homogenates with methanol.  
: Residual nitrogen content in methanol layer after removing the precipitate.  
: Content of nitrogen in the eluate from the precipitation.



## 4) Purification of MHV by methanol precipitation

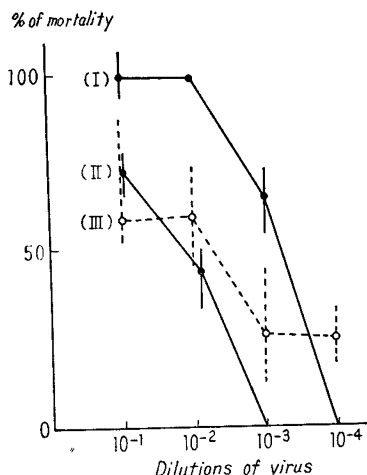
Purification of MHV by methanol as described previously, was carried out to the homogenate (F-0) and the supernatant (F-3), and the methanol concentration was varied from 30% to 40% respectively. These final elutions were estimated the infectivities and nitrogen contents as described previously.

The addition of 30% of methanol to the original homogenate increased the infective titer to the control, and in the other cases the infective titers did not increase and especially in the supernatant fraction (F-3) the infective titer was markedly decreased. But, from the data, specific infective titer per nitrogen was unchanged, in the original homogenate it was rather decreased in the supernatant (F-3). From these experiments purification by methanol seemed to be useful at the concentration of 30%.

## 5) Comparison of mortalities to every dilutions

From the above experiment, every final elutions from the precipitates were inoculated to mice intraperitoneally 0.2 ml per mouse and mortalities were estimated. The results were shown in Figure 3. As shown in the figure, it seemed that purification has been unsuccessful, but variation of

Fig. 3 Comparisons of mortalities to various dilutions. Curve I shows the fraction of 30% methanol, Curve II shows the fraction of 40% methanol and Curve III shows the emulsion of infected mice liver.



mortality to dosis was rather clearly demonstrated. Dose response of mortalities to the dilutions did not clearly paralleled in the original homogenate as shown in Curve 3, but the purified materials both 30% and 40% showed clearly demonstrated the dose response curve as shown in Curve 1 and Curve 2.

The reason has been unknown, but it might be thought that some interfering materials which contaminated in the infected mouse liver was removed by this method.

#### Discussion

It has been known that some viruses produced interferon during the infection into the host. In the case of mouse hepatitis virus, it might be probable that the virus produced interferon, and caused a disturbance of infectious cycles of MHV *in vivo*, so the percent mortalities did not show the dose response curve to the dilutions of virus (ALTUCCI, *et al.* 1959).

The phenomenon made the difficulty of purification of MHV from the infected liver homogenate. On the other hand, tissue culture of MHV *in vitro* has never been successful in spite of many efforts. These reasons made the purification of MHV more difficult than the other viruses.

In our present experiment the direct inactivating effect of methanol on virus was not so strong as shown in Figure 1. The virus titer which was purified from methanol increased only one log of titer but specific activity for nitrogen content did not increase as comparing to the original homogenate.

But, as shown in Figure 3, the mortality to dose response was clearly obtained from the elution of 30% of methanol precipitation. It might be suggested that interference phenomenon might be considered from the two sites of view; one is host cells, and the other one is injected virus itself, which consisted from liver homogenate. Furthermore, the purification of virus which was cultivated in tissue culture method would be able to attribute to these problems.

#### Summary

Effect of methanol on mouse hepatitis virus (MHV EPA 120 isolated in Japan) was studied and following results were obtained;

- 1) At the incubation with 40% of methanol for 3 hours MHV did not completely inactivated.
- 2) By the addition of methanol, liver homogenate gradually precipitated and the residual nitrogen also decreased following the concentrations of methanol and 30% of methanol seemed to be most useful.
- 3) By the fractionation of cellular components virus activity has been remained in the supernatant of cytoplasm without microsomes.
- 4) The eluate from precipitate by methanol virus activity also remained, but the specific activity did not increase.

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