bines with small quantities of S^{35}-6 MP and addition of metals (Table I) enhances the combination. Various metals have been reported to be combined with nucleic acids(5, 6). The interaction of 6-MP with metals in nucleic acid may interfere with cellular metabolism.

Summary. These data indicate that 6-mercaptopurine can combine with ribonucleic acid both *in vitro* and *in vivo*. Binding is enhanced by added metals and the combination is stable to ribonucleic acid isolation procedures.

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Studies on Murine Hepatitis Virus (MHV3) *in vitro*.* (26616)

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The results of several investigations from various approaches(1,2) have focused attention upon murine hepatitis virus and have indicated the possible use of studies of these viruses *in vitro*.

Until recently, knowledge of the murine hepatitis viruses has been based mainly on experiments *in vivo*, since in spite of the observations that these viruses multiply in tissue culture(3-5), no cytopathic effect has been demonstrated. However, Bang(6) has recently shown that MHV2 (Nelson virus) destroys macrophages spreading out from liver explants obtained from a strain of Princeton mice susceptible to hepatitis virus. Parallel observations were made by Bang (7) in macrophage cultures from Princeton mice which were lysed by the virus; macrophages from resistant C3H mice showed no cytopathic effect. This report concerns the behavior of another strain of murine hepatitis (MHV3) in tissue culture, since this strain has been used in experiments designed to show the protective action of antihistamines against liver injury(8,9).

Methods. 1. *Virus.* The MHV3 virus, obtained from Dr. Gledhill, was injected intraperitoneally into adult C57Bl/6 mice, and the infected livers were harvested from moribund mice. The livers were pale and under histological examination appeared to be completely necrotic. For experiments *in vitro* viral pool suspensions were prepared from 10% homogenates of these livers. The homogenate was centrifuged at 1500 rpm for 15 minutes and the supernatant diluted 10-fold with culture medium.

2. *Tissue culture.* Liver explants from 16 to 20-day old fetuses of C57Bl/6 mice were cultivated on reconstituted rat-tail collagen as described by Hillis *et al.*(10). Collagen was prepared and stored according to Ehrmann *et al.*(11). Fetal livers pooled from the same litter were minced with scissors, and 0.5 mm^3 pieces were placed on coverslips on collagen, which were placed in roller tubes. Rotation speed was adjusted to 8 rph. Culture medium consisted of 30% active horse serum, 5% chick embryo extract, and 65% Eagle's basal medium in Earle's balanced salt solution. Tubes were gassed with 5% CO_2 before being stoppered.

In addition, dispersed spleen cell cultures were prepared according to the method of Manaker *et al.*(12).

3. *Passage of virus in tissue culture.* Virus was passed in tissue culture either by using 10-fold diluted infected culture medium or by transferring homogenates from infected explants. This was accomplished by breaking

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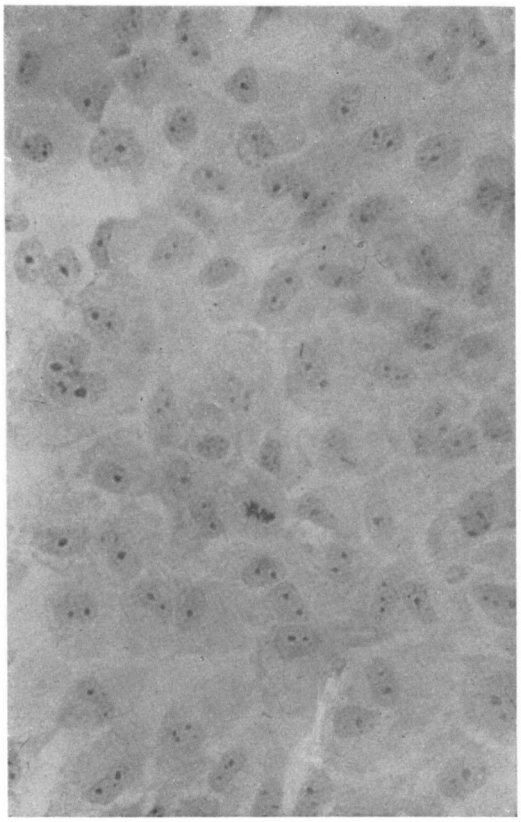

FIG. 1. Epithelial outgrowth from fetal liver explant cultivated on reconstituted collagen. × 250.

the explants, each of which was suspended in 1 ml of saline, in a sonic oscillator. This material was diluted by a factor of 10 and transferred into fresh cultures. Infectious material was carried serially from infected cultures to fresh culture by diluting it directly in culture medium of a new explant.

4. *Inoculation of mice.* Virus from explants was assayed *in vivo* using the infected tissue. Presence of virus in tissue culture passages was determined by inoculation of mice with either media or with washed explants prepared as described earlier. One-tenth ml of the preparation was injected intraperitoneally into adult C57Bl/6 mice.

5. *Preparation of antisera.* Antisera against MHV3 and MHV2 (Nelson virus) were prepared in C57Bl/6 and Princeton (PRI) adult mice by challenging those surviving from the primary infection with an intraperitoneal injection of virus from infected explants. Blood was collected by cardiac puncture one week after the second injection, pooled, and inactivated for one-half hour at 56°C. Serum was separated after overnight refrigeration and was stored at −20°C.

6. *Histological examination.* For histological studies, explants were fixed in Zenker's fluid and stained routinely with hematoxylin and eosin without being removed from the coverslip. This procedure permits detailed study of the outgrowth but not of the explant itself.

Results. Liver explant on collagen. It is known that fetal liver does not survive long when cultivated on a plain glass surface. Collagen enhances outgrowth from liver explants(7,10) and this outgrowth includes either palisades, or sheets of epithelial cells which have the appearance of liver parenchyma (Fig. 1). Growth sequence of cells from the explant is as follows. After one or 2 days in cultivation, fibroblasts start to spread out. Coincidently a number of free cells scatter in the vicinity of the explant, some of which reach and attach themselves to the walls of the culture tube. In subsequent days epithelial cells start to grow out mainly in sheets, sometimes as palisades. Epithelial outgrowth often reaches and passes the edge of the fibroblastic outgrowth. During this time, free cells around the explant increase in number; are phagocytic and motile, and resemble macrophages.

Effect of MHV3 on fetal liver cultures. When a homogenate prepared from an MHV3-infected liver was added to cultures, a cytopathic effect appeared in 3-5 days which was easy to distinguish from the gross appearance of the explant (Fig. 2A & B). Fibroblastic outgrowth appeared to be little affected but epithelial cells tended to form clumps, become detached, and then lysed. Closer examination revealed that most of the epithelial outgrowth was destroyed; the cells showed various stages of pyknosis and in areas of worst damage the liver cells disappeared, leaving the fibroblasts (Fig. 3). When tested with neutral red (1:40,000 dilution) outgrowth from infected cultures picked up the stain far less than that of the control cultures (Fig. 4A & B). As shown

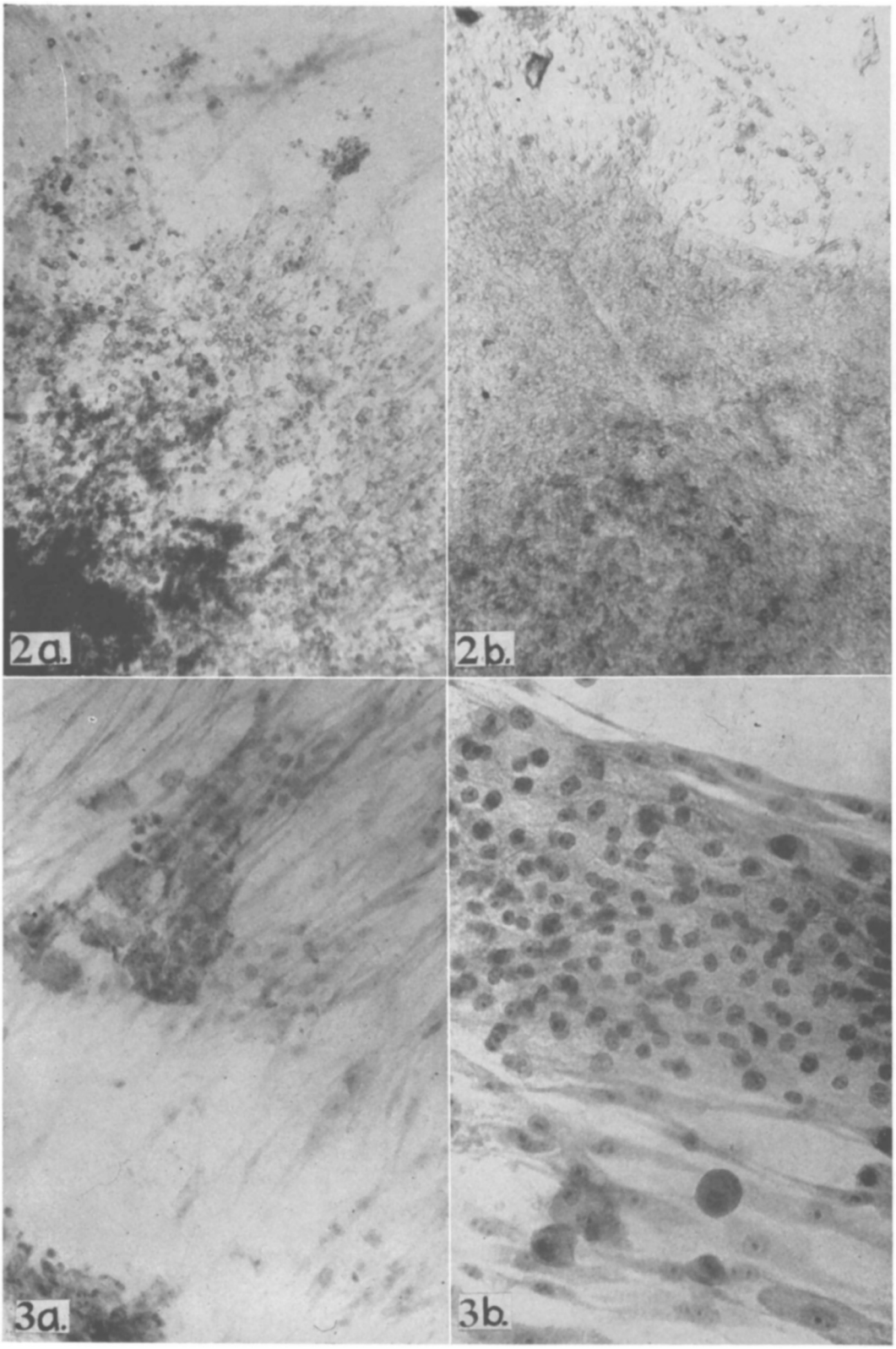

FIG. 2. Gross appearance of liver outgrowth from (a) infected and (b) control cultures. × 35.
FIG. 3. Epithelial outgrowth after 4 days infection with (a) MHV and (b) that of control culture stained with hematoxylin and eosin. × 125.

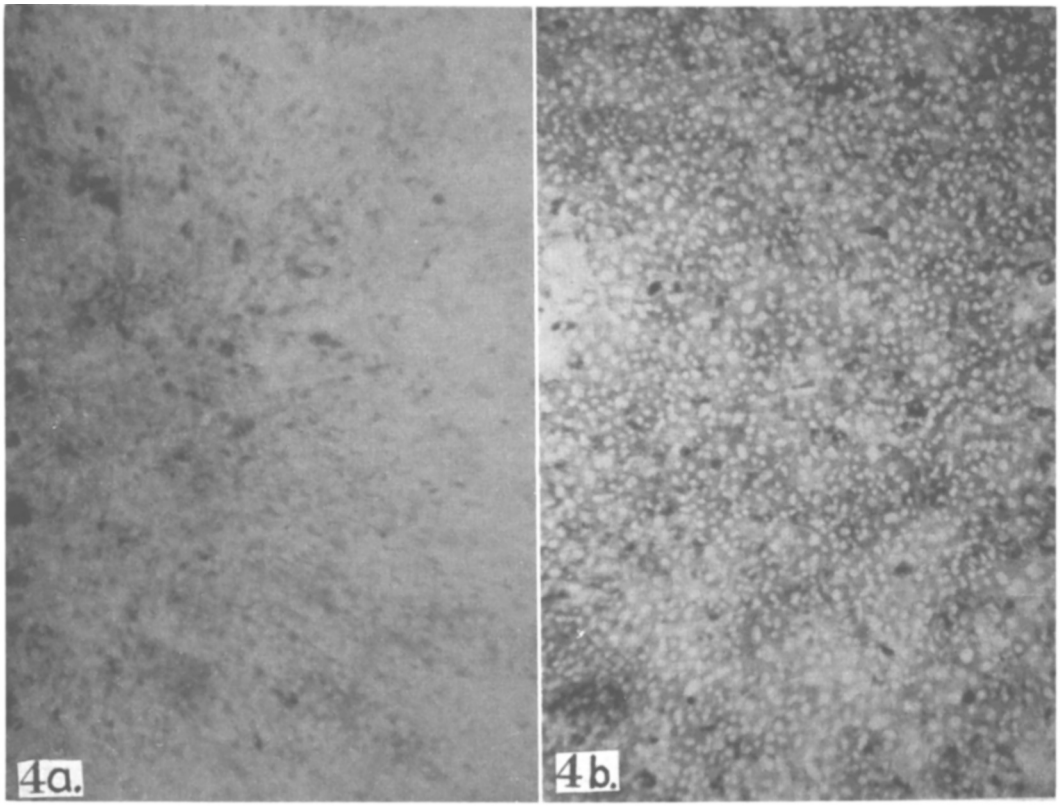

FIG. 4. MHV-infected liver explant culture (5 days from inoculum of virus) (a) stained with neutral red, and (b) control culture. × 125.

by Dick *et al.*(13), pleomorphic basophilic cytoplasmic inclusions were observed by means of the histological technic described (Fig. 5).

As infection advanced in explants, phagocytic cells became affected. To determine whether these cells were attacked directly by virus, macrophage cultures prepared from spleen were exposed to infectious tissue culture fluid from explant cultures. The macrophages were destroyed in 2-3 days.

Transmitting MHV3 virus and assay in vivo. MHV3 virus was carried in fetal liver explant cultures for 22 passages by serial transfer of tissue culture fluids and/or explant homogenate from cultures showing advanced CPE to newly prepared liver explants. Infectious material was usually harvested 3 days after inoculation, but seemed to yield virus at least 5 days thereafter when tested on fresh cultures. From time to time infected culture material was injected intraperitoneally in adult mice and lethal amounts of virus were shown both in the culture medium and in the explant itself (Table I). Some explant cultures were apparently able to support the multiplication of virus, but did not show CPE. It appeared that explants from certain litters were resistant to cytopathic action of the virus. No explanation for this variation is offered because the amount of virus transferred was not measured.

Strain specificity of MHV3. A few strains of mice other than C57Bl/6 were tested and lesions were observed after liver explants from C3H, BRVR, and BSVS fetuses were infected with MHV3. No differences in time of appearance and nature of the lesion were seen as compared with cultures from C57Bl/6 mice. Macrophages derived from spleens of one-month old C3H and C57Bl/6 mice were found to be highly susceptible to MHV3 virus.

TABLE I. Passage of MHV3 in Fetal Liver Explant Cultures and Assay of Infected Culture Material *In Vivo*.

No. passage	Culture material treated with	CPE No. of positive/No. of infected cultures	*In vivo* assay Material	No. mice dead/No. mice inj.
3	None	7/12	Explants	5/5
			Media	9/10
3	"	0/7	Explants	4/5
			Media	5/5
14	"	16/16	Explants	6/10
			Media	9/10
21	Normal mouse serum 1/100	7/7	Explants	3/5
			Media	2/5
21	Mouse anti-MHV3 1/100	0/7	Explants	0/5
			Media	0/5

Inactivation of cytopathic activity of virus. MHV3 virus obtained from liver explants is rapidly inactivated at 37°C. Thus only a few of the tested cultures were destroyed when the infectious material was previously inactivated for 60 minutes at 37°C; and no cytopathic effect was observed after inoculation of infectious material previously maintained at 37°C for 3 hours.

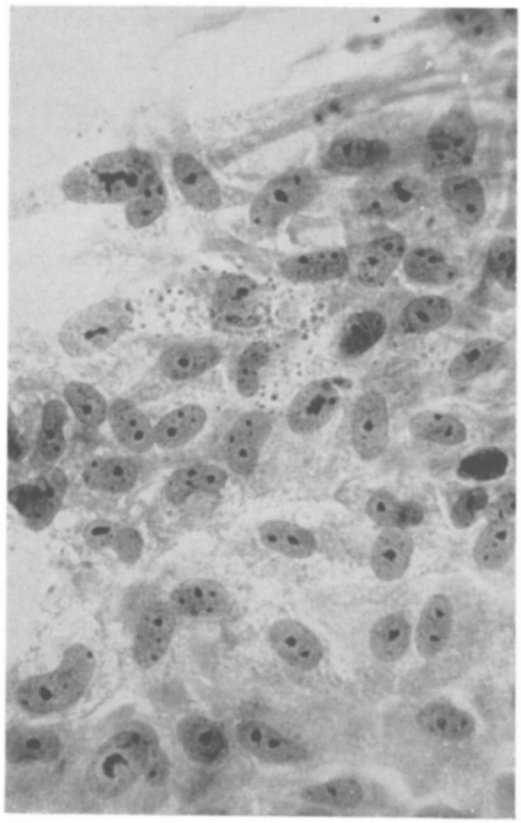

FIG. 5. Basophilic inclusions in epithelial cells after 2 days MHV infection. × 160.

Recovery of virus from infected cultures. Yields of MHV3 virus from infected cultures were low. Only on 2 occasions has a 10^{-2} dilution of a liver explant homogenate given rise to lesions in fresh cultures.

Certain factors did not alter in any way the cell damage caused by MHV3 virus on liver explants. They were: age of fetus from which the explant was prepared; age of the culture itself, if infected before the 14th day of cultivation· use of calf serum instead of horse serum and omission of chick embryo extract from the medium.

Effect of storage on viral activity. Virus in infected explants remained infective after storage at -20°C for at least 2 months. In infected culture medium, viral activity was often lost under these conditions, although occasionally activity was observed after storage of culture medium at –70°C for one month·

Effect of antisera. Contrary to earlier observations(13,14), sera of animals which had been twice injected with infectious material inhibited CPE in high titer. A dilution of 10^{-4} of anti MHV3 mouse serum prevented cytopathic effect of MHV3 virus in liver explants. Control sera from individuals of the same strain occasionally inhibited CPE only when used undiluted. *In vivo* assays of cultures in which viral cytopathogenicity was inhibited (Table I) revealed no virus.

As described earlier(9), pooled serum from mice twice-infected with MHV2 inhibited cytopathic effect caused by MHV3 virus. Under the experimental conditions used, the inhibitory titer of pooled serum from immunized PRI mice was 10^{-2}.

Discussion. Working with Nelson virus (MHV2), Bang *et al.*(6,7) showed that macrophages were destroyed *in vitro*. Although no lesions were found in liver cells, infected cultures produced less acid than the controls. Among other hepatitis viruses, only that of the duck has been shown to damage liver parenchymal cells *in vitro*(15). Human hepatitis virus is unable to affect human fetal liver explant cultivated on reconstituted collagen(10). The cytopathic effect demonstrated in liver parenchymal cells and in the area of epithelial outgrowth of murine fetal liver explant is the only *in vitro* hepatocytocidal effect due to hepatitis viruses in mammals described so far. The only noticeable difference between the technics used by Bang and those used in this work lies in the amount of collagen employed. In the experiment above, the collagenized coverslip instead of collagen slant in roller tube was used. Bang(16) has suggested that the amount of collagen might be of importance in demonstration of the hepatocytocidal effect of hepatitis viruses.

Even though semiquantitative technics were used throughout these experiments, it became evident during the study that MHV3 can multiply in some explant cultures without causing cellular damage. Since this resistance seemed to be a property of the cells from all livers in the same litter it is suggested that liver cell necrosis is indirectly related to viral multiplication, being brought about by the operation of a secondary mechanism which in CPE-resistant livers is not set into motion. It is believed that the mechanism of liver cell necrosis is inhibited by anti-histamine drugs. This possibility is dealt with elsewhere(17).

A low level of activity of antisera against murine hepatitis viruses has been demonstrated so far. The high neutralizing titers demonstrated in the sera of mice immunized against MHV3 were, therefore, unexpected. It remains to be determined whether this high titer of neutralization is due solely to the antibodies or whether some other factors are involved.

Summary. It has been shown that murine hepatitis virus (MHV3) can be propagated serially in liver explant cultures. Cultures were prepared from fetal C57Bl/6 livers and cultivated on rat-tail reconstituted collagen. MHV3 virus caused cytopathic changes in such explants that are mainly in the epithelial outgrowth and macrophages. Virus has been carried for 22 passages and has been recovered by assaying the culture material in mice. Using this system, the sera from mice infected with the virus have been tested and high titers of CPE inhibitory activity were demonstrated.

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