

VIRAL HEPATITIS ASSOCIATED WITH TRANSPLANTABLE MOUSE LEUKEMIA

I. ACUTE HEPATIC MANIFESTATIONS FOLLOWING TREATMENT WITH URETHANE OR METHYLFORMAMIDE*

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PLATES 64 AND 65

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Several workers have observed the incidental occurrence of a viral disease in various strains of transplantable leukemias in mice. Nelson (1-4) has fully described a filterable agent causing acute hepatitis in leukemic mice of the Princeton strain. Although he made repeated efforts to keep the leukemia in passage, it was regularly lost after a few transfers. Marked pathological changes were noted in the livers of some of the mice which died, and a filterable agent capable of inducing similar changes was obtained from the liver. A disease closely related to that described by Nelson had been found by Gledhill and Andrewes (5, 6) in the Webster strain of VS mice, following the injection of liver filtrates from the Parks strain of mice. Later, it was discovered that the full expression of the disease was determined by the presence of *Eperythrozoon coccoides* (7). This finding was subsequently confirmed by Nelson (4), who showed that the virulence of the hepatitis virus in Swiss mice—which have a high natural resistance to infection by the agent,—was greatly enhanced by combined infection with the eperythrozoon. MacDowell *et al.* (8) had also reported the presence of a virus in line I leukemia that caused a mild illness in C58 mice in which the line was carried, but was fatal to Bagg albino mice in which the leukemia did not “take.” Somewhat similar diseases caused by filterable agents were noted by De Bruyn (9) in animals bearing a transplantable lymphoma and by Law and Dunn (10) in a subline of transplantable lymphatic leukemia L 1210 in dba mice. Although these workers (8-10) did not specifically refer to a hepatitis, the signs and symptoms they described are strikingly similar to our findings.

The present report describes the events leading up to the isolation of a virus causing an hepatic disease in mice carrying line I leukemia. The signs during life and the pathological features of the disease which followed upon the administration of urethane or methylformamide and led to isolation of the virus, are here presented as also are certain observations on host susceptibility.

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Materials and Methods

The mice used were F1 hybrids of C58 × Bagg albino crosses (F1); pure Bagg albino; Webster strain Swiss mice; and Princeton Rockefeller Institute mice (PRI). They were from 4 to 6 weeks of age.

The original line I leukemia (11) carried in C58 mice was supplied to the Sloan-Kettering Institute by Dr. E. C. MacDowell in 1950 and since then has been kept in passage by the intraperitoneal injection of ten to twenty million cells from minced spleen of leukemic donors. There was a take of approximately 98 per cent, and the survival time was approximately 5 to 7 days (Fig. 1).

When it was noted that urethane and methylformamide prolonged the survival time of mice with leukemia (12), attempts were made to start additional leukemic sublines which, it was hoped, would eventually become refractory to the treatment. One urethane and one methylformamide treated subline were started from the original line I. F1 mice previously inoculated with leukemia were injected with urethane or methylformamide respectively, in doses sufficient to delay the leukemic process. Urethane was uniformly given in doses of 1000 mg./kg., and methylformamide in doses of 400 mg./kg. In the dosages used, urethane and methylformamide did not cause any pathological changes in the livers of the control animals. "Pretreatment" with urethane was given 120, 72, and 24 hours before inoculation with leukemia. "Treatment" was given at 48 hour intervals beginning 24 hours after inoculation. One animal from each group was sacrificed shortly before its death was expected and a suspension of its spleen was transferred to new animals which were treated in the manner described. Each such passage was designated a transfer generation. No morphological changes from the original line I—an undifferentiated "stem cell" leukemia—were detected in the treated sublines.

After several such transfer generations in treated animals, the survival time of the mice, which had been significantly prolonged in the early transfers, was found to have been shortened as compared with the survival time of the untreated leukemic controls. It was noticed that the treated mice (although dying earlier) had smaller lymph nodes, spleens, and livers than did the untreated controls with the original line I leukemia. Shortly thereafter, the mice in the methylformamide-treated subline started to die 2 to 3 days after inoculation, and presented no macroscopic or microscopic evidence of leukemia at death. All of them exhibited ivory-pale livers thickly covered with hemorrhagic spots. Microscopically, these livers showed extensive areas of necrosis. Since essentially the same picture was found in untreated control mice inoculated with minced splenic or hepatic tissue of the urethane- or methylformamide-treated sublines, a bacterial infection was suspected and numerous cultures of the organs of the diseased mice were taken. These were either negative or inconclusive. The mice were further treated with various antibiotics, all of which proved ineffective. It was at this point that a viral etiology of the disease was considered and filtrates were made of the organs of the sick mice.

The liver and spleen from each animal were ground in approximately 15 ml. of saline. The ground tissue suspension was sedimented at low speed (500 to 1000 R.P.M.) for 5 to 10 minutes. The supernatant fluid was then filtered through Selas 02 or 03 candles. It was found that the hepatic disease could be transmitted in the absence of leukemia to mice inoculated intraperitoneally with 0.1 to 0.5 cc. of the filtrate.

EXPERIMENTAL

Classification of the Disease

The disease has been arbitrarily classified as: (a) *acute* if a high percentage of the F1 hybrid mice died after inoculation with the filtrate; (b) *subacute* if

only a low percentage of the F1 mice died, although a high percentage of the BALB mice died; and (c) *latent* if neither the F1 nor BALB died or became ill.

(a) *The Acute Disease*.—The acute disease appeared in the methylformamide-treated subline after twelve transfer generations, killing the mice within 4 days. (In the original line I this compound prolonged life for 11 to 12 days.) At the same time, all signs of leukemia were lost. The hepatitis, however, could be transferred serially by the injection of either a suspension of minced liver and spleen or a filtrate of these organs and has thus far retained its virulence without further use of the drug. The results of the inoculation of the filtrate into F1, BALB, and the PRI strains of mice are presented in Table I. Prior to death, the mice showed the following symptoms, which are charac-

TABLE I
Host Susceptibility to the Acute Hepatic Disease

Strain	Age	No. inoculated	No. dead	Average survival time
				<i>days</i>
F1	Weanlings*	10	10	3.1
F1	Adults†	12	12	3.6
BALB	Weanlings	10	10	3.4
BALB	Adults	12	12	3.7
PRI	Young adults‡	20	20	3.6

Inoculum: 0.3 cc. intraperitoneally of filtrate of liver and spleen of acutely diseased mice.

* 12 to 15 gm.

† 18 to 25 gm.

‡ 13 to 18 gm.

teristic of the disease: marked weight loss, inactivity, roughing of the fur, tremors, and dark urine. At autopsy, the thymus and lymph nodes were generally reduced in size, but no constant relation was found for the size and weight of the spleen. A few animals showed focal hemorrhages in the lungs. The principal changes were found in the liver, which was pale and varied from light pink to almost white. In addition, it contained numerous hemorrhagic areas. On microscopic examination, an almost complete breakdown of the parenchymal cells was noted. Occasionally, a moderate leukocytic infiltration was present. The large vessels were frequently distended with blood (Figs. 2 and 3). In a subsequent publication a complete pathological study of the organs of affected mice will be presented.

(b) *The Subacute Disease*.—The subacute disease appeared in the urethane-treated subline after eleven transfer generations and in the methylformamide-treated subline after seven transfer generations. The mice died within 6 to 7 days with unusually pale livers. The results of the inoculation of the filtrate

from the livers and spleens of subacutely ill mice of the urethane-treated subline into several strains of mice are shown in Table II. The average survival time was longer than that observed in the acute disease, but the signs of illness and the autopsy findings were identical (Figs. 4 and 5). It can be seen that the various strains tested differ markedly in susceptibility to the agent. The PRI appear to be highly sensitive—a finding also noted by Nelson (2) with his hepatitis virus. In general, infants and weanlings were more susceptible than adults.

All mice which survived showed more or less marked signs of the disease between the 6th and 14th days after inoculation. The most sensitive and

TABLE II
Host Susceptibility to the Subacute Hepatic Disease

Strain	Age	No. inoculated	No. dead	Average survival time
				<i>days</i>
F1	Infants	5	5	5.8
F1	Weanlings*	10	7	6.5
F1	Adults†	60	3	7.2
BALB	Weanlings	10	10	5.2
BALB	Adults	20	14	6.9
Swiss	Infants	8	8	6.8
Swiss	Young adults‡	20	6	5.8
PRI	Young adults	20	20	5.2

Inoculum: 0.3 cc. intraperitoneally of filtrate of liver and spleen from subacutely diseased mice.

* 12 to 15 gm.

† 18 to 25 gm.

‡ 14 to 18 gm.

constant sign was a weight loss of up to 20 per cent. Approximately 3 weeks after inoculation the surviving mice had reached or passed their former weight and were fully recovered. If the animals were sacrificed at the height of the non-lethal disease, focal areas of parenchymal damage were found on microscopic examination in the liver. No pathological changes were found in animals that recovered from the illness and that were subsequently sacrificed.

(c) *The Latent Disease.*—A latent disease was found in the original line I. A filtrate of livers and spleens of these animals was not lethal to infant or adult F1 or BALB mice, nor did these animals show any signs of illness. Evidence, however, is presented below to support the presence of a latent virus.

The Development of Immunity to the Virus

It was of interest to determine whether the mice which had survived the hepatic disease had developed sufficient antibody to resist reinfection with the virus.

(a) *Immunity after Subacute Infection.*—Twenty adult F1 mice which had recovered 22 days after inoculation were challenged by reinoculation of a filtrate from animals with the acute disease. None of these mice died or showed signs of illness, whereas six control non-immune mice died within 4 days (Table III). Essentially the same results were obtained with ten BALB mice which had survived the subacute hepatitis and were then challenged with the virus from subacutely diseased mice.

(b) *Immunity after Latent Infection.*—Twenty BALB mice were injected with a filtrate from the livers and spleens of mice with the original line I leukemia. None of these died or showed signs of the disease. 18 days later these mice were reinoculated with a filtrate from subacutely ill mice. Again, none of these animals died or showed signs of illness; whereas six out of ten control animals which had received the same filtrate died, and the surviving four showed marked signs of the disease and a 15 per cent weight loss (Table III).

The fact that the initial infection with material from line I leukemia had provided sufficient antibody to protect against infection with the virus from

TABLE III
The Development of Immunity after Infection

Strain	No. inoculated	Initial infection	Challenged with:	No. surviving
F1	20	Subacute	Acute	20
F1	6	None	Acute	0
BALB	20	Latent	Subacute	20
BALB	10	None	Subacute	4

Inoculum: 0.3 cc. intraperitoneally of filtrate containing virus.

the subacute disease is interpreted as evidence for the presence of a latent virus carried in line I leukemia.

The Influence of Urethane and Methylformamide on the Evolution of the Disease

It has been shown that only three out of sixty F1 adults died from the subacute disease transmitted by the filtrate (Table I). If urethane or methylformamide were administered to these mice, the severity of the disease increased greatly. The results of an experiment in which one group of F1 were treated with three doses of urethane after inoculation with the filtrate; a second group pretreated with three doses, infected, and then given three further doses of the drug; and a third non-treated control group that received only the filtrate—are summarized in Table IV. Pretreatment and posttreatment, or posttreatment alone, caused a considerable increase in mortality. All animals showed the characteristic signs of the disease, and at autopsy large necrotic areas were found in the livers.

For further investigation of the possibility of a latent asymptomatic disease in the line I leukemia, twelve F1 adult mice were treated with urethane before and after inoculation with a filtrate of liver and spleen from mice with the original line I leukemia. Seven of the twelve animals died with typical liver lesions and the surviving five mice showed signs of illness. Essentially the same

effect was observed in F1 mice after being pre- and posttreated with methylformamide. Seven out of ten mice died when injected with the filtrate from the line I leukemia. None of the infected non-treated controls or the uninfected drug control mice showed any sign of disease. This was interpreted as further evidence that the agent is latent in the original line I, but manifests itself only if a supplementary factor is present. Whether the role of this factor is preparatory, inciting, or damaging remains to be determined. It should be emphasized, however, that in the dosages used urethane or methylformamide did not cause any pathological changes in the livers of control animals.

Since mice which had overcome the subacute disease had been found to be immune to reinfection, fifteen F1 adults which had survived inoculation with the filtrate from subacutely diseased animals 22 days previously were treated

TABLE IV
The Effects of Urethane on the Subacute Disease in F1 Adult Mice

No. animals	Dose urethane prior to filtrate injection	Dose urethane after filtrate injection	No. dead	Average survival time
				<i>days</i>
20	0	0	1	7.0
20	0	1000 mg./kg. × 3	6	7.2
20	1000 mg./kg. × 3	1000 mg./kg. × 3	19	7.2

Inoculum: 0.3 cc. intraperitoneally of filtrate of liver and spleen from subacutely diseased mice.

with urethane before and after reinfection with the filtrate. Eight of these fifteen mice died from the disease and all of the others had shown marked signs of the disease, indicating that the immunity established by the initial infection was broken down by the urethane treatment.

Finally, two new sublines treated with either urethane or methylformamide were started to check whether the enhancement of the agent through passage in the treated mice could be regularly reproduced. In this second series, the subacute disease developed after eight transfer generations in the urethane-treated subline and appeared after six transfer generations in the methylformamide-treated subline. An acute disease had meanwhile developed in the original urethane-treated subline after twenty-five passages.

"Freeing" the Leukemic Cells from the Agent

Leukemic cells of the urethane-treated subline that carried the subacute disease were passed through four transfer generations in F1 mice which had survived infection with the disease. After passage in these immune animals, the spleens and livers of the mice dying of leukemia had attained the same size and color as those in the original line I leukemia and no sign of hepatic necrosis

could be observed. Filtrates of livers and spleens from these animals caused neither death nor illness when inoculated into F1 or BALB. When, however, the F1 were pretreated and then posttreated with urethane after inoculation with these filtrates, five out of ten mice died, thus indicating that the cells were not completely freed of the agent, which had merely reverted to its latent form by passage in immune mice.

DISCUSSION

There is strong evidence that the acute form of the hepatic disease described in this paper is closely related, if not identical, with that described by Nelson (1-4). The manifestations during life and the pathological picture are strikingly similar, with the exception that we have never detected eperythrozoa in the peripheral blood of our mice. A close relation seems to exist also to the disease reported by Gledhill *et al.* (5-7). The subacute form appears to correspond to the disease noted by MacDowell *et al.* (8, 13) and by Law and Dunn (10), although Law and Dunn's disease later turned spontaneously to a more acute form.

It is possible that the agent we have been investigating is the original virus described by MacDowell, since line I leukemia maintained in Sloan-Kettering Institute was supplied by his laboratory. We have been unable to free the leukemic cells from the virus, but have succeeded in inducing a change to a latent asymptomatic form. Since we have employed the same methods as Taylor and MacDowell (13), it is conceivable that the line was never completely freed of the agent, which has been carried for several years in its latent form.

Whatever differences there may be between our findings and those of other workers (8, 9, 10, 13) in regard to the response of the lymphatic system to infection with the agent they are not incompatible nor do they necessarily indicate different diseases. We have encountered similar differences between the acute and subacute forms. There seems to be a direct correlation between the virulence of the agent and the lymphopenic changes. In general, when the virulence of the agent is enhanced and the disease becomes acutely lethal, there is not only a marked hypoplasia of the lymphatic system, but an absence of leukemia (4, 8, 10). This constitutes a very interesting, but as yet obscure, relationship of the latter to the hepatic disease. It is conceivable that the leukemic cells, which presumably act as host of the virus, are rapidly destroyed when its virulence is enhanced.

The agent has been found primarily in transplantable mouse leukemias in all the instances cited, except one in which its origin was not clear (5). While the possibility exists that a contaminating agent may have been passed along with the serial transfer of leukemic cells, the fact that this agent has been in each case hepatitis virus is most suggestive. It will be of interest to examine not only other lines of established leukemias, but also new lines of spontaneous

leukemias for the presence of similar agents. Such investigations might reveal more than just a coincidental relationship between this group of hepatic diseases and leukemia.

The principal result of these investigations is the demonstration of the enhancement of the virulence of a filterable agent by treating each transfer generation with urethane or methylformamide. It should be noted that, once the enhancement of virulence was obtained, it was permanent and independent of further treatment with these drugs. It thus resembled the spontaneous change in virulence which has been observed occasionally in this group of diseases (10, 13). Treatment with urethane has been found to increase the severity of infection with pneumonia virus of mice, but no mention is made of an enhanced virulence of the virus itself (14). The permanent, enhanced virulence which we obtained may be due either to a direct action of urethane or methylformamide on the agent or to one on the host. There is no evidence for the first possibility. The present concept of the relationship between the host and the virus makes the second possibility more likely.

Urethane may cause liver damage (15, 16), and methylformamide has been proven to be hepatotoxic (17, 18). It is, therefore, conceivable that treatment with these compounds leads to liver damage, which is overcome, under ordinary conditions, but which in the case of the latent infection gives the agent a better opportunity to penetrate and multiply in the liver cells.

Another hypothesis to consider is an inhibition of antibody formation or of some other general defense mechanism during the treatment with urethane or methylformamide. Preliminary experiments have shown that nitrogen mustard, which inhibits the formation of antibody (19), may have the same effect in increasing the virulence of the virus as urethane and methylformamide. Cortisone, thus far, has proved ineffective in this respect.

The method of pretreating and posttreating animals with urethane or methylformamide may prove useful in investigating other virus diseases. An attempt to adapt human hepatitis virus to mice which had been given urethane in their drinking water has been reported (20).

SUMMARY

An hepatic disease caused by a filterable agent carried in leukemic mice is described. Ordinarily the virus remains latent and asymptomatic. If, however, the mice are treated with urethane or methylformamide before and after virus inoculation, the disease becomes manifest and is characterized by extremely marked liver necrosis.

Infant mice, a large percentage of weanlings, and adult Bagg albino mice are killed when injected with a filtrate from organs of diseased animals. Adult F1 and Swiss mice show signs of the disease but generally recover. They succumb, however, when simultaneously treated with urethane or methylform-

amide. By continuing the treatment of consecutive transfer generations an acute disease can be induced which finally kills all adult F1 mice without the treatment. At this stage the original leukemia may be lost.

Mice which have recovered from the subacute disease are resistant to the acute disease, and mice injected with the latent form of the agent are immune to the subacute disease. However, even immunized animals lose their resistance if they are treated with urethane. The acute or subacute disease can be reduced to the latent stage by passing the agent through several generations of immunized animals.

The relationship of this hepatitis virus of mice to viruses causing similar diseases is discussed, as is the possibility that these agents are closely related, if not identical.

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EXPLANATION OF PLATES

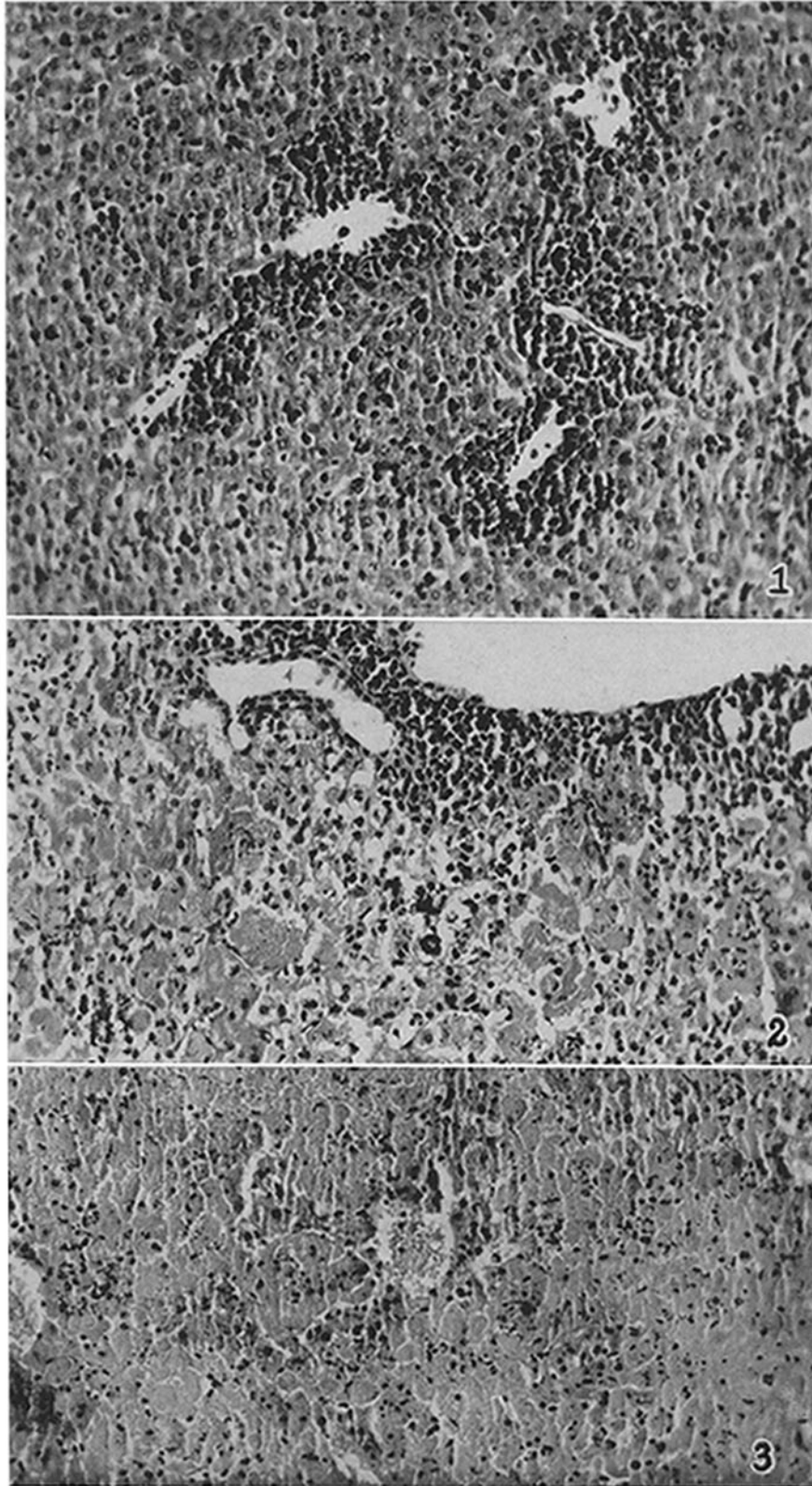
Mouse livers. Hematoxylin and eosin. $\times 120$.

PLATE 64

FIG. 1. Control, 4 days after inoculation with cells of Line I leukemia. Leukemic cells cuffing major vessels and lying free in sinusoids. No necrosis.

FIG. 2. Urethane treated, 4 days after inoculation with leukemia. Acute hepatitis. Marked diffuse necrosis.

FIG. 3. Untreated, 5 days after inoculation with 0.1 cc. of 10^{-1} dilution of filtrate. Acute hepatitis in the absence of leukemia. Marked diffuse necrosis.

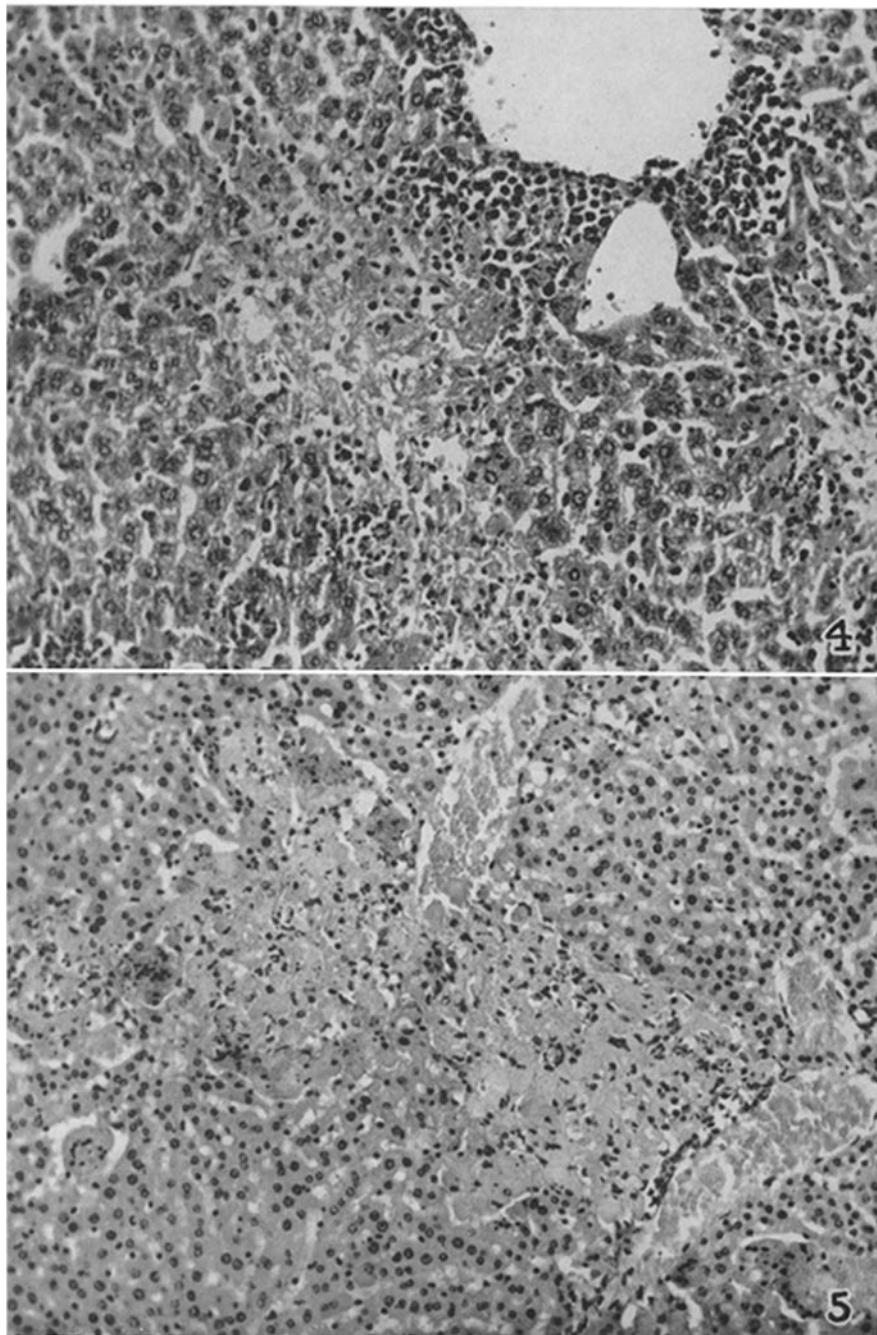


(Braunsteiner and Friend: Latent viral hepatitis in leukemic mice)

PLATE 65

FIG. 4. Urethane treated, 5 days after inoculation with leukemia. Subacute hepatitis. Focal necrosis.

FIG. 5. Untreated mouse, 8 days after inoculation with 0.1 cc. of 10^{-1} dilution of filtrate. Subacute hepatitis in the absence of leukemia. Focal necrosis.



(Braunsteiner and Friend: Latent viral hepatitis in leukemic mice)