SUMMARY

The thesis is divided into two sections. The first of these describes the occurrence and isolation of a strain of ectromelia virus from an epidemic in which skin lesions were not a notable feature. The outstanding feature at necropsy in these cases was a regularly occurring hepatitis. The identity of the agent was confirmed by means of egg inoculation, virus neutralisation and haemagglutination tests, and it was considered that the disease might serve as a useful experimental model of infective hepatitis.

The second section of the thesis deals with the effects of environmental factors on the development of the disease produced by the virus. Protein deficient diets were selected on the basis of their ability to produce dietetic necrosis in rats. Two such diets which differed only in the source of casein used failed to influence adversely the development of hepatitis due to the virus. The effect of a cold environment was next investigated and it was demonstrated that the 50 per cent. lethal dose end-point was much higher in the cold environment. The results were highly significant and indicated that a hundred to a thousand times as much virus is required to kill approximately 50 per cent. of mice in the warm environment used (approximately 75°F.) as is needed to kill the same proportion of mice in the cold environment (approximately 43°F.).

That this effect was due specifically to the action of the virus was shown by the fact that it could be prevented by the prior administration of immune serum. Intravenous inoculation was as effective as intraperitoneal administration in eliciting the effect and it proved that mice adapted from birth to the cold environment were as susceptible as mice exposed to that environment on the day of infection. The time relations of the effect were next investigated. Pre-treatment of mice with cortisone or with thyroid extract failed to reproduce the effect seen in the cold environment. It was not possible to demonstrate significantly greater amounts of virus in mice in the cold environment, using the infectivity of liver suspensions as the index. Examinations were carried out to exclude the presence of Eperythrozoon coccoides.

Histological examination of the livers of mice dying in the two environments revealed that with intermediate doses of virus the hepatic necrosis tended to be confluent and extensive in the cold environment but was focal and of smaller extent in the warm environment. The survival times with the same dose of virus were usually two to three days less in the cold environment. It is considered that the increased mortality of infected mice in the cold environment is due primarily to the increased susceptibility of the liver under these conditions.

UNIVERSITY OF GLASGOW

A PATHOLOGICAL STUDY OF LIVER DISEASE IN ANIMALS

by

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INDEX OF TEXT

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GENERAL INTRODUCTION

A wide variety of factors is capable of producing liver disease. Himsworth (1947) in the Lowell lectures has suggested a classification of such factors. He has emphasised the tentative nature of any such classification because he considers that the outcome of any injury is determined by factors inherent in the liver itself rather than by the nature of the injurious agent. Throughout the lectures he considers not only the way in which the disease-producing factors operate but also the circumstances which determine the type of injury produced. This outlook on liver disease is largely based on a consideration of the background of experimental work in animals. Himsworth is primarily concerned with the pathogenesis of liver lesions, and, of the factors dealt with in his broad classification, the effects of nutrition are discussed at greatest length.

Himsworth gives a bibliography and an account of the development of the work on dietary factors from its inception, and points out that it was not until it was shown that liver lesions could be produced by dietary deficiencies (Weichselbaum, 1935) that the importance of diet became generally recognised. Himsworth and Glynn (1944) showed that there were two kinds of experimental dietetic injury in rats; first an acute massive necrosis, the survivors of which develop post-necrotic scarring/

scarring and nodular hyperplasia, and second a slowly developing diffuse fibrosis. The first type is related to deficiency of protein and tocopherol, the second to choline deficiency followed by fatty infiltration of the liver, and acute necrosis is never a feature of the process. They described in detail the conditions necessary for the production of these lesions in rats.

Rao (1948), working in Bombay and using the methods of Himsworth and Glynn, reported his failure to produce acute dietetic necrosis in rats and suggested that England's colder climate might be an important factor. Naftalin and Howie (1949) had reported the occurrence of "nutmeg" liver with centrilobular necrosis proceeding to central and periportal cirrhosis in suckling and weanling pigs reared in a cold and damp environment. Naftalin (1951) was thus prompted to investigate the effect of environmental temperature on the development of acute dietetic necrosis in rats. He described in detail the amounts of food ingested by individual rats, and he also studied the effects of food restriction at the different environmental temperatures which he used. Contrary to what might have been expected, it was found that liver necrosis could be prevented by a <u>low</u> environmental temperature and that a sufficient degree of food restriction prevented liver necrosis at all the environmental temperatures studied.

This particular condition of acute massive necrosis/

necrosis in rats produced by dietary means has been the subject of a very considerable body of research (see Himsworth, 1947, for bibliography) presumably because the condition, both in its distribution of lesions and in its histology, closely resembles the acute liver strophies of the human subject. It is noteworthy, however, that attempts to reproduce the condition in different laboratories have met with widely varying degrees of success, (Himsworth and Lindan, 1949; Gyorgy et al., 1950; Naftalin, 1951; Rao, 1948). Those authors have put forward a number of suggestions to account for the discrepancies observed.

It should be recalled that Himsworth has himself pointed out that, at least under conditions of western civilisation, it is unlikely that humans are ever required to subsist on diets comparable in deficiency to those required for the production of experimental liver injury in animals, but it is interesting to note that a Swedish worker (Obel Anna-Lisa, 1953) has described a naturally occurring disease in pigs which she designates "hepatosis dietetica" and which appears to be comparable both in pathogenesis and in aetiology with the experimental disease described in rats. She was able to reproduce the disease experimentally by feeding similarly deficient diets. However, it is probably true to say that "hepatosis dietetica", as a naturally occurring condition, represents a comparative

rarity even in the liver diseases of the domestic animals.

It has, however, been recognised for some time that dietary factors play an important part in modifying hepatic necrosis produced by other agents. A great deal of this work is concerned with the effect of dietary factors on hepatic damage produced by poisons. The earlier observations dealt mainly with the effect of carbohydrates or fat. It was shown that carbohydrate-rich feeding reduced the susceptibility to chloroform poisoning, while fat rich diets had the opposite effect. (Davis and Whipple, 1919; Opie and Alford, 1914). A correlation between resistance to such poison and a high liver glycogen content was demonstrated by Graham (1915). Later it was shown that susceptibility to chloroform is closely connected with the state of protein metabolism in the body, and that the comparative resistance to chloroform anaesthesia of pups born of protein-depleted bitches was associated with the high sulphur content of their livers relative to that of their dams, (Miller and Whipple, 1940). One extremely interesting finding is that the sulphur containing amino-acids which control susceptibility to chloroform poisoning are the same as those which protect rats against experimental dietary necrosis. Possibly one of the best examples of the effect of diet in modifying the hepatic lesions of experimental poisoning is in the work of Messinger and Hawkins (1940) who showed that arsphenamine/

readily caused centrilobular necrosis in animals kept on protein-deficient diets while relatively high doses are required to produce even minimal lesions in normal animals.

While there is a fairly extensive literature of experimental findings dealing with the importance of diet in modifying hepatic damage due to poisons, the situation is somewhat different in respect of the infectious hepatitides. There is plenty of good clinical evidence to point to the importance of nutrition, although only two references will be cited here. Findlay (1948) has made a study of infective hepatitis in West Africa. Although the incidence of the disease was 2 to 4 times as great in white as compared with coloured troops, the mortality rate in the latter was 15 times as great - this difference was related to the nutritional levels of the two groups. Fox et al. (1942) described an outbreak of yellow fever vaccine hepatitis in Brazil in which the mortality rate from massive hepatic necrosis was 12 times higher in the local population (poorly fed) than in the well-fed American troops. There is, however, comparatively little literature on the experimental aspects of the association. Wylie (1946) showed that the adminstration of additional methionine produced a statistically significant reduction in the incidence of liver necrosis in guinea-pigs experimentally infected with Leptospira icterohaemorrhagiae.

MacCallum and Miles (1946) demonstrated the enhancing effect of protein deficient diets on a hepatitis in rats. Andersen and Tulinius (1938) describe a similar effect on a hepatitis occurring in pigs. The paucity of the literature on this aspect is all the more surprising when one considers the large amount of experimental evidence that has accumulated with reference to (a) the effects of nutrition on other experimental infections, and (b) the effects of nutrition on the development of liver disease which has been briefly reviewed above.

It was with this back-ground in the literature that the work to be described in this thesis was undertaken. When the opportunity arose for the study of a naturally occurring disease in mice, characterised at post-mortem by the appearance of an acute hepatic necrosis, it was felt that an experimental investigation of the effects of diet and environmental temperature on the course of the disease might provide valuable information.

The thesis is therefore divided into two main sections, the first of which deals with the isolation and identification of the virus responsible for the disease in mice, and the second part which deals particularly with the effect of diet and environmental temperature on the development of the disease.

SECTION ONE

REVIEW OF THE LITERATURE ON MOUSE HEPATITIS

A variety of agents, namely Cysticerci (Taeniae formis), coccidia, bacteria and viruses, may invade the liver of the mouse and produce local changes. The following review, however, will be restricted to those agents which are not cultivable on ordinary bacteriological media.

Liver lesions of unknown etiology characterised by infiltration and necrosis have been reported by Olitsky and Casals (1945) in a percentage of supposedly normal mice of different strains.

A liver disease of particular interest was described by Tyzzer (1917) which occurred as an epidemic in a colony of Japanese waltzing mice, in which mice there was a high rate of mortality. The incubation period varied from 10 - 40 days. At post-mortem examination the lesions were limited to the liver and were in the form of multiple tubercle-like nodules. A non-cultivable spore forming organism named Bacillus piliformis was regularly seen in hepatic cells at the margins of the necrotic areas. The disease was transmissible by contact, feeding and intravenous injection, but not by intra-peritoneal inoculation. Common albino mice were largely resistant. Rights et al. (1947) encountered the same disease in a British strain of white mice. B. piliformis was found in the liver lesions, but the disease could not be transmitted

to/

to Swiss mice by feeding or intraperitoneal injection. Intracerebral injection was followed by local proliferation of B. piliformis which gave rise to paralysis and death. The organism was propagated in embryonated eggs but rapidly became avirulent. These authors pointed out that the relation of B. piliformis to the disease was not clearly established.

American literature contains a number of reports dealing with agents producing ascites and hepatosplenomegaly in mice. Perrin (1943) drew attention to the presence in his mice of a protozoan similar morphologically to that described by Levaditi et al. (1923) and classified it as Encephalitozoon. Jordan and Mirick (1955) and Lackey et al. (1953) were unable to demonstrate such structures in the tissues of mice experimentally infected with the agents they investigated. Morris et al. (1956) have reported a disease which is essentially similar in incubation period, low mortality, splenomegaly, hepatic pathology and in its resistance to heat and to antibiotics. The disease, however, it consistently associated with the presence of intracellular protozoan-like structures which are not often numerous. Morris and his co-workers consider that these structures may have been overlooked by the other authors and that the diseases are very similar if not identical. The protozoan is considered to be different from any previously reported. Jordan and Mirick (1956) and Morris et al. (1956) take/

take particular care to exclude Toxoplasma gondii which can also produce ascites and hepatitis in mice. It is of particular interest that these four reports originate in laboratories where efforts were being made to transmit human hepatitis viruses to laboratory animals. In each case the disease was encountered when human material was inoculated into mice which had been pre-treated with drugs or X-ray irradiation in an attempt to make them susceptible to the human viruses. None of the workers mentioned make any claim that the agents they describe are related to the human disease.

Since 1951 several viruses causing hepatitis in mice have been reported, two of which are of particular interest. A series of papers has appeared relating to each virus.

The infectious agent described by Gledhill and Andrewes (1951) was originally isolated by them from the Parkes (P) strain of mice, maintained by the Medical Research Council Laboratories in London. Mice of this strain are relatively resistant. The agent was designated as MHV (Mouse Hepatitis Virus). Since newly weaned mice of the VS strain (Webster, 1937) were shown to be highly susceptible until they attained a weight of 14 gm., they were used as an indicator of the presence of the agent. When such mice were infected with material from naturally infected P strain mice, the mortality was in/

in the region of 100 per cent. It was observed that the disease could be prevented by treatment with aureomycin or terramycin.

In a later report (Gledhill et al. 1952) these authors found that MHV could be resolved into a stable component (S) and a labile one (L). Both factors were transmissible and filterable through gradocol membranes. Neither factor alone produced evident disease in VS mice but when combined produced fatal hepatitis. The (L) component was sensitive to the above mentioned antibiotics and was destroyed by exposure to room temperature for 24 hours. Mice of the VS strain were protected if treated with the antibiotic on the same day but not if this was injected 24 hours before the virus. In another communication (Niven et al. 1952) the (L) component of MHV which had been thought to be a virus was identified as Eperythrozoon coccoides. MHV1 is now used to refer to the (S) component.

Nelson (1952) described hepatitis associated with a naturally occurring and transmissible mouse leukaemia. After several passages at short intervals in weanling mice of the Princeton strain it was possible to separate the agent producing hepatitis. Marked differences in susceptibility were shown between mice of different strains, experimental transmission giving rise to a mortality of 98 per cent in Princeton weanlings and 4 per cent in Swiss mice./

He compared the disease with that caused by Gledhill's MHV and showed certain differences between the two. The disease was not prevented by prior injection of terramycin and the virus was resistant to exposure to room temperature for a period of 24 hours. Eperythrozoon was not demonstrated in the blood of Princeton mice infected with this virus. The development of Eperythrozoon in mice infected with both agents had no effect on the outcome of the disease in Princeton mice but in the relatively insusceptible Swiss mice the disease was markedly enhanced by combined infection with Eperythrozoon.

More recently Dick et al. (1956) have reported a virus causing hepatitis in mice which was recovered from mice which had been inoculated with serum from a human case of hepatitis followed by the injection of hypertonic glucose solution. The virus is referred to as MHV3 and is unrelated to that of human hepatitis. The virus is in many ways similar to MHV1 but differs in its greater virulence for weanling mice.

The relationship between the viruses has been the subject of several papers and it appears that they may be grouped together on account of many common properties among which is the fact that they produce qualitatively similar histological lesions and that they are both hepatotropic and potentially neurotropic. The following nomenclature has been suggested/

suggested for the viruses noted above:

Gledhill's virus	MHV1
Nelson's virus	MHV2
Dick's virus	MHV3

In addition to the agents dealt with above, Stanley et al. (1953) have described a virus producing hepatitis with jaundice in suckling mice which can be cultivated on the chorioallantois of the chick egg with the production of macroscopic lesions.

It is worthy of mention that lymphocytic chorio-meningitis virus, which is usually considered as a neurotropic agent, is known to include strains which are viscerotropic and may give rise to liver lesions (Traub, 1935).

ECTROMELIA

This disease also is associated with acute necrosis of the liver in a proportion of animals affected. It was first described by Marchal (1930) who gave a very complete account of the clinical signs and the post-mortem picture. Since her original paper the disease has been the subject of a considerable amount of research which has recently been reviewed by Tuffery (1956). The close relationship of ectromelia virus to vaccinia virus was shown by Burnet & Boake (1946). Since that time 'mouse pox' has been used as a model of the acute exanthemata principally/

principally by Fenner (1949) who has studied the pathogenesis of the disease in great detail. De Burgh (1950) and Nossal and de Burgh (1953 and 1954) have also found it useful in studying virus multiplication in animal tissue and the cytochemical changes occurring during infection.

Several authors have noted that in the initial stages of outbreaks skin lesions were not a notable feature (Melnick and Gaylord, 1953; Trentin, 1953). It is also of interest that Trentin commented that the typical inclusion bodies first described by Marchal are by no means as numerous in mouse tissues as the literature would suggest. Gledhill (1951) in discussing the differential features of MHV points out that ectromelia produces liver lesions having a very close resemblance to those brought about by MHV.

In view of the potentially neurotropic character of the MHV group of viruses it is also pertinent that Dalldorf and Gifford (1955), working in America, described the identification of two agents as ectromelia virus which had been sent to them as neurotropic viruses of human origin.

SUMMARY OF MAIN FEATURES

All of the agents dealt with in the preceding short review of the literature have been shown to be potentially neurotropic as well as hepatropic, and not cultivable on artificial media.

Tyzzer's disease and the diseases considered by Morris et al. to be due to a protozoan would appear to have a rather lengthy incubation period compared with the other virus diseases described, with the possible exception of LCM.

The MHV group of viruses have one feature in common, namely, their inability to grow in embryonated eggs.

With the exception of LCM virus all the agents have been shown to have a very narrow host range and most of them can in fact produce disease only in mice.

ORIGIN OF THE VIRUS

In the latter part of 1953 an outbreak of disease occurred among mice kept in the Zoology Department of the University of Glasgow. Four mice which died during this outbreak were submitted to the Department of Veterinary Pathology for post-mortem examination (ref. No. 22887A). The main lesions at post-mortem were in the liver. In two of the mice the liver was very pale and clay-like but in the other two animals the organ was enlarged and showed lobular mottling due to numerous pin-point haemorrhages. There were no significant bacteriological findings. Histological examination revealed an acute hepatic necrosis of rather irregular distribution tending to be zonal in type but of such extent as to merit the description massive. One of the spleen sections examined showed a few focal necroses. No obvious changes were noted in sections of heart muscle, lung, pancreas, small intestine and kidney.

On the basis of these findings the disease was tentatively diagnosed as ectromelia and reported as such.

Further examination of the sections, however, failed to reveal the presence of the typical cytoplasmic inclusions (Marchal, 1930), and it was felt that the disease merited further investigation, particularly in view of the numerous published attempts to transmit the virus(es) of human hepatitis to mice.

Isolation accommodation was not available at this time, but pieces of liver, spleen and lung were stored in a deep-freeze cabinet at -20°C. until such time as it was possible to carry out transmission experiments. In addition a further four live mice (ref. No. 23003), which had been in contact with those previously examined, were obtained from the Zoology Department and these were sacrificed and portions of liver and spleen collected with sterile precautions and stored in the same manner. No gross lesions were noted in this latter group.

Transmission experiments were not carried out until isolation accommodation became available some ten weeks later.

IDENTIFICATION OF THE VIRUS

Material and Methods

Mice:- White Swiss mice were used throughout the experiments. These mice were from two colonies. One of these had been maintained at the Department of Veterinary Pathology, University of Glasgow, over a period of three years immediately preceding their use, without serious intercurrent disease. Most of the earlier experiments were carried out with this strain. The other strain used was originally obtained from an accredited breeder * and had been maintained without serious losses at the Department of Veterinary Pathology, University of Liverpool, for a period of at least a year prior to their use in the experiments to be described. So far as possible, mice weighing 16 to 20 gms. were used and, on those occasions when it was necessary to use older mice for titration of infectivity, care was taken to ensure an equal distribution of these larger mice in the experimental groups.

Diet:- "Rowett" cubes (Diet 41) were fed ad lib. without further supplement. The water bottles and food containers were continously replenished.

Inoculum:- Material to be used for preparing inocula was collected with sterile precautions from moribund mice. Only in the initial passage was material taken from dead mice. All inocula were prepared/

* Schofield, The Mousery, Delph, Near Oldham, Lancs.

prepared with sterile precautions under an inoculating hood.

Sterility tests were carried out using 0.1 ml. of the prepared suspension in McCartney bottles containing glucose broth. The cultures were incubated aerobically and anaerobically at 37°C. and the results read after two days. If, after incubation, there was any doubt as to the presence of cloudiness in the media the suspected culture was sub-cultivated on blood agar plates.

Extracts of liver or liver and spleen were prepared by grinding with sterile sand in a mortar. The tissue was suspended in 10 per cent serum broth so as to form a 10 per cent (w/v) suspension. An average of 5.0 ml. of the diluent was required for the pooled liver and spleen of one mouse. The suspension thus formed was lightly centrifuged so as to deposit gross particles and the supernatant fluid was collected. Even with the precautions mentioned above bacterial contamination was often encountered and early in the work the effect of penicillin and streptomycin on the activity of the agent was investigated. After these were shown to be without effect, antibiotics were added to all inocula so as to give a final concentration of:-

2000 units/ml. of Crystalline Penicillin
200 µg/ml. of Dihydrostreptomycin sulphate.

The treated material prepared in this way is referred to throughout as the standard 10^{-1} dilution. All other dilutions were prepared from this using 10 per cent horse serum broth as diluent.

In the early experiments inoculation was not carried out until the results of the sterility test were available, the standard suspension being kept after preparation in the deep freeze at -20°C. However, with the use of antibiotics at the concentrations stated, bacterial contamination ceased to be a problem and although sterility tests were still routinely carried out, the material was used after it had been allowed to stand for 1 hour following the addition of antibiotic. Except where otherwise stated, all inoculations consisted of 0.1 ml. amounts of the standard suspension and were carried out by intraperitoneal injection using the tuberculin type of all-glass syringe.

First attempts at transmission

An extract was prepared of mouse liver 23003 in the manner described above. 0.25 ml. of this material was injected intraperitoneally into each of five mice. These mice showed no signs of illness and were sacrificed ten days after inoculation. Gross lesions were not observed at post-mortem examination.

Extracts of liver and spleen of the three dead mice (ref.22887A) which had been stored in the deep freeze cabinet were prepared. One of these proved to be bacteriologically sterile and this extract was injected in 0.25 ml. quantities intraperitoneally into a group of ten mice.

Seven days after inoculation two of these mice were moribund and these were sacrificed to yield material for injection into a further group of five mice. The results of the first five passages carried out in this way are summarised in the following table.

Figure 1.

Passage No.	No. of mice injected	No. moribund or dead	Days to death or killing
1	10	2	7
2	5	2	7 - 8
3	5	1	7
4	5	2	6
5	5	4	6 - 9

In all the mice which died or were killed gross liver lesions were present. These are described in detail on page 23.

In addition to the above, a small series of control experiments was carried out. Liver-spleen suspensions of apparently normal stock mice were prepared. Two mice were inoculated with this material. One of the mice was sacrificed seven days after inoculation, the other being left for observation. A liver-spleen extract of the killed mouse was injected into a further two mice and the same procedure repeated. Five passages in this way failed to reveal the presence of lesions and no deaths occurred.

The surviving mice in both infected and control groups were kept under observation for a period of one month. In this first series no deaths occurred after the tenth day following infection.

Some difficulty in obtaining bacteriologically sterile suspensions was experienced in this first series.

Titration of Infectivity

A simple titration of infectivity was carried out using groups of five mice with results that are shown in Figure 2 (page 22). The starting material was from one of the killed mice from the fifth mouse passage shown in Figure 1 (page 20).

The volume of inoculum was not considered in expressing the dilution value.

Figure 2.

log. dose of virus	Alive	Dead	Cumulative Mortalities Alive	Dead
-1	0	5	0	13
-2	1	4	1	8
-3	3	2	4	4
-4	3	2	7	2
-5	5	0	12	0
-6	5	0	17	0

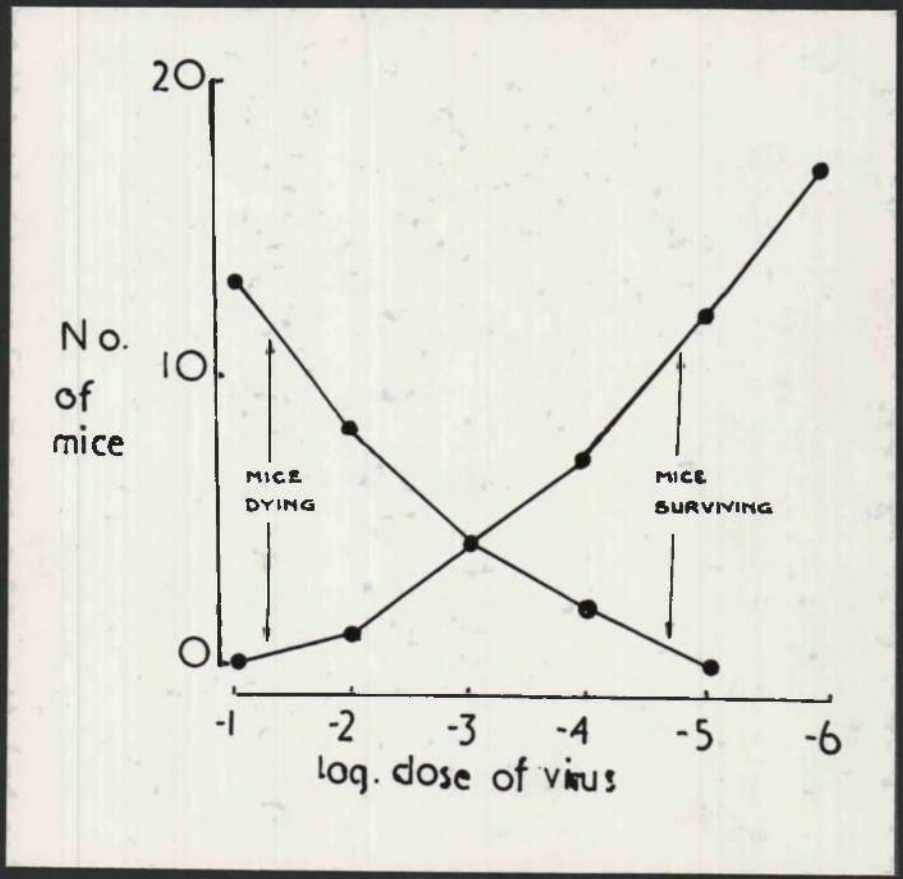


Figure 2 shows results of the first titration of infectivity and the method of calculating the cumulative mortalities.

Figure 3 shows graph of the cumulative mortalities and survivals (ordinates) at different log. dilutions of virus (abscissae). The lines intersect at the 50 per cent. lethal dose end point (after Reed-Meunch, 1938). This is based on the results in Figure 2.

The experimental disease in mice

Clinical signs in affected mice. Infected mice usually appeared in good health until a few hours or a day before death, when their fur became ruffled and a few showed a rather puffy appearance of the face. Trembling of the extremities is then sometimes seen. Other mice may be found prone with their hind legs extended shortly before death and, should they be twirled by the tail, they may go into a tonic spasm. There is usually a loss of weight, amounting sometimes to several grammes; there is no evidence of diarrhoea and no signs of jaundice although some mice may show staining of the perineal region with deeply coloured urine. The incubation period varies in duration but following intra-peritoneal injection it is usually from six to ten days and appears to be related to the size of the infecting dose, i.e. shorter with higher doses.

Changes observed at post-mortem examination. At necropsy some mice show a slight excess of peritoneal fluid but the only constant pathological changes were found in the liver. This organ is usually swollen, the colour varying from light pink to yellowish white and mottled from petechial haemorrhages. This appearance is quite uniform throughout the liver (see Figure 4, page 24). The spleen is usually deep red and may be enlarged with, sometimes, pale focal markings on the capsule. The kidneys are sometimes pale and swollen.

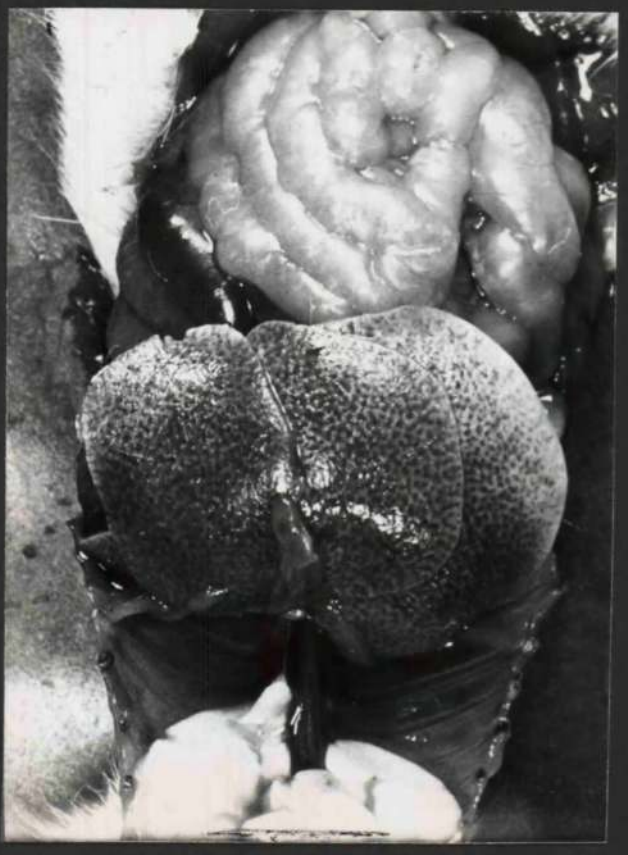


Figure 4 shows the typical appearance at necropsy with the liver swollen and uniformly mottled due to petechial haemorrhages.

Figure 5 shows the gross appearance of the only mouse in the present series which developed a generalised exanthema.

The proximal part of the large intestine is often deeply congested. In the initial series of experiments no gross changes were observed in apparently recovered mice sacrificed at varying intervals from the fourteenth to the thirtieth day following infection.

Histological changes. These will not be referred to in detail at this point but are described later in the text (Part II). It is worthy of mention here that examination of sections of kidney, pancreas, intestine and bladder failed to show the presence of cytoplasmic inclusions such as are described in infectious ectromelia (Marchal,1930). In other respects the lesions are similar, the changes in the liver being essentially those of an acute parenchymal necrosis. The splenic lesions are somewhat variable but there is usually an apparent decrease in the proportion of lymphocytes and the endothelial cells appear more prominent. Occasionally the latter are the seat of necrosis, which varies very largely in extent from one mouse to another. In a very few animals large wedge-shaped areas of spleen are completely necrosed and resemble infarcts.

Early in the work particular care was taken to exclude the possibility of Bacillus piliformis despite the more chronic nature of the disease described by Tyzzer (1917). Giemsa stained sections and/

and smears were examined from representative material in all of the first series of passages. Comparison smears of the organism were available on which the organisms were highly distinctive and it was never observed in smears or sections of livers from mice infected with the agent under investigation.

In view of the association of Eperythrozoon coccoides with MHV a search was made for this organism in films of peripheral blood. The details of these examinations are given later (Part II).

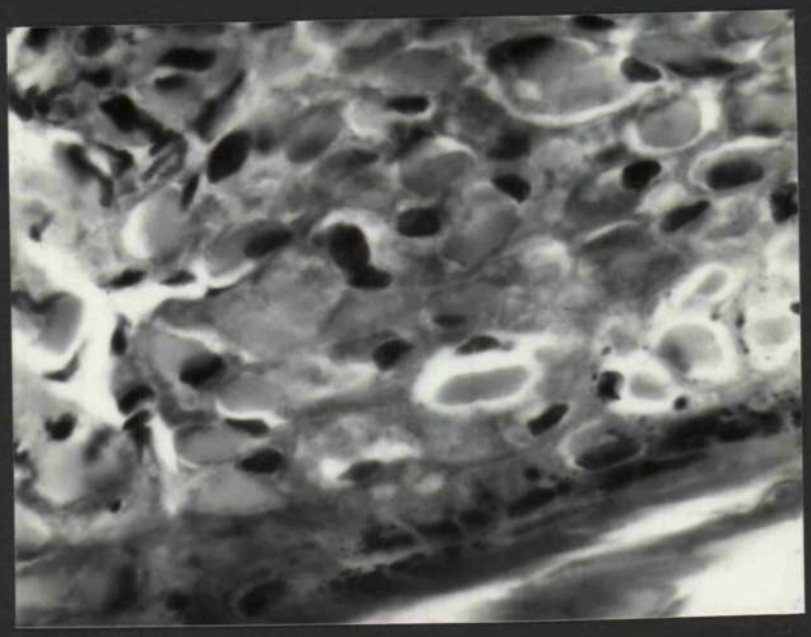
A note on the occurrence of lesions of the skin

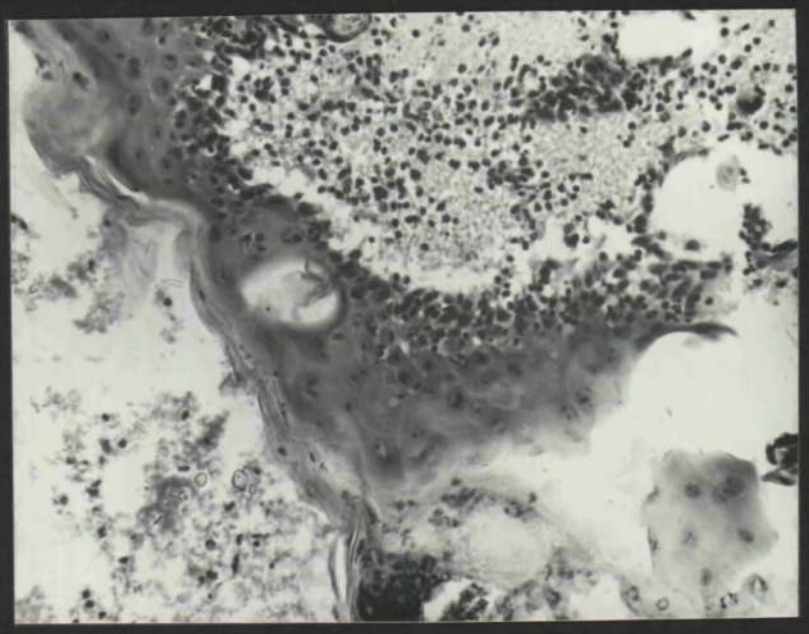
Fenner (1948) has pointed out that cutaneous lesions may not be observed in the acute rapidly fatal form of ectromelia. The primary skin lesion with swelling and oedema was often seen on the face in the present experiments. Secondary skin lesions were never a prominent feature and indeed among the many animals injected only one was noticed to have a generalised exanthema. A photograph of this mouse is shown in Figure 5 (page 24). It must be pointed out, however, that intradermal inoculation was rarely used since my main interest was in the development of the hepatic lesion and the mortality with which it may be associated.

Histological examination of sections of skin from this animal was carried out. The changes seen were essentially those described by Fenner and consisted of oedema of the skin and subcutaneous tissues with widespread lymphocytic infiltration of the/

the dermis. Most of the epidermis was necrotic but a few foci of oedematous epithelium were still visible. The nuclei in these patches were deeply basophilic and the cytoplasm of the cells was replaced by large vacuoles. Eosinophilic cytoplasmic inclusions of the type first described by Marchal (1930) were clearly visible in these areas.

Photographs illustrating these changes are shown in Figures 5B and 5C (page 28).





Resistance

Primarily from the point of view of comparison with the agent described by Gledhill and Andrewes (1951) the effects of heat and ether on the activity of the virus were investigated.

Two groups of ten mice were used. A 10^{-1} suspension of infective mouse liver was used, the LD50 of which was known from a previous experiment to lie between 10^{-3} and 10^{-4}. The suspension was prepared in the usual way in ten per cent. serum-saline but antibiotics were not added. Aliquots of this material were used and treated by (a) heat of 56°C. in a waterbath for 30 minutes and (b) the addition of an equal volume of ether followed by storage overnight at 2°C. 0.25 ml. of each of the above preparations was injected intra-peritoneally into each of the mice. No deaths occurred in any of the inoculated mice.

In a further experiment a similar 10^{-1} suspension was allowed to stand at room temperature (20°C.) for 30 hours before inoculation (0.25 ml.) into each of the mice. Eight of ten mice thus injected died before the twelfth day after inoculation.

Suspensions have been found to retain their infectivity for at least eighteen months at -20°C.

Figure 5B. - Section of skin of the mouse shown in Figure 5 showing oedema of the skin and subcutaneous tissues with underlying lymphocytic infiltration.
Heamatoxylin and eosin. x 160.

Figure 5C. - Section of epidermis showing cytoplasmic inclusions, the nuclei are deeply basophilic and the inclusions in this field have almost entirely replaced the cytoplasm of the cells.
Haematoxylin and eosin. x 800.

The effect of antibiotics

Groups of ten mice were used. A standard 10^{-1} suspension was prepared and treated with penicillin so as to contain a final concentration of 1000 units of Potassium Penicillin G per ml. A similar suspension was treated with Dihydrostreptomycin sulphate so as to contain 200 μg/ml. Both treated suspensions were allowed to stand overnight in the refrigerator after treatment with the antibiotics before being injected. 0.25 ml. amounts of the treated suspensions were injected intraperitoneally into two groups of ten mice.

Eight of the ten mice injected with the penicillin treated suspension and nine of the ten mice injected with the streptomycin treated suspension succumbed within nine days following the injection.

Inoculation of the virus into embryonated eggs

Material and Methods Fertile eggs of the White Leghorn Breed were used and were incubated at 37°C. for twelve days prior to inoculation. Inoculations were made onto the chorio-allantois using Burnet and Boakes' modification of drawing the inoculum (0.1 ml.) into the egg during the process of lowering the chorio-allantoic membrane to form the base of the artificial air sac. The drill holes in the shell were sealed with 'Sello-tape' prior to further incubation.

The initial inoculum consisted of a 10^{-1} suspension of mouse liver prepared as already described. For serial transfer in eggs a 10 per cent. suspension of the membranes in normal saline solution was employed. The membranes were macerated with the aid of a Griffiths tube, and antibiotics added to the same final concentration as that routinely used for mouse tissue suepensions. All dilutions were made in normal saline solutions. Membranes were harvested with sterile precautions after three to four days incubation and removed to a Petri dish for examination under a dissecting microscope.

Results. The results of the first few passages (Series A) are summarised in Figure 6 (page 32). The lesions take the form of small (0.2 to 0.4 mm.) thickenings which tend to be confluent when higher doses of inoculum are used, (see Figure 7, page 35).

Figure 6.

Inoculum	Pass. No.	Log. dose of virus	Temp. of incubation	Results *	Inclusions
Mouse liver	Series A.I	10^{-1}	37°C	3/5	—
Egg membrane	Series A.II	10^{-1} 10^{-2}	37°C	4/6 4/6	—
Egg membrane	Series A.III	10^{-1} 10^{-2} 10^{-3}	35°C	6/6 6/6 5/5	+
Egg membrane	Series A.IV	10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}	35°C	4/5 6/6 4/6 1/6 0/6	+
Mouse liver	Series B.I	10^{-2}	35°C	3/3	+
Egg membrane	Series B.II	10^{-2}	35°C	3/3	+
Egg membrane	Series B.III	10^{-2}	35°C	2/3	+

* The numerator shows the number of eggs with lesions, the denominator the number of eggs injected.

At the third passage the temperature of incubation after inoculation was reduced to 35°C. (Burnet and Lush, 1936) and indeed growth did appear to be more regular at this temperature.

Histological examination of the membrane lesion. Selected chorio-allantoic membranes were fixed in 10 per cent. formol-saline, processed and embedded in paraffin-wax in the usual way. The following staining methods were used:-

Haemalum (Mayer 1903) and Eosin,
Phloxine Tartrazine (Lendrum 1947) and
Mann's Eosin Methyl Blue.

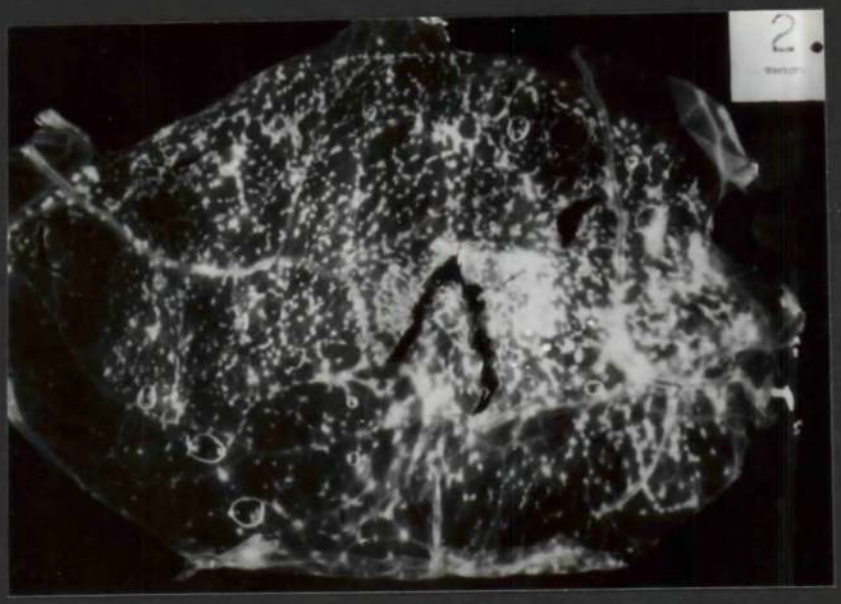
Histologically there is ectodermal proliferation with a mesodermal infiltration of inflammatory cells which is most marked at the periphery of the lesions, (see Figure 8, page 35). This is followed by necrosis of the superficial ectodermal layers, which is present in most membranes incubated for four days after inoculation.

An interesting finding was that the typical cytoplasmic inclusions (Burnet and Lush, 1936) were not observed until the third egg passage, although definite ectodermal proliferations were present in the first two egg passages. It will be observed from the table that this coincides with the reduction in post-inoculation temperature of incubation (Series A).

The procedure was therefore repeated using mouse/

mouse liver as inoculum (suspension 10^{-2}) and incubating the eggs at 35°C. after inoculation. All eight membranes of this series were harvested on the third day and examined histologically. In this series the typical cytoplasmic inclusions appeared at the first egg passage (Series B).

In both series the large cytoplasmic eosinophilic inclusions are readily demonstrable with all three methods of staining used. Reference sections of mouse ectromelia lesions on the chorioallantois were available, and the inclusions were similar to those described above.



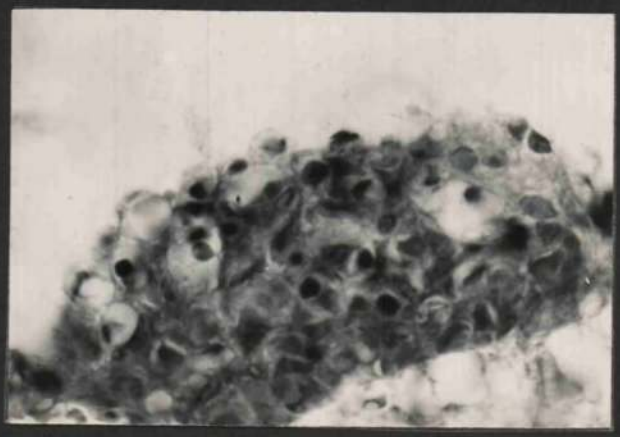


Figure 7 shows the appearance of the lesions on the chorioallantois after four days incubation at 35°C. Discrete foci are numerous at the periphery while at the centre they have become confluent.

Figure 8. - Section of the chorioallantoic membrane shown above showing ectodermal proliferation. Several cytoplasmic inclusions are present in the proliferated cells.
Phloxine Tartrazine. x 520.

Cross neutralisation tests in chick embryos.

McCarthy and Downie (1948) showed that the growth of ectromelia virus on the chorioallantois of developing eggs could be inhibited by anti-vaccinia serum as well as by homologous anti-serum. The technique adopted here is essentially that described by those authors except that the final titre of the anti-serum employed was not measured nor was the 50 per cent. infectious dose endpoint estimated.

Material and Methods. The viruses used were:-

(a) an egg-adapted strain of vaccinia virus originating in the Lister Institute of Preventive Medicine, London,

and (b) the unknown virus from the fourth passage in eggs.

The anti-sera used were:-

(i) hyperimmune vaccinia anti-serum prepared in a rabbit by repeated immunisation with rabbit-passaged virus in the form of purified elementary bodies.

(ii) serum from mice which had survived the intra-nasal inoculation of the unknown virus for a period of sixteen days. The inoculation was then repeated and the anti-sera collected at twelve days after the second inoculation. Convalescent ectromelia serum has poor neutralising ability (Melnick and Gaylord, 1953).

All sera were inactivated at 56°C. for thirty minutes before use.

Dr. Dumbell of the Bacteriology Department, University of Liverpool, kindly provided the vaccinia serum and virus.

The maximum dilution of virus for use in the test was roughly selected as that dilution at which the growth on the chorioallantois ceased to remain confluent i.e. log dose 10^{-3} in the case of the unknown and 10^{-4} in the case of the strain of vaccinia used. Thus serial dilutions of both viruses were prepared as follows:-

10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}.

Equal volumes of serial dilutions of virus and undiluted serum were mixed, held at room temperature for two hours, and 0.1 ml. of this mixture injected into four eggs of each group. Controls consisted of:-

(1) saline + virus at maximum dilution

(2) undiluted normal rabbit serum + virus at maximum dilution

i.e. vaccinia log dose 10^{-4}

unknown log dose 10^{-3}

The two viruses were tested on different days but the results are included together in Figure 9, (page 39). In both cases the eggs were examined four days after inoculation.

Results. The results are shown in the form of a table, Figure 9. The normal serum controls showed some degree of inhibition in that several of the eggs in these groups did not show confluent lesions as compared with the saline controls, but nevertheless obvious growth was present.

It is evident from the above that some cross relationship exists between the two viruses. Neutralisation with a known strain of ectromelia was not sttempted since it was deemed inadvisable to introduce this into the laboratory.

Figure 9.

Results of Cross neutralisation tests between the unknown and vaccinia virus

Serum	* Log. dose of virus	
	unknown	vaccinia
anti-unknown mouse serum.	10^{-1}	10^{-1}
anti-vaccinia rabbit serum.	10^{-1}	10^{-1}
normal mouse serum	10^{-3}	10^{-4}
normal rabbit serum	10^{-3}	10^{-4}
saline control	10^{-3}	10^{-4}

* maximum dilutions at which all four eggs showed obvious lesions.

Haemagglutination

Materials and Methods. The vaccinia used was the "Hart" strain.* Stock suspensions of this virus and the unknown virus were obtained by inoculating a series of eleven-day old chick embryos and harvesting the membranes after three days' incubation at 35°C. The membranes were allowed to drain and then ground with sterile sand in a mortar and pestle. 1.0 ml. of saline per membrane was added and the suspension centrifuged for ten minutes in a small B.T.L. Angle centrifuge at maximum revolutions (approximately 11,000 r.p.m.). The supernatant fluid was used as stock material. Control antigen consisted of normal membranes prepared in the same way.

The technique used in the haemagglutination tests was essentially that of Burnet and Boake (1946), except that the tests were carried out on perspex plates. Dilutions of antigen were made in saline using 0.2 ml. as the standard volume, one volume of saline solution and one volume of 2 per cent. red cells being subsequently added. The mixtures were incubated at 35°C. for one hour before the test was read.

* Dr. Norman Grist of the University of Glasgow kindly supplied this virus.

Red cells from ten fowls were tested for haemagglutinating activity with the vaccinia stock material, and two of these were selected, one from a fowlwhose cells were susceptible to agglutination with the virus, and one from a bird whose cells were insusceptible. The latter proved a convenient check against non-specific agglutination. Red cells were collected from mice by bleeding from the caudal vein. Results of the haemagglutination test are shown in Figure 10 (page 43), from which it can be seen that the unknown agent behaved as ectromelia in that it agglutinated vaccinia-positive fowl red cells and mouse red cells and failed to agglutinate vaccinia-negative fowl red cells.
On the basis of those results an haemagglutination-inhibition test was set up.

Haemagglutination-inhibition.

Antigens. Two dilutions of vaccinia antigen were used, viz., a 1/8 and a 1/16 dilution of the stock material as described under haemagglutination. Only one dilution of the "unknown" antigen was used i.e. 1/4 of the stock material.

Sera. The sera used were:-

A. Vaccinia immune serum prepared in rabbits by repeated injection of purified elementary bodies.

B. Immune serum against the "unknown" virus prepared by injecting a rabbit intravenously with a suspension of infective mouse liver at intervals of ten days with a total of five injections. The serum was collected ten days after the final injection.

C. Serum prepared against normal mouse liver by injecting a rabbit with the same amount of normal mouse liver as that used of infective mouse liver in B, the same number of injections being used.

D. Normal rabbit serum.

In each case the serum was diluted 1/10 in normal saline prior to use.

Red cells. Two sources of red cells were used, one from a fowl whose cells were susceptible to agglutination with vaccinia virus and one from a bird whose cells were insusceptible.

Technique. 0.2 ml. of antigen was mixed with 0.2 ml. of serum in the dilutions described above. Controls consisted of 0.2 ml. of antigen with 0.2 ml. of saline. The mixtures were allowed to stand at room temperature for one hour when 0.2 ml. of a 2 per cent. suspension of red cells was added. The mixtures were then incubated at 35°C. for one hour before the tests were read.

Results. No haemagglutination occurred with vaccinia-negative fowl red cells. The results shown in Figure 11 (page 44) therefore refer to the test in which vaccinia-positive fowl red cells were employed. It is evident that there is good cross inhibition of haemagglutination.

Figure 10.

Vaccinia antigen	Vaccinia Positive fowl RBCs	Vaccinia Negative fowl RBCs	Mouse RBCs
Stock	+	-	-
1:2	+	-	-
1:4	+	-	-
1:8	+	-	-
1:16	+	-	-
1:32	+	-	-
Unknown antigen			
Stock	+	-	+
1:2	+	-	+
1:4	+	-	+
1:8	+	-	-
1:16	-	-	-
Control antigen			
Stock	-	-	-
1:2	-	-	-
1:4	-	-	-

+ = complete agglutination

- = no agglutination.

Figure 11.

	Saline Control	Serum A Vaccinia	Serum B unknown	Serum C Normal Mouse Liver	Serum D Normal Rabbit
Vaccinia antigen					
1/8	+	-	-	+	+
1/16	+	-	-	+	+
Unknown antigen					
1/4	+	-	-	+	+

+ = complete agglutination

- = no agglutination.

CONCLUSIONS

The combined evidence of the post-mortem and histological findings, together with the results of the neutralisation and haemagglutination tests, make it clear that the virus here described is in fact a strain of ectromelia. The only unusual features of the experimental disease were (a) the failure to demonstrate visceral cytoplasmic inclusions and (b) the rarity of secondary skin lesions. The regular occurrence of hepatitis in the inoculated mice suggested its use as an experimental model of that condition.

SECTION TWO

Consideration of the literature reviewed in the general introduction suggested that the disease described in Section One might serve as a useful experimental model on which the effect of environmental factors, particularly those ascribed to dietary deficiency, might be examined.

Two protein-deficient diets were employed in this series of experiments. They were selected on the basis of the work of Naftalin (1951, 1952) who showed that environmental temperature played an important part in the production of acute dietary liver necrosis in the rat. In my hands these diets failed to provoke the condition in rats. The probable reason for that failure as given by Naftalin (1954) was the nature of the casein used as the source of protein.

The experiments can best be described by dividing them under two headings (a) those in rats (Experiments 1 and 2), the sole purpose of which was to repeat the effect described by Naftalin, and (b) those with mice (Experiments 3 and 4) in which the same diets were fed prior to infection with the virus already described.

This method of description is used for convenience although it does not represent the chronological sequence in which the experiments were carried out.

EXPERIMENTS 1 and 2

Object:- the production of dietary liver necrosis in rats.

Methods. Male albino rats were used in each of the following experiments. These animals were from a stock maintained in the University of Glasgow Department of Veterinary Pathology. The weaning age of the rats used was not known exactly but none of the animals was older than seven weeks.

Diet. After weaning and prior to their use the young rats had access to Rowett rat cubes fed ad lib. In both experiments a preliminary high protein diet (Naftalin's No.157) was fed for a period of one week prior to the low protein diet (Naftalin's No.84). The percentage composition of these diets is shown in Figure 13 (page 50).

In none of the groups was any attempt made to restrict the amount of food intake. The food consumption of individual rats was not measured. Two similar experiments were carried out and the only difference in the diets used was in the source of the casein. In Experiment 1 the "low-vitamin" casein of Genatosan Ltd., Loughborough, was used in both the high and the low protein diets, while in Experiment 2 Glaxo casein C of Glaxo Laboratories Ltd., Greenford, Middlesex, was used in both the high and the low protein diets. A copy of the analytical/

analytical figures of the Genatosan "low-vitamin" casein is given in Figure 12 (page 49).

Environment. In each experiment seven-day thermographs were used to record the dry bulb air temperature.

Experiment 1. Two environments were studied. The first of those was provided by an unheated animal house, the temperature of which varied from 50 to 60°F., and the second by a heated room, the temperature of which varied from 68 to 74°F.

Experiment 2. The same rooms were used and the temperature range in the heated room was of the same order as in Experiment 1, but the temperature in the unheated room ranged from 58 to 64°F.

Design. Groups of ten rats were used in both cases. In addition two similar groups of ten rats were kept as controls, i.e. they were kept on the high protein diet throughout the course of the experiment. The plan common to both experiments is shown in Figure 14 (page 50).

Results. In Experiment 1, in which Genatosan casein was used as the source of protein, no deaths occurred within a period of 120 days in any of the groups at either of the environmental temperatures studied. The animals were sacrificed at this time and post-mortem examination failed to reveal any evidence of disease.

Figure 12.

COPY OF THE ANALYTICAL FIGURES OF GENATOSAN

"LOW VITAMIN" CASEIN

LOW VITAMIN CASEIN (ch. 7.8.8.10 107313). H 0365

Appearance	Fine White Powder
Ash	1.34%
Loss at 100°C.	4.73%
Nitrogen	14.66%
Casein	93.53%
Alcoholic acidity	0.036%
pH	5.6
Lactose	No detected
Fat	0.11%
Total P	0.80%

Appearance	Fine White Powder
Aneurine	0.32 γ/gm.
Riboflavin	0.12 γ/gm.
Nicotinic acid	0.25 γ/gm.
Pantothenic acid	0.14 γ/gm.

PASSED
R.G.
10 Oct. 1951.

Figure 13.

Percentage composition of diets used

Constituent	High protein diet	Low protein diet.
Casein	16	8
Yeast *	3	3
Lard	7	7
Sucrose	68	76
McCollum's salt mix. ‡	4	4
Cod-liver oil (B.P.) (c.c.)	2	2
Choline chloride (mg. per 100 g. diet)	200	200

The following B vitamins (mg. per 100 g.diet) were added to all the above diets; aneurin 0.3, riboflavin 0.3, Ca pantothenate 2.0, pyridoxin 0.3, inositol 0.5, nicotinic acid 4.0.

* Dried yeast powder (Pulv. saccharomycetis cerevisiae exsicc.) The Pharmaco-Chemical Products Ltd., London and Burton-on-Trent.

‡ McCollum's salt mixture 185 - 0.22 g. KI per kilo of salt.

Figure 14.

Number of rats.	High protein diet.	Low protein diet.	Environmental temperature.
10	7 days	Till death or killing	High
10	7 days	Till death or killing	Low
10	Throughout experiment		High
10	Throughout experiment		Low

Histological examination of a general set of tissues including spleen, kidney, lungs, heart muscle, pancreas and small intestine likewise failed to reveal obvious abnormality. Several liver sections were examined from each animal and these were also apparently normal.

On the other hand in Experiment 2 in which Glaxo casein was used as the source of protein, five out of ten animals kept at an environmental temperature of 68 to 74°F. on the low protein diet succumbed and at necropsy these showed typical massive hepatic necrosis. Only two of the rats kept at the lower temperature on the low protein diet died and at necropsy these animals also showed massive hepatic necrosis.

Interpretation. These results confirm those of Naftalin (1954). It should be emphasised, however, that the precise date of weaning was not recorded. Nevertheless the results were sufficiantly clear cut to enable the selection of diets for use in mice.

EXPERIMENTS 3 and 4

Object:- to examine the effect of the diets used in Experiments 1 and 2 on the course of the disease produced by the virus of ectromelia.

Materials. Virus. The virus used was that described in Section 1, which is considered as an hepatotropic variant of the ectromelia virus. The methods of preparation of virus suspensions are as in Section 1 except that liver suspensions and their subsequent dilutions were made in normal saline solution. The mice used averaged four to six weeks of age. Sexes were mixed at random. All injections were carried out intra-peritoneally using 0.25 ml. amounts except where otherwise stated.

Environment. The same rooms were used as in Experiments 1 and 2.

Diet. The diets used were of the same composition as in Experiments 1 and 2 and in each case these were fed for one week prior to the injection of the virus dilutions.

Design. Experiment 3. Parallel titrations of infectivity were carried out in groups of five mice per dilution of virus suspension. Five dilutions were used for each titration. In this experiment Genatosan "low protein" casein was used as the dietary source of protein.

Experiment 4. The same dilutions of virus were used as in Experiment 3 and the layout is also identical, the only difference being that Glaxo casein C was used as the dietary source of protein.

The design of the experiments is shown in Figure 16 (page 56).

Where such comparative titrations were carried out the dilutions were prepared as quickly as possible using the reagents at 4°C. and the highest dilutions were injected first, i.e. all groups at 10^{-9} then all groups at 10^{-8} and so on.

Record cards of the kind illustrated in Figure 15 (page 55) were kept for each group of mice injected and proved to be particularly useful where material was collected for histology, as well as facilitating a comparison of the periods of survival. Post-mortem and histological examination was carried out on all mice which died.

The results are shown in Figure 17 (page 57).

Interpretation. In any attempt to show the effect of diet or other stimuli two main criteria are available. Thus, the size of the infective dose required to produce death under the conditions of the experiment may be calculated and, if this can be shown to vary sufficiently with changed conditions, it may not only provide evidence for the existence of the effect but also constitute a measure of its magnitude. Similarly, when a constant/

constant dose of the infective agent is used, if the period of survival can be shown to vary with different experimental conditions, this period may be used as an index. Secondly, post-mortem and histological examinations may reveal differences in the type of lesion produced.

In the experiments described here both approaches were used. The 50 per cent. lethal dose end-points (LD50) were calculated according to the method of Reed and Muench (1938). It seemed likely that any marked difference due to the experimental stimulus would be reflected by difference in the doses thus calculated.

The differences shown in Experiments 3 and 4 (see Figure 17, page 57), are not in fact statistically significant. Nevertheless in both experiments all groups kept at the lower environmental temperature did appear to show a slightly higher LD50. This, however, was obviously not related to the amount of protein in the diet since the same slight difference was present in the control groups given the high protein diet and kept at the lower temperature.

Post-mortem and histological examination of mice dying in Experiments 3 and 4 failed to reveal obvious difference in hepatic pathology.

It was therefore decided to attempt to enhance the effect by further lowering of the environmental temperature and to use the usual "Rowett" cubes (Diet 41) in place of the synthetic diets.

REF.

GROUP	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Date / Day
SERUM		1																
		2																
		3																
VIRUS		4																
		5																
		6																

CONTROL GROUPS

Source of virus .....

Inoculum .....

Strain .....

RESULT

Other procedures

mice have been kept for periods up to two months in the refrigerator described above, without ill effect. These environments are referred to hereafter as the cold and warm environments respectively.

Design. Parallel titrations were carried out using groups of five mice and eight dilutions of virus in each of the environments. 0.25 ml. amounts were injected intra-peritoneally.

Results. A graph of the cumulative totals from this titration is shown in Figure 18 (page 60). This shows a difference between the two environments amounting to 3 log. places in the calculated 50 per cent. lethal dose end-points. The actual results together with their statistical analysis are given in the appendix (page 129). It is sufficient to say here that the results are highly significant and indicate that 100 to 1,000 times as much virus is required to kill approximately 50 per cent. of mice in the warm environment as is needed to kill the same proportion in the cold environment.

The gross changes observed at necropsy were as those already described except that the mottling of the liver was usually more obvious in mice dying in the cold environment. Material for histological examination was collected from at least one of the mice dying in each group. Certain differences/

EXPERIMENT 5

Object:- to examine the effect of a cold environment on the infectivity of ectromelia virus in mice.

Methods. In the previous experiments an unheated room was used to provide the cold environment. The temperatures were recorded on a seven-day thermograph but even in the winter months the temperature rarely fell below 50°F. In addition there was marked daily variation. For this reason the mice were housed in a commercial refrigerator, the door of which was wedged open for approximately half an inch at its free edge, to provide ventilation. By this means eight mouse boxes could be comfortably accommodated. The environmental temperature reached with the dial at its lowest setting and with five or six mice in each box was approximately 43°F. with a relative humidity of approximately 50 per cent. and this temperature was well maintained, the variation not exceeding ± 5°F. except when the door was opened fully and the boxes removed for inspection. Galvanized metal boxes were used with wire mesh lids carrying the food-hopper and the water-bottle.

The control groups of mice were kept in a heated thermostatically controlled room at 75°F. with a variation of ± 7°F. and were fed diet 41 (Rowett cubes unsupplemented). On such a diet mice/

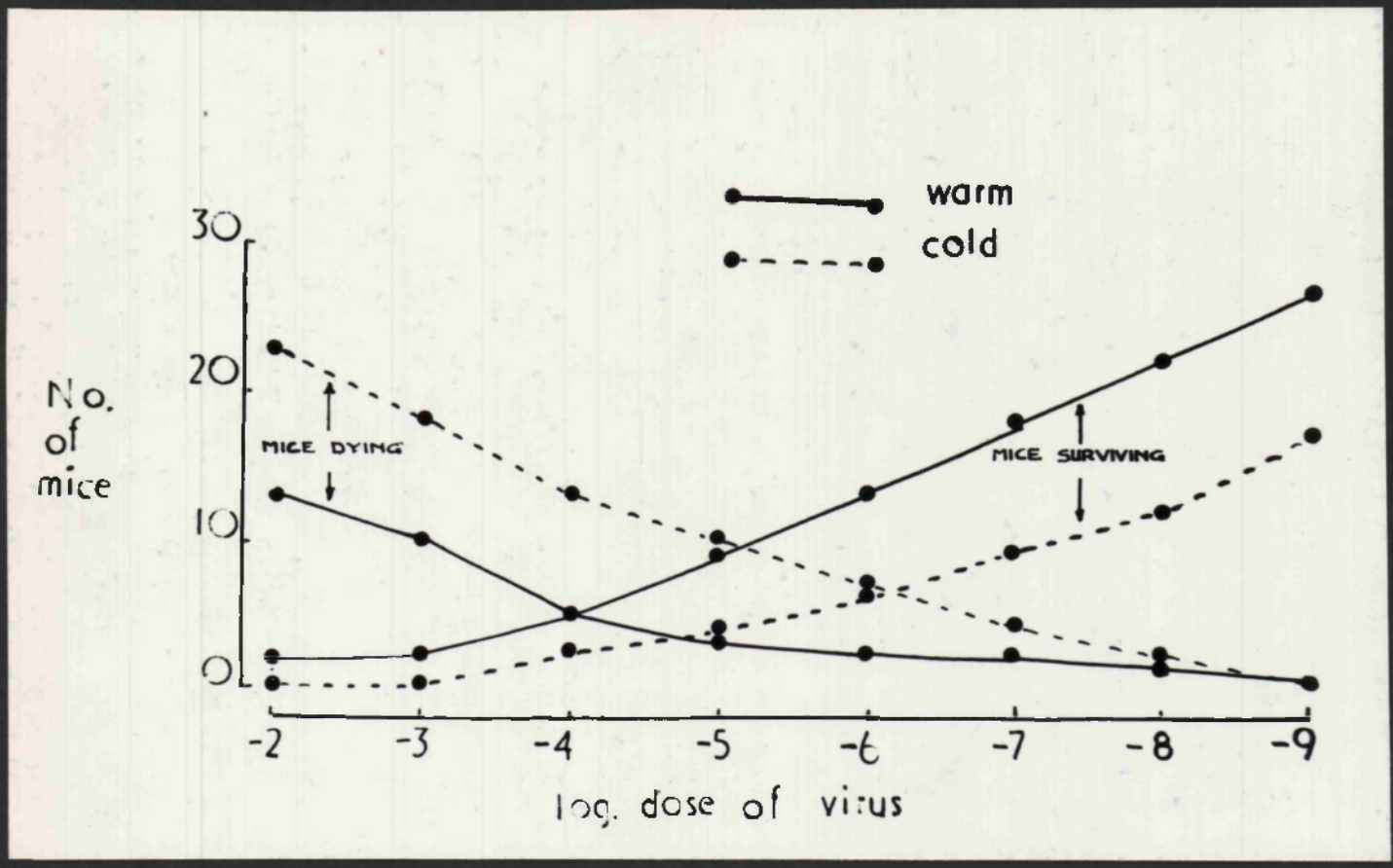

warm
cold
30
20
10
0
No. of mice
MICE DYING
MICE SURVIVING
-2
-3
-4
-5
-6
-7
-8
-9
log. dose of virus

Figure 17.

Group	Log. dose of virus	Number of mice dying / Number of mice injected	LD50
3A	10^{-2}	5/5	$10^{-3.6}$
	10^{-3}	3/5	
	10^{-4}	2/5	
	10^{-5}	1/5	
	10^{-6}	0/5	
2B	10^{-2}	4/5	$10^{-4.3}$
	10^{-3}	4/5	
	10^{-4}	3/5	
	10^{-5}	3/5	
	10^{-6}	0/4	
3C	10^{-2}	5/5	10^{-4}
	10^{-3}	4/5	
	10^{-4}	2/5	
	10^{-5}	1/5	
	10^{-6}	1/5	
3D	10^{-2}	5/5	$10^{-4.5}$
	10^{-3}	5/5	
	10^{-4}	3/5	
	10^{-5}	2/5	
	10^{-6}	1/5	
4A	10^{-2}	3/5	$10^{-3.54}$
	10^{-3}	4/5	
	10^{-4}	2/5	
	10^{-5}	1/5	
	10^{-6}	0/5	
4B	10^{-2}	5/5	$10^{-4.31}$
	10^{-3}	5/5	
	10^{-4}	3/5	
	10^{-5}	1/5	
	10^{-6}	0/5	
4C	10^{-2}	4/5	$10^{-3.54}$
	10^{-3}	4/5	
	10^{-4}	2/5	
	10^{-5}	0/5	
	10^{-6}	0/5	
4D	10^{-2}	4/5	$10^{-3.57}$
	10^{-3}	5/5	
	10^{-4}	2/5	
	10^{-5}	1/5	
	10^{-6}	1/5	

Figure 15. - Specimen record card used in titrations of infectivity to facilitate comparisons of incubation periods.

Figure 16.

Group	Log. dose of virus	Number of mice	High protein diet	Low protein diet	Environmental temperature
A	10^{-2}	5		Throughout the course of the experiment	High
	10^{-3}	5			
	10^{-4}	5			
	10^{-5}	5			
	10^{-6}	5			
B	10^{-2}	5		Throughout the course of the experiment	Low
	10^{-3}	5			
	10^{-4}	5			
	10^{-5}	5			
	10^{-6}	5			
C	10^{-2}	5	Throughout the course of the experiment		High
	10^{-3}	5			
	10^{-4}	5			
	10^{-5}	5			
	10^{-6}	5			
D	10^{-2}	5	Throughout the course of the experiment		Low
	10^{-3}	5			
	10^{-4}	5			
	10^{-5}	5			
	10^{-6}	5			

Figure 18. - Graph of cumulative mortalities constructed from results of experiment 5. The 50% lethal dose end-point is shown in each case where like lines intersect. The actual mortalities on which this graph is based are shown in the appendix.

differences were noted. These were the subject of further study (see page 88).

In addition to the difference in 50 per cent. lethal dose end-points, it was noted that the mice kept in the cold environment tended to die sooner than those maintained under warm conditions. This can be most clearly shown in the form of a graph (Figure 19, page 63), which is based on the sum of the results of Experiments 10A and 10C.

Control tests were also carried out and these were of two types (a) exposure of mice to the cold environment without other treatment for periods of up to two months. Twenty mice were used for this purpose. (b) intra-peritoneal inoculation of 0.25 ml. of a 10^{-1} suspension of normal mouse liver followed by exposure to the cold environment for one month. Thirty mice were treated in this way. No deaths occurred in either group.

It should be noted that mice are capable of reproduction at temperatures lower than those used here. Laurie (1946) has observed that the house mouse, Mus musculus L., can breed in cold stores kept permanently at well below 0°C. Barnett and Manly (1954) have shown that laboratory mice are able to make a physiological adaptation to temperatures below freezing-point without major loss of fertility. Their cages were in fact in a room kept at/

at -2°C. to -4°C.

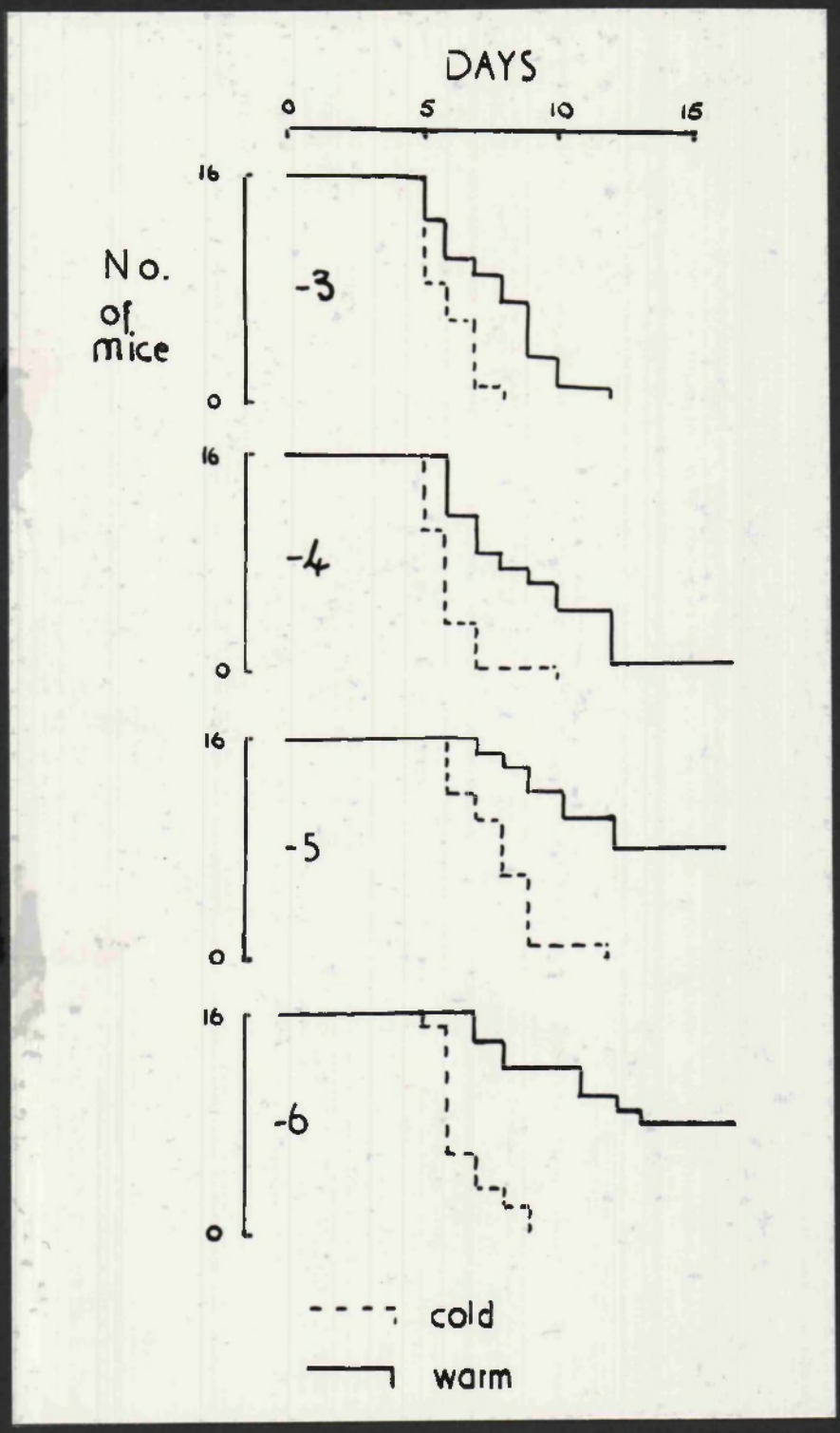

DAYS
0
5
10
15
No. of mice
16
0
-3
-4
-5
-6
cold
warm

Figure 19. - From the data of Experiments 10A (warm environment) and 10C (cold environment). Ordinates indicate the number of mice (out of 16) surviving at different periods of time after infection (abscissae). Large numerals indicate log. dose of virus.

EXPERIMENT 5B.

Object:- to show that the effect of the cold environment on the infectivity of ectromelia virus in mice can be prevented by the prior administration of specific immune serum.

Design. Two groups of ten mice were used. On the day before injection of the virus, one group received by intra-peritoneal injection 0.5 ml. of the hyperimmune vaccinia anti-serum that was employed in the neutralisation tests in chick embryos (see page 36). The other group was given the same amount of normal rabbit serum by the same route. Mice of both groups were infected by intra-peritoneal injection of 0.25 ml. of a 10^{-2} suspension of infective mouse liver. All the mice were then transferred to the cold environment.

Results. No deaths occurred during a period of one month following infection in the group given immune serum, whereas all ten mice pre-treated with normal rabbit serum succumbed within nine days following infection.

Interpretation. The increased mortality shown in Experiment 5 in the cold environment is specifically related to the virus used since deaths at this temperature can be prevented by the prior administration of immune serum.

EXPERIMENT 6

Object:- to examine the method of intravenous inoculation of the virus in the cold and the warm environments.

Design. The same pool of virus as that used in Experiment 5 was prepared and the experiment is essentially the same in that parallel titrations were carried out using groups of five mice and eight similar dilutions of virus in each of the environments, the only difference being that the virus was given intravenously in 0.2 ml. amounts.

Results. As in Experiment 5 a similarly striking difference was seen in the 50 per cent. lethal dose end-points. The graph of the results is shown in Figure 20 (page 66). The calculated titres were $10^{-6.17}$ in the cold environment and $10^{-3.57}$ in the warm environment. The actual results on which these figures are based are shown in the mortality table in the appendix (page 129).

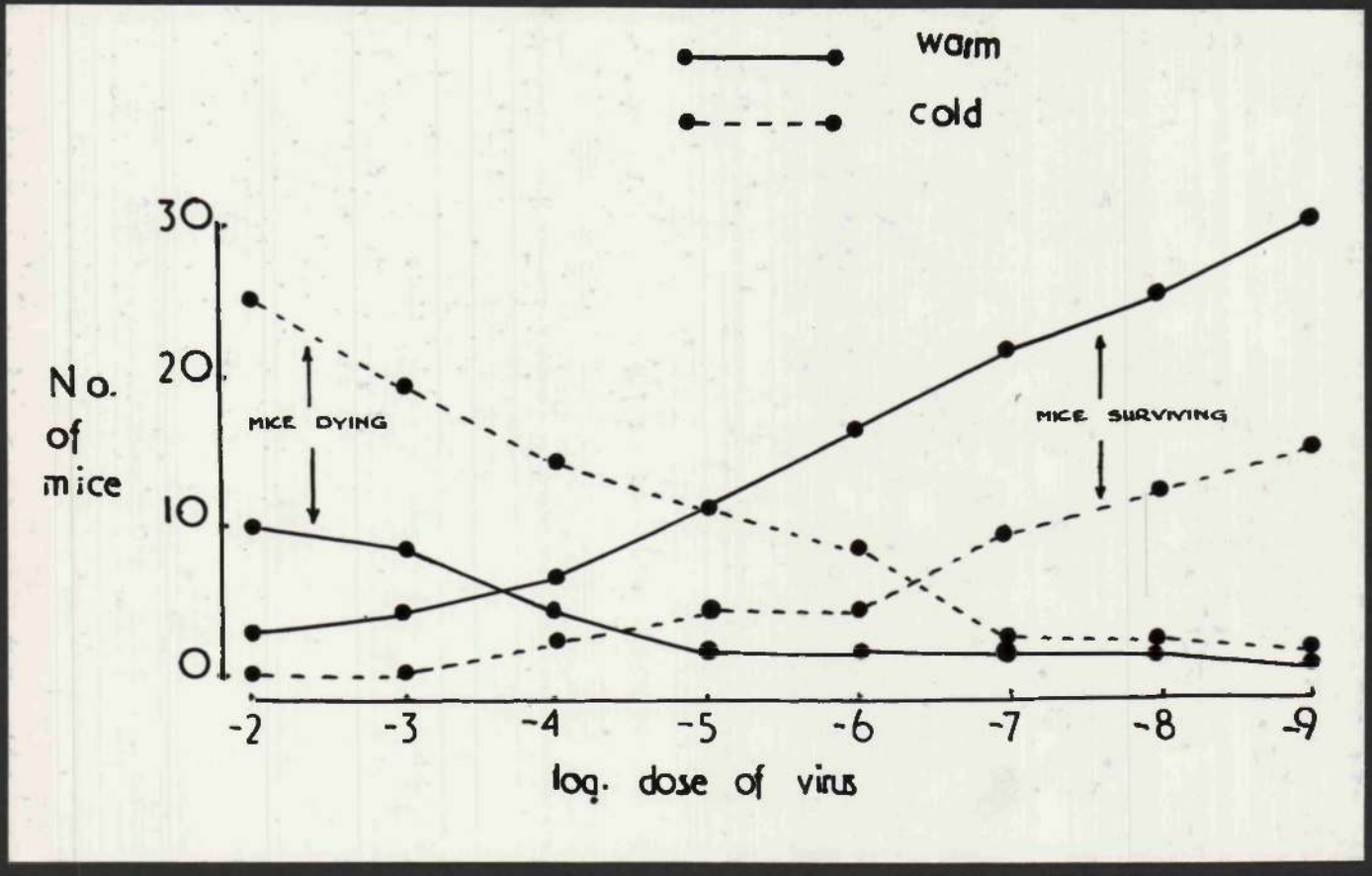

warm
cold
30
20
10
0
No. of mice
MICE DYING
MICE SURVIVING
-2
-3
-4
-5
-6
-7
-8
-9
log. dose of virus

Figure 20. - Graph of cumulative mortalities constructed from the results of Experiment 6. The 50% lethal dose end-point is shown in each case where like lines intersect. The actual mortalities on which this graph is based are shown in the appendix.

EXPERIMENT 7

Object:- to compare the susceptibility of groups of mice adapted from birth and unadapted to the cold environment.

Methods. Twelve adult females were mated and, when they proved to be pregnant, transferred to the cold environment. To provide for nesting the boxes were furnished with cotton wool which was removed when the litters were ten days old. In this way, thirtyfour animals were obtained from the litters born and were used for experiment when they were approximately five to six weeks old at which time their weights varied from 14 to 17 grammes. These are referred to as mice born and reared in the cold environment.

Design. Parallel titrations were carried out using these mice for one group. The other group consisted of mice of similar age and weight which had been born and reared at room temperature. The latter group of mice was exposed to the cold environment on the day of infection while the former group of mice was infected on the same day and returned to the cold environment immediately following the injection.

The pool of virus used was the same as that employed in Experiments 5 and 6. Five dilutions of virus were tested in groups of six mice in each case. The/

The injections were carried out intra-peritoneally using 0.25 ml. amounts. The remaining four mice which had been born and reared in the cold environment were not inoculated but were kept in the cold environment and appeared normal throughout the course of the experiment.

Results. The titres in each case were virtually the same. The calculated 50 per cent. lethal dose end-points were $10^{-6.28}$ and $10^{-6.17}$. The actual results on which these figures are based are shown in the mortality table in the appendix (page 130).

Interpretation. Mice born and reared in the cold environment are quite as susceptible to the enhancing effect of the the cold environment as mice exposed to that environment on the day of inoculation with the virus.

EXPERIMENT 8

Object:- to examine the effect of a smaller difference in environmental temperature than that used in the previous experiments.

Design. Three groups of fifty mice each were used. These were injected with the same pool of virus used in Experiments 5 and 6 diluted so as to kill only a small proportion of animals in the warm environment. The actual dose of virus being 0.25 ml. of a 10^{-6} dilution of infected liver given intraperitoneally.

Three environmental temperatures were studied i.e. 68° to 72°F., 54° to 58°F. and 43°F. As in Experiments 5 and 6 the mice were transferred from the stock animal house (the temperature of which varied from 68° to 74°F.) to the experimental environments on the same day as they were injected with the virus infected liver suspension.

Results. In the group kept at 68° to 72°F. nine of the fifty mice injected died; in the group kept at 54° to 58°F. sixteen mice died and in the group kept at 43°F. thirty-five of the mice died.

Interpretation. These results suggest a direct relationship between the percentage mortality and the degree of cooling to which the mice were exposed following infection. However there is no significant difference between a take of 9/50 as against a take of 16/50. On the other hand the difference/

difference between a take of 16/50 and a take of 35/50 is highly significant ($p<0.001$).

As has already been mentioned, the usual period of observation was three weeks for experiments of this type. The survivors from the warm environments (68° to 72°F. and 54° to 58°F.) in Experiment 8 which had survived for this period were seventy-five in number. These mice were collected and exposed to the cold environment for a further period of three weeks. No deaths occurred in these animals during this period of observation. It was thus desirable to examine the effect of exposure to the cold environment at shorter intervals following infection.

EXPERIMENT 9

Object:- to examine in more detail the time realtions of the effect demonstrated in Experiments 5 and 6.

Design. The same dose of virus was used as in Experiment 8 i.e. 0.25 ml. intra-peritoneally of a 10^{-6} dilution. Two groups of twenty mice were used. One of these, group A, was kept in the warm environment for fourteen days before the day of inoculation, when it was transferred to the cold environment. The other, group B, was kept in the cold environment for fourteen days before the day of inoculation, when it was transferred to the warm environment.

Results. In group A eighteen of the twenty mice injected died whereas in group B only three mice succumbed. From these results it appears that exposure to the cold environment prior to infection with the virus is ineffective.

The effect of exposure to the cold environment at shorter periods after infection was next investigated.

Design. Groups of twenty mice were used and these were injected with the same dose of virus as in Experiment 10A and 10B. Three such groups were kept in the warm environment and infected on the same day. The first group (group C) was transferred to the cold environment on the third day following infection/

infection. The second group (group D) was transferred on the sixth day following infection and the third group (group E) on the ninth day following infection.

Results. In group C fourteen of the mice died within a period of three weeks, whereas in groups D and E only one mouse and four mice, respectively, died during the same length of time.

Interpretation. There is no significant difference between the takes of 1, 3 or 4 our of 20, (groups 9D, 9B and 9E) or between takes of 14 and 18 out of 20, (groups 9C and 9A). On the other hand, the difference between a take of 14/20 and 4/20 is highly significant ($p < 0.01$). From these results it is clear that mice must be present in the cold environment at least during the first three days following infection and that exposure on, or after, the sixth post-infection day is ineffective, at any rate with the prescribed dose of virus used.

EXPERIMENT 10

Object:- to examine the effect of the administration of cortisone on the infectivity of the virus. Cortisone is know to affect the outcome of many infections. It also seemed likely that the effect of exposing mice to a cold environment might be that of a stress producing agent, causing excessive secretion of adreno-cortical steroids. It is known, however, that while injected cortisone will enhance several infections in mice, stimulation of the mouse's own adreno-cortical function with injected ACTH is not usually effective (Southam and Babcock, 1951; Kass et al., 1954).

Methods. Cortisone acetate was used ("Cortone", Merck) and this was given daily by intramuscular injection. From the figures of Patrick (1955) a dose of 2 mg. per 100 gms. body weight per day was selected for the present purpose. Immediately before use, the suspension was diluted with nine times its volume of normal saline solution and hormonal treatment was begun one day before administration of the virus.

Design. Groups of sixteen mice were used in each case. In Experiments 10A, 10B and 10C four dilutions of virus were administered intra-peritoneally in the usual way, the actual dilutions being 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. In Experiment 10D one group of sixteen mice was given the hormone but no virus.

Experiment 10A. Four groups of sixteen mice were given virus and maintained in the warm environment thereafter.

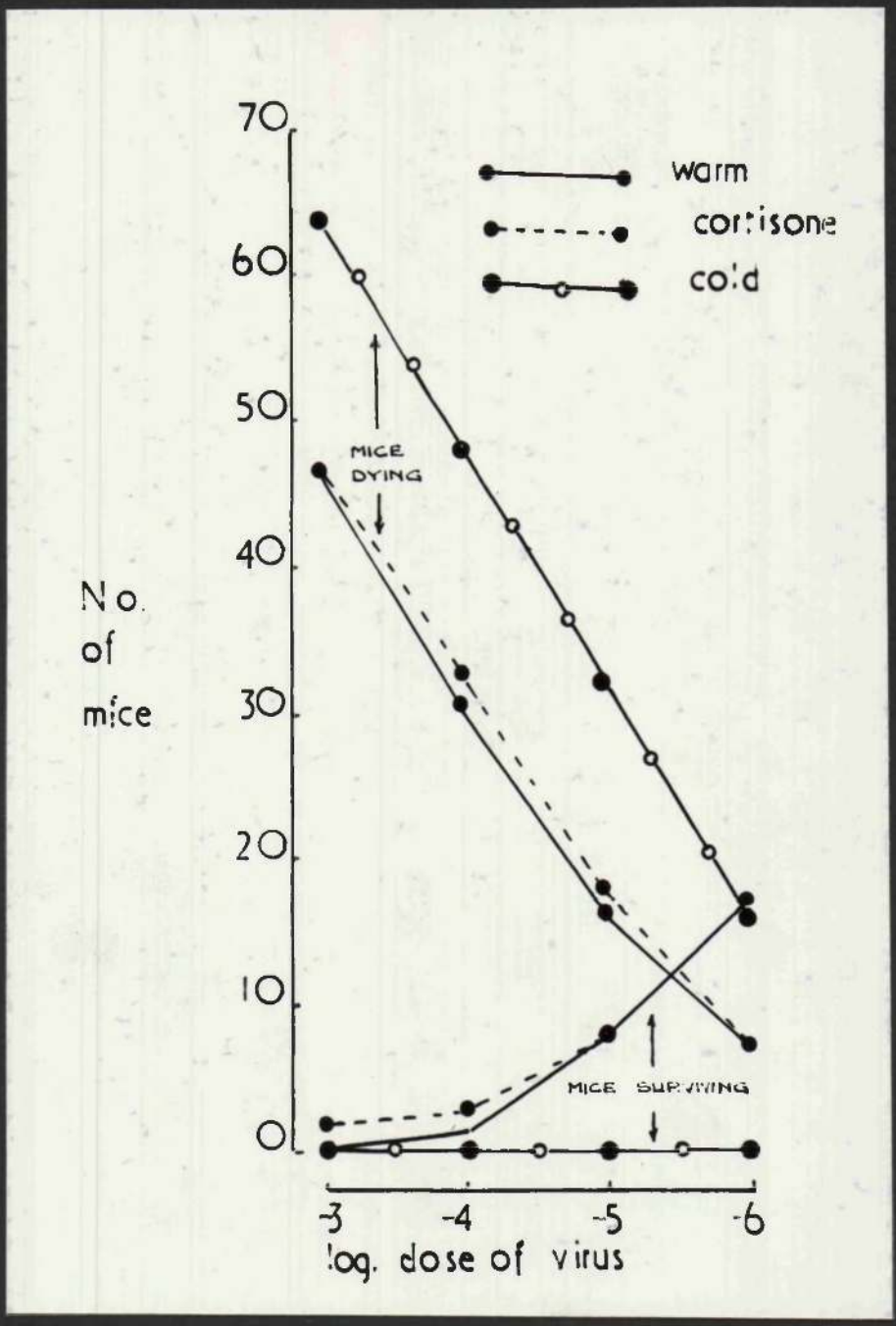
Experiment 10B. Four groups of sixteen mice were given cortisone daily, the injections being started on the day prior to infection. Administration of the virus was carried out on the same day as in Experiment 10A and the mice were kept thereafter in the warm environment.

Experiment 10C. Four groups of sixteen mice were given virus and then exposed to the cold environment, the infection being carried out on the same day as in Experiments 10A and 10B.

Results. It should be emphasised that the mice used in this experiment were given cortisone up to the limit of their tolerance, i.e. towards the end of the duration of the experiment, from the tenth day onwards, the well known signs of cortisone overdosage were apparent (pendulous abdomen, atrophy of the muscles of the back, etc.). These signs were evident in the control group (Experiment 10D) given hormone alone, as well as in the survivors of the experimental group (Experiment 10B).

The results of the titration are shown in the form of a graph (Figure 21, page 75) from which it can be seen that there is no significant difference in titre between mice kept in the warm environment and those kept in the warm environment and given cortisone.

Figure 21. - Graph of cumulative mortalities constructed from the results of Experiment 10. The 50% lethal dose end-point shown in each case where like lines intersect. The actual mortalities on which this graph is based are shown in the appendix. The 50% lethal dose end-point in the cold environment is not included within the dose range used here but is obviously greater than 10^{-6}.


70
60
50
40
30
20
10
0
No. of mice
warm
cortisone
cold
MICE DYING
MICE SURVIVING
-3
-4
-5
-6
log. dose of virus

EXPERIMENT 11

Object:- to examine the effect of the cold environment on the growth rate of normal mice. In preceding experiments it had been noted that mice kept in control groups in the cold environment appeared to eat more of the diet offered than those kept in the warm environment. In order to check this observation the following test was carried out.

Method. Twenty mice were used. They were of the same age and approximately of the same weight as those used in the preceding experiments, i.e. recently weaned animals weighing about 16 gms. They were housed in the usual boxes in groups of five and each group was weighed once daily during the experiment. Ten mice were maintained in the warm environment for a period of ten days, while the remaining ten animals were kept in the cold environment. These animals were not infected and appeared normal throughout. This period of observation was selected since it included the time interval during which the enhancing effect of the cold environment on the infectivity of the virus had been demonstrated. The usual diet in the form of cubes was offered (Diet 41) but the amount given to each group was weighed. No attempt was made to restrict the food or water intake.

Results. The results are shown in the form of a graph (Figure 22, page 78) which relates the food intake/

intake to the growth curves in the two environments. It can be seen that while the rate of weight gain is less in the cold environment, the amount of food eaten is slightly greater. This suggests that the metabolic rate of mice in the cold environment is much increased. It is probable that this is due to increased thyroid activity under these circumstances.

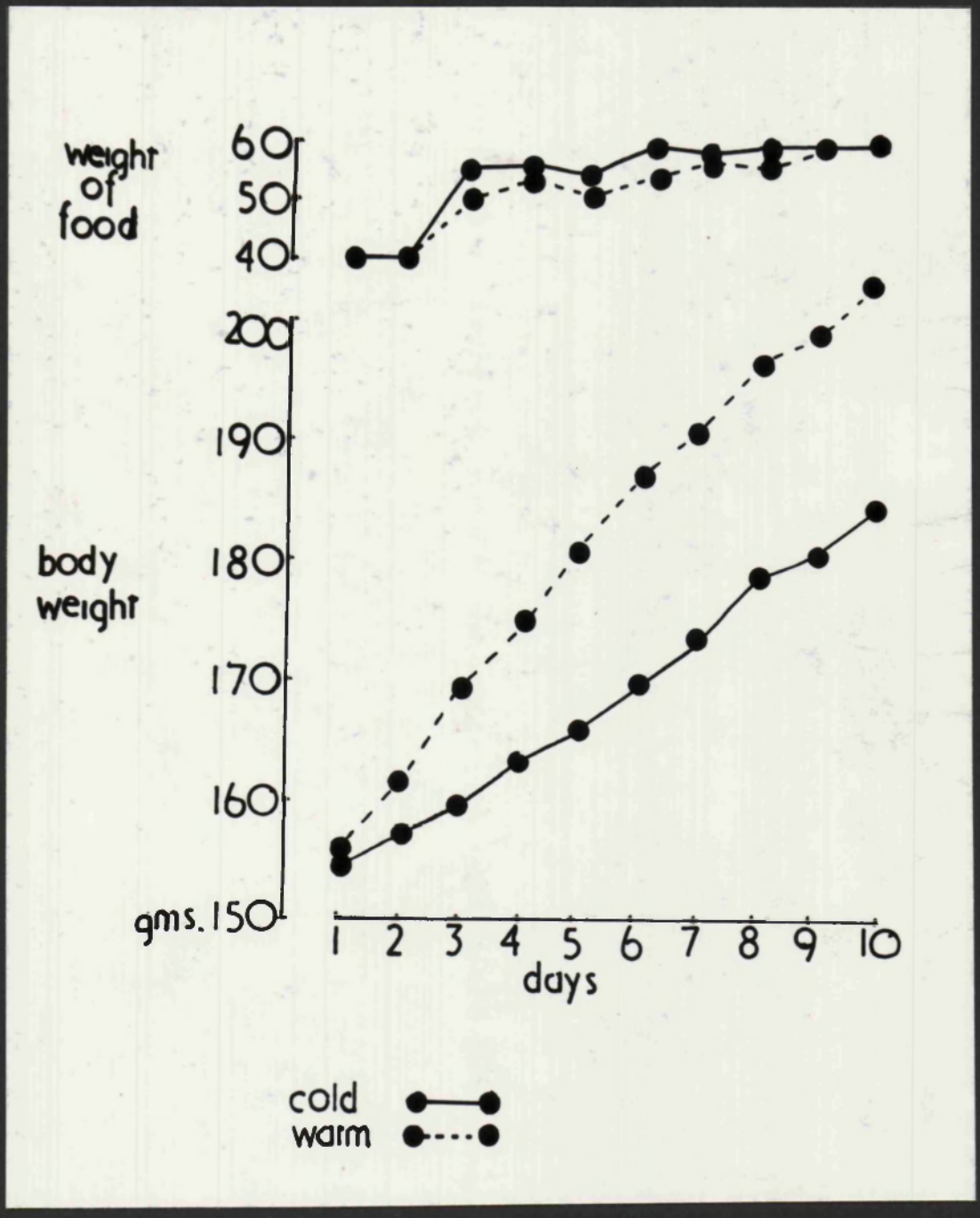

weight of food
60
50
40
200
190
body weight
180
170
160
gms. 150
1 2 3 4 5 6 7 8 9 10
days
cold
warm

Figure 22. - From the results of Experiment 11 the ordinates in the upper part of the figure indicate the amounts of food eaten by groups of ten mice on each succeeding day (abscissae) after exposure to the experimental environments. The lower part of the figure shows a graph of the weight gains for each group of ten mice (ordinates) on each succeeding day (abscissae) after exposure to the experimental environments.

EXPERIMENT 12

Object:- to examine the effect of thyroid extract on the infectivity of the virus.

Design and Methods. Three parallel titrations of infectivity were carried out. In Experiments 12A, 12B and 12C, eight tenfold dilutions of virus were injected intraperitoneally in the usual way, the actual dilutions being from 10^{-2} to 10^{-9}. Groups of six mice were used in each case. In Experiment 12D one group of twelve mice was given hormone without virus.

Experiment 12A. This experiment was carried out as above, the mice being kept in a warm environment. The animals received 0.8 per cent. saline solution as drinking fluid.

Experiment 12B. This group was also maintained in the warm environment. They received a solution (also in 0.8 per cent. NaCl) of 0.05 per cent. thyroid extract B.P. The thyroid extract used was stated to contain not less than 0.09 per cent. and not more than 0.11 per cent. iodine. This concentration was selected on the basis of the work of Dubos (1955) who used thyroid extract in the manner described here and showed that mice thus treated succumbed more quickly than controls when infected with six strains of the tubercle bacillus.

Experiment 12C. In this case the titration was carried out in mice maintained in the cold environment. They/

They also received 0.8 per cent. saline solution as drinking fluid.

Experiment 12D. This consisted of a group of twelve mice which were kept in the warm environment and received a solution of 0.05 per cent. thyroid extract B.P. in 0.8 per cent. saline solution as drinking fluid. These animals were not injected with virus and no deaths occurred in this group.

Results. The cumulative mortalities are shown in the form of a graph (Figure 23, page 81), from which it can be seen that the addition of thyroid extract in this way appeared to enhance the titre of the virus. The calculated LD50 in the warm environment (Experiment 12A) being $10^{-4.17}$ while that in the warm environment with the addition of thyroid extract was $10^{-5.4}$. The mortality in Experiment 12C fell outwith the range of the LD50 as can be seen from the graph.

The difference between an LD50 of $10^{-4.17}$ and and LD50 of $10^{-5.4}$ is not in itself statistically significant, and in any case does not approach that produced by the same dosages of virus in the cold environment.

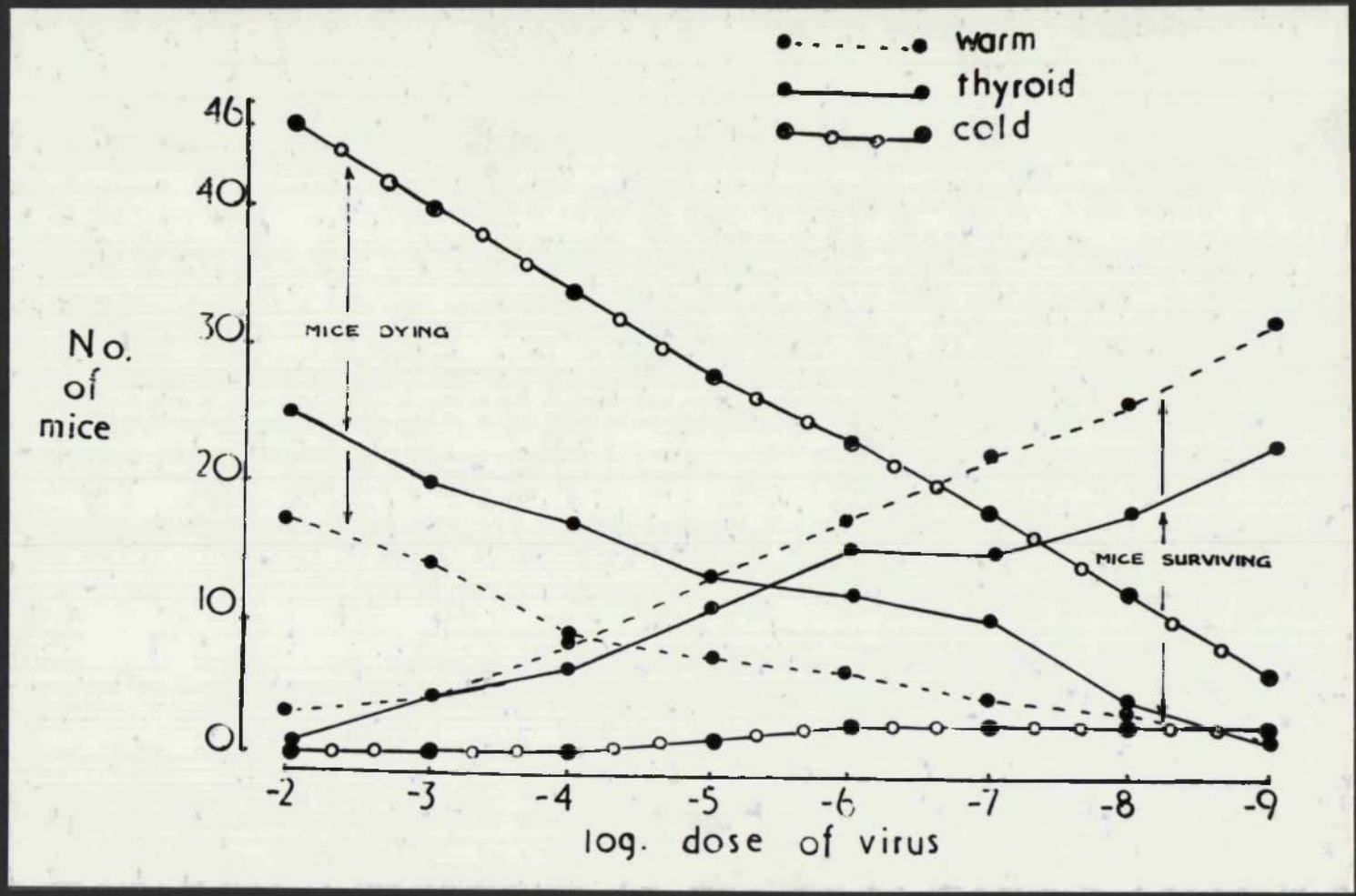

warm
thyroid
cold
46
40
30
20
10
0
No. of mice
MICE DYING
MICE SURVIVING
-2
-3
-4
-5
-6
-7
-8
-9
log. dose of virus

Figure 23. - Graph of cumulative mortalities constructed from the results of Experiment 12. The 50% lethal dose end-points are shown in each case where like lines intersect. The actual mortalities on which this graph is based are shown in the appendix, page 130.

THE INFECTIVITY OF LIVER SUSPENSIONS

The apparent enhancing effect of the cold environment may be due to one or both of two main causes, namely:-

(a) an increased susceptibility to the action of the virus in mice kept in the cold conditions or

(b) an increased growth of virus in the mice kept at the lower temperature.

To distinguish between those possibilities, the following experiment was carried out.

Methods. Two groups of five mice were each injected with the same amount of virus (0.25 ml.) of a 10^{-6} dilution of mouse liver suspension. This dose was selected to show clearly the differences already described. One of these groups was transferred to the cold environment (approximately 43°F.) following injection, while the other was kept in the warm environment (approximately 75°F.). On the fifth day following infection three of the mice in the cold environment were moribund and these were sacrificed by cervical dislocation. Although none of the mice in the warm environment showed obvious signs of illness at this time, three of them were sacrificed. The livers of both groups were collected with sterile precautions and weighed individually as described in Section 1. The three livers from the mice in the cold environment were pooled/

pooled and ground together with sterile sand in a mortar. The tissue was then suspended in 10 per cent. horse serum broth to form a 10 per cent. (w/v) suspension. The three livers from the mice kept in the warm environment were similarly treated. The suspensions so prepared were then titrated in mice using the Reed and Muench (1938) method of calculating the LD50. For this titration six groups of five mice were used in each case and these animals were infected intraperitoneally using six tenfold dilutions of the initial liver suspension. They were maintained in the cold environment following infection.

Results. The LD50 of the mouse liver suspension from animals maintained in the warm environment following infection was in fact higher (LD50 = $10^{-6.4}$) than that in the mice kept in the cold environment following infection (LD50= $10^{-5.8}$).

A second experiment was carried out using the same methods as those above described. Two groups of mice (three mice in each group) were injected with the same amount of virus (0.25 ml. of a 10^{-6} dilution of mouse liver suspension). One group of three mice was transferred to the cold environment following infection, while the other group was kept in the warm environment. One mouse of each group was sacrificed on the second day after infection. The livers were collected with sterile precautions and weighed. They were then transferred to a deep-freeze/

deep-freeze cabinet at -20°C. The same procedure was followed with one mouse of each group on the third and fourth days following infection.

10 per cent. (w/v) suspensions in 10 per cent. horse serum, prepared from the livers of each mouse sacrificed on the second day after infection, were titrated in mice as before.

Similar suspensions from the livers of each mouse sacrificed on the third day after infection were titrated in mice after one month's storage in the deep-freeze cabinet at -20°C.

The suspensions of the livers of each mouse sacrificed on the fourth day after infection were similarly prepared and titrated in mice after ten weeks' storage at -20°C.

Results. The results of these titrations are shown in the accompanying table (Figure 24, page 85) from which it can be seen that the titres of virus in the livers of mice kept in the cold environment and sacrificed on the second, third or fourth day following infection appear to be slightly higher in each case than the corresponding titres from the livers of mice kept in the warm environment. It should be pointed out, however, that in no case is the difference statistically significant.

Figure 24.

Number of mice	Origin of suspension	Length of storage (-20°C.)	LD50
1	2nd day (cold)	7 days	$10^{-3.2}$
1	2nd day (warm)	7 days	$10^{-2.8}$
1	3rd day (cold)	1 month	$10^{-4.1}$
1	3rd day (warm)	1 month	$10^{-3.9}$
1	4th day (cold)	10 weeks	$10^{-4.8}$
1	4th day (warm)	10 weeks	$10^{-4.2}$
3	5th day (cold)	not stored	$10^{-5.8}$
3	5th day (warm)	not stored	$10^{-6.4}$

All titrations were carried out in mice kept in the cold environment using six dilutions of the initial liver suspensions. Five mice were used to test each dilution.

Examination for E. coccoides

The L component of MHV, originally thought to be a virus, was identified by Niven, Gledhill, Dick and Andrewes (1952), as the mouse parasite, Eperythrozoon coccoides, which is filterable and occurs naturally in some strains of mice. Transmission of the parasite to non-carriers is readily accomplished by intra-peritoneal injection of blood. Detection of the organism in carrier mice is favoured by splenectomy which is followed, in three or four days, by the appearance of Eperythrozoon coccoides in the peripheral blood where it can be demonstrated in films stained by Giemsa. Infected mice do not show any signs of illness or of specific post-mortem changes.

Since the enhancing effect of a cold environment on the infective titre of the virus described in this thesis was of the same order as that shown by Gledhill et al. (1955) for E. coccoides on the titre of MHV in weanling mice, it was of particular importance to examine the strain of mice used in the present work for the presence of E. coccoides.

Blood films showing numerous and distinctive organisms were obtained from Dr. Janet S. Niven, of the National Institute for Medical Research, Mill Hill, London.

Attempts were made to demonstrate the organism in Giemsa-stained films of peripheral blood taken daily/

daily from the caudal veins of mice treated in the manner described below:-

In each case the thinnest films were selected and were allowed to dry in air. They were then fixed for one minute in methyl alcohol and stained overnight in a Coplin jar containing a 1 in 50 dilution of Giemsa's stain.* They were next differentiated in distilled water, blotted, allowed to dry and examined under the oil-immersion lens.

Blood films made in this way were examined from:-

(a) five mice which had been splenectomised four days previously.

(b) at least ten mice moribund after infection with the unknown virus and kept thereafter in a cold environment.

(c) at least ten mice moribund after infection with the unknown virus and kept thereafter in a warm environment.

In no case was it possible to demonstrate the occurrence of parasites resembling E. coccoides, although an occasional erythrocyte showing either punctate or diffuse basophilia was quite often seen and in these cases particularly thorough search was made.

* Messrs. Hopkins and Williams.

POST-MORTEM AND HISTOLOGICAL EXAMINATIONS

The histological changes occurring in ectromelia are well documented. Comprehensive accounts of these changes have been given by Marchal (1930) and Fenner (1949). It should be stressed, however, that those descriptions are largely based on the disease produced after intradermal inoculation that is indistinguishable from naturally acquired mouse-pox. The present studies were carried out on mice infected by intraperitoneal injection of the virus and revealed a picture which differs considerably from that observed in the natural disease.

The appearances at necropsy have already been described (page 23) but it should be noted that fibrinous exudates or adhesions were never a feature. In this respect it is also important to observe that the survival times in fatal cases inoculated intraperitoneally are usually two or three days less than when the same dose of virus is inoculated intradermally.

Material and Methods. The following account of the histological changes is based on the examination of material from mice dying in Experiments 3 to 12. Necropsies were carried out on all mice which died in these experiments, but autolytic changes were often present so that material was selected for histological examination from only a proportion of the animals.

The fixative most used was alcohol-formalin (10 per cent. formalin in 60 per cent. alcohol) and sections were routinely stained by two methods (a) haematoxylin and eosin and (b) slow Giemsa. Other techniques used in selected cases were Heidenhain's iron haematoxylin for bile canaliculi and Gordon-Sweet's reticulin-impregnation method.

A general set of tissues taken from each animal included blocks of lung and heart muscle, small intestine and pancreas and kidneys with adrenal glands where possible. In addition sections of brain, skin and voluntary muscle were examined from ten animals in Experiment 3. In all cases (96 animals) at least two blocks of liver were sectioned and one block of spleen through its long axis.

Histological Findings. These can best be considered under three headings (a) changes in the liver (b) changes in the spleen and (c) changes in other organs.

(a) Changes in the liver were always present and the predominant microscopic feature was necrosis of the parenchymal cells. The distribution and extent of the necrotic process varied from many small irregular areas interspersed with apparently normal cells to nearly complete destruction of the parenchyma. This appearance is to some extent dependent on the dose used and hence the survival time.

Livers from mice in both environments which died during the first seven days following infection with the larger doses of virus (10^{-1} and 10^{-2}) usually showed very extensive hepatic necrosis. On the other hand, with intermediate doses of virus (10^{-4}, 10^{-5} and 10^{-6}) certain differences were noted in the two environments. In sixteen out of eighteen such mice in the warm environment the necrotic foci were small, irregular in size and of quite random distribution (see Figure 26, page 100). In marked contrast, extensive necrosis was present in the liver of twenty-two out of thirty mice that had been kept in the cold environment. Closer examination of these sections showed that the necrotic process did not involve the central veins. Although the appearances varied somewhat from mouse to mouse according to the proportion of the liver lobule involved, in general they were remarkably regular in any one liver (see Figure 27, page 101). Some difficulty was experienced in relating the distribution of the necrosis to the normal liver architecture. In cases where the lesions were most extensive, the central veins appeared dilated and the portal triads were themselves involved in the necrotic process (see Figure 28, page 102). The distribution is essentially periportal and this is most readily appreciated by comparison with sections of/

of mouse livers showing centrilobular necrosis such as that produced by poisoning with carbon tetrachloride.

In mice given the lowest doses of virus (10^{-8} and 10^{-9}) the distribution of the necrotic foci was quite haphazard although all of these animals died in the cold environment.

The appearance of the livers of mice pre-treated with thyroxine was essentially similar to that seen in mice kept in the warm environment.

Massive areas of necrosis were encountered in six livers from mice pre-treated with cortisone. These had no relationship to the normal liver architecture, the necrosis having extended to include complete lobules in which no surviving parenchymal cells could be demonstrated (see Figure 31, page 103).

While parenchymal necrosis was by far the most striking feature of the disease process in the liver, other changes were also present and were encountered in the livers of animals from either environment. Groups of infiltrating cells, having the appearance of lymphocytes, were often seen around the portal tracts and in many of the sinusoids. The significance of this is hard to assess since portal accumulations of round cells occurred in the control groups although the cells were much fewer in number. In addition, in livers showing extensive necrosis/

necrosis the sinusoids often appeared grossly dilated and pools of red blood corpuscles were seen in the spaces thus formed. The appearance is somewhat similar to that seen in bovine telangiectasis (see Figure 30, page 103). In sections stained by Gordon-Sweet's method it was not possible to demonstrate deviation from the normal reticulin pattern even in severely affected livers. Six out of ten livers from mice dying after the eighth day showed evidence of regeneration in the form of hyperplasia of bile duct epithelium and multinucleated parenchymal cells. These changes were most obvious at the periphery of the lobules and only occasionally small groups of lymphocytes were seen intralobularly in these cases. In one case several small hyaline connective tissue scars were seen and this liver also showed evidence of repair (see Figure 32, page 104 and Figure 33, page 105).

(b) Changes in the spleen have already been described (see page 25). No qualitative differences were noted in the spleens of mice dying in either environment and it was not possible to relate the extent of the necrotic foci in the spleens to the degree of hepatic necrosis. In six out of thirty mice, which died in the cold environment and showed extensive hepatic necrosis, examination of a longitudinal section of spleen failed to reveal the presence of obvious necrosis. On the other hand,

hepatic/

hepatic necroses were present in all the mice (96) which succumbed and were examined histologically.

(c) Changes in organs - other than liver and spleen. No significant changes were noted in sections of brain or of voluntary muscle (10 animals). Cytoplasmic inclusions of the type described by Marchal (1930) were never encountered in the viscera. The intestines were often congested and the lymphoid follicles appeared hyperplastic. The kidneys and lungs occasionally showed small discrete haemorrhagic foci. The mesenteric lymph nodes were not specifically collected but were seen to contain small areas of necrosis in two out of five sections in which they were included.

Summary. Various tissues are affected following intraperitoneal administration of the virus of ectromelia in mice. In the present series the liver was the only organ which consistently showed necrosis in animals dying of the disease. The findings in respect of the hepatic necrosis can be summarised as follows:- with the highest doses of virus used the lesion is extensive in mice kept at both environmental temperatures. Intermediate doses of virus give rise to lesions which differ, being in the majority of cases focal and haphazard in the warm environment and extensive and periportal in the cold environment. The lowest doses of virus give rise to focal and haphazard necroses even in the cold environment.

It was evident from the histological examination of the tissues of dead and moribund mice, which has been summarised on the preceding page, that differences did occur in the liver lesions particularly in respect of the histological distribution of the hepatic necrosis. However, in view of the fact that post-mortem autolysis is known to produce apparent enhancement of the degree of necrosis existing before death, and that agonal changes may also influence the appearance (van Beek and Haex, 1943; Popper, 1948) it was necessary to confirm the description of the hepatic lesions by examining the tissues of mice which had been sacrificed for this purpose.

De Burgh (1950)and Nossal and de Burgh (1953 and 1954) have given a very full account of the changes induced by the virus of ectromelia and of the associated cytochemical changes. The methods used in the present study are essentially those described by the above authors.

Material and Methods. Stock virus of the same suspension as that prepared for Experiment 8 was used. The material had been stored in suspension in a deep-freeze cabinet at -20°C. during the interval before its use in this experiment. The inoculation was carried out intraperitoneally using 0.1 ml. of 10^{-5} suspension (which was approximately the LD50 of the suspension as measured in mice kept in the warm environment).

The mice were killed by cervical dislocation. The livers were rapidly removed and pieces fixed in cold acetone at 0°C. Sections were made and stained for acid and alkaline phosphatase according to the method of Gomori. The remainder of the liver and a selection of other tissues, including spleen, kidney, pancreas, intestine, lung, cardiac and voluntary muscles were fixed in alcohol-formalin (10 per cent. formalin in 60 per cent. alcohol). This solution was also used for blocks from which serial sections of the thyroid region were made from each mouse and it was usually possible to demonstrate thyroid tissue. Serial sections of the acetone fixed blocks were stained by Mayer's acid haemalum with or without eosin and for glycogen using the periodic acid-Feulgen method of McManus (1948), as well as the phosphatase methods. Inorganic phosphate was demonstrated by treating the deparaffinised sections with the cobalt nitrate and ammonium sulphide solutions of the Gomori method.

To demonstrate ability to form and store glycogen a proportion of the mice were given 0.5 ml. of 20 per cent. (w/v) glucose in water by intra-peritoneal injection. One hour later another glucose injection was given and the animals were killed one hour following the second injection. No preliminary fasting/

fasting was used lest this in itself should interfere with the development of the liver lesion.

In addition to the stains already mentioned slow Giemsa and pyronin-methyl green were used.

Seventy-two mice in all were given virus. These were then divided into two equal groups one of which was placed in the cold environment and the other in the warm environment throughout the duration of the experiment. On the first, second and third days following infection two animals were sacrificed from each environment. One of these in each case received a prior injection of glucose as described above. On the fourth, fifth, sixth, seventh and eighth days following infection six animals were sacrificed from each group. Three of these received glucose prior to sacrifice in each case. In addition sixteen uninfected control mice were assigned to each experimental environment. Two control mice were sacrificed from each environment daily and one of these animals received glucose prior to sacrifice in each case.

Results. The post-mortem changes in these mice were similar to those already described. In both groups from the fifth day onwards the liver was seen to be swollen but mottling and petechial haemorrhages were more marked in most members of the cold group. No gross difference other than this was observed.

Histologically the earliest change was noted about the third day in the form of a patchy loss of cytoplasmic basophilia (Figure 24, page 99) and it seems likely that cells affected in this way later become necrotic. The nuclei of these cells often appear smaller and hyperchromatic and in some cases show actual karyorrhexis. The appearance is best seen in Giemsa-stained sections and occurred in material from mice in both environments. In P.A.S. stained sections there is a loss of glycogen from these areas and this is best observed in tissues from glucose-treated mice. The appearance, however, is never so clearly demarcated as is the loss of basophilia. This change is not seen in tissues from mice kept in the cold environment since these animals appear unable to store glycogen inasmuch as very small amounts are demonstrable in sections from such mice, even when these have not been injected with virus.

Following this lesion from the fifth day onwards there is an abrupt transition to frank necrosis with loss of cell boundaries, pyknosis and karyolysis. The distribution of the fully developed necrotic lesion is the same as that already described, i.e. the foci are haphazard in the warm group and show less tendency to the development of large areas of necrosis. By contrast, all the/

the liver sections from mice in the cold environment show a definite distribution of the necrotic process. Large confluent areas surrounding the portal triads are formed, in many cases extending well into the lobule. In two cases from mice sacrificed on the fifth day following infection numerous focal areas were found adjacent to the larger veins (Figure 26, page 100).

The phosphatase methods gave disappointing results in that the relation of enzyme activity to the developing lesions described by de Burgh (1950) could not be demonstrated.

No significant differences between the two environments were noted in the other tissues examined. In one animal from the warm environment sacrificed on the eighth day following infection massive areas of necrosis were present in the spleen while only minor degrees of portal lymphocytic infiltration were seen in the liver.

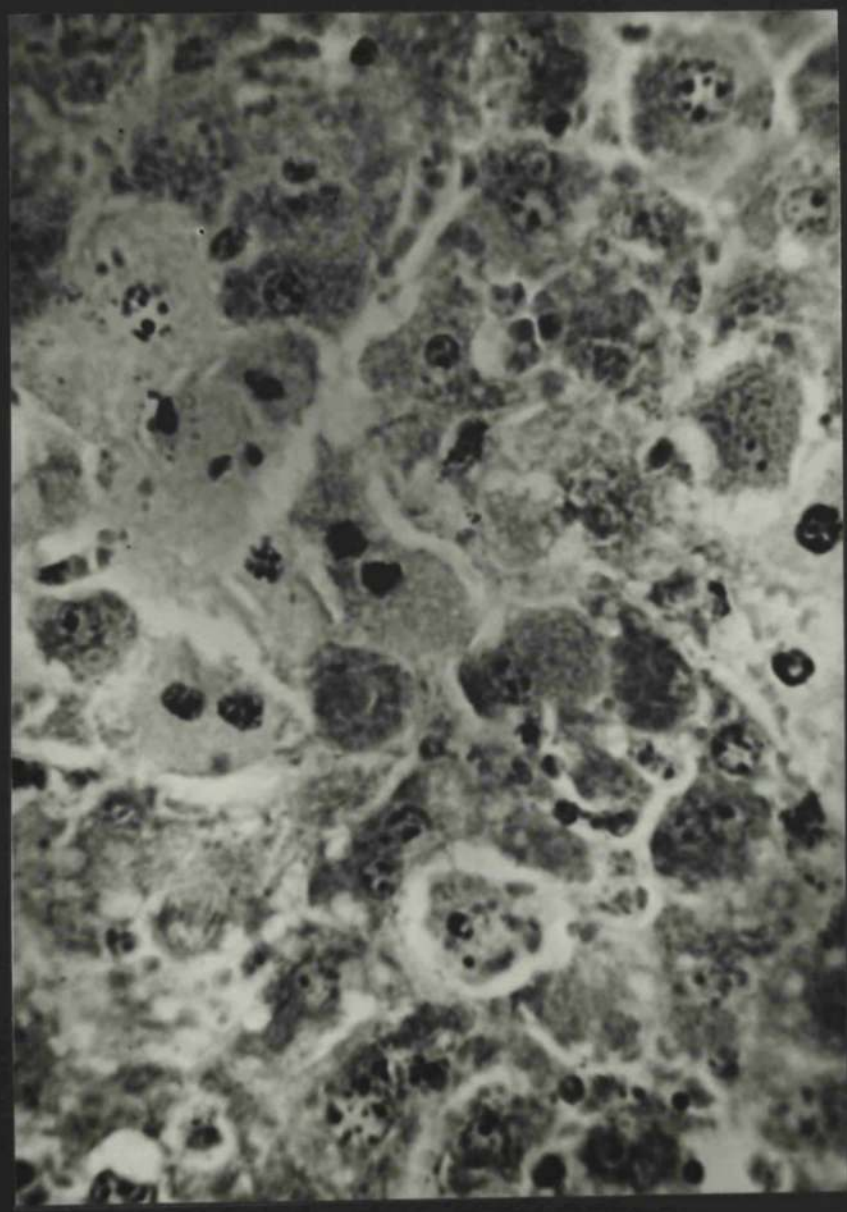
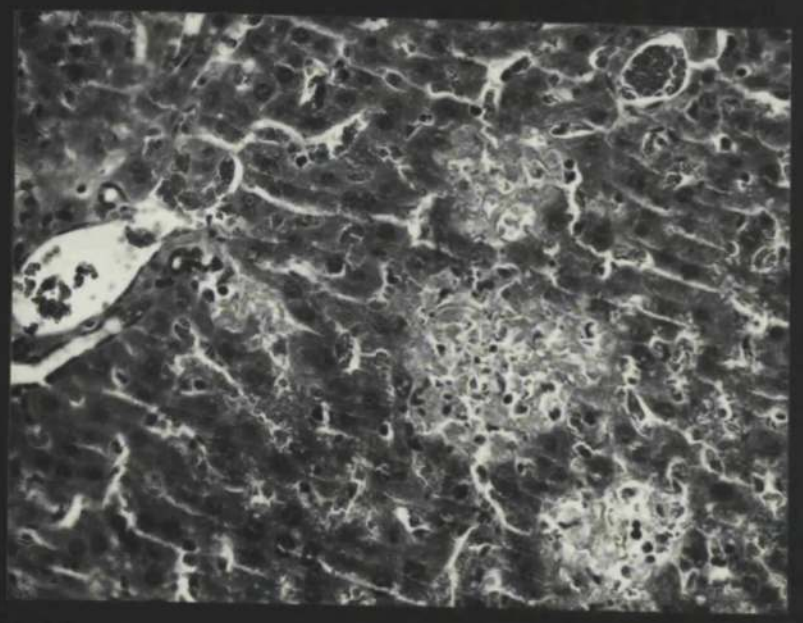


Figure 24. - Section of liver of mouse showing patchy loss of cytoplasmic basophilia.
Slow Giemsa. x 770.



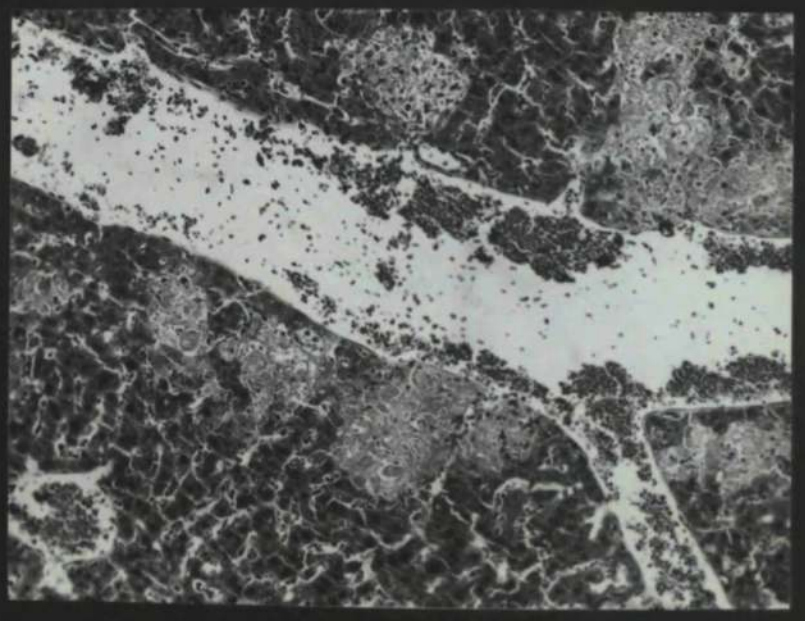


Figure 25. - Section of liver from a mouse in the cold environment showing the predominantly perivascular distribution of the foci of necrosis. Slow Giemsa. x 110.

Figure 26. - Section of liver from a mouse in the warm environment. The necrotic foci are essentially similar to those shown in Figure 25 except that they are random in distribution. Slow Giemsa. x 175.

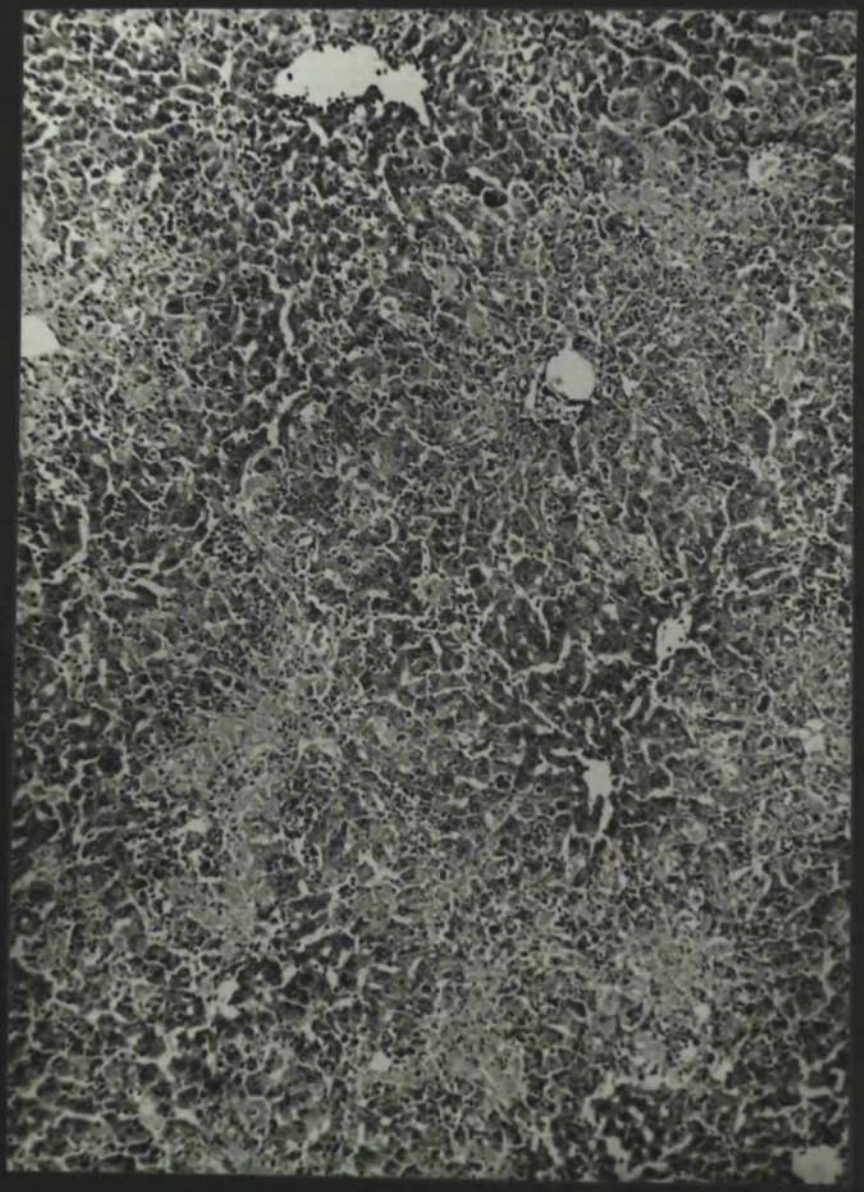
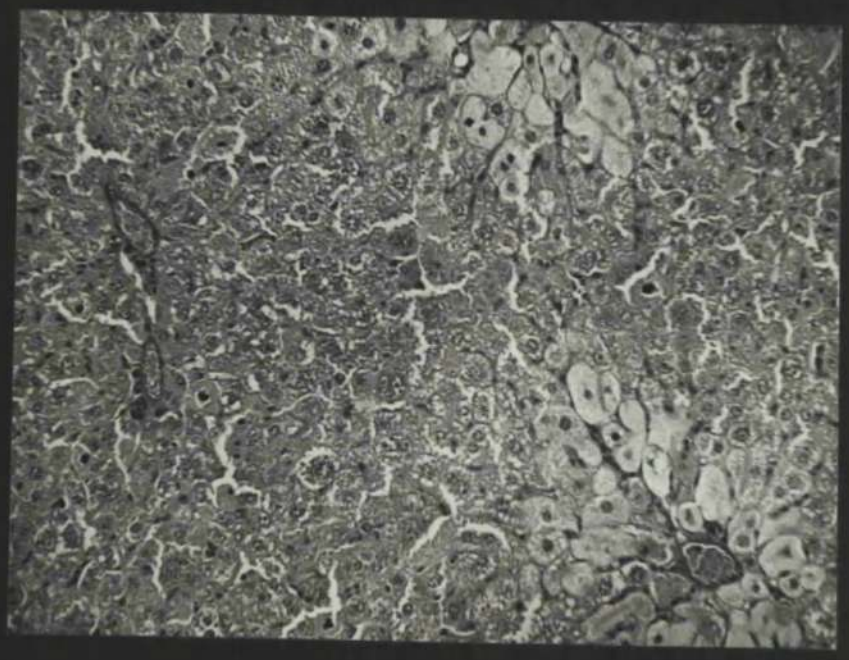


Figure 27. - Section of mouse liver from an animal dying in the cold environment. The darker staining areas of surviving cells regularly occur around the central veins.
Slow Giemsa. x 56.



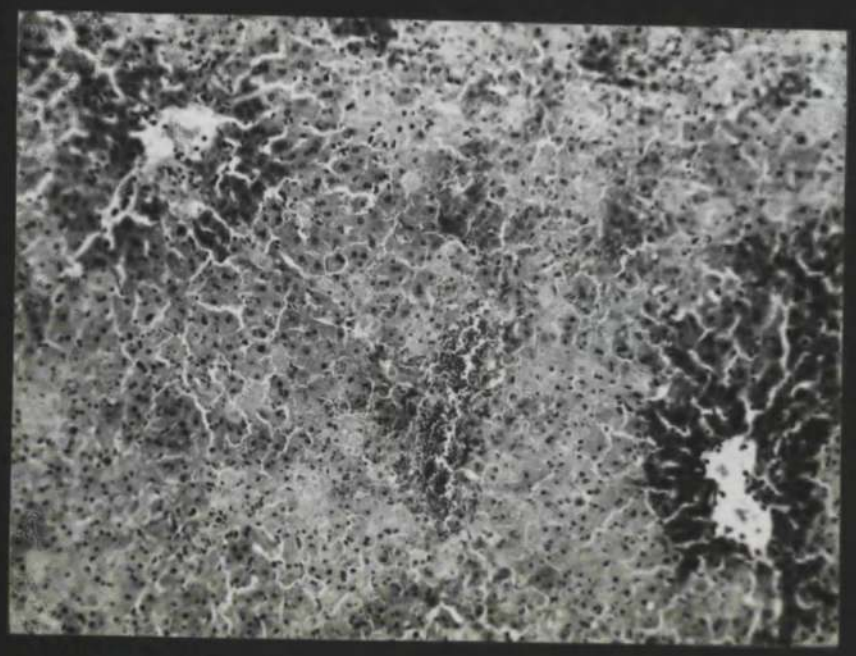
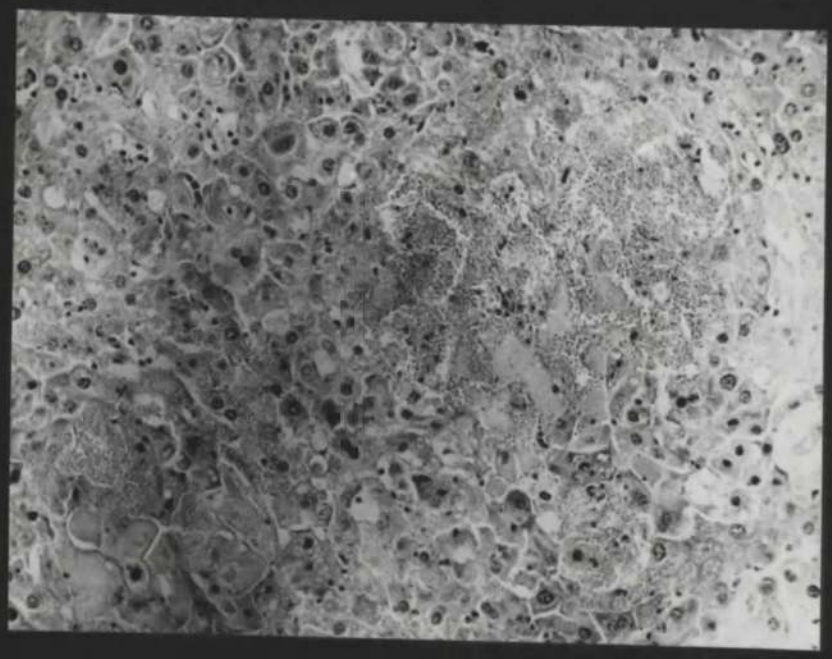


Figure 28. - Section of mouse liver showing the areas of surviving cells around two central veins which are grossly dilated. Between these the remains of a portal tract can be seen.
Slow Giemsa. x 110.

Figure 29. - Section of mouse liver following carbon tetrachloride poisoning. In this case the lesion is centrilobular and this Figure has been included for comparison with the above.
Haematoxylin and eosin. x 160.



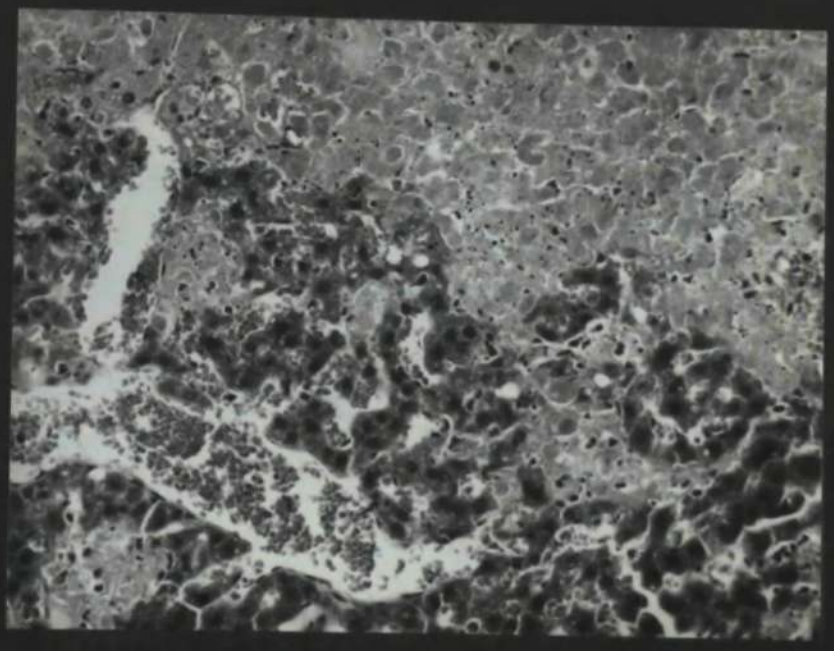


Figure 30. - Section of mouse liver showing dilation of sinusoids and "pooling" of blood at the margins of a necrotic area.
Haematoxylin and eosin. x 125.

Figure 31. - Section of liver of a mouse pre-treated with cortisone showing the margin of an area of "massive" necrosis.
Slow Giemsa. x 150.

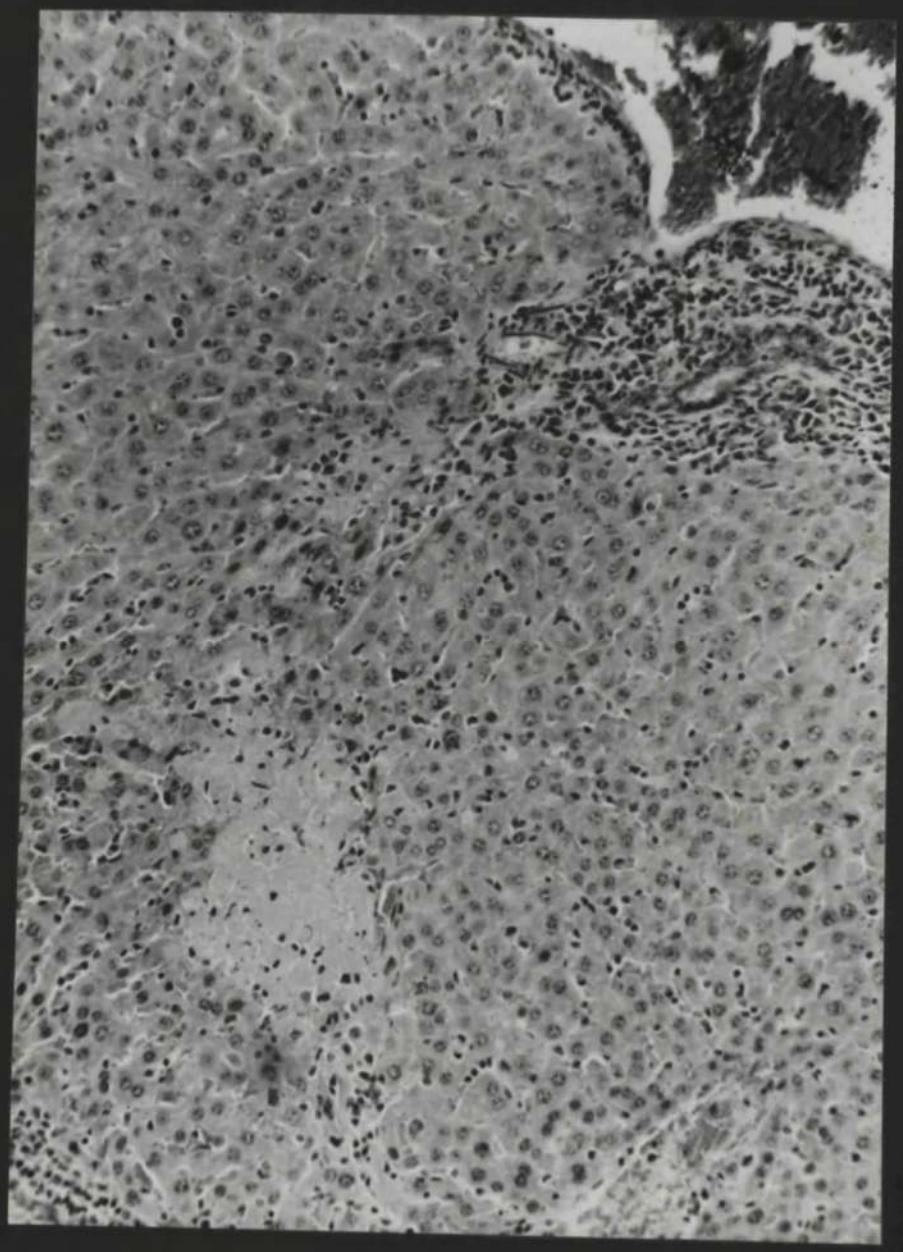


Figure 32. - Section of mouse liver showing an area of hyaline scar-tissue. There is evidence of bile-duct hyperplasia in the upper part of the print.

Haematoxylin and eosin. x 175.

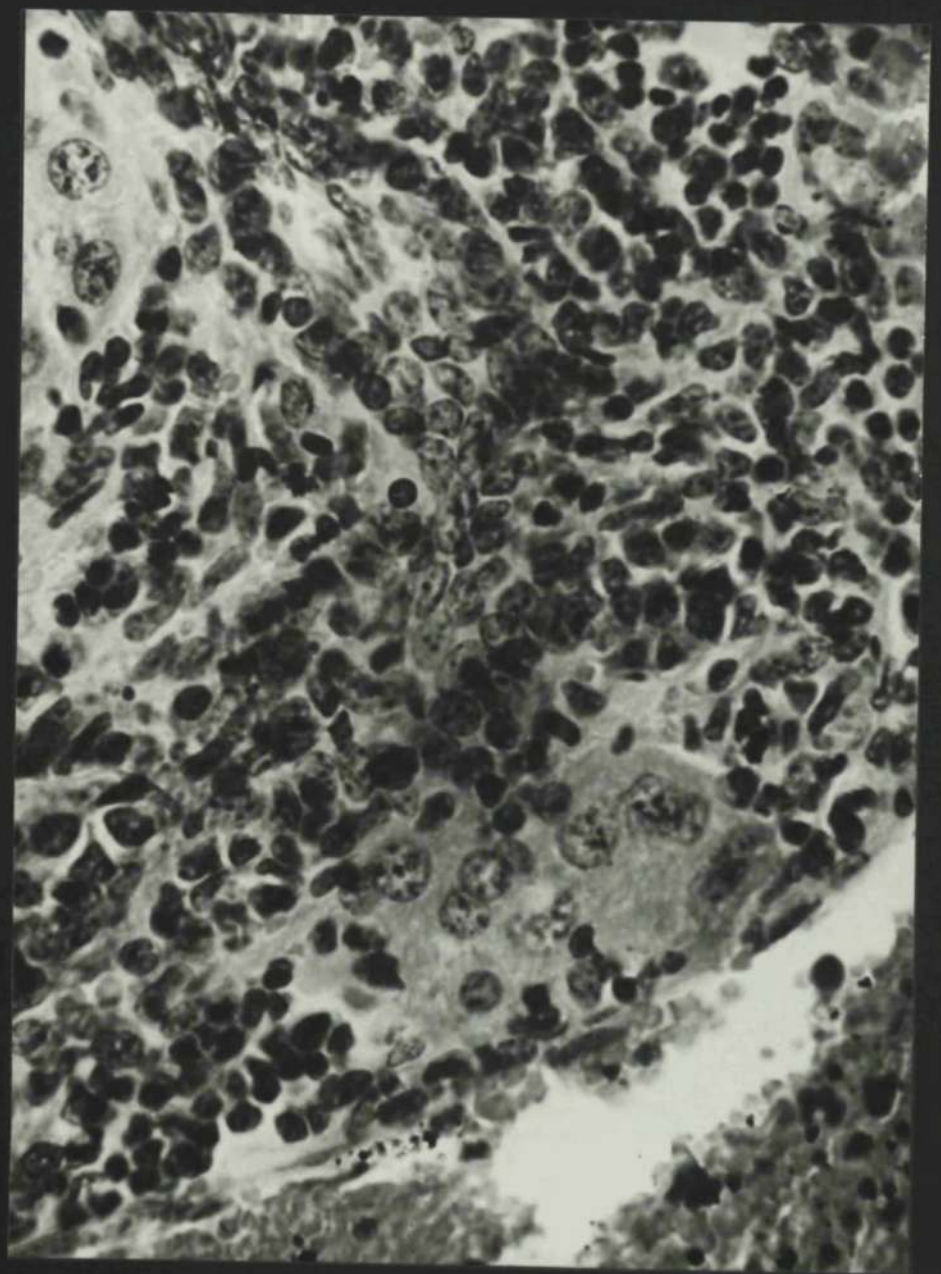


Figure 33. - Section of mouse liver in an area of regeneration showing multi-nucleated parenchymal cells interspersed with hyperplastic bile-duct epithelium.

Haematoxylin and eosin. x 700.

DISCUSSION

It is clear from the work of Gyorgy *et al.* (1950) and Naftalin (1954) that a pure deficiency of systine and vitamin E is not in itself sufficient to produce the condition of massive hepatic necrosis in rats. Nevertheless many authors have attempted to explain similar conditions occurring naturally in other species on the basis of such dietary deficiency.

One of the main difficulties in dietary experiments is that synthetic diets are never completely satisfactory even when complex mixtures of vitamins and amino-acids are included with the basic components. For this reason comparisons between synthetic diets and "natural" diets are of doubtful value.

The particular interest of the findings here reported lies in the fact that the diets used were similar in every respect except in the source of casein used. One of these diets, containing Genatosan "low vitamin" casein, proved quite ineffective in producing hepatic necrosis in rats but the other, containing Glaxo casein C, was highly effective. It can therefore be argued that if the factor(s) which produce the disease in rats are of importance in the development of the hepatic lesion produced by the virus in mice, this should be clearly shown by using these two diets and comparing their effects. Within the limitations of the techniques used here no/

no difference could be shown between the two diets, or between the high and low protein versions of these. It must be admitted, however, that the possibility of such a dietary effect was not disproved since it has been shown with at least two host-virus systems that a single deficient diet constantly administered gives rise to a cyclic change in susceptibility (Sprunt and Flanigan,1956). In both of these examples the cycle is composed of a primary susceptibility increase, a secondary resistance increase and lastly another susceptibility increase. Such differing time relations were not studied since it appeared early in the course of the work reported that the environmental temperature played a large part in determining the outcome of the infection produced by the virus.

For the same reason no attempt was made to compare the effect of the virus on mice fed synthetic diets as opposed to those fed with stock cubes (Diet 41), nor was any restriction of food intake investigated although it is possible that either or both of these factors might be important in conditioning the lesions produced.

The effect of environmental temperature was proved first by demonstrating differing infectivities of the same virus suspension as titrated in mice kept at different temperatures and this criterion was used in an attempt to elucidate the mechanism of the effect.

There have been various reports of the effects of environmental temperature on other host-virus systems. In most cases cold has intensified the results of infection but, in some instances, it appears to have had a protective effect.

Sulkin (1945) showed that mice kept at high environmental temperatures (95°F.) showed less severe lung lesions than those kept a lower temperatures (60°F.), following experimental infection with influenza A virus. This was not, however, reflected in the mortality. His findings agree with the known epidemiology of human influenza.

Briody et al. (1953) showed that a strain of influenza could be more quickly mouse adapted in two strains of mice if these were kept at 5°C. than if kept at room temperature.

Milzer (1943) showed that chilling animals by cold water immersion up to six days after inoculation with the BK strain of polio virus significantly increased the proportion of severe cases of paralysis and Wolf (1935) was able to prevent the development of poliomyelitis in monkeys by inducing pyrexia one or two days after intracerebral inoculation of the virus.

Infection of mice with the virus of herpes simplex is almost always fatal if they are kept at 70° to 80°F. but it has been shown by Armstrong (1942) that/

that death can be largely prevented in mice kept at temperatures of approximately 98°F.

Moragues and Pinkerton (1944) showed that a uniformly fatal rickettsial peritonitis could be produced by intra-peritoneal injection of murine typhus rickettsiae in mice kept at temperatures ranging from 65° to 80°F. while with an environmental temperature of 85° to 98°F. a mortality of less than twenty five per cent. was observed.

On the other hand Griffin et al. (1954) showed that the mortality of mice infected with the virus of vesicular stomatitis was significantly lowered in animals kept at 8°C. than in mice kept at 27° or 35°C. It is of interest that acclimatisation to a low temperature prior to inoculation was essential to influence the survival rate favourably.

By far the most striking results however are those of Boring et al. (1956). These authors have shown that adult mice of the strain they use seldom suffered ill effects from even very large doses of the Conn.5 strain of coxsackie virus but that, if infected mice were kept at 4°C. following inoculation, the infection became uniformly lethal. Since cortisone is known also to produce a similar loss in resistance to this strain , it has been suggested that cold may act as a stress-producing agent, causing excessive secretion of adreno-cortical steroids.

It was for this reason that an attempt was made in the present study to show an enhancing effect with cortisone on the infection produced by the strain of ectromelia used. From the results of Experiment 10 it is clear that, while cortisone may alter the course of the disease, the effect is not nearly so marked as that of the cold environment as judged by increased infectivity, nor is the histological difference reproduced. Cortisone and ACTH have also been shown to influence the development of the liver lesion produced by poisoning with carbon tetrachloride (Patrick, 1955) and the dosage used in Experiment 10 was based on the findings of that author. Boring et al. (1956) also point out that the change in resistance due to a cold environment is unlikely to result from the non-specific effects of stress since the higher temperature (36°C.) they studied represented a severe stress but did not reduce resistance. Indeed, at this temperature deaths occurred after one week of exposure, in the control groups not given virus.

The results of Experiment 7 suggest that the effect is not due to non-specific stress since mice born and bred in the cold environment, and hence presumably well adapted, show the effect quite as clearly as mice transferred from the warm environment on the day of inoculation.

It has been reported that mice subjected to severe stress by scalding, ligation trauma, injection of bacterial polysaccharide, or exposure to cold, show a marked decrease in liver non-protein sulphydryl concentration (Beck and Linkenheimer, 1952). This is particularly interesting since it is probable that the protective action of high protein diets against certain types of liver injury is associated with their ability to provide sulphydryl groups.

Changes in thyroid activity have been shown to influence resistance to infection (Weiss et al., 1952). Thyroid activity is markedly stimulated by a cold environment and one of its effects is a fall in hepatic glycogen (de Minjer, 1952). In the present experiments it was observed that normal mice exposed to cold showed very little, if any, stainable liver glycogen even following the intraperitoneal injection of glucose. In view of the established association between a fall in liver glycogen and increased susceptibility to various poisons it seemed worth while to examine the effect of thyroxine given orally on the course of the virus disease used here. The results of Experiment 12 suggest that at the dosage rate indicated the effect of thyroxine given in this way is to enhance the infectivity of the virus suspension but that the/

the difference is not as marked as that produced by the cold environment. Similarly, the histological picture in mice so treated does not correspond with that shown by the mice in the cold environment.

It is clear that the marked enhancing effect of the cold environment on the infectivity of the virus cannot be fully explained by either of the mechanisms suggested above.

The other interesting difference noted in mice in the cold environment was that the period of survival following infection was shortened. Ipsen (1944, quoted by Fenner, 1949) using the Laigret-Durand strain of mouse pathogenic murine typhus rickettsiae, showed that the time to death of mice after intra-peritoneal inoculation could be used to titrate the agent. It was later found by Packalen (1947) that this strain was in fact pure ectromelia virus. While it was noted in the present studies that the interval to death appeared shorter with higher doses of the virus, the differences were not sufficiently consistent to make this a feasible means of titration. The shortened interval to death in the cold environment could thus be regarded as evidence for an increased growth of virus in mice kept in the cold environment, but it was not possible to demonstrate significant differences in the virus content of mouse livers in such an environment in the experiment described on page 82.

Burnet and Lush (1936) noted that a temperature below the physiological optimum (39.5°C.) for the development of chicken embryos was necessary for the lesions of ectromelia to appear on the chorioallantois. At 36° to 37°C. lesions regularly developed. This was confirmed with the virus used in the present experiment and may suggest that a similar mechanism operates in mice kept in the cold environment. It is of special interest that vesicular stomatitis virus multiplies better in eggs incubated at 35° to 36°C. than at 39° to 40°C. (Sigurdsson, 1943), but that Griffin et al. (1954) showed that the mortality of mice infected with this virus was significantly lower in mice kept at 8°C. than in mice kept at 27° or 35°C.

The other difference shown between the two environments i.e. the difference in the type of lesion produced and demonstrated histologically is much less easily measured. Two main theories have been put forward to explain the way in which hepatotoxic agents bring about the effects. The first of these suggests direct interference with cellular metabolism (Christie and Judah, 1954; Cameron, 1954). The other view has been mainly advanced by Himsworth who suggests that the effects are largely brought about by swelling of the parenchymal cells which thus obstruct the sinusoids and produce cellular ischaemia. This/

This view has been accepted by Elias (1955) to explain toxic centrilobular necrosis and by Glynn et al. (1948) to account for the distribution of the lesions in massive hepatic necrosis produced by abnormal diets in rats. Naftalin and Howie (1949) considered that the liver injury described by them in pigs reared in a cold and damp environment was probably due to circulatory changes in the liver which they interpreted as the result of local cooling of the abdomen against a cold floor.

The distribution of lesions in mice given virus and exposed to the cold environment suggests that vascular factors may play a part. Nevertheless the fundamental cause of the necrosis in mice kept at different environmental temperatures is the same i.e. direct action of the virus. As has been shown by de Burgh (1950), the failure to synthesise glycogen is compatible with the theory that energy derived from oxidation is diverted to synthesis of virus rather than of glycogen. It has also been demonstrated by Pogell (1956) that carbohydrate metabolism is impaired in viral hepatitis in mice. He showed that systems over and above the general decrease in protein included enzymes causing the aerobic disappearance of fructose and conversion of fructose 1-phosphate and fructose 6-phosphate to inorganic phosphate. In addition virus infected mouse liver homogenates were found to inhibit fructose/

fructose disappearance by normal liver homogenates, but this effect was not specific to the virus infection since similar inhibitions occurred in necrotic livers following carbon tetrachloride injection. In the present experiments little, if any, stainable liver glycogen was present in any part of the lobule of the livers of mice kept in the cold environment and thus does not explain the perivascular distribution of the necrosis in this group.

On the other hand, as has been shown in Experiment 9, exposure to the cold environment is effective only if this is begun and maintained within the first four days following infection i.e. when the liver is first invaded by the virus. Vascular factors produced by the cold environment might presumably tend to localize the distribution of the lesions intralobularly at this stage.

The effect of exposure to a cold environment is to bring about a complex series of adaptive changes (Stullken and Hiestand, 1954) and it is unlikely that any one of these can be held responsible for the increased susceptibility demonstrated in the present experiments. It should be stressed, however, that the liver in particular is more severely affected in the cold environment and it is considered that this accounts for the increased mortality.

Gledhill (personal communication) has confirmed that the LD50 of a strain of ectromelia (which was isolated, like my own, from an acute outbreak of disease in which hepatitis was the most prominent lesion) is smaller in mice kept in the cold than in mice kept at normal temperatures and has used this as a guide to the dose of virus which would regularly infect mice without producing symptoms in many of them.

The main practical implication of the findings here reported is that they stress the need for controlled environmental temperatures when mice are used in titrations of infectivity and particularly where experiments are carried out at different times of year. It is unlikely that the cold conditions (approximately 43°F.) would be often used, but it is probable that smaller variations exert an effect and it would thus be desirable to record the environmental temperatures in any description of such experiments.

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APPENDIX

Since the measure of the effect of the altered environment depends upon showing a difference in the cumulative 50 per cent. lethal dose end-points calculated by the method of Reed and Muench (1938), it is very important to estimate the error of individual end-point determinations so as to set up confidence limits within which the true end-point should lie and thus to test the significance of the difference between two end-point determinations.

The error of a single 50 per cent. end-point determination can be calculated from the Reed and Muench method by the technique of Pizzi (1950). This makes possible the direct comparison of two unrelated end-points with greater economy of animals than where the precision of an end-point is estimated by repeated titrations.

Schwerdt and Merrell (1952) have estimated the variation in 50 per cent. end-points calculated by the Reed-Muench method in replicate titrations of Lansing poliovirus in cotton rats. The standard error was in good agreement with that calculated from Pizzi's formula. They point out, however, that this formula deals only with variations arising from simple sampling and that it can only be expected to agree with observed variation in end-point/

end-point determinations if there is no appreciable variation introduced by causes other than simple sampling. For this reason, no attempt has been made to compare the results of experiments carried out at different times, which are here described.

The pool of virus with which most of the work was done gave reasonably consistent titres over a period of three months but, since the lowest storage temperature available was -20°C., it was felt necessary to carry out a control titration with each experiment so that the results would not be influenced by degradation due to storage. Thus, where the Pizzi formula is used in the following account, it should be understood that the computed errors refer to titrations carried out with the same pool of virus on the same day so that the only variation, apart from that arising from simple sampling and estimated by Pizzi's formula, should be due to the experimental stimuli.

By way of illustration an example (Experiment 5) is given of the estimates of the LD50 by the method of Reed and Muench of its standard error by the Pizzi formula.

EXPERIMENT 5A.

Room Temperature

Group No.	Log. dose of virus	Alive	Dead	Totals Alive	Totals Dead	per cent. Mortality
LL 16	-2.00	3	2	3 ↓	10 ↑	75
LL 18	-3.00	1	4	4 ↓	8 ↑	67
LL 20	-4.00	2	3	6 ↓	4 ↑	40
LL 22	-5.00	5	0	11 ↓	1 ↑	8
LL 24	-6.00	5	0	16 ↓	1 ↑	
LL 26	-7.00	5	0	21 ↓	1 ↑	
LL 28	-8.00	4	1	25 ↓	1 ↑	
LL 30	-9.00	5	0	30 ↓	0 ↑	

The 50 per cent. end-point lies between 10^{-3} and 10^{-4}.

Using formula for calculation of the proportionate distance:-

$$\frac{\text{50 per cent. - (Mortality at dilution next below)}}{\text{(Mortality next above) - (Mortality next below)}} = \text{Proportionate distance}$$

$$= \frac{50 - 67}{40 - 67} = \frac{-17}{-27}$$

$$= 0.629$$

The final readings are as follows:-

Logarithm of lower dilution = -3

Proportionate distance X log. dilution factor	
0.629 x 1	-0.6295
Sum (log. of end-point)	-3,6295

EXPERIMENT 5B.

Cold Environment

Group No.	Log. dose of virus	Alive	Dead	Totals Alive	Totals Dead	per cent. Mortality
LL 17	-2.00	0	5	0 ↓	24 ↑	100
LL 19	-3.00	0	5	0	19	100
LL 21	-4.00	2	3	2	14	87.5
LL 23	-5.00	2	3	4	11	73
LL 25	-6.00	0	5	4	8	67
LL 27	-7.00	5	0	9	3	25
LL 29	-8.00	3	2	12	3	20
LL 31	-9.00	4	1	16 ↓	1	

The 50 per cent. end-point lies between 10^{-6} and 10^{-7}.

Using formula for calculation of the proportionate distance:-

$$= \frac{50 - 67}{25 - 67} = \frac{-17}{-42} = 0.405$$

The final readings obtained are as follows:-

Logarithm of lower dilution = -6.00

Proportionate distance X log. dilution factor

0.405 x 1 0.405

Sum (log. of end-point) -6.405

Applying the Pizzi formula:-

$$SE_{LD/50} = \sqrt{\frac{0.79(h)(R)}{n}}$$

Where h = interval on log. scale between dilutions = 1

n = number of animals per dilution = 5

R = interval on log. scale between cumulative 25 per cent. and 75 per cent. positions.

Applying this formula to the results of Experiment 5, it can be seen that in the titration carried out in the warm environment:-

$$LD75 = 10^{-2}$$

$$LD25 = 10^{-4.5}$$

thus the interval on the log. scale between the cumulative 25 per cent. and 75 per cent. positions is 2.5 = R

$$SE_{LD/50} = \sqrt{\frac{0.79(1)(2.5)}{5}}$$

$$= \sqrt{0.39}$$

$$= 0.62$$

Applying the same formula to the results of the titration in Experiment 5 carried out in the cold environment, where LD75 = 10^{-5} and LD25 = 10^{-7} the interval on the log. scale between the cumulative 25 per cent. and 75 per cent. positions is 2.0 = R

$$SE_{LD/50} = \sqrt{\frac{0.79(1)(2)}{5}}$$

$$= \sqrt{0.32}$$

$$= 0.56$$

The standard errors thus calculated may be used to test the difference between the two 50 per cent. end-points by the usual formula:-

$$SE_{diff.} = \sqrt{SE_1^2 \quad SE_2^2}$$

If SE_1^2 = SE of the LD50 in the warm environment

= 0.39

and SE_2^2 = SE of the LD50 in the cold environment

= 0.32

then $SE_{diff.} = \sqrt{0.71}$

= 0.842

and any difference should be more than twice this value to be considered outside the chance range.

In this case

log. LD50 (Warm)	= -3.63
log. LD50 (Cold)	= -6.405
Difference on log. scale	= 2.775

This difference is more than three times the standard error of the difference as calculated above and is thus significant at the 95 per cent. probability level.

MORTALITY TABLE

Experiment Number	Negative log. dilutions of virus 2	3	4	5	6	7	8	9	LD50
3A	5	3	2	1	0	-	-	-	$10^{-3.66}$
3B	4	4	3	3	0	-	-	-	$10^{-4.37}$
3C	5	4	2	1	1	-	-	-	$10^{-3.56}$
3D	5	4	3	2	1	-	-	-	$10^{-4.48}$
4A	3	4	2	1	0	-	-	-	$10^{-3.54}$
4B	5	5	3	1	0	-	-	-	$10^{-4.31}$
4C	4	4	2	0	0	-	-	-	$10^{-3.54}$
4D	4	5	2	1	1	-	-	-	$10^{-3.57}$
5 (WARM)	2	4	3	0	0	0	1	0	$10^{-3.63}$
5 (COLD)	5	5	3	3	5	3	2	1	$10^{-6.4}$
6 (COLD)	5	5	3	3	3	2	2	0	$10^{-6.17}$
6 (WARM)	3	5	2	1	1	0	1	0	$10^{-3.57}$

Five mice were used to test each dilution in the above titrations.

MORTALITY TABLE

Experiment Number	Negative log. dilutions of virus 2	3	4	5	6	7	8	9	LD50
7 (ADAPTED)	-	-	5	5	2	1	0	-	$10^{-6.28}$
7 (NOT ADAPTED)	-	-	6	3	3	3	1	-	$10^{-6.17}$
12 (WARM)	3	5	2	1	2	1	2	1	$10^{-4.17}$
12 (THYROXINE)	5	3	4	1	2	6	3	1	$10^{-5.4}$

Six mice were used to test each dilution in the above titrations, (Experiments 7 and 12).

10 (WARM)	-	16	15	9	7	-	-	-	$10^{-5.43}$
10 (CORTISONE)	-	14	15	11	7	-	-	-	$10^{-5.47}$

Sixteen mice were used to test each dilution in the above titrations, (Experiment 10).

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