the release of nucleases, that are latent *in vivo* and interfere with the *in vitro* measurement of the enzyme. In this respect it is interesting that a significant increase of DNAase 1 was demonstrated by Giusti *et al* in homogenates of livers from mice infected 48 hours previously with MHV-3(11). On the other hand the marked increase of RNA synthesis can be related to an alteration of the metabolic and structural factors regulating this pathway and occurring in the later stages of infection.

It may be inferred that RNA biosynthesis is not a metabolic function that is greatly altered in the early stages of this form of virus hepatitis. Therefore, the mechanism of the initiation and rapid development of the hepatocellular lesion does not appear to derive, initially at least, from an alteration of the RNA synthesis system.

Summary. RNA polymerase activity, measured *in vitro* in the nuclear fraction of livers from mice infected with MHV-3 virus, was decreased 23% and 50% at 24 and 36 hours respectively after inoculation of the virus. The *in vivo* incorporation of orotate radioactivity in the nuclear RNA of liver cells showed no change from the normal during the first 24 hours after infection when viral multiplication had already reached its peak level, but a significant increase was evident at 36 hours. The cytoplasmic RNA of liver cells showed no change in *in vivo* incorporation of orotate radioactivity even at 36 hours after infection when the hepatocellular lesions were already marked.

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A Toxic Factor in *Leptospira pomona*. (32462)

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The icterogenic character of leptospires prompted some investigators to credit them with toxic properties(1,2), but reports of toxins in cultures of leptospires(3,4,5) could not be confirmed by either the classical methods for bacterial endotoxins(6,7) or by the use of cultured mammalian cells. Although cytopathic effects were reported in cultured cells after the addition of leptospires(8,9,10), Miller *et al*(11) observed no cytopathology until after a week or more if the medium was adequate for the survival and growth of both the cells and virulent *Leptospira pomona*.

A new technique for *in vitro* demonstration of bacterial endotoxin(12) was used for the detection of leptospiral toxins. This report concerns a toxic factor(s) in both virulent and avirulent *L. pomona*.

Materials and methods. Strains. Cultures of virulent *L. pomona* strains Ohio and DM-2 were grown in Stuart's medium(13); the avirulent variants were selected and grown in modified Tween synthetic medium (MTSM) (14). The virulence of these strains for guinea pigs(14), hamsters(15), swine(16), and cattle(17) has been described.

Macrophages. Macrophages were obtained from the peritoneal cavity of normal 400- to 500-g guinea pigs and were maintained in Scherer's maintenance solution without added serum as described by Kessel and Braun(12). Macrophages were also harvested from the peritoneal cavity of immune guinea pigs 10 or more days after an infection with virulent *L. pomona*(17).

Cytotoxicity. Cellular permeability. To cultured macrophages was added living or disrupted leptospires or sterile cultural supernatant fluids. Cultures of virulent and avirulent *L. pomona* were diulted to *ca.* 1×10^7 organisms per ml by the addition of Scherer's solution, and 1 ml was added to each of 4 or more tubes of guinea pig peritoneal macrophages. After 6 hours of incubation at 36°C, 0.5 ml of trypan blue (0.5% aqueous solution) was added. After further incubation for 5 to 10 minutes, the medium was decanted and the cells were carefully rinsed with 0.5 ml of Scherer's solution. The tubes were allowed to drain while inverted for a few minutes and the percentage of stained cells was determined(12).

The cytotoxicity of disrupted leptospires and of the supernatant fluids of cultures was investigated. The leptospires were sedimented by centrifugation (12,000 × *g* for 30 minutes), washed once in 0.02 M phosphate buffer (pH 7.3), and stored at −70°C until used. A portion of each crop was placed at 110°C for 16 hours to determine the dried weight. The supernatant cultural fluids were forced through a sterile Millipore filter (100 mμ pores) and dialyzed for 16 hours against Scherer's solution at 4°C. Fluids from the cultures of virulent leptospires were diluted 1:10 in Scherer's solution. After the frozen cells were thawed, they were suspended in Scherer's solution and disrupted either by sonication [10-1 minute pulses with a sonic cell disrupter (Branson Instruments, Inc., Danbury, Conn.) at maximum electrical output] or by 5 cycles of freezing-thawing. Different concentrations (Table I) of disrupted *L. pomona,* or sterile cultural supernatant fluids, or *Escherichia coli* endotoxin (Difco Laboratories, Detroit, Mich.) at a concentration of 10 μg per ml of Scherer's solution were added to macrophages and the cytotoxic effects were determined(12).

TABLE I. Cytotoxicity of Living and Disrupted Virulent and Avirulent *Leptospira pomona* and Cultural Supernatant Fluids for Guinea Pig Peritoneal Macrophages.

Preparation tested	Avirulent *L. pomona*	Virulent *L. pomona*
Viable cultures	68.±10*	75.± 7.*
Killed, disrupted leptospires		
10,000 μg/ml of medium	86.±12.	82.±10.
1,000 " "	66.± 4.	83.± 4.
100 " "	26.± 6.	43.± 8.
10 " "	12.± 2.	23.± 5.
Cultural supernatant fluids	43.± 8.	87.± 9.
Controls:		
Escherichia coli endotoxin	84.± 6.	
Scherer's solution	10.± 3.	
Dialyzed MTSM	18.± 5.	

* Percentage of toxic macrophages.

Cytotoxicity. Cellular respiration. The filtered supernatant fluids from cultures of virulent and avirulent *L. pomona* were compared for their effects on the uptake of oxygen by mammalian cells. Renal, cortical cells were obtained from the minced kidney of 2-week old, specific-pathogen-free pigs by treatment with trypsin (0.25%) and were inoculated into Blake bottles containing Hanks' balanced salt solution with 10% bovine serum and antibiotics (penicillin, 100 units/ml and streptomycin, 100 μg/ml). After 3 days of incubation at 37°C, the medium was changed to Earle's basic salt solution with 10% bovine serum and incubation was continued for 3 more days. After treatment with trypsin, the cells were sedimented by centrifugation and were washed first in Hanks' solution without serum and then in phosphate-buffered, 0.85% saline; they were resuspended in Scherer's solution supplemented with 2% bovine serum at a concentration of 1.8×10^6 cells/ml of medium.

Standard Warburg respiration studies were performed at 36°C in duplicate for 4 hours in air. Each flask contained 2 ml of cell suspension, 1 ml of dialyzed supernatant cultural fluid or Scherer's solution in the side arm, and 0.2 ml of a 3 M solution of KOH in the center well. The contents of the side arms were added after 10 min of equilibration with shaking.

Results. Cellular permeability. Motile lep-

tospires were observed in the medium at the conclusion of the exposure period After exposure for 6 hours with either virulent or avirulent *L. pomona*, 75% and 68%, respectively, of the remaining macrophages were toxic and stained with trypan blue. The relative cytotoxicity of different concentrations of disrupted virulent and avirulent *L. pomona* strain Ohio and of sterile cultural fluids is tabulated (Table I); similar results were obtained with strain DM-2.

Macrophages from immune guinea pigs were not resistant to the toxic factor of leptospiral cultures. Supernatant fluids from virulent and avirulent *L. pomona* damaged 68. ± 4% and 48. ± 3%, respectively, of the macrophages.

The effects of the toxic factor in the dialyzed supernatant fluid of *L. pomona* in MTSM were not specifically neutralized by antiserum (titer of 1:10,000 in the microscopic agglutination test) since antiserum and normal serum were equally protective (Table II).

TABLE II. Effect of Normal Serum and Homologous Antiserum on Leptospiral Cytotoxicity.*

Supernatant fluid plus	Concentration (V/V): 50%	10%	2%
Immune serum	10.± 4.†	43.± 7.	56.± 5.
Normal "	13.± 5.	50.± 5.	62.± 4.

* Guinea pig peritoneal macrophages were incubated in the supernatant fluid of *L. pomona* cultures plus different concentrations of normal or immune porcine serum.

† Percentage of toxic macrophages.

The toxic factor in the supernatant fluid of both virulent and avirulent *L. pomona* strain Ohio was nondialyzable (dialyzed against Scherer's solution without serum for 16 hr at 5°C) and thermolabile (56°C/20 min). After exposure to the dialysate or the heated supernatant fluid, 14. ± 6% and 8. ± 2%, respectively, of the macrophages took up trypan blue.

Cellular respiration. Compared to cultured kidney cells in Scherer's solution, the addition of supernatant fluids of either virulent or avirulent *L. pomona* inhibited the uptake of oxygen. The inhibitory effect was not detected if serum was included at a concentration of 10% (Fig. 1).

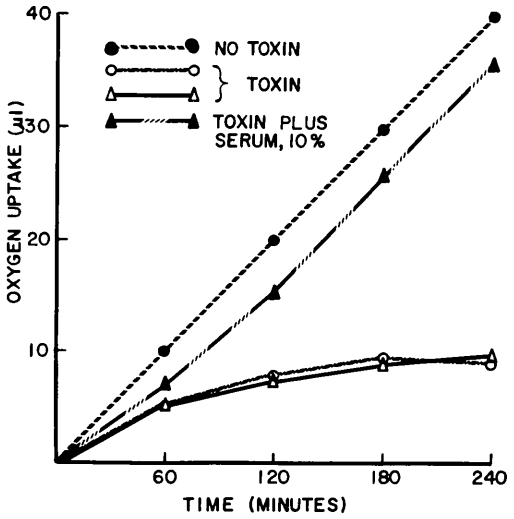

FIG. 1. Uptake of oxygen by porcine kidney cells suspended in sterile Scherer's solution supplemented with 2% calf serum (control) (●) and the effect of additions of dialyzed supernatant fluids of cultures of virulent (○) and avirulent (△) *Leptospira pomona*; lack of inhibition by toxin in medium containing 10% of serum (▲).

Discussion. A toxic factor(s) in *L. pomona* affected the integrity and respiration of mammalian cells in systems containing little or no serum. The effect of the toxin on other types of cells is not known; but it may play a role in the development of the biochemical lesions of acute leptospirosis(18) or in the development of nephritis during persistent, renal leptospirosis. Further studies are also required to show whether cytotoxicity results from the action of the toxin *per se* or if it is mediated by normal antibody adherent to the surface of washed mammalian cells.

In contrast to bacterial endotoxin, the soluble, nondialyzable toxic factor in leptospires is thermolabile. The failure of antiserum to neutralize it specifically, and the full susceptibility of macrophages from immune guinea pigs, suggests that either the toxin is a poor antigen or that the toxin-antibody complex is toxic. Like most pathogenic bacteria, leptospires are toxigenic, but cytotoxicity is not a distinction of virulent strains of *L. pomona*.

Summary. A toxic factor was demonstrated in living and disrupted *Leptospira pomona* and in the supernatant fluids of cultures by alterations in the permeability of guinea pig

peritoneal macrophages; the supernatant fluids also inhibited the uptake of oxygen by porcine kidney cells. Virulent and avirulent *L. pomona* were equally toxic. The soluble toxic factor was nondialyzable and thermolabile; it was not specifically neutralized by antiserum.

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Genetic Regulation of Multiple Forms of Tyrosinase in Mice: Action of *a* and *b* Loci.* (32463)

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The relationship of tyrosinase activity to the qualitative and quantitative attributes of melanin granules (melanosomes) found in the various coat color mutants of mice has received careful attention(1-3). It is generally held that alleles at the *c*-locus regulate tyrosinase activity by direct involvement in the production of tyrosinase molecules(1,2). Alleles at the *b*-locus influence not only tyrosinase activity but also the nature of eumelanin (black *vs* brown) synthesized by melanocytes(1,2). It has been proposed that the *b*-locus controls, at least in part, the production of the protein matrix which forms an intricate component of the melanosome (1,2). The matrix appears to provide binding sites for the tyrosinase units synthesized in conformity with *c*-locus specifications. Alleles at the *a*-locus determine the type of melanin (phaeomelanin *vs* eumelanin) produced within the melanosome and also appear to have influence on the structure of its protein matrix (1,4).

As in a variety of other organisms, the tyrosinase of mammals has been shown to occur in a number of forms separable by electrophoresis(1,5-9). Considerable attention has been directed toward elucidating the properties of tyrosinase in melanomas derived from mice and hamsters(5-12). Recently, Wolfe and Coleman(1) reported the existence of multiple forms of tyrosinase in the normal skin of pigmented mice. In their study, involving allelic substitutions at the *c*-locus, a maximum of two electrophoretically separable forms of tyrosinase were identified. Alleles at the *c*-locus appeared to influence the presence or absence of specific bands of tyrosinase and also their mobility. This first demonstration of an association between genetic mechanisms and the varieties of tyrosinase obtained from normal melanocytes has added a new dimension to mammalian pigment cell research. The obvious need for more complete insight

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