8). While conflicting reports have appeared, it seems probable that intrauterine infection with HSV does occasionally occur in man(9). Our observations in hamsters demonstrate that at least some variants of HSV can cause transplacental infection. Further work is in progress to determine whether this ability is a unique property of LPV XIII. Failure to cause intrauterine infection in mice, reported here, has also been reported by Berry and Slavin(10) who failed to isolate virus from mouse embryos although they could detect virus in the blood of the adult infected mice.

Failure of HSV to induce tumors in either newborn mice or hamsters adds one more virus to those human viruses not found to cause induction of malignancy in these animals(1). It is of course possible that other strains of HSV or other routes of inoculation might yield different results. The techniques used, however, regularly result in tumors when adenovirus types 12(1,11) or 18(1,12) or the papovaviruses SV40(13,14) and polyoma (15) are introduced into newborn animals. It is of interest that the HSV variants employed in this study have been shown to cause selective aberrations of mammalian chromosomes in both diploid Chinese hamster and embryonic human cells(16). It therefore appears likely that ability to damage chromosomes does not necessarily confer oncogenic capability to viruses.

Summary. Variants of herpes simplex virus were shown to differ in virulence for both newborn mice and hamsters. Intraperitoneal introduction of all variants led to a higher death rate than subcutaneous inoculation. The virulent variant caused transplacental infection of hamster, but not of mouse, embryos. Inoculation of 10^5 plaque-forming units of the attenuated variants into newborn hamsters and mice within 24 hours after birth failed to induce tumors within a period of 6 months.

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Received February 17, 1964. P.S.E.B.M., 1964, v116.

Studies of Germfree Animals I. Response of Mice to Infection With Influenza A Virus.* (29249)

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There are few reports of the effects of viruses on germfree (GF) animals. Rous sar-

* In part, these studies were conducted under the sponsorship of the commission on Acute Respiratory Disease, Armed Forces Epidemiological Board, and were supported in part by Office of the Surgeon General, Dept. of the Army.

coma virus(1), and hog cholera virus(2) were shown to produce the characteristic disease in their GF host.

Newborn GF mice were found to be more susceptible than conventional mice to Coxsackie B virus using mortality as the parameter(3). Germfree mice were found to be more susceptible than conventional mice to the Friend leukemia virus using spleen weights, leukocyte counts, and Fe^{59} uptake as parameters(4).

The purpose of these experiments was to compare the LD_{50} of GF animals with conventional animals using geometric doses of respiratory virus. Data from histologic, immunologic, and serologic procedures were analyzed in an attempt to understand the reason for the difference in susceptibility.

Materials and methods. Seven-week-old, fifth generation, GF and conventional ICR mice were purchased from the A. R. Schmidt Co. and fed a sterilized Purina mouse diet and sterile water for 2 weeks before use in the experiment. They were all housed in GF flexible plastic chambers throughout the experiment. The term GF is used by us to designate animals free of bacteria, fungi, and PPLO by the tests described below, and free of external and internal parasites, and of obvious symptoms of infectious disease. The possibility of their being infected with a virus is very low as described in a survey for viruses of GF and conventional mice(5).

The mouse-adapted influenza A virus, strain PR8, was obtained from Dr. John E. Kempf. It had been adapted to mice in 1934 by Francis, by whom it was passed through mice 335 times, then passed 163 times through mice by Loosli(6) and Hamre, and 3 times each by Kempf and ourselves. The virus inoculum was prepared by mincing infected lungs with scissors, grinding with sand, suspending the brei in normal saline solution, centrifugating for 10 minutes at 3,000 rpm, and passing the supernate through coarse, then fine Millipore filters with the last passage through sterile 0.22 μ Millipore filters. All virus suspensions were administered to mice anesthetized with ether. The virus was dropped intranasally through a 25-gauge needle in 3 drops, an average volume of 0.018 cc. The dose of virus was controlled by using 4- or 5-fold dilutions of infected lung. Deaths were recorded daily and the experiment terminated after 21 days. The Miller-Tainter method was used to calculate the LD_{50}(7).

Lungs were examined from the dead animals grossly and were cultured, as were the GF food, water, and feces, in thioglycollate and in brain heart infusion broth at 37°C and 55°C for bacteria, and Sabouraud's agar for fungi. The tubes were examined a week after inoculation. In the experiments described, no bacteria or fungi were detected in our GF animals. Four tests of lungs of 2 GF animals were performed for PPLO on 2.5% tryptic digest agar, with 1.3% tryptic digest broth, 1% yeast extract, and 16% horse serum, aerobically and anaerobically. Madoff's(8) technique was used to inspect the agar after one week; no growth of PPLO was detected.

Lungs from survivors were examined grossly and histologically after H & E staining. The histopathologic findings were also confirmed in this laboratory by Dr. H. Matsumoto, now of Osaka University, Japan.

Immunoelectrophoresis. Microimmunoelectrophoresis(9,10) was used to compare sera for the presence of $gamma_2$ globulin. The electrophoresis was run for 3 hours in order to amplify the $gamma_1$ and $gamma_2$ regions for increased resolution of precipitate lines. Rabbit origin anti-mouse serum was prepared in our laboratory by repeated injections of conventional mouse sera with Freund's adjuvant intradermally and intramuscularly. At later dates, mouse serum was given intravenously until test sera gave a consistent immunoelectrophoretic pattern. This condition appeared within 8 weeks in the 3 rabbits used.

Hemagglutination-inhibition tests. Sera used in these tests(11) were heat inactivated, periodate treated, and adsorbed with a 50% suspension of chicken erythrocytes so that the final serum dilution was 1:10. The antigen was 4 HA units of mouse-adapted PR8. The indicator was chicken erythrocytes. Tests were read after 50 minutes by the pattern method. Pre- and post-infection sera were taken from different individuals.

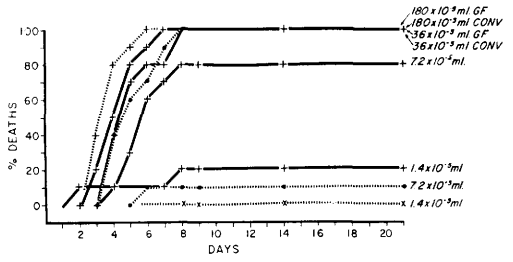

FIG. 1. Mortality of 9-week-old, germfree (——) ICR mice compared with conventional (....) mice after intranasal inoculation with geometric doses of mouse-adapted Influenza A PR8 virus. LD_{50} ratio is 4.2 to 1.

Results. Experiment 1: Fifty GF and 50 conventional nine-week-old male mice were divided into 4 experimental dosage groups of 10 mice each, according to the 4 groups shown in Fig. 1, and one non-inoculated control group.

Dates of death were recorded (Fig. 1) and survivors were exsanguinated on the twenty-first day to obtain sera for the immunoelectrophoretic study.

Experiment 2: The same procedure was followed as for Exp. 1 except that a lower dose and narrower range of a new preparation of a later passage of virus was given to the animals (Fig. 2). Bacterial cultures of lungs of GF animals were negative.

Susceptibility. In Exp. 1, the LD_{50} of conventional mice was 13.5×10^{-5} cc of infected donor lung while the LD_{50} of GF mice was 3.2×10^{-5} cc, a ratio of 4.2 to 1. In Exp. 2, the LD_{50} of conventional mice was 13×10^{-6} cc of infected lung, while the LD_{50} of GF mice was 5.2×10^{-6} cc, a ratio of 2.5 to 1.

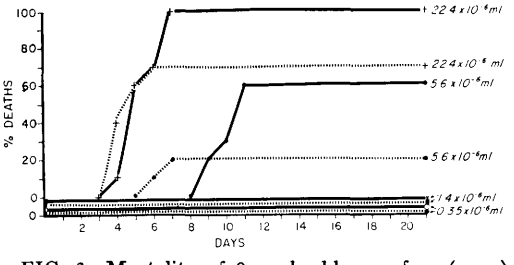

FIG. 2. Mortality of 9-week-old, germfree (——) ICR mice compared with conventional (....) mice after intranasal inoculation with geometric doses of mouse-adapted Influenza A PR8 virus. A newly prepared virus suspension was used which was more potent than that used in Fig. 1. LD_{50} ratio is 2.5 to 1.

Clinical symptoms, dyspnea, diminished water intake, lethargy were more severe in GF mice than conventional mice in groups which received identical infecting doses.

The incidence of deaths in GF animals compared to conventional animals, both given a dose of 7.2×10^{-5} cc, was statistically significant, $p = >0.01$. In all other dosages the difference in incidence of deaths was not statistically significant but the trend was consistently in the direction of greater susceptibility of the GF.

Pathology. Lungs from freshly autopsied, influenza-infected, bacteria-free mice were examined grossly and histologically. The gross appearance was completely red and edematous in those which died before 21 days. An infected lung was photographed to show a normal area adjacent to a pathologic area (Fig. 3).

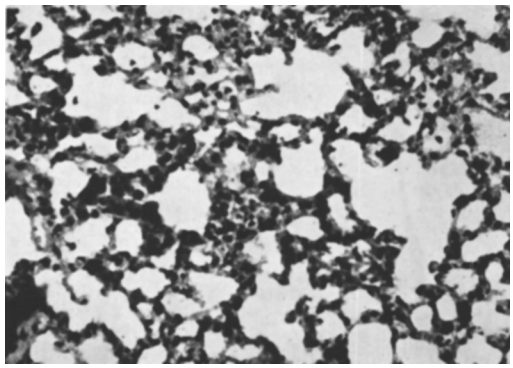

FIG. 3. Interstitial pneumonia of germfree mouse infected with Influenza A virus, with lymphocytic invasion of alveolar walls, at left, compared with nearly normal walls at lower right. H & E stain; × 240.

The alveolar walls were infiltrated with lymphocytes; some of the alveolar spaces were filled with lymphocytes (Fig. 4); some of the alveoli were filled with edematous fluid; a few alveoli were hemorrhagic. No polymorphonuclear leukocyte aggregates were detected.

In contrast, the conventional lungs showed all of the pathologic features of the GF lungs, hemorrhage, lymphocytic infiltration, and edema, and, in addition, areas of polymorphonuclear aggregation within alveoli. In some of these lungs, bacteria were visible (Fig. 5).

Immunoelectrophoresis. Non-infected GF

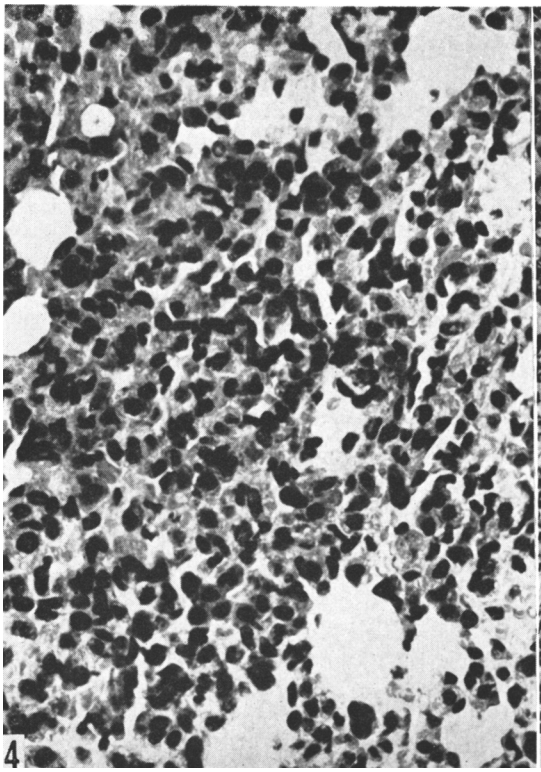

FIG. 4. Lung of germfree mouse infected with Influenza A virus with lymphocytic infiltration of alveolar walls and lymphocytic aggregates within alveoli. H & E stain; × 540.

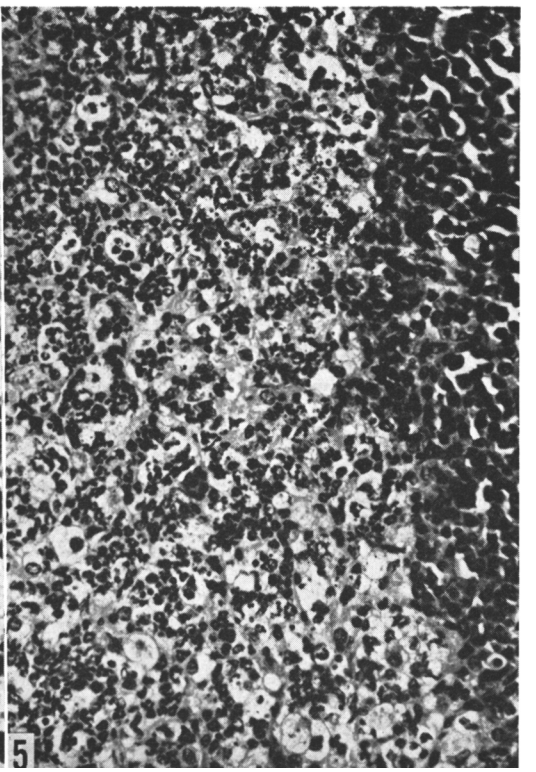

FIG. 5. Interstitial pneumonia of conventional mouse infected with Influenza A virus, with pclymorphonuclear leukocyte aggregates within alveoli, bacteria, and edema, left. Many lymphocytes at right. H & E stain; × 360.

mice showed one faint line in the gamma$_1$ region and no gamma$_2$ line (Fig. 6). At 21 days after infection, regardless of dose of virus, no gamma$_2$ line was seen and no increase was evident in gamma$_1$ in 20 of 23 sera. In 3 cases, a more intense gamma$_1$ line appeared to increase in length into the gamma$_2$ region. Two of these 3 occurred in the lowest dose, the third in the highest dose group in which there were any survivors. Furthermore, in 11 cases of 23, the gamma$_1$ line was slightly longer in the non-infected than in the 21-day convalescent mice.

Conventional mice showed identical immunoelectrophoretic patterns, both in length of lines and their intensity, with precipitate lines present in the gamma$_1$ and gamma$_2$ regions before infection and 21 days later.

The striking difference in the absence of a gamma$_2$ line in GF sera is evident when compared with a conventional serum run simultaneously for 3 hours (Fig. 7).

Serology. The pre-infection sera of GF animals did not inhibit hemagglutination of chicken erythrocytes by the homologous virus in the lowest test dilution of serum, 1:10. No rise in hemagglutination-inhibition (HI) titer occurred at 21 days after infection, in any of the survivors at all of the dose levels (Table I).

Conventional animals did not show any HI antibody at a 1:10 dilution before infection but striking rises were observed in HI antibody 21 days after infection in most of the animals in the 2 highest dose groups.

Discussion and conclusion. Under the experimental conditions described, GF mice were more susceptible than conventional mice to influenza A virus by the clinical parameters of cumulative incidence of mortality at the end of 3 weeks and the greater severity of clinical illness characterized by dyspnea,

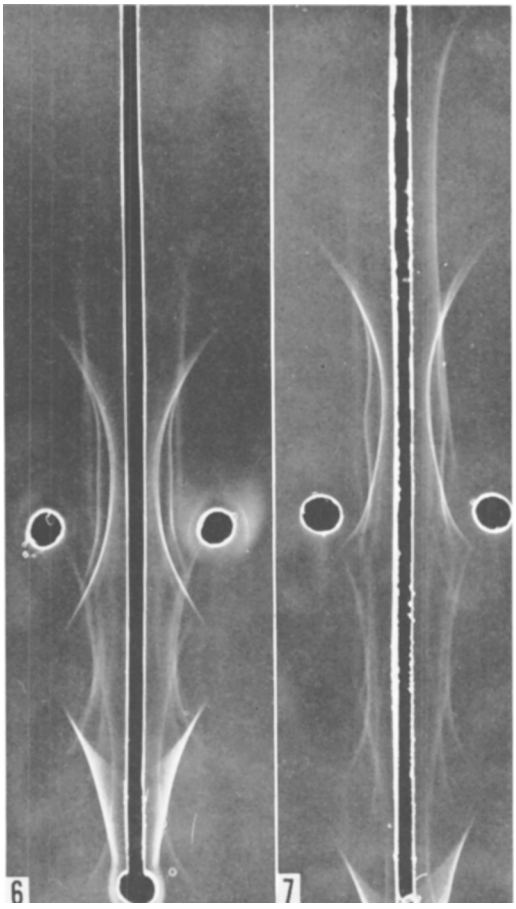

FIG. 6. Immunoelectrophoretic pattern of sera of germfree mice before (left well) and 21 days after (right well) Influenza A infection reacting with rabbit anti-mouse serum in center trough.

FIG. 7. Immunoelectrophoretic pattern of sera of germfree mouse (left well) compared with convensional mouse (right well) reacting with rabbit anti-mouse serum in center trough.

lethargy, and decreased water consumption. It is possible that greater precision in LD_{50} determinations could be obtained by using a nebulizer instead of intranasal droplet instillation (because less mouse to mouse variation in pathology was shown to occur by the nebulizer method)(12). However, we used a much more precise volume in our dose, 0.018 cc, than was used in the comparison of the 2 methods of instillation(12), therefore, the degree of difference in precision would have to be ascertained by further experimentation.

The histologic appearance of lungs taken from GF mice which survived virus infection differed from conventional mice at 21 days after inoculation, in that the predominant type of leukocyte found in GF mice was the lymphocyte while the conventional mice had polymorphonuclear leukocytes in large numbers, probably attracted by the bacteria which were visible. Invading macrophages and lymphocytes were also observed concurrently with antiviral antibodies in the sera.

In view of the greater susceptibility of GF mice, though admittedly the difference was small, we initiated immunoelectrophoretic and HI antibody studies.

Previously, beta and gamma globulins were shown to be lower in GF mouse sera than in conventional mouse sera(13).

Germfree mice showed no $gamma_2$ globulin line upon immunoelectrophoresis (IE) in non-virus-infected animals, and in 20 of 23 virus-infected animals. Germfree mice accidentally infected with bacteria showed complete $gamma_2$ lines in 21 days. Conventional mice showed complete $gamma_2$ lines both before and 21 days after virus inoculation.

Neither GF nor conventional mice had HI antibodies even at the lowest dilutions tested, 1:10 and 1:20 prior to inoculation. However, 21 days after virus inoculation, GF mice did not produce a rise in HI antibody in any of 16 individuals, while 9 of 20 conventional mice showed a striking rise. Hemagglutination-inhibition antibodies were shown to be located predominantly in the 18 S macroglobulin at 3 days after inoculation, and predominantly in the 7 S gamma globulin in 2 weeks after vaccination of conventional mice with formalized influenza virus(14).

The most significant group appears to be the one which received the 56×10^{-7} cc dose in which 4 surviving GF mice had an HI titer of less than 1-10 and the 6 conventional sera had HI titers of 1-80 to over 1-640. The lack of antibody rise in all except one individual in the 2 lower dose groups of the conventional animals was probably due to dilution of virus.

Serum of GF mice was reported to contain only 19 S bactericidal antibody prior to immunization with Salmonella typhosa, but developed both 7 S and 19 S bactericidal anti-

TABLE I. Hemagglutination-Inhibition Antibody of Germfree Mice Compared with Conventional Mice Infected with Mouse-Adapted Influenza A, Strain PR8 Virus.

Group	Exp	Dose of PR8 virus, ml of infected lung	HI end point for individual mice in reciprocal
Germfree	1	None	<10, <10
		7.2×10^{-5}	<10
		1.4×10^{-5}	<10, <10, <10, <10
	2	None	<10, <10, <10, <10, <20*
		224×10^{-7}	No survivors
		56×10^{-7}	<10, <10, <10, <10
		14×10^{-7}	<10, <10, <10, <10, <10, <20
		3.5×10^{-7}	<10, <10, <10, <10, <20, <20
Conventional	2	None	<10, <10, <20, <20†
		224×10^{-7}	<20, 160, 320
		56×10^{-7}	80, 80, 320, >640, >640, >640
		14×10^{-7}	<10, <20, <20,* 640
		3.5×10^{-7}	<10, <10, <10, <20, <20, <20, <20

* Pooled sera of 3 mice.

† Pooled sera of 2 mice.

bodies after repeated injections of *S. typhosa* (15). Germfree chickens produce specific antibodies to bacteria and to soluble proteins (16). The fact that antibodies can be produced by GF mice and chickens in response to bacterial antigens, confirms that the GF mouse has a defense mechanism competent to produce antibacterial antibodies. Therefore, the nature of the antigen or the dose of the antigen, or both, participate in determining the antibody response of the GF mouse.

The observation that GF mice did not produce HI antibody correlates with the lack of gamma$_2$ globulin seen by IE.

The fact that GF mice recover from the infection without producing HI antibodies in these experiments, suggests other mechanisms, non-specific inhibitors such as interferon, may be responsible for the resistance of the animals.

Summary. Germfree mice were found to be more susceptible than conventional mice to mouse-adapted influenza A strain PR8, although the difference in susceptibility is small. Work with more precise dosage methods is indicated to ascertain the nature of the difference. At 21 days after infection, the predominant leukocytic response in GF lungs was lymphocytic infiltration, while conventional mice responded with lymphocytes, polymorphonuclear leukocytes, and macrophages. Gamma$_2$ globulin was not detected by IE prior to infection either in GF mice or in 20 of 23 GF mice at 3 weeks after infection. HI antibody was not detected in GF mice before and after infection, but was detected in conventional mice after infection.

ADDENDUM. Since the submission of this paper, we have been able to elicit HI antibody production in germfree mice after intraperitoneal inoculation of five LD_{50} of live PR8 virus.

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Received February 20, 1964. P.S.E.B.M., 1964, v116.

Mercuric Cyanide Poisoning and Its Treatment in Dogs. (29250)

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Two common cyanides of mercury, mercuric cyanide and mercuric oxycyanide, have some commercial application. The former has been employed in electroplating, and the latter in leather industry. In medicine both have been used in treatment of syphilis and as surgical instrument disinfectants. Bolgert and Levy(1) advocated the combination of penicillin and mercuric cyanide as an ideal antisyphilitic therapy. Although Gettler and Baker(2) emphasized the dominant toxic action of mercury, it is conceivable that cyanides of mercury can cause cyanide and mercury poisoning simultaneously. Indeed Bonciu and Petrovici(3) showed the dual toxicity of mercuric oxycyanide in guinea pigs. The purpose of our investigation was to assess the toxicology of mercuric cyanide in dogs, and to test the efficacy of nitrite-thiosulfate therapy against the CN ions, and that of BAL (British Anti-Lewisite, or 2,3-dimercaptopropanol) against Hg ions. The success of using sodium nitrite and sodium thiosulfate for acute cyanide poisoning has long been known(4,5). The effectiveness of BAL for mercury poisoning was proven by Gilman *et al*(6) and clinically confirmed by Longcope and Luetscher(7). BAL apparently brought about the recovery of a woman who ingested 10 g of mercuric oxycyanide(8).

Methods. Fifty-two dogs were injected subcutaneously with various doses of mercuric cyanide in a 5% aqueous solution. The first group of 32 animals were observed for the appearance of acute toxic signs and their ultimate survival time in days. Sufficient data were obtained to permit calculation of the LD_{50} of the poison. A second group of 15 dogs were subcutaneously injected with larger doses of mercuric cyanide, immediately followed by intravenous injection of sodium nitrite and sodium thiosulfate to detoxify the cyanide portion of the molecule. The smallest dose of the group, 7.5 mg per kg, caused no acute toxic signs, and therefore no cyanide antidote was injected. All the dogs in the second group were treated with BAL, 10% in oil, to detoxify the mercury part of the molecule. In these experiments the first dose of BAL, 7 mg/kg, was injected intravenously at a very slow rate, 30 minutes after mercuric cyanide. Subsequent doses were given by intramuscular injection at 2, 4, 6 and 10 hours after the first dose. All surviving dogs were sacrificed on the 18th day, and their viscera and those of the dead animals were studied grossly and microscopically.

Results and discussion. Thirty-five dogs receiving mercuric cyanide alone ranging from 2 to 5 mg per kg did not develop toxic signs of cyanide poisoning—vomiting, restlessness, rapid respiration, ataxia, convulsions, urination and defecation. However a certain number of them died in an average of 4.1 to 15.6 days (Table I). Two dogs which were injected with a dose of 15 mg/kg had typical signs of cyanide poisoning within 20 minutes. One died of respiratory failure in 2½ hours, the other on the second day, making an average of 1.3 days. By the usual computation(9), the $LD_{50} \pm$ S.E. of mercuric cyanide is 2.71 ± 0.17 mg/kg. The LD_{50} of sodium cyanide was previously