and none developed antibody against either virus. The findings in the experiments did not indicate that immunization against rhinoviruses may be achieved by the procedure used.

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Microneutralization Test for the Reoviruses. Application to Detection And Assay of Antibodies in Sera of Laboratory Animals.* (31025)

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Reoviruses are known to have a very wide natural host range, infections occurring in a variety of wild and domestic animals in addition to man(1,2,3). Type 3 reovirus has been shown to be indigenous in laboratory mice(4,5), and its presence in mouse colonies has caused increasing concern as attention has been focused upon the importance of defining the viral spectra of laboratory rodent colonies employed for virological research, particlularly for studies on viral oncogenesis (*e.g.*, 6,7). Detecting reovirus infections in laboratory animals is also important from the standpoint of choosing animals without pre-existing antibody for the preparation of viral immune sera and for production of guinea pig complement for use in viral serology.

Antibodies to the reoviruses are generally demonstrated in animal sera by hemagglutination inhibition (HI) tests using either kaolin-treated(8) or untreated(4,7) sera. The prevalence of low levels of HI activity for reovirus type 3 in murine sera has aroused speculation as to whether such activity is due to specific antibody or to nonspecific inhibitors(4,6,7). However, kaolin adsorption for removal of nonspecific inhibitors has not received wide acceptance for use with murine sera since it requires a relatively large volume of serum, and also tends to remove specific antibody(4).

When a monitoring program for rodent

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viruses was initiated in this laboratory, it was found that the sera of most of the hamsters, mice and rats examined had HI activity for reovirus type 3 at serum dilutions ranging from 1:8 to 1:256. The problem of determining the specificity of this inhibitory activity together with the need for a sensitive and efficient procedure for screening other laboratory animal sera for the presence of reovirus antibodies led to the development of a microneutralization technic for the reoviruses. This report describes the technic and compares the levels of neutralizing and HI antibody detected in the sera of certain laboratory animals.

Materials and methods. Virus strains. The Lang strain of reovirus type 1, the D5-Jones strain of reovirus type 2 and the Abney strain of reovirus type 3 were employed for both HI and neutralization tests.

Hemagglutinating antigens. Some of the antigens for reovirus type 3 were prepared in the BS-C-1 line of grivet monkey kidney cells by Microbiological Associates, Inc.(7) and were made available to this laboratory by the National Cancer Institute, National Institutes of Health, Bethesda, Md. Antigens for reovirus types 1 and 2, as well as some of the type 3 antigens, were prepared in this laboratory in rhesus monkey kidney cell cultures by a procedure described elsewhere(9). All antigens were used at dilutions containing 4 hemagglutinating units.

Sera examined. Reovirus antibody assays were performed on sera from our colonies of mice [Webster Swiss and inbred Balb/C (Andervont)], hamsters (NIH polyoma-free), rats (inbred Osborne-Mendel), rabbits (New Zealand white), guinea pigs (random-bred English) and rhesus monkeys. Also, certain human sera from our diagnostic files were examined. Rhinovirus immune sera prepared in large animals (bovines, goats, baboons, rhesus monkeys and dogs) were assayed for the presence of reovirus antibodies; these sera were provided by the Research Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

Hemagglutination inhibition (HI) tests. HI tests were conducted in the microtiter system(10). Serum and virus dilutions were prepared in phosphate buffered saline, pH 7.2, and the 1.0% suspension of human group O erythrocytes was prepared in phosphate buffered saline, pH 7.2 containing 0.1% bovine albumin.† Two-fold dilutions of serum were prepared in 0.025 ml volumes, and to each dilution was added 0.025 ml of virus diluted to contain 4 hemagglutinating units. The serum-virus mixtures were incubated at room temperature for 45 minutes, and to each cup was added 0.025 ml of the 1.0% erythrocyte suspension. Tests were incubated at room temperature for 30 to 60 minutes, until the cell controls showed proper settling. Controls to test for possible agglutinating activity of the serum dilutions alone, a control to establish that the antigen had been employed at the proper unitage, a known positive and negative serum, and controls to indicate that the erythrocytes settled properly under the test conditions were included in each run.

All sera were inactivated at 56°C for 30 minutes prior to examination in HI tests. As indicated in the text, certain sera were adsorbed with kaolin according to the method described by Rosen(8), and the sera of bovines, goats, dogs, baboons and monkeys were also absorbed with human group O erythrocytes(8).

Neutralizing antibody assays in tube cultures. The sera of large animals were assayed for the presence of reovirus neutralizing antibodies by our standard procedure in tube cultures of monkey kidney cells(11) as well as in the microneutralization test described below.

Results. Problems encountered in monitoring rodent colonies for the presence of reovirus type 3 antibodies. A murine-virus monitoring program was initiated in this laboratory using the test procedures described by Parker *et al*(7). In general, sera were not adsorbed with kaolin prior to examination for HI antibodies, but they were inactivated at 56°C for 30 minutes. As indicated above, virtually every mouse, hamster or rat serum tested showed HI activity for reovirus type 3. Fig. 1 shows the distribution of reovirus

† Fraction V from bovine plasma, Armour Pharmaceutical Co., Kankakee, Ill.

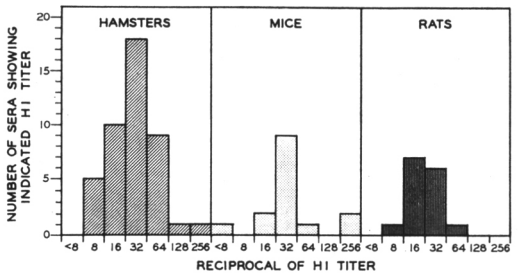


FIG. 1. Distribution of reovirus type 3 hemagglutination-inhibiting titers of *untreated* rodent sera.

type 3 HI titers of some representative groups of animals, and it is seen that the most frequently occurring titers were 1:32 for hamsters and mice and 1:16 and 1:32 for rats. Certain sera showed relatively high titers of 1:64, 1:128 and 1:256. The prevalence of HI activity in these sera led us to question its specificity, and consequently the sera were assayed for the presence of neutralizing antibody to reovirus type 3 and were also reexamined for HI activity following kaolin treatment.

None of the mouse, hamster or rat sera showed neutralizing antibody for reovirus type 3 when tested by the microneutralization technic described below (although antibody was detected in the sera of a variety of other animal species) and, as shown in Fig. 2, kaolin treatment abolished the HI activity of most, but not all, of the sera. Thus, the fairly high levels of HI activity for reovirus type 3 seen with untreated sera apparently represented nonspecific inhibitor rather than specific antibody.

The microneutralization technic described below was developed with the aim of providing a more sensitive and specific procedure for monitoring laboratory animals for the presence of reovirus antibodies.

Development of a microneutralization technic for the reoviruses. Ideally, a neutralization technic for detecting the presence of reovirus antibodies in sera of wild or domestic animals should be one which employs small volumes of sera, since much of its application is to murine sera or the sera of other small animals. The test should also be applicable to large-scale use, as murine-virus monitoring programs require the examination of large numbers of individual animal sera, as does the selection of animals free from antibody for use in immune serum or complement production. To these ends, (a) the microtiter system was employed to permit the use of small volumes of serum and, (b) in order to make the test applicable to large-scale use, a colorimetric (metabolic inhibition) procedure was devised to eliminate the need for microscopic reading of results.

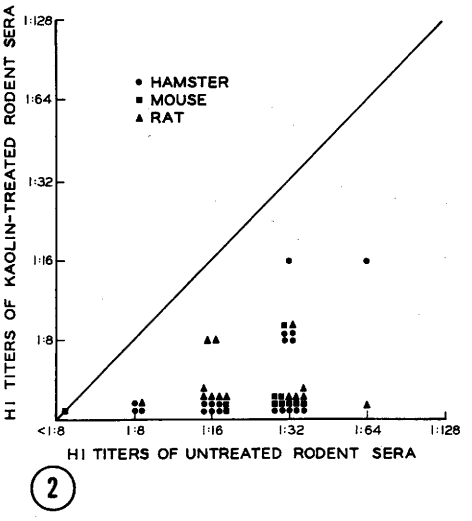
One of the problems inherent in the development of a colorimetric test for the reoviruses is the slow rate at which cytopathogenicity of these viruses is expressed. Thus, in a conventional metabolic inhibition system, the host cells have an opportunity to convert the pH of the medium to acid (with a resultant change in color of the phenol red indicator from red to yellow) before the virus exerts a cytolytic effect, and it is impossible to demonstrate a viral cytopathic effect colorimetrically (by retention of an alkaline pH).

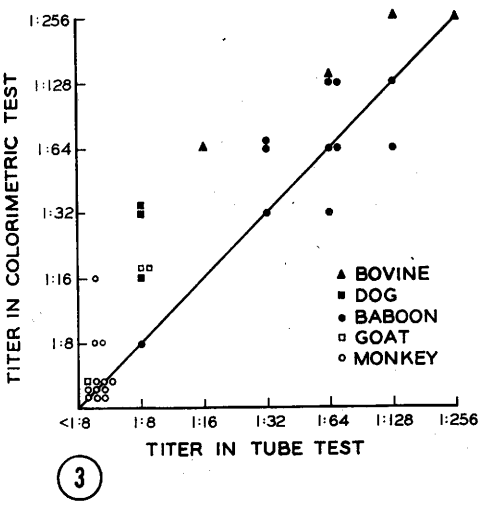
Based upon a number of preliminary experiments, a technic was evolved in which tests are initiated in Leibovitz medium No. 15 (L-15)(12) which contains galactose rather than glucose (eliminating the need for a bicarbonate buffering system) and certain amino acids in the free base form. Cells in culture do not convert the pH of this medium to acid as readily as they do a glucose-containing medium. After several days' incubation, during which time the virus exerts a cytopathic effect on the host cells, a second medium containing glucose and a bicarbonate buffering system is added and the tests are incubated for an additional 2 to 4 days. Cells destroyed by virus fail to convert the pH of this "indicator" medium to acid and the phenol red remains red or purple, while cells protected by neutralizing antibody metabolize, producing acid which converts the color of the medium to yellow. Details of this 2-phase metabolic inhibition technic are as follows.

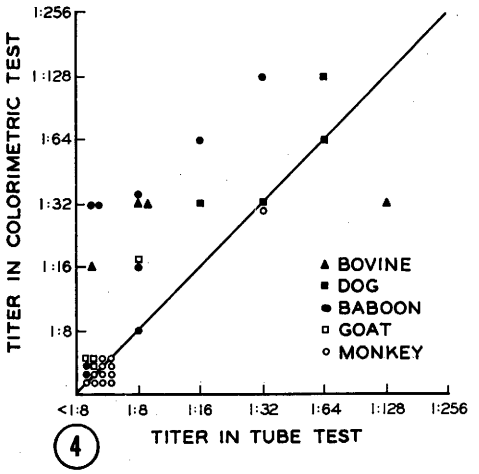
Tests are conducted in cups in disposable plastic microplates (Linbro model IS-MRC-96‡); these are soaked in 95% ethyl alcohol for 2 hours and sterilized by ultraviolet irradiation before use. HeLa cells of the D line

‡ Linbro Chemical Co., Inc., New Haven, Conn.

are employed, and reoviruses of the 3 types were passaged in this cell line several times before they were used for testing. Serum dilutions, virus dilutions and the cell sus-







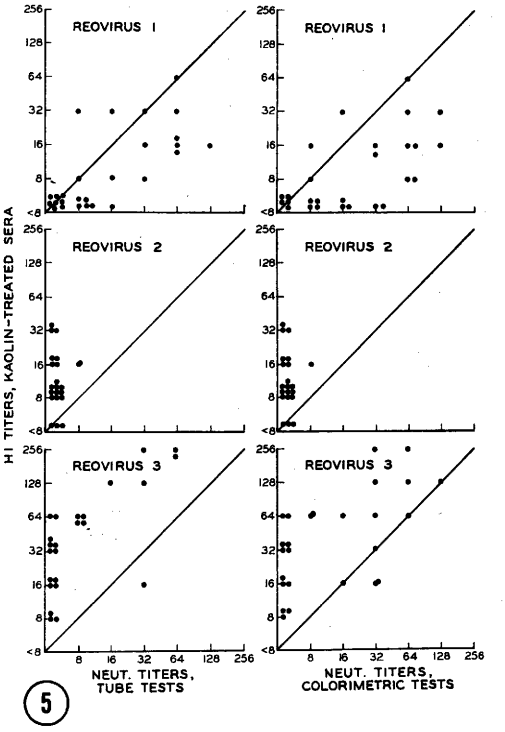


FIG. 2. Effect of kaolin treatment on reovirus type 3 hemagglutination-inhibiting titers of rodent sera lacking reovirus type 3 neutralizing antibody.

FIG. 3. Correlation between reovirus type 1 neutralizing antibody titers obtained in colorimetric tests and in conventional tube neutralization tests.

FIG. 4. Correlation between reovirus type 3 neutralizing antibody titers obtained in colorimetric tests and in conventional tube neutralization tests.

FIG. 5. A comparison of titers to reoviruses obtained in hemagglutination inhibition and in neutralization tests. Sera from dogs, baboons and rhesus monkeys.

pension are prepared in a medium consisting of 10% fetal bovine serum and 90% L-15. Sera are inactivated at 56°C for 30 minutes and 2-fold dilutions are prepared with diluting loops. To each set of serum dilutions is added 0.025 ml of a reovirus type diluted to contain 100 TCD_{50}. As a control to detect possible toxicity of the serum for the cells, dilutions ranging from 1:8 to 1:64 are prepared and to each is added 0.025 ml of medium rather than virus. Serum-virus mixtures are incubated for one hour at room temperature, and to each cup is added 0.025 ml of a suspension of HeLa cells containing 200,000 cells per ml. A concurrent titration of each test virus is performed to establish that the test dose contained approximately 100 viral TCD_{50}. As a check on the viability and number of cells the suspension is added to cups at 2×, 1×, ½× and ¼× the test concentration. Plates are carefully sealed with 3¼″ wide Paklon§ tape and tests are incubated at 36°C.

Tests for reovirus types 1 and 3 are incubated for 4 to 5 days before the second "indicator" medium is added, and for 3 to 4 days after addition of the second medium. Reovirus type 2 is slower to exert a cytopathic effect, and tests with this virus type require 7 days' preliminary incubation in order to permit the demonstration of viral CPE colorimetrically, but results can be read 2 days after addition of the "indicator" medium.

After the appropriate preliminary incubation period 0.05 ml of "indicator" medium consisting of 98% Eagle's minimum essential medium (prepared in Earle's balanced salt solution) and 2%fetal bovine serum is added to each cup. Plates are then resealed and incubated at 36°C until results can be read colorimetrically. A pH of 7.4 or higher is considered indicative of a viral cytopathic effect, while a pH of 7.2 or lower indicates the absence of virus, or viral neutralization.

Correlation between reovirus neutralizing antibody titers obtained in colorimetric tests and in conventional tube neutralization tests. Sera from 5 animal species (bovine, goat, dog, baboon and rhesus monkey) which had been assayed for reovirus antibodies in conventional tube neutralization tests were tested by the colorimetric microneutralization technic. Fig. 3 compares the titers to reovirus type 1 and Fig. 4 compares the titers to reovirus type 3 obtained by the two procedures. Too few sera had demonstrable antibody to reovirus type 2 to permit a valid comparison; however the 2 sera showing titers of 1:8 in tube tests also showed titers of 1:8 in colorimetric tests. In Fig. 3 and 4 it is seen that differences in titers obtained by the two methods rarely exceeded 2-fold, but the colorimetric test appeared to be slightly more sensitive than the conventional technic. Three sera which showed antibody to reovirus type 1, and 3 which showed antibody to reovirus type 3 in colorimetric tests, had titers of $<1:8$ in tube tests.

Comparison of reovirus antibody titers obtained in HI tests and in colorimetric and tube neutralization tests. Large-animal sera which had been examined for the presence of reovirus neutralizing antibody by tube and colorimetric procedures were then assayed for HI antibody to the 3 virus types. All sera were treated with kaolin prior to testing. Although it was unnecessary to absorb mouse, hamster or rat sera with human group O cells to remove natural agglutinins, the large-animal sera required absorption, and even after such treatment the sera of bovines and goats still agglutinated the test erythrocytes. Results presented in Fig. 5, which compares HI titers and neutralizing antibody titers seen in both colorimetric and tube tests, are those obtained on sera of rhesus monkeys, baboons and dogs.

In the case of reovirus type 1, neutralization tests revealed higher antibody levels than did HI tests; a number of sera had neutralizing antibody titers of 1:8, 1:16 and 1:32, but HI titers of $<1:8$. A different picture was seen with reovirus types 2 and 3. Only a single serum included in this comparison had demonstrable neutralizing antibody for reovirus type 2 (titer of 1:8) and it had a titer of 1:16 by HI. However, most of the sera without neutralizing antibody had low levels of HI activity for reovirus type 2. Similarly, a number of sera without neutralizing anti-

§ Minnesota Mining and Manufacturing Co., St. Paul.

TABLE I. Detection of Reovirus Neutralizing Antibodies in Sera of Various Animal Species by a Microneutralization Technic.

Species	Neutralizing antibodies detected to Reovirus 1	Reovirus 2	Reovirus 3
Mouse	0	0	0
Hamster	0	0	0
Rat	0	0	0
Rabbit	0	0	0
Dog	+	0	+
Rhesus monkey	+	+	+
Bovine	+	+	+
Goat	+	+	+
Baboon	+	+	+
Guinea pig	+	+	+
Man	+	+	+

body for reovirus type 3 had HI titers ranging from 1:8 to 1:64. It would appear that inhibitor to reovirus types 2 and 3 was not effectively removed from all of the sera by kaolin treatment. Neutralization tests would appear to be more reliable than HI tests for detecting reovirus antibody in the sera of these laboratory animals.

Presence of neutralizing antibodies to the reoviruses in sera of various species. In addition to the rodent and large-animal sera mentioned above, sera from rabbits, guinea pigs and man were also tested in microneutralization tests with the 3 types of reoviruses. Table I summarizes the results of tests on sera of the various species. None of the mouse, hamster, rat or rabbit sera examined from our colonies possessed neutralizing antibodies for the reoviruses. The dog sera tested showed neutralizing antibodies only for reovirus types 1 and 3, but antibodies to all 3 viral types were detected in sera from bovines, goats, baboons, rhesus monkeys, guinea pigs and man. These findings confirm the need for testing sera of laboratory animals for antibodies to *all three* reovirus types before using the animals for preparation of reovirus typing sera. In this connection, rabbits in this laboratory were found to produce reovirus immune sera which are satisfactory for type-specific identification of isolates in HI tests, while sera produced in guinea pigs and rhesus monkeys have shown little or no type specificity. This is probably a reflection of the fact that rabbits in our colony appear to be free from preexisting antibody to all three of the reovirus types. The extent of infection with reoviruses in different species varies between laboratories. If possible, animals with reovirus antibodies should not be employed for preparation of immune sera to other viruses, since sera produced in such animals may give misleading results in the identification of viral isolates, particularly in laboratories which are not familiar with the distinctive cytopathic effects caused by reoviruses. In any case, the reovirus antibody content of viral antisera should be defined, and the microneutralization test provides an efficient and sensitive tool for this purpose.

Discussion. The studies described here indicate that valid results can be obtained in HI tests for reovirus type 3 with rodent sera only if the sera are treated to remove nonspecific inhibitors. Kaolin treatment did not appear to be entirely effective in the removal of inhibitors; a few rodent sera without neutralizing antibody for reovirus type 3 showed HI titers of 1:8 and 1:16 after such treatment. Similarly, a number of the large-animal sera treated with kaolin still had fairly high levels of HI activity for reovirus types 2 and 3, but possessed no neutralizing antibody for the corresponding viral type.

Treatment of sera with filtrates from a psychrophylic *Pseudomonas* sp. has been found to be as effective as kaolin adsorption for removal of nonspecific inhibitors of echovirus, reovirus type 1 and adenovirus hemagglutinins from human sera; the procedure is also less detrimental to specific antibody(13). Preliminary studies have shown this method to be as effective as kaolin adsorption for inactivation of reovirus inhibitors in murine and other rodent sera. Heating animal sera at 56°C for 30 minutes would appear to be ineffective in reducing nonspecific inhibitors in rodent sera, and other studies reported from this laboratory(14) have shown human serum inhibitors of reovirus type 1 hemagglutination to be heat stable.

The microneutralization technic described herein provides an efficient and specific method for examining animal and human sera for the presence of antibody to all three types of reoviruses. Although results cannot be obtained as rapidly as for HI tests, the technic has other advantages over the HI test in ad-

dition to its sensitivity and specificity. Treatment of sera with kaolin and absorption with the test erythrocytes are time-consuming and wasteful of serum, the latter being of particular importance in working with murine sera. Also, kaolin has been shown to remove varying amounts of specific antibody from sera (9,15,16). Further, it is difficult to prepare reovirus type 3 antigens with sufficiently high hemagglutinating titers for human group O erythrocytes since the factors influencing the production of hemagglutinins in tissue culture systems have not been clearly defined. (Bovine erythrocytes are agglutinated to higher titer than human group O erythrocytes by reovirus type 3, but the use of this cell type necessitates absorption of all test sera with the erythrocytes and the use of two different types of erythrocytes for reovirus HI tests.) Technically the microneutralization test is as easy or easier to perform than HI tests. The only serum treatment required is inactivation at 56°C; preparation of the HeLa cell suspension is no more time-consuming than the preparation of an erythrocyte suspension; and addition of the second "indicator" medium is no more cumbersome than the addition of sensitized cells to a complement fixation test.

Some of the microneutralization technics described to date have utilized the viral cytopathic effect as an indication of unneutralized virus, and results have been read using an inverted microscope(17,18). The technic devised for use with the reoviruses permits reading of results colorimetrically and thus lends itself more readily to large-scale use. Two-phase metabolic inhibition systems would appear to be applicable for use with a variety of the slower-growing human and animal viruses and with other cell types.

Summary. The problem of determining the specificity of hemagglutination-inhibiting activity for reovirus type 3 seen in mouse, hamster and rat sera examined as part of a murine virus monitoring program, together with the need for a sensitive and efficient technic for detection of reovirus antibodies in the sera of other laboratory animals, led to the development of a microneutralization technic. The test, conducted in a HeLa cell system in disposable microplates, is a modified metabolic inhibition procedure which permits reading of results colorimetrically. The results obtained in microneutralization tests showed good correlation with those obtained in conventional neutralization tests conducted in tube cultures of monkey kidney cells. The technic was found to be suitable for detection of antibodies to all 3 reovirus types in sera of a variety of species, and it is particularly useful for monitoring rodent colonies for reovirus type 3 infections. HI tests appeared to possess less specificity than neutralization tests for demonstration of reovirus antibodies in animal sera.

The excellent technical assistance of Miss Katherine Yamamoto, Miss Donna Maher and Mrs. Victoria Nakamura is gratefully acknowledged.

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Effects of Thermal Burn and X-Irradiation on Early Mortality.* (31026)

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Thermal burn applied immediately before X-irradiation significantly increased mortality during the time period characteristic of radiation injury to the gastrointestinal tract(1). The increased mortality was attributed to an inhibitory effect of toxic materials produced at the site of the burn on recovery of the intestinal epithelium from radiation damage. If this were so, the degree of inhibition would be expected to reach a maximum some time after the burn, then decrease. This possibility was tested by a study of early mortality in rats when the interval between thermal burn and X-irradiation was varied. If the synergism between X-ray and thermal burn was due to a nonspecific inhibition of cell proliferation, then it would be expected to occur in bone marrow and be reflected by changes in the formed elements of the blood. This possibility was also studied.

Materials and methods. Mortality studies. Two series of rats were exposed to thermal burn and/or X-irradiation as previously described(1). The first consisted of 6 groups containing 10 animals each. One group received 700 R of X-irradiation alone while another received a standard one-minute burn followed immediately by irradiation. The 4 remaining groups received the burn at 6-96-hour intervals prior to irradiation. The second series was a replication of the first except that the burn was administered at different times after irradiation.

Peripheral blood studies. Four groups of 10 rats each were used. The first was exposed to a one-minute burn and the second to 700 R of X-irradiation. The third group received the burn followed immediately by 700 R of X-irradiation, while the fourth received the burn 96 hours after the irradiation. The cells in the bloods from each group were counted at 4, 7, 10, 14 and 21 days after injury. Bloods samples, taken by cardiac puncture, from at least 5 animals from each group were examined at each time period.

Erythrocyte studies. The erythrocytes in blood diluted ($1:10^5$) in Eagle's solution were counted and a distribution of cell volumes determined employing a Coulter Counter (Model B with plotter). The distribution represented a division of erythrocyte volumes into 25 distinct class intervals, called channels, representing a range of cell volumes from 6 to 160 cubic microns. An analysis of the operation of the Coulter Counter has been published(2).

Leukocyte studies. The leukocytes in blood diluted 1:500 were counted after the erythrocytes had been lysed by Cetavlon(3).

Results. Mortality. Administering the thermal burn at times up to 96 hours before X-irradiation increased mortality in the first 8 days postirradiation, the time period characteristic of gastrointestinal injury (Fig. 1). Mortality was maximum when the burn was administered at 72 hours prior to irradiation. When the burn was administered at times up to 96 hours after irradiation, fewer animals died from gastrointestinal injury but there was an increased mortality in the time period characteristic of bone marrow injury.

Erythrocytes. The frequency distribution of the cell volumes in a group of randomly

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