mice as a test object, the slopes of the dose-response curves for several estrogens appear to fall into 3 or more categories. In addition to estriol and 16-oxoestrone, a substance related structurally to stilbestrol showed a shallow slope, and a Vallestril derivative showed a slope steeper than the impeded estrogens, but not as steep as those of certain standard substances.

1. Huggins, C., and Jensen, E. V., *J. Exp. Med.*, 1955, v102, 335.

2. Hisaw, F. L., Velardo, J. T., and Goolsby, C. M., *J. Clin. Endo.*, 1954, v10, 1134.

3. Rubin, Betty L., Dorfman, Adaline S., Black, Lila, and Dorfman, R. I., *Endocrinology*, 1951, v49, 429.

4. Snedecor, G. W., *Statistical Methods, 4th ed.*, Iowa State College Press, Ames. 1946.

5. Fisher, R. A., *Statistical Methods*, 10th ed., Hafner Pub. Co., New York, 1948.

6. Sturtevant, F. M., *Endocrinology*, 1955, v56, 256.

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Determination of Respiratory LD_{50} from Number of Primary Lesions as Illustrated by Melioidosis.* (22548)

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(Introduced by Albert P. Krueger.)
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Lurie *et al.*(1) have used the number of primary pulmonary foci as a quantitative index of resistance to experimental tuberculosis as well as an index of the virulence of the infecting bacteria. In this report it is shown that in melioidosis, where one primary pulmonary lesion is fatal to mice and hamsters, it is possible to use this lesion-count method to determine the respiratory LD_{50}. This method shows good agreement with the usual titration method and has certain advantages over the latter.

Materials and methods. Mice, Namru strain(2), and golden hamsters were exposed to aerosols of virulent *Malleomyces pseudomallei*, strain 103-67, using a Wells-type atomizer and the method described by Leif and Krueger(3). After the animals died, the lungs were removed and inflated by injecting fixative (FU 48 Technicon) intratracheally. The primary pulmonary lesions were yellow and easily distinguishable from the tan to brown background of the fixed lung parenchyma (Fig. 1).

The number of organisms in the inhaled dose was calculated from colony counts of aerosol aliquots cultured on 4% glycerine beef extract agar. The number of pulmonary lesions was determined either by dissecting the lung if the lesion count was 25 or less, or using the graph shown in Fig. 2 if the lesion count was above 25.

Determination of Respiratory LD_{50} from total number of lung lesions. A single lung lesion in experimental melioidosis in mice and hamsters is always fatal since only progressing pulmonary lesions have been found. Almost without exception, such progressing primary lesions do not occur elsewhere in the body following exposure to aerosols of the organism. It is therefore possible to determine the number of organisms necessary to generate one pulmonary melioidotic lesion, or the "ratio," by dividing the inhaled dose by the number of lung lesions. This "ratio" is an expression of virulence and the respiratory LD_{50} is equal to the "ratio" multiplied by

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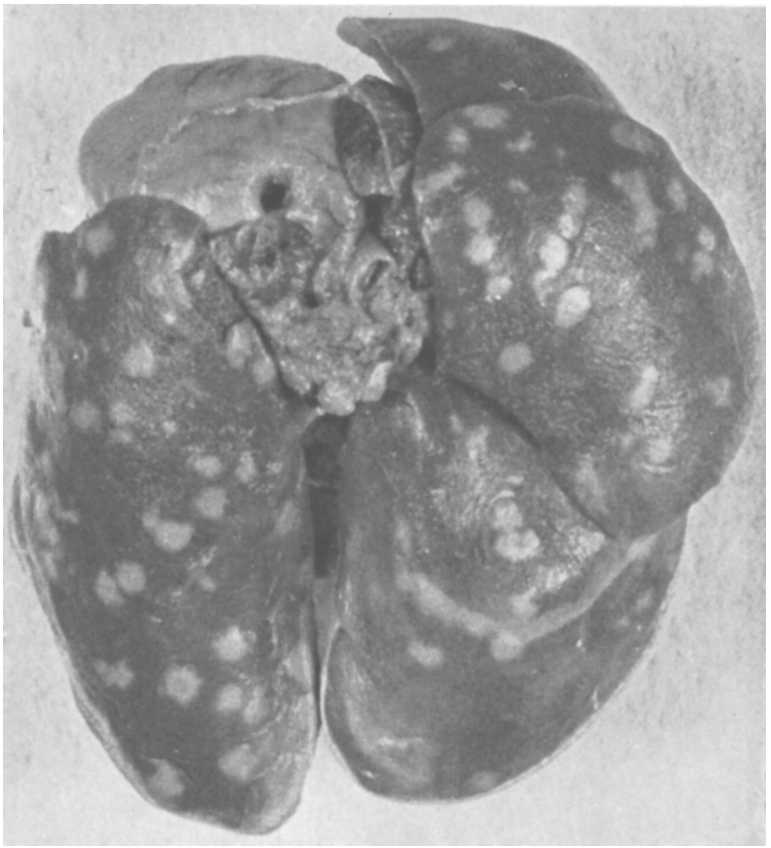

FIG. 1. Lung of a mouse dying 5 days after inhalation of 1.1×10^4 virulent *Malleomyces pseudomallei*, strain 103-67. This was fixed by intratracheal inj. of FU 48 (Technicon).

0.7.‡ This lesion-count method of calculating the respiratory LD_{50}, which seems correct theoretically, is also supported by experimental data. Table I lists mean LD_{50} values obtained by this method as well as by the titration method(4). The 95% confidence intervals for these mean LD_{50} values were determined by using a modification of the binomial distribution(5). The confidence intervals for the lesion-count method were found to overlap those for the titration method. It is probable that an even greater overlap exists than that illustrated, because the total lesion counts which were derived from Fig. 2 were assumed to have the same precision as the direct lesion counts.

Estimation of total number of pulmonary lesions when they are too numerous to count. The accurate counting of about 25 lesions per lung in mice and hamsters is not difficult, but as the number of lesions increases, inaccuracies occur. Reasonable estimates of the latter may be made by assuming that (1) each lesion started as a completely isolated focus of infection regardless of the dose, and (2) the ratio of the number of inhaled organisms to total lesions was the same for high as for low dosages. This is predicated on the observation that the number of alveoli in the lung far exceed the number of bacteria inhaled. Based on actual counts on histological sections, it was estimated that there were approximately 40 million alveoli in mice and 160 million alveoli in hamsters. Maximal doses in experimental melioidosis never exceeded one million organisms. Since approxi-

‡ If all the animals showing lesions had but a single lesion, the average lesion count in a group showing 50% mortality would equal 0.5. Since some animals have more than one lesion, the average is raised to 0.7 lesion, actually 0.693 lesion, because of Poisson's distribution.

TABLE I. Comparison of Respiratory LD_{50} Values Obtained by Lesion-Count and Titration Methods in Mice and Hamsters Infected with *M. pseudomallei*, Strain 103-67.*

No. of animals	Dose inhaled (organisms)	Avg surface lesion count	Total lesions†	Ratio‡	Organisms per LD_{50}: Lesion count method, Ratio × 0.7	Lesion count method, Geometric mean§	Titration method, geometric mean§
			Mice				
8	4.2×10^0		.25	17	12		
7	1.4×10^1		1.0	14	10		
8	1.3×10^2		7.3	18	12		
10	3.3×10^3	33	44	75	53	17 (10–28)	9 (5–17)
10	3.8×10^1		1.6	24	17		
10	2.5×10^2		5.0	50	35		
10	1.3×10^3		21.0	62	43		
10	1.3×10^4	85	325	40	28		
10	2.3×10^5	369	6900	33	23	28 (18–43)	16 (8–32)
7	3.6×10^1		1.0	36	25		
8	3.1×10^2		8.9	35	25		
8	1.8×10^3	34	48	38	27		
4	1.0×10^4	138	880	11	8		
6	1.3×10^5	363	6500	20	14	18 (11–31)	25 (12–59)
			Hamsters				
7	9.3×10^0		0	—	—		
8	4.0×10^1		3.1	12	9		
6	2.9×10^2		7.4	39	27		
6	6.6×10^3	45	85	78	55		
6	8.6×10^3	59	150	57	40	27 (15–42)	22 (9–40)
9	3.4×10^1		1.2	28	20		
10	2.7×10^2		2.2	123	86		
8	2.6×10^4	142	960	27	19		
4	6.6×10^4	186	1700	39	27		
6	7.3×10^4	176	1500	49	34	31 (19–51)	11 (6–20)
8	2.4×10^1		1.3	18	13		
6	9.6×10^1		2.0	48	34		
6	2.9×10^2		4.8	60	42		
7	1.7×10^3	34	48	35	25		
7	1.5×10^4	68	200	75	53		
7	1.1×10^5	509	10000	11	8	24 (15–38)	13 (6–29)

* Incomplete data from experiments employing other strains of *M. pseudomallei* or vaccinated, rather than normal, mice and hamsters also supported the principles presented in this report.

† Counts above 25 estimated by Fig. 2.

‡ Variation observed in ratios is probably inherent in the exposure apparatus rather than in sampling or lesion-counting technics.

§ 95% confidence interval in parentheses.

mately 32§ inhaled organisms were required to produce one lesion in mice (with strain 103-67), the total number of lesions was calculated by dividing the inhaled dose by 32 (Table II). Plotting the total lesions calculated in this way against the surface lesion count in mice resulted in the curve shown in Fig. 2 which in turn serves to estimate the total lesions in mice from any surface lesion count. Fig. 2 also shows a similar curve for hamsters. After an estimate of the total number of lesions is obtained, the ratios may be computed by dividing the calculated inhaled dose of organisms by this estimate. From such a ratio an estimate of the organisms per LD_{50} is determined by multiplying by 0.7.

Discussion. The advantages of the lesion-count method as compared to the titration method for determining the respiratory LD_{50} are that (1) the LD_{50} can be obtained with the former even if all the animals die or are killed; (2) the former is probably more accurate; and (3) fewer animals and exposures

§ 32 is the geometric mean of "ratios" obtained from average lesion counts of 25 or less.

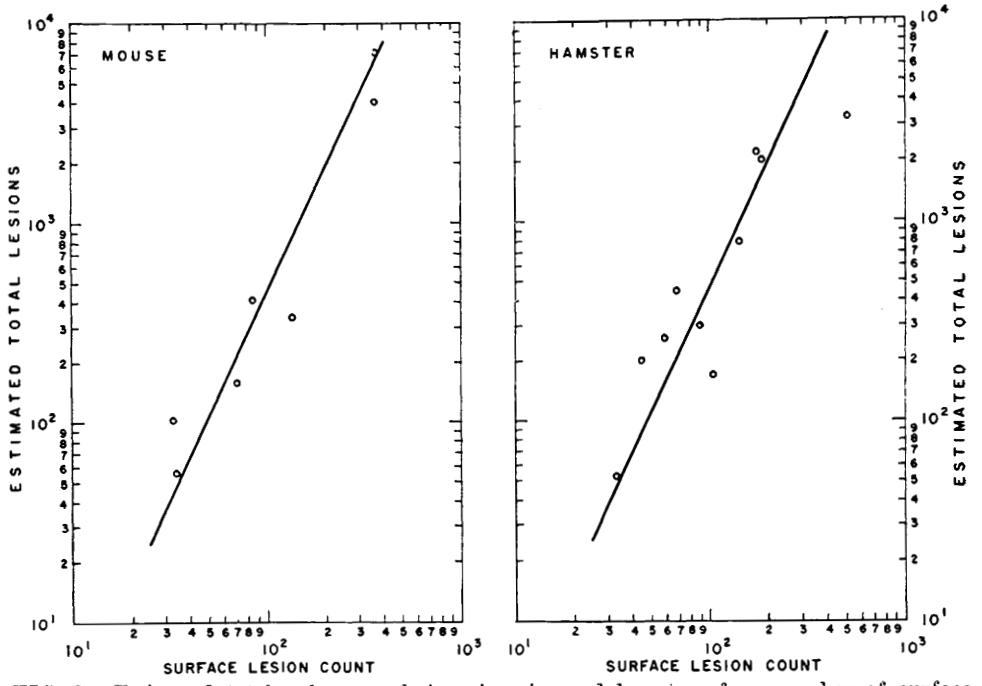

FIG. 2. Estimated total pulmonary lesions in mice and hamsters from number of surface lesions. (See Table II for derivation.)

are required. This latter point can mean considerable financial saving when monkeys are employed in such determinations. It should be mentioned that with the lesion-count method an LD_{50} can be computed from a single cloud exposure and it should therefore be possible to compare many more experimental variables simultaneously. The lesion-count method may also be used in conjunction with the usual titration method. The graphs relating the total number of pulmonary lesions in mice and hamsters to the surface lesion count are only approximations. A certain amount of error would be introduced should the pulmonary lesions be the same in number but smaller in size than those used to construct these graphs since fewer lesions would reach the surface of the lung. Also the converse is true. Nevertheless, these graphs should find application in studies with this and other organisms that produce discrete pulmonary lesions because they yield information from experiments that might otherwise be useless.

Summary. 1. A method is described whereby the respiratory LD_{50} can be obtained from the dose of organisms inhaled and from counts of primary pulmonary lesions, provided a single pulmonary lesion is fatal and progressing primary lesions do not occur elsewhere. This

TABLE II. Estimation of Total Pulmonary Lesions in Mice and Hamsters when Number of Surface Lesions Is Known. (Data for Fig. 2.)

No. of animals in exp.	Dose inhaled (organisms)	Estimated total No. of lesions*	Avg surface lesion count
	Mice		
8	1.8×10^3	56	34
10	3.3×10^3	103	33
15	5.1×10^3	159	70
4	1.1×10^4	340	138
10	1.3×10^4	410	85
6	1.3×10^5	4100	363
10	2.3×10^5	7200	369
	Hamsters		
7	1.7×10^3	52	34
5	5.6×10^3	170	104
6	6.6×10^3	200	45
6	8.6×10^3	260	59
5	1.0×10^4	300	90
7	1.5×10^4	450	68
8	2.6×10^4	790	142
4	6.6×10^4	2000	186
6	7.3×10^4	2200	176
7	1.1×10^5	3300	509

* Dose/32 for mice and dose/33 for hamsters.

method was tested and confirmed in experimental melioidosis in mice and hamsters, and its advantages over the usual titration method were discussed. 2. The number of *M. pseudomallei* necessary to generate one lesion, called the "ratio," is obtained by dividing the inhaled dose by the total number of primary pulmonary foci. This "ratio" multiplied by 0.7 equals the respiratory LD_{50}. Data and graphs are presented to estimate the total number of primary pulmonary lesions when they are so numerous that only the lesions on the surface of the lung can be counted. Estimates of the 95% confidence interval of the respiratory LD_{50} obtained by this lesion-count method overlapped those obtained by the titration method.

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1. Lurie, M. B., Abramson, S., and Heppleston, A. G., *J. Exp. Med.*, 1952, v95, 119.

2. Garber, E. D., and Hauth, F. C., *J. Hered.*, 1950, v41, 122.

3. Leif, W. R., and Krueger, A. P., *J. Infect. Dis.*, 1950, v87, 103.

4. Goldberg, L. J., Watkins, H. M. S., Dolmatz, M. S., Schlamm, N. A., *J. Infect. Dis.*, 1954, v95, 9.

5. Goldberg, L. J., (to be published).

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A Chick Embryo-Derived Complement-Fixing Antigen for Western Equine Encephalomyelitis. (22549)

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Complement-fixing antigens for the diagnosis of certain neurotropic viral infections, for example, Western equine encephalomyelitis, St. Louis encephalitis, Japanese B encephalitis, are in general derived from infected rodent (usually mouse) brain tissue. Howitt(1) used ether-extracted mouse brain as an antigen for the detection of antibodies to the Western equine and St. Louis encephalitis viruses but found that the margin between specific and nonspecific reactions was narrow. Casals and Palacios(2), also using mouse brain as an antigen source, were able to remove some of the nonspecifically reacting substance by means of repeated freezing and thawing. Havens *et al.*(3) used high-speed centrifugation to remove nonspecifically reacting substances from mouse and hamster brain antigens.

In the case of the Western equine encephalomyelitis virus, complement-fixing antigens have also been prepared from homogenized chick embryos. Mohler(4), who used formalinized preparations, found them to be highly anticomplementary and frequently nonspecific. Brown(5), however, found that homogenized embryo suspensions, either uninactivated or inactivated by ultraviolet irradiation, gave specific fixation with guinea pig hyperimmune sera. One of the difficulties with WEE (and EEE) mouse brain antigens has been the low antibody titers obtained with human sera (WEE(6,7)) and horse sera (EEE and WEE(8)), and also the occurrence of nonspecific results(6). DeBoer and Cox(6) found that antigens prepared from homogenized chick embryos or from mouse brains treated by repeated freezing and thawing reacted with syphilitic sera and that this non-specific reactivity could be removed by benzene extraction. España and Hammon(9) modified the method in order to obtain more sensitive antigens. Preparation of mouse brain antigen by the method of DeBoer and Cox requires approximately 4 days(9), and by the method of España and Hammon, approximately 36 hours(9). A simpler and less expensive means of preparing antigens is de-