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Reliability of 50% Egg Infectivity Titrations with Suspensions of Influenza Viruses Prepared from Mouse Lungs.* (20002)

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The reliability of replicate 50% end point determinations, employing the PR8 strain of influenza virus for titration of infectivity in mice or eggs, has previously been described (1,2). Weighted end points, based on pulmonary lesions, and EID_{50} determinations were shown to have approximately the same reproducibility, if equal numbers of mice or eggs were employed in each type of test. It is noteworthy that these studies were carried out with a strain of virus highly adapted to eggs and mice and that purified suspensions of egg passage virus were used in these experiments. The present report describes the precision of duplicate titrations of infectivity in eggs executed with crude suspensions of mouse lungs infected with recently isolated strains of Type A-prime and Type B influenza viruses. These lines of virus had been passed relatively few times in eggs or mice, and might be considered to be less well adapted than the PR8 strain for growth in either host. Moreover, a variable period of time extending up to 3 months intervened between the first and the second determination, and during this interval the lung suspensions were stored in the frozen state. Nevertheless, it will be shown that under these circumstances the reliability of 50% end point determinations in eggs was approximately equal to that described for similar determinations, in eggs or in mice, with purified suspension of the PR8 strain of virus.

Materials and methods. Viruses. The Rhodes strain of Type A-prime and the Allen

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TABLE I. Standard Error of the Difference between Duplicate Titrations (EID_{50}) with Influenza Viruses.

Strain	Type	Passage	1st titration	2nd titration	Diff. (Δ)	Diff. from the mean* ($\Delta-\bar{\Delta}$)	Square of diff. from the mean $(\Delta-\bar{\Delta})^2$
Allen	B	F1E19M1	4	4.6	+.6	+.6	.36
		"	4.5	4.7	+.2	+.2	.04
		"	4.3	4	—.3	—.3	.09
		"	4.5	4.5	0	0	0
		19M2	5.3	5	—.3	—.3	.09
		"	5.3	4.5	—.8	—.8	.64
		19M5	4.7	4.2	—.5	—.5	.25
		23M7	3.3	3.5	+.2	+.2	.04
		23M9	5	4.5	—.5	—.5	.25
		19M11	4.7	4.7	0	0	0
		"	4.7	4.6	—.1	—.1	.01
		19M13	6.5	6.5	0	0	0
		"	6.7	6.7	0	0	0
		"	6.5	6.5	0	0	0
		23M13	3.5	4	+.5	+.5	.25
		19M19	5	5.6	+.6	+.6	.36
		"	6	6.5	+.5	+.5	.25
		19M21	6.2	6	—.2	—.2	.04
		F1M22	4	4.5	+.5	+.5	.25
Warner	B	E11M1	5.3	4.6	—.7	—.7	.49
		"	5	5.3	+.3	+.3	.09
		E11M10	7.5	6.7	—.8	—.8	.64
Rhodes	A'	F4M22	7	7	0	0	0
			7	7	0	0	0
			7	7.2	+.2	+.2	.04
			5.5	5.7	+.2	+.2	.04
			7	7	0	0	0
	Total = 27				—.4		4.22

* Mean difference between duplicate titrations $= \frac{-.4}{27} = -.015$, *i.e.* $0 = \bar{\Delta}$.

$$\text{Stand. error of the difference} = \sqrt{\Sigma\frac{(\Delta-\bar{\Delta})}{N-1}} = \sqrt{\frac{4.22}{26}} = .40.$$

strain of Type B influenza virus were isolated in this laboratory in 1947 and 1945, respectively. The Warner strain of Type B influenza virus was isolated in Australia in 1945 and obtained through the courtesy of F. M. Burnet. The passage history of each line is given by a formula in which the letters F, M, and E refer to ferret, mouse, or egg passage. The subscript refers to the number of transfers made, and the order of the letters refers to the sequence in which the passages were carried out. *Viral suspensions.* Lungs were harvested from mice aseptically at variable intervals after inoculation. Ten per cent suspensions were prepared by grinding lungs in a mortar with sterile alundum, employing 2 ml of diluent per lung. The suspensions were lightly centrifuged, the supernates were decanted and used, and the sediments were discarded. *Titrations of infectivity in eggs.* Serial 10-fold dilutions of virus suspensions were made in diluent chilled in an ice-water bath. A volume of 0.2 ml of the desired dilution of virus was inoculated in the allantoic cavity of fertile chicken eggs which had been incubated at 38°C for 9 or 10 days prior to inoculation. Four eggs were used for each dilution. In titrations with Type B influenza virus the eggs were incubated at 35°C for 72 hours before harvesting. With Type A-prime virus a 48-hour period of incubation was chosen. Allantoic fluid from each egg was tested for the presence of virus by agglutination of chicken erythrocytes, and the 50% in-

fectivity titer (EID_{50}) was calculated(3). End points were calculated to two decimal places, and the values rounded to one decimal place. Titers are expressed as the logarithm of the reciprocal of the dilution calculated to represent one EID_{50}. *Solutions.* Saline refers to 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate. Ten per cent normal horse serum in saline containing 600 units of penicillin and 1.25 mg of streptomycin per ml were used as diluent for the preparation of 10% suspensions of lung and for the dilution of lung suspensions used for titration. Normal horse serum was heated at 56°C for 30 minutes prior to use.

Experimental. Standard error of the difference (S.E. diff.) between duplicate titrations. In the course of experiments designed for other purposes 27 duplicate titrations were performed with 24 different suspensions of mouse lung each containing one of 5 different lines of Type A-prime or Type B influenza viruses. These determinations were carried out by a single operator over a period of 3 years. In general, all suspensions had been stored in the frozen state at either —70°C or —50°C prior to titration. The results of these experiments and a tabulation of the calculations are shown in Table I. It is important to note that a difference in the end points obtained with virus suspensions are pertinent only when comparisons are made between rows in Table I (horizontal), as these figures represent the results of duplicate titrations. Differences within the columns (vertical) are attributed to the fact that either different viruses or different concentrations of virus were used as inocula, or that the lungs were harvested at different intervals of time after inoculation with the same suspension of virus. Inspection of the data indicates that the error of duplicate titrations was distributed normally. This impression was substantiated by demonstrating that if the differences between paired titrations were plotted on normal probability paper(4), a straight line relationship was evident. These findings validate the use of the Standard Error of the difference between duplicate titrations to assess the significance of variation between end points. The mean difference between

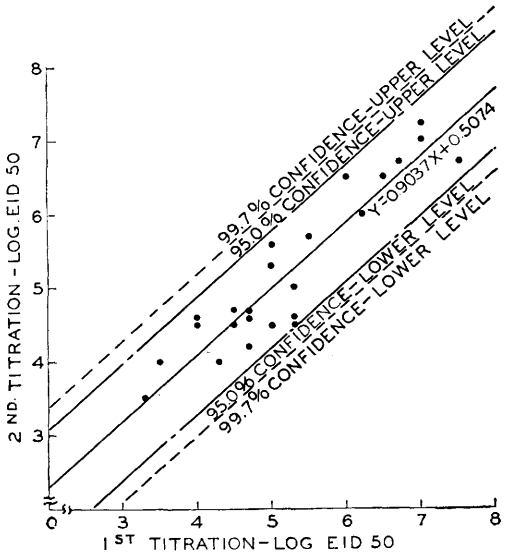

FIG. 1. Correlation of values observed in first determination and second determination.

duplicate titrations was —0.015. For practical purposes this value was considered as zero. The S.E. of the difference between duplicate titrations was 0.40. Thus variation between 2 end points of more than twice the S.E. Diff., *i.e.*, .80 may be considered statistically significant at the 95% level of confidence. Variation between 2 end points of more than 3 times the S.E. Diff., *i.e.*, 1.2 may be considered significant at the 99.7% level of confidence.

Analysis of the data by linear correlation. If the exact value of the first titration could be duplicated in the second, and if the values of the pairs of determinations were plotted on arithmetic grid so that ordinate distances represent values obtained in the first determination while abscissa distances those in the second, a straight line with a slope of one would be obtained on which all of the points would fall. An analysis of deviations from this theoretic ideal, in data derived experimentally, provides another measure of the reproducibility of these end points. In Fig. 1 the pairs of values recorded in Table I were plotted as described above. A line of best fit was calculated with equations derived from the theory of least squares(5). The slope was found to be 0.904. This value is not strikingly different from the theoretical slope of

TABLE II. Comparison of End Point Differences Required for Significance at Various Levels of Probability.

Probability (%)	End point difference required: Purified suspensions of Type A influenza virus — Mouse titration* (5 mice)	Purified suspensions of Type A influenza virus — Embryo titration† (5 eggs)	Crude suspensions of lungs infected with Type A′ and Type B influenza viruses — Embryo titrations (4 eggs)
95	.73	.62	.8
99.7	1.11	.93	1.1

* Recalculated from data of Lauffer & Miller(1).
† " " " " " Knight(2).

one. Inspection of the deviations of the points from the line of best fit indicates that the error of these determinations is distributed normally. By a mathematical analysis of the deviations from this line of best fit, or expected value, one may calculate a standard error of the estimate (S.E. Est.)(6) of the agreement between duplicate titrations. The S.E. Est. proved to be 0.38. Since the slope of the line of best fit is so close to 1.0, it would be expected that the value of a second determination would be approximately equal to the value of the first except for the influence of errors due to chance. Hence a difference between any 2 end point determinations greater than twice the S.E. Est. ($2 \times 0.38 = 0.76$ or .8) may be considered significant at the 95% level of confidence and a difference 3 times that value, or 1.1, significant at the 99.7% level. These limits are indicated in Fig. 1. Attention is called to the similarity of the value of the difference between any 2 titrations required for significance, as derived in this and in the preceding section. This is not surprising since the assumptions underlying the 2 methods are the same.

Comparison of the variation of end point determinations with other strains of influenza virus and with other methods of titration. In Table II a summary is presented of the difference required for significance in end point determinations employing influenza viruses, as described in this and in previous reports(1,2). The data concerning titrations of purified suspensions of the PR8 strain of influenza virus in mice and in eggs have been recalculated, so that probability is expressed in per cent. The differences between successive end point determinations required for significance at various levels of confidence are remarkably similar even though the data were compiled from the results of different investigators employing different suspensions of influenza viruses in titration of infectivity in eggs or in mice. These observations suggest that in general when standardized techniques are followed, the reliability of EID_{50} determinations is a function of errors inherent in these methods. The reliability appears to be independent of the strain of virus used, the source of virus suspension employed, or the host used for infectivity titration.

Summary. 1. The use of 2 different methods for analyzing the reliability of 50% end point determination has been illustrated. The data were available because duplicate titrations of a number of virus suspensions were required for the purposes of other experiments. Data of comparable significance could have been obtained by a similar number of replicate titrations of the same virus suspension. The latter type of procedure is tedious, expensive, and is seldom carried out. It would seem likely that the records of other investigators would contain data suitable for analysis by the methods used in this report. 2. In the present study the reliability of 50% egg infectivity titrations was determined with crude suspensions of lungs infected with recently isolated strains of Type A-prime and Type B influenza viruses. The reliability of these determinations was similar to that previously described for titrations of infectivity for mice or for eggs with purified suspensions of the PR8 strain of influenza virus.

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Effect of Acetoacetate and β-Hydroxy Butyrate on Riboflavin and Nicotinic Acid. (20003)

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It has recently been reported(1) that sodium acetoacetate on prolonged injection in rabbits brings about several diabetic symptoms associated with increase of blood sugar, decrease of glucose tolerance and depletion of glycogen storage in liver and muscle. This substance has also been found to cause depletion of plasma ascorbic acid and increase of blood lactic acid(2), disturbance in the balance of hepatic glycogenesis and glycogenolysis(3) and increase in blood urea and inorganic phosphate(4). It has also been shown(5) that acetoacetate and β-hydroxy butyrate, when injected intraperitoneally for a considerable period cause enormous depletion of vit. B_1 in blood and urine thus accounting for the observed neuritic conditions in the old cases of diabetic patients. Besides development of neuritic conditions through deficiency of vit. B_1, other symptoms such as glossitis and stomatitis are also frequently noticed in diabetics suffering for a long period, which have been found by Sydenstricker *et al.* (6) to be cured by nicotinic acid. Several investigators showed how members of the vit. B_2 complex have a beneficial role in carbohydrate metabolism. Gordon(7) noticed a drop of blood sugar by oral administration of nicotinic acid amide in diabetic patients and Ricciute(8) reported its importance in raising the low glycogen content of muscle and liver. Schroeder(9) has shown that riboflavin is helpful in relieving hyperglycemia and according to the findings of Collazo and Bayo(10) the biochemical functions of riboflavin consist in promoting the accumulation of liver and muscle glycogen from administered glucose or lactic acid produced in the organism.

Since carbohydrate metabolism is disturbed by the ketone bodies and since nicotinic acid amide and riboflavin are the constituent parts of coenzymes I and II and the yellow enzyme, the presence of which is essential for proper utilization of carbohydrate, it was thought worthwhile to ascertain whether the substances like acetoacetate and β-hydroxy butyrate have any destructive influence on these vitamins *in vivo*. The present paper will show how both the substances on injection to rabbits cause tremendous depletion of nicotinic acid as well as riboflavin in blood and urine.

Experimental. Twenty healthy rabbits each weighing approximately 2 kg were selected. They were kept on a diet of germinated gram (*Cicer arietinum*). They were divided into 3 groups of 8, 8, and 4. Group 1 was given daily injection of sodium acetoacetate. Group 2 was injected with sodium β-hydroxy butyrate intraperitoneally in doses shown in Table I. The third group was not given any injection (control). For riboflavin estimation in blood, the blood sample was first deproteinized by trichloroacetic acid and kept overnight in the incubator. After incubation, the riboflavin contents of the blood were estimated according to the method of Burch *et al.*(11). Urine riboflavin was estimated according to