

# THE APPLICATION OF WALKER'S INDEX OF FUNCTIONAL NORMALITY TO A STUDY OF DEVELOPING CHICK EMBRYOS INFECTED WITH THE LEVADITI STRAIN OF VACCINIA

RACHEL E. HOFFSTADT AND HELEN B. TRIPI

*Department of Microbiology, University of Washington School of Medicine,  
Seattle 5, Washington*

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Two methods are generally employed for analyzing results obtained in studies of the growth of viruses on developing chick embryos: (1) counting of pocks and changes in the structure of the egg membranes and (2) estimation of the percentages of deaths among the embryos. No attempts have previously been made to record the changes of functional normality of the developing chick during the experiments. In most cases the morphology of embryos observed when the eggs were candled has been used to determine the age and development of the embryos. This procedure has not always been reliable because the source of the eggs is often a commercial hatchery and the eggs are not from controlled flocks.

Walker (1938), in a study of normal stages of the development of chick embryos, suggested the use of the crown-rump length in conjunction with the wet weight, expressed as index values, as a criterion for determining variation in functional normality. He used the formula  $I = \frac{1,000\sqrt[3]{W}}{L}$  to calculate these values. In the study of 361 normal Barred Plymouth Rock chick embryos of 7 to 19 days' incubation age, he found a reasonably good frequency curve could be set up, with a mean represented by index values in frequency classes 333 to 335 and a range with extreme limits in frequency classes of 315 to 317 and 351 to 353. Any embryos outside this range he considered abnormal. In our studies of developing chick embryos inoculated by the allantoic route with the Levaditi strain of vaccinia, the Walker formula was applied as a means of analyzing some of the data obtained.

## EXPERIMENTAL METHODS

All of the eggs in these experiments were from New Hampshire Red chickens and were obtained from a commercial hatchery where they had been incubated in a Buckeye "forced draft" type incubator. Eggs were candled at the hatchery and were packed at incubator temperature in containers heavily lined with cotton. They were then transferred to the laboratory incubator, the temperature of which was 39.5 C. On the day prior to use, the eggs were opened by the Goodpasture and Buddingh technique (1935) and returned to the incubator.

In these experiments two lots of eggs were used. They will be designated as series A and B. Series A contained 223 normal embryos and 90 infected embryonated eggs. The range in age for normal and infected eggs was the same, 10 to 18 days. The normal embryos were used in part as incubator controls for the

infected eggs and the rest as a source of allantoic and amniotic fluid in another phase of the experiment. Ninety embryonated eggs were inoculated by the allantoic sac method, with 0.1 ml of a 1:150,000 dilution of the Levaditi strain of vaccinia, in order to ensure six living embryos for each of the 9 days of the experiment. Fifty-four infected eggs were studied.

Series B contained 174 normal eggs and 179 infected eggs. The latter were used to subculture the virus in an experiment to determine the effect *in vitro* of allantoic fluid and amniotic fluids on the virus. They were of the same age at the time of inoculation, 10 days, and were 12 days old when sacrificed. The chorio-

TABLE 1  
*Tabulation of average values reported for normal chick embryos*

DAY	WEIGHT			LENGTH		pH ALL. FL.		pH AMN. FL.		LN. WT		
	(1)	(2)	(3)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(3)
10	2.4	2.0	2.6	3.8	3.7	7.68	7.49	7.83	6.90	0.81	0.64	0.93
11	3.4	2.8	3.4	4.3	4.2	8.71	7.31	7.75	6.83	1.22	1.04	1.34
12	5.2	3.8	5.2	5.0	4.7	7.96	7.39	7.72	6.65	1.63	1.34	1.64
13	7.7	5.2	7.3	5.8	5.2	7.98	7.39	7.52	6.58	2.03	1.64	1.99
14	9.8	7.4	10.4	6.2	5.8	8.00	6.79	7.54	6.54	2.27	2.01	2.34
15	13.3	10.0	13.8	6.9	6.4	7.37	5.87	6.90	6.40	2.58	2.31	2.62
16	15.3	12.8	15.7	6.9	7.1	6.80	5.75	6.80	6.92	2.72	2.54	2.76
17	17.5	14.9	18.8	7.5	7.4	6.28	5.60	6.89	7.12	2.85	2.70	2.94
18	22.5	17.3	22.3	8.2	7.8	5.85	5.71	7.17	7.48	3.11	2.85	3.10

Investigators are indicated by number at the heads of the columns as follows: (1) Hoffstadt and Tripi; (2) P. A. Walker; (3) Romanoff and Romanoff.

Legend: All. Fl. indicates allantoic fluid; Amn. Fl. indicates amniotic fluid; LN. Wt indicates the natural logarithm of the weight.

allantoic membrane route of Goodpasture and Buddingh (1935) was used for the inoculations in series B.

When the eggs were to be sacrificed, the allantoic and amniotic fluids were removed with a 2-ml syringe fitted with a 27-gauge  $\frac{1}{2}$ -inch needle, and extreme care was taken not to puncture blood vessels. The embryos were then placed in sterile petri dishes and freed of their extraembryonic membranes, wet weights were taken, and crown-rump length measurements were made. The pH's of the allantoic and amniotic fluid were determined for all embryos by the use of the Beckman pH meter.

#### EXPERIMENTAL RESULTS

*Rate of growth.* In comparing the results obtained in this study of normal New Hampshire Red embryos with those of Walker (1943a, 1943b, 1943c) for normal embryos of Barred Plymouth Rocks, it was found that the crown-rump lengths correlated more closely than did the weights. The average daily weight of the embryos was more nearly that reported by Romanoff and Romanoff (1933) for White Leghorns (table 1).

Despite the discrepancies noted for daily gain in weight, the over-all percentage gain showed a fairly close correlation: Walker, 31 per cent; Romanoff and Roman-

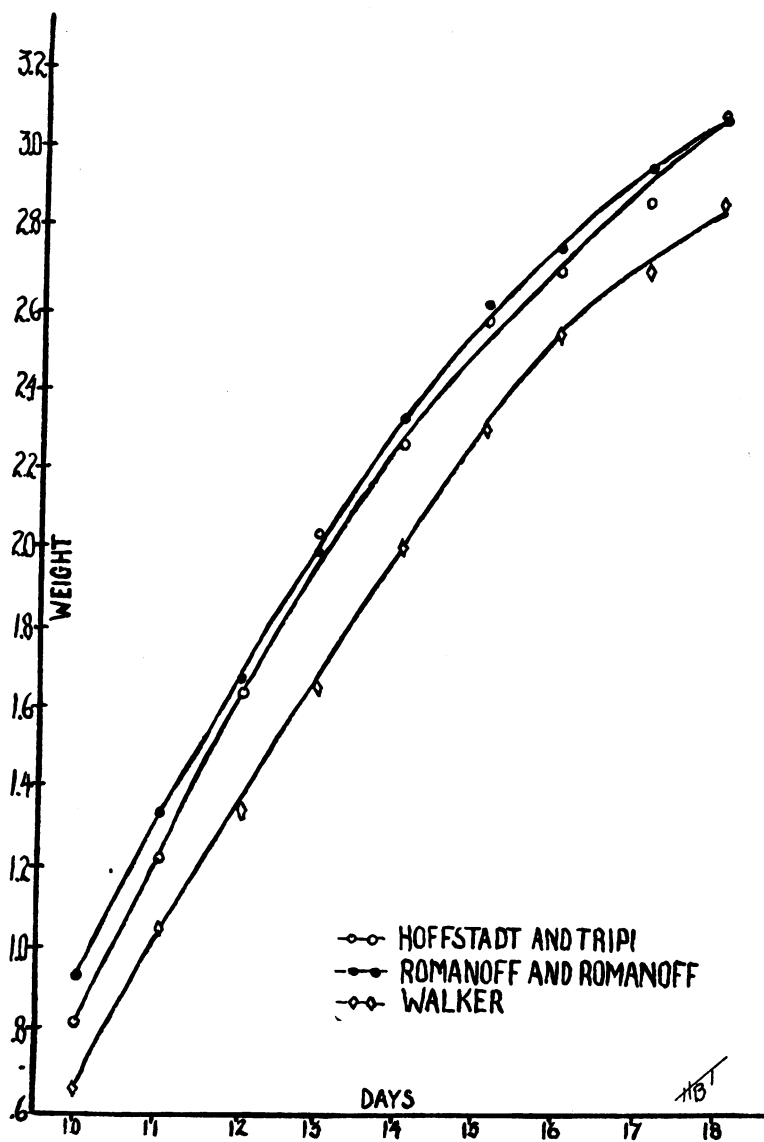


FIG. 1. CUMULATIVE GROWTH CURVES FOR NORMAL EMBRYOS  
Weight expressed as its natural logarithm

off, 32.5 per cent; and that obtained by us, 32 per cent. This may be better demonstrated by the representation of the cumulative weights of embryos expressed as the natural logarithms of the average weights (figure 1). It is readily apparent that all three curves are similar in shape and slope.

*Functional normality.* The index values for functional normality for 223 normal embryos ranging from the age of 10 to 18 days were calculated by the formula as suggested by Walker:  $I = \frac{1,000 \sqrt[3]{\text{wet weight in grams}}}{\text{crown-rump length in cms}}$ . The results of application of the index are illustrated in the histogram (figure 2). Embryos used in this experiment differed in breed from those used by Walker, were from a smaller age group, and probably came from a number of different flocks of chickens. As is to be expected under these conditions, greater variations in the indexes were encountered, yet the absolute range, 290 to 414, differed but 2 units from the 294 to 416 of Walker. Because fewer embryos (223) were available for this determination, and because there was greater variation toward the two extremes, an interval of 5 units, rather than 3 units as used by Walker, was selected to show index frequency. Absolute limits were 290 and 414.

From the histogram, it is apparent that a reasonably good frequency curve could be constructed. Since the indexes tend to be concentrated toward the higher values, the curve would be positively skewed and would be characterized by the following values:

Arithmetic mean.....	347.72
Median.....	347.02
Mode.....	346.22
Standard deviation.....	17.76
Coefficient of variation.....	5.11
Coefficient of skewness.....	0.0845
Standard error of the mean.....	1.19
Range of normality (including approximately 95.5 per cent of all embryos).....	310-384

The range of normality was determined by using 2 standard deviations rather than the 3 standard deviations usually used, because of the wide frequency distribution, which was assumed to be due to the lack of control of the source of the eggs.

Two factors may account in part for this variation: (1) the age of the embryos (7- to 19-day-old embryos were used in Walker's experiment and 10- to 18-day-old embryos were used by us); (2) the breed of the embryos (embryos of New Hampshire Reds were uniformly heavier than the embryos of Barred Plymouth Rocks used by Walker).

*Rate of growth of the embryos following inoculation of vaccinia (Levaditi strain) into the chorioallantoic fluid.* Of the 90 eggs inoculated with the virus, 6 living embryos were sacrificed daily from the 10th to 18th day of incubation of the embryos. A total of 54 embryonated eggs was studied (table 3). The embryos were weighed and measured, and the allantoic fluids and organ extracts were each subcultured on three eggs. Both the crown-rump length and the wet weight showed fluctuations from the normal. The over-all percentage gain in daily weight was 26.9 per cent as compared with 32 per cent for the normal embryos. The actual difference in the final average weight was 6.5 grams less for the infected embryos than the normal ones. Cumulative growth curves for normal and in-

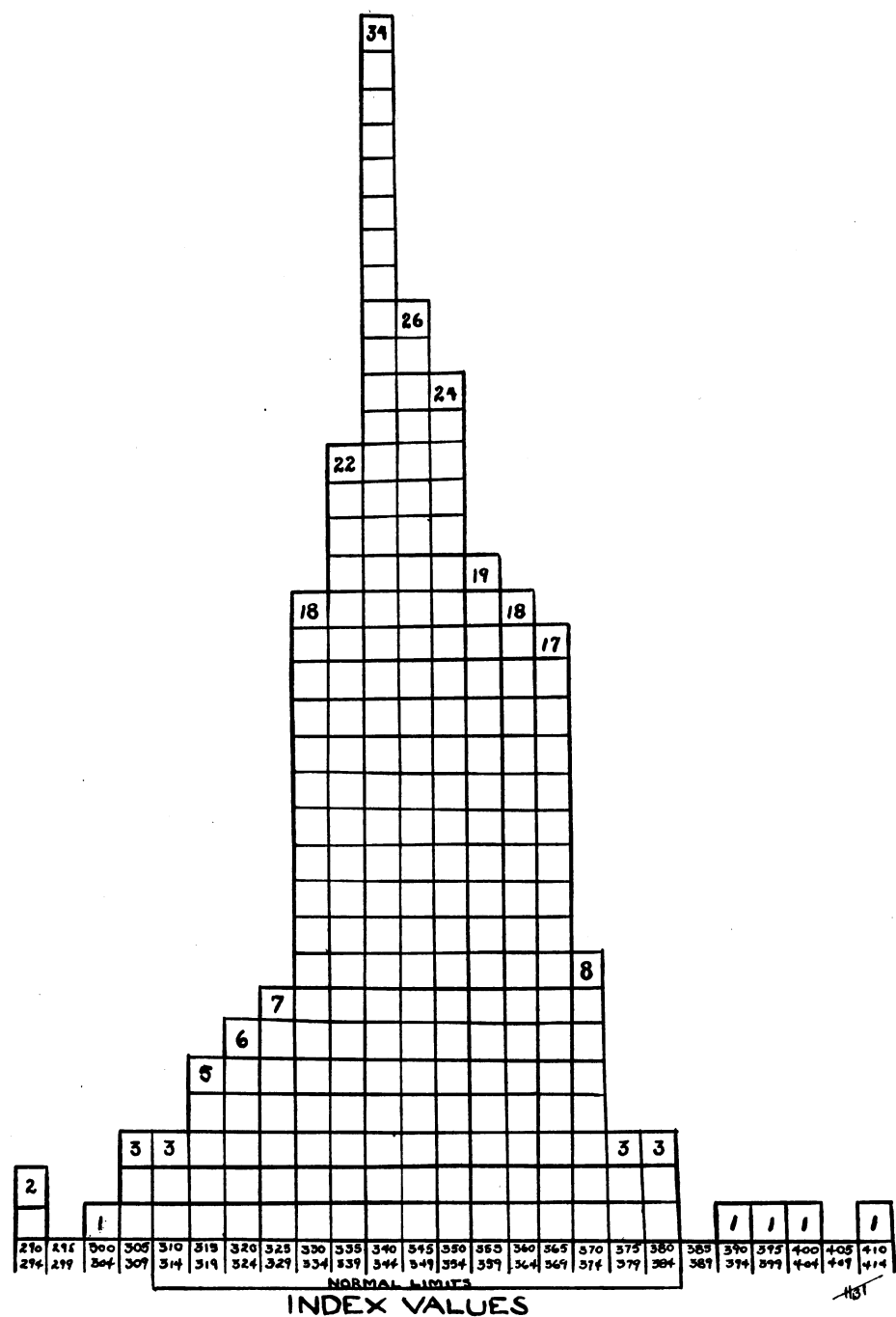


FIG. 2. HISTOGRAM RESULTING FROM APPLICATION OF WALKER'S INDEX OF FUNCTIONAL NORMALITY TO NORMAL EXPERIMENTAL EMBRYOS, SERIES A

fectured embryos are shown in figure 3. The smallest gains in weight by the embryos were on the 12th, 15th, and 18th days.

Walker (1943) reported a definite shift to the acid side in the pH of the allantoic fluid in normal embryos, and this was verified by our results, although the drop was not so sharp as that reported by Walker (table 1). This increase in

TABLE 2  
*Average normal values for chick embryos\*—series A*

DAY	WEIGHT	LENGTH	ALL. FL. pH	AMN. FL. pH	INDEX	LN. WT	% GAIN OF WT
	gms	cms					
10	2.4	3.8	7.68	7.83	349	0.81	—
11	3.4	4.3	7.71	7.75	348	1.22	41.6
12	5.2	5.0	7.96	7.72	348	1.63	53.0
13	7.7	5.8	7.98	7.52	342	2.03	48.1
14	9.8	6.2	8.00	7.54	343	2.27	27.3
15	13.3	6.9	7.37	6.90	343	2.58	35.7
16	15.3	6.9	6.80	6.80	356	2.72	15.0
17	17.5	7.5	6.28	6.89	348	2.85	14.3
18	22.5	8.2	5.85	7.17	346	3.11	28.6

Legend: Same as for table 1.  
\* Averages of 25 embryos per day.

TABLE 3  
*Average values for infected chick embryos\*—series A*

DAY	WEIGHT	LENGTH	ALL. FL. pH	AMN. FL. pH	INDEX	LN. WT	% GAIN OF WT
	gms	cms					
10	2.2	3.5	7.99	7.84	370	0.80	—
11	3.3	4.1	8.33	7.31	360	1.18	48.0
12	3.8	4.3	8.29	7.59	364	1.34	17.1
13	5.0	4.8	7.96	7.08	353	1.61	31.3
14	7.8	5.4	7.76	7.24	368	2.05	56.1
15	9.0	6.1	8.02	7.16	342	2.19	14.0
16	11.9	6.5	7.10	6.67	353	2.47	32.7
17	14.8	7.2	6.17	6.68	341	2.68	24.4
18	16.0	7.4	6.53	7.41	341	2.76	7.5

Legend: Same as for table 2.  
\* Average of 5 embryos per day.

acidity foreshadows the conditions seen in adult avian urine (Takamatsu, 1935). A similar change in the pH of the amniotic fluid was observed on the 14th and 15th days (table 3). The average difference in length between the normal and infected embryos was 1.8 cm (tables 2 and 3).

*Functional normality.* Walker's index for functional normality was calculated for the infected embryos. Figure 4 is the histogram obtained for the infected embryos of series A. Three embryos were outside the normal range for functional

normality previously established. Calculations made upon these frequencies characterize a curve with the following values:

Arithmetic mean.....	353.33
Median.....	352.92
Mode.....	352.10
Standard deviation.....	15.38
Coefficient of variation.....	4.35
Coefficient of skewness.....	0.080
Standard error of the mean.....	2.11
Range of normality, including approximately 95.5 per cent of all embryos..	325-384

Comparison of the foregoing values with those obtained for normal embryos shows the following relationship:

Standard error of differences between means ( $\sigma_D$ ).....	2.42
Critical ratio ( $T$ ).....	$2.73\sigma_D$

The critical ratio,  $2.73\sigma_D$ , is above the 1 per cent level of significance ( $2.576\sigma_D$ ) and it may be concluded, therefore, that there is a significant difference between the two means which cannot be attributed to random errors of sampling. Actually, the probability that differences so large could occur as the result of random causes is less than 1 in 100. From application of the index of functional normality to the experimental material and comparison with normal values it is concluded that the functional normality of the infected embryos was altered. The most significant factor was the shift of the mean from the 340-to-344 to the 350-to-354 frequency class.

*Series B embryos.* Since it was apparent that the Walker index could be applied for the interpretation of data over a period of several days of embryonic growth, it was applied to embryos of a single age group. The infected eggs, 179 in number, were inoculated by the chorioallantoic membrane route on the 10th day and were sacrificed on the 12th day. The normal controls were 174 in number. The results of the application of Walker's index of functional normality to the experimental material are shown by the histograms (figures 5 and 6) and by calculated values for the frequency curves as follows:

	<i>Normal</i>	<i>Infected</i>
Arithmetic mean.....	347.7	353.4
Median.....	347.3	352.4
Mode.....	346.5	350.4
Standard deviation.....	19.51	19.98
Coefficient of variation.....	5.61	3.66
Coefficient of skewness.....	0.112	0.150
Deviation of the mean.....	1.88	1.50
Range of normality including approximately 95.5 per cent of all embryos.....	308.7-386.7	313.4-393.4
Standard error of differences between means ( $\sigma_D$ ).....	2.107	
Critical ratio.....	$2.70\sigma_D$	

With the critical ratio of  $2.70\sigma_D$ , inspection of the probability tables reveals the fact that the probability that the differences between means could be so great as

a result of random errors of sampling would be less than 1 in 100. The difference may, therefore, be said to be a significant one. It is of significant in-

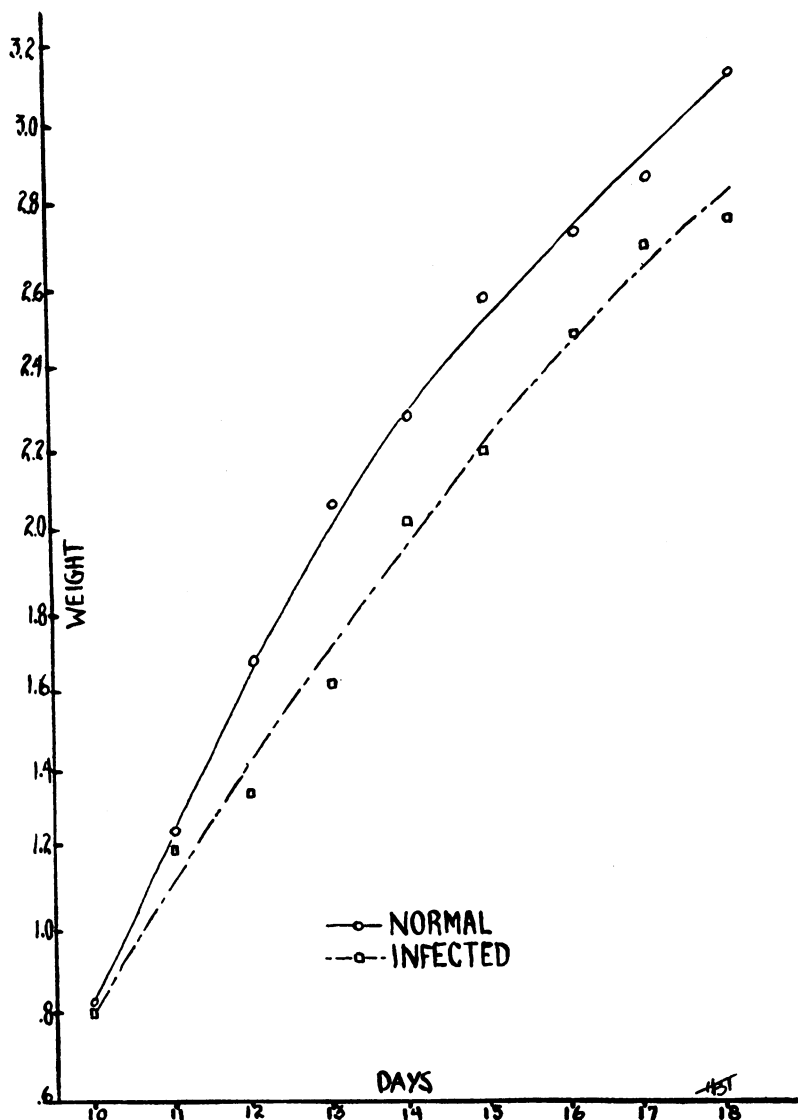


FIG. 3. CUMULATIVE GROWTH CURVES FOR NORMAL AND INFECTED EMBRYOS  
Weight expressed as its natural logarithm

terest that these values closely approximate those calculated for the experimental material of series A embryos.

#### SUMMARY

The over-all daily rate of growth, expressed as percentage gain in weight of allantoic-sac-infected embryos, was 26.5 per cent, as compared with the normal



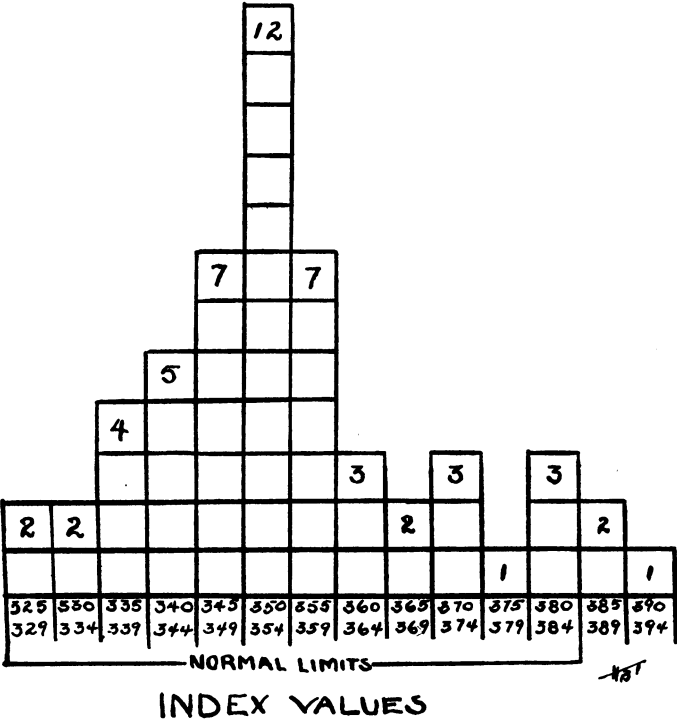


FIG. 4. HISTOGRAM RESULTING FROM APPLICATION OF INDEX TO INFECTED EXPERIMENTAL EMBRYOS, SERIES A

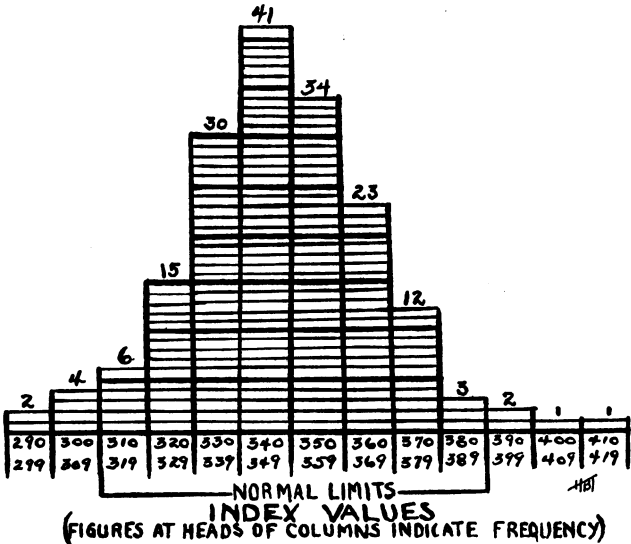


FIG. 5. HISTOGRAM RESULTING FROM APPLICATION OF INDEX OF FUNCTIONAL NORMALITY TO NORMAL EXPERIMENTAL EMBRYOS SERIES B

rate of 32 per cent. A normal rate of growth was maintained for allantoic-sac-infected embryos for the 10- to 11-day period but was followed by a loss of rate of growth in the 12th, 15th, and 18th days. The final average weight of infected embryos on the 18th day did not equal that of normal 17-day-old embryos. The actual final discrepancy between the infected and normal embryos was 6.5 grams.

Comparison of the functional normalities of infected embryos and normal embryos, as expressed by the Walker index, indicates an alteration of functional normality for the infected embryos.

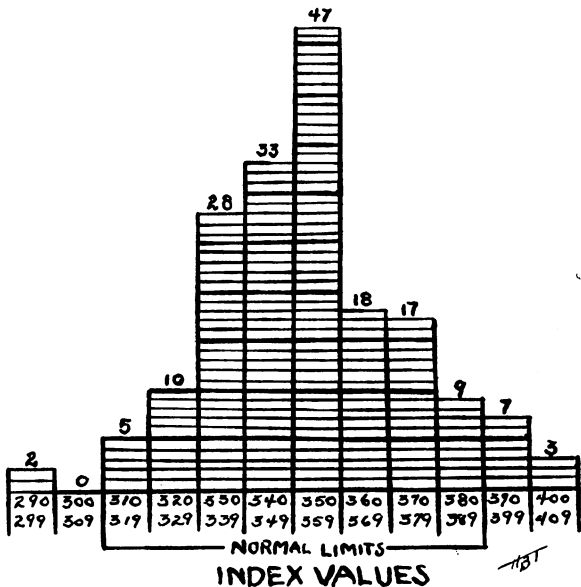


FIG. 6. HISTOGRAM RESULTING FROM APPLICATION OF INDEX OF FUNCTIONAL NORMALITY TO EXPERIMENTALLY INFECTED EMBRYOS, SERIES B

Comparison of the functional normalities of embryos infected by either the allantoic fluid or the chorioallantoic membrane route indicates no difference in the degree of alteration of functional normality obtained by either method of inoculation.

Comparison of the results of application of the index of functional normality to 10- to 18-day embryos and to 12-day embryos indicates that the Walker index is applicable to embryos of a single age or of a range of ages.

The numbers of eggs used for these determinations varied and may, in part, account for some discrepancies in calculations, but the discrepancies were all within the limits of error.

From these results it may be concluded that the Walker index is of value for interpreting the functional normality of infected embryos.

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