EFFECT OF ENZYME INHIBITORS AND ACTIVATORS ON THE MULTIPLICATION OF TYPHUS RICKETTSIAE

III. Correlation of Effects of PABA and KCN with Oxygen Consumption in Embryonate Eggs*

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INTRODUCTION

In previous papers of this series (1, 2) it was shown that the growth of typhus rickettsiae in the entodermal cells lining the yolk sac of the egg is inhibited by para-aminobenzoic acid (PABA), by dyes of the toluidin blue group, and by increasing the temperature of incubation from 37.5° to 40.0°C. Potassium cyanide, in doses tolerated by the embryos, was found to neutralize completely the rickettsiostatic action of increased temperature but to have no apparent effect on the action of PABA or toluidin blue.

Potassium cyanide is known to depress cellular respiration while increased environmental temperature and dyes of the toluidin blue group are known to increase the oxygen uptake of many types of tissue. The effect of PABA on the respiration of cells was unknown.

It therefore seemed desirable to make accurate measurements of the oxygen uptake of fertile eggs after injecting these compounds, and to correlate their effects on cellular respiration with their ability to inhibit or accelerate rickettsial growth. The apparatus and technics used for this purpose will be described in detail, since they form a basis for subsequent studies of a similar nature. For the same reason, the statistical analysis of our experimentally determined figures will be given rather fully.

Material and Methods

Methods for infecting fertile chick eggs with typhus rickettsiae, injecting the agents to be tested, and determining the degree of infection were described in detail in Papers I and II of this series.

In the present work oxygen consumption was measured by direct gas analysis. The apparatus to be described was designed to measure the oxygen consumption of eggs in groups of fifteen to twenty, so that the figure obtained in each analysis represents the average utilization

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of oxygen per egg per hour. Reasons for making analyses of eggs in groups rather than individually will be given below.

Groups of eggs were removed from the incubator and placed in air-tight containers of approximately 7 liters capacity. These containers were buried in a constant temperature water bath for the duration of the analysis. Aluminum desiccators were found to be the most suitable containers for this purpose. The atmosphere within the desiccators was stirred continuously by a propeller mounted on the end of a tuberculin syringe plunger. A portion of the barrel of the syringe served as the bearing. The barrel was inserted into a rubber stopper which in turn was sealed into the lid of the desiccator by means of sealit (Fisher Scientific Co.). Heavy rubber tubing fitted snugly around the external portion of the barrel and plunger prevented water from coming into contact with the bearing and also aided in forming an air

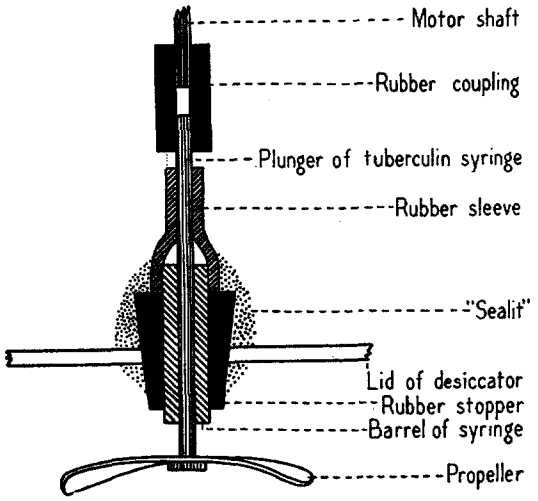

FIG. 1. Details of propeller assembly sealed into lid of desiccator.

tight seal. The end of the plunger projecting to the outside was attached to a motor, mounted on the lid, by means of a rubber coupling (see Fig. 1).

The bearing and shaft were lubricated with glycerine. During the course of the experiment more glycerine was added through the top of the rubber sleeve by injection with a hypodermic syringe. This assembly permitted a pressure differential of 140 mm. of mercury (either positive or negative) while the motor was running at 1750 R.P.M. There was no loss of pressure after several hours. A stop-cock, thermometer, mercury manometer, and serum vial stopper were also sealed into the top of each desiccator.

The water bath designed for these experiments holds four desiccators simultaneously and maintains a temperature of 37.5° ± 0.02°C.

Preliminary experiments (to be discussed below) demonstrated that the accumulation of CO_2 and the increasing humidity during the course of the analysis affected oxygen consumption and viability. Therefore a beaker containing a measured amount of ascarite was placed in the container. This not only removed the CO_2 evolved by the eggs, but also reduced the relative humidity to approximately zero. Neither of these environmental changes appeared to affect the viability of the eggs during their relatively short stay in the respirometer. The use of a dry CO_2 absorbent made it unnecessary to correct for dissolved oxygen.

After placing the eggs to be tested in the container, its ground edges are coated with stop-cock grease and the lid is fastened in place by the use of a 4 inch rubber cuff. One inch of the rubber band encircles the body of the container, the remainder projecting over the lid and thereby sealing it (see Fig. 2). The container is then placed in the water bath. After the air within the containers reaches a temperature of 37.3°C. (as determined by the built-in ther-

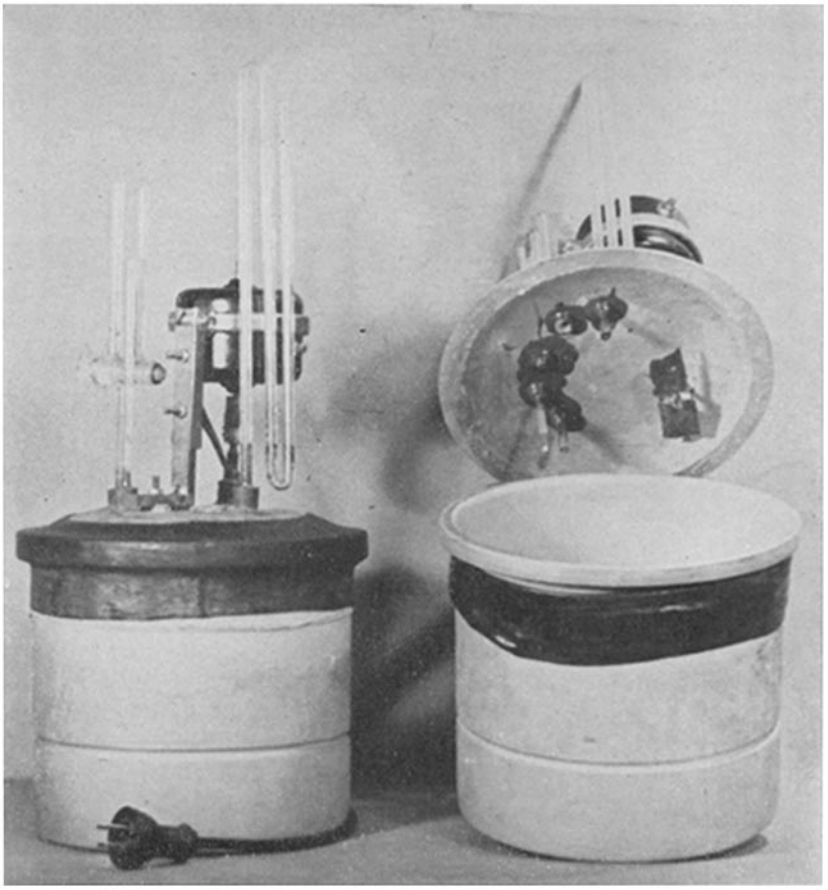

FIG. 2. Details of respirometer chamber to hold twenty eggs, constructed from aluminum desiccator.

mometer) the stop-cock is closed. Five minutes later the first sample of gas is removed. The needle of a 10 cc. syringe, with its plunger closed, is inserted through the serum vial stopper in the lid. The barrel and plunger are coated with heavy stop-cock grease to prevent loss or contamination of the gas sample. An 11 cc. sample of gas is drawn into the syringe. After withdrawal, the needle is quickly removed and the syringe placed in a test tube containing enough mercury to cover the tip of the syringe barrel. A small amount of gas (0.5 cc.) is expelled by depressing the plunger and the assembly is put aside for a half-hour period to permit the entrapped gas sample to reach room temperature. The second sample is

removed when the manometer registers approximately 10 mm. of negative pressure. The elapsed time between samples is noted. The eggs are then removed from the container and candled to determine if any embryos have died. For purposes of calculating oxygen consumption, it is assumed that the occasional embryos which die have lived half of the elapsed time that the eggs were in the respirometer chamber. Oxygen consumption determination is based on the average number of live eggs in the container. When dealing with embryos less than 8 days old, the second sample of gas is removed at the end of 2 hours, regardless of the manometer reading. It is necessary to adopt this arbitrary procedure because of the length of time (6 to 8 hours) required under these conditions for the atmosphere to reach 10 mm. of negative pressure.

The gas samples are analyzed for oxygen by means of a Haldane-Henderson gas analyzer. The results obtained with this apparatus are independent of temperature, barometric pressure, and wet or dry state of the gas sample (3). In order to shorten the time required for an analysis of oxygen consumption oxorbent (Burrel) is used to absorb the oxygen instead of alkaline pyrogallol. Readings which check within 0.002 ml. are obtained in 10 to 15 passes with the former instead of the 30 to 40 required with the latter.

The volume of the eggs, necessary for determining the corrected volume of the respirometer chamber, is found for each setting of eggs by placing a group of them in a 1000 ml. graduate, and pouring water from a volumetric flask (1000 ml.). The amount of water left in the volumetric flask divided by the number of eggs gives the average egg volume.

The volume (in cubic centimeters) of oxygen consumed per egg per hour is calculated from the following:

$$\text{Cc. } O_2\text{/egg/hr.} = \frac{[V_c - (V_E + V_B + V_A)]\left[\frac{R_{CO_2} - R_{O_2}}{S_i} - \frac{R'_{CO_2} - R'_{O_2}}{S'_i}\right]}{\left[\frac{E_1 + E_2}{2}\right](T_2 - T_1)}$$

In certain preliminary experiments in which the amount of CO_2 evolved was measured, the CO_2 absorbent was omitted, and the following formula used for the determination.

$$\text{Cc. } CO_2\text{/egg/hr.} = \frac{[V_c - (V_E + V_B + V_A)]\left[\frac{S'_i - R'_{CO_2}}{S'_i} - \frac{S_i - R_{CO_2}}{S_i}\right]}{\left[\frac{E_1 + E_2}{2}\right](T_2 - T_1)}$$

Where:

V_c = volume of respirometer container;
V_E = total volume eggs;
V_B = volume of beaker holding ascarite;
V_A = volume of ascarite;
R_{CO_2} = volume of gas after absorption of CO_2 (first sample);
R_{O_2} = volume of gas after absorption of O_2 (first sample);
S_i = volume of first gas sample;
R'_{CO_2} = volume of gas after absorption of CO_2 (second sample);
R'_{O_2} = volume of gas after absorption of O_2 (second sample);
S'_i = volume of second gas sample;
E_1 = number of live eggs placed in respirometer;
E_2 = number of live eggs removed from respirometer;
T_1 = time of taking first gas sample;
T_2 = time of taking second gas sample.

Reliability of Results

Groups of untreated eggs were used to determine the reliability of experimentally obtained figures for oxygen consumption. A commonly used and important criterion for judging the significance of differences between control and treated groups of individuals is the consistent recurrence of similar differences in repetitions of the original experiment. As a further check, and in order to avoid the necessity of repeating each experiment in which small differences were found, it seemed worth while to employ methods of statistical analysis to determine the magnitude of the differences which should be considered as having definite significance. Values for the minimum differences in cubic centimeters of oxygen consumed per egg per hour necessary for significance, are given in the last column of Table VI. The causes for variation in

TABLE I

Variations in the Measurement of Oxygen Consumption due to Errors Inherent in the Technique Employed

Age of embryos	Oxygen consumption per egg per hr.					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
5	0.38	0.35	0.43	0.61	0.47	0.52
9	2.01	2.54	2.49	2.58	2.49	2.26
13	8.95	9.47	8.97	9.36	9.42	9.44
17	16.21	16.63	16.11	16.15	16.58	16.37

the figures may be considered under two headings: the errors inherent in the technique of analysis and the variations resulting from the lack of biological uniformity in the groups of eggs.

Accuracy of the Technique.—Using eggs of the White Rock variety, six samples of gas were removed for analysis at intervals of approximately 10 seconds. Table I shows the results of carrying out this procedure at four different stages of embryonic development. It will be noted that although the absolute variations did not differ markedly at these different stages, they ranged from 40 per cent of the total figure in early embryos to 2 per cent in older embryos. In the statistical technique eventually adopted, variations of less than 70 per cent in younger embryos and 4.8 per cent in older embryos were considered not to be significant.

Biological Variations in Eggs.—There are numerous variables which modify the stage of development of the embryos, and consequently the oxygen consumption of individual eggs in any lot (4–6). In the first place, although White Rock eggs laid within an 8 hour period were used in most experiments, there was obviously a variation in the time of laying of individual eggs. Since the initiation of ontogenesis by fertilization occurs in the upper reaches of the

oviduct, and egg formation in the hen usually takes from 18 to 30 hours, the development of the blastoderm with differentiation of the three germ layers has already taken place by the time the egg is laid. Thus no two eggs contain embryos of the same age at the time of laying. Another factor is the variable temperature of the environment in which the eggs are maintained before they reach the incubator. In order to average out the above differences in embry-

TABLE II

Oxygen Consumption in Embryonate Eggs of the White Rock Variety (Fall Setting)

Age of embryos	Oxygen consumption per egg per hr.					
	Series 1	Series 2	Series 3	Series 4	Series 5	Series 6
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
5	0.89	1.06	0.95	1.39	1.12	0.91
7	1.84	1.42	1.51	1.39	1.52	1.44
9	2.23	2.17	2.68	2.24	2.32	2.61
11	2.99	3.51	3.48	3.20	3.11	3.42
13	8.88	9.01	9.13	9.36	8.91	9.03
15	13.86	13.94	14.36	14.03	14.26	13.92
17	19.50	19.00	19.46	19.56	19.32	19.22

TABLE III

Oxygen Consumption in Embryonate Eggs of the White Rock Variety (Winter Setting)

Age of embryos	Oxygen consumption per egg per hr.					
	Series 1	Series 2	Series 3	Series 4	Series 5	Series 6
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
6	0.48	0.71	0.34	0.59	0.29	0.57
8	0.82	1.15	1.35	1.01	0.99	0.92
10	2.01	2.48	2.37	2.46	2.17	2.18
12	6.49	6.37	5.98	6.01	6.11	6.36
14	9.87	10.00	9.97	10.39	10.24	10.16
16	13.66	13.41	13.37	13.97	13.82	13.55
18	17.53	17.36	17.74	17.22	17.68	17.34

onic age and development, fifteen to twenty eggs are placed in each respirometer.

Statistical Analysis.—As has been stated the figures obtained in these experiments represent the mean oxygen consumption per egg per hour. It was therefore impossible to employ the statistical methods ordinarily used for comparing populations composed of individuals.

The statistical technique evolved was as follows: Variations in the oxygen consumption between groups of untreated eggs were first found and tabulated. These differences were used as a standard for determining the significance of

differences later found between the control and treated groups of eggs. The eggs used for these preliminary experiments were from the same flock (White Rock variety) and oxygen consumption was measured on settings obtained in the fall, winter, and spring. Each setting was divided into a number of series. Oxygen uptake at different ages was measured simultaneously on groups of twenty eggs from each series. Once used the eggs were discarded. The results are shown in Tables II (fall), III (winter), and IV (spring).

The mean differences were obtained by making all possible combinations of oxygen consumption of embryos of the same age among the several series from

TABLE IV

Oxygen Consumption in Embryonate Eggs of the White Rock Variety (Spring Setting)

Age of embryos	Oxygen consumption per egg per hr.					
	Series 1	Series 2	Series 3	Series 4	Series 5	Series 6
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
5	0.42	0.70	0.66	0.91	0.60	0.38
6	1.31	1.62	1.49	1.37	1.78	1.68
7	1.97	1.73	2.20	2.15	1.99	2.41
8	2.50	2.33	1.92	2.47	2.28	2.07
9	2.77	2.99	2.46	2.53	3.01	2.87
10	3.82	4.12	3.67	4.10	3.64	4.02
11	4.99	4.67	4.83	4.91	4.58	4.63
12	5.47	5.96	5.63	5.59	5.45	5.84
13	8.91	8.85	9.22	9.35	9.01	8.97
14	11.22	11.68	11.23	11.74	11.47	11.33
15	13.53	13.68	13.62	13.30	13.78	13.80
16	14.23	14.62	14.54	14.16	14.18	14.49
17	16.07	16.21	16.00	16.51	16.33	16.33
18	17.69	17.55	17.67	17.82	18.03	17.93

each setting of eggs. Thus from Table II, the six oxygen readings for 5 day old embryos give 15 (6^C2) mean differences; *viz.*, 0.11 (1.06 − 0.95), 0.17 (1.06 − 0.89), 0.06 (1.12 − 1.06), etc. These differences were treated as absolute values; *i.e.*, without regard to sign. The mean of the mean differences (M_{md}) was found from the formula:

$$M_{md} = \frac{\Sigma md}{N}$$

where Σmd = the summation of all possible mean differences of the several series of a given setting of embryos of the same age, and N = the number of mean differences.

In order to compare mean differences it was also necessary to find the standard error of the mean differences (σ_{md}). This was calculated from the following:

$$\sigma_{md} = \sqrt{\frac{\Sigma(M_{md} - md)^2}{N}}$$

Although biological distributions in general follow a cocked-hat form with the normal law as a central type, they frequently show some degree of departure from normality. The type of distribution of oxygen consumption of developing chick embryos was not known. However, it is known that the means of a series of samples drawn from a non-normally distributed parent population will show a close approximation to a normal distribution. From the practical

TABLE V

Mean of Mean Differences and Standard Error of Mean Differences of Oxygen Consumption Determinations in Embryonate Eggs of the White Rock Variety

Age of embryos	Fall setting (Table II)		Winter setting (Table III)		Spring setting (Table IV)	
	M_{md}	σ_{md}	M_{md}	σ_{md}	M_{md}	σ_{md}
days						
5	0.21	0.15			0.24	0.14
6			0.20	0.11	0.23	0.12
7	0.17	0.15			0.28	0.17
8			0.22	0.14	0.28	0.16
9	0.25	0.17			0.29	0.17
10			0.23	0.12	0.26	0.15
11	0.36	0.24			0.20	0.12
12			0.26	0.16	0.23	0.15
13	0.21	0.14			0.23	0.14
14			0.24	0.13	0.27	0.15
15	0.24	0.16			0.22	0.14
16			0.29	0.16	0.24	0.15
17	0.25	0.15			0.23	0.13
18			0.25	0.14	0.21	0.12

point of view in biology, this deviation of the distribution of means and mean differences from the normal form is in general so small as to be of trivial consequence. The form of the above distribution is characterized by the mean of the mean differences (M_{md}) and the standard error of the mean differences (σ_{md}). Table V gives the aforementioned statistics for the several settings.

Differences between the means of mean differences were tested for statistical significance by dividing the difference by the standard error of the difference.

$$\text{The difference in standard units (sigmas)} = \frac{M_{md_1} - M_{md_2}}{\sqrt{\sigma^2_{md_1} + \sigma^2_{md_2}}}$$

This test was made between embryos of the same age but different settings. In no case was there a difference between means of the mean differences that remotely approached significance (3 sigma or greater). Therefore the means

of the mean differences and the standard errors of the mean differences for embryos of the same age but of different settings were combined and weighted to give greater value to the more reliable statistic.

$$\bar{M}_{md} = \frac{N_1 M_{md_1} + N_2 M_{md_2} + \cdots + N_K M_{md_K}}{N_1 + N_2 + \cdots + N_K}$$

$$\bar{\sigma}_{md} = \sqrt{\frac{N_1 \sigma^2_{md_1} + N_2 \sigma^2_{md_2} + \cdots + N_K \sigma^2_{md_K}}{N_1 + N_2 + \cdots + N_K - K}}$$

Where:

$\bar{\bar{M}}_{md}$ = the weighted mean of the mean differences.

$\bar{\sigma}_{md}$ = the weighted standard error of the mean differences. $N_1 + N_2$ etc. = number of individuals in the several samples.

K = number of samples.

TABLE VI

Statistical Analysis of Figures for Oxygen Consumption of Eggs of the White Rock Variety at Different Embryonic Ages

Age of embryos	Weighted mean of mean differences* $\bar{\bar{M}}_{md}$	Weighted standard error of mean differences* $\bar{\sigma}_{md}$	Weighted standard error of mean of mean differences* $\bar{\sigma}M_{md}$	Upper value of the five sigma confidence level* $\bar{\bar{M}}_{md} + 5\bar{\sigma}M_{md}$	Minimum differences necessary for significance* $\bar{\bar{M}}_{md} + 5\bar{\sigma}M_{md} + 3\bar{\sigma}_{md}$
days					
5	0.23	0.148	0.028	0.37	0.82
6	0.22	0.118	0.022	0.33	0.69
7	0.23	0.166	0.030	0.38	0.96
8	0.25	0.155	0.029	0.39	0.87
9	0.27	0.175	0.033	0.43	0.97
10	0.25	0.140	0.026	0.38	0.80
11	0.28	0.195	0.036	0.46	1.06
12	0.25	0.160	0.030	0.40	0.88
13	0.22	0.144	0.027	0.35	0.78
14	0.26	0.145	0.027	0.39	0.84
15	0.23	0.155	0.029	0.37	0.85
16	0.27	0.160	0.030	0.42	0.90
17	0.24	0.145	0.027	0.37	0.82
18	0.23	0.135	0.026	0.36	0.78

* All figures represent cubic centimeters of O_2 consumed per egg per hour.

Table VI gives the foregoing statistics.

As was pointed out previously, the means of a series of samples drawn from a parent population will be approximately normally distributed. Also the mean of the means will approach very closely in magnitude the mean of the parent population. However, when the mean of the parent population is not known, then the best guess as to the true mean is that it will lie somewhere within the range expressed by the mean ± 5 standard errors of the mean. In fact we

would expect the true mean to lie outside of the foregoing range only once in a million times. This is known as the 5 sigma confidence level. Thus in our experiments the expression $\bar{M}_{md} + 5\bar{\sigma}_{M_{md}}$ expresses the upper level of the confidence band and the true mean difference could only be expected to be beyond this value once in a million times. The weighted standard error of the mean of mean differences ($\bar{\sigma}_{M_{md}}$) was found as follows:

$$\bar{\sigma}_{M_{md}} = \frac{\bar{\sigma}_{md}}{\sqrt{N-1}}$$

where N = the number of individuals in the sample. The values of the upper limit of the 5 sigma confidence level are given in Table VI. It was these values which were used in determining the existence of significant or non-significant differences in oxygen consumption between two groups of embryos of the same age.

The following test for significance was used:

$$\text{Mean difference in sigmas} = \frac{\bar{X}_1 - \bar{X}_2 - (\bar{M}_{md} + 5\bar{\sigma}_{M_{md}})}{\bar{\sigma}_{md}}$$

Where:

$\bar{X}_1$ and $\bar{X}_2$ = the figures for oxygen uptake per egg per hour for the two samples of embryos of the same age.

$\bar{M}_{md} + 5\bar{\sigma}_{M_{md}}$ = the upper limit of the 5 sigma confidence band of the mean difference in oxygen consumption to be expected for ebmryos of the same age as the samples.

$\bar{\sigma}_{md}$ = the weighted standard error of the mean difference of oxygen consumption of embryos of the same age as the samples.

Only when the above difference was a positive 3 sigma or greater (expected to occur less than two times in a thousand by chance alone) was the difference between the two samples considered significant.

The figures in the last column of Table VI, obtained by adding three weighted standard errors of the mean differences to the upper value of the 5 sigma confidence level, represent the experimentally determined minimum differences in oxygen consumption considered necessary for significance at each embryonic age.

Preliminary Experiments

Before adopting the above described technique for determining oxygen uptake, certain preliminary experiments were carried out. The method which it was eventually decided to use was chosen as a result of information gained from these experiments.

The Effect of Oxygen and Carbon Dioxide Tensions.—Relatively few observations have been made on the effect of changes in oxygen and carbon dioxide tensions on cellular respiration. In general, however, it has been observed that wide differences in oxygen tension do not influence oxygen consumption.

Slight increases in carbon dioxide tension have been shown to cause a rise in the rate of oxygen uptake, while further increases markedly lower respiration. Since the oxygen consumption of our eggs was measured while they were in closed containers, the effect of CO_2 accumulation required consideration. On the other hand, if the carbon dioxide was absorbed as it was liberated a lowering of the oxygen tension would result. It was therefore necessary that the effect of this factor on oxygen uptake be determined.

Experiment.—A setting of fertile White Rock eggs was divided into two groups. The first group was used to determine the effect of oxygen tension on the oxygen consumption of the developing embryos. The carbon dioxide was removed by placing a beaker of ascarite in the respirometer chamber. This compound also removed water vapor from the atmosphere, reducing the relative humidity to zero.

The second group was used to determine the effect of carbon dioxide tension on oxygen consumption. A beaker containing anhydrone was placed in the container holding the eggs. This compound reduced the relative humidity to zero. In order to prevent the anhydrone from absorbing small amounts of carbon dioxide, it was first saturated with this gas. Excess gas was removed by gentle heating.

As the atmosphere surrounding the eggs was not renewed during the course of an oxygen consumption determination, the carbon dioxide tension varied continuously. The average partial pressure of the gas was calculated from the following:

$$\text{Average partial pressure of } CO_2 = \frac{\text{per cent } CO_2' \cdot P' - \text{per cent } CO_2 \cdot P}{2}$$

Where: P' and P = the barometric pressures at the time of removal of the second and first samples respectively.

The eggs were allowed to remain in the respirometer chambers for a period of 5 hours. An initial gas sample was removed for analysis as soon as temperature equilibrium became established (about 20 minutes after sealing the containers). Thereafter, hourly samples were removed and analyzed.

Discussion.—The hourly differences in oxygen consumption for embryos of a given age which were tested in a carbon dioxide–free atmosphere were statistically insignificant (Table VII). Parnas and Krasinska (7) showed that fertilized eggs of amphibia developed normally in an atmosphere of pure oxygen. Hasselbalch (8), Bohr and Hasselbalch (9), and Krogh (10) in their classical experiments on oxygen consumption of the developing chick embryo, bathed the eggs being tested in a carbon dioxide–free atmosphere and instantly removed any carbon dioxide liberated. They found that the absence of this gas in no way affected either oxygen uptake or carbon dioxide production. It might also be pointed out that the methods of Warburg, Barcroft, Dixon and others used so frequently in the measurement of oxygen consumption of tissues all involve the absorption of carbon dioxide. Thus the absence of carbon dioxide would appear in general to have little effect upon oxygen uptake.

The results shown in Table VII indicate also that the decrease in oxygen concentration resulting from the absorption of CO_2 did not affect the rate of

oxygen uptake. Riddle (11) has reported that for its entire developmental period the embryo presumably requires a concentration of oxygen of not less than 14 per cent. Wesselkin (12) has shown that embryos will live and develop for a limited time in approximately 12 per cent oxygen. Warburg and his coworkers have also shown that the rates of respiration of isolated cells and tissues were independent of oxygen tension within wide limits (13–15). The concentration of oxygen in the respirometer containers in our apparatus was never below 16 per cent. Thus it can reasonably be assumed that the diminished oxygen tension did not affect the respiration of the developing embryos.

The effect of increased carbon dioxide tension on the oxygen consumption of fertile eggs is shown in Table VIII. The 5 day old eggs showed an increase

TABLE VII

Oxygen Consumption of Embryonate Eggs in the Absence of Carbon Dioxide

Age of embryos	Oxygen consumption per egg per hr.				
	1st hour	3rd hour	2nd hour	4th hour	5th hour
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
5	0.38	0.32	0.61	0.42	0.29
8	1.35	0.98	1.42	1.01	1.19
10	2.12	2.36	1.99	2.21	2.00
12	5.48	5.61	5.13	5.52	5.50
15	10.64	10.38	10.37	10.49	10.25
17	16.04	16.48	16.33	16.21	16.29

in the rate of oxygen utilization with increased carbon dioxide tension. Although the individual differences were not statistically significant, they probably should be considered significant because of their uniform trend. The 8 and 10 day old embryos showed the same response, and the individual differences were significant. The 12, 15, and 17 day old eggs all showed a significant decrease in oxygen uptake with increasing carbon dioxide tension. The reason for this is not clear.

Root (16, 17) using sea urchin eggs, paramecium, and nerve sections and Burfield (18) using flounder eggs have reported that slight elevations of the carbon dioxide concentration increased oxygen uptake in the foregoing organisms, while large increases in the concentration of the gas greatly lessened oxygen consumption. Romanoff (19) has shown that increased carbon dioxide tension (0.4 per cent) stimulates early growth of the chick embryo. With the advancement of incubation, however, the size of the embryo diminished and at hatching time, many of the embryos were abnormal. Where the atmosphere contained 6.0 per cent carbon dioxide all embryos grew slowly and soon died.

Murray (20) has pointed out that estimations of carbon dioxide production of the developing chick embryo are subject to a number of unknown variables determinable with difficulty. These include variations in the concentration of carbon dioxide in the embryo, albumin and yolk, and contributions made by the carbonates dissolved from the shell.

From the foregoing discussion, it would appear that the most accurate measurement of oxygen consumption of fertile eggs is obtained in a carbon dioxide–free atmosphere.

TABLE VIII

The Effect of Carbon Dioxide Tension on the Oxygen Consumption of Embryonate Eggs

Age of embryos	1st hr.		2nd hr.		3rd hr.		4th hr.		5th hr.	
	V_{O_2}*	P_{CO_2}‡	V_{O_2}	P_{CO_2}	V_{O_2}	P_{CO_2}	V_{O_2}	P_{CO_2}	V_{O_2}	P_{CO_2}
days	*cc.*	*mm.*	*cc.*	*mm.*	*cc.*	*mm.*	*cc.*	*mm.*	*cc.*	*mm.*
5	0.33	1.18	0.54	1.85	0.57	2.67	0.56	4.59	1.03	5.49
8	0.73	4.01	1.32	5.34	1.35	6.83	1.35	8.84	1.80	10.69
10	2.02	4.68	2.06	4.72	2.37	9.42	2.99	11.30	3.31	13.59
12	5.03	3.78	4.82	6.30	4.58	8.97	4.32	11.95	3.62	14.98
15	10.28	11.72	8.53	15.43	7.61	17.21	5.82	20.10	4.11	21.95
17	16.28	11.20	15.01	20.40	12.32	23.61	10.59	28.40	8.64	34.25

* Cubic centimeters of oxygen consumed per egg per hour.

‡ Average partial pressure of carbon dioxide in millimeters of mercury.

The Effect of Relative Humidity on the Oxygen Consumption of Fertile Eggs.—Romanoff (21) has shown that a definite relationship exists between relative humidity and the growth, calcium metabolism, and mortality of the developing chick embryo. High relative humidity (80 per cent) favored growth and calcium metabolism but caused heavy mortality before hatching. Low relative humidity (40 per cent) retarded growth and calcium metabolism but had no effect on mortality. However, no reports appear to be available concerning the effect of relative humidity on the oxygen uptake of fertile eggs.

Experiment.—A setting of eggs was divided into two groups. One group was placed in a respirometer chamber containing a beaker of ascarite. This resulted in a relative humidity

of 0 per cent and also removed carbon dioxide. The other group was tested in the presence of 30.0 per cent solution of potassium hydroxide. This solution removed the carbon dioxide formed and also produced a relative humidity of 73.8 per cent.

Discussion.—The results obtained are shown in Table IX. There were no significant differences in oxygen uptake for embryos of the same age. Thus the relative humidity appears to have no effect of importance on oxygen consumption. However, it was observed that older embryos displayed a higher mortality with increased humidity, approximately 20 per cent dying within 36 hours after being removed from the respirometer and returned to the incubator. In view of the results of these experiments, ascarite, which absorbed CO_2 and reduced the relative humidity to approximately zero, was adopted in our routine technique.

TABLE IX

The Effect of Relative Humidity on the Oxygen Consumption of Embryonate Eggs

Age of embryos	Oxygen consumption per egg per hr.	
	High relative humidity (73. 8 per cent)	Low relative humidity (0.0 per cent)
days	*cc.*	*cc.*
5	0.61	0.44
8	1.07	1.37
10	2.36	2.85
12	4.99	5.42
15	10.37	10.42
17	15.81	16.25

The Effect of Rickettsial Infection

Gildenmeister and Haagen (22) in 1940 reported the presence of a toxin, lethal to mice, in the yolk sacs of typhus-infected fertile eggs. Kligler and Oleinik (23), and Olitzki and his coworkers (24) have demonstrated the existence of thermolabile and thermostable toxins in typhus-infected yolk sacs. Bengston, Topping, and Henderson (25) have shown that there is a toxic substance closely associated with rickettsial bodies.

In our method of infecting eggs with typhus rickettsiae, yolk sac material containing unwashed rickettsiae was injected. Thus with each portion of inoculum the eggs presumably received the above mentioned toxins. A series of experiments was carried out to determine the immediate effect of these toxins in the inoculum, as well as the subsequent growth of rickettsiae, on oxygen consumption.

Experiment.—A setting of White Rock eggs was divided into two groups. One group received 0.1 cc. of a 1:50 heavily infected (++++++) suspension of yolk sac material.

The other group served as a control. Oxygen consumption was measured 24 hours before the injections of the yolk sac material, shortly after inoculation (1 hour), and at intervals thereafter. On removal from the respirometer chamber, five of the infected eggs were smeared and stained in order to follow the course of the developing infection.

Discussion.—Table X gives the results obtained in a typical experiment. The oxygen consumption of both series increased steadily from the 6th to the 11th day. The differences between figures for embryos of a given age were not significant. From the 12th day to the 16th day the oxygen uptake of the control group continued to increase in a normal manner. In the typhus-infected group, however, the increase in oxygen uptake with advancing age was at a

TABLE X

The Effect of Infection with Typhus Rickettsiae on Oxygen Consumption of Embryonate Eggs

Age of embryos	Oxygen consumption per egg per hr.		
	Uninfected eggs (control)	Typhus eggs (infected)	Degree of infection
days	*cc.*	*cc.*	
6	1.23	1.18	0, 0, 0, 0, 0
6.9*			
7	1.56	1.27	0, 0, 0, 0, 0
9	2.08	2.16	0, (+), 0, 0, 0
11	4.65	5.11	0, (+), 0, 0, (+)
12	7.22	5.49	1, 1, (+), 2, 1
13	8.95	6.34	3, 2, 4, 3, 4
14	10.83	7.11	5, 6, 4, 6, 4
16	14.26	2.60	7, 6, 6, 7, 5

* Rickettsiae injected.

much lower rate than in the controls. On the 16th day this group showed a sharp decline in the rate of oxygen uptake. The differences in oxygen consumption between the two groups for embryos of a given age were highly significant.

The presence of the previously discussed toxins in the inoculum appeared to have little effect upon the oxygen consumption of the developing chick embryos from ages 6 to 10 days. However, it was possible that the effect of these toxins was too small to be demonstrable by our technique.

Definite rickettsiae became evident in yolk sac smears on the 12th day and the degree of infection increased steadily, reaching a peak on the 16th day. Thus there appears to be an inverse correlation between the rate of rickettsial growth and the rate of oxygen consumption. The decline in oxygen uptake of the developing embryos appears to be due to the toxins produced by the rickettsiae, as this value declines several days before the degree of infection is great enough to cause the death of large numbers of cells. The low figure obtained for the

16 day old infected embryos may be due to the death of many cells as well as to the effect of rickettsial toxins. At this point, embryonic death was occurring in many eggs.

Although several variables entered into this experiment, it is clear that the presence of rickettsiae in the entodermal cells of the yolk sac, even before fetal death begins to occur, lowers the oxygen consumption of the eggs. The results suggest that the toxins produced by rickettsiae have a depressing effect on cellular respiration, and thus favor the development of the infection. This may be a factor of importance in explaining the favorable effect of antitoxin in typhus.

On careful analysis of the figures obtained in eight experiments (four of which are shown in Tables X, XI, XII, and XIV) it became evident that a slight and temporary rise in oxygen consumption invariably occurred before the more marked and prolonged fall. This effect was noted on the 4th day of infection, at a time when, relatively speaking, very few rickettsiae were present (less than one per high power field). Although the increase in oxygen uptake was on the average only about half as great as the figure which it was decided to regard as statistically significant, its occurrence in all eight experiments makes it clear that it is a real effect. It seems probable then, that the mild concentrations of rickettsial toxins which are present in the early stages of cell invasion have a stimulating effect on cellular respiration. In the higher concentrations which develop rapidly, the effect of these same toxins is apparently a marked reduction in the respiratory rate, and eventual embryonic death.

The Effect of PABA, MABA, and OABA

Nothing was known concerning the effect of PABA, MABA (meta-aminobenzoic acid), and OABA (ortho-aminobenzoic acid) on the respiration of tissues. In the first two papers of this series, we reported that PABA was rickettsiostatic, while MABA and OABA did not influence rickettsial growth. We suggested the possibility that PABA might inhibit rickettsial growth by increasing the metabolic rate of the host cells. The fact that PABA is usually regarded as a member of the B group of vitamins suggested that it might have an effect on intracellular metabolism. It was however possible that all three benzoic acid compounds might affect the metabolism of the host cells in a similar manner and that PABA might act specifically and directly on the metabolism of rickettsiae.

Experiment.—A setting of eggs of the White Rock variety was divided into two series. One series was inoculated with typhus rickettsiae. Each series was further divided into four groups. One group of the non-infected series and one group of the infected series were used as controls. The other groups received 6.6 mg. of PABA, MABA, and OABA respectively. Oxygen determinations were made within 2 hours after the eggs were inoculated with rickettsiae or injected with the aforementioned compounds and at intervals thereafter. Because of the great numbers of eggs required in this experiment it was necessary to use some of the eggs

several times for oxygen determinations. These eggs, however, were never used until they had been in the incubator for several days.

Results.—Table XI shows results typical of those obtained in several experiments. There were no significant differences in oxygen consumption of the developing chick embryos from ages 6 to 11 days. From the 12th day of incubation onward the oxygen uptake of the infected controls and infected egg injected with MABA and OABA was found to decline steadily. These values for embryos of a given age were not significantly different from each other but

TABLE XI

The Effect of PABA, MABA, and OABA on the Oxygen Consumption of Non-Infected and Typhus-Infected Embryonate Eggs

Oxygen consumption per egg per hr.

Age of embryos	Oxygen consumption per egg per hr.							
	Non-infected series				Infected series			
	Control	6.6 mg. PABA	6.6 mg. MABA	6.6 mg. OABA	Control	6.6 mg. PABA	6.6 mg. MABA	6.6 mg. OABA
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
6	1.23	1.29	1.68	1.42	1.18	1.27	1.33	1.41
6.9					Eggs inoculated with typhus rickettsiae			
7	1.53	1.53	1.63	1.46	1.38	1.28	1.16	1.49
8.9		PABA injected	MABA injected	OABA injected		PABA injected	MABA injected	OABA injected
9	2.02	2.58	2.42	2.33	1.98	2.14	2.21	2.00
11	4.67	5.50	5.01	4.82	5.17	4.63	5.12	4.75
12	7.20	6.90	7.12	7.35	5.48	6.88	5.92	5.18
13	8.95	12.24	8.86	8.11	6.39	11.35	6.48	6.17
14	10.81	16.00	10.75	9.93	7.14	23.00	7.03	6.57
16	14.21	15.44	13.97	13.03	2.61	18.01	2.37	1.85
18	17.80	16.58	14.87	12.39	—	17.00	—	—

all were significantly lower than the values for non-infected controls of the same age.

The values for oxygen consumption of the non-infected control eggs and non-infected eggs injected with MABA and OABA were not significantly different for 6 to 14 day old embryos. Sixteen day old embryos infected with OABA showed a metabolic rate significantly lower than the controls and those injected with MABA. The values for oxygen consumption of 18 day old embryos injected with MABA and OABA were significantly lower than the values for the controls. Also the metabolic rate of the group injected with OABA was significantly lower than that of the group injected with MABA.

The PABA-treated eggs of both the infected and non-infected series displayed a significant increase in oxygen consumption over the non-infected

controls from the 13th to the 16th days of incubation. The values obtained for 14 and 16 day old PABA-injected eggs of the infected series were significantly greater than those of the non-infected series. Non-infected, PABA-treated, 18 day old embryos were found to have a significantly lower oxygen consumption than the non-infected controls. The values obtained for infected, PABA-treated 18 day old embryos were not significantly different from those found in the non-infected controls.

Discussion.—In summarizing the results of several experiments of this type, it may be said that PABA increased the oxygen consumption of normal eggs, the effect being first seen on the 4th day after its injection, lasting for 4 days and reaching its height at about the middle of this 4 day period. Thereafter, the oxygen consumption fell below that of the untreated eggs. In typhus-infected eggs, PABA affected oxygen consumption in a similar way, the principal difference being that oxygen consumption at its height was much greater than in the uninfected eggs.

This stimulating effect of PABA on respiration is of particular interest, since this finding harmonizes well with previous observations which suggest that rickettsial growth is inversely proportional to the metabolic rate of the host cells (26–28). MABA and OABA, which are not rickettsiostatic, do not increase oxygen consumption; in fact they lower it somewhat. This observation may be explained on the theory that these compounds compete with PABA for linkage in some enzyme system. The detrimental effect of the sulfonamides in rickettsial diseases has been explained in this way.

The fact that PABA raises the oxygen consumption to an even higher level in the presence of rickettsiae than it does in their absence was confirmed repeatedly. The simplest explanation of this observation—and quite possibly the correct one—is that the relatively small numbers of rickettsiae which are present in PABA-treated eggs produce a concentration of rickettsial toxin just sufficient to stimulate cellular respiration. This stimulating effect would persist, since rickettsiae are never completely destroyed, and would be added to the stimulating effect of PABA. The fact that rickettsial infection in untreated eggs causes a slight and temporary increase in oxygen consumption in the early stages of infection has already been brought out.

The Effect of Potassium Cyanide

In our previous work (2) we found that KCN stimulated rickettsial growth. This was presumably the result of a decrease in the metabolic rate in the host cells.

Experiment.—A setting of fertile eggs of the White Rock variety was divided into four groups. One group was untreated and served as a control. The second and fourth groups were inoculated with 0.1 cc. of a suspension of rickettsiae. The third and fourth groups were injected with 10^{-4}M concentration of KCN (based on 20 cc. of yolk).

Results.—Tables XII and XIII show the results typically obtained. The typhus-inoculated eggs gave a curve for oxygen consumption and degree of infection similar to that previously described.

TABLE XII

The Effect of Potassium Cyanide on the Oxygen Consumption of Non-Infected and Typhus-Infected Embryonate Eggs

Age of embryos	Oxygen consumption per egg per hr.			
	Controls (non-infected)	Typhus-infected	Potassium cyanide	Typhus-infected plus potassium cyanide
days	*cc.*	*cc.*	*cc.*	*cc.*
6	0.37	0.42	0.36	0.36
6.2		Typhus inoculated		Typhus inoculated
7.9			KCN injected	KCN injected
8	1.11	1.20	0.75	0.95
10	2.28	2.46	1.41	1.28
13	4.75	3.48	3.50	1.46
15	8.50	1.63	7.50	
18	22.86		14.28	

TABLE XIII

The Effect of Potassium Cyanide on the Oxygen Consumption of Non-Infected and Typhus-Infected Embryonate Eggs

Age of embryos	Oxygen consumption per egg per hr.			
	Controls (non-infected)	Typhus-infected	Potassium cyanide	Typhus-infected plus potassium cyanide
days	*cc.*	*cc.*	*cc.*	*cc.*
5	0.38	0.63	0.44	0.97
6		Rickettsiae in-		Rickettsiae in-
7.8		jected		jected
			KCN injected	KCN injected
8	1.49	1.99	1.01	0.12
10	3.99	3.29	2.29	2.16
12	5.07	4.05	4.12	2.87
15	10.88	1.68	11.80	
17	14.28		14.71	

The injection of KCN caused an immediate decrease in oxygen uptake. The difference between the control group and the KCN-injected group was not statistically significant. However, this immediate effect has been observed in eight experiments and the difference, therefore, is probably real. Two days after injection of the reagent the oxygen consumption of treated eggs was significantly lower than that of the controls and remained so for the duration of

the experiment (see Table XII). In some experiments the decrease in oxygen uptake was present for several days after the injection of KCN but at later embryonic ages the metabolism of the two groups was not significantly different (see Table XIII).

The fertile eggs inoculated with typhus rickettsiae and subsequently injected with KCN showed essentially the foregoing picture. The decrease in metabolic rate was more pronounced and the embryos died about 24 hours sooner than those inoculated only with rickettsiae. In several experiments the inoculated eggs receiving KCN showed an immediate and significant drop in oxygen consumption. In one experiment (Table XIII) the rate of oxygen consumption in the infected eggs given KCN was only a small fraction of the rate in non-infected and infected untreated controls.

Discussion.—The depressing effect of KCN on the respiration of uninfected eggs is in harmony with known facts concerning the action of this compound. The amount of KCN injected was the largest amount which could be injected without injury to the developing embryos. Quantitatively, the effect was somewhat less than might have been anticipated. It was however, sufficient to explain the enhancement of rickettsial growth which this compound has been observed to cause (2) when injected into eggs in which conditions were unfavorable for rickettsial growth.

In most of our experiments, a single injection maintained the respiratory rate at a lowered level for at least 9 days.

The Effect of PABA Plus KCN

Experiment.—A setting of eggs of the White Rock variety was divided into two groups. Each group was subdivided into four series. One group was inoculated with typhus rickettsiae. One series of each group was used as a control. The second, third, and fourth series of each group received KCN in an amount necessary to give a 10^{-4}M concentration (based on 20 cc. of yolk), 6.6 mg. of PABA, and PABA + KCN respectively. The great numbers of eggs required in this experiment made it necessary to use some of them several times for respiration studies. These eggs were never reused unless they had been in the incubator for several days.

Results.—Table XIV summarizes the results obtained in a typical experiment. The individual effects of rickettsial infection, KCN, and PABA were similar to those already presented.

In the non-infected eggs, PABA caused a striking increase in oxygen consumption. This effect first appeared about 4 days after the injection of the compound, and lasted for 3 days. KCN caused a moderate decrease in oxygen consumption, beginning immediately after injection and lasting for about 9 days. When both PABA and KCN were injected, the stimulating effect of PABA on repiration predominated, so that eggs receiving both compounds showed a marked increase in oxygen consumption as compared with the controls. The rate of oxygen consumption was, however, significantly lower than it was in the eggs receiving PABA alone.

In the infected eggs, the effects of PABA, KCN, and PABA plus KCN were qualitatively similar to the effects of these compounds in the absence of rickettsiae. The stimulating effect of PABA on respiration was, however, quantitatively greater than in the case of the uninfected eggs. KCN caused a decrease in oxygen consumption, the rate even falling significantly below that in the infected controls. When both PABA and KCN were injected, the

TABLE XIV

The Effect of PABA Plus KCN on the Oxygen Consumption of Non-Infected and Typhus-Infected Fertile Eggs

Age of embryos	Oxygen consumption per egg per hr.							
	Non-infected				Typhus-infected			
	Non-infected control	KCN	PABA	PABA plus KCN	Typhus-infected control	KCN	PABA	PABA plus KCN
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
6	1.35	1.47	1.48	1.17	1.18	1.47	1.37	1.62
6.9					Eggs inoculated with typhus rickettsiae			
7	1.64	1.42	1.74	1.81	1.51	1.58	1.30	1.65
8.9		KCN injected	PABA injected	PABA plus KCN injected		KCN injected	PABA injected	PABA plus KCN injected
9	2.13	1.73	2.70	2.63	2.11	1.95	2.25	1.84
11	4.79	3.92	5.60	5.42	5.28	4.10	4.75	5.09
12	7.33	6.08	6.98	7.01	5.57	4.53	6.99	6.77
13	8.97	7.89	12.36	11.13	6.50	3.72	12.71	11.15
14	11.92	10.78	17.00	15.11	7.63	2.17	22.14	19.32
15	14.32	12.28	16.71	15.45	1.89	—	17.22	16.42
16	16.91	14.58	16.01	16.12	—	—	17.87	17.74
18	18.94	17.99	17.69	17.06	—	—	18.11	18.37

stimulating effect of PABA again predominated, but the respiratory rate was again significantly lower than that of the infected eggs receiving PABA alone.

Effect of Folic Acid

In view of the fact that PABA forms a part of the folic acid molecule, it was decided to test folic acid for a possible effect on rickettsial growth and on cellular respiration in infected and uninfected eggs.

Single injections of 21 mg. of folic acid, an amount corresponding approximately to the optimum dosage of PABA (6.6 mg.), caused a mortality of 80 per cent in the embryos. Injections of half of this amount, however, were well tolerated, and were given on the 2nd, 4th, and 6th days after injecting with

rickettsiae. In other experiments, the eggs received 5 mg. of folic acid daily for 9 days, and in still other experiments 1 mg. and 0.1 mg. daily for 9 days. In none of these experiments did folic acid show any evidence of rickettsiostatic action, or cause any significant increase in oxygen consumption in the uninfected eggs. In one experiment, the larger doses of folic acid appeared to lower oxygen consumption slightly but significantly in the uninfected eggs.

SUMMARY AND CONCLUSIONS

A technique is described for measuring the oxygen uptake of embryonate eggs. Statistical analysis has shown that the method is reliable and accurate. Determinations were made on groups of 15 to 20 eggs, in order to average out individual biological variations. Reduction of the CO_2 tension and relative humidity to approximately zero previous to analysis has been found to be desirable. The oxygen consumption of normal and typhus-infected eggs, untreated and treated with agents previously found to inhibit or enhance rickettsial growth has been studied.

Rickettsial infection caused a slight but significant increase in the rate of oxygen consumption on the 4th day after inoculation, followed by a rapid drop in the rate as the infection developed. The evidence suggests that low concentrations of rickettsial toxins may stimulate respiration, while higher concentrations depress respiration and lead eventually to embryonic death.

PABA, which is rickettsiostatic, markedly increased the oxygen uptake of normal eggs, the effect appearing 4 days after injection and lasting for about 4 days. Thereafter, the rate fell below that of the untreated eggs. In typhus-infected eggs, PABA had similar effects, but the oxygen consumption reached much higher levels. A possible explanation of this fact is suggested. MABA and OABA, which are not rickettsiostatic, did not increase oxygen uptake; in fact they depressed cellular respiration moderately, OABA being more active in this way than MABA. These two compounds may compete with PABA for a position in some respiratory enzyme system.

Potassium cyanide, which enhances rickettsial growth, caused, in concentrations not lethal to the embryos, a moderate drop in the oxygen consumption of normal eggs, the effect starting almost immediately after injection and lasting usually for 9 days. In infected eggs, its effect was more striking. It is probable that rickettsial toxins and KCN act synergistically to depress cellular respiration.

When PABA and KCN were injected simultaneously, the stimulating effect of PABA on respiration predominated. The resulting level of oxygen consumption, though lower than that resulting from PABA alone, was still high enough to inhibit rickettsial growth.

As far as our results go, they support the hypothesis that, within certain limits. rickettsial growth is inversely proportional to the respiratory rate of the

host cells, regardless of the factors which determine that rate. It is not yet clear that PABA owes its rickettsiostatic action to its ability to increase cellular respiration, but this assumption seems reasonable as a working hypothesis. The respiratory mechanism in which PABA participates is not as yet known.

Although PABA forms part of the folic acid molecule, folic acid itself, in concentrations corresponding to effective doses of PABA, did not increase cellular respiration or show rickettsiostatic action.

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