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

II. Temperature, Potassium Cyanide, and Toluidin Blue

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The work on which this report is based is a continuation of that set forth in Paper I of this series (1). In this first paper, penicillin and para-aminobenzoic acid were shown to inhibit markedly the multiplication of typhus rickettsiae in the entodermal cells lining the yolk sac of the fertile egg. Reasons were given for believing that certain rickettsiostatic agents may act indirectly, by altering the metabolism of the cells in which they grow. In attempting by means of the present studies to obtain specific information concerning the enzymatic mechanisms involved in the intracellular multiplication of rickettsiae, agents with known action on cellular metabolism are to be employed. The positive effects on rickettsial growth obtained by altering the environmental temperature, and by introducing sublethal concentrations of potassium cyanide and of toluidin blue will now be presented. Negative results obtained with certain other agents will be mentioned briefly.

Factors affecting the degree of infection in the control eggs will also be discussed, since the ability to obtain heavily or lightly infected eggs has proven to be advantageous in carrying out certain of the experiments.

Material and Methods

A murine strain of typhus in its 45th passage was used. After preliminary incubation for 5 days, the eggs were injected with yolk sac suspensions containing rickettsiae obtained from an infected egg of the preceeding series. Unless otherwise indicated, all eggs were incubated at 37.5° C. The agents to be tested were introduced into the yolk sacs at varying intervals, usually subsequent to the injection of rickettsiae. Estimates of the degree of infection were made from Giemsa-stained films of the yolk sac membranes. Techniques for these procedures were described in detail in paper I of this series. These techniques were followed in the present experiments, with the following exception: For the purpose of puncturing the air sac end of the egg shells, for the subsequent introduction of the needle, a Burgess Vibro-tool (sold by several scientific supply houses) is now used. This has been found to save considerable time, since it makes it unnecessary to remove the eggs from the egg holders.

Factors Affecting Degree of Infection in Control Eggs

In Paper I, it was pointed out that the control eggs, even when injected with material containing large numbers of rickettsiae, occasionally failed to show

demonstrable rickettsiae, or showed only very few organisms. In such eggs, fetal death did not occur. In other experiments, the appearance of rickettsiae in the yolk sac was delayed for several days, but eventually infection reached a moderate or even a high level. It seemed possible that these irregular results might be due either to the use of eggs having greater resistance to infection or to variations in the effective rickettsial content of the inoculum. It has been possible to show that both of these assumptions are true, and to isolate two definite factors which influence the degree of infection in untreated eggs.

Blending Time.—In our early experiments, including those reported in Paper I, the yolk sac membrane was emulsified in the Waring Blendor by running the motor for 4 minutes. Heavy infection usually occurred in the controls, but in about every third or fourth experiment only a few rickettsiae were found. In later experiments the time of blending was reduced to 10 seconds, and it became clear that this resulted in heavier infection or in the earlier development of infection. In order to evaluate this factor quantitatively, parallel series of eggs were run in which the blending time was varied.

In some experiments, the blending time had no demonstrable effect, while in other experiments, more prolonged blending resulted in delayed rickettsial multiplication. In several experiments, eggs injected with material blended for 10 seconds became heavily infected, while eggs injected with the same material blended for several minutes showed only a few rickettsiae. A striking example of the effect of the time of blending is shown in Table I, where each symbol indicates rickettsial growth in an individual egg. All eggs in this series were injected with the same inoculum, the only difference being in the time of blending.

Strain of Egg.—Certain strains of eggs were found to be more susceptible to rickettsial infection than other strains. Here again, certain experiments showed no definite differences in the behavior of different strains, but in other experiments strking differences were seen. The results obtained when strains of lower susceptibility were used varied from a delay of 1 to 3 days in the development of infection to almost complete failure of rickettsial growth. A typical example of delayed infection is seen in Table I. Table II shows almost complete inhibition of rickettsial growth in eggs of the Wyandotte strain, while eggs of the White Rock strain, inoculated in parallel series, developed heavy though somewhat delayed infection.

Discussion.—It is believed that heavy infection occurs only when large numbers of entodermal cells are invaded by viable rickettsiae contained in the original inoculum. Prolonged blending, by separating the rickettsiae from their film of protective cytoplasm, probably reduces the number of viable organisms, and also reduces the number of organisms which can survive long enough in the yolk sac to gain entrance to entodermal cells.

Another variable is the number of viable organisms originally present in the yolk sac membrane used as a source of inoculum. Even when smears show

TABLE I

Effect of Time of Blending and Strain of Egg on Rickettsial Multiplication

Day following inoculation	White Rock eggs		New Hampshire Red eggs	
	Blended 10 sec.	Blended 2 min.	Blended 10 sec.	Blended 2 min.
4	−	−		
5	− −	−	−	−
6		−		
7	++		−	− − −
8	+++ +++		(+)	(+)
9	++++ +++++ ++ +++++ ++++ +++ ++++++			
10	+++ +++ ++++++		+++++	
11		− −* −*	+++++ ++++ ++++	+* +*
12		−* (+)* +++*	+++++ ++++++ +++ ++++++ ++++	−* −*
13		(+)* −* −*		− − (+)*
14		−* −*		−*

− no rickettsiae recognizable with certainty.
(+) less than one rickettsia per oil immersion field.
+ 1–10 rickettsiae per oil immersion field.
++ 10–100 rickettsiae per oil immersion field.
+++ 100–1000 rickettsiae per oil immersion field.
++++ 1000–5000 rickettsiae per oil immersion field.
+++++ 5000–8000 rickettsiae per oil immersion field.
++++++ 8000–12,000 rickettsiae per oil immersion field.
* Embryo alive at time of examination.

numerous rickettsiae, there is no way of estimating the number of living rickettsiae present.

The factors determining the strain variation in susceptibility are as yet unknown. The important practical point brought out by the above experiments

TABLE II

Effect of Strain of Egg on Rickettsial Growth

Blending time 2 minutes.

Day following inoculation	White Rock	Wyandotte
3		−
7	+*	−*
	−*	−*
8	++*	
	++*	
9	(+)*	−*
10		−
		−
11	+*	−
	++++++	−
12	+++++	−*
	++++++	−*
13	++++++	−*
	+++	(+)*
	+++++	
14	+++	(+)*
	+++	−*
	+++	−*
	++++*	−*
	+*	−*

For explanation of symbols see Table I.

is that an inoculum blended 10 seconds and injected into White Rock eggs results almost uniformly in early infection of maximum intensity, while an inoculum blended 2 minutes or longer and injected into Wyandotte eggs frequently gives infection of minimal degree. These facts have been made use of in the following experiments. It has been possible to obtain at will heavily infected eggs, which are most satisfactory when agents which inhibit rickettsial growth are to be studied, or lightly infected eggs which are advantageous in studying

agents which enhance rickettsial growth. For initial experiments on agents of unknown action, both heavily and lightly infected series of eggs have been employed.

Environmental Temperature

It is well known that an increase in the environmental temperature of living organisms, within certain tolerated limits, causes increased metabolic activity, increased oxygen consumption, and an increased growth rate. We have shown previously (2) that typhus-infected mice kept at a high environmental temperature (85–98° F.) show a survival rate of about 90 per cent, as contrasted with a survival rate of zero among mice in parallel series kept at a temperature of 65–72° F.

Cox (3) has shown that the titre of infectivity of yolk sac tissues infected with spotted fever rickettsiae is much higher when the eggs are incubated at 35° C. than when incubated at 39° C.

Table III summarizes the results of an experiment in which an environmental temperature of 40° C. caused almost complete suppression of rickettsial growth in the yolk sac, while eggs in parallel series maintained at 37.5° C. showed the usual picture of uniformly heavy infection. In this experiment White Rock eggs injected with inoculum blended for 10 seconds were used.

Discussion.—Brody and Henderson (4) used the rate of growth of developing embryos at different environmental temperatures as a measure of total metabolic activity. It was found that on any given day during development the weight attained was greater the higher the environmental temperature. On the 17th day the mean weight at 35.0° C. was 5.5 gm.; at 37.5° C., 12.6 gm.; and at 40.5° C., 20.4 gm.

Romanoff (5) found that the developing chick embryo, although producing heat, at first behaved as a poikilothermic or "cold-blooded" animal, but later became homeothermic. True homeothermy presumably is not acquired by the chick until the 4th or 5th day after hatching. The rise in temperature of the developing egg tends to be periodic. The temperature of the embryo reaches that of its environment on the 4th day and rises steadily until the 9th day, when it is about 1° C. above that of its environment. During the next 5 days it falls nearly to that of its environment, and then rises again, reaching a point 2° above that of the incubator on the 19th day. Heavy rickettsial infection in our eggs occurs 7 to 9 days after inoculation (12th to 14th days of incubation), corresponding to the period of falling embryonic temperature.

The inability of rickettsiae to multiply freely in eggs kept at 40° C. is probably the result of increased metabolic activity in the entodermal cells, rather than a direct effect of the higher temperature on rickettsial division.

Potassium Cyanide

Potassium cyanide was chosen as an agent to be tested because of its well known inhibitory action on the respiration of cells of many different types.

TABLE III

Effect of Temperature on Rickettsial Growth

Day following inoculation	37.5° C.	40° C.
4	−	−
	−	
	−	
5	−	−
		−
6		−
		−
7		−
8	++	−
	++	++
	++++	−
	+++++	
	+++	
	+++++	
	++++	
	+++++	
	++++++	
9	++++	−
	++++	−
	+++	
	++++++	
10		(+)
		−
11		−
		(+)*
12		−*
		+*

For explanation of symbols see Table I.

Eggs receiving KCN in such amounts as to give a concentration of approximately 10^{-3}M in the yolk showed embryo death within 24 hours. Concentrations of 10^{-4}M or less were in general well tolerated. The KCN was dissolved in distilled water and injected into the yolk sac, 72 hours after the

injection of inoculum containing rickettsiae. In calculating the amounts to be injected, the yolk was considered to have a volume of 20 cc. Some of the chemical was undoubtedly absorbed by the other components of the eggs, so that the true concentration in the yolk was not known.

TABLE IV

Effect of KCN

Day following inoculation	Control	KCN 8×10^{-6} M	KCN 10^{-6} M	KCN 2×10^{-6} M
4	−	−	−	−
			−	−
			−	−
				−
				−
5		−		−
6		−		
7	(+)*	+*	(+)*	++*
	−*	+++	+++*	+++*
8	(+)		++	−
9	−*	++*	++*	(+)
	−*	+*	+*	+
10	−*	+	−*	++++
	−*			
11	(+)*	+++++	++++++	++*
	(+)*	++	++	++*
12	+	+++*	++++++*	++
	+	+++++	++++++	
13	−*	++++++*	+++*	
	−*	++++*	+*	
	−*	+++		

For explanation of symbols see Table I.

Many series of eggs were injected with varying concentrations of KCN. In those series in which the controls developed maximal infection, rickettsial growth was not appreciably affected by KCN. In all experiments in which only minimal infection developed in the controls, however, a marked enhancement of rickettsial growth as a result of the injection of KCN was observed. A typical experiment in which this effect was seen is represented by Table IV.

In this experiment 60 Wyandotte eggs were injected with inoculum prepared from a yolk sac membrane showing a +++++ infection, and blended for 3 minutes. Fifteen eggs served as controls, while 3 other groups of 15 eggs each received KCN in such doses as to produce theoretical concentrations of 2×10^{-4}M, 10^{-4}M, and 8×10^{-5}M.

Discussion.—Warburg's experiments in 1923 (6) led him to the conclusion that cellular respiration was due entirely to the reaction between molecular oxygen and a complex intracellular iron compound (the respiratory enzyme). He believed that all cellular respiratory enzymes were cyanide-sensitive (7).

Dixon and Elliot (8) later showed that the respiration of tissue suspensions and slices could not be completely inhibited by cyanide. They concluded that about one-third of the respiration of animal tissues was carried out by enzyme systems which were insensitive to cyanide. The flavoprotein enzymes, in which riboflavin forms an essential component, probably are largely responsible for this cyanide-resistant portion of cellular oxidation processes (9).

Keilin in 1925 (10) showed that the intracellular iron porphyrin proteins known as cytochrome a, cytochrome b, and cytochrome c were characterized by their ability to undergo reversible oxidation and reduction. Later Keilin and Hartree (11) isolated a fourth component of this system, namely cytochrome oxidase. This enzyme catalyzes the oxidation of the cytochromes by molecular oxygen. Warburg's cyanide-sensitive respiratory enzyme was probably identical with cytochrome oxidase.

There is a rough parallelism between the respiratory activity of aerobic cells and the concentration of the cytochromes and cytochrome oxidase. Cyanide inhibits the action of cytochrome oxidase, but there is good evidence that the cytochromes are cyanide-insensitive (12).

From the above brief discussion of the action of cyanide, it is evident that its main effect upon cells is to lower their metabolic rate by inhibiting cytochrome oxidase. The stimulation of rickettsial growth, under certain conditions, by KCN is then presumably the result of a decrease in the metabolic rate in their host cells, brought about by this specific mechanism.

Toluidin Blue

The use of toluidin blue in this series of experiments was suggested by the work of Peterson (13), who found this dye to be chemotherapeutically effective in experimental murine typhus infection. This part of the work was further stimulated by the reported observation of Axmacher (14) that toluidin blue approximately doubled the oxygen uptake of thin slices of rat nerve. In view of the similar effect of methylene blue on mammalian erythrocytes reported by Harrop and Barron (15), this dye was also tested.

For the purpose of testing these dyes, experimental conditions leading to maximal infection in the control eggs were used. The dyes were injected 72 hours after the injection of the rickettsial inoculum, in such amounts as to give a theoretical concentration in the yolk sac of 10^{-4}M or 10^{-5}M. In some experiments, the dyes were given in two doses 72 and 144 hours after rickettsial infection.

These two dyes were found to have a similar effect on rickettsial growth. In the majority of experiments, these dyes caused a delay of about 3 days in the development of heavy rickettsial infection. In only one experiment was there evidence that the degree of infection eventually attained in the treated eggs was somewhat lower than in the controls.

A typical experiment showing the effect of toluidin blue is pictured in Table V. Thirty-three White Rock eggs were injected with rickettsial inoculum blended for 10 seconds. Eighteen eggs served as controls, while 15 eggs, 72 hours after injection with rickettsiae, were given an injection of toluidin blue calculated to produce a concentration in the yolk sac of 5×10^{-5}M. A second similar injection of the dye was given 144 hours after injection with rickettsiae.

Discussion.—The effect of methylene blue and other dyes of the same class on biological oxidation has been discussed recently by Ball (9). The increased oxygen consumption caused by these dyes appears to depend on their ability to act as hydrogen acceptors and to combine with molecular oxygen. With intact cytochrome enzyme systems, they apparently furnish a supplementary route for oxidation, and thus increase the total oxygen utilization.

The failure to produce inhibition of rickettsial growth of a degree comparable to that shown by penicillin and para-aminobenzoic acid may be due to the limitations of our experimental methods. The dyes became decolorized after about 48 hours in the eggs, and it was not practical to re-inject them after the 6th day of the experiment because of the large number of traumatic deaths of the embryos caused by injection at this stage.

Temperature Plus KCN

Since the effect of an increase in environmental temperature is to increase the oxygen consumption of living organisms, it is a reasonable assumption that the inhibition of rickettsial growth observed when the eggs were kept at 40° C. is the result of increased activity of the respiratory enzymes. The effect of KCN on rickettsial growth at this higher temperature was therefore studied in a series of experiments.

In a typical experiment of this series, 48 Rhode Island Red eggs were injected with 0.1 cc. each of yolk sac inoculum blended for 10 seconds. These eggs were placed in an incubator maintained at 37.5° C. Seventy-two hours later 32 of these eggs were transferred to an incubator kept at 40° C. Sixteen of these eggs kept at 40° C. were immediately injected with KCN to give an estimated concentration of 10^{-4}M.

The control eggs, incubated at 37.5° C. developed a normal pattern of maximal infection, and were all dead by the 9th day. Only minimal infection developed in the untreated eggs at 40° C.; five of these were killed on the 10th day. The eggs incubated at 40° C. and receiving KCN showed a pattern of rickettsial growth essentially like that of the eggs kept at 37.5° C. The results of this experiment are shown in Table VI.

Discussion.—Presumably the inhibition of cytochrome oxidase by KCN reduces the respiratory activity of the entodermal cells in eggs kept at 40° C.

TABLE V

Effect of Toluidin Blue Alone and in Combination with KCN

Day following inoculation	Control	Toluidin blue 5 × 10^{-6}M 2 injections	Toluidin blue 10^{-4}M (2 injections) + KCN 10^{-4}M
4	−	−	−
	−	−	−
	−		−
5	−	(+)	−
		−	−
		−	−
			−
6		−	
7		−	−
8	++		(+)
	++++		−
	+++++		−
	++		
	+++		
	+++++		
	++++		
	+++++		
	++++++		
9	++++	++++	+
	++++		
	+++		
	++++++		
	++++		
10		+++++*	++++*
		++	+++++*
11		−	
		+++++*	++++*
12		+++	++++
		++	
		++++	

For explanation of symbols see Table I.

to approximately the level maintained in untreated eggs incubated at 37.5° C. thus allowing free rickettsial growth in spite of the higher environmental temperature. The multiplication of rickettsiae in experiments of this type appears to be a function of the respiratory activity of the entodermal cells in which they grow.

Toluidin Blue Plus KCN

In several experiments toluidin blue (rickettsiostatic) was combined with KCN, which was shown to enhance rickettsial growth under certain conditions. In these experiments, one of which is summarized in Table V, the moderate rickettsiostatic action of the toluidin blue was not neutralized by KCN.

TABLE VI
Effect of Temperature Plus KCN

Day following inoculation	37.5° C.	40° C.	40° C. + 10^{-4} M KCN
5	(+) +	(+) ++ (+)	+ +
6	+++ (+)	−	++ +++ ++++
7	++++++ ++ ++++	−	++ ++++ ++ ++++
8	++++ ++++ ++++ ++++++	(+) (+) ++ +	+++++ +++++ +++++
9	+++++ +++++ ++++++	+ (+)	+++
10		−* −* −* +* +*	+++++ +++ +

For explanation of symbols see Table I.

Discussion.—Dyes of this type, as has been brought out, are believed to furnish an alternative route for biological oxidation, by-passing the cyanide-sensitive cytochrome oxidase system. Barron (16) has furnished evidence that the methylene blue route of oxidation is not blocked by cyanide. The results of our experiments appear to harmonize with this view. Since cyanide had no effect on rickettsial growth in experiments showing heavy infection in the control eggs, it would not be anticipated that it would neutralize the rickettsiostatic action of the dye under similar conditions, unless the dye itself worked in a system which was cyanide-sensitive.

Since the rickettsiostatic action of elevated environmental temperature is neutralized by KCN, while the rickettsiostatic action of toluidin blue is not

TABLE VII

Effect of PABA in Combination with KCN

Day following inoculation	Control	10^{-4} M KCN	6.6 mg. PABA	10^{-4} KCN plus 6.6 mg. PABA
4	−		−	−
				−
	−	+	−	−
				−
5	+		++	
	+			
6	++	++++	+	+
	++++	++++	++	
		+++		
		+++		
		+++		
7	++++++	+++++	+	+
	+++++	++++	(+)	−
		+++++	−	
		++++		
8	++++++++	+++++	+	(+)
	+++++++	++++++	++	++
	++++++	+++++++	++	
	++++++	++++	+++	
	++++++++			
	+++++			
9	+++++	+++++	(+)	++
10			−	+
				−
				−
11			(+)*	+*
				−*

For explanation of symbols see Table I.

affected, it must be concluded that the mechansims of action of these two rickettsiostatic agents are not identical.

Temperature elevation presumably acts by increasing the activity of *cyanide-sensitive* cytochrome oxidase: cyanide would be expected, therefore, to neutralize the effect of temperature elevation. Toluidin blue acts by furnishing an

alternative *cyanide-insensitive* route of oxidation: cyanide therefore, would not be expected to neutralize the effect of toluidin blue.

PABA Plus KCN

Several experiments, one of which is summarized in Table VII, indicated quite clearly that the previously described rickettsiostatic action of para-aminobenzoic acid (PABA) was not neutralized by KCN. Since the inhibition of rickettsial growth by PABA is incomplete, it was thought unnecessary to carry out experiments titrating PABA against KCN.

Discussion.—The biologic action of PABA is obscure. It is believed by some workers to be a member of the B group of vitamins. It is thought to be an essential bacterial metabolite.

From the experiments reported here, it may be concluded that the rickettsiostatic action of PABA is exerted by a mechanism differing from that of the rickettsiostatic action caused by temperature elevation. Whether PABA acts in a manner similar to toluidin blue and methylene blue or in some other way remains to be determined. It is important to determine whether PABA increases the oxygen uptake of cells: if not, the possibility of a direct action on rickettsial multiplication must be considered.

Negative Results

Several compounds related to *p*-aminobenzoic acid (PABA) were tested for their rickettsiostatic action by the technique described above. The figures following each agent indicate the amount injected into each egg. *p*-Aminophenylacetate (Na salt), a homologue of PABA, 6.6 mg.; *p*-aminobenzamide, the amide of PABA, 6.6 mg.; sodium *N*-(phenyl-4-carboxylic acid) glycineamide, a nitrogen substituted derivative of PABA, 6.6 mg.; *p*-thioureabenzoic acid (Na salt), a nitrogen substituted derivative of PABA, 6.6 mg. No appreciable effect on rickettsial growth was produced by any of these compounds.

Dinitrophenol, injected in such amounts as to give concentrations of 10^{-4} and 10^{-5}M, likewise showed no demonstrable effect on rickettsial growth.

SUMMARY

The time of blending the rickettsial inoculum, as also the strain of hen's egg employed, influenced the degree of infection which developed in fertile eggs after the injection of typhus rickettsiae into the yolk sac. By varying these factors, maximal or minimal infections could be obtained.

Eggs incubated at 40°C. developed only minimal rickettsial infection, whereas control eggs incubated at 37.5°C. became heavily infected. Potassium cyanide markedly enhanced rickettsial growth in experiments in which the control eggs developed only minimal infection. Under circumstances such

that the control eggs became heavily infected, KCN had no appreciable effect.

Toluidin blue and methylene blue delayed the development of rickettsial infection in the yolk sac, but their rickettsiostatic action under the conditions of these experiments was less marked than that of penicillin and para-aminobenzoic acid.

The rickettsiostatic action resulting from temperature elevation was neutralized by KCN, and hence is believed to be due to the increased activity of the cyanide-sensitive respiratory enzyme (cytochrome oxidase) in the entodermal cells in which the rickettsiae multiply.

The rickettsiostatic action of toluidin blue and methylene blue, though probably also resulting from increased metabolic activity in the entodermal cells, was not neutralized by KCN. This observation is in harmony with the reported observation that dyes of this type furnish an alternative mechanism for intracellular oxidation which is cyanide-insensitive.

The rickettsiostatic action of para-aminobenzoic acid was not neutralized by KCN. No conclusions can be reached at present concerning the mechanism of action of this compound.

Dinitrophenol and several compounds related to para-aminobenzoic acid gave negative results.

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