BIOCHEMICAL STUDIES ON THE PHENOMENON OF VIRUS REPRODUCTION

III. The Inhibition of Coliphage T2r+ Multiplication by Sodium Salicylate and Sodium Gentisate

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The appropriate use of inhibitors of known biochemical action on the growth and metabolism of bacteria has resulted in marked advances in the fields of bacteriology and biochemistry during recent years. Only a few attempts have been made, however, to apply this approach to studies on the bacterial viruses although the technics for such studies are equally simple.

It was demonstrated by Spizizen (1943) that the inhibition of a coliphage (P1) multiplication by the glycine analog, amino methane sulfonic acid, could be prevented by the addition of xanthine. Fitzgerald and Lee (1946) showed that inhibition of the multiplication of a coliphage by 2-amino-9-(*p*-aminophenyl) acridinium chloride could be relieved by ribonucleic acid. Cohen and Anderson (1946) reported that the inhibition of the growth of coliphage T2 by 5-methyl tryptophan was reversed by tryptophan. Wooley and Murphy (1949) showed that an analog of pyridoxine, desoxypyridoxine, inhibited the multiplication of coliphage T2 and that this inhibition could be reversed by pyridoxine and by a number of compounds involved in carbohydrate metabolism.

These reports indicate that an attempt may be made to identify the metabolites involved in the cellular reactions leading to virus multiplication by the use of inhibitors and that further investigations are justified. The specificity of the inhibitor on the virus system may be appraised by information derived from data on, first, the ratio of the concentration of inhibitor required to delay the growth of uninfected host cells to that required for retardation of virus growth, and second, the kind of substances that can reverse or prevent such inhibitions.

The present paper describes a simple procedure for obtaining these data by means of one-step growth curves. A study of the effects of sodium salicylate and sodium gentisate on the multiplication of phage T2r+ and on the growth of uninfected cells illustrates the applicability of the method.

METHODS

A synthetic medium (*SM8*) with the following composition was used for the growth of the host cells *Escherichia coli*, strain B:

	Per cent
NH_4Cl	0.05
$(NH_4)_2SO_4$	0.005
Glucose	0.4
NaCl	0.01
$MgCl_2 \cdot 6H_2O$	0.01
Na_2HPO_4 anhyd	0.6
KH_2PO_4	0.3

Prior to infection, the host cells which had been propagated in test tubes containing 4.5 ml of *SM8* medium, with aeration for 24 hours at 37 C, were transferred to fresh medium for 3 to 4 hours so that the culture would reach a concentration of about 5×10^7 to 10^8 bacteria per ml. Then 0.5 ml of T2r+ bacteriophage prepared in *SM8* medium (containing approximately 5×10^5 plaque-forming particles) was added to the actively growing culture and mixed. The phage-bacterial mixture was held at 37 C for 5 minutes to permit adsorption of the virus to the cells, and then diluted 100-fold in *SM8* containing the substances to be tested (final volume 5 ml). The test mixtures were incubated at 37 C and assayed for phage at intervals by mixing 0.5 ml with 4.5 ml of nutrient broth containing about 10^8 bacteria per ml. One-tenth ml of this final mixture was spread on each of two nutrient agar plates, incubated for 24 hours at 37 C, and examined for plaques (Ellis and Delbrück, 1939). One-step growth curves were obtained as described by Delbrück and Luria (1942).

For the studies on bacterial growth, the cells were treated similarly, except that no infection was made with phage. Viable counts on nutrient agar and turbidity determinations in a Klett-Summerson colorimeter were used for following growth.

The data obtained by the use of a series of concentrations of an inhibitor were expressed in terms of: (a) the lowest concentration of inhibitor that retarded multiplication by at least 25 per cent in 60 to 80 minutes, referred to as the minimal phage inhibiting concentration (MPIC); (b) the lowest concentration of inhibitor that delayed bacterial growth in 2 to 24 hours, designated as minimal bacterial inhibiting concentration (MBIC); (c) the activity ratio of the compound (AR). This value (AR) represents the ratio between the amount of the compound required to inhibit the growth of uninfected cells to that required to inhibit phage multiplication.

$$\left(\frac{\text{MBIC}}{\text{MPIC}} = \text{AR}\right)$$

When this figure is greater than 1.0, the compound can be considered to be more active against the phage system than the bacterial growth.

EXPERIMENTAL RESULTS

Studies with sodium salicylate. Sodium salicylate was found to delay both the multiplication of phage T2r+ and the cell growth, with a minimal phage inhibiting concentration of 7.5×10^{-4} and a minimal bacterial inhibiting concentration of from 4.5×10^{-3} M to 7.5×10^{-3} M, so that the activity ratio was approximately 6 to 10. Hence it would appear that phage growth is more sensitive to inhibition by salicylate than is the cell growth. This differential effect could be due to any of the following factors: (a) a direct action on the phage particle itself, that is, inactivation, (b) an effect on the adsorption of the virus to the cell, or (c) an effect on the invasion, multiplication, and release of the virus from the host cell.

Further experiments showed that no inactivation of phage occurred during a time interval of 80 minutes with concentrations of salicylate of from 7.5×10^{-4} M to 7.5×10^{-3} M. Neither did the rate of adsorption diminish with 1.5×10^{-3} M salicylate (table 1), as determined by assays of the supernatant of phage-bacterial mixtures after centrifugation for 5 minutes at 3,000 rpm at two different time intervals. Hence the salicylate effect must be presumed to be on some process involved in the invasion, growth, and release of the phage.

In order to study the nature of this interference, a large number of compounds were tested to determine which substance, if any, would prevent inhibition of

TABLE 1

Rate of phage adsorption with and without salicylate in SM8 medium at 37 C

Initial phage count: 10^6/ml
Coli count: 5×10^7/ml

PER CENT ADSORPTION		
Time, min	Salicylate (1.5×10^{-3} M)	Control
5	75	80
10	91	90

TABLE 2

Effect of tryptophan and its biological precursors on the salicylate inhibition of phage T2r+

COMPOUND	FOLD INCREASE OF PHAGE IN 70 MIN	PER CENT INHIBITION
Control	99	
Salicylate (1.5×10^{-3} M)	13	86
Salicylate + DL-tryptophan (10^{-3} M)	102	0
Salicylate + indole (10^{-6} M)	140	0
Salicylate + anthranilic acid (10^{-6} M)	130	0
DL-tryptophan (10^{-3} M)	120	
Indole (10^{-6} M)	100	
Anthranilic acid (10^{-6} M)	60	

the phage multiplication in the presence of 1.5×10^{-3} M salicylate. Very slight relief of inhibition (about 20 per cent) was obtained with coenzyme I (20 μg per ml) and nicotinic acid (1 mg per ml). On the other hand inhibition could be prevented entirely with approximately 10^{-3} M DL-tryptophan, indole, or anthranilic acid (table 2 and figure 1). The similarity of the structure of these compounds to salicylate is striking (figure 2), and suggests a possible metabolite-antimetabolite relationship.

Since the growth of the host cells was also inhibited by salicylate, but at higher concentrations, it was of some interest to determine whether bacterial inhibition also could be prevented by tryptophan. With relatively high concentrations of DL-tryptophan (10^{-5} M), inhibition of bacterial growth by salicylate at concen-

trations from 4.5×10^{-3} M to 7.5×10^{-3} M was unaffected, but pantothenate (4×10^{-4} M) prevented this inhibition to some extent (table 3).

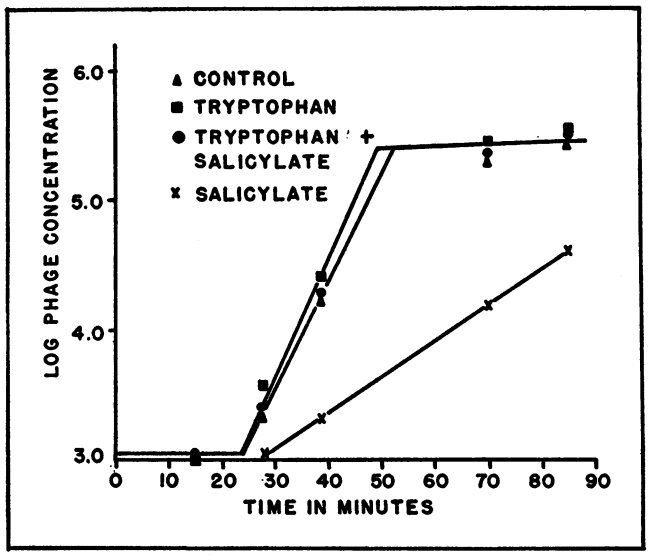

Figure 1. Graph showing tryptophan reversal of salicylate inhibition of phage T2r+ multiplication.

DL-tryptophan, 10^{-3} M.
Sodium salicylate, 1.5×10^{-3} M.

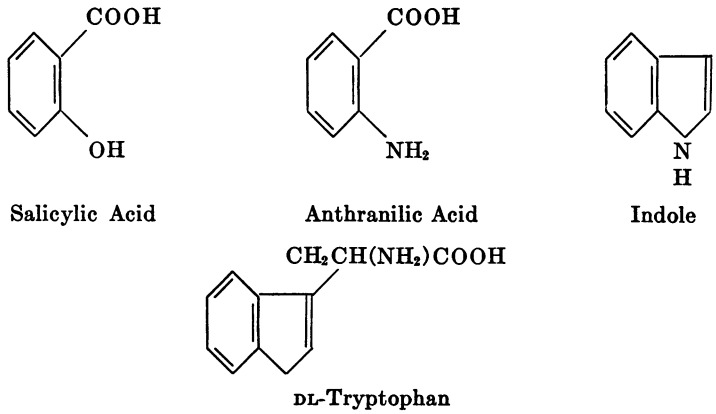

Figure 2. Showing structural relationship between salicylate and reversal agents in the phage system.

Studies with sodium gentisate. Since a metabolic relationship has been suggested for gentisate and salicylate (Meyer and Ragan, 1948; Kapp and Coburn, 1946; and Lutwak-Mann, 1942), the effects of gentisate were also investigated.

Here it was found that the lowest phage inhibiting concentration of gentisate was 1.5×10^{-4} M but that at least 1.5×10^{-2} M was required to inhibit the bacterial growth. Thus the activity ratio was 100.

A large number of compounds were tested for their ability to prevent the gentisate inhibition of phage multiplication. During the course of these experiments it was observed that solutions of sodium gentisate upon standing in the cold for

TABLE 3

Effect of pantothenate and DL-tryptophan on the salicylate inhibition of bacterial growth

Turbidity readings in Klett-Summerson colorimeter, blue filter. Growth in 20 hours at 37 C in *SM8* medium, unaerated, with or without added substances.

Tubes Containing	READINGS: No Added Substances	With Added Pantothenate (3.6×10^{-4} M)	With Added DL-tryptophan (10^{-5} M)
SM8 medium	170	148	170
SM8 medium + salicylate 7.5×10^{-3} M	18	74	27
SM8 medium + salicylate 6.0×10^{-3} M	27	71	27
SM8 medium + salicylate 4.5×10^{-3} M	41	80	12

TABLE 4

Effect of tyrosine, methionine, and ribonucleic acid on phage T2r+ inhibition by gentisate

Gentisate solution kept 2 weeks at 4 C

COMPOUND	FOLD INCREASE OF PHAGE IN 70 MIN	PER CENT INHIBITION
Control (*SM8*)	403	
Gentisate (6×10^{-3} M)	1	100
Gentisate + L-tyrosine (100 μg/ml)	351	10
Gentisate + DL-methionine (100 μg/ml)	164	59
Gentisate + Ribonucleic acid (2,000 μg/ml)	226	44
Gentisate (1.5×10^{-3} M)	178	56
Gentisate + L-tyrosine (100 μg/ml)	350	10
Gentisate + DL-methionine (1,000 μg/ml)	301	25
Gentisate + Ribonucleic acid (2,000 μg/ml)	320	20
L-tyrosine (100 μg/ml)	300	
DL-methionine (1,000 μg/ml)	301	
Ribonucleic acid (2,000 μg/ml)	317	

a few days became brownish in color, indicating the possible formation of a quinone. With such aged solutions, the inhibition of phage growth by gentisate was prevented by the following compounds: L(+)tyrosine (100 μg per ml), purified ribonucleic acid (200 μg per ml), and DL-methionine (1,000 μg per ml) (table 4).

L-tyrosine was the only compound tested that could prevent the inhibition of phage multiplication by freshly prepared solutions of sodium gentisate that contained little or no brown color (figure 3).

Studies on the growth of the host cells showed that only L(+)tyrosine could prevent the bacterial inhibition by the compound.

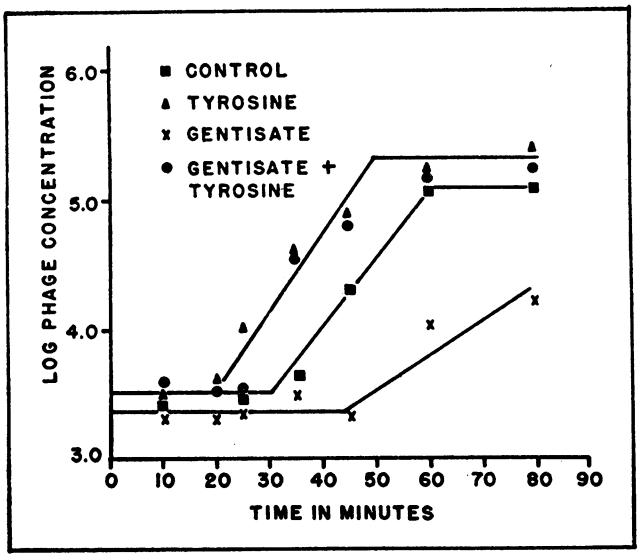

Figure 3. Graph showing inhibition of phage growth by gentisate and the protective action of L-tyrosine.

L-tyrosine, 5.5×10^{-4} M.
Sodium gentisate, 4.8×10^{-4} M.

DISCUSSION

These experiments were designed to test the possibility of using selective inhibitors of virus growth to determine the nature of essential reactions for virus multiplication. It has been shown that salicylate can delay phage multiplication in a single cycle of growth at much lower concentrations than is required to inhibit the growth of uninfected host cells. The inhibition of phage can be prevented entirely by DL-tryptophan, indole, or anthranilic acid while the inhibition of the growth of the host organisms is unaffected by these compounds. Pantothenate, which has been shown by Ivánovics and co-workers (1942, 1948) to prevent salicylate inhibition of bacterial growth, has no effect on the inhibition of the virus. These facts indicate that tryptophan or its biological precursors (Tatum *et al.*, 1944) may be essential for phage T2r+ propagation in a rather specific fashion, in confirmation of Cohen and Fowler (1947).

Although it required 100 times less gentisate to inhibit phage T2r+ multiplication than to inhibit the growth of the host cells, both inhibitions could be prevented by L-tyrosine. This indicates that L-tyrosine may be more critical for the virus system than for the host metabolism.

SUMMARY

A method for the study of the requirements of the virus system has been proposed and tested. The method calls for the determination of an activity ratio for an inhibitor, which indicates how much more of the inhibitor is required to delay the growth of the uninfected host cells than to inhibit phage multiplication.

Inhibitions of a virus-host system by salicylate and gentisate in a synthetic medium were studied as examples of the method. The activity ratio for salicylate was found to be 6 to 10. The activity ratio for gentisate was 100.

DL-tryptophan, indole, or anthranilic acid was found to prevent salicylate inhibition of phage multiplication, but not that of bacterial growth, whereas pantothenate partially prevents the host cell growth inhibition.

L-tyrosine prevented both phage and bacterial growth inhibitions by gentisate. Inhibition of phage multiplication by old solutions of gentisate was reversed also by DL-methionine and ribonucleic acid.

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