CHEMICAL STUDIES ON HOST-VIRUS INTERACTIONS*

III. Tryptophane Requirements in the Stages of Virus Multiplication in the Escherichia coli-T2 Bacteriophage System

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(Received for publication, March 18, 1947)

INTRODUCTION

In previous papers in this series (1, 2), some effects of the adsorption of the T2 and T4 bacteriophages on the multiplication and metabolism of the infected host, *Escherichia coli* B, have been presented. It was shown that the inhibition of the ability of a virus to undergo multiplication within its host, owing to the adsorption of irradiated virus went hand-in-hand with two other characteristics, namely, inhibition of the host's ability to multiply itself, and no apparent change in its respiration or R. Q. The similarity in these respects of the *E. coli*-T systems and the chorioallantoic membrane-influenza virus system was also noted.

The tryptophane analogue, 5-methyl tryptophane, inhibited the multiplication of *E. coli* B without change in the rate of oxygen consumption or R. Q. of the organisms. The establishment of these conditions by 5-methyl tryptophane resulted in the inhibition of the ability of *E. coli* B infected with T2 to synthesize virus. Thus the action of this compound simulated in these respects the interference phenomenon produced by the adsorption of irradiated, inactivated virus.

In contrast to the production of an irreversible inhibition with irradiated virus, the action of 5-methyl tryptophane (5MT) appeared to be reversible. Virus plaques appeared when 5MT was diluted and the infected bacteria were plated on nutrient agar containing tryptophane. It was considered that some knowledge of the tryptophane requirements of virus multiplication might be obtained by a study of the precise conditions of this reversible phenomenon.

Materials and Methods

The preparation and assay of bacteriophage and bacteria employed in these studies have been described previously (1, 3). The studies were made in the defined lactate-NH_4Cl medium F (1, 3). Concentrates of the bacterial virus, $T2r^+$, prepared from F medium, and con-

* The work described in this paper was assisted by the Office of Naval Research.

centrated by ultracentrifugation, were used in these experiments. T2r$^+$ and T4r$^+$ had been used in previously published studies in this series (1, 2). All reference to T2 virus in this paper refers to the r$^+$ type.

One-step growth curves were determined by the method of Delbrück and Luria (4). The detailed protocols are presented to describe the precise nature of the experiments. The ordinate "virus particles per infected bacterium" in the figures is determined as is burst size (2); *i. e.*, final titer minus initial free virus titer divided by initial titer minus initial free virus titer.

We are greatly indebted to the Winthrop Chemical Company for a generous supply of 5-methyl tryptophane (5).

EXPERIMENTAL

The Effect of Tryptophane on Virus Synthesis in F.—The simple medium, F, does not contain tryptophane. Since the 5MT inhibition was most readily interpretable as an interference with the utilization of synthesized tryptophane, it appeared desirable to know whether the addition of tryptophane to F would alter T2 synthesis on this medium. The one-step growth experiment presented in Table I yielded the results in Fig. 1. It may be seen that 5×10^{-4} M tryptophane did not appreciably affect the latent period, the distribution of time of bursting of infected cells, or the number of virus particles liberated per infected bacterium (burst size).

Of interest is the considerable inactivation of T2 appearing towards the end of the curve (3). This appears to be a common feature of virus production in F. It should be noted that the latent period in these systems was 22 to 23 minutes. This may vary from 21 to > 30 minutes, depending on the particular culture of B used, although the organisms in all the experiments were grown to approximately the same concentration under the same conditions (3).

The Addition of 5MT during the Latent Period.—In experiments described previously (2), infection was accomplished in the presence of 5MT. In the following experiment the inhibiting action of this compound was determined by the addition of 5MT to the system at different times during the latent period. It was observed that addition of 5MT up to 12 minutes in the latent period produced total inhibition of virus liberation. Table II and Fig. 2 present an experiment of this type. In the absence of the compound, the virus was liberated in 25 minutes with a burst size of 31. Addition of 5MT at 15 minutes resulted in a slow liberation of virus with a burst size of less than 4. Addition at 18 minutes still impeded virus liberation. The burst size in this instance was only 7 or less than 25 per cent of the control.

Reversal of 5MT Inhibition with Tryptophane.—Two types of experiments were attempted. The first involved the reversal of 5MT inhibition which had been established simultaneously with infection. The second type established the inhibition by 5MT during the latent period and subsequently reversed the inhibition by the addition of tryptophane.

Table III and Fig. 3 present an experiment of the first type. Control

bacteria were infected in the presence of tryptophane. They had a latent period of 25 minutes and a burst size of 24.

Bacteria were infected in the presence of 5MT and in the absence of added tryptophane. After 30 minutes, tryptophane was added and in 25 minutes

TABLE I

Schedule of One-Step Growth Experiment in the Presence of Added Tryptophane

Time	
min.	
−120	0.025 cc. of 24 hour culture of B in F (B_F) inoculated into 5 cc. F and incubated at 37° with aeration. All tubes were incubated at 37°.
−1	Assay for B. B = 6.8×10^7 per cc.
0	0.70 cc. B + 0.20 cc. T2 + 0.1 cc. 5×10^{-3} M tryptophane in F (try.) (tube A) 0.70 cc. B + 0.20 cc. T2 + 0.1 cc. F (tube β) T2 = 2.8×10^8 per cc.
5	Add 0.1 cc. tube A to 8.9 cc. F + 1.0 cc. try. (tube 5A) 0.1 cc. tube 5A + 0.8 cc. F + 0.1 cc. try. α Add 0.1 cc. tube β to 9.9 cc. F (tube 5β) 0.1 cc. tube 5β + 9.9 cc. F β Centrifuge α and β 5 min. at 4000 R.P.M.
8	Add 0.1 cc. tube 5A to 8.9 cc. F + 1.0 cc. try. I Add 0.1 cc. tube 5β to 9.9 cc. F II
10	0.1 cc. aliquots from I and II were assayed
12	Add 0.2 cc. I to 3.6 cc. F + 0.2 cc. try. III Add 0.2 cc. II to 3.8 cc. F IV
15	Assay supernatant fluids α = 1.90×10^4 free T2 per cc. β = 1.80×10^4 free T2 per cc. Assay periodically on nutrient agar.

virus was liberated with a burst size of not quite 27. Thus a 30 minute inhibition in 5MT did not interfere with the subsequent process of virus multiplication and liberation in the presence of tryptophane.

Attempted Inhibition of Virus Multiplication by 5MT in Presence of Tryptophane.—It was found that the addition of 5MT at the 12th minute during the latent period was unable to stop virus liberation in the presence of traces of

tryptophane. In one experiment, wherein the control infection had a latent period of 23 minutes and a burst size of 32 in the presence of 5×10^{-4} M tryptophane, the addition of 5MT to a final concentration of 5×10^{-4} M 5MT and

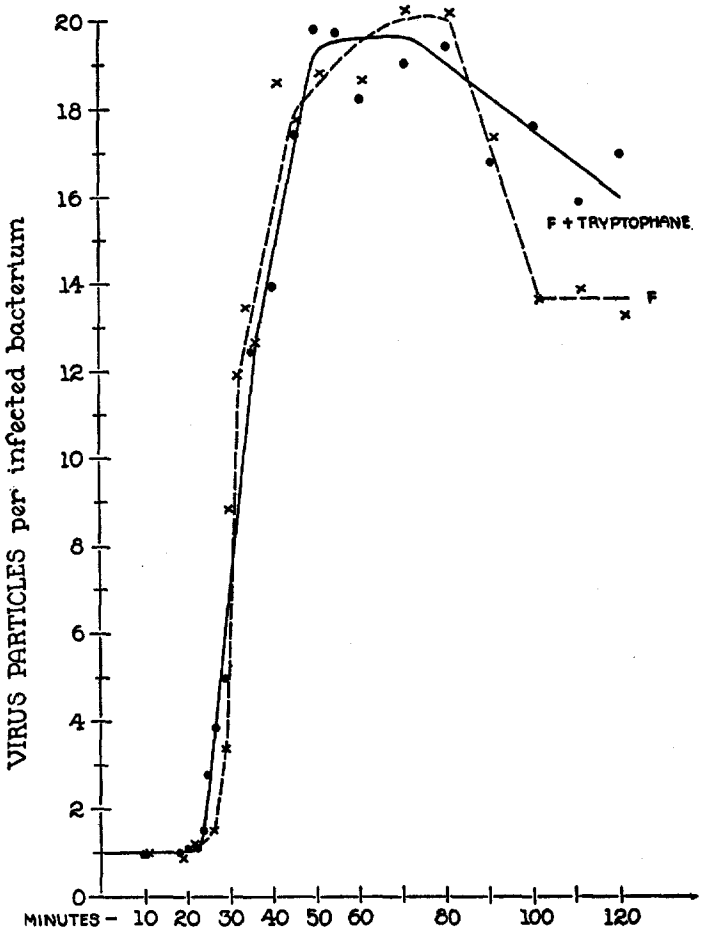

FIG. 1. The growth of T2r$^+$ in *E. coli* B in F in the presence and absence of added tryptophane.

5×10^{-6} M tryptophane did not alter the latent period nor the rate of virus liberation. The burst size, however, was reduced to 26. It is difficult to say whether this slight reduction is significant.

The Specificity of Reversal of 5MT.—It was found that tryptophane promptly reversed the inhibitory action of 5MT without an appreciable change in the latent period or other characteristics of the one-step growth curve. Further-

more, a concentration of 5×10^{-6} M tryptophane prevented the establishment of the 5MT inhibition at 5×10^{-4} M 5MT. An examination of a few compounds indicated their relative inability to duplicate these tryptophane effects.

TABLE II

Schedule of One-Step Growth Experiments on 5MT Addition during the Latent Period

Time	
min.	
−120	0.025 cc. of 24 hour culture of B_F inoculated into 5 cc. F and incubated at 37° with aeration. All tubes were incubated at 37°.
−1	Assay for B. B = 6.16×10^7 per cc.
0	0.95 cc. B + 0.05 cc. T2 at 2.5×10^8 per cc. (tube A)
5	0.1 cc. tube A + 9.9 cc. F (tube 5A) 0.1 cc. tube 5A + 9.9 cc. F. I Centrifuged 1 cc. I 5 min. at 4000 R.P.M.
7	0.2 cc. I + 3.8 cc. F II
10	Assay I
12	0.2 cc. Tube 5A + 8.9 cc. F + 1.0 cc. 5×10^{-3} M 5MT in F III
13	Assay III
15	0.1 cc. tube 5A + 8.9 cc. F + 1.0 cc. 5MT IV
16	Assay supernatant I in duplicate. Contains 4.9×10^2 free T2 per cc.
17	Assay IV
18	0.1 cc. tube 5A + 8.9 cc. F + 1.0 cc. 5MT V
19	Assay V
	Assay periodically on nutrient agar

Experiments were performed similar to that described in Table III and Fig. 3. The inhibitory action of 5×10^{-4} M 5MT was established at infection and continued for 30 minutes. In contrast to the prompt reversal by 5×10^{-4} M tryptophane at 5×10^{-6} M 5MT, 5×10^{-4} M phenylalanine or indole-3-acetic acid were unable to effect the liberation of virus.

In a system in which 5×10^{-4} M tryptophane reversed 5MT after 30 minutes with a latent period of 26 minutes and burst size of 24, 5×10^{-4} M indole-3-acetic acid was inactive. However, 5×10^{-4} M α-napthalene acetic acid

reversed 5MT but only with a latent period of *ca.* 55 minutes and a burst size of 3.4.

Resumption of an Interrupted Latent Period.—It appeared possible to test in this system whether interference with tryptophane utilization by 5MT prevented virus multiplication or merely inhibited virus liberation. The former choice was suggested by the experiment presented in Fig. 3 wherein it

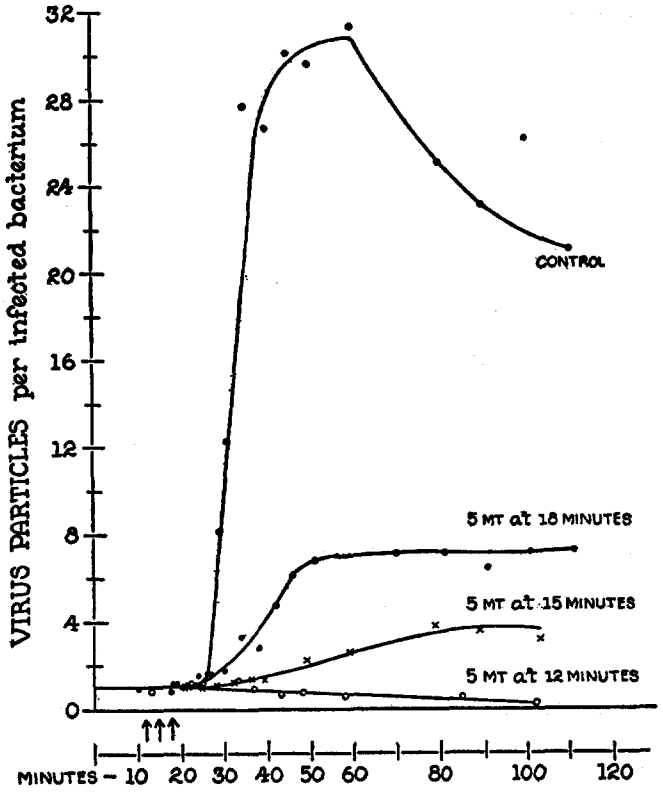

FIG. 2. The inhibition of virus production by the addition of 5MT during the latent period.

was demonstrated that infection in the presence of 5MT required a complete latent period after reversal by tryptophane. This signified that virus multiplication had been inhibited at a very early stage, since if some virus multiplication had progressed, reversal should have resulted in an appreciably shortened latent period. The possibility was not obviated, however, that the inhibitory effect of 5MT added well within the latent period might be due to an inhibition of the burst. Thus, on that hypothesis, tryptophane might be an essential for the complex processes only at the initiation of virus multiplication and during lysis. It does not appear likely that tryptophane is essential

for lysis since inhibition with 5MT at 15 and 18 minutes did not totally prevent lysis.

The most likely hypothesis was that tryptophane was essential for the processes of virus multiplication. This could be tested by establishing an

TABLE III

Schedule of One-Step Growth Experiment on Reversal of 5MT Inhibition by Tryptophane

Time		
min.		
−120	0.05 cc. of 24 hour culture of B_F inoculated into 10 cc. F and incubated at 37° with aeration.	
−1	Assay for B. B = 7.52×10^7 per cc.	
0	0.85 B + 0.05 cc. T2 + 0.1 cc. try.	A
	0.85 B + 0.05 cc. T2 + 0.1 cc. 5MT	β
5	0.1 cc. A + 8.9 cc. F + 1.0 cc. try.	5A
	0.1 cc. β + 8.9 cc. F + 1.0 cc. 5MT	5β
5.5	0.1 cc. 5A + 8.9 cc. F + 1.0 cc. try.	I
	0.1 cc. 5β + 8.9 cc. F + 1.0 cc. 5MT	II
6	Centrifuge 1 cc. of I and II at 4000 R.P.M. for 5 min.	
8	0.2 cc. I + 3.7 cc. F + 0.1 cc. try.	III
10	Assay I, II	
15	Assay supernatant fluid of	I
16	Assay supernatant fluid of	II
30	0.1 cc. 5β + 8.9 cc. F + 1 cc. try.	IV
	0.2 cc. IV + 3.7 cc. F + 0.1 cc. try.	V
	Assay periodically on nutrient agar	

inhibition with 5MT during the latent period, reversing after a suitable time with tryptophane, and examining the ensuing characteristics of the one-step growth curve.

An experiment of this type is presented in Table IV and Fig. 4. The latent period of a typical infection was interrupted at 12 minutes by the addition of 5MT. After 15 minutes of inhibition, reversal was accomplished by the addition of tryptophane and the liberation of virus was followed. In the

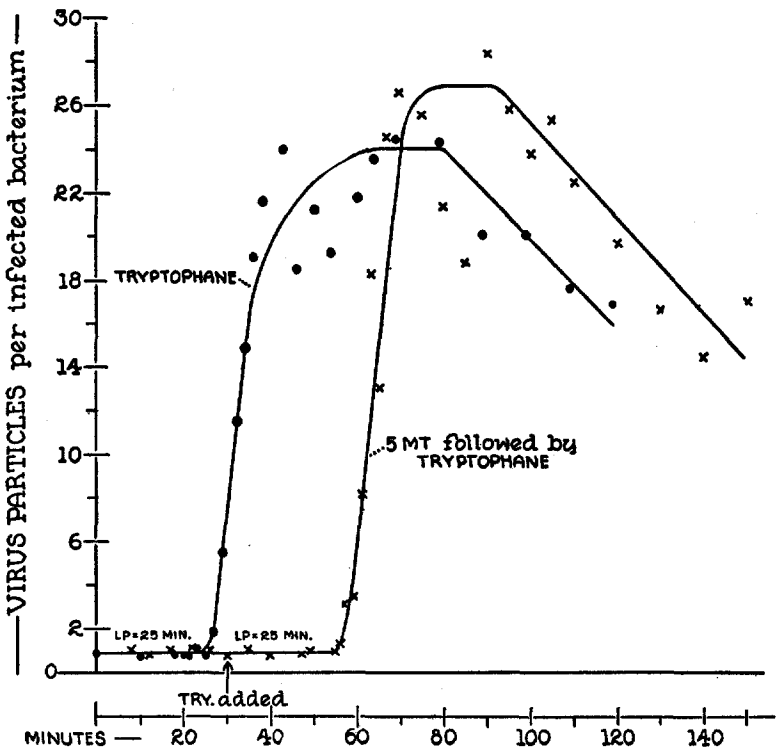

FIG. 3. The inhibition by 5MT at infection and its reversal by tryptophane. LP = latent period.

TABLE IV

Schedule of One-Step Growth Experiment on Resumption of a Latent Period Interrupted by 5MT

Time	
min.	
−120	0.025 cc. of 24 hour culture of B_F inoculated into 5 cc. F and incubated at 37° with aeration.
−1	Assay for B. B = 6.60×10^7 per cc.
0	0.95 cc. B + 0.05 cc. T2 — A
5	0.1 cc. A + 9.9 cc. F. — 5A 0.1 cc. 5A + 9.9 cc. F. — I Centrifuge 1 cc. of I at 4000 R.P.M. for 5 min.
7	0.2 cc. I + 3.8 cc. F — II
10	Assay I
12	0.1 cc. 5A + 8.9 cc. F + 1.0 cc. 5MT — III
15	Assay supernatant fluid of I
27	0.4 cc. III + 3.2 cc. F + 0.4 cc. try. — IV
	Assay periodically on nutrient agar

control infection, in the absence of 5MT and tryptophane, the latent period was 26 to 27 minutes with a burst size of *ca.* 33. In 14 to 15 minutes after reversal, or after a complete but interrupted latent period of 12 plus 15 or 27 minutes, lysis occurred with a burst size of 32. It is clear, therefore, that processes of virus multiplication requiring tryptophane were sharply stopped

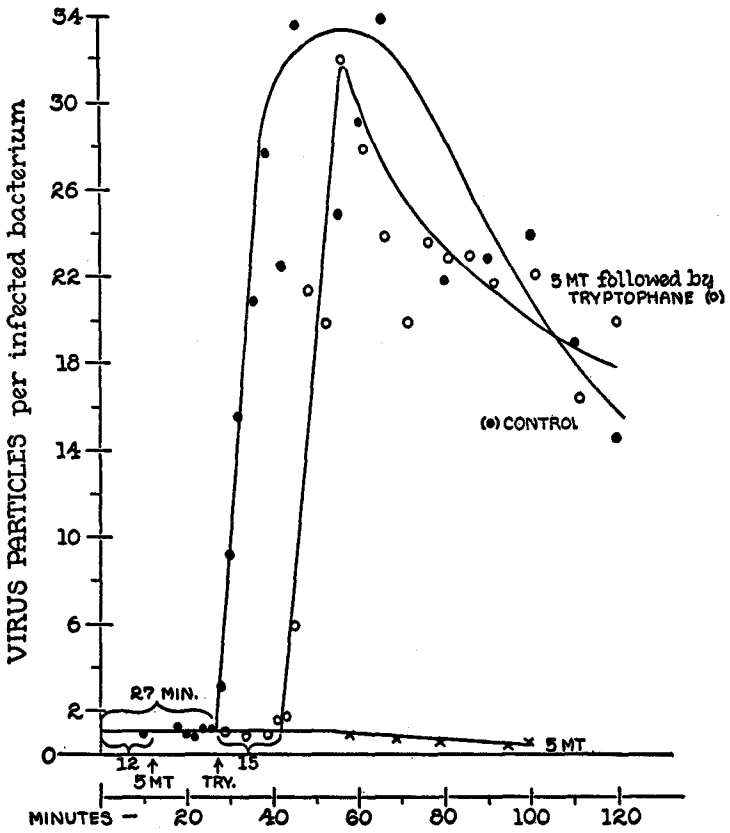

FIG. 4. The resumption of a latent period interrupted by 5MT.

at 12 minutes for a considerable period and as precisely resumed when the effects of 5MT were overcome with tryptophane.

The "Killing" Effect of 5MT on Infected Bacteria.—In Fig. 4 is presented a curve in which infected bacteria are held in the presence of inhibiting concentrations of 5MT for the entire course of the experiment. It may be seen that after *ca.* 55 minutes the number of assayable plaques falls, eventually to the number of free virus particles. This irreversible inactivation has been noted previously (2). The irreversible inactivation will be termed a "killing" effect since neither bound virus nor infected bacterium can go on to reproduce virus

or bacteria. Unpublished data obtained in this laboratory have suggested also the progressive loss of the ability to consume O_2.

In numerous experiments, killing of the *E. coli*-T2 system by 5MT has proven to be quite variable. In several experiments, killing did not occur

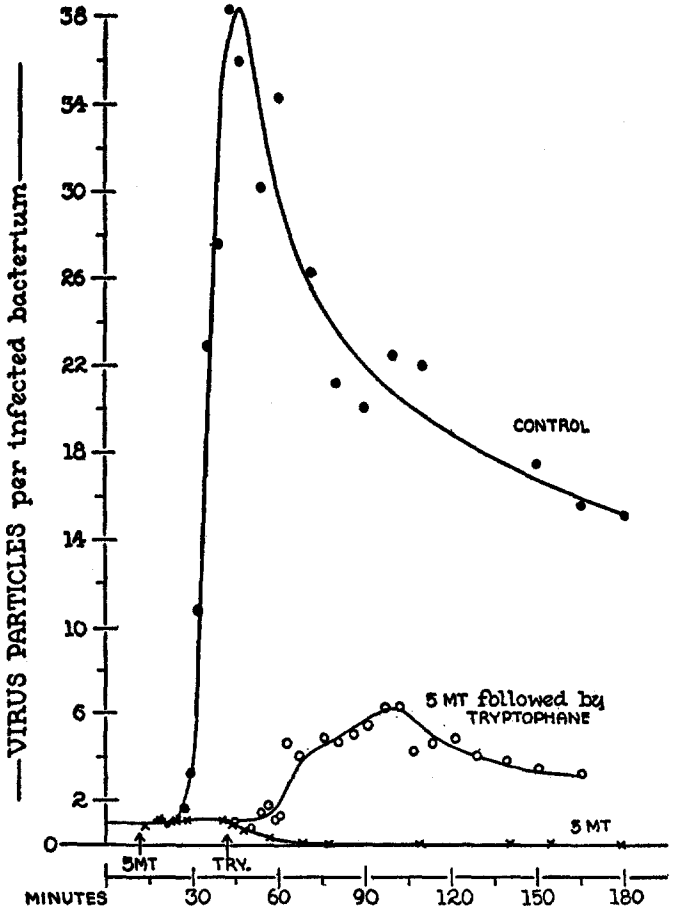

FIG. 5. The reversal of 5MT inhibition during the killing effect.

over a 2 hour period. In most instances, however, the killing effect began at *ca.* 50 to 70 minutes after infection. Thus inhibition or interruption of virus multiplication for too long a period killed the infected bacterium.[1]

If reversal of 5MT by tryptophane was accomplished so that the end of the

[1] The inactivation curve of B-T2 presented in Fig. 3 of the second paper of this series (2) should be redrawn in the light of these data. Killing did not appear in that experiment until after 40 minutes.

latent period arrived during the killing effect, the time of the latent period was unchanged but the burst size was considerably reduced and the liberation of virus occurred over a considerable period. An experiment of this type

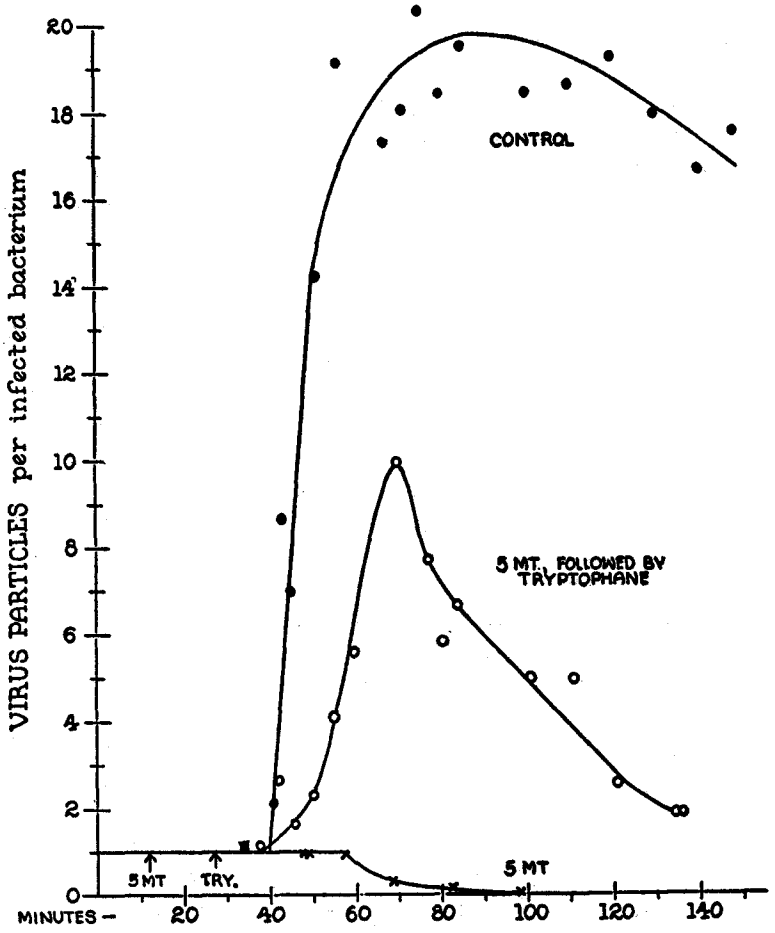

FIG. 6. Reversal of 5MT inhibition with abnormal bacteria; the resumption of a latent period interrupted by 5MT.

is presented in Fig. 5. The schedule of the experiment was identical with that of Table IV except that addition of tryptophane to reverse inhibition occurred at 42 minutes.

5MT-Tryptophane Relations in Abnormal Bacteria.—It has been noted that organisms which have an abnormally slow multiplication time in F, may have an abnormally long latent period after infection. In one such case, presented in Fig. 6, it was noted that the control had a latent period of 40 minutes. The

schedule in Table IV applies to this experiment. Interruption of the latent period at 12 minutes with 5MT, and reversal of the inhibiton at 27 minutes resulted in a burst at 38 minutes. Thus under these conditions the organisms had a total latent period of 23 minutes after subtraction of the 15 minute period of inhibition in contrast to the 40 minute latent period of the control.

It seems reasonable to suppose that the culture which was used for this experiment was not synthesizing tryptophane at the normal rate for division and virus synthesis. The addition of tryptophane to this infected culture therefore significantly reduced the period of multiplication to a more nearly normal value. It would appear that tryptophane synthesis is a critical point in the normal functioning of *E. coli* in F.

DISCUSSION

Some bacteriostatic agents tested as metabolite antagonists may be inhibited in their action by several naturally occurring metabolites. However, there are several reasons to believe that the 5MT effects herein described are due to specific interference with stages of tryptophane metabolism or utilization. In the first place these effects may be overcome specifically by tryptophane. Thienyl alanine, which is bacteriostatic for *E. coli*, may have its toxicity overcome by either phenylalanine or tryptophane to about the same degree of effectiveness per mol of added metabolite (6). In the *E. coli*-T2 system, the reversal of 5MT inhibition did not occur with phenylalanine at concentrations far greater than those at which tryptophane readily overcame 5MT action.

The time relationships and burst size are also important criteria of specificity. Tryptophane shows no lag period or diminution of burst size in overcoming 5MT action. This is in marked contrast to α-naphthalene acetic acid which has a slight activity in overcoming 5MT action, requiring a very long latent period resulting in the production of little virus. A slow conversion of α-naphthalene acetic acid to tryptophane may explain this effect. Surprisingly, indole-3-acetic acid was totally inactive, thereby indicating that this compound was not converted to tryptophane under these conditions.

Therefore, 5MT appears to be specific in blocking the utilization of tryptophane in virus synthesis. The tryptophane requirement is manifested in all stages of reproduction throughout the latent period. However, the tryptophane requirement does not appear to be essential to the adsorption (7) or the liberation of T2. Examination of the systems (Fig. 2) to which 5MT was added late in the latent period indicates that lysis may occur even when tryptophane utilization is inhibited, when enough virus has been synthesized within the organism to start the complex events leading to lysis. Although lysis does not appear to be inhibited, it is possible that some infected bacteria are completely inhibited with respect to proceeding to lysis while others are not. The apparently decreased burst size would then represent the average of both

inhibited and uninhibited infected cells although the amount of virus actually liberated per cell might be in the normal range. In the inhibited systems, the distribution of the time of lysis was much broader than in uninhibited systems, suggesting that the initiation of lysis at least was somewhat repressed.

It is apparent from Fig. 1 that normally the tryptophane requirement is completely supplied by the synthetic mechanisms of the host. The amount of tryptophane available within the cell at any one time is minute, since a small amount of added tryptophane serves to prevent the establishment of 5MT inhibition, whereas this may be established with ease in the absence of added tryptophane.

Tryptophane may be defined in this system as a synthesized metabolite essential for virus multiplication, which need not be present as a component of the host's external environment. Nevertheless, it must be present in the environment subject to organization by the virus. The detection of essential metabolites of this type which need not be added to the medium and which are synthesized in amounts optimal for virus multiplication may be accomplished in general by the method described in this paper. Thus requirements for phenylalanine, glycine, etc., may be studied in this system by the use of the appropriate metabolite antagonist.

The same basic approach would appear applicable to other viruses grown under controllable conditions. There is no reason to believe that influenza virus, for instance, will grow only on chorioallantoic membranes immersed in media which contain all the compounds utilized during reproduction of influenza virus. Compounds essential to the synthesis of influenza virus may be synthesized by the membrane, in the absence of the compound in the medium. The technique described in this paper could be applied in this way to the discovery of these requirements in virus reproduction on hosts in defined media.

The question naturally arises as to the specific nature of the tryptophane requirement in the synthesis of T2. It has been shown that T2 contains tryptophane (1). It is probable that the enzymes involved cannot synthesize virus peptides containing 5MT. It has also been observed that 5MT completely blocks the stimulated synthesis of desoxyribose nucleic acid that occurs during infection of B by T2 (8). This inhibitory action of 5MT is explicable as being due to the inhibition of the synthesis of virus peptides essential for the reception and organization of virus nucleotides.

Finally, the "killing" effect is of interest from the point of view of the chemotherapy of virus diseases because, if the effect described here is found in animal virus systems, it should be possible to inactivate a virus irreversibly merely by inhibiting its multiplication for a suitable period while it is bound to a host cell. Since it may be expected that free virus in the blood, for instance, would be rapidly cleared by cellular receptors, the treatment of a virus disease may be

considered in part as treatment of virus-infected tissue rather than of free virus. The "killing" effect could become a major attack upon a given virus disease, insofar as the virus-infected tissue might be susceptible to this effect. However, it is clear that the use of this principle does not permit the survival of the infected cell, at least in these systems.

SUMMARY

The inhibition of virus multiplication by 5-methyl tryptophane can be specifically reversed by tryptophane. The conditions of reversal indicate that 5MT specifically interferes with tryptophane utilization. The tryptophane requirements of virus multiplication appear to exist throughout the latent period and determine the time of lysis and amount of virus liberated. A latent period may be interrupted for 15 minutes or more and be resumed on addition of tryptophane. Extended inhibition with 5MT results in a somewhat variable "killing" effect, the extent of which determines aspects of the reversal of the inhibition by tryptophane. The implications of these phenomena have been discussed.

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