*INHIBITION OF DEOXYRIBONUCLEIC ACID-DIRECTED RIBONUCLEIC ACID POLYMERASE IN ESCHERICHIA COLI AFTER INFECTION WITH BACTERIOPHAGE T4**

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Astrachan and Volkin[1] have observed that a metabolically labile form of RNA[2] with a base composition comparable to that of the invading phage DNA is formed early in phage infection. At least from a quantitative point of view, the turnover of this RNA (messenger RNA)[3–5] decreases with increasing time after infection.[1] According to current concepts,[3–5] the production of messenger RNA and hence phage-specific proteins should be directly related to the activity of DNA-directed RNA polymerase.[6–10] One such group of phage-specific proteins are the "early enzymes,"[11] which are related to the formation and incorporation of 5-hydroxymethyl deoxycytidylate[12] into phage DNA. These enzymes appear shortly after phage infection but their production abruptly ceases at about 12–15 min.[13, 14]

We have found that under the conditions of our assay the level of the activity of DNA-directed RNA polymerase in *E. coli* B decreases markedly during the first 10–12 min after infection with T4 bacteriophage. A relatively heat-stable substance is produced that can effectively inhibit purified RNA polymerase of uninfected cells. The production of the inhibitor is arrested by the addition of chloramphenicol to cells shortly after their infection with bacteriophage. In a corresponding although not similar situation Franklin and Baltimore[15] have previously reported the lowering of the DNA-directed RNA polymerase activity of L-cell nuclei following infection with the RNA-containing mengovirus.

Materials and Methods.—The bacteriophage T4 and its mutant *am* N82[16, 17] were grown in *E. coli* B and *E. coli* CR 63, respectively. After complete lysis the debris was removed by centrifugation for 30 min at 5000 × *g*. The bacteriophage were sedimented by centrifugation at 20,000 × *g* for one hour at 0°C; the pellets were covered with buffer solution (0.001 *M* potassium phosphate, pH 7.0, 0.001 *M* $MgCl_2$, gelatin, 100 mg/l) for several days and finally suspended by stirring. Bacteriophage DNA was prepared by phenol extraction as described by Davison and Freifelder.[18, 19]

C^{14}-labeled ATP and CTP were purchased from Schwarz BioResearch, Inc. and the unlabeled nucleoside triphosphates, ATP, CTP, GTP, and UTP from Pabst Laboratories, Milwaukee, Wisconsin. The preparation of C^{14}-labeled dHMP has been described.[17] Chloramphenicol and streptomycin sulfate were obtained from Parke, Davis and Company and E. R. Squibb and Sons, respectively. Sephadex G-25 was obtained from Pharmacia, Uppsala, Sweden.

Preparation of extracts from infected bacteria: Cells of *E. coli* B were grown in the medium of Fraser and Jerrel[21] to a concentration of 10^9 per ml and were then infected with T4 or T4 *am* N82 at the desired multiplicity. In some instances 4 phage per bacterium were added, and 4 min later, the same amount of phage was added again, to give a final multiplicity of 8. At various intervals after infection, a 400-ml aliquot of cell suspension, containing about 5 gm of cells, was siphoned off into 400 ml of frozen, crushed Fraser and Jerrel medium, and was then centrifuged at 5000 × *g* for 40 min in a refrigerated Stock centrifuge. The packed cells from each sample were frozen after the addition of 5 ml of buffer A (0.01 *M* Tris buffer, pH 7.9, 0.01 *M* $MgCl_2$, and 0.0001 *M* EDTA), and then ruptured in a Hughes press.[22] The pressed material was weighed, thawed, and centrifuged at about 20,000 × *g* for 30 min at 0°C. The ribosomes were then sedimented by further centrifugation for 120 min at 34,000 rpm in the size 40 head of the model L Spinco preparative centrifuge, and the supernatant fluid was carefully removed. A 14.3 *M* solution of 2-

mercaptoethanol was added to the supernatant fluid to give a concentration of 0.01 *M*, and 0.05 ml of a 10% solution of streptomycin sulfate (w/v) was added for each ml of supernatant fluid to remove nucleic acids. After standing 15 min at 0°C, the solution was centrifuged for 30 min at 20,000 × *g*. The supernatant fluid was used in the assays for RNA polymerase and dHMP kinase after an aliquot had been removed for a protein determination by the method of Lowry *et al.*[23]

Purified RNA polymerase from uninfected cells: The enzyme was purified from *E. coli* B according to the procedure described by Chamberlin and Berg.[10] The most active fractions from the DEAE-cellulose chromatography of the enzyme in the final purification step were stored at 0°C until used. This enzyme fraction will be referred to as "highly purified" enzyme to differentiate it from RNA polymerase of extracts that had been treated with streptomycin sulfate to remove nucleic acids. This latter enzyme preparation will be designated as "partially purified" RNA polymerase.

Enzyme assays: The assay for dHMP kinase has been described earlier.[17] The standard assay for RNA polymerase was essentially that of Chamberlin and Berg[10] except that a Nuclear-Chicago scintillation counter was used for counting the radioactive samples. The reaction mixture contained 10 μmoles of Tris-Cl buffer, pH 7.9, 0.25 μmole of $MnCl_2$, 1.0 μmole of $MgCl_2$, 100 mμmoles each of ATP, CTP, GTP, and UTP, 70–340 mμmoles of bacteriophage DNA (expressed as nucleotide phosphorus and equivalent to 0.4–1.9 optical density units at 260 mμ), 3.0 μmoles of β-mercaptoethanol and enzyme in a volume of 0.25 ml. ATP or CTP contained C^{14}, approximately 400–700 cpm per mμmole. If necessary, the enzyme was diluted with a solution containing 0.01 *M* $MgCl_2$, 0.01 *M* β-mercaptoethanol, 5×10^{-5} *M* EDTA, and 1 mg per ml of crystalline bovine serum albumin. Incubation was at 37°C and for 10 min unless otherwise indicated.

Results.—Studies on the virus-induced inhibition of E. coli RNA polymerase with time after infection: Figures 1*A* and 1*B* demonstrate the changes in RNA polymerase activity in *E. coli* B as a function of time after infection with wild-type T4 at a multiplicity of 2 and with *am* 82 T4 at a multiplicity of 8, respectively. The RNA polymerase activity, as measured by the incorporation of C^{14}-ATP into polymer form, decreases rapidly in the partially purified extracts to 15–20 per cent of the preinfection activity within 12 min after infection. Parallel assays for dHMP kinase activity, one of the "early enzymes" of T-even phage infection, indicated a normal progress of virus infection. In experiments not reported in Figure 1, the same rapid decrease in polymerase activity was seen if crude bacterial extracts without streptomycin treatment were used in the assays.

In order to identify further the enzyme under study as the DNA-directed RNA polymerase, an experiment similar to that described in Figure 1*A* and *B* was performed with C^{14}-CTP instead of C^{14}-ATP. Again a sharp decrease in RNA polymerase activity to about 10 per cent of the preinfection activity was reached by 12 min after infection (Fig. 2, curve *A*). The very low enzyme activities of the

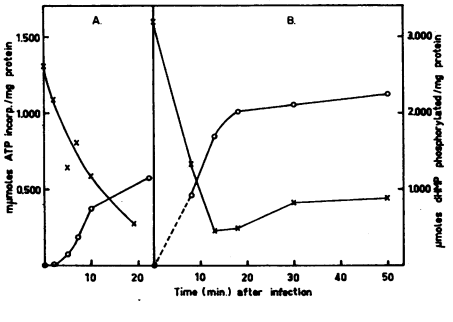

FIG. 1.—RNA polymerase activity of *E. coli* B after phage infection as measured by the incorporation of C^{14}-labeled ATP into RNA. (*A*) Infection with wild-type T4 at a multiplicity of 2. 0.75 A_{260} unit per assay of T4-phage DNA was used as template. (*B*) Infection with T4 *am* N82 at a multiplicity of 8. 0.42 A_{260} unit per assay of T4 *am* N82 DNA was used as template. The zero-time samples were withdrawn from the bacterial cultures before the phage infection. ×—× represents mμmoles of ATP incorporated per mg of protein. ○—○ represents μmoles of dHMP phosphorylated per mg of protein.

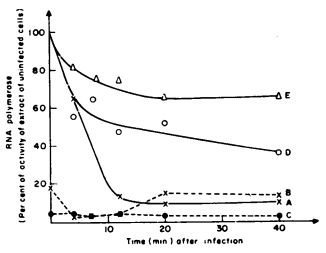

FIG. 2.—RNA polymerase activity of *E. coli* B after phage infection as measured by incorporation of C^{14}-labeled CTP into RNA; effect of chloramphenicol on the production of the inhibitor of RNA polymerase by *E. coli* B infected with phage T4. *Curve A:* RNA polymerase in cells infected with wild-type T4 at a multiplicity of 8. *Curve B:* Conditions were the same as for curve *A* but the assays of RNA polymerase were carried out in the absence of added template DNA. *Curve C:* Conditions were the same as for curve *A* but the assays of RNA polymerase were carried out in the absence of ATP, GTP, and UTP. *Curve D:* Conditions were the same as for curve *A* except that chloramphenicol was added to the cell culture at a concentration of 60 μg/ml 3 min after infection with T4. *Curve E:* Conditions were the same as for curve *A* except that chloramphenicol was added to the cell culture at a concentration of 80 μg/ml 1 min after infection with T4. Standard assay conditions were employed. The assays represented by curves *A*, *D*, and *E* contained per assay 0.87, 0.95, and 0.95 A_{260} units, respectively, of T4 phage DNA. 100% activity is equivalent to the incorporation of 0.54, 1.46, and 0.87 mμmoles of C^{14}-CTP into RNA per mg of protein in curves *A*, *D*, and *E*, respectively.

control assays without added DNA (Fig. 2, curve *B*) indicate the DNA-dependence of the RNA polymerase activity studied. Furthermore, omission of ATP, GTP, and UTP greatly reduced the incorporation of the fourth radioactive nucleoside triphosphate (CTP) into RNA by extracts from infected cells (Fig. 2, curve *C*).

The incorporation of C^{14}-ATP into insoluble polynucleotides by other enzymes known to decrease after infection of cells by T4 phage infection is avoided under the conditions of the assay.[24] For example, the ATP-polymerizing enzyme reported by August *et al.*[25] is bound to ribosomes, and this fraction has been removed from our extracts. The activity of the DNA-dependent ATP-polymerizing enzyme reported by Chamberlin and Berg[10] is severely inhibited by the presence of the other nucleoside triphosphates, the presence of which is necessary for the activities measured here.

Characterization of the phage-induced enzyme inhibition: There are several explanations that could account for the observed decrease in the activity of the DNA-directed RNA polymerase after phage infection. These possibilities include the phage-induced formation of: (*a*) a ribonuclease, which could have digested the labeled product in the assay; (*b*) a deoxyribonuclease, which could have destroyed the template DNA; (*c*) phosphatases, which could have degraded the nucleoside triphosphate substrates; (*d*) an inhibitor of the RNA polymerase activity itself. To distinguish among these possibilities a series of experiments was performed, where the DNA-directed RNA polymerase was assayed in various mixtures of extracts from uninfected and phage-infected cells.

In order to determine whether the low RNA polymerase activity in extracts of infected cells (vessels 3 and 4 of Table 1) resulted from a depletion of DNA by degradative enzymes, the amount of DNA template of the assay system was doubled (vessel 5). This increase in the DNA template level had little or no effect on the enzyme activity.

In vessels 6 and 7, Table 1, two different amounts of RNA polymerase in extracts of uninfected cells were incubated with DNA template and nucleoside triphosphate substrates for 2 min prior to addition of the extract of infected cells. The incubation was then carried out for another 8 min. Conversely, in vessels 8 and 9 the components of the assay system were first incubated with extract of infected cells for 2 min at which time RNA polymerase in extracts of uninfected cells was added

TABLE 1

EFFECT OF THE ORDER OF ADDITION OF COMPONENTS OF REACTION ON THE INHIBITION OF RNA POLYMERASE BY EXTRACTS FROM T4-INFECTED *E. coli* B

Vessel	*E. coli* B extract, mg protein added	Infected *E. coli* B extract, mg protein added	CTP incorporated, mμmoles
1	0.54	—	0.24
2	1.08	—	0.49
3	—	0.46	0.04
4	—	0.92	0.09
5	—	0.92*	0.10
6†	0.54 (preliminary incubation, 2 min)	0.46	0.26
7†	1.08 (preliminary incubation, 2 min)	0.46	0.57
8‡	0.54	0.46 (preliminary incubation, 2 min)	0.13
9‡	1.08	0.46 (preliminary incubation, 2 min)	0.26

0.97 A_{260} unit per assay of T4-phage DNA was used as template DNA.
* The concentration of the DNA template of vessel 5 was doubled.
† The components of the assay system were incubated for 2 min with extract of *E. coli* B; extract of infected *E. coli* B was then added, and the incubation was continued for another 8 min.
‡ The components of the assay system were incubated for 2 min with extract of infected *E. coli* B; extract of *E. coli* B was then added, and the incubation was continued for another 8 min.

at two levels and the incubation was continued for 8 min. In all instances the amount of radioactive nucleoside triphosphate incorporated into polymer form was directly proportional to the amount of enzyme under a given set of conditions. In addition, the RNA polymerase activity of vessels 6 and 7 in which the extracts of the infected and uninfected cells were incubated together was approximately equal to that for the sum of the extracts incubated separately. These experiments seem to rule out the involvement of phage-induced ribonucleases or phosphatases that would have interfered with the polymerase assay by dissipation of the product or the substrates, respectively.

On the contrary, these data suggest that the inhibition results from the interaction of RNA polymerase with an inhibitory substance produced as a result of phage infection. The experiments in Table 1, as well as those reported in Figure 3, also show that demonstration of the action of the inhibitor depends on the order of interaction of RNA polymerase of uninfected cells with the DNA template and the inhibitory substance of infected cells (hereafter referred to simply as "inhibitor"). The three possible combinations of enzyme, inhibitor, and DNA template have been studied. (1) When partially purified RNA polymerase (see *Materials and Methods*) was incubated with DNA template and the nucleoside triphosphates prior to addition of the inhibitor (vessels 6 and 7, Table 1), there was no evidence of the action of an inhibitory substance. (2) When inhibitor was first incubated with DNA template and the nucleoside triphosphates prior to addition of the partially purified RNA polymerase, substantial inhibition was observed (vessels 8 and 9, Table 1). This experiment was then repeated with the slight modification that varying amounts of the inhibitory extract of infected cells were first mixed with a constant amount of DNA template and nucleoside triphosphates, and the highly purified preparation of RNA polymerase was then added. Again substantial inhibition of the RNA polymerase activity was observed (curve *C*, Fig. 3). As a control varying amounts of uninfected extract were mixed with DNA template and the nucleoside triphosphates prior to addition of the highly purified enzyme. No inhibition of the enzyme activity was noted (curve *A*,

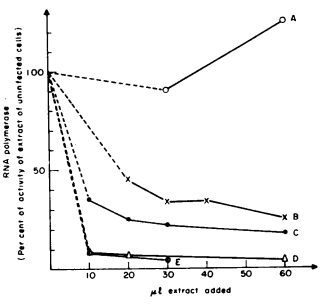

FIG. 3.—Effect of heating of extracts of infected cells on inhibitory activity; effect of the order of addition of components of reaction on inhibition of highly purified RNA polymerase and noninhibitory activity of extracts from uninfected cells. *Curve A:* Nucleoside triphosphates, template DNA, and indicated amounts of extract from uninfected *E. coli* B were incubated at 37°C for 2 min. 0.04 ml of highly purified RNA polymerase was added and the incubation carried out for another 18 min. *Curve B:* Same conditions as for curve *A* except that preliminary incubation was performed with the indicated amount of heat-treated extract of *E. coli* B harvested 12 min after infection with T4 phage at a multiplicity of 8. Extract had been heated for 10 min on a boiling waterbath and then cooled prior to its addition to the assay system. *Curve C:* Same conditions as for curve *B* except that extract from infected cells was not heat-treated. *Curve D:* Nucleoside triphosphates, 0.04 ml of highly purified RNA polymerase and the indicated amounts of an extract of *E. coli* cells that had been infected for 12 min with wild-type T4 at a multiplicity of 8, were incubated at 37°C for 2 min. Template DNA was then added and incubation carried out for another 18 min. *Curve E:* Same conditions as for curve *D* except that half the amount of template DNA was used in the assays. As template 1.93 A_{260} units of phage T4 DNA were used in the assays of curves *A–D*. In curve *E*, 0.97 A_{260} units of the same DNA was used. Standard assay conditions were employed except that the final volume was 0.32 ml. 100% activity is equivalent to the incorporation of 0.49 mμmole of C^{14}-CTP into RNA during a 20-min incubation in the presence of 0.04 ml of highly purified RNA polymerase.

Fig. 3). (3) Finally, when highly purified RNA polymerase was incubated with varying amounts of inhibitor and the nucleoside triphosphates for a period of two minutes prior to addition of DNA template, a severe inhibition of the RNA polymerase activity was observed even at the lowest level of extract of cells that had been infected for 12 min (compare curves *E* and *D* to curve *C* in Fig. 3).

The results of these three sets of experiments fortify the conclusion that the observed decrease in RNA polymerase activity results from the phage-induced formation of an enzyme inhibitor. The inhibitor seems to be abundant in extracts from phage-infected cells and can be assayed with purified RNA polymerase. The observed effect of the order of addition of enzyme, template, and inhibitor is interpreted to mean that the binding of template DNA to RNA polymerase protects the enzyme from inhibition.

Effect of chloramphenicol on production of the inhibitor: Chloramphenicol, a well-established inhibitor of protein synthesis in bacteria,[26] was used in the experiments described in Figure 2 to determine whether protein synthesis after phage infection is required for production of the RNA polymerase inhibitor. Extracts from three cultures of T4-infected *E. coli* B were compared regarding the changes in RNA polymerase activity as a function of time after infection. Chloramphenicol (60 μg/ml final conc.) was added 3 min after infection in one culture and 1 min after infection in another (80 μg/ml final conc.). The presence of chloramphenicol resulted in a cessation of the process that ultimately is reflected in the inhibition of RNA polymerase. Our present interpretation of these experiments is that chloramphenicol, by its interference with protein synthesis, prevents the formation of protein material, which is either the inhibitor or catalyzes the production of the inhibitor.

In an experiment not reported in Figure 2, the extract from T4-infected, chloramphenicol-treated (80 μg/ml, 1 min after infection) cells, harvested 12 min after infection, was tested for its ability to inhibit RNA polymerase. Highly purified polymerase was used, and other experimental conditions were similar to those

illustrated in Figure 3, curve *C*. No inhibitory effect was detected under conditions, which with an extract from phage-infected cells not treated with chloramphenicol gave an inhibition of 90–95%. Therefore, we have concluded that in a normal infection an inhibitory substance is produced that in effect combines with or "titrates" the enzyme, RNA polymerase. At about 12 min after infection the combination of enzyme with inhibitor is complete, and there is excess inhibitor remaining that can react further with active RNA polymerase if it is provided from another source. In the presence of chloramphenicol only a limited amount of inhibitor is produced (during the first minute of infection), in fact not enough to react with all of the RNA polymerase of the infected cells.

Physical properties of the inhibitor: Experiments reported in Figure 3 also relate to the relative stability of the inhibitor to heating. Curve *C* shows the extent of inhibition of highly purified RNA polymerase by graded amounts of extracts from phage-infected cells. Curve *B* illustrates identical experiments but the extract from infected cells had been heated at 100°C for 10 min. The assays were performed by incubation of the triphosphates, template DNA, and the inhibitory extract together for 2 min before the highly purified polymerase was added. Although comparisons between curves *B* and *C* are only semiquantitative, they indicate that some of the inhibiting material, at least, can survive heat treatment at 100°C for 10 min.

In an attempt to obtain information about the approximate molecular size of the inhibitor, an extract from T4-phage-infected cells, harvested 12 min after infection, was passed through a Sephadex G-25 column. The fractions collected were assayed for inhibitory activity with highly purified RNA polymerase. The inhibitory activity was found in the fractions collected just after the void volume was eluted from the column. This pattern of elution indicates that the inhibitor, or the inhibitor complex, had a minimum molecular weight of approximately 5,000.

Discussion.—The data presented in the foregoing section have described the inhibition of the DNA-directed RNA polymerase of *Escherichia coli* B that results from infection of the cells with bacteriophage T4. The extent of the inhibition increases with time until at 12 min after infection the level of RNA polymerase activity may be only 10 per cent of that of the uninfected cell.

Preliminary studies have been made on the nature of the inhibitory material. From the evidence presented it seems difficult to attribute the inhibition of the RNA polymerase activity observed after phage infection to factors that would dissipate the template, products, or substrates of the assay system. On the contrary, the evidence points to the production of a specific inhibitor in excess of that needed to inactivate the RNA polymerase of the infected cell. Since the production of the inhibitory substance is arrested by the presence of chloramphenicol, a phage-induced protein (or enzyme) must be involved either directly or by its ability to catalyze the formation of the inhibitor.

The inhibitor is probably of macromolecular proportions and at least to some degree can withstand heating at 100°C. Of the macromolecular compounds that have been studied, Tissières *et al.*[27] have demonstrated that certain ribonucleic acids can inhibit DNA-directed RNA polymerase of *E. coli* and that the degree of

inhibition is dependent on the order of addition of RNA and DNA template to the enzyme.

In our experiments we have also shown that a relationship exists between the inhibitor and DNA template and that prior reaction of the enzyme with template protects the enzyme from loss of activity in the presence of the inhibitor. In all of the studies reported herein the DNA template used in the assay was obtained from T4 bacteriophage by the method of phenol extraction. An extensive study of the relationship of the source of the DNA template used in the assay of RNA polymerase to the activity of the enzyme after phage infection is required.

Franklin and Baltimore[15] have reported that when L cells are infected with the RNA-containing mengovirus there is a relatively rapid loss in DNA-directed RNA polymerase activity. Since the disappearance of this enzyme activity is prevented when L cells are incubated with puromycin shortly after infection, they likewise have concluded that during a normal infection an inhibitor of protein nature is formed.

The present communication, however, reports a loss of a DNA-directed RNA polymerase as a result of infection by a DNA phage and the first demonstration of the direct inhibitory effect of extracts from infected cells on the activity of purified DNA-directed RNA polymerase from uninfected cells. The question of the physiological significance of the production of the RNA polymerase inhibitor and its relationship to the regulation of the sequential steps of phase production naturally arises. Three phenomena occurring after phage infection are specifically considered; first, the immediate cessation of bacterial protein synthesis as expressed by the cessation of the induced synthesis of β-galactosidase;[28, 29] secondly, the gradual decrease in the turnover of "messenger RNA" during the first 15 min after infection; and thirdly, the characteristic shut-off of synthesis of "early enzymes" at approximately 12–15 min after infection. It is unlikely that the shut-off of the bacterial protein synthesis results from appearance of this RNA polymerase inhibitor, unless the synthesizing system for bacterial protein is unusually sensitive to the small amount of inhibitor formed within the first minute or so.

However, the decrease in RNA turnover and cessation of "early enzyme" synthesis could well be related to the almost complete inhibition of RNA polymerase by the first 12 min after infection. This drastic decrease in RNA polymerase activity could result in a marked decrease in messenger RNA synthesis and subsequently in the abrupt halt of "early enzyme" synthesis which occurs at 12–15 min after infection. However, a mechanism must exist for the production of virus protein in relatively large quantity during the latter stages of infection. Quite possibly either another RNA polymerase is formed after phage infection that has a different template specificity than the RNA polymerase of uninfected *E. coli* cells, or the phage DNA formed during replication is somehow different in its function as template and can direct RNA polymerase activity under conditions where template extracted from mature T4 cannot.

Note added in proof: We should like to acknowledge a paper by R. B. Khesin, M. F. Shemyakin, Zh. M. Gorlenko, S. L. Bogdanova, and T. P. Afanas'eva [*Biokhimiya*, **27**, 1092 (1962)] that came to our attention after submission of our manuscript. Their paper entitled "RNA polymerase in *Escherichia coli* B infected with T2 phage" describes the inhibition of RNA polymerase of *E. coli* following ienfction with T2 bacteriophage.

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[2] The following abbreviations are used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; dCMP, deoxycytidine-5′ monophosphate; ATP, CTP, GTP, and UTP, the 5′ triphosphates of adenosine, cytidine, guanosine, and uridine, respectively; dHMP, deoxy-5-hydroxymethyl cytidine-5′ phosphate; Tris, tris (hydroxymethyl) aminomethane.

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