Single-stranded DNA from the Kilham Rat Virus

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SUMMARY

The nucleic acid of the Kilham rat virus was found to be single-stranded DNA. This was demonstrated by the lack of a distinct melting curve of the viral DNA, lack of reaction of actinomycin D with viral DNA, and the reaction of both intact virus and viral DNA with formaldehyde. The hydroxymethyl groups, added by the formaldehyde, did not interfere with complete recovery of the secondary structure of the extracted DNA.

Electron micrographs of the DNA also showed pooling and sharp folds and turns which are characteristics of single-stranded DNA.

When rat virus was centrifuged to equilibrium in cesium chloride density gradients, three peaks of haemagglutinating activity were found at densities of 1.43, 1.38 and 1.32 g./cm.3. The examination of material from these peaks showed the peak at 1.43 g./cm.3 contained full viral particles, the peak at 1.38 g./cm.3 contained a mixture of full and empty viral particles, and the peak at 1.32 g./cm.3 contained large amounts of debris in which an occasional viral particle was visible. It was concluded from these data that the intact viral particle had a density of 1.43 g./cm.3, and the protein coat had a density of 1.32 g./cm.3. The density of the DNA was found to be 1.72 g./cm.3; therefore, the intact viral particle contained 34% DNA.

The average length of the DNA molecule extracted from the rat virus was found to be 1.30 μm., which gave a molecular weight of $1.2 \times 10^6$ for the DNA. With this value and the 34% value found for the DNA content of the viral particle, the molecular weight of the virus particle was estimated to be $3.6 \times 10^6$.

INTRODUCTION

The Kilham rat virus was isolated in rat embryo cell cultures from tumours of conditioned rats (Kilham & Olivier, 1959). Payne, Beals & Preston (1964) noted that the x-14 strain of rat virus was similar to the φX-174 coliphage in size, shape, and buoyant density. Since the φX-174 phage is known to contain single-stranded DNA (Sinsheimer, 1959b), the characteristics of the nucleic acid of rat virus became of interest to us. Several investigators (Rabson, Kilham & Kirschstein, 1961; Cochran & Payne, 1964; Whalley, 1965) have reported rat virus to be a DNA-containing virus and from acridine-orange staining studies Jamison & Mayor (1965) concluded that the DNA of rat virus was single-stranded.

In a further study utilizing acridine-orange staining of infected cells, Mayor & Jordan (1966) found an unusual type of green-yellow staining at the periphery of the nucleus of infected cells while the virus stained flame red. They postulated that the virus within the cell was double-stranded but was being unstranded during purification. May et al. (1967) recently reported that the DNA of the rat virus is double-
stranded. The results to be reported here are not in agreement with their findings, as our evidence indicates that rat virus contains single-stranded DNA.

**Methods**

The RV-13 strain of rat virus used in this investigation was obtained from the American Type Culture Collection and its passage history included 12 passages in rat embryo cell cultures and 34 passages in suckling hamsters. In our laboratory it has undergone one passage in suckling hamsters and one passage in rat embryo cell cultures. A single lot of stock virus was produced in primary rat embryo cell cultures from this material and was used for inoculation of all production lots of virus. This stock lot and all of the production lots were identified as rat virus by haemagglutination-inhibition tests using rat virus immune serum generously furnished by Dr L. Kilham. Since haemagglutination (HA) can be used as an assay method for the virus (Cochran & Payne, 1964; Whitman & Hetrick, 1967), the HA test was routinely employed to detect and quantify rat virus in the various procedures to be described. The test was performed as described by Kilham & Olivier (1959).

**Virus purification**

Virus was produced by infecting monolayer cultures of 6th to 8th passage rat embryo cell cultures propagated in 32 oz bottles. Following two cycles of freezing and thawing, the crude harvests were clarified by centrifugation at 20,000g for 30 min. The pellets were then treated in an ice bath for 5 min. at 20 kc/sec. with a Mullard Ultrasonic disintegrator, extracted with 10 times their volume of Hanks's balanced salt solution and centrifuged as above. The supernatant fluids from both centrifugations were pooled and the virus sedimented by centrifugation at 106,000 av-g for 4 hr in the Spinco Model L Preparative ultracentrifuge using the 40 rotor.

After ultrasonic treatment of the pellets they were treated with an equal volume of nuclease solution (0·5 mg./ml. ribonuclease (RNase) and 0·1 mg./ml. deoxyribonuclease (DNase) for 1 hr at 37°. An equal volume of papain solution (0·6 mg./ml. in 0·1 M-sodium acetate, +0·1 M-cysteine, +0·05 M-EDTA, pH 5·1) was then added to the above mixture followed by incubation for an additional 30 min. at 37°.

A 5 ml. sample of the mixture was layered on a 10% to 40% sucrose gradient. Following centrifugation at 64,000 av-g for 5 hr in a SW-25·1 rotor, 2 ml. fractions were collected and assayed for virus by HA tests. The fractions containing appreciable amounts of virus were pooled and vacuum dialysed against 0·005 M-EDTA. The dialysed virus suspension was adjusted with the aid of an Abbe-3L refractometer to a density of 1·30 to 1·40 g./cm.³ by the addition of solid CsCl. The gradients were then centrifuged for 24 hr at 115,000 av-g in the SW-39 rotor after which 0·02 ml. fractions were removed from the bottom of the tube and the virus located by microtitre HA tests. The refractive index of each fraction was measured and converted to density (Lifft, Voet & Vinograd, 1961) and the density curves derived by the method of least squares (Li, 1965).

The virus peaks which occurred at densities of 1·42 to 1·44 g./cm.³ were pooled for each lot and vacuum dialysed against a single saline citrate solution. This material represented purified virus and was stored at −70°.
Single-stranded DNA from the Kilham rat virus

Studies on intact virus

Buoyant density. In order to determine the buoyant density of the virus before treatment with enzymes and rate zonal centrifugation, crude virus pellets were suspended in saline citrate and treated by ultrasonic vibration for 5 min. in an ice bath. The virus suspension was adjusted to a density of 1.39 to 1.40 g./cm.³ by the addition of solid CsCl and centrifuged for 24 hr at 115,000 av-g in the Spinco Model L ultracentrifuge using the SW-39 rotor. The refractive indices and HA titres were determined for each of the 0.2 ml. fractions collected from the bottom of the gradient. The densest viral bands from three gradients were pooled, treated by ultrasonic vibration for 1 min., adjusted to the proper density and centrifuged in CsCl under the same conditions as before. The gradients were again separated into 0.2 ml. fractions and the same parameters measured.

Formalin reaction curves. Following the addition of 10% buffered formalin (Fisher Scientific Company) to a final concentration of 1.8% formaldehyde, the extinction coefficients of samples of purified virus were automatically read at 15 min. intervals at a wavelength of 260 nm. with a Gilford model 2000 Absorbence Recorder at room temperature.

Nucleic acid extraction. Purified virus used for nucleic acid extraction was quantified by standardizing its optical extinction (E) at a wavelength of 260 nm. with a Beckman DU spectrophotometer. A 2 ml. quantity of purified virus with an E of 10 or greater was mixed with an equal volume of papain solution and held for 1 hr at 37°, when 1 ml. of 5% sodium lauryl sulphate was added and the mixture incubated for an additional 30 min. at 37°. Protein was then removed by adding 5 ml. of 80% re-distilled phenol with 20% saline citrate (pH 7.8). The virus phenol mixture was gently agitated for 15 min. at room temperature and centrifuged for 5 min. at 4000 g. The aqueous phase was removed and extracted three additional times. To remove the phenol, a volume of ether equal to twice the volume of aqueous material was agitated with the aqueous phase for 3 min., allowed to separate, and was discarded. The ether extraction was repeated ten times and was then removed by bubbling nitrogen gas through the aqueous phase for 10 min. Salmon sperm DNA (highly polymerized, A grade, sodium salt, Calbiochem, Los Angeles) with the same optical extinction was treated similarly to serve as control material and is subsequently referred to as re-extracted polymerized DNA. The extracted nucleic acid was stored over chloroform at 4° and was stable for at least 2 weeks.

Studies on nucleic acid

Buoyant density. The buoyant density of the extracted DNA was determined by adjusting a nucleic acid solution with an E260 of 1.00 to a density of 1.71 to 1.73 g./cm.³ by adding solid CsCl. This mixture was centrifuged for 24 hr at 115,000 av-g in a Spinco model L preparative ultracentrifuge using the SW-39 rotor. Gradients were fractionated by removing 0.2 ml. from the bottom of the tube and the E260 and the refractive index were measured for each fraction.

Thermal denaturation curves. All nucleic acid melting curves were constructed with a Gilford model 2000 Multiple Sample Absorbence Recorder attached to a Beckman DU optical system. The temperature was increased 1°/min. with a Haake model F heater driven at 2 rev./min. by an electric motor. The samples were placed in Beckman
standard silica 0.1 ml. microcells which were sealed with rubber stoppers to prevent evaporation at high temperatures. Controls, which were run during every determination, included one cuvette containing saline citrate and one cuvette containing re-extracted polymerized DNA. The $E_{260}$ of the cuvette which contained saline citrate was subtracted from the sample reading to correct for thermal expansion. Readings were made automatically every 15 sec. After the temperature had reached 100° either the motor was reversed and the DNA allowed slowly to anneal or the cuvettes were quenched in an ice bath.

**Formalin reactions.** Nucleic acid lots were scanned from 240 to 320 nm. on a Beckman DB Spectrophotometer with a Beckman Linear Potentiometric Recorder before neutralized formalin was added to a final concentration of 1.8% formaldehyde. The samples were scanned again immediately after addition of the formalin and subsequently. In addition, thermal denaturation curves were determined for viral DNA reacted with formalin as previously described. Controls included saline citrate and polymerized DNA with 1.8% formaldehyde. Other samples with controls were reacted with 1.8% formaldehyde at room temperature and the reaction followed in the Gilford model 2000 Multiple Sample Absorbence Recorder. The $E_{260}$ of the samples were automatically read every 15 min. for a period of 24 hr.

**Enzyme reactions.** Ribonuclease (Nutritional Biochemicals Corporation) was diluted in phosphate-buffered saline to contain 40 µg/ml and heated to 80° for 10 min. to inactivate deoxyribonuclease. Deoxyribonuclease I (bovine pancreas, Worthington Biochemical Corporation) was diluted with 0.005 M-MgCl₂ prepared in phosphate-buffered saline to a final concentration of 40 µg/ml. Equal volumes of ribonuclease or deoxyribonuclease and viral nucleic acid were mixed and the $E_{260}$ changes followed for 24 hr at 30° in a Gilford model 2000 Absorbence Recorder.

**Studies with Actinomycin D.** An actinomycin D solution with an $E_{442}$ of 0.400 or greater was mixed with 0.25 ml. of a rat virus nucleic acid solution containing 20 µg. of nucleic acid and immediately scanned from 350 to 500 nm. with a Beckman DB spectrophotometer. Controls included 20 µg. of polymerized DNA in 0.25 ml. and 0.25 ml. of saline citrate added to 2.75 ml. samples of the actinomycin D solution. Scans were re-run at selected intervals after addition of the actinomycin D.

**Chemical assays.** The method of Lowry et al. (1951) was used to determine the protein concentrations during purification with the modification that the extinction coefficient of the sample was determined at a wavelength of 700 nm. rather than 650 nm. The Burton method (1956) was used to measure DNA levels.

**RESULTS**

**Studies with intact virus**

The identity of the stock virus and all of the production lots of rat virus prepared in rat embryo cell cultures was confirmed by serological tests. Vasquez & Brailovsky (1965) used DNase treatment during their purification of rat virus without loss of infectivity. Neither DNase nor RNase had any effect on rat virus in our procedure either and the papain treatment actually increased titres probably by disrupting clumps of virus and releasing virus from cellular material. The partially purified virus was layered on a sucrose rate zonal gradient. Following centrifugation, fractions with peaks of virus activity were pooled. This virus material was then banded in CsCl
a, Electron micrograph of purified virus negatively stained with uranyl acetate. Occasional virus particles appear to lack a nucleic acid core. b, Electron micrograph of the dense (1·43 g./cm.²) HA activity peak from CsCl equilibrium centrifugation of pelleted crude virus. c, Electron micrograph of the intermediate (1·38 g./cm.²) HA activity peak from CsCl equilibrium centrifugation of pelleted crude virus.

D. M. ROBINSON AND F. M. HETRICK

(Facing p. 272)
a, Electron micrograph of the light (1.32 g./cm.³) HA activity peak from CsCl equilibrium centrifugation of pelleted crude virus. Few, if any, particles can be identified as virus particles. b, Electron micrograph of viral DNA. The DNA was mixed with a solution containing 0.01% cytochrome c and 1 M-ammonium acetate and floated on 0.1 M-ammonium acetate. The grids were circularly shadowed with platinum.

D. M. ROBINSON AND F. M. HETRICK
Single-stranded DNA from the Kilham rat virus

Electron micrographs of the dense band showed a mixture of full and empty virus particles (Plate 1a). The 3 HA activity peaks corresponded with densities of 1.43, 1.38 and 1.32 g./cm.³. These results are very similar to those reported by Crawford (1966) for the minute virus of mice, which also has a single-stranded DNA and is approximately the same size as rat virus, being 19 to 26 nm. in diameter. Crawford reported peaks of virus activity at densities of 1.43, 1.38 and 1.35 g./cm.³.

![Isopycnic gradient centrifugation of pelleted rat virus in CsCl gradients.](Fig. 1)

Fig. 1. Isopycnic gradient centrifugation of pelleted rat virus in CsCl gradients. (Results from five gradients.)

![Isopycnic gradient centrifugation of the most dense HA activity peak (density of 1.42 g./cm.³ or greater) recovered from gradients described in Fig. 1.](Fig. 2)

Fig. 2. Isopycnic gradient centrifugation of the most dense HA activity peak (density of 1.42 g./cm.³ or greater) recovered from gradients described in Fig. 1.

Electron micrographs of our bands showed the dense band to be composed of full virus particles relatively free of extraneous material (Plate 1b); the intermediate band contained empty and full virus particles associated with other debris (Plate 1c) and the light band was made up of large amounts of debris in which an occasional virus particle could be seen (Plate 2a). When the dense HA peak (1.43) was dialysed and

J. Virol. 4
recentrifuged in a CsCl gradient, the presence of several peaks at lesser densities indicated that the virus was breaking down with repeated CsCl centrifugation (Fig. 2). Since further CsCl gradients did not increase the 260:280 absorbence ratio found with the initial CsCl gradient, the production lots of virus were purified by sedimentation followed by enzyme treatment, sucrose, and CsCl density-gradient centrifugations in sequence.

**Formalin reactions**

Five different lots of purified virus were treated with formalin and an average reaction curve constructed (Fig. 3). The increase varied from lot to lot by less than 4 % and the curve for the intact rat virus was always intermediate between the curve for polymerized DNA and acid degraded DNA used as controls.

![Fig. 3. The effect of formaldehyde on the absorbance of rat virus (Δ--Δ), polymerized DNA (●—●) and degraded DNA (○—○).](image)

Josse & Eigner (1966) have described the secondary structure of single-stranded nucleic acids as a single alpha helix. The stability of this molecule would depend on the linear stacking of the bases in the helix, and this stacking could lead to masking of some of the amino groups and their unavailability to react with formaldehyde. The DNA degraded by perchloric acid had none of this structure, and all of the amino groups were available for reaction with formaldehyde. The polymerized DNA should have had no free amino groups on the molecule to react with formaldehyde, and the small increase which was seen (2 %) was compatible with increases found by Randall *et al.* (1966) with double-stranded DNA.

The formaldehyde was capable of reacting with the intact virion to give an increase in $E_{260}$ of 6 % to 12 % which was less than the 21 % reported by Sinsheimer (1959a) or the 18 % reported by Crawford (1966).
Studies on extracted viral nucleic acid

Buoyant density. The DNA extracted from rat virus banded at a density of 1.72 g./cm.³ in a CsCl gradient (Fig. 4). This material was assumed to be DNA only since there was no detectable protein or RNA. The proportion of DNA in the virion was estimated to be 34% from the solution of the following equation (Breedis, Berwick & Anderson, 1962):

\[ V_p = \left( \frac{V_{DNA} \times \% DNA + V_{protein} \times \% protein}{100} \right) \]

where \( V_p \) = 0.698, partial specific volume of the virus (reciprocal of observed density); \( V_{DNA} \) = 0.581, partial specific volume of the DNA and \( V_{protein} \) = 0.758, partial specific volume of viral protein.

![Fig. 4. Isopycnic density centrifugation of rat virus DNA in CsCl gradients for a period of 24 hr at 115,000 g.](image)

Reactions with nucleases. In two experiments RNase and DNase were reacted with polymerized DNA and viral DNA (Fig. 5). When the polymerized DNA was not reextracted the increase in \( E_{260} \) averaged 10% with RNase treatment. The DNase reactions had a long half-time because of the unfavourable Mg:P ratio present in the reaction mixture. The DNA was suspended in phosphate-buffered saline, and the DNase was prepared in 0.005 M-MgCl₂. The long reaction time made the difference in rates between the two DNAs more definite than when they were suspended in saline, and the DNase was in 0.05 M-MgCl₂. The DNA extracted from rat virus was degraded quicker than the native double-stranded DNA, as Sinsheimer (1959b) also found for φX-174 DNA measured by light scattering.

Thermal denaturation curves. Fig. 6 illustrates the results of five thermal denaturation experiments which were made with DNA extracted from five different lots of purified virus. The DNA concentrations, as measured by diphenylamine reactions, ranged...
Fig. 5. The effect of DNase I on the $E_{260}$ of the DNA extracted from rat virus ($\triangle$--$\triangle$) and polymerized DNA ($\circ$--$\circ$).

Fig. 6. The effect of heating on the absorbence of DNA extracted from rat virus ($\triangle$--$\triangle$) and polymerized DNA ($\bullet$--$\bullet$).
Single-stranded DNA from the Kilham rat virus from 14 to 32 \mu g./ml., and no differences were noted in the curves for different concentrations. As little as 10 \mu g./ml. of polymerized DNA gave a typical melting curve. The \( T_m \) curve of the DNA of rat virus had no sharp rise in it comparable to the double-stranded DNA curve. The gradual hyperchromicity began at 50\(^\circ\) to 60\(^\circ\), depending on the lot of viral DNA, and continued to increase in an almost linear manner until the experiments were terminated at 100\(^\circ\). The maximum hyperchromicity at 100\(^\circ\) ranged from 14\% to 26\%, and when the DNA from rat virus was either quenched in an ice bath or cooled slowly, the maximum residual hyperchromicity was 5\%, and three lots had none. In contrast to this, the known double-stranded DNA, which had been re-extracted, showed small increases in hyperchromicity from 50\(^\circ\) to 80\(^\circ\) and a sharp rise of approximately 40\% in \( E_{260} \) from 80\(^\circ\) to 92\(^\circ\). The method of cooling made a great difference to the residual hyperchromicity of the double-stranded DNA. When the DNA was quenched in an ice bath, 30\% to 35\% of the hyperchromicity was retained, but when the heating unit was adjusted to 70\(^\circ\) and the DNA allowed to renature, only 8\% to 15\% was retained. These results (lack of a \( T_m \) and low residual hyperchromicity) also indicated that the DNA of rat virus existed in the single-stranded configuration. However, the increasing hyperchromicity with increasing temperature suggested that the molecule did have some secondary structure.

**Formalin reactions.** When the extracted nucleic acids (both rat virus and polymerized) were reacted with formaldehyde, two distinctly different curves were obtained. The curve for rat virus (Fig. 7) showed an increase in optical extinction and a shift.
in the maximum extinction toward 280 nm. when scanned on the spectrophotometer. The original adsorption maximum was 264 nm., which had shifted to 270 nm. after 20 hr formalin treatment. The increase could be measured as early as 15 min. after addition of the formalin, but the maximum increase did not occur until approximately 18 hr after formalin addition. The increase at 260 nm. for three separate lots of DNA extracted from rat virus averaged 16.4% compared to the 6% to 12% increases found when formalin was reacted with intact virus.

The formaldehyde reaction was also followed in relation to time, and five determinations were averaged (Fig. 8). This technique gave an average maximum increase of 15.6% comparing very well with the 16.4% found in the scanning experiments. When the nucleic acid extracted from rat virus was heated in the presence of formalin, the method of cooling made little difference in the residual hyperchromicity. Each sample decreased by 15% to 20%, and no sample uniformly changed more than any other.

Reactions with actinomycin D. The reaction of actinomycin D with double-stranded DNA (Fig. 9), occurred very rapidly. The cuvettes were scanned 5 min. after the addition of the actinomycin D, when the reaction was 75% complete. The DNA from rat virus did not react with the actinomycin D at all as shown by the lack of change in the optical extinction at 442 nm., the absorption peak of actinomycin D. Since actinomycin D binds exclusively to double-stranded nucleic acids (Goldberg, Rabinowitz & Reich, 1962), these results again indicated a single-stranded configuration for the viral DNA.

Electron microscopy. When extracted DNA was examined in the electron microscope (Pl. 2b), it was found to be extensively pooled, and the isolated molecules con-
Single-stranded DNA from the Kilham rat virus

Single-stranded DNA from the Kilham rat virus (Inman, 1966). Many circular molecules were seen along with smaller fragments, but it was impossible to determine whether the molecule in its natural configuration was circular or whether this represented an artifact formed during the extraction and preparation for electron microscopy. The average length obtained from measuring 20 molecules was 1.30 μm, with a standard deviation of ± 0.14. Using half the value of 192 daltons/Å of double-stranded DNA (MacHattie & Thomas, 1964) a value of 1.2 × 10\(^6\) daltons was calculated from the DNA of rat virus. Since the virus contains 34% DNA, it can be estimated that its molecular weight is 3.6 × 10\(^8\).

DISCUSSION

The broad viral band observed in the sucrose rate zonal centrifugations indicated a heterogeneous viral population. Whether this heterogeneity came from differences in size or density was investigated in the equilibrium centrifugation experiments conducted on the pelleted virus. No virus-like particles other than the typical 20 to 22 nm. rat virus were seen in the CsCl gradients examined. Additionally, empty and full virus particles were found in all areas of the gradient lighter than 1.40 g./cm.\(^3\). Therefore, the source of the heterogeneity was the presence of particles with and without nucleic acid cores, but it was not ascertained whether the empty particles were formed by the cell or by loss of nucleic acid from the full particle after release from the cell. It would appear from Fig. 2 that the intact dense particles lose their nucleic acid and become light particles with HA activity, but this may be an artifact of the repeated CsCl centrifugations.

Formaldehyde reacted with the virion causing an increase in optical extinction at 260 nm. Although this increase was not as great as that reported by Sinsheimer (1959a) it would not be necessary for all native single-stranded DNA viruses to react to the same extent. The reaction would be dependent on the masking of the amino groups caused by the secondary structure of the DNA and the involvement of the DNA in the integrity of the protein coat of the virus. The disruption of the protein coat and the destruction of the secondary structure of the DNA during the extraction procedures allowed the formaldehyde to react maximally with the amino groups. Under these conditions the increase in \(E_{260}\) from the formaldehyde reaction was the same as that reported for other single-stranded DNA viruses.

Single-stranded DNA, whether produced by artificial means or isolated from natural sources, tends to form large clumps and lose its hyperchromicity when either slowly cooled or quenched. This phenomenon was illustrated by the lack of hyperchromicity of rat virus in the heating experiment and by examination with the electron microscope. However, the lack of knowledge about the structure of single-stranded DNA in its native state cannot be emphasized too strongly. Josse & Eigner's (1966) suggestion that the secondary structure of single-stranded DNAs could be attributed to base stacking and hydrophobic forces rather than areas of complementary base pairing in the chain was used to explain the relationship found in the cooled samples which had been reacted with formaldehyde.

The total of the percentage increases for viral DNA with formaldehyde and heating separately should be comparable to the increase with heating in the presence of formaldehyde. The total of 39% (or 34.6% depending on which absorption average
for heating was used) compared very well with the 35% increase in optical extinction found with both heating and formaldehyde. However, the important observation was the retention of 15% to 18% of the hyperchromicity when the viral DNA, which had been heated in the presence of formaldehyde, was either quenched or cooled. When this figure was compared with the 17% increase in optical extinction found with 24 hr formaldehyde treatment, it was concluded that the presence of additional hydroxymethyl groups on the molecule did not inhibit the complete recovery of the secondary structure of the extracted single-stranded DNA molecule. Therefore, complementary base pairing was not important to the secondary structure of the extracted DNA. However, the masking of approximately 50% of the amino groups of the DNA in the intact virion would be useful in explaining its restricted reaction with formaldehyde. It may be that the DNA in the virion did not have the same secondary structure as the DNA after the extraction procedures.

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