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# CDDP induces conformational changes in BTV ds RNA rather than forming protein-protein and/or protein-RNA crosslink

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**Abstract** : cis-Diamminedichloroplatinum(II)(CDDP), an antitumor drug, did not generate crosslink between bluetongue virus (BTV) capsid protein at moderate concentration. Cesium chloride density gradient centrifugation study revealed that protein-RNA crosslink was not detectable in CDDP treated BTV. CDDP treated BTV ds RNA showed remarkable change in the migration pattern in polyacrylamide gel electrophoresis. These results suggest that the reduction of BTV core associated transcriptase activity is most likely by the CDDP adduction to the genomic ds RNA rather than by the protein-RNA crosslink and/or protein-protein crosslink.

Bluetongue is a disease of domestic and wild ruminants caused by bluetongue virus (BTV), a member of family Reoviridae. The capsid of BTV is composed of four major and three minor polypeptides, which contains ten genomic segments of ds RNA size ranged from $0.5 \times 10^6$ to $2.8 \times 10^6$ daltons (Verwoerd et al., 1972). The ten segments of RNA are roughly distributed into three groups namely, large (segment 1, 2, and 3), medium (segment 4, 5, and 6), and small (segment 7, 8, 9, and 10), based on their size (Verwoerd and Huismans, 1972 : Van Dijk and Huismans, 1980).

CDDP has been established as a potent antitumor chemotherapeutic agent, but it's molecular mode of action has not been clearly elucidated yet (Roberts and Pera, 1983). It has been shown that CDDP interacts with biomacromolecules especially with DNA and to the lesser extent with protein (Lippard and Hoeschele, 1979) and has been proposed that CDDP adduction to DNA, most likely by intrastrand crosslinks and monoadducts (Girault et al., 1982 and Marcellis et al., 1982) is responsible for the observed inhibition of DNA replication for the effectiveness as an antitumor activity (Pinto and Lippard 1985).

We have found that relatively impure preparations of BTV infectivity and the transcriptase activity of the BTV core were reduced to an undetectable level after treat the virus and core with 100 μM CDDP (manuscripts submitted for publication). It was suggested that CDDP binding to the template RNA blocks chain elongation of the virion bound transcriptase that is ultimately responsible for the inactivation of BTV infectivity. However, the possible mode of action of CDDP on BTV macromolecules that is responsible for the inactivation of BTV core associated transcriptase activity has been remained to be solved. In this study we have investigated the possible mode of action of CDDP on BTV capsid protein and genomic ds RNA.

Key words : CDDP, BTV, crosslink, ds RNA

## Materials and Methods

### Virus and cell

BHK 21 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY). BTV grown in BHK 21 cells was harvested after 24 to 48 hr postinfection. Cells were pelleted, resuspended in 2 mM Tris-HCl buffer (pH 8.8), sonicated three times, and centrifuged at 11,000×g for 10 min. To the supernatant an equal volume of twice concentrated lysis buffer (1% Triton X−100, 0.2 M KCl, 0.02 M $MgCl_2$, 0.0072 M $CaCl_2$, 0.002 M Tris-HCl, pH 8.8) was added, incubated for 15 min at 37℃, and centrifuged at 11,000×g for 10 min. The supernatant was layered over a 40% sucrose cushion and centrifuged in a Beckman SW 27 rotor at 24,000 rpm for 2 hr. The pellet was resuspended in 2 mM Tris-HCl buffer (pH 8.8), loaded on top of preformed 10 to 40% sucrose gradient, and centrifuged in a Beckman SW 41 rotor at 24,000 rpm for 70 min. The light scattering band was collected, diluted in 2 mM Tris-HCl buffer (pH 8.8), pelleted in a Beckman SW 41 rotor at 24,000 rpm for 100 min, and resuspended with 2 mM Tris-HCl buffer (pH 8.8).

### $^3H$ and $^{32}P$ labeling of BTV RNA

After 1 hr of adsorption on BHK cells, unadsorbed virus was washed out and DMEM supplemented with 2% FBS and 0.5 μg/ml of actinomycin D was added. After 4 hr of incubation, maintenance medium was replaced with fresh DMEM with 2% FBS containing 10 μCi/ml of tritiated uridine (NEN, Boston, MA, 35−50 μCi/mmol). For $^{32}P$ labeling, confluent BHK 21 cells were infected with BTV and DMEM consisting of 0.1× phosphate concentration was added. After 1 hr of incubation, phosphate free DMEM with 50 μCi of carrier free $^{32}P$ (NEN, Boston, MA) was added. Virus purification and RNA extraction was done as described in the other section.

### Extraction of BTV RNA

Purified BTV disrupted with 0.5% SDS for 2 min at 60℃ and exposed to protease K (1 mg/ml) for 2 hr at 37℃. RNA was extracted twice with phenol : chloroform (1 : 1). The aqueous phase was washed twice with chloroform : isoamylacohol (24 : 1) and the residual phenol was removed by dialysis against TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.2). RNA was then ethanol precipitated by addition of NaCl to 0.1 M and ethanol to 70% and chilling at −70℃ for 1 hr. The precipitated RNA was pelleted at 11,000×g for 5 min at 4℃, washed with 70% ethanol, and vacuum dried.

### CDDP treatment

Purified BTV was diluted 1 : 4 with 20 mM $KH_2PO_4$ (pH 7.2). An equal volume of CDDP of twice the final concentration, prepared in 20 mM $KH_2PO_4$ (pH 7.2), was added. The reaction mixture was incubated in the dark for 1 hr at 37℃, diluted 1 : 3 with 2 mM Tris-HCl (pH 8.8), and pelleted through 40% sucrose cushion in a Beckman SW 50.1 rotor at 40K rpm for 45 min. The supernatant was carefully removed and the pellet was resuspended in 2 mM Tris-HCl (pH 8.8).

### Polyacrylamide gel electrophoresis

BTV was disrupted by boiling for 3 min in electrophoresis sample buffer (0.06 M Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 3% dithiothreitol, 0.005% bromophenol blue). Samples were applied to 10% acrylamide gel (acrylamide/bisacrylamide weight ratio, 37.5 : 1) and subjected to electrophoresis as described in Laemmli (1970). $^{32}P$ labeled RNA was visualized by exposing the dried gel to Kodak XR-5 film.

### Cesium chloride density gradient centrifugation

$^3H$-uridine and $^{35}S$-methionine double labeled BTV was treated with CDDP, disrupted with SDS, and layered on top of preformed CsCl gradient. It was

then centrifuged by a Beckman SW 50.1 rotor at 40K rpm for 48 hr. Fractions were collected by puncturing the bottom of the tube and radioactivity was counted by liquid scintillation counter.

## Results and Discussion

### CDDP does not generate protein-protein cross-link

We have found that BTV infectivity and the transcriptase activity of the BTV core were reduced to an undetectable level after treat the virus and core with 100 μM CDDP. It was suggested that CDDP binding to the template RNA blocks chain elongation of the virion bound transcriptase that is ultimately responsible for the inactivation of BTV infectivity. The possible mode of interaction between CDDP and BTV macromolecules that is responsible for the inactivation of BTV core associated transcriptase activity was examined.

To examine interactions between CDDP and BTV capsid protein, BTV treated with various concentrations of CDDP was analysed in 10% SDS polyacrylamide gel electrophoresis. As shown in Figure 1, pro-

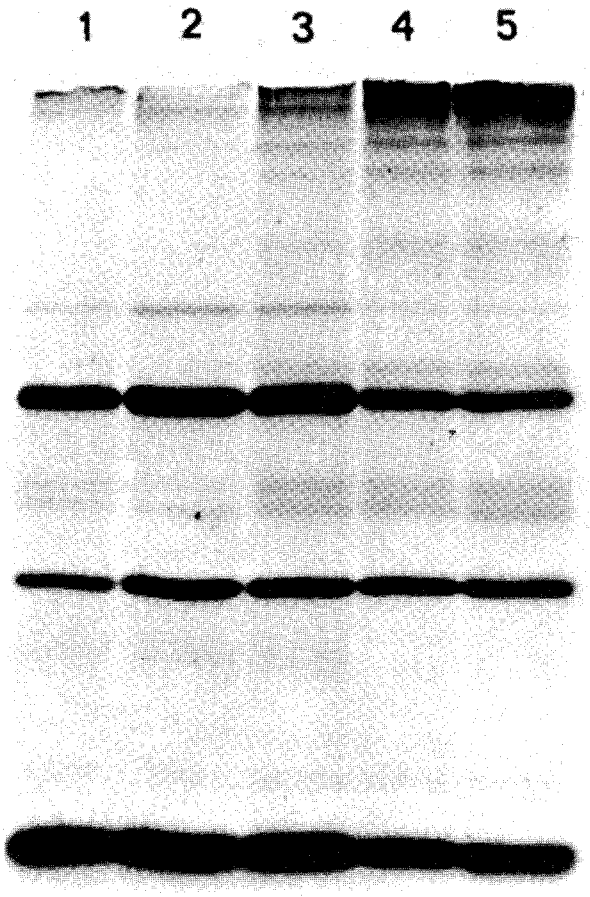


Fig. 1. 10% SDS polyacrylamide gel electrophoresis of BTV.

BTV(40 μg) treated with various concentrations of CDDP was dissolved in Laemmli sample bufffer and ran in 10% SDS PAGE. (1) BTV; (2) BTV treated with 10 μM CDDP; (3) BTV treated with 100 μM CDDP; (4) BTV treated with 500 μM CDDP; (5) BTV treated with μM CDDP.

tein-protein crosslink was not detectable unless BTV treated with 500 μM of CDDP. TDDP, a trans isomer of CDDP, has two chloride groups whose distance is little bit longer than that of CDDP. The relatively longer distance between two chloride groups let TDDP be superior to CDDP for the generation of protein-protein crosslink. BTV treated with various concentrations of TDDP, was analyzed in 10% SDS PAGE. As shown in Figure 2, TDDP did not generate

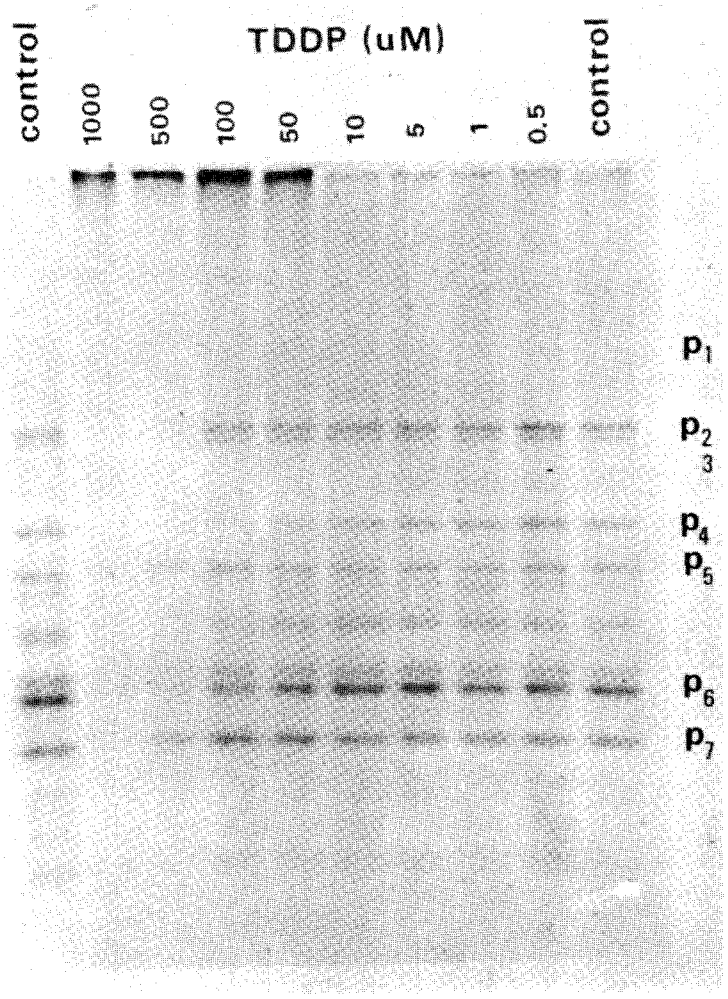


Fig. 2. 10% SDS polyacrylamide gel electrophore sis of BTV.

BTV (40 μg) treated with various concentrations of CDDP was dissolved in Laemmli sample buffer and ran in 10% SDS PAGE. Control : BTV not treated with TDDP.

protein-protein crosslinks at moderate concentrations. Since 10 μM of CDDP is more than enough to inactivate BTV infectivity and transcriptase activity of the BTV core, any protein-protein crosslink induced by CDDP would not be a probable cause for the reduction of the BTV infectivity and BTV core associated trancriptase activity.

### CDDP does not generate protein-RNA crosslink

Since the ten BTV genomic RNAs are confined within the limited space of the core, they are probably in close association with capsid protein. The close association between genomic RNA and capsid protein would generate RNA-protein crosslink in the presence of crosslinking agent such as CDDP. To elucidate the possible RNA-protein crosslink, the capsid protein of BTV was labeled with $^{35}$S-methionine and BTV genomic RNA was labeled with $^{3}$H-uridine.

The double labeled BTV was purified, treated with CDDP, disrupted by treating with SDS, and layered on top of the preformed CsCl density gradient. Since materials are separated in the CsCl density gradient centrifugation based on its density, the crosslinked RNA-protein complex would have a peak at intermediate density value of RNA and protein. Figure 3 shows that high density RNA (labeled with $^{3}$H) peaks at the bottom of the tube while low density protein (labeled with $^{35}$S) peaks at the top of the tube. The absence of new peak with the intermediate density (labeled with both $^{3}$H and $^{35}$S) suggests that the RNA-protein crosslink was not generated to detectable level.

### CDDP adduction to BTV ds RNA

The adduction mode of CDDP on nucleic acid has been hypothesized as monoadduct, intrastrand crosslink, and interstrand crosslink of which monoadduct is the most probale mode. As psoralen adduction to reovirus ds RNA generated kinked conformation, it is very likely that the CDDP adduction to BTV ds RNA also generates kinked conformation.

To confirm CDDP adduction to BTV ds RNA, BTV ds RNA was treated with CDDP and analysed in polyacrylamide gel electrophoresis. When CDDP treated BTV RNA was run in polyacrylamide gel electrophoresis, the CDDP-treated RNA migrated more slowly and formed diffused bands compared to that of the control RNA (Fig. 4).

Conformational change of CDDP-treated RNA into compact form would give a larger cross section than control RNA of the same contour length. Since the pore size of 10% polyacrylamide gel is only a few multiples of the diameter of the double helix (Cooper, 1977), compact RNA would encounter difficulty in passing through the gel interstices. RNA treated with 500 μM CDDP did not migrate into the running gel (Fig 4, lane 4) presumably because the RNA conformation was so compact and rigid that its effective cross section exceeded the diameter of the gel pores of the running gel.

This result proves indirectly that the CDDP addution to BTV ds RNA generates kinks resulting in the conformational change into compact form. The lack of protein-protein and protein-RNA crosslinks and the conformational changes of ds RNA generated by CDDP implies that the transcription inhibition by CDDP adduction to ds RNA lead to the physical block of the transcription. The transcriptase falls off from the template DNA and generates prematurely terminated transcripts.

## Acknowledgments

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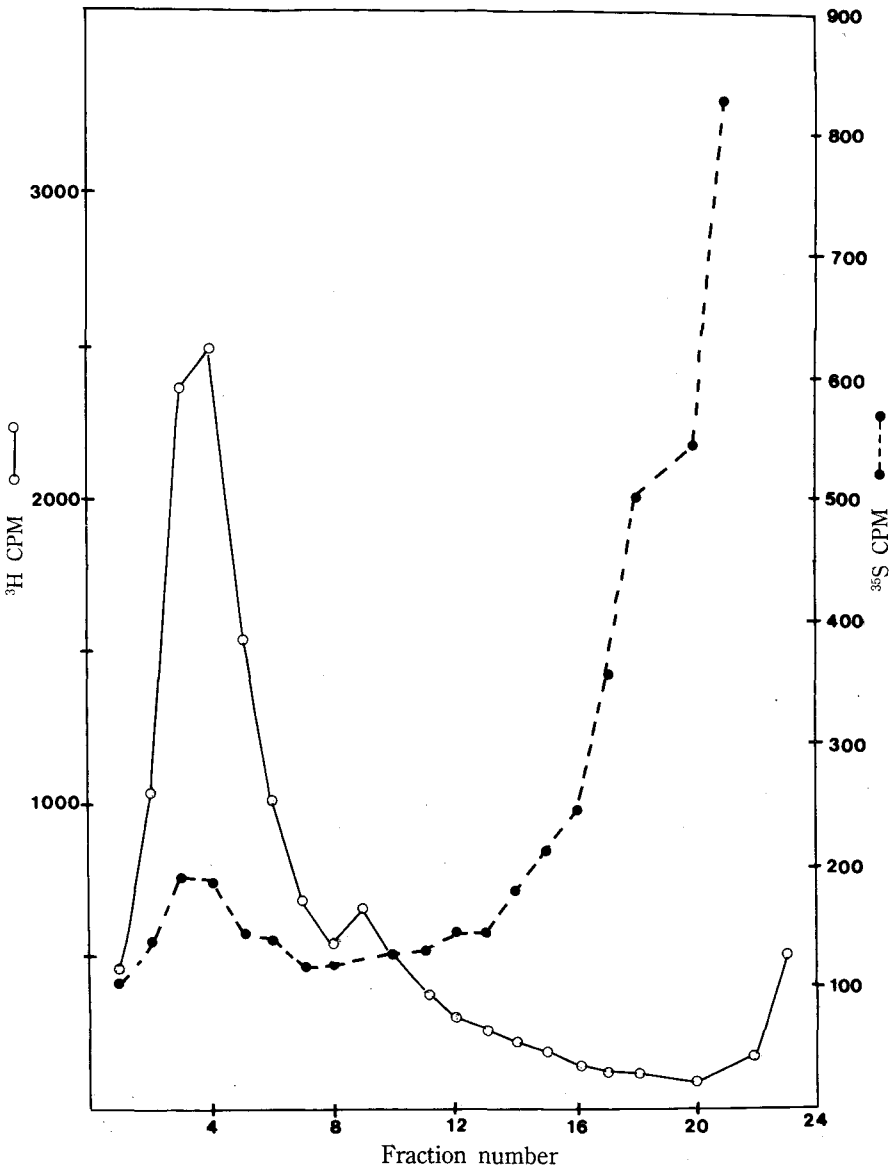


Fig. 3. CsCl density gradient profile of $^{3}$H, $^{35}$S labeled BTV.

BTV labeled with $^{3}$H-uridine and $^{35}$S-methionine was treated with 100 μM CDDP and disrupted by adding 10% SDS to the final concentration of 1%. The sample was loaded on top of preformed CsCl gradient and centrifuged in a Beckman SW 50.1 rotor for 48 hr at 40 k rpm. After centrifugation fractions were collected from the bottom of the tube. The radioactivity of each fraction was determined by liquid scintillation counter.

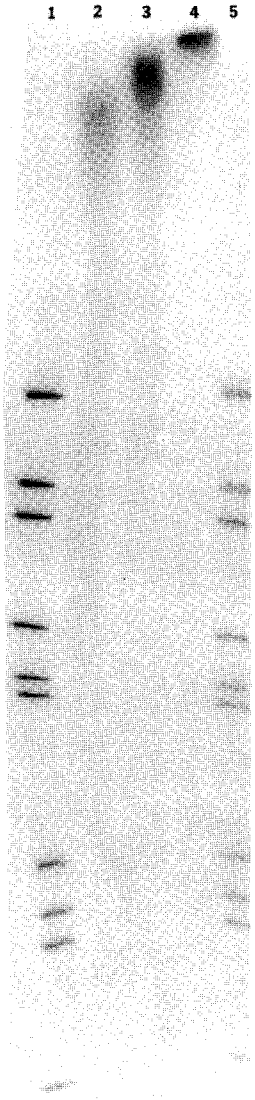


Fig. 4. Electrophoresis of CDDP-treated BTV in 10% polyacrylamide gel.

$^{32}P$ labeled BTV RNA was treated with CDDP at 0 μM (lane 1 and 5) ; 20 μM (lane 2) ; 100 μM (lane 3) ; 500 μM (lane4) and analyzed in 10% polyacrylamide gel. RNA bands were visualyzed by drying the gel and exposed to Kodak XR-5 film.

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## cis-Diamminedichloroplatinum(II)(CDDP)에 의한 불루텅 바이러스 이중가닥 RNA의 구조변화

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**초록** : CDDP는 100 μM 이하의 농도에서 BTV 캡시드 단백질에 crosslink를 형성하지 않는다. 밀도구배초원심분리 결과에 의하면 캡시드 단백질과 RNA사이의 crosslink도 일어나지 않는다. CDDP를 처리한 BTV RNA를 폴리아크릴아마이드 겔에 전기영동하면 RNA이동 패턴이 변한다. 이 결과는 BTV core에 있는 RNA 중합효소의 불활성화가 CDDP에 의한 BNA RNA의 구조변화에 의함을 가리킨다.