BREAKAGE OF DNA-MEMBRANE COMPLEX BY BLEOMYCIN

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The "DNA-membrane complex" (1200S-400S) obtained by lysis of rat ascites hepatoma cells in a low concentration of sodium dodecyl sarcosinate-sodium deoxycholate sedimented through neutral sucrose gradient more rapidly than the "free DNA" (200S) which was released by a high concentration of sodium dodecyl sulfate. The former was decomposed by Pronase in the gradient but the latter was not affected by this enzyme. Density of the complex was not more than 1.55 in CsCl equilibrium centrifugation and the free DNA (density 1.70) was liberated from the DNA-membrane complex by incubation of the cells with a low concentration of bleomycin which hardly caused double-strand scission in the free DNA molecule. Post-incubation of the cells regenerated the complex but the repair was incomplete within a short period. It is suggested that a low concentration of bleomycin decomposes the complex both by destruction of membrane material and by a very small amount of DNA strand scission near the membrane attachment site prior to random strand breakage of DNA.

Bleomycin causes a single-strand scission of DNA *in vitro* and *in vivo*[1~5] and we have demonstrated that bulk DNA in sensitive cells was highly damaged by this antibiotic compared to that of cells insensitive to bleomycin[6]. In general, accumulation of the single strand scission of DNA results in the double-strand breakage of DNA and inhibition of DNA synthesis, and this seems to be correlated with the sensitivity of the cells in S-phase[7]. Cell division has been found to be very sensitive to bleomycin since it is inhibited by a low concentration of the antibiotic which does not suppress DNA synthesis[8,9]. A small amount of single-strand breakage produced by a low concentration of bleomycin was reparable within a short post-incubation period even in the sensitive cells[6] and, hence, single-strand scission alone does not appear to be the reason for inhibition of cell division. Attachment of DNA to the annuli of nuclear membrane has been postulated in animal cells using electron microscopy[10,11] and contribution of the membrane for segregation of DNA during cell division has been described[12]. ORMEROD and LEHMAN showed the attachment of DNA to nuclear membrane in L5178Y cells employing lysis of cells in a dilute solution of a detergent followed by sedimentation of the DNA-membrane complex through sucrose gradient[13]. We applied this procedure to examine the release of DNA from the DNA-membrane complex by a low concentration of bleomycin which did not change the sedimentation profile of free DNA, in relation to the sensitivity of cell division to the antibiotic.

Materials and Methods

Labeling of cells

The ascites hepatoma cells, AH-66 or AH-66F, were labeled overnight with 50 μCi of ^{3}H-thymidine (25 Ci/mmol) in the abdominal cavity of a Donryu rat. The cells were withdrawn

and suspended in EAGLE's minimum essntial medium (MEM) to a concentration of 2×10^6 cells/ml after being washed with saline. The cells were incubated with bleomycin A_2 (Cu free, Nippon Kayaku Co.) at 37°C for 30 minutes, washed three times with MEM, and a part of the treated cells were reincubated without bleomycin at 37°C for 30 minutes. For double labeling, the cells labeled by ^{3}H-thymidine were incubated with 20 μCi/ml of ^{14}C-acetate (49 mCi/mmol, Daiich Pure Chemicals), ^{14}C-choline (41 mCi/mmol, New England Nuclear), ^{14}C-leucine (282 mCi/mmol, Daiich Pure Chemicals), or ^{14}C-uridine (53 mCi/mmol, New England Nuclear) for 0.5 to 2 hours.

Cell lysis and sucrose gradient centrifugation

The cells (4×10^4/0.1 ml) were lysed over 0.5 ml of 0.2% sodium dodecyl sarcosinate-0.08% sodium deoxycholate-0.1 M NaCl-0.01 M sodium citrate-0.02 M EDTA (pH 9.0) (SDSa-DOC) layered on 16 ml of 5~20% (w/v) sucrose gradient containing 0.1 M NaCl and 0.01 M sodium citrate (pH 9.0). To obtain free DNA, 2% sodium dodecyl sulfate-0.01 M Tris-0.01 M EDTA (pH 8.0) (SDS) was used as a lysing solution. The tubes were left at 20°~28°C for 15~120 minutes and centrifuged at 25,000 rpm for 60 minutes at 20°C in a Hitachi SW-25.3 rotor. It took 20 minutes to reach 25,000 rpm and 20 minutes to stop and, therefore, average speed of centrifugation was 19,000 rpm and the time was 80 minutes. The gradients were fractionated from the top of the tubes and aliquots were placed on Whatman 3 MM paper discs which were washed with cold 5% trichloroacetic acid and ethanol, dried, and their radioactivity was counted in a toluene scintillator. When the cells were labeled with ^{14}C-acetate or ^{14}C-choline the discs were washed with water instead of ethanol, or fractions were evaporated to dryness and extracted with chroloform-methanol or butanol and radioactivity in the extract was measured after evaporation of the solvent.

Sucrose gradient containing Pronase

The 5~20% sucrose gradient (pH 7.0) contained 0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, and 90~200 μg/ml of Pronase-P (Kaken Kagaku) which was incubated at 37°C for 60 minutes before use to inactivate DNase. The cells (2×10^4/0.1 ml) were placed on 0.5 ml of the lysing solution containing SDSa-DOC or SDS layered over 16 ml of sucrose gradient and, after being kept for 60 minutes at 28°C the tubes were centrifuged at 25,000 rpm for 120 or 180 minutes in a Hitachi SW-25.3 rotor.

CsCl equilibrium centrifugation

The cells (4×10^4/0.1 ml) were lysed over 0.2 ml of SDSa-DOC which was layered on 4 ml of CsCl solution of density 1.76 or 1.50. The tubes were kept at 28°C for 60 minutes, filled with liquid paraffin, and centrifuged at 50,000 rpm for 44 hours at 20°C in Beckman 50 Ti rotor. The fractions were collected from the bottom of the tube and the radioactivity was measured as described above.

Results

Effect of Bleomycin on Sedimentation Profile of DNA-Membrane Complex in Neutral Sucrose Gradient

AH-66 or AH-66F cells were incubated with bleomycin and directly placed on the top of a sucrose gradient since any handling of DNA would introduce breaks by mechanical shearing. The sedimentation coefficient of DNA released from untreated cells by 0.2% SDSa-0.08% DOC varied depending on the lysis temperature and time as shown in Table 1. Cell lysis at 20°C for 15 minutes yielded a peak of about 1200S and that at 20°C for 30~60 minutes gave 800~600S. The highly reproducible peak at 400S was obtained by lysis in the same solution at 20°C for 60~120 minutes or at 28°C for 30~60 minutes or longer, and was the smallest size of DNA complex released by this lysing solution. Exhaustive lysis of the cells by 2% SDS at 28°C for

1~18 hours released the "free DNA" which had a reproducible sedimentation coefficient of about 200S. All DNA from untreated cells sedimented as a single peak. Treatment of AH 66 cells with a low concentration of bleomycin (10~20 μg/ml) did not change the size of the 200S molecule (Fig. 1 g~i). Number of single breaks in one strand of DNA by such a low concentration of bleomycin was 1~2 break/molecule because the single-stranded DNA was broken to half size as previously demonstrated by alkaline sucrose gradient centrifugation[6]. It seemed reasonable that double-strand scission was hardly observed in the free DNA by treatment with 10~20 μg of bleomycin. In contrast to "free DNA", "DNA-membrane complex" (400S) released by SDSa-DOC, was broken even by 10 μg of bleomycin and the damaged DNA-complex sedimented almost the same distance as the free DNA from untreated cells which was released by SDS (Fig. 1 d~f). The DNA complex of 600S, which was obtained by lysis under milder conditions, was also reduced in size by 10~20 μg of bleomycin but S-value was slightly larger than that of free DNA due to incomplete lysis of the attachment site which is sensitive to SDSa-DOC (Fig. 1 a~c). The "DNA-membrane complex" from AH-66F cells was also decomposed by bleomycin which caused neither single-strand

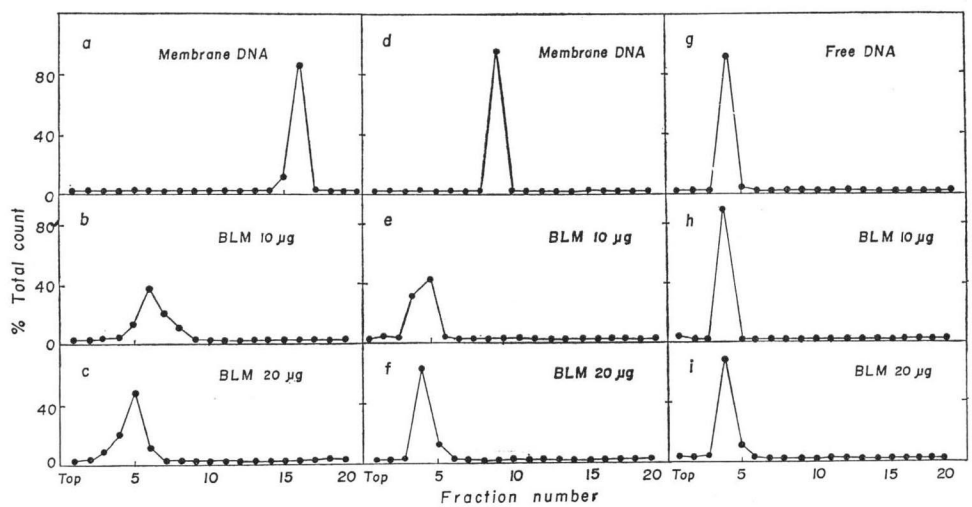
Table 1. Rapidly sedimenting DNA in neutral sucrose gradient centrifugation

Detergent	Lysis conditions		S-value of DNA-membrane complex
	Temperature	Time (minutes)	
SDSa-DOC (0.2%) (0.08%)	20°C	15	1150 S
〃	20	30	800
〃	20	60	600
〃	20	120	400
〃	28	30~60	400
SDS (2%)	28	30~60	200

The cells labeled with ^{3}H-thymidine were lysed under the indicated conditions and centrifuged at 25,000 rpm for 30~60 minutes.

Fig. 1. Effect of bleomycin on sedimentation profile or membrane DNA or free DNA from AH-66 in neutral sucrose

AH-66 cells labeled by ^{3}H-thymidine were incubated with bleomycin at 37°C for 30 minutes and centrifuged in the neutral sucrose gradient at 25,000 rpm for 60 minutes after lysis at 20°C for 60 minutes by SDSa-DOC (a~c), at 28°C for 60 minutes by SDSa-DOC (d~f) or SDS (g~i). Concentration of bleomycin: (a), (d) and (g) 0; (b), (e) and (h) 10 μg/ml; (c), (f) and (i) 20 μg/ml.



scission nor double breakage of the "free DNA", but AH-66F cells were less sensitive to the chemical than AH-66 for breakage of the complex (Fig. 2).

Fig. 2. Effect of bleomycin on sedimentation profile of membrane DNA or free DNA from AH-66F in neutral sucrose

AH-66F cells labeled by ^{3}H-thymidine were incubated with bleomycin at 37°C for 30 minutes and centrifuged in the neutral sucrose gradient at 25,000 rpm for 60 minutes after lysis at 28°C for 60 minutes by SDSa-DOC (a~c) or SDS (d~f). Concentration of bleomycin: (a) and (d) 0, (b) and (e) 10 μg/ml, (c) and (f) 20 μg/ml.

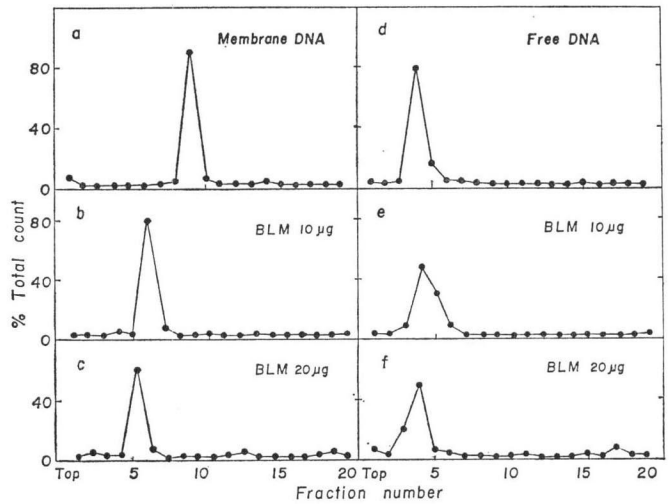
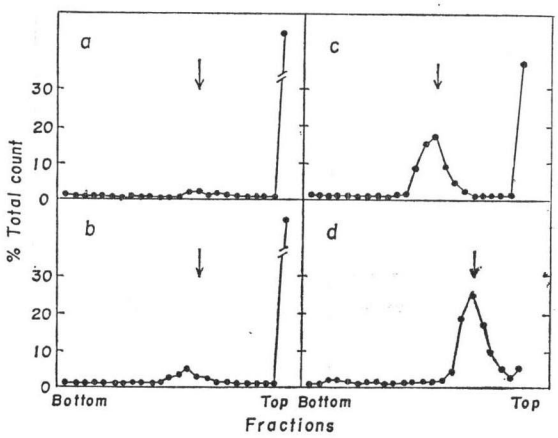
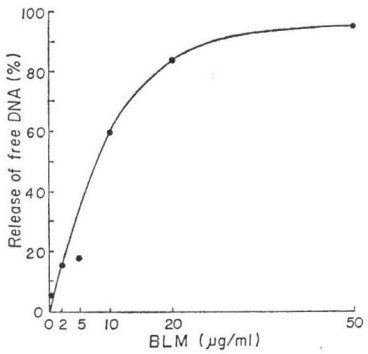


Fig. 3. Release of DNA from DNA-membrane complex by bleomycin in CsCl equilibrium centrifugation

AH-66 cells labeled by ^{3}H-thymidine were incubated with bleomycin at 37°C for 30 minutes and lysed at 28°C for 60 minutes by SDSa-DOC on top of CsCl solution and the tubes were centrifuged at 50,000 rpm for 44 hours. Arrow indicates the position of density 1.70 in CsCl gradient. Concentration of bleomycin: (a) 0, (b) 2 μg/ml, (c) 10 μg/ml, (d) 20 μg/ml. (e) The relative amount of radioactivity at density 1.70 expressed in percent of recovered total activity was plotted.





Effect of Bleomycin on the Release of Free DNA from the Complex in CsCl Equilibrium Centrifugation

When the cells were lysed by SDSa-DOC under the same conditions as those liberating 400S-peak in sucrose gradient, all radioactivity of DNA from untreated AH-66 cells floated on top of the dense CsCl gradient (density 1.75~1.67). Incubation of the cells with 10 μg of bleomycin led to the release of free DNA which banded at a density of 1.70. In parallel with the result from sucrose gradient centrifugation analysis, 20 μg of bleomycin released all DNA as indicated in Fig. 3. Although recovery of DNA was not quantitative, relative amount of the free DNA to the complex increased according to concentration of the chemical. When the DNA-membrane complex from untreated cells was centrifuged in the light CsCl gradient (density 1.55~1.42), 80% of total radioactivity was banded near the bottom and 20% was at a density of 1.48. Treatment of the cells with 10 μg of bleomycin resulted in the appearance of three minor peaks at 1.48 (4% of the total count), 1.44 (15%), and 1.43 (21%) in the light CsCl gradient (not shown), and 60% seemed to be free DNA from the pattern in the dense CsCl gradient (Fig. 3). In the case of cell lysis by SDS such experiments were unsuccessful since the precipitate of SDS floated on top of the CsCl gradient.

Repair of Breaks

Reincubation of the cells treated with 10 μg of bleomycin regenerated the DNA-membrane complex in sucrose gradient centrifugation but the repair was incomplete within 30 minutes at 37°C with respect to 400S-peak and 600S-peak (Fig. 4). These conditions were sufficient for repair of the single-strand scission by 10 μg of bleomycin[6].

Nature of DNA-Membrane Complex

AH-66 cells were incubated with ^{14}C-labeled compounds for 30~180 minutes after pre-

Fig. 4. Reformation of membrane DNA by post-incubation of cells treated with bleomycin

AH-66 cells labeled by ^{3}H-thymidine were incubated with 10 μg/ml of bleomycin at 37°C for 30 minutes and re-incubated without bleomycin at 37°C for 30 minutes. The cells were lysed by SDSa-DOC at 20°C (a and b) or 28°C (c and d) for 60 minutes.

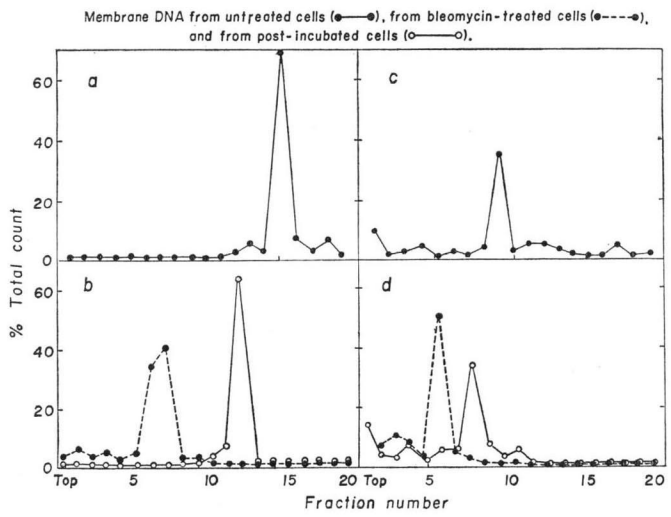


Fig. 5. Incorporation of ^{14}C-precursor into membrane DNA

AH-66 cells labeled by ^{3}H-thymidine were incubuted with 20 μCi/ml of the ^{14}C-compound and DNA-membrane released from the double-labeled cells by SDSa-DOC was sedimented through neutral sucrose gradient. (a) ^{14}C-choline, 120-minute incubation, (b) ^{14}C-leucine, 90 minutes, (c) ^{14}C-uridine, 30 minutes.

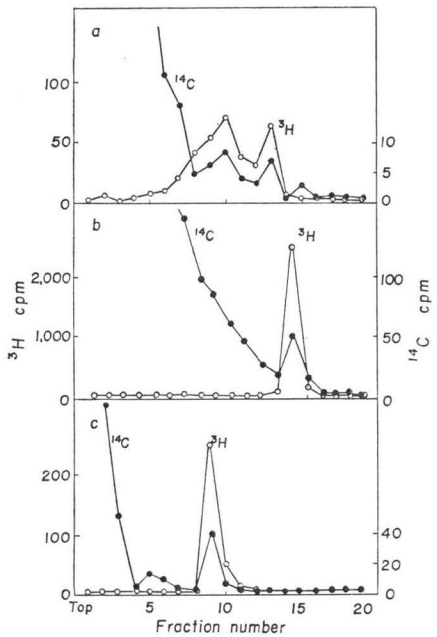
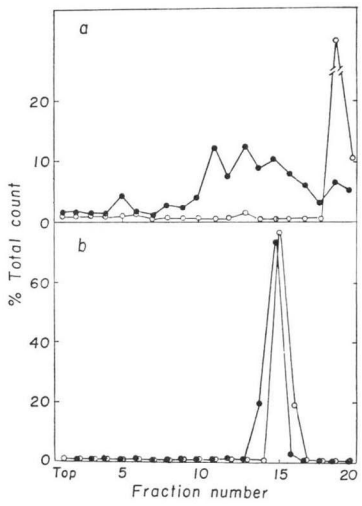


Fig. 6. Effect of Pronase on sedimentation profile of membrane DNA or free DNA

AH-66 cells labeled by ^{3}H-thymidine were lysed on top of the neutral sucrose gradient containing 100 μg/ml of Pronase and the tubes were centrifuged at 25,000 rpm at 20°C. (a) Lysis by SDSa-DOC and centrifugation for 120 minutes, (b) lysis by SDS and centrifugation for 180 minutes. (◦——◦) Control, (•——•) Pronase.



labeling by ^{3}H-thymidine overnight to examine components of the rapidly sedimenting peak (Fig. 5). Only 3% of the incorporated ^{14}C-acetate was associated with 800S-peak and less than 1.6% with 400S when the cells were incubated with 20 μCi/ml of ^{14}C-acetate for 120 minutes. The amount of ^{14}C-choline at 800S-peak was 1.7% of total radioactivity incorporated which was soluble in warm chloroform-methanol solution and the activity associated with 400S-peak was not more than 0.3% of the total. Treatment of the cells with ^{14}C-choline decomposed the peak of DNA complex but the reason was not clear. Incorporation of ^{14}C-leucine into the complex during 90 minutes was about 0.5% of the total ^{14}C-activity in the cells. Labeling of the cells with ^{14}C-uridine for 30 minutes resulted in the association of 4~6% of total ^{14}C-activity with the 800S or 400S peak. Despite the low incorporation of ^{14}C-leucine to the complex, 400S-peak released by SDSa-DOC was decomposed by Pronase in sucrose gradient during centrifugation for 120 minutes, while the free DNA released by SDS was not affected by the same enzyme in gradient during 180 minutes (Fig. 6). The DNA-membrane complex from untreated cells floated on top of the dense CsCl gradient (1.75~1.65) and banded near the bottom in light CsCl gradient (1.55~1.42) as already described. These results suggested that 400S and the larger complex contained lipid, protein, and RNA, amounts of which were assumed to be rather small.

Discussion

Complete lysis of cells by a neutral high concentration of SDS released the "free DNA" which had a sedimentation coefficient of about 200S, while the "DNA-membrane complex" with larger S-value (400S~1200S) was liberated by a low concentration of SDSa-DOC from untreated AH-66 or AH-66F cells. The latter appeared to contain membrane components such as lipid, protein, and RNA from the results of double labeling experiments. Although the radioactivity associated with the DNA complex was less than 2% of the respective ^{14}C precursors incorporated into the cells, density of the complex (1.55) in CsCl gradient indicated that DNA was not present as a free molecule. Sensitivity of the complex to Pronase also suggested inclusion of protein. It is also possible that protein and lipid in the DNA complex are component of chromatin, but ORMEROD and LEHMAN[18] suggested the DNA-lipid attachment in the complex released by dilute SDSa-DOC solution. It has been demonstrated by electron microscopy that chromatin fibers have attached to the annuli of nuclear membrane in mammalian interphase nuclei[10,11] and that the complex released by SDSa-DOC probably includes the annuli material. BURREL *et al.*[14] have described a complex with density of 1.50~1.60 which contained less than 2% of total active label for RNA, protein, or lipid and temporarily appeared as an intermediate during the repair of double-strand break by X-irradiation in the large DNA-membrane complex (density 1.40) from *Micrococcus*. Similar density was reported for unfragmented DNA released from spheroplast of *Escherichia coli* by dilute SDS and protease[15].

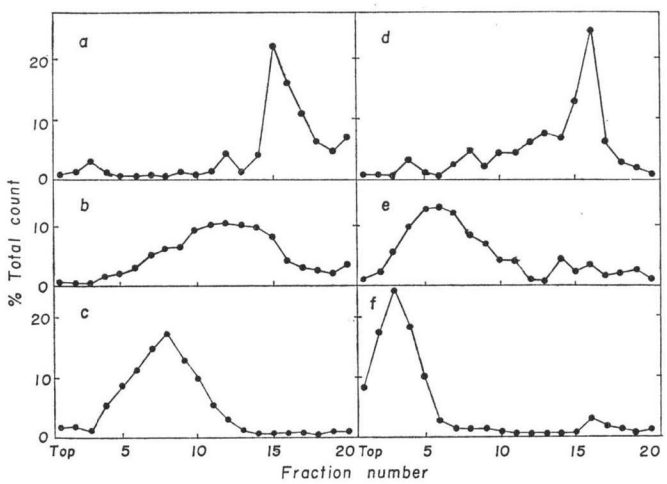
The free DNA banded at a density of 1.70 in CsCl gradient was released from "DNA-membrane complex" and S-value of the complex in sucrose gradient was decreased at the same time by incubation of AH-66 cells with a low concentration of bleomycin which did not cause double strand scission in the "free DNA". The attachment sites of DNA to membrane, being stable to SDSa-DOC but labile to SDS, seemed to be decomposed by the treatment with this chemical. After release of free DNA (density 1.70) by 10 μg of bleomycin, the residual DNA was banded in CsCl gradient at densities of 1.44~1.43 which were lower than the initial density of the complex (1.55). This result suggests that bleomycin causes double-strand scission in DNA near the membrane attachment site, at which the break hardly changes the S-value of total free DNA, and small pieces attaching to membrane material were banded at the lower density. While, the membrane-DNA complex from the cells treated with the same concentration of the chemical sedimented through the neutral sucrose gradient at a rate similar to or somewhat more rapid than the free DNA, indicating that most of the complex was decomposed by 10 μg of bleomycin. Such difference in amount of free DNA between banding in CsCl and sedimentation in sucrose seemed to be due to difference in the conditions of cell lysis between the two analytical procedures. The cells were lysed by SDSa-DOC layered on high concentration of CsCl in equilibrium centrifugation (Fig. 3) but the cells were lysed by SDSa-DOC layered over neutral sucrose in sedimentation analysis (Fig. 1).

Number of single-strand break in DNA was 1 per strand when AH-66 cells were treated with 10 μg of bleomycin[6]. Repair of the damage in DNA-membrane complex was incomplete after 30 minutes of post-incubation which was sufficient for repair of a single-strand scission. If release of free DNA was due only to the single-strand break, regeneration of the complex would be completed within 30 minutes, similar to the repair of a single break. The ratio of double-strand breaks to single-strand breaks produced by X-irradiation *in vitro* was about 1:10[16] and that for DNA in *Micrococcus in vivo* was 1:13[14]. The frequency of double-strand scission by bleomycin *in vitro* was 13- to 17-fold lower than that of a single break per DNA double helix (Fig. 7). It is reasonable that the amount of double break was extremely small *in vivo* as demonstrated previously[6] and no break of free DNA was observed in neutral sucrose by treatment of the cells with 10 μg of bleomycin (Fig. 1). Calculation by ORMEROD and LEHMAN shows that 50% release of DNA from the membrane complex occur when three random double breaks are introduced by X-ray in DNA between adjacent membrane attachment

Fig. 7. Double and single strand scission of DNA by bleomycin *in vitro*

^{3}H-λ-DNA was incubated with bleomycin under the same conditions as described[18] and centrifuged in 5~20% neutral (a~c) or alkaline (d~f) sucrose gradient at 20,000 rpm for 16 hours.

Concentration of bleomycin: (a) and (d) 0, (b) and (e) 10 μg/ml, (c) and (f) 1 μg/ml+10 mM mercaptoethanol.



site. Release of at least 60% free DNA by a far smaller number of breaks than that expected for X-irradiation indicates that bleomycin acts not only on DNA strand but also on the membrane material. About 20% of the radioactivity of ^{14}C-bleomycin incorporated into the nuclei of AH-66 was associated with the protein fraction of phenol extraction and 2% was bound to DNA[17]. This result suggests that bleomycin possibly affects nuclear proteins including a membrane component. In a similar manner as decomposition of the DNA-membrane complex by acting on the membrane material, bleomycin may break the mitotic apparatus such as centriols and spindles, resulting in the inhibition of mitosis.

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