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Detection of the Recovery Substance for Cell Divison in UV-Irradiated *Escherichia coli* B

——Stabilization of the Active Substance by Magnesium——

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紫外線 照射한 大腸菌 B株의 細胞分裂 回復活性物質

——Magnesium에 依한 活性物質의 安定化——

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(1979년 6월 20일 수리)

Abstract

Recovery component for cell division in UV-irradiated *E. coli* B was detected with use of the cell extract of *E. coli* B/r which is a resistant mutant of *E. coli* B against UV-irradiation. The active substance was non-dialyzable and increased the activity by adding ß-NAD remarkably. One more factor for increasing or promoting the restoration recognized was magnesium. Magnesium was effective to stabilze the substance in procedure of isolation. Two active substances were obtained from sucrose gradient centrifugation. One of them was recovred from the bottom area and the other from top area just below below surface. the former was not stabilized by magnesium, while the latter stabilized the activity by it remarkably. The former which did not require magnesium was insensitive to protease and the latter which required magnesium was sensitive to it. Both were insensitive to RNase and DNase. Recovery ratio was doubled by using nitrogen gas than aeration in purification process. DNA-ligase less mutant was revealed same activity on it's recovery ratio with the parent strain of *E. coli* K-12. The active substance stimulating the filament cell may exist as a complex which is inactivated easily in the dissociated state and requrie ß-NAD or magnesium.

Introduction

Escherichia coli B or lon-mutant of *E. coli* K-12 form long, moribund filaments with nonseptated and multinuclea after exposure to low dose of UV irradiation, although their synthesis of protein and nucleic acid continue normally[1,2].

Since reported the phenomenon termed as "neighbor restoration" by Delarpote[3] in 1956 which cell division is much more likely to occur in UV irra-

diated *E. coli* B if the cells aretightly grouped on agar plate, isolation of the substance stimulating or promoting cell division activity to cytokinesis in radiation damaged individuals was studied hardly.

K. S. Korgaonker *et al.*[4] reported the active substance from a nucleoprotein fraction in cell freeextract by lysis. H. I. Adler *et al.*[5] described the presence of another division stimulating substance whic was membrane -associated sprerical particle with density of 1.22 and a sedimentation coefficient of 100S.

Recently, Y. Yoshiyama *et al.*[6] identified ß-NAD from cell extracts as a cofactor for the active substance. Some kinds of high molecular substance which can not permate collodion membrane react as an active substance to cytokinesis on UV damaged filament cells under the presence of ß-NAD on agar plate.

The above finding clerifies that Mg^{++} should be needed for protecting the aciivity of cell free extract and two active bads are distributed in sucrose density gradient centrifugation. The extract of ligase less mutant was tested as a division stimulating substance to detect whether membrane protein fraction has the activity or not.

Materials and Methods

Organisms; The filament-forming test organism was *Escherichia coli* B and the source of cell division stimulating extract was UV resistant strain of *Escherichia coli* B/r storaged in the laboratory of microbiology, Department of Agricultural Chemistry, The University of Tokyo.

Medium; The liquidymedium was 1.2% of nutrient broth (Kyokutou, Japan). Agar plating medium was added 0.9% of Difco agar. The organism was grown in liquid media with shaking at 37°C for 2 or 3 hr to 2×10^8 cell/*ml* after inoculation with 0.1% of cell suspension, and then culturedo vernight at the same condition.

Irradiation: Late logarithmic cultures of *E. coli* B in above condition were dilted to 100 with 0.067 M phosphate buffer (pH 6.0). Four ml of the diluted suspension were added to petri dish (2×10cm) in three casfs each of which was exposed to 15W germicidal UV-lamp at a distance of 50cm for 20 seconds. For standardization, three cases cell suspensions were mixed together.

Aossay procedure; Assay for the activity of division stimulating substance was carried out by buring the cell suspension of irradiated *E. coli* B in bouillon agar plate (2×6cm) with or without ß-NAD(Oriental Yeast Co.) and the test materials which were passed through a 0.45u membrane filter (Sartorius Co.) to remove contaminating cells and counting the macrocolonies after irradiation.

Final concentration of about 0.3mg of ß-NAD per ml of plating medium and 1–2m*l* of the extract was added to the petri dish, and then 0.1m*l* of the irradiated cell suspension was noculated just before pouring 4m*l* of bouillon agar medium made previously in test tube at 45°C. The plate was swirled gently to ensure mixing of the test organism with other components. It was transferred to an incubator immediatly after hardening and counted the coleonies after 48 hr incubation at 37°C. 0.067M potassium phosphate buffer (pH 6.8–7.0) containing 2mM of Mg^{++} was used throughout this experiment.

The cell division activity of the substance was reported in terms of "*division index*" which was the ratio of the number of macrocolonies in the presence of a test material to that in the absence of it.

Preparation of division stimulating extract; The cells of UV resistant strain of *E. coli* B/r were grown at 37°C with aeration in nutrient broth and harvested by centrifugation at the late logarithmic phase, then washed twice with cold 0.067M phosphate buffer (pH6.8) and stored −20°C One gram of packed cells was suspended to 2m*l* of the same buffer, then disrupted by sonic treatment for 15 min. after suspending one min. of Waring blender treatment.

The lysed cell by sonication was centrifuged at 20,000 rpm for 1 hr with a Hitachi RP-32 rotor. The supernatant fluid was used as the starting material for further purification.

Sonic treatment was performed under 2000V, 300mA by Toyorica Sonicator(JAPAN) and cont-

rolled pH to 6.8—7.0 with ammonium water just after sonic treatment.

Purification on sucrose gradient centrifugation; The sonic lysed supernatant was charged on the surose gradient centrifugation[7,8]. 1—1.5m*l* of the sample was loaded onto 14.5 m*l* of 10-30% sucrose gradient with 55% cushion. The gradient contained 0.067M phosphate buffer (pH 6.8). Centrifug eruns 20,000 rpm for 18 to 20 hr at 4°C by Hitachi RPS-3A-25 rotor. The gradient was fractionated in 1 to 1.2 m*l* (20 drops) of sample by LKB fraction collector at 4°C, then determined UV adsorption by Hitachi-double Spectrophotometer. The fractionated gradient was dialysed in cellophane tube against 0.067M phosphate buffer (pH6.8) for 6-10 hr at 4°C.

Enzyme treatment; The division stimulating extract was treated with RNase, DNase and pronase for determining inaction or inhibition of biological activity. The incubation mixture was consisted of the fraction with 0.067M phosphate buffer and final concentration of 100 ug/m*l* of each enzyme solution. Incubation was carried out at 37°C for 30 min. In each case, buffer was used to replace either enzyme or stimulating substance as a control. (DNase and RNase used were products of the Sigma Chem. Co. N. Y., extract from beef pancreas, and pronase was a product of Kaken Kagaku Co.)

Preparation of membrane protein; The membrane protein was fractionated from cell extract by the method of Inouye *etal*[9].

Newly cultured cells of *E. coli* B/r in 100m*l* of 1.2% nutrient broth by shaking was gathered after centrifugation at 10,000 rpm for 10 min and washed with 0.067M phosphate buffer. The pellet was sonicated (Ohtake Co. 7A) for 3 min in 10 m*l* of buffer, then removed the pellet by centrifuging at 5000 rpm for 10 min at 4°C. The membrane fraction was held as pellet by centrifuging at 40,000 rpm for 30 min with Hitachi RP-65 rotor, then suspended in 5 m*l* of phosphate buffer. All preparation were carrried out in 4°C.

Preparation of the extract of ligase less mutant; The strains used were KH-39, KN-321 and KN-350 from T. Nagata *etal.*[10] The relevant genoytpe and DNA ligase activity of the crude extract was like that: KH-39; dnaL$^+$ sup-126; 1.00, KN-321; dnaL 321 sup-126; 0.12, KN-350; dnaL321 sup-126 sup F$^-$; 0.55. The ligase activity was demonstrated as a relative activity of joining 5′-^{32}P in nicked dAdT and cultured temperature was 30°C. Cell extract was used from the supernatant of 40,000 rpm for 30 min after sonic treatment of cell suspension that about one gram of wet cells of each strain was dissolved with 3 m*l* of phosphate buffer. 0.7∼1.5 m*l* of cell extract was assayed.

Results

Sensitivity to UV irradiation; To determine sensitivity and optimal dose of irradiation, the survial ratio of *E. coli B* was tested in bouillon agar after exposure to UV light for a given time, and was compared to that of *E. coli* B/r, a resistant mutant of UV. Most of the *E. coli* B cells were affected in 20 sec. exposure under UV irradiation. Within 10 to 30 seconds, *E. coli* B showed about one hundredth of *E. coli* B/r in survivors. (Fig. 1)

Cell division stimulating activity; Colony formation, in case of the extract added to the medium with ß-NAD was promoted remarkably compared to the medium with added extract only. The maximal division index of colony formation was shown in

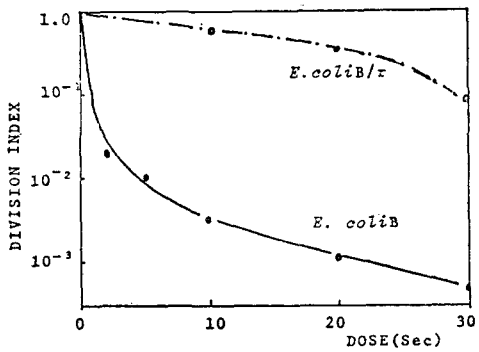

Fig. 1. Survival of *E. coli* B and *E. coli* B/r after UV Treatment.

Late logarithic cultures of *E. coli* B and it's resistant mutant of *E. coli* B/r was irradiated by UV, and survival ratio was determined by colony formation on agar plate. See precicely in Materials and Methods.

about 0.07m*l* of the extract per ml of plating medium.

Out of the plates with different concentration of ß-NAD in the added extract, the one with 0.3 mg of ß-NAD/m*l* of plating medium showed the maximal division index. (data not shown). Division promoting activity was to be double the plate with added ß-NAD only. The division activity of extract was 20 to 30 times more in the maximal multiplication condition and it was possible by adding ß-NAD together.

Sephadex G-100 column chromatography; The active fraction was recovered at late elute of main protein band sharply by Sephadex G-100 column chromatography (2.5×40 cm). But the activity band was shown as two peaks in large size of column (5.0×50 cm). Both of the active peaks in large column showed shimilar pattern in it's protein distribution on sucrose gradient centrifugation. About to 80% of the total activity was recovered by this method. When the extract was chromatograpged in Sephadex G-100 column after previous fractionation by sucrose gradient or precpiitation with ammonium sulfate or acetone, the recovery ratio of active substance reduced remarkably.

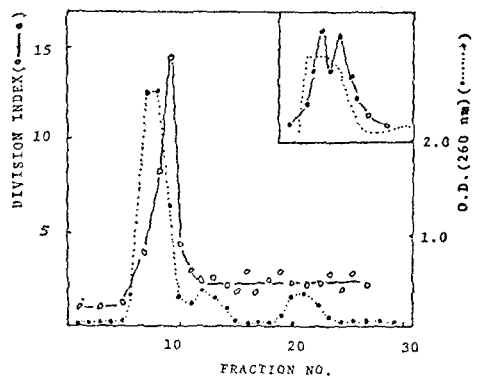

Fig. 2. Sephadex G-100 Column Chromatorgraphy.
The cell extract of *E. coli* B/r was chromatographed on Sephadex-100 column (2.5×40 cm) stabilized with 0.067M phosphate buffer previously and each fraction was checked out the recovery activity on agar plate. The activity peak devided two bands sharply on large column (5.0×50 cm).

Centrifugation in sucrose gradient centrifugation; Two of the active fraction were recovered from sucrose gradient centrifugation. One of the was distributed in bottom of the tube and the other just below the surface in the 11th fraction as in the case of the egg albumin as a marker.

Approximately 80% of biological activity was recovered. Centrifugation of sucrose gradient in Hitachi RPS-25-3A rotor for 20 and 48 hr indicated same distribution, and the division promoting activities is isopycnic in sucrose gradient.

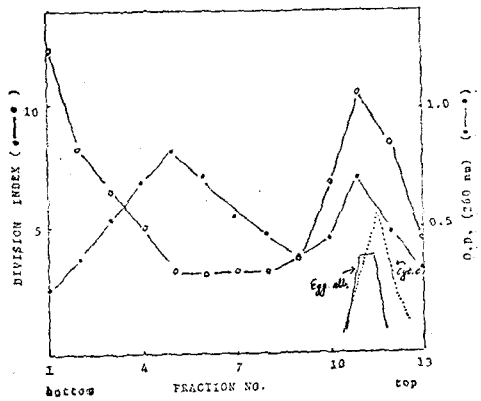

Fig. 3. Sucrose Gradient Centrifugation of Crude Extract.
Centrifugation of 10-30% of sucrose gradient runs 20,000 rpm for 18 to 20 hr at 4°C by Hitachi RPS-3A-25 rotor. 1~1.2 m*l* of sample was fractioned at 4°C, then determined the activity after dialyzed with cellophane tube. Two active band was recognized by this procedure. One of then was recovered on bottom area and the other top area which was similar zone with egg albumin (MW 45,000) as a marker.

Sensitivity to enzymes; Both of the active fractions in sucrose gradient were not sensitive to RNase and DNase from beef pancreas as shown in table 1. But, lower molecular substance was sensive to pronase; inactivated about 50%. As a control, pronase itself had no effect on survival when added directly to the plate after same treatment.

The membrane protein, prepared by the method of Inouye *etal.*[9] did not show the cell division promoting activity. (Table 1)

Table 1. Sensitivity of Active Fractions to Enzymes.

case	division index lower m.	higher m.	memb.	enzy.
DNase	15.74	2.06	1.14	1.10
RNase	16.44	2.67	1.56	1.72
pronase	8.20	2.33	1.03	1.14
none	16.98	2.17	1.50	—
no treat	16.32	2.09	1.06	—

Both fractions after sucrose gradient centrifugation and membrane fraction were incubated with 100 ug /m*l* of DNase, RNase and pronase, respectively, at 37 °C for 30 min, and added to agar plate as an active component. Enzyme solution only added as a control. lower m; lower molecular substance (upper fraction), higher m; higher molecular substance (bottom fraction), memb; membrane fraction, enzy; enzyme solution only.

Effect of metal ions on the activity; Division promoting activity was decreased in the process of purification; gel column chromatography or dialysis with collodion or cellophane membrane. So, it suggested that some kind of dializable material may be related to the activity excepting NAD. As shown in Table 2, Fe^{++}, Fe^{++}, Fe^{+++}, Na^{+} didn't effect to the activity. Mg^{++} and Mn^{++} enhanced the activity, whereas Zn^{++} and Ca^{++} induced inhibitory effect.

Each material was dissolved in distilled water and added to the plate after controlling pH to 7.0 in the final concentration of 10^{-3} M/m*l* with dialyzed cell extract by cellulose tube for 12 hr at 4°C.

Table 2. Effect of Metal Ions to Dialysed Crude Extract.

metal ions (1mM)	colony number	survial ratio
Fe^{++}	172	1.1
Fe^{+++}	180	1.2
Mg^{++}	280	1.8
Zn^{++}	37	0.2
Ca^{++}	74	0.4
Na^{+}	197	1.3
Mn^{++}	580	3.7
none	156	1.0

10^{-3}M/m*l* of each metal solution was added to the plate after control pH to 7.0 with dialyzed cell extract by cellophane tube for 12 hr at 4° C. Magnesium and mangane were react as an activator, whereas Zn^{++} and Ca^{++} as an inhibitor.

Effect of magnesium ion on the activity; Division promoting activity was tested by using various concentrations of magnesium ion in order to determine the optimal concentration. The activity was decreased remarkably in high concentration of Mg^{++} compared to the increase in lower concentration. About 2 mM of Mg^{++} was the optimal concentration in the extsact. (Fig. 4)

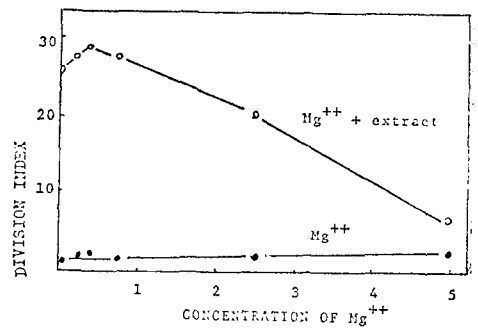

Fig. 4. Effect of Mg^{++} on Crude Extract.
Each concentration of magnesium mixed with non-dialyzable fraction of extract, and then poured out into agar plate in order to determine the optimal concentration of magnesium for highest division index. 2 mM of magnesium was shown the optimal condition and in the case of adding magnesium only was not effected.

In order to know the effect of Mg^{++}, so-called protection or activation, experiment was performed in the presence of Mg^{++} or not from the first step of preparation of cell extracts. As a result, the diffference between presence and absence of Mg^{++} was recognized remarkably.

In sucrose gradient, one of the active fraction distributed in upper layer was masked, but the other in bottom area was revealed sharply in the absence of Mg^{++}, while both of them demonstrated sharply in the presence of Mg^{++}. The upper peak required Mg^{++} essentially for the activity and the bottom peak did not require Mg^{++} (Fig. 5).

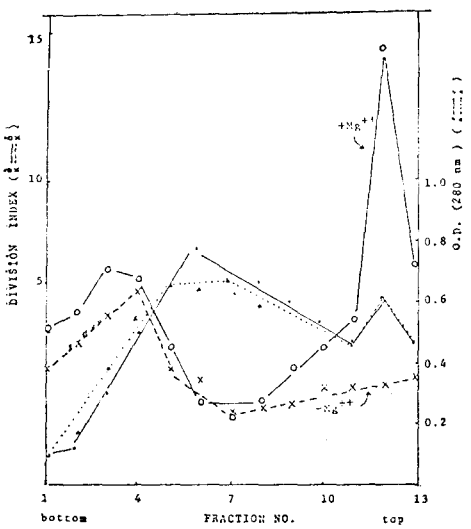

Fig. 5. Effect of Mg^{++} on Sucrose Gradient Centrifugation.

As shown in fig. 6, the cell extract was column chromatographed The recovery component of *E. coil* B/r extract was isolated by 10~30 % of sucrose density gradient centrifugation in the presence of 2 mM of Mg^{++} or not. Bottom fraction (higher molecular substance) was not effect on it's activity by addion of magnesium, while the most activities of lower moleclar substance was masked in the upper

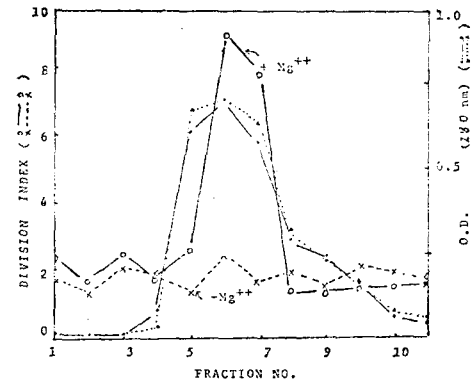

Fig. 6 Effect of Mg^{++} on Sephadex G-100 Column Chromatography.

The active peak was masked by absence of magnesium. If 2 mM of magnesium was added to cell extract of *E. coli* B/r, main active peak reveled sharply. Disvision index: (○——○) added Mg^{++}, (×·····×) not added Mg^{++}, OD 280 nm: (●——●) added Mg^{++}, (▲·····▲) not added Mg^{+}r. fraction absence of magnesium.

The active peak was not observed in absence of Mg^{++}, on Sephadex G-100 in both the presence and absence of Mg^{++}. whereas that was revealed sharply in the presence of Mg^{++}.

Effect of nitrogen gas on the substance solution; Nitrogen gas was used instead of aeration in preparations of sucrose gradient mixture, fractioning after centrfugation and dialyzing the outer solution. In the presence of nitrogen gas, each step recove-

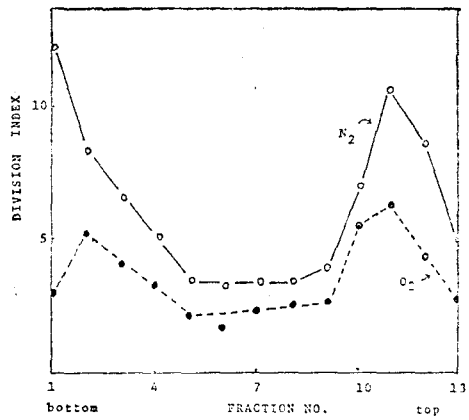

Fig. 7. Effect of Nitrogen Gas on the Process of Sucrose Gradient Centrifugation. If nitrogen gas was used in preparations of sucrose gradient mixture, fractioning after centrifugation and dialyzing the outer solution, the activity was doubled in both fractions compared to aeration process. (○——○) nitrogen gas used, (●·····●) aeration process.

Table 3 Division Promoting Activity of Ligase Less Mutant.

strain	relevant genotype	ligase activity	vol (ml)	division index
KH-39	*dna* L^{+}	1.00	7.7	7.3
	sup-126		1.5	25·3
KN-321	*dan L(am)321*	0.12	0.7	5.4
	sup-126		1.5	26.4
KN-350	*dna L(am)321*	0.55	0.7	7.4
	sup-126, susIII^{+}		1.5	26.6

Cell extract of DNA-ligase less mutant of *E. coli* K-12 was added to the medium. Ligase less mutants; KN-350 and KN-321 are shown same division index with the parent strain; KH-39.

red about 2 times of activity in both fractions compared to that in the aeration process. (Fig. 7)

Division stimulating activity ofligase less mutant: To determine whether the division stimulating substance is related to ligase or not, the extract of an amber mutant (KN-321) of E. coli K-12 affected DNA ligase was used as a source of cell extract. In table 3., ivision index of lig mutant was not different from that of lig$^+$strain, a parent strain. (Table 3)

DISCUSSION

There are many reasons as to why bacteria form filaments. Filaments are formed due to the low dose of agents which inhibit growth[11,12,13,14,25,30], in the case of fast growth in rich media[16,17,18,19,20,21] and by reversing growth suddenly slowly by chloramphenicol[21,22,23], nutritional shift down[19] and liquid holding[24].

And under UV irradiation, bacteria can be devided into two groups like as growth-inhibited and division-inhibited group[25]. The former can not grow appreciably in rich media after lost of irradiation, while the latter can grow, but fail to devide, what is called, filaments formers. Phenethyl alcohol and butyl alcohol like pantoyl lactone have been proposed that they reverse the UV damaged filament formers to devide by suppressing the initiation of DNA replication and by stimulating activities[26,27,28].

Since known a "diffusible substance" from doner cell extract which can devide by stimulating to UV inactivated filaments and which originated from the"neighbor restoration"phenomenon, may researchers have been trying to isolate the active substance[29].

The fact that many kinds of cell extracts were cappable to stimulate the cell division of filaments, non-specifically, makes this approach somewhat more attractive. Division index of *E. coli* B extract to UV irradiated *E. coli* B itself was 26.9, while that of *E. coli* B/r which is a resistant mutant to UV was 29.1[31].

Many kinds of biochemical methods for isolation of cell division stimulating substance were introduced. Among them, the sucrose gradient centrifugation method was the most effective tool for recovering the activity. Gel colum chromatography by Sephadex G-100 or Sephalose 4-B was introduced at the first step, too. But, in this case the purity was less and the ratio of inactivation was higher than sucrose gradient centrifugation.

Two of the active fractions were distributed in sucrose gradient. One of them was at the bottom of the gradient, the other other near the peak of egg albumin (molecular weight:about 45000) just below upper layer. When purified in the presence of Mg^{++}, two of active fractions were observed in the sucrose gradient, whereas only one active fraction was revealed in the absence of Mg^{++}. The upper fraction was masked in the absence of Mg^{++}. Therefore, the bottom fraction did not require Mg^{++} for the activity compared to the upper fraction required Mg^{++}. Adler *etal.*[5] reported the active substance which was distributed in bottom area only, because the purification was conducted in the absence of Mg^{++}. The lower molecular substance as an active substance was effected remarkably by Mg^{++} for preservation and activation of the cell division stimulating activity. In the absence of Mg^{++}, the activity peak was not observed in Sephadex G-100 column chromatography. Here, Mg^{++} was essential for the activity like NAD, and may react as a considerably important factor for maintenance of the cell division promoting activity.

Mg^{++} is important biologically for preventing dissociation of ribosome and membrane fraction, and protecting or catalyzing some kinds of enzyme activity. For example, membranes obtained by osmotic pressure shock from the protoplast of *Pseudomonas* differed, whether magnesium ions were present or not, for it's preservation of original state. Membranes obtained in the absence of magnesium ions did not oxidize succinate and malate; but those, obtained in a medium containing Mg^{++}, oxidized these substrates readily[32]. And it has been suggested that the membrane obtained from halophilic bacteria prevents itself from breaking down into small fragments in the presense of

Mg^{++}[33]. D. Kennel *etal.*[34] reported the effect of Mg^{++} on the survival of *Aerobactor aerogenes* on the aspect of cytochemical change.

According to Adler *etal.*, the active fraction located in bottom area in sucrose gradient was suggested as a particle-associated substance because of it's localization in sucrose gradient. The chemical analysis of the bottom partiole did not show RNA or DNA and the activity was not inhibited by the tseatment of RNase or DNase[5].

K. S. Korgaonker *etal* reported that the active itca vesubstance detected in the nucleoprotein fraction of the cell-free extract by lysis, did not appear to be identical with that isolated by H. I. Adler *etal.* The nucleoprotein fraction was inactivated perfectly by DNase or RNase treatment, but the purified RNA or DNA from the nucleoprotein fraction pid not show the activity[4].

By the enzyme treatment of both fractions separately, the bottom fraction was found to be insensitive to pronase, while the upper fraction was sensitive to it. Both were insensitive to DNase and RNase in this experiment.

Considering the two reports, the active fraction from bottom area in sucrose gradient may be correlated with the active fraction reported by Adler et al, but the upper fraction near surface is not clear whethr it correponds with the active fraction reported by Korganonker et al or not. And it can not be clarified whether active substances in both fractions under consideration are identical with each other or not.

In the culture with the added cell extract containing little ligase activity, was prepared from ligase less mutant of *E. coli* K-12 instead of *E. coli* B/r, the division promoting activity of this lig-mutant was similar to that in the case of parent strain. So, the activity in non-dialyzable fraction of the cell extract may not be ligase.

The active substance was labile to heat and acid or alkaline pH range. Majority of the active substance was precipitated in 33~50% of acetone fraction and in 30~50 % of ammonium sulfate fraction. (Data not shown) When the precipitate after fractionating with ammonium sulfate or acetone charged to sucrose gradient, the distribution of protein was similar with the pattern of original solution, although ribosomal band was somewhat reduced.

Recovered fraction by the treatment of ammonium sulfate precipitation or gel column chromatogaphy reduced the activity about 30~40% rompared to the original extract. Continuec purification process by ion-exchange coldmn chromatography or preparative electruphoresis after fractionation with precipitaoion, gel column chromatography, and sutcrose gradient centrifugation, the activity was reduced remar kably or was not detactable. The effect of Mg^{++} as a cofactor in protecting the activity may be related to those phenomenon importantly. And if feasible, the recovery ratio would be better using nitrogen gas than aeration in all processes of purification.

Adler's report suggests that suggests that the active substance may be a particle associated, especially with membrane-associated, on account of low density of the division promoting activity and it's sensitivity to phospholipase, lipase and detergents. But the membrane protein fraction obained by the method of Inouye *et al* was not effective to the stimulation of cell division. It would be inappropriate to suggest the location of the active substance in cell at this state of knowledge.

The active substance stimulating the filament cell may exist as a complex which is inactivated easily in the dissociated state and require ß-NAD or Mg^{++}.

Acknowledgments

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要 約

紫外線照射한 大腸菌B株의 細胞分裂回復活性分을 究明코저 紫外線耐性菌인 大腸菌 B/r 株의 超音波抽出液으로부터 活性成分을 分離한 結果 β-NAD가 關與함이 發表되었다. 本稿에서는 β-NAD 以外 Magnesium이 活性物質의 安定化가 重要한 役割을 나타냄을 究明하였으며 10~30%의 庶糖密度勾培遠心分離에依해 2個의 새로운 活性部分이 있음을 確認하였다. 2個의 活性物質 가운데 하나는 遠心管의 最下部에 位置하였으며 또 다른 하나는 上部의 分子量 45,000 部位에서 回收되었다. 下部에 位置한 活性劃分은 Mg^{++}이 그 活性에 無關하였으나 上部의 低分子 活性部分은 Mg^{++}을 添加하지 않으면 回收가 不可能하였다. 低分子活性部分은 pronase에 對해 感受性이었으며 DNA-ligase는 아님이 推定되었다. 超遠心分離過程에 N_2 gas를 處理할 境遇 aeration에 比해 約 2倍의 活性이 나타났다. Mg^{++}은 β-NAD에 또하나의 回復活性 및 必須的因子로 要求된다고 생각된다.

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