Acta Medica Okayama

Volume 28, *Issue* 1 1974 *Article* 2

FEBRUARY 1974

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Abstract

Gornin was extracted from bovine liver. The effects of cornin on DNA synthesis were compared with its effects on cell growth using L cells growing in suspension. As the first step of this experiment, a simple method of suspension culture was established with a new modification of YLE medium. Both effects of cornin paralleled with dosage. And the properties of the inhibitory factor of DNA synthesis are the same as those of growth inhibitor in respect to the heat stability and impermeability against dialyzing membrane. The inhibitor of DNA synthesis could not be separated from that of growth by gel filtration with Sephadex G-75.

*PMID: 4276080 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **28**, 7—17 (1974)

STUDIES ON THE CORNIN EXTRACTED FROM BOVINE LIVER
II. INHIBITORY EFFECT OF THE CORNIN ON DNA SYNTHESIS AND CELL GROWTH OF L CELLS CULTURED IN SUSPENSION

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Received for publication, August 21, 1973

Abstract: Cornin was extracted from bovine liver. The effects of cornin on DNA synthesis were compared with its effects on cell growth using L cells growing in suspension. As the first step of this experiment, a simple method of suspension culture was established with a new modification of YLE medium. Both effects of cornin paralleled with dosage. And the properties of the inhibitory factor of DNA synthesis are the same as those of growth inhibitor in respect to the heat stability and impermeability against dialyzing membrane. The inhibitor of DNA synthesis could not be separated from that of growth by gel filtration with Sephadex G-75.

It has been reported that "cornin", a fraction extracted from adult animal tissues with the method of NISIDA and MURAKAMI (1) has inhibitory effects on the growth of cells (2). It has also been demonstrated that some of the cornin fractions examined, such as those extracted from skeletal muscle and cornea, have inhibitory effects on the synthesis of DNA (3, 4, 5, 6). However, it still remains obscure whether in cornin fraction, the inhibitory factor of growth is identical with that of DNA synthesis. To solve this question, the purification of the factor is needed, because the cornin is not a pure substance (7) and its composition differs slightly depending on tissues (7, 8, 9). The purification method of the growth inhibitory factor in liver cornin has been established in the previous paper (10). This time the investigation was carried out to see whether some fraction obtained during the course of purification of growth inhibitor in liver cornin has also an inhibitory effect on the synthesis of DNA and whether the effect on the growth parallels with that on the DNA synthesis.

MATERIALS AND METHODS

The methods for extraction, dialyzation and biological assays of liver cornin were the same as those reported previously (10). The aqueous solution of 50 mg undialyzable part of liver cornin was loaded on a column Sephadex

G-75 (3.5×42 cm) and eluted with distilled water. The effluent was fractionated to 4 ml each. A part of each fraction (0.2 ml) was diluted 20 times with distilled water. The optical densities of each diluted fraction were measured at 260 mμ and 280 mμ. The rest of the undiluted effluent from the 19th to 75th fractions was divided into 3 groups (F I to F III). Each group was then lyophilized.

The contents of DNA, RNA and protein were estimated by the methods as described previously (10). GOMORI's method was employed for the determination of inorganic and organic phosphates (11).

Nucleotides were separated by ascending method in paper chromatography after hydrolyzation with acid (1 N HCl) at 100°C for one hr or alkali (0.3 N KOH) at 37°C for 18 hr. The solvent used for development was composed as follows; isopropanol 68 and 6 N HCl 33.3 were made to 100 by addition of water. The spots of nucleotides were detected under an ultraviolet lamp.

L cells originating from mouse fibroblasts were cultured in suspension. The cell suspension was sealed in an Elremyer flask with a silicon stopper and was shaken at the frequency of 40-50 rev/min in a water bath at 37°C. Culture medium was freshly replaced every 4 or 5 days. The composition of the medium is shown in Table 1. The inoculum size was adjusted from 1.0×10^5 to 1.5×10^5 cells per ml by the time of medium replacement, using a hematocytometer. The inhibitory factors dissolved in the medium were sterilized by Millipore filtration and were added on the cell suspension.

For the estimation of DNA synthesis in the cells, the following two methods for labeling were employed. 1) ^{3}H-thymidine (specific activity 25 Ci/mM) was added on the cell suspension after two hr of preincubation with or without inhibitors. 2) The radioisotope was added at 6, 12 and 24 hr after starting the treatment by inhibitors. In both cases, the concentration of the radioisotope was 0.2 μCi/ml. The aliquot of the cell suspension (5×10^5 cells) was then collected on a sheet of glass fiber filter by suction at a given time after the addition of the radioisotope, and washed thoroughly with ice cold 5% perchloric acid, 80% ethanol and absolute ethanol. The cells on the filter were dried under an infrared lamp.

Radioactivities were measured by a liquid scintillation counter (Packard, 3,000 series). The scintillation liquid used was composed of 5 g PPO (2,5-Diphenyloxazole), 0.05 g dimethyl-POPOP (1,4 bis-[2(4-Methyl-5-phenyloxazoyl)]-Benzene) and 1,000 ml toluene. Some cells were fixed by a solution composed of acetic acid 1 and ethanol 3 in proportion and spread on a slide glass. After the extraction with 5% of ice cold perchloric acid, the cells on the slide glass were washed with ethanol and dried. The autoradiogram was made with Sakura NR-M2 emulsion.

RESULTS

For the studies on the biochemical process in the cells, such as DNA synthesis, it is more convenient to use the cells cultured in suspension than in monolayer, because any desired number of the cells could be harvested from

the same batch of the culture at a given time. Thus as the first step of this work, a simplified method of suspension culture was used by modifying the composition of YLE medium (EARLE's solution (12) containing yeast extract and lactalbumin hydrolysate). However, cells aggregated to each other in the suspension culture. To overcome this problem, calcium and magnesium ions were removed from culture medium (13) or a small amount of trypsin was added on the medium (14). To modify the composition of the medium, the concentration of phosphates must be high enough to buffer the medium (13) and the precipitation of the materials, occurring on the addition of the serum to the suspension, can be avoided in the presence of methyl cellulose (15).

The culture medium used in this experiment lacked calcium ion and contained methyl cellulose and high concentration of phosphates (Table 1).

TABLE 1 COMPOSITION OF THE MEDIUM

NaCl	6.80 g	Yeast extract	1.0 g
KCl	0.4	Lactalbumin hydrolysate	5.0
$MgSO_4 . 7H_2O$	0.2	Methyl cellulose	1.0
$NaH_2PO_4 . 2H_2O$	1.41	Phenol red	0.02
$NaHCO_3$	2.0	Penicilin	10^5 Units
Glucose	4.5		

1,000 ml
Supplimented with 10% of bovine serum

In this medium, cell aggregation was prevented. Methyl cellulose also protected the cells from damages due to shaking. With this medium, any special apparatus such as the culture vessels devised by SALTZMAN (16) and a gyrotary shaker used by NAGLE, Jr., *et al.* (17), was not necessary. Only Elremyer flasks with silicon stoppers and a water bath with a shaker moving in a linear fashion were good enough for preparing the culture. However, the ratio of suspension volume to the capacity of the flask was another important factor for the cell growth. When the ratio was lower than 0.1, the cells adhered to the glass wall at the boundary of the medium and air then soon died. In the experiment reported here, the ratio was maintained at the value over 0.2.

Under the conditions mentioned above, the cells grew exponentially after a lag phase of about 12 hr and ceased to proliferate 4 or 5 days after the beginning of the culture (Fig. 1). In the following experiments, the cells in the logarithmically growing phase were used, in which the generation time was about 32 hr.

The growth rate of the cells was depressed at least for 2 days after addition of bovine liver cornin at the concentration of 0.1, 0.2 and 0.5%

(Fig. 2). The degree of depression was proportional to the concentration of liver cornin. At the concentration of 0.5%, liver cornin stopped the growth of the cells almost completely. But the cells treated with 0.1 and 0.2% of liver cornin were released from depression at the time between days 2 and 3 of the treatment and showed even higher growth rate than the control group. In the cells treated with 0.5% of liver cornin, nuclear picnosis was observed by day 5.

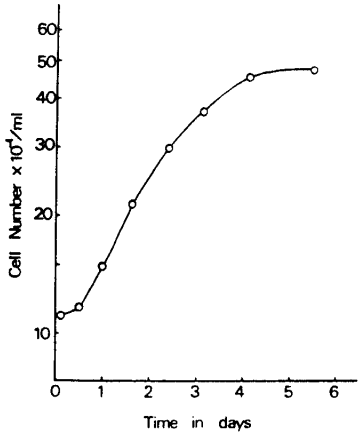

Fig. 1. Growth curve of L cells.
The cells were cultured in suspension at 37°C. They were subcultured every 4 or 5 days by the renewal of the medium. Inoculum size at the subculture was 1.0×10^5 -1.5×10^5 cells per ml. In the following experiments, the cells in logarithmically growing phase were used.

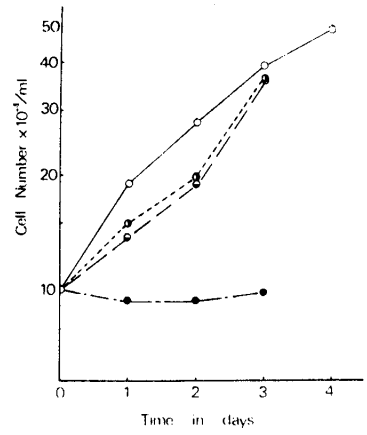

Fig. 2. The effect of bovine liver cornin on the growth of L cells in suspension.
Testing materials dissolved into the culture medium were added on the cell suspension at 0 time of this and the following figures, illustrating the growth of the cells. Final concentrations of liver cornin were; ○ : 0% (control), ◑ : 0.1%, ◒ : 0.2% and ● : 0.5%.

As shown in Fig. 3, liver cornin had inhibitory effects on a short term synthesis of DNA in the cells. The inhibition was almost complete at the concentration of 0.5%. Here again, the inhibiton and dosage paralleled. The percentage of inhibition caused by 0.1, 0.2 and 0.5% of liver cornin ranged 18–25%, 46–51% and 96–96%, respectively in 3 successive experiments (Fig. 3). No detectable change was seen microscopically within 5 hr even in the cells treated with 0.5% of liver cornin.

Heat treatment caused precipitates in cornin solution, though at the first step of cornin-extraction, the debris was removed after boiling of the materials. Thus, the effect of heat treatment on the inhibitory activity of liver cornin solution on DNA synthesis was tested. Bovine liver cornin was dissolved into saline at the concentration of 2.5% and heated 100°C for

10 min. Precipitates appeared upon heating were removed by centrifugation. The supernatant was added on 9 volumes of the cell suspension. Liver cornin solution without heat treatment (2.5%) and the same volume of saline were added in the same way on the second and third groups. As shown in Fig. 4,

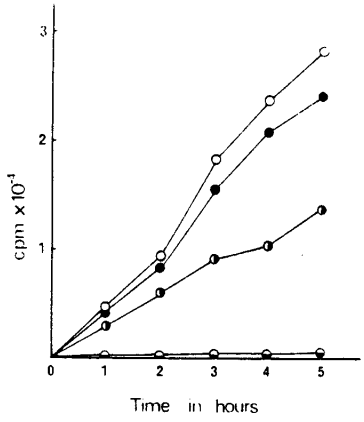

Fig. 3. The effect of bovine liver cornin on the synthesis of DNA in L cells.

Bovine liver cornin was added on the cell suspension two hr before the addition of ^{3}H-thymidine. The time after the addition of the radioisotope is shown in the abscissa of this and the following figures illustrate the synthesis of DNA, except for Figs. 13 and 14. Final concentration of liver cornin; ○ : 0% (control), ● : 0.1%, ◑ : 0.2%, ◒ : 0.5%.

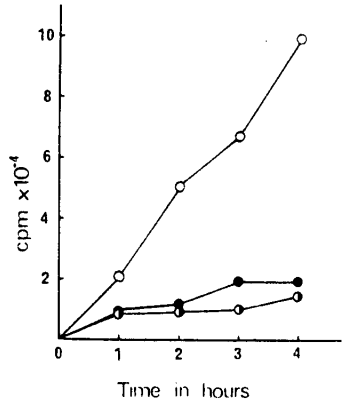

Fig. 4. The effect of heat treatment on the inhibitory activity of liver cornin on the DNA synthesis.

Bovine liver cornin dissolved in saline was heated at 100°C for 10 min. and its activity on the synthesis of DNA in the L cells was compared with that of unheated one. ○ : control; ● : 0.25% of liver cornin, untreated; ◑ : 0.25% of liver cornin, heat treated.

the heat treatment caused hardly any effects on the activity of liver cornin as regards the inhibitor of DNA synthesis. The specific activity of the inhibitor may be higher after heat treatment than before it.

Liver cornin was separated into two fractions, *i. e.* dialyzable (D-) and undialyzable (U-). The absorption maximum of D-fraction in the ultraviolet range was seen at 257 mμ and that of U-fraction at 262 mμ (Fig. 5). The peak in the absorption spectrum was steeper in D-fraction than in U-fraction. At the concentration of 0.2%, U-fraction inhibited the growth of L cells more strongly than D fraction (Fig. 6) and the inhibitory effect of U-fraction (0.25%) was even more marked on the synthesis of DNA in the cells (Fig. 7).

Nucleotides in U-fraction were compared with those in yeast RNA by ascending paper chromatography after hydrolyzation of the materials with acid (Fig. 8) or alkali (Fig. 9). Upon development four fluorescent spots appeared in both the U-fraction and the yeast RNA. The Rf values of each

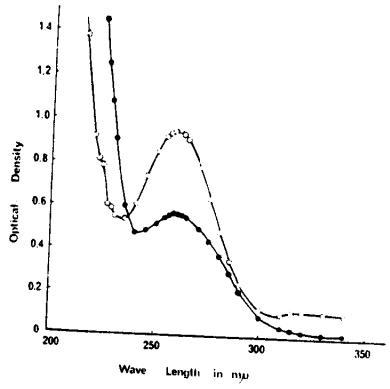

Fig. 5. The ultraviolet absorption spectra of the fractions of liver cornin. ○ : dialyzable fraction, ● : undialyzable fraction.

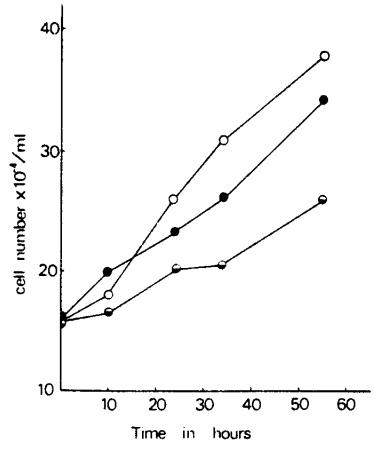

Fig. 6. The effect of dialyzable and undialyzable fraction of liver cornin on the growth of L cells.
○ : control, ● : 0.2% dialyzable fraction, ◒ : 0.2% undialyzable frctioan.

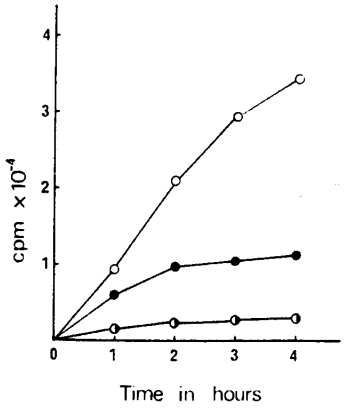

Fig. 7. The effect of dialyzable and undialyzable fraction of liver cornin on the synthesis of DNA in L cells.
○ : control, ● : 0.25% dialyzable fraction, ◑ : 0.25% undialyzable fraction.

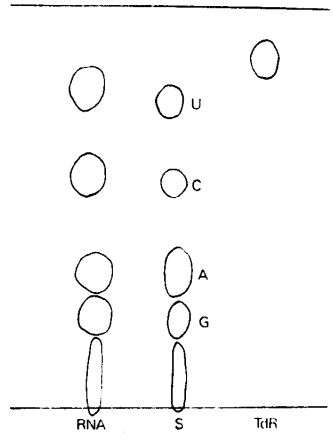

Fig. 8. Paper chromatogram of nucleotides in undialyzable fraction of liver cornin and of yeast RNA, hydrolyzed with 1 N HCl.
Solvent ; isopropanol : 6 N HCl : water (68 : 33.3 : made 100 by addition of water). RNA : yeast RNA, S : U-fraction of liver cornin, TdR : thymidine, G : guanine, A : adenine, C : cytidylic acid, U : uridylic acid.

spot in the two series coincided, suggesting that nucleotides in U-fraction were the same as those in yeast RNA ; namely, uridine, cytidine, adenine

and guanine. The cochromatogram of the two samples confirmed this (Fig. 9). The spot corresponding to thymidine was not detected (Fig. 8).

Upon the gel filtration of U-fraction with Sephadex G-75, two peaks were separated consistently with 8 trials (Fig. 10, lower figure). The eluates

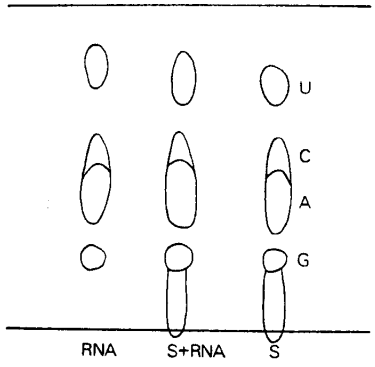

Fig. 9. Paper chromatogram of nucleotides in undialyzable fraction of liver cornin and of yeast RNA, hydrolyzed with 0.3 N KOH.

Solvent is the same as in Fig. 8. RNA: yeast RNA, S: U-fraction of liver cornin, S+RNA : mixture of the above two, G : guanylic acid, A : adenylic acid, C : cytidylic acid, U : uridylic acid.

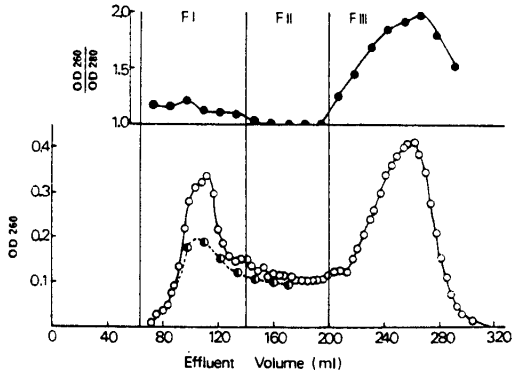

Fig. 10. Gel filtration of undialyzable fraction of liver cornin.

Lower figure: Elution pattern of undialyzable fraction of liver cornin on the gel filtration with Sephadex G-75.
○ : elution pattern at 260 mμ before removal of precipitats, ◑ : elution pattern at 260 mμ after removale of precipitates. Upper figure: The ratio of optical density at 260 mμ/at 280 mμ (every 3 fractions of the lower figure).

were therefore divided into 3 groups, *i. e.* the first peak (F I), interval between the two peaks (F II) and the second peak (F III). White precipitates appeared in F I were removed by centrifugation. The ratio of optical density at 260 mμ/at 280 mμ was higher than one in F I and FIII and one in F II (Fig. 10, upper figure). The percentages of the material recovered by this method was over 92% in weight, the lyophilizates of F I, F II, F III and the precipitates in F I consisting of 28.6, 33.9, 19.0 and 12.5%, respectively.

Absorption spectra of F I, F II and F III were shown in Fig. 11. F I had no peak in the range measured. Whereas F II and F III showed absorption maxima at 275 mμ and 260 mμ, respectively.

The inhibitory activity of each fraction on the cell growth was tested at 0.2% (Fig. 12). F I and F II had hardly any effect on the growth but F III had a strong inhibitory effect on it. The effects of the fractions on the DNA synthesis were compared at 6, 12 and 24 hr of the treatment after the addition of each fraction at the concentration of 0.2% (Fig. 13). In this instance, F III also showed the highest inhibition of the three, this inhibition became higher

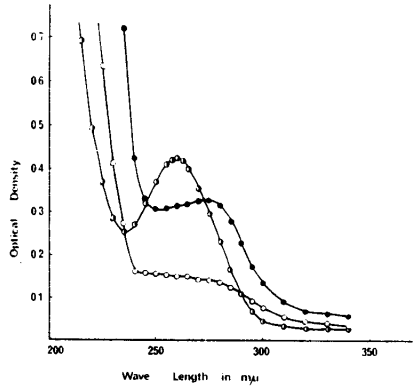

Fig. 11. Ultraviolet absorption spectra of the fractions separated by gel filtration. ○ : FI, ● : FII, ◑ : F III.

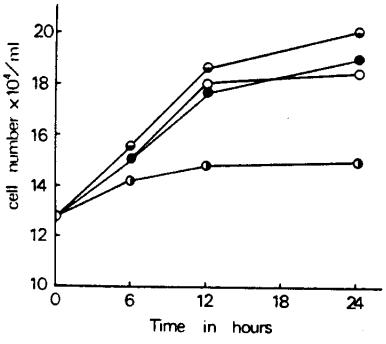

Fig. 12. The effect of fractions of liver cornin on the growth of L cells.
○ : control, ● : F I, ◒ : F II, ◑ : F III.
Final concentration of each fraction was 0.2%.

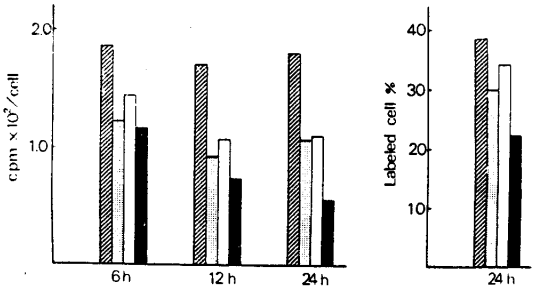

Fig. 13. (left) The effects of fractions of liver cornin on the synthesis of DNA in L cells.

The fractions were added on the cell suspension at the concentration of 0.2%. At 6, 12 and 24 hr after the addition of each fraction, ^{3}H-thymidine was added on the suspension at final concentration of 0.2 μCi/ml. The radioactivities incorporated into the acid-insoluble fraction of the cells for 30 min were measured by a liquid scintillation counter.

▨ : control, ▩ : F I, □ : F II, ■ : F III.

Fig. 14. (right) The effect of fractions of liver cornin on the percentage of the cells in DNA synthetic phase in the cell cycle.

The cells were incubated with each fraction at 0.2% for 24 hr and were labeled with ^{3}H-thymidine (0.2 μCi/ml) for 30 min. Percentages of the labeled cells were counted autoradiographically.

▨ : control, ▩ : F I, □ : F II, ■ : F III.

with the lapse of time, while inhibitory effects of F I and F II remained almost constant. Thus, ^{3}H-thymidine incorporated into DNA after 24-hours treament with F III was 30% of the control. Microscopically all the fractions caused no structural changes in the cells within 24 hr.

In the radioautographical experiments, the labeled indices of the cells treated with all the three fractions for 24 hr were lower than those of the control series, the lowest value (60%) had been obtained with F III (Fig. 14).

The contents of protein, RNA and phosphates in the fractions of liver cornin were shown in Table 2. No detectable amount of DNA was contained in all the samlpes tested. Of the three fractions from U-fraction F I and F II were proteins or polypeptides, while F III contained protein and RNA. The ratio of protein to RNA in F III was about 2.3.

TABLE 2 CONTENTS OF PROTEIN, PHOSPHATES AND RNA IN THE FRACTION OF BOVINE LIVER CORNIN.

	Protein	Total P	Inorg. P	RNA
Cornin	44.6	7.87	6.87	
D-fraction	11.0	11.3	11.9	
U-fraction	94.1	0.55	—	6.17
F I	92.0	—	—	—
F II	97.7	—	—	—
F III	66.6	2.09	—	28.4

percentage in w/w. — : not detectable

DISCUSSION

Cornin extracted from bovine liver shows recognizable inhibition to the growth and DNA synthesis at 0.1% and these processes are inhibited almost completely at 0.5% if the cells are suspended (Figs 2 and 3). If they are spread in monolayer, however, it has been reported that liver cornin has no effect on the growth at 0.1% and does not stop the growth even at 0.6%(10). It may be concluded that the sensitivity of the cells to the inhibitor is higher in suspension than in monolayer. The suspention culture is not only more useful than monolayer culture in this respect, but also it is convenient to harvest an alipuot of the cells at any given time.

At the concentration of liver cornin lower than 0.2%, recovery of the growth rate is observed (Fig, 2). This phenomenon has been known in cornin extracted from tissues other than liver (6, 18), because cells may acquire resistance to the inhibitor (18); this may be the case in the present experiment.

The results mentioned above suggest that cornin extracted from bovine

liver contains two fractions which inhibit cell growth and DNA synthesis, and that the inhibitory activities of both factors are in parallel with the dosage. As shown in Fig. 4, the inhibitory factor of DNA synthesis in liver cornin is heat stable. Similarly, the heat stability of the growth inhibitor has been known (10). The similarity of the two factors is also shown in the impermeability against the dialyzing membrane (Figs. 6 and 7).

It is reported that on the gel filtration of U-fraction of liver cornin with Sephadex G-200, two peaks are separated in the elution pattern and that the fractions in the region of the second peak show the strongest inhibition on the growth of the cells among all the others (10). Similar tendencies as regards the elution pattern (Fig. 10) and the restriction of the inhibtory factor around the second peak (Fig. 12) have been obtained with Sephadex G-75. The inhibitory factor may have better separated and more purified by gel filtration with Sephadex G-75 than G-200, since the factor in the U-fraction appears to be relatively small in molecular size. Therefore, the fraction of F III, obtained in this experiment, may be included in F IV ; the strongest inhibitory fraction of the growth separated with Sephadex G-200 in the previous paper (10), and may be more purified than F IV. But the growth inhibitor cannot be separated from the inhibitor of DNA synthesis by the gel filtration with Sephadex G-75.

The inhibitory activity of F III on DNA synthesis is not due to thymidine, because the substance is not included even in U-fraction (Fig. 8).

Which phase of the cell cycle is affected by F III? If the cell cycle was stopped at a phase other than the S phase, the labeled index would have been reduced to the same extent as the reduction of the rate of DNA synthesis. However, the percentage of inhibition on the synthetic rate of DNA (Fig. 13) is higher than that of reduction in the labeled index (Fig. 14) when the cells are treated with F III for 24 hr. It may be considered that the fraction, F III, reduces the rate of DNA synthesis at S phase as well as retards the process in other phases.

Acknowledgement: I am gratefull to Prof. I. NISIDA and Dr. T. H. MURAKAMI for encouragement throughout the course of this work.

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