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Molecular Transformation of Tumor Adenylosuccinate Synthetase and Characteristics of Its Converting Factor

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ABSTRACT

The properties of adenylosuccinate synthetase [inosine monophosphate:L-aspartate ligase (guanosine 5′-diphosphate-forming), EC 6.3.4.4]) of rat Yoshida sarcoma ascites tumor cells changed during purification. The isoelectric points (pI) of the crude and purified enzymes were 5.0 and 5.9, respectively. The K_m values of the crude enzyme for inosine monophosphate, aspartate, and guanosine 5′-triphosphate were calculated to be 99 ± 1 (S.E.), 870 ± 40, and 27 ± 2 μM, respectively, while those of the purified enzyme were 410 ± 10, 980 ± 50, and 69 ± 5 μM, respectively. These data indicate that the crude enzyme should be more effective for activity than the purified one. It was found that the change in pI occurred during diethylaminoethyl cellulose column chromatography and that, during this step a compound, named pI-converting factor (ICF), was separated from the enzyme molecule. On addition of ICF, the pI of the purified enzyme changed from 5.9 to 5.0, indicating that the pI conversion was dependent on ICF and was reversible. ICF was nondialyzable, heat stable, and partly precipitated with 1 N perchloric acid but was not affected by 1 N KOH. It was partially degraded by DNase I. These results suggest that ICF is a DNA-like compound.

INTRODUCTION

In neoplastic tissues, it has been demonstrated (13) that *de novo* nucleotide biosynthesis increases with neoplastic progression. Adenylosuccinate synthetase is considered to be one of the key enzymes of purine nucleotide biosynthesis. Jackson *et al.* (4) compared the activities of this enzyme in various hepatomas with that in normal rat liver, finding that the activities were significantly increased in all tumors but that the properties of the enzyme in rapidly growing hepatoma 3924A were similar to those of the enzyme in normal liver.

Recently, Matsuda *et al.* (7) found 2 types of adenylosuccinate synthetase (type L and type M) in rat liver and suggested that they might play different physiological roles; *i.e.*, type L might contribute to adenine nucleotides biosynthesis, while type M might be involved in the purine nucleotide cycle proposed to be an ammonia-liberating system by Lowenstein (5). Data on changes in the contents of the isozymes in rat liver after partial hepatectomy (8) and under various dietary conditions (1) have substantiated the idea that type L enzyme is correlated with purine biosynthesis. Matsuda *et al.* (9) also purified adenylosuccinate synthetase from rat Yoshida sarcoma ascites tumor cells to characterize its regulatory and kinetic properties in neoplastic tissue. They found that the tumor enzyme seemed to be similar to type L enzyme in normal liver, which is consistent with our previous suggestion that type L enzyme mainly works where nucleotide biosynthesis is active.

The present work, however, revealed that the tumor adenylosuccinate synthetase appeared to be a modified form of the type L enzyme and that the modification was reversible and dependent upon some factor(s).

MATERIALS AND METHODS

Yoshida Sarcoma Ascites Tumor Cells. The cells were kindly supplied by Dr. Shirasaka, Division of Regulation of Macromolecular Functions of this Institute. The tumor cells were grown i.p. in 5-week-old Donryu rats and were harvested 5 to 7 days after inoculation. The ascitic fluid was washed with 0.9% (w/v) NaCl solution to remove lysed RBC, and then the tumor cells were collected by low-speed centrifugation and stored at −20°.

Assays. The activity of adenylosuccinate synthetase was measured spectrophotometrically, as described previously (9). Protein concentration was determined by the method of Lowry *et al.* (6) with bovine serum albumin as a standard. DNA concentration was determined by the method of Burton (2) with calf thymus DNA as a standard.

Isoelectric Focusing with a Sucrose Density Gradient Column. The method of Matsuo *et al.* (10), which is a modification of the method of Vesterberg (12), was used. The sample was applied to a column (120 ml) of a linear density gradient formed with 0 and 50% (w/v) sucrose solutions containing 0.75 and 2.25 ml of 40% (w/v) ampholine, respectively, and subjected to electrofocusing at 700 V for 42 hr. Then the sample solution was drained out, and fractions of 30 drops were collected. The enzyme activity and pH value of each fraction were measured.

Isoelectric Focusing with a Polyacrylamide Gel Column. A modification of the method of Wrigley (14) was used. Polyacrylamide gel containing 5% (w/v) acrylamide, 0.25% (w/v) *N,N′*-methylenebisacrylamide and 2% (w/v) ampholine was prepared in glass tubes of 5 × 100 mm. Photopolymerization was adopted in the preparation of the gel using riboflavin without ammonium persulfate. The sample mixed with an equal volume of 60% (v/v) glycerol:4% ampholine solution was applied to the gel column, and protecting solution composed of 15% glycerol, 2% ampholine, and 0.8% (w/v) glutamine was layered on to it. The gel tubes were placed in the electrophoresis apparatus, in which the upper (anode) and lower (cathode) electrode vessels were filled with 0.02 M H_3PO_4 and 0.1 M NaOH, respectively. The voltage was gradually raised to 200 V, maintaining a current of less than 1 ma/tube. After electrofocusing at 200 V for 5 hr, the gel was cut into 60 to 70 sections 1 mm wide. The enzyme activity in the gel was assayed by incubating pairs of gel slices in the standard reaction mixture at 37° for several hr or overnight. The pH gradient in the gel was determined by soaking pairs of slices in 0.5 ml of distilled water at 4° overnight and measuring the pH of the extracts. The pH gradient was also determined with pI marker colored proteins (Oriental Yeast Co., Japan).

Enzymatic Digestion of ICF.[2] Each enzyme was added at a concentration of 1 mg/ml to ICF solution dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and incubated at 37° overnight. Then the reaction mixtures were boiled in a water bath for 2 min to stop the

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[2] The abbreviation used is: ICF, isoelectric point converting factor.

digestion, and ICF activity in the supernatant was determined. For digestion with DNase I, 20 mM $MgCl_2$ was added in the reaction mixture.

RESULTS

Occurrence of pI Conversion. Chart 1 shows the results of isoelectric focusing of crude and purified adenylosuccinate synthetase from Yoshida sarcoma ascites tumor cells. The peak of activity of the purified enzyme was observed at pH 5.9, while that of the crude enzyme was at pH 5.0. Although the contents of the column became slightly turbid due to precipitated proteins in the region of pH 4 to 5 during electrofocusing of the crude extract, the peak of activity was sharp enough to exclude the possibility that the turbidity affected the migration of enzyme protein.

Differences in Kinetic Properties. The crude and purified enzymes also had different kinetic properties. As shown in Table 1, the K_m values for IMP and GTP of the crude enzyme were significantly lower than those of the purified enzyme, although the K_m values for aspartate were similar. Thus, it can be concluded that adenylosuccinate synthetase has higher affinities for the substrates in the crude extract than in the purified state and consequently that the crude enzyme is more effective for activity.

Change in pI during Purification. As reported previously (9), adenylosuccinate synthetase was purified by ammonium sulfate fractionation, DE52 column chromatography, Ultro Gel AcA 34 column chromatography, and Hadacidin-Sepharose 4B column chromatography. We examined the step at which the pI conversion occurred. The pI of the enzyme at each purification step was determined by polyacrylamide gel disc isoelectric focusing, as described in "Materials and Methods." Results showed that the change occurred at the step of DE52 column chromatography (data not shown). The pI values of crude and purified enzymes determined by this method were the same as those determined by isoelectric focusing in sucrose density gradient columns (Chart 1). The alteration of pI during purification of tumor adenylosuccinate synthetase was found to be reproducible, whichever method was used.

In addition to the 2 forms with pI 5.0 and 5.9 mentioned above, a third form with a pI of 5.5 to 5.6 appeared under certain conditions as described later. Accordingly, we named these multiple forms of type L adenylosuccinate synthetase L_1 (pI 5.0), L_2 (pI 5.5 to 5.6), and L_3 (pI 5.9) according to their pI values.

Separation of the Converting Factor. Since the pI conversion of adenylosuccinate synthetase during DE52 column chromatography seemed to be caused by separation of some factor from the enzyme molecule, we next attempted recombination experiments. Chart 2*A* shows the elution profiles on DE52 column chromatography. The crude extract separated into 3 fractions, Fraction I, which was not adsorbed on the DE52 column, and Fractions II and III, which were adsorbed on the column but eluted with 0.2 and 0.5 M KCl, respectively. The enzyme activity was found only in Fraction II.

Chart 2*B-a* shows that Fraction II contained the L_3 form enzyme; its activity peak appeared at pH 5.9 on gel disc isoelectrofocusing. When a mixture of Fractions I and II was subjected to isoelectric focusing, no significant change in the pI was observed, as shown in Chart 2*B-b*. On the other hand,

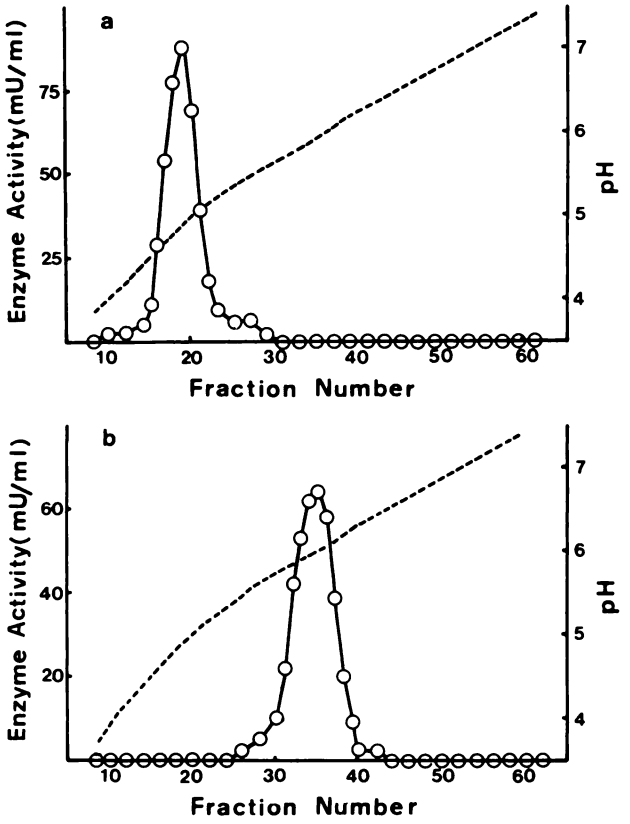

Chart 1. Difference between pI of crude and purified preparations of adenylosuccinate synthetase. Twenty ml of crude extract of tumor cells (protein, 400 mg) and 2 ml of purified enzyme solution (protein, 0.5 mg), respectively, were applied to sucrose density gradient columns containing 1% ampholine (pH range, 4 to 7) and subjected to isoelectric focusing as described in "Materials and Methods." The crude extract and the purified enzyme were prepared as reported previously (9). Ampholine of pH 4 to 7 was prepared by mixing equal volumes of ampholines of pH 4 to 6 and 5 to 7. ——, enzyme activity; - - - -, pH gradient; *a*, crude enzyme; *b*, purified enzyme; *mU*, milliunits.

Table 1

Comparison of K_m values of crude and purified adenylosuccinate synthetase

Substrate	Crude enzyme (μM)	Purified enzyme (μM)
IMP	99 ± 1[a]	410 ± 10[b]
GTP	27 ± 2	69 ± 5[b]
Aspartate	870 ± 40	980 ± 50[c]

[a] Means ± S.E.M. for 4 samples.
[b] Difference between the crude and purified enzymes, $p < 0.001$.
[c] Difference between the crude and purified enzymes, not significant.

addition of the Fraction III resulted in conversion of the L_3 form to the L_1 form (Chart 2*B-c*). Similar conversion of the L_3 form to the L_1 form was observed when the crude extract or the fraction obtained by ammonium sulfate fractionation (40 to 65% saturation) was added to the purified enzyme. No preincubation was necessary for this conversion. These findings substantiate the previous suggestion that the crude extract contains a converting factor(s). We named this factor(s) ICF. Addition of unsufficient ICF to the L_3 form changed the pI to 5.5 to 5.6 (the L_2 form). In this case, however, the peak of activity was broad, suggesting that the L_2 form might be an intermediate product. This finding also suggests that abundant ICF was present in the crude extract of tumor cells.

Effects of Various Treatments on ICF. The properties of ICF were investigated by subjecting ICF prepared by DE52 column chromatography, as described above, to various treat-

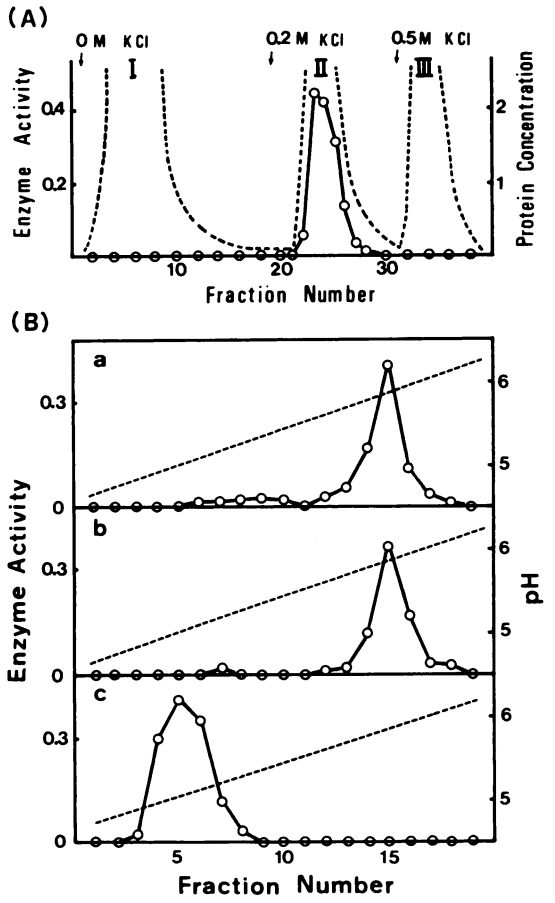

Chart 2. Separation and reconstitution of adenylosuccinate synthetase and ICF. *A*, fractionation of crude extract by DE52 column chromatography. The crude extract (25 ml) dialyzed against 10 mM potassium phosphate buffer (pH 7) containing 0.1 mM dithiothreitol was applied to a DE52 column (5 ml) equilibrated with the same buffer. After washings with 10 mM potassium phosphate buffer (pH 7), stepwise elutions were carried out with 0.2 M and 0.5 M KCl. ——, enzyme activity expressed as increase in absorbance at 280 nm; - - - -, protein concentration expressed as absorbance at 280 nm. *B*, change in pI of the enzyme on recombination of fractions obtained by DE52 column chromatography. Fraction II containing the enzyme activity was mixed with 2 volumes of Fractions I and III (Fraction I, II, and III are defined as described in the text), respectively. As a control, it was mixed with 2 volumes of 10 mM potassium phosphate buffer (pH 7.0). Then 100 μl of the mixture were applied to polyacrylamide gel column containing 2% ampholine (pH 4 to 7) and subjected to isoelectric focusing as described in "Materials and Methods." An ampholine of pH 4 to 7 was prepared by mixing ampholines of pH 4 to 6, 5 to 7, and 3.5 to 10 at a ratio of 2:2:1. The migration profiles of Fraction II plus buffer (*a*); Fraction II plus Fraction I (*b*); and Fraction II plus Fraction III (*c*) are shown as in Chart 1, except that enzyme activity is expressed as increase in absorbance at 280 nm.

ments. Dialysis of a solution of ICF against 3 changes of 500 volumes of 10 mM potassium phosphate buffer (pH 7) for 3 days did not affect its converting activity. The dialyzed ICF preparation was also stable when boiling for 30 min in a water bath. These findings indicate that ICF is nondialyzable and heat stable. On addition of 1 N perchloric acid to the ICF preparation, much insoluble materials appeared, and the converting activity in the supernatant, which was neutralized with KOH after centrifugation, was considerably reduced. However, the activity was recovered by suspending and neutralizing the resulting precipitate in a small volume of water. These findings indicate that part of the ICF was reversibly denatured and precipitated by acid treatment. On the other hand, when the ICF solution was boiled for 30 min in the presence of 1 N perchloric acid, the converting activity was decreased and could not be recovered from the precipitate. The ICF solution was markedly resistant to incubation in the presence of 1 N KOH at 37° for 20 hr.

When the ICF solution was treated with a mixture of chloroform:methanol (2:1, v/v) (3), the converting activity remained in the aqueous phase. This indicates that ICF is not hydrophobic.

These results suggest that ICF resembles DNA in chemical properties.

Next, we investigated the effects of various enzymes on ICF activity. Trypsin, proteinase K, neuraminidase, lipase, and RNase A had no significant effect, but DNase I reduced the converting activity considerably. These findings also suggest that ICF is DNA or a DNA-like substance.

Molecular Size of ICF. The molecular size of ICF was estimated by gel filtration in a high-pressure liquid chromatograph equipped with an SW 2000 G column (Toyo Soda, Japan). Chart 3 shows the elution pattern. Each fraction eluted from this column was desalted by dialysis and added to the L_3-form enzyme, and its converting activity was checked by gel disc isoelectric focusing. As shown in Chart 3, the activity was recovered in a broad region (shaded portion), between the elution positions of creatine kinase and malate dehydrogenase. From this pattern, its molecular weight was calculated as 60,000 to 80,000 assuming that it is a globular protein. In addition, weak activity was widely distributed in later fractions. These results suggest that the molecular size of ICF is not uniform.

The heterogeneity in the molecular size of the ICF was confirmed by ultrafiltration through a Membranfilter SM 121-36 (Sartorius, West Germany), which separates substances with molecular weights of below 10,000. When the ICF solution was subjected to ultrafiltration, most of the converting activity was recovered in the remaining solution, but some was lost in the filtrate.

ICF in the Nuclear Fraction. For the experiments thus far described, ICF was prepared from the soluble fraction obtained by centrifugation of the cell homogenate at 100,000 × *g* for 60 min, because adenylosuccinate synthetase is present only

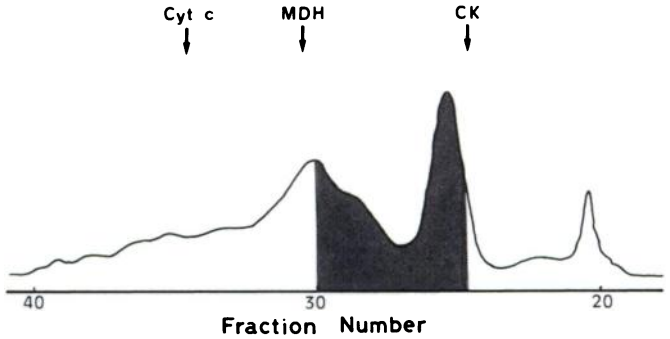

Chart 3. Gel filtration of ICF by high-pressure liquid chromatography. Two hundred μl of ICF solution prepared by DE-52 column chromatography (Chart 2*A*, Fraction III) were applied to an SW 2000 G column (5 × 600 mm), and the column was eluted with 10 mM potassium phosphate buffer (pH 7) containing 1 M KCl at a pressure of 15 to 20 kg/sq cm^2. The effluent was passed through a flow-through cell to measure the absorbance at 254 nm, and then fractions of 0.5 ml were collected. The fractions were dialyzed to remove KCl, and their converting activity was examined. ——, elution profile expressed as the absorbance at 254 nm. The fractions with comparatively strong activity are shown by shading. Marker proteins for determination of the molecular weight were eluted in the same manner. *CK*, creatine kinase; *MDH*, malate dehydrogenase; *Cyt c*, cytochrome *c*.

in the cytoplasmic fraction. However, DNA is mainly present in the nucleus. Next, we tested whether ICF was also present in the nuclear fraction.

The nuclear fraction was prepared as follows; 5 g of Yoshida sarcoma ascites tumor cells were disrupted in 5 volumes of 0.25 M sucrose solution containing 5 mM $CaCl_2$ by ultrasonic vibration at 20 kHz for 20 sec. The homogenate was centrifuged at 900 × *g* for 10 min, and the resulting pellet was suspended in 10 ml of the sucrose solution described above. This suspension was again subjected to ultrasonic vibration for 60 sec to break the nuclear envelops and boiled in a water bath for 3 min to denature proteins. Then it was centrifuged, the supernatant was added to L_3 form, and the pI was determined. The result showed that ICF is present in the nuclear fraction, too.

The converting activity in the nuclear fraction was greatly reduced by digestion with DNase I, as in the case of ICF in the cytoplasmic fraction. Moreover, nuclear ICF was also precipitated in part by treatment with 1 N perchloric acid, was significantly inactivated by boiling for 15 min in the presence of 1 N perchloric acid, and was resistant to alkali treatment.

ICF Activity of Commercial DNA. Commercial DNA isolated from calf thymus (Sigma) was used to see if DNA shows nonspecific converting activity. This DNA preparation also caused conversion of the L_3 enzyme to L_1 form, as shown in Chart 4. The conversion was dependent on the amount of DNA added, but more than 50 μg were required for complete conversion of 5 milliunits of the L_3 enzyme, whereas the DNA content of the cytoplasmic ICF preparation required to exert a similar effect was not more than 2 μg. This difference suggests structural specificity of ICF.

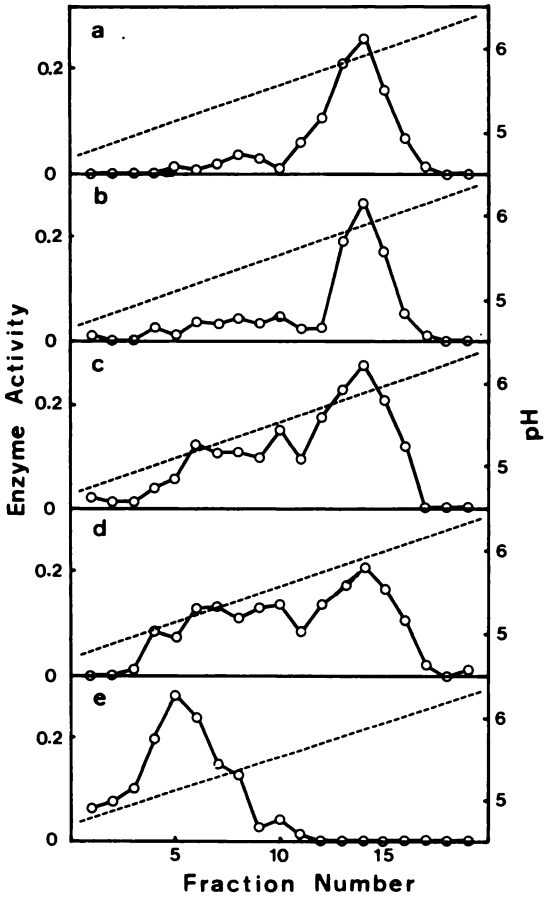

Chart 4. Change in the pI of adenylosuccinate synthetase by calf thymus DNA. Various amounts of calf thymus DNA were added to 5 milliunits of the L_3 enzyme in a total volume of 150 μl. Then 100-μl aliquots of the mixtures were subjected to gel disc isoelectric focusing as described in Chart 2. ——, enzyme activity; - - - -, pH. The enzyme activity is expressed as described in Chart 2*B*. *a*, without DNA; *b*, 3 μg DNA; *c*, 10 μg DNA; *d*, 30 μg DNA; *e*, 90 μg DNA.

The converting activity of calf thymus DNA was also lowered by digestion with DNase I; the inactivation was time dependent, and a long time was required for complete inactivation even with excess of DNase. These findings on the actions of calf thymus DNA support the idea that ICF may be DNA fragments.

DISCUSSION

The physiological significance of the presence of these distinct molecular forms of type L enzyme awaits further investigation. As mentioned previously (7, 8), the type L enzyme may contribute to purine nucleotide biosynthesis, and the L_1 form in the crude tumor extract showed higher affinities for substrates than did the L_3 purified enzyme (Table 1). This characteristic of the L_1 form favors the active formation and supply of ATP in growing tissue such as tumors.

The properties of ICF resemble those of DNA fragments, although the detailed characteristics of ICF are still unknown. If ICF is DNA fragments, the question arises of whether there is a significant quantity of DNA in the cytoplasmic fraction, where adenylosuccinate synthetase exists. Schneider and Kuff (11) found that about 2% of the total cellular DNA in rat liver was present in the cytoplasmic fraction. Moreover, DNA was detected in our ICF preparation by the diphenylamine method (2), and only a small amount of it was required for conversion of the L_3 to the L_1 form, as mentioned above. One or 2 orders of magnitude higher concentrations of DNA of calf thymus DNA than of ICF were necessary for similar converting effects. These findings suggest that specific DNA fragments are present in the cytoplasm and that the action of ICF is specific.

The data on the molecular size of ICF are quite unusual. As shown in Chart 3, ICF was widely distributed in fractions eluted from an SW 2000 G column on gel filtration chromatography. Moreover, ICF was partly precipitated by acid treatment (1 N perchloric acid, 0°) and partly passed through a Membranfilter SM 121-36. These findings indicate diversity of ICF molecules and suggest that some critical structure might be required for exerting converting activity.

The ICF activity was reduced by digestion with DNase I but was not completely lost even on treatment with excess DNase I (1 to 2 mg/ml) for a long time (37°, overnight), although calf thymus DNA was degraded with the loss of converting activity under the same conditions. However, ICF became more susceptible to DNase digestion after alkali treatment (incubation with 1 N KOH at 37° overnight) or acid treatment (1 N perchloric acid, 0°), although it is not known yet how it was influenced by these treatments. These results suggest that ICF might have some specific conformational difference from DNA.

ICF was also found in the nuclear fraction and showed properties similar to those of DNA. These findings also support the idea that ICF is DNA fragments. On the other hand, it is possible that the multiple forms of type L adenylosuccinate synthetase are artifacts formed by the action of DNA fragments which leak out of the disrupted nuclei during homogenization

of the cells. However, even when the tumor cells were disrupted in such a mild way as freezing and thawing, only the L_1 enzyme, which is supposed to be composed of the L_3 form and ICF, was found in the crude extract. Therefore, it seems very likely that modification of the enzyme actually occurs in tumor cells.

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