DNA Breakage by Hydrolyzable Tannins in the Presence of Cupric Ion

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DNA breakage by hydrolyzable tannins of known structures was investigated by agarose or polyacrylamide gel electrophoretic analysis. Hydrolyzable tannins could cause both double-strand and single-strand breakages in λ DNA in the presence of Cu^{2+} . The breaking reaction was strongly suppressed at concentrations higher than $100\ \mu\text{M}$ of hydrolyzable tannins. DNA breakage by various tannins was dependent upon their sorts, numbers, and binding sites of the constituent acids and polyalcohols. Metallic ions other than Cu^{2+} had little effect on the breaking reaction.

Tannins are widely distributed in the plant kingdom and are significant to humans because of their use as tanning agents, dyes, and drugs in addition to their wide distribution as astringent components in many foodstuffs. The chemical structures of tannins have been studied by a number of investigators for a long time, but their extremely complex and unstable structures have inhibited the progress of research. Therefore, crude tannins have been used in most studies dealing with the biological activities of tannins, which made it impossible to discuss in detail the relationship between the activity and chemical structure. However, recent developments in purification techniques and analytical methods finally made it possible to determine the chemical structures of many tannins and to test the biological activities of tannins of known structures. Especially, a few hundred kinds of tannins have been separated from various crude drugs or plants and new physiological activities have been discovered one after another.¹⁾ The research in this field is

expected to progress rapidly in the near future.

Although binding of tannins to proteins or metals is known to be their physiological activity, little information has been obtained about their action on nucleic acid. Tannins show strong reducing activity and belong to the aromatic reductones, because they have many enetriol or enediol groups per molecule. Since aromatic reductones such as pyrocatechol, catecholamines, and lignins have in common both antitumor activity and DNA or RNA breaking activity,^{2~6)} tannins are also expected to have such biological activities. This paper describes DNA breakage by various hydrolyzable tannins of different molecular structures.

MATERIALS AND METHODS

1. *DNA and reagents.* Double-stranded DNA from phage λ was purchased from Biotech Co. Cells of *E. coli* strain RRI were cultured in essentially the same way as the method of Norgard *et al.*⁷⁾ Plasmid pBR322 DNA was

prepared from this strain by the procedure of Colman *et al.*⁸⁾ Metal sulfates and gallic acid were products of Nakarai Chemicals Co. All hydrolyzable tannins used were isolated from various kinds of plants and their chemical structures were determined as reported previously: of the galloyl glucoses, mono: 6-mono-galloyl glucose,⁹⁾ di: 1,6-digalloyl glucose,⁹⁾ tri: 1,2,6-trigalloyl glucose,¹⁰⁾ 3,4,5-trigalloyl glucose,¹¹⁾ tetra: 1,2,3,6-tetragalloyl glucose,¹²⁾ 1,2,4,6-tetragalloyl glucose,¹¹⁾ 2,3,4,6-tetragalloyl glucose,¹¹⁾ penta: 1,2,3,4,6-pentagalloyl glucose¹³⁾; hexa-, hepta-, octa-, nona-, and decagalloyl glucoses¹³⁾ were mixtures of positional isomers; of other gallotannins, 2',3,5-trigalloyl hamamelose,¹⁴⁾ 3,4,5-trigalloyl proto-quercitol,¹⁵⁾ and 3,4,5-trigalloyl quinic acid¹⁵⁾; of ellagitannins, ellagic acid,¹⁶⁾ eugenin,¹⁶⁾ and sanguisorbic acid dilactone¹⁷⁾; of other tannins, trapain.¹⁸⁾

2. *DNA breakage test.* Unless otherwise noted, the conditions of the DNA-breaking reaction were as below. A mixture of 1 μ g λ DNA, tannins of varying concentrations from 1 to 400 μ M, and metallic sulfate was incubated in 30 μ l of 8 mM phosphate buffer (pH 7.0) at 37°C for 2 hr. The reaction was stopped by adding 20 μ l of 15 mM EDTA (pH 7) containing 0.05% bromophenol blue and 15% sucrose. The sample was analysed by gel electrophoresis. As ellagic acid and sanguisorbic acid dilactone were practically insoluble in water, they were dissolved in dimethyl sulfoxide (DMSO) and the breaking reaction was carried out in 20% DMSO. The pH dependence was tested in 50 mM buffer. We used acetate buffer for pH 4 and 5, phosphate buffer for pH 6, 7, and 8, Tris-HCl buffer for pH 9 and carbonate-bicarbonate buffer for pH 10. Oxidation of tannins in these buffers was followed by measuring the visible spectrum using Shimadzu UV-240 spectrophotometer equipped with a constant-temperature cell holder. A reaction mixture containing 60 μ M tannin, 200 μ M Cu^{2+} , and 8 mM buffer was incubated at 37°C in a quartz microcell and the change of the visible spectrum accompanying the oxidation of tannin was examined.

3. *Gel electrophoresis.* Electrophoresis was done in 0.3 or 0.7% agarose slab gel (Nakarai Chemicals Co.) using a horizontal electrophoretic apparatus (20.5 $\times$ 16 $\times$ 0.3 cm gel) or in 8% polyacrylamide gel (Nakarai Chemicals Co.) using quartz glass tubes (0.8 $\times$ 8 cm gel). The buffer for electrophoresis was 89 mM tris, 8.9 mM boric acid and 2.5 mM EDTA. Na_2 (TBE buffer) containing 0.5 μ g/ml ethidium bromide for agarose or 1.0 μ g/ml for polyacrylamide gel electrophoresis. DNA fragments larger than 3 $\times$ 10⁵ daltons were analyzed by agarose gel electrophoresis at 50 V/10 mA for 15 hr and smaller DNA fragments by polyacrylamide gel electrophoresis at 200 V/20 mA/tube for 1 hr at room temperature. For alkaline gels to detect single-strand breaks, a buffer (pH 12.6) containing 67 mM NaOH, 33 mM glycine, 33 mM NaCl, and 2 mM EDTA was used in both the gel and running buffer.¹⁹⁾ Electrophoresis was done at 50 V/10 mA for 15 hr using 0.02% bromo-

cresol green as a migration marker. After electrophoresis, gels were stained with 1 μ g/ml ethidium bromide in TBE buffer for 4 hr.

4. *Estimation of mean molecular weights of DNA fragments.* After electrophoresis, DNA was visualized under irradiation with a short-wave ultraviolet light source. In order to quantitate DNA mass by densitometry, photographs were taken on Neopan Film using a Minolta camera with UV and red filters (Kenko Co.). Negatives were traced on a Shimadzu UV-240 spectrophotometer equipped with a gel scanner (GSC-3 type). If the exposure and development are suitable, the optical density of the developed film is known to be proportional to the amount of DNA.^{20,21)} The quantitateness of this method was confirmed with known amounts of DNA. A calibration curve of molecular weights was made using λ DNA or pBR322 DNA fragments cleaved with restriction enzymes *Hind*III²²⁾ or *Hae*III²³⁾, respectively. Molecular sizes of these marker DNA fragments ranged from 4.6 $\times$ 10³ to 1.6 $\times$ 10⁷ daltons. Mean molecular weights of DNA fragments yielded by treatment with tannins were computed by a microcomputer M223 mark III (Sord Computer Systems Co.) using the calibration curve and the densitometric data on the film. Since the correspondence between molecular weights of DNA fragments and distances migrated is logarithmic, DNA fragments having larger molecular weights tend to be overestimated.²⁴⁾ Therefore, the relative molecular numbers of DNA fragments were calculated by dividing the optical density on the film by the molecular weight and the mean molecular weights and the mean breakage numbers were computed according to the equations below,

$$M_n = \Sigma n_i M_i / \Sigma n_i \quad \text{MBN} = M/M_n - 1$$

where M_n : mean molecular weight of DNA fragments, n_i : relative molecular number of DNA fragments having molecular weight of M_i , M_i : molecular weight of DNA fragments at each measurement, MBN: mean breakage number, M : molecular weight of native DNA molecules.

RESULTS

1. Double-stranded DNA breakage by hydrolyzable tannins

Hydrolyzable tannins fit into three general groups according to the sorts of constituent acids, as shown in Fig. 1. The first group is gallotannins, constituted of gallic acid. The second is ellagitannins, containing hexahydroxydiphenic acid. The third group is other tannins having other acids. Double-stranded DNA breakage by these hydrolyzable tannins were examined with λ DNA. Since λ DNA has a homogenous molecular size of 3.2 $\times$ 10⁷ dal-

tons, it is a favorable material to detect the activity of DNA-breaking reagents.

DNA breakage by galloyl glucoses was first investigated. The number of galloyl groups in

a galloyl glucose molecule ranged from one to ten. Figure 2 is the electrophoregram of DNAs which were treated with various concentrations of heptagalloyl glucose in the presence of $200\text{ }\mu\text{M}$ Cu^{2+} at 37°C for 2 hr. As is evident from lanes 6, 7, and 8 in Fig. 2, heptagalloyl glucose caused double-strand breaks in DNA in the presence of Cu^{2+} . Twenty-five μM heptagalloyl glucose had the strongest breaking activity. Double-stranded DNA breakage by tannins was compared by measuring the decrease of the mean molecular weight of the double-stranded DNA fragments by electrophoretic analysis. DNA breakage by galloyl glucoses is summarized in Fig. 3. Although gallic acid, a constituent of galloyl glucose, showed the strong activity depending upon its concentration, the activity of monogalloyl glucose was so weak that no breakage was detected at the concentration of $400\text{ }\mu\text{M}$. When the number of galloyl groups in a molecule increased from two to ten, DNA breakage was scarcely detected at concentrations higher than $100\text{ }\mu\text{M}$ of tannin. Each galloyl glucose showed a characteristic pattern of breakage which had a peak in the

FIG. 1. Structures of Hydrolyzable Tannins Examined.

FIG. 2. Agarose Gel Electrophoregram of λ DNA Treated with the Heptagalloyl Glucose- Cu^{2+} Mixture. λ DNA was treated with various concentrations of heptagalloyl glucose (1, 3, 6, 12, 25, 50, 75, 100, 200, and $400\text{ }\mu\text{M}$) in 8 mM phosphate buffer ($\text{pH } 7.0$) in the presence of $200\text{ }\mu\text{M}$ Cu^{2+} at 37°C for 2 hr. After addition of 6 mM EDTA, 0.02% bromophenol blue (BPB), and 6% sucrose, 0.7% agarose gel electrophoresis was done at $50\text{ V}/10\text{ mA}$ for 15 hr in a Tris-borate-EDTA (TBE) buffer containing $0.5\text{ }\mu\text{g}/\text{ml}$ ethidium bromide. Lane 1, DNA-*Hind*III fragments. The concentration of heptagalloyl glucose: lane 2, $0\text{ }\mu\text{M}$; lane 3, $1\text{ }\mu\text{M}$; lane 4, $3\text{ }\mu\text{M}$; lane 5, $6\text{ }\mu\text{M}$; lane 6, $12\text{ }\mu\text{M}$; lane 7, $25\text{ }\mu\text{M}$; lane 8, $50\text{ }\mu\text{M}$; lane 9, $75\text{ }\mu\text{M}$; lane 10, $100\text{ }\mu\text{M}$; lane 11, $200\text{ }\mu\text{M}$; lane 12, $400\text{ }\mu\text{M}$.

FIG. 3. The DNA-Breaking Activity of Galloyl Glucose. λ DNAs were treated with the galloyl glucoses and Cu^{2+} mixture, and electrophoresed in the same conditions as in the legend to Fig. 2. The mean molecular weights of the DNA fragments produced were calculated by a micro-computer as described in MATERIALS AND METHODS. The origins of the ordinate indicated the molecular weight of native λ DNA (3.2×10^7 daltons). In the tests of tri- and tetragalloyl glucose, the kinds of the positional isomers used are shown by the arrows. Unless otherwise noted, the experimental conditions were the same as in the following figures and table.

FIG. 4. The DNA-Breaking Activity of 2',3,5-Trigalloyl Hamamelose, 3,4,5-Trigalloyl Quinic Acid, and 3,4,5-Trigalloyl Proto-quercitol.

●—●, 2',3,5-trigalloylhamamelose; ▲---▲, 3,4,5-trigalloyl quinic acid; ■---■, 3,4,5-trigalloyl proto-quercitol.

FIG. 5. The DNA-Breaking Activity of Ellagitannins and Other Hydrolyzable Tannins.

The experimental conditions were the same as in Figs. 2 and 3, except that ellagic acid and sanguisorbic acid dilactone were tested in 20% DMSO because of their insolubility in water. Ellagitannins: ●—●, ellagic acid; ▲---▲, sanguisorbic acid dilactone; ■---■, eugenin. Others: ●—●, trapain.

range of concentrations from 25 to 75 μM . Positional isomers of tri- and tetragalloyl glucose had different activity patterns.

The activity also differed according to the sort of the constituent polyalcohols, as shown in Fig. 4. 2',3,5-Trigalloyl hamamelose had the strongest activity at the concentration of 75 μM and 3,4,5-trigalloyl quinic acid and 3,4,5-trigalloyl proto-quercitol at the concentration of 50 μM . The maximum activity decreased in the order of 2',3,5-trigalloyl hamamelose, 3,4,5-trigalloyl quinic acid and 3,4,5-trigalloyl proto-quercitol.

The results of the tests with ellagitannins were shown in Fig. 5. Although no breakage was detected for ellagic acid and sanguisorbic acid dilactone, eugenin had maximum breaking activity at the concentration of 50 μM . Since ellagic acid and sanguisorbic acid dilactone were practically insoluble in water, the breaking reaction was done in 20% DMSO. In order to examine the effect of DMSO on DNA breakage by hydrolyzable tannins, λ DNAs were treated with a mixture of 25 μM hexagalloyl glucose and 200 μM Cu^{2+} in 8 mM phosphate buffer containing 0, 1, 5, 10, 20, 30, and

40% DMSO for 2 hr. The DNA breaking reaction was extremely suppressed in 1% DMSO and completely inhibited in higher concentrations of DMSO than 5%. Thus, it was obscure whether ellagic acid and sanguisorbic acid dilactone had no DNA-breaking activity or their activity was inhibited by DMSO. Trapain, one of the "other tannins" group, had rather strong activity at lower concentrations and a maximum activity was found at the same concentration as for eugenin.

As is evident from lanes 6, 7, and 8 in Fig. 2 and the decrease of the mean molecular weight of the double-stranded DNA fragments in Figs 3, 4, and 5, hydrolyzable tannins caused double-strand breaks in the presence of Cu^{2+} . Double-stranded DNA breakage by hydrolyzable tannins changed variously according to the sorts, numbers, and binding positions of the constituent acids and polyalcohols.

2. Course of DNA breakage by hexagalloyl glucose

We examined DNA breakage by hydrolyzable tannins in more detail in a mixture of 25 μM hexagalloyl glucose (HGG) and 200 μM Cu^{2+} . Although double-strand breakage was scarcely detected in the first hour of reaction the average breakage number then gradually increased and reached 3.6 after 4 hr (Fig. 6).

Single-strand breakage was examined by alkali gel electrophoresis. Although no single-strand breakage was detected in the first 30 min, obvious breaks were observed after 1 hr in spite of the lack of double-strand breaks, so hydrolyzable tannins first broke single strands, and the accumulation of single-strand breaks resulted in double-strand breaks. The fact that even single-strand breaks couldn't be detected at the initial stage of the reaction suggested that some activation of hydrolyzable tannins might be necessary for

FIG. 6. The Course of DNA Breakage by Hexagalloyl Glucose.

DNAs (4.2 μg) were incubated with a mixture of 25 μM hexagalloyl glucose (HGG) and 200 μM Cu^{2+} in 75 μl of 8 mM phosphate buffer for various periods of time. The reaction was ended by the addition of 30 μl of 30 mM EDTA. After addition of 0.02% BPB and 6% sucrose to 40 μl of the reaction mixture, 0.7% agarose gel electrophoresis was done using TBE buffer (pH 8.3) in the same conditions as in Figs. 2 and 3. The rest of the reaction mixture, after the addition of 0.02% bromocresol green and 6% sucrose, was used for alkaline gel electrophoresis using 0.7% agarose gel in the alkaline buffer (pH 12.6) in order to detect single-strand breaks. (A): (a) neutral gel electrophoresis (pH 8.3). (b) alkaline gel electrophoresis (pH 12.6). Lane 1, DNA-HindIII fragments; lane 2, pBR322 DNA-HaeIII fragments. The reaction time: lane 3, 0 min; lane 4, 1 min; lane 5, 5 min; lane 6, 10 min; lane 7, 20 min; lane 8, 30 min; lane 9, 1 hr; lane 10, 2 hr; lane 11, 3 hr; lane 12, 4 hr. (B): The course of double-stranded DNA breakage by HGG.

the appearance of the DNA-breaking activity.

3. Requirement of Cu^{2+} for DNA breakage by HGG

The effect of Cu^{2+} on the DNA-breaking reaction by hydrolyzable tannins was investigated. DNA was treated with a mixture of various concentrations of HGG and Cu^{2+} at 37°C for 2 hr and DNA breakage was examined. As shown in Fig. 7, no breakage by HGG was observed in the absence of Cu^{2+} (0 mM). However, strong activity emerged in the presence of Cu^{2+} (0.2, 0.5, 1.0, and 2.0 mM). When the Cu^{2+} concentration was increased from 0.2 mM, the strong breaking activity appeared even at HGG concentrations of 75 and $100\ \mu\text{M}$, at which there had been no breakage observed, so possibly the suppression of breakage at higher concentrations of tannins is caused by a deficiency of Cu^{2+} .

4. Effects of various kinds of metallic ions and pH on DNA breakage by HGG

Cu^{2+} was absolutely required for DNA breakage by HGG. The effects of other metallic ions on the breakage were examined using HGG. The breakages caused by treatment with a mixture of various concentrations of HGG (1 to $400\ \mu\text{M}$) and 0.5 mM metallic ions (as sulfates) for 2 hr were determined. The results are shown in Table I. Cu^{2+} caused about 50.2 double-strand breaks per DNA molecule, but Mn^{2+} , Zn^{2+} , and Fe^{3+} had extremely weak activity and Fe^{2+} , Ni^{2+} , Co^{2+} , and Mg^{2+} had no effect on the breaking reaction. This remarkable effect of Cu^{2+} might be related to the excellent catalysis by Cu^{2+} of the oxidation of aromatic reductones, as is evident from the fact the oxidizing enzymes of aromatic reductones are almost all copper enzymes.

The effects of pH on the DNA-breaking reaction were investigated. As shown in Fig. 8, the breaking activity of the HGG- Cu^{2+} mixture was weak in acidic conditions and enhanced with increasing pH. As the possibility that DNA was broken by Cu^{2+} alone at higher pH was considered, DNA was treated with

FIG. 7. Requirement for Cu^{2+} by the DNA-Breaking Activity of Hexagalloyl Glucose.

λ DNAs were treated with various concentrations of HGG at 37°C for 2 hr in the absence or presence of Cu^{2+} and the mean number of breaks were examined. The Cu^{2+} concentrations: ●—●, 0 mM; ▲—▲, 0.2 mM; ■—■, 0.5 mM; *—*, 1 mM; ○—○, 2 mM.

TABLE I. THE EFFECT OF METALLIC IONS ON THE DNA-BREAKING ACTIVITY OF HEXAGALLOYL GLUCOSE

λ DNAs were treated with various concentrations of HGG (0, 1, 3, 6, 12, 25, 50, 75, 100, 200, and $400\ \mu\text{M}$) in the presence of 0.5 mM metallic ions (as sulfates) at 37°C for 2 hr and the mean breakage numbers were examined. Maximum values of the mean breakage numbers are shown in Table I.

Metallic ions	Mean breakage number
Cu^{2+}	50.2
Mn^{2+}	<0.1
Zn^{2+}	<0.1
Fe^{3+}	<0.1
Fe^{2+}	u.d.
Ni^{2+}	u.d.
Co^{2+}	u.d.
Mg^{2+}	u.d.

u.d., undetected.

Cu^{2+} alone at each pH. However, no DNA breakage occurred upon treatment with Cu^{2+} alone in the pH range examined. The HGG solution was yellow at alkaline pH and the reaction was accelerated by Cu^{2+} . The oxidation rate of HGG was expressed in Fig. 8 as the increase in absorbance at 420 nm after the incubation at 37°C for 20 min. Since the oxid-

FIG. 8. Effects of pH on the DNA-Breaking Activity and the Oxidation of Hexagalloyl Glucose.

λ DNAs were treated with the mixture of 25 μ M HGG and 200 μ M Cu^{2+} in 50 mM buffers having various pH values at 37°C for 2 hr and the decrease of the mean molecular weights of DNA fragments was investigated. Oxidation of HGG was followed by measuring the increase of absorbance at 420 nm after the incubation of the mixture of 60 μ M HGG, 200 μ M Cu^{2+} and 8 mM buffers. We used acetate buffer for pH 4 and 5, phosphate buffer for pH 6, 7, and 8, Tris-HCl buffer for pH 9 and carbonate-bicarbonate buffer for pH 10.

ation of HGG was accelerated in alkaline conditions, the enhancement of the breaking activity of HGG with increasing pH was supposed to be correlated with the increasing rate of the Cu^{2+} -catalyzed oxidation of tannins. The zigzag relation between the breaking activity or oxidation rate of tannin and pH was assumed to be related to differences in the buffers used, because the breakage or oxidation rate of HGG increased at higher pH in the same sort of buffer. Since the buffer can interact with metallic ions as well as protons, it may influence the breakage by tannins in the presence of metallic ions.

DISCUSSION

DNA breakage, especially double-stranded DNA breakage, is assumed to influence many functions of cells. Although single-strand breaks caused by epinephrine, an aromatic reductone, are rapidly repaired, double-strand

breaks are retained for a long time, resulting in changes of cell functions.²⁵⁾ There is a hypothesis that appropriate breakages of DNA can initiate carcinogenesis, carcinostasis, cyto-differentiation, mutation, or virogenesis.²⁶⁾ We found double-strand breaks caused by pure hydrolyzable tannins. The activity changed variously according to the sorts, numbers, and binding positions of the constituent acids and polyalcohols.

A prominent feature of the DNA breakage by hydrolyzable tannins was the suppression of the activity at concentrations higher than 100 μ M of tannins. Since the suppression was released by the increase of Cu^{2+} concentration, a deficiency of Cu^{2+} which could participate the breaking reaction was supposed to be the cause of the suppression. Tannins are known to be potent chelators of metallic ions, and form insoluble precipitates with Cu^{2+} .²⁷⁾ When Cu^{2+} is chelated by higher concentrations of tannins in such a form that Cu^{2+} can no longer be used in the breaking reaction, the breaking reaction might be suppressed.

Because no breakage was observed by Cu^{2+} alone, it was obvious that both tannins and Cu^{2+} were indispensable for the DNA-breaking reaction. The complex behavior of hydrolyzable tannins in the breaking reaction compared with that of gallic acid may be associated with the complex structures of tannins carrying many binding sites for Cu^{2+} . HGG exhibited a maximum activity at the Cu^{2+} concentration of 0.5 mM. The activity was weakened at higher concentrations of Cu^{2+} than 0.5 mM. Such a tendency is known in the case of ascorbic acid.^{28,29)} This suggested that Cu^{2+} didn't work only as the catalyzer of the oxidation of tannins. Because Cu^{2+} can bind to DNA,^{30,31)} the interaction of DNA, Cu^{2+} and tannins may be important in the breaking reaction.

In the metal-catalyzed oxidation process of tannins, such secondary radical products as $\cdot\text{OH}$, $\cdot\text{O}_2^-$, and H_2O_2 are supposed to be produced concomitantly and are assumed to take part in the DNA breaking reaction. The fact that DMSO, which is known as a scavenger for

·OH radicals, strongly inhibited DNA breakage by HGG suggested the participation of ·OH radicals in the breaking reaction. However, since the oxidation process of hydrolyzable tannins is very complex, the identification of the intermediate responsible for the DNA breakage is not easy. The detailed mechanism of the DNA-breaking reaction by hydrolyzable tannins is under investigation.

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REFERENCES

- 1) I. Nishioka, *Yakugaku Zasshi*, **103**, 125 (1983).
- 2) K. Yamafuji and H. Murakami, *Enzymologia*, **35**, 139 (1968).
- 3) K. Yamafuji, H. Murakami and M. Shinozuka, *Z. Krebsforsch.*, **73**, 195 (1970).
- 4) K. Yamafuji, Y. Nakamura, H. Omura, T. Soeda and K. Gytoku, *Z. Krebsforsch.*, **76**, 1 (1971).
- 5) S. Shirahata, H. Murakami, K. Yamada and H. Omura, *Nippon Nôgeikagaku Kaishi*, **56**, 1027 (1982).
- 6) H. Murakami, S. Shirahata, C. Hori, H. Yoshii, K. Yamada, K. Shinohara and H. Omura, *Agric. Biol. Chem.*, **43**, 1619 (1979).
- 7) M. V. Norgard, K. Emigholz and J. J. Monahan, *J. Bacteriol.*, **138**, 270 (1979).
- 8) A. Colman, M. J. Byers, S. B. Brimrose and A. Lyons, *Eur. J. Biochem.*, **91**, 303 (1978).
- 9) G. Nonaka and I. Nishioka, *Chem. Pharm. Bull.*, **31**, 1652 (1983).
- 10) G. Nonaka and I. Nishioka, *Chem. Pharm. Bull.*, **29**, 2862 (1981).
- 11) unpublished data.
- 12) M. Nishizawa, T. Yamagishi, G. Nonaka and I. Nishioka, *J. Chem. Soc., Perkin Trans. I*, 961 (1983).
- 13) M. Nishizawa, T. Yamagishi, G. Nonaka and I. Nishioka, *J. Chem. Soc., Perkin Trans. I*, 2963 (1982).
- 14) G. Nonaka, K. Ishimaru, T. Tanaka and I. Nishioka, *Chem. Pharm. Bull.*, **32**, 483 (1984).
- 15) H. Nishimura, G. Nonaka and I. Nishioka, *Chem. Pharm. Bull.*, **32**, 1741 (1984).
- 16) G. Nonaka, M. Harada and I. Nishioka, *Chem. Pharm. Bull.*, **28**, 685 (1980).
- 17) G. Nonaka, T. Tanaka and I. Nishioka, *J. Chem. Soc., Perkin Trans. I*, 1067 (1982).
- 18) G. Nonaka, T. Tanaka and I. Nishioka, *J. Chem. Nishizawa and T. Yamagishi*, *Chem. Pharm. Bull.*, **29**, 1184 (1981).
- 19) S.-H. Shiou, *J. Biochem.*, **94**, 1259 (1983).
- 20) D. E. Pulleyblank, M. Shure and J. Vinograd, *Nucleic Acids Res.*, **4**, 1409 (1977).
- 21) A. Prunell, F. Strauss and B. Leblanc, *Anal. Biochem.*, **78**, 57 (1977).
- 22) K. Murray and N. E. Murray, *J. Mol. Biol.*, **98**, 551 (1975).
- 23) R. J. Roberts, *Nucleic Acids Res.*, **10**, 117 (1982).
- 24) C. P. Ordahl, T. R. Johnson and A. I. Caplan, *Nucleic Acids Res.*, **3**, 2985 (1976).
- 25) H. Murakami, T. Miyoshi, M. Koga, K. Yamada and H. Omura, *Agric. Biol. Chem.*, **39**, 795 (1975).
- 26) K. Yamafuji, "Food, Cancer and Cytodifferentiation," Shukosha, Fukuoka, Japan, 1970.
- 27) T. Okuda, K. Mori, M. Shiota and K. Ida, *Yakugaku Zasshi*, **102**, 35 (1982).
- 28) K. Shinohara, M. So, M. Nonaka, K. Nishiyama, H. Murakami and H. Omura, *J. Nutr. Sci. Vitaminol.*, **29**, 491 (1983).
- 29) K. Shinohara, M. So, M. Nonaka, K. Nishiyama, H. Murakami and H. Omura, *J. Nutr. Sci. Vitaminol.*, **29**, 489 (1983).
- 30) Ch. Zimmer, G. Luck, H. Fritzsche and H. Triebel, *Biopolymers*, **10**, 441 (1971).
- 31) H. Richard, J. P. Schreiber and M. Daune, *Biopolymers*, **12**, 1 (1973).