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INTERACTION BETWEEN ACTIVATED NORDIHYDROGUAIARETIC ACID AND DEOXYRIBONUCLEIC ACID

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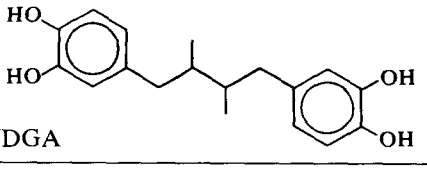
Abstract—Nordihydroguaiaretic acid (NDGA) is a natural product of the lignan family that has been shown to possess antimicrobial and antineoplastic properties in a variety of test systems. NDGA was observed by u.v.-visible spectroscopy to be unstable in an aqueous environment; however, by these same techniques NDGA was shown to be stable in the presence of mercaptoethanol. It is suggested that an activated NDGA is an intermediate in the O_2-mediated oxidation of NDGA and that the activated NDGA forms a stable complex when reacted with duplex DNA. This DNA-activated NDGA complex was detected and studied by both fluorescence spectroscopy and CsCl density gradient techniques. The addition of DNA quenched the fluorescence of activated NDGA in a concentration-dependent fashion. Furthermore, the exposure of activated NDGA lowered the buoyant density of DNA, also, in a concentration-dependent manner. Since mononucleotides did not quench the fluorescence of activated NDGA, and heat-denatured DNA was less effective than fully duplex DNA in its ability to interact with activated NDGA, the duplex structure of DNA was determined to be important in the complex formation. Whereas activated NDGA bound to both poly dG · poly dC and poly (dA · dT), there appeared, by one method of analysis, to be a preference for poly dG · poly dC. Activated NDGA–DNA complex was stable to dialysis, but dissociated in the presence of Sarkosyl. No changes in the viscosity or melting temperature of DNA was induced by the addition of activated NDGA. These data suggest a mechanism in which the activated NDGA was bound to the more apolar regions of duplex DNA that are located in either the major and/or minor grooves.

Nordihydroguaiaretic acid (NDGA), a natural product of the lignan family [1], was used for years as an antioxidant to inhibit the development of rancidity in the fats of food products [2] and as a stabilizer of pharmaceutical preparations, perfumery oils, rubber and other industrial products [3]. Because of its potential toxicity to animal systems, it is no longer used in this fashion.

NDGA has been reported to have antimicrobial effects on a variety of organisms [4–8], and antineoplastic potential, *in vitro*, has been observed in Ehrlich ascites, K-2 ascites, and leukemia L-1210 cell lines [9]. Preliminary studies, *in vivo*, have shown NDGA to have therapeutic activity against two separate cancers [10, 11].

With the knowledge that NDGA is a potent antioxidant in biological systems [2] and that this compound is active in a number of *in vitro* oxidation–reduction enzyme catalyzed reactions [12–14], it is reasonable to suggest that any biological action of NDGA treatment could result from either NDGA itself or from one of its oxidation products. Preliminary studies, *in vitro*, showed that an O_2-mediated product of NDGA (activated NDGA) formed a stable complex with DNA. Since many antimicrobial and antineoplastic agents are known to function by interacting with DNA and subsequently affecting nucleic acid metabolism [15–18], it became important to further investigate the interaction of activated NDGA and DNA. This report documents that activated NDGA is formed by an O_2-dependent oxidation of NDGA and that this intermediate forms a stable complex with DNA.



NDGA

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MATERIALS AND METHODS

Impure NDGA was obtained from the W. J. Strange Co. (Chicago, IL) and from Columbia Organic Chemicals, Inc. (Columbia, SC). [^{3}H]NDGA and Sarkosyl NL 97 were from ICN Pharmaceuticals, Inc. (Plainview, NY). Highly polymerized calf thymus DNA, 2′-dAMP, 2′-TMP, 2′-dGMP and 2′-dCMP were purchased from the Sigma Chemical Co. (St. Louis, MO). 3,5-Diaminobenzoic acid (DABA) was a product of the Aldrich Chemical Co. Inc. (Milwaukee, WI). Optical grade cesium chloride was obtained from the Harshaw Chemical Co. (Solon, OH). Polydeoxyguanylate · polydeoxycytidylate [poly (dG) · poly (dC)] and polydeoxyadenylate-thymidylate [poly (dA · dT)] were obtained from Miles Laboratories, Inc. (Elkhart, IN). A stock of T-7 phage was supplied by Dr. R. E. Harrington, University of Nevada, Reno. The Union Carbide Corp. (New York, NY) was the

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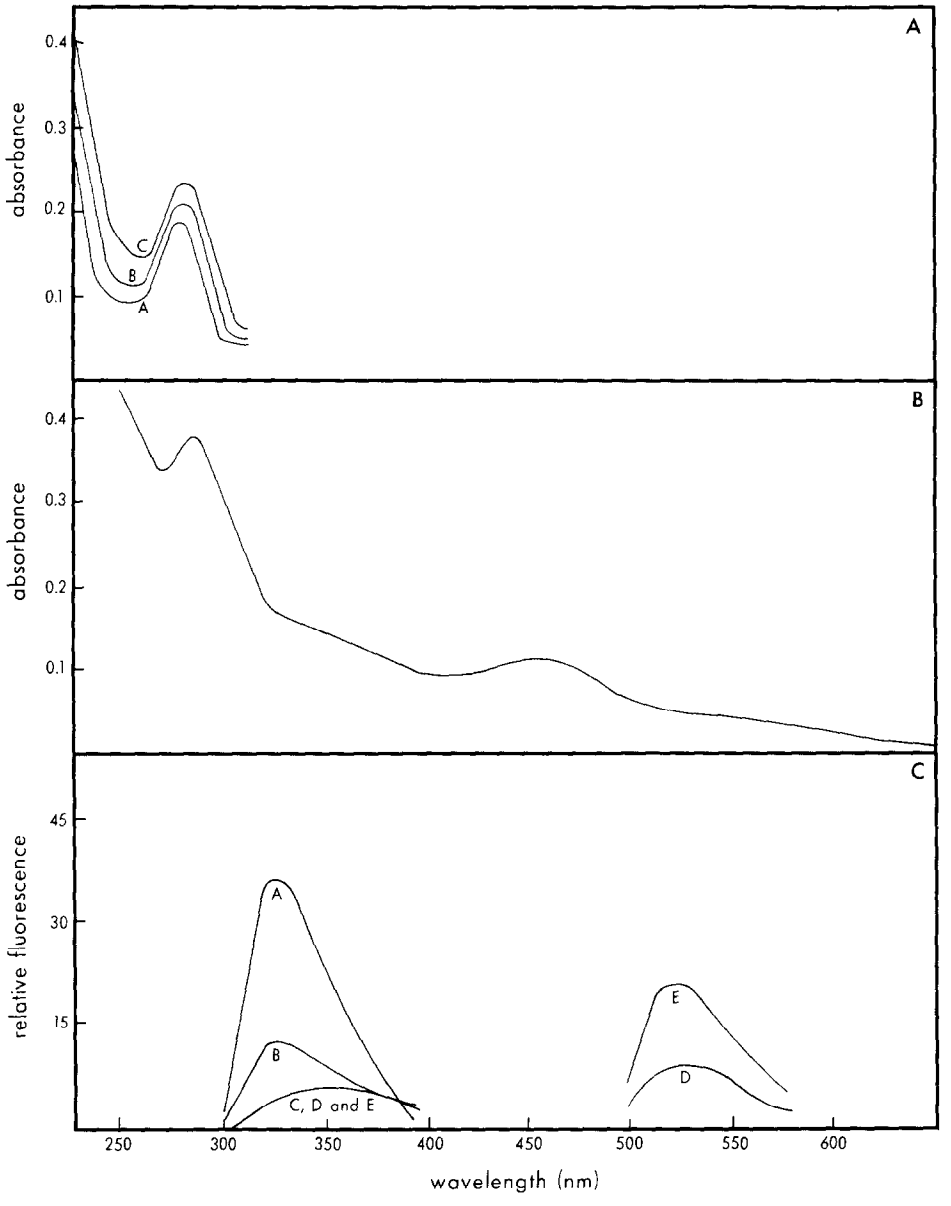

Fig. 1. Spectral characteristics of NDGA and activated NDGA. (A) Time-dependent changes in the u.v. absorbance spectrum of NDGA. NDGA was dissolved in SSC at a concentration of 30 μM. Spectra were obtained for A in less than 5 min; B, at 1 hr; and C, at 2 hr. (B) Ultraviolet and visible spectral characteristics of NDGA products after 72 hr. NDGA was dissolved in SSC at a concentration of 50 μM. (C) Fluorescence characteristics of NDGA with time. NDGA was dissolved in SSC at a concentration of 30 μM. Visible and u.v. spectra were obtained at various times as NDGA underwent chemical changes. For fluorescence in the region of 325 nm, activation wavelength was 280 nm, and for fluorescence in the region of 530 nm, activation wavelength was 460 nm. Times for samples measurement were A, 1 hr; B, 24 hr; C, 48 hr; D, 96 hr; and E, 144 hr.

source of polyethylene glycol 6000, and ethidium bromide was from the Boots Pure Drug Co. (UK). All other chemicals were reagent grade.

Purification of NDGA. Impure NDGA was suspended in 20% aqueous acetic acid at a concentration of 30 mg/ml. Upon heating, the NDGA dissolved and, to the brownish solution, decolorizing charcoal was added. The decolorized solution was recovered by pressure filtration and allowed to cool slowly to room temperature. Ivory white crystalline NDGA obtained from this procedure melted at 184–185°.

[^{3}H]NDGA was purified by preparative silica gel chromatography. The compound was chromatographed utilizing the system developed by Gisvold and Thacker [19], which consists of benzene–isopropyl alcohol–acetic acid–water (25:5:2:10, by vol). NDGA was detected with an ultraviolet lamp, scraped from the plates, and extracted with 95% ethanol.

Nucleic acid preparations. Highly polymerized calf thymus DNA was phenol extracted and dialyzed against 0.15 M sodium chloride, 0.015 M sodium cit-

rate (standard saline-citrate, SSC) [20]. Using this procedure, protein content was not detectable by the method of Lowry *et al.* [21] and orcinol tests [22] indicated that the RNA content was less than 0.01 per cent.

DNA concentrations were determined by measuring optical density at 260 nm, followed by calculation of the concentration using an extinction coefficient of 0.02 $cm^2/\mu g$ duplex DNA [20]. A molar extinction coefficient of 6600 liters/mole of duplex DNA-nucleotide is obtained if the average sodium mononucleotide molecular weight was taken to be 330 g/mole.

T-7 DNA was isolated from bacteriophage grown in infected *Escherichia coli* B cells. After cell lysis and centrifugation at 5000 *g* at 4° to remove debris, the phage was concentrated by the method of Yamamoto *et al.* [23] using polyethylene glycol 6000. The precipitated phage were then collected by centrifugation at 5000 *g* and the concentrate was layered on a CsCl gradient of range 1.25 to 1.70 g/ml. This procedure separated the polyethylene glycol from the virus. After centrifugation at 0° for 24 hr (22,000 rpm) in a Spinco 25.1 rotor, a sharp band of T-7 virus was removed from the gradients.

DNA was isolated from the purified T-7 virus by double phenol extraction followed by dialysis in SSC buffer, pH 7.5 to 8.5 [20]. DNA banded in CsCl at 1.704 g/ml and had a molecular weight of 25 million daltons, as determined by viscosity measurements. Both the buoyant density [24] and molecular weight [25] agreed with reported values.

Addition of NDGA to DNA solutions. NDGA has a maximum water solubility of 330 μM [26]. Therefore, more concentrated solutions of NDGA were prepared in 95% ethanol and aliquots of these solutions were added to aqueous preparations. Samples prepared in this fashion were clear and colorless. The final alcohol concentration in all experiments. ranged from 0.7 to 1.0%.

Fluorescence measurements. Relative fluorescence intensities were determined with an Aminco–Bowman spectrophotofluorometer at ambient temperature in SSC buffer. The absorbance of excitation light by DNA at 280 nm with fluorescence at 325 nm was corrected by multiplying the fluorescence intensity by the antilog of one-half the sample optical density [27].

Sedimentation equilibrium studies. Cesium chloride density gradients were prepared according to the method of Grossman *et al.* [28]. The length of the runs was from 40 to 50 hr at 36,000 rpm and 25°, in a Spinco SW 50.1 rotor using nitrocellulose tubes (3.0 ml). Densities were determined by refractometry at 25% and the u.v. absorbance of all fractions was obtained spectrophotometrically.

3,5-DABA was purified as described by Hinegardner [29] and used to determine the presence of DNA in density gradient experiments. An aliquot of each fraction from the gradients was added to an equal volume of 2 M DABA and incubated for 30 min in a water bath at 60°. To the samples was then added 1.8 ml of 1.5 N HCl, and fluorescence intensities were determined (activation wavelength 415 nm, fluorescence wavelength 505 nm). It was found that the presence of CsCl did not alter the fluorescence intensities of the samples studied.

Viscosity measurements. A Gill and Thompson low shear rotating cartesian-diver viscometer was used for viscosity measurements of T-7 DNA solutions [30]. Measurements were carried out in SSC buffer at 25°. The total volume of the DNA solutions was less than 1.0 ml.

Melting profiles. Thermal denaturation studies were carried out with a Zeiss PMQ II spectrophotometer equipped with a programmed heating and cooling water bath. The heating rate was 0.5°/min. A teflon probe was immersed in the sample cuvette for accurate temperature measurements, and the DNA samples were measured in 0.1X SSC buffer [31].

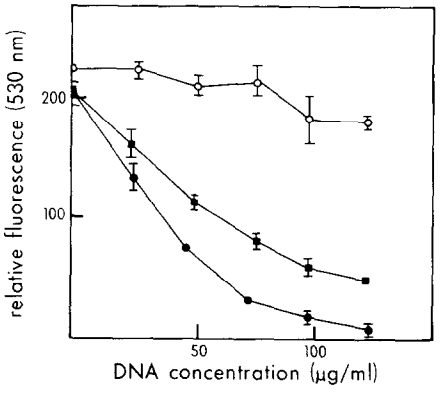

Fig. 2. Fluorescence of activated NDGA. NDGA was dissolved at a concentration of 20 μM in SSC buffer (pH 8.3) containing DNA (●), increasing concentrations of an equimolar mixture of the four deoxymononucleotides (○), or heat-denatured calf thymus DNA (■). Samples stood for 72 hr before readings were obtained.

RESULTS

Instability of NDGA in aqueous solutions. As evidence that NDGA in SSC buffer undergoes a time-dependent chemical alteration, the u.v.-visible spectrum of the solution was monitored at various times. After standing for 2 hr the u.v. absorption of samples increased in intensity at its absorption maximum and shifted from a maximum of 280 to 285 nm as shown in Fig. 1. After 72 hr the newly formed reddish-brown color was of sufficient intensity to be detected spectrophotometrically, and a visible absorbance maximum was apparent at 460 nm. This is in addition to the 285 nm absorbance maximum.

The absorbance maxima at 280 nm for fresh NDGA solution and 460 nm for 48 hr or older solutions were found to be excitation maxima for fluorescence at 325 and 530 nm respectively. As shown in Fig. 1, the u.v. fluorescence intensity of NDGA at 325 nm decreased to roughly one-third its initial value after 24 hr. After 48 hr the trend continued and the spectrum shifted slightly to the red; the u.v. fluorescence, however, did not change further after 48 hr. At 96 hr, fluorescence developed at 530 nm, and this peak was increased in intensity at 144 hr. That compound which fluoresced at 530 nm was defined as activated NDGA.

Interaction between DNA and activated NDGA.

Perturbation in the fluorescence spectrum of NDGA was employed as a measure of interaction between DNA and NDGA and/or activated NDGA. To determine possible interactions of DNA and NDGA, fresh samples of NDGA at a concentration of 100 μM were prepared, and to those solutions was added calf thymus DNA over a concentration range of 0–216 μg/ml. After 1 hr there was no alteration of the NDGA mediated fluorescence at 325 nm. This observation indicates either a lack of interaction between DNA and NDGA or an inability of the technique to detect such an interaction.

On the other hand, when calf thymus DNA was added to NDGA solutions and those solutions were allowed to stand for 72 hr (a time sufficient to convert NDGA to activated NDGA), quenching of the fluorescence of activated NDGA at 530 nm was detectable. The results of a typical experiment (DNA ranging in concentration from 0 to 125 μg/ml and NDGA at 20 μM) are illustrated in Fig. 2. These results suggest that an interaction between the DNA and activated NDGA had occurred.

The interaction of activated NDGA and DNA could take place by one of two routes. Activated NDGA might be either a transient intermediate with the ability to interact with DNA, or it might accumulate in the 72-hr reaction. To determine which of these two possiblities took place, NDGA was allowed to stand for 72 hr after which calf thymus DNA was added to the samples. Both the calf thymus DNA concentration range and the initial NDGA concentration were comparable to those of Fig. 2. DNA treated in this fashion did not alter the fluorescence of activated NDGA. Therefore, it is concluded that it was necessary for NDGA to be activated in the presence of DNA and that this activated NDGA was trapped by DNA. Furthermore, it was concluded that the 72-hr product of NDGA was inactive. In all further experiments where activated NDGA will be described, both NDGA and nucleic

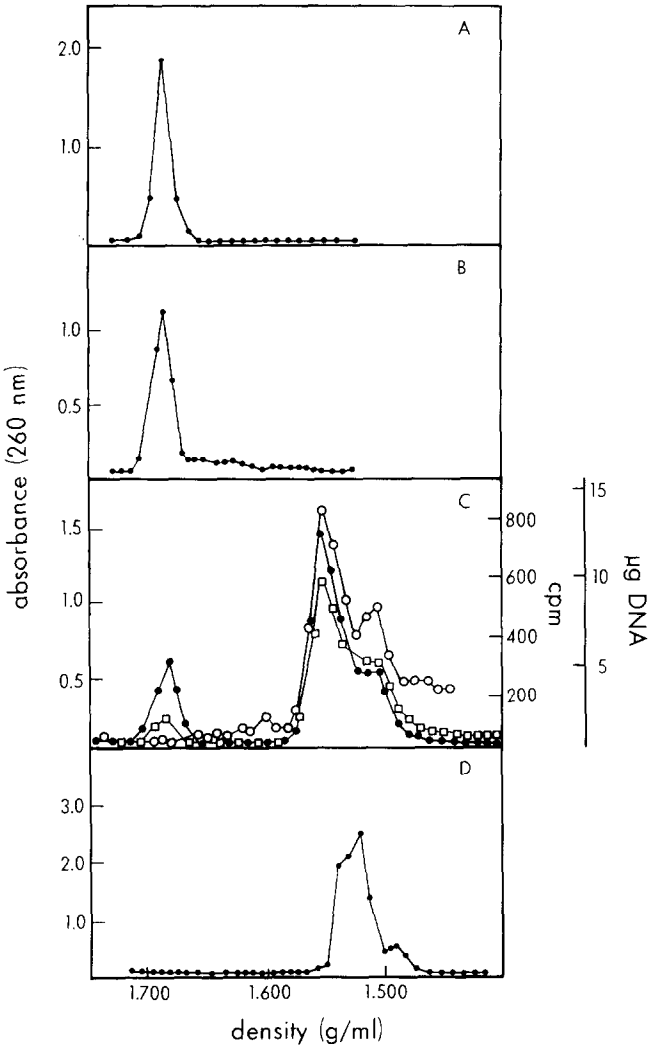

Fig. 3. CsCl sedimentation of activated NDGA–DNA complex. NDGA was added to 0.7 μmole nucleotide equivalents of calf thymus DNA. The NDGA/DNA nucleotide molar concentration ratios were: (A) 0, (B) 0.14, (C) 1.22, and (D) 2.1. In panel C, [^{3}H]NDGA was added to 0.7 μmole nucleotide equivalents of calf thymus DNA to give a final [^{3}H]NDGA/DNA nucleotide molar concentration ratio of 1.22. [^{3}H]NDGA, cpm —○—; DNA, μg/fraction as assayed by the DABA method [29], —●—; E_{260nm} —□—.

acid were present during the activation time preiods.

Binding studies. A second technique used to detect the interaction between activated NDGA and DNA was CsCl buoyant density centrifugation. When radioactive NDGA was added to calf thymus DNA in a CsCl solution and centrifuged to equilibrium, part or all of the DNA that normally banded in the region of native DNA was shifted to areas of lower buoyant density. As indicated in Fig. 3C, both activated [^{3}H]NDGA and DNA sedimented at densities of 1.575 g/ml and 1.535 g/ml. Native DNA was detected in the gradients at a buoyant density of 1.700 g/ml; no radioactive NDGA, however, was detected in this position.

The patterns of the low density bands were commonly of variable shapes and buoyant densities. For thirty-four runs, with initial molar concentration ratios (NDGA/DNA nucleotide) in the range 0.14 to 2.1, the following observations were made. First, at an initial NDGA/DNA nucleotide molar concentration ratio of 0.14, a negligible amount of DNA-activated NDGA complex was detected at lower densities. An example of this is shown in Fig. 3B. Second, as the initial NDGA/DNA nucleotide molar concentration ratio was increased, greater amounts of the complex were seen at lower density and a simultaneous decrease of native DNA was seen at its position in the gradients; a representative gradient of this type is shown in Fig. 3C. The low density banding pattern was rather broad in this gradient with a maximum at 1.575 g/ml and a strong shoulder at 1.535 g/ml, thus indicating density heterogeneity. Third, upon further increasing the initial NDGA concentration relative to constant DNA to give NDGA/DNA nucleotide molar concentration ratios of 2.1 and greater, no optical density was detected in the gradients at the position of native DNA. Figure 3D illustrates a gradient with an initial NDGA/DNA nucleotide molar concentration ratio of 2.1. This illustration shows a peak at 1.522 g/ml, a shoulder at 1.539 g/ml, and a second peak at 1.488 g/ml.

For this series of thirty-four experiments. the precise character of the low density bands obtained when DNA was centrigued in the presence of NDGA was not reproducible. The breadth, number, amplitude, and buoyant densities of these DNA-activated NDGA complexes were not identical from run to run; however, the variable patterns of complexes fell in the density range of 1.510 to 1.600 g/ml. The predictable aspect of these experiments was that addition of NDGA perturbed the bouyant density of DNA and that there was an inverse relationship between the initial NDGA/DNA nucleotide molar concentration ratio and the amount of DNA remaining at the buoyant density of native calf thymus DNA.

Effects of mercaptoethanol. As evidence that mercaptoethanol retards the chemical alteration of NDGA in aqueous solutions, 1.0 mM mercaptoethanol caused the complete inhibition of the reddish-brown color formation that normally occurred when 100 μM NDGA was in solution for 72 hr. Likewise, when these samples were scanned spectrophotometrically from 760 to 300 nm, no absorbance was detectable in this range, and the original u.v. spectrum of fresh NDGA was maintained throughout the 72-hr period. Thus, mercaptoethanol maintained the integrity of NDGA and prevented the formation of activated NDGA.

As described earlier, the fluorescence of fresh solutions of NDGA at 320 nm was not altered by DNA. It was desirable to test by a second method whether or not NDGA itself would interact with DNA. In these studies, mercaptoethanol was added to the experimental samples to maintain the NDGA concentration. This treatment of DNA with NDGA (protected from alterations by mercaptoethanol) did not alter the buoyant density of the DNA in CsCl gradients. Therefore, NDGA itself does not appear to interact with DNA in a fashion similar to that observed for activated NDGA.

Effects of the 72-hr NDGA products. To test the influence of the final products formed during a 72-hr time period, two centrifuge tubes were filled with CsCl solution and NDGA. To one of the centrifuge tubes was added sufficient calf thymus DNA to produce an initial NDGA/DNA nucleotide molar concentration ratio of 1.22, and to the other there was no addition of DNA. The samples stood for 72 hr after which calf thymus DNA was added to the second centrifuge tube. The samples were then centrifuged to equilibrium in CsCl and a characteristic peak of activated NDGA–DNA complex was obtained where DNA and NDGA were both present during the 72-hr preincubation period. However, there was no shift of the native DNA in the sample in which DNA was added to the NDGA product after 72 hr. Thus, the concept of activated NDGA as a transient species with an affinity for DNA is strengthened by these studies.

DNA structure and activated NDGA binding. To determine if the four deoxymononucleotides that comprise the building blocks of polymeric DNA possessed the capacity to interact with activated NDGA, an equal molar mixture of the four compounds was tested for their abilities to quench the fluorescence of activated NDGA. When NDGA was exposed to the nucleotides for 72 hr over a concentration range of 0–125 μg/ml, no significant alteration of the fluorescence intensity of activated NDGA was observed (Fig. 2).

The importance of the duplex structure of DNA in the binding reaction was tested by adding activated NDGA to samples of calf thymus DNA which had been heat denatured. Heat denaturation followed by rapid cooling converts duplex DNA to a structure consisting of both single and double stranded regions. As indicated in Fig. 2, the fluorescence of activated NDGA was altered to a lesser extent by heat-denatured DNA than by duplex DNA. Therefore, it appears that the double-standard structure of DNA is important for an optimal interaction.

Template binding specificity. Solutions of activated NDGA and poly (dG)·poly (dC) and poly (dA·dT) were analyzed by CsCl density gradient centrifugation techniques to determine whether complex formation exhibited a base sequence specificity. Figure 4 illustrates that activated NDGA alters the buoyant density of both poly (dG)·poly (dC) and poly (dA·dT) and did not show a preference for either base sequence. When template specificity was measured by spectral methods (Fig. 5), however, poly

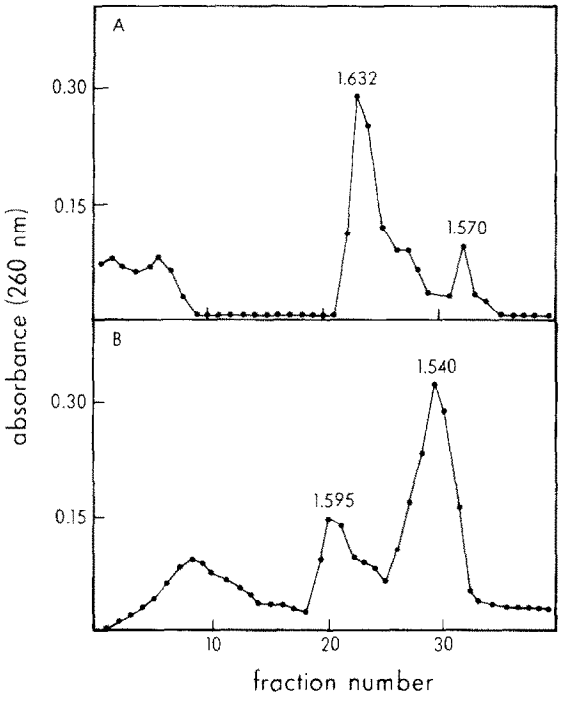

Fig. 4. Complex formation between activated NDGA and synthetic polynucleotides. (A) NDGA was added to 0.12 μmole nucleotide equivalents of poly (dG)·poly (C) to give a final NDGA/poly (dG)·poly (dC) molar concentration ratio of 2.0. CsCl was added to give an initial density of 1.614 g/ml, and the sample was then centrifuged to equilibrium. (B) NDGA was added to 0.12 μmole nucleotide equivalents of poly(dA·dT) to give a final NDGA/poly (dA·dT) molar concentration ratio of 2.5. CsCl was added to give an initial density of 1.571 g/ml, and the sample was then centrifuged to equilibrium.

(dG)·poly (dC) displayed a greater effect on the fluorescence of activated NDGA than did poly (dA·dT).

Noncovalent binding of activated NDGA. Two appraoches were taken to study whether the binding of activated NDGA to DNA was reversible or irreversible. In one method, fractions from a density gradient that contained DNA-activated NDGA complex (fractions representing complex between densities 1.637 g/ml and 1.560 g/ml) were pooled and dialyzed versus SSC buffer. The dialysis period lasted for 48 hr with frequent changes of the dialysis buffer. When the contents were recentrifuged in CsCl, only a small amount of DNA banded at 1.680 g/ml (native DNA), while the vast majority of the complex remained at the original low density region of 1.583 g/ml. Thus, it is evident that the complex was stable to dialysis.

In a second method the anionic detergent Sarkosyl was used to dissociate the complex. In this experiment, regions of DNA-activated NDGA complex (densities 1.543 g/ml through 1.553 g/ml) were isolated from a CsCl density gradient, pooled and divided into two equal parts. To one part was added Sarkosyl to a final concentration of 2.5%. To the other part no additions were made. These samples, as well as a sample of pure DNA containing 2.5% Sarkosyl, were centrifuged to equilibrium. After centrifugation the buoyant density of the pure DNA sample (1.688 g/ml) was not appreciably altered by the Sarkosyl, but the detergent caused the dissociation of the DNA-activated NDGA complex, resulting in the subsequent sedimentation of DNA to the region of native DNA (1.686 g/ml). When a separate sample of activated NDGA–DNA complex was recentrifuged in the absence of Sarkosyl, no dissociation was observed and the DNA complex sedimented at its characteristic low density site. That Sarkosyl dissociated the complex was confirmed by u.v. spectral analysis of the various fractions of sedimented DNA resulting from treated and untreated samples.

Thus, Sarkosyl is capable of liberating DNA from the activated NDGA–DNA complex and the liberated DNA has a buoyant density similar to that of a native DNA. Therefore, the complex appears to be the result of a reversible, non-covalent interaction between activated NDGA and DNA.

Viscosity measurements. Viscosity measurements at low shear rates (0.01 to 1.0 per sec) were carried out to determine the effects of activated NDGA on the reduced specific viscosity of DNA solutions. An increase in the viscosity would be expected if an intercalation-dependent lengthening or stiffening of the DNA occurred. A decrease in viscosity may indicate collapse of the DNA structure—for example, degradation of the strands. It was found that activated NDGA at NDGA/DNA nucleotide ratios up to 1.7 was ineffective in either increasing or decreasing the reduced specific viscosity of T-7 DNA.

Thermal denaturation studies. Thermal denaturation studies were carried out as another means to categorize the interaction of activated NDGA with DNA. An increased T_m would be indicative of increased stabilization of the DNA by activated NDGA, and a decreased T_m would be consistent with destabilization of the helical structure.

Calf thymus DNA at a concentration of 60 μM in nucleotides was added to NDGA at 90 μM in 0.1 SSC buffer and allowed to stand for 72 hr. The above concentrations produced an activated NDGA/DNA nucleotide molar concentration ratio of 1.5. Under these conditions no change in the T_m was observed.

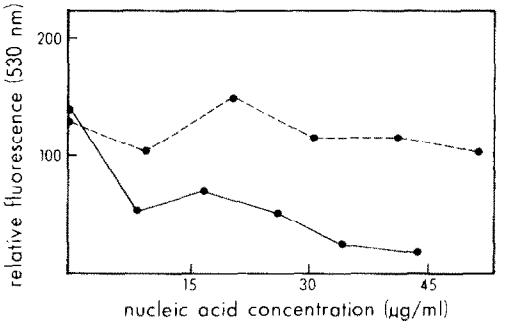

Fig. 5. Effects of synthetic DNA polymers on activated NDGA fluorescence. NDGA was dissolved at a concentration of 20 μM in SSC buffer, pH 8.0, containing poly (dA·dT) (-------) or poly (dG)·poly (dC) (———). Samples stood for 72 hr before readings were obtained. Activation wavelength was 460 nm.

DISCUSSION

An activated intermediate resulting from the chemical alteration of NDGA in aqueous media has been found to interact with DNA; NDGA itself, however, does not form a complex with DNA. This lack of interaction is evidenced by the inability of DNA to alter the fluorescence of NDGA in SSC buffer, as well as the inability of NDGA in the presence of mercaptoethanol to alter the buoyant density of DNA.

The sequence of events that leads to an interaction of activated NDGA (NDGA*) with DNA seems to follow the scheme shown below:

$$\text{NDGA} + O_2 \rightarrow \text{NDGA}^*$$
$$\text{NDGA}^* + \text{DNA} \rightarrow \text{DNA} - \text{NDGA}^*$$
$$\text{NDGA}^* + O_2 \rightarrow \text{inactive}$$
$$\text{NDGA}^* + \text{NDGA}^* \rightarrow \text{inactive}$$

According to the above scheme, only when DNA was present during the sequential chemical change of NDGA to its 72-hr products was there an interaction between the two molecules. Thus, the activated NDGA produced during the 72-hr period is a transient species that subsequently loses its affinity for DNA.

That active intermediates arise from the oxidation of NDGA is supported by studies of Hathway and Seakins [32]. They showed that color formation that occurs in aqueous solutions of catechol, catechin, and flavins (molecules with structural characteristics similar to NDGA) is due to oxidative processes. Further, increased color formation is consistent with an increased amount of oxidative product. Since the u.v. and visible spectra of NDGA products resemble the spectra determined by Hathway and Seakins for the oxidative polymers of catechol, catechin, and flavins produced by slow aqueous oxidation at neutral pH, it might be expected that NDGA undergoes similar oxidative changes. This impression is strengthened by the fact that the antioxidant mercaptoethanol inhibits the 72-hr color formation in solutions of NDGA. Furthermore, experiments conducted under anaerobic conditions also resulted in a lack of color production in NDGA solutions after 72 hr. From these data, molecular oxygen, with its high electron affintiy and thus its ability to abstract hydrogen atoms from NDGA, could be considered responsible for initiating oxidative changes in NDGA solutions.

Studies with catechin in aqueous solution by Brown and Whiteoak [33] have indicated that, in its oxidation at neutral pH, the loss of hydrogen atoms from the catechol R group produces an intermediate quinone or radical that subsequently couples in a head-to-tail fashion with other catechin molecules and other radicals forming a polymeric colored product. It has been determined by electron paramagnetic resonance studies* that in the presence of O_2 NDGA undergoes formation of a relatively long lived radical intermediate.

The data in this report suggest a model for the binding of activated NDGA to duplex DNA. Fluorometric evidence does not support the concept that mononucleotides interact with activated NDGA and heat-denatured DNA is less effective than fully duplexed DNA in the formation of complex. With respect to base specificity, the results show that a G-C base preference exists; however, both G-C and A-T base pairs react with activated NDGA. The activated NDGA–DNA complex is not the result of covalent bond formation; rather it appears to be due to a hydrophobic interaction that is stable to dialysis and to dissociation by high concentrations of CsCl but is dissociated by the anionic detergent Sarkosyl. Since the hydrophobic region of DNA is located in the inner core of the duplex, this suggests that the binding of activated NDGA occurs in the major and/or minor grooves of the duplex structure. This concept is supported by the observation that complex formation is favored by the duplex structure of DNA. In support of this model are the studies by Liquori *et al.* [34] as well as by Boyland and Green [35], which indicated that DNA solubilizes polycyclic hydrocarbons. The solubilization was attributed to hydrophobic exclusion of the hydrocarbons from the aqueous environment within the structure of DNA. In this study dilution did not dissociate the complex but detergent did; thus, it might be expected that there was a competition between a hydrophobic binding site in DNA and the detergent for the activated NDGA ligand. Thus, sequestration of an activated NDGA intermediate in the hydrophobic core of duplex DNA is a possibility to explain how the two molecules interact.

A shift to higher density in the CsCl sedimentation studies would be expected for a mechanism producting denaturation [36]. Since the direction of the shift of DNA buoyant density was not toward higher density, this suggests that activated NDGA did not denature the DNA. That DNA was not denatured was also the conclusion of thermal transition studies, which showed the inability of activated NDGA to alter the melting characteristics of DNA. Also, since there was no change in viscosity of activated NDGA–DNA complex, this further supports the concept that denaturation did not occur.

That activated NDGA may complex with DNA by intercalation is unlikely. Two methods that indicate whether or not a molecule is capable of intercalating the base pairs of DNA were used to test this possibility. The first of these methods was thermal transition (T_m). The elevation in the T_m of DNA has been found for many intercalative compounds by Lerman [37], but activated NDGA did not increase the T_m of calf thymus DNA. The second method is that of low shear viscosity. This property is quite sensitive to the hydrodynamic characteristics of DNA. Intercalation of a molecule between base pairs is expected to lengthen and stiffen the DNA duplex, thus increasing the viscosity of the DNA [37]. Activated NDGA did not increase the reduced specific viscosity of T-7 DNA samples, and thus appeared incapable of the lengthening and stiffening of DNA.

* J. H. Nelson, personal communication.

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REFERENCES

1. K. Weinges and R. Spanig, in *Oxidative Coupling of Phenols* (Eds. W. I. Taylor and R. R. Battersby), p. 323. Marcel Dekker, New York (1967).
2. J. R. Chipault, in *Autoxidation and Antioxidants* (Ed. W. O. Lundberg), Vol. 2, p. 396. Interscience Publishers, New York (1962).
3. E. P. Oliveto, *Chem Ind.* **17**, 677 (1972).
4. G. Kajimoto, *J. Jap. Soc. Fd. Nutro.* **12**, 385 (1960).
5. S. S. Epstein, I. B. Saporoschetz and S. H. Hunter, *J. Protozool.* **14**, 238 (1967).
6. H. P. Kaufman and A. K. S. Ahmad, *Fette Seifen Anstr-Mittel* **69**, 837 (1967).
7. K. Hirose, Y. Ose and J. Kitamura, *Gifu Yakka Daigaku Kiyo* **6**, 66 (1956).
8. T. Nagata, *Vitamins, Kyoto, Japan* **5**, 580 (1952).
9. D. Burk and M. Woods, *Radiat. Res. (Suppl.)*, **3**, 212 (1963).
10. C. R. Smart, H. H. Hogle, R. K. Robins, A. D. Brown and D. Bartholemew, *Cancer Chemother. Rev.* (I) **53**, 147 (1969).
11. R. S. Pardini, T. Lambert and G. Winston. *Proc. NW. Reg. Am. chem. Soc. Meeting* **1**, 9 (1973).
12. A. L. Tappel and A. G. Marr, *J. agric. Fd Chem.* **2**, 554 (1954).
13. R. S. Pardini, J. C. Heidker and D. C. Fletcher, *Biochem. Pharmac.* **19**, 2695 (1970).
14. M. C. DeGraw, W. A. Skinner, M. C. Theisen and C. Mitoma, *J. med. Chem.* **10**, 64 (1967).
15. V. N. Iyer and W. Szybalski, *Proc. natn. Acad. Sci. U.S.A.* **50**, 355 (1963).
16. R. D. Wells and J. E. Larsen, *J. molec. Biol.* **49**, 319 (1970).
17. H. Umezawa, in *Cancer Medicine* (Eds. J. F. Holland and E. Frei, III), p. 817, Lea & Febiger, Philadelphia (1973).
18. N. R. Cozzarelli, in *Annual Review of Biochemistry* (Eds. E. E. Snell, P. D. Boyer, A. Meister and C. C. Richardson), Vol. 46, p. 641. Annual Reviews, Inc., Palo Alto, CA (1977).
19. O. Gisvold and E. Thacker, *J. pharm. Sci.* **63**, 1905 (1974).
20. C. A. Thomas and J. Abelson, in *Procedures in Nucleic Acid Research* (Eds. G. L. Cantoni and D. R. Davies), Vol. 1, p. 569. Harper & Row, New York (1966).
21. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
22. J. H. Parish, in *Principles and Practice of Experiments with Nucleic Acids*, London (Eds. p. 172. Longman Group, Ltd. (1972).
23. K. R. Yamamoto, B. M. Alberts, R. Benzinger, L. Lawhorne and G. Treiber, *Virology* **40**, 734 (1970).
24. W. Szybalski and E. H. Szybalski, in *Procedures in Nucleic Acid Research* (Eds. G. L. Cantoni and D. R. Davies), Vol. 1, p. 311. Harper & Row, New York (1966).
25. D. M. Crothers and B. H. Zimm, *J. molec. Biol.* **12**, 525 (1965).
26. J. R. Chipault, in *Autoxidation and Antioxidants* (Ed. W. O. Lundberg), Vol. I, p. 517. Interscience Publishers, New York (1962).
27. G. Weill and M. Calvin, *Biopolymers* **1**, 407 1(963).
28. L. I. Grossman, R. Watson and J. Vinograd, *J. molec. Biol.* **86**, 271 (1974).
29. R. T. Hinegardner, *Analyt. Biochem.* **39**, 197 (1971).
30. S. J. Gill and D. S. Thompson, *Proc. natn. Acad. Sci. U.S.A.* **57**, 562 (1967).
31. M. Mandel and J. Marmur, in *Methods in Enzymology* (Eds. L. Grossman and K. Moldave), Vol. XII, Part B, p. 195. Academic Press, New York (1968).
32. D. E. Hathway and J. W. T. Seakins, *J. chem. Soc.* 1562 (1957).
33. B. R. Brown and R. J. Whiteoak, *J. chem. Soc.* (Suppl.) 6084 (1964).
34. A. M. Liquori, B. D. Lerma, F. Ascoli, C. Borte and M. Transciatti, *J. molec. Biol.* **5**, 521 (1962).
35. E. Boyland and B. Green, *Br. J. Cancer* **16**, 507 (1962).
36. W. Kersten, H. Kersten and W. Szybalski, *Biochemistry* **5**, 236 (1966).
37. L. S. Lerman, *J. cell. comp. Physiol.* **64**, (Suppl. 1), 1 (1964).