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The Reaction of Guanine Derivatives with 1,2-Dicarbonyl Compounds*

Robert Shapiro and John Hachmann†

ABSTRACT: The reaction product of glyoxal with guanosine has been isolated. On the basis of its spectral properties, it has been assigned a structure (I) in which a new ring is formed involving the 1 and N^2 positions of the guanine ring and both carbonyls of glyoxal. Ninhydrin has been found to react smoothly with guanine and guanosine, and analogous structures have been postulated for the products of these reactions. The ninhydrin–guanine adduct reacts with sodium metaperiodate to give a product identified as N^2-phthalonylguanine. While the glyoxal–guanosine and ninhydrin–guanosine adducts react with nitrous acid to give xanthosine, phthalonylguanine is resistant to nitrous acid. These reactions are of potential value as methods for modification of the guanine residues of the nucleic acids.

Aldehydes have shown particular promise as reagents for the modification of nucleic acids. A large amount of attention has been directed especially to the use of formaldehyde for this purpose. It is an antiviral agent, and readily reacts with and inactivates the ribonucleic acid (RNA) of tobacco mosaic virus (Fraenkel-Conrat, 1954; Staehlin, 1958). The reaction has been explored with a variety of nucleic acids. The site of reaction in each case is believed to be at the amino groups of those adenine, cytosine, and guanine residues that are not involved in hydrogen bonding. The linkages formed are labile and are readily broken upon dialysis (Staehlin, 1958; Haselkorn and Doty, 1961). This reaction has been used to prevent the renaturation of thermally denatured deoxyribonucleic acid (DNA) (Grossman *et al.*, 1961) and to study the secondary structure of soluble RNA (s-RNA) (Penniston and Doty, 1963a,b; Zubay and Marciello, 1963). The effect of formaldehyde upon the coding properties of synthetic polynucleotides has been investigated (Michelson and Grunberg-Manago, 1964). Formaldehyde has also been used to elaborate the structure of a complex between polyinosinic acid and protonated poly-

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cytidylic acid (Giannoni and Rich, 1964). A mutagenic effect upon the larvae of *Drosophila melanogaster* is shown by formaldehyde in the presence of adenylic acid (Alderson, 1960).

Glyoxal, and related 1,2-dicarbonyl compounds, have also been shown to have significant biological properties. A number of them have proved to be effective antiviral agents (Tiffany *et al.*, 1957; Moffett *et al.*, 1957; Wright *et al.*, 1957) and to have carcinostatic properties (French and Freedlander, 1958; Freedlander and French, 1958a,b). A recently isolated bacterial growth inhibitor, retine, is believed to be related in structure to methylglyoxal (Együd, 1965; Együd and Szent-Györgi, 1966). It was shown that both glyoxal and Kethoxal[1] (β-ethoxy-α-ketobutyraldehyde), like formaldehyde, react with and inactivate the RNA of tobacco mosaic virus (Staehlin, 1959). In the same paper, the interaction between α-ketoaldehydes and the nucleic acid components was examined, primarily by ultraviolet spectroscopy. Unlike formaldehyde, glyoxal and Kethoxal were shown to be specific for guanine derivatives. The products found were found to revert readily to the starting materials under alkaline conditions. No well-defined adducts of guanine derivatives with glyoxal or Kethoxal were prepared, however, although a 1:1 isocytosine–Kethoxal adduct was isolated. A definite assignment of structure was not made for this compound. In contrast to the situation with formaldehyde, no additional chemical studies and few biochemical applications of these reactions have been reported. It has been shown that reaction with glyoxal protects the dinucleoside phosphate, guanylyl-(3′→5′)-cytidine, from hydrolysis by Takadiastase ribonuclease T_1 (Whitfeld and Witzel, 1963). The reaction of neoguanosine with glyoxal was of use in establishing its structure as N^2-ribosylguanine (Shapiro and Gordon, 1964).

The 1,2-dicarbonyl compounds seem to us to have considerable potential, as yet unexploited, for the chemical modification of nucleic acids. Their greater specificity should make them a useful adjunct to those studies using formaldehyde. It has been suggested that it would be useful to have chemical methods of isotopically labeling nucleotides in s-RNA that are not involved in the secondary structure (Zubay and Marciello, 1963). A specific reagent of this type might be found among the glyoxals. Attention has been directed recently to the development of reagents such as carbodiimides (Gilham, 1962) which can increase the specificity of enzymes in cleaving nucleic acids. A reference that established the potential suitability of glyoxal for this purpose has been mentioned above. 1,2-Dicarbonyl compounds might also be useful in increasing the specificity of chemical reagents for the nucleic acids. Nitrous acid, for example, is known to deaminate the adenine, cytosine, and guanine residues of nucleic acids (Schuster and Schramm, 1958). It is believed that the reaction with guanine is not mutagenic and may be lethal (Vielmutter and Schuster, 1960). It has also been shown that guanosine undergoes a side reaction with nitrous acid which introduces a nitro group at the 2 position (Shapiro, 1964). It would obviously be of value to have an acid-stable blocking group which would protect the guanine residues of nucleic acids from attack by nitrous acid, and could subsequently be removed by mild alkaline treatment.

For these reasons, and because of the important biological properties of 1,2-dicarbonyl compounds, the authors felt it desirable to study further the chemistry of the reactions of these compounds with guanine derivatives. The authors wanted to isolate and establish the structure of several of these compounds, and to investigate their chemical properties with reference to the possible uses discussed above.

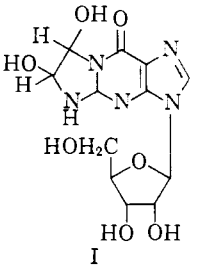
Results and Discussion

The reaction of guanosine with a large excess of aqueous glyoxal at pH 4 was first examined. The progress of the reaction was followed by thin layer chromatography and ultraviolet spectroscopy. It was found that the reaction went to completion in 3 hr at 65° or 6 days at room temperature. Attempts to separate the glyoxal–guanosine adduct from the excess of glyoxal and its polymerization products on cellulose columns failed. Only a partial purification was achieved by a bisulfite anion-exchange resin. Separation was best accomplished by passing the reaction mixture through a column of strongly acidic cation-exchange resin. The glyoxal–gluanine adduct was retained, while the excess glyoxal passed through. The product was best eluted by extrusion of the resin from the column and treating it with ammonium bicarbonate solution. Care was taken that the pH did not rise above 7 during this process, as this would have caused some reversion of the product to guanosine and glyoxal. The solution was then treated with a weakly acidic ion-exchange resin to remove traces of ammonium bicarbonate, filtered, and lyophilized. (It was found that merely concentrating the solution under vacuum at room temperature caused some reversion of the labile product to guanosine and glyoxal, even when the pH was maintained near 4 by a stream of carbon dioxide.) The product obtained by this procedure was a white powder, contaminated with amounts of guanosine varying, on different runs, from 0.6 to 3%. As recrystallization attempts led to decomposition, this material was used directly for further studies.

An analysis indicated that the product contained 1 mole of guanosine:1 mole of glyoxal, with 1 H_2O of hydration. Attempts to remove this water by heating under vacuum led to decomposition of the compound. Its ultraviolet spectrum was similar to that of guanosine, with the major difference being that the intensity of the shoulder at 275 mμ, relative to the main absorbtion at 252 mμ, had diminished. This indicated that no large change in the chromophore had occurred. The carbonyl absorption in the infrared (5.82 μ) was at a

[1] Kethoxal is the registered trademark of the Upjohn Co., Kalamazoo, Mich.

wavelength close to that of guanosine (5.80 μ). Prominent peaks were also seen at 6.24 and 6.44 μ which, as it turned out, were characteristic for this series of compounds. The most conclusive evidence about the structure of the glyoxal-guanosine adduct was obtained from the low-field portion of the nuclear magnetic reasonance spectrum (Figure 1). Whereas guanosine had given an absorbtion at τ −0.80, ascribed to the 1 proton, and another at τ 3.40, integrating as two protons and assigned to the amino group (Gatlin and Davis, 1962), the glyoxal–guanosine adduct showed three peaks, τ 1.13, 2.73, and 3.33, and integrating for one proton each. When D_2O was added to the solution (Figure 1) these peaks exchanged and disappeared, and two new CH peaks could be seen that had previously been hidden by the absorbtion of the sugar hydroxyl protons. These occurred at τ 4.29 and 4.85, which indicated that they were hemiacetal-type protons, rather than aldehydic ones. On the basis of this information, structure I was assigned to the guanosine–glyoxal adduct. This structure is consistent



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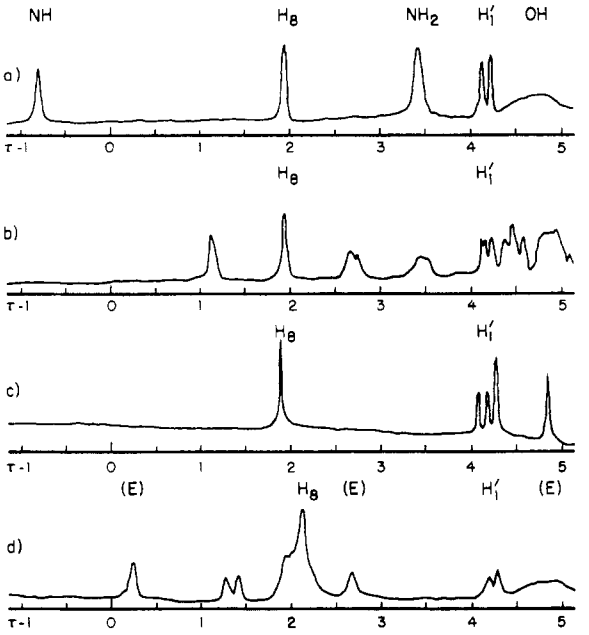


FIGURE 1: Low-field portion of the proton nmr spectra in $(CD_3)_2SO$ of (a) guanosine; (b) glyoxal–guanosine adduct I; (c) glyoxal–guanosine adduct I in $(CD_3)_2SO$ with added D_2O; (d) ninhydrin–guanosine adduct III (R = β-D-ribofuranosyl). In d, those portons labeled (E) exchanged and disappeared on addition of D_2O and thus represent NH or OH protons. Tetramethylsilane was used as internal standard.

with the fact that N^2-methylguanine reacts with glyoxal, whereas 1-methylguanine does not (Staehlin, 1959).

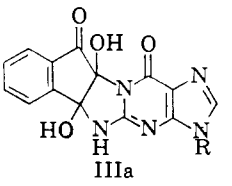
The rate of reversion of I to guanosine and glyoxal, at 25°, was measured in solutions of varying pH. The adduct was stable for days at pH values below 6, but some decomposition was detectable after 21 hr at pH 6.6, or 2 hr at pH 7.8. After 21 hr at pH 7.8, the compound had reverted entirely to guanosine. The reaction of I with nitrous acid was also studied, to determine whether glyoxal could be used to protect the guanine residues of nucleic acids from attack by nitrous acid. The glyoxal–guanine adduct reacted quickly with this reagent, however, and yielded xanthosine. As no guanosine could be detected after partial reaction, the conversion presumably proceeds *via* direct attack of nitrous acid on I.

The reaction of guanosine with biacetyl was next examined, with the hope that the adduct in this case might prove more resistant to attack by nitrous acid. This reaction proceeded quite sluggishly however, and unreacted guanosine could be detected even after 11 days at 75°. Thin layer chromatography revealed that the reaction mixture contained a number of ultraviolet-absorbing products. Some of these turned out to be derived from biacetyl alone. After evaporation of the solution, extraction of the resulting oil by organic solvents, and fractionation of the insoluble residue by chromatography on a large silicic acid column, only one new crystalline, nitrogen-containing product (II) could be isolated, in low yield. This, however, had been produced by a rather complex reaction, as its analysis and nuclear magnetic resonance spectrum indicated that it contained 1 mole of guanine, 2 moles of biacetyl, and no ribose. This product was not investigated further. It was concluded that the two methyl groups of biacetyl so diminished the reactivity of its 1,2-dicarbonyl system toward guanosine as to make it useless as a reagent for the modification of nucleic acids.

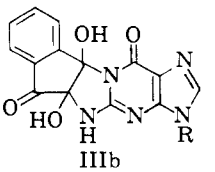
A survey was made of the reactivity of a variety of carbonyl compounds toward guanosine. The reactions were run for 18 hr at 60° with a large excess of the carbonyl compound, and followed by thin layer chromatography. Very little or no reaction was observed in the following cases: hydroxyacetone, diethyl oxalate, diethyl mesoxalate, 1,2-naphthoquinone-4-sulfonic acid, and pyruvaldehyde. The result with hydroxyacetone disagrees with those reported earlier (Staehlin, 1959). Two other compounds, phenylglyoxal and di-3-pyridylglyoxal, were seen to form compounds with guanosine under our conditions, but the reaction was slow and only partial conversion was achieved. Two additional reagents, Kethoxal and ninhydrin, were found to be suitable for modification of nucleic acids, as they converted guanosine essentially quantitatively to single new products. The reaction of ninhydrin with nucleic acid components has not previously been reported. Apparently, activation by electron-withdrawing groups

is a necessary requirement if a 1,2-dicarbonyl compound is to be an effective reagent for guanosine. It is interesting to compare these results with the reported efficiency of the same compounds as antiviral agents (Tiffany *et al.*, 1957). Glyoxal Kethoxal, phenylglyoxal, and ninhydrin were highly active in this respect. Biacetyl and quinones were inactive. Hydroxyacetone was active, but this was ascribed to prior oxidation of this compound by the host. The only discrepancy with our results is the case of pyruvaldehyde, which was found to be a highly active antiviral agent.

A more detailed study was made of the reaction with ninhydrin. The excess ninhydrin was removed from the reaction mixture by ether extraction, and the ninhydrin–guanosine adduct isolated by evaporation of the resulting solution. While this compound was even more labile under neutral or alkaline conditions than the glyoxal–guanosine adduct, it was less susceptible to decomposition in other ways. It could be purified by recrystallization. An analysis indicated that it was composed of 1 mole of ninhydrin:1 mole of guanosine, with one water of hydration. Its ultraviolet spectrum was quite similar to that of the glyoxal–guanine adduct. The infrared spectrum showed an additional carbonyl peak at 5.92 μ, in addition to those reported above for I. This peak is presumably due to a ketone conjugated with the benzene ring of the ninhydrin moiety. The low-field portion of the nuclear magnetic resonance spectrum of the ninhydrin–guanosine adduct is given in Figure 1. A peak attributed to the H_8 proton of the guanine ring appears at τ 2.13, superimposed on a broader absorbtion due to three of the aromatic protons of the benzene ring. The fourth benzene proton is represented by a doublet (with further splitting not well resolved) centered at τ 1.35. This is analogous to the nmr[2] spectrum of phthallide (Bhacca *et al.*, 1963) in which a multiplet at lower field than the absorbtions of the remainder of the aromatic protons is ascribed to the proton *ortho* to the carbonyl group. In the present case, we presumably also have a situation in which a carbonyl group and a saturated substituent are adjacent on a benzene ring and the downfield absorbtion can be ascribed to the proton *ortho* to the carbonyl group. The nmr spectrum of ninhydrin itself shows a single peak at τ 1.81 representing the four aromatic protons and another at τ 2.3 due to the two hydroxyl protons. In addition to the peaks already described, the low-field portion of the nmr spectrum of the ninhydrin–guanosine also showed exchangeable peaks, each representing one proton, at τ 0.25 and 2.68. Integration of the peaks due to aromatic protons before and after addition of D_2O indicated that the absorbtion of a third proton that exchanged had been masked by the broad aromatic band. On the basis of these spectral properties, it seemed likely that the ninhydrin–guanosine adduct had a structure (IIIa or b, R = β-D-ribofuranosyl) analogous to the glyoxal–guanine adduct I.



IIIa

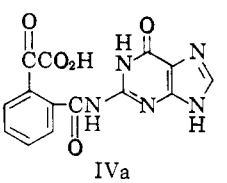


IIIb

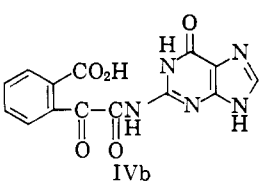
The adduct of ninhydrin and guanine was also prepared. Its infrared, ultraviolet, and low-field nmr spectra were similar to those of the ninhydrin–guanosine adduct. In the higher field portion of the nmr spectrum, only a broad peak at τ 6.5, integrating for two protons and ascribed to the water of hydration, was seen. This adduct was assigned the structure IIIa or b (R = H).

The reactions of ninhydrin with deoxyguanosine and guanosine 2′-(3′)-phosphate were examined by thin layer chromatography. Both reacted completely and gave two spots. In both cases the compounds were separated by preparative paper chromatography and their ultraviolet spectra were examined. They were quite similar to each other and to the other ninhydrin adducts. In the case of the nucleotide, the two spots probably represent the 2′- and 3′-phosphate isomers, as the parent compound also divided into two spots upon thin layer chromatography. In the case of deoxyguanosine, the spots may represent diastereomers or positional isomers corresponding to IIIa and b.

The ninhydrin–guanosine adduct, like the glyoxal–guanosine adduct, was found to react with nitrous acid to give xanthosine. In a further effort to obtain a more stable blocking group, resistant to attack by nitrous acid, the reaction of the ninhydrin–guanine adduct with periodate was examined. It was found to react completely, giving a new yellow fluorescent spot on thin layer chromatography. The product was isolated and purified, and analyzed as $C_{14}H_9N_5O_5 \cdot H_2O$. Its ultraviolet spectrum was considerably different from its precursor, with maxima as 244, 270, and 296 mμ. Its nmr spectrum revealed little, as the OH and NH protons appeared as a single broad peak at τ 3.03, presumably because they interchanged rapidly. The product was acidic, as spectrophotometric titration revealed pK_a values at about 3.5 and 6.5, and it moved as an anion when subjected to paper electrophoresis at pH 4.5. It was unchanged after treatment with nitrous acid, and gave guanine when treated with 1 N NH_3 for 3 days at room temperature. Ninhydrin itself is known to consume 1 mole of periodate to give a product assumed to be phthalonic acid (Sprinson and Chargaff, 1946). On the basis of the above data, our new product was assigned a N^2-phthalonylguanine structure IVa or b. The unreactivity to nitrous acid



IVa



IVb

[2] Abbreviation used: nmr, nuclear magnetic resonance.

and the strength of the second acidic dissociation make an alternative linkage of the phthalonyl group to the 1 position of guanine less likely. The two products formed from the reaction of deoxyguanosine with ninhydrin also reacted with periodate to give the same substance, as judged by mobility on electrophoresis and thin layer chromatography. This makes it more likely that the products were diastereomers, rather than positional isomers. The mixture of products formed from ninhydrin and guanosine 2′-(3′)-phosphate reacted to give two products, presumably 2′- and 3′-phosphates.

Reaction with ninhydrin, followed by periodate treatment, would therefore seem a promising method for attaching, under mild conditions, an acyl residue to the amino group of a guanine derivative or a guanine within a nucleic acid. The acyl residue would be stable under neutral conditions, and would protect the amino group from attack by nitrous acid, but could be removed by treatment with ammonia. One limitation to this procedure would be that the guanine derivative should not contain free *cis*-2′- and 3′-hydroxyl groups, as this would also be attacked by periodate.

Experimental Section

Infrared spectra were obtained with a Perkin-Elmer Infracord spectrophotometer. Nuclear magnetic resonance spectra were determined in hexadeuteriodimethyl sulfoxide using a Varian A-60 spectrophotometer and are reported on the τ scale with tetramethylsilane (τ 10.00) as internal standard. Ultraviolet spectra were obtained with a Perkin-Elmer 202 spectrophotometer. Microanalyses were performed by Mr. George I. Robertson, Jr., Florham Park, N. J. Guanine, deoxyguanosine, and guanosine were purchased from Schwarz Bioresearch Inc., Orangeburg, N. Y., and were found to be chromatographically homogeneous in the solvent systems indicated. Guanosine 2′-(3′)-phosphate was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Thin layer chromatography was carried out on 0.25-mm thick layers of MN-cellulose powder 300 (Machery Nagel and Co.). Paper chromatography was carried out by the descending technique on Whatman No. 40 paper. After development of the paper or thin layer chromatogram, ultraviolet-absorbing materials were located with the aid of an ultraviolet lamp equipped with a short-wavelength filter. The chromatographic solvent systems used and the R_F values obtained on thin layer chromatography are given in Table I.

TABLE I: R_F Values.[a]

Compound	Solvent System[b] A	B	C	D
Guanine	0.48		0.26	0.77
Guanosine	0.49	0.63	0.15	0.58
Deoxyguanosine	0.63	0.61		
Guanosine 2′-(3′)-phosphate	0.38 0.48			
Xanthosine	0.58	0.76	0.13	0.48
Glyoxal–guanosine adduct I	0.62	0.78	0.19	0.58
Phenylglyoxal–guanosine adduct	0.92		0.63	
Kethoxal–guanosine adduct	0.88		0.52	
Biacetyl product II	0.78	0.83		
Ninhydrin	0.89	0.80	0.33	
Ninhydrin–guanosine adduct III (R = β-D-ribofuranosyl)	0.75	0.68	0.41[c]	0.50
Ninhydrin–guanine adduct III (R = H)	0.73		0.35[c]	
Phthalonylguanine (IV)	0.60		0.09[c]	0.62
Ninhydrin–deoxyguanosine adduct	0.48 0.80	0.51 0.69		
Ninhydrin–guanosine 2′-(3′)-phosphate adduct	0.67 0.76			

[a] All data are for thin layer chromatography on cellulose unless otherwise noted. [b] Solvent systems are: A, 2-propanol–water (7/3); B, water; C, 1-butanol–water (86/14); D, isobutyric acid–ammonia–water (66/4.7/29.3) (all proportions v/v). [c] This value is for paper chromatography.

Preparation of the Glyoxal–Guanosine Adduct I. In 1030 ml of H_2O were dissolved 11.8 g (155 mmoles) of glyoxal monohydrate (British Drug Houses), 496 mg (1.65 mmoles) of guanosine·H_2O, and 2 ml of acetic acid, and the solution was heated at 65°. The progress of the reaction was followed by thin layer chromatography in solvent A and by ultraviolet spectroscopy. In the latter method, the ratio of absorbances at 280 and 250 mμ was seen to change from 0.58 to 0.47 and the ratio of absorbances at 280 and 270 changed from 0.82 to 0.98. The reaction went to completion in 3 hr at 65° or 6 days at room temperature. A column of 100 ml of Amberlite CG 120 cation-exchange resin (H^+ form) was prepared in the following manner just before use. The resin was washed repeatedly in a beaker with ammonium bicarbonate solution and decanted. This was done until the supernatant washings were clear. The resin was then packed into a column, converted to the acid form with HCl, and washed with water. The crude reaction mixture was then added on top of the column, and the column was washed with H_2O until ultraviolet-absorbing material had ceased to come off. The resin was extruded from the column, covered with water in a beaker, and treated with portions of ammonium bicarbonate, with stirring. This was done at a rate sufficiently slow so that the pH of the solution did not rise above 6. The elution of the product from the column was followed by measuring the absorption at 250 mμ

in the ultraviolet of aliquots of the supernatant solution. When elution was complete, the solution was filtered, and the pH was brought to about 5 by adding portions of Amberlite CG-50 cation-exchange resin (H^+ form). This resin had been pretreated in the manner described above. The resin was removed by filtration and the solution was lyophilized to give 356 mg (60%) of I as a white powder, which contained 3% of guanosine. The identification of this impurity was done by isolating it by preparative paper chromatography in solvent A, and comparing its R_F values in solvents A–C and ultraviolet spectra at pH 1, 7, and 11, with authentic guanosine. On another run, when the contact of the resin with ammonium bicarbonate solution was lessened, the product contained only 0.6% of guanosine, but the yield fell to 150 mg. The product was stored under refrigeration as it slowly decomposed on standing at room temperature, giving faster spots upon chromatography in solvent A. The product I had the following characteristics: the infrared spectrum (KBr) showed prominent bands at 5.82, 6.24, and 6.44 μ; ultraviolet maximum (H_2O) 251 and 275 mμ (sh), $\epsilon_{275}/\epsilon_{251}$ = 0.56. The substance decomposed from 185 to 200° without melting upon heating. A sample was dried for analysis by heating under vacuum at 65° for 5 hr. Heating at 118° under vacuum led to decomposition.

Anal. Calcd for $C_{12}H_{15}N_5O_7 \cdot H_2O$: C, 40.12; H, 4.77; N, 19.49. Found: C, 40.06; H, 4.76; N, 19.20.

Reversion of I to Guanosine. When placed in 1 N ammonia solution, I was rapidly converted to a product identified by its ultraviolet spectrum and R_F in solvent A as guanosine. Samples of I were dissolved in water, pH 4.4, 1 N sodium acetate buffer, pH 5.7, and 1 M sodium phosphate buffers of pH 6.6 and 7.8. The change in the 270/250 and 280/270 absorbance ratios in the ultraviolet were followed as a measure of the reversion of I to guanosine. The results were as described in the Discussion.

Reaction of I with Nitrous Acid. To 1 ml of 1 N sodium acetate–acetic acid buffer, pH 4.5, was added 5 mg of I and 0.5 ml of 4 N sodium nitrite solution. The solution was allowed to stand at room temperature for several hours. Thin layer chromatography revealed a spot corresponding in R_F to xanthosine in solvents A, B, and D. A portion of this material was isolated by preparative paper chromatography in solvent A. It had an ultraviolet spectrum identical with that of xanthosine at pH 3, 8.4, and 14. A smaller amount of unreacted I was also observed, but no guanosine could be detected.

Reaction of Guanosine with Biacetyl. To 1 g (3.3 mmoles) of guanosine·H_2O suspended in 500 ml of water were added 34 ml (390 mmoles) of biacetyl and 1 ml of acetic acid. The reaction mixture was heated at 75° with stirring for 11 days. An additional 17 ml (195 mmoles) of biacetyl was added during the course of the reaction. The solution was concentrated under vacuum, giving a brownish oil. This was extracted with ether and filtered and the insoluble materials were retained. The ether solution was evaporated, and the residual oils were treated with ethyl acetate and water. The aqueous fraction was separated and evaporated, and the residue was washed with ethyl acetate and combined with the previous crop of insoluble material. A total of 804 mg of yellow powder was obtained. Thin layer chromatography in solvent B indicated that this was a complex mixture of substances. A 581-mg portion was applied to a column of 200 g of silicic acid (Mallinckrodt, 100 mesh) and elution was conducted with ethyl acetate and increasing quantities of methanol. The ultraviolet absorption of the eluate was measured continuously at 267 mμ, and eight distinct peaks were observed and collected. The fourth peak, eluted with 7.5% methanol, contained the most material, 150 mg. Thin layer chromatography showed it to be composed of one ultraviolet-absorbing substance. Repeated recrystallization from methanol–ethyl acetate gave a pure sample of II: the infrared (KBr) included peaks at 5.88, 6.20, 6.39, and 6.50 μ; ultraviolet maxima (H_2O) at 247 and 283 mμ, minima at 228 and 268 mμ, $\epsilon_{283}/\epsilon_{247}$ = 0.57; the nmr included peaks at τ 2.16 (1 H) (guanine H_8), 6.90 (3 H) (CH_3OH), 8.00, 8.08, 8.64, and 8.80 (each 3 H), but no peak near τ 3.80 (H_1' of guanosine). When heated, the substance started to decompose at 165° and melted with decomposition from 185 to 210°. A sample of II was dried for 5 hr at 65° for analysis.

Anal. Calcd for $C_{13}H_{17}N_5O_4 \cdot CH_3OH$: C, 49.85; H, 5.68; N, 20.76. Found: C, 49.12; H, 5.99; N, 20.75.

Product II was allowed to stand in 1 N NH_3 solution. Thin layer chromatography (solvent B) indicated that it was unchanged after 3 hr, but partly converted to a new product, R_F 0.37, after 20 hr. To 1 mg of II, 1 ml of 1 N sodium acetate buffer, pH 4.5, and 0.3 ml of 1 N $NaNO_2$ were added. After several hours, thin layer chromatography in solvent B indicated only one substance, of R_F corresponding to the starting material. A portion of this was isolated by preparative paper chromatography in solvent A and had the same ultraviolet spectrum as starting material.

Another peak from the silicic acid column chromatography of the biacetyl reaction had been eluted with 15% methanol in ethyl acetate. It contained 67 mg of a substance whose ultraviolet spectra at pH 1, 7, 11, and R_F values on thin layer chromatography in solvents A, C, and D were identical with those of guanosine. The remaining fractions from the silicic acid column contained less than 50 mg each and were mixtures of substances.

Attempted Reaction of Guanosine with Hydroxyacetone, Diethyl Oxalate, Diethyl Mesoxalate, 1,2-Naphthoquinone-4-sulfonic Acid, and Pyruvaldehyde. To 2.5-mg samples of guanosine·H_2O in separate test tubes was added a 100-fold molar excess of each of the carbonyl compounds listed above, and the volume was brought to 5 ml with H_2O. The solutions were heated for 18 hr at 60°. The naphthoquinonesulfonic acid experiment was worked up in a manner similar to that done with glyoxal. The others were merely extracted several times with ether. A control reaction, without guanosine, was run in each case. Each reaction mixture was examined by thin layer chromatography in several solvent systems. In no case

was any new ultraviolet-absorbing spot seen that was not present in the control. In the case of diethyl mesoxalate, the reaction was also worked up by preparative paper chromatography. The major component had the same ultraviolet spectrum as guanosine at pH 1, 7, and 11. A minor component, which fluoresced lightly on paper, had an ultraviolet spectrum similar to that of diethyl mesoxalate. The diethyl oxalate and pyruvaldehyde reactions were also rerun in the presence of 1 drop of acetic acid. This did not affect the result.

Reaction of Guanosine with Phenylglyoxal. Guanosine·H_2O (0.01 mmole, 3 mg) was heated with 67 mg (0.5 mmole) of phenylglyoxal and 5 ml of 50% aqueous ethanol at 60° for 18 hr. The solvent was concentrated under a stream of N_2 and the remaining solution washed several times with ether. The residue was examined by thin layer chromatography in solvents A and C, as was the residue from a control reaction, run without guanosine. A new fluorescent spot, absent in the control, was seen in both solvents, in addition to unreacted guanosine.

Reaction of Guanosine with Di-3-pyridylglyoxal. To 2.5 mg (0.008 mmole) of guanosine was added 212 mg (1 mmole) of di-3-pyridylglyoxal (Aldrich Chemical Co., Milwaukee, Wis.). The volume was brought to 5 ml with H_2O, and the pH was adjusted to 4.5 with acetic acid. The mixture was heated at 60°, and a duplicate run was conducted at room temperature. Aliquots were withdrawn from time to time, extracted with ether, and examined by thin layer chromatography in 50% 2-propanol–50% 2 N HCl. Control experiments, run without guanosine, were treated in a similar manner. The controls showed several spots arising from decomposition of the di-3-pyridylglyoxal. The reactions with guanosine, after 1 day, showed an additional spot of R_F 0.30. This was best visualized by exposure of the plate to NH_3 vapor, upon which the product appeared as a bright yellow fluorescent spot. In the reaction at 60°, a second product, of R_F 0.72, appeared after 2 days of heating. After 5 days of reaction, at both temperatures, unreacted guanosine could clearly be demonstrated by thin layer chromatography in solvent B. Reactions were also run under conditions (pH 2) in which di-3-pyridylglyoxal (pK_a = 3.7) was protonated. These, however, gave more decomposition of the di-3-pyridylglyoxal, and less guanosine product, than the reactions run at pH 4.5.

Reaction of Guanosine with Kethoxal (3-Ethoxy-2-ketobutyraldehyde). This was conducted in the same manner as the reaction with hydroxyacetone. Thin layer chromatography in solvents A and C showed complete conversion of the guanosine to a single new product. The ultraviolet spectrum of the reaction mixture was obtained, using the control reaction as a blank. The spectrum obtained was similar to that of I: maxima at 248 mμ and 278 mμ (sh), $\epsilon_{273}/\epsilon_{248} = 0.51$.

Preparation of the Ninhydrin–Guanosine Adduct III (R = β-D-Ribofuranosyl). To 500 mg (1.66 mmoles) of guanosine·H_2O, 8.65 g (49.6 mmole) of ninhydrin and 500 ml of water were added, and the solution was heated at 60° for 18 hr. Thin layer chromatography of the reaction mixture in solvents A and B revealed the presence of a single new product, with only a trace of guanosine remaining. The reaction mixture was extracted with ether until no color was produced when glycine was added to a few drops of the aqueous solution. The solution was then evaporated at 30° under vacuum to give 766 mg (100% crude yield) of the adduct as a solid with a light purple discoloration. A sample was purified in the following manner. Sufficient hot acetone was added to partly dissolve the solid, and the suspension was filtered. Evaporation of the filtrate gave material free of guanosine but still discolored. This was recrystallized from 95% ethanol with the addition of a small amount of charcoal, to give a white solid III (R = β-D-ribofuranosyl), which on heating decomposed at 185–200° without melting; the infrared spectrum (KBr) showed prominent absorbtion peaks at 5.80, 5.92, 6.24, and 6.42 μ; ultraviolet maxima (H_2O) at 253 (ε 24,500) and 280 (sh, 7800), minimum at 230 mμ (11,000). A sample for analysis was dried under vacuum at 64° for 18 hr.

Anal. Calcd for $C_{19}H_{17}N_5O_8 \cdot H_2O$: C, 49.46; H, 4.15; N, 15.18. Found: C, 49.68; H, 4.66; N, 15.20.

Reversion of the Ninhydrin–Guanosine Adduct to Guanosine. This was conducted in the manner described for the glyoxal–guanosine adduct I. The ninhydrin–guanosine adduct was found to revert completely to guanosine quite rapidly in 1 N NH_3, in 2 hr in sodium phosphate buffer, pH 7.8, and in about 3 hr in sodium phosphate buffer, pH 7.0. It was stable for 22 hr in water at pH 4.5.

Reaction of the Ninhydrin–Guanosine Adduct with Nitrous Acid. This was conducted in the same manner as the corresponding reaction with I. The results were the same. The product was identical with xanthosine in its R_F value in solvent A and its ultraviolet spectra at pH 3, 8, and 14.

Preparation of the Ninhydrin–Guanine Adduct III (R = H). To 300 mg (2 mmoles) of guanine suspended in 500 ml of H_2O were added 8.6 g (48 mmoles) of ninhydrin, and the mixture was heated at 70° for 18 hr. The guanine gradually went into solution. Thin layer chromatography in solvent A revealed the presence of a single new product with only a trace of guanine remaining. The reaction mixture was worked up in the same manner as the ninhydrin–guanosine reaction. The product was obtained as a solid, 638 mg (97% crude yield), with a light purple discoloration. It was best purified by dissolving in dimethyl sulfoxide, pouring the solution into a 1:1 acetone–ether mixture, and washing the precipitate five times with ether. This procedure was repeated several times to give the ninhydrin–guanine adduct III (R = H) as a white solid, which decomposed without melting when heated above 200°; the infrared spectrum (KBr) showed prominent absorbtion peaks at 5.79, 5.90, 6.24, and 6.40 μ; ultraviolet maxima (H_2O) at 252 mμ and 283 mμ (sh); $\epsilon_{283}/\epsilon_{252} = 0.37$; the nmr spectrum showed a broad peak at τ 0.50, a doublet centered at 1.30 (H *ortho* to carbonyl or benzene ring), a broad peak

from 1.83 to 2.33 (three protons of benzene ring) superimposed upon a sharper peak at 2.03 (H_8 of guanine), a broad area of absorbtion from 2.33 to 2.83, and a broad peak at 6.50 (H_2O of hydration). Upon addition of D_2O, the absorbtion at τ 0.50 and 2.33–2.83 disappeared.

Preparation of Phthalonylguanine (IV). To 300 mg of the ninhydrin–guanine adduct III (R = H) was added 50 ml of saturated sodium metaperiodate solution and the reaction mixture was stirred overnight. Thin layer chromatography in solvent A indicated that the starting material had reacted completely. The white precipitate that had formed was collected by centrifugation and washed with water until the washings gave a negative test with starch–iodide paper. The precipitate was then washed with acetone to give 149 mg (48%) of phthalonylguanine (IV) as a white solid; mp 298–300° (dec); the infrared spectrum (KBr) showed prominent absorbtion peaks at 5.81 and 6.39 μ; ultraviolet maxima (H_2O) at 244, 270, and 298 mμ; $\epsilon_{244}/\epsilon_{270}/\epsilon_{298}$ = 1/0.62/0.70; minima were at 260 mμ and 270 mμ; the nmr showed a complex band from τ 1.67 to 2.34 (aromatic protons) with a peak discernable at 2.12 (H_8 of guanine), and a broad peak centered at 3.03 (OH and NH protons). Spectrophotometric titration revealed pK_a values for IV of approximately 3.5 and 6.5. The substance moved 3.2 cm as an anion upon paper electrophoresis in 0.1 N sodium acetate buffer, pH 4.5 (21 v/cm, 1 hr). It appeared as a yellow fluorescent spot upon thin layer chromatography or paper electrophoresis. An analytical sample was prepared by dissolving IV in dimethyl sulfoxide and precipitating it with water, then redissolving in dimethyl sulfoxide and reprecipitating with a 1:1 acetone–ether mixture. It was washed with acetone and then with ether. As it was hygroscopic, it was dried under vacuum for 4 hr at 78° immediately before analysis.

Anal. Calcd for $C_{14}H_9N_5O_5 \cdot H_2O$: C, 48.70; H, 3.21; N, 20.28. Found: C, 49.03; H, 3.77; N, 20.00.

Phthalonylguanine was treated with nitrous acid in the same manner used for the glyoxal–guanine adduct I. Thin layer chromatography in solvents A and D revealed only unchanged starting material. The reaction mixture had the characteristic ultraviolet spectrum of IV.

Reaction of Phthalonylguanine (IV) with NaOH and NH_3. Phthalonylguanine (1 mg) was allowed to stand in 1 N NaOH for 18 hr. The reaction mixture was worked up by preparative paper chromatography in solvent A. The fastest band, on elution, had ultraviolet spectra at pH 1, 7, and 11 identical with those of guanine. Its R_F values in solvents A, C, and D were identical with those of guanine. A band corresponding to unreacted IV, and another one, presumably sodium phthalonate or a decomposition product, were also seen. Phthalonylguanine was seen in similar fashion to react completely with 1 N NH_3 in 3 days at room temperature to give guanine.

Reaction of Deoxyguanosine with Ninhydrin. To 26.7 mg (0.1 mmole) of deoxyguanosine a solution of 861 mg (4.84 mmoles) of ninhydrin in 50 ml of H_2O was added and the solution was heated for 18 hr at 60°. The solution was extracted several times with ether to remove ninhydrin. Thin layer chromatography in solvent B showed that the deoxyguanosine had reacted completely and two new substances had been formed. The mixture was worked up by means of preparative paper chromatography in solvent A. The faster band showed, in the ultraviolet, maxima at 254 and 282 mμ (sh), with $\epsilon_{282}/\epsilon_{254}$ = 0.40. The slower band showed, in the ultraviolet, maxima at 254 and 283 mμ (sh), with $\epsilon_{283}/\epsilon_{254}$ = 0.35. A portion of each solution was added to saturated sodium metaperiodate solution. Each reaction showed, upon thin layer chromatography in solvent A, a yellow fluorescent spot of R_F 0.55. Similarly, each reaction showed, upon paper electrophoresis in 0.1 M phosphate buffer, pH 6.8 (21 v/cm, 30 min), a spot that traveled 3.9 cm as an anion.

Reaction of Guanosine 2-'(3')-Phosphate with Ninhydrin. To 40.7 mg (0.1 mmole) of guanosine 2'-(3')-phosphate was added 861 mg (4.84 mmoles) of ninhydrin in 50 ml of H_2O. The reaction was conducted in the same manner as the deoxyguanosine–ninhydrin reaction. On thin layer chromatography in solvent A, the two spots (negative to periodate–benzidine spray) corresponding to starting material were not seen and two new spots (positive to periodate–benzidine spray) had appeared. A portion of the reaction mixture was worked up by means of preparative paper chromatography in solvent A. The faster band showed, in the ultraviolet, maxima at 249 and 281 mμ (sh) while the slower band showed maxima at 253 and 280 mμ (sh). Another portion of the reaction mixture was allowed to react with saturated sodium metaperiodate solution for 18 hr. Two new yellow fluorescent spots of R_F 0.20 and 0.47 were seen upon thin layer chromatography in solvent A.

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