The virus stocks were stored at −70 °C. Prior to use, 8% glycerol was added, and the virus samples were further kept at 4 °C during the course of the experiments.

Reference Compounds. Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridium bromide) was obtained from Calbiochem (Los Angeles, Calif.), whereas tilorone dihydrochloride [2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one dihydrochloride] was supplied by Richardson-Merrell Inc. (Cincinnati, Ohio). Berenil (diminazene diaceturate) was a product of Hoechst A.G. (Frankfurt/Mainz, Federal Republic of Germany), whereas stilbamidine isethionate (4,4′-stilbenedicarboxamidine diisethionate) and pentamidine isethionate (4,4′-diamidino-α,ω-diphenoxypentane isethionate) were products of May and Baker Ltd (Dagenham, England).

Heat-Induced Formation of α,β-Unsaturated Nucleoside Dialdehydes and Their Activity with Adenosine Deaminase

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Application of heat to aqueous solutions of nucleoside dialdehydes (periodate-oxidized nucleosides) affords the corresponding α,β-unsaturated aldehydes. The reaction was first discovered during studies with adenosine deaminase and was initially investigated enzymatically until the nature of the chemical transformation was determined. A UV peak at 230–250 nm, characteristic of the α,β-unsaturated aldehyde group, was obtained by difference spectroscopy and affords a more practical means to study the reaction. Adenosine dialdehyde, obtained by periodate oxidation of adenosine, afforded the same product upon heating as obtained by periodate oxidation of 9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine. Further proof of identity was obtained by reduction of these compounds with sodium borohydride and comparison of the dialcohols obtained to each other and to a known unsaturated dialcohol. Heating of nucleoside dialdehydes at any time is not recommended. The exact composition of nucleoside dialdehydes used in previous and current biological studies is open to question.

Nucleoside dialdehydes have potential use as chemotherapeutic agents for the treatment of neoplastic diseases.[2-5] They are obtained by periodate oxidation of nucleosides.[3,6,7] Especially important is inosine dialdehyde (1, Chart I), which has been tested clinically and for which the results of a phase I study have been published.[2] Although represented as structures like 1, it is generally recognized that nucleoside dialdehydes are really complex equilibrium mixtures of cyclic and acyclic hydrates and hemiacetals.[8-10] In fact, spectroscopic techniques do not even detect the aldehyde groups in solution,[9,11] a situation akin to that of reducing sugars, such as glucose, which are similarly defined as poly(hydroxyaldehydes).

While studying the deamination of adenosine dialdehyde (2) as catalyzed by adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) from calf intestinal mucosa, it was observed that application of a small amount of heat from a steam bath to aid in dissolution of the dialdehyde in phosphate buffer resulted in erratic kinetic results. Whereas 2 was a weak substrate for the enzyme, substrate activity was lowered appreciably or lost entirely upon heating. Moreover, 2, which was an extremely poor competitive inhibitor, became a rather strong inhibitor after heating. It appeared that a transition was taking place. This is a serious problem because the solubilities of some of the nucleoside dialdehydes are rather low and require heat to effect dissolution. Furthermore, preparative procedures for nucleoside dialdehydes often include extraction of the product from salts with hot alcohols and drying of the lyophilized powders in a drying pistol with heat.[9,11-13]

Table I. Comparison of the Inhibitor Constants of Solutions of Nucleoside Dialdehydes, Before and After Heating, with Adenosine Deaminase[a]

nucleoside dialdehyde	K_i, μM: before heating[b]	K_i, μM: after heating[c]
1		
2	520	5
3	310	8
4	28	30
5	780	800
6	46	30
7	430	24
8		69
9		38
10	150	20
11	370	21
12		
13	160	100

[a] 0.05 M sodium phosphate buffer (pH 7.0). [b] Most values are reported in ref 7 and 15. [c] Heated to 100 °C; see text for details.

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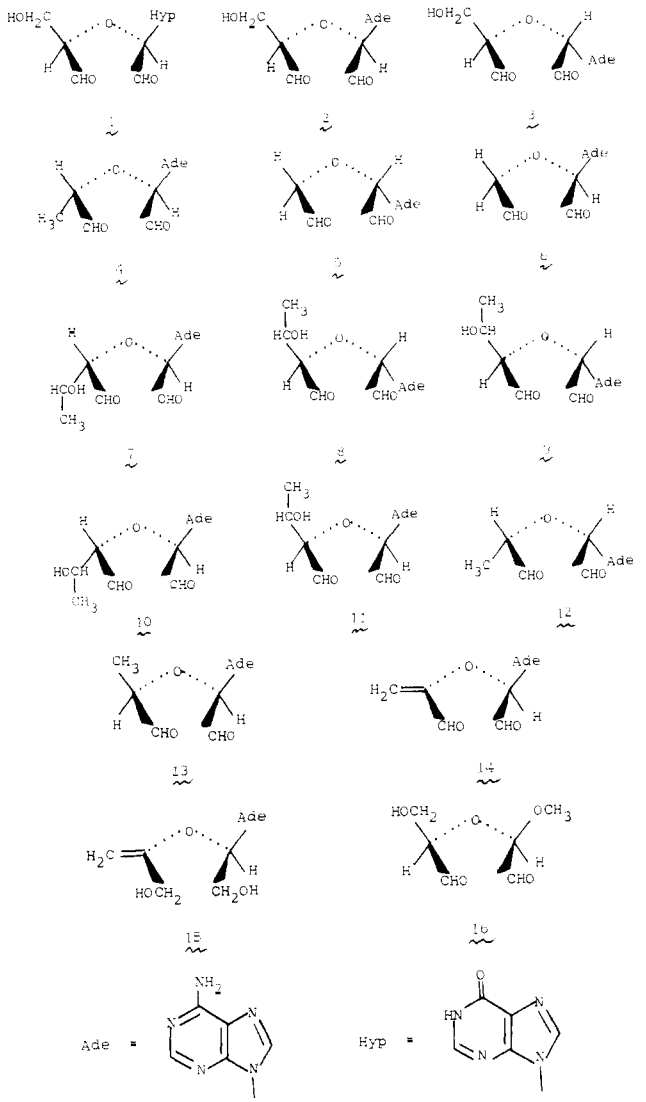
Chart I



Tremendous differences in the solubilities of 2 have been reported by different groups,[9,10] and the reasons for this may, in fact, be explained by our discovery concerning the effect of heat on these compounds.[14]

When the change in apparent K_i (inhibitor constant) with adenosine deaminase was followed as a function of time during heating of an aqueous solution of 2 in phosphate buffer at 100 °C, the value for K_i was found to become constant after about 1 h. Simultaneously, all substrate activity was lost. This change in K_i value, as seen in Table I, was very dramatic. The effects of heat on solutions of other nucleoside dialdehydes were also investigated and are reported in Table I as a comparison of the values for the inhibitor constants with adenosine deaminase before and after heat treatment. The length of time required to obtain constant K_i values was 0.5–1 h for the compounds undergoing change. Compounds that resisted change were heated for 2 h or more. Chart I shows the structures of the periodate-oxidized nucleosides in the dialdehyde form. It is evident that, in addition to 2, dialdehydes 3 and 7–11 were 10- to 100-fold more powerful inhibitors after heating than they were before heating. Compounds 8 and 9, which were not inhibitors before heat treatment, became rather respectable inhibitors after heating. Both before and after heating, the inhibition followed competitive kinetics in all cases. All nucleoside dialdehydes that were substrates before heating lost all substrate activity afterward. The adenine-containing dialdehydes 4, 8, 9, and 12 did not have substrate activity before heating either.[7,15] Dialdehydes 1, 4–6, and 13 showed no significant changes in K_i values. Compounds 1 and 12, which were not inhibitors before heating, were still not inhibitors after heating. As shown below, this lack of enzyme activity did not mean that a chemical transformation was not taking place.

The distinguishing feature of the dialdehydes that exhibited a dramatic change in enzyme activity after heat treatment was the hydroxyl group on the carbon atom vicinal to the distal[16] carbon atom. This suggested the possibility of an elimination reaction in which a molecule of water is removed to yield an unsaturated product. With the exception of inosine dialdehyde (1), adenosine dialdehyde (2) is the most readily available nucleoside dialdehyde in this group of compounds. It was the best substrate for adenosine deaminase and exhibited a 100-fold increase in enzyme inhibition. It was also used or has been used by a number of investigators for chemical and biological studies. Therefore, it seemed the best candidate for a study of the nature of the chemical transformation. A sample of 2 was heated to 100 °C in distilled water in order to obtain the substance free of salts after lyophilization. The rate of transformation was considerably slower in water than in phosphate buffer. The chromatographic mobilities of 2 and heated 2 on paper strips were different in two solvent systems. The solubilities of 2 and heated 2 in water at room temperature were dramatically different, heated 2 being much more soluble than unheated 2. Examination of the infrared spectrum of heated 2 revealed that a small peak at 4.3 μm, which appeared in nearly all of the nucleoside dialdehydes we have prepared thus far, was not present.[17] One of the nucleoside dialdehydes prepared earlier that did not have this peak was the unsaturated dialdehyde 14, obtained by periodate oxidation of 9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine.[7] In fact, the IR spectra of 14 and heated 2 were identical, signifying that they were the same compound. In addition, 14 was similar to heated 2 in other ways. It was not a substrate for adenosine deaminase, was a strong inhibitor of this enzyme with an inhibitor constant (K_i = 4 μM) identical with heated 2 (K_i = 5 μM), and chromatographed on paper identically to heated 2. In order to confirm that heated 2 was 14, it was treated with sodium borohydride to effect reduction of the aldehyde groups to alcohols. The product was compared to a known sample of dialcohol 15, a compound that had been prepared several years earlier.[18] Thanks to the kindness of Dr. P. T. Gilham of Purdue University, who provided us with a sample of 15, we were able to show that the compound obtained by reduction of

(13) Any exposure of nucleoside dialdehydes to alcohols is not to be recommended, since addition products (acetals) readily form. See Dvonch, W.; Alburn, H. E. U.S. Patent 4 000 137, *Chem. Abstr.* **1977**, *86*, 155900*t*; U.S. Patent 4 041 037, *Chem. Abstr.* **1977**, *87*, 202037*z*.

(14) It had previously been reported[10] that the solubility of nucleoside dialdehydes could be increased by heating with no detectable effect on the compounds.

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(16) In past publications,[7,15] we have called the carbon atom that was originally the anomeric carbon atom of the sugar the *proximal* carbon atom, and the one bonded to the bridge oxygen, one aldehyde group, and containing the tail end of the molecule, the *distal* carbon atom.

(17) We do not know the meaning of this peak.

(18) Schwartz, D. E.; Gilham, P. T. *J. Am. Chem. Soc.* **1972**, *94*, 8921.

heated 2 was identical with 15 (melting points, IR spectra, paper chromatography). Furthermore, the inhibitor constants with adenosine deaminase were 0.9 and 1 μM, respectively. A sample of 14 obtained from periodate oxidation of 9-(5-deoxy-β-*erythro*-pent-4-enofuranosyl)-adenine was also reduced to the same dialcohol 15. Therefore, heated 2 is 14 and arises as a result of a β elimination of a molecule of water.

α,β-Unsaturated aldehydes are known to be only slightly hydrated in aqueous solution.[19] Therefore, one would expect the aldehyde group at the distal carbon atom of 14 to exist in solution as a real aldehyde. An NMR spectrum of 14 confirmed this with a singlet at δ 9.00 for the aldehydic proton. In addition, a carbonyl peak at 5.9 μm was obtained in the IR spectrum of solid 14, typical for the α,β-unsaturated aldehydes.[20] In the UV, α,β-unsaturated aldehydes give absorption peaks between 220 and 250 nm.[20] The spectrum of 14 was obtained using an equimolar concentration of 2 as the reference to blank out the nucleoside absorption. The UV difference spectrum of 14 had a peak at 232 nm. The UV difference spectrum of heated 10 had a peak at 242 nm, and heated 1 gave a broad peak between 230 and 240 nm. Dialdehyde 4, which has no hydroxyl group next to the distal carbon atom, gave no peak upon being heated. Heated dialdehyde 16 likewise gave a peak at 236 nm. It was not necessary to run heated 16 against anything but water as the reference, since 16 contained no chromophoric group. It was clear then that the purine ring had no role in the elimination. This UV technique is a simple and rapid procedure for following the course of the elimination reaction without dependence upon an enzyme assay or chromatography. In addition, it provides the necessary information directly for the determination of the α,β-unsaturated aldehyde even in cases where no enzyme activity is observed either before of after heating. Examples are 1 and 16, both of which were converted to the α,β-unsaturated aldehydes upon heating but whose reactions cannot be detected enzymatically. We have repeated the extraction of adenosine dialdehyde from periodate salts with ethanol[13] as described by Jones et al.[9] and have found that between 20 and 30% conversion to the unsaturated aldehyde occurred, as determined by the UV difference spectrum technique. However, powders of the dialdehydes can be safely heated in a drying pistol at 100 °C without any change.

In view of the rather extensive work already reported on the antitumor activity, biological effects, enzyme interactions, and cross-linking of nucleoside dialdehydes to proteins, it is necessary to examine critically the exact structure of the compounds used in these studies with special consideration being given to application of heat during their preparation or during subsequent handling.

Experimental Section

Instrumentation and Methods. Melting points were determined on a Kofler hot stage. UV spectra and kinetic studies were recorded on either a Beckman DK-2 or Beckman 25 spectrophotometer. Difference spectra were obtained by running the spectrum of the α,β-unsaturated nucleoside dialdehyde in 0.05 M sodium phosphate buffer (pH 7.0) against an equimolar solution of the precursor dialdehyde in the reference cell. IR spectra were obtained on a Perkin-Elmer Model 21 spectrophotometer, and NMR spectra were obtained on a Varian T-60A spectrometer. Evaporations were performed under reduced pressure on a rotary evaporator. Paper chromatography was performed on descending strips (63 cm) of Whatman no. 1 paper, and spots were located with a Mineralight lamp that emitted UV radiation at 254 nm.

Table II

nucleoside dialdehyde	R_f: *n*-butyl alcohol–water (86:14)	R_f: *n*-butyl alcohol–ethanol–water (5:1:4, upper phase)
2	0.50	0.61
14 (heated 2)	0.75	0.72

Nucleoside Dialdehydes. A general procedure for the synthesis of nucleoside dialdehydes as performed in this laboratory has appeared.[7,15] The procedure requires no application of heat or contact with alcohols.[13] The preparation of 1 has been described in several papers,[3,12,21] but we have utilized the same procedure as above.[7] Similarly, 16, which has also been reported before,[3] was prepared from methyl β-D-glucopyranoside. Details concerning the chemical properties and enzymatic interaction of dialdehydes 2–6, 12, and 13 appear in ref 7; details for 7–11 are in ref 15.

Enzyme Assay and Kinetic Measurements. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), purchased from Sigma Chemical Co. (Type 1), had a specific activity of 220 units/mg of protein. One unit is defined as the amount of enzyme that will deaminate 1 μmol of adenosine per minute at pH 7.5 and 25 °C. The rate of change in absorbance at 265 nm was measured at 25 °C in 0.05 M sodium phosphate buffer, pH 7.0.[22] Inhibitor constants were determined by addition of the enzyme solution (0.1 mL) containing 0.25 unit to 3 mL of buffered adenosine solution containing the nucleoside dialdehyde. The adenosine concentration was 30–70 μM and the dialdehyde concentration was 3–190 μM, depending upon the activity of the compound. A minimum of five substrate concentrations and two inhibitor concentrations were used for each assay. The K_i values were obtained by the reciprocal plot method of Lineweaver and Burk,[23] and the best lines were obtained by use of the least-square analysis of Wilkinson.[24]

Enzymatic Assay of Heated Nucleoside Dialdehydes. A small sample of nucleoside dialdehyde (5–10 mg) was added to 80 mL of 0.05 M sodium phosphate buffer, pH 7.0. The flask was heated under reflux and 5-mL aliquots were withdrawn at 15-min intervals. Each aliquot was quickly cooled in ice and then used to prepare the solutions for determination of the inhibitor constants with adenosine deaminase. The K_i values obtained are reported in Table I.

Preparation of 14 by Heating of 2. Adenosine dialdehyde (2, 1 g) was heated under reflux in 70 mL of water until either the K_i became constant or the UV peak at 232 nm stopped increasing (ca. 1 h). Evaporation and lyophilization gave a white powder that was dried further in an Abderhalden drying pistol under high vacuum over P_2O_5 at room temperature. Paper chromatography showed that there was a significant difference in the mobility of 2 and heated 2 (14) (Table II).

The IR spectrum of heated 2 and a sample of 14 obtained by periodate oxidation of 9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine[7] were identical: IR (KBr) 5.9 μm (α,β-unsaturated aldehyde carbonyl); NMR (H_2O, DSS added as internal reference) δ 9.00 (aldehyde proton). The UV spectrum of 14 against water gave the typical nucleoside peak at 260 nm and considerable absorption in the 230–240-nm region not present in 2. The UV difference spectrum against an equimolar solution of 2 in the reference cell gave a peak at 232 nm (ε 6000). These UV spectra of heated 2 were also identical with those of 14 obtained directly from the nucleoside.

Preparation of Nucleoside Dialcohol 15. Adenosine dialdehyde (2, 100 mg) was converted to 14 by heating in water as

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described above. After the solution was chilled in an ice bath, 100 mg of sodium borohydride was slowly added, and the mixture was kept at 4 °C for 18 h. The solution was neutralized with Amberlite IR-120 (H^+) ion-exchange resin. The resin was removed by filtration and washed with water. The filtrate was evaporated to near dryness, and preparative TLC was used to purify the product. Two precoated 2-mm-thick silica gel plates, Sil G-200 with UV indicator (Machery-Nagel and Co., Germany), were used. The solvent system was 5:1:4 *n*-butyl alcohol–ethanol–water (upper phase). The main band at R_f 0.44, located with a UV lamp, was scraped off the plates and the gel was extracted with ethanol. Evaporation gave a residue that was dissolved in water, from which a white solid (**15**; 68 mg) was obtained, mp 173 °C (lit.[19] 176 °C). The IR spectrum was identical with the reference sample **15** supplied by Dr. Gilham. The mobilities on TLC were identical, as were the K_i values with adenosine deaminase (0.9 μM, 1 μM). Dialcohol **15** was also prepared by reduction of a sample of **14** that had been obtained earlier[7] from 9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine, and it was identical with the other two preparations in every way.

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Novel Antitumor Nitrosoureas and Related Compounds and Their Reactions with DNA

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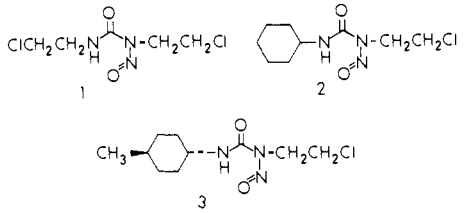
A number of nitrosoureas have been synthesized in order to examine those structural features which lead to optimum interstrand DNA cross-linking as this property may relate to their cytotoxicity and antitumor effects. The compounds include 2-chloroethyl trifluoromethanesulfonate, **5**, 2-chloroethyl *p*-nitrobenzenesulfonate, 2-chloroethyl *p*-toluenesulfonate, and 2-chloroethyl methanesulfonate. Of these compounds, **5**, bearing the most reactive leaving group, cross-links 11% of DNA after 6 h, presumably by way of an intermediate chloroethonium ion. A second group of compounds designed to favor low activation S_N2 displacement by biological nucleophiles included *N*-(2-chloroethyl)-*N*-nitrosoacetamide (**12**), 5-[3-(2-chloroethyl)triazenyl]imidazole-4-carboxamide (**13**), and ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**14**) which cross-link DNA to the extent of 44, 33, and 12% in 6 h, respectively. The extent of DNA cross-linking produced by ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**14**) is increased in the presence of thiol nucleophiles which favor decomposition to give the alkylating species. This result led to the design of 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyl disulfide (**17**) which after reductive activation undergoes intramolecularly activated decomposition to an electrophile, leading to enhanced rates of DNA cross-linking. Compound **17** is active against leukemia in mice (T/C = 136). Based on the preceding analysis, novel nitrosoureas were synthesized which were designed to produce internally activated alkylating species. The lead compound, 1-[2-[(2-chloroethyl)thio]ethyl]-3-cyclohexyl-1-nitrosourea (**22**), produces 90% interstrand cross-linked DNA in 10 min under physiological conditions and in vivo a percent T/C value of 194 against leukemia L1210 in rodents. Comparison of the reactivity of compound **22** toward DNA with that of the modified nitrosoureas **30–32** supports the interpretation of the high reactivity of **22** being due to side-chain activation by the neighboring sulfur atom.

2-Haloethylnitrosoureas, including BCNU [*N,N*′-bis(2-chloroethyl)-*N*-nitrosourea (**1**)], CCNU [*N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea (**2**)], and MeCCNU [*N*-(2-chloroethyl)-*N*′-(4-methylcyclohexyl)-*N*-nitrosourea (**3**)], are of clinical value in the treatment of a range of



malignant diseases.[1-6] Previous papers in this series have examined various aspects of the chemistry of the nitrosoureas, including aqueous decomposition, alkylation and interstrand cross-linking of DNA (which appear to underlie their molecular mechanism of action), and DNA strand scission.[7-10] The results of several investigations suggest that alkylation and DNA cross-linking by nitrosoureas require the generation of a chloroethyl alkylating agent upon aqueous decomposition.[7,11-14] Chloroethylation of an appropriate base in DNA is followed by labilization of the C–Cl bond and a second alkylation to complete the

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