and terminates on Day 15.

Several studies linked the alteration of events common to palatogenesis and bone formation, including glycosaminoglycan (23) and collagen formation (24, 25), and the activities of enzymes (alkaline phosphatase, acid phosphatase, and β-glucuronidase) (26) and hormones (parathyroid hormone and calcitonin) (27) to the production of cortisone-induced cleft palate. Because of these relationships and the similarities that exist between cortisone- and phenytoin-induced skeletal malformations, it can be concluded that, qualitatively, the teratogenic mechanism of action of these agents in the formation of skeletal anomalies is similar.

A graphical approximation of the regression lines of the mean percent of cleft palate per litter on log-dose for cortisone- and phenytoin-treated groups appears in Fig. 1. These lines are the result of the second iteration of the method of probit analysis described by Finney (8). The original iteration was performed on lines fitted independently from the results obtained from the test groups without constraint of parallelism. The results from the second stage were employed to calculate the common slope, which was used to construct the curves in Fig. 1. Heterogenicity and parallelism were both examined by χ^2 tests. Because the dose–response regression lines for cortisone- and phenytoin-induced cleft palate did not deviate from parallelism, the teratogenic mechanism of action of these agents in causing this anomaly may be similar. The relative potency of phenytoin as a cleft palate-inducing teratogen was calculated, according to the method of Finney (8), to be from 14.54 to 69.18% that of cortisone.

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Oxidative Degradation of 6-Selenoguanosine in Aqueous Solutions

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Abstract □ The degradation of 6-selenoguanosine (NSC 137679)(I) in water and in various buffer systems was investigated. Drug degradation in aqueous media was monitored by high-pressure liquid chromatography. Some kinetic aspects of the degradation of I in various buffer systems at 25° also were studied spectrophotometrically. The degradation, which requires oxygen, involves autoxidation of I to the corresponding diselenide, which produces a selenide and metallic selenium in the presence of oxygen. This degradation pathway differs from that reported for the oxidation of related thio compounds.

Keyphrases □ 6-Selenoguanosine—oxidative degradation in aqueous solutions, kinetics □ Autoxidation, aqueous—6-selenoguanosine, selenium compounds, pharmacokinetics □ Kinetics—6-selenoguanosine, oxidative degradation in aqueous solutions, selenium compounds □ Antineoplastics—6-selenoguanosine, selenium compounds, oxidative degradation in aqueous solutions, kinetics

During a study of selenium analogs of physiologically active sulfur compounds, numerous selenium analogs were synthesized for biochemical investigation. 6-Selenopurine was synthesized (1) and found to be active as an antileukemic agent (2), but it was unstable and toxic to the host (3). Subsequently, 6-selenopurine-9-β-D-ribonucleoside was prepared, but it was even less stable than 6-selenopurine under the same conditions (3).

In a search for a more stable analog, 6-selenoguanosine (NSC 137679) (I) was synthesized (4); it was found to be an active inhibitor of several experimental tumors *in vitro* (5, 6) and *in vivo* (7, 8). Although I was reported to be more stable than preceding analogs (4, 9), it still undergoes sufficiently rapid degradation in aqueous solutions such that its formulation in a dosage form suitable for evaluation of anticancer activity presented serious problems (10).

While considerable information has been published

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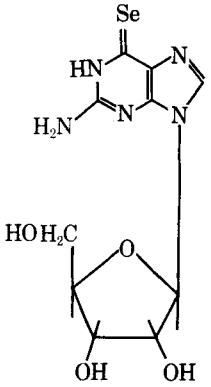


I

relative to the oxidation and other solution reactions of sulfur-containing compounds, including thiopurines and thionucleosides (11, 12), there is a lack of such information for related selenium compounds. The present work examined the nature of the degradation, including its reaction products, and investigated the factors affecting the degradation rate of I in solutions.

RESULTS AND DISCUSSION

When I was dissolved in water (0.75 mg of I/ml of water) and air was continuously bubbled through the solution, cloudiness developed within 5 hr. The cloudy solution was filtered at 6 hr, and the solid dark-yellow residue was dissolved in methanol. When the methanolic solution was examined by high-pressure liquid chromatography (HPLC), a major peak was eluted at 21.5 ml (capacity factor $k' = 7.08$). An authentic sample of the diselenide of 6-selenoguanosine in methanol (II) also eluted at 21.5 ml. Results of the elemental analysis of the solid residue were in good agreement with those of the authentic II sample. Additionally, the UV spectra of methanolic solutions of the solid residue and authentic II also were identical. Thus, it was concluded that the cloudiness was due to II precipitation. When the filtrate of the cloudy solution at 5 hr was examined by HPLC, only a very small peak (D, Fig. 1) corresponding to II was observed; it was attributed to the fact that II is only very sparingly soluble in water (~0.1 μg/ml) and little remains in solution.

The compounds responsible for the remaining peaks in the filtrate chromatogram shown in Fig. 1 also were examined. Peak A ($k' = 1.46$) was due to I itself. Peak B ($k' = 3.92$), which was only present in small amounts, was characterized as guanosine (III) based on the fact that it coeluted with an authentic guanosine sample.

The compound responsible for peak C ($k' = 4.40$) was more difficult to characterize. In chromatograms of freshly prepared solutions of I, peak C was not observed. But on standing with exposure to molecular oxygen, peak C increased in intensity. After standing for ~10 days, the reaction appeared to reach completion; the chromatogram of the reaction mixture filtrate showed only a small peak B (corresponding to III) and a large peak C, with no remaining peak A or D. When a dilute aqueous suspension of II (0.2 mg of II/ml of water) was prepared and allowed to stand exposed to air, peak C again was formed at the expense of II. The sediment remaining after the aqueous solution of I was exposed to oxygen for 10 days was red. Separation and analysis of the solid demonstrated that it was selenium metal.

The filtrate obtained at 10 days was evaporated to dryness under reduced pressure, and the compound corresponding to peak C was isolated and recrystallized. HPLC analysis of the recrystallized solid indicated that recrystallization had not resulted in any significant compound degradation, although some contaminants, including III and selenium metal, appeared to be present in the final product. Subsequent attempts to purify the samples further resulted in some degradation, as evidenced by visual inspection and HPLC analysis. Elemental analyses of the samples obtained indicated 11–13% selenium. When the sample was treated with trimethylsilane and analyzed by chemical-ionization mass spectrometry, the parent ion ($MH^+ = 1190$) and the two major fragments (mass 635 and 555) suggested the structure of the silylated compound to be IV*a*.

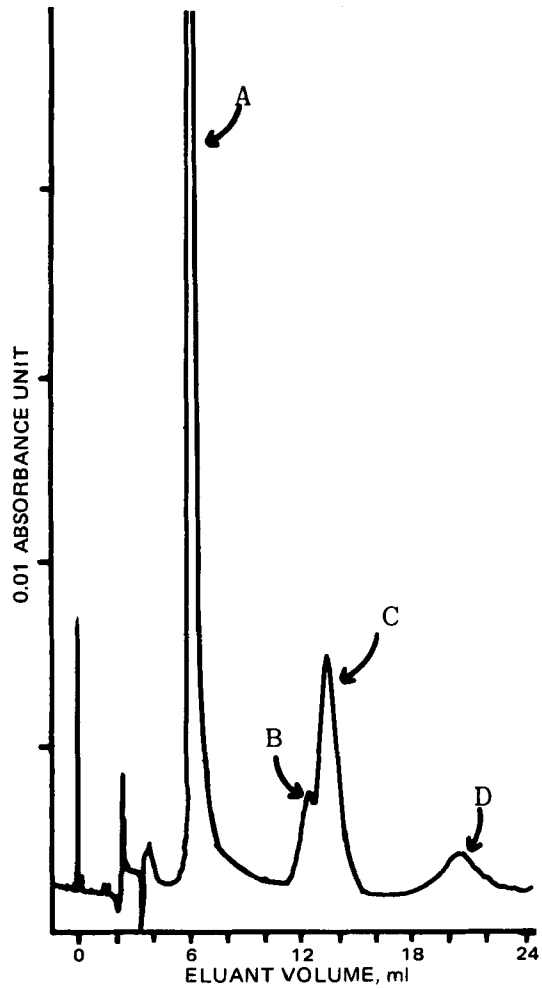
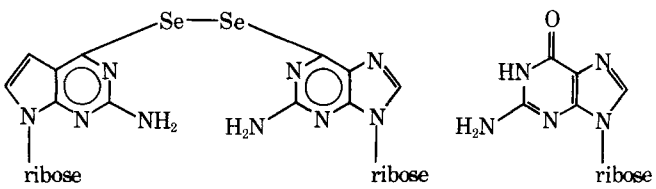


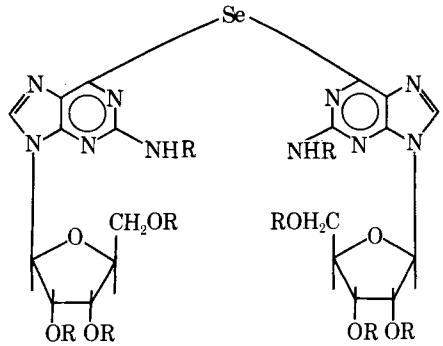
Figure 1—*Chromatogram (HPLC) of a solution of 6-selenoguanosine in water (pH ~4–5) after 5 hr at room temperature.*

While the carbon, hydrogen, and nitrogen analysis of the isolated material did not agree closely with that of IV, the discrepancy may be attributed to an inability to obtain a pure sample, even when recrystallization from various solvents was attempted. The UV spectrum of IV



II

III



IV: R = H, bis(2-amino-9-β-D-ribofuranosyl-6-purinyl) selenide

IV*a*: R = trimethylsilyl

showed a maximum at 325 nm, indicating more conjugation in the purine ring system and the absence of the selenone group.

When I was dissolved in water that had been recently boiled and cooled (under argon or nitrogen) and the reaction mixture was kept under argon (or nitrogen), no degradation products were detected by HPLC and the solution remained clear for >6 days. Similarly, a II suspension in oxygen-free water stored under an inert gas showed no IV formation. However, when these same systems were subsequently exposed to oxygen, decomposition again was apparent within a few hours.

These data demonstrate the need for oxygen in conversion of I to II and of II to IV. These results support the following scheme:

$$2\mathrm{I} \xrightarrow{O_2} \mathrm{II} \xrightarrow{O_2} \mathrm{IV} + \mathrm{Se}$$

Scheme I

where the conversion of I to II involves a simple oxidation while the conversion of II to IV is a more complex transformation. While the production of IV from II appears to be a disproportionation reaction, the necessity of atmospheric oxygen precludes such a simple explanation.

Although a free radical process may be involved, available data do not provide a specific mechanistic interpretation of the conversion of the diselenide to the selenide and metallic selenium.

As already mentioned, in the degradation of I and II, traces of III were present in the solutions. It is not clear whether III occurs as a result of a minor side reaction of the decomposition of I and/or II or as a product of the slow degradation of IV. In any event, it represents only a small fraction of the products.

While II has a very low water solubility of ~1 μg/ml, both I and IV are much more soluble, with the solubility of I being ~2–3 mg/ml and that of IV being ~3.5 mg/ml.

Spectrophotometric changes observed during the described reaction also supported Scheme I. In simple aqueous solution (pH 4–5), the absorbance maximum of I was at 358 nm while in 0.1 *N* NaOH, λ_{max} was ~330 nm. This marked change in λ_{max} may be ascribed to tautomerization and ionization as shown in Scheme II.

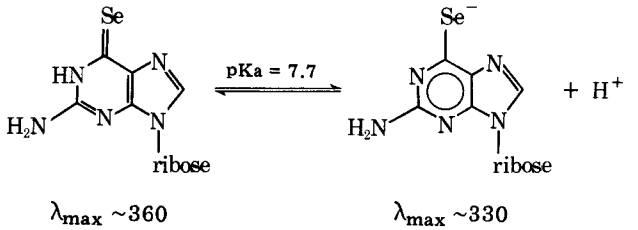
Methanolic solutions of I, II, and IV yielded λ_{max} values of 365 (log a 4.29), 318 (4.30), and 327 (4.31) nm, respectively. The chemical structures of both II and IV are related more closely to I*a* than to I and thus would be expected to exhibit λ_{max} values similar to those of I*a*. When oxidation of I in water was followed spectrophotometrically at pH <7, the reaction was accompanied by a substantial time-dependent decrease in absorbance at 350–360 nm and a corresponding increase at 315–330 nm. In alkaline media of pH >10, the maximum absorbance was at 315–330 nm; little change in absorbance occurred as the reaction proceeded (as monitored by HPLC).

Similar spectral behavior has been reported and rationalized for numerous related 6-thiopurine derivatives including the corresponding disulfides and the 6-alkylmercapto compounds (12–16). In all cases, loss of the thione or selenone form resulted in a hypsochromic shift.

The initial degradation studies were carried out on solutions prepared by dissolution of I in water. The resulting solutions were in the pH 4–6 range. Subsequent efforts were directed toward determining the effects of pH on the degradation rate of I.

The rates of loss of I from aqueous buffered solution exposed to air were studied at pH 2–11 using UV spectrophotometry and/or HPLC. Over this entire pH range, the degradation rate of I appeared to be a first-order process, with linear semilog plots obtained for more than two half-lives in all cases and for up to five half-lives in most instances.

The rate data are summarized in Table I; I apparently was most stable at pH 4–5. At values of pH 7–11, there appeared to be no significant change in the rate of loss of I, which is surprising since the pKa of I was found by spectrophotometric determination to be 7.68 ± 0.05. Normally, it might be expected that the ionization involving the selenium atom would alter the rate significantly. Table I also shows that the buffers used may substantially influence the degradation rate. This finding is particularly obvious at pH 5, where acetate and citrate buffers were used and the rate in citrate was more than fourfold greater than that in acetate at concentrations of 1×10^{-2} *M*.



I Ia

Scheme II

Table I—Rate Data Obtained for the Loss of 6-Selenoguanosine [a] for Aqueous Buffered Solution at 25° and Various pH Values

pH	Buffer	λ_{max}, nm	$t_{1/2}$, hr	k_{obs} (× 10^2), hr^{-1}
11.0	Sodium hydroxide[b,c]	330	6.6	10.6
9.0	0.010 *M* borate[b]	330	3.9	17.8
8.0	0.010 *M* borate[b]	335	5.0	13.9
7.9	0.005 *M* phosphate	335	2.5	27.7
7.0	0.005 *M* phosphate	357	4.7	14.7
5.0	0.010 *M* acetate[b]	358	40.8	1.7
			(43.8)[c]	(1.6)[c]
5.0	0.010 *M* citrate[b]	358	10.1	6.9
4.0	0.010 *M* acetate[b]	358	44.9	1.5
			(49.3)[c]	(1.4)[c]
2.0	Hydrochloric acid	360	27.7	2.5

[a] Initial concentration was ~5×10^{-5} *M*. [b] Solution contained 0.1 *M* KCl. [c] Data obtained by HPLC studies with initial concentration of I of 2×10^{-4} *M*.

Additional absorbance studies were carried out to determine if citrate enhanced degradation or if acetate acted as a stabilizer. With both citrate and acetate buffers (pH 5), the rate of absorbance loss increased when the buffer concentrations increased, indicating that both buffer systems enhanced the rate of loss of I.

With acetate buffers, the rate enhancement due to buffer appeared to be rather small and a linear relationship appeared to exist between buffer concentration and the observed rate constant. The effect of total citrate concentration on the observed rate constant was nonlinear, exhibiting a decreasing rate dependence on citrate as the buffer concentration was increased from 1×10^{-3} to 0.10 *M*. The precise cause for the observed asymptotic relationship is not understood but presumably involves a reversible interaction between I and citric acid (or citrate ion) to yield a species that is less stable than I itself. Studies on the effects of citrate on I degradation are continuing.

At pH 4 and 5 in acetate buffers, the rates of loss of I as determined by both spectrophotometry and HPLC appeared to accelerate at about two half-lives (60–90 hr). Whether this result was due to product catalysis or another competing side reaction was not determined. Such acceleration was not observed at other pH values or even at pH 4 and 5 when citrate buffers were used. However, in those cases, the half-lives of the reaction being monitored were much shorter and, consequently, the loss of I was largely complete in 50–60 hr.

While the oxidation of I in water is rather facile, the oxidation rates in dimethyl sulfoxide and dimethylacetamide were much faster. Within 1 hr at ambient temperature, complete conversion of I to II occurred. However, there did not appear to be any subsequent diselenide degradation in either of these organic solvents over the several days during which the solutions were monitored. Similar oxidation reactions in these solvents were reported for sulfur compounds (17).

CONCLUSION

In a reaction analogous to the autoxidation of thiols and thionucleosides such as thioguanosine, 6-selenoguanosine (I) is oxidized in aqueous media in the presence of air to yield the corresponding diselenide. However, unlike the disulfides, which are relatively stable except in alkaline media or in the presence of potent oxidizing agents, the diselenide (II) of I undergoes a facile reaction to yield the more soluble selenide and selenium metal. Whether this is a general reaction of diselenides was not addressed in this study but may merit investigation as a simple means of preparing seleno ethers from organic selenols.

Previous reports that I was more stable than some of its analogs were based on absorption changes in aqueous solution (9). However, the comparisons made were between a solution of I in an alkaline medium (where loss of I is not accompanied by significant changes in absorption) and solutions of 6-selenopurine and related compounds at neutral pH (where loss of the parent substance is evidenced by large absorption changes). When solutions of I were studied at neutral pH values, relatively rapid and large absorbance changes were observed. Consequently, the stability of I clearly does not differ substantially from that of its closely related predecessors.

In the absence of oxygen, I was quite stable, and aqueous solutions gave no evidence of drug loss over several days. In the presence of oxygen, the minimum rates of oxidation appeared to occur at pH 4–5. Further sta-

bilization can be afforded by the incorporation of antioxidants such as ascorbic acid and dithioerythritol (10).

EXPERIMENTAL

Materials—6-Selenoguanosine[1] (I) was purified by recrystallization from aqueous ascorbic acid as previously described (10). All other chemicals were analytical or chromatographic grades.

Preparation of I Solutions for Kinetic Studies—A stock solution of I was prepared by dissolving the drug (15–17 mg) in 100 ml of 0.1 *M* KCl. The solution obtained was filtered (0.45-μm membrane filter), and 10 ml of the clear filtrate was diluted to 100 ml with the selected buffer to yield I concentrations of $\sim 5 \times 10^{-5}$ *M*. The resulting solution was kept in a tightly closed flask and maintained at 25° in a water bath. Aliquots were withdrawn at intervals, and the UV absorption at λ_{max} (358 nm in pH 4–5 acetate buffer, ~330 nm in more alkaline buffers, and 360 nm in acid buffers) was monitored spectrophotometrically[2].

For kinetic studies monitored by HPLC, solutions were prepared similarly, but the final concentrations of I were $\sim 2 \times 10^{-4}$ *M*. Aliquots (20 μl) were removed and analyzed by HPLC.

pKa Determination of I—The pKa was determined spectrophotometrically at 25° by the method of Albert and Serjeant (18). Buffers were 0.01 *M*, and calculations were based on absorbances monitored at 255, 330, and 358 nm. At all wavelengths, the value obtained was pKa = 7.68 ± 0.05.

Chromatography—HPLC was performed as described previously (10). Although the methodology used was unchanged, the elution volume and, consequently, the capacity factors were much smaller. These factors were observed to decrease as column use continued.

Isolation and Characterization of Degradation Product (IV) of I in Aqueous Solution—Compound I (300 mg) was dissolved in deionized water (400 ml). Air was continuously bubbled through the solution, and the degradation was followed chromatographically (1 ml of the reaction mixture was diluted to 10 ml with methanol and 10 μl of solution was injected) until the reaction was complete (~10 days). Water loss due to evaporation (which was accelerated by the passage of air through the solution) was compensated for by daily addition of water to a constant total volume. Such replacement of water was necessary to keep the reactants in solution.

After 10 days, the reaction mixture was filtered (0.45-μm filter), and the filtrate was evaporated to dryness under reduced pressure at ~40°. The residue was treated with absolute ethanol (30 ml, to facilitate the complete removal of water) and was again evaporated to dryness. The product was dissolved in boiling methanol (25 ml), filtered, and poured on cold ether (400 ml). The resulting liquid, containing a yellow precipitate, was kept in a refrigerator overnight. The precipitate was separated by filtration (glass filter, 4–4.5 μm), washed with cold ether (3 × 25 ml), and dried under vacuum for 24 hr to give 195 mg of IV (65% yield) as a yellow solid, which decomposed on melting at 220–222° (uncor.).

Anal.—Calc. for $C_{20}H_{24}N_{10}O_8Se$: C, 39.29; H, 3.96; N, 22.91; Se, 12.91. Found[3]: C, 37.77; H, 4.1; N, 21.09; Se, 11.92.

Chemical-ionization mass spectrometry[4] of the trimethylsilylated compound gave evidence of a molecular ion (MH^+) of 1190 and two major fragments at masses of 555 and 635, corresponding to fragmentation of the compound (IV*a*) between the selenium atom at position 6 of the purine rings. Both the parent ion and the mass 635 peaks exhibited the typical selenium cluster. These results agreed with the molecular weight of 612 for Structure IV*a*.

[1] Obtained from the National Cancer Institute.

[2] Cary model 14 or 15, Varian Instruments, Palo Alto, Calif.

[3] Analyses were performed by Schwarzkopf Microanalytical Laboratory, and by the Medicinal Chemistry Department, University of Kansas.

[4] Mass spectrometry was performed at the Pharmaceutical Chemistry Department, School of Pharmacy, University of California at San Francisco.

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