Abstracted in part from a dissertation submitted by G. Subba Rao to the Horace H. Rackham School of Graduate Studies, University of Michigan, in partial fulfillment of Doctor of Philosophy degree requirements.

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Note added in proof: An isolation and antisweet evaluation of the gymnemic acids which includes their methyl derivatives was recently reported [K. Kurihara, Y. Kurihara, and L. M. Beidler, in "Olfaction and Taste," C. Pfaffmann, Ed., Rockefeller University Press, New York, N. Y., 1969, p. 450; K. Kurihara, *Life Sci.*, **8**, 537(1969)].

Reactions of Germine-3,16-diacetate in Aqueous Solution

EDWARD M. COHEN and REZSO ACZEL

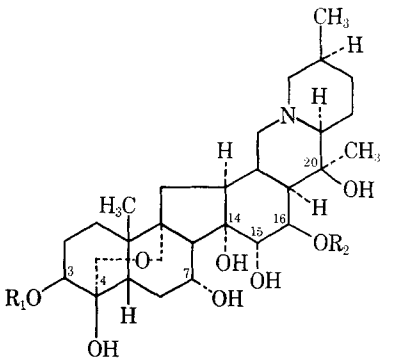
Abstract □ Germine-3,16-diacetate degrades rapidly in aqueous solution above pH 7 at room temperature ($t_{90\%} \sim 1$ hr. at pH 7.2 and $t_{1/2} < 1$ hr. at pH 9.5). The compound undergoes ester hydrolysis to form germine monoacetate and germine, as well as conversion to two as yet unidentified germine acetate esters. TLC was used to monitor these changes. A kinetic model describing the degradative behavior is proposed. Directions are given for preparing an aqueous solution stable for at least 3 days at room temperature.

Keyphrases □ Germine-3,16-diacetate reactions—aqueous solution □ Degradation—germine-3,16-diacetate in aqueous solution □ Stability profile and pH effect—germine-3,16-diacetate □ TLC—degradation monitoring □ IR spectrophotometry—identification

Recent work in this laboratory has revealed that germine-3,16-diacetate (GDA-3,16) degrades rapidly in aqueous solution at its natural pH, 9–10. The relatively rapid rate of hydrolysis of GDA-3,16 was first detected when titrating the compound as a base with standard acid. Consistently low results were obtained as the result of liberation of acetic acid during the dissolution of the compound in water prior to titration (about 40 min.). A concurrent observation of significance, reflecting this hydrolysis, was that the pH of a 0.1% GDA-3,16 solution decreased from an initial value of about 9.5 to about 6.5 in 3.5 days at room temperature.

These findings are of considerable importance because several publications have described the pharmacology and clinical experience obtained with unbuffered aqueous solutions of the drug, presumed to be GDA-3,16 (1). The significance of GDA-3,16 instability in connection with previously reported biological data is given by Torchiana *et al.* (2). The purposes of this publication are fourfold:

1. To alert investigators using GDA-3,16 about the chemical instability of this compound and recommend a method for preparing solutions that avoid the initial decomposition.
2. To describe a TLC system to assess the integrity of GDA-3,16 in solution.
3. To report preliminary findings about the influence of pH and solvent composition on the stability of GDA-3,16.
4. To report the conversion of GDA-3,16 into Unknowns 1 and 2.



structure of germine compounds

I

Compound	R_1	R_2
G	—H	—H
GDA-3,16	—$COCH_3$	—$COCH_3$
GMA-3	—$COCH_3$	—H
GMA-16	—H	—$COCH_3$

EXPERIMENTAL

Chemicals—GDA-3,16, germine-3-monoacetate (GMA-3), and germine-16-monoacetate (GMA-16) were synthesized,[1] starting from commercially available germine (G). The structure of germine and the acetate esters discussed is given in Structure I. The physical and chemical constants obtained for these compounds were in accord with either literature or expected values (3, 4). All of the germine compounds were reduced in particle size to less than 100-mesh to facilitate dissolution of the solids. All other chemicals used in these studies were reagent grade or better and were not further purified.

TLC—Glass plates were coated by standard techniques (5) with a 250-μ thick layer of a commercial adsorbent.[2] The TLC plates were air dried for about 15 min. and then further dried at 105° for 30 min.; they were stored in a desiccator prior to use.

An ethyl acetate–methanol–concentrated ammonia (80:15:5) mobile solvent resolved all of the germine compounds. Some typical R_f values observed by ascending TLC in a vapor-saturated developing chamber follow:

Compound	R_f
Unknown 1	0.80
GDA-3,16	0.75
GMA-3	0.60
Unknown 2	0.45
GMA-16	0.40
Germine	0.25

[1] Merck Sharp & Dohme Research Laboratories.
[2] Absorbosil-1, Applied Science Labs., Inc., State College, Pa.

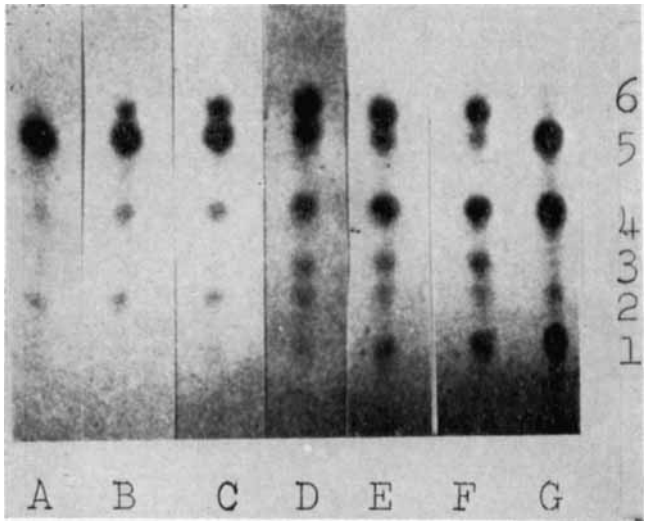


Figure 1—*TLC pattern obtained for solution of GDA-3,16 in water* versus *time. A, B, C, D, E, and F are solutions at 1, 5, 30, and 90 min. and 3 and 24 hr., respectively. G is a mixture of GDA-3,16 (5), GMA-3 (4), and germine (1). Spot 2 = GMA-16; Spot 3 = Unknown 2; and Spot 6 = Unknown 1.*

The exact R_f's obtained varied slightly from time to time, but the relative R_f's observed for these compounds were reasonably constant throughout a number of experiments.

The separated germine compounds were identified on the TLC plate by comparison of their R_f values with those exhibited on the same plate by the available authentic specimens—*viz.*, GDA-3,16, GMA-3, GMA-16, and germine, after visualization with iodine vapor. Semiquantitative estimates of the amounts of separated germine compounds were made by visual comparison of the size and intensity of the sample spots with the spots given by known amounts of the reference compounds.

No significant *in situ* degradation of GDA-3,16 occurred during TLC development when two-dimensional TLC was done as described by Penner *et al.* (6).

Aqueous Stability Profiles—A 1-mg./ml. solution of GDA-3,16 was prepared by dissolving the compound in water. One-milliliter aliquots were taken periodically and "quenched" by adding 1 drop of 0.1 *N* HCl to each sample. This lowered the pH to between 2 and 3, and at that pH no change in solution composition occurred during the subsequent TLC experiments. Fifty microliters of each "quenched" solution was spotted on the TLC plate, and the solvent front was allowed to ascend 12 cm. from the origin (about 45 min. for a 20 × 20-cm. TLC plate). The plate was then removed from the tank, dried until the mobile solvent odor dissipated, and then visualized with iodine vapor. The pH of the stock GDA-3,16 solution was monitored occasionally during the run. Similar experiments were done with aqueous solutions of GMA-3, GMA-16, and germine.

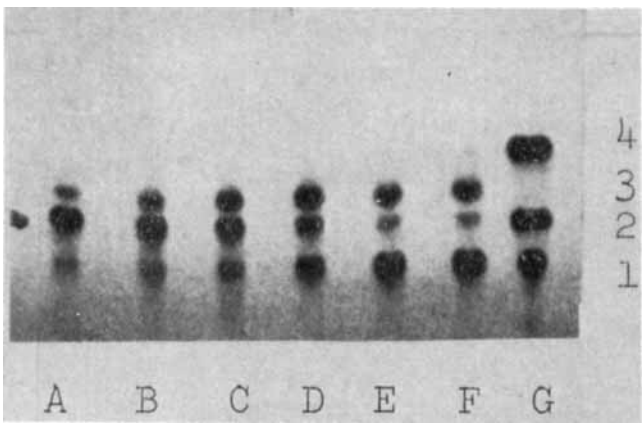


Figure 2—*TLC pattern obtained for solution of GMA-16 in water* versus *time. A, B, C, D, E, and F are solutions at 1, 10, and 30 min. and 2, 20, and 24 hr., respectively. G is a mixture of GMA-3 (4), GMA-16 (2), and germine (1). Spot 3 = Unknown 2.*

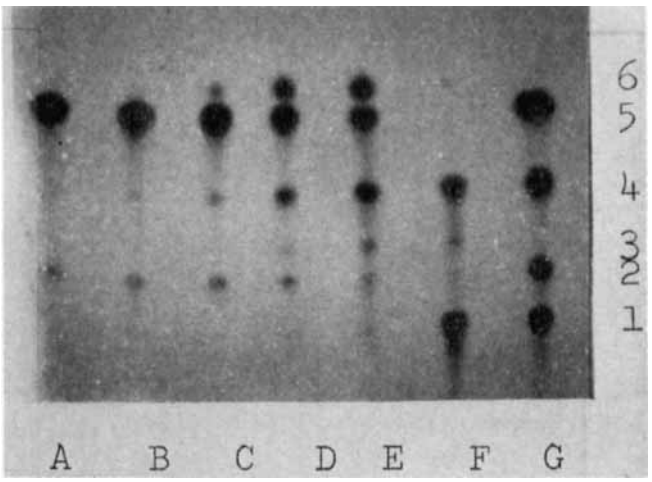


Figure 3—*TLC pattern obtained for GDA-3,16 solutions* versus *time at pH 4.6 (A = 1 hr., B = 24 hr.), pH 7.2 (C = 1 hr., D = 24 hr.), and pH 9.5 (E = 1 hr., F = 24 hr.). G is a mixture of GDA-3,16 (5), GMA-3 (4), GMA-16 (2), and germine (1). Spot 6 = Unknown 1, and Spot 3 = Unknown 2. GMA-16 occurs as an impurity in the GDA-3,16 sample used in these studies.*

Conversion of Unknowns 1 and 2 to Germine—A degraded aqueous solution of GDA-3,16 (initial concentration 1 mg./ml.), which had a pH of 8 and contained both Unknown 1 and Unknown 2, was adjusted to above pH 13 with sodium hydroxide and stored at room temperature for 2 hr. After this alkaline treatment, only one spot was detected in the solution by TLC, corresponding in R_f to germine. The alkaline solution was saturated with sodium chloride and extracted with several portions of chloroform. A white solid was obtained after evaporating the combined chloroform extracts to dryness with a nitrogen stream. The IR spectrum of the solid (KBr mull) was essentially the same as the spectrum of an authentic sample of germine.

Stability of GDA-3,16 *versus* pH and Solvent Composition—For the pH studies, 0.1% solutions of GDA-3,16 were prepared by directly dissolving the solid in the following solvents: 0.1 *N* HCl, pH 4.6 (0.1 *M* acetate buffer), pH 7.2 (0.1 *M* phosphate buffer), pH 9.5 (0.1 *M* ammonia buffer), and 0.1 *N* NaOH. The solutions were stored at room temperature and samples were removed periodically and evaluated by TLC.

The stability of GDA-3,16 in the following solvents was assessed in a similar fashion: methanol, methanol–water (1:1 by volume), alcohol USP, alcohol–water (1:1 by volume), chloroform, acetone, acetonitrile, pyridine, and methylene chloride.

RESULTS AND DISCUSSION

Aqueous Stability Profiles—Figure 1 shows the degradation profile *versus* time for GDA-3,16 in water. An examination of the TLC plate revealed the following:

1. There was very rapid conversion of GDA-3,16 to Unknown 1 (R_f > GDA-3,16). The amount of Unknown 1 continued to increase while the GDA-3,16 continued to decrease with time. At some point (90 min. *versus* 3 hr.), the amount of Unknown 1 decreased.
2. The formation of GMA-3 also occurred very rapidly, and its level increased with time.
3. There was a gradual buildup of Unknown 2 and germine with time.
4. There did not appear to be any significant change in the GMA-16 level with time. As is evident in the reference migration zone, GMA-16 occurred as an impurity of GDA-3,16.

The corresponding solution stability profile for GMA-3 is very simple, with the only observed degradation product being germine. Unknowns 1 and 2 were not detected by TLC.

Figure 2 gives the solution composition as a function of time for a water solution of GMA-16. The salient features of this chromatogram are:

Table I—Stability of GDA-3,16 (0.1%) *versus* pH at Room Temperature

Degradation Products Observed	Solution Composition[a]									
	Concentrated HCl, 2 hr.	0.1 *N* HCl, 1 hr.	0.1 *N* HCl, 10 days	pH 4.6, 1 hr.	pH 4.6, 24 hr.	pH 7.2, 1 hr.	pH 7.2, 24 hr.	pH 9.5, 1 hr.	pH 9.5, 24 hr.	0.1 *N* NaOH, 1 hr.
G	++	−	−	−	−	−	−	−	+++	+++[b]
GMA-16	+++	−	++	−	−	+	+	+	−	−
Unknown 2	−	−	−	−	−	−	+	+	+	−
GMA-3	+	−	−	−	−	+	+	++	++	−
Unknown 1	−	−	−	−	−	+	++	++	−	−
Intact GDA-3,16	10%	100%	90%	100%	100%	90%	60%	20%	0	0

[a] Amount of degradate present estimated as −(<1%), +(<10%), ++(<50%), and +++(>50%). [b] Solution completely degraded in less than 1 hr.

1. There was rapid conversion of GMA-16 into a compound whose R_f is identical with Unknown 2 noted in Fig. 1.
2. Accompanying the buildup of Unknown 2 was a somewhat slower buildup of germine. A slight amount of germine was present initially as an impurity in the GMA-16 sample used for these studies.
3. After 2 hr., germine buildup was accompanied by a decline in the amount of Unknown 2, suggesting conversion of the latter to the former.

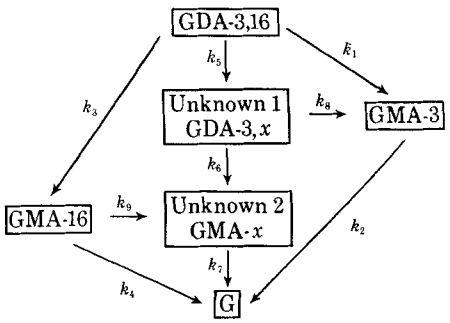
The data from the GMA-16 experiment indicate that Unknown 2 can be made from GMA-16 at a rate that is comparable to the rate of formation of Unknown 1 in GDA-3,16 solutions (Fig. 1). Furthermore, since Unknown 1 was not observed here (Fig. 2), it almost certainly is derived exclusively from GDA-3,16.

An additional experiment conducted in this series was to observe the behavior of germine itself in water. No change in the TLC pattern or solution pH was observed after 24 hr. Thus, as expected, neither Unknown 1 nor 2 was produced from germine.

In all of these experiments, with the exception of those starting with germine, the pH of the stock solutions decreased with time, reflecting the hydrolysis of the acetate ester linkage.

Structures of Unknowns 1 and 2—A consideration of the relative R_f's on the TLC plates shows that Unknown 1 lies near GDA-3,16 and that Unknown 2 lies near GMA-3, suggesting a germine diacetate structure for the former and a germine monoacetate structure for the latter. The observations that Unknown 1 was derived exclusively from GDA-3,16 and Unknown 2 was derived from GMA-16, coupled with the fact that both Unknowns 1 and 2 were converted to germine in alkaline solution, lend further support to a GDA structure for Unknown 1 and a GMA structure for Unknown 2. Experiments are currently in progress to isolate and characterize both Unknowns 1 and 2.

Proposed Kinetic Model for GDA-3,16 Degradation—The observed aqueous stability profiles (Figs. 1 and 2) suggest the following model (Scheme I) for the degradation of GDA-3,16 in water.



Scheme I

The rate constants in the model reflect the following chemical changes:

$$\frac{\text{hydrolysis of } C_3\text{-acetoxy}}{k_2,\ k_3,\ k_6} \qquad \frac{\text{hydrolysis of } C_{16}\text{-acetoxy}}{k_1,\ k_4}$$

$$\frac{\text{hydrolysis of } C_x\text{-acetoxy}}{k_7,\ k_8} \qquad \frac{\text{conversion of GDA-3,16 to GDA-3,}x}{k_5}$$

$$\frac{\text{conversion of GMA-16 to GMA-}x}{k_9}$$

The continual decrease in pH with time in the aqueous stability profile precluded any attempt to estimate rate constants at this time, since each rate constant described is very likely to be pH dependent (Table I).

Influence of pH on Stability of GDA-3,16—Table I summarizes the stability patterns obtained for GDA-3,16 in buffered aqueous solutions at room temperature; Fig. 3 shows the TLC chromatograms obtained for pH's 4.6, 7.2, and 9.5.

The overall stability of GDA-3,16 increases with decreasing pH and apparently reaches a maximum below pH 7, since the degradation occurs faster in both concentrated HCl and pH 7.2 than it does in 0.1 *N* HCl and pH 4.6. Not enough data were obtained in these studies to ascertain the pH of maximum stability.

Above pH 7, conversion to Unknowns 1 and 2 as well as hydrolysis occurred in solutions of GDA-3,16 (1 hr. stability data, Table I). Moreover, both types of reactions are evidently base catalyzed; in 0.1 *N* NaOH, GDA-3,16 degraded completely to germine within 1 hr. In 0.1 *N* HCl and concentrated HCl, GMA-16 was the initial degradation product obtained from GDA-3,16. This observation suggests that the conversion reaction is not acid catalyzed and, in addition, that the C_3-acetoxy hydrolysis (k_3) is faster than the C_{16}-acetoxy hydrolysis (k_1) in strongly acidic media (3). As time permits, the individual rate constants will be evaluated in more detail and the data reported.

Recommended Preparation of Extemporaneous GDA-3,16 Solutions—Germine-3,16-diacetate solutions, which are stable for several days at room temperature, can be conveniently prepared by dissolving the compound in water containing a quantity of organic or inorganic acid such that the final pH of the solution is between 4.5 and 6. A typical solution preparation is as follows. Dissolve 200 mg. of GDA-3,16 (0.34 mmole) in 100 ml. of water containing 27 mg. of anhydrous citric acid (0.12 mmole). This solution had a final pH of 5.8. Of the several acids tried in this laboratory (hydrochloric acid, phosphoric acid, citric acid, and lactic acid), citric acid was found to be most convenient. It can be weighed out as needed and, because of its overlapping ionization constants in the desired pH range, will not result in drastic pH changes for small variations in the ratio of GDA-3,16 to citric acid.

Table II—GDA-3,16 Stability *versus* Solvent Composition

Solvent	Storage Time at Room Temperature	Observations
Chloroform	1 month	Stable[a]
Methylene chloride	1 month	Stable[a]
Acetone	1 day	Stable[a]
Acetonitrile	1 day	Stable[a]
Pyridine	1 day	Stable[a]
Alcohol USP	4 days	Unstable[b]
Alcohol USP–water, 1:1 by volume	<3 hr.	Unstable[b]
Methanol	3 hr.	Stable[a]
Methanol–water, 1:1 by volume	3 hr.	Unstable[b]

[a] No extraneous spots detected by TLC. [b] TLC pattern similar to that observed in aqueous stability profile. The instability of GDA-3,16 in alcohol is particularly important since alcohol might be used by some workers to prepare GDA-3,16 stock solutions for subsequent dilution with water prior to use.

Stability of GDA-3,16 *versus* Solvent Composition—Some data accumulated to date on short-term stability of GDA-3,16 in some solvents are given in Table II. These observations were obtained during the course of experiments to devise efficient reaction media for the conversion of GDA-3,16 to either Unknown 1 or GMA-16.

SUMMARY

GDA-3,16 degrades rapidly in water at its natural pH (about 9.5). In addition to the expected ester hydrolysis to germine monoacetate and germine, rapid conversion of GDA-3,16 to another as yet unidentified GDA occurred. Degradation of GDA-3,16 solution was accompanied by a drop in pH, reflecting the ester hydrolysis. A kinetic model describing these changes was proposed. TLC was used to monitor the chemical changes of GDA-3,16.

Overall stability of GDA-3,16 increases with decreasing pH ($t_{1/2}$ <1 hr. at pH 9.5, $t_{90\%}$ ~ 1 hr. at pH 7.2) and reaches a maximum near pH 4.6 (no instability detected after 24 hr.). Directions are given for preparing an aqueous GDA-3,16 solution stable for several days at room temperature.

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Interaction of Bishydroxycoumarin with Human Serum Albumin

M. J. CHO*, A. G. MITCHELL, and M. PERNAROWSKI†

Abstract □ The binding of bishydroxycoumarin to human serum albumin was studied by means of spectrophotometry, solubility analysis, and equilibrium dialysis. The data were interpreted on the basis of the theory of multiple equilibria. The human serum albumin–bishydroxycoumarin association is exothermic and occurs spontaneously under the experimental conditions. The α,β-unsaturated lactone structure in bishydroxycoumarin is involved in the complexation. A heterogeneity of binding sites on human serum albumin was observed. The average number of the first-type binding sites is approximately three. The corresponding intrinsic association constant is equal to 1.7×10^5 l./mole at 40° and 3.5×10^5 l./mole at 20°. The binding sites are believed to be hydrophobic regions, probably located in the interior of the human serum albumin molecule whose van der Waals' contour approximates and thus, in a sense is selective for, the bishydroxycoumarin molecule. The selectivity of the binding sites was supported by the large contribution of $\Delta H°$ to $\Delta G°$. The main binding energy is derived from nonionic sources.

Keyphrases □ Bishydroxycoumarin–human serum albumin—interaction □ Serum albumin, human—bishydroxycoumarin binding □ Solubility analysis—human serum albumin □ Equilibrium dialysis—analysis □ UV spectrophotometry—analysis

The binding of bishydroxycoumarin to human serum albumin was first reported about 20 years ago (1). However, the mechanism of interaction has not been extensively studied. The present work was designed to evaluate the mechanism.

EXPERIMENTAL

Materials—Bishydroxycoumarin USP[1] and human serum albumin Fraction V[2] were used in this study. Tris(hydroxymethyl)aminomethane was obtained commercially. Cellophane dialysis tubing[3] was cut to the size of the dialysis cell, washed thoroughly with distilled water, and stored in the refrigerator.

Buffer System—The effect of pH and ionic strength on the apparent solubility of bishydroxycoumarin was investigated (2). Solubility increased rapidly with increase in pH from 7.6 to 8.0 at a constant ionic strength of 0.2. This is the pH range where bishydroxycoumarin starts to undergo the second ionization. It is believed that completely ionized bishydroxycoumarin has a strong ion–dipole interaction with water molecules, which results in a marked increase in solubility.

The solubility also increased as the ionic strength, provided by chloride ion, was increased at a constant pH of 7.2. Frank and Wen (3) claim that halide ions, except fluoride, are "breakers" of water structure. The number of "unbound" water molecules is thus greater in the presence of chloride ion than in the ordinary liquid water, and this will provide more cavities for the solution of hydrocarbon (4, 5).

In the biologically significant pH range, there is little choice of buffer system. Phosphate buffer interferes with the protein binding of small molecules (6). Tris(hydroxymethyl)aminomethane has intramolecular hydrogen bonds (7) and, hence, is expected to be relatively inert. Chloride ion may interfere with the human serum albumin–bishydroxycoumarin interaction. However, its competitive

[1] Abbott Laboratories, Montreal, Quebec, Canada.
[2] Pentex Inc., Kankakee, Ill.
[3] Fisher Scientific Co., No. 8-667-2, 4.41-cm. (1.735-in.) flat width.