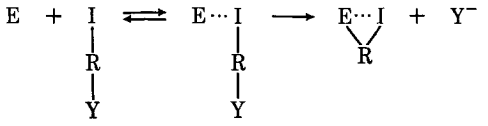
Enzyme Inhibitors XXI

Hydrophobic and Hydroxylic Binding Sites of Adenosine Deaminase

By HOWARD J. SCHAEFFER and CHARLES F. SCHWENDER

Recent studies have shown that the *ortho* and *para*-isomers of 9-(bromoacetamidobenzyl)adenine are good irreversible inhibitors of adenosine deaminase. In an attempt to prepare other types of irreversible inhibitors of this enzyme, a series of 9-(ω-bromoacetoxyalkyl)adenines were synthesized. However, none of these compounds were active as irreversible inhibitors although they were reversible inhibitors of this enzyme. Thus, it appears that the alkyl chain of the 9-(ω-bromoacetoxyalkyl)adenines does not complex with the enzyme in a manner identical to the 9-substituent of 9-(*p*- or *o*-bromoacetamidobenzyl)adenine. When the 9-(ω-hydroxyalkyl)adenines were evaluated as reversible inhibitors of adenosine deaminase, it was found that as the alkyl chain was lengthened, the compounds became initially more potent inhibitors, then less potent, and finally more potent again as the alkyl chain was lengthened to octyl. These results can be rationalized by assuming two different modes of binding. The short chain compounds bind to a hydroxyl binding site whereas the long chain compounds bind to a hydrophobic region which based on other data appears to extend approximately seven or eight carbons from the 9-position of adenine.

One of the prime requirements of an active-site-directed irreversible inhibitor is that the enzyme and inhibitor (1) form a reversible complex prior to the reaction in which the irreversible inactivation occurs (see Scheme I).

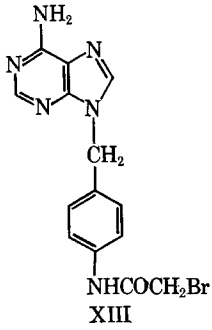


Scheme I

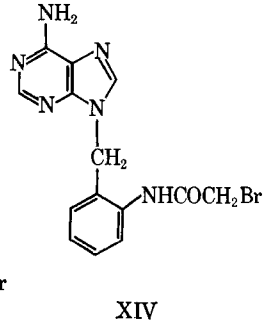
Thus, knowledge concerning types of binding sites on the enzyme required for reversible complexation are essential for the design of active-site-directed irreversible inhibitors. It has previously been shown that adenosine deaminase has both a polar and a nonpolar area that are important in binding certain substituents of a 9-substituted adenine (2). A successful attempt to utilize the hydrophobic region of adenosine deaminase for the preparation of active-site-directed irreversible inhibitors resulted in the synthesis of some 9-benzyl- and 9-phenethyladenines in which the benzene ring carried an alkylating agent (3–6). In order to determine if certain 9-alkyladenines bearing an alkylating group on the alkyl substituent could irreversibly inhibit adenosine deaminase, the authors decided to prepare some 9-(ω-bromoacetoxyalkyl)adenines where the alkyl group varies from ethyl to octyl. The present paper describes the synthesis and enzymic evaluation of these compounds as well as a study of the hydrophobic and hydroxyl binding sites of adenosine deaminase with some 9-(ω-hydroxyalkyl)adenines.

DISCUSSION

An examination of Table I reveals that the compounds prepared for this study were capable of causing reversible inhibition of adenosine deaminase. However, when the potential irreversible inhibitors (VII–XI) were incubated with the enzyme for periods of 5 hr. at 37°, no irreversible inactivation was observed. The incubations were performed with concentrations of inhibitors such that the amount of the reversible E· · ·I complex was at least 0.5 E_t. Under similar conditions, 9-(*p*-bromoacetamidobenzyl)adenine caused extensive irreversible inactivation of adenosine deaminase (3, 4). Comparison of the rates of reaction of VII–XI with iodoacetamide using 4-(*p*-nitrobenzyl)pyridine as the nucleophilic reagent revealed that VII–XI were approximately 4–5 times more reactive than iodoacetamide. Thus, VII–XI exhibit approximately the same rates of reaction as do 9-(*p*- and *o*-bromoacetamidobenzyl)adenines (XIII and XIV) with 4-(*p*-nitrobenzyl)pyridine (3–5).



XIII



XIV

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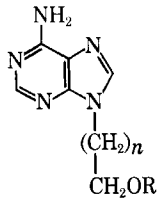
TABLE I—INHIBITION OF ADENOSINE DEAMINASE BY



Compd.[a]	n	mM Concn. for 50% Inhibition[b]	$(I/S)_{0.5}$[b]	Relative Reactivity
R = H				
I	1	0.070 ± 0.004[c]	1.1 ± 0.05[c,d]	...
II	2	0.046 ± 0.005	0.70 ± 0.08[d]	...
III	3	0.128 ± 0.009	1.94 ± 0.14[d]	...
IV	4	0.200 ± 0.007	3.0 ± 0.12[d]	...
V	5	0.112 ± 0.010	1.7 ± 0.14	...
VI	7	0.042 ± 0.001	0.64 ± 0.02	...
R = $-COCH_2Br$				
VII	1	0.122 ± 0.006	1.8 ± 0.09	4.0
VIII	2	0.106 ± 0.002	1.6 ± 0.04	4.2
IX	3	0.060 ± 0.004	0.90 ± 0.05	4.1
X	5	0.046 ± 0.002	0.71 ± 0.04	4.8
XI	7	0.026 ± 0.002	0.39 ± 0.02	4.8

[a] None of these compounds served as substrates of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 mM. In no experiment of reversible inhibition did the concentration of inhibitor exceed 0.12 mM. In those cases where a higher concentration is shown for 50% inhibition, the value was obtained by extrapolation of a plot of V_0/V *versus* (I). [c] Average deviation. [d] Data taken from *Reference 2*. [e] Ratio of the rates of reaction of the various 9-(ω-bromoacetoxyalkyl)adenines to iodoacetamide with 4-(*p*-nitrobenzyl)pyridine.

The fact that VII–XI form reversible complexes with adenosine deaminase and possess good alkylating activity but nevertheless are not irreversible inhibitors of the enzyme is evidence that the 9-substituent of these compounds does not complex with the enzyme in a manner identical to the 9-substituent of XIII and XIV. In addition, it was found that 9-(2-bromoacetoxypropyl)adenine was a poor reversible inhibitor and did not cause irreversible inactivation of adenosine deaminase. Moreover, when the stability of the bromoesters to phosphate buffer was evaluated, it was found that less than 10% of the alkylating ability of these compounds was lost during a 2-hr. incubation at 37°. The lack of irreversible inactivation of adenosine deaminase by VII–XI substantiates the selectivity that one may obtain with active-site-directed irreversible inhibitors (1). An examination of molecular models reveals that VII–XI may assume several conformations identical with XIII or XIV. Even though all of these compounds form reversible complexes with the enzyme and have nearly equal alkylating activity, only 9-(*o*- and *p*-bromoacetamidobenzyl)adenines and 9-(*m*-bromoacetamidophenethyl)adenine are good irreversible inhibitors of adenosine deaminase.

An interesting phenomenon was observed when a comparison was made of the reversible inhibition of adenosine deaminase by some 9-(ω-hydroxyalkyl)adenines and some 9-alkyladenines (see Fig. 1). As the alkyl chain of the 9-alkyladenines is lengthened, the ability of the inhibitor to form a complex with the enzyme is increased. It was on these data that we previously concluded that the hydrophobic area of adenosine deaminase extends from approximately three to seven carbon atoms (2). The authors have recently prepared 9-nonyl- and 9-decyladenines which unfortunately were not

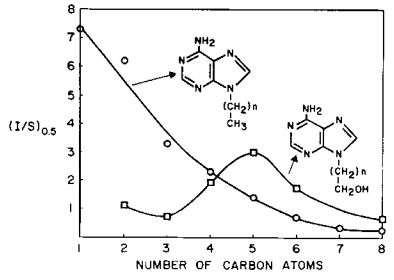


Fig. 1—A plot of the index of inhibition vs. *the number of carbons in the 9-substituent of some 9-(alkyl)- and 9-(hydroxyalkyl)adenines.*

sufficiently water-soluble to allow an accurate measurement of their inhibition index. Nevertheless, when these compounds were evaluated as inhibitors of adenosine deaminase in phosphate buffer containing 40% dimethylsulfoxide, they were weaker inhibitors than was 9-octyladenine evaluated under the same conditions. From these data, it would appear that the hydrophobic area on adenosine deaminase does terminate at a length of approximately seven or eight carbon atoms. However, an examination of a plot of $(I/S)_{0.5}$ *versus* chain length for a number of 9-(ω-hydroxyalkyl)adenines reveals that as the alkyl group is lengthened, the inhibitors become more potent, then less potent, and finally more potent again. The authors believe that these data conclusively demonstrate that two types of forces are involved in the formation of a complex with the 9-substituent of 9-(ω-hydroxyalkyl)adenines. When the 9-position of adenine bears either a 2-hydroxyethyl or a 3-hydroxypropyl group, both

the hydroxyl site and the hydrophobic region on the enzyme are making a contribution to binding. It has previously been shown that the hydroxyl site and the large hydrophobic site are not related to each other in an unbranched manner; *i.e.*, a straight carbon chain compound, such as 2-hydroxyheptyl, cannot bridge to both sites (7). This observation is further supported by the study of the compound with a 4-hydroxybutyl group at the 9-position of adenine. In this case, the 9-substituent cannot bind to both areas on the enzyme at the same time. When the butyl group binds to the hydrophobic region of the enzyme, there is some repulsion caused by proximity of the terminal hydroxyl group to the nonpolar area. However, by extensive folding of the butyl chain which is energetically unfavorable, it is possible for the alcohol function to bind to its hydroxyl binding site. The repulsive factor on the one hand is almost equally balanced by the second binding site so that the inhibition caused by III is almost equal to that caused by 9-butyladenine. The substituent of 5-hydroxypentyl represents a case where the hydroxyl group apparently does not make a contribution to binding. In fact, its major effect is a repulsion from the hydrophobic region. Finally, in the case of the 6-hydroxyhexyl and especially in the case of 8-hydroxyoctyl, the hydroxy group has essentially lost its repulsive effect. This occurs because the hydrophobic region has a length of only seven or eight carbon atoms. Thus, when the octyl group of 9-(8-hydroxyoctyl)adenine is complexed with the enzyme, the hydroxyl group extends beyond the hydrophobic region and is no longer repelled. Consequently, 9-octyladenine and 9-(8-hydroxyoctyl)-adenine approach each other in their inhibitory properties. Finally, a plot of $(I/S)_{0.5}$ *versus* the number of carbons in the alkyl chain of the 9-(ω-bromoacetoxyalkyl)adenines reveals a reasonably smooth curve with the inhibition becoming greater as the chain is lengthened. We interpret these data to mean that there is only one type of interaction important in binding the 9-substituent of the 9-(ω-bromoacetoxyalkyl)adenines and that this type of interaction is mainly hydrophobic.

CHEMISTRY

The synthesis of 9-(6-hydroxyhexyl)adenine was accomplished by a modification of a procedure (8) in which adenine was alkylated with 6-chloro-1-hexanol in dimethylacetamide in the presence of potassium carbonate. 9-(8-Hydroxyoctyl)adenine was synthesized by condensing 8-hydroxy-1-octanol with 5-amino-4,6-dichloropyrimidine. The resultant substituted pyrimidine was cyclized with trimethyl orthoformate to the desired 6-chloro-9-(8-hydroxyoctyl)purine which on treatment with methanolic ammonia gave 9-(8-hydroxyoctyl)adenine.

Several procedures were investigated to prepare the 9-(ω-bromoacetoxyalkyl)adenines using bromoacetyl bromide and bromoacetic anhydride, but the yields were consistently low. Finally, it was found that the bromoacetoxy derivatives could best be prepared by simple esterification of the hydroxyl group with bromoacetic acid catalyzed by ethanesulfonic acid. In this way 9-(ω-bromoacetoxyalkyl)adenines were prepared where the alkyl group was ethyl, propyl, butyl, hexyl, and octyl. In addition 9-(2-bromoacetoxypropyl)adenine was prepared by this general procedure.

EXPERIMENTAL[1]

9-(6-Hydroxyhexyl)adenine (V)—A mixture of 9.92 Gm. (58.0 mmoles) of adenine dihydrate, 15.8 Gm. (116 mmoles) of 6-chloro-1-hexanol, and 8.01 Gm. (58.0 mmoles) of anhydrous potassium carbonate in 40 ml. of dimethylacetamide was heated at 125° for 43 hr. under an atmosphere of nitrogen. The reaction mixture was filtered, and the filtrate was evaporated *in vacuo* to a residual solid, 6.01 Gm., which was placed on a column (35 × 900 mm.) of 450 Gm. of neutral alumina and eluted with a methanol–chloroform mixture collected in 100-ml. fractions. The desired material was obtained from fractions 51–66 (4% methanol in chloroform). Removal of the volatile material *in vacuo* gave a crude solid, 3.64 Gm. (37.4%), m.p. 184–187°, which was shown by TLC on silica gel to be one component, R_f 0.67 (20% methanol in chloroform).

8-Amino-1-octanol—To a stirred suspension of 19.9 Gm. (125 mmoles) of 8-aminooctanoic acid in 250 ml. of absolute ethanol at 0°, was slowly added 16.7 Gm. (140 mmoles) of thionyl chloride. The mixture was then refluxed for 8 hr. The reaction mixture was evaporated *in vacuo* and gave crude solid ethyl 8-aminooctanoate hydrochloride in a quantitative yield. The crude material was dissolved in 150 ml. of chloroform and washed with cold 5% sodium hydroxide solution (3 × 10 ml.). After the chloroform solution had been dried with anhydrous magnesium sulfate, the chloroform was evaporated *in vacuo* and gave the crude ethyl 8-aminooctanoate, 16.5 Gm. (71.2%). To a suspension of 10.6 Gm. (280 mmoles) of lithium aluminum hydride in 1 L. of anhydrous ether was added 16.5 Gm. of crude ethyl 8-aminooctanoate in 1 L. of ether. The reaction mixture was refluxed for 6 hr. and then water (45 ml.) was added to the cooled mixture. The resulting suspension was filtered, and the residual solid was washed with chloroform (500 ml.). The filtrates were combined and evaporated *in vacuo;* yield, 13.4 Gm. of crude 8-amino-1-octanol, m.p. 54–57°. Recrystallization of the material from benzene–hexane gave the pure white product; yield, 11.6 Gm. (63.8%), m.p. 54–59°. ν in cm.$^{-1}$ (KBr): 3450 (OH); 3300, 1550 (NH).

Anal.[2]—Calcd. for $C_8H_{19}NO$: C, 66.17; H, 13.19; N, 9.64. Found: C, 66.26; H, 13.11; N, 9.87.

This compound has previously been prepared by a different procedure and a m.p. 45° was reported (9).

5-Amino-4-chloro-6-(8-hydroxyoctylamino)pyrimidine—A mixture of 692 mg. (4.23 mmoles) of 5-amino-4,6-dichloropyrimidine, 644 mg. (4.44 mmoles) of 8-amino-1-octanol, and 504 mg. (5.00 mmoles) of triethylamine in 35 ml. of 1-butanol was heated at reflux for 23.5 hr. under nitrogen. The mixture was evaporated *in vacuo* and gave a residual syrup which was crystallized from a mixture of ethanol and water; yield, 940 mg. (81.0%), m.p. 80–85°. Recrystallization of the material from

[1] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer model 202 spectrophotometer; the enzyme studies were done on a Gilford instrument model 2000 spectrophotometer. The melting points, unless otherwise noted, were taken in open capillary tubes on a Mel-Temp apparatus and are corrected.

[2] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

TABLE II—PHYSICAL CONSTANTS AND ANALYTICAL DATA FOR SOME PURINES AND THEIR INTERMEDIATES

Compd.	Recrystn. Solvent[a]	M.p., °C.	Yield, %	Formula	C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found	Hal. % Calcd.	Hal. % Found	Cosolvent	Ultraviolet $\lambda_{max.}$ ($\epsilon \times 10^{-4}$) 0.1 *N* HCl	H_2O	0.1 *N* NaOH
V	*A*	190–192	37.4	$C_{11}H_{17}N_5O$	56.10	56.01	7.28	7.48	29.77	29.60			10% EtOH	261 (1.24)	263 (1.27)	262 (1.24)
VI	*B*	207–210	59.2	$C_{13}H_{22}ClN_5O$	52.08	51.81	7.37	7.42	23.36	23.29	11.83[b]	11.66	5% EtOH	260 (1.48)	262 (1.42)	262 (1.42)
VII	*C*	190–192 dec.	18.7	$C_9H_{10}BrN_5O_2$	36.01	35.82	3.34	3.40	...	...	26.63[c]	26.54		261 (1.28)	263 (1.28)	263 (1.26)
VIII	*C*	182–184 dec.	56.5	$C_{10}H_{12}BrN_5O_2$	38.22	38.54	3.86	3.96	...	...	25.43[c]	25.29	10% EtOH	262 (1.22)	264 (1.30)	263 (1.50)
IX	*D*	139 dec.	31.0	$C_{11}H_{14}BrN_5O_2$	40.25	40.40	4.30	4.35	...	...	24.35[c]	24.60	7.5% EtOH	262 (1.28)	264 (1.35)	263 (1.48)
X	*C*	173–178 dec.	32.7	$C_{13}H_{18}BrN_5O_2$	43.86	43.62	5.08	5.09	19.66	19.90	22.44[c]	22.50	1% EtOH	262 (1.31)	263 (1.37)	263 (1.31)
XI	*E*	152–155 dec.	48.2	$C_{17}H_{24}BrN_5O_6$[d]	43.01	43.00	5.10	5.14	14.76	14.71	16.85[c]	16.90	12% EtOH	262 (1.42)	262 (1.45)	262 (1.39)
XII[e]	*C*	203 dec.	68.9	$C_{10}H_{12}BrN_5O_2$	38.22	38.42	3.81	3.99	22.29	22.53	25.47[c]	25.62	8% EtOH	258 (1.24)	261 (1.18)	261 (1.23)

A, ethanol; *B*, methanol–ether; *C*, acetone–hexane; *D*, ethyl acetate–hexane; *E*, 1% oxalic acid in methanol. [b] Chlorine analyzed. [c] Bromine analyzed. [d] Isolated as oxalate salt. [e] Compound XII is 9-(2-bromoacetoxypropyl)adenine.

ethanol–water gave the analytical product; yield, 855 mg. (73.7%), m.p. 84–87°. ν in cm.$^{-1}$ (KBr): 3400 (OH); 3300 (shoulder), 1640 (NH); 1580 (C=C, C=N). $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 12% EtOH/0.1 *N* HCl, 305 (1.20); 12% EtOH/H_2O, 265 (1.13), and 287 (1.08); 12% EtOH/0.1 *N* NaOH, 265 (0.982) and 295 (1.00).

Anal.—Calcd. for $C_{12}H_{21}ClN_4O$: C, 52.83; H, 7.75; Cl, 13.00; N, 20.54. Found: C, 52.42; H, 8.00; Cl, 12.72; N, 20.59.

9-(8-Hydroxyoctyl)adenine Hydrochloride (VI)—A mixture of 5.34 Gm. (19.6 mmoles) of 5-amino-4-chloro-6-(8-hydroxyoctylamino)pyrimidine, 268 mg. (2.43 mmoles) of ethanesulfonic acid, and 25 ml. of trimethyl orthoformate was stirred at room temperature for 23.5 hr. The reaction mixture was evaporated *in vacuo* to give a syrup which would not crystallize. The crude 6-chloro-9-(8-hydroxyoctyl)purine (17.6 mmoles) was heated at 80° with 60 ml. of methanolic ammonia solution in a steel bomb for 66 hr. The reaction mixture was cooled and a solid which precipitated was collected by filtration; yield, 3.68 Gm. (79.7%), m.p. 186–192°. For purification the product was converted into the hydrochloride salt which was recrystallized from methanol–ether.

9-(ω-Bromoacetoxyalkyl)adenines—These materials were prepared by the same general procedure. Therefore, only one procedure will be presented. The physical constants and analytical data for these compounds are given in Table II.

9-(6-Bromoacetoxyhexyl)adenine (X)—A mixture of 258 mg. (1.10 mmoles) of 9-(6-hydroxyhexyl)-adenine, 3.06 Gm. (22.0 mmoles) of bromoacetic acid, and 138 mg. (1.25 mmoles) of ethanesulfonic acid in 20 ml. of chloroform was heated at reflux for 23 hr. The chloroform volume was increased to 40 ml. and then washed with a saturated sodium bicarbonate solution (8 × 1 ml.). The chloroform phase was dried with anhydrous magnesium sulfate and evaporated *in vacuo* to a residual solid. The crude material after recrystallization from acetone–hexane gave 322 mg. (82.3%) of crude X, m.p. 174–177° dec. Further recrystallizations from acetone–hexane gave the analytical product; yield, 128 mg (32.7%), m.p. 173–178° dec.

CHEMICAL REACTIVITY OF THE ALKYLATING AGENTS

The procedure has previously been described (5) and is a modification of a literature procedure (10–12).

ENZYME ASSAY AND REAGENTS

Adenosine deaminase (type I, calf intestinal mucosa) was purchased from the Sigma Chemical Co. The assay procedure for reversible inhibitors is a modification of the method developed by Kaplan (13).

The method used to study the irreversible inactivation of adenosine deaminase at 37° has been described (5) and is a modification of the general procedure of Baker (14).

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Modified Hofmann Degradation for the Analysis of *n*-Alkylbenzyldimethylammonium Chlorides by Gas Chromatography I

C_{14}- to C_{18}-Alkyl Compounds

By E. C. JENNINGS, JR., and H. MITCHNER*

The Hofmann degradation of quaternary ammonium compounds has been studied for *n*-alkylbenzyldimethylammonium chlorides. Gas chromatography of the reaction products provides resolution of the benzyldimethylamine and the corresponding alkene. Quantitative analysis of quaternary content and qualitative evaluation of impurities have been obtained for a number of commercial products.

OF THE METHODS for splitting of C—N bonds in amines, such as the von Braun with cyanogen bromide (1), the Hofmann degradation by pyrolysis of quaternary ammonium hydroxides (1), the Emde reduction of quaternary ammonium salts with sodium amalgam (2), and catalytic hydrogenation (2, 3), only the Hofmann and catalytic hydrogenation methods lend themselves readily to the analysis of *n*-alkylbenzyldimethylammonium chlorides. Warrington (3) has determined the alkyl distribution in benzalkonium chloride by the gas chromatographic analysis of the amines produced by catalytic hydrogenation. The determination of impurities was not feasible by this method, and the quantitative assay of the benzalkonium chloride was not afforded. Metcalfe (4) attempted the analysis of long chain quaternary ammonium compounds by a Hofmann degradation with direct injection on an alkaline treated gas chromatographic column. The reaction did not follow the expected course, and the results were not sufficiently precise for quantitative assay. Changing of the column with use also altered the alkyl distributions.

The Hofmann degradation for the analysis of *n*-alkyltrimethylammonium halides (5), and the catalytic hydrogenation of *n*-alkylbenzyldimethylammonium chloride (6, 7) have been reported in connection with gas chromatography for the determination of the long chain alkyl group. It has been suggested that in the Hofmann degradation (5) a disproportion reaction results, giving rise to both alkenes-1 and the corresponding alkanols, although this was not established by quantitative data.

Catalytic hydrogenation requires special equipment and does not afford a ready means of quantitatively determining volatile impurities. The Hofmann reaction suggested a simpler analytical approach. This work was directed toward determination of optimum reaction conditions with subsequent gas chromatography analysis of the reaction products. Assay of volatile impurities prior to Hofmann degradation combined with analysis of the products of the reaction provided a means of complete assay of commercial C_{14} to C_{18} alkylbenzyldimethylammonium chlorides.

APPARATUS AND REAGENTS

An F&M 810R-12 gas chromatograph equipped with a linear temperature programmer and dual hydrogen flame ionization detectors was employed with dual columns, 5 ft. × 1/8 in. (0.101 in. i.d.),

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* Present address: Quality Control Division, Syntex Laboratories, Inc., Palo Alto, CA 94304