Table I shows the precision of this assay method. The indicated 1Σ standard deviation calculated from these data is ± 0.025. This amounts to about $\pm 3\%$ of the amount present.

Table II shows a comparison between the U.S.P. chemical assay and the X-ray absorption edge method. The X-ray values are all single assays, the chemical assays are the final resul. reported by the Lilly control division and are averages of several assays.

CONCLUSION

The X-ray absorption edge assay is a useful method. In the case of the iodine control of thyroid extract the values obtained by X-ray are comparable to those obtained by the chemical method.

The X-ray method requires, in our hands, about 1 hour. This allows about 0.5 hour for measurements and 0.5 hour for preparation and calculations.

It is important to realize that the method may be applied to other elements. Using the relatively simple apparatus described here, elements having an absorption edge in the region from about 0.3 Å. to about 2.0 Å., that is, from cerium to manganese in atomic number can be determined. With a more complicated apparatus, the list of elements can be expanded.

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Kinetics and Mechanism of Isomerization and Hydrolysis of 4,6-Diamino-1-(3,5)-dichlorophenyl-1,2-dihydro-2,2-dimethyl-1,3,5-triazine in Dilute Aqueous Solution

By D. H. SZULCZEWSKI, C. M. SHEARER, and A. J. AGUIAR

Rate measurements, concurrent with reaction product identification, indicate that there are three routes by which inactivation of the title compound can occur: (*a*) unimolecular isomerization of free triazine; (*b*) unimolecular isomerization of protonated triazine; (*c*) acid catalyzed hydrolysis of protonated triazine. The relative importance of these reactions on drug decomposition for various pH values is determined. Likewise, rate studies at critical pH values and several elevated temperatures enable accurate estimation of rate of degradation at any temperature. Equations are derived relating specific rate to both pH and temperature. Identification of reaction products by means of thin-layer chromatography shows that besides the 6-dichloroanilino isomer and 3,5-dichlorophenyl-biguanide hydrolysis product, *N*-3,5-dichlorophenyl-*N'*-guanylurea is formed as a secondary product of degradation.

I N RECENT YEARS a variety of substituted dihydrotriazines has been reported in several areas of chemotherapeutic research (1–3). Although many dihydrotriazines have been prepared and screened, there is little information available concerning their stability in aqueous systems. Likewise, information concerning the likely route and rate of degradation is meager.

In a communication by Carrington *et al.* (3) concerned with isolation, identification, and synthesis of the active metabolite of chlorguanide hydrochloride,[1] isomerization and hydrolysis of 1-phenyl-dihydrotriazines to nonactive products was noted. This suggests that loss of chemotherapeutic activity is possible and that relative

rates of destruction need to be known to determine the feasibility of liquid formulations having a suitable shelf life.

The following study, concerned with the kinetics of isomerization and hydrolysis of 4,6-diamino - 1 - (3,5) - dichlorophenyl - 1,2 - dihydro-2,2 - dimethyl - 1,3,5 - triazine[2](I) in aqueous buffered solution, was carried out to determine these rates and to determine the chemistry of the degradative process.

RESULTS AND DISCUSSION

Order and Nature of Degradative Process.— Representative data obtained on the rate of degradation of I in aqueous buffered solution at 45° is shown in Fig. 1. This, together with studies at other temperatures, indicates that in aqueous buffered solution the decomposition of I is first order with respect to I over a broad pH range.

The rate of degradation was not influenced by changes in concentration of buffer (Table I). Likewise, the observed rate remained constant in

[2] Parke, Davis ABT-15, 251.

extremely alkaline solutions despite a tenfold change in base concentration (NaOH) from 0.05 to 0.5 N. However, neutral salt did cause changes in the rate of reaction when carried out in the presence of strong acid (Fig. 2).

The nature of the product or products of the degradative reaction at various pH's was determined by thin-layer chromatography. This was necessary since the ultraviolet absorption spectra (Fig. 3) of both primary and secondary reaction products were too similar to permit spectrum analysis in mixtures. Chromatograms obtained on samples drawn from solutions held at 45° (Fig. 4) indicated that the isomer, 4-amino-6-(3,5)dichloroanilino-1,2-dihydro-2,2-dimethyl-1,3,5-triazine (II) is the only product of degradation over the broad pH range of 13 to 5. From pH 5 to 2 mixtures of 3,5-dichlorophenyl-biguanide (III) and isomer (II) are found, whereas in extremely acid systems (pH 2 or less) only the biguanidine or its hydrolysis product N-3,5-dichloro-phenyl-N'-guanyl urea (IV) are observed.
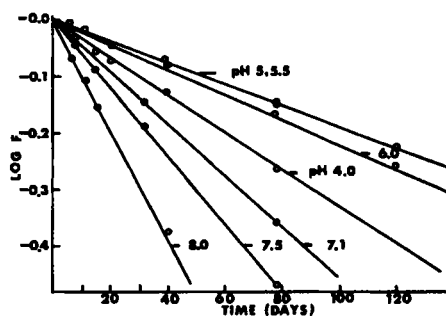

Fig. 1.—Degradation of I at 45° C. in aqueous solution at various pH's (F = fraction I remaining).

TABLE I.—EFFECT OF BUFFER CONCENTRATION ON OBSERVED RATE OF DEGRADATION AT 65° C.

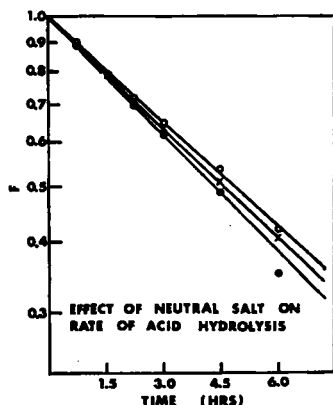| Buffer Molarity	0.1	0.2
pH 8.8 (borate)	$k_{obs} = 0.238$ hr.$^{-1}$	$k_{obs} = 0.236$ hr.$^{-1}$
pH 7.0 (phosphate)	$k_{obs} = 0.009$ hr.$^{-1}$	$k_{obs} = 0.009$ hr.$^{-1}$

Fig. 2.—Rate of acid catalyzed hydrolysis at various ionic strengths but constant pH. pH = 1.4 maintained with HClO$_4$. Ionic strength varied by changing concentration of NaClO$_4$.

Fig. 3.—Ultraviolet spectra in pH 7 phosphate buffer. Key: I, 4,6-di-amino - 1 - (3,5) - dichlorophenyl-1,2-dihydro - 2,2 - di-methyl - 1,3,5 - triazine; II, 4-amino-6 - (3,5) - dichloro-anilino-1,2-dihydro-2,2 - dimethyl-1,3,5-triazine; and III, 3,5-dichlorophenyl-biguanide.
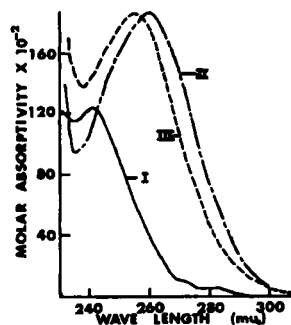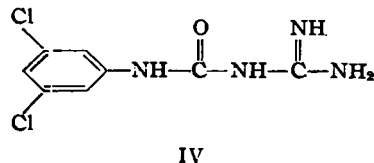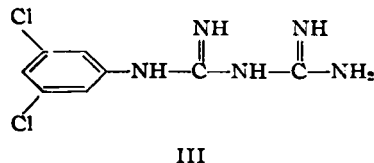

Fig. 4.—Thin-layer chromatograms obtained on solutions of partially degraded I at 45°. pH refers to pH of aqueous solution of I. Solution spotted directly after approximately two half-lives. Top boundary represents solvent front. Bottom boundary represents point of application.
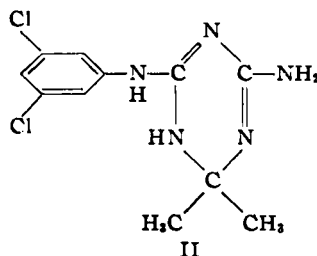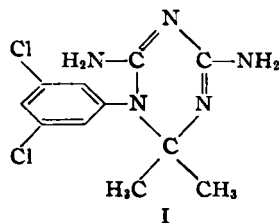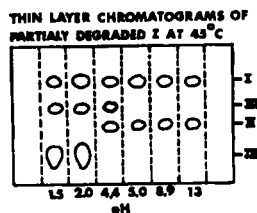
Variation of Reaction Rate with pH.—Variation of the observed first-order rate constant with pH is shown in Fig. 5 for two temperatures. In brief, the pH profile is characterized by the following regions (at 65°): *A*, pH 1 to 2.5 straight line, slope −1; *B*, pH 4 to 5.5 plateau region; *C*, pH 8 to 9.5

Fig. 5.—Plot of the logarithm of the observed specific rate *vs.* pH over the entire pH range. 65° solid line represents results of calculating observed specific rate by Eq. 13. Open circles represent experimentally determined values.

TABLE II.—k_1 VALUES CALCULATED AT 65° C. pKa' = 10.0

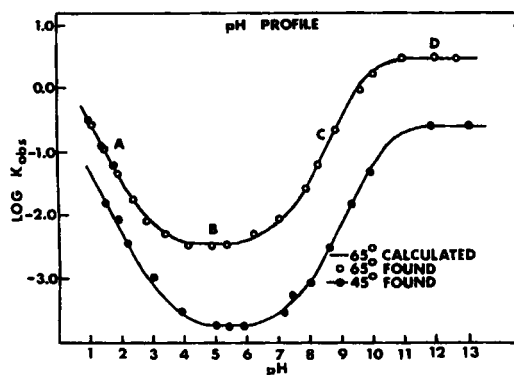| pH	log k_{obs}	log k_1
9.6	0.00	0.40
8.8	−0.62	0.58
8.2	−1.19	0.61
7.9	−1.55	0.45
	Av.	0.51

straight line, slope +1; *D*, pH 10.5 to 13 plateau region.

Region D.—The independence of rate on concentration of alkali suggests two possibilities for the reaction occurring in region *D*: *a*, unimolecular isomerization or *b*, base-catalyzed isomerization of monoprotonated I.

The rate law governing the first premise would simply be

$$-d \ln I/dt = k_1 \qquad \text{(Eq. 1)}$$

while that for the second

$$-d \ln I/dt = k_{OH^-} \cdot \{(H^+)/[(H^+) + Ka']\} \cdot (OH^-) \qquad \text{(Eq. 2)}$$

where Ka' = acid dissociation constant of IH^+, which under conditions where $(H^+) \ll Ka$ reduces to

$$-d \ln I/dt = k_{OH^-} \cdot K_w/Ka' = k^* \qquad \text{(Eq. 3)}$$

Both reactions would yield identical behavior with respect to the influence of pH on rate and would therefore be kinetically indistinguishable. However, the second possibility, involving attack of negatively charged hydroxyl ion on a protonated species bearing a positive charge, would be expected to show a primary salt effect (4). Since no salt effect is observed, Eq. 1 is favored.

The first possibility requires that pH affect the rate of degradation only by controlling the relative proportion of protonated and free I, not by fixing the concentration of a catalytic specie. For total *drug the* applicable rate law would be

$$-d \ln I_T/dt = k_1 \{Ka'/[(H^+) + Ka']\} \qquad \text{(Eq. 4)}$$

where I_T = total amount of *I* present regardless of ionic condition. From Eq. 4, in buffered solution

$$k_{obs} = k_1 \cdot Ka'/[(H^+) + Ka'] \qquad \text{(Eq. 5)}$$

where k_{obs} = observed first-order rate constant. At 65° where pKa' = 10.0, if pH ≥ 11.5, Eq. 4 simplifies to

$$k_{obs} = k_1 \qquad \text{(Eq. 6)}$$

or

$$\log k_{obs} = \log k_1;$$

and if pH ≤ 9.5, Eq. 4 may be expressed as

$$\log k_{obs} = \log k_1 - pKa' + pH \qquad \text{(Eq. 7)}$$

Equation 6 would predict that at pH's equal to or greater than 11.5, k_{obs} should be essentially independent of hydroxyl ion concentration. Equation 7 predicts a straight line with slope +1 at pH's less than 9.5. As seen from Fig. 5 (regions *C* and *D*) these conditions are realized.

Equations 6 and 7 provide a check for consistency since k_1 can be independently calculated from two different regions of the pH profile. From the plateau in the extremely alkaline region k_1 at 65° is, $k_1 = 3.14$ hr.$^{-1}$ or log $k_1 = 0.50$. Table II lists values of k_1, as calculated from Eq. 7, for the pH region 8 to 9.5 (region *C*, Fig. 5).

It is evident that the average value of k_1 calculated from Eq. 7 is in good agreement with the value of k_1 obtained by extrapolating values of k_1, determined directly at lower temperatures, to 65°.

Region B.—The plateau region, pH 4 to 6 at 65°, is apparently due to unimolecular isomerization of monoprotonated I or $IH^+ \xrightarrow{k_2} IIH^+$.

The rate law consistent with the data from pH 13 to 4 would become

$$-d \ln I_T/dt = k_1 \{Ka'/[Ka' + (H^+)]\} + k_2 \{(H^+)/[Ka' + (H^+)]\} \qquad \text{(Eq. 8)}$$

which reduces to

$$k_{obs} = k_2 \qquad \text{(Eq. 9)}$$

in the region of interest.

Region A.—Continuing to lower pH's (4 to 1) an increase in log k_{obs} is seen. The reaction responsible for this increase appears to be acid catalyzed hydrolysis of I to the biguanide $IH^+ + H_2O \xrightarrow[H^+]{k_3}$ $IIIH^+$ + acetone. If this were the case, the rate law applicable would be (pH 6 to 1) with water in great excess:

$$-d \ln I_T/dt = k_2 + k_3 (H^+) \qquad \text{(Eq. 10)}$$

so that $k_{obs} = k_2 + k_3 (H^+)$ and at sufficiently low pH so that $k_3 (H^+) \gg k_2$

$$\log k_{obs} = \log k_3 - pH \qquad \text{(Eq. 11)}$$

The above expression leads to the expectation of a region with a slope of −1 in the pH profile.

Fig. 6.—Log k_{obs} as a function of the square root of ionic strength at 65° C. at pH 1.85 and pH 1.40. pH maintained with HClO$_4$, ionic strength changed by varying concentration of NaClO$_4$.
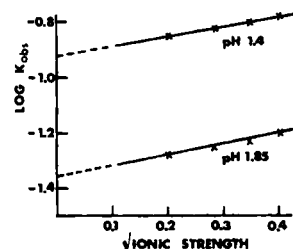
TABLE III.—CALCULATED VALUES AT TWO TEMPERATURES

Temp., °C.	Ka'	k_1 (hr. $^{-1}$)	k_2 (hr. $^{-1}$)	k_3 (hr. $^{-1}$)
65	1×10^{-10}	3.16	5×10^{-3}	3.24
45	3.16×10^{-11}	2.18×10^{-1}	1.9×10^{-4}	5×10^{-1}

TABLE IV.—HEATS OF ACTIVATION

pH or pH Range	Log Frequency Factor	ΔH_a (Kcal. mole^{-1})
12 or greater	17.84	26.7
10	20.14	30.7
8.7	23.62	37.5
8.2	23.83	38.5
7.0	21.06	35.5
4.4	17.70	31.0
1.4	13.79	22.6

In this acidic region the reaction is reversible (3). However, under the experimental conditions of low concentration and great excess of water, the concentration of IH$^+$ at equilibrium is extremely low so that the reaction may be considered to be irreversible. Plots of log F versus time over a period of at least one half-life showed no deviation from linearity.

The rate of acid catalyzed hydrolysis would be expected to show a positive primary salt effect since two positively charged ions would be involved in the formation of the activated complex (4). As seen in Fig. 6, this is observed experimentally. Since region A of the pH profile is the only region in which a salt effect is observed, further evidence for the reaction as written is obtained.

The over-all relationship of k_{obs} as a function of pH for the range 1 to 14 would then be

$$k_{obs} = k_1 \{Ka'/[Ka' + (H^+)]\} + \{(H^+)/[Ka' + (H^+)][k_2 + k_3(H^+)]\} \quad \text{(Eq. 12)}$$

Values of Ka', k_1, k_2, and k_3 for two temperatures are listed in Table III. In this equation, the effect of ionic strength on k_3 is ignored because although real, it is of minor importance compared to the large effect of pH on the over-all rate.

Substitution of the values from Table III in Eq. 12 gives a relationship which closely approximates the observed results over the whole pH range (Fig. 5).

Temperature Dependence.—Arrhenius plots at representative pH's were made and the information in Table IV obtained.

The assumption that the same reaction occurs over the pH range 14 to 6 or 7 requires that the heats of activation determined at various pH's over this range be the same. As is evident from Table IV, the observed activation energies at pH 8.2 and 8.7 are considerably higher than the values obtained for pH 12 or greater.

The equation for k_{obs} as a function of pH and pKa' in region C (straight-line portion, slope +1) is

shown by Eq. 7 while that in the alkaline plateau region D is shown by Eq. 6. Differentiating with respect to 1/T, holding pH constant,

$$\{\partial \log k_{obs}/[\partial/(1/T)]\}_{pH} = \{\partial \log k_1/[\partial/(1/T)]\}_{pH} + \{\partial pKa'/[\partial/(1/T)]\}_{pH} \quad \text{(Eq. 13)}$$

$$\{\partial \log k_{obs}/[\partial(1/T)]\}_{pH} = -\{\partial \log k_1/[\partial(1/T)]\}_{pH} \quad \text{(Eq. 14)}$$

Equations 13 and 14 indicate that the apparent heat, as determined in region C, should differ from that as determined in region D by the heat of ionization of IH$^+$, i.e.,

$$[\partial pKa'/\partial(1/T)] = \Delta H_i/2.3 \, R.T.$$

Attempts were made to determine the heat of ionization by an independent procedure. Unfortunately, conventional spectrophotometric determination of this quantity is very difficult. The instability of the drug at pH's near the pKa' (10 to 11) precludes accurate spectrophotometric measurement at elevated temperatures. Also, there are inherent changes in absorptivity with temperature, not due to changes in sample composition, which further complicate the determination. In spite of these difficulties, an approximate value of 7 Kcal. mole^{-1} was obtained spectrophotometrically.

Although not providing an independent measurement, a better value is obtained from rate data: rearranging Eq. 5 gives

$$k_{obs}(H^+)/k_1/[1 - (k_{obs}/k_1)] = Ka' \quad \text{(Eq. 15)}$$

which under pH conditions such that $k_{obs}/k_1 \ll 1$ gives

$$pKa' = pH - \log k_{obs}/k_1 \quad \text{(Eq. 16)}$$

This provides for a check of consistency, because if Eq. 16 is valid, it should give the known pKa' at room temperature from rate data obtained at elevated temperatures.

The values of k_1 and k_{obs} (pH 8 to 9) can be calculated at any temperature from the data in Table III: $k_1 = 17.84 - 26,700/2.3 \times 1.98 \times T$ at pH 8.2; $k_{obs} = 23.83 - 38,500/2.3 \times 1.98 \times T$ at pH 8.7; $k_{obs} = 23.62 - 37,500/2.3 \times 1.98 \times T$. Table V gives the values of pKa' obtained by calculation at various temperatures.

The value obtained at 25° is in good agreement with that obtained spectrophotometrically (11.0) at room temperature. This agreement would strongly suggest that the 11 Kcal. difference between the heats of activation as determined in region D

TABLE V.—CALCULATED pKa' VALUES

Temp., °C.	—log k_1 — log k_{obs}—		pKa' as Determined from Data— at pH		
	pH 8.2	pH 8.7	8.2	8.7	Av.
65	1.75	1.27	10.0	10.0	10.0
45	2.30	1.82	10.5	10.5	10.5
25	2.80	2.12	11.0	10.8	10.9

Fig. 7 —Absorb-
ancy ratio $(A\lambda_{240}/A\lambda_{265})$ as a func-
tion of fraction
drug remaining
Solvent: pH 7
phosphate buffer.
Key: —O—, mix-
tures of I and II;
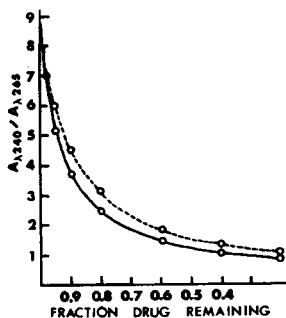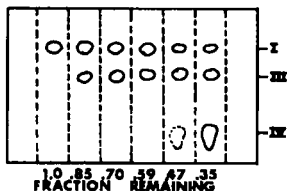---O---, mixtures of
I and III.

FRACTION DRUG REMAINING

Fig. 8.—Thin-
layer chromato-
grams obtained on
acidic (0.04 N
H_2SO_4) solutions of
I concurrent with
analysis.

FRACTION REMAINING

and C is, in fact, due to the heat of ionization of
IH^+ and that the true heat of activation is the same
in both regions.

EXPERIMENTAL

Kinetic Procedure.—A standard solution (approx.
0.001 M with respect to I) was prepared. This was
diluted tenfold into various buffers held at the
temperature of the run. Samples of the thermo-
stated solutions were withdrawn periodically and
diluted tenfold into pH 7 phosphate buffer for
spectrophotometric analysis of intact drug. The
pH was maintained by using dilute $HClO_4$ at pH's
below 2, glycine-HCl for pH 2 to 4, phosphate from
4 to 8, borate buffers in the region 8 to 10, carbonate
for 10 to 11, and dilute NaOH (carbonate free) for
pH's above 11. The actual pH of the buffered
solution was measured at the temperature of the
run with a Beckman model G pH meter. It did
not vary more than 0.1 pH units during the course
of a run.

Analysis.—The solution of partially decomposed
drug was diluted tenfold into pH 7 phosphate buffer.
These dilutions were scanned immediately using a
Cary model 14 spectrophotometer. The ratio of
absorbances at 240 mμ to 265 mμ was determined
and the fraction of I remaining was read off a
standard plot (Fig. 7). This procedure gives a
sensitive measure of intact drug in the region 0 to
40% loss which is quite adequate for rate studies on
pharmaceutical systems where the greatest emphasis
is on an accurate estimate of 10% loss.

The similarity in ultraviolet spectra of the
biguanide and its hydrolysis product makes a
separate analytical procedure unnecessary. In
addition, thin-layer chromatograms obtained con-
current with analysis (Fig. 8) demonstrated that
the rate of conversion of biguanide to substituted
urea was small compared to the rate of hydrolysis of
I. Thus no N-3,5-dichlorophenyl-N'-guanylurea
could be detected until approximately 50% of the
drug was hydrolyzed.

Thin-Layer Chromatographic Analysis.—Solutions
of I, 0.1% in various buffers were prepared and
stored at 45° for approximately two half-life periods.
Five microliters of these solutions was spotted

directly on chromatographic plates prepared with
aluminum oxide Fluka D5 containing a fluorescent
indicator.

The plates were developed using pH 6 phosphate
buffer until the solvent front had moved 6.5 cm.
When viewed under a short wave ultraviolet lamp,
the ultraviolet absorbing components appeared as
dark spots on the yellow fluorescing background.
The R_f values were not completely reproducible,
but the various spots were identified by spotting
knowns on the same plate.

**Characterization of Secondary Hydrolysis
Product.**—One gram of I was dissolved to 20 ml.
1 N HCl and heated on the steam bath for 1 day.
Likewise, 50 mg. of 3,5-dichlorophenylbiguanide was
dissolved in 0.5 ml. 1 N HCl and treated similarly.
In both cases, crystalline material was precipitated
whose infrared spectra were identical. The pre-
cipitates had an R_f equal to that of the slow moving
component observed during kinetic runs at pH's 3
or less. Physical data on the crystalline precipitate
from I are as follows: U.V.: (in pH 7 phosphate
buffer) λ 254 ϵ = 19,060; m.p. 189–191 (hot stage).

Anal.—Calcd. for $C_8H_8N_4OCl_2 \cdot HCl \cdot H_2O$: C,
32.0, H, 3.6; N, 18.6; Cl, 35.5; H_2O, 6.0. Found:
C, 32.1; H, 3.4; N, 18.4; Cl, 36.6; H_2O, 5.0.

N-3,5-Dichlorophenyl-N'-guanylurea was syn-
thesized by Henry's procedure (5) and found
identical with the above precipitates.

SUMMARY AND CONCLUSIONS

The kinetics of degradation of 4,6-diamino-1-
(3,5)-dichlorophenyl-1,2-dihydro-2,2-dimethyl-1,3,5-
triazine in dilute aqueous solutions have been in-
vestigated.

Variations of specific rate with pH gave a profile
consistent with the expression

$$k_{obs} = k_1\{Ka'/[Ka' + (H^+)]\}$$
$$+ \{(H^+)/[Ka' + (H^+)] [k_2 + k_3(H^+)]\}$$

derived on the basis of: (*a*) unimolecular isomeriza-
tion of both protonated and free drug, and (*b*) acid
catalyzed hydrolysis of protonated drug.

Variation of specific rate with temperature was
determined and found to comply with

pH	
11 or greater	log k = 17.84 − 26,700/2.3 *R.T.*
10	log k = 20.14 − 36,700/2.3 *R.T.*
8.7	log k = 23.62 − 37.500/2.3 *R.T.*
8.2	log k = 23.83 − 38,500/2.3 *R.T.*
7.0	log k = 21.06 − 35,500/2.3 *R.T.*
4.4	log k = 17.70 − 31,000/2.3 *R.T.*
1.4	log k = 13.79 − 22,600/2.3 *R.T.*

At 45° the isomer (4-amino-6-(3,5)-dichloro-
anilino-1,2-dihydro-2,2-dimethyl-1,3,5-triazine) was
found to be produced in the pH range 13 to 5.
Between pH's 5 and 3 both isomer and 3,5-dichloro-
phenyl-biguanide are products, while at more acid
pH's only biguanide or its hydrolysis product
N-3,5-dichlorophenyl-N'-guanylurea are observed.

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