# Colorimetric Methods for the Determination of Simazine and Related Chloro-*s*-triazines

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Four sensitive, rapid, and reliable methods for chloro-*s*-triazines based on their reactivity in the Zincke reaction and condensation of the unstable, yellow reaction product with ethyl cyanoacetate, barbituric acid, or 2-thiobarbituric acid have been developed. Respective regions of maximum absorbance for the four methods are 436.5, 550, 582, and 625 mμ. The sensitivities of the four methods are 0.033, 0.020, 0.025, and 0.017 p.p.m., respectively. The latter three methods are superior in color stability and reproducibility and in residue analysis for less interference from yellow-colored coextractives. All methods obey Beer's law over a wide concentration range.

The widespread use of chloro-*s*-triazine herbicides after the introduction of simazine in 1955 necessitated the development of sensitive, quick, and reliable methods for determination of their residues. Ragab (1959, 1960, 1964b) developed the pyridine-alkali method which involves the Zincke reaction (1904) and also three related methods by which the unstable yellow reaction product was converted into other chromophores of better stability and sensitivity which were more suitable for residue analysis. Knüsli *et al.* (1964) reviewed the pyridine-alkali method and its three related modifications. Radke *et al.* (1966) evaluated the pyridine-alkali and the ethyl cyanoacetate methods of Ragab and reinvestigated conditions relating to color stability and reproducibility. Knüsli *et al.* (1964) reviewed also the ultraviolet method which is lengthy, subject to many interferences from plant extracts or soil leachates, and suffers from irreproducibility. Matson *et al.* (1965) discussed the gas-liquid chromatographic methods for triazine herbicides. A sensitivity of 0.05 p.p.m. was obtained using the Dorhmann microcoulometer. Semiquantitative procedures were reported for paper chromatography (Major, 1962), fluorescent paper chromatography (Ragab, 1964a), and thin-layer chromatography (Ragab, 1966c) of chloro-*s*-triazines. Polarographic methods (Hayes *et al.*, 1967) for *s*-triazines have been reported.

The present study describes the pyridine-alkali method and its three modifications, the reaction conditions involved in the color development, and the mechanism of the reactions involved.

## APPARATUS AND REAGENTS

Borosilicate glass test tubes 30 ml. (12 × 150 mm.) fitted with air condensers (about 5 × 300 mm.) drawn out to constricted tips to minimize evaporation loss of solutions during heating were used.

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The simazine stock solution was 250 μg. per ml. in absolute ethanol. The simazine standard solutions were prepared from stock solution by dilution with water or buffers prepared according to Clark and Lubs (1958).

Pyridine, analytical reagent grade, was purified by refluxing over KOH (70 grams per liter) for one hour, cooling, decanting into a dry flask, and redistilling.

Pyridine, 70% (v./v.), was prepared from purified pyridine by dilution with water.

Seventy per cent pyridine saturated with glycine was prepared by saturating the 70% pyridine with glycine; the excess glycine was filtered.

All pyridine solutions were kept in amber bottles with polyethylene screw caps or glass stoppers.

## METHODS

**Pyridine–Alkali Method.** Color Development. A 5-ml. aliquot of simazine solution (5 μg. per ml.) was pipetted into a series of 30-ml. test tubes, and 1 ml. of 70% pyridine or 70% pyridine saturated with glycine was added. The solutions were mixed, the air condensers attached, tubes placed in a boiling water bath for 30 minutes, cooled to room temperature in another water bath, and the air condensers removed. One milliliter of 9*N* NaOH was added, the tubes were removed from the cooling bath, and their contents were mixed rapidly. The resulting brilliant yellow color was measured one minute after the addition of alkali at 436.5 mμ in a Beckman DU spectrophotometer fitted with a photomultiplier and 1-cm. Corex cells against a reagent blank prepared in the same way.

By use of the above procedure, the following variables were studied.

Preparation of Absorption Spectra. Because of the rapid fading of the color, freshly developed test solutions (5 μg. per ml.) were used for each wavelength tested, and the time between addition of the alkali and color measurement was very carefully controlled. This time should be equal in all measurements but should not exceed 2 minutes if it is inconvenient to maintain it at one minute.

Preparation of Standard Curve. Five milliliters of simazine solution (0 to 5 μg. per ml.) were used for color development and measurements were made at 436.5 mμ.

Color Stability Curve. Color was developed using 5-ml. solutions (5 μg. per ml.), and the absorbance was

measured at 436.5 mμ at various intervals at room temperature.

EFFECT OF PYRIDINE AND NaOH REAGENTS ON COLOR INTENSITY. Color was developed using 5-ml. solutions (5 μg. per ml.). Pyridine concentrations in water were varied from 10 to 100% and NaOH from 0.1*N* to 10*N*.

EFFECT OF INITIAL pH OF REACTION SYSTEM ON COLOR INTENSITY. Color development was made using 5 ml. of simazine standard solutions in buffer (5 μg. per ml.), 70% pyridine, and 9*N* NaOH.

**Pyridine–Alkali–Ethyl Cyanoacetate Method.** One milliliter of ethyl cyanoacetate was added to the yellow reaction product of the pyridine-alkali method as soon as it was developed. The contents of the tubes were mixed, 1 ml. of ethanol was added to bring all of the resulting pink color into solution, and absorbance measured at 550 mμ against a reagent blank.

**Pyridine–Alkali–Barbituric Acid Method.** The yellow reaction product of the pyridine-alkali method was diluted to 25 ml. with a solution of barbituric acid in glacial acetic acid (approximately 1% v./v.). After 30 minutes, the resulting reddish violet color was measured at 582 mμ against a reagent blank.

**Pyridine–Alkali–2-Thiobarbituric Acid Method.** Substituting 2-thiobarbituric acid for barbituric acid in the pyridine–alkali–barbituric acid method, a blue color with a maximum absorbance at 625 mμ was obtained. This was measured one hour after the addition of the 2-thiobarbituric acid.

Absorption spectra and standard and stability curves were also studied in the latter three methods.

## RESULTS AND DISCUSSION

The three related methods were basically modified from the pyridine-alkali method following the development of its yellow color. Therefore, the conditions affecting the development of this color were found to affect similarly the colors produced in the three methods. The most intense yellow color (and subsequently, the other three colors) was obtained when simazine and pyridine were heated first in a boiling water bath and cooled to room temperature before adding the alkali. Reproducibility was obtained when cooling was rapid and maintained as constant as possible until the spectrophotometric reading was taken. This could be accomplished by cooling in a water bath and working in a well-ventilated, air-conditioned room. The colorless reaction product between simazine and pyridine was stable either at room temperature or under refrigeration. This was observed from the absorbance of the yellow color produced by adding the alkali to samples stored for two days. Prolonged storage resulted in some turbidity in the solutions. Maximum intensity of colors was obtained with a pyridine concentration of 70% and 9*N* sodium hydroxide. Higher concentrations of both pyridine and alkali caused turbidity or two layers. Glycine increased color intensities with no change in the absorption maxima (Figures 1, 3, and 4). The yellow color (Figure 1) faded in a rectilinear manner very rapidly, and hence the time interval between the addition of alkali and spectrophotometric reading must be maintained equal and kept to the shortest. Absorbance readings should always be computed with reference to standards made at the same time and under the same conditions as the unknown. The speed and manner with which the alkali is added and mixed with the reaction product of simazine and pyridine may contribute, at least in part, to the daily fluctuation. Standard curves showed that colors in all four methods obeyed Beer's law over the concentration range used.

The reaction between simazine and pyridine was pH dependent. Figure 2 shows that the intensity of the yellow color, as an example, increased as the pH of the reaction system decreased. The decreased intensity of the yellow color in more alkaline systems might also mean that simazine and pyridine reacted and some of the color formed but faded during the heating. This observation was confirmed when the yellow color was developed by the Fujiwara (1916) procedure. A less intense yellow color was formed and almost disappeared during cooling to room temperature before the absorbance reading was taken.

These observations indicate that the nature of the reaction between simazine and pyridine in the Zincke procedure is similar to that in the Fujiwara procedure, since in both, yellow colors with the same absorption maxima are produced, and that the Fujiwara procedure is not as efficient for the determination of simazine as the Zincke procedure. In the light of these findings, the increased intensity of colors in all methods in the presence of glycine, while absorbance maxima remained the same, might be related to the pH difference between the two pyridine reagents. In

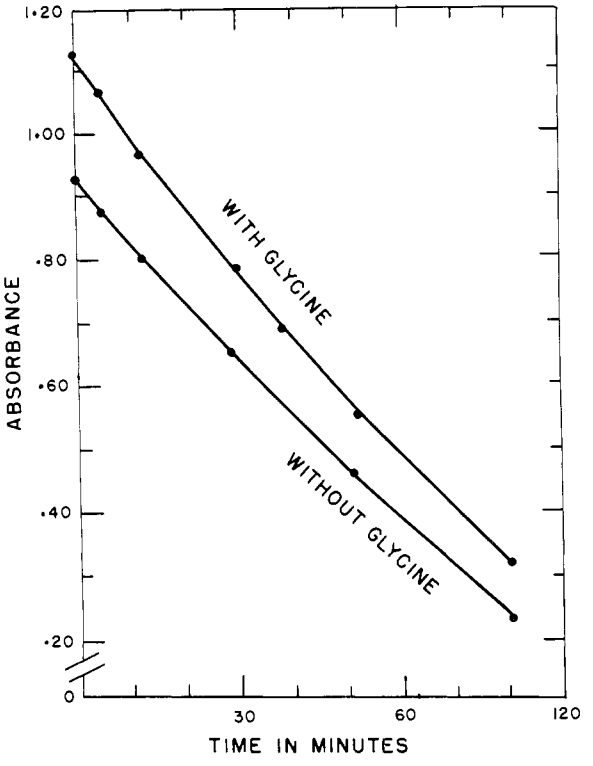


**Figure 1. Stability curves for the yellow reaction product formed in the pyridine-alkali method**

3.56 μg. of simazine per ml. of the final test solution

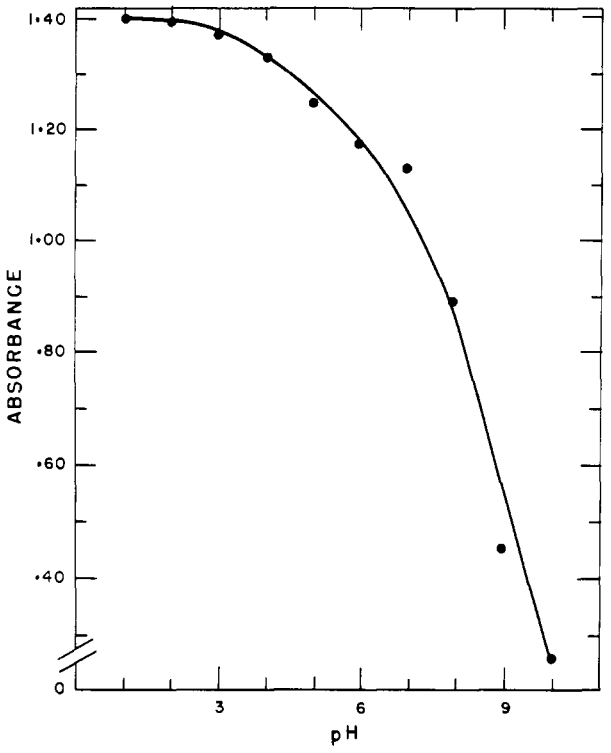


**Figure 2. Effect of initial pH on the reaction between simazine and 70% pyridine as measured by the absorbance of the yellow product**

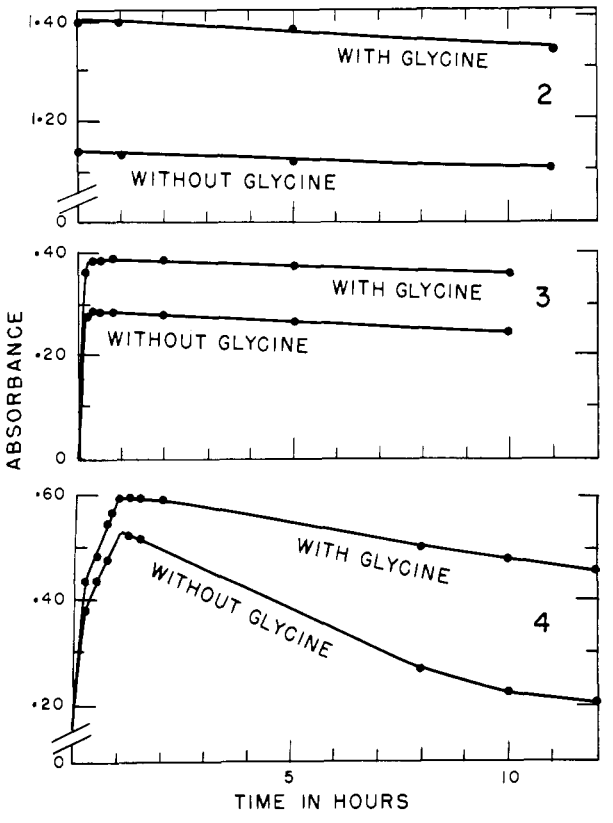


**Figure 3. Stability curves for the colored reaction products formed in the pyridine–alkali–ethyl cyanoacetate, pyridine–alkali–barbituric acid, and pyridine–alkali–2-thiobarbituric acid methods**

2.78, 1.00, and 1.00 μg. of simazine, respectively, per ml. of the final test solution

other words, the lower pH of the glycine-saturated pyridine enhances the electrophilic attack of simazine at the pyridinium nitrogen displacing the chlorine. Radke *et al.* (1966) reported similar results relating to pH and also its influence in improving color stability and reproducibility. In the determination of dyrene employing the Zincke reaction, Burchfield and Schuldt (1958) stated that the reason for enhancement of the yellow color by the use of glycine was unknown. The present authors believe that this effect can, probably, also be related to pH.

In the pyridine–alkali–ethyl cyanoacetate method, maximum intensity of the pink color was obtained when ethyl cyanoacetate was added immediately after the yellow color was formed. Very little color was formed when this reagent was added before the alkali. Treating with acids or heating before or after adding ethyl cyanoacetate either prevented the formation of or destroyed the pink color, respectively. Methyl cyanoacetate gave similar response but cyanoacetic acid gave some color which faded when the concentration of the acid was increased. Under the same conditions ethyl phenylacetate or ethyl acetoacetate produced no colors. The stability of the pink color produced in this method (Figure 3) was much greater than the yellow color of the pyridine-alkali method.

Replacement of ethyl cyanoacetate by barbituric acid or 2-thiobarbituric acid in the simazine-pyridine yellow solution produced no new color. However, when acetic acid, which discharges the yellow color, was also added, a reddish violet (pyridine–alkali–barbituric acid method) or blue color (pyridine–alkali–2-thiobarbituric acid method), respectively, was formed. Maximum intensity of those two colors was obtained when barbituric acid or 2-thiobarbituric acid in acetic acid was added following the development of the yellow color. Figure 3 shows the stability of those two colors.

Even though the additional reagents reduced the final concentration of simazine in the test solutions, the three modified methods were more sensitive than the pyridine-alkali method (Figure 4). If the minimum detectable difference in absorbance is assumed to be 0.01, the sensitivities of the four methods are 0.033, 0.020, 0.025, and 0.017 p.p.m., respectively. Obviously, the sensitivities can be increased by reducing the final volume of test solutions. The four methods were applicable also to the other chloro-*s*-triazine herbicides with differences in absolute absorbance but without changes in absorption maxima. The three modified methods are superior to the pyridine-alkali method not only in sensitivity, color stability, and reproducibility but also in residue work for less interference from yellow coextractives.

Because of its simplicity and rapidity, the ethyl cyanoacetate method was preferred and has been used in studies on chloro-*s*-triazine herbicide residues in water, soils, and agricultural crops; results will be published in ensuing papers. However, the barbituric and 2-thiobarbituric acids methods are also very useful. The former has a unique advantage in that its solution exhibits strong fluorescence in ultraviolet light and has been adapted for the

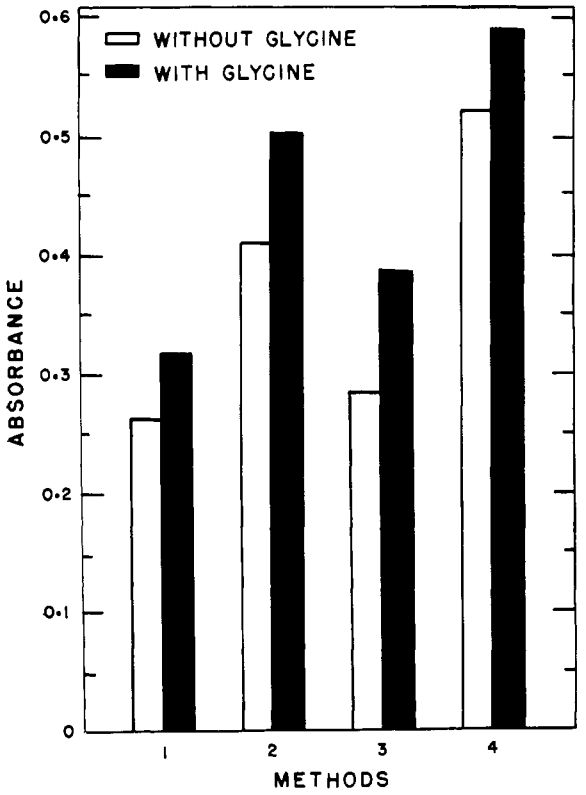


**Figure 4. Absorbance of solutions of simazine analyzed by the four colorimetric methods**

1 μg. per ml. final volume

fluorescent detection of chloro-*s*-triazines (Ragab, 1964a) and several chlorinated organic pesticides (Ragab, 1964c) on paper chromatograms. The method employing 2-thiobarbituric acid may be favored if more sensitivity is required.

Qualitative tests have shown that the three modified methods are also applicable to chlorinated organic pesticides and related compounds reactive in the pyridine-alkali techniques (Fujiwara, 1916; Zincke, 1904). Quantitative procedures for kelthane, thiodan, dyrene, and trichloroacetic acid (Ragab, 1963), dalapon (Ragab, 1966a), and chlorophenoxy acid herbicides after nitration to activate the chlorine atoms (Ragab, 1966b) have been developed. Burchfield and Schuldt (1958) reported that 2,4-D did not give colored products in any of the pyridine-alkali reactions they reviewed.

The conditions required to carry out the pyridine-alkali method indicate that the nature of the intermediates is, probably, similar to those first described by Zincke (1904), presented by Mosher (1950), and reviewed by Burchfield and Schuldt (1958) for the reaction between pyridine and 1-chloro-2,4-dinitrobenzene. Thus, Figure 5 shows that an electrophilic attack occurs between simazine (I), through its strongly electron-attracting chlorine, and pyridine (II) at the unshared electron pair of nitrogen, forming the quaternary salt (III) which undergoes addition of a hydroxyl group resulting in the carbinol base (IV). Treatment with alkali results in hydrolyzing IV and destroying the aromaticity of the pyridine ring yielding a monoanil of glutaconic aldehyde (V) which, probably, exists in equilibrium with its tautomeric form (VI). Evidence in favor of the open-chain structure (V) or its enol form (VI) is its color. The latter structure has completely conjugated double bonds and would, therefore, be expected to show strong absorption of visible light. Since structure III is colorless, structure IV, in which the conjugation of pyridine has been destroyed, should undoubtedly be colorless too. The rapid fading of the yellow color of VI as a result of heating, addition of an acid, or storage at room temperature could be ascribed to its hydrolysis to 2-amino-4,6-bis(ethylamino)-*s*-triazine (VII) and glutaconic dialdehyde (VIII).

The probability that glutaconic dialdehyde is one of the final products is indicated by the formation of new colors when the unstable yellow product reacted with phenylmethylpyrazolone (Epstein, 1947), *p*-aminobenzoic acid, or benzidine under acidic conditions. These were probably produced by the formation of dianils of glutaconic aldehyde.

The formation of highly colored solutions by addition of ethyl cyanoacetate, barbituric acid, or 2-thiobarbituric acid also elucidates the nature of the mechanism of the pyridine-alkali reaction. Ethyl cyanoacetate changed the yellow color of compound VI into a pink color having the probable structure IX. Since very little color was formed by adding ethyl cyanoacetate to the simazine-pyridine solution before the addition of the alkali, the yellow color in the pyridine-alkali reaction cannot be the quaternary salt (III) or the carbinol base (IV). Furthermore it cannot be the free glutaconic dialdehyde (VIII), as no color was formed when ethyl cyanoacetate was added after discharging the yellow color. This latter observation may also exclude a structure such as IX′ for the pink complex. The presence of the nitrile group (—C≡N) may contribute to the pink color, as a similar response was noticed with methyl cyanoacetate, some response with ethyl cyanoacetic acid, but not with phenyl ethylacetate or ethyl acetoacetate. Fading of the pink color with prolonged heating, standing at room temperature, or adding acids indicates that the ester group in structure IX may be hydrolyzed to the acid. Acidity effect on the pink color was noticed when the increased amount of cyanoacetic acid was substituted for ethyl cyanoacetate. Similarity between the 1-chloro-2,4-dinitrobenzene–pyridine reaction of Zincke and simazine-pyridine reaction is indicated by a similar response towards ethyl cyanoacetate. The red-violet color of the former reaction and the yellow color of the latter reaction were both changed into a pink color.

Since barbituric acid or 2-thiobarbituric acid did not form a new color with the yellow simazine-pyridine-alkali (VI) unless it was discharged with acid presumably forming VIII, structures $X_a$ and $X_b$ are proposed for the reddish violet color of barbituric acid and blue color of 2-thiobarbituric acid methods, respectively. Those two compounds ($X_a$ and $X_b$) may be considered resonance hybrids of structures $XI_a$ and $XII_a$ and $XI_b$ and $XII_b$, respectively. Structures such as $X'_a$ and $X'_b$ are not likely to form under these conditions.

The three modified methods help to explain the pyridine-alkali reaction for organic compounds containing active halogens and so improve techniques for their chemical analysis.

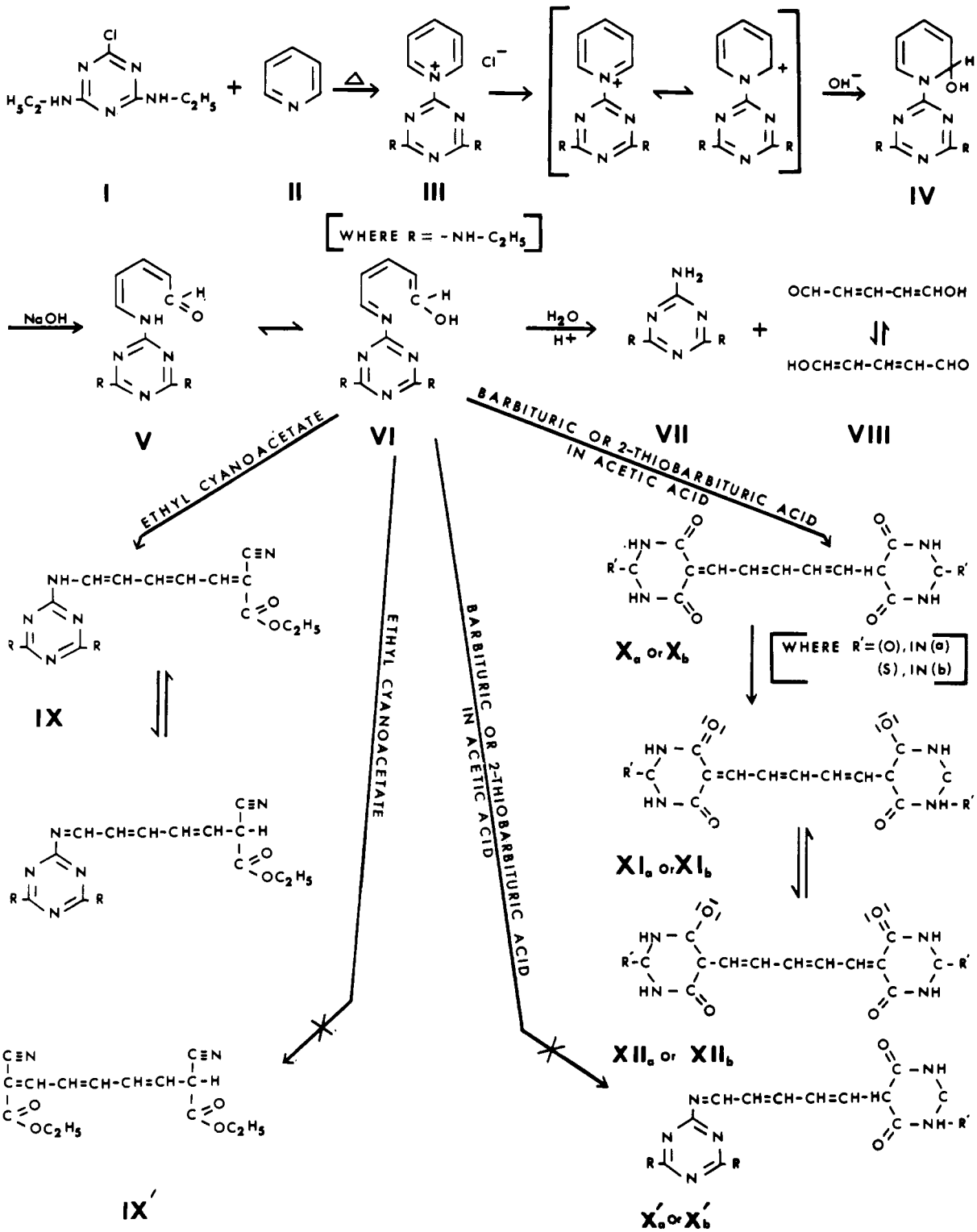


Figure 5. Reaction mechanism proposed in the four methods

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