# Technology Section

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## DENSITOMETRIC METHOD FOR THE DETERMINATION OF 2-METHYL-4-AMINO-5-AMINOMETHYL PYRIMIDINE

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A thin layer densitometric method was used for the estimation of 2-methyl-4-aminomethyl pyrimidine in the irradiated mixture of thiamine hydrochloride. The method is based on the chromatographic separation of pyrimidine by using a thin layer of silica gel $HF_{254}$ and the solvent mixture chloroform – methanol – ammonia 30% (70:30:1). No interference from other photodecomposition products was observed. The standard deviation for the method was 0.33. Using this method, preliminary kinetic studies for the formation of pyrimidine during the irradiation of thiamine hydrochloride were also carried out.

Denistometery in TLC is a method in which the intensity of the colour of a substance is measured by comparing the incident and reflected light directly on the chromatogram. This method was first used for measuring the concentration of amino acids separated by means of electrophoresis [1]. In the present investigation, this method was applied successfully for the determination of 2-methyl-4-amino-5-aminomethyl pyrimidine - a photodecomposition product of thiamine hydrochloride.

UV,

## EXPERIMENTAL

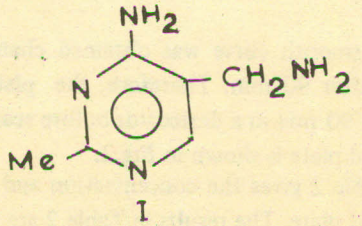
### *Materials*

(a) Silica gel $HF_{254}$ plates (20X20 cm); (b) solvent mixture: chloroform – methanol – 30% ammonia (70:30:1), CdAc ninhydrin spray reagent.

### *Apparatus*

Agla micrometer syringe (Wellcome Research Laboratories) Joyce Lobel Chromoscan with TLC attachment. The following instrument settings were used: filter 465; and aperture 1005.

The compound 2-methyl-4-amino-5-aminomethyl pyrimidine (I) ($C_6H_{10}N_4$), mol. wt 138 was supplied by Roche Products Ltd.




I

Its m.p was 212–215°, dipicrate –225°. IR:1020, 2200 $cm^{-1}$, 3340 $cm^{-1}$ and 1400–1700 $cm^{-1}$ multiple bands.

The NMR spectra showed three singlet at $\tau$7.5$\tau$ (3H), $\tau$6.15 (2H), and 20 (1H).

This compound was used in our laboratory for the synthesis of 2-methyl-4-amino-5-hydroxymethyl pyrimidine a thermal decomposition product of thiamine hydrochloride, It is one of the intermediates in the synthesis of thiamine HCl.. Earlier workers have isolated and identified it as one of the ammonia cleavage product of thiamine hydrochloride.

*Procedure.* Solutions of different concentrations of aminopyrimidine ($0.25–1.5X10^{-3}M$) were prepared. 20 $\mu$l of each solution was spotted on silica gel $HF_{254}$ plates of 0.25 mm thickness. For the applications of spots on thin layer plates an Agla micrometer syringe was used, in order to have the spot size as small as possible and also to ensure constant area of all the spots. The syringe outfit was assembled as described by Hasan [2]. The tip of the needle was coated with a silicone fluid in order to facilitate the terminal drop formation. A volume of 2 $\mu$l was delivered at a time, dried for exactly 15 sec and the process repeated until a total volume of 20 $\mu$l was applied in each spot. The plate was then run in a tank saturated overnight with the solvent system.

After developing, the plate was dried by a hot air current for 5 min and then dried in an oven at 100° for 15 min, The drying treatment prior to spraying is essential to remove any ammonia vapours which could interfere with the sparying reagent and affect the development of colour by giving a coloured background to the plate. For spraying the plate, cadmium acetate ninhydrin reagent was

used. Blackburn [3], while studying the colour reaction of amino acids with ninhydrin, pointed out that if ninhydrin only is used for staining, it suffers several disadvantages, and reagents incorporating a metal ion such as Cu(II) or Cd(II) are preferable. The original reagent of Barollier [4] incorporated cadium chloride, which was later replaced with cadmium acetate to avoid interference of chloride ion on the colour development. The reagent first used by Alfield and Morris [5] and later by Blackburn and Lee [6] was used for spraying the plates in this work. The composition of solution A was cadmium acetate (1 g), water (100 ml), acetic acid (20 ml), and acetone (1000 ml). One gram ninhydrin was dissolved in 112.0 ml of solution A and used for spraying. After spraying, the plate was heated for 30 min at 50° in an oven, cooled for 15 min, and then scanned on Joyce Lobel Chromoscan with TLC attachment.

*Determination of Standard Deviations.* For determining the standard deviations the experiment was repeated six times and the slopes of concentration of pyrimidine vs peak area plots were used to calculate the standard deviation using the following formula.

$$\text{Standard deviations} = \sqrt{\frac{(\text{Sum(obs)}^2 - (\text{sum obs})^2/n)}{n-1}}$$

## RESULTS AND DISCUSSIONS

The factors which caused variations in the final spot on the plate were investigated by Fairbairn [7]. To minimize these errors, the tank was saturated overnight with the solvent system. The initial volume of the solvent was kept at 1% of the tank volume. The same tank under identical conditions was used during the course of the present study. To minimize variations due to plate coating and adsorptivity, all the samples to be studied were spotted together with the standard solution on the same plate. The moisture content of each plate, which affects adsorbtivity, was controlled as far as possible by activating the plate at the same temperature and for the same period. The ninhydrin colour reaction can be carried out under a wide variety of conditions, the rate of colour development being greatly affected by such factors as humidity and temperature. In order to ensure reproducable results these factors were controlled closely.

During the premilinary runs it was noted that the intensity of the yellow colour changed with time. In order to find the relation between the change in colour intensity and time, another plate was treated in exactly the same way as has been described earlier. This time the plate was scanned immediately after drying and then at 10, 25, 40, 60, 90, 120 and 150 min intervals. The areas of the curves were calculated using a plainimeter. The results are given in Table 1. Fig 1 shows the increase in area plotted against time.

Table 1

| Time (min) | Area ($cm^2$) | Increase in area ($cm^2$) |
|---|---|---|
| 0 | 2.32 | – |
| 10 | 2.57 | 0.25 |
| 25 | 3.16 | 0.84 |
| 40 | 3.46 | 1.14 |
| 60 | 3.65 | 1.33 |
| 90 | 3.82 | 1.50 |
| 120 | 3.83 | 1.51 |
| 150 | 3.82 | 1.50 |

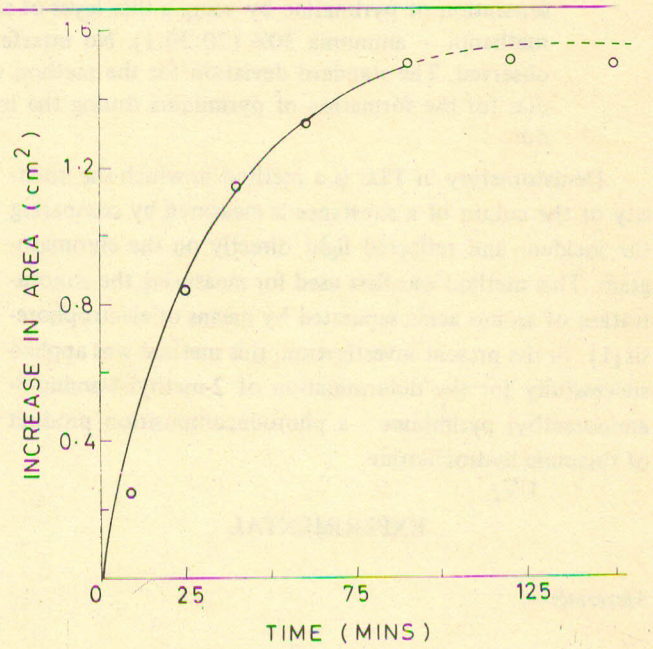



Fig. 1. Time vs increase in area.

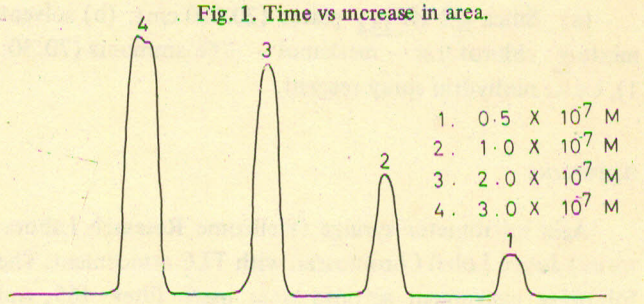



Fig. 2. Standard plate: Peak 1. $0.5 \times 10^{-7}$ *M* pyrimidine, peak 2. $1.0 \times 10^{-7}$ *M* pyrimidine, peak 3. $2.0 \times 10^{-7}$ *M* pyrimidine, and peak 4. $3.0 \times 10^{-7}$ *M* pyrimidine.

A smooth curve was obtained changing to a constant value after 90 min. Therefore, the plate after drying was left for 90 min in a desiccator before scanning. A scan for a standard plate is shown in Fig.2.

Table 2 gives the concentration and peak areas for the standard plate. The results in Table 2 are plotted in Fig.3.

A linear relationship was observed between peak area and concentration. The standard deviation for the method came out to be 0.33. The advantage of this method over

Table 2

| Concn of pyrimidine X10$^7$*M* | Peak area (cm)$^2$ |
|---|---|
| 0.5 | 1.25 |
| 1.0 | 3.20 |
| 2.0 | 6.16 |
| 3.0 | 9.16 |

Table 3

| Time sec | PyX10$^3$*M* | $\delta py/\delta t$X10$^5$ | ThX10$^3$*M* | (Th)$^2$X10$^6$*M* |
|---|---|---|---|---|
| 10 | 0.195 | 2.70 | 2.239 | 5.013 |
| 20 | 0.442 | 2.14 | 2.039 | 4.158 |
| 30 | 0.612 | 1.65 | 1.862 | 3.467 |
| 40 | 0.775 | 1.43 | 1.677 | 2.812 |
| 50 | 0.900 | 1.19 | 1.514 | 2.292 |
| 60 | 1.00 | 0.97 | 1.366 | 1.866 |

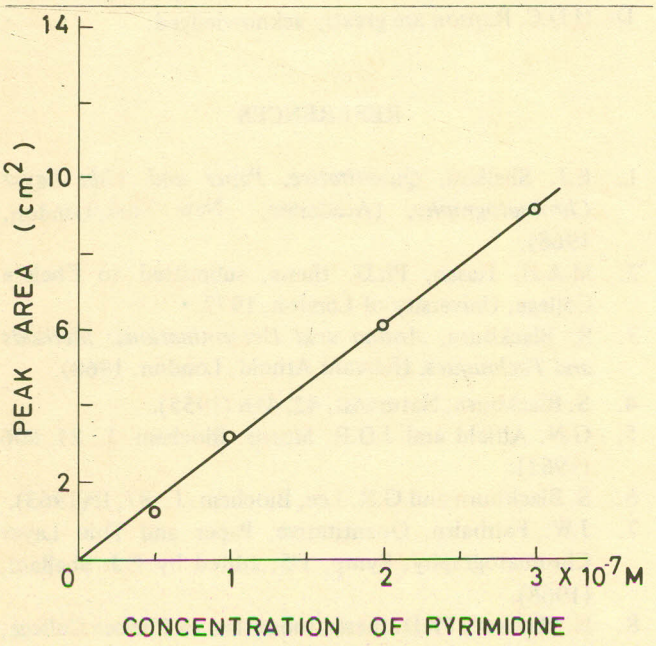



Fig. 3. Peak area vs concentration

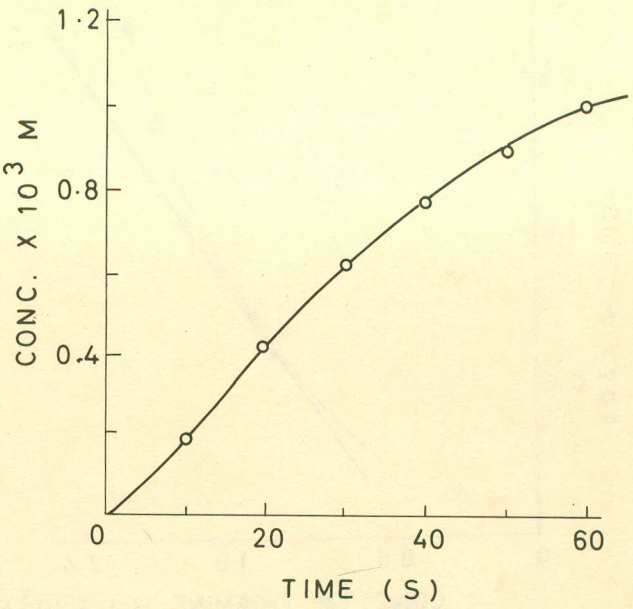



Fig. 4. Appearance of pyrimidine.(Concn vs time).

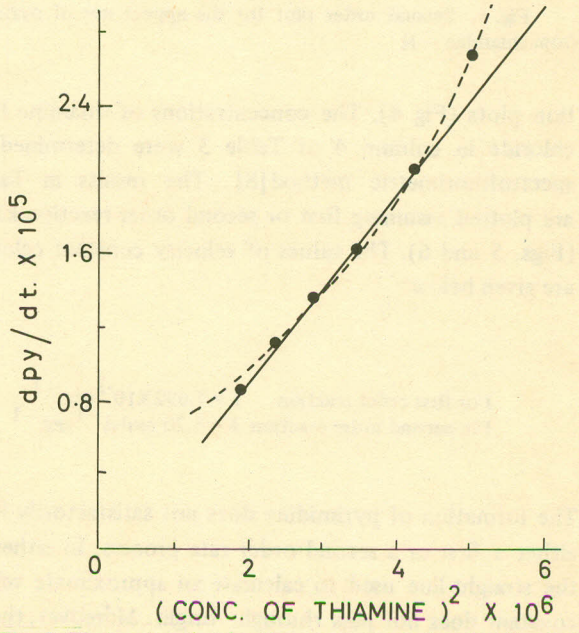



Fig. 5. First order plot for the appearance of pyrimidine from thiamine - HCl.

other analytical procedures is that many analyses can be carried out extremely rapidly as several samples can be applied on the same plate to ensure identical treatment.

In the present study this method was used to estimate 2-methyl-4-amino-5-aminomethyl pyrimidine in the irradiated reaction mixture of thiamine hydrochloride. For this purpose a 2.5 X 10$^{-3}$*M* solution of thiamine hydrochloride at pH 4 was irradiated for 60 sec and the samples were taken at the interval of 10, 20, 30, 40, 50 and 60 sec, 20 µl of each sample alongwith the standard solution of 2 methyl-4-amino-5-aminomethyl pyrimidine were applied on thin layer plates, and the plates scanned after developing and spraying. The amount of pyrimidine in each sample was calculated by comparing it with the standard solution, and is given by the formula :

$$\text{Amount of pyrimidine in the sample} = \frac{\text{Pa (samle)} \times C\text{(standard)}}{\text{Pa (standard)}}$$

where Pa, peak area; and *C* concentration.

The results obtained are recorded in Table 3.

Figure 4 shows the appearance of pyrimidine plotted against time. A smooth curve is obtained; only the $t_{10}$ point does not fall in. The values of $\delta py/\delta t$ were obtained by taking the slope at different points on time vs concentra-

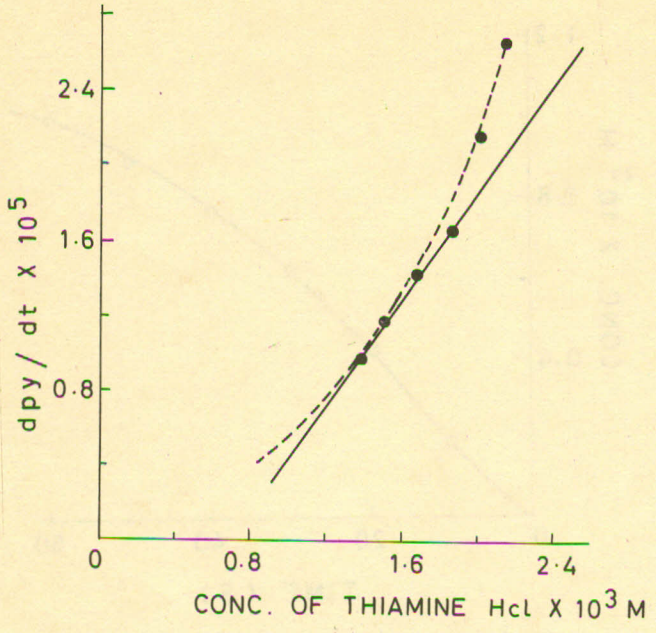



Fig. 6. Second order plot for the appearance of pyrimidine from thiamine – H

tion plots (Fig 4). The concentrations of thiamine hydrochloride in column 4 of Table 3 were determined by a spectrofluorimetric method [8]. The results in Table 3 are plotted assuming first or second order reaction kinetics (Figs. 5 and 6). The values of velocity constant calculated are given below:

For first order reaction $K = 7.692 \times 10^{-3} sec^{-1}$
For second order reaction $K = 3.20$ moles$^{-1}$ $sec^{-1}$

The formation of pyrimidine does not satisfactorily follow either a first or a second order rate process. In either case the straight-line used to calculate an approximate velocity constant does not pass through origin. Moreover, the plot of concentration against time suggests an induction period, and hence the probability of formation of this compound through some intermediate. At higher concentration of thiamine, deviation from linearity is also observed and the straight-line in both the cases show systematic deviation from experimental data, and could very well be curves (Fig. 5, 6). From the above observations it can be suggested that the formation of 2 methyl-4-amino-5-aminomethyl pyrimidine during the photodecomposition of thiamine hydrochloride is not a simple process but proceeds through some intermediate stage. More precise arguments could only be given unless some more experiments are carried out between $t_0$ and $t_{20}$ and at lower concentration of thiamine hydrochloride. Time did not permit us to carry these experiments. Our work was restricted only in devising of an analytical procedure and applying it to preliminary kinetic study.

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