Estimation of Tryptophan Content by Spectrophotometric Methods: Analysis of the Interaction Between Alpha-Ketoglutaric Acid and Tryptophan

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ABSTRACT

The interaction between L-tryptophan and a-ketoglutaric acid (a-KGA), first reported by Chu and Clydesdale (1,2), was used as the basis for development of a method for estimation of tryptophan content. Analysis of reaction mixtures with ultraviolet spectrophotometers revealed the development of absorbance with a wavelength of maximum absorbance (λ max) at 358 nm. Four parameters were manipulated to increase the rate and amount of chromophore formed. These parameters were: concentration of HCl, concentration of a-KGA, concentration of sodium nitrite, and temperature.

Tryptophan is an essential amino acid. This implies that it cannot be manufactured by the human body in sufficient amounts to satisfy demand, and therefore it must be ingested to maintain positive nitrogen balance. An important function of tryptophan is its role as the precursor of niacin. If enough tryptophan is ingested in the diet, the requirement for niacin is met, and thus the disease pellagra can be prevented.

Due to the importance of tryptophan and its metabolites, various analytical methods have been developed to assay for its presence in both its free and peptide-bound forms. To date, however, most procedures have been time-consuming and/or unreliable. Friedman and Finley (3) wrote an excellent review article on this subject.

The purpose of this investigation was to explore the interaction between tryptophan and a-ketoglutaric acid (a-KGA). Chu and Clydesdale (1,2) reported that colored derivatives were produced by the interaction of tryptophan and a-KGA. The possibility of using this reaction as a quantitative spectrophotometric method for determination of tryptophan content was examined.

MATERIALS AND METHODS

From preliminary studies it was found that when acidic solution of a-KGA (Eastman Kodak Co.) was allowed to interact with 2.0-mM solutions of L-tryptophan (Aldrich Chemical Co.), a yellow-brown color developed. On the basis of this observation certain standardized experiments were designed to individually test the effects of four parameters (concentration of HCl, concentration of a-KGA, concentration of sodium nitrite, and temperature) on the rate and amount chromophore produced.

Preparation of standard tryptophan solutions

A 2.0-mM solution of L-tryptophan was prepared in a one liter volumetric flask. The flask was wrapped with aluminum foil and stored in the refrigerator (8°C) to minimize destruction of tryptophan. However, because of its known lability, new stock solutions were prepared for each set of experiments.

Measurement of color formation

In preliminary studies the increase in absorbance due to formation of chromophore was monitored by both visible and ultraviolet spectra. The General Electric Recording Spectrophotometer (GERS) was used for the visible spectrum and the Perkin Elmer Model 450 UV-VIS NIR Spectrophotometer (PE-450) was used for the ultraviolet spectrum.

To maintain the readings on scale the reaction mixture had to be diluted 1:5 with distilled water.

Effect of HCl concentration

Stock solutions containing 14.52 g of a-KGA were made up to volume in 200-ml volumetric flasks (0.5 moles a-KGA/liter) with each of the following: distilled water, 3 N, 6 N, 9 N, and 12 N HCl. Duplicate sample solutions were prepared in 20-ml sample bottles containing equal amounts of the a-KGA reagent and either stock tryptophan solution (for the test samples) or distilled water (for the blanks). All samples were incubated in an oven maintained at 40°C (± 2°C). Aliquots of the test samples and blanks were taken every 8 h for the first 80 h and then after 92, 116, and 140 total hours of incubation. These were then diluted and measured on both the PE-450 and HPE-139.

Effect of a-KGA concentration

Solutions containing 0.05, 0.10, 0.30, 0.50, and 0.70 moles a-KGA/liter of 6 N HCl were prepared in 200-ml volumetric flasks. As in the previous experiment duplicates were prepared for each test sample and blank and incubated at 40°C. Aliquots were taken for analysis at 12-h intervals for the first 168 h and then at 24-h intervals for the last 72 h. Thus the total reaction time was 240 h.

Effect of temperature

Test temperatures: 20°C, 40°C, and 60°C. A set of both 0.1 M and 0.5 M a-KGA were made in duplicate with 6 N HCl for each of the three test temperatures. Appropriate test samples and blanks were made and incubated at 20°C, 40°C, or 60°C. Aliquots from all three sets were taken for analysis every 12 h for the first 120 h and then after 144, 168, and 192 h.
of total incubation.

Test temperatures: 60 and 80°C. Two sets of samples containing 0.1 M 0.5 M, and 0.01 M α-KGA were made up in 6 N HCl. Test samples and blanks were prepared in duplicate for each temperature and concentration of α-KGA used. Incubation temperatures were 60 and 80°C. Aliquots were taken for analysis every 6 h for the first 30 h and a final set was taken after 42 total hours of incubation.

Test temperature: 100°C. Duplicate sets of samples containing 0.10, 0.05 and 0.01 M α-KGA were made up in 6 N HCl. Test samples and blanks were prepared and incubated at 100°C. Aliquots were taken for analysis every 6 h for a total of 60 h.

Effect of oxidizing agents
Effect of NaNO₂ and NaClO₄•H₂O. Separate solutions of 1.00, 0.50, 0.10, and 0% NaNO₂ and NaClO₄•H₂O were prepared with distilled water. Also a 0.05 M solution of α-KGA in 6 N HCl was mixed with an equal amount of stock tryptophan solution and incubated at 80°C. Samples from this reaction mixture were taken after 3, 6, 12, and 24 h of incubation. To 1-ml aliquots of these samples was added 1, 2, or 4 drops of the oxidizing solutions. These samples were placed in the dark for 15 min and then analyzed for the development of chromophore.

Effect of other oxidizing agents. One percent solutions of NaNO₂, NaNO₃, KNO₂, KNO₃, NaMnO₄, Mg(NO₃)₂, and NaClO₄•H₂O were prepared with distilled water. The reaction mixture used in the previous experiment was also utilized for this experiment. After 4 h of incubation at 80°C, seven 5-ml aliquots were taken and 5 drops of each of the oxidizing solutions were added. Chromophore development was then analyzed as previously outlined.

Effect of increasing concentration of NaNO₂. One percent, 5.0%, and 10.0% solutions of NaNO₂ were prepared with distilled water. Sixteen 5-ml aliquots were taken from the reaction mixture used in the previous two experiments. To these was added 1 drop, 5, 10, or 20 drops either of distilled water or the oxidizing solutions. Again chromophore development was analyzed.

RESULTS AND DISCUSSION

Measurement of color formation

Figures 1 and 2 illustrate the fact that when a 0.5 M solution of α-KGA dissolved in 6 N HCl was allowed to interact for 48 h at 40°C with an equivalent amount of stock tryptophan solution (2.0 mM), there is production of a chromophore which has peak absorbance at 358 nm. Both the PE-450 and HPE-139 correlated well in support of this observation. There was no absorption in the visible range.

\[ \text{Sample} \quad \text{Blank} \]

Figure 1. Spectral curves of a sample (α-KGA + tryptophan) and a blank (α-KGA + H₂O) at time 0 obtained using the PE-450.

\[ \text{Sample} \quad \text{Blank} \]

Effect of HCl concentration

The production of chromophore with λ max at 358 nm was found to be catalyzed by acid. Figure 3 clearly illustrates that when 0.5 moles of α-KGA were dissolved per liter of 3, 6, 9, or 12 N HCl rather than in distilled water, greater amounts of chromophore were produced when allowed to react with stock solutions of tryptophan at 40°C.

When the α-KGA was dissolved in 6 N HCl the yield of chromophore and the rate of its production were at a maximum. Production of chromophore leveled off after 48 h and was stable for an additional 40 h. Thus 6 N HCl was used in subsequent experimentation.

Effect of α-KGA concentration

Figure 4 shows that the lower (0.05 and 0.10 M) concentrations of α-KGA produced the greatest increase in absorbance at 358 nm. However, the reaction took over 200 h to complete. As the concentration of α-KGA was increased, the initial reaction rate was greatly increased. The sample containing 0.70 M α-KGA exhibited the fastest rate of production but this ceased...
after 34 h, yielding only approximately two-thirds as much chromophore as did the sample containing 0.10 M a-KGA.

The sample containing 0.50 M a-KGA produced somewhat more chromophore than did the sample containing 0.70 M a-KGA. In both instances the chromophore production was stable for almost 60 h. Inasmuch as the two reactions were similar with respect to the rate, amount, and stability of chromophore produced, the lower concentration (0.5 M) was selected for further testing to conserve reagent.

Effect of temperature

Test temperatures: 20, 40, and 60 C. Analysis of chromophore production revealed that at the higher temperature, 60 C, samples containing 0.1 M a-KGA produced more chromophore and at a faster rate than did the sample containing the 0.5 M a-KGA solution (See Fig. 5 and 6). In both instances chromophore production peaked after approximately 24 h and started to decrease within a few hours thereafter.

At 40 C the sample containing the lower concentrations of a-KGA (0.1 M) yielded higher amounts of chromophore but the production had not leveled off even after 192 h of incubation. Again, the sample containing 0.5 M a-KGA leveled off after 48 h of incubation and maintained a stable level for 60 additional hours. At 20 C all samples failed to cease chromophore production even after 192 h of incubation.

Test Temperatures: 60 and 80 C. The results of the previous experiment indicated that at higher temperatures, samples containing relatively low concentrations of a-KGA produced the greatest amount of chromophore. Therefore 0.10, 0.05, and 0.01 M solutions of a-KGA dissolved in 6 N HCl were used in these experiments. Explanations for this behavior were not attempted.

At both temperatures, 60 and 80 C, samples containing 0.01 M a-KGA yielded the least amount of chromophore at the slowest rate (See Fig. 7 and 8). Since production of chromophore had not leveled off by the time the experiment was halted, it may be implied that these lower concentrations of a-KGA would eventually produce the greatest amount of chromophore. This hypothesis was based on the earlier results.

The samples containing 0.05 M a-KGA produced almost equivalent amounts of chromophore at approximately the same rate at both temperatures. Since the sample incubated at 80 C showed more of a plateau region after 22 to 30 h of incubation, those conditions were chosen for further experimentation.
The samples containing 0.10 M α-KGA produced less chromophore than did the samples containing 0.05 M α-KGA at both temperatures. The rate of chromophore production was faster for the 0.10 M α-KGA. This supports the observation that at higher temperatures, samples containing more α-KGA produced lower yields of chromophore but the rate of production was faster and there was a long time period when production was stable.

Test temperature: 100 C. When samples containing 0.10, 0.05, and 0.01-M solutions of α-KGA dissolved in 6 N HCl were incubated at 100 C, production of chromophore was limited (Fig. 9). All three levels showed a fast initial rate of chromophore production with the sample containing the least amount of α-KGA (0.01 M), producing the most chromophore.

At all concentrations tested, at least 18 h were required for production of chromophore to level off. Since the total amount of chromophore produced at 100 C was significantly lower than the amounts produced at lower temperatures, no further work was done at this temperature.

Selection of parameters for further analysis

The purpose of this investigation was to investigate the possibility of utilizing α-KGA in a quantitative analysis of tryptophan. In view of the objective, two sets of reaction conditions, named RC-1 and RC-2, were chosen because they produced a measurable amount of chromophore within a reasonable time. Also of prime importance was the fact that chromophore production was stable for a relatively long period and the absorbance produced was reproducible.

RC-1 was defined as a 0.5 M solution of α-KGA made up in 6 N HCl which was reacted with equal amounts of solutions containing free tryptophan at 40 C for 48 h.

RC-2 was defined as a 0.05 M solution of α-KGA made up in 6 N HCl which was reacted with equal amounts of solutions containing free tryptophan at 80 C for 24 h.

Effect of oxidizing agents

Many of the early methods developed for analysis of tryptophan involve the reaction between tryptophan and various aliphatic or aromatic aldehydes in acid media. One of the most successful reactions utilizes p-dimethylaminobenzaldehyde (p-DMB). It has been theorized that the reaction involves two separate reactions. The first one is a fast condensation reaction between tryptophan and p-DMB. The second reaction is a slow oxidation of this colorless condensation product which forms the chromophore. This reaction has been accelerated through the addition of oxidizing agents, NaN0₂ or light (4-9).

Since α-KGA contains a carbonyl group and the interaction between it and tryptophan is a slow one which
is catalyzed by acid, the possibility existed that this reaction was similar to the p-DMB reaction. Therefore, various oxidizing agents were tested to determine their possible use in speeding up the reaction time.

Effect of NaNO₂ and NaClO₄·H₂O. Figure 10 illustrates the effects that different concentrations of NaNO₂ and NaClO₄·H₂O have on the reaction involving RC-2 and tryptophan. NaClO₄·H₂O had no effect as can be seen by the fact that despite the various amounts and concentrations of NaClO₄·H₂O added, the amount of chromophore production was identical to that produced when water alone was added.

When various concentrations of NaNO₂ were added, there was an increase in absorbance which was almost proportional to the amount of NaNO₂ added.

Since the NaClO₄·H₂O produced no change in absorbance, it was hypothesized that the reaction involving α-KGA and tryptophan was dissimilar to the p-DMB tryptophan reaction. The NaNO₂ was thought to have some other effect on the reaction which was not due to its oxidizing ability.

Effect of other oxidizing agents. Several other oxidizing agents were tested for their effect on chromophore production. Also, nitrate ions and KNO₂ were tested to determine if the increased absorbance was due to the action of the nitrite ions.

Table 1 reveals that production of increased absorbance is due to the nitrite ion. Both NaNO₂ and KNO₂ elicit the same behavior whereas NaNO₃, KNO₃, and other oxidizing agents have no effect.

Effect of Increasing Concentrations of NaNO₂. Figure 10 suggests that there might be an upper limit to the amount of NaNO₂ added that will effect any further change in absorbance. This hypothesis was tested by adding more concentrated solutions of NaNO₂ to a reaction mixture. Table 2 illustrates that no upper limit was found. In fact the absorbance was found to proceed off-scale when increased amounts of NaNO₂ were added. Therefore, nitrite ions were considered to be an interfering substance and were not a means to speed up the reaction time.

This investigation showed that by the appropriate manipulation of pH, temperature, time and concentration of α-KGA, reproducible amounts of a stable chromophore could be produced. These results indicate that a procedure for quantitative analysis of tryptophan might be developed on this basis in future work.

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<th>% NaNO₂</th>
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aO = addition of H₂O

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