DEVELOPMENT OF PROCEDURES FOR THE DETERMINATION OF TRYPTOPHAN

by

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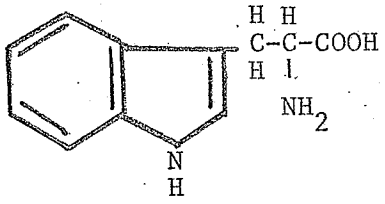
ABSTRACT

Experiments were designed to develop a reliable method for the determination of tryptophan by means of a modified Hopkins-Cole test. The most reliable procedure developed was to hydrolyze samples in 4 N NaOH in an autoclave from 15 minutes to 6 hours. The hydrolyzate was adjusted to a pH of approximately 7.0 and diluted to 25 ml with distilled water. This was centrifuged and from the supernatant 1 ml and 2 ml aliquots were tested by the following modified Hopkins-Cole test: The aliquots were placed in a 25 ml beaker in an ice bath. Then acetic acid-iron glyoxylic acid solution and sulfuric acid were added with constant agitation. The colored solution that developed was removed from the ice bath and allowed to stand at room temperature for 15 minutes. Tryptophan was determined spectrophotometrically at 545 mμ. Results obtained for tryptophan in casein were 1.1%, Soy X 0.86%, Broiler Strontium 60M, Ration 0.48%, and Egg 0.73%.

INTRODUCTION

The biological importance of tryptophan as an essential amino acid and the difficulties involved in its accurate quantitative determination account for some of its contentious history over the years. Since its discovery by Hopkins and Cole in 1901, it has been analyzed and investigated many times, often leading man to error in seeking a reliable method of determination. No chemical tests have, as yet, proven 100% satisfactory. Of course, inroads into its very nature have been made over the years.

Tryptophan, alpha-amino beta-indole propionic acid, has an empirical formula, $C_{11}H_{12}O_2N_2$, and contains 64.69% carbon, 5.92% hydrogen, 15.67% oxygen, and 13.72% nitrogen, with a molecular weight of 204.23. Its structural formula is:



Tryptophan is the only natural amino acid containing an indole nucleus. It is the ability of this nucleus to form colored compounds with a variety of reagents upon which most of the chemical determinations for tryptophan have been based. Its reaction with aromatic aldehydes, such as p-dimethylaminobenzaldehyde or vanillin, is typical of this property of the indole nucleus. Thus, all my research on

tryptophan was made in this direction. All procedures were based on a modification of the Hopkins-Cole reaction and with various changes.

Tryptophan is easily destroyed in an acid medium, hence it cannot be readily liberated intact from proteins by acid hydrolysis for determination on an amino acid analyzer. Thus, the reason a search for other reliable methods for its determination has continued.

REVIEW OF THE LITERATURE

Colored derivatives of proteins were reported as early as 1831 by Tiedemann and Gmelin and by Bernard in 1856. In 1874, Adamkiewicz reported a striking color reaction of proteins when he mixed glacial acetic acid with a solution of albumin and treated with concentrated sulfuric acid. Hopkins and Cole, in 1901, first isolated tryptophan and for a characteristic test they used the one of Adamkiewicz (1874). They proved that the formation of color was due to the presence of glyoxylic acid in the acetic acid used.

The announcement by Hopkins and Cole (1901) triggered many investigations for the accurate determination of tryptophan, most of which proved unreliable or worthless. Several modifications of the Hopkins-Cole test that proved somewhat successful were reported by Cary (1926), Brice (1933), Winkler (1934), and Shaw and McFarlane (1938). In 1934, Winkler showed that small amounts of copper sulfate greatly enhanced the amount of color given by the Hopkin's glyoxylic acid method. Four years later Shaw and McFarlane (1938) further improved the analysis by hydrolyzing the protein with 5 N sodium or barium hydroxide; or by dissolving the protein in 10 to 20% NaoH or in 5% formic acid before reaction with glyoxylic acid. In 1960, Fischl developed a mofidication by using persulfate or peroxide to oxidize acetic acid in the presence of thioglycolic acid. The Polish workers, Opienska-Blauth, Charezinski, and Berbec (1963) described a method in which iron in the presence of

H_2SO_4 converted acetic acid to glyoxylic acid, which in turn produced the color of the Hopkins-Cole reaction.

In 1905, Voisenet showed that indole, skatole, etc., gave highly colored solutions when treated with KNO_3, concentrated HCl and various aliphatic and aromatic aldehydes. That same year, Rhode (1905) reported that proteins gave a red color with 5% p-dimethylaminobenzaldehyde (Ehrlich's Reagent) in 10% H_2SO_4 when concentrated HCl was added. Other aromatic aldehydes such as vanillin and p-nitrobenzaldehyde can be used in place of Ehrlich's reagent. Thus it was Voisenet (1905) who pointed out that oxidizing agents, such as KNO_3, increase color formation; while Thomas, in 1920, demonstrated the importance of meticulously-controlled conditions not only with respect to reagents but temperature as well. Since that time more tryptophan procedures have been based on reactions with aldehydes than with any other reactant. A few modifications of the Voisenet-Rhode (1905) method that followed were those of Furth and Dische (1924), Komm (1926), Tomiyama and Shigematsu (1934), Rapoport and Eichinger (1936), and Sullivan and Hess (1944).

Graham and Smith (1947), Spies and Chambers (1948, 1949), and Spies (1950, 1967, 1968) reported modifications of the p-dimethylaminobenzaldehyde method with both hydrolyzed and intact proteins using light or sodium nitrite for oxidation. Since H_2S and chloride and bisulfite ions interfere in the Spies dimethylaminobenzaldehyde method, he eliminated this interference by the appropriate use of silver sulfate.

Because tryptophan is destroyed in an acid medium and proteins are usually hydrolyzed in acid before their determination on an ion exchange amino acid analyzer, this method has not proved very satisfactory in analyzing for tryptophan. However, Matsubara and Sasaki (1969) discovered that moderate concentrations of thioglycolic acid greatly decreased tryptophan's destruction and yielded recoveries of 80-90%. Mercaptoethanol has also proved somewhat effective in this direction. However, neither compound has produced the results needed for complete recovery of tryptophan.

Microbiological methods for tryptophan analysis are laborious, time consuming and often less precise than chemical methods, although chemical and biological methods sometimes agree closely. However, microbiological assays have been used extensively since the middle 1940's with mutants of Escherichia coli, Bacillus subtilis, and Streptococcus zymogenes. Rather than employing microorganisms for bioassay, Harwood and Schrimpton (1969) used chick growth as an index of tryptophan present in intact protein.

To date most procedures have inherent disadvantages with respect to accuracy, reproducibility, sensitivity, convenience, and general applicability to the determination of tryptophan present in soluble and insoluble proteins, feeds, foodstuffs, and tissues. Hence a need still exists for a fast, accurate, generally applicable method for determining tryptophan. A possible solution suggested by Friedman and Finley (1971) is a specific chemical modification of tryptophan residues in proteins so that they can be determined in an acid hydrolysate

simultaneously with the other amino acids by ion-exchange or gas-liquid chromatography, or individually by mass spectroscopy. Several attempts of this kind have been made. Koshland and Barman (1967) have used 2-hydroxy-5-nitrobenzyl-bromide as a reagent for tryptophan which lead to two types of tetracyclic indoline derivatives. However, whether similar compounds would result from tryptophan residues in proteins has not been proven. Tryptophan residues are oxidized by proflavin-sensitized photo-oxidation to kynurenine (Galiazzo and Scaffone 1968; Lapuk, Christyakova, and Kravchenko 1968). Since kynurenine has an absorption maximum near 360 mu and also can be eluted from the column of an amino acid analyzer as a discrete peak before lysine, conditions could be devised for quantitative transformation of tryptophan to kynurenine residues which could be estimated spectrophotometrically or by ion-exchange chromatography.

These authors suggested a tryptophan determination measuring quantitatively the acid degradation products of tryptophan, although at present the identity of these products is not known.

ESTABLISHING A STANDARD CURVE

A modified Hopkins-Cole test was used for the determination of tryptophan. This test utilizes the violet color reaction produced in an acid medium between the indole ring and an aldehyde. In the preliminary trials, glycolic acid was substituted for the glyoxylic acid. This was done because it was speculated that glyoxylic acid was oxidized to glycolic acid which then formed a chromophore with tryptophan. The chemical formula for glycolic acid is $HO-CH_2-COOH$ and that of glyoxylic acid is $O=\overset{H}{C}-COOH$. Also necessary for the experiment were the following:

1. 50 mg D-L tryptophan in 1 liter distilled H_2O--Standard
2. Acetic Acid Solution:
   0.27 gms $FeCl_3$ dissolved in 0.5 ml of H_2O and diluted with glacial acetic acid to 1 liter
3. Concentrated sulfuric acid (sp gr 1.84)--18M
4. 50 mg glyoxylic acid in 50 ml of acetic acid solution
5. 50 mg glycolic acid in 50 ml of acetic acid solution

In this procedure various aliquots of tryptophan were diluted to 2 ml with distilled H_2O. To these was added 4 ml of the acetic acid-Fe+++ glyoxylic (or glycolic acid) solution, followed by 4 ml of concentrated sulfuric acid. The samples were shaken vigorously and allowed to stand for 15 minutes.

First attempts at establishing a standard curve at an optical density of 545 mμ on a Coleman Junior Spectrophotometer, Model 6A, were erratic and inconsistent (Figure 1, curves 1, 2, 3, 4, 5, and 6). On curve 6 plain acetic acid was heated to the boiling point with no worthwhile effects. Also, fresh acetic-iron solutions were made but with no better results.

In the next two attempts to establish a standard curve a 10 mg or 50 mg portion of glyoxylic acid was added to 50 ml of acetic-Fe+++ solution. The resulting curves were again erratic. Six more determinations were attempted with glycolic acid but with little success. In one of these tests added butanol gave no noticeable effect.

Up to this time the tryptophan standard and the acetic-iron glycolic acid solution (or glyoxylic acid), plus H_2SO_4 were combined and immediately put into a 10 ml volumetric flask and allowed to stand for 15 minutes. At this point the procedure was changed. The reaction mixture was allowed to stand in a 50 ml beaker for 15 minutes. This caused an increase in optical density, probably due to increased oxidation in the open beaker.

Next, the entire reaction mixture was chilled in ice water until the time for reading on the spectrophotometer at 545 mμ. The result was a drop in absorpbancy. Also, at the same time glyoxylic acid was substituted for glycolic acid and was used throughout the remainder of the experiments. The original Hopkins-Cole test called for glyoxylic acid.

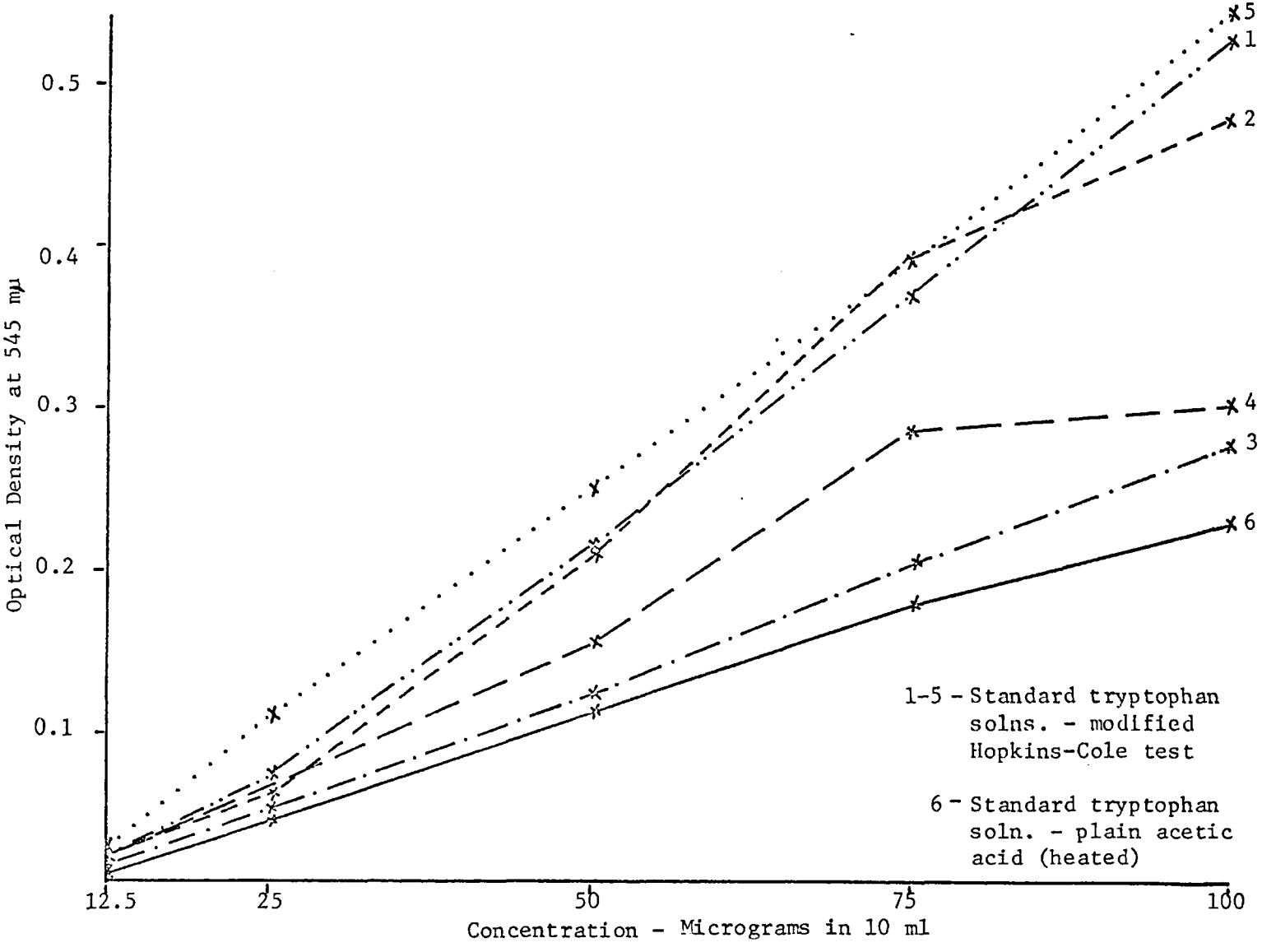


Figure 1. Standard Tryptophan Solutions

The next step was to chill ONLY the acetic-Fe+++ glyoxylic acid mixture; and the final reaction mixture was allowed to stand for 15 minutes at room temperature. This procedure seemed to give the most consistent results (Figure 2, curves 1 and 2). Undoubtedly this chilling prevented the oxidation of the glyoxylic acid in the acetic-Fe+++ solution before its reaction with tryptophan and H_2SO_4. Henceforth, this procedure was followed throughout all the experiments.

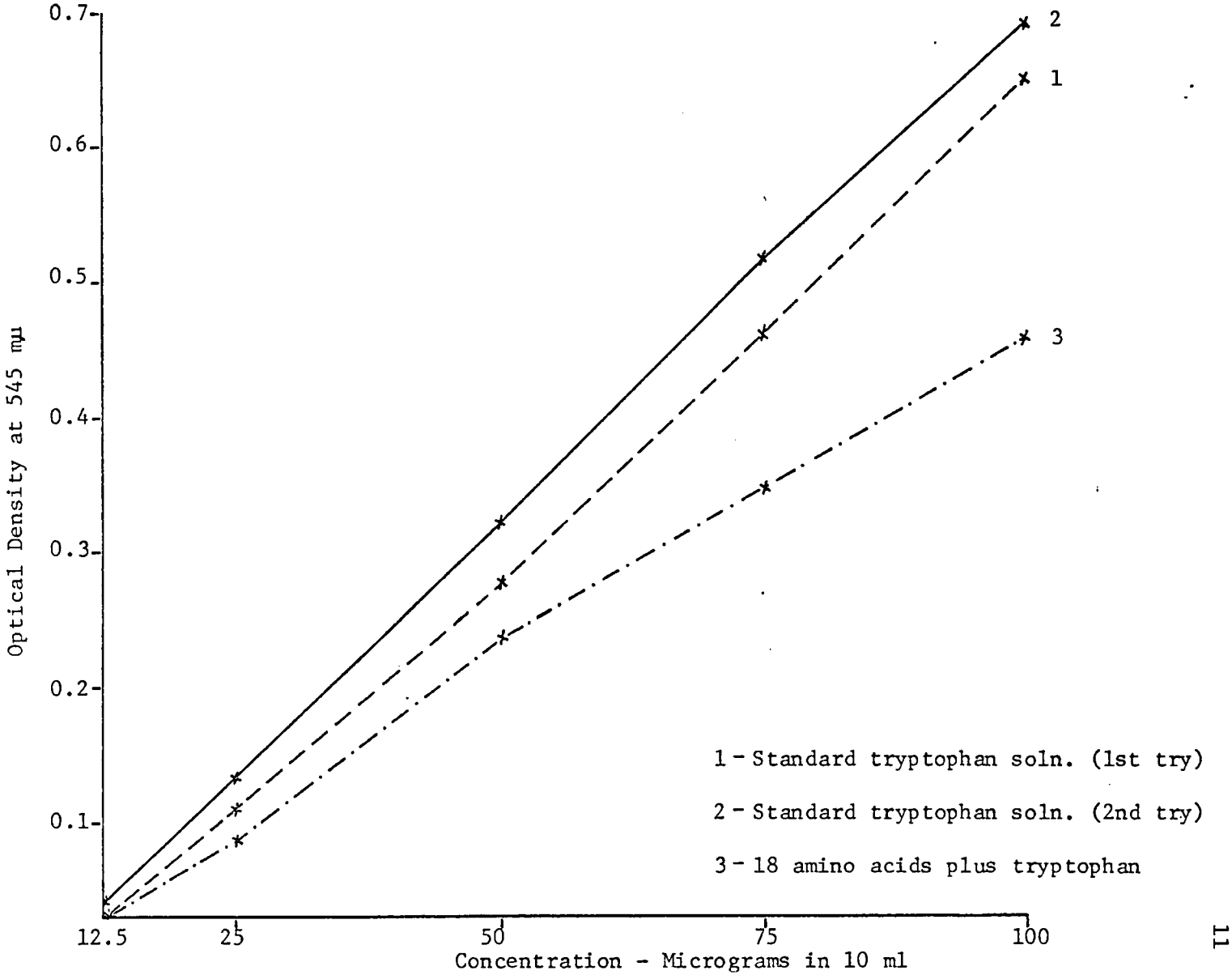


Figure 2. Standard Tryptophan Curves Plus Amino Acid Curve

THE EFFECT OF CARBOHYDRATE UPON THE PROCEDURE

To determine the effects of carbohydrate upon this procedure 10 mg sucrose and 50 mg tryptophan were dissolved in a 1000 ml H_2O solution. A linear relationship (Figure 3, curve 1) was achieved but with a somewhat lowered tryptophan value. Next, one gram of sucrose was added to the standard tryptophan solution. There was a marked change in the curve (Figure 3, curve 2) which probably was due to browning, which occurred when H_2SO_4 was added to the tryptophan-sugar-acetic-Fe+++ glyoxylic acid solution. In order to prevent browning, the above solution was chilled while adding the H_2SO_4. A fairly consistent curve was the result (curve 3). In order to improve upon this, the solutions were agitated during the addition of the H_2SO_4. This procedure resulted in the most consistent curve achieved (curve 4). Hence, if browning was completely eliminated by cooling and agitation a consistent curve for tryptophan was obtained (curve 4).

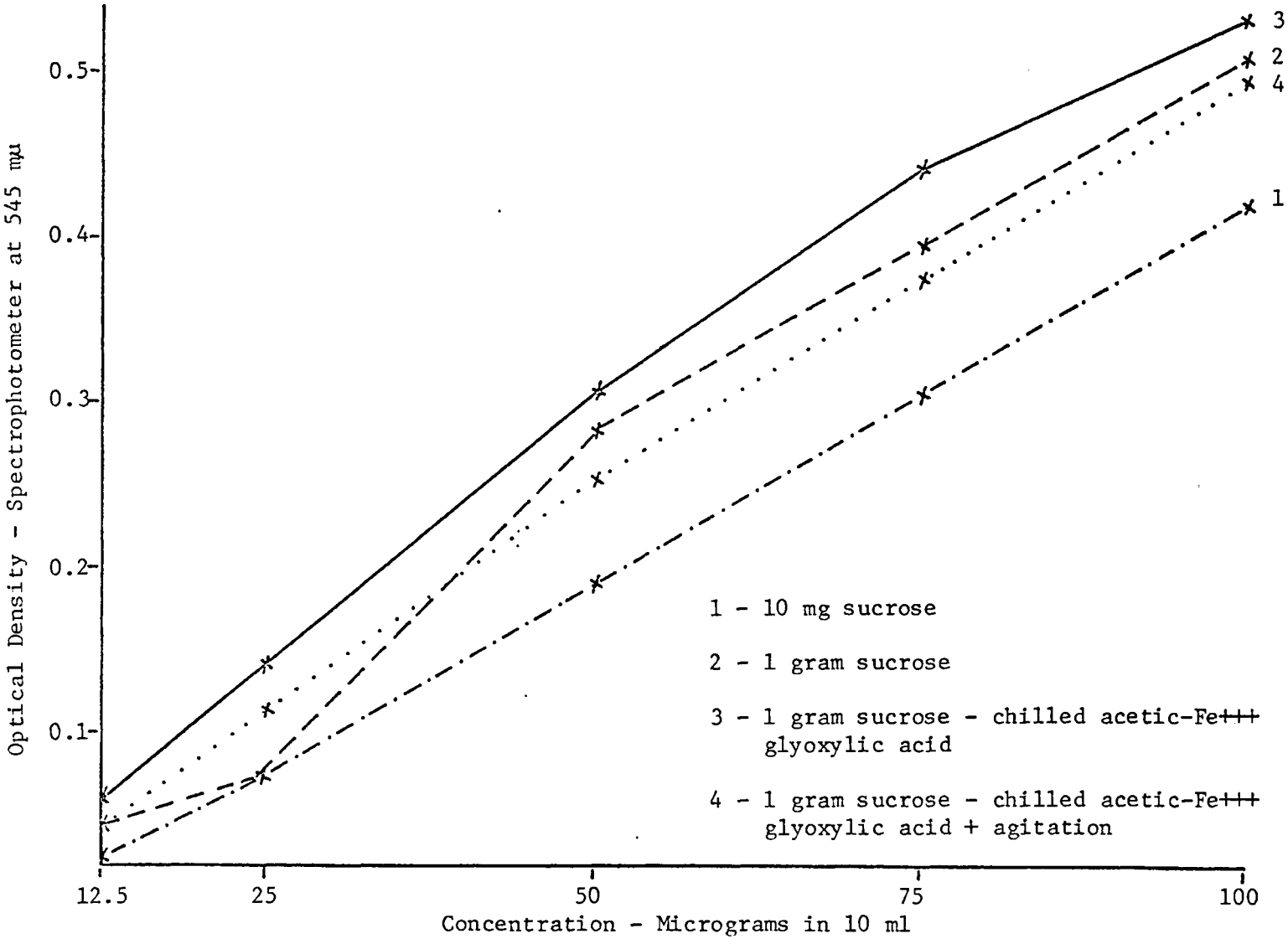


Figure 3. Tryptophan Plus Sucrose Standard Curve

ANALYSIS FOR TRYPTOPHAN IN VARIOUS FOODS AND FEEDS

The first experiment was to determine the effect of other amino acids upon the assay. Ten mg each of 17 amino acids plus 10 mg of tryptophan were dissolved in water and diluted to 200 ml. The amino acids were:

arginine
alanine
aspartic acid
cystine
glutamic acid
glycine
histidine
isoleucine
leucine
lysine
methionine
phenylalanine
proline
serine
threonine
tyrosine
valine

The result of this test was satisfactory with a fairly straight curve obtained (Figure 2, curve 3) and a 105% recovery of tryptophan. However, this curve had a lesser slope than the curve for the tryptophan standard alone.

The second experiment was to determine the amount of tryptophan in casein. A great deal of inconsistency occurred. Most of these tests were made with 100 mg of casein hydrolyzed in 10 ml NaOH solution, varying in normality from 1-4. The best results were obtained with a 4 N solution. The casein samples were hydrolyzed in an autoclave at 15 lbs pressure (approximately 121 degrees C) in covered 50 ml beakers for periods ranging from 15 minutes to 17 hours. After autoclaving,

the mixture was adjusted to a pH of 6 to 7. No definite conclusions could be reached, since no consistent estimations for tryptophan were obtained (Table 1).

Also, 100 mg of casein was autoclaved for two hours with 4 N $Ba(OH)_2$, the barium precipitated with H_2SO_4, and then neutralized and diluted to 25 ml. The tryptophan value was low. At another time various concentrations of acetic acid were used: 50%, 25%, and 10% in place of the NaOH solution. Very low tryptophan values were obtained. These mixtures were hydrolyzed for 15 minutes; therefore, it is doubtful that any tryptophan was destroyed. The protein probably did not go into solution.

Attempts were made with dried egg, Soy X, and Broiler Strontium 60M ration. One hundred mg of each was autoclaved for 15 minutes in 10 ml of a 4 N NaOH solution. Each sample was neutralized and diluted to 25 ml before being analyzed for tryptophan. Considerable browning resulted upon the addition of the acetic-Fe+++ glyoxylic acid and sulfuric acid.

Next attempts were made with the hydrolysis for 15 minutes of dried egg in an alkaline solution. The first test made was not effective as considerable egg had not dissolved. This was corrected by placing the egg-NaOH mixture in a large test tube, then breaking the egg up with a stirring rod and covering the test tube with aluminum foil so that it would not boil out during the autoclaving. Also, the mixture was allowed to stand for 20 minutes before autoclaving in order to dissolve the egg, but there was no noticeable improvement. Results

Table 1. Test on Casein

Optical Density at 545 Millimicrons							
Autoclave: 15 minutes		Autoclave: 1 hour		Autoclave: 2 hours		Autoclave: 3 hours	
1 N NaOH							
1 ml	2 ml	1 ml	2 ml	1 ml	2 ml	1 ml	2 ml
0.170	0.327	0.194	0.395	0.170	0.401	0.181	0.359
0.174	0.369	0.142	0.332	0.175	0.388		
0.178	0.409						
2 N NaOH							
0.215	0.370			0.181	0.380	0.180	0.414
0.194	0.340			0.169	0.352	0.181	0.382
0.242	0.391					0.186	0.400
4 N NaOH							
0.193	0.309	0.226	0.465	0.163	0.385	0.210	0.420
0.215	0.313	0.198	0.405	0.190	0.390	0.163	0.359
0.235	0.430	0.152	0.356				

were inconsistent. When plain acetic acid was used in place of the acetic-Fe+++ glyoxylic acid mixture, results were low. Thus, in order to improve the procedure various concentrations of Na_2SO_3 were added to the egg mixture. The concentrations varied from 20 mg to 100 mg added before autoclaving, and 40 mg and 50 mg portions after autoclaving. This procedure improved results, with the best results occurring with the addition of Na_2SO_3 after hydrolysis. Forty to 50 mg of sodium sulfite seemed to be the best amount to use (Table 2).

In autoclaving Soy X and testing for tryptophan results were higher than should be expected, and there was considerable browning. Addition of 50 mg, 100 mg, and 200 mg of Na_2SO_3 reduced the browning (Table 2). Hence, the Soy X acetic-Fe+++ glyoxylic acid mixture was put in an ice bath and agitated upon the addition of H_2SO_4. This procedure prevented browning although there was no appreciable change in results in the few tests that were run. No more tests were done at this time; hence this testing is incomplete and inconclusive.

Time did not permit adequate testing on Broiler Strontium 60M. Several of the tests that were made on Broiler Strontium 60M with sodium sulfite caused less browning. No conclusion can be reached as to the correct amount of Na_2SO_3 to be added although at this time the addition of 40 mg after hydrolysis appeared to give the best results. This is consistent with the amounts and method in the previous two samples. A standard curve was made using the standard tryptophan solution plus 20 mg as well as 50 mg of Na_2SO_3. The modified Hopkins-Cole procedure was used and the results can be seen in Figure 4. It appears that Na_2SO_3 reduced the amount of color formed.

Table 2. Test on Egg, Soy X, and Broiler Strontium X

Egg			Soy X			Broiler Strontium X		
Na_2SO_3 added	% Tryptophan		Na_2SO_3 added	% Tryptophan		Na_2SO_3 added	% Tryptophan	
None	0.44	0.59	None	0.81	0.73	None	0.47	0.40
	0.56	0.65		0.81	0.75		0.54	0.54
	0.63	0.55		0.75	0.73		0.55	0.47
Na_2SO_3 added before hydrolysis								
20 mg	0.50	0.44	50 mg	1.37	1.14	40 mg	0.84	0.81
40 mg	0.64	0.53	50 mg	1.23	1.22	100 mg	0.90	0.88
60 mg	0.53	0.47	100 mg	1.23	1.14	200 mg	0.90	0.78
80 mg	0.47	0.40	200 mg	1.24	1.20			
100 mg	0.69	0.59						
Na_2SO_3 added after hydrolysis								
40 mg	1.30	1.17	40 mg	1.62	1.60	40 mg	1.06	0.94
50 mg	1.11	1.04	50 mg	1.37	1.20			

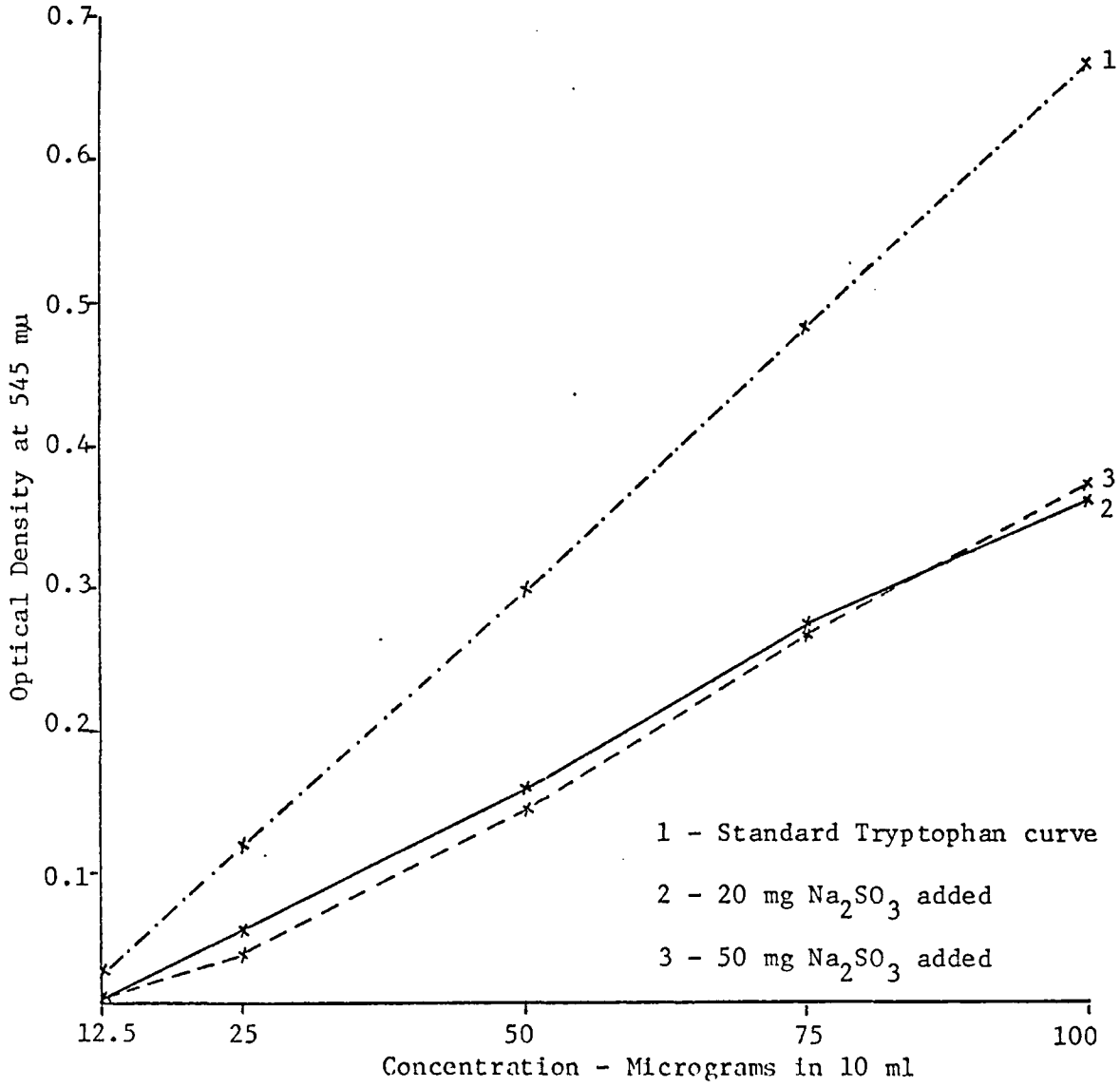


Figure 4. Tryptophan Plus Sodium Sulfite

In another attempt to eliminate browning completely, 10% mercuric sulfate in 5% sulfuric acid was used to precipitate the tryptophan. This was added to 1 ml and 2 ml aliquots of the pH adjusted hydrolyzate. In order to determine the correct amounts of $HgSO_4$ solution to use, tests were made on standard tryptophan solutions. Best results were obtained with 4 ml of mercuric sulfate solution and 4 drops of H_2SO_4. Upon their addition to the tryptophan solution a good precipitate was formed. The mixture had to be centrifuged approximately 15 minutes at 1000 RPM in order to separate the precipitant. After separation, the Hopkins-Cole test was made on the precipitate. To determine whether this procedure could be improved, the tryptophan-mercuric sulfate mixture was put in a hot water bath for 5, 10, and 15 minutes. Most consistent results occurred with 10 minutes of heating (Figure 5).

Further tests were made on casein with varying and inconclusive results. Considerable cloudiness occurred upon the addition of the $HgSO_4$-H_2SO_4 to the casein hydrolyzate. When heating in a hot water bath, centrifuging, and following with a Hopkins-Cole test, results were high and inconsistent. Evidently salts were formed so that no consistency or accuracy could be established.

Thus the final testing was done by merely hydrolyzing the food or feed to be tested in a 4 N NaOH solution and bringing this mixture up to a pH near 7 and diluting with distilled H_2O to 25 ml. Since a precipitate was formed in every solution when returning it to a near neutral pH, it was centrifuged for 30 minutes. The resulting clear

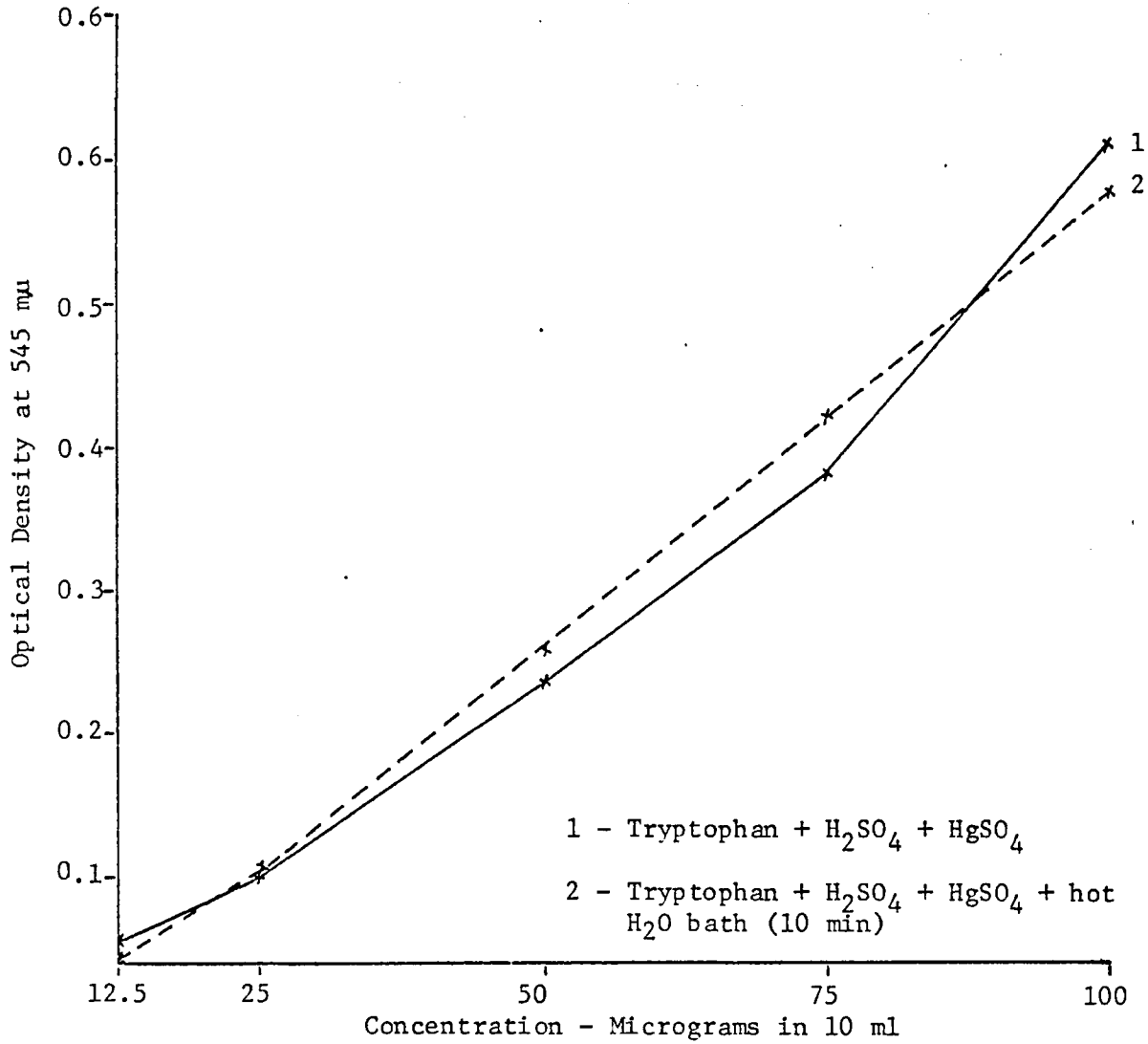


Figure 5. Tryptophan Plus Mercuric Sulfate Plus Sulfuric Acid

supernatant was tested via a modified Hopkins-Cole test. This included doing all procedures in an ice bath plus constant agitation of the resultant mixtures. This prevented all browning which especially had occurred in the Broiler Strontium 60M, Soy X, and egg mixtures unless these steps were taken. However, after the addition of the acids, the solutions were allowed to stand at room temperature for 15 minutes. The curve obtained with this procedure on a standard tryptophan solution is shown in Figure 6. Results for the various compounds are in Table 3.

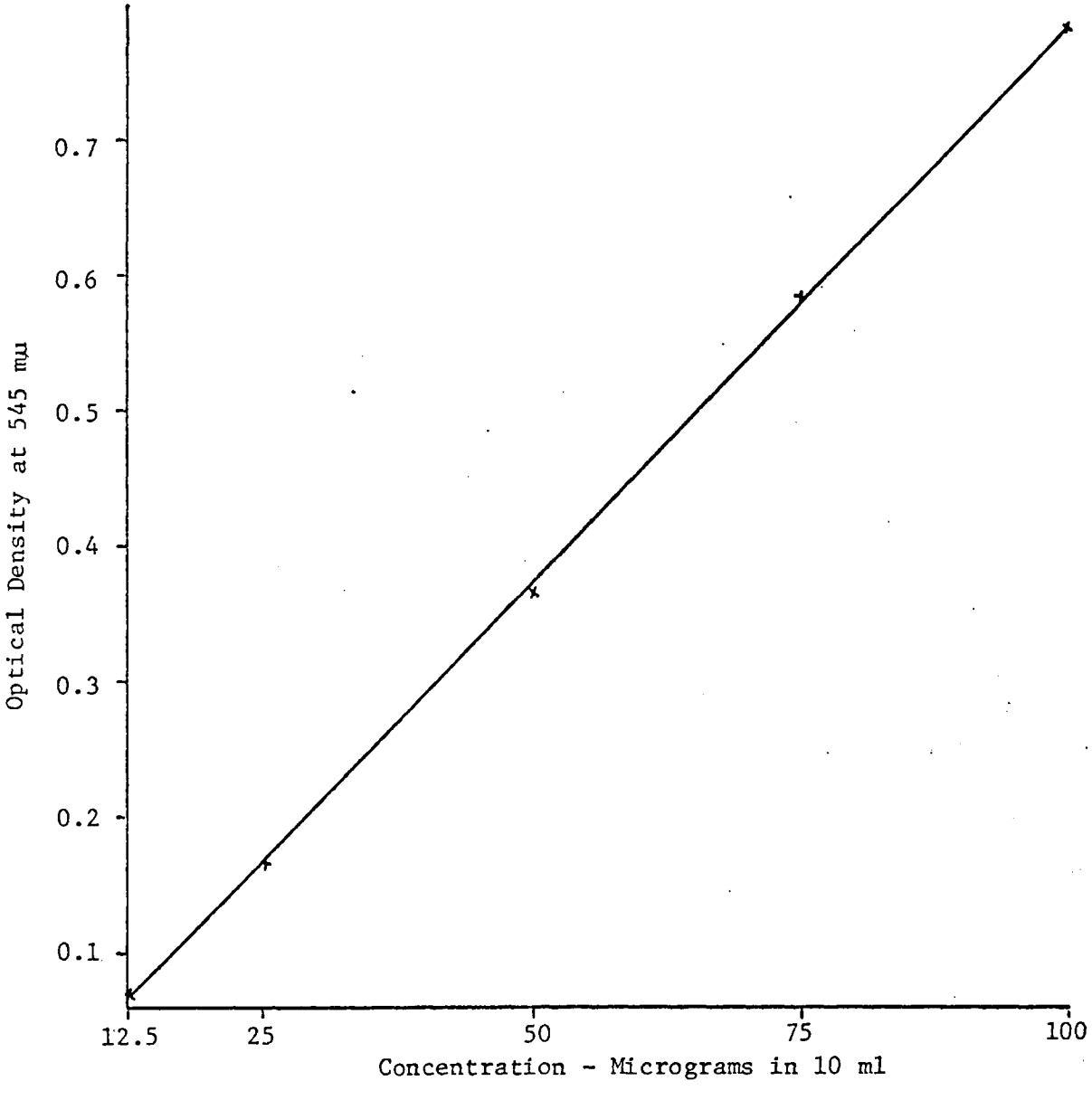


Figure 6. Standard Tryptophan Solution--Ice Bath Plus Constant Agitation

Table 3. Tryptophan in Foods or Feeds

Food or Feed	pH	Hydrolysis (minutes)	Optical Density 1 ml	% Tryptophan	Optical Density 2 ml	% Tryptophan
Casein*	6.9	15	.170	0.625	.449	0.75
	6.8	30	.182	0.67	.478	0.79
	6.9	120	.252	0.89	.620	1.02
	7.0	210	.250	0.89	.632	1.03
	6.6	240	.327	1.13	.700	1.13
	6.5	360	.300	1.05	.620	1.02
Soy X	6.9	15	.191	0.70	.465	0.76
	6.3	30	.223	0.79	.507	0.84
	7.0	120	.225	0.81	.530	0.88
	7.0	210	.219	0.78	.498	0.83
	6.5	240	.212	0.77	.448	0.75
	6.6	360	.240	0.86	.535	0.88
Broiler Strontium 60M Ration	6.5	15	.117	0.47	.207	0.38
	7.0	30	.108	0.44	.226	0.41
	6.8	60	.120	0.48	.222	0.40
	7.0	120	.101	0.41	.228	0.41
Egg** (dried)	6.8	15	.178	0.65	.400	0.67
	6.8	30	.169	0.63	.367	0.63
	6.7	60	.198	0.72	.431	0.72
	7.0	120	.193	0.71	.404	0.68

* Casein contains approximately 1.2% tryptophan.

** Egg contains approximately 0.9% tryptophan.

CONCLUSION

The foregoing tests have proved that the quantitative estimation of tryptophan is still consistently inconsistent. However, that it can be controlled and improved has been shown. Each time some controllable factor was added, i.e., sodium sulfite, mercuric sulfate plus sulfuric acid, an ice bath, agitation, more consistent results were obtained for the amount of tryptophan present. Of these the ice bath plus constant agitation of the mixture proved to be the most reliable. Although the tryptophan value for egg did not produce anticipated results (0.9% tryptophan), all resulting values were in a narrow range, hopefully proving that the testing was accurate and that this just may be the true tryptophan value in this dried egg product. A near possible true value for tryptophan in casein (1.1%) was achieved upon hydrolyzing for four hours. No values for tryptophan content of the Soy X and Broiler Strontium 60M are known, therefore no comparisons could be made. However, in the procedure of an ice bath plus agitation all browning was eliminated while with the addition of sodium sulfite this was not so.

Thus, what seems to be necessary is the prevention of tryptophan reacting with other elements in a solution to form unknown compounds. This occurred most rapidly when the hydrolyzed tryptophan from a food or feed had the acid added to it. For it was at this point that browning occurred, and in certain foods and feeds more than in others.

However, the ice bath plus constant agitation prevented this from happening, and therefore, seemingly allowed for more accurate analysis of tryptophan content.

Since tryptophan is an extremely labile amino acid, it is undoubtedly undergoing constant change while in solution; hence the reason for the variable results. The fact that these results fell into a somewhat narrow margin with the ice bath and agitation procedure is encouraging.

It was observed that tryptophan loss after hydrolysis due to air oxidation is slow and relatively small, but large and rapid losses occur when a strong acid, such as H_2SO_4, is added. Tryptophan alone does not show this marked change, but when other compounds are present, such as carbohydrate, its destruction occurs very rapidly.

Hence, with carefully controlled conditions and testing as rapidly as possible, after hydrolysis, a fairly accurate analysis for tryptophan was obtained. To make this 100% accurate tests undoubtedly should be controlled under very rigid conditions.

Method for Tryptophan Analysis

The most reliable procedure developed from this was the following: 100 mg of a food or feed was hydrolyzed for 15 minutes to 6 hours in 10 ml of a 4 N NaOH solution. (Time of hydrolysis depended upon the food or feed.) The pH was adjusted to approximately 7. This was brought up to 25 ml with distilled H_2O and centrifuged for 20-30 minutes. One ml and 2 ml aliquots of the supernatant were taken for a Hopkins-Cole test. This consisted of bringing the 1 ml supernatant up to 2 ml

with H_2O and placing in an ice bath, followed by addition of 4 ml of the acetic-Fe+++ glyoxylic acid with constant agitation. Then 4 ml of sulfuric acid was added with constant agitation. The mixture was removed from the ice bath and allowed to stand at room temperature for 15 minutes. Reading on a spectrophotometer was at 545 mμ.

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