CLXXVII. THE COLORIMETRIC DETERMINATION OF THE TYROSINE AND TRYPTOPHAN CONTENT OF VARIOUS CRUDE PROTEIN CONCENTRATES.

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IN the course of investigations on poultry nutrition, it was desired to know the tyrosine and tryptophan content of the protein of buttermilk powder, fish meal, cod-liver meal, and two abattoir by-products, meat meal and tankage. A rapid and reliable method for the estimation of tyrosine and tryptophan in food-stuffs would be an extremely valuable asset, not only in regard to our particular problem, but in a multitude of nutritional investigations.

At the present time it is doubtful whether any of the known methods for determining the tyrosine, and particularly the tryptophan, content of purified proteins are absolutely reliable. Onslow [1924], summarising the values given for the tryptophan content of caseinogen by different investigators, has pointed out the great discrepancies in the results obtained. Though the colorimetric methods have sometimes given low values, and are open to the usual objections to colorimetric methods in general, they are the only methods which could be satisfactorily applied to the determination of tyrosine and tryptophan in a routine way, such as we contemplated.

It was hoped that after making as thorough a purification of these materials as was possible, results, of sufficient accuracy to compare the tyrosine and tryptophan content of one material with another, might be obtained by colorimetric methods. Fürth and Lieben [1921] and Ide [1921], using a colorimetric method based on Voisenet's reaction [1905], have determined the tryptophan content of many of the common foodstuffs. The unreliability of the Voisenet formaldehyde reaction has been shown by Hunter and Borsook [1923], who state that glycytryptophan gives a far deeper and redder colour than pure tryptophan. This reaction is thus affected both as to hue and intensity, not only by the presence of tryptophan, but by the mode of combination of tryptophan in the protein molecule. Kretz [1922] in endeavouring to use this method for localising tryptophan in plant cells has concluded that it did not give accurate results because of the susceptibility of the reaction to many destructive influences. The colorimetric method of Folin and Ciocalteu [1927]

for determining tyrosine and tryptophan commended itself. The two determinations are combined into one procedure, which is reasonably rapid, and the accuracy has been subjected to considerable study in so far as its application to purified protein is concerned. Whether accurate determinations of the tyrosine and tryptophan content of these crude protein materials could be made by this method, or by other methods discussed in the text, remained to be determined.

EXPERIMENTAL.

Representative samples of these protein supplements were prepared for analysis as follows. They were ground in a Wiley mill to pass through a 100 mesh sieve and then dried *in vacuo* at 100° and 5 mm. for 24 hours. The dried samples were then extracted with sodium-dried ether in a large Soxhlet extractor until fat-free, which usually took about 48 hours. The residues were placed in 250 cc. centrifuge bottles and extracted with two volumes of absolute alcohol by shaking for 12 hours. After centrifuging, the alcohol was decanted and the extraction repeated three times as before. The residues were again dried in the vacuum oven and finally stored in tightly stoppered bottles. All subsequent weighing of samples for analysis was made by weighing the stoppered tube before and after the transfer. Total nitrogen was determined (Kjeldahl) and all subsequent determinations are expressed as % of the total crude protein ($N \times 6.25$). The tyrosine and tryptophan determinations were first made by Folin and Ciocalteu's method. A sample of purified caseinogen on repeated analysis was consistently found to contain 6.62 % tyrosine and 1.37 % tryptophan. The values obtained by Folin and Ciocalteu for a highly purified caseinogen were 6.55 % tyrosine and 1.40 % tryptophan.

In each case a weighed quantity of the protein material representing the equivalent of 1 g. of crude protein was used in duplicate determinations. The alkali digests, excepting the caseinogen digest, were highly coloured and in the subsequent determinations of tyrosine it was found to be impossible to make accurate colour comparisons against the standard solution in the colorimeter. It was then decided to decolorise the hydrolysates by shaking with 1 g. of kaolin for 12 hours and centrifuging. By similar treatment of standard solutions of tyrosine and tryptophan it was determined that no loss of these amino-acids by absorption took place. While norite and animal charcoal made much better decolorising agents, a very considerable loss, particularly of tyrosine, was found to take place. The results of the determinations on the decolorised hydrolysates, expressed as % of the total crude protein ($N \times 6.25$), were as follows:

	Tyrosine	Tryptophan
Meat meal	2.93	1.34
Tankage	3.02	0.67
Fish meal	2.61	1.30
Cod-liver meal	—	2.04
Buttermilk powder	5.46	1.80

With the phenol reagent (tryptophan) the colour comparison with the unknown and the standard was excellent in every case and duplicate determinations gave practically the same results. With Millon's reagent (tyrosine) the colour comparison was not perfect, the unknown having a yellowish red colour and being slightly cloudy, while the standard was bright red in colour and perfectly clear. The readings were made as carefully as possible but leave some doubt as to their accuracy. It was found impossible to determine the tyrosine content of the cod-liver meal protein by this procedure as, on boiling the tyrosine-containing solution with HgSO_4 and cooling, the solution became very cloudy. Careful regulation of the acidity of the solution at this point, varying the acidity in repeated determinations, or carefully avoiding undue delay in making the colour comparisons, did not prevent this fine grey cloudy precipitate from forming with the other hydrolysates. Making the solution (after heating with HgSO_4) up to volume with $N \text{ H}_2\text{SO}_4$ instead of water slightly improved the readings, especially in the case of the fish meal hydrolysate.

Hanke [1928], in a criticism of Folin and Ciocalteu's method for determining tyrosine, maintains that tyrosine cannot be determined by this method when cystine is present. Cystine gives a precipitate and this precipitate contains tyrosine. He has proposed a method for determining tyrosine which consists in hydrolysing the protein with H_2SO_4 and then removing the H_2SO_4 with baryta. The tyrosine is precipitated, even from a mixture of amino-acids and such other substances as might be present in a protein hydrolysate, by boiling in acetic acid solution with mercuric acetate and treating the mixture with NaCl . The mercury is removed by H_2S and the tyrosine determined in the filtrate colorimetrically with Millon's reagent, as in the Folin and Ciocalteu procedure. More accurate determinations of tyrosine in these crude protein materials might be expected by actual precipitation of the tyrosine in the hydrolysate, rather than by a colorimetric determination in the presence of all possible interfering substances.

The tyrosine content of these proteins was next determined by Hanke's method with the following results:

	% tyrosine	
	Hanke's method	Folin and Ciocalteu's method
Caseinogen	6.59	6.62
Meat meal	2.35	2.93
Tankage	2.51	3.02
Cod-liver meal	3.60	—
Fish meal	2.64	2.61
Buttermilk powder	5.28	5.46

In every case perfect colorimeter readings were obtained by this method. Excepting in the fish meal proteins, the tyrosine determination by Folin and Ciocalteu's method was, in every case, higher than that obtained by Hanke's method. Higher values were almost invariably obtained by Hanke, using Folin and Ciocalteu's procedure on purified proteins.

The tyrosine content of meat meal, tankage, fish meal and cod-liver meal is

much lower than that of the milk proteins. Ingvaldsen [1929] reports for fish meals from different sources and processed at different temperatures a tyrosine content of the proteins ($N \times 6.25$) varying from 3.85 to 4.43 % determined by Folin and Ciocalteu's method. He does not report encountering any difficulties in the application of this method to fish meal hydrolysates. With the possible exception of tankage, these protein materials appear to be well supplied with tryptophan when compared with the value for caseinogen. The question still remains as to whether these figures represent the actual tryptophan content of these materials.

Hanke [1928] states that "a crude protein hydrolysate, either acid or alkaline, appears to contain something other than tyrosine or tryptophan that reacts with both the phenol reagent and Millon reagent of Folin and Ciocalteu. This may be one substance or there may be several; but whatever it is, it does not react in an identical manner with both reagents." In criticising the use of the phenol reagent for determining tryptophan, Kraus [1925] has pointed out that indole and skatole give a blue colour with the phenol reagent so that decomposition products of tryptophan would also be included. Indole and skatole, however, are extremely soluble in ether, and were they present in the original materials as the result of putrefaction they would have been removed in the preparation of these products for analysis. It is possible, however, that in the alkali-digestion of these crude protein materials considerable reduction of tryptophan may take place. Indole and skatole, according to Kraus, may be removed quantitatively from the hydrolysates by extraction with toluene.

The original decolorised hydrolysates were next extracted with toluene by continuous shaking for 12 hours, allowed to stand for 2 hours in separating funnels and then separated from the toluene. That tyrosine and tryptophan are not removed from solution by toluene was determined by a similar treatment of standard solutions. The tyrosine and tryptophan contents of the toluene-extracted hydrolysates were next determined by Folin and Ciocalteu's method with the following results:

	In original decolorised hydrolysate		After extraction of hydrolysate with toluene	
	Tryptophan	Tyrosine	Tryptophan	Tyrosine
Caseinogen	1.37	6.62	1.23	6.55
Meat meal	1.34	2.93	0.90	2.31
Tankage	0.67	3.02	0.59	2.92
Fish meal	1.30	2.61	1.17	1.83
Cod-liver meal	2.04	—	0.77	—
Buttermilk powder	1.80	5.46	1.51	5.40

A considerable reduction in the values for the tryptophan and tyrosine content in all cases was found. A reduction in the apparent tryptophan content of cod-liver meal of 1.27 % was very surprising. If the substances giving the reactions of tryptophan (*i.e.* precipitated in acid solution with $HgSO_4$ and giving a blue colour with the phenol reagent) but removable with toluene, are indole and skatole, it would seem that they must have been produced during the alkali-digestion of the protein material.

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It was next decided to determine if tyrosine and tryptophan added to these protein materials could be recovered quantitatively in the alkali hydrolysates. Weighed quantities of Pfanstiehl C.P. tyrosine and tryptophan were added to weighed quantities of the prepared meat meal and fish meal as used in the previous determinations. The alkali-digestion and the subsequent determination of tyrosine and tryptophan were carried out as before. The results were as follows:

	Tyrosine		Tryptophan	
	Added (mg.)	Recovered (mg.)	Added (mg.)	Recovered (mg.)
Meat meal	45.0	40.3	4.0	3.37
Fish meal	40.1	44.0	6.4	7.0

It will be observed that in some cases more tyrosine and tryptophan was recovered than had been added. This can only be explained by the slight improvement in the readings obtained where tyrosine and tryptophan had been added. In these readings the colour of the unknown was more nearly proportional to the colour developed by the standard solution. The results, however, indicate that there was no appreciable destruction of added tyrosine or tryptophan during the alkali-digestion of these two crude protein materials. Results obtained after extraction of the meat meal and fish meal hydrolysate with toluene gave practically the same recovery of added tyrosine and tryptophan as recorded above. Evidently, only negligible amounts of decomposition products of tryptophan or tyrosine soluble in toluene were formed as the result of alkali-digestion.

The next endeavour was to determine what the substance (or substances) present in the alkali hydrolysates of these materials was, which gave the reactions of tryptophan but which could be extracted by toluene. The toluene extracts of all the hydrolysates gave a blue colour of varying intensity with the phenol reagent. The blue colour was particularly deep in the case of the toluene extract of the cod-liver meal hydrolysate, and least in the toluene extract of the caseinogen hydrolysate. Evidently the unknown substance was present in the greatest amount in the hydrolysate of cod-liver meal. That the original cod-liver meal contained no indole was determined by applying Bergeim's [Hawk and Bergeim, 1927] method for the quantitative determination of indole in faeces. Negative results were also obtained on the alkali hydrolysate of cod-liver meal.

The toluene extract of these hydrolysates gives Millon's reaction, which indicates that this unknown substance may be phenolic. Negative results were obtained for free and conjugated phenols by Tisdall's [1920] modification of the Folin and Denis method. Uric acid gives a blue colour with the phenol reagent; it is soluble in NaOH but is precipitated by H_2SO_4 . However, sufficient remains in solution to give a fairly heavy precipitate with HgSO_4 . While uric acid was found to be insoluble in toluene it may be that some other purine base, or some pyrimidine base, set free by decomposition of nucleoprotein is

contributing to the blue colour given by tryptophan. The actual purine content of the ox-liver as reported by Vogel [1911] is only 0.099 %.

While the colour given by tyrosine with the phenol reagent is a different shade of blue from that given by tryptophan, the colour given by indole is identical with that given by tryptophan. The comparative solubilities of indole did not indicate that, even if indole were present in the hydrolysates, it would increase the tryptophan reading. To confirm this 0.0833 g. indole was added to 0.9327 g. caseinogen and the determination of tryptophan carried out in the usual manner. A reading representing 2.46 % tryptophan was obtained while caseinogen contains 1.37 % tryptophan. The apparent tryptophan content of caseinogen was increased by 1.09 % by adding 8.9 % indole. The presence of indole in the hydrolysates would, undoubtedly, increase the tryptophan reading. The colour given by Millon's reagent, in determining tyrosine in presence of indole, was not rose-pink but a brownish yellow, and was somewhat typical of the colour given by the crude protein hydrolysates with Millon's reagent. Inorganic substances, such as ferrous iron or any sulphite, produce a deep blue colour with the phenol reagent. It would seem, as Gortner and Holm [1920] have pointed out, that any substance which is easily oxidised will react with the phenol reagent and produce a blue colour.

That some substance is present in these alkali hydrolysates which shows the reactions of tryptophan, but which evidently is not this amino-acid, has been reported by Hanke [1928] and by Kraus [1925], independently, and is confirmed by the above findings. The evidence does not point to this substance as being indole as Kraus has concluded. Until this unknown chromogenic substance has been identified, the results obtained after extraction of these crude protein hydrolysates with toluene would appear to represent the true tyrosine and tryptophan content of these protein materials.

An endeavour was made to determine the tryptophan content of these materials by the method of Komm [1926], based upon the reaction of tryptophan with *p*-dimethylaminobenzaldehyde. A value for caseinogen of 2.39 % tryptophan was obtained. While the tryptophan standard gave a uniform blue colour, the colour given by caseinogen was more of a purplish tinge. The colour comparison was not perfect and as a result the figure for caseinogen is probably too high. All the other determinations were a complete failure, owing to the deep brown colour produced on adding concentrated H_2SO_4 which prevented any possible colour comparison.

THE TYROSINE AND TRYPTOPHAN CONTENT OF THESE CRUDE PROTEIN MATERIALS AS DETERMINED BY ENZYMIC HYDROLYSIS.

A weighed quantity of the crude protein material (approximately the equivalent of 1 g. crude protein) was digested for 10 hours at 37° in 50 cc. 0.1 *N* HCl containing 0.2 % pepsin. The digest was neutralised with 5 cc. *N* NaOH and 5 cc. of a 6 % solution of trypsin in 0.1 *N* NaOH added. Digestion was then continued for 24 hours. Toluene was added as a preservative. The

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enzyme activity was destroyed by heating on a water-bath for 5 minutes at 75°–80°. The digests were then acidified with H_2SO_4 , diluted to 100 cc. and filtered. The addition of a small amount of kaolin greatly facilitated obtaining a clear filtrate. Tyrosine and tryptophan determinations were then made on an 8 cc. aliquot of the filtrate by Folin and Ciocalteu's procedure. Correction was made for the tyrosine and tryptophan content of the enzyme blank, obtained by adding known amounts of standard solutions of tyrosine and tryptophan to the blank pepsin-trypsin digest and so determining the tyrosine and tryptophan content of the latter by difference. The following results were obtained:

	Amino-N %	Tyrosine %	Tryptophan %
Caseinogen	8.31	7.59	1.54
Buttermilk powder	7.60	6.36	1.25*
Meat meal	4.97	3.02	0.95
Tankage	4.06	2.42	0.88
Cod-liver meal	2.25	—	0.77
Fish meal	5.43	2.92	1.38

* This solution was persistently cloudy and accurate colour comparison was extremely difficult. The result is, therefore, very doubtful.

A much higher reading was obtained for caseinogen by this method than was obtained by alkali-hydrolysis. The results obtained for meat meal were somewhat the same as before, while the tryptophan content of tankage was slightly higher. Fish meal would seem to contain considerably more tryptophan than meat meal and tankage. It was interesting to find that the tryptophan content of cod-liver meal, as determined by this method, was exactly the same as that obtained after extracting the alkali hydrolysate with toluene. This may have been simply a coincidence, but the fact that the results were very much of the same order suggests strongly that the unknown chromogenic substance present in the alkali hydrolysate of cod-liver meal in particular was formed during alkali-digestion.

Ragins [1928, 1] has recently reported the perfecting of her method for the determination of tryptophan by means of the vanillin-HCl method, so that it may be applied to proteins. She claims that this reaction for tryptophan is more sensitive than other colour reactions and that it is more specific. The method has been applied with evident success to the tryptic hydrolysates of highly purified proteins. An endeavour to determine the tryptophan content of these crude protein materials by this method and to compare the results with those obtained by Folin and Ciocalteu's method on the same hydrolysate was next made.

1 g. of the protein material and 0.25 g. U.S.P. trypsin were weighed into 100 cc. Erlenmeyer flasks containing 25 cc. 0.4 % Na_2CO_3 . The solutions were preserved with toluene and incubated for 5 days at 37°. The enzyme was destroyed by heating at 75°–80° on the water-bath for 5 minutes. The digest was then acidified with 1 cc. 14 *N* H_2SO_4 , diluted to 100 cc. and filtered. Tryptophan determinations were then made on an 8 cc. aliquot of the digest by Folin and Ciocalteu's method and on 3 cc. by the vanillin-HCl method.

	Tryptophan %		Remarks on colour with vanillin-HCl reagent
	Phenol reagent	Vanillin- HCl	
Caseinogen	1.25	1.30	Not identical with the standard but good colour comparison
Meat meal	0.52*	—	Reddish blue colour, impossible to match but qualitative indication that tryptophan was present
Tankage	—*	—	Red colour—no indication of blue colour
Cod-liver meal	1.21*	—	Deep brownish red colour
Fish meal	0.59*	—	Reddish blue colour as with meat meal digest
Buttermilk powder	1.87	1.73	Colour identical with that developed with caseinogen digest

* 2 cc. standard tryptophan solution (0.59 mg. tryptophan) were added to 8 cc. of the digest and tryptophan determined by difference. In the tankage digest the same amount of tryptophan was recovered as was added. The filtrate from the HgSO_4 precipitate in every case gave a negative test with the Hopkins-Cole reagent.

Tryptophan determinations by the phenol reagent in the tryptic digest of meat meal and fish meal were much lower than the previous results obtained by peptic-tryptic digestion. The preliminary digestion with pepsin resulted in a very considerable increase in the amount of tryptophan liberated. The negative result with the tankage digest, and more particularly the higher value obtained for cod-liver meal, would seem to be impossible to interpret.

Except in the case of the milk products, the results with the vanillin-HCl reagent were a uniform failure even when the determinations were repeated after decolorising the hydrolysates with kaolin. Some other substance present was found to develop a red colour with the reagent which made colorimeter readings impossible. The result for caseinogen is in fair agreement with that obtained by Ragins, *i.e.* 1.23 % tryptophan.

Waterman and Jones [1921, 1922] have found their determinations of the relative digestibility of proteins *in vitro* to correspond with protein digestibility trials *in vivo*. It was next decided to repeat these digestions by their method and to determine the tryptophan content.

0.5 g. of the protein material was digested for $1\frac{1}{2}$ hours at 37° in 50 cc. 0.1 *N* HCl containing 0.2 % pepsin¹. The digest was neutralised with 5 cc. *N* NaOH and 5 cc. of a 6 % solution of trypsin¹ in 0.1 *N* NaOH added. Digestion was continued for $2\frac{1}{2}$ hours. The enzyme was destroyed by heating on a water-bath for 5 minutes at 75° – 80° . The digest was then acidified with 1 cc. 14 *N* H_2SO_4 , diluted to 100 cc. and filtered. A 3 cc. aliquot was taken for the amino-nitrogen determinations (Van Slyke) and a 5 cc. aliquot for the tryptophan determinations by the vanillin-HCl procedure. Tryptophan in concentrations of less than 0.2 mg. in a 5 cc. aliquot cannot be precipitated quantitatively under the conditions described. Therefore, 2 cc. of the standard tryptophan solution (the same amount as the standard) were added to each digest and tryptophan determined by difference, allowance being made for the tryptophan determined in the blank enzyme digest. The colours developed by the digests were much the same as already described; the interfering red colour making

¹ Products of the Digestive Ferments Co., Detroit, Mich.

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accurate comparison with the standard impossible. It was found that by placing a blue-green (minus red) filter, which only admitted light of wave lengths 3800 Å.-5800 Å., between the colorimeter lamp and the Duboscq colorimeter excellent colorimeter readings could be obtained. The results were as follows:

	Amino-N %	Tryptophan %
Caseinogen	4.99	1.72
Buttermilk powder	3.18	2.95
Meat meal	3.96	1.78
Tankage	2.86	0.92
Fish meal	4.08	1.62

The results obtained were much higher than in all previous determinations but, comparatively, they fell into the same order as before. Preliminary digestion of these crude protein materials with pepsin, followed by trypsin, and limitation of the duration of digestion have given higher values for tryptophan liberated. Ragins [1928, 2] has found that treating the purified proteins which she has studied (caseinogen, edestin and squash seed-globulin) first with pepsin and then with trypsin, does not hasten or increase the liberation of tryptophan; also that only the enzymes in U.S.P. pancreatin liberate tryptophan from the proteins studied. Frankel [1916] found that proteins after having been acted upon by pepsin are much more readily digested by trypsin or erepsin. No definite interpretation can be placed on the results obtained with these crude protein materials until much more comprehensive experiments, determining the rate at which tryptophan is liberated with changing time of peptic and tryptic digestion, have been made. Excepting caseinogen and buttermilk powder, a considerable amount of these crude protein materials remains in suspension during enzyme digestion, so that accurate results cannot be obtained by removing aliquots as digestion proceeds.

SUMMARY.

1. The tyrosine and tryptophan content of the proteins of buttermilk powder has been found to be much higher than that of the other crude protein materials investigated. No very appreciable difference in the tyrosine content of fish meal, cod-liver meal, meat meal and tankage was found.

2. Conflicting results as to the tryptophan content of the proteins of fish meal and meat meal, depending upon the method of determination, have been found. In general, the tryptophan content of fish meal was found to be higher than that of meat meal. The tryptophan content of tankage has invariably been found to be much lower than that of fish meal and meat meal.

3. The limitations in the methods described for determining tryptophan leave some doubt as to the actual tryptophan content of any of these materials, particularly in the case of the cod-liver meal proteins. The alkali digest of these crude protein materials contains some substance, or substances, precipitable by HgSO_4 and giving a blue colour with the phenol reagent which, unlike tryptophan, is soluble in toluene. This unknown chromogenic substance does

not appear to be indole as Kraus has concluded. The results obtained after extraction of the alkali hydrolysates with toluene would appear to represent the true tyrosine and tryptophan content of these protein concentrates.

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