ON TYROSINE AND TRYPTOPHANE DETERMINATIONS IN PROTEINS.

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I. Preliminary Observations on the Merits and Limitations of Tyrosine and Tryptophane Determinations by Means of the Phenol Reagent.

The original purpose of this investigation was to subject the Folin-Looney (1) methods for tyrosine and tryptophane determinations to a more critical study than they have yet received. A number of investigators have found those methods satisfactory, but others have condemned them on general principles, and some have been utterly unable to get any reasonable figures with them. In the meantime, the analytical figures obtained by the Folin-Looney methods have been used extensively by Cohn (2) in his calculations of the molecular weights of proteins. In these circumstances it seemed well worth while to try to clear up any uncertainties or flaws that may legitimately be ascribed to those methods. But the original purpose of an investigation may be almost lost and forgotten before a research is finished. Such has been our experience in this instance.

An elementary yet a most important question which must

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always be determined in connection with any colorimetric method is that concerning the amount of the unknown substance which can be determined with a given quantity of the chromophoric reagent. This question is usually covered by studies of the range of true proportionality obtainable from different amounts of the pure substances to be determined—in this case, tyrosine and tryptophane.

In studies of the range of true proportionality obtainable in any quantitative color reaction, it is often important to try to find out the reason why the proportionality fails to hold true beyond a given point. If the failure is due merely to unequal dilution of the colored compound, then it is usually not possible to increase the range by altering the conditions. It is comparatively seldom that one encounters the dilution phenomenon as a cause of limited proportionality within the ranges measured by the ordinary colorimeters, when the standard is set at 20 mm. The two most important causes of limited proportionality are, first, that the chemical reaction between the reagent and the substance to be determined is not quite quantitative, and, second, that the reagent used by itself yields a color.

In practical colorimetry one usually has to be satisfied with a true range of proportionality between 66 per cent and 150 per cent of substance when the standard, frequently 1 mg., is taken as 100 per cent. In the methods of Folin and Looney this range of proportionality is very nearly attained when working with pure tyrosine or tryptophane. There are two reasons why the proportionality is limited to this range; the reaction between tyrosine or tryptophane and the phenol reagent is not quite complete, and the reagents alone yield an appreciable amount of blue color. It is necessary to discuss these two factors so as to make the situation clear.

The active ingredient in the phenol reagent, a phosphotungstic phosphomolybdic acid of the 1:18 series, according to Wu's nomenclature, is very unstable and quickly decomposed in alkaline solutions, and it reacts with tyrosine only in solutions sufficiently alkaline to bring about the rapid destruction of the reagent. From this combination of circumstances it follows that a relatively enormous excess of the reagent must be used in order to get complete reaction and a maximum amount of color from a given quantity

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of tyrosine or tryptophane. For this reason, 0.5 mg. of tyrosine will give somewhat more than one-half as much color as is obtained from the standard, 1 mg., and 2 mg. will give a little less than twice as much.

In order to overcome or reduce this limitation one would naturally try to use greater amounts of the phenol reagent. But if materially larger amounts than those employed by Folin and Looney are used, precipitates and turbidities similar to those encountered in uric acid determinations are obtained, and it is often very difficult to secure perfectly clear filtrates from such mixtures. The formation of the insoluble sodium salts, representing the turbidity, can fortunately be prevented in this case, just as in the case of the uric acid reagent, by the addition of lithium salts.

The phenol reagent containing the requisite amount of lithium sulfate is prepared as follows:

Transfer 100 gm. of sodium tungstate, $Na_2WO_4 \cdot 2H_2O$, and 25 gm. of sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$, together with 700 cc. of water to a 1500 cc. Florence flask. Add 50 cc. of 85 per cent phosphoric acid and 100 cc. of concentrated hydrochloric acid. Connect with a reflux condenser by means of a cork or rubber stopper wrapped in tin-foil, and boil gently for 10 hours. At the end of the boiling period add 150 gm. of lithium sulfate, 50 cc. of water, and a few drops of liquid bromine. Boil the mixture, without the condenser, for about 15 minutes to remove the excess bromine. Cool, dilute to 1 liter, and filter. The finished reagent should have no greenish tint, as this means the presence of blue reduction products which will lessen the range of true proportionality between different *small amounts* of tyrosine or tryptophane. The reagent should be kept well protected against dust, as organic materials will gradually produce slight reductions.

By means of the phenol reagent described above we have solved the turbidity difficulty and can now use practically any desired amount of reagent. 5 cc. of this reagent seem to give the maximum color obtainable, and with 5 cc. one obtains perfectly clear solutions and true proportionality in color from 1 mg. of tyrosine, the standard, and 4 mg. of tyrosine on the one hand or 0.5 mg. on the other.

From the figures shown in Table I it will be seen that the color reaction obtainable from the phenol reagent and pure tyrosine is one of the most perfect reactions to be found in the field of modern colorimetry, when the color reaction is developed under suitable

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conditions. In these determinations the standard tyrosine was kept in approximately 2 N sulfuric solution, and to equalize the acidity in the flasks when different volumes of the standard were

TABLE I.

Illustrating the Proportionality of Color Obtained from Different Amounts of Tyrosine.

The standard, 1 mg., was set at 20 mm.

Tyrosine taken.	Volume.	Colorimetric reading.	Tyrosine found.
mg.	*cc.*	*mm.*	*mg.*
0.25	50	39.6	0.255
0.50	100	39.4	0.51
0.7	100	28.2	0.71
0.8	100	25.0	0.80
1.4	100	14.3	1.40
1.6	100	12.6	1.59
1.8	100	11.0	1.82
2.0	100	10.0	2.0
2.2	100	9.1	2.2
2.4	100	8.4	2.38
2.6	100	7.7	2.6
2.8	100	7.1	2.8
3.0	100	6.7	2.98
4.0	100	5.0	4.0

TABLE II.

Showing That True Proportionality Is Obtained between Colors Derived from Widely Different Amounts of Tryptophane.

Tryptophane found.	Colorimetric reading.	Tryptophane found.
mg.	*mm.*	*mg.*
0.5	39.4	0.508
0.75	26.9	0.744
1.50	13.3	1.50
2.00	10.0	2.0
3.00	6.7	2.98
4.00	5.0	4.0

taken all were diluted to 5 cc. with 2 N sulfuric acid. 40 cc. of water and 25 cc. of sodium carbonate were then added and finally 5 cc. of the phenol reagent. 30 minutes were allowed for the development of the color.

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Similar experiments were made with tryptophane. Here, also, as may be seen from the figures recorded in Table II, the proportionality is perfect within the same wide range, 0.5 mg. and 4 mg.

It has already been mentioned that the reaction between the phenol reagent and tyrosine is inevitably accompanied by much destruction of the phenol reagent by the alkali. But the reaction between tyrosine and the phenol reagent is more rapid than the reaction between tryptophane and the reagent. A larger proportion of the reagent is, therefore, necessarily destroyed by the alkali and lost when one is working with tryptophane. This difference is adequately provided for in the determinations recorded in Tables I and II by the use of 5 cc. of the phenol reagent. With only 2 cc. of the reagent, and particularly when working in the presence of smaller volumes of water, the destruction of the reagent is so rapid that maximum color and a wide range of proportionality cannot be obtained with tryptophane.

Under the conditions employed by Folin and Looney, 1 mg. of tryptophane gives about 58 per cent of the color obtained from 1 mg. of tyrosine. But under the new conditions, involving the use of 5 cc. of phenol reagent, we obtain more color from tryptophane, in fact the maximum color; and we now find that 1 mg. of tryptophane gives from 88 per cent to 92 per cent of the color given by 1 mg. of tyrosine. One sample of tryptophane gave us consistently 88.5 per cent of the color obtained from tyrosine. The molecular weight of tyrosine, 181.17, is 88.7 per cent of the molecular weight of tryptophane, 204.17. The theoretical chromophoric value of tryptophane in terms of tyrosine should, therefore, be 88.7 per cent. It is clear, therefore, that we are now securing the theoretical color values within remarkably small limits of experimental error.

The finding of this value should leave no room for doubt about the fact that the chemical reaction taking place between the phenol reagent and tyrosine or tryptophane is a thoroughly sound one as a basis for quantitative measurements.

This theoretical value should also be practically useful. Tyrosine is easily obtained or can be bought, is readily purified, and in acid solutions keeps indefinitely. Tryptophane, on the other hand, is very expensive if it can be bought at all, is seldom obtained strictly pure, and standard solutions of tryptophane deteriorate

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appreciably in the course of a few weeks. By means of pure standard tyrosine solutions it is now possible to assay with accuracy the purity of a given tryptophane preparation or the tryptophane content of a given solution. Tyrosine standards in fact can now be used for the estimation of the tryptophane in the protein hydrolysates.

Our tyrosine was recrystallized as follows:

Transfer 20 gm. of tyrosine to a Florence flask (capacity 2 liters). Add 200 cc. of N hydrochloric acid and 200 to 400 cc. of water. Heat to about 70°C., and shake until the tyrosine has dissolved. Add more water, 500 to 800 cc., and shake the solution with 2 to 3 gm. of bone-black which has been washed with dilute hydrochloric acid. Filter.

To the filtrate add the calculated quantity of sodium bicarbonate, a little at a time, with shaking. The tyrosine comes down at once. Filter on a Buchner funnel and wash with water.

The moist tyrosine is then dissolved again in a known quantity of N hydrochloric acid (150 cc.), and water as before. The solution this time should be water-clear. Neutralize as before and filter.

This process can be repeated if desirable without involving much loss of tyrosine, but two recrystallizations should be enough, unless the starting material is very impure.

The wide range of true proportionality between different amounts of tyrosine and tryptophane shown in Tables I and II cannot be obtained unless the reagents employed for developing the color fail to yield any perceptible color in blank, control experiments. When 5 cc. of our phenol reagent are added to a mixture of 5 cc. of 5 per cent sulfuric acid, 40 cc. of water, and 25 cc. of sodium carbonate solution, a water-clear colorless solution is obtained as soon as the reagent is destroyed by the alkali. This ideal outcome is unfortunately not attainable under the conditions prescribed by Folin and Looney. Even the very best grades of sodium cyanide which may give no trace of color with the uric acid reagent always give some color when used with the phenol reagent.

The cyanide is so handy and so useful a feature of the Folin-Looney methods that we have made many attempts to circumvent its undesirable color-producing properties, but without success. The more we have worked with sodium cyanide the more doubtful have we become as to the wisdom of retaining it in these particular methods. The trouble with the cyanide is not only

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that the best grades give a color with the phenol reagent, but the poorer grades give much more, and will thus prove an incalculable source of error. The complete failure of the Folin-Looney methods in the hands of J. Warkany, for example, whose work is reported by Fürth (3), is almost certainly due to the use of an exceptionally unsuitable sample of sodium cyanide.

One particularly treacherous feature of the cyanide in this connection is that the blue color which it gives with the phenol reagent alone is no criterion as to the amount of color it will give in the presence of a mercuric salt. 4 cc. of 5 per cent sodium cyanide solution plus 1 cc. of 10 per cent mercuric sulfate solution will give several times as much color as is obtained in the absence of the mercury. Curiously enough, almost any other metallic salt, such as cadmium sulfate, and even zinc sulfate, has the same effect of greatly increasing the blue which sodium cyanide yields with the phenol reagent.

Notwithstanding these serious drawbacks to the cyanide, it is possible to use it and obtain excellent analytical results; but as it stands, the method probably can never become a standard method, because it does not adequately represent the well nigh perfect character of the underlying color reaction.

II. On the Hydrolysis of Proteins with Alkalies.

At first and in fact for a long time we were not able to secure the same tryptophane values as have been reported by Folin and Looney, except by materially altering the conditions for the precipitation of the tryptophane with mercuric sulfate. The reasons for these divergent results were finally cleared up, but only after considerable work. In the course of this study I finally decided to reexamine the hydrolysis process. For the hydrolysis of the protein for tryptophane determinations, Folin and Looney used the technique recommended by Annie Homer; namely, 48 hours boiling with 25 cc. of a 14 per cent solution of crystallized barium hydroxide per gm. of protein. While all the tryptophane is probably set free by this process it is, of course, a fact, recognized by Homer, that this relatively mild process does not produce complete hydrolysis. By adding a very little copper acetate at the end of 48 hours boiling, one obtains an intense, heat-stable, biuret reaction. One can then continue the boiling for at least 2 days

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more before the biuret reaction finally disappears. It is obvious that one cannot obtain a uniform degree of hydrolysis in 48 hours under such conditions. The result will depend on the speed of the boiling and on the amount of barium hydroxide rendered inert by absorption of carbonic acid and by combination with silicic acid from the flask. The presence of peptones is most conveniently proved by adding 5 cc. of the phenol reagent to 5 cc. of hydrolysate. There seemed to be excellent reason for using just this hydrolysis. From Homer's (4) work, and Herzfeld's (5), it would appear that barium hydroxide is the only alkali that can be used for the hydrolysis of proteins without destroying tryptophane. Both of these investigators found that sodium carbonate, even in weak concentration (0.5 per cent) produces notable destruction of tryptophane, and Herzfeld found that 9 per cent sodium hydroxide is very destructive. Fürth later found that even barium hydroxide will destroy pure tryptophane, although it does not destroy it in the protein hydrolysate, while Kraus (6) claims that even in the hydrolysates there is some tryptophane destruction—with indole formation.

Tryptophane is far more stable in alkaline solutions and less stable acid solutions than has heretofore been recognized. The only practical limit as to the strength of alkali which may be used for the hydrolysis of proteins for tryptophane determinations, is the loss of material through holes in the Kjeldahl flasks.

For the hydrolysis process described below one must use clean, *new*, Kjeldahl flasks of Pyrex glass. The only reason why Pyrex flasks are best is that they are so thick that they usually can withstand one such boiling experiment. The used flasks are perfectly good for other purposes, but cannot be used for a second prolonged digestion with sodium hydroxide. If the glass were not porous these flasks could probably be used a great many times, because the bottoms are seemingly as thick and strong as ever. But the alkali always seems to find capillary channels through which it etches invisible, but disastrous leaks.

By means of a long slender test-tube transfer into a *new*, clean, *dry* Kjeldahl flask (250 cc.) about 1 gm. of thoroughly dried protein material. The exact weight is obtained by weighing the tube before and after the transfer. Then introduce into the flask 2 cc. of butyl alcohol (to prevent foaming), a couple of short spirals made

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from silver wire or silver foil (to prevent bumping), and finally 4 gm. of sodium hydroxide, in the form of 20 per cent solution. Insert into the neck of the flask a condenser made from a test-tube of such a size that it fits very loosely, yet rests firmly on the flask by means of its flange.

The mixture should be boiled for 18 to 20 hours. For this boiling it is inadvisable to apply the flame directly to the bottom of the flasks. One may succeed that way, perhaps a dozen times in succession, but it may also happen that two or three consecutive digestions are ruined because of leaks. Some form of improvised air bath should be used to secure an even application of heat. An iron crucible (diameter 7 cm.) is satisfactory. The boiling will continue perfectly smoothly, if the silver coils are right and provided that the condenser continues to function so that the butyl alcohol is not lost. It is not necessary to boil hard.

At the end of the boiling period, remove the condenser, add 10 cc. of water, and continue the boiling for 10 minutes to remove the alcohol. Then remove the flame and, from a pipette, add immediately to the hot solution, drop by drop, but rather fast, 10 cc. of 14 N sulfuric acid (200 cc. of concentrated H_2SO_4 diluted to 500 cc.). It is quite essential that the first 10 cc. of acid should be introduced into the alkaline solution while the latter is still quite hot. The addition of acid should in fact produce boiling. Unless the mixture becomes very hot the silicic acid is apt to remain in colloidal solution and the mixture will have to be discarded.

The first 10 cc. of acid are more than enough to neutralize the alkali in the flask. After the addition of 10 cc. of acid shake thoroughly and cool. Then add 5 cc. more of the 14 N acid to produce the required acidity; rinse the contents into a 100 cc. volumetric flask, dilute to volume, shake thoroughly, and filter. The filtration is slow and the funnel should be covered with a watch-glass during the 2 hour period required to get about 60 cc. of filtrate.

If more than 60 cc. of filtrate is desired, it is best to start with 2 gm. of protein material. In that case, one should add 8 gm. of sodium hydroxide and for neutralization and acidification should use 20 cc. and 10 cc. of 14 N sulfuric acid. The acidified digest is then diluted to 200 cc. before filtering.

The acidified protein hydrolysates should be kept in an ice box, or at least in the dark, unless all the desired determinations can

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be started rather promptly, for if the hydrolysates stand around exposed to light at room temperatures for many days they soon grow dark in color due to decomposition of the tryptophane. These secondary decomposition products are precipitated with the tryptophane and give a blue color with the phenol reagent, but they also impart a violet tint to the solutions so that it becomes difficult to make exact colorimetric readings.

The alkali protein hydrolysates are, of course, more or less colored without reference to the subsequent decomposition of tryptophane in the acidified filtrates. It is perhaps hazardous to try to explain the origin of these colored decomposition products except on the basis of detailed investigation, but the amount of color obtained is certainly quite unrelated to the amounts of tyrosine or of tryptophane present. Edestin, for example, yields hydrolysates which are only slightly more colored than the hydrolysates obtained from gelatin while pure egg albumin yields quite deep colored hydrolysates. To us it seems altogether probable that the discoloration obtained is due to the carbohydrate content of the different proteins.

Nearly all of the color can be easily removed by shaking the acidified filtrates with a little kaolin; but as the analytical results obtained are changed little if at all by this treatment it does not seem worth while to recommend it as a regular or required procedure.

III. A New Colorimetric Method for the Estimation of Tyrosine in Protein Hydrolysates.

In the course of extended critical studies of the tryptophane precipitation by means of mercuric sulfate it soon became apparent that the empirical directions given by Folin and Looney were not entirely satisfactory. If to 8 cc. of a protein hydrolysate whose acidity is approximately normal one adds 2 cc. of a 10 per cent solution of mercuric sulfate in 2 N sulfuric acid and centrifuges at the end of 2 hours, one obtains a clear supernatant liquid. This mother liquor should contain all the tyrosine and no tryptophane. Within a few minutes after decantation this mother liquor again becomes turbid. This same phenomenon of a second precipitation is encountered whether one centrifuges and decants after 1, or 2, or several hours. At no time does one obtain a

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mother liquor in which there is going on no visible further precipitation. From this phenomenon the conclusion was drawn that one step at least in the Folin-Looney process, namely the washing of tryptophane sediment with sulfuric acid and throwing away this wash liquid, could not be right. Moreover, it seemed that Folin and Looney had depended too much on the data ascertained for the separation of pure tyrosine and tryptophane. After many fruitless attempts to secure visibly sure and certain separation of tryptophane and tyrosine in protein hydrolysates we finally tried to use Millon's reaction as a test for the presence or absence of tyrosine in the tryptophane precipitates.

Tyrosine as is well known reacts only very slowly with Millon's reagent except on heating. We were, therefore, surprised to obtain immediate positive reactions for tyrosine without any heating. The tyrosine reactions obtained were like those obtained with unsubstituted phenol. A number of different experiments were then made for the purpose of discovering the cause of the prompt phenol reaction obtained with Millon's reagent, and it was soon found that prolonged contact with mercuric sulfate was the responsible factor. A preliminary short heating with mercuric sulfate was even more effective. It was also found that the original Millon's reagent was no better if as good as the sodium nitrite originally recommended by Nasse.

This discovery was extremely welcome. While extensive use has been made of the phenol reagent of Folin and Denis for the estimation of tyrosine (and tryptophane), one has always had to reckon with the fact that some careful investigators have persistently held, with Abderhalden, that the tyrosine values obtained with the phenol reagent must be very much too high, and that the only really acceptable values are those represented by the actual isolation of tryosine. If a dependable quantitative method for tyrosine based on Millon's reaction could be found, the results obtained with protein hydrolysates ought at least to show whether the older colorimetric results have any justification. Those who have worked with gravimetric isolation methods for tyrosine have also used Millon's reaction as guide to the presence or absence of tyrosine, because this is the most specific reaction for tyrosine.

As Millon's reaction has always been used it is to be sure not perfectly selective for tyrosine because tryptophane will give a

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somewhat similar color when heated with Millon's reagent. But in the form here developed tryptophane gives no color. If present at all it will merely give a precipitate with the mercuric sulfate.

The idea of using Millon's reaction for quantitative tyrosine determinations is, of course, far from new. Several investigators have published papers on that subject. The different methods based on this reaction have given widely different results and that they could not be dependable follows from the fact that the colored compound obtained is comparatively unstable, yet has always been obtained by the aid of heat. The situation becomes entirely different, however, if the color can be developed practically instantly and at ordinary room temperature.

The development of the new method was not quite so simple a task as it seemed at first. The colored compound produced with tyrosine resembles in some of its properties the red compound obtained from Nessler's reagent and ammonia. The intensity and still more the shade of the color is determined partly by the reaction of the mixture and to a very large extent by the concentration of mercuric sulfate present. As the acidity is reduced the intensity of the color increases and the shade becomes more violet. Similar results are obtained by increasing amounts of mercuric sulfate. For accurate quantitative comparisons it is, therefore, essential that the standard and the unknown shall be substantially identical in acidity and in mercuric sulfate content. Significant inequality in these respects is revealed by the fact that the standard and the unknown are seen to have different shades when the color comparison is made. All such determinations must be discarded.

The most troublesome feature of the reaction is the tendency for the colored compound to give turbidities instead of crystal-clear solutions. It is impossible, for example, to obtain any dependable solutions in the presence of more than minute traces of tryptophane. It is, therefore, not possible to apply the reaction directly to the alkali protein hydrolysates without first removing substantially all of the tryptophane. But as this separation of tryptophane was the main object on hand the difficulty due to tryptophane was easily overcome.

The tyrosine determination on the protein hydrolysates whose preparation has been described, is as follows:

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Transfer to a 15 cc. centrifuge tube 8 cc. of the protein hydrolysate and add, drop by drop, from a height of about 3 cm. 4 cc. of a 15 per cent solution of mercuric sulfate in 6 N sulfuric acid. No stirring is necessary. Let the mixture stand for 2 to 3 hours and centrifuge fairly hard for 5 minutes. Decant the supernatant liquid into a 100 cc. volumetric flask, draining thoroughly and rinsing the edge of the centrifuge tube with about 2 cc. of 0.1 N sulfuric acid. The amount of tyrosine remaining with the tryptophane is perhaps a shade more than could be accounted for on the basis of the amount of mother liquor in the tube. To the sediment in the tube add 10 cc. of a solution containing 1.5 per cent of mercuric sulfate in 2 N sulfuric acid. Stir with a fine glass rod and let stand for 10 minutes. Traces of precipitated tyrosine dissolve fairly easily in 2 N acid and the added mercuric sulfate prevents the solution of any tryptophane. At the end of 10 minutes rinse the stirring rod with 2 cc. of the same 1.5 per cent mercuric sulfate solution. Centrifuge again and transfer this wash liquid to the flask containing the original mother liquor, not omitting to rinse the edge of the centrifuge tube.

The standard is prepared as follows: Introduce into a second 100 cc. volumetric flask 5 cc. of a standard tyrosine solution in 2 N sulfuric acid containing 1 mg. of tyrosine per cc. Add 4 cc. of the 15 per cent mercuric sulfate solution and 12 cc. of the 1.5 per cent mercuric sulfate solution and about 7 cc. of 0.1 N sulfuric acid.

To the standard and the unknown must further be added 6 cc. of 7 N sulfuric acid, for the total acidity in each flask should be approximately equivalent to 100 cc. of normal acid. Heat the two flasks in boiling water for 15 minutes and then cool in cold water approximately to room temperature. Next add to each flask, with shaking, 1 cc. of 2 per cent sodium nitrite solution. Dilute to volume at once and make the color comparison without undue delay, always, of course, first reading the standard against itself so as to adjust the colorimeter, or the eye.

If the standard is set at 20 mm. then 20 divided by the reading of the unknown multiplied by 1.25 and by 5 gives the per cent of tyrosine, provided that the hydrolysate represents exactly a 1 per cent protein solution.

A few explanatory remarks concerning the tyrosine method described above may be helpful.

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1. It has been made clear that there should be no undue delay in finishing the determination after the heating of the mixtures with mercuric sulfate. The reason for this limitation is the fact that after such heating with so much mercuric sulfate the protein hydrolysates always show a tendency to give another slight turbidity. A great deal of time was wasted on the tryptophane precipitation, because of the occurrence of this turbidity in the heated tyrosine-containing filtrates. As the turbidity could not be due to tyrosine it was long thought that it might represent tryptophane which had escaped the precipitation. It finally became clear, however, that the turbidity does not represent tryptophane and its only importance lies in the fact that it tends to interfere a little with the color comparison in the tyrosine determination unless that comparison is made with reasonable promptness. The turbidity is anyway very slight.

2. The reader will note that a number of solutions representing different strengths of sulfuric acid are used. Thus 14 N sulfuric acid is used for acidifying the protein hydrolysate. And we use 7 N and 0.1 N sulfuric acid, and also 6 N and 2 N sulfuric acid, for the preparation of the two mercuric sulfate solutions. No great accuracy of normality is required for these solutions and all the sulfuric acids are obtained by suitable dilutions of the 40 volume per cent or 14 N sulfuric acid employed for neutralizing and acidifying the alkaline hydrolysates.

The 6 N sulfuric acid used for the preparation of the 15 per cent mercuric sulfate solution would not have come in at all if undiluted 7 N sulfuric acid could be used, but the latter will not take up 15 per cent of mercuric sulfate. The 15 per cent mercuric sulfate solution is prepared as follows: Transfer 30 gm. of the salt to a 200 cc. volumetric flask by the help of about 80 to 90 cc. of 7 N sulfuric acid. Then add 31 cc. of water and shake or stir until complete solution is obtained. Then fill to the mark with 7 N sulfuric acid. This solution is slightly supersaturated and after a time may need to be filtered.

The weaker, 1.5 per cent, mercuric sulfate solution is made by diluting 10 cc. of the 15 per cent mercury solution plus 10 cc. of 14 N sulfuric acid with water to 100 cc.

The 0.1 N sulfuric acid, finally, is used instead of water for rinsing purposes only because water would tend to precipitate basic mercuric sulfate.

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3. It will be noted that an unusually large amount of tyrosine (5 mg.) is used as the standard in this colorimetric method. Different practical reasons led to this selection. The color produced in the reaction is intense enough to permit the use of 1 mg. in 100 cc. flasks, but the brilliant red obtained is extremely difficult to read with accuracy in such dilutions. By using a 5 mg. standard one gets a color that is easy to read in the colorimeter and this standard is just about right for use with all of the tyrosine present in 8 cc. of protein hydrolysate. By using the whole of the mother liquor and washings from the tryptophane precipitation the analytical process is simplified, and, more important, the accuracy is increased.

4. The proportionality of the color derived from different amounts of tyrosine is excellent. The standard and the unknown can be at least 100 per cent apart and still yield true proportionality.

5. It may be pointed out that the new form of Millon's reaction represented in this colorimetric method is not without theoretical interest. The fact that prolonged preliminary contact, or heating, with mercuric sulfate is an essential step in the reaction clearly indicates that some organic combination between tyrosine and mercury must be an intermediary product essential to the reaction with nitrous acid. This conclusion is more or less in harmony with some recent remarks by Gibbs (7) on the same subject.

The thought will doubtless occur to some that a very convenient method for tryptophane determinations in protein hydrolysates might be obtained by determining the "total tyrosine" in the hydrolysates by means of the phenol reagent, and the true tyrosine by the process described above. The difference between the two should give the tyrosine equivalent of the tryptophane. It is unfortunately a fact, however, that tryptophane does not develop its full chromophoric power when much tryosine is present even when a large excess of the phenol reagent is added. Accurate tryptophane results cannot, therefore, be obtained in that manner.

The suggested combination of colorimetric estimations is rather useful for another purpose, however. It supplies a fairly conclusive and reasonably accurate means of determining how much there is of other materials than tyrosine and tryptophane in protein hydrolysates which react with the phenol reagent.

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IV. On Tryptophane Determinations.

The colorimetric method for the determination of tyrosine described in the preceding section is given as though it were intended solely for tyrosine determinations. It is obvious, however, that the tryptophane-mercury precipitate left in the centrifuge tubes after the removal of the tyrosine with the mother liqour and washings can be used for determination of the precipitated tryptophane. The one important question to be considered, however, is whether all the tryptophane is in this sediment. This question cannot be answered off hand in the affirmative, because small amounts of tryptophane might be present in the tyrosine mother liquor without interfering with the tyrosine determination. It is not worth while to discuss the very many experiments which have been made to find the maximum amount of tryptophane absolutely free from tyrosine.

This problem was finally condensed to the following definite and simple proposition: The described tyrosine method gives all the tyrosine in the protein digest and no tryptophane value determined by means of the phenol reagent can be accepted as correct unless the mother liquor and washings also give the same, full tyrosine value.

As a matter of fact the tryptophane is precipitated as rapidly, and seems to be precipitated as completely, under the conditions selected for the given tyrosine method as under any other practical conditions. One can lengthen the time allowed for the tryptophane precipitation to 5 or 6 hours without incurring demonstrable loss of tyrosine, but by doing so one also does not get an increase of tryptophane. On the other hand, by lengthening the precipitation period to 24 hours (with casein hydrolysates) one encounters a very small loss (0.05 to 0.08 per cent) of tyrosine and the increase in the yield of tryptophane is so small (not over 0.1 per cent) that one must conclude that this extra "tryptophane" is only tyrosine.

Tryptophane Method.—The precipitation of the tryptophane is made exactly as described under the tyrosine determination.

Two different processes are available for the determination (aside from the cyanide method of Folin and Looney) and for both of these 1 mg. of tyrosine is used as the standard.

First Method.—To the tryptophane sediment tube add 10 cc.

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of N hydrochloric acid; stir with a fine glass rod (diameter 3 mm. or less). Heat in a beaker of boiling water for 10 minutes and then pass into this hot solution hydrogen sulfide in a slow current through a glass tube having a capillary point so long that only the capillary part can get into the liquid in the centrifuge tube. The hydrogen sulfide treatment takes only a few seconds, but must be continued till *black* sulfide of mercury is obtained. The fine stirring rod still in the centrifuge tube is then taken out and placed inside of the hydrogen sulfide delivery tube and both are washed with 2 cc. of 10 N sulfuric acid. The centrifuge tube is then stoppered and set aside for an hour. The 1 hour waiting period introduced at this stage is scarcely necessary, for one obtains the same tryptophane values when it is omitted. It is introduced partly as an additional precaution and partly to draw attention to the fact that tryptophane, like tyrosine, yields organic mercury compounds which are decomposed less rapidly than ordinary mercury salts.

Centrifuge and decant into a 100 cc. volumetric flask of Pyrex glass which can stand boiling. Wash off the edge of the tube with 2 cc. of 0.1 N sulfuric acid. Add 10 cc. of 0.1 N acid to the Hg_2S residue; stir, rinse the stirring rod with 2 cc. of 0.1 N acid, and centrifuge once more. Add this wash liquid to the mother liquor in the 100 cc. flask. Finally add about 15 cc. of water and boil rapidly for 4 to 5 minutes, to remove the H_2S. Cool.

Transfer 5 cc. of a standard tyrosine solution containing 1 mg. of tyrosine to another 100 cc. flask and dilute with 35 cc. of water. Add 25 cc. of saturated sodium carbonate to each flask. Dilute the contents of the boiled flask to 65 or 70 cc. Then add to each flask, with shaking, 5 cc. of the phenol reagent and let stand for 30 minutes. Dilute to volume, mix, and make the color comparison.

Calculation.—When the standard is set at 20 mm. $\frac{20}{R} \times 12.5$ gives in mg. the tyrosine equivalent of the tryptophane in the 1 gm. of protein used for the hydrolysis. This figure divided by 0.887 gives the value as tryptophane.

This colorimetric comparison is not absolutely perfect. The color given by the tryptophane is just a little less bright than that obtained from tyrosine. But the difference is so small that one

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need never be in doubt about the correct reading if one has first read the standard against itself.

The saturated carbonate solution must, of course, be free from hydrogen sulfide. As sodium carbonate rapidly takes up sulfides from rubber, the carbonate solution must not be allowed to touch rubber tubing or rubber stoppers.

The standard tyrosine solution is made by diluting 20 cc. of the strong tyrosine solution, used for tyrosine determinations, to 100 cc. with 2 N sulfuric acid.

Second Method.—In this process the washed tryptophane mercury precipitate is stirred with 10 cc. of 0.1 N sulfuric acid and the stirring rod is washed with 2 cc. of 0.1 N acid. The sediment is then thrown down once more by means of the centrifuge and the wash liquid is removed by decantation. The purpose of this extra washing is to remove the excess mercuric sulfate left from the first washing. This second washing is added to the flask containing the tyrosine and a similar amount of 0.1 N sulfuric acid is then also added to the standard.

To the sediment are then added 10 cc. of N hydrochloric acid as in the first process and the stirred mixture is heated in boiling water for 30 minutes.

The sediment dissolves at once in hot hydrochloric acid, but the long heating is necessary to secure decomposition of the organic tryptophane compound.

At the end of 30 minutes heating, cool, rinse into a 100 cc. volumetric flask with 30 cc. of water, and add 25 cc. of saturated sodium carbonate solution.

Prepare the standard tyrosine solution just as in the first process and then add 5 cc. of the phenol reagent to each. Let stand for 30 minutes. Then add 2 or 3 cc. of 5 per cent sodium cyanide solution to each flask, dilute to volume, and make the color comparison as before.

The sodium cyanide as here used does not affect the color, as the phenol reagent is gone. The cyanide is here added only to clear the solution from the mercury which tends to come out in the alkaline solution. If desired, 10 cc. of 10 per cent sodium sulfocyanide may be used instead of the cyanide.

Calculation.—The calculation of the tryptophane from its tyrosine equivalent is a little different in this case because of the mer-

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cury. By means of many experiments with different amounts of tryptophane it was found that the liberated mercury salt diminishes the depth of color obtained from a given amount of tryptophane by 5 per cent. Instead of dividing the tyrosine equivalent of the tryptophane with the theoretical factor 0.887 the divisor must be reduced 5 per cent so that the divisor in this case is 0.843.

The two methods give substantially identical values on freshly prepared hydrolysates.

V. Tyrosine and Tryptophane in Proteins.

This is primarily a paper on the technique of tyrosine and tryptophane determinations, and practically all the check work has been done on Kahlbaum's casein ("Nach Hammarsten"). But some pure proteins, obtained from Dr. Cohn, have been analyzed by the same process.

Casein.—The vast majority of analyses on casein recorded in the literature are based on Kahlbaum's material. In this casein Folin and Looney found 1.54 per cent of tryptophane and 5.32 per cent of tyrosine. The corresponding figures now obtained are 1.4 per cent of tryptophane and 6.37 per cent of tyrosine. Hundreds of tryptophane determinations have been made on this casein and at one stage it was thought that the Folin-Looney value (1.54) was too low instead of too high. But that was because some tyrosine was constantly included in the tryptophane determinations. It is not strange that the Folin-Looney value for tryptophane should be somewhat too high since not less than 1 per cent of tyrosine remained unaccounted for in their determinations. The higher tyrosine values now found are, of course, the result of more complete hydrolysis. Casein is far more resistant to the hydrolytic action of alkalies than are the other proteins examined and it is only here that the new methods give so much higher tyrosine values.

A highly purified casein obtained from Dr. Cohn was also analyzed. The filtered hydrolysate obtained from this casein had no more color than the very slight straw-yellow which one obtains in gelatin hydrolysates. The other casein hydrolysates are light brown. This difference indicates that Kahlbaum's casein probably contains some milk sugar. The tryptophane-mercury precipitate from Cohn's casein has almost the pure canary-yellow color

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that is obtained from colorless tryptophane solutions. Cohn's casein contained 1.4 per cent of tryptophane and 6.55 per cent of tyrosine.

Egg Albumin.—In ovalbumin Folin and Looney found 4.2 per cent of tyrosine and 1.23 per cent of tryptophane. Here the barium hydroxide hydrolysis has evidently set free all the tyrosine as well as the tryptophane. Here also the lower tyrosine content reduces the danger of getting tyrosine in the tryptophane precipitate; but, on the other hand, the tryptophane precipitation is not quite complete. The figures now obtained for ovalbumin are 1.3 per cent tryptophane and 4.0 per cent tyrosine. It may be remarked in passing that even before the introduction of the hydrolysis with sodium hydroxide or the new tyrosine method, Dr. Ciocalteu had obtained 1.33 per cent tryptophane and 4.0 per cent tyrosine in ovalbumin. At that time the hydrolysates were made 2 N in acidity and the tryptophane was precipitated in 24 hours after the addition of 2 cc. of 2 N sulfuric acid containing 10 per cent of mercuric sulfate.

In the case of egg albumin it is advisable to decolorize with about 1 gm. of kaolin. The figures recorded above were obtained on such decolorized filtrates.

Edestin.—According to Folin and Looney, edestin should contain 1.40 per cent tryptophane and 5.7 per cent tyrosine. The latter figure must be due to some serious error, for the Folin-Looney process could not possibly give such a tyrosine figure for edestin. In his recent paper on the subject, Looney records the tyrosine of edestin as 4.58 per cent and the tryptophane as 1.52 per cent. These figures are substantially identical with the values obtained by the methods described in this paper—tryptophane 1.51 per cent, tyrosine 4.53 per cent.

The second decimals in these values are not, of course, to be insisted upon. Edestin contains 1.5 per cent of tryptophane and 4.5 per cent of tyrosine.

Gliadin.—In gliadin Folin and Looney found 1.14 per cent of tryptophane and 3.4 per cent of tyrosine. These figures were later revised by Looney to 1.1 per cent of tryptophane and 3.04 per cent of tyrosine. The corresponding values now obtained are 0.84 per cent of tryptophane and 3.1 per cent of tyrosine.

Looney's results represent the average of several determinations

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and we have made a considerable number of determinations, using in part the same samples of gliadin as had been used by Looney. While his and our tyrosine values agree very well there is a quite material difference in our respective tryptophane figures. It is perhaps best not to try to explain these differences at present. If we were dealing with analyses of a less experienced investigator in this field than Looney, it might be permissible to assume that here was a case where the cyanide-mercury combination of the Folin-Looney process had conspired to introduce an excessive deviation from true proportionality in the color comparison. If our figure is correct there would be only about 0.7 mg. of tryptophane in the 8 cc. of hydrolysate used, and this would be compared against the 1 mg. tryptophane standard. Any error that could creep in would, therefore, certainly be on the plus side. It seems scarcely possible, however, that an error of the magnitude here involved could be due only to a fluctuating cyanide-mercury blank. But it is particularly in cases of this kind where the tryptophane content is low, that we should expect the process described in this paper to yield more dependable figures.

Zein.—The tyrosine determinations were made in the usual manner on 8 cc. of hydrolysate from which the tryptophane had been removed by a 2 hour precipitation with mercuric sulfate. For the tryptophane determinations 25 cc. of hydrolysate plus 12 cc. of 15 per cent mercuric sulfate were used (in 50 cc. centrifuge tubes). The standard contained only 0.4 mg. of tyrosine. The zein used in these analyses came originally from Osborne's laboratory. It contains 0.17 per cent of tryptophane and 5.9 per cent of tyrosine.

Folin and Looney referred to the tryptophane content of zein, as of gelatin, only in tabular form and gave it as 0, instead of a trace, as they well might have done purely on the basis of the fact that a turbidity is obtained on the addition of mercuric sulfate to the hydrolysates. At that time such small traces seemed unimportant. Now the situation is different. The smaller the quantity of a given amino acid the greater is its significance for molecular weight calculations, provided that the analyzed material is pure.

Incidentally it may be mentioned that a precipitation method which is capable of yielding 0.17 per cent of tryptophane from 1

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per cent zein hydrolysates should be very dependable when applied to corresponding hydrolysates from other proteins which contain from 5 to 8 times as much tryptophane as does zein. In this case, 2½ hours were allowed for the tryptophane precipitation and the precipitate was washed as usual with 2 N acid containing 1.5 per cent of mercuric sulfate. The tryptophane figure, 0.17 per cent, should probably be regarded as a minimum value, since one cannot ascribe absolute insolubility to the tryptophane-mercury precipitate.

VI. The Preparation of Pure Mercuric Sulfate.

In the preceding pages we have pointed out that different samples of sodium cyanide give different amounts of color with the phenol reagent, and that the color thus obtained is greatly intensified in the presence of mercuric sulfate. We have also found that different samples of mercuric sulfate give different amounts of color with a given solution of sodium cyanide. The reason for these differences is evidently the fact that practically all brands of mercuric sulfate contain more or less of mercurous sulfate, and may contain other reducing salts such as ferrous sulfate.

We have examined a large number of mercuric sulfate samples, including one French and one German preparation. The sample manufactured by Baker and Adamson was different from all the others, and the only one which was substantially pure. It contained only traces of iron and of mercurous sulfate.

Since it is important that only pure mercuric sulfate should be used for the quantitative estimation of tryptophane, we set ourselves the task of working out a suitable method for recrystallizing it. We have found no useful information, bearing on this point, in the literature. The method described below is based on the discovery that mercuric sulfate, in the presence of the right amount of sulfuric acid, is extremely soluble in water, up to more than 50 per cent. From such concentrated solutions it is almost quantitatively precipitated, in snow-white crystalline condition, by the addition of more sulfuric acid. The process given below is based on the use of 1 kilo of the crude, bought material; but the proportions given, 1 cc. of concentrated sulfuric acid to 6 gm. of the salt, will work equally well with smaller amounts.

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Transfer to a 4 liter Pyrex beaker 1 kilo of mercuric sulfate, and 150 cc. of concentrated sulfuric acid. Stir until a uniform paste is obtained. Then add water slowly with constant stirring (heavy glass rod or porcelain spoon) until none of the original heavy salt remains at the bottom of the beaker. More or less black dirt and mercurous sulfate will remain suspended in the solution. 1700 to 1800 cc. of water will be required to dissolve the mercuric sulfate. Filter through a good quality heavy filter paper into a 4 liter flask. This filtration is slow; the first part of the filtrate may be a little turbid and should be poured back on the funnel. The color of the filtrate is largely determined by the amount of iron present in the original salt.

To the filtrate in the flask add gradually, with shaking, 450 cc. of concentrated sulfuric acid. Considerable heat is, of course, generated during the addition of the acid, and we prefer to keep the flask in a large kettle of cold water during the process. The snow-white precipitate comes out at once, and settles rapidly. Remove by decantation about 100 cc. of the supernatant liquid and to it add 10 cc. of concentrated sulfuric acid. If more than a very slight precipitate is obtained, add more sulfuric acid to the original mixture: 450 cc. should be enough, however. Cool, and filter on a Buchner funnel through a hardened filter paper. Wash with 400 cc. of 25 volume per cent of sulfuric acid. Drain thoroughly by suction with a water pump.

Blow the precipitate back into a dry 4 liter beaker and remove the filter paper. Add 150 cc. of concentrated pure sulfuric acid, stir to a paste, and dissolve, as before, by the gradual addition of water.

Filtration at this stage is superfluous, but should not be omitted unless the solution is *really* clear. Repeat the precipitation by the addition of sulfuric acid just as was done with the first filtrate. Cool, filter, and wash with cold dilute sulfuric acid (1 volume of acid to 3 volumes of water).

Blow the precipitate into a *dry* beaker, pick out the hardened filter paper, and add to the precipitate, gradually and with stirring, 500 cc. of an equal mixture of alcohol and ether. Stir very thoroughly and filter the mixture on a Buchner funnel. In this final filtration it is not necessary to use a hardened filter. A little additional alcohol-ether mixture may be used for rinsing the last of the precipitate in the beaker onto the funnel, but none should be used for washing the precipitate. It takes very little water or moisture to give insoluble yellow basic sulfate, when practically all the sulfuric acid has been thus removed, but if the directions are followed no basic sulfate is obtained at any stage.

Transfer the precipitate to a new, porous, clay plate. Place the plate on a double layer of filter paper, away from too much atmospheric moisture, and allow to dry, at room temperature. Yield 750 gm.

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