CLXXVII. ON THE USE OF MERCURIC SALTS AND NITROUS ACID IN THE COLORIMETRIC DETERMINATION OF TYROSINE AND TRYPTOPHAN PRESENT IN SOLUTION

By JOSEPH WILLIAM HENRY LUGG

From the Nutrition Laboratory of the Commonwealth Council for Scientific and Industrial Research, University of Adelaide, South Australia

(*Received 30 June 1937*)

The work recorded in this article was initiated owing to unsuccessful attempts to estimate the tyrosine contents of some hydrolysates of plant-leaf proteins by the method of Folin & Ciocalteu [1927], which is based upon the Millon [1849] reaction. The unknown colour solutions were cloudy, and on centrifuging them to remove the suspensions it was noticed that the precipitates were deeply coloured and had presumably carried down some of the red colour complex.

The appearance of "cloud" was partly overcome by the adoption of von Deseö's [1934] recommendation of diluting the colour solutions with dilute sulphuric acid instead of with water, and completely overcome by more radical changes in procedure. These embraced mercuration of the tyrosine and precipitation of any tryptophan present in one step instead of two and dilution of the reaction mixture with a solution approximating closely to it in composition.

Later, the effects of extraneous substances in test solutions were investigated more generally with a view to their elimination, and a fairly thorough search was made for the most satisfactory means of applying the Millon and associated reactions to the estimation of tyrosine. It has long been known that tryptophan will interfere by contributing a spurious coloration. Folin & Ciocalteu [1927] made use of the Hopkins & Cole [1901] acid mercuric sulphate reagent to remove the tryptophan. In the course of the work here recorded, the colour reactions between nitrous acid and tryptophan and between nitrous acid and the tryptophan mercurial, were investigated briefly. Under appropriate conditions the second reaction was found to provide a delicate test for tryptophan, and further work resulted in it being made the basis of a very simple colorimetric method for the estimation of this amino-acid.

Reactions between tyrosine, mercuric salts and nitrous acid

Millon's reagent for phenols (a solution of mercury in nitric acid) was employed by Hoffmann [1853] in testing for tyrosine. Despite Meyer's [1864] demonstration that Hoffmann's test depended upon the presence of a little nitrous acid, Nasse [1879] was of the opinion at first that nitration constituted an essential step in the reaction. Nickel [1890] expressed the view that nitrosophenols were first formed and were transformed into red dyes, a view endorsed by Gibbs [1926; 1927] who has very ably reviewed the historical side.

The experiments of Lintner [1900] suggest the proper sequence of steps in the Millon reaction, but their significance appears to have escaped him. Still more clearly is the sequence revealed in the work of Folin & Ciocalteu and their support of Gibbs's view is not readily comprehended.

The following points have been elicited in the present work.

Reaction (1) occurs with any of the "ionized" mercuric salts (sulphate, acetate, nitrate) and the particular phenol employed, a mercurial, substance I, being produced. It is reasonably stable. It is generally not very soluble in the acid solution, its solubility increasing with the acidity.

Reaction (2) occurs when substance I is treated with nitrous acid, substance II being produced. In the case of the substituted phenol, tyrosine, one molecule of nitrous acid reacts with one of the phenol in the form of substance I. Substance II, which is responsible for the red Millon colour, is not very stable and is generally more soluble than substance I. It appears to depend for its existence upon the presence of at least a little ionized mercuric salt.

Reaction (3). If to a solution of substance II is added some "unionized" mercuric salt (e.g. chloride or cyanide), the red coloration changes in tint, intensity and stability, substance III being produced. The reaction is very rapid with chloride but of measurable rate with cyanide. Substance III appears to exist in equilibrium with II.

Reaction (4). If excess of ionized chloride or cyanide (e.g. the sodium salt) is added to a solution of substance II or III, substance IV is produced, the red coloration being fairly sharply replaced by a yellow which is generally more stable.

Reaction (5). When substance I, present in acid solution, is treated with excess of ionized chloride or cyanide, substance V is produced. It is quite stable and generally sparingly soluble.

Reaction (6). Substance V forms substance IV when treated with nitrous acid.

Reaction (7). When substance IV in solution, resulting from reaction (4) or (6), is treated with excess ionized mercuric salt, substance III is produced.

The substance numerals refer to classes of substances, the precise composition probably depending upon the particular acid radical of the mercuric salt. The substance I obtained from ordinary phenol and mercuric sulphate is an almost white powder, sparingly soluble in acid mercuric sulphate solutions and virtually insoluble in alcohol. It is slowly hydrolysed by water to a yellow substance. The substance V obtained from this substance I by the action of excess hydrogen chloride is white and is sparingly soluble in alcohol. Unlike substance I it contains only a trace of sulphur and appears to escape hydrolysis even at 100°. The red-yellow colour change, accompanying the conversion of the corresponding substance II, via III, into IV, is obtained when there is added the stoichiometric amount of HCl for the conversion of the $HgSO_4$ present into $HgCl_2$. The substance V must be different from the mercurichlorophenols described by Lefeve & Desgretz [1935], which are yellow or orange in colour and mixtures of which fail to give the typical red coloration with nitrite in acid $HgSO_4$ solution.

Vaubel [1900] states that di-*ortho*- and di-*meta*-substituted phenols do not give the Millon reaction. This must have a bearing on any detailed theory of the reactions listed above and no such theory can be presented yet. In general terms, however, it would seem that substance II is an *o*-quinone monoxime whose existence in acid solution depends upon a bonding with an attached ionized mercury atom, that in reaction (3) the polar nature of substance II is diminished to give III which is still an *o*-quinone monoxime, and that substance IV is a nitrosophenol. Tyrosine and the other phenols form nitroso-derivatives in the absence of mercuric salts, but the subsequent addition of mercuric sulphate will not convert these yellow derivatives into red complexes, and they are therefore not of the substance IV class.

It is not known how many mercury atoms are associated with each original phenol molecule in the different classes of substances listed above, nor is it known whether the number is constant or variable with the class of substance and the particular phenol. Incomplete analysis of the substance V obtained from phenol, mercuric sulphate and hydrogen chloride, suggests that two H atoms have been replaced by two HgCl radicals.

Reactions between tryptophan, mercuric salts and nitrous acid

Hopkins & Cole [1901] found that tryptophan could be precipitated from mixed amino-acids in sulphuric acid solution by mercuric sulphate. In general I find that the solubility of the precipitate and the rate of destruction of the compound (which must depend materially upon the solubility) increase with increasing acidity and temperature, and that the rate of precipitation of the compound, and therefore probably its rate of formation, increase markedly with the temperature.

The yellow precipitate is fairly soluble in solutions of the well ionized cyanides and chlorides, and in sulphuric acid at acidities greater than $10N$. When the precipitate is treated with dilute hydrochloric acid it rapidly turns white and slowly dissolves, but Folin & Ciocalteu [1927] state that the mercury is completely removed from combination only on heating. When fairly concentrated sulphuric acid is the solvent, however, the yellow precipitate dissolves without apparent change in composition to give a pale yellow solution.

If nitrous acid is added to the sulphuric acid solution an intense reddish brown coloration rapidly develops. The substance responsible for it is unstable. The tint, intensity and stability of the coloration depend upon the acidity, the temperature, the concentration of mercuric sulphate and other factors, and generally, the coloration is more intense if the acidity and mercuric sulphate concentration are increased, whilst the stability decreases with increasing temperature and acidity.

If excess hydrogen chloride is added to the colour solution the coloration fades at a greatly enhanced rate, whereas the addition of mercuric chloride slightly changes the tint and intensity and slightly diminishes the fading rate. The characteristic reddish brown coloration cannot be obtained by adding nitrous acid to a hydrochloric acid solution of the yellow precipitate (a pale orange coloration develops), nor can it be obtained by treating with mercuric sulphate the orange coloration that is developed when nitrous acid is added to a solution of tryptophan in fairly concentrated sulphuric acid, but it is obtained if the mercuric sulphate is added before the nitrous acid. Nitric acid cannot replace nitrous acid in the reaction, but it does not seriously interfere. If mercuric formate or acetate in a solution of the corresponding acid be used in place of the mercuric sulphate-sulphuric acid reagent, a very inferior reaction is obtained.

Indole itself will give the reaction and it is presumed that other derivatives besides tryptophan will give it, though it may be that the α-hydrogen in the pyrrole ring must not be substituted. The reactions involved are even more obscure than are the phenol reactions already described, for whilst some of these can be recognized as conforming with known types of reactions very little work appears to have been done with the indole mercurials [see Goddard & Goddard, 1928].

Experimental

Colour intensity comparisons were made with the aid of a tested "Hellige" moving-cup colorimeter. In the case of the phenol reactions the particular phenol (usually tyrosine) was made up to 5 ml. with H_2SO_4 at some definite acidity. To this were added 5 ml. of a reagent containing H_2SO_4 and $HgSO_4$ and sometimes other salts as well. The mixture was maintained at a definite, somewhat elevated, temperature for a time sufficient to bring about at least 99% of the necessary mercuration, cooled to about room temperature and diluted to 24·5 ml. with a solution made by mixing equal volumes of the reagent and of H_2SO_4 of the same acidity as the first 5 ml. volume of liquid. 0·5 ml. of a nitrite solution was added, and at intervals after mixing the colour intensity was measured against a constant artificial standard. In the case of the indole reactions the particular indole (usually tryptophan), either dissolved in a very small quantity of dilute acid or alkali or in the form of the precipitated indole mercurial, was treated with 10 ml. of the reagent (fairly concentrated H_2SO_4 containing $HgSO_4$ and sometimes $HgCl_2$ also). The mixture was maintained at a definite temperature to bring about mercuration or to dissolve the solid mercurial, cooled to about room temperature and diluted to 24·5 ml. with more of the reagent. 0·5 ml. of a nitrite solution was added and colour comparisons against a constant artificial standard were made at intervals after mixing.

In the various figures the intensities of coloration in arbitrary units (ordinates) have been plotted against time in minutes (abscissae).

The compositions of the solutions are recorded by stating the approximate number of millimoles of the various substances present in the 25 ml. of solution. Reagents that contain less H_2SO_4 than is sufficient stoichiometrically to convert all secondary metal sulphates present into primary salts very readily deposit basic mercuric sulphate. The solutions to be described were all more acid than this and the metal sulphates are recorded as acid salts, but the adjustment required by the addition of the metal nitrite is small and has been ignored. With the second ionization of the acid largely repressed and the first taken as complete, the hydrogen ion concentration may be roughly equated with the excess H_2SO_4 molarity.

Phenol reaction

With all of the reagents employed tyrosine is mercurated to within 99% of completion by heating at 100° for 5 min. or 60° for 30 min., and prolonged heating at 100° (60 min.) causes negligible destruction. There is detectable decomposition with sulphonated tyrosine in 30 min. at 100°. With ordinary phenol and *p*-hydroxybenzoic acid the reactions are virtually complete in 15 min. at 100°.

Varying nitrous acid. Curves *a*1, *a*2 and *a*3 in Fig. 1 show the colorations developed by 4 mg. tyrosine, 3·5 millimol. $Hg(HSO_4)_2$, 16·5 millimol. H_2SO_4, with 0·0290, 0·0145 and 0·0072 millimol. $NaNO_2$ respectively, at 20°; curves *a*4, *a*5 and *a*6 are for 2, 1 and 0·5 mg. tyrosine respectively, with 0·0290 millimol. $NaNO_2$ and the same reagent at the same temperature. These curves all have substantially the same development and fading rates in relation to their respective maxima. In the series *a*1, *a*4, *a*5, *a*6, the coloration is closely proportional to the tyrosine at any selected time, *a*1/*a*6 being 7·7 at the peaks instead of 8·0, and in the series *a*2, *a*3, the coloration is closely proportional to the nitrite (the tyrosine being in excess). It is therefore permissible to compare the two series and to establish the stoichiometry of the reaction with nitrous

acid, and it is found to within 2% that 1 mol. of nitrous acid reacts with one of tyrosine in the form of the mercurial. Curve *a*7 was obtained like *a*4 except that the tyrosine was first sulphonated. The coloration was more pink.

Curve *a*3 is unaffected by 100 mg. glycine, and 50 mg. arginine reduce the maximum by only 2%, the amino-acids being first brought to *p*H 1 in H_2SO_4 solution, but there is a large depression with urea, which becomes deaminated rapidly.

Curves *b*1, *b*2 and *b*3 in Fig. 2 show the colorations developed with 1 mg. tyrosine, 2·5 millimol. $HgCl_2$, 6·25 millimol. $Hg(HSO_4)_2$, 12·5 millimol. $NaHSO_4$, 6·25 millimol. H_2SO_4, by 0·5, 0·125 and 0·031 millimol. $NaNO_2$ respectively, at 23°.

Curves *c*1, *c*2, *c*3 and *c*4 show the colorations developed with 1 mg. tyrosine, 2·5 millimol. $HgCl_2$, 3·12 millimol. $Hg(HSO_4)_2$, 12·5 millimol. $NaHSO_4$, 12·5 millimol. H_2SO_4, by 1, 0·5, 0·25 and 0·125 millimol. $NaNO_2$ respectively, at 23°. Curves *c*5 and *c*6 were obtained like *c*2 and *c*4 respectively except that the temperature was 17°, and *c*7 like *c*2 except that the temperature was 14°. Curve *c*8 shows the effect of 1 mg. cystine upon *c*2. Curve *c*9 was obtained like *c*2 but with 1 mg. *p*-hydroxybenzoic acid instead of 1 mg. tyrosine. It had not reached its peak in 45 min. and the coloration was more pink than that of *c*2. The curve obtained in the same way, but with 0·1 mg. ordinary phenol, is not shown. Its peak is reached in about 1 min. and the coloration, which is more orange than that of *c*2, fades at a lower rate. Curves *c*5, *c*6, *c*7, *c*8 and *c*9 are shown only in the inset, in relation to *c*2 and *c*4 reproduced there.

It will be noticed that the maxima are reached more rapidly and are of greater value as the nitrite concentration is increased, a result due to the fact that the rate of development increases more rapidly than the rate of fading. Both rates are decreased by a fall in temperature. The proportionality between coloration and tyrosine in members of the *b* and *c* series of higher nitrite concentration is phenomenally good, exceeding that of the *a* series.

For comparison, curve *a*5 is shown transferred from Fig. 1 to the ordinates of Fig. 2. Whilst variation in nitrite concentration does not alter the tint, variations in other variables do alter it, and it is therefore not possible to compare tyrosine members of the *a*, the *b* and the *c* series accurately. The same applies to a change from one phenol to another. Such comparisons, shown or implied in the different figures, may be accepted as "intensity matchings" to a normal eye when the colorimeter is illuminated with a "daylight" type of filament lamp.

Effects of chloride, cyanide and cysteine. Curve *a*5, which is obtained with 1 mg. tyrosine, 3·5 millimol. $Hg(HSO_4)_2$, 16·5 millimol. H_2SO_4, 0·029 millimol. $NaNO_2$, at 20°, is shown transferred from Fig. 1 to the ordinates of Fig. 3. Curves *d*1 and *d*2 are obtained when 1 and 2 millimol. NaCl respectively are added before the heating period. Curves *d*3 and *d*4 are obtained by adding 0·85 and 1·7 millimol. $HgCl_2$ respectively, the addition being made before, after or half before and half after, the heating period. The colorations, particularly that of *d*2, are decidedly more pink than that of *a*5, and the peaks of *d*3 and *d*4, as well as may be judged for different tints, are higher by 11 and 15% respectively. Curves *d*5, *d*6 and *d*7 were obtained by adding 1 ml. of 0·5*M* Na_2SO_4, *M* KCl and *M* KCN respectively to 10 ml. of the solution responsible for curve *a*5, 13 min. after the addition of the nitrite. Curve *d*5 clearly simulates *a*5, the colour intensity being almost exactly 10/11 times that of *a*5. The KCl is seen to exert its effect almost instantly, whereas the KCN acts but slowly, the solution slowly becoming slightly more pink than that of *a*5 or *d*5.

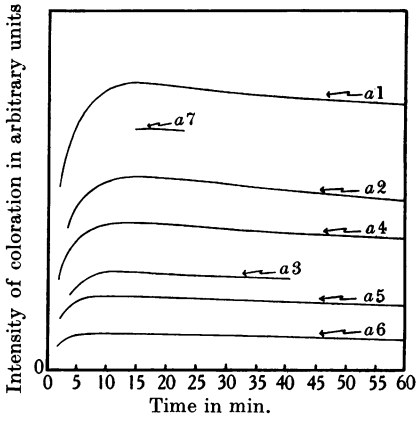

Fig. 1. Phenol reaction.

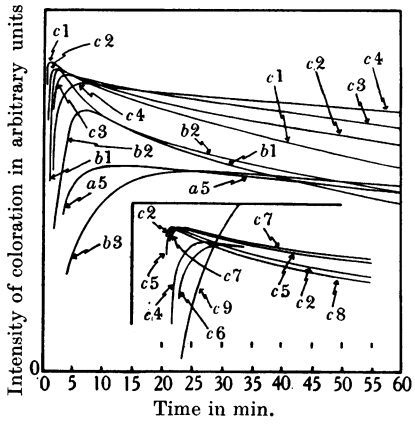

Fig. 2. Phenol reaction.

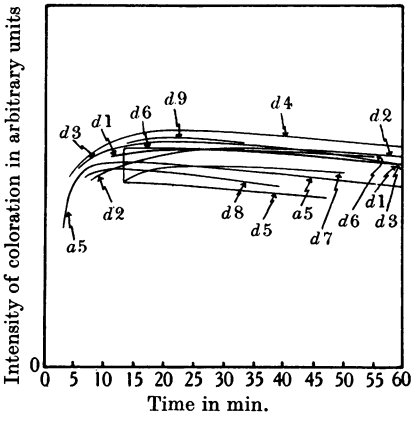

Fig. 3. Phenol reaction.

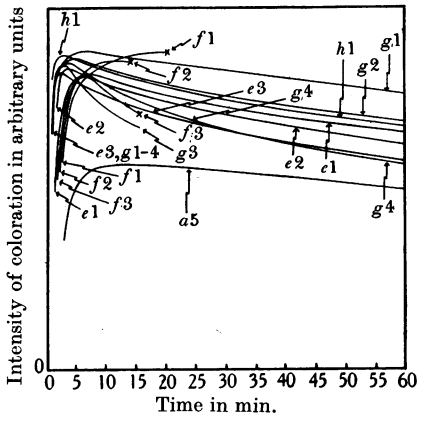

Fig. 4. Phenol reaction.

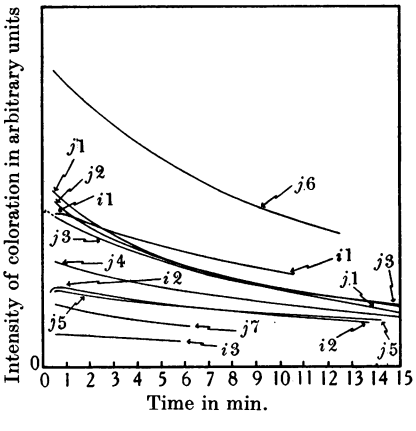

Fig. 5. Indole reaction.

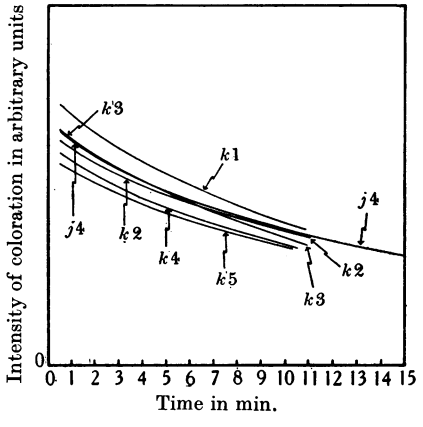

Fig. 6. Indole reaction.

The effects of cysteine and cystine are entirely similar in that the coloration does not reach as great a maximum, slowly changes to a slightly browner tint and fades more rapidly. It is well known that cysteine, as the mercaptide, is a major dismutation product of cystine through the action of mercuric salts, and it was found that cysteic acid, another product, had no effect in small quantities. Many misdirected attempts to eliminate the effect were made before it was recognized as due to a type (3) reaction. Small quantities of cysteine or cystine give no precipitate but larger quantities do, particularly at low acidities and low room temperatures; and if there is sufficient to form a precipitate during the heating period the precipitate is likely to contain tyrosine mercurial if the temperature of heating has been high. Curve $d8$ was obtained by adding 2·5 mg. cystine, and curve $d9$ by adding 2·5 mg. cystine and 1·7 millimol. $HgCl_2$ to the curve $a5$ solution before the heating period. Comparing with curves $a5$ and $d4$ it is found that the $HgCl_2$ only slightly reduces the depression of the maximum.

Varying H_2SO_4, $Hg(HSO_4)_2$ and $NaHSO_4$. Curve $a5$ is shown in Fig. 4 for comparison as usual. The solutions responsible for all the other curves in Fig. 4 have, as common constituents, 1 mg. tyrosine, 2·5 millimol. $HgCl_2$ and 0·5 millimol. $NaNO_2$, and the temperature is 23°. Curves $e1$, $e2$ and $e3$ are obtained with 5·0 millimol. $Hg(HSO_4)_2$ and with 6·25, 12·5 and 18·5 millimol. H_2SO_4 respectively. Curves $f1$, $f2$ and $f3$ are obtained with 3·12 millimol. H_2SO_4 and with 2·5, 3·75 and 6·25 millimol. $Hg(HSO_4)_2$ respectively. Curves $g1$, $g2$ and $g3$ are obtained in the same way as corresponding members of the f series but with 12·5 millimol. $NaHSO_4$ present in addition in each case, and curve $g4$ belongs to the g series containing 5·0 millimol. $Hg(HSO_4)_2$. Curve $h1$ is obtained in the same way as $f1$ but with 25 millimol. $NaHSO_4$ present in addition. Crosses on $f1$, $f2$ and $f3$ indicate the incidence of "cloud".

In general, increases in H_2SO_4, $Hg(HSO_4)_2$ and $NaHSO_4$, each and severally, make the coloration slightly browner or less pink, and as shown by curves in Fig. 2 as well as Fig. 4, the fading rate is increased, whilst the rate of development is increased with H_2SO_4 and $NaHSO_4$ but not significantly with $Hg(HSO_4)_2$. In passing it should be mentioned that with very large amounts of H_2SO_4 (80 millimol. or more) the predominant tint changes from red to brown.

Indole reaction

It was found that the coloration developed by 1 mg. tryptophan, 150 millimol. H_2SO_4, 1 millimol. $Hg(HSO_4)_2$ and 0·025 millimol. $NaNO_2$ at 20° was the same whether the tryptophan were initially in the free state or in the form of the mercurial, provided that the solutions before the addition of the nitrite were allowed to stand for 20 min. at 20° or were heated at 50° for a few minutes and then cooled. It was also found that the tryptophan mercurial solution in absence of nitrite deteriorated by some 8 or 9 % in 20 hr. at 20°. As the coloration fades (curve $i1$ in Fig. 5) it becomes more yellow in tint, and the plotted variation of intensity with time is based upon "intensity matchings" to a normal eye under "daylight" filament illumination as described earlier. This applies to all the curves in Figs. 5 and 6 as well as to comparison of one curve with another of different characteristics.

Varying nitrous acid. Curves $i1$, $i2$ and $i3$ in Fig. 5 are obtained with 1, 0·5 and 0·2 mg. tryptophan as such respectively, in the presence of 150 millimol. H_2SO_4, 1 millimol. $Hg(HSO_4)_2$ and 0·025 millimol. $NaNO_2$ at 20°. The proportionality between coloration and tryptophan (the peaks are reached within 1 min. and $i1/i3$ is 4·5 instead of 5·0) becomes poorer with fading. Curves $j1$,

*j*2, *j*3, *j*4 and *j*5 are for 0·5 mg. tryptophan as such, 150 millimol. H_2SO_4, 1 millimol. $Hg(HSO_4)_2$ and 0·8 millimol. $HgCl_2$ with 2, 1, 0·5, 0·125 and 0·031 millimol. $NaNO_2$ respectively, at 20°. Curves *j*6 and *j*7 are obtained in the same way as *j*3 but with 1 and 0·2 mg. tryptophan respectively. The development and fading rates and the maxima are all increased with increase in nitrite, and there is increasing tendency to formation of small gas bubbles. The coloration becomes slightly more grey. The peaks of *j*3, *j*6 and *j*7 are reached within about 10 sec. and the proportionality is good (after 1 or 2 min. *j*6/*j*7 is 4·8 instead of 5·0).

Effects of chloride, cyanide and cysteine. Curve *k*1 in Fig. 6 is for 0·5 mg. tryptophan, 150 millimol. H_2SO_4, 1 millimol. $Hg(HSO_4)_2$ and 0·125 millimol. $NaNO_2$ at 20°. Curve *k*2 is obtained in the same way as *k*1 but with 1·2 millimol. $HgCl_2$ in addition, and curve *j*4 (0·8 millimol. $HgCl_2$) is shown transferred to these ordinates from Fig. 5. The $HgCl_2$ makes the coloration slightly more grey, and as well as may be judged the intensities of *j*4 and *k*2 are 12 and 15% respectively less than that of *k*1 after 0·5 min. and 5 and 7% respectively after 10 min. The effect of 1·2 millimol. $Hg(CN)_2$ is disastrous, the coloration being replaced by a pale greenish brown.

Curves *k*3, *k*4 and *k*5 show the effects of 3 mg. cystine upon *k*1, *j*4 and *k*2 respectively. In all cases the cystine makes the coloration rather more grey, slightly increases the fading rate and reduces the intensity, as well as may be judged after 1 or 2 min., by some 10%.

Varying H_2SO_4 and $Hg(HSO_4)_2$. When H_2SO_4 and $Hg(HSO_4)_2$ concentrations are both relatively low, the coloration is rather pale. Increase in H_2SO_4 alone increases the intensity of coloration, makes it more grey, and increases the fading rate. Under favourable circumstances $Hg(HSO_4)_2$ can be increased in strongly acid solutions beyond the point of saturation with respect to $HgSO_4$, and more intense colorations can be obtained with these unstable reagents of high acidity. Thus the colorations developed at 20° by 0·3 mg. tryptophan, 160 millimol. H_2SO_4, 0·025 millimol. $NaNO_2$ and 0·5, 1, 1·25 and 2 millimol. $Hg(HSO_4)_2$ respectively (the last three being supersaturated) are progressively less grey, and as well as may be judged their intensities after 1 or 2 min. are in the ratios 70 : 87 : 90 : 100. With reagents of increasing acidity (4–8 *M* H_2SO_4) and saturated with respect to $HgSO_4$, there is a progressive increase in the development and fading rates and the colorations are more grey but are of much the same intensity after 10 min. when 0·5 millimol. $NaNO_2$ is used. Incidentally, indole itself gives colorations much more stable than those of tryptophan.

Solubility of $HgSO_4$ in H_2SO_4 solutions

The solubility was measured by saturating the H_2SO_4 solutions with known amounts of $HgSO_4$ in about 100% excess, dissolving the residues in *N* H_2SO_4 and titrating with standard HCl solution using the phenol mercurial in presence of a little HNO_2 as indicator. At 20° the solubility of $HgSO_4$ in g. per l. of acid was found to be 105, 39, 13, 5·5 and 2·2 in 4, 5, 6, 7 and 8 *M* H_2SO_4 respectively; at 15° the solubility in 6 *M* H_2SO_4 was found to be 12·5 g. per l.

Solubility of the mercurials in various reagents

Generally, the higher the temperature and the more acid the solution, the more soluble are the mercurials in it. 4 mg. tyrosine, mercurated by heating with reagent at 60° for 30 min., remain in solution in 10 ml. of the diluted reagent responsible for the *c* series of curves for more than 1 hr. at 25°, whereas only about 2·5 mg. remain in solution after 1 hr. at 15°. If the diluted reagent responsible for the *b* series of curves is employed instead, the mercurial begins

to separate before the temperature has fallen to 25°. With ordinary phenol the solubilities are very small.

Mercuration of tryptophan by all the diluted reagents mentioned under "Phenol reaction" appears to be complete within a few hours at 20° or 10 min. at 60°. Provided that its solubility is exceeded the mercurial first appears as a yellow cloud that will flocculate most readily if there is much of it and if the temperature is high. With very little tryptophan there may be a faint cloud only on cooling or no precipitate at all. In solutions each containing in 10 ml. 2 millimol. $Hg(HSO_4)_2$, 1 millimol. $HgCl_2$ and 2·5, 5, 7·5 and 12·5 millimol. H_2SO_4 respectively 0·01 mg. tryptophan gave clouds in the first three at 50° after 10, 15 and 20 min. respectively. After 30 min. at 50° and 3 hr. at 20°, nephelometric estimation showed that precipitation in the first two was virtually complete and that 0·002 mg. tryptophan remained in solution in the third. There was no precipitate in the fourth. With 10 ml. of the diluted reagent responsible for the *a* series of curves 0·01 mg. tryptophan gave no cloud in 30 min. at 60°, and after cooling for 2 hr. at 20° some 0·006 mg. remained in solution. 1 millimol. $HgCl_2$ and 50 mg. glycine were each without effect. With the diluted reagent responsible for the *c* series of curves there was a faint cloud in 30 min. at 60°, and after cooling for 1 hr. at 20° some 0·003 mg. remained in solution. Under the same conditions the diluted reagent responsible for the *b* series of curves gave virtually complete precipitation. 10 ml. of solution containing 1·5 millimol. $Hg(HSO_4)_2$ and 50 millimol. H_2SO_4 will hold about 0·9 mg. tryptophan as the mercurial in solution at 50°, and 10 ml. of the reagents responsible for the *i* and *j* series of curves will hold more than 1 mg. in solution at 20°.

With 10 ml. of the diluted reagent responsible for the *c* series of curves 1·5 mg. cystine just fails to give a precipitate after heating at 60° for 30 min. and cooling at 20° for 2 hr., and the limiting amount of cystine when the diluted reagent responsible for the *a* series of curves is used instead is about 2·5 mg.

Methods

Reagents and conditions that are best suited for the estimation of tyrosine or tryptophan in a given solution can be selected from the experimental section. Those described below are generally satisfactory for the estimation of both amino-acids in the same aliquot of test solution at room temperatures of 15–25°. The aliquot must contain not more than 2 mg. tyrosine and 1 mg. tryptophan and preferably not less than 0·5 mg. tyrosine and 0·25 mg. tryptophan. It may contain several millimol. $NaHSO_4$ and 0·25 milliequiv. of chloride without seriously affecting the colorimetry, but it must be free from nitrates and nitrites and from other halides, phenols and indoles. At *p*H 1·0, extraction with 2 vol. of ether will remove 95 % of any *p*-hydroxybenzoic acid and 98 % of any ordinary phenol that may be present in a test solution without affecting the tyrosine or tryptophan.[1] Extraction with toluene at *p*H 6–7 will not affect the tyrosine or tryptophan, and Kraus [1925] has shown that indole and skatole are removed by toluene.

Reagents

Solution A. 5*N* H_2SO_4 solution (25 g. of 98 % H_2SO_4 per 100 ml.).

Solution B. 75 g. $HgSO_4$, 55 g. $HgCl_2$, and 70 g. Na_2SO_4 are dissolved in 850 ml. water plus 125 g. 98 % H_2SO_4 and diluted to 1 l.

[1] It seems superfluous to add that an extracting solvent is removed and the solution adjusted to a new volume, but see Shinohara [1935]. Differential extraction of phenols containing amino- but no carboxyl groups might be effected at *p*H 8.

Solution C. This is made by diluting a volume (600 ml. for convenience) of solution B with an equal volume of N H_2SO_4 solution.

Solution D. 12 g. $HgSO_4$ and 9 g. $HgCl_2$ are dissolved in 600 ml. water plus 100 g. 98% H_2SO_4. A further 500 g. H_2SO_4 are added with cooling, and the mixture is diluted to 1 l.

Solution E. M $NaNO_2$ solution (6·9 g. $NaNO_2$ per 100 ml.).

Standard tyrosine and tryptophan solutions. These contain 0·25–1 mg. of tyrosine or tryptophan per ml., with 0·1 N H_2SO_4 or 0·05 N NaOH as solvent for tyrosine, and water for tryptophan. The tryptophan solution deteriorates by 1% in a week at 20°, but the tyrosine not appreciably in several months.

Procedure

Liquids are separated from suspended solids by centrifuging, 10 min. in a field of 1500 times gravity being generally sufficient. A glass rod of 2 mm. diameter and slightly bent at the end is used to stir solutions and to suspend precipitates in them. Barely moistened with octyl alcohol it serves as a whisk to force solids at the air-liquid interface beneath the surface before centrifuging. It is rinsed down with a few drops of the appropriate solution.

In a 15 ml. centrifuge tube (conical bottom type), the aliquot of up to about 3 ml. of test solution together with sufficient of A to bring it to pH 0·3 (from titration of a separate aliquot, using an indicator such as brilliant cresyl blue), is diluted to 5 ml. with N H_2SO_4 or with the appropriate volumes of A and of water. 5 ml. of B are added and the tube is maintained at 60–65° in a water-bath for 30 min. It is then cooled in a water-bath for 1 hr. at 1 or 2° below room temperature, and after centrifuging, the clear liquid is drained into a 25 ml. graduated cylinder. 10 ml. of C are run into the centrifuge tube, any precipitate is well stirred up for a minute or so, and the contents are again centrifuged. The liquid is drained into the cylinder and the contents are diluted with C to 24·5 ml. in readiness for the tyrosine estimation. The standard is prepared simultaneously with the unknown and in an entirely analogous manner. The precipitate remaining in the centrifuge tube is used in the tryptophan estimation.

For the estimation of the tyrosine the contents of the graduated cylinders should be employed within an hour, as cloudiness may develop on long standing. 0·5 ml. of E is run slowly into each cylinder so as to float on top, and as soon as possible thereafter the cylinders are shaken simultaneously. Colorimetric comparison of the unknown with the standard should be made 3 min. after the mixing.

For the estimation of the tryptophan the solid mercurial, which may be left moist in the tube for a day without detectable destruction, is well rubbed up with 10 ml. of D, and the tube is maintained at 40–45° in a water-bath for 15 min. with occasional rubbing of any solid that settles out. It is then cooled in a water-bath for 30 min. at 1 or 2° below room temperature, and after centrifuging, the clear liquid is drained into a 25 ml. graduated cylinder. A further 10 ml. of D are run into the tube, the contents are stirred and any solid is well rubbed for a few minutes, and after centrifuging, the liquid is drained into the cylinder and the volume made up to 24·5 ml. with D. The standard is prepared simultaneously and in precisely the same way. Within an hour or so 0·5 ml. of E is run into each cylinder so as to float on top, and as soon as possible thereafter the cylinders are shaken simultaneously and colorimetric comparison is made with the least delay. The coloration peaks are reached within some 10 sec. but it is seldom possible to compare within 1 min.

Each ml. of the standard tyrosine and tryptophan solutions requires about 0·25 ml. of A to bring it to *p*H 0·3. The colour standards are conveniently prepared from suitable amounts of them mixed together. They should preferably be within 70 and 150 % of the intensities of the unknowns. If their necks are not so narrow as to hinder mixing, 25 ml. standard flasks can be used in place of the cylinders, and the final volume of colour solution may be 25·5 ml. throughout instead of 25 ml. without appreciably altering the development and fading rates.

Substances in the test solutions may contribute adventitious coloration to the tyrosine and tryptophan unknowns before the addition of the nitrite. Correction can be made by employing appropriate blanks (substituting water for nitrite) with standards and unknowns in a compensating colorimeter. If, apart from adventitious coloration or the effects of large quantities of cystine, standards and unknowns differ in tint or are found to develop and/or fade at different rates, the presence of other phenols or indoles may be inferred. Incidentally, differences in rates can sometimes be enhanced by using another reagent, such as that responsible for the *b* series of curves for the tyrosine estimation.

Results of tests

Curves *c*2 and *j*3 respectively are representative of these tyrosine and tryptophan colour solutions. Generally, substances that might otherwise influence the tryptophan colour reaction are washed away into the tyrosine solution. The effect of 1 mg. cystine upon the estimation of 1 mg. tyrosine is shown by curve *c*8 (1 % low after 3 min.). With up to some 2·5 mg. cystine the error remains with the tyrosine estimation and is roughly in proportion; larger amounts begin to cause error in the tryptophan estimation. One object of the inclusion of $HgCl_2$ in reagent B (and consequently C) is to increase its scope, and it is needed in D because variable amounts of C may be left with the precipitated tryptophan mercurial. There is no appreciable error in the estimation of tyrosine or tryptophan in a test solution aliquot that contains 0·25 milliequiv. of chloride, 3 millimol. NaOH (which is of course converted into $NaHSO_4$), 1 millimol. $ZnSO_4$, 100 mg. glycine, 30 mg. glycine plus 10 mg. phenylalanine, 1 mg. histidine or 5 mg. methionine.

Analysis of an alkaline hydrolysate of gelatin indicated 0·02 % tyrosine and 0·01 % tryptophan calculated on original protein. Analytical recoveries of 1 mg. tyrosine and 0·5 mg. tryptophan, added to an amount of hydrolysate representing 0·1 g. of original protein, were perfect to within the errors of comparison (< 1 % for tyrosine, < 2 % for tryptophan).

Analytical recoveries of 1 mg. tyrosine and 0·5 mg. tryptophan, added to the soluble products of acid and alkaline hydrolysis of 40 mg. of several carbohydrates, were likewise perfect to within the errors of comparison. The slight adventitious colorations required correction.

Summary

The Folin and Ciocalteu method (based on the Millon reaction) for estimating tyrosine was found to be unsatisfactory when applied to the hydrolysates of plant-leaf proteins, as the unknown colour solutions were cloudy. The difficulty has been overcome by radical changes of procedure, embracing mercuration of the tyrosine and precipitation of any tryptophan present in one step

instead of two, and dilution of the reacting mixture with a solution approximating closely to it in composition. Data are provided concerning the Millon and associated colour reactions of tyrosine, including the effect of extraneous substances in test solutions.

During the course of the work the colour reactions between nitrous acid and tryptophan and between nitrous acid and the tryptophan mercurial were investigated. Under appropriate conditions the second reaction was found to provide a delicate test for tryptophan, which has been made the basis of a very simple colorimetric method for the estimation of this amino-acid.

Methods of estimating tyrosine and tryptophan in solution are described, and the errors discussed.

REFERENCES

Folin & Ciocalteu (1927). *J. biol. Chem.* **73**, 627.
Gibbs (1926). *Chem. Rev.* **3**, 291.
—— (1927). *J. biol. Chem.* **71**, 445.
Goddard & Goddard (1928). Text Book of inorganic Chemistry, Vol. **11**, Part 1. (London: Griffin and Co., Ltd.)
Hoffmann (1853). *Liebigs Ann.* **87**, 123.
Hopkins & Cole (1901). *J. Physiol.* **27**, 418.
Kraus (1925). *J. biol. Chem.* **63**, 157.
Lefeve & Desgretz (1935). *C.R. Acad. Sci., Paris*, **200**, 762.
Lintner (1900). *Z. angew. Chem.* p. 707.
Meyer (1864). *Liebigs Ann.* **132**, 156.
Millon (1849). *C.R. Acad. Sci., Paris*, **28**, 40.
Nasse (1879). *Ber. naturf. Ges. Halle*, p. 23.
Nickel (1890). Die Farbenreaktionen der Kohlenstoffverbindungen, 2nd ed. (Berlin.)
Shinohara (1935). *J. biol. Chem.* **110**, 263.
Vaubel (1900). *Z. angew. Chem.* p. 1125.
von Deseö (1934). *Biochem. Z.* **271**, 142.