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The Colorimetric Determination of Dipeptides by means of Oxygenated Cobalt Complexes

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Numerous methods have been proposed for the determination of dipeptide hydrolysis (Martin & Synge, 1945), but no single one of them satisfies the dual requirements of rapidity and accuracy. Procedures based on acid-base titration lack precision because of the large blank due to the substrate; this is true also of the colorimetric-ninhydrin (Schwartz & Engel, 1950) and ammonia-ninhydrin (MacFadyen, 1944) methods. Measurement of the carbon dioxide evolved in the ninhydrin reaction has been made the basis of quantitative procedures (Van Slyke, MacFadyen & Hamilton, 1941; Van Slyke, Dillon, MacFadyen & Hamilton, 1941); these have the advantages that, since simple dipeptides evolve no carbon dioxide, amino acids are specifically determined. A disadvantage of these methods is the laborious and time-consuming technique required. Methods based on the formation of copper complexes (Spies & Chambers, 1951; Spies, 1952) are imprecise because, even under the most favourable conditions, dipeptides give 70–80% of the extinction produced by their constituent amino acids. The Van Slyke nitrous acid technique has many disadvantages, notably the time required per determination and the non-stoichiometric reaction.

In view of these difficulties it was considered desirable to explore the possibility of utilizing other reactions for analytical purposes.

Smith (1948) observed that, when glycylglycine was incubated for several hours at room temperature and pH 8 with Co^{2+} ions, a pink colour developed which was more intense than the colour produced by an equivalent quantity of glycine. The reaction is not restricted to glycylglycine and similar colour production from other dipeptides

has been observed. Gilbert, Otey & Price (1951) made the significant discovery that atmospheric oxygen is involved in the colour-forming reaction and that the coloured compound is an oxygen-containing cobaltous complex. Suitable conditions have been found for the utilization of this reaction as a quantitative analytical method by using hydrogen peroxide as oxygenating agent and a suspension of cobaltous phosphate in borate buffer as the source of cobaltous ions (Crook & Rabin, 1954). Techniques have been evolved for the determination of dipeptide hydrolysis at two levels: these are designated the 'macro' and 'micro' methods and the choice of either depends on the availability and cost of the substrates.

MATERIALS AND METHODS

Glycyl-DL-alanine, glycyl-L-tryptophan, DL-alanyl-DL-alanine, glycyl-DL-phenylalanine, glycyl-L-tyrosine and acetyl-glycine were obtained from L. Light and Co., Colnbrook, Bucks.

Glycylglycine, glycyl-DL-leucine, DL-alanylglycine, DL-leucylglycine and diglycylglycine were obtained from Roche Products Ltd., Welwyn Garden City, Herts.

Sarcosylglycine and *N*-ethylglycylglycine were prepared by the method of Levene, Simms & Pfaltz (1924) and reprecipitated twice by the addition of ethanol to an aqueous solution.

Glycyl-L-proline was prepared by the method of Bergmann, Zervas, Schleich & Leimert (1932). The amino acids used were commercial preparations available in this laboratory.

Absorption spectra were measured in a Unicam Model SP. 500 quartz spectrophotometer with cells of 1 cm. path length.

Colorimetric determinations were made in a Gallenkamp Direct-Reading colorimeter with cells of 1 cm. path length.

Preparation of suspension of cobaltous phosphate in borate buffer (pH 9.1). The suspension is prepared by adding 3 vol. of 0.2M-cobaltous chloride solution to 2 vol. of 0.2M-trisodium phosphate solution, mixing thoroughly and diluting with 10 vol. of 0.1M-borax solution. The suspension should be shaken well before use; it can be used immediately but keeps for several weeks after preparation.

'Macro' method. The sample, placed in a flask graduated at 25 ml., should contain approx. 80 μ moles of dipeptide or hydrolysis products. The sample may have any volume up to 10 ml. without significantly affecting the colour development. Ethanolic thymolphthalein (0.1%, 3 drops) is added and the solution made blue by the addition of 0.5N-NaOH. The cobaltous phosphate suspension is then added from a pipette with a wide tip, and the solution placed in a boiling-water bath. At the end of each of three successive 10 min. intervals, hydrogen peroxide (0.5 ml. of '2 volumes', AnalaR or equivalent) is added slowly with constant shaking. The flask is removed from the bath for the addition of the peroxide and replaced when the addition is completed. After the final addition of peroxide, the heating is continued for 30 min., the flask is then cooled to room temperature and made up to the mark with water. The solution is filtered (Whatman no. 2 paper) and read in a colorimeter with an Ilford 624 (green) filter. The colour is stable for at least 3 weeks. When the method is used in enzyme assays, the reaction may be stopped by the addition of perchloric acid and a sample taken for analysis. In order to avoid undue dilution, a volume of 10N-NaOH equivalent to the quantity of perchloric acid present is added; the final adjustment to a blue colour is made with 0.5N-NaOH as above.

'Micro' method. The sample, placed in a test tube graduated at 5 ml., should contain approx. 20 μ moles of dipeptide or hydrolysis products. Ethanolic thymolphthalein (0.1%; 1 drop) is added and the solution made blue by the addition of 0.1N-NaOH. Cobaltous phosphate suspension (2 ml.) is added. The remaining procedure is as described for the 'macro' method, with three successive additions of 0.25 ml. of 1 volume hydrogen peroxide. After making up to the mark it is preferable to centrifuge rather than filter the solution.

RESULTS

Factors influencing colour production

The addition of hydrogen peroxide in stages rather than in a single batch has the advantage that

colour production is greater, more reproducible and linear with quantity of dipeptide. No advantage is gained from adding the peroxide in a greater number of increments than three, since reproducibility within the limits of use of the photo-electric colorimeter can be obtained by this technique. It is important to add the peroxide slowly, otherwise low results of poor reproducibility are obtained.

A systematic investigation of the effect of temperature on the colour-forming reaction was not attempted; however, unless the colour formation is carried out in a boiling-water bath, the solutions obtained are difficult to filter or to centrifuge. Furthermore, lowering the temperature causes increased colour production from glycine; this is disadvantageous since it reduces the precision of the method.

The effect of varying the time of incubation after the final addition of peroxide was investigated; increasing this reduces the colour production from solutions containing large amounts of glycine. Since no further reduction was found after 30 min., this was chosen as the most suitable time.

The effect of a number of added substances was also investigated. Some of the results obtained are shown in Table 1. It can be seen that the method is suitable for use in enzyme experiments in barbiturate buffers and for assaying preparations of sarcosomes and mitochondria, since added sucrose has no effect. A slight depression of colour formation is observed by the addition of 200 μ moles of phosphate. The readings can be corrected by simple proportion to bring them back to a calibration curve constructed in the absence of phosphate. There was no detectable depression of colour formation on the addition of 100 μ moles of phosphates.

Chromogenic compound. The absorption spectra of solutions obtained by carrying out the 'macro' method on 80 μ moles of glycylglycine and on 160 μ moles of glycine are shown in Fig. 1. The dipeptide complex has an absorption peak at 520 m μ with a smaller peak at 390 m μ . Alanylglycine, glycylalanine, alanylalanine and leucylglycine give

Table 1. *Colorimeter readings on mixtures of glycylglycine and glycine which simulate different degrees of hydrolysis of 80 μ moles of glycylglycine*

Addition	Colorimeter reading		
	0 % hydrolysis	50 % hydrolysis	100 % hydrolysis
None	50.0	21.3	1.8
CoCl ₂ (10 μ moles)	49.4	21.6	1.9
Na ₂ HPO ₄ (200 μ moles)	47.8	20.5	1.9
Na ₂ HPO ₄ (200 μ moles) corr.*	50.0	21.4	1.9
2 % Sodium barbiturate (1 ml.)	49.5	21.6	—
1:5 Rat-muscle homogenate (6 mg./ml.; 1 ml.) +	50.2	—	1.8
20 % perchloric acid (2.5 ml.)			
20 % (w/v) Sucrose (1 ml.)	49.2	21.4	1.3

* Multiplied by 50.0/47.8, the ratio of the colorimeter readings before hydrolysis in the absence and presence of phosphate.

Fig. 1. Absorption spectra of oxygenated cobalt complexes of: —, 80 μ moles of glycylglycine; ---, 160 μ moles of glycine.

absorption spectra similar to glycylglycine. Methylation of the amino group, as in sarcosylglycine, reduced colour production and shifts the wavelength of maximum absorption to 530 $m\mu$. Very little colour is given by glycinamide. Of the remaining dipeptides tested, all except those containing tyrosine residues have absorption maxima at 520 $m\mu$. The colour production from a number of dipeptides is illustrated in Table 2. The optical density at 520 $m\mu$ does not always parallel the colorimeter reading with an Ilford 624 filter (maximum transmittance at 520 $m\mu$); this is illustrated by the figures quoted for alanylglycine and leucylglycine in Table 2. One reason for this is that the absorption spectra produced from different dipeptides differ in the breadths of their absorption peaks; since the 624 filter has a rather broad transmittance curve, the colorimeter reading represents the integrated absorption over quite a wide spectral range and thus reflects the shape of the absorption peaks as well as their height.

The colour production of a number of amino acids and related compounds is illustrated in Table 3; it can be seen that the colour production from most of these substances is negligible. However, atypical colours are produced from some substituted amino acids and peptides; these are listed in Table 4. The absorption spectra produced from some peptides giving atypical results are shown in Fig. 2; DL-leucylglycine is included for the purpose of comparison.

Table 2. Colour production obtained from 80 μ moles of some simple dipeptides

Substance	Colorimeter reading (624 filter)	Optical density (520 $m\mu$)
Glycylglycine*	50.3	0.560
DL-Alanylglycine*	51.8	0.584
DL-Leucylglycine*	52.9	0.574
Glycyl-DL-leucine*	32.5	—
Glycyl-DL-alanine*	50.4	0.560
DL-Alanyl-DL-alanine*	47.8	0.532
Glycyl-L-tryptophan	34.0	0.389
Glycyl-DL-phenylalanine*	36.5	0.411
Sarcosylglycine	38.3	0.408†
N-Ethylglycylglycine	19.4	—
Glycylproline	0.0	—

* These compounds give essentially the same absorption spectrum as glycylglycine.

† 0.420 at wavelength of maximum absorption (530 $m\mu$).

Table 3. Colour production from 80 μ moles of some representative amino acids and related substances, with an Ilford 624 filter

Substance	Colorimeter reading
Glycine	2.7
Alanine	5.6
Valine	4.8
Leucine	2.7
Serine	3.2
Proline	2.5
Phenylalanine	1.2
Arginine	3.4
Aspartic acid	4.9
Glutamic acid	3.8
Asparagine	6.0
Glycinamide	9.3
Acetyl glycine	0.2
Ammonium chloride	0.0

Linearity of colour production

For dipeptides alone, the graphs of colorimeter readings plotted against quantities of dipeptides are linear, either the 'macro' or 'micro' techniques (e.g. Fig. 3 being used). Deviations from the straight line give a measure of the precision of the method: in no case are deviations greater than 0.5 of a colorimeter unit obtained. This linearity is destroyed by the addition of amino acids, and hence, in any application of the method for observing the hydrolysis of dipeptides, calibration curves must first be constructed with known mixtures of the dipeptide and its hydrolysis products. Some examples of such curves are shown in Fig. 4; these curves are quite reproducible.

In order to investigate this non-linearity of the calibration curves, the effect of glycine on the colour production from a constant amount of glycylglycine was determined (Fig. 5). It can be seen that glycine reduces the amount of colour produced and that this reduction is very nearly

Table 4. Colour production from 80 μ moles of some substances giving atypical results

Substance	Colour produced	Colorimeter reading (624 filter)	Optical density (520 $m\mu$)	Wavelength of max. absorption ($m\mu$)	Optical density at max. absorption
Diglycylglycine	Orange	29.0	0.383	487	0.532
Glycyl-L-tyrosine	Orange-yellow	27.7	0.322	505	0.331
Sarcosylglycine	Bluer than glycylglycine	38.3	0.408	530	0.420
L-Tyrosine	Orange-yellow	30.3	—	—	—
L-Histidine	Orange-yellow	14.8	—	—	—
L-Tryptophan	Yellow	15.1	—	—	—
L-Cysteine	Yellow-green	42.8	—	—	—

Fig. 2. Absorption spectra of oxygenated cobalt complexes of 180 μ moles of: —, DL-leucylglycine; ----, diglycylglycine; ····, sarcosylglycine; - · - ·, glycyl-L-tyrosine; - - - -, glycyl-L-tryptophan.

Fig. 3. Linearity of colour production with dipeptides. Graphs have been displaced vertically by the number of colorimeter units indicated in parentheses: 1, glycyl-DL-alanine (0); 2, glycylglycine (0); 3, DL-alanyl-glycine (10); 4, DL-leucylglycine (20); 5, glycyl-DL-alanine (30); 6, DL-alanyl-DL-alanine (40); 7, glycyl-DL-leucine (60); 8, sarcosylglycine (80).

proportional to the concentration of glycine present. Since the quantity of glycine present varies continuously along the calibration curve, the immediate cause of non-linearity in the calibration curves is apparent. The reduction in colour produced by added glycine might be due to the inhibition of the colour-forming reaction or to the formation of mixed complexes of amino acids and dipeptides. Spectrophotometric results have been obtained which suggest that the latter explanation is the correct one (Fig. 6). In the region 280–460 $m\mu$ the experimental solution has a greater extinction, and from 460 to 700 $m\mu$ a smaller extinction than is predicted by assuming additivity of extinction of the individual components. The simplest explanation of these results is that mixed complexes of amino acids and dipeptides are formed, and that these have a higher absorbency at wavelengths less than 460 $m\mu$ and a smaller

extinction at wavelengths greater than 460 $m\mu$ than the sum of the corresponding quantities of simple complexes. Similar results have been obtained with alanyl-glycine-alanine and glycyl-alanine-glycine-alanine mixtures.

Further evidence of a qualitative nature has been obtained for the formation of mixed complexes. The addition of the cobaltous phosphate suspension to a mixture of a dipeptide and an amino acid causes the production of an olive-brown colour; this is not observed for either dipeptides or amino acids alone and it might well be associated with the formation of mixed complexes. This colour production is labile and disappears on heating in a boiling-water bath.

A disadvantage of non-linear calibration curves arises when the calibration curve based on one initial quantity of dipeptide has been determined, but is required for another initial quantity. However, since the non-linearity is probably due to the formation of mixed complexes, then provided that the ratio [amino acid]:[dipeptide] is held constant, the colorimeter reading should be proportional to [dipeptide], since the relative amounts of the complexes should be independent of the absolute concentration of dipeptide. Thus a graph of colorimeter reading against quantity of dipeptide is linear in the presence of amino acids provided that the ratio [dipeptide]:[amino acid] is held constant. For two calibration curves, each based on a

Fig. 4. Calibration curves for the determination of hydrolysis based on an initial 80 μ moles of dipeptides. Graphs are displaced vertically by the number of colorimeter units shown in parentheses.

Fig. 5. Effect of glycine on colour production from 40 μ moles of glycylglycine.

Fig. 6. Absorption spectrum obtained from 40 μ moles of glycylglycine + 80 μ moles of glycine: —, experimental; ---, theoretical obtained by adding together the individual extinctions of the components.

Fig. 7. Inter-relation of calibration curves based on different initial quantities of glycylglycine. Initial quantity of glycylglycine: ●, 80 μ moles; ○, 60 μ moles; ■, 50 μ moles. Experimental points based on the initial 60 and 50 μ moles were converted into the curve for 80 μ moles by simple proportion.

different initial quantity of dipeptide, the ratio [dipeptide]:[amino acid] at any given percentage hydrolysis is constant and independent of the initial quantity of dipeptide. Since the colorimeter reading is linear with quantity of dipeptide in the presence of amino acids when the ratio [dipeptide]:[amino acid] is constant, it follows that all the calibration curves based on different initial quantities of dipeptide should be interconvertible by simple proportion. To do this it is essential to use the 100% hydrolysis solution as a blank since amino acids alone give a non-stoichiometric colour production. An example of such interconversion is shown in Fig. 7, where colorimeter readings of artificially prepared hydrolysis mixtures based on an initial 50 and 60 μ moles of glycylglycine have been converted so as to fall on a calibration curve based on an initial 80 μ moles of glycylglycine.

DISCUSSION

The success of the present method results from the use of cobaltous phosphate suspensions. No stoichiometric relation between colour and amount of dipeptide was obtained with suspensions of cobaltous hydroxide in various buffers, including borate, or in the presence of glycerol; various oxidizing reagents other than hydrogen peroxide were also investigated. The chief merit of phosphate appears to lie in its ability to retard the oxidation of Co^{2+} ions and thus to allow full opportunity for oxygenation of the cobaltous complexes.

The method is rapid and accurate. Thirty or more estimations can be performed in one batch in 2–3 hr. Its reproducibility is such that individual determinations in the one colorimeter, but with different batches of reagents, have a standard deviation of 1.5–2% of the colorimeter reading. With care the percentage hydrolysis of dipeptides can be determined within the limits of precision of the colorimeter. Where precision of a lower order suffices, simplifications can be made; the time of final incubation can be cut and the hydrogen peroxide can be added in a single batch. Thus the method has obvious advantages over other methods which are available for observing hydrolyses, although its application is restricted, in the main, to dipeptides of simple, non-substituted amino acids.

Most amino acids give colours initially, but these are not stable during the subsequent heating. Very little colour is obtained from glycylglycine, although this substance forms complexes with Co^{2+} ion of stability comparable with those of sarcosylglycine

(Datta & Rabin, 1956). It is probable therefore that a free carboxyl group is essential for colour formation. No colour is obtained from glycylproline, although this substance forms complexes with Co^{2+} ion of stability greater than those of glycylglycine (Datta & Rabin, 1956). A possible explanation of this is that, in the formation of a coloured complex, ionization of the peptide hydrogen occurs as with the copper complexes of these compounds (Datta & Rabin, 1956).

SUMMARY

1. A colorimetric procedure for the determination of dipeptides is described.
2. It depends upon the formation of oxygenated cobalt complexes of the dipeptides in the presence of alkaline cobaltous phosphate suspensions.
3. It is applicable to a large number of dipeptides and has advantages of rapidity and accuracy.
4. It can be used for following the hydrolysis by acids, bases or enzymes of dipeptides of mono-aminomonocarboxylic acids, but not for those containing cysteine, tyrosine, tryptophan or histidine, since these amino acids themselves give appreciable colours under the conditions of the assay. Most other amino acids and ammonium salts cause no appreciable interference.

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