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The Ninhydrin Reaction and its Analytical Applications

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The colorimetric or spectrophotometric measurement of the absorption produced by the purple reaction product (ANP) between α-amino acids and ninhydrin at 570 mμ has become one of the most widely used methods for the quantitative determination of small quantities of α-amino acids. Its analytical application is, however, sometimes adversely affected by one or more of the following factors. Since the ANP has not been prepared in the solid state, its extinction coefficients could not be determined; in consequence it has been necessary to express analytical results in terms of 'colour yield' in relation to that given by known quantities of a reference standard reacting under defined conditions (Moore & Stein, 1948). It has often been observed that the ANP yield was not constant owing to the occurrence of oxidative side reactions decomposing it to colourless products. Moore & Stein (1948) sought to overcome this effect by the addition of reducing agents like stannous chloride or hydrindantin to the reaction systems. The absorption maximum of the ANP was found to vary within the 500–580 mμ range, thus making impossible absorption measurements at a fixed wavelength. A method devised to eliminate this effect, by treating with alkali spots of ANP on chromatograms, was described by Kay, Harris & Entenman (1956).

The sensitivity of the reaction, i.e. the colour yield by a given amino compound produced under defined conditions, is significantly influenced by the composition of the solvent in which the reaction takes place. It is highest in organic solvents and decreases in solvent mixtures containing water (Yanari, 1956).

The experiments reported in this paper were designed to investigate the properties and behaviour of ANP, especially its absorption spectrum and its changes under different conditions and to correlate the changes in ninhydrin reactivity with measurable properties of this compound and, in turn, with structural changes undergone by the ninhydrin molecule in different solvents.

MATERIALS AND METHODS

All chemicals used were of analytical grade. The disodium salt of ethylenediaminetetra-acetic acid (EDTA) was recrystallized from water before use. Hydrindantin was

prepared according to West & Rinehart (1942) by reduction of ninhydrin with ascorbic acid.

Solvents. The absorption spectra of ninhydrin and hydrindantin were measured in absolute ethanol. In all other experiments 95·5% (w/v) ethanol was used. All water used was twice distilled. Spectrophotometric measurements were performed in a Beckman quartz spectrophotometer model DU, in 1 cm. cells.

Preparation of ANP. A solution of 0·5 g. of DL-alanine, 0·2 g. of ninhydrin hydrate and 0·1 g. of EDTA in 50 ml. of water was refluxed for 8 min. and cooled to room temp. Small quantities of bis-diketoindanyl (Moubasher & Ibrahim, 1949) were filtered off. The filtrate was then cooled to 2° and acidified with cold (2°) 20% (w/v) H_2SO_4. The reddish precipitate formed was filtered off and washed with ice-cold water as rapidly as possible, until the filtrate showed a strong purple colour. The precipitate was then sucked dry and suitable fractions of the still slightly wet material were taken for nitrogen determination and spectrophotometric measurements. Another weighed portion was dried to constant weight at 105°. All analytical results were calculated with this dry-weight determination. The nitrogen contents of a wet sample, recalculated to dry weight, and that determined after previous drying were identical, so that no loss of material had occurred on drying (Found: N, 4·5; 4·7. $C_{18}H_9O_4N$ requires 4·6%). It was, however, found that a loss of 10–12% of absorption occurred on drying, indicating that a colourless non-volatile substance had begun to form, presumably hydrindantin (Moubasher & Ibrahim, 1949). The absorption spectrum is shown in Fig. 1.

Ninhydrin blue. A solution of the blue compound formed by ninhydrin with concentrated alkali (ninhydrin blue) suitable for spectrophotometric measurements was prepared by dissolving 13 mg. of ninhydrin hydrate in 3 ml. of 6N-NaOH. A blue colour is rapidly formed which is stable to air and heating, but disappears when the solution is diluted with water.

Hydrindantin blue. A solution of the blue compound formed by hydrindantin with alkali (hydrindantin blue) was prepared by dissolving 10 mg. of hydrindantin in 25 ml. of 0·1N-NaOH. For spectrophotometric measurements the solution was rapidly transferred to the absorption cell and covered with a layer of liquid paraffin to protect it from air.

General procedure. The reaction mixture, consisting of 0·1 ml. of an aqueous solution of the nitrogen compound to be tested, 0·2 ml. of 0·3% aq. EDTA soln. and 4 ml. of 0·2% ninhydrin soln. in the solvent employed in the respective experiment was refluxed for 20 min. in a 25 mm. × 200 mm. Pyrex test tube fitted with a cold-finger condenser reaching to within 3 cm. above the boiling solution. The cooled solution was filtered, if necessary, from crystals of EDTA through a small wad of cotton wool and measured in the spectrophotometer at 578 or 408 mμ, if necessary after dilution with an appropriate volume of solvent. The reagent blank produced by ninhydrin with recrystallized EDTA is negligible when measurements are taken at 578 mμ, but at 408 mμ the reagent blank is significant owing to the absorption of ninhydrin at this wavelength (see Fig. 2).

For experiments in which the dependence of ninhydrin reactivity with amino acids upon the pH of the reaction mixture was determined: reaction mixtures of constant ninhydrin content and varying pH were prepared by bringing 25 ml. of a 0·4% aq. ninhydrin soln. to approximately the desired pH by the addition of 0·1N-HCl or NaOH and adding water to make the total volume 50 ml. The final pH of 4 ml. of this solution, to which 0·1 ml. of water containing 500 μg. of DL-alanine but no EDTA was added, was then accurately determined.

For experiments to test the availability of the 'general procedure' for the quantitative determination of amino nitrogen 95·5% (w/v) ethanol was used as the solvent.

(*a*) Determinations of colour yield at 578 and 408 mμ at different concentrations were carried out for all the natural amino acids and glycylglycine, glycyl-leucine, leucylglycine, triglycine and leucylglycylglycine. They were added as aqueous solutions containing from 0·2 to 9 μg. of α-amino nitrogen/0·1 ml.

(*b*) The influence of buffers normally used was determined in a parallel series of experiments, under the same conditions except that the amino acids and peptides were dissolved in acetate, or citrate or phosphate buffers (0·1M, pH 6·5).

(*c*) The reactivity of nitrogen-containing buffers was tested by adding 5 mg. of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) or sodium diethylbarbiturate in 0·1 ml. of water to a reaction mixture not containing other nitrogen compounds.

(*d*) Ninhydrin reactivity with free ammonia, methylamine, ethylamine and ethylenediamine was tested in reaction mixtures containing quantities of each nitrogen compound equivalent to 10 μg. of nitrogen. Ammonium chloride and the hydrochlorides of the amines mentioned were added in quantities equivalent to 2 mg. of nitrogen.

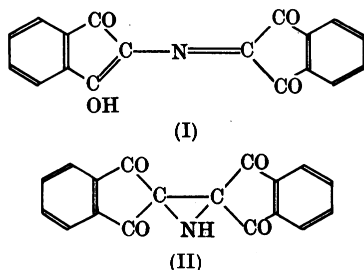
All experiments mentioned under (*a*) and (*d*) were also carried out with hydrindantin instead of ninhydrin.

Kinetic experiments. The desired amount of DL-alanine was dissolved in 4 ml. of water, 8 ml. of a 0·3% aq. EDTA soln. was added, followed by 160 ml. of ethanol containing the desired amount of ninhydrin. This reaction mixture was brought to the boil in a cylindrical separating funnel of 250 ml. capacity, externally wound with electrical-heating tape and fitted with a reflux condenser. The reaction mixture was stirred by a vigorous stream of nitrogen gas. Samples (3 ml.) were withdrawn through the tap of the funnel into test tubes kept in ice–water at 20 sec. intervals during the first 2 min. of reaction time (counting from the beginning of boiling), at 30 sec. intervals during the following 6 min., and then every 3 min. until the end of the reaction time (50 min.). Spectrophotometric measurements were taken at 578 and 408 mμ; if necessary, the samples were diluted with an equal volume of ethanol. All reactant concentrations are given in Table 1.

RESULTS

Properties of ANP. In the solid state ANP is a reddish substance, easily soluble in all solvents containing alcoholic or phenolic hydroxyl groups and insoluble in ether, chloroform and non-polar organic solvents. Attempts to recrystallize it from water failed owing to decomposition on warming. It completely decomposes in dilute aqueous solution if kept for some hours, and almost immediately when such solutions are acidified and slightly warmed. Alkaline ANP solutions and those containing 0·1% of EDTA are stable for some days. Solutions of purified ANP in ethanol or *n*-butanol, stabilized by the addition of EDTA, can be kept for

several weeks. A characteristic property of ANP, which can also be used to purify the compound, is its specific absorption by anion exchangers. The effect can easily be demonstrated by shaking aqueous ANP solutions with De-Acidite or Amberlite IR-4 B in their OH^- forms. Elution can be effected with aq. N-NaCl soln. or with ethanol. The nitrogen content of the ANP fits the structures proposed by Ruheman (1911) (I) and by Woker & Antener (1937) (II). Both (I) and (II) are assumed to be anionic structures (Clarke, 1948; Wieland, 1949).



The absorption spectrum of pure ANP shows two well-defined bands, with $\lambda_{max.}$ 578 and 408 mμ and $E^{1\%}_{1\,cm.}$ 660 and 724 respectively (Fig. 1, curve 2). The maximum at 408 mμ is especially characteristic for ANP, since it does not appear in the absorption spectra of the nitrogen-free compounds ninhydrin blue and hydrindantin blue (Fig. 5) which both absorb strongly in the 570–580 mμ region. The absorption maximum at 408 mμ does not form a part of the absorption spectrum of murexide, which is sometimes regarded (Clarke, 1948) as a structural analogue of ANP (Fig. 1, curve 3).

Since the extinction coefficients of ANP have been determined, it can be decided whether ANP

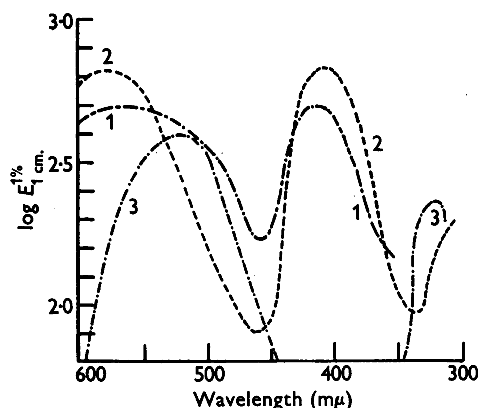


Fig. 1. Absorption spectra: 1, ANP (10 mg./l.) in *n*-butanol; 2, ANP (10 mg./l.) in ethanol or water, containing 1% (v/v) of 0·3% aq. EDTA soln.; 3, murexide (10 mg./l.) in water.

contains all the nitrogen originally present in the amino compound from which it had been formed. A solution of 9·2 mg. of ANP/l. gave $E_{1\,cm.}$ at 578 mμ, 0·600. The same extinction was measured in an ANP solution in which the ANP had been formed by the reaction of 11·62 μg. of alanine with ninhydrin. The reaction mixture (total vol. 4·3 ml.) contained therefore 1·83 μg. of amino nitrogen, which corresponds, calculated to a nitrogen content of the ANP of 4·62%, to an ANP concentration of 0·2 mg./l. All the alanine nitrogen has therefore been converted into ANP nitrogen.

The absorption spectrum of ANP formed in a reaction mixture in *n*-butanol in the complete absence of water is shown in Fig. 1, curve 1. It can be seen that absorption at both characteristic wavelengths is depressed, but enhanced at all others up to 530 mμ. The colour of the material produced in butanol is reddish instead of the deep-purplish blue. When minute amounts of water are present in the reaction mixture, absorption spectra are produced which vary in appearance between the extreme shown and that of the normal ANP spectrum. When the water content of the reaction mixture reaches or exceeds 5%, the normal ANP spectrum is always produced. Since the existence of an anionic structure in the ninhydrin ANP molecule is probable, it seems reasonable to assume that the normal ANP spectrum is due to the existence of the ANP anion, whereas the spectra produced in the absence of water are characteristic of the undissociated form of ANP.

Kinetics of the formation of ANP. The results of the kinetic experiments are given in Table 1. Colour formation was preceded by a latent period during which no colour was formed. The colour-formation reaction itself, when measured at any of the ninhydrin concentrations given in Table 1 and at alanine concentrations varying from 0·0129 to 0·103 m-mole/l., followed (within an experimental error of 5%) unimolecular kinetics, the rate of ANP formation being independent of the initial alanine concentration. Measurements taken at constant alanine and varying ninhydrin concentrations showed, however, that both the latent periods and the reaction half-times were not independent of

Table 1. *Kinetic data on the formation of ANP by the reaction of ninhydrin with* DL-*alanine*

Alanine concentration was 0·0258 m-mole/l.

Concn. of ninhydrin (m-moles/l.)	Molar concn. ratio (alanine:ninhydrin)	Latent period (sec.)	Reaction half-time (min.)
5·82	1:225	61	5
11·64	1:450	30	2·5
17·76	1:675	15	2·0
22·38	1:900	6	1·7
56·56	1:1800	6	1·7

ninhydrin concentration, as should be expected with the large excesses of ninhydrin used (see Table 1). It can be seen that both latent period and reaction half-time become independent of ninhydrin concentration only when the molar-concentration ratio alanine:ninhydrin equals or exceeds 1:900. This result seems to show that, of the total ninhydrin concentration present, only a small part is available for colour formation.

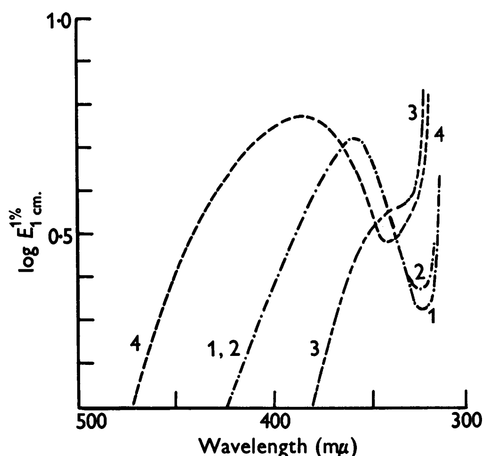


Fig. 2. Absorption spectra: 1, ninhydrin (1 g./l.) in absolute ethanol; 2, hydrindantin (1 g./l.) in absolute ethanol; 3, ninhydrin (1 g./l.) in water; 4, ninhydrin (1 g./l.) in aq. NaOH soln. (pH 10·25).

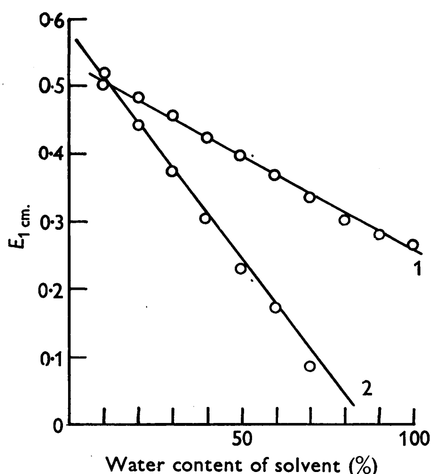


Fig. 3. Changes of absorption of ninhydrin at 356 mμ (1) and of the colour value of the product of its reaction with amino acids (2) with increasing water content of the solvent. For (2), 10 μg. of DL-alanine was used in 0·2% ninhydrin soln.; other conditions are as described under Materials and Methods.

Factors affecting the reactivity of ninhydrin. Success in applying the ninhydrin reaction to analytical problems depends to a large measure upon the state of this compound in solution, since the sensitivity of the reaction is much greater in organic solvents than in water. Another factor greatly affecting sensitivity is the H^+ ion concentration (McFadyen, 1948), especially in aqueous solution. As ninhydrin solutions are known to deteriorate with time, a method of standardizing such solutions to constant activity would be desirable.

The following experiments were an attempt to provide evidence for the existence of an active form of ninhydrin, the concentration of which varies with different conditions and determines the sensitivity of the ninhydrin reaction. It was found that reactivity changes of ninhydrin, especially those occurring upon changing the composition of the solvent, can be correlated with changes in its absorption spectrum happening at the same time. The absorption spectrum of ninhydrin in ethanol is shown in Fig. 2, curve 1. The absorption maximum at 356 mμ almost completely disappears when the solvent is water (Fig. 2, curve 3). When absorption at 356 mμ was measured in ninhydrin solutions where the ethanol was gradually replaced by water, it could be shown that the decline in absorption is linearly proportional to the increase in water content (Fig. 3, curve 1). Concurrently, the colour yield obtained with alanine also declined in linear proportion with increasing water content of the solvent (Fig. 3, curve 2).

Since both absorption at 356 mμ and the ANP-producing activity of ninhydrin are linearly proportional to the water content of the solvent, a linear-functional relationship exists between the two factors. The colour-producing activity of ninhydrin in any solvent mixture can therefore be expressed in terms of the extinction coefficient measured at 356 mμ. Ninhydrin solutions can thus be standardized to their maximum or any given percentage activity, either by the addition of water to the ethanolic ninhydrin solutions or by varying the ninhydrin concentration.

The 0·2% ninhydrin soln. in ethanol generally used in the experiments reported in this paper was standardized to an extinction value of 1·00 at 356 mμ, measured in a 1 cm. cell.

Apart from the composition of the solvent, the sensitivity of the ninhydrin reaction is most influenced by the H^+ ion concentration in the solvent. In aqueous solutions with a pH of below 4 no colour formation took place. Reactivity, as expressed by colour yield, increases sharply to reach its peak at pH 6·18, declined then to half its maximum value at pH 8·8 and disappeared completely at pH 10 and above (Fig. 4, curve 1). The absence of reactivity in

acid solutions is due to the instability of ANP in acid. When solutions containing ANP were acidified, the ANP colour disappeared almost instantaneously in solutions of pH of below 4 and more slowly in less acid solutions. It can therefore be concluded that in such solutions the colour yield observed depends on the ratio between the rates of formation and destruction of ANP. No such effect is possible in alkaline solutions in which ANP is stable; reactivity changes in alkaline solutions have therefore to be explained by other effects, such as changes in the ninhydrin molecule. Evidence for such an effect can be seen in the significant changes occurring in the ninhydrin-absorption spectrum in alkaline solution, in contrast with acid conditions where no changes in the absorption spectrum of ninhydrin could be detected at different degrees of acidity. A ninhydrin solution in water to which increasing quantities of alkali are added turns yellow and shows a new absorption maximum at 380 mμ. Extinction at this wavelength reached half its maximum value at pH 8·8 and its maximum value at pH 10·25 (Fig. 4, curve 2). (The shape of the absorption spectrum at this point is shown in Fig. 2, curve 4.) Extinction at 380 mμ declines then rapidly and disappears completely at pH 12 and higher.

The potentiometric titration curve of ninhydrin (Fig. 5) shows that ninhydrin behaves like an acid with a pK of 8·8. When the alkali concentration of aqueous solutions containing ninhydrin is increased to 6N, the colour of ninhydrin blue appears. Its absorption spectrum (Fig. 6) shows, in addition to the maximum at 580 mμ, a pronounced absorption maximum at 356 mμ resembling that shown by ninhydrin in ethanol (Fig. 2, curve 1). It should be noted that the absorption spectrum of ninhydrin blue is very similar to that of hydrindantin blue, a compound formed when hydrindantin, the reduction product of ninhydrin, is dissolved in 0·1N-NaOH (Fig. 6).

Hydrindantin has been shown to react with amino acids in an aqueous medium and to yield the same reaction products, including ANP (Moubasher & Ibrahim, 1949). We found that in ethanolic solution hydrindantin produces amounts of ANP equal to those obtained with ninhydrin under similar conditions.

Quantitative determination of amino acids

Complete conversion of the α-amino nitrogen into ANP nitrogen, i.e. a theoretical colour yield, was obtained with all the amino acids and peptides

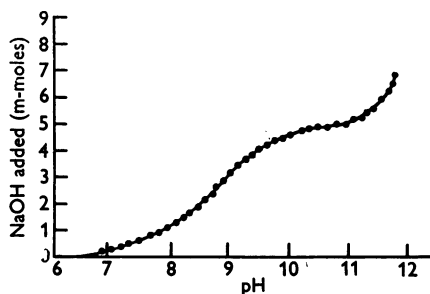


Fig. 5. Titration curve of ninhydrin (4·92 m-moles titrated).

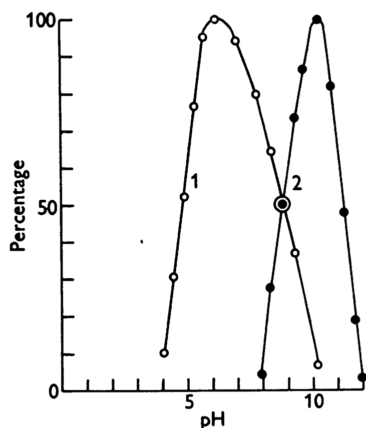


Fig. 4. Changes in the colour-producing activity of ninhydrin with amino acids (1) and of its absorption at 380 mμ (2) with pH in aqueous solution, expressed as percentages of the respective maximum values. Absorption of ninhydrin was measured in 0·2% soln. and colour values were determined with 500 μg. of DL-alanine in 0·2% aq. ninhydrin soln.; other conditions as described under Materials and Methods.

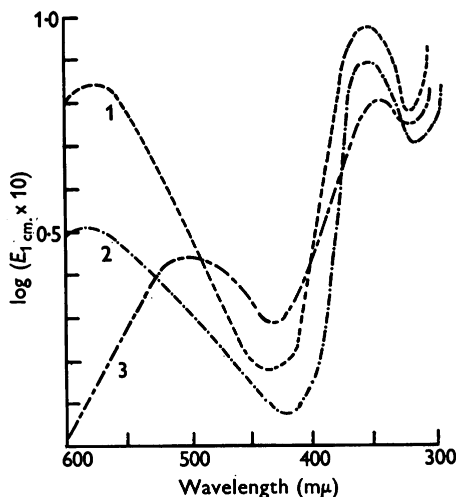


Fig. 6. Absorption spectra: 1, hydrindantin blue (from 10 mg. of hydrindantin in 25 ml. of 0·1N-NaOH); 2, ninhydrin blue (from 13 mg. of ninhydrin in 3 ml. of 6N-NaOH); 3, hydrindantin in ethanol (1 g./l., freshly prepared solution).

tested, with the exception of proline, hydroxyproline, phenylalanine, tyrosine and tryptophan. Hydroxyproline and proline cannot be determined by any method measuring ANP production, since their reaction product with ninhydrin differs in structure and absorption characteristics from ANP (Grassmann & Arnim, 1934). Phenylalanine, tyrosine and tryptophan yield about 70 % of the theoretically expected colour value. They can, however, be determined with the use of special calibration curves, since the colour values given by them were reproducible. In all other cases the construction of a single calibration curve is sufficient, or the α-amino nitrogen content of the sample analysed can be calculated directly by

$$\mu\text{g. of } \alpha\text{-amino nitrogen in sample} = 100Ava/(E^{1\%}_{1\,\mathrm{cm.}} \times l),$$

where A is the extinction (optical density) value measured for the sample, v is the total volume of the reaction mixture (4·3 ml.), a is the nitrogen content of ANP (in %), $E^{1\%}_{1\,\mathrm{cm.}}$ is the specific extinction coefficient of ANP at the wavelength used and l is the cell length in cm.

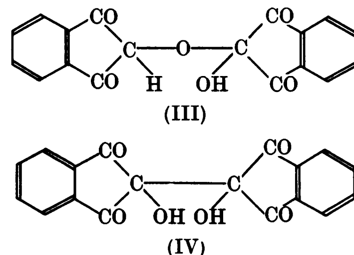
It should be noted that the method is strictly specific for α-amino nitrogen; lysine, arginine, ornithine, citrulline and histidine were all found to react with their α-amino groups only.

Strictly linear dependence of extinction (optical density) on concentration was observed with samples containing amounts of α-amino nitrogen ranging from 0·5 to 6 μg. The accuracy of the method is ± 3 %. The same results were obtained when the compounds tested were dissolved in acetate, citrate or phosphate buffers. The nitrogen-containing buffers tris and diethylbarbituric acid did not react with ninhydrin.

Both ammonia and the amines tested, when present as free bases, reacted in ethanolic solution with ninhydrin. They yielded 17–30 % of the amount of ANP theoretically expected, but the results obtained varied within very wide limits. The interference of ammonia and amines with the determination of amino acids can, however, easily be eliminated by converting them into their salts, since ammonium and amine salts did not yield measurable amounts of colour.

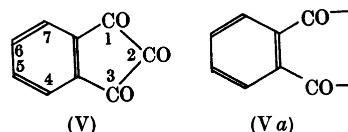
DISCUSSION

Structure of ninhydrin. Since it has been shown (Fig. 2) that the absorption spectra of ninhydrin and hydrindantin are the same in the region most connected with activity changes, it may be concluded that both compounds owe their reactivity with amino compounds to similar structures. According to its discoverer, Ruheman (1911), hydrindantin is a condensation product of ninhydrin (V) with its reduction product 1:3-dioxo-2-hydroxyindane and has the structure (III). Schönberg & Moubasher (1949) favoured structure (IV). Hydrindantin does

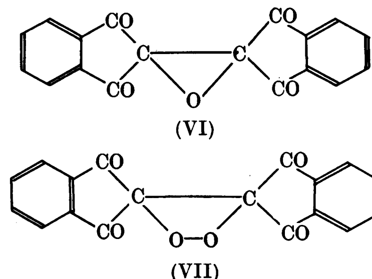

(III)

(IV)

not therefore contain the carbonyl group in the 2-position to which the reactivity of ninhydrin (V) with amino compounds is generally believed to be due (Clarke, 1948). It is therefore difficult to see how hydrindantin could react with amino compounds at all, and why ninhydrin, usually represented as (V), should show exactly the same absorption characteristics in the 300–400 mμ region as its reduction product, hydrindantin.


(V) (V*a*)

The system (V*a*), common to both hydrindantin and ninhydrin, cannot be responsible for this effect since the absorption spectrum of ANP, which also contains (V*a*), does not show any absorption maximum at 356 mμ. It is therefore assumed that hydrindantin, on dissolving in organic solvents, loses one molecule of water thereby forming structure (VI), and that ninhydrin forms a similar

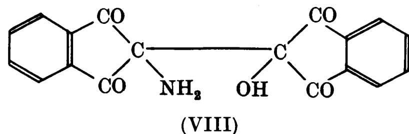

(VI)

(VII)

structure (VII) by losing two molecules of water. This structural change is in hydrindantin accompanied by absorption changes. Freshly prepared hydrindantin solutions show a red colour with an

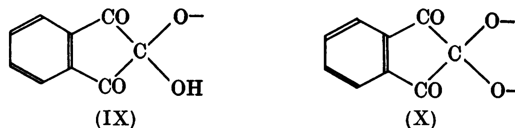
absorption maximum at 510 mμ (Fig. 6, curve 3). When kept the colour fades and the absorption spectrum then assumes the form shown in Fig. 2, curve 2. Absorption at 356 mμ is due to the presence of (VI) and (VII), which are the reactive forms of hydrindantin and ninhydrin respectively, but appear in solution only in small equilibrium concentrations. This assumption is borne out by the fact that a very large excess of hydrindantin and of ninhydrin is necessary to make them react with amino compounds. The dependence of both absorption at 356 mμ and reactivity of ninhydrin become now easily explainable by assuming that, in the presence of water, (VII) is split into two molecules of (non-reactive) ninhydrin hydrate. Evidence for the existence of a condensed form of ninhydrin can be seen in the fact that hydrindantin has been prepared by Schönberg & Moubasher (1949) by the action of ninhydrin and magnesium in the presence of magnesium iodide in ether. This is essentially the method for the synthesis of pinacols from aromatic ketones published by Gomberg & Bachmann (1927), which is believed (Wheland, 1953) to lead through the intermediate stage of the often highly coloured metal ketyls. It is suggested that the deep-blue compounds ninhydrin blue and hydrindantin blue both formed by the action of alkali on the parent compounds could be regarded as analogous to the metal ketyls. The fact that ninhydrin blue has an absorption spectrum very similar to that of hydrindantin blue (Fig. 6) can also be taken as evidence for the existence of a condensed form of ninhydrin, since hydrindantin blue is most probably to be regarded as a derivative of the condensed structure (IV).

Simple reaction mechanisms for explaining the ninhydrin reaction, the reactivity of amino compounds with ninhydrin and hydrindantin, can be constructed with the use of structures (VI) and (VII), the complete oxidative deamination of the amino compounds being performed by one molecule of ninhydrin or two molecules of hydrindantin, leading to the common intermediate (VIII) from which either (I) or (II) could be formed.



(VIII)

Structure of ninhydrin in aqueous solution. The greatly diminished reactivity of ninhydrin in aqueous solution, due to the prevalence of the non-reactive form (V), is still further reduced and finally abolished when such solutions are made alkaline (Fig. 4). This effect can be explained by the assumption that ninhydrin (V), which in aqueous solution can still form a small amount of the active form (VII) of ninhydrin, can not do so if converted by alkali into the univalent ion (IX). This ion is



(IX) (X)

capable of forming resonance structures and therefore shows absorption (at 380 mμ). At pH 8·8, the pK of the acid form of ninhydrin, ninhydrin reactivity has half the value it has under conditions excluding the presence of (IX). Concurrently, absorption at 380 mμ, which is due to the formation of (IX), has half its maximal value at the same pH. At pH 10·25 all ninhydrin has been converted into (IX); accordingly, absorption at 380 mμ is maximum and reactivity is completely abolished. When the solution is made still more alkaline, the bivalent ion (X) is formed which is incapable of forming resonance structures. There is then no absorption at 380 mμ (Fig. 4).

Analytical conclusions

The high sensitivity of the ninhydrin reaction can be fully utilized when ethanol is used as the solvent, since in ethanol the formation of the active form of ninhydrin (VII) is favoured and the formation of its inactive forms (V), (IX) and (X) is largely suppressed. Sensitivity is not measurably diminished by the amount of water present, which is necessary to avoid the formation of atypical ANP spectra and to hold a sufficient amount of EDTA in solution. Ethanol also largely obviates any interference likely to be caused by the pH of the solution to be analysed, except in extreme cases. Solutions containing the amino acid to be determined may vary from pH 4·5 to 8. When ammonia or amines are present, care should be taken to neutralize them completely.

Interfering factors. Strong oxidizing or reducing substances destroy the ANP. Trichloroacetic acid seriously interferes with ANP formation and must therefore quantitatively be removed by ether extraction from solution to be analysed. The interference caused by metal ions present in the concentrations usually found in biological systems is eliminated by the use of EDTA. No other interfering factors have so far been found.

The method described is primarily intended for the determination of amino acids and peptides in column eluates or cell extracts. It can be adapted to the determination of amino acids on paper chromatograms; the zones containing the amino

acids are cut out and refluxed in a reaction mixture consisting of 4 ml. of ninhydrin solution and 0·2 ml. of EDTA solution and 0·1 ml. of water.

The method has been so far successfully employed for the determination of amino acids in ether-treated trichloroacetic acid and warm-water extracts of yeast cells (Benziman, 1956) and for following the enzymic decomposition of polylysin (M. Rigbi, personal communication).

SUMMARY

1. The coloured reaction product of amino acids with ninhydrin (ANP) was prepared in the solid state. Extinction coefficients for its absorption maxima at 578 and 408 mμ were determined.

2. By using the fact that ANP is greatly stabilized in the presence of ethylenediaminetetra-acetic acid, uniform absorption spectra are obtained and oxidative side reactions leading to the decomposition of ANP are avoided.

3. The α-amino nitrogen of amino acids is quantitatively transferred to ANP.

4. The changes in the reactivity of ninhydrin with amino compounds in different ethanol–water mixtures and in aqueous solutions at different H^+ ion concentrations, and the accompanying changes in its absorption spectrum, are explained by structural changes in the ninhydrin molecule. A structure for the reactive form of ninhydrin and hydrindantin is suggested.

5. An analytical method for the spectrophotometric determination of 0·5–6 μg. of α-amino nitrogen, contained in amino acids or peptides, is described.

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Blood Lipids

1. PLASMA LIPIDS OF THE LACTATING COW: CHROMATOGRAPHY ON SILICIC ACID

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Arteriovenous studies (for review see Folley, 1956) have demonstrated that no component of blood lipids except triglycerides and possibly cholesterol esters is absorbed from the blood plasma by the lactating ruminant udder. It is therefore of some importance to study the fatty acid composition of these classes of lipid, though it is first necessary to separate them from each other and from other plasma lipids.

The separation of mixtures of lipids into their component classes was restricted for many years to their segregation into acetone-insoluble lipids (phospholipids) and acetone-soluble lipids (glycerides, sterol esters, free sterols and fatty acids), from which mixture the last two components could each be recovered chemically. However, no means of distinguishing between fatty acids in combination as glycerides and as sterol esters was devised until Kelsey (1939) used pig pancreatic lipase for the selective hydrolysis of glycerides in the presence of cholesterol esters. This procedure was employed by Kelsey & Longenecker (1941) in an investigation of the component fatty acids of the plasma lipids of lactating cows. As has been discussed by Lovern (1956), it seems that the enzymic procedure is unsatisfactory in that it does not effect the complete hydrolysis of glycerides. Clément, Clément-Champougny & Louedec (1954) found that the method given by Kelsey (1939) did not effect more than 67 % hydrolysis of the glycerides from various