

THE NINHYDRIN REACTION WITH AMINO-ACIDS AND AMMONIUM SALTS.*

BY VICTOR JOHN HARDING AND FRANCIS H. S. WARNEFORD.

(From the Biochemical Laboratory, McGill University, Montreal.)

(Received for publication, April 22, 1916.)

Among the many biological reactions which have attracted attention during recent years, the one bearing the name of Abderhalden¹ has been prominent. The simple nature of the underlying idea, that a circulating foreign protein induces in the blood a corresponding and specific defensive ferment which accomplishes its hydrolysis, combined with the obvious importance of its applications, has rendered the reaction the object of numerous investigations. Both theory and results have been assailed from many directions and the reaction is still a matter of doubt, though evidence is steadily accumulating against it.²

The detection of the hydrolysis products of the circulating foreign protein is usually carried out by the dialysis method, and depends ultimately on the detection of these products after dialysis, by the now so called ninhydrin reaction. This latter reaction was discovered by Ruhemann³ who found that α -amino-acids when warmed with triketohydrindene hydrate gave a fine blue color. β - and γ -amino-acids hardly reacted at all, and α -amino-acids when substituted on the amino or carboxyl group, gave a negative reaction. Abderhalden and Schmidt⁴ confirmed and extended Ruhemann's observations, and, as far as is known, there is no amino-acid obtained from protein hydrolysis which does not

* A preliminary note on this and the succeeding communication was published by Harding, V. J., *Tr. Roy. Soc. Canada*, 1915, series 3, ix, 33.

¹ Abderhalden, E., *Handb. biochem. Arbeitsmethoden*, 1912, vi, 223; *Münch. med. Woch.*, 1912, lix, 1305, 1939, 2172.

² Van Slyke, D.D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 377. Hulton, F., *J. Biol. Chem.*, 1916, xxv, 163.

³ Ruhemann, S., *J. Chem. Soc.*, 1910, xcvi, 2030; 1911, xcix, 798.

⁴ Abderhalden, E., and Schmidt, H., *Z. physiol. Chem.*, 1911, lxxii, 37.

respond readily to the test. Proteins and their hydrolysis products were also found to give a positive reaction. One or two ammonium salts and a few bases were examined with negative results, and thus the test began to be regarded as characteristic of proteins and their hydrolysis products.

Herzfeld,⁵ however, noticed that ammonium carbonate and ammonium oxalate when evaporated to dryness with triketohydrindene hydrate gave a positive reaction. It was, however, due to Neuberg⁶ that the fact was discovered that the ninhydrin reaction with ammonium salts was a general one, thus contradicting the experiments and conclusions of Abderhalden and Schmidt. Neuberg also discovered the positive nature of the ninhydrin reaction with many organic bases and these results will be discussed in the succeeding paper.

The results of Neuberg destroy at once the specific nature of the ninhydrin reaction, and we wish in this paper to discuss the probable cause of the discrepancies and endeavor to gain some insight into the reaction.

The Analogy between the Ninhydrin Reaction and the Formation of Murexide from Alloxan.

This analogy was pointed out by Ruhemann,⁷ who showed conclusively that the blue coloring matter produced by heating amino-acids and triketohydrindene hydrate was similar in properties and in method of preparation to murexide. Basing his ideas on the constitution of murexide advanced by Piloty and Finckh,⁸ and by Slimmer and Stieglitz,⁹ and the analogy between triketohydrindene hydrate and alloxan, he prepared the coloring matter in the following way.

Alloxan and triketohydrindene hydrate on reduction with mild reducing agents yield alloxantin and hydrindantin respectively, to which he gave the following constitutions.

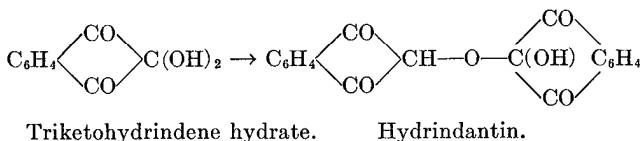
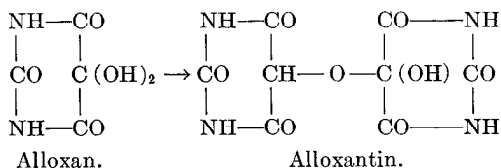
⁵ Herzfeld, E., *Biochem. Z.*, 1914, lix, 249.

⁶ Neuberg, C., *Biochem. Z.*, 1913, lvi, 500.

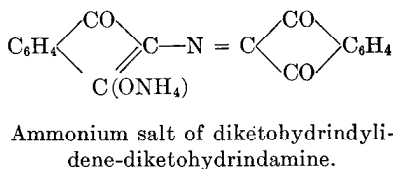
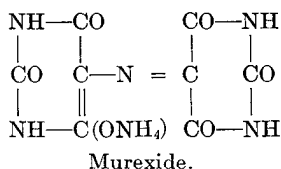
⁷ Ruhemann, *J. Chem. Soc.*, 1911, xcix, 792, 1486.

⁸ Piloty, O., and Finckh, K., *Ann. Chem.*, 1904, cccxxxiii, 22.

⁹ Slimmer, M., and Stieglitz, J., *Am. Chem. J.*, 1904, xxxi, 661.



On warming with a mixture of ammonium carbonate and ammonium acetate these gave murexide and the ammonium salt of diketohydrindylidene-diketohydrindamine respectively.



The ammonium salt of diketohydrindylidene-diketohydrindamine prepared in this way through the intermediate hydrindantin was found to have a blue coloration identical with that formed by heating alanine and triketohydrindene hydrate, the alanine being oxidized to carbon dioxide and acetaldehyde. Triketohydrindene hydrate and glycine were shown to give formaldehyde, carbon dioxide, and the ammonium salt of diketohydrindylidene-diketohydrindamine. Strecker¹⁰ had shown that alanine and alloxan, when heated together in aqueous solution, produced acetaldehyde, carbon dioxide, and murexide. Thus a close analogy between the two reactions was established.

The identity of the ammonium salt of diketohydrindylidene-diketohydrindamine was established by Ruhemann by means of analysis and chemical reactions. As a further means of identification, especially in dilute solution, we have observed three properties which can be used for the detection of the compound.

¹⁰ Strecker, A., *Ann. Chem.*, 1862, cxxiii, 363.

1. The coloring matter gives a broad absorption band in the visible spectrum when examined in dilute solution. This band extends from the red into the green part of the spectrum, blocking out almost entirely the whole of the yellow.¹¹

2. The blue color changes to a purple when viewed in artificial light, resembling very much the color of dilute potassium permanganate when viewed in daylight.

3. The color is quantitatively resistant to oxidation by the passage of a rapid current of air.

Prepared by either of the two methods—by the action of ammonium carbonate and acetate upon hydrindantin, or by the interaction of alanine and triketohydrindene hydrate—the coloring matter shows the same characteristics in dilute solution. It is also of interest to note that murexide gives a similar absorption band,¹² thus supporting the analogy drawn by Ruhemann.

The Interaction of Ammonium Salts and Triketohydrindene Hydrate.

It has just been stated that various contradictory results had been obtained by the earlier observers on the positive or negative nature of the ninhydrin reaction with ammonium salts. This we believe has been due to the use of ammonium salts of different concentrations. In our experiments we have used the pure ammonium salts obtainable from Kahlbaum or Schuchardt. Where these were not available we have taken a known weight of the pure acid, and added the theoretical amount of ammonia from a standardized solution of ammonium hydroxide. In this way we have made sure of the purity of our salts. The purity of the Kahlbaum or Schuchardt specimens was controlled by a determination of their ammonia content by means of Nesslerization.

The process of carrying out the ninhydrin reaction has been to take 1.0 cc. of ammonium salt and 1.0 cc. of 1 per cent ninhydrin solution, and heat them for 20 minutes in a rapidly boiling water bath.

¹¹ We have found, since writing the preliminary report, that the existence of this absorption band was first noticed by Deniges, G., *Bull. Soc. Pharm. Bordeaux*, 1914, liv, 49, of whose work we were unaware at the time.

¹² Compare Hartley, W. N., *J. Chem. Soc.*, 1887, li, 153.

The following table shows the results obtained:

TABLE I.

Salt.	Concentration.	Reaction.
	<i>per cent</i>	
Chloride.....	1.0	No reaction.
Nitrate.....	1.0	“ “
Sulfate.....	1.0	“ “
Hydrogen phosphate....	1.0	Intense violet red; blue on dilution.
Sodium hydrogen phosphate.....	1.0	“ “ “ “ “ “
Formate.....	0.92	Deep violet red.
Acetate.....	0.28	Violet.
Carbonate.....	1.0	Intense violet red; blue on dilution.
Succinate.....	1.0	Deep reddish violet.
Glycollate.....	1.0	Violet.
Lactate.....	0.8	“
Malate.....	0.2	“
Tartrate.....	1.0	Reddish violet; blue on dilution.
Citrate.....	1.0	Violet.
Oxalate.....	1.0	Deep reddish violet; blue on dilution.
Benzoate.....	1.0	Violet.
Thiocyanate.....	1.0	Deep reddish violet.

A glance at the table will show that all the ammonium salts tested, with the exception of those of the strong mineral acids, give a positive ninhydrin reaction. By increasing the concentration, however, even the salts of these acids can be made to show a positive test. Thus in a concentration of 5 per cent, ammonium sulfate gives a decided pinkish color. The chloride and the nitrate, however, require to be nearly saturated before responding, faintly but unmistakably, to the test, and it certainly seems significant that the salts of the strongest acids require to be used at the highest concentration before they show the ninhydrin reaction. Our own list contains seventeen ammonium salts: the two of Neuberg⁶ contain nineteen other salts, including salts of many rare thio-acids, and together they include almost every type of acid, so that we feel justified in stating that the ninhydrin reaction is given by all ammonium salts, provided the concentration be sufficiently high. If, however, the concentration is very low

a negative reaction is obtained in all cases. Thus when the concentration of the ammonium salt is such that 1.0 cc. = 0.05 mg. ammonium nitrogen, then, in all cases examined (twenty), with but one exception, a perfectly colorless solution resulted on heating them with 1.0 per cent aqueous ninhydrin solution (Table II). The one exception is ammonium sodium hydrogen phosphate, which gives a very faint trace of violet color under those conditions and the probable cause of this exception will be discussed later.

In the majority of cases we have been able to identify the blue or violet coloration as due, at any rate in part, to the ammonium salt of diketohydrindylidene-diketohydrindamine. We used the three tests mentioned on page 322, but in no case was sufficient color present to be determined quantitatively by the Harding-MacLean colorimetric method.¹⁵ The discrepancies of previous observers can thus be accounted for; in concentration of about 1 per cent the ammonium salts of the weak organic acids give an unmistakable ninhydrin reaction, which is not the case when the concentration is extremely low (1.0 cc. = 0.05 mg. ammonium nitrogen).

In view of the results it became of interest and importance to determine whether ammonium salts in very dilute solution gave a ninhydrin reaction in the presence of pyridine. This modification of the ninhydrin reaction discovered by Harding and MacLean¹³ results in a quantitative decomposition of the α -amino nitrogen of amino-acids. How far, if at all, does it affect the decomposition of ammonium nitrogen when in similar concentration? The method of investigation was as follows:

Standard solutions of different ammonium salts were prepared of such a strength that 1 cc. of solution contained 0.05 mg. of ammonium nitrogen, thus being equivalent in strength to the standard alanine.

1 cc. of the ammonium salt solution together with 1 cc. of 10 per cent aqueous pyridine and 1 cc. of 1 per cent ninhydrin were heated for a period of 20 minutes in a rapidly boiling water bath. A strong blue color developed. The contents of the test-tube were cooled, diluted to 50 cc., and the amount of color was measured against a standard alanine color simultaneously prepared.

¹⁵ Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1915, xx, 217.

For reasons given in a previous paper¹⁴ the amount of decomposition of alanine, etc., is assumed to be quantitative. It will be seen by a glance at Table II that the decomposition of ammonium nitrogen under these conditions is constant and independent of the acid attacked, the two columns representing duplicate determinations.

TABLE II.

Ammonium salt.	Without pyridine.	With pyridine. Nitrogen decomposed.	
		mg.	mg.
Chloride.....	No color.	0.019	0.020
Nitrate.....	" "	0.018	0.018
Sulfate.....	" "	0.017	0.016
Sodium hydrogen phosphate.	Very pale violet color.	0.016	0.015
Hydrogen phosphate.....	No color.	0.019	0.019
Carbonate.....	" "	0.018	0.018
Formate.....	" "	0.019	0.019
Acetate.....	" "	0.020	0.020
Oxalate.....	" "	0.017	0.018
Succinate.....	" "	0.020	0.019
Glycollate.....	" "	0.019	0.019
Lactate.....	" "	0.019	0.019
Malate.....	" "	0.020	0.019
Tartrate.....	" "	0.018	0.019
Citrate.....	" "	0.015	0.015
β -Oxybutyrate.....	" "	0.020	0.020
Benzoate.....	" "	0.020	0.022
Cinnamate.....	" "	0.020	0.020
Opianate.....	" "	0.018	0.018
Thiocyanate.....	" "	0.019	0.019

This decomposition of ammonium nitrogen is constant at about 0.018 mg. in 0.05 mg., or about 36 per cent, and the identification of the coloring matter as the ammonium salt of diketohydrindylidene-diketohydrindamine is readily effected by the use of the three afore mentioned tests.

We next investigated the effect of varying amounts of ninhydrin and pyridine upon the reaction under conditions which parallel those in our studies on the decomposition of alanine. This is shown in Table III.

¹⁴ Harding and MacLean, *J. Biol. Chem.*, 1916, xxiv, 503.

TABLE III.

Ammonium salt. 1 cc. = 0.05 mg. N.	Pyridine concentration (per cent).				Ninhydrin concentration (per cent).		N decom- posed. <i>mg.</i>
	5	10	15	25	1	2	
	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	
Chloride.....	1				1		0.010
“		1			1		0.017
“			1		1		0.019
“				1	1		0.020
“		1				1	0.024
“		1			2		0.025
Sulfate.....	1				1		0.010
“		1			1		0.018
“			1		1		0.019
“				1	1		0.020
“		1				1	0.025
“		1			2		0.026
Nitrate.....		1			2		0.025
Acetate.....		1			2		0.025
Benzoate.....		1			2		0.026

1 cc. of the ammonium salt was heated with the amount of pyridine and ninhydrin stated, in a boiling water bath for a period of 20 minutes, and the amount of ammonium nitrogen decomposed was found in the usual way. It will be seen that concentrations of pyridine higher than 10 per cent have very little influence, though there is to be noted a small but distinct rise. In this respect the reaction of an ammonium salt differs from that of alanine. An increase in the amount of ninhydrin, even if the ninhydrin be used in the same concentration, causes a marked increase in coloring matter. With alanine, no such increase is noted.

We have not made any extensive studies on the influence of time on this reaction. At the end of 20 minutes, however, the reaction is mainly complete. Thus an isolated observation on ammonium β -hydroxybutyrate showed that an hour's heating at 100°C. only increased the decomposition of the nitrogen from 0.020 to 0.027 mg.

In summing up the experimental results, it will be seen that all ammonium salts under the right conditions are capable of giving

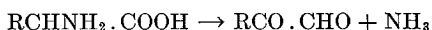
the ninhydrin reaction. Those conditions appear to be a faint alkalinity of solution, brought about either by the ammonium salt itself or by the presence of pyridine. The coloring matter formed agrees in properties with the ammonium salt of diketohydrindylidene-diketohydrindamine prepared either synthetically or by the interaction of alanine and triketohydrindene hydrate, and there thus remains the task of showing how ammonium salts in general can interact with Ruhemann's reagent and give an end-product identical with that produced from amino-acids. The simplest and most obvious explanation, that amino-acids pass into ammonium salts, is inadequate, for if so, the decompositions should either parallel one another or ammonium salts should be more reactive than amino-acids, statements which are not in agreement with experimental facts. Of the two, amino-acids are by far the more reactive. They react in much more dilute solutions than ammonium salts and, in the presence of pyridine, are nearly three times as strong in their action.

The Mechanism of the Ninhydrin Reaction.

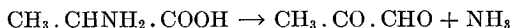
Ruhemann's⁷ view of the interaction of amino-acids and triketohydrindene hydrate involves the oxidation of the amino-acid to carbon dioxide and an aldehyde possessing a carbon atom less than the amino-acid with the simultaneous reduction of the triketone to hydrindantin and the condensation of the hydrindantin with the ammonia liberated by the oxidation of the amino-acid, forming the blue colored ammonium salt of diketohydrindylidene-diketohydrindamine. Such a mechanism, while satisfactory for amino-acids, does not explain the positive ninhydrin reaction with ammonium salts, especially of inorganic acids. Adopting Ruhemann's view of the constitution of the blue coloring matter, we have sought to determine the mechanism whereby the amino-acid acts as a reducing agent and to see whether any parallel reaction was possible with an ammonium salt.

In this respect we have utilized the theory of Dakin and Dudley,¹⁵ who have postulated that amino-acids undergo either a dissociation or a decomposition into ammonia and the corresponding glyoxal.

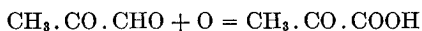
¹⁵ Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, 1913, xv 127.



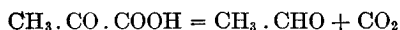
Their later work has inclined them to the belief that the reaction expressed is not a true chemical dissociation. In the case of alanine this would lead to the formation of methyl glyoxal and ammonia.



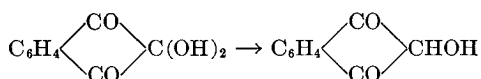
The glyoxals as a class are distinguished by being powerful reducing agents; they reduce ammoniacal silver oxide and Fehling's solution even in the cold. Thus it would be expected that a reduction of triketohydrindene hydrate would take place, giving rise to a substituted glyoxylic acid,



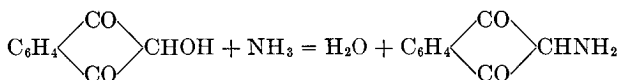
and that this would decompose into carbon dioxide and an aldehyde.



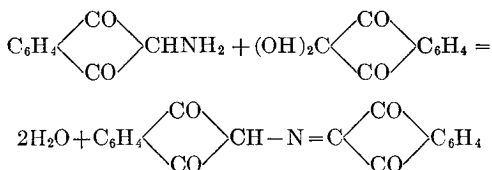
The triketohydrindene hydrate would be reduced to 1, 3-diketohydrindol



which on condensation with the ammonia from the amino-acid would give 1, 3-diketohydrindamine.

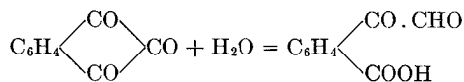


This compound readily condenses with aldehydes and ketones, is readily oxidized to a deep blue coloring matter,^{3,7} and consequently would be expected to condense with a molecule of triketohydrindene hydrate to give diketohydrindylidene-diketohydrindamine, the ammonium salt of which is the required blue coloration.



Such a scheme accounts satisfactorily for the end-products of the reaction. It differs from that outlined by Ruhemann by not regarding hydrindantin as an intermediate product. The direct action of ammonia upon hydrindantin to produce diketohydrindylidene-diketohydrindamine would be expressed much better by a series of simple reactions involving the hydrolysis of hydrindantin to diketohydrindol and triketohydrindene hydrate. Moreover, we believe that we possess a certain amount of evidence against the view, for the presence of hydrindantin in large amounts reduces the amount of diketohydrindylidene-diketohydrindamine formed from an ammonium salt (page 333).

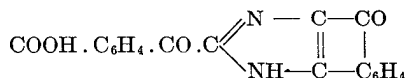
Again, as a working hypothesis, the intermediate formation of a glyoxal is attractive to us because it enables us to explain the ninhydrin reaction with ammonium salts. It is to be noticed that the ninhydrin reaction in the presence of pyridine is the same for all ammonium salts, being entirely independent of the acid attached; without pyridine it is most evident with the ammonium salts of the weak organic acids; *i.e.*, those salts which are the most readily hydrolyzed into their basic and acidic components, and with those salts which are faintly alkaline in reaction; *e.g.*, sodium ammonium hydrogen phosphate. Any reduction of the triketohydrindene hydrate under these circumstances must come from itself and the first action of the pyridine, or the weakly alkaline or dissociated salt must be the action of the hydroxyl ions upon the triketone. Ruhemann has shown conclusively that the first action of alkalis upon triketohydrindene hydrate is the formation of phenylglyoxal-*o*-carboxylic acid.



In other words, under the influence of weak alkalis the triketone itself can furnish the necessary glyoxal and the reaction would then proceed in the manner previously indicated. The formation of the yellow colored glyoxal can readily be observed on adding a few drops of 10 per cent aqueous pyridine to the triketone.

A necessary consequence of the hypothesis is that ammonia itself should give the ninhydrin reaction. Ruhemann investigated the action of ammonia upon the triketone and found

that he obtained a deep brownish red solution, from which by acidification he obtained a compound he regarded as 2-*o*-carboxy-benzoylindoglyoxaline,



The formation of the phenylglyoxal-*o*-carboxylic acid is evident, and this undergoes condensation with ammonia and a second molecule of triketohydrindene hydrate. We have found, however, that in very dilute solution the reaction with ammonia follows the reaction with ammonium salts. In a concentration of 1 cc. = 0.05 mg. ammonia nitrogen no reaction is obtained on heating 1 cc. with 1 cc. of 1 per cent ninhydrin for 20 minutes in a boiling water bath. In the presence of 1 cc. of 10 per cent aqueous pyridine, the ammonium salt of diketohydrindylidene-diketohydrindamine is formed and the decomposition of the ammonia nitrogen is 0.013 mg., a figure not far removed from the constant given by ammonium salts.

It is also easy to see why β - and γ -amino-acids have been found to give a faint ninhydrin reaction, and why acids with an amino group attached to a benzene ring fail to give the test, though we should expect that even here those aromatic amino-acids of which the amino group is readily hydrolyzed, would give a feeble positive reaction.

The Action of Reducing Agents on the Ninhydrin Reaction with Ammonium Salts.

It is evident that reducing reagents which assist the production of diketohydrindol from triketohydrindene hydrate should increase the amount of coloring matter formed with ammonium salts. And this in general we have found to be the case. We have examined a number of organic reducing agents which from their known reactions might be expected not to interfere with the condensations which take place, and measured their influence on the amount of coloring matter formed.

1 cc. of the standard solution of ammonium salt, 1 cc. of 10 per cent aqueous pyridine, 1 cc. of 2 per cent ninhydrin solution, and 1 cc. of the

reducing agent of the strength shown in Table IV were heated together in a boiling water bath for 20 minutes and the amount of nitrogen decomposed was estimated in the usual way. Control experiments on the reducing agent and ninhydrin in presence of pyridine gave negative results.

TABLE IV.

Ammonium salt.	Reducing agent.	Concentration of reducing agent.	N decomposed.
		per cent	mg.
Chloride.....	Ethylene glycol.	10	0.025
“	“	100	0.035
“	Glycerol.	10	0.027
“	“	100	0.032
“	<i>p</i> -Hydroxybenzaldehyde.	1	0.029
“	<i>p</i> -Hydroxybenzaldehyde.	3	0.032
“	Glucose.	0.5	0.025
“	“	5.0	0.032
Nitrate.....	“	0.5	0.027
“	“	5.0	0.031
Sulfate.....	“	0.5	0.026
“	“	5.0	0.032
Acetate.....	“	0.5	0.026
“	“	5.0	0.032
Benzoate.....	“	0.5	0.026
“	“	5.0	0.032
Constant for ammonium salt.....	1 cc. water.		0.025

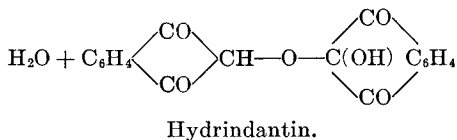
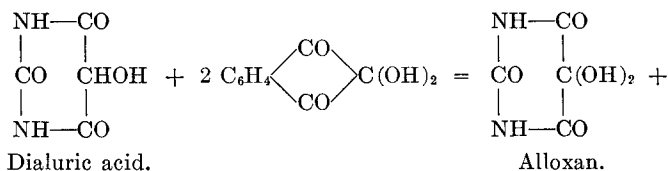
It will be seen that reducing agents have a distinct influence on the decomposition of ammonium nitrogen, though it is not so great as might have been expected, and in all cases a high concentration is necessary before the effect is shown. Thus a 0.5 per cent solution of glucose is without any influence and a 5 per cent solution only raises the decomposition from 50 to 64 per cent.

The results with ethylene glycol and glycerol are of interest, as they have a distinct bearing on the claims of Halle, Loewenstein, and Příbram¹⁶ that these substances themselves give a ninhydrin reaction. They appear to act as very weak reducing

¹⁶ Halle, W., Loewenstein, E., and Příbram, E., *Biochem. Z.*, 1913, lv, 357.

agents only, but this will be discussed more fully in another communication. The poor results obtained go to show that the reducing agent which takes part in the ninhydrin reaction must be either a very specific one or exceptionally powerful.

Of all the reducing agents we have examined dialuric acid is by far the most interesting. This acid was shown by Ruhemann¹⁷ to give excellent yields of hydrindantin when heated with triketohydrindene hydrate, and we have repeated the observation several times in this laboratory; indeed, it is not necessary to heat the solutions, for in the concentrations at which we were working the hydrindantin rapidly crystallized out at the ordinary temperature.



A cold saturated solution of dialuric acid was prepared by dissolving the acid in hot water, cooling, and allowing the excess of acid to crystallize out over night (A). A second solution was prepared by dissolving dialuric acid in hot water, of such a strength that 1 cc. was capable of converting the whole of the triketohydrindene hydrate present in 1 cc. of a 2 per cent solution into hydrindantin according to the above equation (B). Such a solution of dialuric acid is supersaturated at ordinary temperatures, but as the dialuric acid does not crystallize out immediately, no difficulty is experienced in its use. Table V shows the effect of dialuric acid as a reducing agent on the ninhydrin reaction with ammonium salts, the experimental details being the same as those described on pages 330–331, and the figures representing mg. of ammonium nitrogen decomposed.

¹⁷ Ruhemann, *J. Chem. Soc.*, 1911, xcix, 1310.

TABLE V.

Ammonium salt.	+ 1 cc. of water.	Dialuric acid.	
		(A)	(B)
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Chloride.....	0.025	0.045	0.031
Nitrate.....	0.025	0.044	0.031
Sulfate.....	0.026	0.043	0.031
Acetate.....	0.025	0.045	0.032
Benzoate.....	0.026	0.042	0.031

The striking influence of small amounts of dialuric acid is at once apparent. The cold saturated solution of the acid raises the decomposition of the ammonium nitrogen to nearly 90 per cent. The effect of the second solution of dialuric acid is not so marked. Here the solution contains a large excess of hydrindantin and its effect is not so great as that of smaller amounts; it is this fact that has induced in us the belief that hydrindantin probably does not form an intermediate step in the ninhydrin reaction. Moreover, the colors produced in the second series are of a very red shade, making the matching with the standard very difficult. In order to see if these red shades of color were due to the presence of alloxan or any derivative of it, or murexide, the hydrindantin formed by the interaction of the dialuric acid and the triketohydrindene hydrate was filtered off, washed on the filter with a little cold water, suspended in 2 cc. of distilled water, and a quantitative ninhydrin reaction was carried out with it. The solution obtained was extremely red in color and was found to contain only 0.013 mg. of decomposed nitrogen. A second experiment with pure crystallized hydrindantin gave an almost exactly similar result. The production of the reddish yellow colorations, then, is brought about by the excess of hydrindantin which does not decompose readily to give the ammonium salt of diketohydrindylidene-diketohydrindamine by the action of ammonia.

As the action of the cold saturated solution of dialuric acid had raised the decomposition of the ammonium salt to nearly 90 per cent, it became of interest and perhaps of practical importance to see if conditions could not be found under which the decomposition would become quantitative. Consequently a series of ex-

periments were performed by heating 1 cc. of standard ammonium chloride solution with 1 cc. of 10 per cent aqueous pyridine and 1 cc. of 2 per cent ninhydrin solution, with 1 cc. of dialuric acid solution of various strengths for varying periods of time in a rapidly boiling water bath. The results are shown in Table VI.

TABLE VI.

Dialuric acid per 1 cc.	Time of heating.	N decomposed.
<i>mg.</i>	<i>min.</i>	<i>mg.</i>
0.000	20	0.025
0.514	20	0.032
1.028	20	0.043
1.542	20	0.045
2.056	20	0.046
2.570	20	0.045
3.084	20	0.044
4.112	20	0.042
5.140	20	0.039
2.056	10	0.029
2.056	15	0.037
2.056	20	0.045
2.056	30	0.045
2.056	40	0.044

The decomposition of ammonium nitrogen by triketohydrindene hydrate in the presence of pyridine never becomes complete. The optimum decomposition occurs in the presence of a solution of dialuric acid containing 2.056 mg. per cc. when the time of heating is 20 minutes. These conditions are practically those of Table V with the cold saturated solution of dialuric acid (A). Increase in time of heating does not affect the amount of coloring matter, but with larger amounts of dialuric acid the color gradually decreases and also acquires a strong reddish shade which renders its comparison with the alanine standard very difficult and uncertain.

SUMMARY.

1. Methods of detection of the ammonium salt of diketohydrindylidene-diketohydrindamine are given.
2. In a concentration of 1 per cent the ammonium salts of weak acids react positively with ninhydrin.

3. In very high concentration the ammonium salts of the strong mineral acids give a positive ninhydrin reaction.

4. In a concentration of 1 cc. = 0.05 mg. of ammonium nitrogen, no reaction is obtained.

5. In the presence of pyridine and in a concentration of 1 cc. = 0.05 mg. of nitrogen, all ammonium salts react positively with triketohydrindene hydrate. The amount of decomposition of the ammonium nitrogen was found to be independent of the acid radicle attached and possessed a constant value of 0.018 mg. nitrogen; the blue coloration is due to the ammonium salt of diketohydrindylidene-diketohydrindamine.

6. A mechanism of the ninhydrin reaction with amino-acids is suggested, depending on the decomposition of the amino-acid into ammonia and the corresponding glyoxal.

7. The formation of phenylglyoxal-*o*-carboxylic acid by the hydrolysis of triketohydrindene hydrate is supposed to be the first stage in the mechanism of the ninhydrin reaction with ammonium salts.

8. Reducing agents increase the ninhydrin reaction with ammonium salts.