In all these proteolytic reactions, as in enzymic changes in general, there must be at least three factors, the substrate, the ferment, and the acid or alkali accelerator. Temperature may enter as a fourth modifying factor. Now it is evident that with a difference of molecular structure the proteins may fall apart with different degrees of facility, and this fact must enter to modify the extent or conditions of ferment action. It is not the ferment alone which must be considered as dependent on the reaction of the medium, but the ferment in relation to the substrate. These two constitute a new complex and the optimum reaction may therefore be expected to vary with the substrate. From this point of view other proteins are now under investigation.

Conclusions.

Trypsin acts on fibrin and fibrin peptone most energetically at a hydrogen-ion concentration between 10^{-8} and 5×10^{-9}. Our work on the former leads to results closely agreeing with those of Michaelis and Davidsohn for the latter.

For casein the optimum hydrogen concentration is distinctively greater, and within the limits 3×10^{-6} to 5×10^{-7}. In addition to this the digestion proceeds at a degree of acidity much greater than that for the beginning of the fibrin digestion.

It is probable that for each type of protein substance there is a distinct range for the optimum activity. It is suggested that it may be the enzyme plus the substrate rather than the enzyme alone which is affected by the reaction.

CHICAGO, ILL.

[CONTRIBUTION FROM THE OTHO S. A. SPRAGUE MEMORIAL INSTITUTE AND THE PATHOLOGICAL LABORATORY OF THE UNIVERSITY OF CHICAGO.]

THE MECHANISM OF THE NINHYDRIN REACTION.

A CONTRIBUTION TO THE THEORY OF COLOR OF SALTS OF ALLOXANTINE-LIKE COMPOUNDS.[1]

BY J. M. RETINGER.

Received November 27, 1916.

Ninhydrin is the name given by Abderhalden to triketohydrindenehydrate, which because of its peculiar color reaction with amino acids and amines, is used by him as indicator in his dialysis method for detection

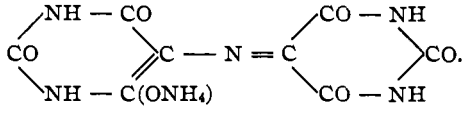
[1] The experimental work upon which this article is based was completed at the University of Leipzig in 1913 under the direction of Prof. A. Hantzsch and appears among the publications of that institution. Out of courtesy to Prof. Hantzsch with whom it is impossible to communicate at present, the author does not feel justified in quoting in detail from the protocols.

After submission of this paper for publication Prof. J. Stieglitz called my attention to a publication by G. N. Lewis in the Proceedings of the National Academy of Sciences, 1916, p. 586–92, in which a very similar idea for color production in special cases

of the activity of specific ferments in the animal organism under pathologic conditions.

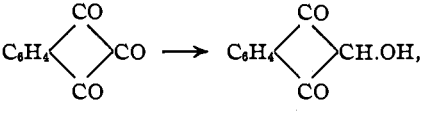
In all the recent theoretical papers dealing with the mechanism of the ninhydrin reaction a parallel is drawn between the substance causing the blue coloration and murexide. The substance having the purple color is said to be diketohydrindylidene-diketo-hydrindamine, although it has been neither isolated nor identified as such. The only evidence supporting that assumption is the similar way of preparing that compound and the qualitative identity of the absorption in the visible part of the spectrum.[1]

Alloxan with ammonia gives murexide

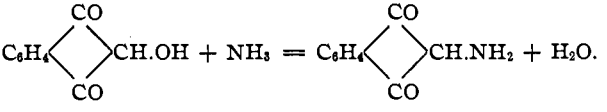


The reaction between triketohydrindene and amino acids is similarly explained:

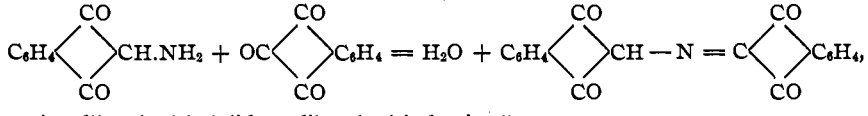
"The nitrogen atom is supplied by the decomposition of the amino acid into the corresponding glyoxal and ammonia, the former causing a reduction of the triketone to 1,3-diketohydrindole



which condenses with loss of water with the ammonia



A second molecule of triketohydrindene hydrate then condensed with the 1,3-diketo-hydrindamine



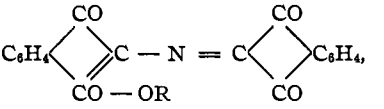
to give diketohydrindylidene-diketohydrindamine."

is advocated. In discussion of the problem of the color of triphenylmethyl, G. N. Lewis makes, on page 589, a statement that "there seem, therefore, to be every reason to believe that triphenylmethyl is colored not because it assumes the quinoid form, but because it possesses an odd electron which, as in every other known substance of this class except NO, has a sufficiently low natural frequency to absorb visible light." In other words the colorless hexaphenylethane dissociates into two triphenylmethyls because of overloading of the affinities of the middle carbons with heavy groups as shown by Schlenck (*Ann.*, **272**, 1 (1910)) and the presence of a free valence or odd electron determines the color. That, of course, applies also to alloxantine and hydrindantine and explains the reaction in an identical way from the electronic point of view.

[1] Harding and Warneford, *J. Biol. Chem.*, **25**, 319–336 (1916); Harding and McLean, *Ibid.*, **25**, 337–350 (1916).

The authors themselves see a difficulty in explaining the reaction with organic amines because the question of supplying the necessary unsubstituted nitrogen for the bridge would necessitate a splitting off of the amino group, which is known to be a matter of great difficulty if not an entire impossibility under these conditions.

Likewise the similarity of absorption in the visible spectrum, when the absorption as stated, is not selective, *i. e.*, composed of several bands all over the spectrum, but uniform going from red to green, cannot be a criterion of identity. Any two substances dissolving with the same color and shade would have the same absorption in the visible spectrum, provided the absorption is not selective, because then the visible color would be the result of mixing of many single colors. In this case the absorption would be represented by one single broad absorption band. But even here, a number of Abderhalden reactions, which will be the subject of another paper, have given evidence that not only the shade but also the color would not always be identical. Its tint varies from blue to purple, a fact which could hardly be explained by assuming the murexide-like formula for the coloring matter



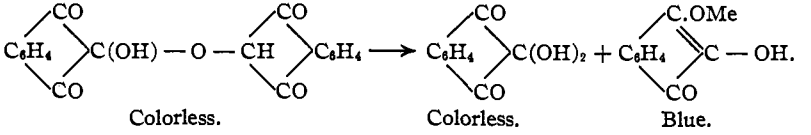
because in that case the change of color would be caused by a colorless cation R while we know that esterification or salt formation does not as a rule deepen or lighten the absorption. The difference in the constitution of the amine can of course have no influence on the bridge-nitrogen as all its three valencies are necessary for the bridge connection and a pentavalent nitrogen would behave in general in a very different way.

The diketohydrindylidene-diketohydrindamine explanation of the ninhydrin reaction not being sufficient for elucidation of all facts connected with it, an attempt may be made to explain it in a quite different way, basing the theory on observations made by S. Ruhemann[1] on hydrindamine and on experimental work by the author on the colored salts of alloxantine and hydrindantine.[2]

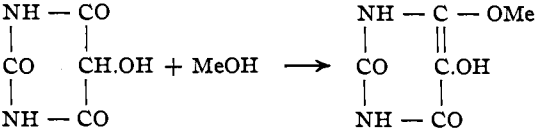
In 1911 S. Ruhemann made the observation that hydrindantine gives a red coloration with potassium carbonate and a blue one with free potassium hydroxide. But this coloration was so unstable that all attempts to isolate the colored substance failed. He declared the red product to be a true salt of hydrindantine, but the blue substance to be a salt of the dioxindone. According to his theory by splitting from hydrindantine, triketohydrindene and dioxindone are formed, and the latter is enolized by the alkali and gives the chromo-salt

[1] S. Ruhemann, *J. Chem. Soc.*, **99**, 792, 1306 (1911).

[2] J. M. Retinger, *Dissertation*, Leipzig (1913).



Ruhemann gives no proofs to support his views but nevertheless takes it as base for a conclusion that alloxantine is decomposed in the same way by barium hydroxide, that it is split into alloxan and dialuric acid and that the latter is enolized and gives the purple chromo-salts.[1]

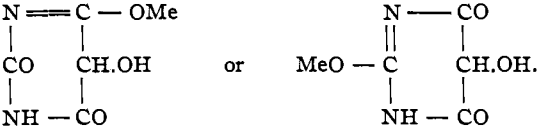


The explanation is untenable according to my experimental work on the purple salts of alloxantine previously cited.[2] In addition to the Ba salts, also the K, Na, and Rb salts of the simple alloxantine, its two dimethyl derivatives, and of malic acid, *i. e.*, tetramethyl-alloxantine, were prepared, though not all in a state of purity suitable for analysis. These salts regenerate, on acidification under exclusion of oxidation, alloxantine and not dialuric acid. In the same way potassium dialurate when treated with an excess of barium hydroxide does not yield the violet supposed to be basic dialurates.

On acidifying the blue alkaline solution of hydrindantine no hydrindantine is obtained, a fact which is explainable by the great lability of the colored substance. After disappearance of the coloration, dioxindone and *o*-carboxyl-mandelic acid remain in the liquid just as from alloxantine and aqueous potassium hydroxide dialuric and alloxanic acids are formed. Even in the alloxantine series the purple salts remain undestroyed only, when they are insoluble, as the Ba salts in the alkaline mother liquor, or as the alkali salts in alcohol. As soon as they go into solution (as the alkaline salts in water) they are destroyed.

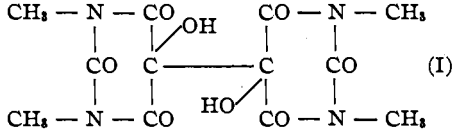
Of the colored salts the following were prepared in analyzable state of purity: The Na and Ba salts of the simple alloxantine and of its two dimethyl homologs; all those salts have four metallic equivalents for every molecule of alloxantine and are, of course, formed by rearrangement of two NH-CO groups into the enolic form N = COH.

[1] He gives the colorless dialurate the very unbelievable formula



[2] *Loc. cit.*

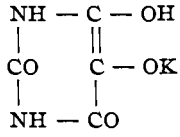
Amalic acid treated with $Ba(OH)_2$ under all precautions gives also blue but very perishable salts, although as a tetramethyl-alloxantine,



(I)

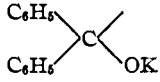
it contains only two substituable hydrogens—those in the hydroxyls.[1]

From this fact the conclusion can be drawn, that the color of those salts is not due to the enolization of the NH-CO groups but to primary salt formation on the free hydroxyls and especially on the second one because monometallic salts are colorless.[2] The enolic hydroxyl of the formula by Piloty cannot be considered as giving the coloration to the salts because the potassium salt of dialuric acid



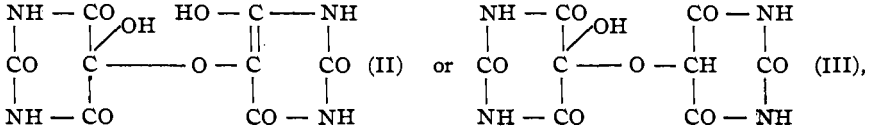
remains colorless in alkaline liquid although it contains an identical configuration with the other part of alloxantine.

On the basis of newer researches a satisfactory, though radical explanation, can be given to the formation of color in these salts. Schlenk and Zeickel[3] ascribe to the blue potassium salt of benzophenone the formula with a trivalent carbon or as we may say with a free valency



In salts of alloxantine we would assume the affinity of the two middle carbons becomes so loosened by the vicinity of four negative oxygen-holding groups that their connection is severed very easily, even by substitution of both of their hydroxyls by metals.

[1] I ascribe to alloxantinethe old Formula I with carbon-binding between the two parts and not that of Piloty (*Ann.*, **333**, 62 (1904)) II or Slimmer and Stieglitz III (THIS JOURNAL, **31**, 661 (1904)),

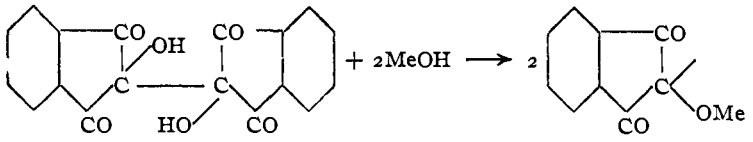


because in that case we would have a quinhydrone-like compound which ought to be deeper colored than its "quinoid" component, while according to the work of Bilz (*Ber.*, **45**, 3659 (1912)) the anhydrous alloxan is yellow while alloxantine is colorless.

[2] J. M. Retinger, *Dissertation*, Leipzig (1913).

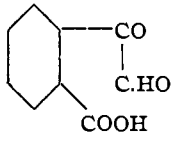
[3] Schlenk and Zeickel, *Ber.*, **44**, 1182 (1911); Beckmann and Paul, *Ann.*, **266**, 1 (1891); K. H. Meyer, *Ibid.*, **377**, 62 (1904).

The same explanation applies to the blue salts of hydrindantine

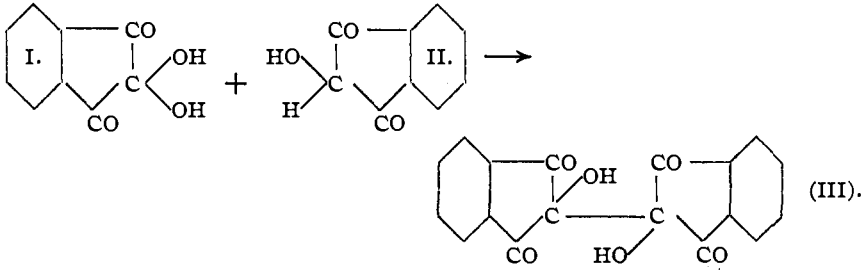


because in the salt formation with hydrindantine also only the grouping CO — C(OH) — CO takes action while the benzole rest which is connected with it has no significance at all, just as the urea rest in alloxantine is of no importance for the chromo-salt formation.

Ruhemann has shown that triketohydrindene hydrate partly hydrolyzes into *o*-carboxyl-phenylglyoxal



which as a rather strong reducing agent, on account of its two carbonyl groups, will reduce one molecule of triketohydrindene to dioxindone (II). This cannot exist in a free state in the presence of triketohydrindene (I), and consequently will combine with the excess of it to give hydrindantine (III).



Hydrindantine in its turn when reacted upon by an amino acid will give the salts in which on account of overloading of three valencies of the bridge carbons with heavy groups the remaining valencies will not be sufficiently strong to hold the two halves of the molecule together. The two halves will split apart and remain free, thus forming two independent molecules or radicals. The great instability of the colored substances in all the cases mentioned is a great support to the theory. This free valency would have to be responsible for the color of the combination and there can hardly be any objection to the use of the idea of a trivalent carbon after the magnificent work of Gomberg in that field.

It was mentioned already that in different ninhydrin reactions the color of the liquid ranges from pink to deep blue-violet and it was pointed out that the simple esterification or salt formation could not account for the

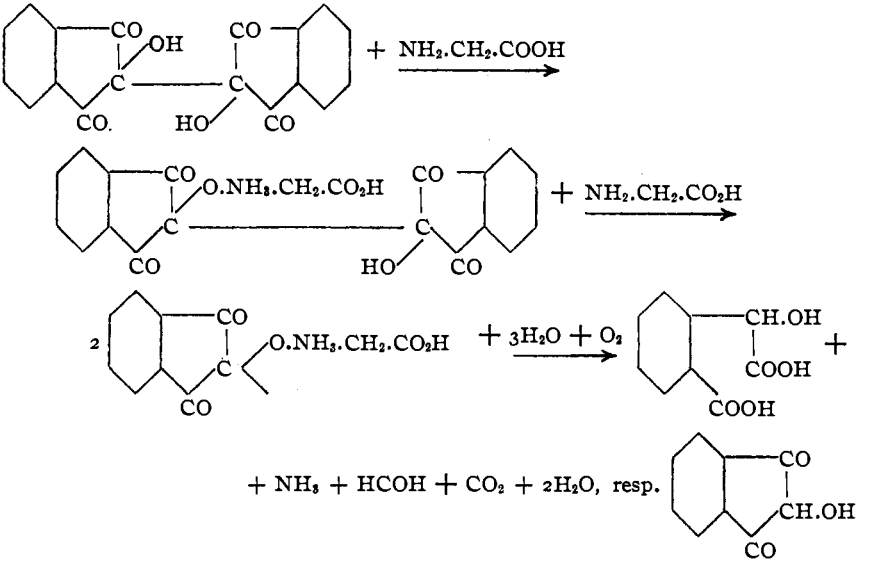
difference in color since the cations in that case are colorless and could not produce that effect. In our explanation there is also a salt formation but the conditions vary. The groupings connected with the trivalent carbon are of different sizes and consequently take up different amounts from the sum of its valencies; the remaining free affinity varies in size or, we may say, has a different potential, and produces a different optical effect in every instance.

The colored liquids resulting from a positive ninhydrin reaction, if left exposed to the air for several days, fade and finally become colorless. If we accept the diketohydrindylidene-diketohydrindamine explanation for the ninhydrin reaction there is no reason for this fading, as this compound is stable. The trivalent carbon formula explains this fact just as well as the instability of the blue salts of alloxantine.

The chromo-salt of hydrindantine with the amino acid undergoes hydrolysis and oxidation giving *o*-carboxyl-mandelic acid, ammonia, carbon dioxide and an aldehyde which in turn is partly used up for reducing the other part of the molecule to dioxindone. After acidifying the solution, we have *o*-carboxyl-mandelic acid and dioxindone, just as in the case of alloxantine alloxanic and dialuric acids.

Summary.

The whole ninhydrin reaction would then proceed as follows: The triketohydrindene hydrate hydrolyzes during boiling giving *o*-carboxyl-glyoxal which reduces part of the triketohydrindene to dioxindone, which in turn combines with another molecule of triketohydrindene to give hydrindantine. The amino acid or amine derived from enzyme action



gives first, as shown in the alloxantine series on alkaline salts, a monobasic salt which is colorless; further boiling produces a dibasic neutralization, and the molecule splits into equal parts with trivalent carbon—a free valency—as cause of the absorption in the visible spectrum. Exposure to air in water solution decomposes the split molecules further, giving *o*-carboxyl-mandelic acid, ammonia, carbon dioxide, water and an aldehyde, resp., dioxindone through reduction.

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, VANDERBILT MEDICAL COLLEGE.]

THE EFFECT OF THE INGESTION OF ALUMINUM UPON THE GROWTH OF THE YOUNG.

BY J. T. LEARY AND S. H. SHEIB.

Received February 7, 1917.

During the investigations carried on by the Board of Scientific Experts of the U. S. Department of Agriculture,[1] to determine the effects of alum baking powder upon man, it was noted by Dr. Taylor that the administration of aluminum caused a distinct decrease of phosphates in the urine and a corresponding increase of phosphates in the stools. The extent of this change was said to be too slight to have any material meaning or effect.

The members of the Referee Board were unable to detect the presence of resorbed aluminum in the blood of four men who had been placed upon a diet of small and known phosphorus content to which aluminum was added. This finding is at variance with the results obtained by Kahn[2] and Steele[3] using dogs. In each of these cases blood was removed from the living animal and aluminum determined quantitatively.

The object of our work was twofold: First, to ascertain the possibility of inhibiting the growth of young animals by diverting the phosphate content of the food to the intestine by means of an aluminum compound, hence limiting the amount of this essential constituent capable of being utilized by the animal; and secondly, to determine whether aluminum was resorbed under these conditions.

The aluminum compound used was aluminum hydroxide, made by the action of sodium bicarbonate upon sodium aluminum sulfate, the resulting product being washed as free as possible from adhering soluble salts.

The experimental animals used were puppies and young white rats. A series of experiments was also run upon full grown white rats. Each animal was fed a diet of low and known phosphorus content. The excreta were analyzed in case of the puppies in order to determine the extent

[1] U. S. Dept. Agr., *Bull.* 103.

[2] Kahn, *Biochem. Bull.*, 1911, I, 235.

[3] Steele, *Am. J. Physiol.*, 28, 94 (1911).