Application of the Ninhydrin and Alloxan Reactions to Tissue Sections*

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The ninhydrin and alloxan reactions have been criticized as being nonspecific and unreliable for histochemical studies due to the diffusion, solubility, and fading of their reaction products. This paper presents some modifications of the techniques that appear to render the reactions more reliable for the identification of proteins and peptides possessing terminal α-amino acids. The resulting color complexes are also stable.

In 1862 Strecker demonstrated that a mixture of alloxan and α-amino acid in aqueous solution reacted with the generation of carbon dioxide and the concomitant development of a deep blue color which could be attributed to the formation of murexide (acid ammonium purpurate). The latter product was isolated as purple-red crystals from the reaction mixture which, in addition, yielded an aldehyde containing one less carbon atom than the parent amino acid. Hence an oxidative deamination of the amino acid was instigated by the alloxan reagent (Greenstein and Winitz, 1961).

The cytochemical application of the alloxan reaction to tissue sections was criticized by Romieu (1925) on grounds of lack of specificity, and by Giroud (1929) because of color diffusibility. Serra (1946) quotes Winterstein (1933) as saying that the test was insensitive with fixed material. Vercauteren (1951) maintained that the reaction product behaved as an anionic dye that stained tissue components having an affinity for such dyes.

Ruhemann (1910, 1911) observed that other reagents may behave as oxidizing agents toward amino acids leaving similarly colored end products. Ninhydrin (triketohydrindene hydrate) reacts with α-amino acids in neutral aqueous solution, resulting in the evolution of carbon dioxide, the formation of an aldehyde with one less carbon atom than the parent amino acid, and the development of a deep blue coloration. This is due to the formation of a colored complex, the ammonium salt of diketohydrindylidene diketohydrindamine (Greenstein and Winitz, 1961).

The ninhydrin reaction, as employed in biochemical studies, was applied to tissue sections by Berg (1922). Later, both this reaction and the alloxan reaction were applied to fixed tissue sections by Serra (1946). Using formalin-fixed material, Serra was able to demonstrate a weak alloxan reaction, and further showed that the ninhydrin reaction, which could only be produced with prolonged excessive heat, faded rapidly. Vercauteren (1951) indicated that the reaction products of ninhydrin were, to some extent, soluble in water, and thus may be absorbed on cellular structures which are not the site of the reaction. Hutton (1953) was able to stabilize the reaction product for about a week in unerupted molars of hamsters, fixed only by their incidental embedding in celloidin. Sections sprayed with a 0.25% ninhydrin solution in n-butanol required three to four days incubation at 55 C to develop maximum color.

Notovny and Owens (1960) prevented diffusion of amino acids, and their ninhydrin reaction products, from original sites by fixing and dehydrating the tissues in anhydrous,

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peroxide-free dioxane. The color produced by α-amino acids faded within 24 hours, while that produced by protein was stable for seven days or longer. Vainer and Bona (1963) used various fixatives, such as Carnoy and methyl alcohol, and different concentrations of ninhydrin varying from 0.1% to 0.4% to study its reaction on peripheral blood. Their reactions were carried out at 80 C for 5 to 30 minutes, and the staining remained intact for "a long time."

Yasuma and Ichikawa (1953) stained the aldehyde groups by the Schiff reagent in an oxidative deamination reaction brought about by ninhydrin. Alloxan was also used as an oxidative deamination reagent. A serious objection to such use of the Schiff reagent is its tendency to stain other tissue components (direct Schiff reaction), thus confusing the recognition of true protein sites in the sections.

Both the ninhydrin and the ninhydrin-Schiff histochemical reactions have been criticized. Recently, Puchtler and Sweat (1962), reviewed the literature and concluded that the ninhydrin-Schiff method was unreliable for demonstrating proteins. Kasten (1962) took issue with this conclusion and stated that, in the ninhydrin reaction, as distinct from the ninhydrin-Schiff reaction, ammonia reacts with reduced ninhydrin (hydrindantin) to form a violet product which is diffusible, unstable, and offers little possibility for histochemical application. He observed that acetylating and deaminating agents block the ninhydrin and alloxan reactions by masking the carboxyl or the amino groups of the α-amino acids. This indicates that the action of ninhydrin in tissues is at the amino group, and qualifies the ninhydrin reaction as an histochemical test for proteins and polypeptides with free and reactive amino groups.

Materials and Methods

Pieces of rat kidney, liver, small intestine, skeletal muscle, and cardiac muscle were removed after decapitation. Different concentrations of aqueous and alcoholic solutions of trichloroacetic, phosphotungstic, phosphomolybdic, and acetic acids were used as fixatives for 24 hours at room temperature. Tissues fixed in aqueous solutions were dehydrated in a series of alcohols, cleared in xylene, and embedded in Paraplast. Tissues fixed in 80% alcoholic solutions were dehydrated in 95% and absolute alcohol, cleared in xylene, and embedded in Paraplast.

Both aqueous and alcoholic solutions of phosphomolybdic and phosphotungstic acids proved to be poor penetrants and their use was discontinued.

Sections of tissues fixed in 5% acetic acid in 80% alcohol were treated with ninhydrin, but, only a weak color developed. The following detailed procedure was followed: Tissues were fixed in 5% trichloroacetic acid in 80% alcohol for 24 hours at room temperature. They were then transferred to 95% alcohol and absolute alcohol for one hour each, and after clearing in xylene, were embedded in Paraplast. Paraffin sections were mounted on slides with starch paste, instead of egg albumin, to avoid false localization. Deparaffinization of sections in xylene was followed by going through two changes of absolute alcohol. Sections were then treated in 1% ninhydrin in absolute alcohol at 37 C from one to three hours. Overtreatment tended to weaken the reaction. After rinsing in two changes of absolute alcohol, and clearing in xylene, the sections were mounted in Permount. Both 0.1% and 0.5% stannous chloride were added to the ninhydrin solution.

Pepsin and trypsin digestion procedures were used as well as acetylation and deamination blocking reactions (Pearse, 1961). Alternate sections were treated with 1% alloxan in absolute alcohol instead of ninhydrin in the above procedures.

Comparable sections of the kidney, liver, muscle, and intestine were stained by the ninhydrin-Schiff and alloxan-Schiff methods for protein-bound amino groups (Yasuma and Ichikawa, 1953), the chloramine-T Schiff method for protein-bound amino groups (Burstone, 1955), and the acid solochrome cyanine method for basic proteins (Pearse, 1961).

Photomicrographs of rat tissue sections fixed in 5% trichloroacetic acid in 80% alcohol and treated with alcoholic ninhydrin or alloxan.

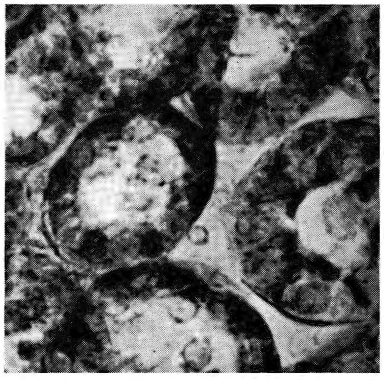

Fig. 1—Kidney tubules following alloxan treatment (× 700).

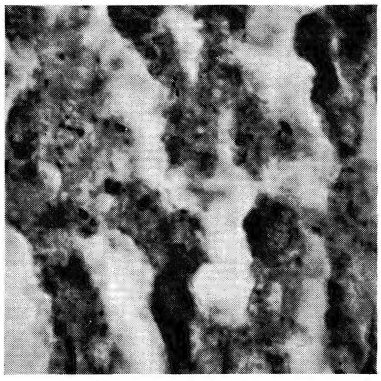

Fig. 2—Liver cells following the ninhydrin reaction (× 700).

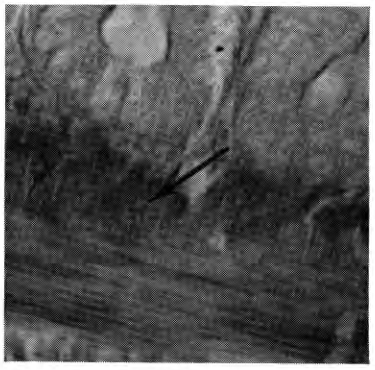

Fig. 3—Smooth muscle fibers in the small intestine following the alloxan reaction. Arrow points to darkly colored submucosa (× 700).

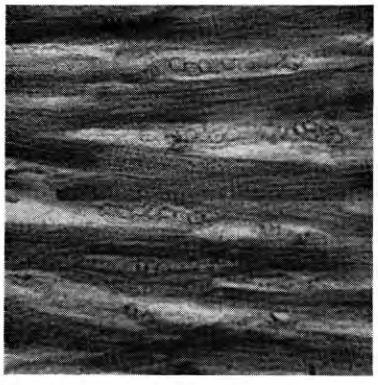

Fig. 4—Cardiac muscle colored by the ninhydrin reaction (× 700).

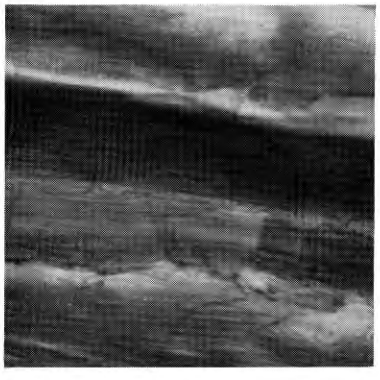

Fig. 5—Striated muscle colored by the alloxan reaction (× 700).

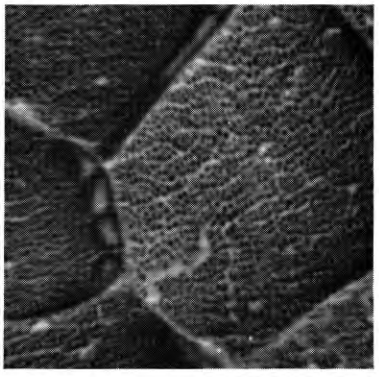

Fig. 6—Striated muscle demonstrating ninhydrin-reaction (× 1400).

Results and Discussion

We observed an intense bluish-violet color in trichloroacetic acid-fixed sections after the ninhydrin treatment. Alloxan stained the tissues a rich red color. Both ninhydrin and alloxan produced intense coloration of proximal renal tubular epithelium and basement membranes (fig. 1). Some parenchymal liver cells (fig. 2) were well-stained while others in the same area failed to bind the color complex. Smooth muscle in the small intestine (fig. 3) stained strongly in contrast to the epithelium. The myofibrils and striations in both cardiac (fig. 4) and skeletal muscles (fig. 5 and 6) were intensely stained. The connective tissue fibers of the muscle, liver, and kidney were poorly stained, except in the submucosa of the small intestine where they were intensely stained.

Pepsin and trypsin digestion, prior to ninhydrin and alloxan treatment, completely blocked the reaction by digesting the proteins. Both acetylation and deamination blocked the ninhydrin and alloxan reactions and prevented color development, thus establishing that the ninhydrin and alloxan act on the terminal amino acids of the protein and peptides chains.

According to Moore and Stein (1948), the addition of stannous chloride to the ninhydrin solution reduced for ninhydrin, thus intensifying the reaction; however, we found that the addition of 0.1% or 0.5% stannous chloride completely abolished the reaction.

The localization and color obtained by the ninhydrin reaction or the alloxan reaction were superior to those obtained by the ninhydrin-Schiff or the alloxan-Schiff reactions.

The chloramine-T Schiff method, where chloramine-T acts as a dicarbonyl compound like alloxan and ninhydrin, stained the tissues with the same intensity and localization as those of ninhydrin-Schiff and alloxan-Schiff, but was less intense than that of alloxan or ninhydrin alone.

The acid solochrome cyanine method stained the tissues rather weakly.

Summary

The ninhydrin (triketohydrindene hydrate) reaction, used extensively in biochemical work, has not been successfully applied cytochemically, due to lack of specificity, fading, and diffusion artifacts. These faults are effectively overcome by avoiding aqueous media and by fixing the tissues in a 5% solution of trichloroacetic acid in 80% alcohol, a good precipitant for proteins possessing free NH_2 groups. A fixation of 24 hours is followed by customary embedding in paraffin, and deparaffinized sections are passed through two changes of absolute alcohol, then treated in 1% ninhydrin in absolute alcohol for one to three hours at 37 C. The presence of free NH_2 groups in the peptide and protein chains produces a blue-violet color. Treated sections retained their color for up to six months. Alternate sections were used for the alloxan procedure; the resulting red color showed the same reaction sites.

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