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On the Chemistry of Hammarsten Test for Indican in Urine

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Abstract

Physiological indican, indoxyl sulphate, is an analyte since higher levels of this compound indicate stomach dysfunction and intestinal dysbiosis. The Hammarsten test for indican in urine is interesting from the Biochemistry point of view as for Organic Chemistry since the chemistry involved in this test has not been described. The test employs a solution of calcium chlorohypochlorite added to acidulated urine with fuming hydrochloric acid, in the presence of chloroform. The latter turns blue due to indigo formation. The colour can be pale, bright, or dark, in accordance with indican concentration. This swift indigo-synthesis in acidic medium, via chlorinated intermediates, differs notably from the oxygenation process in alkaline medium that takes place during the obtention of indigo from vegetable source. We provide the route from physiological indican to indigo blue and to indigo red (indirubin), giving the electron flow, step by step, in this reaction series.

1. Introduction

There are two connotations for indican: one is the glycoside of indoxyl, the other refers to the physiological product excreted in the urine, and known as indoxyl-sulphuric acid, [1]. This name is incorrect because it means protonation to yield a salt, but pyrrole and therefore indole are not basic [2].

The product is an ester of indoxyl and sulphuric acid, an aryl sulphate [3, 4]. Indoxyl sulphate is found as its potassium salt. Other name for indoxyl-sulphuric acid is indoxyl

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sulfonic acid [5]; however a sulpho group on oxygen is a sulphate. Figure 1.

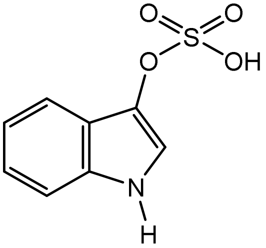


Figure 1. Physiological indican: indoxyl sulphate.

Gut bacteria convert tryptophan to indole which is then oxidized to indoxyl and transformed to sulphate in the liver. Indican levels are directly associated with bacterial activity in the intestines. Elevated levels indicate intestinal toxemia or overgrowth of anaerobic bacteria. Elevated indican may also indicate indigestion and hypochlorhydria (stomach dysfunction). Indican excretion is reduced when the gut is colonized with beneficial bacterial strains, [6, 7].

In this paper we provide the chemistry of the Hammarsten test for indican in urine. He employed fuming hydrochloric acid and chlorinated lime in the presence of chloroform, which turns blue. The test differs substantially from the process for obtaining the natural indigoids in alkaline medium, [8].

This communication is a follow up of our studies on reaction mechanism, [9-12].

2. Antecedents

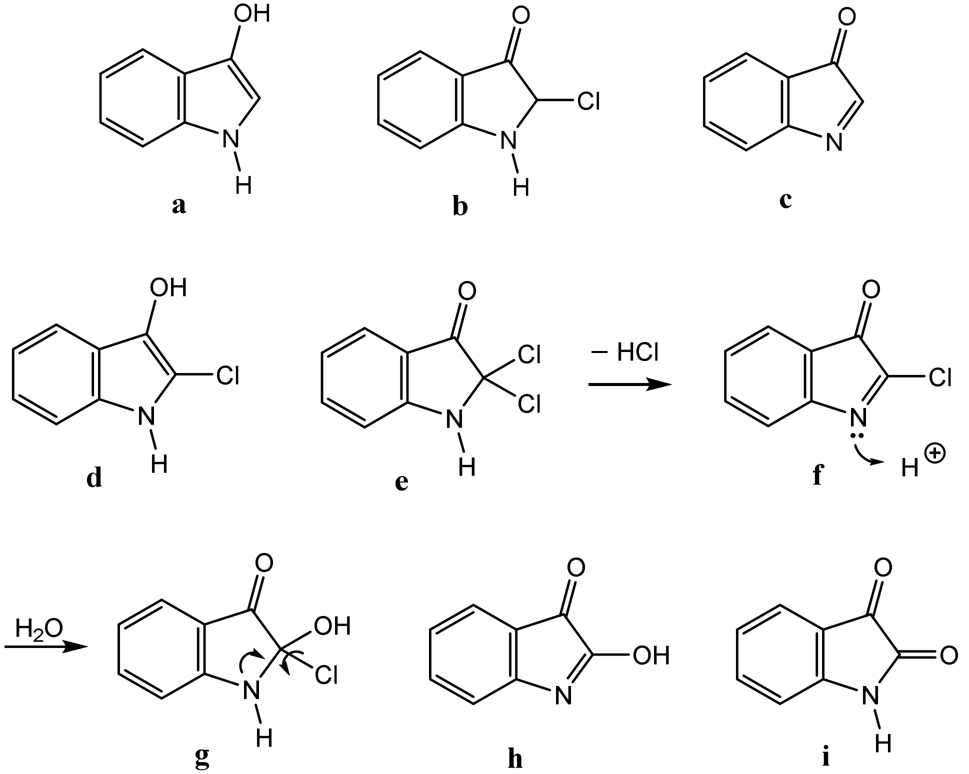
The test under study is due to Olof Hammarsten (1841-1932), Swedish professor of medical and physiological chemistry at Uppsala University, [13]. The test is based on the blue colour developed when chlorinated lime is added to strong acidulated urine containing indican.

Chlorinated lime is obtained by passing chlorine over slaked lime, $Ca(OH)_2$. The reaction product is calcium chlorohypochlorite, CaCl(OCl), [14]. This compound has also been named calcium oxychloride, $CaOCl_2$, but this term does not distinguish between the two anions present in the compound. In this preparation calcium hydroxide still remains. When this solution is added to concentrated hydrochloric acid, hypochlorous acid is formed and it is the precursor of the reactive species.

The test is as follows: mix 10 ml of the urine with 10 ml of fuming hydrochloric acid, add chlorinated lime solution drop by drop and shake out the mixture with 5 ml of chloroform. The latter takes up indigo resulting from the indican and is coloured blue. Excess of chlorinated lime solution is to be avoided, [15, 16].

3. Discussion

The first reaction occurring in this test is the acid hydrolysis of indoxyl sulphate. The liberated indoxyl, **Figure 2**, **a**, is chlorinated by hypochlorous acid generated in situ. The reactive species may be protonated-hypochlorous acid, a halogen containing complex, which can react as such or deliver chloronium ion [17-19]. In this way 2-chloro-indolin-3-one is formed, **b**. This intermediate is a halo analog of hemiaminal and is labile due to the presence of the electrodotic [20] nitrogen and the good leaving chlorine, both in ipso position. Therefore 3-oxo-indolenine, **c**, is produced by hydrogen chloride loosening (oxidation step). In the cross conjugated molecule there has been polarity inversion (Umpolung) at C—2. This position is free of steric hindrance and is preferred for reaction. This way the key intermediate for indigo blue has been formed.



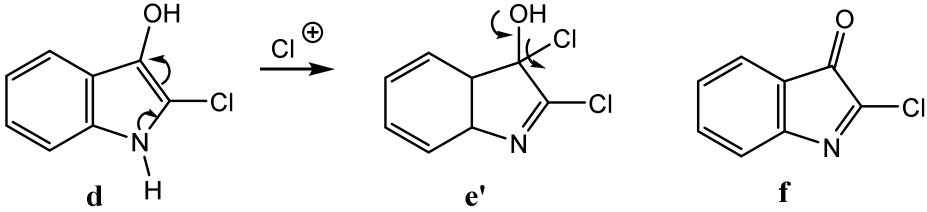


Figure 2. Formation of the key intermediates for indigo blue and indigo red (indirubin).

However, protonation of the keto group in 2-chloro-3-indolinone restores enolization, **d**, and permits attack of a chloronium ion to the double bond. A second chlorination can be at C—2 as before, **e**, ipso attack. Cf. (21). The electron donor nitrogen eliminates hydrogen chloride and the resulting chloro imine, **f**, (isatin chloride), very reactive, is hydrated to a chloro hemiaminal, **g**. This intermediate loses hydrogen chloride yielding the lactim form, **h**, of isatin, 2,3-dioxoindoline, **i**.

Isatin is the key intermediate for indigo red formation (indirubin). This compound is always the secondary product, but not a by-product. This is because there are more steps involved to produce isatin.

There is also other route to isatin chloride and therefore to isatin. The enamine in 2-chloro-indoxyl, **d**, can direct chlorination at C—3 and unstable *gem*-chlorohydrin results, **e'**, which releases hydrogen chloride and a ketone is formed, that is isatin chloride, **f**. Two factors favour chlorination at C—3: nitrogen as donor is less electronegative than oxygen, and the hydroxyl at C—3 is less bulky than the chlorine atom at C—2 for ipso reaction.

Isatin chloride in anhydrous conditions can react with indoxyl to form leucoindigo. However, some leucoindigo can be formed from this chloride in the Hammarsten test before hydration of the imidol chloride.

Reaction of 3-oxo-indolenine with indoxyl yields leucoindigo, **Figure 3**. Chlorination of one unit of this bis-indole intermediate, followed by HCl elimination affords indigo blue: (2E)-Δ-2,2'-biindole-3,3'-dione.

Indirubin results by combination of isatin and indoxyl, that is, water elimination from the keto group of the first and the methylene group of ψ-indoxyl. Indirubin is 2-(oxindol-3-ylidene) indolin-3-one.

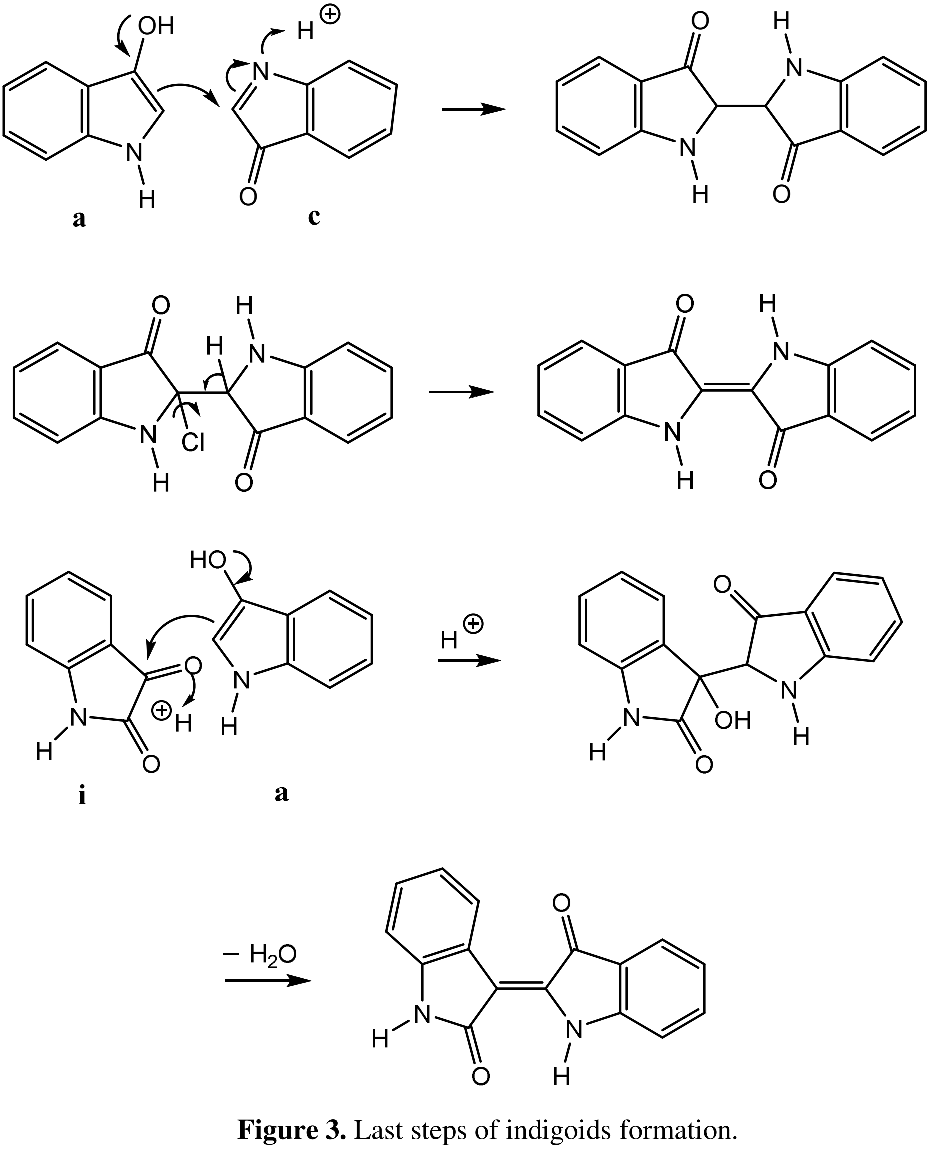


Figure 3. Last steps of indigoids formation.

4. Conclusion

The transformation of indoxyl to indigo blue and indigo red (indirubin) from vegetable source is accomplished by an oxygenation process in alkaline medium. In the Hammarsten test the reactions take place in acidic medium and there is oxidation by electron loss. In several steps a chloronium ion is captured and afterwards expelled as chloride ion.

In the Hammarsten test there are ipso attacks to aromatic intermediates, and double bond reacting as enol or as enamine to yield, steps later, the same key intermediate.

The oxidation of leucoindigo is accomplished not by hydrogen peroxide elimination but by hydrogen chloride detachment. The formation of indirubin has been explained also, and why this companion is always a secondary product.

In the reaction series taking place during the test we provided the electron flow step by step.

Acknowledgements

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