NON-NITROGENOUS NINHYDRIN-POSITIVE COMPOUNDS IN ION-EXCHANGE CHROMATOGRAPHY

IDENTIFICATION OF LEVULINIC ACID IN HYDROLYZED PLANT FRACTIONS*

R. M. ZACHARIUS AND E. A. TALLEY

*Eastern Regional Research Laboratory***, Philadelphia, Pa (U.S.A.)*

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Employing ion-exchange chromatographic procedures[1] to crude protein hydrolysates, SCHRAM *et al.*[2] have described a red peak resulting from the ninhydrin reaction with earlier fractions eluted from the ion-exchange column. They were able to demonstrate that this peak could be produced from carbohydrates in the absence of amino acids under the conditions employed on acid hydrolysis.

In our laboratory a similar peak had been noted from acid hydrolysates of the non-protein nitrogen fraction of potato tubers. More recently during studies of the components of the non-protein as well as bulk protein fractions of the mature kidney bean seed, the red peak was observed following hydrolysis and ion-exchange chromatography. Column chromatography carried out by the SPACKMAN *et al.*[3] procedure on the Phoenix Automatic Amino Acid Analyzer*** with a low, constant base line revealed, in addition, a number of small peaks, of which only one was of sufficient size to be noteworthy. Since its absorption was largely at 440 mμ, it was designated the "yellow" peak.

Nitrogen-free glucose was acid treated under the same conditions as that employed in the hydrolysis of the plant fractions. Analytical column chromatography showed the pattern of ninhydrin-positive peaks also found in the plant fractions.

When the analytical separations were carried out on the Phoenix Amino Acid Analyzer using 0.2 *N* pH 3.25 sodium citrate buffer at 30°, the red peak maximum appeared at 80–83 ml (Fig. 1). The instrument's spectrophotometer which measures at 440 and 570 mμ showed a somewhat greater absorption at 440 mμ (1.35 : 1.0). Since the magnitude of this peak compared favorably with that of the amino acids in larger amount, the compound responsible for the red peak was isolated.

A scaled up version of the analytical column with a capacity 24 times as great

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*** Mention of this name does not imply endorsement of this product by the Department of Agriculture over other similar products not named.

was employed. Because of availability the column consisted of Dowex-50-8 X screened through 200 mesh[4]. The bean seed non-protein nitrogen fraction was hydrolyzed in a sealed vial with 10% HCl for 10 hours in an autoclave at 120° and concentrated under vacuum until most of the HCl had been removed. The hydrolysate was separated on the preparative column with pH 2.2 sodium citrate buffer. The lower pH (than on the

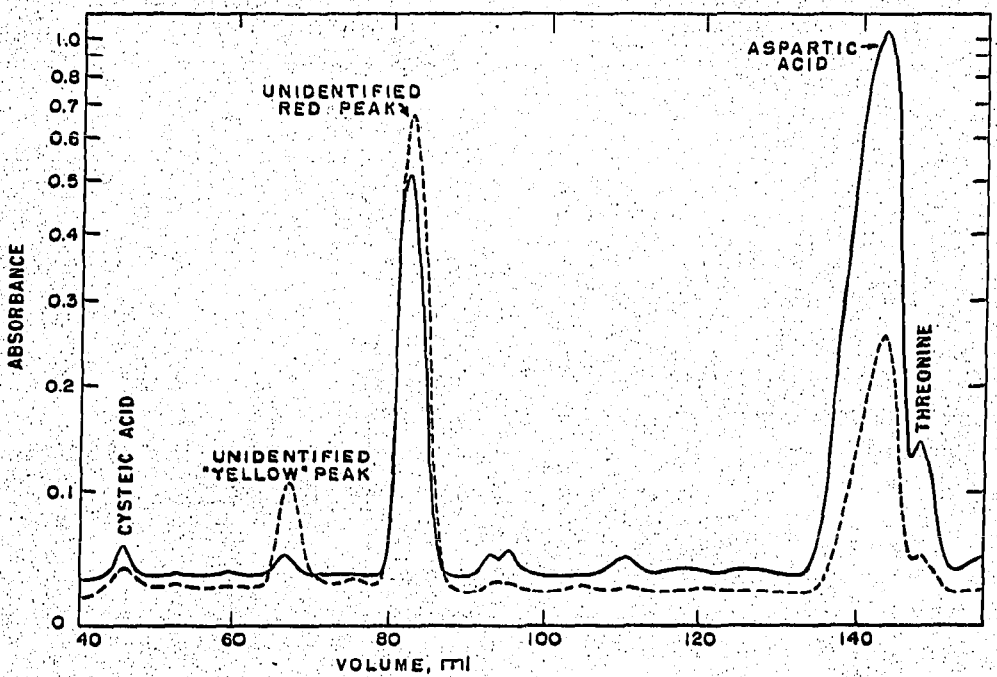

Fig. 1. Acid hydrolyzed soluble N fraction of bean seed. 150 × 0.9 cm Amberlite IR 120 column, 30° 0.2 *N* Na citrate[3]. ——— 570 mμ; – – – – 440 mμ.

analytical column) was used in order to facilitate the separation. Fractions of 24 ml were collected in the fraction cutter and 0.5 ml aliquots of each fraction were assayed with MOORE AND STEIN ninhydrin reagent[5] in order to locate the red peak and the "yellow" peak which precedes it. The fractions of each peak were combined and desalted on Dowex-2-8 X (OH^-) less than 200 mesh, eluting the compounds with *N* acetic acid. The desalted eluate was concentrated under reduced pressure at 40° and dried over H_2SO_4. Rechromatography of the isolated compound responsible for the red peak on the amino acid analyzer revealed a very small contamination by the "yellow" peak.

The isolated material was a near colorless liquid weighing nearly 800 mg although only a 8–10 mg yield of the peak compound had been expected based on the assumption that the ninhydrin color factor was almost equivalent to that of leucine.

A Kjeldahl determination demonstrated that the isolated compound did not contain nitrogen. Assuming that this compound arose from one of the hexose sugars or quantities of starch present in the seed, a possible degradation product is 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural). A test for the furfural ring with aniline acetate, however, proved negative.

The preparation of levulinic acid had been described from starch by RISCHBIETH[6]

and from glucose by the action of HCl[7]. Moreover levulinic acid was produced from 5-hydroxymethyl-2-furaldehyde with HCl by TEUNISSEN[8]. The isolated compound gave a positive iodoform reaction and yielded a crystalline 2,4-dinitrophenylhydrazone, m.p. 204–205°. A mixed m.p. with the 2,4-dinitrophenylhydrazone of authentic levulinic acid produced no depression. The two derivatives gave matching infrared absorption curves and matching X-ray diffraction patterns.

Levulinic acid reacts with the MOORE AND STEIN ninhydrin reagents to produce a red color, and with chromatography on the analytical resin column produced a single red peak when mixed with the isolated compound. The foregoing evidence demonstrates rather conclusively that the compound responsible for the red peak is levulinic acid. It is of interest that SCHRAM *et al.*[2] had noted that levulinic acid produced a red color with ninhydrin but did not isolate or characterize the compound.

The absorption curve for the ninhydrin reaction product of levulinic acid is somewhat different than that of the amino and imino acids. There is no clear cut maximum although greatest absorption lies somewhere between 510–520 mμ. However, the high ratio of the 570:440 mμ absorption of this peak shows a marked resemblance to some imino acids, *e.g.* pipecolic acid. The color factor for levulinic acid with the instrument employed, yields a value at 570 mμ of 0.183 whereas leucine is 17.1; this is approximately 1/100 the sensitivity of leucine at this wavelength. At 440 mμ the color factor for levulinic acid is 0.249 as compared with 4.0 for proline. The low sensitivity of levulinic acid to the ninhydrin reagent explained the unexpected high yield of isolated compound. Calculation of the levulinic acid from its peak in the hydrolysate of the non-protein nitrogen fraction revealed the presence of 37 mg per g of kidney bean seed. By comparison, pipecolic acid, in largest amount of any ninhydrin-positive nitrogen constituent was present to the extent of 8.5 mg per g of seed.

The maximum of the smaller "yellow" peak mentioned above occurs at 66-69 ml of the column effluent. When an aliquot of the isolated fraction from the preparative column was rechromatographed on the analytical column, its elution volume had increased to 102 ml. This indicated some alteration of the compound in the isolation process.

According to the old carbohydrate literature, the two isomeric angelicalactones can be produced from levulinic acid by distillation. β,γ-Angelicalactone was synthesized according to THIELE *et al.*[9]; the compound was redistilled, b.p. 46–47°/9.4 mm. α,β-Angelicalactone was prepared by heating levulinic acid with a mixture of redistilled acetic anhydride–acetyl chloride (9:1) at 100° overnight[10]; the lactone distilled, b.p. 82–84°/10.3 mm. Neither of the isomeric angelicalactones had the same effluent volume as the "yellow" peak on chromatography, although the α,β-compound may be responsible for one of the several minor peaks found in the plant extracts and in glucose treated with hot acid.

The more unstable β,γ-angelicalactone is approximately twelve times more sensitive (0.22) to the ninhydrin reagent than the α,β-isomer (0.018) although both provide colored products whose 570:440 absorption values are virtually identical

and not unlike the "yellow" peak. These ratios for the unidentified peak are identical with that given by proline.

In a further effort to discover the origin of the "yellow" peak, consideration was given to the possible effects of ammonia in the hydrolysate on the angelicalactones. An 8.6 mg sample of freshly prepared β,γ-angelicalactone was permitted to stand in contact with 1.0 ml 0.15 M NH_4OH at room temperature overnight. After neutralization, the reaction mixture was chromatographed on the analytical column. In addition to some levulinic acid, a very large peak and shoulder, one of which is presumably levulinamide[11] appeared at the same effluent volume as the unknown. However, the absorption at 570 mμ exceeded that at 440 mμ for the synthetic compound(s).

A similar treatment of the α,β-angelicalactone yielded some of the double peak found above for the isomeric lactone, levulinic acid, an unidentified peak and 44 % of the original lactone. The results suggest the possible partial conversion of the α,β-lactone to the β,γ-isomer and hence to the reaction products found.

Other possible sources of the "yellow" peak were investigated. Acetyllevulinic acid was prepared according to HELBERGER[12] and the crystalline product, m.p. 78–79°, chromatographed on the analytical column. The acetyllevulinic acid peak maximum with a 116–117 ml effluent volume has a 570:440 mμ color absorption ratio nearly identical with levulinic acid; thus it is not the identity of the "yellow" peak. Chromatography indicated that acetyllevulinic acid decomposes in solution to levulinic and acetic acids.

γ-Valerolactone fails to give a colored reaction product with ninhydrin. A crude preparation of γ-chlorovalerolactone was prepared by the action of acetyl chloride on levulinic acid[13]. The preparation was not distilled because of its instability. Ion-exchange chromatography revealed a large amount of levulinic acid and a small peak with an effluent volume of 122 ml and high 570:440 mμ absorption, presumed to be the chloro derivative.

Although the identity of the compound responsible for the "yellow" peak has eluded us, the studies made so far suggest that it arises through the intermediate and relatively unstable β,γ-angelicalactone.

During an early part of the investigation, a few other keto acids were examined in view of the fact that levulinic acid gave a ninhydrin color product. The 2-ketogluconic acid gave a ninhydrin color product absorbing more at 570 mμ whereas the 5-ketogluconic and 2-ketoglutaric acids produced products absorbing more at 440 mμ. Both 2-keto acids appeared as shoulders on the cysteic acid peak (53–55 ml) and the 5-keto acid was eluted at 60 ml. All three compounds were of a low order of sensitivity with the reagent. It is reasonable to conclude that other related keto acids or their lactones would behave similarly. It is conceivable that the condensation reaction with ninhydrin occurs in these compounds through their active methylene groups resulting from proximity to a carbonyl or position in the lactone ring[14]. This work points up the danger of assigning all ninhydrin-positive peaks to products of nitrogen compounds.

SUMMARY

Ion-exchange chromatography of acid hydrolyzed kidney bean seed fractions revealed two major non-nitrogenous ninhydrin-positive peaks. The larger of the two was isolated and characterized as levulinic acid. Its sensitivity to the ninhydrin reagent at 570 mμ is approximately 1/100 that of leucine while its 570:440 mμ absorption ratio resembles that of some imino acids.

Behavior on the ion-exchange column was noted for the isomeric angelicalactones, acetyllevulinic acid, levulinamide, γ-chlorovalerolactone, 2-ketogluconic acid, 5-ketogluconic acid and 2-ketoglutaric acid. Each gave a low order ninhydrin-positive reaction.

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