ionization of $-N_1H-$ (pK' 9.6) disrupting the hydrogen bonding and hence the ring.

The *Ve* of 1-methylguanine (pH 7) is less than that of guanine. Substitution of the hydrogen atom at $N_{(1)}$ of guanine with a methyl group leads to a decrease in adsorption, presumably due to lack of ring formation.

Hence, although hydrogen bonding has been considered the principal mechanism by which substances adsorb to PVP, the hydrogen bonding may be concerted with a nucleophilic attack at the pyrrolidone carbonyl carbon, resulting in ring formation and increased adsorption. The feasibility of the stereo-structural relationships shown in Fig. 1 has been confirmed by use of molecular models.

As a practical application of altering the elution pH, cytosine and thymine can be separated by PVP chromatography at pH 3.5, since the $-N_1H-$ of thymine is hydrogen bonded to PVP at this pH and elutes at 36 ml. The two bases may also be resolved at pH 10.3 where the $-N_1H-$ of thymine is ionized (pK' 9.8), disrupting the hydrogen bonding and allowing thymine to be eluted first.

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Behavior of uronic acids and acid-treated uronic acids on an automatic amino acid analyzer

SCHRAM *et al.*[1] reported briefly that ninhydrin reacted with non-nitrogen-containing carbohydrate material in a protein hydrolysate. More recently, the reaction of ninhydrin with non-nitrogenous compounds such as carbohydrates and related compounds has been investigated by ZACHARIUS AND TALLEY[2], ZACHARIUS AND PORTER[3], and SCHILLING *et al.*[4,5].

The kinds of compounds reported to give positive peaks on an amino acid analyzer include hydroxy acids, keto acids, hydroxy aldehydes, keto aldehydes, aldonic and uronic acid derivatives, hydroxy ketones, and some monosaccharides. In general, the reaction products from these compounds absorb more at 440 mμ than at 570 mμ. This high 440:570 mμ absorption ratio is similar to that found for the

imino acids, proline and hydroxyproline. The sensitivity of the reaction with ninhydrin is much less for these compounds than for amino acids, which means considerably more of the compounds must be applied to the analyzer in order to detect them.

In the course of analyzing acid hydrolysates of protein preparations of plant origin containing polysaccharide, several previously unidentified peaks were observed with high 440:570 absorption ratios. It was originally considered that these peaks were due to isomers of hydroxyproline. Subsequent investigation revealed they were from the acid decomposition products of pectic materials in the original sample. This paper reports the results of applying some uronic acids and other carbohydrates to an amino acid analyzer, both directly and after treatment with 6 *N* HCl at 110°. Different positive responses on the amino acid analyzer are reported from these non-nitrogenous precursors before and after treatment with 6 *N* HCl.

*Materials and methods**

A Phoenix Precision Instrument Company model K-8000 amino acid analyzer with Beckman type AA-15 and PPI type X-15 resins was used in this work. Operation was under usual conditions[6] for accelerated analyses[7] with buffer and ninhydrin flow rates of 30 and 15 ml/h, respectively.

Chemicals were obtained as follows: 5-hydroxymethylfurfural from K and K Chemical Company; furfural from Baker and Adamson (distilled prior to use, b.p. 53–54° 15 mm uncorr.); pectin and the monosaccharides from Sigma Chemical Company. Plant material containing galacturonic acid was obtained from the media in which plant tissue cultures had been grown. It was obtained from the media by dialysis and lyophilization[8].

Treatment with acid was done at concentrations of 1 to 2 mg sample/ml 6 *N* HCl in nitrogen purged, evacuated, sealed ampules. Ampules were heated at 110° for 20–22 h unless otherwise indicated. Where considerable humin formed, samples were filtered through sintered glass prior to removal of the HCl under vacuum at 50°.

TABLE I

BEHAVIOR OF SOME URONIC ACIDS ON AN AUTOMATIC AMINO ACID ANALYZER

Samples applied to analyzer without prior acid treatment; elution temperature 52.5°.

Compound	*Sample size (μmoles)*	*Elution volume (ml)*	$(H \times W)_{440}$	*440:570 mμ absorption ratio*
Galacturonic acid	25	23.5	0.77	2.64
Glucuronic acid	25	22.0	3.12	2.90
Hydroxyproline	1.0	49.0	4.17	2.00

Results and discussion

As pointed out by ZACHARIUS AND PORTER[3], the response of non-nitrogenous compounds to give ninhydrin-positive reactions on an amino acid analyzer is deter-

* Reference to a company or product name does not imply endorsement by the U.S. Department of Agriculture to the exclusion of others which may also be suitable.

mined in large measure by the conditions used. Thus because of the generally low response of these materials to ninhydrin, it is important to designate the amount of material necessary to produce a peak. Normally, aliquots of protein hydrolysates containing *ca.* 10 μmoles of amino acids (0.5 μmole/amino acid, total material applied *ca.* 1 mg) are applied to the analyzer. In order to analyze plant material containing small amounts of protein for its amino acid content, it is necessary to hydrolyze and apply the residue from larger amounts of material. A practical arbitrary amount of carbohydrate to apply to the analyzer for detection purposes is about 5 mg of material. At these levels both galacturonic and glucuronic acid (applied directly to the analyzer without prior acid treatment) gave peaks on the analyzer (Table I). Under the same conditions neither galactose, glucose, 5-hydroxymethylfurfural nor furfural gave any peaks.

Neither galacturonic nor glucuronic acid would be expected to survive treatment with HCl under hydrolysis conditions[9,10]. It is not surprising, therefore, that application of the residue from such treatment produces a different response on the analyzer (Table II). Residues from both hexuronic acids give the same response, namely, peaks at elution volumes of 27 and 38 ml compared to the elution volume of 22–24 ml for the untreated acids. The color response from the residues is also larger than for the untreated acids. No response is observed in the absence of ninhydrin showing that the peaks are the result of reaction with this reagent.

A similar response is observed for galacturonic acid, for galacturonic acid plus protein, and for pectin. Protein does not alter these peaks and pectin is apparently degraded to the same compound(s) as the untreated acid. The peak eluting at 37–38 ml shifts to 40–42 ml if the elution temperature is lowered from 52.5° to 33°. No significant shift was observed for the peak eluting at 27 ml with this change in column elution temperature. That the two peaks are related is concluded from the fact that on standing after acid treatment or extension of the duration of acid treatment ($H \times W$) for the peak eluting at 38 ml decreases while ($H \times W$) for the peak eluting at 27 ml increases an approximately equivalent amount (Table II).

After similar acid treatment both galactose and glucose gave peaks on the analyzer at 30 and 38 ml. The peak eluting at 30 ml is the major peak and corresponds to that for levulinic acid. This peak has a 440:570 mμ absorption ratio of 1 compared to 3 for the peak at 38 ml. The 440:570 mμ ratio for the peak at 38 ml from the uronic acids is also about 3 while that for the peak at 27 ml is about 5.

In the analysis of a carbohydrate-rich sample from plants for the amino acid composition of the protein present, peaks occurred on the analyzer corresponding to the 27 and 38 ml elution peaks obtained with galacturonic acid (Table II, Fig. 1). Subsequent analysis of the plant material for pectic-like materials by the carbazole procedure showed that it did contain 18% galacturonic acid in polymer form[8]. This is approximately what one might expect using the ($H \times W$) values from acid-treated galacturonic acid. However, no attempt has been made to quantitate the response. Indeed, it would not appear to be a very direct measurement of uronic acid because the response is in two areas which change with time.

The material responding is one of the decomposition products of galacturonic acid. It is not furfural or hydroxymethylfurfural since these compounds do not respond in the analyzer at the levels under consideration. Furfural would not be expected in the hydrolysate residue in any event because it would have been removed

TABLE II

BEHAVIOR OF SOME URONIC ACIDS AND SELECTED OTHER MATERIAL ON AN AUTOMATIC AMINO ACID ANALYZER AFTER TREATMENT WITH 6 *N* HYDROCHLORIC ACID

Elution temperature 52.5° except where noted; treated with 6 *N* HCl, 20–22 h at 110° in nitrogen-purged, evacuated ampules.

Compound	*Sample size (μmoles)*	*Elution volume (ml)*	$(H \times W)_{440}$	*440:570 mμ absorption ratio*
Galacturonic acid	25	26.5	2.78	4.63
2 days after hydrolysis		37.5	12.17	2.87
Galacturonic acid	25	27	4.57	4.86
6 days after hydrolysis		37.5	12.18	2.95
Galacturonic acid	25	27	16.93	4.05
5 months after hydrolysis				
Glucuronic acid	25	27	1.01	4.69
1 day after hydrolysis		38	11.69	3.04
Pectin	5mg	26.5	2.15	3.26
1 day after hydrolysis		37.5	11.88	2.83
Galacturonic acid	25	27	1.81	6.03
+ 1 mg α-chymotrypsin		37.5	12.86	2.77
Glucuronic acid	25	27	2.38	4.41
+ 1 mg α-chymotrypsin		38	10.6	2.86
Galactose	30	29.5[a]	11.5	1.13
		37	3.84	2.95
Glucose	30	30.0[a]	17.3	1.09
		37.5	1.70	2.83
Galacturonic acid[b]	25	27	17.4	3.75
Plant material[b]	15 mg	26.5	2.70	4.09
21-h hydrolysis		32[a]	12.1	1.11
		40	6.37	2.88
Plant material[b]	15 mg	27.5	3.25	4.17
40-h hydrolysis		32.5[a]	11.1	1.12
		40	6.35	2.94
Plant material[b]	12.5 mg	27.5	6.94	4.36
70-h hydrolysis		33[a]	9.13	1.13
		41	1.24	2.17

[a] Response from levulinic acid.
[b] Elution temperature 33°.

with the HCl and water. The formation of 5-carboxy-2-furfural by acid treatment of galacturonic acid has been reported[10,11]. The compound prepared according to the procedure of STUTZ AND DEUEL[11] gave the characteristic color of the carbazole test

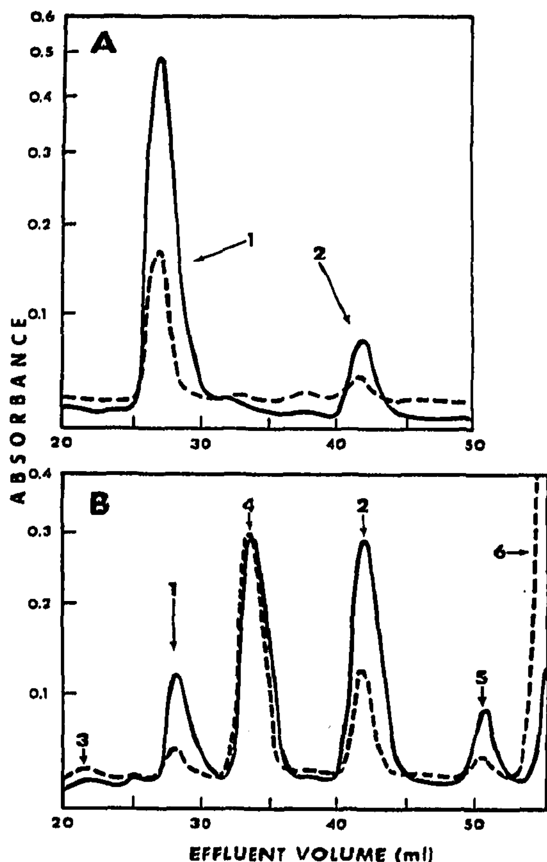

Fig. 1. Chromatogram of (A) acid-treated galacturonic acid (25 μmoles) and (B) hydrolysate from 16.6 mg of plant material containing a large amount of galacturonic acid. See text for details. Peaks 1 and 2 from galacturonic acid, 3 from cysteic acid, 4 from levulinic acid, 5 from hydroxyproline, and 6 from aspartic acid. (———) 440 mμ; (— — —) 570 mμ.

for hexuronic acids. The compound failed to give any response with ninhydrin at levels comparable to those applied to the amino acid analyzer in the above study. The identity of the compound(s) from galacturonic acid responsible for the production of color with ninhydrin is not known.

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