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A MODIFIED URONIC ACID–INDOLE REACTION*

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ABSTRACT

Modification of the conditions of the reaction of carbohydrates with indole in acidic medium made this reaction specific for uronic acids. A preliminary heating at 100° for 30 min in 3M hydrochloric acid eliminated the interference of DNA and other 2-deoxyribose derivatives. The reaction was positive for a concentration above 17 nmol/mL of final solution, and the Lambert and Beer law was satisfied up to 86 nmol/mL. The spectrum of the reaction is identical to that obtained with DNA, with a maximum at 492 nm and a shoulder at 465 nm. D-Galacturonic acid gave the highest value, while D-mannuronic acid gave 50%, and D-glucuronic acid only 14% of the value given by D-galacturonic acid. The reaction mechanism may be explained by dehydration of the uronic acid in 3M hydrochloric acid at 100°, condensation of the dehydrated product with indole in the cold, and color formation after a second heating at 100°.

INTRODUCTION

The discrepant results obtained by Baltus *et al.*[1] and by Dawid[2] for the localization of cytoplasmic DNA in amphibian oocytes and by us[3] for the same material prompted us to study, in a systematic way, the Ceriotti reaction[4] for the colorimetric determination of 2-deoxyribose (2-deoxy-D-*erythro*-pentose) of DNA and the possible interferences. At first, the possibility that DNA from yolk platelets is contaminated by glycosaminoglycuronans present in the outer coat of those structures was considered[5]. During systematic research on nucleotides and sugar nucleotides from oocytes and embryos in the early development of *Bufo arenarum*, the presence of UDP-glucuronic and UDP-galacturonic acids was always found[6–9]. This may indicate that both uronic acids are present in the glycosaminoglycuronans from yolk platelets and interfere in the Ceriotti reaction for DNA. We found that D-galacturonic

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acid, and to a lower extent, D-glucuronic and D-mannuronic acids react with indole in an acidic medium and form a colored product having an absorption spectrum identical with that produced by DNA.

The present study has established the optimal conditions of the reaction, and the interference of 2-deoxyribose from DNA and similar compounds was eliminated by a previous heating in the presence of 3M hydrochloric acid before color development. The specificity for uronic acids and stability of the color were also determined.

EXPERIMENTAL

Materials and methods. — D-Glucuronic acid and D-galacturonic acid (puriss.) were purchased from Fluka A.G., Chemical Fabrik, Buchs S.G. (Switzerland). D-Mannuronic acid was kindly donated by Dr. Marcelo Danker (Instituto de Investigaciones Bioquímicas, Fundación Campomar, Buenos Aires, Argentina). Pentoses, hexoses, and all the other sugar derivates tested were of analytical grade. Indole (m.p. 52–53°, puriss.) was obtained from Fluka A.G. Chloroform and hydrochloric acid were analytical reagents.

6M Hydrochloric acid. — The concentration was verified by titration with a standard solution of M sodium hydroxide. The use of concentrated hydrochloric acid was avoided because of changes of the concentration with time.

10mM Indole. — A solution was prepared in warm, distilled water and the concentration verified by measuring the absorbance at 225 nm ($E^{M}_{1\,cm}$ 25 000). The solution was stable at 4°, at least for one month.

Indole–hydrochloric acid. — The reagent was prepared immediately before use. 6M Hydrochloric acid (1 vol.), 10mM indole (0.9 vol.), and distilled water (0.1 vol.) were mixed; 2 mL of this mixture was added to 1 mL of the uronic acid solution in 3M hydrochloric acid in order to obtain a final concentration of 3M hydrochloric acid and 3mM indole.

Ceriotti reaction. — The sample (0.75 mL) containing D-glucuronic acid (0.5–2.0 μmol). D-galacturonic acid (0.1–0.5 μmol), or D-mannuronic acid (0.2–0.6 μmol) was mixed with the same volume of 0.04% indole (0.85mM final), and with 6M hydrochloric acid (1.5 mL; 3M final). The samples were heated in a boiling-water bath for 10 min. The tubes were cooled under running water, and the solutions were extracted three times with chloroform (1 mL each) and centrifuged to give a completely clear water-phase. Absorbance of the water phase was read at 490 nm against a control containing only the reagents and which had been treated in the same way.

Modified reaction. — The uronic acid solution in 3M hydrochloric acid (1 mL) was heated for 30 min in a boiling-water bath. After the reaction mixture had been cooled to room temperature, the indole reagent (2 mL) was added. Color development was obtained after a 30-min additional heating in a boiling-water bath. Three extractions with chloroform (1 mL each) were performed in order to eliminate the pinkish color formed. After centrifugation, the absorbances were read at 490 nm against a control having only the reagents and which had been treated in an identical manner.

RESULTS AND DISCUSSION

Uronic acids and Ceriotti reaction. — The uronic acid solutions were treated with indole–hydrochloric acid under the conditions described by Ceriotti[4]. All solutions gave a positive reaction, and the absorption spectrum of the yellow-brownish product obtained was identical with that produced by 2-deoxyribose of DNA, with a maximum

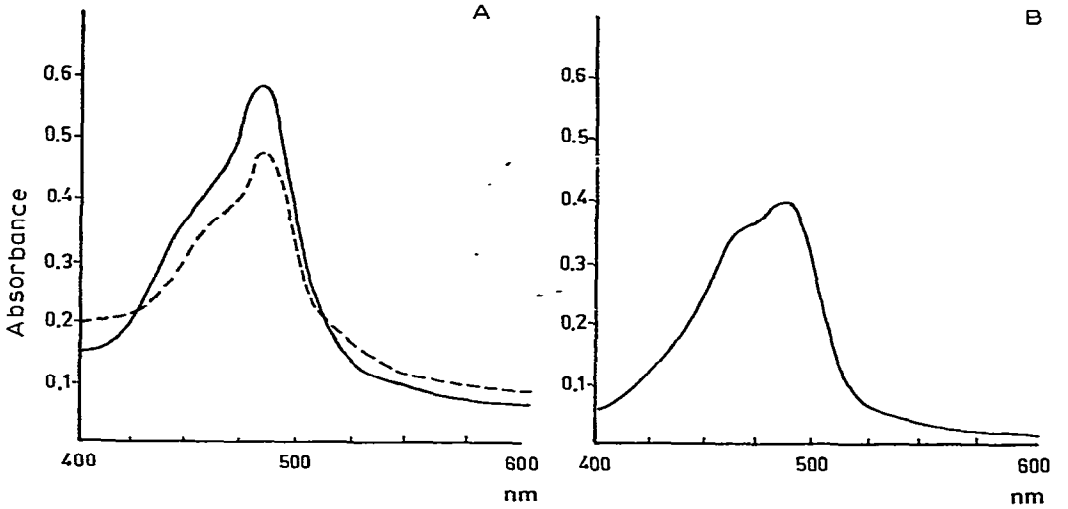

Fig. 1. Absorption spectra of the colored products formed in the reaction between uronic acids and 0.85mM indole in the presence of 3M hydrochloric acid for 10 min at 100°: A, (—) D-galacturonic acid (0.4 μmol) and (---) D-mannuronic acid (0.6 μmol); B, (—) D-glucuronic acid (2.5 μmol).

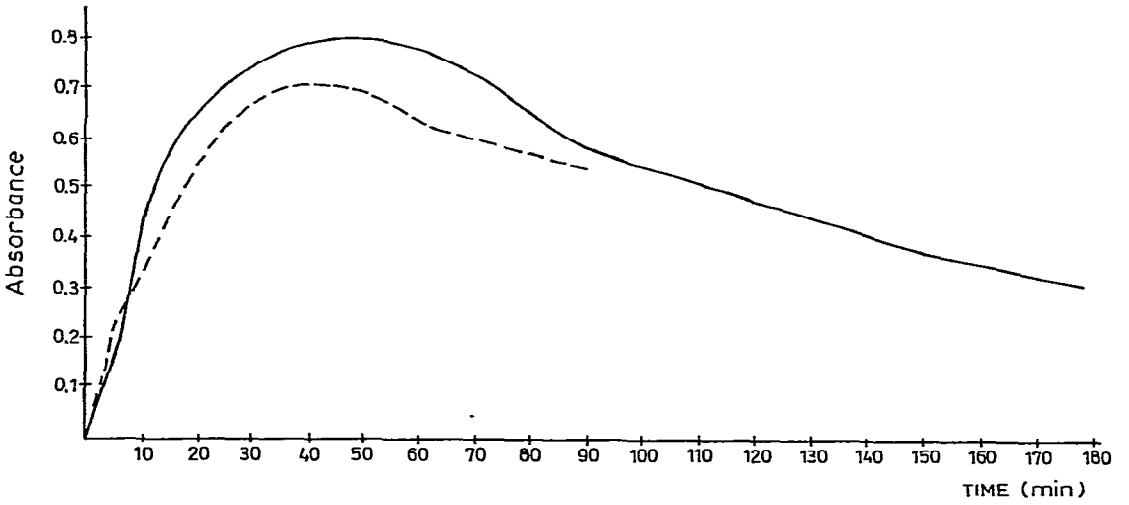

Fig. 2. Time course of the reaction of uronic acids with indole–hydrochloric acid. Other conditions as given in legend to Fig. 1: (—) D-galacturonic acid (0.3 μmol) and (---) D-mannuronic acid (0.5 μmol).

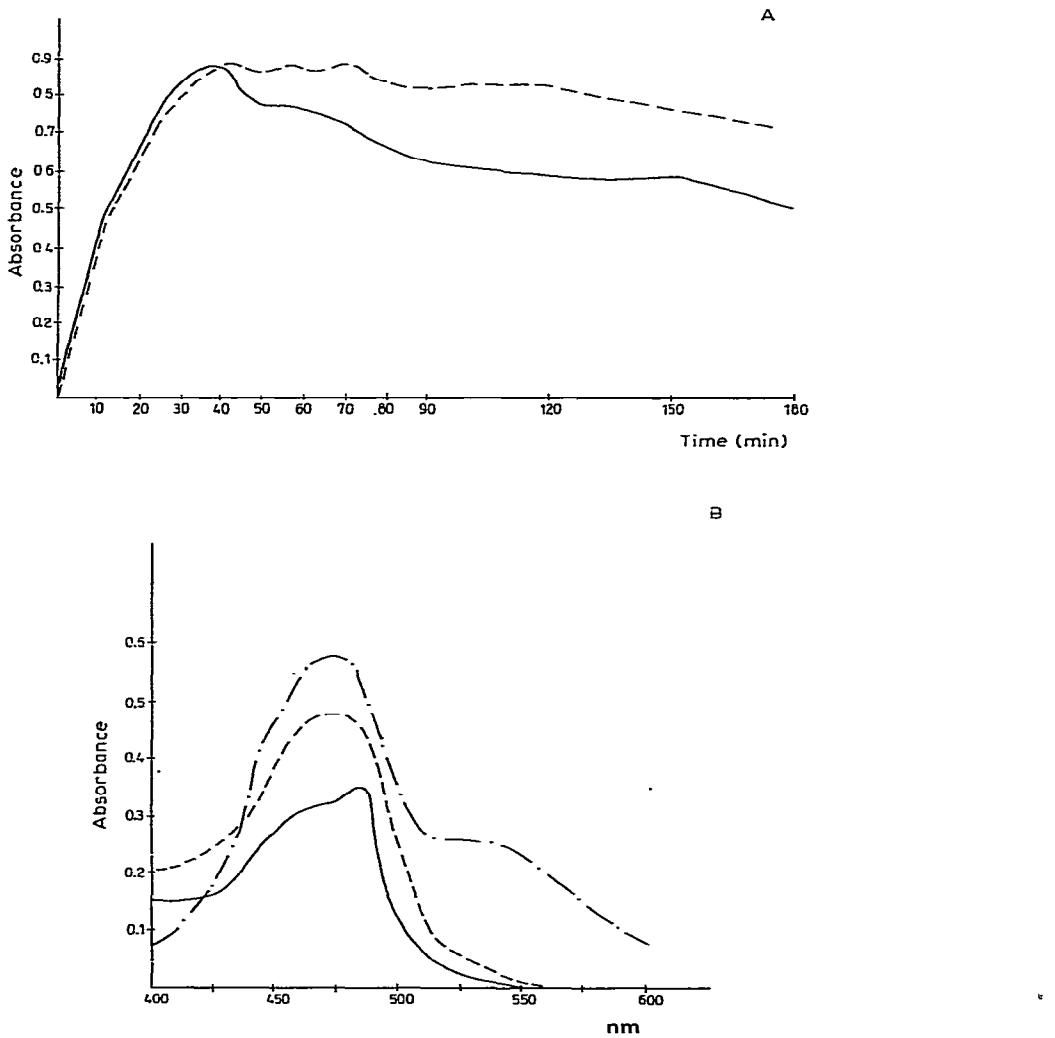

Fig. 3. Time course of the reaction of D-glucuronic acid with indole–hydrochloric acid. Other conditions as given in legend to Fig. 1: A, comparison between A_{490} (—) and A_{460} (– – –); B, absorption spectra at various times: (—) 10, (– – –) 30, and (–·–·–) 180 min.

absorbance at 490 nm and a small but constant shoulder at 460 nm. However, in the case of D-glucuronic acid, the ratio of the absorbance at 490 to that at 460 nm was lower than those given by the other compounds (see Fig. 1A and B).

Heating time and color development. — The uronic acid solutions were heated in a boiling-water bath for different times, under the conditions described by Ceriotti[4]. The results are illustrated in Figs. 2 and 3. For D-galacturonic and D-mannuronic acid (Fig. 2), a maximum was reached after 40–50 min of heating. For both compounds, the spectra of the colored products did not change during the range of times studied. For D-glucuronic acid, the optimum heating-time was 35 min (Fig. 3A). The absorp-

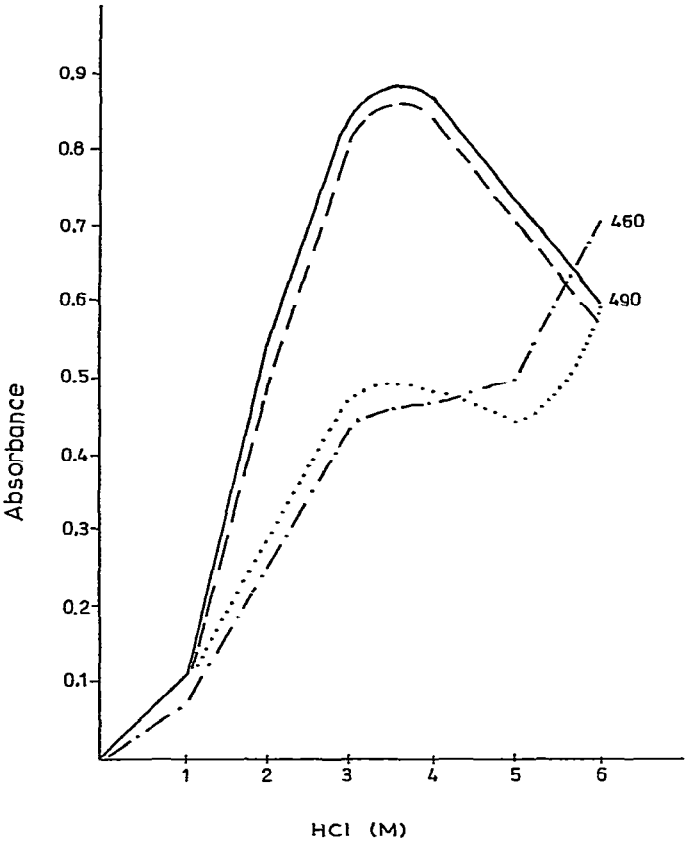

Fig. 4. Concentration of hydrochloric acid and color development for an indole concentration of 0.85mM and the optimum heating time illustrated in Figs. 2 and 3: (—) D-galacturonic acid (0.3 μmol), (– – –) D-mannuronic acid (0.6 μmol), (–·–·–) D-glucuronic acid (2.5 μmol) at 460 nm, and (·····) D-glucuronic acid (2.5 μmol) at 490 nm.

tion spectrum of the colored products formed in the reaction changed during the heating time, with a slow increase of the shoulder at 460 nm until it predominated over the 490-nm peak (Fig. 3B). This is better illustrated by Fig. 3A, which shows the time pattern for the absorbances at 460 and 490 nm. The two curves cross each other at 40 min.

Hydrochloric acid concentration and color development. — The uronic acid solutions were heated in a boiling-water bath, in the presence of 0.85mM indole, for the optimum time determined earlier, but increasing amounts of hydrochloric acid were introduced. The maximum color formation was always reached with 3–4M hydrochloric acid (Fig. 4). Again, for D-glucuronic acid, the curves for absorbances at 490 and 460 nm cross each other, in this case when the concentration of hydrochloric acid exceeded 4M. The spectra of the colored products were also deformed at a concentration of hydrochloric acid greater than 4M; consequently, a concentration of 3M was selected as the optimum.

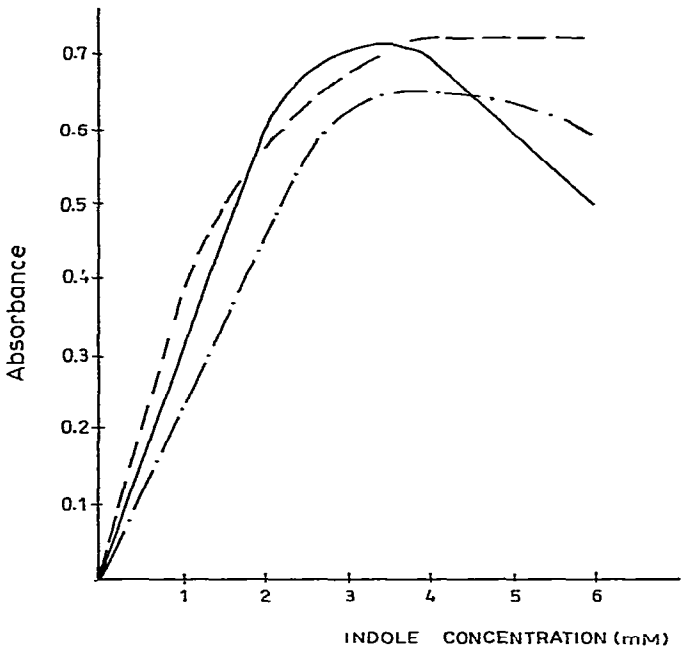

Fig. 5. Indole concentration and color development for a heating time and a hydrochloric acid concentration as illustrated in Figs. 2, 3, and 4: (—) D-galacturonic acid (0.3 μmol), (– – –) D-mannuronic acid (0.6 μmol), and (–·–·–) D-glucuronic acid (2.5 μmol).

Indole concentration and color development. — The uronic acid solutions were heated in a boiling-water bath during 40 min with 3M hydrochloric acid in the presence of increasing amounts of indole. In all cases, the maximum color formation was reached with indole concentrations of 3–4mM (Fig. 5). The absorption spectra of the colored products formed did not change appreciably over the range of indole concentrations tested.

Interferences. — In order to eliminate the interference from 2-deoxyribose of DNA and similar compounds, and taking into account the extreme lability of 2-deoxy sugars, a preliminary heating with 3M hydrochloric acid before the addition of indole was tested. As Fig. 6A shows, a heating time of 30 min was necessary to decrease to nearly zero the production of color by 77.4 μg of DNA. For deoxyadenosine and deoxyguanosine (Fig. 6A), 2-deoxy-D-glucose, and 2-deoxy-D-galactose (Fig. 6B), only 10 min was necessary. Under the same conditions, the color formation for uronic acids was decreased by only 5% (Fig. 6C).

Selection of indole concentration after a preliminary heating for 30 min in 3M hydrochloric acid. — D-Galacturonic acid solutions in 3M hydrochloric acid were heated for 30 min in a boiling-water bath. After the solution had been cooled, color was developed by heating for between 10 and 60 min in the presence of 3M hydrochloric acid and increasing amounts of indole ranging from 1.25 to 6mM (Fig. 7). With 1.25mM

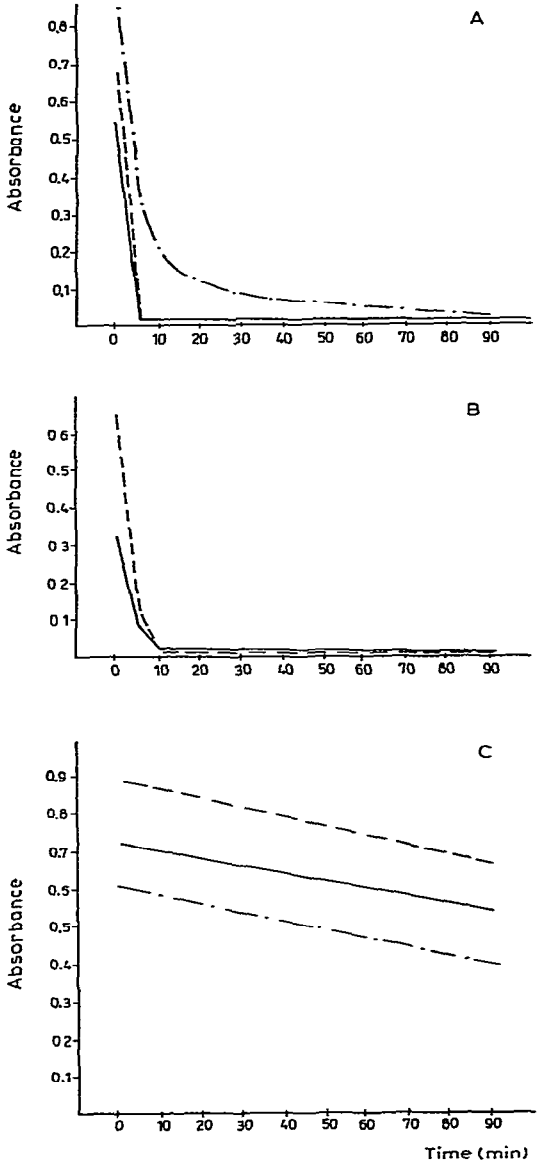

Fig. 6. Effect of a preliminary heating in the presence of 3M hydrochloric acid on the reaction of uronic acids and other compounds with indole–hydrochloric acid. The heating time, and hydrochloric acid and indole concentrations are as illustrated in Figs. 2, 3, 4, and 5: A, (–·–·–) DNA (77.4 μg), (– –) deoxyadenosine (0.2 μmol), and (—) deoxyguanosine (0.2 μmol); B, (—) 2-deoxy-D-*arabino*-hexose (0.2 μmol) and (– – –) 2-deoxy-D-*lyxo*-hexose (0.4 μmol); and C, (– – –) D-galacturonic acid (0.2 μmol), (—) D-mannuronic acid (0.4 μmol), and (–·–·–) D-glucuronic acid (2 μmol).

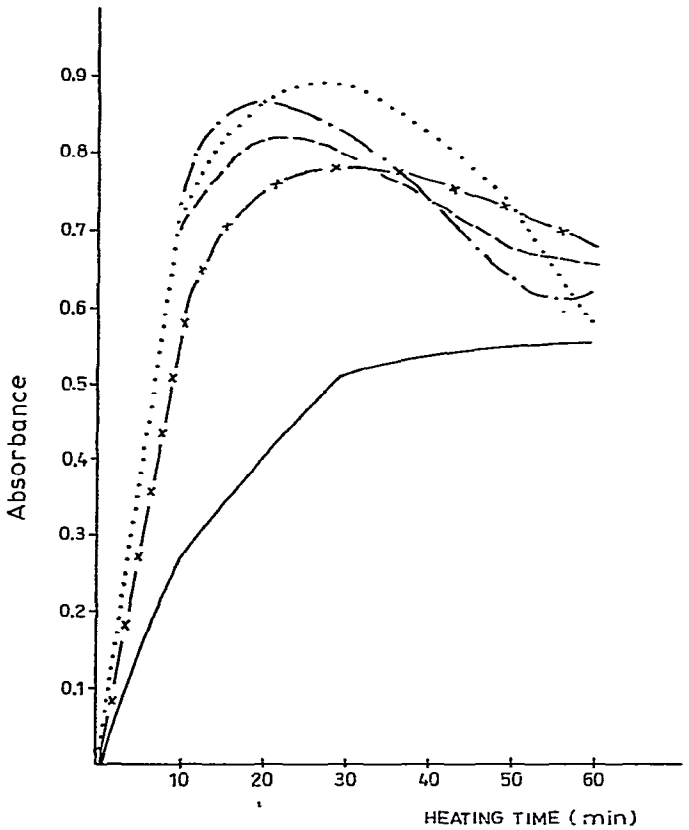

Fig. 7. Optimum heating time and indole concentration after a heating time of 30 min in the presence of 3M hydrochloric acid: (—) 1.25mM, (—×—) 3.00mM, (– – –) 4.00mM, (–·–·–) 5.00mM, and (·····) 6.00mM indole.

indole, a plateau was reached after 40 min. Optimal heating-time was found to be 30 min for 3mM indole, and 20 min for higher concentrations.

Sensitivity. — A standard calibration curve for D-galacturonic acid was established by use of the optimal conditions selected in the previous experiments: 3M hydrochloric acid, 3mM indole, and 30-min heating time. In each case, the uronic acid sample was heated for 30 min at 100° in 3M hydrochloric acid before the addition of indole. The results are shown in Fig. 8, which indicates that the extent of reaction is proportional to concentration between 0.015 and 0.08 μmol/mL of final solution for D-galacturonic acid.

Specificity. — Pentoses, hexoses, 6-deoxyhexoses, 2-deoxyhexoses, and several other sugar derivates were tested under the conditions established for uronic acids (Table I). Pentoses and hexoses gave a color reaction the intensity of which ranged between 0.95% for D-glucose and 17% for D- and L-arabinose of that given by D-galacturonic acid. Ketoses gave values near to zero; they developed a very strong, reddish color which was extracted by chloroform.

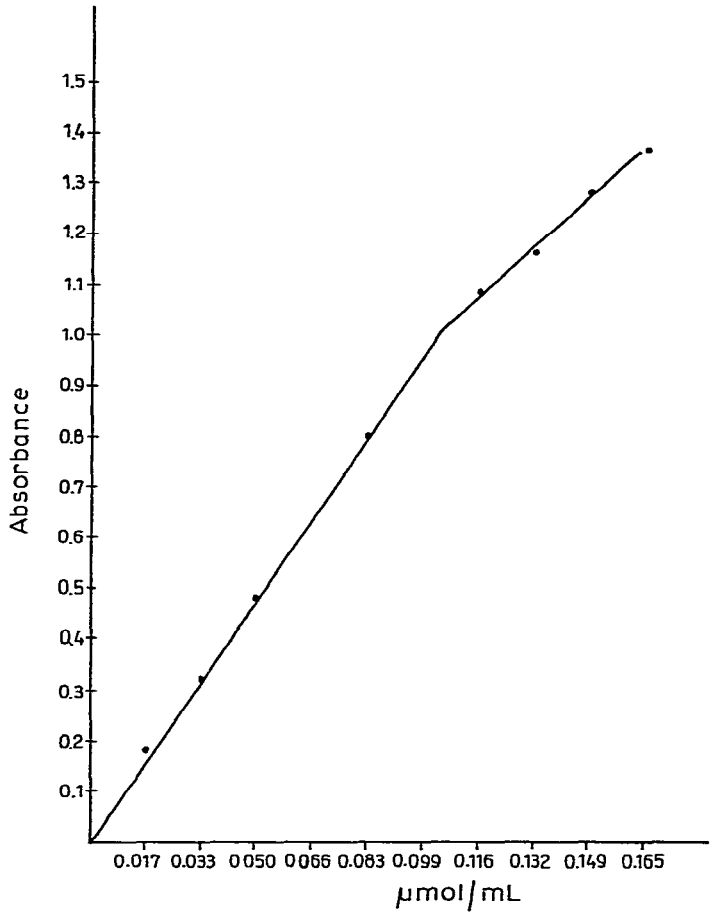

Fig. 8. Typical standard calibration curve for D-galacturonic acid determined by the modified reaction (see section on Methods).

Even at a concentration of 10 μmol, hexosamines and *N*-acetylhexosamines gave no color with indole–hydrochloric acid under the conditions optimal for uronic acids. As mentioned earlier, the interference of 2-deoxy sugars was eliminated by the preliminary heating with 3M hydrochloric acid.

Stability. — The color developed in the reaction of uronic acids with indole–hydrochloric acid is very stable. Readings at 490 nm did not change after the solution had been kept for 24 h at room temperature, and decreased by only 10% after 64 h. The absorption spectra remained unchanged, even after 64 h.

Reaction mechanism. — On treatment with 3.29M hydrochloric acid, D-galacturonic acid undergoes decarboxylation, ~1 mol of carbon dioxide being produced per mol of acid[10]. 2-Furaldehyde and reductic acid are the main products of the reaction[11]. This suggests three steps for the mechanism of the reaction of uronic acids with indole in acid medium: (*a*) In order to react with indole, uronic acids must be

TABLE I

SPECIFICITY OF THE REACTION WITH INDOLE–HYDROCHLORIC ACID AFTER A PRELIMINARY HEATING IN 3M HYDROCHLORIC ACID FOR 30 MIN AT 100°

Compound	*Concentration (μmol)*[a]	*Color produced (%)*[b]
D-Galacturonic acid	0.2	100.00
D-Mannuronic acid	0.4	50.00
D-Glucuronic acid	2.5	14.00
D-Ribose	2.0	2.80
D-Arabinose	1.0	17.00
L-Arabinose	1.0	17.00
D-Xylose	2.0	6.80
D-Lyxose	1.0	12.50
Deoxyadenosine	1.0	0.00
Deoxyguanosine	1.0	0.00
Deoxyribonucleic acid	0.22[c]	0.00
Deoxyribonucleic acid	1.0[d]	7.00
D-Glucose	10.0	0.95
D-Galactose	2.0	9.00
D-Mannose	2.0	8.50
D-Fructose	5.0	0.00
L-Sorbose	5.0	0.00
D-Tagatose	5.0	0.00
2-Deoxy-D-*arabino*-hexose	2.5	0.00
2-Deoxy-D-*lyxo*-hexose	2.5	0.00
L-Fucose	2.0	5.50
L-Rhamnose	2.0	5.50
2-Amino-2-deoxy-D-glucose	10.0	0.00
2-Amino-2-deoxy-D-galactose	10.0	0.00
2-Acetamido-2-deoxy-D-glucose	10.0	0.00
2-Acetamido-2-deoxy-D-galactose	10.0	0.00
2-Acetamido-2-deoxy-D-mannose	10.0	0.00
D-Gluconic acid	1.0	0.00
D-Galactonic acid	1.0	0.00
2-Amino-2-deoxy-D-gluconic acid	1.0	0.00
L-Ascorbic acid	1.0	0.00
N-Acetylneuraminic acid	1.0	0.00
Mucic acid	1.0	0.00
Ribitol	10.0	0.00
Galactitol	10.0	0.00
4-Oxopentanoic acid	10.0	0.00

[a]Concentration of sugar (μmol) in 3 mL of final solution. [b]Percent relative to the color produced by D-galacturonic acid (100%). [c]72.5 μg. [d]362.5 μg.

first dehydrated. As Fig. 9 shows (upper curve), the spectrum of indole did not change when D-galacturonic acid was added in the cold to a solution of 0.2mM indole in 3M hydrochloric acid. When the uronic acid solution had been previously heated for 5 or 10 min at 100° in 3M hydrochloric acid, the spectrum typical of indole disappeared after the addition (Fig. 9, middle and lower curve). (*b*) Disappearance of the spectrum

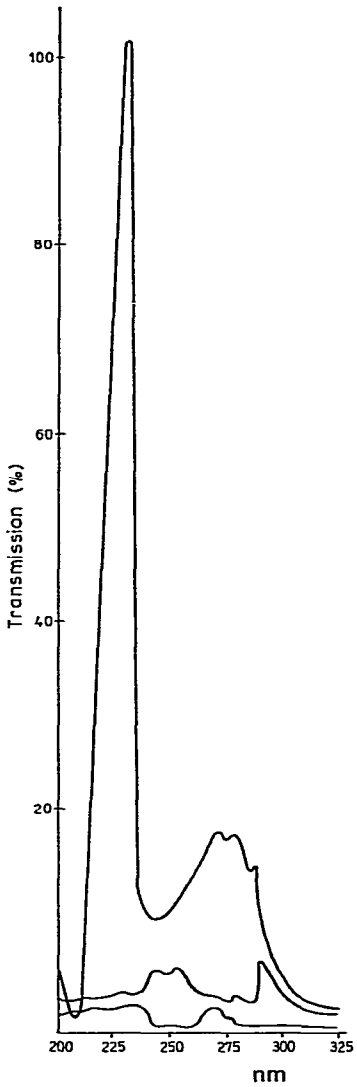

Fig. 9. D-Galacturonic acid and its reaction with indole. Upper curve: u.v. absorption spectrum of a 0.3mM indole solution in 3M hydrochloric acid with and without addition of D-galacturonic acid in the cold. Middle curve: u.v. absorption spectrum of an 1.25mM indole solution in 3M hydrochloric acid after the addition of 0.3 μmol of D-galacturonic acid in 3M hydrochloric acid previously heated for 5 min at 100°. Lower curve: same conditions as given for middle curve but with a heating time of 10 min at 100°.

for indole after addition of indole in the cold to a solution of uronic acid previously heated suggests that, in a second step, condensation of indole with the dehydrated sugar occurs. (*c*) Finally, a second heating at 100° is necessary to develop the color characteristic of the reaction.

Comparison with other methods. — As compared with the classical carbazol

method for uronic acids[12,13], the reaction with indole–hydrochloric acid abolishes completely the interference from D-glucose (Table I). In addition, the stability of the color is much higher. As compared with the method of Blumenkrantz and Asboe-Hansen[14], which uses 3,3′-biphenyldiol for the reaction with uronic acids, our method is a little less sensitive, but D-glucuronic acid gave only 14% of the color produced by D-galacturonic acid. D-Mannuronic acid, the other uronic acid tested, gave 50% of the color produced by D-galacturonic acid. The method with 3,3′-biphenyldiol gave the same sensitivity for D-glucuronic and D-galacturonic acids. A combination of both methods would allow the determination of galacturonic and glucuronic acids in biological fluids.

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