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THE USE OF TRIPHENYLTETRAZOLIUM CHLORIDE FOR THE QUANTITATIVE ANALYSIS OF SUGARS AND SUGAR DERIVATIVES REPORTED IN GLYCOPROTEINS

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SUMMARY

Three different types of sugars, reported to be present in the carbohydrate moiety of glycoproteins, were estimated on paper chromatograms using 2,3,5-triphenyltetrazolium chloride. The use of steam for the visualization of the sugars on the chromatograms diminished the reaction time from the usual 15 min to 2 min with equal sensitivity and better reproducibility.

INTRODUCTION

In the characterization of glycoproteins the determination of the sugar components has always been a problem[1]. In glycoproteins many different types of sugars were found, ranging from the acidic uronic acids to the basic amino sugars[2,3]. For the quantitative analysis of these sugar components three interrelated factors must be considered: separation, sensitivity and accuracy. All three factors may be satisfied for any given group of chemically related sugars by various chromatographic procedures such as gas–liquid, thin-layer and paper chromatography[4–6]. Uronic acids, neutral sugars and amino sugars are separated by paper chromatography in a single run and without having to form derivatives[7,8].

Of the many reagents available for the detection of reducing sugars on paper chromatograms, 2,3,5-triphenyltetrazolium chloride (TTC) proved to be sensitive at the 1–2 μg level for many sugars reported to be present in glycoproteins[9]. This reagent was adapted for colorimetry by others[10–12] for the estimation of carbohydrate components in polysaccharides and glycoproteins. This reaction was non-stoichiometric according to FAIRBRIDGE *et al.*[13] and conditions of colour development had to be strictly adhered to for reproducible results. The rate and extent of the reduction of TTC to the red-coloured formazan varied with temperature, humidity, reaction time and to a lesser extent alkalinity[14,15].

The present paper introduces the use of steam for the visualization of sugar spots on chromatograms treated with TTC. Using a predetermined steam flow, variations in reaction time and in concentration of sodium hydroxide were investigated in order to establish optimum working conditions. The result was a more rapid and sensitive method than hereto reported.

EXPERIMENTAL

All chemicals were analytical reagent grade and were used without further purification. Sheets of 41.5 × 19 cm Whatman No. 1 paper were used for chromatography with the origin 7 cm from the short side of the paper. Solutions which contained 20 × 10^{-9} moles/μl were made up in distilled water for each of the following sugars: D-glucuronic acid, D(+)-galactosamine·HCl, D(+)-glucosamine·HCl, D(+)-galactose, D(+)-mannose and D(—)-ribose.

Two series of experiments, A and B, were employed for different purposes: series A for studying the effect of sodium hydroxide concentration and reaction time, series B for quantification of a synthetic mixture. In series A three spots were placed 4 cm apart and in the order given in Table I. In series B a fourth spot was added which contained quantities of sugar and sugar derivatives corresponding to those found in the composition of untreated skeletal muscle mucoprotein according to McINTOSH[16].

TABLE I

Compound	*Quantity applied (moles × 10^{-9})*	
	Series A	*Series B*
D-Glucuronic acid	80, 160, 240	80, 160, 240, 230
D(+)-Galactosamine·HCl	80, 160, 240	52
D(+)-Glucosamine·HCl		80, 160, 240, 138
D(+)-Galactose	80, 160, 240	20, 40, 80, 26
D(+)-Mannose		20, 40, 80, 14
D(—)-Ribose	80, 160, 240	40, 80, 160, 46

Descending chromatography was carried out in two solvent systems. Series A was chromatographed in *n*-butanol–pyridine–water (10:3:3) for 25 h at room temperature (approximately 25°). Subsequent work with a number of solvent systems showed that ethyl acetate–pyridine–water (12:5:4) for 16 h at 10° was better for quantification, because of superior separation. It was used for series B. After chromatography the papers were left to dry in the fumehood until only a faint solvent odour remained (approximately 4 h).

Prior to colour development water was drained from the steam line at maximum rate of steam flow. The steam chamber shown in Fig. 1 was placed on a pyrex tray. The following procedure was used to standardize the rate of steam flow entering the chamber. The flow of steam was measured directly from the line by leading the steam into a 300 mm Allihn condensor connected in series to a 600 mm Graham condensor. Satisfactory results were obtained with a flow of steam which gave 120 ml of condensed water per minute. The steam valve was marked accordingly.

To determine the effect of alkalinity and reaction time on colour development, the chromatograms of series A were dipped in fresh solutions of 0.5 % TTC in methanol, varying in NaOH concentrations from 0.05 to 0.75 *N*. Optimum conditions of alkalinity and reaction time, established with series A, were selected for subsequent work in series B. Papers were dipped in a 0.5% solution of TTC in 0.25 *N* NaOH in methanol and developed for 2 min in the steam chamber.

The wet papers were held between two paper clamps and hung with the origin up in the steam chamber. The upper opening of the chamber was covered with a stainless steel sheet and the chromatogram steamed for various lengths of time. After development the papers were immediately transferred to a chromatographic jar filled with tap water and washed for 1 h by gentle overflow. After washing the chromatograms were dried in a fumehood (approximately 3 h).

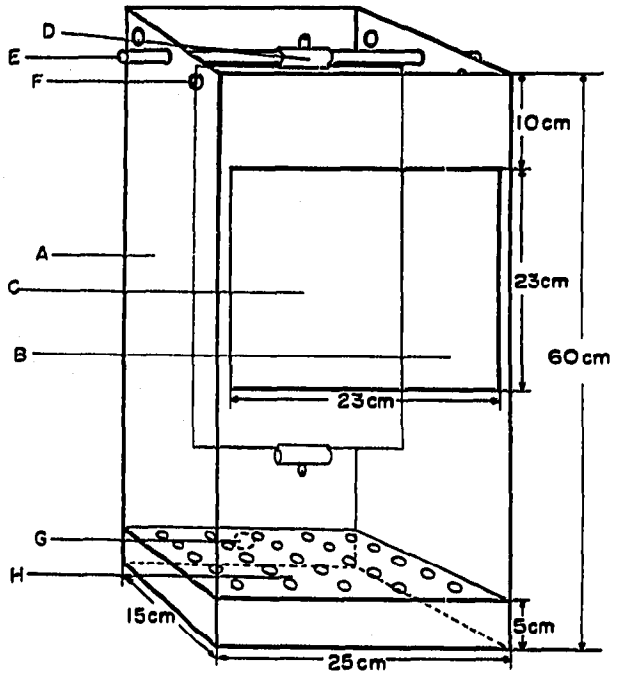


Fig. 1. Steam chamber for the colour development of sugar spots on paper chromatograms. A = stainless steel chamber; B = glass window; C = paper chromatogram; D = clamp; E = glass rod; F = steam outlet; G = steam inlet; H = perforated stainless steel plate.

To quantitate the amount of formazan dye formed by the reaction of sugars with TTC the following procedure was followed. A rectangle was drawn around the coloured spot of greatest area for each sugar or sugar derivative separated by paper chromatography and the same area was used for all other concentrations of the same sugar. Background colour was estimated by drawing the same area adjacent to the series of coloured spots of the same R_g value. The measuring of the areas was greatly facilitated by the use of a celluloid template with various sizes of rectangles. The rectangular areas were cut from the paper and further reduced in size to fit into 25 ml test tubes.

The formazan was extracted in 10 ml of methanol–acetic acid (8:1, v/v) for 24 h with vortex agitation at the beginning and the end of this period. The optical densities of the extracts were read at 482 mμ in a spectrophotometer. The optical density readings were corrected for background colour. The corrected optical densities obtained in series A were plotted *versus* time.

The following design was used in series B to assess the quantitative aspects of the TTC method. Of the four sugar concentrations in Table I, the first three were used to establish a standard curve by plotting optical density *versus* moles of sugar applied. From the optical density obtained with the formazan eluted from the fourth spot, a

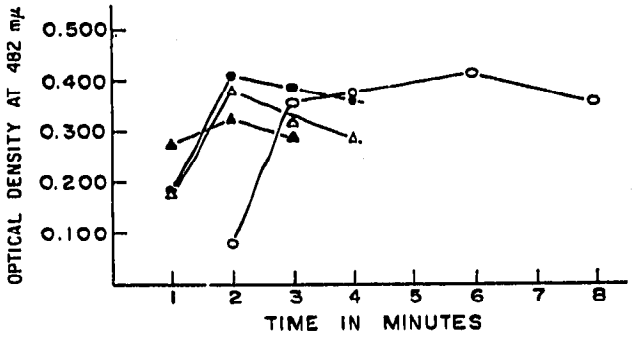
quantitative value was interpolated from the standard curves for comparison with that quantity actually spotted.

For the determination of the stoichiometric relationship of the reaction, formazan crystals were prepared according to FAIRBRIDGE *et al.*[13]. Its molecular weight, melting point and molar extinction coefficient were checked against those values reported in the literature[13,17,18].

RESULTS AND DISCUSSION

The effect of reaction time and alkalinity of the TTC solutions on colour development for D-glucuronic acid is shown in Fig. 2. Maximum colour development was reached at 2 min for 0.25 to 0.75 *N* NaOH solutions and at 6 min for 0.05 *N* NaOH. These maxima were within ±5% of their average value for the different alkaline strengths used, except in the case of 0.75 *N* NaOH, where a marked decrease in maximum optical density was observed. In quadruplicate determinations the variation in optical density value for any given point in Fig. 2 was not more than ±10%. D(+)-Galactosamine·HCl, D(+)-galactose and D(−)-ribose gave the same pattern of development as D-glucuronic acid. Maximum colour development with the least background interference was obtained with 0.25 *N* NaOH at a reaction time of 2 min; this reaction time and concentration of sodium hydroxide were therefore chosen for quantitative work, since its relatively low blank favoured detection of low concentrations of sugars or sugar derivatives.

The standard curves used for the quantification of individual sugars in series B are shown in Fig. 3. Each point was the mean of four measurements. The range of concentrations used for the different standard curves varied according to the antic-



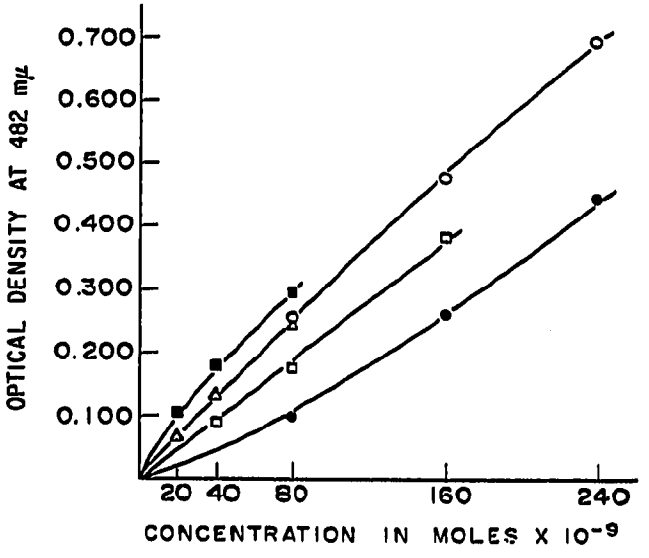


Fig. 2. The effect of alkalinity and reaction time on colour development for the reaction of D-glucuronic acid and TTC. ○—○, 0.05 *N* NaOH; ●—●, 0.25 *N* NaOH; △—△, 0.50 *N* NaOH; ▲—▲, 0.75 *N* NaOH.

Fig. 3. Standard curves for sugars in a synthetic mixture corresponding to that of skeletal mucoprotein[16]. ○—○, D-glucuronic acid; ●—●, D(+)-glucosamine·HCl; ■—■, D(+)-galactose; △—△, D(+)-mannose; □—□, D(−)-ribose.

ipated amounts of sugar present in a glycoprotein hydrolysate. Ribose gave a linear relationship between optical density and moles applied. For all other sugars and sugar derivatives a linear relationship was observed over only a limited range of concentrations and the deviation from linearity was greatest for those sugar spots closest to the origin. It was important to determine whether this effect was caused by non-uniform steam distribution or chromatographic conditions. Preliminary work on the design of the steam chamber showed that individual spots of the same concentration and R_g value differed in intensity of colour development unless the steam entered the chamber perpendicular to the surface of the chromatogram. To determine if there was a difference in colour development along the length of the paper the chromatogram was hung with the origin down. No detectable improvement in linearity of the calibration curves was noticed.

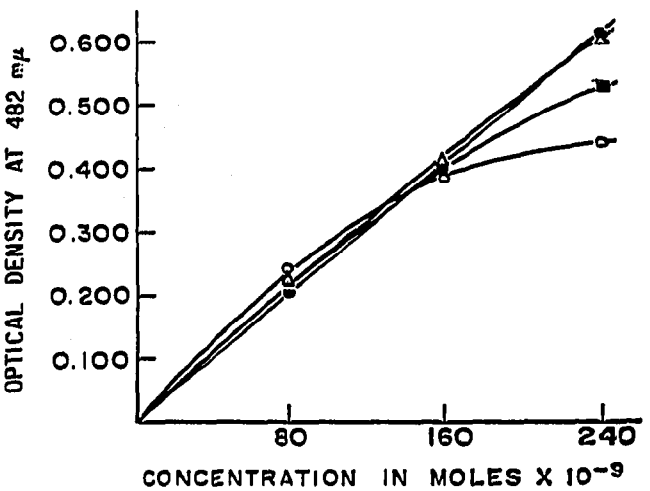


Fig. 4. The effect on standard curves of ribose with concentration and distance travelled on the chromatogram. ○—○, 28 mm; ■—■, 54 mm; △—△, 94 mm; ●—●, 139 mm.

The effect on optical density of sugar concentration and distance travelled on the paper was investigated (Fig. 4). Using ribose only, five chromatograms were prepared and run in the same manner as those of series A. When 80 to 160 $\times$ 10^{-9} moles of ribose were applied no change was observed in the optical density with distance travelled. At the highest level of ribose there was a marked effect on optical density with distance travelled. Apparently the deviation from linearity of ribose was related to the amount of sugar per unit area as affected by the direct relationship of diffusion with distance travelled. This effect was also observed when different chromatographic systems were used. The *n*-butanol–pyridine–water system gave more diffused spots than the ethyl acetate–pyridine–water system, which tended to concentrate the spots. The advantage of better resolution in the latter system was offset by the tendency to non-linearity of the Beer's law plot (Fig. 3) for sugars such as galactose and mannose at low levels of application, while those two sugars gave a linear relationship at higher levels in *n*-butanol–pyridine–water.

The formazan (m.p. 172°; $E_{1M}^{1cm} = 15,560$; mol. wt. = 300) prepared in our laboratory, when in solution, obeyed Beer's law. However, the reaction of sugars or sugar derivaties with TTC appeared to be non-stoichiometric as reported earlier by

FAIRBRIDGE *et al.*[13]. On the expectation that 1 mole of reducing sugar would yield 1 mole of formazan we found that under the conditions applied, most sugars gave approximately 1.5 times the expected value.

The precision and relative accuracy of the TTC method for quantification of the various sugars and sugar derivatives are given in Table II. The composition of the standard mixture in series B was chosen so that the greatest possible variation in quantity and type of sugar was encountered and yet had practical application[16]. Under these conditions the precision of the TTC method was well within 10% and the ratio of quantity of sugar applied to that found was close to 1.00.

TABLE II

PRECISION AND ACCURACY OF THE TTC METHOD FOR THE QUANTIFICATION OF SUGARS

Compound	*Composition of synthetic mixture*[a] *in series B in moles* $\times 10^{-9}$		
	Applied	*Found*[c]	*Found/applied*
D-Glucuronic acid	230	230 ± 3%	1.00
Hexosamines[b]	190	188 ± 2%	0.99
D(+)-Galactose	26	27 ± 9%	1.04
D(+)-Mannose	14	16 ± 9%	1.14
D(−)-Ribose	46	52 ± 3%	1.13

[a] Composition as found in skeletal muscle mucoprotein[16].
[b] Expressed as D(+)-glucosamine·HCl.
[c] Average of four determinations.

Because no paper chromatographic system tried in this laboratory would completely separate galactosamine and glucosamine in the presence of other sugars, these two amino sugars were expressed as hexosamines.

The loss of formazan during washing of the chromatograms was estimated by spotting equal volumes of different concentrations of the dye on Whatman No. 1 paper. The papers were steamed for 2 min and then washed in tap water for 1 h. The loss of dye was found to be negligible. The coloured solution of formazan in methanol–acetic acid was stable under artificial light in the laboratory for at least 48 h.

CONCLUSION

2,3,5-Triphenyltetrazolium chloride may be used for the quantitative estimation of uronic acids, neutral sugars and hexosamines on paper chromatograms under controlled conditions. The use of steam for colour development on chromatograms gave distinct advantages such as shorter reaction time, improved sensitivity, and greater reproducibility.

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