would be compatible with those of KAYE for the ITP also since no Zn-ITP complex is observed and it could not compete for the zinc present thereby releasing the ATP, nor is ITP configurationally satisfactory, with or without Zn ion, to substitute directly for the ATP.

ACKNOWLEDGEMENT

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AN IMPROVED METHOD FOR THE DETECTION OF N-ACETYLAMINO SUGARS ON PAPER CHROMATOGRAMS

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SUMMARY

The detection of N-acetylamino sugars on paper chromatograms has been made more sensitive by the use of an ethanol–borate spray, followed by steaming and spraying with a modified DMAB spray. As little as 1 μg of N-acetylglucosamine can be detected on paper. By extracting the coloured complexes from the paper, the method can be adapted for quantitative studies. The method was applied to the detection of acetylamino sugar compounds in partial acid hydrolysates and lysozyme digests of bacterial cell walls.

INTRODUCTION

PARTRIDGE[1] demonstrated that the reagents used in the MORGAN AND ELSON[2] reaction for N-acetylamino sugars could be adapted for the detection of these compounds on paper chromatograms. The stability and yield of the coloured complex formed under the conditions of the N-acetylamino sugar reactions is susceptible to such factors as the duration of heating with alkali, the presence of salts, the concentration of hydrochloric acid in the *p*-dimethylamino benzaldehyde reagent[2–4]. It is therefore not

References p. 312.

surprising that the reaction on paper has not always been reliable. The colourimetric methods for determining N-acetylamino sugars have been modified in recent years[3,4] and advantage of these improvements has been taken in the development of a more reliable and sensitive method for detecting these compounds on paper chromatograms. The method has been successfully applied to the detection of N-acetylamino sugar substances in partial acid hydrolysates and lysozyme digests of bacterial cell walls.

METHODS

Paper chromatographic procedures and spray reagents

N-acetylglucosamine (Light and Co. and Nutritional Biochemicals Corporation preparations were used) solutions were applied to Whatman paper No. 1 and the papers were irrigated with a variety of solvent systems (pyridine–water, 4:1, v/v; methanol–water, 4:1, v/v; *n*-butanol–glacial acetic acid–water, 3:1:1 by volume). After evaporating the solvents, the following steps were used for the detection of the acetylamino sugars on the paper chromatograms:

1. Paper chromatograms were sprayed with an ethanol–borate mixture (95% ethanol–0.05 *M* $Na_2B_4O_7$, 1:1 by volume), care being taken to see that the papers were fully moistened, yet not flooded.

2. The papers were freely suspended from glass rods in a steam oven (bacteriological steamers proved most convenient for the purpose). The chromatograms were steamed for 10 min.

3. The steamed papers were then sprayed with a *p*-dimethylamino benzaldehyde (DMAB) reagent spray having the following composition: 10 ml of a 2% w/v solution of DMAB in glacial acetic acid + 30 ml *n*-butanol + 0.4 ml conc. HCl.

For most of the expts. reported below, both the ethanol–borate mixture and the DMAB spray reagent were made up freshly each day. However, satisfactory results were also obtained with reagents that had been stored at room temperature for one week.

Development and extraction of the N-acetylamino sugar coloured complex

Before the optimum conditions for the formation of the coloured complexes could be determined, a suitable solvent system had to be developed for the extraction of these complexes from the paper chromatograms. The coloured products formed on reaction with the N-acetylamino sugar spray reagents described above were unstable in water, and mixtures of acetone or methanol or ethanol with acetic acid. Rapid extraction was achieved with a mixture containing ethyl acetate–glacial acetic acid–water, 3:1:1 by volume. Reproducible results were obtained when the coloured areas on chromatograms were extracted individually and the optical densities of the solutions immediately determined. The colour in the ethyl acetate–acetic acid–water mixture also faded but less rapidly than in some of the other solvents tried. However, the colour could also be extracted much more slowly in glacial acetic acid. Extraction in a closed tube with frequent shaking took about 1 h at room temperature. Once extracted the colour remained quite stable in the glacial acetic acid for several hours.

To determine the conditions for maximal colour development, the time course of the reaction on paper was followed at 20°, 35° and 97°. For this purpose, a solution of N-acetylglucosamine to give 250 μg acetylamino sugar on each chromatogram

References p. 312.

"spot", was applied to Whatman No. 1 paper and ascending chromatography was then performed in methanol–water 4:1, v/v. The paper chromatograms were sprayed according to the procedure outlined above. Colour development at the three temperatures was followed by cutting out duplicate "spots" at various intervals of time. From these papers, the colours were extracted individually with 4 ml ethyl acetate–glacial acetic acid–water mixture and the optical densities determined in a Model DU Beckman spectrophotometer at a wavelength of 585 mμ. The results are summarized in Fig. 1.

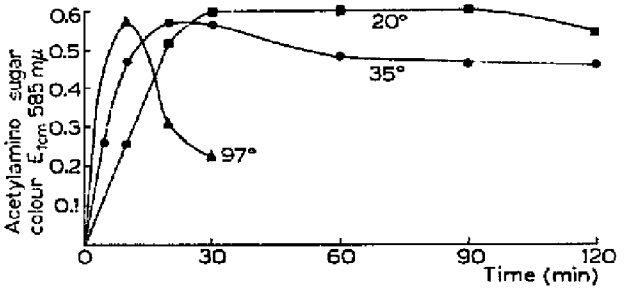

Fig. 1. The effect of temperature on the development of the N-acetylamino sugar colour reaction on paper chromatograms as determined by the spraying and extraction techniques described in the text.

When the colours formed on paper chromatograms at 20° and 35° were extracted and examined in the spectrophotometer, they were found to possess spectra identical to those obtained by reaction of N-acetylamino sugars in solution with the modified MORGAN AND ELSON reagents[3,4]. Heating at 97° for more than 10 min resulted in a blue-purple colour. The complex so formed was unstable in the extraction mixture and a residual blue-grey pigment could not be removed from the paper. Thus for quantitative estimation of N-acetylamino sugars from paper chromatograms, development of the colour should be carried out at temperatures below 50°.

Sensitivity of the method

With the procedure described in this paper, as little as 1 μg of N-acetylglucosamine or N-acetylgalactosamine can be detected on paper chromatograms and the purple spots given by these quantities of acetylamino sugars were visible for several days. 25 μg N-acetylglucosamine gave chromatogram "spots" of sufficient intensity to be visible for at least one week. For extraction from the paper, about 10 μg acetylamino sugar was required to give a suitable reading at 585 mμ. The coloured complex derived from 10 μg N-acetylglucosamine on extraction into 4 ml ethyl acetate–acetic acid–water mixture, gave an $E_{1\,cm}$ of approximately 0.1, at 585 mμ.

N-acetylneuraminic acid reacted very slowly on paper chromatograms, when treated under the conditions of the present method. However, 15 μg of synthetic N-acetylneuraminic acid (kindly provided by Dr. J. W. CORNFORTH) was readily detectable and the colour when formed, remained stable for several weeks.

Application of the method to the detection of acetylamino sugar compounds in partial acid hydrolysates and lysozyme digests of bacterial cell walls

Cell walls of *Micrococcus lysodeikticus* were hydrolysed with 12 *N* HCl for 6 days at 4° and for 1, 2 and 7 days at 37°; the HCl was removed *in vacuo* over H_2SO_4 in

References p. 312.

the presence of NaOH pellets. Lysozyme digests were prepared as previously described[5]. The hydrolysates and digests were then subjected to separation by the "fingerprinting" technique of combined paper electrophoresis and chromatography described by INGRAM[6]. The concentrated dialysable products of lysozyme digested walls and the acid hydrolysates were applied to Whatman No. 3MM paper after prior moistening with a volatile buffer of pyridine–glacial acetic acid–water, 10:0.4:90, by volume[7]. Electrophoresis was performed at pH 6.4 for 2 h at 14 V/cm and after drying the papers, ascending chromatography in *n*-butanol–glacial acetic acid–water, 3:1:1, by volume, was carried out overnight. The fingerprints were dried at room temperature, sprayed as described above and the colours allowed to develop at 35°. Duplicate chromatograms prepared in this way were sprayed with ninhydrin and ammoniacal silver nitrate. Only the N-acetylamino sugar compounds that were well separated from the free amino sugars or free amino acids were selected for further identification. For this purpose duplicate fingerprints were prepared and the areas corresponding to the purple "spots" on the sprayed fingerprints were eluted, hydrolysed for 16 h at 107° with 6 *N* HCl and examined for the constituent amino sugars and amino acids by two dimensional chromatography using pyridine–water, 4:1, v/v in the first direction and *n*-butanol–acetic acid–water, 6:1:2, v/v, in the second direction.

In all of the partial acid hydrolysates, N-acetylamino sugars, free amino sugars and free amino acids were detected. N-acetylglucosamine and a smaller amount of N-acetylmuramic acid were liberated by hydrolysis for 6 days at 4° and in addition an acidic compound of N-acetylglucosamine and N-acetylmuramic acid was also present. Hydrolysis for 1 and 2 days at 37° gave intense spots for both N-acetylglucosamine and N-acetylmuramic acid; the amounts of both acetylamino sugars decreased on hydrolysis for 7 days at 37° due to deacetylation. A number of N-acetylamino sugar-reacting substances possessing negative charges at pH 6.4 have been detected in the 2 day hydrolysates, but these have not yet been identified.

The dialysable fraction prepared from lysozyme-digested walls of *M. lysodeikticus* contained a substance composed of N-acetylglucosamine and N-acetylmuramic acid[5]. With the increased sensitivity of the present method, an additional compound possessing a strong acetylamino sugar reaction has now been detected. The compound was separated as a neutral substance at pH 6.4. On hydrolysis it yielded glucosamine, muramic acid, alanine, glycine, glutamic acid and lysine in the following approximate proportions: 3:1:2.4:1:1:1.4 (as determined by ninhydrin colour). If the ninhydrin colour gives a satisfactory measure of the ratios of amino sugars, then these results suggest that lysozyme can break the glycosidic bonds as close as 3 amino sugar units from the muramic acid–peptide part of the molecule. The results from enzymic digestion and partial acid hydrolysis show that repeating sequences of N-acetylglucosamine–N-acetylmuramic acid form important structural "backbone" or "bridge" units in the cell walls of *M. lysodeikticus*. Although the behaviour of the compound (disaccharide?) of N-acetylglucosamine–N-acetylmuramic acid on the fingerprints is fairly similar for both products obtained by partial acid hydrolysis and enzymic digestion, it is not known whether the substances are identical. PERKINS AND ROGERS[8] found a deacetylated compound of glucosamine and muramic acid under their conditions of hydrolysis.

The location of some of the N-acetylamino sugar substances on fingerprints of

References p. 312.

hydrolysates and lysozyme digests is illustrated in Fig. 2 and some of the products detected are listed in Table I.

The spray reagents used in this study have also been useful in detecting the N-acetylamino sugar residues of the non-dialysable components of the lysozyme-digested walls of *M. lysodeikticus*, when these substances have been separated by electrophoresis on glass fibre "paper".

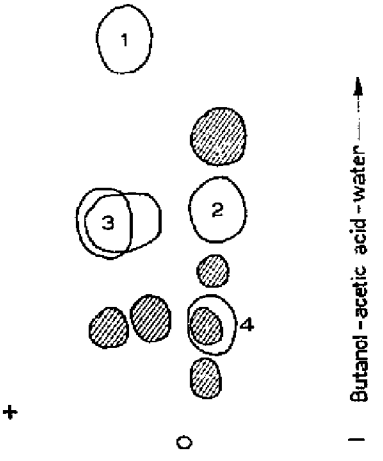

Fig. 2. The location of N-acetylamino sugar compounds on "fingerprints" of partial acid hydrolysates and lysozyme digests of *M. lysodeikticus* cell walls. Electrophoresis, chromatography and spraying performed as outlined in the text. 1. N-acetylmuramic acid; 2. N-acetylglucosamine; 3. N-acetylmuramic acid – N-acetylglucosamine compound present in hydrolysates and dialysable fractions of lysozyme digests; the substance from the latter preparations generally showed more streaking in the direction of electrophoresis. 4. N-acetylamino sugar – peptide complex from the dialysable products of lysozyme digested walls. Unidentified N-acetylamino sugar-reacting substances shown by shaded "spots".

TABLE I

SOME OF THE PRODUCTS OF PARTIAL ACID HYDROLYSIS AND LYSOZYME DIGESTION OF THE WALLS OF *Micrococcus lysodeikticus*, DETECTED BY THE "FINGERPRINT" TECHNIQUE

Hydrolysis and digestion conditions	*Compounds detected*
12 *N* HCl, 6 days at 4°, 1 and 2 days at 37°	N-acetylglucosamine; N-acetylmuramic acid; a compound of N-acetylmuramic acid and N-acetylglucosamine; unidentified substances giving positive acetylamino sugar reactions, free amino acids and free amino sugars.
Lysozyme, 24 h at 37° Dialysable fraction	A compound of N-acetylmuramic acid and N-acetylglucosamine; a neutral acetylamino sugar – peptide complex containing glucosamine, muramic acid, alanine, glycine, glutamic acid and lysine in the proportions 3:1:2.4:1:1:1.4; unidentified substances giving positive acetylamino sugar reactions.

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