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AN AUTOMATED ELSON–MORGAN ASSAY FOR 2-AMINO-2-DEOXY-HEXOSES, WITH INCREASED SENSITIVITY

CHARLES A. WHITE*, STEWART W. VASS**,
National Institute for Biological Standards and Control, Hampstead, London NW3 6RB (Gt. Britain)
AND JOHN F. KENNEDY***
Research Laboratory for the Chemistry of Bioactive Carbohydrates and Proteins, Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (Gt. Britain)

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ABSTRACT

The Elson–Morgan assay for 2-amino-2-deoxyhexoses, despite its many modifications, can still give variable results because of slight variations in reaction conditions. An automated method is reported which uses microgram samples and provides greater sensitivity than hitherto possible. The use of sodium orthophosphate and optimisation of the concentrations of the reagents provide conditions that are more stable, and results that are more reliable, than any previously reported.

INTRODUCTION

The current interest in bacterial polysaccharides and the introduction of purified bacterial-polysaccharide vaccines emphasises the need for analytical methods suitable for the routine determination of the components of such materials. Many of these polysaccharides contain 2-acetamido-2-deoxy sugars; since no method exists for their determination in the intact polymer molecule, the monosaccharides must first be released, usually by acid hydrolysis which also effects *N*-deacetylation.

2-Amino-2-deoxy sugars can be determined by using such reagents as ninhydrin[1], but the removal of such interfering compounds as proteins and amino acids is an added manipulation for a manual assay. However, this method has been fully automated, allowing quantification of individual 2-amino-2-deoxy sugars, using amino acid analysers[1]. More-specific methods are available, including the Dische–Borenfreund procedure[2] and the 3,5-diaminobenzoic acid assay[3], but the most widely used procedure is the Elson–Morgan assay[4] and its modifications, particularly those of Rondle and Morgan[5] and Boas[6].

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*Present adress: Vincent Kennedy Ltd., 47 Conchar Road, Sutton Coldfield, West Midlands, B 72 1LL, Gt. Britain.
**Present address: Leo Laboratories Ltd., Princes Risborough, Aylesbury, Buckinghamshire, Gt. Britain.
***To whom correspondence should be addressed.

Analysis of a large number of samples by the Elson–Morgan assay is tedious, and variable results may be obtained because of the loss of volatile chromogens produced during the condensation reaction[7] with acetylacetone (2,4-pentanedione). Automation of this assay removes many sources of error by eliminating the above problems, but no suitable system has been reported. The problem caused by evolution of carbon dioxide in the assay was overcome by Balazs *et al.*[8], who reported a simple system that was unsatisfactory due to high consumption of sample and reagent and a sinusoidal base-line[8].

We now report an automated system that overcomes these problems and permits the analysis of microgram quantities of 2-amino-2-deoxy sugars.

EXPERIMENTAL

Equipment. — Technicon AA1 Autoanalyser modular-equipment was used. All of the modules were placed as close together as possible, and linked *via* Acidflex sleeving with minimal lengths of glass tubing of the same inside and outside diameters as that used for the various coils. The debubbler after the first heating-stage was placed close to the peristaltic pump, in order to prevent diffusion of the non-segmented stream before it was mixed with the 4-(dimethylamino)benzaldehyde reagent. All mixing and cooling coils consisted of only 5.5 turns (2.5 mL), in order to diminish the delay time in the analysis system. Reaction coils of 31 turns (14 mL) were used which, with the flow rates described below, gave heating times of 14 (at 95°) and 7.5

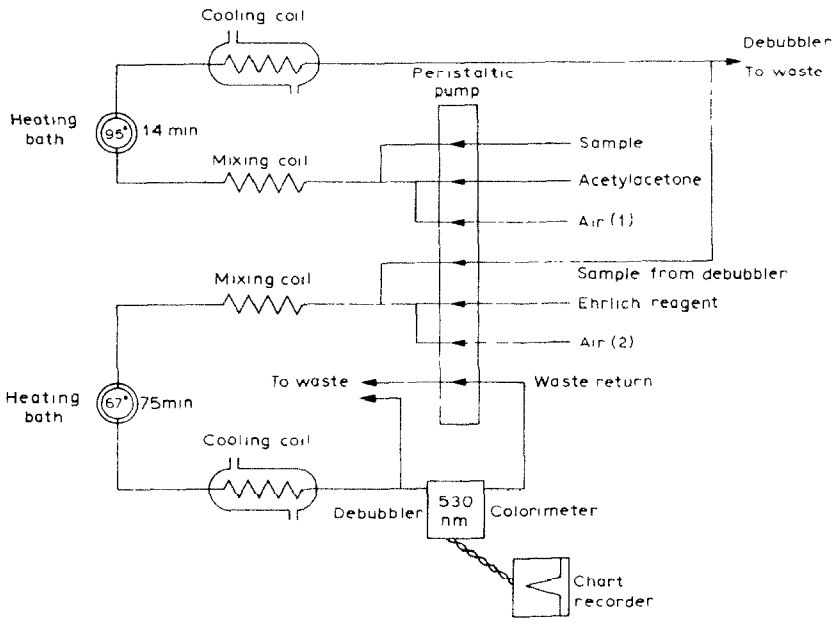

Fig. 1. Schematic representation of the automated Elson–Morgan assay. Pump tubing and composition flow-rates are given in Table I.

TABLE I

TYPE OF PUMP TUBING AND OPTIMAL FLOW-RATE FOR THE AUTOMATED SYSTEM

Solution	*Type of tubing*	*Flow rate (mL/min)*
Sample	Tygon	0.16
Acetylacetone reagent	Tygon	0.10
Air (1)	Tygon	0.10
Sample from debubbler	Tygon	0.16
Ehrlich reagent	Acidflex	0.34
Air (2)	Tygon	0.16
Waste return	Acidflex	0.34

min (at 67°). Absorbance of the coloured product was determined at 530 nm with a flow cell having a path length of 15 mm. The arrangement of the modules used is shown in Fig. 1.

Absorption spectra of the coloured product were recorded with a Hilger and Watts Ultrascan spectrophotometer, using a cell having a path length of 10 mm.

Reagents. — (*a*) *Acetylacetone.* Sodium orthophosphate (6 g) was dissolved in distilled water (50 mL), and acetylacetone (1.8 mL) was added with gentle shaking.

(*b*) *Modified Ehrlich reagent.* 4-(Dimethylamino)benzaldehyde (0.8 g) was dissolved in ethanol (170 mL), and conc. hydrochloric acid (30 mL) was added cautiously with cooling.

(*c*) Standard solutions of 2-amino-3-*O*-[1-(*S*)-carboxyethyl]-2-deoxy-D-glucose (muramic acid), 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, and 2-amino-2-deoxy-D-mannose were prepared freshly as required.

Optimisation of assay conditions. — (*a*) *Acetylacetone reagent.* Reagents con-

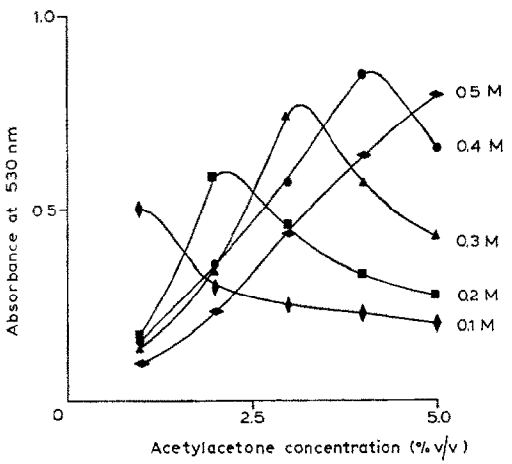

Fig. 2. Effect of acetylacetone concentration on the absorbance in the automated assay for various concentrations of sodium orthophosphate.

taining 1–5% of acetylacetone in 0.1–0.5M trisodium orthophosphate were used in the automated system, and the absorbance produced for a standard solution of 2-amino-2-deoxy-D-glucose (50 μg/mL) was observed. The results are shown in Fig. 2.

(*b*) *Ehrlich reagent.* Solutions containing 3–5% of 4-(dimethylamino)benzaldehyde and 10–20% of hydrochloric acid in ethanol were used, and the absorbance produced for a standard solution of 2-amino-2-deoxy-D-glucose in the automated system was recorded. No significant variations in absorbance were observed.

(*c*) *Ethanol.* Aliquots (200 μL) of 4-(dimethylamino)benzaldehyde (0.8 g) in ethanol (30 mL) and conc. hydrochloric acid (30 mL) were added, together with ethanol (0–1200 μL), to a solution (400 μL) of the amino sugar and acetylacetone reagent. Using a manual version of the assay, the absorbances obtained on subsequent heating are recorded in Table II.

TABLE II

THE EFFECT OF ETHANOL CONCENTRATION ON THE COLOUR PRODUCED IN A MANUAL SIMULATION OF THE AUTOMATED ASSAY[a]

Volume of ethanol added[b] (μL)	*Ethanol concentration* (% v/v)	*Absorbance at 530 nm*	
		Measured	*Corrected for dilution*
1200	72.2	0.225	0.225
1000	68.8	0.266	0.236
800	64.3	0.303	0.236
600	58.3	0.350	0.233
400	50.0	0.393	0.218
200	37.5	0.445	0.198
0	16.7	0.262	0.160

[a]Each solution contained 200 μL each of sample, acetylacetone reagent, and Ehrlich reagent. [b]The Ehrlich reagent contained an additional 100 μL of ethanol.

TABLE III

COMPARISON OF THE QUANTITIES OF MATERIALS USED IN THE RONDLE AND MORGAN MANUAL-ASSAY AND THE AUTOMATED ASSAY

Material	*Quantity per mL of sample solution*	
	Manual assay	*Automated assay*
Buffer (mL)	1.0 (carbonate)	1.0 (orthophosphate)
Water (mL)	1.0	0
Acetylacetone (μL)	20.0	21.0
4-(Dimethylamino)benzaldehyde (mg)	13.3	13.6
Conc. hydrochloric acid (mL)	0.5	0.5
Ethanol (mL)	6.5	2.9

(*d*) *Heating times and flow rates.* Initially, these were chosen to give the reaction conditions used in the Rondle and Morgan manual-system[5] (see Table III) which have proved to be satisfactory for the automated system. No significant increases in absorbance were observed when heating times were increased or the flow rate of the acetylacetone reagent was reduced. A small increase was observed when the flow rate of the Ehrlich reagent was reduced, but this was accompanied by an increase in chart-recorder noise. Reduction in the heating times and increases in reagent flow rates caused decreases in the observed absorbances. The optimal heating-times and flow rates are those shown in Fig. 1 and Table I.

DISCUSSION

The Elson–Morgan assay[4] for 2-amino-2-deoxyhexoses is extremely sensitive to several variables that must be carefully controlled, and this makes the method unattractive for routine use. The first stage of the reaction, namely, the formation of the chromogen, is dependent on pH, time, temperature, and the presence of salts, and has been much investigated[5–7,9]. Volatile chromogens are produced during this stage of the reaction, so that extreme care is required in subsequent manipulations, in order to prevent losses and, thereby, variable results. Precautions that must be taken in the manual assay include the use of sealed tubes during heating with acetylacetone reagent, cooling to 0° before breaking the seals, and careful rinsing of the tubes in order to minimise variations in the results.

Automation presents an opportunity to increase the sensitivity and reliability of the assay, not only by ensuring that all chromogen is converted into chromophoric product, but also by removing the need for the rinsing stage which dilutes the colour produced in the manual assay. The major problem in the automation of the assay is the neutralisation after the first stage of the reaction, prior to the colour-forming reaction. When the acetylacetone reagent is prepared in sodium carbonate, as in the manual method, carbon dioxide is evolved on addition of the Ehrlich reagent. Since evolution of a gas causes breakdown of the discrete bubble pattern in the automated assay, an alternative buffer system is required.

The optimum pH[7] of the acetylacetone reagent is between 9.6 and 9.9, and the most acceptable of the buffer systems that have been investigated[10] is sodium orthophosphate. Variation of the concentrations of sodium orthophosphate and acetylacetone affect the absorbance produced by 2-amino-2-deoxy-D-glucose (see Fig. 2); increasing the concentration of the buffer salt required an increase in the acetylacetone concentration to produce the maximum absorbance. This process is limited by the solubility of sodium orthophosphate and acetylacetone in the reagent, but the pH of the reagent which produced the maximal absorbance was ~10, irrespective of the concentration of components used. From Fig. 2, it can be seen that the optimum concentration of sodium orthophosphate was between 0.3 and 0.35M, and that of acetylacetone between 3.25 and 3.75%; because of the variation in absorbances obtained within this range, it was not possible to identify accurately a single, optimum

set of concentrations. The reagent reported herein represents one within the range that can be prepared easily on a routine basis.

Attempts to simplify the automated assay by using a single-step system, with the addition of the Ehrlich reagent directly into the segmented stream, caused instability in the system and resulted in a very noisy chart-recording, and the two-step system shown in Fig. 1 had to be used. In order to minimise the diffusion between successive samples, it is essential to ensure that the volume of the unsegmented portion of the sample stream (between the debubbler and the "sample from debubbler" tube) is kept to a minimum.

The basis for the automated assay, described herein, is the manual method[5] of Rondle and Morgan; the Boas method[6], which was chosen by Balazs *et al.*[8], has a much longer reaction time and is less specific. The mixing ratios for the various solutions were varied in an attempt to increase the sensitivity and minimise the volume of reagents consumed, but the system described in Fig. 1 represents the optimum system in terms of sensitivity and stability.

Using the optimum conditions for the automated assay, calibration curves for 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, 2-amino-2-deoxy-D-mannose, and muramic acid were obtained (see Fig. 3). 2-Amino-2-deoxy-D-glucose and -D-galactose gave similar responses in the assay (Balazs *et al.*[8] reported different responses), whereas 2-amino-2-deoxy-D-mannose and muramic acid had different and lower responses. The λ_{max} and spectrum shape were similar for the 2-amino-2-deoxy sugars, but the ε value for 2-amino-2-deoxy-D-mannose was lower than those of 2-amino-2-deoxy-D-glucose and -D-galactose (*cf.* Fig. 3). Substitution of HO-3, as in muramic acid, produced a different spectrum with a lowering of the value of λ_{max}.

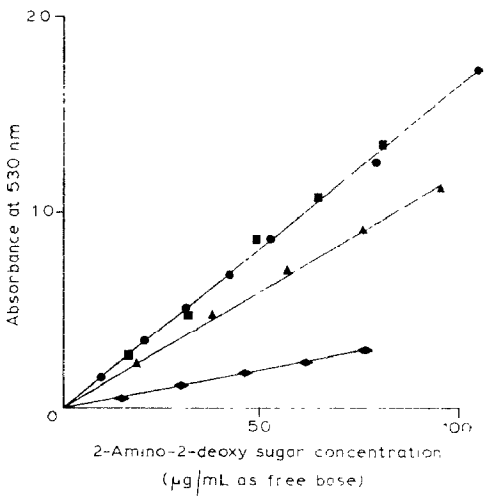

Fig. 3. Calibration curves using the automated assay: 2-amino-2-deoxy-D-glucose (--●--), 2-amino-2-deoxy-D-galactose (-- ■ --), 2-amino-2-deoxy-D-mannose (– ▲ –), and muramic acid (-◆-).

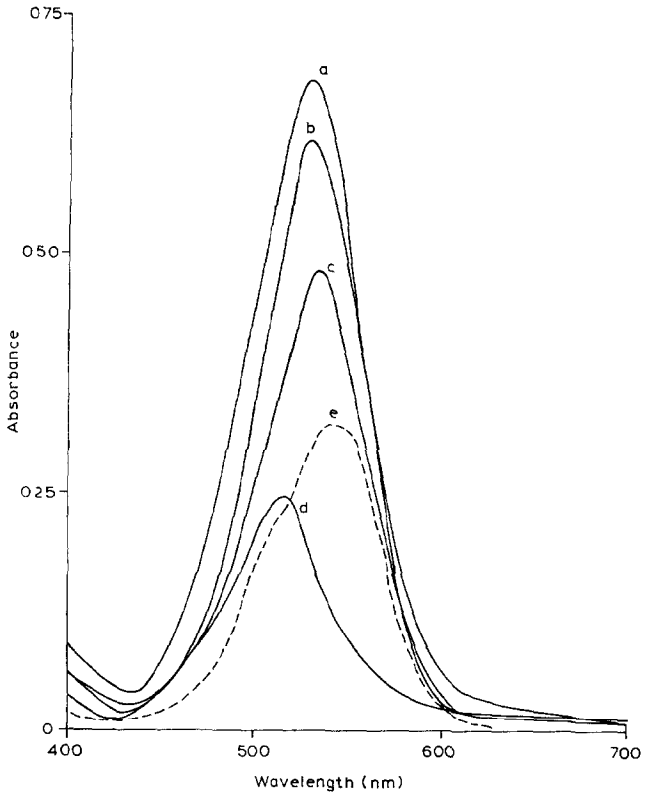

Fig. 4. Absorption spectra produced by (*a*) 2-amino-2-deoxy-D-glucose (52 μg/mL), (*b*) 2-amino-2-deoxy-D-galactose (48 μg/mL), (*c*) 2-amino-2-deoxy-D-mannose (57 μg/mL), (*d*) muramic acid (49 μg/mL) using the automated assay, and (*e*) 2-amino-2-deoxy-D-glucose (53 μg/mL) using the Rondle–Morgan version of the manual assay[5].

Comparison of the ε values for the coloured products obtained by the automated and manual assays (Fig. 4) shows that the former is more sensitive by a factor of 2; when compared with the automated assay described by Balazs *et al.*[8], the sensitivity is increased by a factor of 4 with linearity extending to an absorbance of > 1.8 ($\equiv$ 100 μg of 2-amino-2-deoxy-D-glucose/mL) (*cf.* 0.7 in ref. 8).

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