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The Determination of Glucosamine by Alkaline Decomposition

By M. V. TRACEY

Rothamsted Experimental Station, Harpenden, Hertfordshire

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Estimation of free glucosamine is usually done by the method of Elson & Morgan (1933) or by modifications of this method. Some workers have found difficulty in getting reproducible results unless conditions are most rigorously standardized (Ogston & Stanier, 1950; Johnston, Ogston & Stanier, 1951; Schloss, 1951). This may be explicable on the grounds that both the initial reactants, the amino sugar and acetylacetone, are unstable under the conditions of reaction and that several products are formed, including a number of chromogens (Schloss, 1951). The specificity of the method is not complete; acetylglucosamine gives a similar colour and so do mixtures of amino-acids and sugars (Sideris, Young & Krauss, 1938; Horowitz, Ikawa & Fling, 1950; Immers & Vasseur, 1950). Urea and glyceraldehyde have also been reported as interfering (Lutwak-Mann, 1941). Recently a new colorimetric method in which deamination of the amino sugar precedes its estimation by heating with indole and hydrochloric acid has been proposed by Dische & Borenfreund (1950). This has been reported as giving high results in the presence of other sugars (McCrea, 1951). It has been found that an essentially similar method which had been developed using orcinol for colour development after deamination also gave high results in the presence of other sugars.

The method presented here which has been communicated in a preliminary form (Tracey, 1951) is

based on the observation by Morgan (1936) that glucosamine and galactosamine 'yield practically the whole of their nitrogen in the form of ammonia on heating for a few minutes with normal alkali at 100°'. Conditions under which the yield of ammonia is quantitative and in which interference by other substances is minimal are described.

EXPERIMENTAL

Reagents

Phosphate-borate mixture. Saturated Na_3PO_4 is saturated with $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ at room temperature (at 14°, 1 l. of saturated Na_3PO_4 will dissolve about 42 g. of borate).

Nessler's reagent. This was prepared after Vanselow (1940). 45.5 g. HgI_2 and 34.9 g. KI are dissolved in as little water as possible, 112 g. KOH added and the solution diluted to 1 l.

Procedure

The sample, which should be 2 ml. or less in volume and not strongly acid, is introduced into a Markham (1942) still. The sample is washed in with a total of 5 ml. of the phosphate-borate mixture. Distillation is then carried out slowly (about 3 ml. distillate/min.), the distillate being collected in a test tube matched for use in a photoelectric colorimeter and graduated at 10 ml. Before use 1 ml. of 2% (w/v) boric acid is placed in each tube. Distillation is stopped when the distillate reaches the 10 ml. mark on the receiving tube. Blanks are distilled at the beginning and end of a set of distillations, and also solutions of $(\text{NH}_4)_2\text{SO}_4$

of suitable concentration to act as standards. All tubes of distillate are brought to the same temperature by standing in a vessel of water, and 1 ml. of Nessler's reagent added to each. After mixing, the tubes are allowed to stand 30 min. and are read in a photoelectric colorimeter, using a filter (Ilford 623) with maximum transmission in the region of 490 μ . With $(\text{NH}_4)_2\text{SO}_4$ standards a straight line relation between the instrument reading and the amount of NH_3 distilled is obtained over a range of 5–40 μ g. N (60–500 μ g. amino sugar). Alternatively, the NH_3 may be titrated with dilute HCl in the presence of a suitable indicator (Conway & O'Malley, 1942) using the photoelectric colorimeter to get an exact and reproducible end point.

The sensitivity may be increased either by using a colorimeter in which the light path through the nesslerized distillate is longer (an increase by a factor of 2.5 was obtained by using a 4 cm. cell) or by reducing the volume of distillate collected. Quantitative recoveries of NH_3 were achieved from glucosamine, collecting only 4 ml. of distillate, by preheating the phosphate-borate glucosamine mixture before distillation. Steam was allowed to pass through the still jacket for 3 min. before beginning a slow distillation.

Choice of alkali. Yields of NH_3 on distillation of glucosamine with 0.5N- Na_2CO_3 , saturated Na_2CO_3 , and saturated $\text{Na}_2\text{B}_4\text{O}_7$ were about 50, 80 and 25%. Borate buffers (approx. 0.2M) of pH 10.0, 11.1 and 12.2 gave yields of 20–30%. The phosphate-borate-NaOH mixture of Pucher, Vickery & Leavenworth (1935) gave results similar to those of borate buffers. Saturated Na_3PO_4 (pH approx. 11.4) gave theoretical yields of NH_3 . When glucose is distilled with saturated Na_3PO_4 volatile products are formed that give a colour with Nessler's reagent and a cloudiness that increases with time. Saturation of the phosphate solution with borate stops this interference while causing little change in the pH of the solution and allowing quantitative NH_3 recoveries from glucosamine and galactosamine. Quantitative recoveries are also obtained from biosynthetic phosphoglucosamine (Long, 1951).

Specificity. No NH_3 or other product giving a colour with Nessler's solution under the conditions described is evolved by acetylglucosamine, aspartic acid, glutamic acid, serine, threonine, citrulline, arginine, lysine, thymine, uracil, guanine, adenine, xanthine, allantoin, alloxan, creatinine, creatine, hydantoin, sarcosine, betaine, choline, ethanolamine, hyaluronic acid, or chitin. No NH_3 is evolved from a crude preparation of canavanine from jack bean meal. NH_3 is evolved in the presence of ammonium salts, glutamine, asparagine, nicotinamide, or urea. Interference by ammonium salts can be allowed for by a separate estimation using the Conway microdiffusion method (Conway, 1950). Glucosamine is decomposed at room and higher temperatures by half-saturated K_2CO_3 but not at all at 5°. A period of 4 hr. at 5° was found sufficient for complete diffusion of NH_3 . Recoveries from glucosamine (100 μ g. N) in this period at varying temperatures were 15% (35°), 4% (26°), 2% (20°), 1% (14°, using 500 μ g. glucosamine N), and nil at 5°. Recoveries from galactosamine at 5° were also nil. The amide N of glutamine and asparagine appear as NH_3 after hydro-

lysis with N- H_2SO_4 at boiling point for 3 hr. (Pucher *et al.* 1935), and the same treatment was 80% effective with nicotinamide. Urea is only decomposed to the extent of 40% by this treatment. If the hydrolysed fluid is treated with urease before distillation and estimation of free NH_3 , interference from this source will be eliminated. This is, however, only necessary in the presence of large amounts of urea as the yield on distillation is only about 0.5% of the urea N. Recoveries on distillation from solutions containing 1–2 mg. asparagine N or nicotinamide N are about 3% and from solutions containing 0.1–0.2 mg. glutamine N are about 10–20%. NH_3 is also formed by the distillation of some proteins under the conditions described. 10–20% of the amide N of glutenin and edestin were recovered in this way. After a preliminary hydrolysis with N- H_2SO_4 the N yields by Conway estimation at 5° and distillation were identical and agreed well with published figures for the amide N of these proteins. Thus the hydrolysis procedure seems equally effective in eliminating interference by both free and combined glutamine and asparagine.

Interference by mixtures of sugars and amino-acids occurs in this method as in the method of Elson & Morgan (1933) and its modifications. A direct comparison between the original method of Elson & Morgan and the method described in this paper was made. Using a 1.5M solution of glucosamine hydrochloride the increase in apparent glucosamine caused by the presence of 2.5M-lysine hydrochloride and 2.5M-glucose was 18% for the first method and 5% for the second.

DISCUSSION

The method proposed has been used for more than a year in these laboratories for the analysis of chitin and products containing it. With relatively pure samples of chitin it has the advantage that decomposition of glucosamine on hydrolysis (Ogston & Stanier, 1950; Folkes, Grant & Jones, 1950) is simultaneously allowed for, if ammonia is not estimated at 5°, or measured if it is. It is not suggested that the method is completely satisfactory used alone, but in conjunction with one of the modifications of the Elson & Morgan method the two methods provide very useful safeguards against the acceptance of false results.

SUMMARY

1. A method for the estimation of amino sugars in the range 50–500 μ g., by alkaline decomposition and estimation of the ammonia produced, is described.
2. Elimination of interference by other nitrogen-containing compounds is discussed.

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The Metabolism of Methyl Carbamate

By E. BOYLAND AND D. PAPADOPOULOS*
The Chester Beatty Research Institute, London, S.W. 3

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Urethane or ethyl carbamate, which is carcinogenic (Nettleship & Henshaw, 1943), mutagenic (Vogt, 1948) and used in the treatment of leukaemia (Paterson, Ap Thomas, Haddow & Watkinson, 1946), is considered to be a radiomimetic agent. It is destroyed relatively slowly in the bodies of normal rats and mice and even more slowly by tumour-bearing animals (Boyland & Rhoden, 1949; Skipper *et al.* 1951).

Methyl carbamate is not a radiomimetic agent, being neither carcinogenic nor mutagenic (Larsen, 1947). The rate of destruction of methyl carbamate by rats has been followed by estimation of the concentration of the compound in the tissues at different times after injection.

EXPERIMENTAL

Estimation of methyl carbamate. Methyl carbamate was determined by the method used for urethane by Boyland & Rhoden (1949). The sample containing less than 0.40 mg. methyl carbamate in 2 ml. 2N-KOH was placed in the outer

ring of a Conway unit (Conway, 1947), the central compartment of which contained 1 ml. of 0.025N- $K_2Cr_2O_7$ in 10N- H_2SO_4 . The unit, along with a blank unit in which m- K_2CO_3 replaced the 2N-KOH solution, was heated to 37° for 16 hr. At the end of that time, KI was added to the $K_2Cr_2O_7$, and the liberated I_2 titrated with 0.1N- $Na_2S_2O_3$ from a Conway burette. The difference between the titration figures of the KOH and K_2CO_3 units was proportional to the methyl carbamate present. As in the estimation of ethyl carbamate, the reaction was stoichiometric and reproducible under constant conditions (as shown in Table 1). All determinations were carried out in duplicate, and duplicate sets of blank units with reagents alone were set up with each set of estimations. One ml. 0.1N- $Na_2S_2O_3$ is equivalent to 1.87 mg. methyl carbamate.

Table 2. *Excretion of methyl carbamate in urine of rats dosed with 50 mg./100 g. body weight*

Weight of rat (g.)	Methyl carbamate (mg.) excreted in urine		Percentage of dose excreted in 24 hr.
	During first 4 hr. after injection	Between 4 and 24 hr. after injection	
150	2.3	1.4	4.9
200	2.2	7.6	9.8
190	1.8	7.0	9.2
160	1.4	2.7	5.1

Urinary excretion of methyl carbamate by rats

Adult rats weighing between 100 and 200 g. were dosed by intraperitoneal injection with 50 mg./100 g. body weight of methyl carbamate. The animals were kept in metabolism cages and the urine collected for estimation of the excreted carbamate. The cages were washed 4 and 24 hr. after injection and the washings added to the collected urine.

Table 1. *Estimation of known quantities of methyl carbamate*

Methyl carbamate taken (mg.)	0.1N- $Na_2S_2O_3$ used (ml.)	Titre as % of theory
0.05	0.0262	96
0.10	0.0547	103
0.20	0.1188	96
0.30	0.1551	98
0.40	0.2232	103

* Present address: The Greek Anti-Cancer Institute, Athens.