mediate does not exclude the possibility that the dehydrogenase is capable of reducing it.

It is not known why the cytochrome b_1 band is not fully removed by DHA. It may be either that the residual level of dehydrogenase activity in our experiments was sufficient to keep the cytochrome 70% reduced or, alternatively, that the oxidation-reduction potential of the AA/DHA system, as constituted in this case, was the same as that of 70% reduced cytochrome b_1 .

The inability of many organisms containing cytochrome b_1 to effect the reduction raises a further problem. As there is no evidence that this is due either to their cytochromes or cytochrome-reducing mechanisms, we suppose it to be due to lack of a mechanism linking oxidation of the cytochrome with reduction of DHA. In the previous paper (Eddy, 1952) theoretical considerations were given in favour of an enzyme activating DHA so that it can be reduced. The failure of some coliform bacteria to reduce DHA lends weight to these suggestions, and preliminary experiments have shown that the oxidation of leuco-Nile blue by DHA varies both in rate and magnitude, according to whether DHA-reducing or DHA-non-reducing strains of bacteria are present.

SUMMARY

1. In cell-free dehydrogenase preparations from *Escherichia coli*, which are unable to reduce dehydroascorbic acid (DHA), the cytochromes are either absent or greatly decreased in amount.

2. When DHA is added anaerobically to a suspension of *Escherichia coli* the intensity of the fully reduced cytochrome b_1 band becomes diminished by approximately 30%.

3. Amongst organisms examined so far, ability to reduce DHA is confined to members of the genus *Staphylococcus* and the family Enterobacteriaceae, these being the groups in which the b and c cytochrome bands are replaced by the b_1 band.

4. Approximately half the members of the Enterobacteriaceae tested failed to reduce DHA, and it is suggested that a DHA-activating enzyme may be necessary in addition to cytochrome b_1 .

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The authors wish to express their gratitude to Dr E. F. Hartree and Dr T. Mann, F.R.S., for help and advice received from them.

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Studies in Immunochemistry

11. THE ACTION OF DILUTE ALKALI ON THE N-ACETYLHEXOSAMINES AND THE SPECIFIC BLOOD-GROUP MUCOIDS

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(Received 21 September 1951)

The increasing use within recent years of the colorimetric method introduced by Morgan & Elson (1934) for the detection and quantitative determination of the *N*-acetylhexosamines calls for a more detailed study of the conditions necessary to obtain reliable and reproducible results and demands a more thorough understanding of the structural changes involved before the results can

be applied to complex structures containing *N*-acetylhexosamine components, such as the blood-group mucoids.

Ehrlich (1901) observed that mucins and mucoid substances give rise to an intense purple colour after heating with dilute alkali for a short time and the addition of an acid solution of *p*-dimethyl-aminobenzaldehyde. If the alkali treatment is

omitted no colour formation takes place. At about the same time Müller (1901) found that penta-acetylglucosamine after heating with dilute potassium hydroxide gave a similar colour on the addition of Ehrlich's *p*-dimethylaminobenzaldehyde reagent, and Zuckerkandl & Messiner-Klebermass (1931) subsequently suggested that a pyrroline derivative was formed from *N*-acetylglucosamine by the action of alkali, and that this substance reacted with Ehrlich's reagent to yield a coloured complex. Morgan & Elson (1934) and Morgan (1938) considered that the formation of a pyrrol derivative was difficult to reconcile with the fact that *N*-trimethylacetyl-, *N*-*o*-bromobenzoyl- and *N*-benzoyl-glucosamines gave a similar colour after treatment with dilute alkali and Ehrlich's reagent and it was suggested that *N*-acetylglucosamine formed an oxazole or oxazoline derivative under these conditions. Some oxazole preparations, made by condensing amides with α -halogenoketones (Blumlein, 1884; Lewy, 1887) gave, without previous alkali treatment, a strong colour with the *p*-dimethylaminobenzaldehyde reagent, and the production of an immediate colour by these substances seemed to support the suggestion that *N*-acetylglucosamine was converted to a heterocyclic structure of this kind. It has been known for some time, however, that the positive colour reaction given by the oxazole preparations is due to the presence of an impurity of unknown nature in the materials examined and that if the oxazole is purified by precipitation as a complex with mercuric chloride (Cornforth & Cornforth, 1947), it fails to give a positive colour reaction with *p*-dimethylaminobenzaldehyde.

A number of authentic oxazoline derivatives were found by Morgan (unpublished observations) to yield no colour with the *p*-dimethylaminobenzaldehyde reagent, but nevertheless White (1940), on the basis of the results of methylation experiments, concluded that the formation of a glucosaxoline (2-methyl-4:5-glucopyrano- Δ^2 -oxazoline) occurred when *N*-acetylglucosamine was treated with alkali. Morgan (1938) pointed out that two molecules of *N*-acetylglucosamine might condense together and form a diglucopyrazine structure similar to that proposed earlier by Stolte (1908), who claimed to have obtained ditetrahydroxybutylpyrazine from glucosamine.

In an attempt to understand more fully the structural changes which occur when the *N*-acetylhexosamines are treated with dilute alkali under controlled conditions, such as those employed for their colorimetric determination, a study has been made of the ultraviolet absorption spectra given by these substances after treatment with dilute sodium carbonate or with different buffer systems at known pH values, and of the light absorption of

the systems after the development of the purple colour following the addition of the *p*-dimethylaminobenzaldehyde reagent. A number of purified human blood-group substances which are mucoids and are known to behave with alkali and Ehrlich's reagent as do the *N*-acetylhexosamines, have been examined in the same manner. The isolation and identification of the substance which arises from the simple *N*-acetylhexosamines on treatment with alkali and which is responsible for giving the colour with Ehrlich's reagent is being undertaken in collaboration with Dr J. W. Cornforth.

EXPERIMENTAL

Materials and methods

N-Acetylglucosamine. A standard solution of *N*-acetylglucosamine (1%) was kept at 2° and from this, suitable dilutions were made immediately before use.

N-Acetylchondrosamine. The substance was prepared by the acetylation of chondrosamine hydrochloride with acetic anhydride in the presence of silver acetate. (Found: C, 42.9; H, 6.7; N, 6.3; $C_8H_{15}O_5N$ requires C, 43.4; H, 6.8; N, 6.3%) $[\alpha]_{D_{461}}^{20} + 131^\circ \rightarrow 98^\circ$ (*c*, 0.5 in water); m.p. 162–164° (uncorr.). The material was found to be chromatographically homogeneous, a single component being observed when a solution of the substance was run in collidine, butanol, butanol-acetic acid, butanol-ethanol or in phenol according to the standard techniques used in paper chromatography. The R_F value of the material in these solvents is very similar to that of *N*-acetylglucosamine run simultaneously. After hydrolysis with 0.5*N*-HCl for 16 hr. at 100°, the material gave the full hexosamine colour corresponding to an equivalent weight of glucosamine hydrochloride and chromatographic analysis of the hydrolysate revealed the presence of one component only, which had all the properties of chondrosamine hydrochloride. A 1% solution of *N*-acetylchondrosamine was kept at 2° and the appropriate dilutions made immediately before the experiment.

p-Dimethylaminobenzaldehyde reagent (DMAB). A.R. grade *p*-dimethylaminobenzaldehyde was further purified by the method of Adams & Coleman (1944) and finally fractionated by the addition of water to an ethanol solution of the material. The crystals separating from solution within the range 50–75% water are almost colourless and are suitable for use. The reagent was prepared by dissolving 2 g. of the material in 100 ml. of A.R. glacial acetic acid which contains 2.5% (v/v) A.R. 10*N*-HCl. The solution, which possessed a very pale greenish yellow colour, was stored at 0° in small glass-stoppered bottles. A fresh bottle was opened for each experiment. The use of material which has been repeatedly thawed and frozen or allowed to stand at room temperature should be avoided.

Reducing sugars. Determined according to Somogyi (1937).

Fucose. Determined by the method of Dische & Shettles (1948).

Glucosamine and chondrosamine. Determined as described by Elson & Morgan (1933) using hexosamine concentrations of 10–40 μ g. as standards, a photoelectric colorimeter and a green filter (max. transmission 550 $m\mu$), or a Hilger Uvispek spectrophotometer.

Buffer solutions. As described by Vogel (1939).

A substance. The material was obtained from a pseudomucinous cyst fluid (no. 122) according to the isolation procedures described by Aminoff, Morgan & Watkins (1950). $[\alpha]_{5461} + 11^\circ$. N, 5.5% (Kjeldahl). Total fucose, 18.3%. Reducing power, expressed as glucose after acid hydrolysis, 54%. Hexosamine, as base, 32%.

B substance. The material was prepared from an ovarian cyst fluid by Mr R. A. Gibbons. The substance showed general chemical properties similar to those recorded for group A substance. N, 5.8%. Total fucose, 18%. Reducing power, expressed as glucose after hydrolysis, 50%. Hexosamine, as base, 20%.

H substance. Prepared according to Morgan & Waddell (1945) and Annisson & Morgan (unpublished) from cyst fluids obtained from 'secreters' belonging to group O. The material was found to be similar in general chemical properties to the groups A and B substances. N, 5.3%. Total fucose, 14%. Reducing power, expressed as glucose after hydrolysis, 54%. Hexosamine, as base, 31%. $[\alpha]_{5461} - 30^\circ$ (c, 0.5 in water).

'Lewis' Le^a substance. Prepared as described by Annison & Morgan (1951) from a cyst fluid obtained from a 'Lewis-positive' patient. The substance, $[\alpha]_{5461} - 41^\circ$, contained N, 5.4%, total fucose, 13% and gave, after hydrolysis with 0.5N-HCl at 100°, 57% reducing sugars (as glucose) and 32% hexosamine (as base).

Optimal conditions for the colorimetric determination of the N-acetylhexosamines

A re-examination of the general procedure used by Morgan & Elson (1934) for the estimation of the *N*-acetylhexosamines was first undertaken.

Period of heating with alkali. *N*-Acetylglucosamine (20 μ g.) was measured into each of several test tubes (20 $\times$ 1 cm.) graduated at 10 ml. capacity which were carefully selected to possess walls of equal thickness. The sides of the tubes were washed down with water to yield a total volume of 1 ml., and 0.1 ml. of 0.5N-Na₂CO₃ was added and the contents thoroughly mixed. Each tube was closed by a glass ampoule, the sealed neck of which was inserted into the tube. The ampoules contained 2-3 ml. of water and acted as condensers which prevented loss by evaporation during the short heating periods studied. The tubes were heated up to the level of the liquid contents in a vigorously boiling water bath, and at pre-arranged times up to a maximum period of 15 min., pairs of tubes were withdrawn from the bath and immediately cooled in water at 0° and kept there until all tubes had been heated and cooled. Glacial acetic acid (A.R.) was then run into each tube from a separating funnel to yield a total volume of about 7 ml. and the DMAB reagent (1 ml.) was added. The volume of liquid in each tube was made up to 10 ml. by the addition of glacial acetic acid, was thoroughly mixed and the whole series of tubes allowed to stand at 20° in the dark. A tube containing all the reagents other than the *N*-acetylhexosamine was included to serve as a reagent blank in each experiment. The colour intensity of each solution reached a maximum value after standing for 1.5 hr. at 20°. For routine purposes the intensity of the colour developed is conveniently measured in a simple photoelectric colorimeter using a green filter (max. transmission 550 m μ .) and optical cells 2 cm. deep. In our experience it is advisable to mix the

contents of each tube again a few minutes before they are examined in the colorimeter or spectrophotometer.

The relationship between the time of heating with carbonate solution and the intensity of the colour subsequently produced with a fixed amount of DMAB reagent and expressed directly in terms of the galvanometer readings is given in Fig. 1. Under the conditions described the maximum colour was given by a solution which had been heated for 4 min. and it is evident that after this time the structure which gives rise to the coloured complex with the DMAB reagent undergoes fairly rapid destruction, and it is therefore important that the period of heating with alkali should be accurately controlled. The length of the heating period which gives the optimal colour production on the addition of the DMAB reagent varies somewhat according to the thickness and shape of the glass tubes selected, the heating conditions etc., and must be determined for each outfit.

Fig. 1. The influence of the time of heating with Na₂CO₃ on the intensity of the colour produced on the addition of DMAB reagent. ○—○, *N*-acetylglucosamine; △—△, *N*-acetylchondrosamine.

A similar curve (Fig. 1) was obtained for *N*-acetylchondrosamine, but it was found that nearly five times as much of this amino sugar was required to give a solution of equal colour intensity. Several preparations of *N*-acetylchondrosamine gave similar results. The *N*-acetylchondrosamine chromogen which gives rise to the coloured DMAB complex is apparently less rapidly destroyed than is that derived from *N*-acetylglucosamine. The 4 min. heating period was optimal for quantities from 10 to 50 μ g. of the two *N*-acetylhexosamines treated as described above.

Concentration of alkali. The influence of the concentration of Na₂CO₃ and of the period of heating on the amount of colour subsequently developed by *N*-acetylglucosamine after the addition of the DMAB reagent was next examined. Amounts (20 μ g.) of *N*-acetylglucosamine were placed in a series of tubes with a solution of Na₂CO₃ of known strength and the total volume of the solution made up to 1.1 ml. with water. The tubes were then heated in a boiling-water bath for predetermined periods, removed in pairs, cooled and treated with glacial acetic acid and DMAB reagent. Duplicate tubes containing 10, 20 and 30 μ g. of *N*-acetylglucosamine were likewise heated for 4 min. to act as standards and the maximum colour intensity reached by each pair of tubes, expressed in terms of that given by the *N*-acetylglucosamine standard, was plotted against the time of heating with alkali. The results obtained when three series of tubes containing 0.1, 0.4 and 0.8 ml. of 0.125N-Na₂CO₃ respectively were examined are given in Fig. 2. *N*-Acetyl-

chondrosamine (100 $\mu\text{g.}$) was treated in the same manner and the results, expressed in terms of the colour given by *N*-acetylchondrosamine heated for 4 min., are given in Fig. 3. It appears that under these conditions *N*-acetylchondrosamine requires a slightly shorter period of heating with alkali to yield subsequently its maximum colour than does *N*-acetylglucosamine.

Fig. 2. Effect of the concentration of Na_2CO_3 on the rate and intensity of the colour produced from *N*-acetylglucosamine on the addition of DMAB reagent. □—□, 0.1 ml. 0.125 $\text{N-Na}_2\text{CO}_3$; ○—○, 0.4 ml. 0.125 $\text{N-Na}_2\text{CO}_3$; △—△, 0.8 ml. 0.125 $\text{N-Na}_2\text{CO}_3$.

Conditions for the standard test. The results of these experiments allowed a set of conditions, the so-called 'standard' test conditions, for the estimation of the *N*-acetylhexosamines to be established. Thus, to a neutral solution of the test material contained in a total volume of 1.0 ml., 0.10 ml. of 0.5 $\text{N-Na}_2\text{CO}_3$ is added and the solution is heated for 4 min. in a vigorously boiling water bath, cooled and treated as described above. Standard solutions each containing 10, 20 and 30 $\mu\text{g.}$ of *N*-acetylglucosamine or 50, 100 and 150 $\mu\text{g.}$ *N*-acetylchondrosamine, according to the amino-hexose being examined, are included in each determination made. Under the conditions described the pH of the reactants during the heating period is about 10.8.

Influence of different buffer systems and pH values on the rate and intensity of colour development. The effect of heating *N*-acetylglucosamine with different buffer systems at the

same pH (10.8), and with the same buffers at different pH values, on the maximum amount of colour finally obtained was investigated, using 0.2 ml. of buffer solution in place of the 0.1 ml. of 0.5 $\text{N-Na}_2\text{CO}_3$ used in the 'standard' test. Where the heating period necessary to yield the maximum colour exceeded 30 min. the heating was carried out in sealed glass ampoules which contained a total volume of

Fig. 3. Effect of the concentration of Na_2CO_3 on the rate and intensity of the colour produced from *N*-acetylchondrosamine on the addition of DMAB reagent. □—□, 0.1 ml. 0.125 $\text{N-Na}_2\text{CO}_3$; ○—○, 0.4 ml. 0.125 $\text{N-Na}_2\text{CO}_3$; △—△, 0.8 ml. 0.125 $\text{N-Na}_2\text{CO}_3$.

approximately 1.3 ml. and, subsequently, amounts (1.1 ml.) of the cooled reaction mixture which contained exactly 20 $\mu\text{g.}$ of *N*-acetylglucosamine were pipetted into calibrated test tubes, glacial acetic acid and the DMAB reagent added, and the colours allowed to develop in the usual way. The results obtained are given in Table 1. It was observed many years ago that the use of borate buffer at about pH 10.8 in place of Na_2CO_3 at the same pH gave rise to considerably more colour, and the results set out in Table 1 confirm this early observation. The amount of colour obtained on addition of DMAB reagent to *N*-acetylglucosamine heated with glycine buffer at pH 10.8 was almost identical with that obtained with Na_2CO_3 .

The experiments were repeated using *N*-acetylchondrosamine, and the results (Table 1) revealed at once that the two amino sugars behaved qualitatively in a similar manner

Table 1. Amount of colour given by the *N*-acetylhexosamines after treatment at 100° with different buffer solutions and the addition of DMAB reagent

		<i>N</i> -Acetylglucosamine		<i>N</i> -Acetylchondrosamine	
		Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by <i>N</i> -acetylglucosamine under standard conditions	Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by <i>N</i> -acetylglucosamine under standard conditions
Alkaline system	pH				
	'Standard' procedure	4	100	3-4	23
	Potassium borate + KOH				
	9.0	45	152	50	40
Glycine buffer	10.0	12	155	15	35
	11.0	7	156	10	30
	10.0	110	97	90	25
	10.9	40	100	40	25
	12.2	2	77	1-2	12

with the different buffer systems but that in each instance *N*-acetylchondrosamine gave rise to a maximum amount of colour equivalent to about a fifth of that given by an equal weight of *N*-acetylglucosamine.

Concentration of *p*-dimethylaminobenzaldehyde. The influence of the concentration of *p*-dimethylaminobenzaldehyde in the DMAB reagent on the intensity of the colour formed was investigated by the standard procedure. The DMAB reagents used in the test series contained 1, 2 and 4% (w/v) *p*-dimethylaminobenzaldehyde. The results indicated that increasing concentrations of *p*-dimethylaminobenzaldehyde in the Ehrlich's reagent give rise to a small increase in colour for a constant amount of the *N*-acetylhexosamine. The advantage gained by the development of an increased intensity of colour when using a more concentrated solution of DMAB, however, is offset to some extent by a corresponding increase in the correction necessary to compensate for the enhanced yellowish green coloration given by the reagent's 'blank'.

Fig. 4. Absorption spectrum of coloured complex derived from *N*-acetylglucosamine after treatment with Na_2CO_3 and DMAB reagent. Absorption after A, 1.5 hr., B, 4 hr. and C, 24 hr.

Effect of concentration of NaCl on the colour obtained. To a series of tubes each containing *N*-acetylglucosamine (20 μg . in 0.1 ml.) and 0.2 ml. of 0.125 *N*- Na_2CO_3 , was added (a) water, (b) 1% NaCl, (c) 3% NaCl, (d) 5% NaCl or (e) 10% NaCl to yield a total volume of 1.1 ml. The resulting solutions were then treated by the standard procedure and the intensity of the colours finally obtained were read on the photoelectric colorimeter 0.5, 1, 1.5, 2 and 24 hr. after the addition of the DMAB reagent. The concentrations of NaCl used in the experiments (b) and (c) gave rise to no appreciable change in the rate or amount of colour obtained as compared with that which arises using the 'standard' conditions. The colour development in the presence of the NaCl concentrations (d) and (e) was slower, and the maximum colour intensity obtained was less.

Absorption spectrum of the N-acetylhexosamines after alkali treatment and the addition of DMAB reagent

Amounts of *N*-acetylglucosamine (20 μg .) and *N*-acetylchondrosamine (100 μg .), selected so that solutions of approximately the same colour intensity would finally result, were treated according to the standard procedure and, after standing for 1.5 hr. at 20°, examined in the spectrophotometer over the wavelength range 350–610 $\text{m}\mu$. Control solutions were treated in the same manner except that the *N*-acetylhexosamine and alkali mixture was not heated and no colour developed. The results are given in Figs. 4 and 5 and show that the *N*-acetylhexosamines give rise to a coloured complex with *p*-dimethylaminobenzaldehyde which shows light absorption with maxima at 550 and 590 $\text{m}\mu$. and a minimum at about 570 $\text{m}\mu$. No absorption peaks were observed between 350 and 500 $\text{m}\mu$. and this part of the curve is omitted in Figs. 4 and 5.

The rate of development and fading of the colour was also followed, and it was observed that the maximum intensity of

Fig. 5. Absorption spectrum of coloured complex derived from *N*-acetylchondrosamine after treatment with Na_2CO_3 and DMAB reagent. Absorption after A, 1.5 hr., B, 4 hr. and C, 24 hr.

absorption of both peaks (550 and 590 $\text{m}\mu$.) was reached at 20° in about 1.5 hr. after the addition of DMAB reagent. On standing the colour fades, but the rate of fall in the absorption intensity at 590 $\text{m}\mu$. was faster than that at 550 $\text{m}\mu$. The absorption intensity observed after the coloured solutions had stood for 4 hr. and 24 hr. for each *N*-acetylhexosamine is given in Figs. 4 and 5.

The two absorption peaks could be due to the interaction of one complex chromogenic structure or of two distinct chromogens with the *p*-dimethylaminobenzaldehyde. In order to determine if one of the absorption maxima arises from the presence of an intermediate product of the action of alkali on *N*-acetylglucosamine, specimens of this substance were heated at 100° for 1, 2, 4, 8 and 12 min. with 0.1 ml. of 0.5 *N*- Na_2CO_3 , treated with glacial acetic acid and DMAB reagent in the usual manner and allowed to stand for 1.5 hr.

at 20°. The absorption curves obtained for a selected number of heating times, Fig. 6, demonstrate the similarity of the absorption after the different periods of heating, and show that the extent of heat treatment is reflected equally in the intensity of the absorption at 550 and at 590 m μ . Similar results were obtained with *N*-acetylchondrosamine.

The coloured products obtained by heating *N*-acetylglucosamine and *N*-acetylchondrosamine with different buffer systems at 100°, such as with potassium borate (pH 11) or glycine buffer (pH 10.9) in place of Na₂CO₃, and subsequent treatment with the DMAB reagent, have the same absorption characters. In order to obtain a maximum colour with these buffers, however, it was necessary to heat the borate and glycine buffer systems for 7 and 40 min., respectively, in place of the 4 min. heating required when Na₂CO₃ was employed (see Table 1).

Fig. 6. Effect of varying the time of heating *N*-acetylglucosamine with Na₂CO₃ on the absorption spectrum of the coloured complex formed with DMAB. A, 2 min. heating; B, 4 min. heating; C, 8 min. heating.

The green filter used in the photoelectric colorimeter employed for the routine measurement of the intensity of the colour in the quantitative determination of the *N*-acetylhexosamines gives a maximum transmission of 42% at 550 m μ , whilst at 570 and 590 m μ , the transmissions are about 34 and 20%, respectively. Thus the filter measures predominantly the intensity of absorption at 550 m μ .

The exact amount of colour given by *N*-acetylchondrosamine in terms of *N*-acetylglucosamine was determined by measuring the colour developed from a series of standard amounts of *N*-acetylglucosamine (50, 60, 70 and 80 μ g.) and from 300 μ g. of *N*-acetylchondrosamine. Measurements were made on the Uvispek spectrophotometer at 550 and 590 m μ , using cells 2 cm. deep. The results showed that at 550 m μ , 71 $\pm$ 2 μ g. of *N*-acetylglucosamine gave a solution of the same colour intensity as 300 μ g. of *N*-acetylchondrosamine. At 590 m μ , 68 $\pm$ 2 μ g. of *N*-acetylglucosamine were equivalent to 300 μ g. of *N*-acetylchondrosamine.

Ultraviolet absorption of the *N*-acetylhexosamines after heating with alkali

N-Acetylglucosamine (300 μ g. in 3 ml. water) was run into a 15 ml. volumetric flask, 1.5 ml. of 0.5N-Na₂CO₃ was added and the contents immediately made up to 15 ml. Half of the solution was heated for 4 min. at 100° and cooled; the remainder was unheated. The solutions were examined at once in the Uvispek spectrophotometer over the wavelengths 215–270 m μ , the unheated portion of the solution, which itself showed a strong absorption at 217 m μ , being used as the reference solution. The results are given in Fig. 7 and show that there is only a single absorption maximum and that this occurs at about 230 m μ . The intensity of absorption at this wavelength is greatest after 4 min. heating, that is, after the time of heating necessary to yield subsequently the maximum colour following the addition of the DMAB reagent. The intensity of the absorption at 230 m μ is directly proportional to the amount of

Fig. 7. Absorption in the ultraviolet region of *N*-acetylglucosamine and *N*-acetylchondrosamine after heating with Na₂CO₃. *N*-Acetylglucosamine, $\circ$ — $\circ$, after about 20 min.; $\circ$ — $\cdots$ — $\circ$, after 4 hr. *N*-Acetylchondrosamine, $\triangle$ — $\triangle$, after about 20 min.; $\triangle$ — $\cdots$ — $\triangle$, after 4 hr.

N-Acetylglucosamine heated with alkali and decreases on standing, with a slight shift in the position of the absorption maximum toward a longer wavelength. The absorption recorded after the alkali-treated *N*-acetylhexosamine solution had stood for 4 hr. at room temperature is also shown in Fig. 7. It is evident that the chromogenic structure is alkali-labile. The immediate addition of the DMAB reagent after the optimal time of heating with alkali is essential if maximum colour production is to be obtained subsequently in the colorimetric determination.

N-Acetylchondrosamine treated with dilute Na₂CO₃ under conditions identical with those described gives a similar absorption curve (Fig. 7) which likewise shows a maximum absorption at about 230 m μ . It is to be noted, however, that the maximum intensity of the absorption at 230 m μ for *N*-acetylchondrosamine was considerably less than that given by an equal quantity of *N*-acetylglucosamine.

Solutions (20 $\mu\text{g./ml.}$) of methyl-L-fucopyranoside, *N*-methyl-L-glucosamine hydrochloride, *p*-tolyl-D-isoglucosamine, *N*-acetylglucosaminol, methyl-*N*-acetylglucosaminide, L-fucose, D-glucose, D-galactose, L-rhamnose, digitoxose, L-fucodesose, lactose, *N*-acetyl-L-aminoglucose and D-ribose were treated with alkali under the same conditions, but none of these substances gave an absorption curve similar to that obtained with the *N*-acetylhexosamines with a single maximum absorption at 230 $m\mu$.

The absorption spectrum of glucose-lysine mixtures after alkali treatment and after alkali treatment and the addition of DMAB reagent

The finding by Vasseur & Immers (1949) that mixtures of certain amino-acids, especially lysine, and simple sugars develop, after treatment with alkali and DMAB reagent, a colour similar to that given by the *N*-acetylhexosamines, suggested that the visible and ultraviolet absorption characteristics of lysine-glucose mixtures should be examined.

Fig. 8. The absorption spectrum of a mixture of glucose and lysine after treatment with Na_2CO_3 and DMAB.

Glucose (30 mg.) and lysine (15 mg.) were added separately and together to 1.5 ml. quantities of 0.5 *N*- Na_2CO_3 and the total volume in each instance was immediately made up to 15 ml. with water. The three solutions were divided into equal parts and one half of each was heated for 4 min. at 100° and then cooled. The solutions of glucose and of glucose + lysine developed a yellow colour, the solution of lysine remained colourless. The ultraviolet absorption of the solutions was then determined using the unheated portions as reference solutions. The results indicated that only the heated glucose and the heated glucose-lysine mixture showed any appreciable general absorption and in both instances the characteristic absorption maximum at 230 $m\mu$. given by the *N*-acetylhexosamines was absent.

The heated and unheated test solutions (1 ml. of each) were then treated with glacial acetic acid and DMAB reagent (1 ml.) according to the usual procedure. The yellowish brown coloration of the reaction mixtures which contained heated glucose and glucose-lysine mixture disappeared on the addition of glacial acetic acid. The heated and unheated glucose and lysine solutions and the unheated glucose-lysine mixture developed a pale-yellow

colour only, whereas the heated glucose-lysine mixture (3 mg.) slowly developed a pink coloration. The absorption curve of the coloured solution is given in Fig. 8 and shows only a single absorption maximum at about 560 $m\mu$.

Absorption spectra of the blood-group substances after treatment with (a) alkali and (b) alkali and DMAB reagent

The human blood-group mucoids after treatment with alkali and DMAB reagent give rise to a colour which appears to be identical with that formed by *N*-acetylglucosamine after similar treatment. It is possible, however, in view of

Fig. 9. Absorption in the ultraviolet of blood-group mucoids after treatment with dilute alkali at 100°, $\bigcirc$ — $\bigcirc$, A substance; $\square$ — $\square$, B substance; $\times$ — $\times$, H substance; $\triangle$ — $\triangle$, Le^{A} substance.

the observations of Vasseur & Immers (1949) that several component molecules of the group mucoids other than the *N*-acetylhexosamines, such as galactose and fucose and the individual amino-acids, especially if free, will combine in alkaline solution to form a substance which will give a coloured complex with DMAB. It became of interest, therefore, to examine not only the amount of colour given, but also the absorption characteristics of the alkali-treated blood-group substances and coloured products obtained from them on the addition of the DMAB reagent.

Alkali alone. The group substances A, B, H and Le^{A} (3 mg. in 1.5 ml. 0.5 *N*- Na_2CO_3) were each made up to 15 ml. with water and divided into two approximately equal parts. One part was heated at 100° for 12 or 15 min. (see below), whilst the remainder of the solution was kept as an unheated control. The heated solution was cooled and examined immediately in the spectrophotometer, and its absorption with reference to the unheated material was measured over the wavelength range 215–270 $m\mu$. The results obtained are given in Fig. 9 and show that in each

instance a structure is formed which gives a single absorption maximum at about 230 $m\mu$. Solutions of equal concentration (200 $\mu\text{g./ml.}$) of dextran, glycogen, gum arabic, mannan, galactose, and the specific polysaccharides of *Shigella shigae* and pneumococcus Type II gave no comparable amount of absorption over the same wavelength range and showed no characteristic absorption maximum at 230 $m\mu$. after treatment with alkali under the same conditions as were used for the group mucoids.

Fig. 10. Absorption spectrum of coloured complex derived from blood-group mucoids. $\circ$ — $\circ$, A substance; $\square$ — $\square$, B substance; $\times$ — $\times$, H substance; $\triangle$ — $\triangle$, Le^a substance.

Alkali and DMAB reagent. The group substances (500 $\mu\text{g.}$) contained in 1 ml. water were heated with 0.1 ml. of 0.5 $\text{N-Na}_2\text{CO}_3$ for 12 or 15 min. (see below), cooled and treated according to the standard procedure. The absorption spectrum of each group substance was measured over the range 500–610 $m\mu$. using the unheated group substance, which in no instance gave a purple colour under the test conditions, as the reference solution. Group mucoids so treated sometimes develop a slight opalescence which should be removed by the addition of 1 ml. water to all solutions just before the absorption intensities are measured. The results are given in Fig. 10 and indicate that absorption

curves similar to those given by the *N*-acetylhexosamines were obtained. The points of maximum absorption intensity were again at about 550 and 590 $m\mu$. with a minimum at 570 $m\mu$.

The influence of the time of heating of the group mucoids with alkali on the intensity of the colour subsequently obtained on the addition of the DMAB reagent was investigated using the 'standard' procedure, and the results obtained (Table 2) in one instance (A substance) showed that the optimal period of heating is 12 min. Somewhat longer times, about 15 min., were required by B and H substances. It seems that a minimum time of 12 min. heating should be employed when measuring the '*N*-acetylhexosamine' colour given by the group mucoids. The polysaccharides used as control materials and mentioned above give no colour after heating with alkali for 15 min. and the addition of the DMAB reagent.

The amount of colour obtained with A substance, after treatment with dilute Na_2CO_3 or one of the other buffers, is recorded in Table 2 where the results are expressed in terms of the absorption given by an equal weight of *N*-acetylglucosamine. The values obtained may be compared with those given for *N*-acetylglucosamine or *N*-acetylchondrosamine (Table 1) and show that heating the A substance with borate buffer gives rise to a material which shows about the same intensity of absorption as is obtained with glycine, or Na_2CO_3 , in contrast to the behaviour of the *N*-acetylhexosamines which give an enhanced colour when heated in borate buffer. The group substances give maximum colour intensities equivalent to the absorption given by 7–10% of their weight of *N*-acetylglucosamine.

The influence of oxidation of A substance on the absorption spectrum

Oxidation with sodium periodate. A maximum intensity of absorption was observed at 230 $m\mu$. with A substance oxidized with periodate at pH 5 for 7 hr. (Aminoff & Morgan, 1951) and treated with alkali alone. The addition of the DMAB reagent gave rise to the production of a purple colour which showed two absorption maxima, at 550 and 590 $m\mu$. A quantitative determination of the amount of '*N*-acetylglucosamine' colour given by the oxidized A substance showed that there was probably no significant

Table 2. Amount of colour given by blood-group A substance after treatment at 100° with different buffer systems and the addition of DMAB reagent

Alkaline system	pH	Period of heating for maximum colour production (min.)	Amount of colour expressed as % of amount given by <i>N</i> -acetylglucosamine*	
			(Observed)	(Extrapolated value)
0.1 ml. 0.125 $\text{N-Na}_2\text{CO}_3$	10.8	35	7.8	9.2
0.4 ml. 0.125 $\text{N-Na}_2\text{CO}_3$	10.8	12	6.8	9.1
0.8 ml. 0.125 $\text{N-Na}_2\text{CO}_3$	10.8	10	8.4	10.7
Potassium borate + KOH	9.0	†	—	—
	10.0	80	6.2	7.1
	11.0	45	6.2	7.9
Glycine buffer	10.0	†	—	—
	10.9	150	7.1	8.5
	12.2	5	6.2	7.9

* Under 'standard' conditions.

† Maximum colour not reached after 4 hr. heating.

change in this property as a result of oxidation with periodate. In these respects, therefore, the oxidized A substance behaves as does the untreated blood-group substance.

Oxidation with hypiodite. The procedure described by MacLeod & Robison (1929) was used. A substance (5 ml. of 0.5%) was added to 3.0 ml. of 0.1 N- I_2 and 2.0 ml. of 0.5 N- Na_2CO_3 contained in a 50 ml. glass-stoppered flask. The solution was kept at 22° in the dark for 7 hr., after which time oxidation was essentially complete and the serological activity of the A substance was largely destroyed. The oxidized mucoid was dialysed against distilled water at 0°, made up to a known volume and 500 μ g. were treated with alkali according to the conditions described in the standard procedure for the determination of *N*-acetylglucosamine. The oxidized and alkali-treated material showed a maximum absorption at 230 $m\mu$, but the intensity of absorption was less than that given by an equal quantity of unoxidized material. The addition of the DMAB reagent gave rise to a purple-coloured solution which showed two absorption maxima, at 550 and at 590 $m\mu$. A quantitative measurement of the '*N*-acetylglucosamine' colour revealed that the A substance after oxidation with hypiodite gave rise to about 80% of the colour intensity reached by the original A substance. A detailed account of the oxidation of the blood-group mucoids with hypiodite will be given elsewhere.

DISCUSSION

The conditions usually employed for the colorimetric determination of the *N*-acetylhexosamines have been re-examined in some detail, and it is evident from the results obtained that these substances undergo rapid structural change on treatment for a short time with hot dilute alkali or alkaline buffers, and that in each instance a substance is formed which shows strong light absorption between 220 and 250 $m\mu$. with a maximum absorption at about 230 $m\mu$. The gradual disappearance of the absorption maximum observed, however, indicates that the substance formed by the action of alkali is itself unstable in alkaline solution.

The substances formed from the *N*-acetylhexosamines by the action of dilute alkali condense readily with *p*-dimethylaminobenzaldehyde in acetic acid to yield a solution of intense purple colour which shows characteristic absorption maxima at 550 and 590 $m\mu$. with a minimum at about 570 $m\mu$. (cf. Osaki & Turumi, 1947; Gottschalk & Lind, 1949). In view of the unstable nature of the chromogenic material, it is essential for the quantitative determination of *N*-acetylhexosamine that the *p*-dimethylaminobenzaldehyde reagent should be added and the test completed immediately the solutions under examination have been heated with alkali.

Treatment of the *N*-acetylhexosamines with alkali under different conditions has revealed that increasing the concentration of Na_2CO_3 results not only in a more rapid formation of the chromogenic structure, but also brings about its more rapid destruction and, although the period of heating

necessary to yield the maximum colour after the addition of the *p*-dimethylaminobenzaldehyde reagent becomes shorter with increasing alkali concentration, the actual amount of colour obtained decreases with higher concentration of alkali. Extrapolation of that part of the curve which measures the rate of destruction of the chromogenic material gives a corrected value for the maximum colour intensity found. The corrected value obtained, however, is of no great accuracy. It will be noticed that the alkali concentration finally recommended as suitable for the quantitative determination of the *N*-acetylhexosamines is not that which gives rise to the minimum destruction of the chromogenic substances and consequently to greatest colour intensity per unit weight of the *N*-acetylhexosamines. The alkali concentration finally chosen was selected so that any slight buffer action of the solution under test would not alter significantly the pH of the solution during treatment with alkali which should be close to pH 10.8. Treatment of the *N*-acetylhexosamines with borate buffer at pH 10.8 gives rise to an enhanced colour production as compared with that obtained using sodium carbonate at the same pH.

The interaction in an alkaline medium of simple hexoses and certain amino-acids with the formation of a substance that gives with *p*-dimethylaminobenzaldehyde reagent a colour which closely resembles that given by *N*-acetylglucosamine after similar treatment was first reported by Vasseur & Immers (1949) and subsequently confirmed by Gottschalk & Partridge (1950). The light absorption given by a mixture of lysine and glucose after treatment with dilute alkali has now been determined, and it has been found that within the range 210–300 $m\mu$. there is no characteristic absorption maximum similar to that given by the *N*-acetylhexosamines. The purple-coloured solution which is formed on the addition of the *p*-dimethylaminobenzaldehyde reagent to the alkali-treated glucose-lysine mixture shows a single absorption maximum at 560 $m\mu$. and not two maxima at 550 and 590 $m\mu$. as is shown by the *N*-acetylhexosamines after identical treatment. The product of the interaction of glucose and lysine, therefore, although giving a reddish purple colour with *p*-dimethylaminobenzaldehyde is nevertheless readily distinguished from that which arises from the *N*-acetylhexosamines. The colour given in the 'Direct Test' of Osaki & Turumi (1947) appears to be due to an amino-acid-sugar complex and not, as they suggest, to a different ring structure associated with *N*-acetylhexosamine.

Morgan & King (1943) reported that a purified mucoid which was obtained from hog gastric mucin and which possessed intense human blood-group A activity, gave a colour reaction after treatment with

dilute alkali and *p*-dimethylaminobenzaldehyde similar to that given by *N*-acetylglucosamine. Subsequently it was suggested (Morgan & Waddell, 1945; Morgan, 1947) that this colour reaction was a characteristic property of the blood-group mucoids. It is now known that solutions of essentially homogeneous preparations of the blood-group mucoids which are responsible for the group characters A, B, the recently discovered 'Lewis' Le^a property and the so-called O (H) character of the tissue fluids and secretions, all develop a purple colour after treatment with dilute alkali and the addition of the *p*-dimethylaminobenzaldehyde-hydrochloric acid reagent. In each instance the maximum light absorption of the coloured solution is found to be at about 550 and 590 m μ ., whereas the solutions of the material after alkali treatment alone show a single absorption maximum at 230 m μ . In both these respects, therefore, the group substances behave as do the *N*-acetylhexosamines.

The blood-group substances require a minimum of about 12 min. heating with alkali to yield the maximum amount of colour with the *p*-dimethylaminobenzaldehyde reagent in place of the 4 min. heating necessary for the *N*-acetylhexosamines. The intensity of the colour given by the group substances under these conditions varies somewhat for each group substance investigated, but is found to be equivalent to between 7 and 10 % of the colour intensity given by an equal weight of *N*-acetylglucosamine. It is to be noted that there is no enhancement of colour production when a borate buffer of the same pH value is employed.

Group A substance rendered serologically inactive by oxidation with sodium periodate at pH 5 continues to yield a strong purple colour after treatment with alkali and the addition of DMAB reagent (Aminoff & Morgan, 1951). It is now shown that A substance after oxidation with periodate and treatment with alkali shows a single absorption maximum at 230 m μ . and that the purple colour which subsequently arises after the addition of the DMAB reagent also shows absorption maxima of undiminished intensity at 550 and 590 m μ . The A substance after oxidation with periodate therefore behaves as does the unoxidized material. Aminoff & Morgan (1951) considered that oxidation of the A substance with periodate left unchanged the reducing group and the *N*-acetyl group of the *N*-acetylchondrosamine end unit, to allow for the formation of the chromogenic structure on treatment with alkali, and the results of the absorption studies now reported with A substance oxidized with periodate appear to support this suggestion.

Oxidation of the A substance with sodium hypiodite, on the other hand, forms a substance which gives a reduced '*N*-acetylglucosamine' colour

value after alkali treatment and the addition of *p*-dimethylaminobenzaldehyde. It seems probable that oxidation of the A substance with hypiodite converts the aldehyde group of the *N*-acetylchondrosamine residue present as a reducing end group into a carboxyl group. Such a change would result in the end residue being unable to form a chromogenic structure on treatment with alkali and in consequence the oxidized A substance would yield with *p*-dimethylaminobenzaldehyde a solution of diminished colour intensity. As *N*-acetylchondrosamine gives about one-fifth of the amount of colour given by *N*-acetylglucosamine it must be concluded that *N*-acetylglucosamine contributes to the total colour given by the A substance. Aminoff & Morgan (1949, 1951) suggested that the terminal *N*-acetylchondrosamine residue is linked to C atom 1 of its neighbouring *N*-acetylglucosamine residue and that treatment of the A substance with alkali under the conditions used for the determination of *N*-acetylhexosamine hydrolyses this glycoside linkage and thus transforms the penultimate *N*-acetylglucosamine into a reducing end group and renders it susceptible to the action of alkali with the consequent formation of a chromogenic structure which gives a coloured complex with *p*-dimethylaminobenzaldehyde (cf. Morgan, 1946). If the total colour given by the A substance after treatment with alkali and *p*-dimethylaminobenzaldehyde arises in this way from *N*-acetylchondrosamine and *N*-acetylglucosamine units then the decrease in the colour intensity to be expected from A substance after treatment with hypiodite will be about 18 % of the original value expressed in terms of *N*-acetylglucosamine. A decrease of this order has been found. The action of alkaline hypiodite and of dilute alkali on such complex materials as the blood-group mucoids is, however, far from straightforward, and the results of further work must be awaited before the changes brought about can be fully understood or the results obtained used to establish the presence of definite structures within the blood-group mucoids.

SUMMARY

1. The conditions for the colorimetric determination of the *N*-acetylhexosamines have been re-investigated.

2. The period of heating necessary, and the maximum amount of colour obtained, when *N*-acetylglucosamine is treated with alkali and the *p*-dimethylaminobenzaldehyde reagent, depends not only on the pH of the system but also on the nature of the buffer employed. Borate buffer gives an enhanced colour production.

3. *N*-Acetylchondrosamine behaves similarly, but the intensity of the colour given is about 23 %

of that obtained with an equal weight of *N*-acetylglucosamine.

4. The *N*-acetylhexosamines after treatment with alkali show a single maximum absorption at 230 m μ . and after the addition of the *p*-dimethylaminobenzaldehyde reagent the purple colour which develops shows two absorption maxima, at 550 and at 590 m μ .

5. A mixture of glucose and lysine heated with sodium carbonate shows no characteristic absorption maximum at 230 m μ . The purple colour obtained on the subsequent addition of *p*-dimethylaminobenzaldehyde reagent shows a single absorption maximum at about 560 m μ .

6. The human blood-group mucoids yield between 7 and 10 % of the colour intensity given by an equal weight of *N*-acetylglucosamine, and there is no enhanced colour production when a borate buffer is employed. The absorption characteristics of the chromogenic substances which arise from the group substance after heating with alkali, and of the coloured product obtained on the addition of the

p-dimethylaminobenzaldehyde reagent support the belief that the colour given by the group substances arises from reducing *N*-acetylchondrosamine end groups and closely linked *N*-acetylglucosamine residues, which are readily liberated by the action of alkali.

7. The A substance, after oxidation with hypiodite, gives rise to a coloured complex on treatment with alkali and *p*-dimethylaminobenzaldehyde which shows a reduced absorption intensity compared with that given by the unoxidized material. A substance oxidized with periodate and treated under similar conditions shows no decrease in absorption. The significance of these findings is discussed.

The investigation was supported by personal grants made to two of the authors (W.M.W. and D.A.) by the Medical Research Council and the Governing Body of the Lister Institute respectively. The authors are indebted to the University of London Research Fund for a grant to purchase the Uvispek spectrophotometer used. We wish to acknowledge our indebtedness to Miss V. Hayes and Miss J. Pugh for valuable technical assistance.

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A Study of the Breakdown of Ribonucleic Acid in Tobacco-leaf Extracts

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(Received 18 October 1951)

There is an extensive literature on the preparation of ribonucleic acids and their degradation products from animal tissues and micro-organisms. By contrast, apart from the isolation of certain plant virus nucleoproteins, little work has been undertaken until recently, on the preparation of ribonucleic acids from plant material.

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Foreman (1938) suggested the presence of nucleoprotein in leaves of perennial rye grass, to explain the fact that cytolysed leaves yielded only 30 % of their phosphorus on extraction with boiling water, whereas leaves previously dried at 85° yielded all their phosphorus by cold water extraction. The occurrence in, and isolation of, ribonucleic acid from leaves of barley, rye and spinach has been reported by von Euler & Hahn (1947, 1948). These