SUMMARY

1. The excretion of neutral 17-ketosteroids in eight normal young men and twenty normal young women has been estimated in duplicate both by the polarographic method and by the Zimmermann-Callow colorimetric technique.

2. The colorimetric method gave somewhat higher results than the polarographic method and its standard deviation was also higher. There is some evidence that the polarographic method shows a lower variation due to the errors of technique than the colorimetric method.

3. These values, compared with available data published by other workers on normal 17-ketosteroid excretion, would seem to be higher than any other figures published.

The authors would like to express their thanks to the subjects who co-operated in the collection of 24 hr. specimens.

REFERENCES

Barnett, J., Henly, A. A. & Morris, C. J. O. R. (1946). *Biochem. J.* **40**, 445.
Baumann, E. J. & Metzer, H. (1940). *Endocrinology*, **27**, 664.
Cahen, R. L. & Salter, W. R. (1944). *J. biol. Chem.* **152**, 489.
Callow, N. H., Callow, R. K. & Emmens, C. W. (1938). *Biochem. J.* **32**, 1312.
Callow, N. H., Callow, R. K., Emmens, C. W. & Stroud, S. W. (1939). *J. Endocrinol.* **1**, 76.
Chou, C. Y. & Wang, C. W. (1939). *Chin. J. Physiol.* **14**, 151.
Cuyler, W. K. & Baptist, M. (1941). *J. Lab. clin. Med.* **26**, 38.
Cuyler, W. K., Hamblen, E. C., Baptist, M. & Salmon, A. A. (1942). *J. clin. Endocrinol.* **2**, 318.
Fraser, R. W., Forbes, A. P., Albright, F., Sulkowitch, H. & Reifenstein, E. C. (1941). *J. clin. Endocrinol.* **1**, 234.
Oesting, R. B. (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 524.
Oesting, R. B. (1938). *Proc. Soc. exp. Biol., N.Y.*, **39**, 76.
Patterson, J., McPhee, I. & Greenwood, A. W. (1942). *Brit. med. J.* **1**, 35.
Pincus, G. (1943). *Endocrinology*, **32**, 176.
Pincus, G. & Hoagland, H. (1943). *J. Aviat. Med.* **14**, 173.
Salter, W. R., Cahen, R. L. & Sappington, T. S. (1946). *J. clin. Endocrinol.* **6**, 52.
Wolfe, J. K., Hershberg, E. B. & Fieser, L. F. (1940). *J. biol. Chem.* **136**, 653.
Zimmermann, W. (1935). *Hoppe-Seyl. Z.* **233**, 257.
Zimmermann, W. (1936). *Hoppe-Seyl. Z.* **245**, 47.

An Analysis of the Products Formed During the Hydrolysis of Hyaluronate by Enzymes and Acids, with Observations on the Nature of the Amino-sugar Released from the Polysaccharide

By H. J. ROGERS (Beit Memorial Research Fellow)
The Lister Institute of Preventive Medicine, Elstree, Herts.

(*Received* 31 *May* 1946)

It has been shown (Rogers, 1945, 1946) that after the hydrolysis at pH 7·0 of purified potassium hyaluronate by either partially purified or crude hyaluronidase from bull testis, substances, non-diffusible through cellophan, remain in the hydrolysate which can adaptively increase hyaluronidase production by multiplying streptococci. No such substances remain after hydrolysis of the polysaccharide at pH 7·0 by hyaluronidase produced by either *Cl. welchii* or streptococci.

In the present paper these non-diffusible constituents of the hydrolysates are shown to differ chemically according to the source of the enzyme used for hydrolysis. This result indicates the probability that more than one enzyme is involved in the complete hydrolysis of hyaluronate. A similar conclusion had been reached previously by several authors (Madinaveitia & Quibell, 1940; East, Madinaveitia & Todd, 1941; Meyer, Chaffee, Hobby & Dawson, 1941; Hahn, 1945*a*). Since the work described in the present paper was completed, however, definite evidence has been published (Hahn, 1945*b*, 1946*a*, *b*) which indicates the presence in crude preparations from mammalian testis of two enzymes which, when acting at pH 4·6, can together degrade hyaluronate to monosaccharides. The pH of 4·6 has been shown to be near the optimum for the activity of testicular hyaluronidase when concentrated buffers are used (Madinaveitia & Quibell, 1940; Meyer *et al.* 1941; McClean, 1943). Hydrolysis was carried out at pH 7·0 and in dilute buffer and salt solutions in the present work, because these were the conditions used in the biological studies (Rogers, 1945, 1946) and have been shown (McClean, 1943)

to be close to the optimum for the viscosity reducing activity of at least two of the three hyaluronidases at present being studied. The work to be reported suggests the possibility that the viscosity reducing enzyme, active under McClean's (1943) conditions, is different from either of the enzymes studied by Hahn (1945*b*; 1946 *a*, *b*) which together degrade hyaluronate to monosaccharides.

During the course of these investigations the observations of Meyer *et al.* (1941) and of Humphrey (1946) concerning the release of amino-sugars during enzymic hydrolysis of hyaluronate were confirmed. These authors observed that the apparent amount of *N*-acetylglucosamine released and measured colorimetrically was much greater than could be accounted for, either by the amount of reducing sugar liberated, or even sometimes by the total hexosamine content of the polysaccharide. Humphrey (1946) also showed that substances were present in enzymic hydrolysates which gave a colour with the *p*-dimethylaminobenzaldehyde reagent (to be called Ehrlich's reagent) used in Morgan & Elson's (1934) colorimetric estimation of *N*-acetylhexosamines before alkali treatment. Hahn (1945*a*) observed that the products of enzymic hydrolysis of hyaluronate give a purple colour with Ehrlich's reagent after very much milder treatment with alkali than does pure *N*-acetylglucosamine. In the absence of exact knowledge of the nature of the amino-sugar derivative present in enzymic hydrolysates of hyaluronate and of the reactions involved in its colorimetric estimation, it will be referred to in this paper as *N*-acetylhexosamine.

METHODS

For the estimation of glucosamine and reducing sugar the standard methods referred to elsewhere were used (cf. Rogers, 1946). The amount of colour produced by the hydrolysates, or fractions from them, when tested by the unmodified Morgan & Elson (1934) test, has been compared with that formed, under the same conditions, by known amounts of a sample of synthetic-*N*-acetylglucosamine (Zuckerkandl & Messiner-Klebermass, 1931); the buffer concentration present in the samples and in the *N*-acetylglucosamine standard solution was adjusted to be the same throughout any one test. The value obtained will be referred to as mg. of *N*-acetylhexosamine.

The potassium hyaluronate used was isolated from umbilical cords and purified as described previously (Rogers, 1945).

The preparation of enzymic hydrolysates

A solution of purified hyaluronate was mixed with 0·1 M-phosphate-citrate buffer (pH 7·0) containing 0·6 M-NaCl, the appropriate hyaluronidase preparation added and the mixture incubated at 37° in the presence of chloroform. The enzymes were purified by the methods described elsewhere (Rogers, 1946). Table 1 shows the amount of the three enzymes used and their degree of purity. When testicular enzyme was used incubation was continued until there was no further release of reducing sugars. In order to bring about a comparable degree of hydrolysis by the three hyaluronidases the action of the two bacterial enzyme preparations was stopped as soon as about 50 % of the theoretical amount of reducing sugar had been released from the polysaccharide. The hydrolysates obtained will be called HT (testicular enzyme used), HS (streptococcal enzyme used) and HW (*Cl. welchii* enzyme used). At least two samples of HT, HS and HW were prepared and analyzed. The results reported are the mean of the figures obtained.

The hydrolysates prepared as described above were placed in 'Visking' sausage casing and dialyzed against changes of distilled water at 0–4° until no further fall in the amount of reducing sugars present in the dialysate (i.e. material inside the dialysis sac) could be detected. The combined diffusates were then concentrated *in vacuo* (25°) to the initial volume of the hydrolysates. The enzymes were not inactivated before dialysis since the heat treatment

Table 1. *The preparation of the hydrolysates used, by the action of different preparations of enzymes on hyaluronate*

The degree of hydrolysis of the substrate was measured by the amount of reducing sugar liberated.
Substrate mixture: 0·25 g. hyaluronate in 50 ml. water + 12·5 ml. 0·1 M-phosphate-citrate buffer (pH 7·0) containing 0·6 M-NaCl.
Incubation at 37°; chloroform present.
V.R.U. = viscosity reduction units (McClean & Hale, 1941).

Source of enzyme	Amount of enzyme added (V.R.U.)	V.R.U./mg. total N	Hydrolysis of hyaluronate (%)	Symbol for the corresponding hydrolysate*
Bull testes	4800	300	52·3	HT
Streptococcus C 7	4350	4,550	45·8	HS
Cl. welchii S 107	4400	58,100	52·8	HW

* Symbols used in text and in Tables 2 and 3.

necessary for this might change the substances in the hydrolysates. Balance sheets after dialysis proved that further hydrolysis had not proceeded during the period of dialysis.

Estimations made on the hydrolysates

The amounts of reducing sugar, *N*-acetylhexosamine and glucosamine were determined in the hydrolysates and in the diffusates and dialysates derived from them. The '*N*-acetylglucosamine' values were corrected for the *N*-acetylhexosamine equivalent of the colour produced when the enzymic hydrolysates and fractions from them were mixed with glacial acetic acid and Ehrlich reagent (Morgan & Elson, 1934), without pretreatment with alkali (Humphrey, 1946). The amount of colour produced under these conditions accounted for 10–25 % of the total amount of colour produced by the diffusates from the various hydrolysates in the ordinary Morgan & Elson (1934) test.

RESULTS

The non-diffusible components

Table 2 records the amounts of reducing sugar, glucosamine and *N*-acetylhexosamine remaining inside the dialysis sacs as a percentage of the total amounts in three typical hydrolysates. The total amounts of reducing sugar and *N*-acetylhexosamine present in the hydrolysates were calculated from the glucosamine content of the hyaluronate sample used.

Table 2. *The amounts of non-diffusible substances present in enzymic hydrolysates of hyaluronate recorded as percentages of the total amounts in the hydrolysates*

	Total amounts in hydrolysate			Non-diffusible substances		
	Glucosamine (mg./ml.)	Reducing sugar (mg./ml.)	*N*-Acetylhexosamine (mg./ml.)	Glucosamine (%)	Reducing sugar (%)	*N*-Acetylhexosamine (%)
HT*	1·04	1·26	0·81	46·7	19·2	51·5
HS†	1·27	1·42	2·27	20·4	$\ll 0{\cdot}5$	$\ll 1$
HW‡	1·27	1·54	1·62	55·0	9·8	16·3

* Testicular hyaluronidase used for hydrolysis.
† Streptococcal hyaluronidase used for hydrolysis.
‡ *Cl. welchii* hyaluronidase used for hydrolysis.

The non-diffusible components in the hydrolysate HT are obviously different from those present in HS. HT contains units which, though too large to diffuse through cellophan, still react with Ehrlich's reagent after pretreatment with 0·04 M-Na_2CO_3 under the conditions of Morgan & Elson's (1934) test giving a deep purple colour. These non-diffusible units in HT appreciably reduce the Somogyi (1937) alkaline copper reagent. Hydrolysis by streptococcal enzyme leaves non-diffusible units which fail to react with Ehrlich's reagent under these conditions, and which do not reduce Somogyi's (1937) reagent. Only 20·4 % of the hexosamine originally present in the polysaccharide is non-diffusible in HS compared with 46·7 % in HT and 55·0 % in HW. This very much lower result for HS in the absence of a greater liberation of reducing substances from the hyaluronate can only be explained by supposing the formation of diffusible units larger than simple monosaccharides. Such units, containing sugar derivates linked through their 1-position, would have a diminished reducing power as compared with free monosaccharides. This suggestion is supported by an examination of the diffusible constituents in HS as will be shown later.

The non-diffusible components were also examined in other hydrolysates prepared by the use of streptococcal and testicular enzymes but with somewhat different conditions for hydrolysis. For example, when testicular hyaluronidase acted at pH 7·0 upon partially purified hyaluronate (McClean, 1943) in the absence of added salt only 18–20 % of the theoretical amount of reducing sugar was liberated when hydrolysis had ceased. Of the *N*-acetylhexosamine and reducing sugar liberated, 60·4 and 58·3 % respectively were non-diffusible. In other hydrolysates prepared with this enzyme, but with it acting in the presence of 0·12 M-NaCl, and in very high concentration, 78–80 % of the theoretical amount of reducing sugar was liberated. In such a hydrolysate 20·9 % of the *N*-acetylhexosamine and 23·8 % of the reducing sugar were non-diffusible. In hydrolysates prepared with crude streptococcal enzyme acting under the usual conditions, but in which incubation was continued until hydrolysis had ceased, 80–83 % of the theoretical amount of reducing sugars were liberated, of which only a trace was non-diffusible; the amount of non-diffusible glucosamine was again lower than in the hydrolysate prepared by testicular enzyme, which contained a similar amount of reducing sugar.

The non-diffusible units remaining in HW show properties intermediate between those of the substances present in HT and HS.

The diffusible components

The diffusates from all three hydrolysates in which about 50% of the theoretical amount of reducing sugar had been liberated gave more colour in the *N*-acetylglucosamine test than would have been expected from either the amount of hexosamine estimated after hydrolysis with 4 N-HCl or from reducing sugar estimations (see Table 2). As Humphrey (1946) pointed out, the amount of *N*-acetylhexosamine varied according to the source of the enzyme which had been used for hydrolysis of the hyaluronate. In all three hydrolysates, the substance reacting to give a colour with Ehrlich's reagent without treatment with alkali (Humphrey, 1946) was present in the diffusible fractions and not in the dialysates.

If the diffusible substances in the hydrolysates consist only of monosaccharides, then the ratios of the amount of glucosamine released by strong acid hydrolysis of the diffusates to the reducing sugar content should be 1/2·38 (potassium hyaluronate contains 41·7% glucosamine). Table 3 shows the ratios actually obtained. It will be observed that this ratio in the diffusate from HS is lower than that for this fraction in either HT or HW. This result confirms the presence therein of composite saccharide units as had already been suggested by the low total amount of hexosamine remaining non-diffusible in HS.

Table 3. *The ratios (A) apparent/calculated amount of N-acetylglucosamine and (B) the amount of reducing sugar/the amount of glucosamine, in diffusates from enzymic hydrolysates of hyaluronate. Column C shows the effect upon ratio A of heating the diffusates at 99° in* N*-acetic acid*

Hydrolysates from which diffusate was produced	(A) *N*-Acetylhexosamine / *Calculated *N*-acetylglucosamine	(B) Reducing sugar / Glucosamine	(C) (A) after heating in N-acetic acid†
HS‡	2·40	1·60	0·97
HW‡	2·07	2·24	0·95
HT‡	1·26	2·18	1·04
Theory	1·0	2·38	—

* Calculated from the amount of hexosamine present in the diffusate.
† Diffusate heated for 4 hr. with N-acetic acid.
‡ See Table 1.

The course of hydrolysis of hyaluronate by mild treatment with acids

When hyaluronate is hydrolyzed by 2–4 N-HCl at 100°, glucosamine and an organic acid which is probably acetic acid are rapidly released. The hexuronic acid constituent of the polysaccharide is broken down to CO_2, furfuraldehyde and other degradation products. In the course of experiments described previously (Rogers, 1946) a less destructive method of hydrolysis was required. Morgan (1936) showed that hydrolysis of the *B. dysenteriae* (Shiga) (*Shigella dysenteriae*) polysaccharide by 0·01 N-HCl at the temperature of a boiling water-bath, yields *N*-acetylglucosamine. Hydrolysis of hyaluronate with N-acetic acid under reflux, at the temperature of a boiling water bath, was found similarly to yield a substance reacting to give a purple colour with Ehrlich's reagent under the conditions used by Morgan & Elson (1934). Fig. 1 shows the course of hydrolysis of a solution (0·5 g./100 ml.) of partially purified potassium hyaluronate (containing 70·8% of the theoretical amount of glucosamine) by N-acetic acid when heated in sealed ampoules at the temperature of a boiling water-bath. The degree of hydrolysis of the polysaccharide was estimated by the amount of colour produced by 0·2 ml. samples

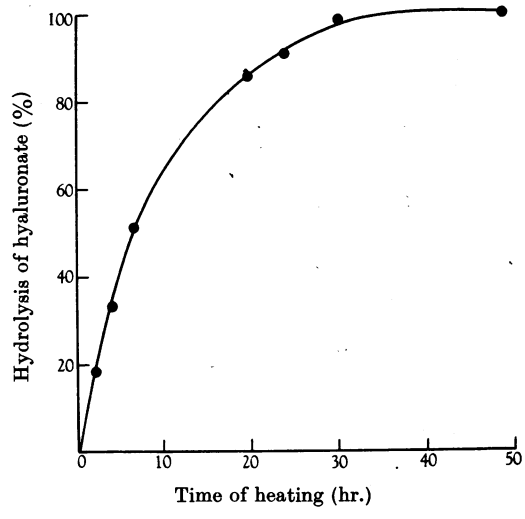

Fig. 1. The hydrolysis of potassium hyaluronate (solution containing 0·45 g./100 ml. of a partially purified preparation estimated to be 70% pure) by N-acetic acid at 99°. The degree of hydrolysis is expressed as the percentage of the expected amount of *N*-acetylglucosamine released. The pH value of the reaction mixture used was 2·66.

taken from the mixtures, neutralized, adjusted to a volume of 1 ml. and compared in the Morgan & Elson (1934) test with the colour formed by known amount of *N*-acetylglucosamine dissolved in neutralized 0·2 N-acetic acid. The pH of the hydrolysis mixtures for which this curve was constructed was found by the use of the glass electrode to be 2·66. It will be seen that after about 30 hr. treatment under these conditions 95–100 % of the *N*-acetylglucosamine contained in the polysaccharide had been liberated. It was found that only 54 % of the theoretical amount of reducing sugar was released after 30 hr. hydrolysis. The interpretation of this result is, however, difficult since it was found that when a solution containing pure glucuronic acid and *N*-acetylglucosamine was heated under the same conditions (i.e. with N-acetic acid adjusted to pH 2·7) the amount of reducing sugar decreased by 47 %. This fall was entirely due to destruction of the glucuronic acid. There was no decrease in the amount of *N*-acetylglucosamine present in the mixture after heating.

The nature of the N-acetylhexosamine

Hahn (1945*a*) has shown that some products of the hydrolysis of hyaluronate by *Cl. welchii* or testicular hyaluronidase form maximum amounts of colour with Ehrlich's reagent after 3·4 min. heating at 100° with 0·05 M-$NaHCO_3$. *N*-Acetylglucosamine he found to require 30–60 min. treatment under these conditions for maximum colour formation. This difference the author attributed to more rapid ring formation by the substance in the enzymic hydrolysates. Morgan (1936, 1938) and White (1940) have shown that when heated in dilute alkalis *N*-acetylglucosamine forms a heterocyclic ring compound which is probably an oxazoline or oxazole. It is most probably a substance of this type which reacts with Ehrlich's reagent to form a purple colour.

An examination was made of the rate of formation of substances giving the colour with Ehrlich's reagent when fractions from HS, HW and HT were treated with 0·04 M-$NaHCO_3$ at the temperature of a boiling water-bath. A solution of $NaHCO_3$ (0·1 ml. of 0·5 M) was added to each of four sets of tubes (set Nos. 1–4) containing the following amounts of fractions from the hydrolysates or of *N*-acetylglucosamine:

dialysate from HT 0·48 ml. (set No. 1),
diffusate from HS 0·06 ml. (set No. 2),
diffusate from HW 0·16 ml. (set No. 3),
N-acetylglucosamine 0·2 mg. (set No. 4).

The volume in each tube was adjusted to 1·0 ml. before the addition of the alkali. Each tube had a small condenser attached. Pairs of tubes from each set were removed from the bath at intervals (see Fig. 2) and cooled at once for 5–10 min. in ice-cold water. Glacial acetic acid and Ehrlich's reagent (Morgan & Elson, 1934) were then added. After a period of 1 hr. the amount of colour which had developed in the tubes heated for various times were compared with that in suitable selected tubes from a set (No. 5) containing various amounts of *N*-acetylglucosamine which had been treated with 0·04 M-Na_2CO_3 for 4 min. by the standard Morgan & Elson (1934) technique. The tubes were selected from set No. 5 so that the colour therein corresponded within ± 20 % of that in the particular test solution. Thus the results obtained for colour developments in set Nos. 1–4 could be expressed in terms of the amount of colour given by known amounts of *N*-acetylglucosamine treated by the usual procedure for estimating this substance colorimetrically. Fig. 2 shows the results obtained from these experiments. It will be seen in confirmation of Hahn's (1945) work that the maximum amount of colour is produced by the hydrolysate fractions after only 3–4 min. heating. *N*-Acetylglucosamine, in contrast, required about 30 min. The results recorded in Fig. 2 are expressed in terms of *N*-acetylglucosamine.

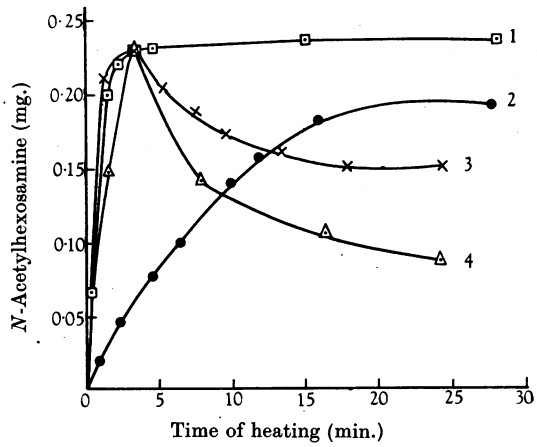

Fig. 2. The rate of production of substances forming colour with Ehrlich's reagent from synthetic *N*-acetylglucosamine and various fractions from enzymically hydrolyzed hyaluronate during heating with 0·04 M-$NaHCO_3$ at 98°. 1, dialysate from HT ⊡—⊡; 2, synthetic *N*-acetylglucosamine ●—●; 3, diffusate from HS ×—×; 4, diffusate from HW △—△.

Hydrolysis of hyaluronate by mild treatment with acids releases exactly 100 % of the amount of *N*-acetylglucosamine to be expected from the hexosamine content of the polysaccharide, yet hydrolysis with *Cl. welchii* or streptococcal hyaluronidase appears to release two to three times as much *N*-acetylhexosamine (see Table 3) as would be expected. When the diffusates from HS, HW and HT were heated in a boiling water-bath with N-acetic acid

(pH of these mixtures was 2·60) the reason for this discrepancy became apparent. Fig. 3 shows that this treatment rapidly decreases the amount of colour given by the substances with Ehrlich's reagent after treatment with alkali, as in the Morgan & Elson (1934) test.

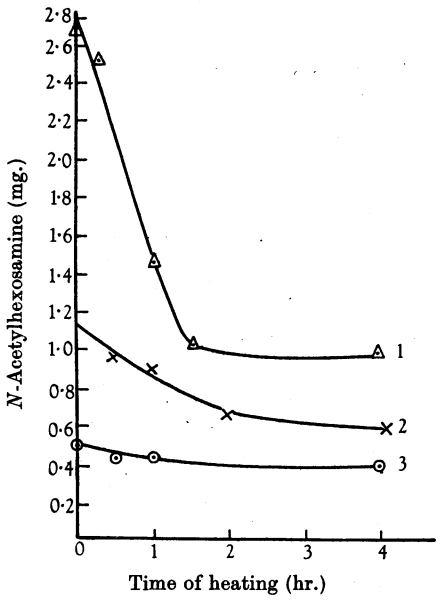

Fig. 3. The effect of heating the diffusible fractions from enzymically hydrolyzed hyaluronate with N-acetic acid upon the amount of colour produced in the Morgan & Elson (1934) test. The pH value of the mixture used was 2·70. 1, diffusate from HS △—△; 2, diffusate from HW ×—×; 3, diffusate from HT ⊙—⊙.

It is probably significant that in all three samples the amount of colour obtained in the *N*-acetylglucosamine test after 4 hr. heating with N-acetic acid corresponded to within 5% of the amount of *N*-acetylglucosamine calculated to be present from the amount of glucosamine present in the completely hydrolyzed diffusates.

Table 3 summarizes these results. The value for the ratio of observed to calculated amounts of *N*-acetylglucosamine is very much lower in the untreated diffusate from HT. When this diffusate was heated with N-acetic acid there was only a slight fall in the amount of colour produced in the Morgan & Elson (1934) test.

When the diffusates, which had been treated for 4 hr. with N-acetic acid in a boiling water-bath, were heated with 0·04 M-$NaHCO_3$, the rate at which substances which form the colour with Ehrlich's reagent were produced corresponded with that for pure *N*-acetylglucosamine.

DISCUSSION

The enzymic hydrolysis of macromolecules, such as those present in a solution of hyaluronate, is likely to be a complex process. The results obtained from studies of the enzymic hydrolysis of other polysaccharides, such as starch, have indicated that the substrate may consist of units joined into different configurations (e.g. amylose and amylopectin) and that several enzymes may be responsible for the ultimate degradation of the polysaccharide to simple mono- or disaccharides (e.g. α and β amylase and maltase). In this paper the products formed during the hydrolysis of hyaluronate at pH 7·0 by hyaluronidase from one mammalian and two bacterial sources, have been examined. In the various hydrolysates the degree of hydrolysis of the polysaccharide was adjusted so that about 50% of the theoretical amount of reducing sugar had been released (i.e. assuming that the polysaccharide consists of equimolar amounts of *N*-acetylglucosamine and glucuronate). The results obtained show that under the conditions used the three hyaluronidases liberate units of different average sizes with various reducing capacities. The streptococcal enzyme, for example, releases freely diffusible reducing sugar and leaves no reducing oligosaccharides, whereas testicular hyaluronidase leaves non-diffusible units which account for as much as 20% of the liberated reducing sugar. Thus no simple unit of enzyme activity based on liberated reducing sugar can satisfactorily be used as a comparison of the potency of hyaluronidases from different sources.

Hahn (1945*b*, 1946 *a*, *b*) found that at pH 4·6 one testicular enzyme degraded hyaluronate to disaccharides whilst a second completed hydrolysis to monosaccharides. Both of these products were, of course, freely diffusible, and so different from the products formed during hydrolysis at pH 7·0 with the salt concentrations used here. The most probable explanation of this difference seems to be that each preparation of hyaluronidase consists of several enzymes responsible for the various stages of the hydrolysis of hyaluronate. The optimal pH and salt concentration for the activity of each of these enzymes may vary according to the source from which it is isolated. In support of this it may be pointed out that Hahn (1945*b*) says that at pH 4·6 *Cl. welchii* hyaluronidase preparations do not degrade the polysaccharide to monosaccharides, i.e. these preparations contain no 'oligosaccharase'. At pH 7·0, however, and with the salt and buffer concentrations used in the present studies, a large proportion of the reducing sugar liberated by this enzyme appears to be monosaccharides. Moreover, Meyer *et al.* (1940) showed that their *Cl. welchii* preparation liberated 91% of the theoretical amount of reducing sugar when acting at pH 6·0 close to the

optimum pH of 5·8 determined under their salt and buffer conditions. Thus the hyaluronidases with optima around pH 7·0 (McClean, 1943), which decrease the viscosity of hyaluronic acid, may well be distinct from either of the polysaccharases studied by Hahn.

The rate of production of substances which form the colour with Ehrlich's reagent, during treatment of the hydrolysates with $NaHCO_3$ confirm the results obtained by Hahn (1945*a*) and suggest the possibility that colour formation is not due to *N*-acetylglucosamine in its usual form. The results obtained here suggest that this substance may be readily converted into *N*-acetylglucosamine by mild treatment with acids. It is important to note, however, that although the conditions of acid treatment used would not perceptibly de-acetylate *N*-acetylglucosamine, acetylhexosamine was released from hyaluronate under these same conditions and so glycosidic bonds involving *N*-acetylglucosamine would most probably be hydrolyzed; the amount of colour given by *N*-acetylglucosamine when combined, for example, into a disaccharide through some bond not involving carbon atom 1, is as yet unknown. The isolation by Meyer & Palmer (1936) of 55% of the theoretical amount of glucosamine from hyaluronate after hydrolysis with 2 N-HCl does not necessarily conflict with the suggestion that the constituent *N*-acetylhexosamine of the original polysaccharide is not *N*-acetylglucosamine itself, since it may have been changed into this amino-sugar derivative by the drastic acid treatment necessary for hydrolysis. Recently, however, Morgan (private communication), has shown that solutions of pure *N*-acetylglucosamine itself give different amounts of colour with Ehrlich's reagent, according to the pH used for pre-treatment. Moreover, he finds that the rate and intensity of colour production are different when different buffers of the same pH are used. Therefore, without further study of the changes undergone by *N*-acetylglucosamine in alkaline solution, far-reaching conclusions are not justified.

SUMMARY

1. The hydrolysis of hyaluronate at pH 7·0 by hyaluronidase obtained from one mammalian and two bacterial sources yields products which are chemically distinguishable. These results correlate with the different adaptive enzyme effects on micro-organisms of the various hydrolysates.

2. The relation of the results obtained to the possible complexity of the enzyme system hitherto known as hyaluronidase is discussed.

3. Observations are recorded of the apparent differences between the behaviour of the substance called here acetylhexosamine, present in enzymic hydrolysates of hyaluronate; and *N*-acetylglucosamine itself.

I wish to thank Dr W. T. J. Morgan for his interest and encouragement during this part of the work.

REFERENCES

East, M. E., Madinaveitia, J. & Todd, A. R. (1941). *Biochem. J.* **35**, 872.
Hahn, L. (1945*a*). *Ark. Kem. Min. Geol.* **19** A, no. 33.
Hahn, L. (1945*b*). *Ark. Kem. Min. Geol.* **21** A, no. 1.
Hahn, L. (1946*a*). *Ark. Kem. Min. Geol.* **22** A, no. 1.
Hahn, L. (1946*b*). *Ark. Kem. Min. Geol.* **22** A, no. 2.
Humphrey, J. H. (1946). *Biochem. J.* **40** (in the Press).
McClean, D. (1943). *Biochem. J.* **37**, 169.
McClean, D. & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
Madinaveitia, J. & Quibell, T. H. H. (1940). *Biochem. J.* **34**, 625.
Meyer, K., Chaffee, E., Hobby, G. L. & Dawson, M. H. (1941). *J. exp. Med.* **73**, 309.
Meyer, K., Hobby, G. L., Chaffee, E. & Dawson, M. H. (1940). *J. exp. Med.* **71**, 137.
Meyer, K. & Palmer, J. W. (1936). *J. biol. Chem.* **114**, 689.
Morgan, W. T. J. (1936). *Biochem. J.* **30**, 909.
Morgan, W. T. J. (1938). *Chem. & Ind.* **57**, 1191.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Rogers, H. J. (1945). *Biochem. J.* **39**, 435.
Rogers, H. J. (1946). *Biochem. J.* **40**, 583.
Somogyi, M. (1937). *J. biol. Chem.* **117**, 771.
White, T. (1940). *J. chem. Soc.* p. 428.
Zuckerkandl, F. & Messiner-Klebermass, L. (1931). *Biochem. Z.* **236**, 19.