The ethereal solution of the distillate was titrated, with shaking, in a separating funnel with *N* NaOH. No pure product was isolated from the neutral ether; a carbonyl group was present since reaction occurred with 2:4-dinitrophenylhydrazine but no identifiable hydrazone was isolated; a semicarbazone could not be formed.

The aqueous solution gave a sodium salt (0.875 g.), equiv. 135. This was dissolved in water (55 ml.), acidified (H_2SO_4) and distilled. The first 20 ml. and the next 29 ml. of distillate gave Na salts, 0.380 g., equiv. 136, and 0.317 g., equiv. 132, respectively. The former was converted into its *p*-bromophenacyl derivative which had m.p. 71.5–72° and mixed m.p. 71–72° with authentic *p*-bromophenacyl caproate (m.p. 71.5–72°).

SUMMARY

1. The results of ozonolysis and of acetone- $KMnO_4$ oxidation of methyl arachidonate are in agreement with the constitution (II) $\Delta^{5,8,11,14}$ -eicosatetrenoic acid, for arachidonic acid, which was proposed by Dolby *et al.* [1940].

2. Contrary to the results of Takahashi [1921] ozonolysis of ethyl linoleate yields products in accordance with the generally accepted structure for linoleic acid, $\Delta^{9,12}$ -octadecadienoic acid (IV).

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Studies on Diffusing Factors

10. NOTE ON THE FORMATION OF VISCOUS MATERIALS BY *CLOSTRIDIUM BUTYLICUM*

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The enzyme system known as hyaluronidase, which exists in some tissues and bacteria, hydrolyses polysaccharides of the hyaluronic acid type. Hyaluronic acid is built up from equimolecular amounts of acetylglucosamine and uronic acid residues [Meyer, 1938]. In the hyaluronidase complex two well-defined individual components have so far been detected [East, Madinaveitia & Todd, 1941]. One of them reduces the viscosity of the viscous polysaccharide without liberating any appreciable amount of reducing sugar. The other is a glucosaminidase which does not attack the viscous polysaccharide but which may play a role in the later stages of the hydrolysis of the polysaccharide by splitting glucos-

aminic linkages. It is possible that a third component also exists, a uronidase which would hydrolyse the uronic linkages of the polysaccharide. No evidence has so far been obtained for the presence of such an enzyme in the preparations assayed. The substrate used was menthol glucuronide.

The presence of diffusing factors has been demonstrated in all the sources of hyaluronidase as yet known (e.g. in mammalian testicle, leech extract, snake venoms and some bacteria). It has been claimed that hyaluronidase and diffusing factor are the same substance [Chain & Duthie, 1940; McClean, 1941*b*; McClean & Hale, 1941]. There is, however, a discrepancy between the viscosity-reducing power

and the diffusing potency of various samples assayed [Madinaveitia, Todd, Bacharach & Chance, 1940]. There is no relation at all between the diffusing potency and glucosaminidase activity.

Mammalian testicle possesses, besides hyaluronidase, an enzyme system which deaminates and oxidizes amino sugars [Lutwak-Mann, 1941]. This enzyme system is also present in kidney, brain and in some bacteria. In testicle we find a complete mechanism to utilize the glucosamine present in hyaluronic acid according to the following scheme:

The components of this system which hydrolyse hyaluronic acid have been detected in culture filtrates of bacteria that produce diffusing factor. In this connexion the work of McClean [McClean & Hale, 1941; McClean, 1941*b*] is perhaps significant. He has found that inclusion of hyaluronic acid in the culture media of *Clostridium welchii* and some strains of *Streptococcus* and *Pneumococcus* enhances the formation of both diffusing factor and hyaluronidase. In the case of *Clostridium welchii* it also increases bacterial growth to about the same extent as glucose. It is of interest to note that no N-acetyl glucosamine could be detected in the medium supplemented by hyaluronic acid in which *Cl. welchii* had grown [McClean & Hale, private communication]. This indicates that the micro-organism has enzymes which, besides hydrolysing hyaluronic acid, also utilize the free glucosamine thus formed. McClean [1941*a, b*] has also observed that addition of hyaluronidase may modify the cultural characteristics of some bacteria. Certain strains of *Streptococcus* which in young culture have capsules become non-capsulated when grown in the presence of hyaluronidase. The addition of hyaluronidase to a capsulated strain immediately destroys the capsules. From these capsulated strains a polysaccharide has been isolated [Kendall, Heidelberger & Dawson, 1937] which is similar, or perhaps identical with hyaluronic acid, and accordingly inclusion of hyaluronidase in the culture medium does not affect capsulation.

The production of polysaccharides by micro-organisms is by no means always connected with capsule formation. A number of bacteria, for ex-

ample, elaborate viscous substances which they secrete into the culture medium. Whilst conducting biotin assays in connexion with other work in this laboratory, using as test organism *Cl. butylicum* [Snell & Williams, 1939; Peterson, McDaniel & McCoy, 1940] one of us observed that this spore-bearing anaerobe makes the medium in which it is growing viscous. *Cl. butylicum* grows in relatively simple glucose media using asparagin as the only source of nitrogen. Biotin is an essential factor for its growth; this can be supplied either as the pure substance [Snell & Williams, 1939; Woolley quoted by du Vigneaud, Hofmann & Melville, 1941] or in the form of peptone [McDaniel, Woolley & Peterson, 1939].

Since certain of the cultural characteristics of this micro-organism are fairly easily reproducible, it seemed of interest to investigate the factors affecting the production of viscous materials. While we have not been able to obtain evidence for the presence of glucosamine in the viscous material, nor is the viscosity of the latter destroyed by hyaluronidase, yet the growth of *Cl. butylicum* and the formation of viscous material are both inhibited to an appreciable extent by growing the organism in the presence of purified hyaluronidase preparations.

The formation of viscous materials is, however, much more markedly affected by other factors such as the initial concentration in the medium of glucose and of the Bactopeptone which was used in our experiments as a source of biotin. There are optimal concentrations of both glucose and peptone for bacterial growth and for production of viscous materials (Fig. 1). At any given concentration of peptone the optimal concentrations of glucose to obtain maximal growth and maximal formation of viscous materials are not very different. However, if the amount of sugar is constant the concentration of peptone which leads to the formation of most viscous material is much lower than that which produces maximal growth. At the highest concentrations of peptone investigated, practically no viscous material was formed, irrespective of the amount of sugar present, and yet the optimal concentration of peptone for bacterial growth had not been reached. This cannot be attributed to all the glucose being metabolized by the bacteria; at high concentrations of glucose a large proportion of the sugar remained unused.

EXPERIMENTAL

Influence of hyaluronidase on the growth of Cl. butylicum. Aseptic conditions were observed throughout this work.

The strain of *Cl. butylicum* used (kindly supplied by Prof. Elizabeth McCoy, Wisconsin, U.S.A.) had been kept for some time in the form of dry spores.

The preparation of hyaluronidase used was prepared from aqueous extracts of bulls' testicle by Pb acetate precipitation followed by $(\text{NH}_4)_2\text{SO}_4$ fractionation and dialysis [Madinaveitia, 1941]. The solutions were sterilized by filtration through a Seitz filter.

A series of tubes each having 15 ml. medium containing 1 % bactopectone, 2 % glucose, 0.2 % asparagine, Speakman's [1923] salt mixture and a trace of reduced iron was prepared. Five of the tubes to be used as controls received 1 ml. of a hyaluronidase solution, 10 mg./ml., before being sterilized in the autoclave. It is known that hyaluronidase is thermolabile. Another five tubes received the hyaluronidase after they had been sterilized. All tubes were inoculated with a loopful of a 24 hr. old culture and incubated in a McIntosh and Fildes jar for 60 hr. at 37°. Then the contents of the tubes were centrifuged until clear. The precipitate was washed with water and the washings rejected. The N content of the washed precipitate was determined and this 'Bacterial N' assumed to be a measure of the number of bacteria present. The supernatant was heated 5 min. at 100° and made clear again by filtration through paper. The viscosity was now determined in an Ostwald viscometer (temp. 25°). The reducing material in the supernatant, as determined by the method of Bertrand, was assumed to be the unused glucose. The figures obtained are given in Table 1.

Table 1

	Bacterial N mg./ml.	Specific viscosity	% sugar consumed
Medium + inactivated hyaluronidase (control)	1.75	0.71	87
	1.68	0.79	92
	1.85	0.93	100
	1.86	0.87	97
	2.01	0.77	97
Medium + hyaluronidase	1.23	0.43	67
	1.29	0.46	79
	—	0.44	79
	1.29	0.51	75
	1.18	0.50	94

Influence of glucose and peptone on the growth of Cl. butylicum. To obtain an indication of the time of incubation required to attain maximal growth, the following experiment was carried out: A series of tubes containing 10 ml. medium (2 % A.R. glucose, 0.6 % Bactopectone, 0.2 % asparagine, Speakman's salt mixture and reduced iron) was inoculated with a loopful of a 24 hr. old culture of *Cl. butylicum*. All tubes were incubated at 37° in the same McIntosh and Fildes jar. Lots of three tubes were removed from time to time and anaerobiosis re-established before proceeding with the incubation. As soon as the tubes were removed the culture was centrifuged and the 'Bacterial N', specific viscosity and the

amount of glucose consumed were estimated as previously indicated. The results obtained are recorded in Table 2; mean values are printed in *italics*.

Table 2

Time of incubation hr.	Specific viscosity	% glucose consumed	Bacterial N mg./10 ml.
14	0.02, 0.04, 0.04 <i>0.03</i>	7.8, 11 <i>9.4</i>	0.35, 0.62, 0.60 <i>0.52</i>
21	0.22, 0.22, 0.45 <i>0.30</i>	32.5, 37, 39 <i>36.2</i>	0.62, 0.69 <i>0.65</i>
40	1.15, 1.06, 2.69* <i>1.10</i>	85, 92, 77 <i>85</i>	0.75, 1.03, 1.11 <i>0.96</i>
99	1.50, 1.18, 1.48 <i>1.39</i>	96, 99.5, 97 <i>97.5</i>	1.15, 1.33, 1.08 <i>1.20</i>

* Not included in the mean.

From Table 2 one can see that the lag phase is about 10 hr. and that the end of the logarithmic phase is reached in about 2 days.

To determine the influence of glucose and of peptone on the growth of *Cl. butylicum* a series of tubes containing 10 ml. medium of various concentrations of Bactopectone and of glucose was prepared. They all had 0.2 % asparagine, Speakman's salt mixture and reduced iron. They were sterilized, inoculated, and after 70 hr. incubation at 37°, the bacterial N, viscosity and amount of sugar used were determined. All these operations were carried out as previously described. The concentrations of glucose and of peptone which were examined, and whether or not growth was observed in each of the tubes, are recorded in Table 3.

Table 3

Bacto- peptone mg./ml.	Glucose mg./ml.						
	20	30	50	60	80	150	300
2.5	+	.	+	.	.	—	—
5	.	+	.	+	+	.	.
10	.	+	.	+	+	.	.
12.5	+	.	+	.	.	—	—
20	.	+	.	+	+	.	.
25	+	.	+	.	.	—	—
30	.	+	.	+	+	.	.
40	.	+	.	+	+	.	.
50	+	.	+	.	.	—	—

+ = growth; — = no growth.

Fig. 1 is a graphic representation of the values obtained for bacterial N, specific viscosity and glucose consumed. It is a contour map of the surfaces resulting from plotting these values along an axis perpendicular to the plane of the paper. On this plane the concentrations of glucose and of peptone are represented.

SUMMARY

Cl. butylicum produces viscous materials when grown in a glucose-asparagine-mineral salts medium

The effect upon some cultural characteristics of *Cl. butylicum* of the initial concentrations of glucose and peptone in the medium has been studied.

Fig. 1. Effect of glucose and Bactopeptone on *Cl. butylicum*. — Viscosity of medium. - - - - Bacterial N (mg./ml.). - · - · - Glucose consumed (mg./ml.).

containing Bactopeptone as a source of biotin. Both the growth and the production of viscous polysaccharides are inhibited to a certain extent by inclusion of hyaluronidase concentrates in the medium.

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