The Role of Glutamine in the Biosynthesis of Hyaluronate by Streptococcal Suspensions

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Hexosamine-containing high-molecular-weight substances form major constituents of many animal and bacterial products, yet only during the last few years has considerable attention been paid to the mechanism of their biosynthesis. Hyaluronate has been known for many years to be formed by strains of Lancefield Groups A and C *Streptococcus haemolyticus* (Kendall, Heidelberger & Dawson, 1937; Seastone, 1939). This bacterial mucopolysaccharide is likely to be identical with the mammalian product originally isolated by Meyer & Palmer (1936). It seems, therefore, reasonable to use this convenient system to obtain preliminary information about mucopolysaccharide synthesis. Recently, Roseman, Moses, Ludowieg & Dorfman (1953, 1954) and Topper & Lipton (1953) have studied the structural relationship between the hexosamine and hexuronic acid moieties of hyaluronate, synthesized by cultures of Group A haemolytic streptococci, and the glucose present in the culture medium. They concluded that both hexose derivatives arose without rearrangement of the glucose carbon atoms. Meanwhile, our interest (Lowther & Rogers, 1953) centred on the mechanism by which the streptococci introduced the amino group into the 2-position of glucose to give hyaluronate glucosamine. Reliance on whole cultures would have complicated this work owing to the complex nutritional requirements of streptococci, but washed suspensions of the organisms synthesized hyaluronate in the presence of glucose, phosphate, magnesium and simple nitrogen sources such as glutamine. Such a system was clearly much simpler than most of those hitherto used in the study of mucopolysaccharides. The earlier work (Lowther & Rogers, 1953) suggested that the amide group of glutamine was transferred to form hyaluronate glucosamine, and during the course of this work Leloir & Cardini (1953) showed that glutamine was also involved in the biosynthesis of hexosamine by extracts of *Neurospora*. The latter result raises hope that the mechanism described in this paper for the biosynthesis of hyaluronate glucosamine may have general application to the biosynthesis of hexosamine. A preliminary report of this work has already appeared (Lowther & Rogers, 1955).

METHODS

Cultivation of the organisms and preparation of suspensions

A single strain of *Streptococcus haemolyticus* (Lancefield Group A), kindly supplied by Dr R. E. O. Williams (Central Public Health Laboratory, Colindale Avenue, London, N.W. 9), called T13, has been used throughout the present work. The medium used for growth of the organism was Hedley Wright broth (Wright, 1933) containing 1% of glucose, 2.5% of sodium β -glycerophosphate and 10% of normal horse serum. The supplements were added aseptically as sterile solutions to the autoclaved broth. The organisms were grown in this medium at $35 \pm 0.5^\circ$ without aeration. In order to avoid loss of the hyaluronate-producing capacity of the strain, various storage and subculture conditions were examined. The most satisfactory procedure was to dry cultures, grown for 18 hr. in the above medium, by the Stamp (1947) method and to revive these dried cultures in Hedley Wright broth containing 10% of sterile whole horse blood. The blood-broth cultures, which could be stored for several months at $0-4^\circ$, were streaked on to blood-agar plates which were incubated and the colonies were examined for purity of the strain and their mucoid character. To grow the rather large volumes of culture necessary for the present work a small volume (200–300 ml.) of the broth-glycerophosphate medium, warmed to 35° , was inoculated with 1.0 ml. of the blood-broth culture and incubated overnight at 35° . This culture was streaked on to blood-agar plates as a check on purity and added to ten times its volume of the same medium. Cell suspensions were prepared from the final culture. The age of the final culture was found to be important for the activity of the cell suspension. The harvested cells showed maximum activity when growth in the culture was equivalent to a bacterial N content of 0.033 mg./ml. Growth was measured by the optical opacity of the cultures using a Spekker absorptiometer with filters H 508 and 609. The equivalent bacterial N content was read from a curve relating optical opacity to bacterial N. The latter was determined on the organisms centrifuged from 10 ml. volumes of cultures of known opacity and washed twice with 10 ml. volumes of distilled water. When growth had reached the desired level in the bulk cultures (usually in about 4 hr.), the flasks were cooled by immersion in iced water and the cultures streaked on to blood-agar plates to check for contamination. The culture was then centrifuged and the cells washed four times with a solution containing 5 g./100 ml. of sodium β -glycerophosphate and 0.04 M- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; all these operations were carried out at $0-4^\circ$. This repeated washing with the slightly alkaline glyce-

phosphate solution (pH 7.8) was necessary to remove as much of the hyaluronate from the surface of the organisms as possible. Finally the organisms were suspended in a solution of sodium β -glycerophosphate (5 g./100 ml.) and magnesium sulphate (0.04M) to give an optical opacity equivalent to 1 mg. of bacterial N/ml. These suspensions were stored at 0–4° until used. Immediately before use they were centrifuged again, and the cells resuspended in the same volume of the glycerophosphate–magnesium solution.

Conditions for incubation of the suspensions. Volumes (0.5 ml.) of a solution containing 0.056M glucose, 0.05M phosphate buffer (Sørensen) at pH 7.0 and any other required additions were pipetted into test tubes and warmed to 37°. An equal volume of the suspension prepared above, also warmed to 37°, was then added; the final pH was 7.0. The mixtures were incubated in open test tubes for 2 hr. at 37° with gentle shaking, then cooled in iced water, centrifuged at 0–4° and the supernatant fluid was withdrawn. If estimations were not to be made immediately, these fluids were stored at –20°.

Analytical methods

The hyaluronate present in the supernatant fluids from incubated suspensions was estimated by measuring (a) the turbidity formed when they were mixed with acidified, diluted, normal horse serum according to Tolksdorf, McCready, McCullagh & Schwenk (1949) and (b) the amount of hexosamine liberated from them on acid hydrolysis.

In the turbidity method, the amount of hyaluronate, which corresponded to a given optical opacity, was interpolated from a curve relating opacity to weight of a sample of hyaluronate of known purity prepared from umbilical cord and dissolved in the fluid used for suspending the organisms. The purity of the standard sample was calculated from its hexosamine content assuming that the pure mucopolysaccharide consists of equal molecular proportions of *N*-acetylglucosamine and glucuronic acid (Meyer & Palmer, 1936). The relationship between optical opacity and concentration of hyaluronate was approximately linear over the range of 0.1–0.6 mg. Therefore, whenever possible, the supernatant fluids were diluted to contain between 0.2 and 0.5 mg. If less than 0.2 mg. of hyaluronate was available in a sample an approximate answer only could be obtained. The specificity of this method was improved by incubating critical samples with ten turbidity-reducing units (Tolksdorf *et al.* 1949; Rogers, 1953, 1954) of partially purified streptococcal hyaluronidase (Rogers, 1946) for 10 min. at 37° and pH 6.0. This enzyme is known (e.g. Chauncey, Lionetti & Lisanti, 1953) to be more specific than hyaluronidase preparations from mammalian testes. When, after this incubation, no turbidity was given on addition of the acidified serum reagent it was considered strong additional evidence in favour of the original turbidity being entirely due to the reaction between hyaluronate and the serum reagent.

The amount of hexosamine liberated by acid hydrolysis of the supernatant fluids was estimated as follows: the supernatant (0.5 ml.) fluid was mixed with 8N-HCl (0.5 ml.) in an ampoule which was sealed and heated in a boiling-water bath for 4 hr. The HCl was then removed by repeated evaporation *in vacuo* and the amount of hexosamine was estimated by the Elson & Morgan (1933) technique; the

colour formed was read with a Unicam spectrophotometer set at 535 m μ . All results are expressed as μ moles of hexosamine hydrochloride/ml. of the supernatant fluid. Sodium β -glycerophosphate, at concentrations greater than 0.5 g./100 ml. in the final test, increased colour formation caused by known amounts of glucosamine-HCl. Therefore, supernatants were always diluted so that less than this concentration of it was present.

Examination of the supernatant fluids by the Elson & Morgan (1933) method before acid hydrolysis gave negative results; thus free glucosamine was not present. Paper-chromatographic examination of the supernatant fluid with neutral phenol as solvent confirmed the negative results; no *N*-acetylglucosamine or other sugars were detected on the chromatograms, which were sprayed with Ehrlich's reagents, aniline phthalate and anisidine hydrochloride (Partridge, 1948, 1949; Hough, Jones & Wadman, 1950). It was also shown that, after quantitative precipitation of the material in the supernatant fluid with acidified normal serum, as in the ordinary turbidity method for estimating hyaluronate, no hexosamine could be detected in the acid-hydrolysed filtrate. If, however, the supernatant fluids had first been treated with streptococcal hyaluronidase all the hexosamine remained in the filtrate after the addition of acidified serum reagent. The results of these experiments are consistent with the supposition that the hexosamine liberated on acid hydrolysis has all been present as part of hyaluronic acid. The quantitative agreement, however, between the amount of hyaluronate estimated from the turbidity test and from the amount of hexosamine liberated on hydrolysis (later referred to as 'bound glucosamine') was not completely satisfactory. In the work to be described the turbidity reaction was used to obtain qualitative information about the substances affecting hyaluronate synthesis, whilst the estimation of bound glucosamine was relied upon in the more crucially quantitative aspects. The discrepancies between the two methods are probably due to factors such as the influence of different degrees of polymerization of the mucopolysaccharide upon the turbidity reaction.

Other methods of analysis used were: ammonia, as described by Conway (1940); glucuronic acid, by Ogston & Stanier (1951) and Dische (1947); and glutamic acid, by the decarboxylase of whole *Clostridium welchii* strain SR12 cells, as described by Gale (1945). Ash was determined by heating at 600° to constant weight, and moisture by drying to constant weight at 105°. Total N was estimated either by the micro-Kjeldahl procedure, using copper selenite as catalyst, or by the method of Conway (1940), according to the amount of material available.

Samples of amino acids, including glutamine and asparagine, were checked for ninhydrin-reacting contaminants by paper chromatography.

The methionine sulphoxide was synthesized as described by McIlwain, Roper & Hughes (1948); on chromatography in neutral phenol, phenol–ammonia, and butanol in equilibrium with 1% aqueous heptanoic acid, it showed only a single spot with ninhydrin.

Identification of the hexosamine as glucosamine. The hexosamine liberated on acid hydrolysis of the supernatant fluids and of isolated hyaluronic acid was identified as glucosamine by means of: (i) its rate of travel on paper chromatograms, in neutral phenol, phenol–ammonia and butanol–heptanoic acid; (ii) its rate of travel on a column

of the ion-exchange resin Dowex-50 developed with 0.3N-HCl (Gardell, 1953); (iii) agreement of the absorption curve of the colour given by acid hydrolysates of the supernatant fluids in the Elson & Morgan (1933) test with that given by pure glucosamine; and (iv) by isolation of the hexosamine hydrochloride by method (ii) above and crystallization from aqueous ethanol mixtures. The crystals were then examined by X-ray crystallography by Mrs O. Kennard of this Institute, to whom we are indebted for help in this matter. Plate 2 shows the picture obtained alongside that of a sample of known glucosamine hydrochloride.

RESULTS

Preliminary experiments showed that when washed suspensions of streptococci were incubated for 2 hr. at 37° with an equal volume of a 1:5 dilution of Hedley Wright broth and a final concentration of 0.5 g./100 ml. of glucose, substances were subsequently found in the supernatant fluid which gave turbidity with the acidified serum reagent (see Methods section). With four different batches of cells, the turbidity obtained was equivalent to 0.2–0.4 mg./ml. of potassium hyaluronate. When broth was omitted the values were reduced to 0.04–0.1 mg./ml. The presence or absence of glucose made little difference when broth was not present; in the presence of broth, glucose was essential for the formation of the material giving turbidity. Incubation of the supernatant fluid with streptococcal hyaluronidase abolished its turbidity-forming properties. It was, therefore, likely that the suspensions were synthesizing hyaluronate in the presence of diluted broth and glucose. No detectable increase in bacterial nitrogen occurred. The hyaluronate in the supernatant fluids from suspensions incubated with broth and glucose did not appear to arise from decapsulation of the organisms. Washed cells of strain T 13, either before or after incubation, were not visibly encapsulated when examined microscopically using the indian ink staining method, despite their ability to form large amounts of extracellular mucopolysaccharide. Likewise, if the cells were suspended in 0.1M phosphate (Sørensen) buffer at pH 7.0 at the same density as that used for the incubation and heated at the temperature of a boiling-water bath for 10 min., no significant amounts of hyaluronate were extracted. Seastone (1939) has used this method for extracting capsular hyaluronate from streptococci. The heat treatment also extracts small amounts of substances soluble in organic solvents and resistant to the action of hyaluronidase. The turbidity given by these substances with the acidified serum reagent obscures the turbidity given by any small amounts of capsular hyaluronate which may also be extracted. It is not possible, for this reason, to be sure of the origin of the small amount of hyaluronate which

appeared in the supernatant fluid when the suspensions were incubated at 37° in the absence of broth. Our interest was in analysing the stimulatory effect of broth, and since the values obtained in the absence of added nitrogen source were not usually more than 10–15% of those in its presence, they were subtracted as a 'blank value' and their origin was not further investigated.

It has been shown (Morison, 1941; McClelland, 1954; Faber & Rosendal, 1954) that even encapsulated hyaluronate-producing strains, including T 13, can form hyaluronidase. Before proceeding with a more detailed examination of the mechanism of the effect of broth, it was necessary to ensure that sufficient hyaluronidase was not formed by the strain, under the suspension conditions used, to lower the apparent concentration of hyaluronate measured by the turbidity method. To test for the presence of hyaluronidase activity, 0.4 ml. of suspension was added to 0.5 ml. of a solution containing 1 mg. of bacterial hyaluronate. This mixture was incubated for 2 hr. at 37°. The supernatant fluid from this suspension was then analysed for hyaluronic acid content by the turbidity method. No change in concentration was found. Further test of the suspensions by the very sensitive viscosity-reducing test (McClean & Hale, 1941; McClean, 1943) showed that a very slight hyaluronidase activity was present, but it was apparently too small to affect the turbidity caused by the mucopolysaccharide although sufficient to lower viscosity appreciably in 1–2 hr. incubation. This small enzymic activity could be safely disregarded.

Incubation of the suspensions with broth and glucose for longer than 2 hr. led to no change in the amount of hyaluronate. Therefore, this period of incubation was used in all the later work.

Replacement of broth by defined nitrogen sources

In a series of experiments the broth addition to the suspensions was replaced in turn by twenty-one different amino acids, asparagine, glutamine, glucosamine and ammonium sulphate, chloride and acetate. A final concentration of 0.5 g./100 ml. of glucose was always present. All these compounds were tested at a final concentration equivalent to 10 µg. atoms of nitrogen/ml. After incubation under the usual conditions the supernatant fluids were assayed for hyaluronic acid by the turbidity method. Table 1 summarizes the results obtained in five such experiments. It will be seen that glutamine allowed the synthesis of the largest amounts of hyaluronate in each of the five experiments. Ammonium salts, serine and asparagine were active with some batches of cells, but not with others. The remainder of the compounds tested were always inactive.

Plate 1. X-ray powder diagram of bacterial hyaluronate glucosamine (a) compared with that of an authentic sample of glucosamine isolated from chitin (b).

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Further examination of the relationship between ammonium salts and the synthesis of hyaluronate showed that, if the cell suspensions were examined within 24 hr. of preparation, they were active with the inorganic nitrogen source. This ability was lost as the suspensions stood at 0–4°, but could be partially restored by the addition to the system of the supernatant fluid from the stored suspension. The cells used for the experiments recorded in Table 1 had always been first centrifuged and resuspended in fresh glycerophosphate solutions. It thus seemed likely that some substance essential for the activity of the suspensions, when only

storage, yet synthesis in the presence of L-glutamine remained unaffected. The addition of L-glutamate, together with ammonium ions, completely restored the ability of the aged preparations to use inorganic nitrogen. This phenomenon has been examined in a considerable number of experiments, always with similar results, but the following variations from the pattern in Table 2 have been noted. (1) Ammonium ions are not always as effective as glutamine for suspensions 24 hr. old. (2) The amount of hyaluronate synthesized in the presence of glutamine also sometimes falls during ageing. These phenomena have not been examined.

Table 1. *Influence of nitrogen-containing substances upon the synthesis of hyaluronate by streptococcal suspensions*

The suspensions were prepared as under Methods and contained final concentrations of 0.5 g./100 ml. of glucose, 0.02M-MgCl₂ and sufficient of the nitrogen source to give a final concentration of 10 µg. atoms of N/ml. Incubation was for 2 hr. at 37°.

Nitrogen source Expt. no. ...	Hyaluronate (mg./ml.)				
	1	2	3	4	5
NH ₄ ⁺ (as sulphate, chloride or acetate)	0.27	—	0.38	0.32	—
Glucosamine-HCl	0.09	—	—	—	—
Amino acids* A†	approx. 0.04	approx. 0.04	—	—	—
B‡	—	—	—	—	0.04
Serine	0.27	—	—	—	0.04
Asparagine	0.27	0.17	approx. 0.03	0.33	approx. 0.03
Glutamine	0.54	0.40	0.48	0.61	0.37

— Not examined.

* Amino acids tested separately.

† A: glutamic acid, proline, hydroxyproline, threonine, alanine, methionine, tyrosine, glycine, cystine.

‡ B: aspartic acid, lysine, cysteine, leucine, norleucine, isoleucine, valine, histidine, ornithine, citrulline, phenylalanine.

ammonium ions and glucose were present, was leaking from the cells during the storage period. Glutamine was consistently active with both fresh and aged preparations of cells, whilst L-glutamic acid was never active. Glutamic acid might have been lost from the cells during standing, and fresh cells with an adequate internal pool of the amino acid might be able to synthesize glutamine from the added ammonium ions, whereas aged preparations of cells from which some glutamate might have leaked would be unable to do so. To test this supposition, a suspension 24 hr. after preparation, and a suspension of these same cells after standing for 4 days in the usual sodium β-glycerophosphate solution, were centrifuged and resuspended in fresh solution. Three duplicate 0.5 ml. volumes of the suspensions were incubated with (a) ammonium chloride, (b) ammonium chloride together with an equimolar concentration of L-glutamate, and (c) L-glutamine. The final concentration of ammonium chloride in (a) and (b) was 5 µmoles/ml. as was that of L-glutamine in (c). Ammonium ions alone were as effective for the fresh suspension as was L-glutamine (see Table 2). No hyaluronate, however, was synthesized by the preparation from inorganic nitrogen alone after a further 3 days

Table 2. *Effect of storing suspensions of streptococci at 0–4° upon the synthesis of hyaluronate in the presence of different nitrogen sources*

The suspensions all contained a final concentration of 0.5 g. of glucose/100 ml. and 0.02M-MgCl₂. The nitrogen sources were present at a concentration of 5 µmoles/ml. Incubation was at 37° for 2 hr.

Nitrogen source	Hyaluronate synthesized (µMG/ml.*) with suspension aged	
	1 day	4 days
L-Glutamine	0.28	0.27
Ammonium L-glutamate	0.35	0.27
Ammonium chloride	0.31	0

* µmoles of glucosamine/ml. liberated on acid hydrolysis.

In other experiments the fluid in which the cells had stood at 0–4° for several days was added to suspensions made in fresh glycerophosphate solution. This addition was partially able to restore the ability of the cells to synthesize hyaluronate in the presence of ammonium ions alone. Thus the activity of some of the original preparations of cells and not others towards inorganic nitrogen seemed to be reasonably explained. Serine, asparagine and

arginine were also sometimes active, and it seemed possible that ammonia was being liberated from these compounds. If this were so, then the addition of glutamate together with serine, asparagine or arginine should lead to the synthesis of hyaluronate by preparations of the cells which were inactive when tested with the amino acids alone.

confirmed. Proof of this was obtained in two ways: (1) by demonstrating that a total net increase in the amount of bound glucosamine occurs when the suspensions are incubated in the presence of simple nitrogen sources; and (2) by isolating and characterizing the hyaluronate from the supernatant fluid of the suspensions.

Table 3. *Influence of adding glutamate together with asparagine and serine upon hyaluronate synthesis by streptococcal suspensions*

The concentrations of each of the amino acids was 5 μ moles/ml. The cells had stood in suspension for 4 days at 0–4° before use. Incubation was at 37° for 2 hr.

Additions	Hyaluronate synthesis			
	Expt. 1		Expt. 2	
	μ MG/ml.*	mgH/ml.†	μ MG/ml.*	mgH/ml.†
L-Asparagine	0.018	0	0.06	0.02
L-Asparagine + L-glutamate	0.70	0.36	0.59	0.26
L-Serine	0	0	0.06	0.01
L-Serine + L-glutamate	0.50	—	0.78	0.30
Ammonium chloride	0.01	0.03	0.06	0.01
Ammonium chloride + L-glutamate	0.60	0.31	0.52	0.40

* μ moles of glucosamine hydrochloride/ml. liberated on acid hydrolysis.

† mg. of hyaluronate/ml. as measured by the turbidity reaction.

Table 3 summarizes the results obtained when suspensions which had been stored for 4 days at 0–4° were incubated with two of these compounds at a final concentration of 5 μ moles/ml., in the presence and absence of 5 μ moles/ml. of L-glutamate. Arginine behaved in a similar manner when tested with glutamate. The supernatant fluids were examined for the concentration of hyaluronate by both the turbidity reaction and by the estimation of bound glucosamine. Both serine and asparagine behave similarly to ammonium ions and allow synthesis of hyaluronate only in the presence of L-glutamate. Previous examination (Hills, 1940) of the deaminases of haemolytic streptococci had revealed a powerful arginine dehydrolase, but not a serine or asparagine deaminase, nor, for the latter compound, a deamidase. Examination of both aged and fresh suspensions, however, showed (Table 4) that in the presence of glucose, but not in its absence, ammonia was formed from these compounds by our strain of organisms. These results are similar to those obtained for the deamidation of glutamine by McIlwain *et al.* (1948). Examination by paper chromatography of the supernatant fluid from suspensions incubated with glucose and asparagine revealed the presence of aspartic acid. Thus the organisms had deamidated the amide.

Confirmation of the synthesis of hyaluronate by the suspensions

At this point in the work, net synthesis by the suspensions of a compound with properties similar to those of hyaluronate from other sources was

Table 4. *Liberation of ammonia from serine and asparagine by streptococcal suspensions in the presence and absence of glucose*

The concentration of the nitrogen compounds was 5 μ moles/ml. and (when added) that of glucose was 0.5 g./100 ml. Other conditions of the suspension were as for the study of the synthesis of hyaluronate. Incubation was for 2 hr. at 37°.

Nitrogen compound	Glucose	NH ₃ liberated (μ mole/ml.) with preparation aged	
		1 day	4 days
Serine	—	0.17	0.10
Serine	+	0.83	0.84
Asparagine	—	0.13	0.14
Asparagine	+	0.92	0.37
— No glucose present.		+ Glucose present.	

An increase in bound glucosamine. Volumes of a cell suspension (24 hr. old) were incubated with a final concentration of 5 μ moles/ml. of L-glutamine, ammonium L-glutamate or ammonium chloride under the usual conditions. Samples of the original suspension before and after incubation, without added nitrogen source, were included as controls. The cells were removed by centrifuging and washed three times with 0.1M (Sørensen) phosphate buffer pH 7.0, and samples were hydrolysed with 4N-HCl as in the ordinary method for the estimation of bound glucosamine. Samples of the supernatant fluids were also hydrolysed. Table 5 shows results obtained. It will be seen that in the presence of both glutamine and ammonium

glutamate, there is a large increase in the amount of hexosamine present in the suspensions. There is also a significant increase in the presence of ammonium chloride, but on incubation in the absence of nitrogen source there is no significant change in the hexosamine content. Thus the added nitrogen sources lead to a net synthesis of hexosamine, and examination of Table 5 shows that the whole of this increase takes place in the supernatant fluid of the suspensions. The hexosamine content of the cells does not increase, but shows a very slight fall of dubious significance.

drying procedure was repeated on all samples immediately before analysis. The dry powder was extracted successively with absolute ethanol, chloroform and ether, each for 1 hr. in a Soxhlet apparatus. This extraction led to a removal of a lipid material which formed 10–20% of the weight of the sample. The polysaccharide analyses recorded in Table 6 show the composition of lipid-free material. Both these samples were converted into the free acid as follows: 10 mg. were dissolved in water (3 ml.) and shaken with 400 mg. of Dowex-50 (acid form, previously washed with water until the

Table 5. *Effect of incubating cell suspensions with nitrogen sources upon the total amount of hexosamine in the suspension*

The conditions of incubation of the suspensions (24 hr. old) were as before. The nitrogen sources were present at a final concentration of 5 μ moles/ml.

Nitrogen source	Hexosamine (μ moles/ml. of suspension)		
	In cells	In supernatant fluid	Total
None (cells before incubation)	0.77	0	0.77
None (cells after incubation)	0.64	0.35	0.99
L-Glutamine	0.67	1.86	2.53
Ammonium L-glutamate	0.58	1.40	1.98
Ammonium chloride	0.58	0.79	1.37

Table 6. *Analysis of samples of streptococcal hyaluronic acid*

The samples were prepared as described in the text. The stated values have been corrected for the ash content of the samples. For this purpose only the metal ion of the ash (supposed as K_2CO_3 or Na_2CO_3) was taken into account.

Sample	Ash (%)	Hexosamine		Glucuronic acid		Total N		Optical rotation $[\alpha]_D$
		(%)	Mol.prop.*	(%)	Mol.prop.*	(%)	Mol.prop.*	
P17 ₁	3.9	35.5	0.78	36.0	0.74	4.57	1.28	-116.5°
P17 _{1-v}	10.2	39.1	0.88	44.0†	0.94	2.81	0.80	-97°
				45.2‡	0.97			

* Molecular proportion of substance per repeating unit of *N*-acetylglucosamine and glucuronic acid.

† Determined by the method of Ogston & Stanier (1951).

‡ Determined by the method of Dische (1947).

Isolation of hyaluronate. A large volume of suspension (100 ml.) was incubated in the presence of 5 μ moles/ml. of ammonium L-glutamate and 0.5 g./100 ml. of glucose for 2 hr. at 37° and the organisms were removed by centrifuging. The supernatant fluid was then placed in supported cellophan tubing and the pressure inside the tube was raised to 60 cm. Hg. Distilled water (6 l.) flowed around the outside of the cellophan during 48 hr. at 0–4°. This process both removed the salt and concentrated the mucopolysaccharide solution. The dialysed concentrated contents of the tube were then centrifuged to remove a small amount of insoluble material and the solution was dried from the frozen state. Remaining moisture was removed from the powder by heating at 80° and 15 mm. pressure over P_2O_5 followed by storage at room temperature over P_2O_5 at 0.1 mm. pressure. This

washing water was between pH 5.0 and 7.0). The resin was removed by filtration and the free hyaluronic acid (7 mg.) was dried from solution as before. Analysis of this product is given opposite P17₁ in Table 6.

Hyaluronate prepared in this way, but not treated with resin to remove salts, was then fractionated with ethanol as follows: 100 mg. were dissolved in 20 ml. of distilled water and chilled to 0°. Ethanol (46 ml.) containing 10% of potassium acetate, chilled to -10° and at pH 9.0, was added and the mixture was allowed to stand for 30 min. at -10°. The precipitate was collected, washed three times with 10 ml. volumes of ethanol, dried and redissolved in 10 ml. distilled water. This precipitation was repeated three times. Finally, the purified product was converted into the free acid by passing a solution of it through a Dowex-50 column

(5 × 1 cm.). The effluent solution was pressure-dialysed and dried for analysis. In Table 6 this sample is called P17_{1-v}.

Examination of the ultraviolet absorption spectrum of a solution of the sample P17₁ showed no specific absorption at 260 m μ ., indicating the absence of nucleic acid, but showed an inflexion at 280 m μ . Therefore the high nitrogen content of the sample before fractionation suggests that the hyaluronic acid is contaminated by about 10% of its weight of protein. This suggestion is supported by the presence in acid hydrolysates of a limited number of substances reacting with ninhydrin. The rate at which these substances travelled down a chromatograph paper in a butanol-acetic acid solvent system suggested that glutamic acid, arginine and alanine were the chief constituents. This small protein contamination was removed by alkaline ethanol fractionation and 93% by weight of the purified material was accounted for by *N*-acetylglucosamine and glucuronic acid. It is noteworthy, however, that the optical rotation of -97° for the purified material remains greater than values previously found for bacterial hyaluronate (i.e. -67.5° by Topper & Lipton, 1953, and by Roseman *et al.* 1953, and -53° to -86° by Kendall *et al.* 1937), or for mammalian hyaluronate, the accepted value for which is about -76° .

An infrared spectrum of the unfractionated material was kindly made for us by Dr S. F. D. Orr of the Chester Beatty Research Institute. The result he obtained agreed well with his previously published figure (Orr, 1954) for mammalian hyaluronate, although the ratio of absorption at the $-\text{CO}_2\text{H}$ position to that at the $-\text{CO.NH}_2$ position was 1.10 compared with 1.00 for the mammalian umbilical cord preparation. An electrophoretic examination was also made of an 0.2% solution of the unfractionated hyaluronate in buffers of pH 5.0 and 7.0 and ionic strength 0.1 μ . Only single peaks were observed in both buffers during a 1.5 hr. run. The peaks were not, however, completely symmetrical.

Relationship between glutamine synthesis and hyaluronate formation

Glutamine and ammonium L-glutamate were added to suspensions to give final concentrations of from 0.25 μ mole up to 1.0 μ mole/ml. and after incubation under the usual conditions the amount of hyaluronate present in the supernatant fluids was measured, by both the turbidity reaction and the amount of bound glucosamine present. It will be seen from Fig. 1 that, between these limits, the amount of hyaluronate synthesized is directly proportional to the concentration of either of the compounds added. In other experiments, in which more than 1 μ mole/ml. of either glutamine or

ammonium L-glutamate was added, no more mucopolysaccharide was synthesized. It may be noted that the plots of the values for bound glucosamine (expressed as mg./ml.) and of the values for hyaluronate estimated by the turbidity reaction are exactly parallel: this provides further evidence that both methods measured the concentration of the same substance, hyaluronate.

Fig. 1. Influence of glutamine and ammonium glutamate concentration on the synthesis of hyaluronate (measured by the turbidity reaction) and of bound glucosamine by suspensions of streptococci. Conditions of incubation as under Methods. ●, mg./ml. hyaluronate as measured by the turbidity reaction; ○, mg./ml. × 10⁻¹ bound glucosamine in supernatant fluid; ×, μ moles/ml. bound glucosamine in supernatant fluid.

More detailed examination of the relationship between the uptake of nitrogen by the cells and the synthesis of hyaluronate was undertaken. Volumes of suspension (16 ml.) were incubated under the usual conditions, with ammonium glutamate (0.74–1.25 μ moles/ml.) and glutamine (1.25 μ moles/ml.). The concentrations of ammonia, glutamate and bound glucosamine were then estimated in the supernatant fluids. The results obtained are shown in Table 7, where it will be seen that approximately equal molecular proportions of ammonia and glutamate disappear from the solution during incubation. Concomitantly, from 0.48 to 0.89 mole of hyaluronate glucosamine appears per mole of ammonia disappearing; in five out of the eight

experiments recorded this ratio was between 0.85 and 0.89. If the concentration of the nitrogen source was raised above 1.25 μ moles/ml., less than this molecular proportion of bound glucosamine always appeared. These results seemed to be most simply explained by supposing that, with the lower concentrations of available nitrogen, about half was converted into hexosamine. It had to be assumed in the experiments yielding smaller

amide. The influence of methionine sulfoxide (α -amino- γ -methylsulphonylbutyric acid) in the system was examined. Methionine sulfoxide, as is well known (Elliott & Gale, 1948; McIlwain *et al.* 1948), inhibits the synthesis of glutamine from ammonium glutamate by a variety of both mammalian and bacterial systems. Various concentrations of this substance were added to the usual system containing either glutamine or ammonium

Table 7. *Relation of the uptake of ammonia and glutamate by cells to the production of hyaluronate glucosamine*

Cell suspensions were incubated for 2 hr. at 37° with the stated amounts of the nitrogen sources, and the concentrations of the various substances in the supernatant fluids were measured. Each experiment was performed with a different batch of cells.

Experiment	Nitrogen source	Concn. (μ moles/ml.)	Hyaluronate glucosamine (μ moles/ml.)	Loss from supernatant fluid	
				Ammonia (μ moles/ml.)	Glutamate (μ moles/ml.)
1	L-Glutamine	1.25	0.33	0.68	0.59
	Ammonium L-glutamate	1.25	0.35	0.68	0.73
		1.25	0.70	0.59	0.68
		0.74	0.42	0.47	0.49
2	L-Glutamine	1.25	0.70	0.81*	—
		1.25	0.70	0.78*	—
	Ammonium L-glutamate	1.25	0.70	0.84	—
		1.25	0.33	0.57	—
3	Ammonium chloride	1.25	0.33	0.57	—

* These figures represent disappearance of amide nitrogen from the supernatant fluid as estimated by the method of Krebs (1948).

Table 8. *Influence of α -amino- γ -methylsulphonylbutyric acid (methionine sulfoxide) on the synthesis of hyaluronate glucosamine*

The suspensions were prepared in the usual manner, were mixed with the appropriate concentrations of nitrogen source and methionine sulfoxide and incubated 2 hr. at 37°. The concentrations of the various substances were measured in the supernatant fluid after centrifuging. The concentration of glucose was 0.5 g./100 ml.

Nitrogen source	Concn. (μ moles/ml.)	Methionine sulfoxide concn. (μ moles/ml.)	Remaining in supernatant fluid		Hyaluronate glucosamine (μ moles/ml.)	Inhibition of glucosamine synthesis (%)
			NH ₄ ⁺ (μ moles/ml.)	Glutamate (μ moles/ml.)		
L-Glutamine	1.25	0	0.47	0.45	0.42	—
	1.25	1.25	0.45	0.50	0.42	0
Ammonium L-glutamate	1.25	0	0.66	0.54	0.70	—
	1.25	1.25	1.22	0.81	0.14	80

amounts of hexosamine that, despite the low concentration of nitrogen available, some other factor limited hyaluronate synthesis. Since ammonium glutamate and glutamine behaved in all ways identically, it seemed possible that glutamine was an intermediate in the process of hexosamine synthesis, and that only the amide or the α -nitrogen atom was used.

If glutamine is a necessary intermediate between ammonium glutamate and hexosamine, synthesis in the presence of the ammonium salt should be sensitive to substances inhibiting synthesis of the

L-glutamate, and the amount of bound hexosamine synthesized was measured. The amount of ammonia, glutamine and glutamate remaining at the end of the incubation period was also measured. Suitable control suspensions, containing, in addition to the usual constituents, either methionine sulfoxide only or one of the two nitrogen sources, were also incubated. The types of results obtained are illustrated in Table 8. A molecular ratio of methionine sulfoxide to nitrogen source of 1:1 inhibits the synthesis of bound hexosamine from ammonium glutamate by 80%, but has no effect

when glutamine is used. Likewise, the disappearance of ammonia in the presence of the ammonium salt, is inhibited by 95% by methionine sulphoxide. In other unquoted experiments some inhibition was recorded in the presence of glutamine, even with the ratio of methionine sulphoxide to nitrogen source of one. If this ratio was increased above one, considerable inhibition of synthesis in the presence of glutamine occurred and with a ratio of 3.0 it amounted to 74%. Synthesis from ammonium glutamate, however, was always very much more sensitive than from glutamine. These results very strongly support the intermediary role of glutamine in the biosynthesis of hyaluronate glucosamine.

*Incorporation of ^{15}N into
bound hexosamine*

So far no available evidence suggested which of the two possible nitrogen atoms in glutamine was incorporated into the hexosamine. Moreover, evidence for the incorporation of nitrogen from the added compounds was still indirect. If it were to be shown that isotopically labelled ammonia in the presence of unlabelled glutamate was incorporated

24 hr. of harvesting. In the other, the cells stood at 0–4° in the usual sodium β -glycerophosphate solution until no synthesis of hyaluronate occurred in the presence of ammonium ions alone. After incubation, the cells were removed by centrifuging and the hyaluronate was isolated from the supernatant fluid as described previously. The isolated mucopolysaccharide was hydrolysed for 4 hr. with 4N-HCl at the temperature of a boiling-water bath and the glucosamine was isolated from the vacuum-dried hydrolysate, using a column of Dowex-50 (Gardell, 1953). The hexosamine was wet-ashed with concentrated 'Microanalytical Standard' sulphuric acid. The ammonia was distilled from this solution into 5 ml. of 0.05N-HCl by the technique of Rittenberg (1946). The ammonia content of the solution was measured on a sample of the distillate. The remainder was then evaporated to dryness *in vacuo*, redissolved, and the nitrogen content of the sample was adjusted with unlabelled NH_4Cl to contain 1 mg./ml. of nitrogen. The excess of ^{15}N in the sample was estimated with the mass-spectrometer by Mr C. Dickinson of this Institute, for whose help we are grateful. The results of the two experiments are summarized in Table 9. It will be

Table 9. *Incorporation of ^{15}N into hyaluronate glucosamine*

Cells were grown and suspensions prepared in the usual way, and incubation was carried out in the presence of 1.25 $\mu\text{moles/ml.}$ of ammonium L-glutamate, containing 30% excess ^{15}N in the ammonium ions. In the first experiment the cells were examined within 24 hr. of preparing the suspension. In the second the cells were stored until no synthesis in the presence of NH_4Cl alone took place.

	Expt. no.	
	1	2
Total nitrogen of hyaluronate hexosamine isolated	0.36 mg.	0.56 mg.
Amount of non-isotopic nitrogen (as NH_4Cl) used for dilution	1.095 mg.	0.56 mg.
Isotopic excess in the diluted sample	5.86%	10.32%
Therefore isotopic excess in hyaluronate glucosamine nitrogen	23.8%	20.6%
Correction factor for unlabelled hyaluronate present as blank*	1.04	1.47
Corrected isotope excess in the synthesized hyaluronate glucosamine	24.3%	30.4%
Incorporation of ^{15}N into hyaluronate glucosamine	80%	101%

* This represents the amount of hyaluronate glucosamine nitrogen present in supernatant fluids of control suspensions, incubated in the absence of nitrogen source. It is calculated from the glucosamine, estimated by the method of Elson & Morgan (1933), in acid-hydrolysed supernatant fluids. This glucosamine was not isolated.

into the glucosamine of hyaluronate without considerable dilution, both of these questions might be settled. If the α -amino group of the intermediary glutamine were used, dilution of the labelled ammonia would be expected from the α -amino group of the added unlabelled glutamate.

Two experiments with [^{15}N]ammonium L-glutamate as nitrogen source were carried out. Both the cell suspensions were incubated under the usual conditions in the presence of 1.25 $\mu\text{moles/ml.}$ of ammonium L-glutamate in which nitrogen of the NH_4^+ contained 30% excess of ^{15}N . In one experiment the suspension was examined within

seen that in Expt. 1, in which freshly prepared cells were used, the isotopically labelled ammonia was diluted to the extent of 20%. In the second experiment, no dilution at all occurred, although it must be noted that the correction for non-synthesized hyaluronate was abnormally high in this experiment. In neither, however, is the dilution sufficient to suggest that the unlabelled α -amino group of the glutamate is the important nitrogen donor. Thus glutamine is synthesized from ammonium glutamate and the amide group is likely to be incorporated into the hexosamine of hyaluronic acid.

*Synthesis of bound glucosamine by
cell-free preparations*

Work with cell-free preparations of *Streptococcus haemolyticus*, strain T13, is only of a preliminary nature and no evidence has yet been obtained to show whether the hexosamine synthesized is part of a larger molecule. It is included here as a final demonstration that glutamine is a necessary intermediate in the synthesis of hexosamine, thus eliminating the possibility that formation of the amide was simply necessary to transport ammonia nitrogen into the cell.

The extracts were made from suspensions prepared in the usual way, excepting that they were washed three times with 0.1M Sørensen phosphate buffer at pH 7.0, instead of with glycerophosphate, and stored for the minimum time at 0–4°. The cells were crushed by means of the Hughes (1951) press

DISCUSSION

The evidence presented in this paper shows that the mucopolysaccharide synthesized by *Streptococcus haemolyticus* T13 consists of glucosamine and glucuronic acid residues and has its physical properties altered by streptococcal hyaluronidase in the same way as hyaluronate isolated from mammalian sources. The isolated mucopolysaccharide has the same infrared spectrum as mammalian hyaluronate, and approximately the same chemical composition. Ultracentrifugal (Ogston & Lowther, unpublished) and electrophoretic analysis of isolated material showed no evidence of more than one polydisperse substance. It therefore seems reasonable to suppose that we are studying the biosynthesis of hyaluronate and that all the hexosamine liberated on hydrolysis of the supernatant fluids came from this mucopolysaccharide.

Table 10. *Biosynthesis of hexosamine by cell-free extracts prepared from streptococci*

Extracts (0.5 ml.) were incubated for 2 hr. at 37° with the additions shown. Total volume in each tube was 1.0 ml. The complete system contained 5 μ moles of either ammonium L-glutamate or L-glutamine.

Nitrogen source	Omission	Glucosamine (μ moles/ml.)			
		1	2	3	4
Ammonium L-glutamate	Expt. no. ...				
	None (i.e. complete system)	0.14	—	—	0.16
	Mg ²⁺ , cysteine, ATP	0.05	—	—	—
L-Glutamine	ATP	0.03	—	—	—
	None (i.e. complete system)	0.91	0.38	1.34	0.68
	Mg ²⁺	—	—	—	0.04
	Cysteine	—	—	—	0.03
	Mg ²⁺ , cysteine, ATP	0.07	—	—	—
	ATP	0.08	0	0.67	0.14
	Glucose	—	0	0.10	0.03
	Glutamine	—	0.09	0.10	—

working at –20° and the cell mush was centrifuged at 0° and 11 000 *g* for 30 min. The supernatant fluid (1 ml. volume) was then supplemented by final concentrations of 0.001M adenosine triphosphate (ATP) as the sodium salt, 0.02M magnesium sulphate, 0.022M cysteine, 0.5 g./100 ml. of glucose, and 5 μ moles/ml. of either glutamine or ammonium glutamate. The final volume of the samples was adjusted to 2 ml. and they were incubated for 2 hr. at 37°. At the end of this time the samples were all hydrolysed for 4 hr. with 4N-HCl, as in the usual estimation of hyaluronate hexosamine. The results obtained and the relative importance of some of the additions can be assessed from Table 10. It will be seen that synthesis occurred only in the presence of glutamine; ammonium glutamate was ineffective. Presumably the glutamine synthetase of the cells was not present in the extracts and in its absence glutamine is a necessary intermediate in the synthesis of hexosamine by streptococci. ATP and glucose are also essential.

The following steps are involved in the synthesis of glucosamine in this hyaluronate from ammonium ions and glucose, by the streptococcal suspensions:

Glutamine + glucose or glucose derivative $\rightarrow$
hyaluronate glucosamine.

The work of Roseman *et al.* (1953, 1954) and Topper & Lipton (1953) shows that the carbon skeleton of the glucose derivative must be close to that of glucose itself. The results of Leloir & Cardini (1953) make it seem reasonable to suppose that this derivative will prove to be a phosphorylated hexose.

In the region of limiting nitrogen supply the conversion of the amide of L-glutamine into the amino group of glucosamine by the suspensions is remarkably efficient, approaching 100%. We have always measured the total amount of hyaluronate formed and have not concerned ourselves with the initial rates of formation. It is not, therefore,

possible to deduce to what extent hexosamine formation limits the more complicated process of mucopolysaccharide synthesis by the cell. Moreover, the proved absence of accumulation of free hexosamine or acetylhexosamine cannot help in this, since it is known that streptococci can rapidly use free amino sugars (Rogers, 1949).

It is not without interest that Reider (1953), who studied the origin of the glucosamine in ovomucoid of hens' eggs, concluded that ammonia was a much nearer precursor than amino acids and considered the possibility that glutamine might be involved but dismissed this idea. Very recently Boström, Rodén & Vestermark (1955) and Boström, Jorpes, Månsson, Rodén & Vestermark (1955) have shown that glutamine specifically stimulates the *in vitro* sulphur exchange of the chondroitin sulphate in cartilage slices.

SUMMARY

1. Washed suspensions of streptococcus T13 will synthesize hyaluronate from glucose and L-glutamine in the presence of phosphate and magnesium.

2. L-Glutamine can always be replaced by an equivalent molar concentration of ammonium L-glutamate, or by L-serine, L-arginine and L-asparagine, provided L-glutamate is also added. When freshly prepared, the cell suspensions can synthesize hyaluronate from glucose and ammonium ions alone, or from amino acids, giving rise to ammonia.

3. In the presence of ammonium L-glutamate, synthesis is much more sensitive to methionine sulphoxide than when L-glutamine is added.

4. Cell-free extracts of streptococci will synthesize glucosamine from glutamine but not from ammonium glutamate.

5. Highly efficient conversion of labelled ammonium ions into hyaluronate glucosamine is effected by streptococci in the presence of unlabelled L-glutamate.

6. Glutamine is proposed as an essential intermediate in the synthesis of hyaluronate glucosamine by *Streptococcus haemolyticus*, strain T13.

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