Hyaluronic Acid Utilization by Hemolytic Streptococci in Relation to Possible Hyaluronidase Function in Pathogenesis. (18526)

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Various polysaccharides have been shown to form the prosthetic groups of biologically-active proteins. In consequence, it is frequently the specific immune response to a particular polysaccharide which constitutes a major body defense to an infectious disease agent. In the main, attention has been focused on the polysaccharide as a functional unit. Little consideration has been given to the possibility that polysaccharides, *per se*, and their degradation products may also function in pathogenesis as a source of energy for disease-producing micro-organisms. Our concern in this paper is to consider this possibility in hemolytic streptococcal infections by utilizing the naturally-occurring polysaccharide, hyaluronic acid, which is the principal intercellular cementing substance of tissues derived from the mesenchyme. Upon hydrolysis, hyaluronic acid yields equimolar fractions of N-acetylglucosamine and glucuronic acid.

Material and methods. In our investigation of the effect of hyaluronic acid and its hydrolytic products upon the respiration of group A streptococci, we separated the latter into two groups: group I, which included 3 streptococcal strains not producing hyaluronidase in neopeptone, beef heart infusion broth and group II, containing 4 strains which did produce the enzyme in this medium. As we have reported elsewhere(1), the group II organisms have been found to produce higher percentage mortalities in chick embryos than group I organisms. All cell suspensions were washed once in phosphate buffer and standardized turbidimetrically using an Evelyn colorimeter. Oxygen uptake was measured in the Warburg apparatus in the usual fashion using a pH 7.4 phosphate buffer and 0.2 ml of a 20% KOH solution in the center well for CO_2 absorption. Oxygen uptake figures when reported by group represent combined averages for the individual strains comprising the group. Hyaluronidase activity was assayed by the turbidimetric method of Kass and Seastone as modified by Meyer(2). Purified hyaluronic acid was prepared from umbilical cords by the method of McClean as modified by Rogers(3). Hyaluronidase was prepared by ammonium sulphate fractionation of a culture of a group A type 4 streptococcus grown in dextrose, neopeptone beef heart infusion broth.

Results. In Fig. 1 it can be seen that groups I and II streptococci had low endogenous respirations. With hyaluronate as substrate, group I respiration increased an average 2.5-fold whereas group II organisms showed an oxygen uptake 5.2 times greater than the endogenous. The respiration of a virulent (for chick embryos) group II streptococcal strain (No. 421) was then measured in the presence of hyaluronate and with

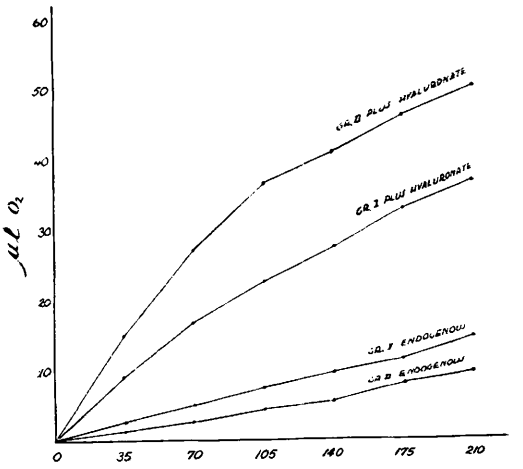

FIG. 1.
Hyaluronate stimulation of respiration in hyaluronidase-nonproducing and producing strains.

1. Sallman, B., and Birkeland, J. M., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1062.

2. Meyer, K., *Physiol. Rev.*, 1947, v27, 335.

3. Rogers, H. J., *Biochem. J.*, 1945, v39, 435.

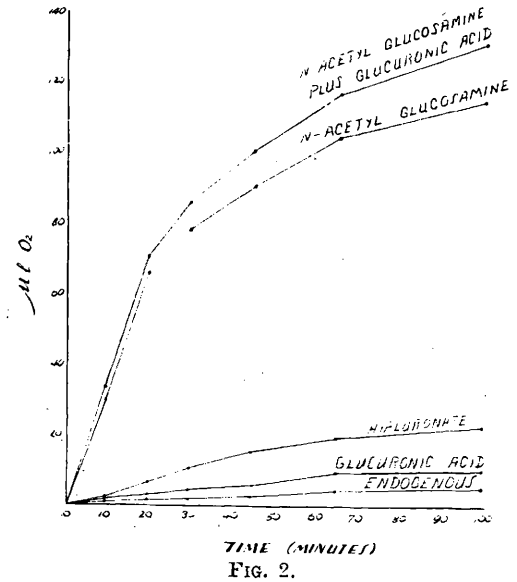

FIG. 2.
Stimulation of respiration (strain 421) by hyaluronate and hyaluronate breakdown products.

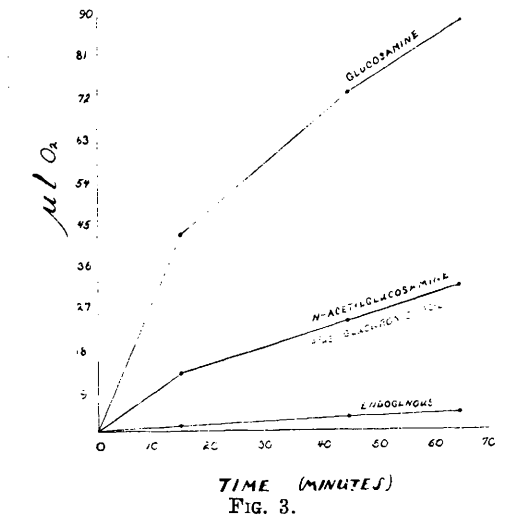

FIG. 3.
Stimulation of respiration (strain Sa) by hyaluronate hydrolytic products and glucosamine.

amounts of N-acetylglucosamine and glucuronic acid equivalent to their concentrations in the unhydrolyzed hyaluronate sample tested. The results in Fig. 2 show that in 100 minutes hyaluronate stimulated oxygen uptake 4-fold, N-acetylglucosamine 20-fold and glucuronic acid but slightly over the endogenous respiration. The addition of N-acetylglucosamine and glucuronic acid simultaneously resulted in an oxygen uptake equivalent approximately to the sum of their individual stimulations. This stepwise degradation of hyaluronate associated with a source of increased available energy suggested that the process might be continued with the deacetylated product, glucosamine. In Fig. 3 it can be seen that an equivalent amount of glucosamine stimulated respiration of another group II organism (strain Sa) to an extent 3 times as great as did N-acetylglucosamine plus glucuronic acid.

Since hemolytic streptococci are frequently found as co-invaders in the host with other pathogenic micro-organisms, the possible synergistic utilization by the latter of hyaluronic acid was investigated. A number of organisms frequently found associated with group A streptococci *in vivo* were tested with hyaluronate alone as a source of energy and with hyaluronate in the presence of streptococcal strain No. 421. From Table I it is evident that all of the organisms tested, with the exception of *N. catarrhalis, C. diphtheriae* and possibly *S. aureus*, utilized hyaluronate for respiration. In the presence of a group A streptococcus, each micro-organism without exception accomplished a more efficient utilization as demonstrated by the streptococcal: hyaluronate ratios ranging from 1.2 to 5.5 for the nonpathogens and 1.1 to 5.8 for the potential pathogens.

It is important to know whether an organism which utilizes hyaluronate for respiration attacks the molecule directly or whether enzymatic hydrolysis into the component N-acetylglucosamine and glucuronic acid, presumably through the agency of hyaluronidase, must occur prior to its utilization. From our findings, it appears that hyaluronate itself is not utilized but that the resultant oxygen uptake in the presence of hyaluronate can be accounted for by the products formed in its hydrolysis. The results of a typical experiment designed to answer this question are given in Table II. Here, it is seen that within 2 hours *E. coli* consumed 502 μl of oxygen with 0.5 mg of hyaluronate as a substrate. When 165 Turbidity-Reducing Units of hyaluronidase were added, respiration was increased to 574 μl. When a similar

TABLE I. Aerobic Utilization of Hyaluronate by Micro-organisms Frequently Found Associated with Group A Streptococci.

Organism	Endogenous respiration	Hyaluronate	Group A streptococcal synergism*	Strep.: hyaluronate ratio
	Nonpathogens			
E. coli 'R' communior	77†	383	460	1.2
N. catarrhalis	17	18	61	3.4
B. subtilis PCI 220	68	131	721	5.5
	Potential pathogens			
D. pneumoniae, type I, Abbott	60	76	82	1.1
S. aureus 209-P	70	77	123	1.6
M. tuberculosis H 37 Ra‡	66	413	828	2.0
C. diphtheriae	99	101	307	3.0
H. influenzae H-2040	57	96	406	4.2
H. pertussis	70	84	490	5.8

* Corrected for streptococcal respiration in the presence of hyaluronate.
† μl O_2.
‡ Eighteen-day growth in Dubos' basal medium without Tween 80.

TABLE II. Mechanism of Hyaluronate Utilization in the Respiration of *E. coli*.

	μl O_2 consumed in 2 hours	
Endogenous	107	
Hyaluronate (.5 mg)	502	
" (.5 mg) + hyaluronidase (165 TRU*)	574	
Hyaluronate (.5 mg) + hyaluronidase (165 TRU) previously incubated 37°C 45 min.	577	
N. acetylglucosamine (.266 mg)	302	572
Glucuronic acid (.234 mg)	270	
Hyaluronidase:hyaluronate ratio	1.14	

* Turbidity-reducing units of the streptococcal enzyme.

amount of hyaluronate was incubated with hyaluronidase prior to being added to the Warburg flask, an equivalent amount of oxygen was taken up (577 μl). Amounts of N-acetylglucosamine (0.266 mg) and glucuronic acid (0.234 mg) equal to their concentration in the unhydrolyzed hyaluronate yielded a total oxygen consumption of 572 μl. The disparity between oxygen uptake with hyaluronate (502 μl) and hyaluronate plus hyaluronidase (574 μl) can be explained by assuming that hyaluronidase production by the organism was insufficient for complete hydrolysis of the substrate. The likelihood of this explanation is enhanced by the fact that the addition of 10 TRU in place of the 165 TRU given in Table II will accomplish the same oxygen uptake.

Evidence that hyaluronidase action is also responsible for the synergistic utilization of hyaluronate may be gleaned from the fact that the streptococcal:hyaluronate ratio of the *E. coli*-group A streptococcus complex (Table I) is 1.2, essentially the same as the hyaluronidase:hyaluronate ratio for *E. coli, viz,* 1.14 (Table II). Similarly, the streptococcal:hyaluronate ratio of the *M. tuberculosis*-group A streptococcus complex is 2.0 compared to a hyaluronidase:hyaluronate ratio of 2.2.

Discussion. When the culture supernatants of 7 of the organisms listed in Table I (*H. influenzae* and *H. pertussis* were grown on solid media) were assayed for hyaluronidase by the turbidimetric and mucin-clot-prevention methods, no enzyme was found. Four of these organisms, however, were able to utilize hyaluronate, apparently producing the enzyme adaptively in the presence of the specific substrate. This behavior cannot be considered anomalous since we have observed repeatedly that many strains of hemolytic streptococci, which do not produce hyaluronidase in the usual culture media, do produce the enzyme after one of more serial transfers in media containing hyaluronate. This fact would indicate that previous surveys(4) of organisms, grown in media free of hyaluronate, for hyaluronidase production should be revaluated since the substrate is available during infection in the host. Another point which should be mentioned at this time is the relative in-

4. Crowley, N., *J. Path. Bact.*, 1944, v56, 27.

sensitivity of the turbidimetric and MCP tests for hyaluronidase assay. Small amounts of hyaluronidase (considerably less than 1 TRU/ml) could be detected manometrically when included with hyaluronate and a suitable micro-organism in the Warburg flask but could not be measured by the turbidimetric and MCP methods. The failure of the latter procedures was also evident in our inability to demonstrate hyaluronidase titers in Warburg vessels during active hyaluronate utilization by the various bacterial organisms.

Apparently, when hyaluronate is present as a substrate, group I streptococci, not producing hyaluronidase in infusion broth, do produce the enzyme in an adaptive fashion. The extent of such adaptive enzyme formation, however, is less than the amount of enzyme normally produced by group II streptococci. This is evident from the fact that twice as great a stimulation of respiration occurred with the group II organisms, indicating that the latter streptococci made greater amounts of N-acetylglucosamine and glucuronic acid available as sources of energy for the respiring cells. The wide distribution of hyaluronic acid in the body may serve as an energy reservoir for bacterial invaders possessing hyaluronidase in their metabolic repertoire. Furthermore, in the presence of such an invader, it is probable that other micro-organisms producing disease may benefit from the degradation products, N-acetylglucosamine and glucosamine, so produced. In this connection it may be well to recall to mind how frequently hemolytic streptococci are found as invaders causing primary, concurrent or secondary diseases. Such associations might be considered synergistic, the streptococci providing a ready source of energy and receiving in return a debilitated body defense due to the activities of the associated organism.

In evaluating the likelihood that just such a state of affairs might exist in the invaded host, let us consider briefly the fate of N-acetylglucosamine in the body. Here, N-acetylglucosamine is deacetylated only with difficulty if at all(5). Deamination is accomplished by kidney, testis, brain and certain muscle tissues only under aerobic conditions. The liver contains no enzymes able to oxidize or deaminate the amine sugars. Thus, N-acetylglucosamine produced and liberated in the body will not be removed or acted upon very readily. This represents an ideal situation for its utilization by invading bacteria. In contrast, many bacteria are able to deacetylate and deaminate N-acetylglucosamine as well as oxidizing both it and glucosamine rather rapidly under anaerobic as well as aerobic conditions.

From the clinical point of view, information is slight but still suggestive. In various infections, the adult blood glucosamine levels have been found to exceed by far the normal range of 76 to 110 mg %. The highest glucosamine titers have been observed in cases of rheumatic fever, rheumatoid arthritis, hemolytic streptococcal pharyngitis, pneumococcal pneumonia, staphylococcal infection and tuberculosis(6). All of the organisms presumably causing these diseases have been shown to be able to utilize hyaluronate and, in many cases, to produce appreciable amounts of hyaluronidase.

Hitherto, the possible role of hyaluronidase in pathogenesis has been visualized solely as a means of increasing the local tissue invasiveness of hyaluronidase-producing micro-organisms. We are of the opinion that further investigation of the role of hyaluronidase in the respiration of hyaluronidase-producing organisms and of the function of hyaluronic acid hydrolytic products in the metabolism of associated agents of infection will yield a more comprehensive picture of a mechanism of disease production.

Summary. Hemolytic streptococcal strains, virulent for chick embryos, were found able to utilize hyaluronic acid for respiration to a greater extent than relatively avirulent strains. With the micro-organisms tested, it appears that the hydrolytic products and not hyaluronate itself are utilized in respiration, such hydrolysis occurring presumably through the agency of hyaluronidase. Pathogenic bacteria frequently found associated with group

5. Lutwak-Mann, C., *Biochem. J.*, 1941, v35, 610.

6. West, R., Clarke, D. H., and Kennedy, E. M., *J. Clin. Invest.*, 1938, v17, 173.

A streptococci *in vivo* had a synergistic relationship with the latter in utilizing hyaluronate as a substrate for energy. On the basis of these findings, a mechanism of pathogenesis by hemolytic streptococci alone and with associated agents of infection was proposed. Certain clinical findings, interpreted as evidence for the existence of such a process, were cited.

It is a pleasure to acknowledge the assistance of Miss Clara Boyd.

Received February 6, 1951. P.S.E.B.M., 1951, v76.

A New Dialyzer for Use as an Artificial Kidney. (18527)

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In the wake of pioneer experimental studies of Abel, Rowntree and Turner(1), the process of dialysis is receiving steadily increasing attention in the treatments of patients with acute anuria(2-8), and in the study of hypertension and renal disease(9,10). Our special interest has been in the application of continuous extracorporeal dialysis of blood for the correction of disturbed ionic equilibrium and the removal of accumulated nitrogenous constituents of the body water as occurs in uremic states. The instrument that was designed incorporates the following desirable features: (1) sterilization by autoclaving, (2) complete visibility of blood and wash channels during operation, (3) provision for rapid exclusion and short-circuiting of defective tubing, (4) true countercurrent flow, (5) surface area adaptable to needs, (6) relative constancy of blood volume, (7) optional and controlled pressure differential between blood and wash electrolyte solution compartments, and (8) reasonable compactness.

A complete dialyzer* is made up of a variable number of units according to the surface area desired, an assembled unit being shown in Fig. 1. Each unit has a cellophane surface area of approximately 4000 cm^2. Fig. 2 and 3 are diagrammatic representations of the top and cross sectional views of such a unit. Top and bottom are formed from a heavy, thermal stress-resistant sheet of plate glass (A), the steel frame (B) is in the middle, and clear plastic gasket sheets (I) are placed between glass and steel. As seen in Fig. 1 the steel frame of each unit forms two double channels, each about one meter in length. After preparation of the cellophane tubing (Visking Corp., 36/32 in. inflated diameter) by repeated boiling in water, and testing with air pressure for leaks, a length of tubing (D) is connected to the nylon fittings (E), sandwiched between the flexible steel, woven, chain-linked screens (C) and placed into a channel. The nylon fittings (cross section Fig. 2) are constructed to provide smooth flow without dead space, folding or leakage. Attachment is made by drawing the end of the cellophane tubing over the inner nylon piece and slipping the steel collar on top. The nylon fittings are then inserted into the openings at both ends of the channel. Turn-

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4. Alwall, N., *Acta med. Scand.*, 1947, v128, 317.

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* Made by Brosites Machine Co., New York City.