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I hereby recommend that the thesis prepared under my supervision by Sanford O. Byers

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Approved by: Myrlain A. Logan

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THE PRODUCTION AND PROPERTIES OF BACTERIAL HYALURONIDASE (SPREADING FACTOR)

by

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EPITOME

The enzyme system hyaluronic acid-hyaluronidase is of general biologic importance since it occurs in vertebrates, insects, and bacteria; it is thought to be part of the means whereby Cl. perfringens type A invades animal tissue.

Hyaluronic acid may be prepared by digesting human umbilical cords with trypsin, dialyzing, filtering, and precipitating with alcohol. It may be further purified by redissolving, precipitating impurities with barium, dialyzing and reprecipitating.

A method for the determination of uronic acids by measurement of evolved CO_2 in the Van Slyke-Neill gasometric apparatus is described.

When hyaluronic acid is used as sole source of carbohydrate in media containing vitamins, salts, and pancreatic digests of beef or veal, Cl. perfringens (and D.pneumoniae) secretes high concentrations of hyaluronidase into the medium.

Hyaluronidase may be assayed by methods which measure the rate of its hydrolysis of hyaluronic acid.

Hyaluronidase is a relatively stable enzyme which may be ultrafiltered, dialyzed, filtered, adsorbed, precipitated and stored, with little loss. It is inactivated by formaldehyde and by X-radiation but not strongly affected by antibiotic drugs.

When injected into animals and man, hyaluronidase or formaldehyde treated hyaluronidase give rise to the production of specific

antibodies which neutralize the *in vitro* action of the enzyme and, in optimal quantities, precipitate it from solution.

ABSTRACT

1. A good yield (4.5 - 6 grams per 100 grams of dried cords) of white potassium hyaluronate of high viscosity (0.20% neutral solutions were 30 - 74 times as viscous as water) was prepared by digesting human umbilical cords with trypsin, dialyzing to remove amino acids and other small molecules, clearing by filtration under gentle suction with "Supercel" and reprecipitating with saturated alcoholic solution of potassium acetate. The preparation contains 1.45% of sulfur and 0.09% of phosphorus. On the assumption that hyaluronidase hydrolyzes 90% of the hyaluronic acid present to molecules of dialyzable dimensions, the preparation is considered as consisting of 55% potassium hyaluronate, 19% chondroitin sulfuric acid, 0.97% nucleic acid, 6.5% unknown high molecular weight substances, and about 19% of dialyzable salts.

The concentration of potassium hyaluronate may be raised to 70% by dialyzing the preparation, and to 88% by precipitating sulfonated polysaccharides and nucleic acid with barium.

2. A short, convenient micro method for the determination of galacturonic acid (and presumably of other uronic acids) is to hydrolyze the sample for 3 hours at 130° C. with 1 N H_2SO_4 in a sealed bomb tube and subsequently to determine the quantitatively evolved CO_2 with the aid of a special side tube attached to the

Van-Slyke-Neill gasometric apparatus. Samples are chosen so as to evolve a convenient quantity (0.025 mM) of CO_2. Any number of samples may be hydrolyzed simultaneously, stored at room temperature, and analyzed when convenient.

3. Two published methods for the assay of hyaluronidase are described in detail and others more generally. The viscosity reduction method assays hyaluronidase by measuring the rate at which it decreases the viscosity of hyaluronic acid solutions. The mucoportein clot prevention test measures the least amount of enzyme which in 20 minutes will hydrolyze hyaluronic acid to such an extent that it no longer clots with protein on acidification. An attempt to find a simple straight line relation for use in the viscosity reduction test, through manipulation of the Schütz-Borrissov law was not successful.

4. D. pneumoniae type III, CHA strain is able to produce hyaluronidase on a semi-synthetic casein hydrolysate medium. The hyaluronidase is produced as a strictly "adaptive" enzyme, none appearing unless the specific substrate is present in the medium; the additional presence of glucose reduces hyaluronidase production to less than 1/50th of the value occurring when potassium hyaluronate is the sole source of carbohydrate. The most rapid appearance of hyaluronidase coincides with the disappearance of culture turbidity. Soft X-rays are able to inactivate pneumococcus hyaluronidase.

5. When potassium hyaluronate is used as sole source of carbohydrate in media containing vitamins, salts, and pancreatic digests of beef heart or veal, and incubation is continued for over 60 hours,

Cl. perfringens type A will produce up to 1400 V.R.U. of hyaluronidase per ml. of medium without detectable amounts of alpha antigens. Filtrates from such cultures should be useful in serological and immunological studies. A comparison of the hyaluronidase production of 6 strains of Cl. perfringens type A revealed that strains BP6K, BP365, BP364 and BG6 were good producers and that strains WX and 1508 Ad were non-producers. Added iron is necessary for best production on casein hydrolysate media but has no effect on meat digest media under the conditions of the experiment.

6. Two strains of Cl. septicum were found to produce 1-10 V.R.U. per ml., while Gebrüder-Mayer yeast, an unknown species of Penicillium glaucum, Penicillium notatum, Trichophyton interdigitale, Cl. sordelli, Cl. novvi and B. tularensis were not found to produce hyaluronidase in the small number of cultures tried.

7. Cl. perfringens hyaluronidase is stable to storage in the cold or at room temperature, and to ultrafiltration, dialysis, filtration through Berkfeld and Mandler candles, adsorption, evaporation, and precipitation by alcohol. It is inactivated by X-radiation when pure, and by formaldehyde, but not strongly affected by most sulfone drugs or penicillin.

8. Hyaluronidase is neutralized by specific antisera. A non-specific serum inhibition, occasionally present, may be removed by diluting the serum in question. Similar dilutions of specific sera retain their neutralizing power.

The antisera particulate and flocculate with undiluted hyaluronidase. The supernatant fluid at equivalence points was completely

or almost completely free from hyaluronidase activity, indicating that flocculation took place at or near the neutral point.

9. When injected into animals and man Cl. perfringens hyaluronidase or formalized hyaluronidase cause the appearance of specific neutralizing antibodies in the serum of about 75% of those treated. The highest antibody titer occurred in man, the next highest in dogs, and, in order of descending titer, rabbits, mice, and guinea pigs.

10. Suggestions for future work on the various subjects studied are included. A bibliography of 170 references and an appendix containing 131 descriptions of published methods of preparation of hyaluronic acid are given.

I. GENERAL INTRODUCTION

Much of the work described in this thesis was done with an immediate purpose in view: to obtain knowledge necessary to the production of a toxoid for active immunization of military personnel against gas gangrene. For this reason the work is, perhaps, less well rounded than if it had been undertaken solely as an academic study. Nevertheless, the scope of the research is not excessively narrow and it embodies much of the preliminary work necessary to a more academic inquiry.

Investigation of the enzyme system hyaluronidase-hyaluronic acid is justified for several reasons: (a) it is intimately concerned in the physiological problem of the permeability of the connective tissue and probably also in capillary permeability; (b) it is part of the mechanism of production of such normal body secretions as the synovial and ocular fluids and of such abnormal secretions as those of tumors and inflamed tissue; (c) the presence of hyaluronidase at the site of infection enhances the pathogenic effect of every infectious agent, so far observed, which acts in or upon the connective tissue; (d) the presence of hyaluronidase increases the invasiveness of animal poisons, and (e) hyaluronidase plays a role in mammalian fertilization.

Connective tissue permeability

Although at one time there was a question as to whether or not there is an intercellular ground substance in connective tissue (140, 141), it is now generally accepted that such a substance exists (8, 59, 103, 169). The ground substance is viscid (8, 21) and variation in this viscidity is the main determinant of the permeability of the tissue (39). A similar ground substance is found in the adventitia of the blood capillaries (39, 164, 165, 170). A mucopolysaccharide currently called hyaluronic acid (116) is the chief contributor to the viscidity of the connective tissue ground substance. This is disclosed by the loss of viscosity of tissue secretions attacked by a specific hydrolytic enzyme called hyaluronidase (119), by the isolation of hyaluronic acid from viscous tissue secretions in amounts sufficient to account for the viscosity of the original secretion (39, 128), and by the simultaneous destruction of hyaluronic acid and viscosity in tissue secretions attacked by hyaluronidase (18). This destruction of hyaluronic acid results in a large increase in connective tissue permeability (4). Hyaluronidase also increases capillary permeability (3, 38, 148).

Occurrence of Hyaluronic Acid

Hyaluronic acid has been isolated or obtained from almost every animal tissue, from many animal secretions, and from some bacteria. In the early literature it is not designated hyaluronic acid but its presence is evident from the mode of preparation and properties of

substances designated as mucoids, animal gums, mucine, mucoitine, glucoproteins, hyaloids, and mucoproteins. Some of these sources of hyaluronic acid are tendon (13, 20), synovial fluid (124), vitreous and aqueous humor (116) pleural fluid (128), edema fluid (114), fowl and human tumor fluid (126), rabbit and pig skin (18, 24), rabbit fasciae (150), umbilical cord (122), kidney, pancreas, and liver (101), prostate (1), and streptococcus(124).

Hyaluronidase in Infection

Hyaluronidase was first noted because of its ability to enlarge the tissue area attacked by a given dose of virus (28, 31). Infectious agents and their toxins do not leap from cell to cell but pervade, and are propagated in, the intercellular ground substance which is hydrolyzed and made permeable by hyaluronidase.

Enchancment of infection by hyaluronidase has been shown for 12 strains of filterable virus including vaccina (30, 36, 57, 100), vesicular stomatitis (67), rabbit fibroma (36), Rous sarcoma (35, 65), and probably also for poliomyelitis (159); for about 50 types of bacteria including streptococci (34) and staphylococci, both of which have been shown to produce hyaluronidase (34, 154), tubercle bacilli (166), pneumococcus (33), and members of the gas gangrene group such as C. perfringens and Vibrion septique (114); and for infection of rats with a bacillus isolated from lepers (89).

Specific antisera against the hyaluronidase produced by different types and species of bacteria will prevent the enhancing effect (89, 114).

Animal Poisons

Russel's viper venom destroys hyaluronic acid (107) as do the venom of *Vipera aspis*, *V. ammodytes*, *Bothrops jaraca*, *Crotalus terrificus*, and many other poisonous snakes (48). This hydrolytic power is neutralized by specific antisera. Leech head extracts also contain hyaluronidase (48) and the enzyme has been demonstrated in bee stings (18). Two species of scorpions show invasive power, as do species of poisonous fish (158). A nematode, parasitic in humans, possesses this invasive power (12) and it is present in extracts from poisonous insects such as spiders, wasps, and mosquitoes, but not in extracts from non-poisonous insects (37).

Hyaluronidase in Fertilization

The presence of hyaluronidase in spermatozoa has recently been shown to bring about the dispersal of a viscous gel which surrounds the egg of the rat, and thus to facilitate the penetration of the egg (111).

II. THE PREPARATION OF POTASSIUM HYALURONATE

INTRODUCTION

Hyaluronic acid is a widespread and biologically important constituent of animal and bacterial tissue, where it probably plays a protective and architectural role (39). Its preparation with greater ease, in better yield, or purer form is therefore a step towards solution of the problem of the composition of living matter. Further, hyaluronic acid is necessary to the preparation of the highest yields of bacterial hyaluronidase. In this connection, the ease of preparation of reasonable quantities of hyaluronic acid is an important factor, since investigation of bacterial hyaluronidase has been limited by the difficulty of obtaining adequate quantities of substrate. The method of preparation described in the experimental part of this chapter results in excellent yields of an undegraded, highly polymerized, purified product, suitable for use as a carbohydrate substrate in bacterial media.

HISTORY

Orientation

Hyaluronic acid is a complex polysaccharide which occurs in nature probably bound to protein in an easily dissociable salt linkage (121). When so bound it falls into the group of compounds

variously known as "mucins," "mucoids", "hyaloid," "animal gum," or "sinistrin;" and would be considered a prosthetic group in the sense in which that term is said to have been introduced, that is, to mean the non-protein part of a complex protein molecule (83).

Mucins, Mucoids, and Their Polysaccharides

In the light of retrospect it is apparent that "mucin," "mucoid," "hyaloid" and similar terms found in the older literature may be taken to mean only the products of general methods of preparation and not definitely characterized chemical entities. The history of these compounds probably begins even before 1854 when Boedeker (9) is said to have prepared a sulfur containing carbohydrate by hydrolysis of cartilage. This preparation was probably contaminated chondroitin sulfuric acid. Rollet (151) in 1859 was the first to show that tendon contains mucin-like material, a finding which was verified by Eichwald (42). Other early preparations are those of Eichwald (42) in 1865 who obtained a mucin from the small Helix Pomatia by methods which would conserve hyaluronic acid or mucoitin if such were present; of Landwehr (77) in 1882 who obtained a purer material from Helix Pomatia which he called "animal gum;" and of Hammarsten (61) in 1888 who obtained a protein containing mucin of low sulfur content from submaxillary glands. The animal gum of Landwehr is of interest as this author removed protein chemically and finally obtained a product which exhibits one of the characteristics of the purest hyaluronic acid made today, that is, it failed to precipitate in ethyl alcohol without the presence of added salt. He was therefore the

first to isolate a polysaccharide from mucin. In 1891 and 1898 Jernstrom (69) prepared "funis mucin" from umbilical cords. C. Th. Morner (136) obtained a mucoid from vitreous humor and from cornea in 1894, and from human urine in the following year (137), when he appears to have been the first investigator to effect a purification by shaking the mucoid with chloroform. Folin (52) working in Hammarsten's laboratory, published a work on animal gum in 1897. More detailed description of the work of these and other authors may be found in Appendix I, where 131 publications on the preparation of mucins are particularized.

Interest in these compounds was to some extent stimulated by the medical practice of determining whether or not a body fluid was of inflammatory origin by acidifying the fluid in question, usually with dilute acetic acid. Under this treatment exudates of secretory or inflammatory origin were said to yield a precipitate or to become turbid, while transudates remained clear (68, 75). The precipitate yielded is of a mucoid character. In this connection, it is of interest to note that in recent times McClean (114) has shown the presence of hyaluronic acid in edema fluid at the site of inflammation and has noted that such edema fluid was more localized within the area of inflammation than were fluids in which the hyaluronic acid was destroyed. Hyaluronic acid has also been found in tumor fluid (71) and in fluid from cancernus pleura (128). In the cases of these fluids, and of synovial fluid (124) it is a hyaluronic acid-protein which precipitates upon acidification and furnishes the basis for the diagnostic test.

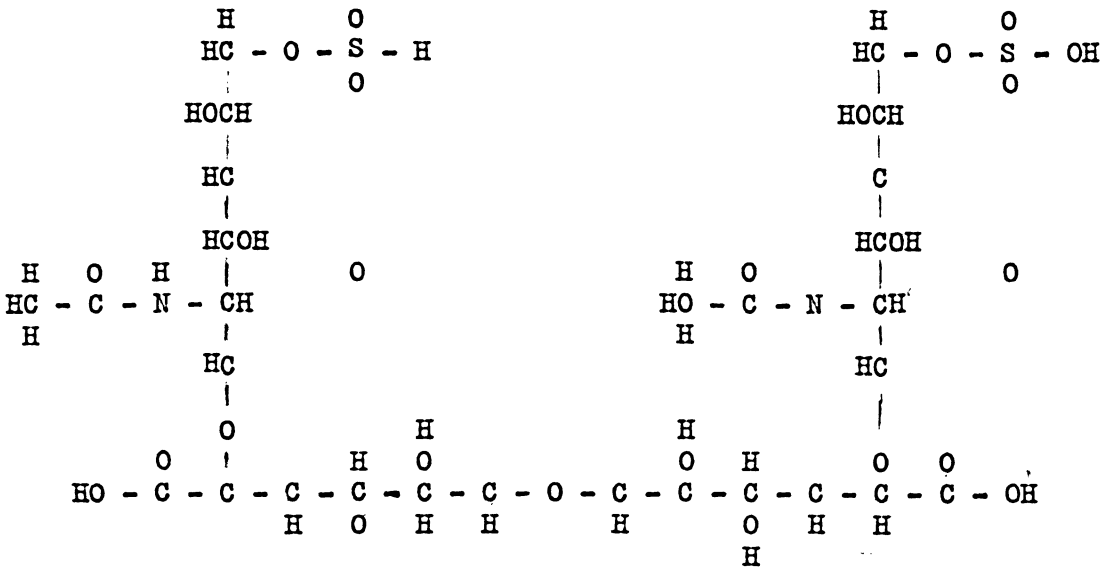
A question which repeatedly arises in dealing with the older work and which has not entirely vanished with the advent of modern means of analysis is well phrased by Levene (86). He asks, "Were the different groups of investigators dealing with different mucoproteins, containing different complex carbohydrates, or did the individual workers isolate the same substance in different degrees of purity"? For instance, van Lier (88) obtained from skin what he considered a sulfuric ester of carbohydrate while Meyer (131) has shown that both chondroitin sulfuric acid and hyaluronic acid are extracted together from skin. Both Landwehr (78, 79) and Schmiedeberg (152) obtained sulfur-free substances from mucin by similar methods; the products were called "animal gum" and "hyaloidin," respectively. From similar mucins, however, Levene (82) obtained sulfuric esters. Other examples might be cited, but are, perhaps, unnecessary. Appendix I contains the details of 131 published preparations of mucoproteins, slimes, and complex carbohydrates, together with the analysis given by their authors. Reference may be made to this appendix if it is desired further to ascertain to what extent the so-called isolations overlap. In general it may be said that, if the presence of a mixture of complex polysaccharides is presumed in the tissue or fluid used as raw material, then such a mixture must be presumed in the final preparation. A few modern authors have recognized this and effected purification by fractionation with barium, brucine, or hyaluronidase (70, 131).

Mucoitin Sulfuric Acid

Evidence of the nature of the carbohydrate part of mucoproteins was built up gradually during the years 1890-1925 by the analyses of various workers already cited. A large part of this work is to be attributed to Levene (82). Sulfuric acid was determined as acid-hydrolyzable sulfate, the acetyl radical was identified as the silver salt, hydrolyzed and quantitatively determined by titration; hexosamine was isolated; and glucuronic acid was identified through its p-bromphenylhydrazine derivative or as saccharic acid or other derivative, and determined by distillation and estimation of furfural or by the evolution of weighing of the carbon dioxide split off an acid hydrolysis. According to Furth (55) the aminosugars in the mucoproteins are monoacetylated.

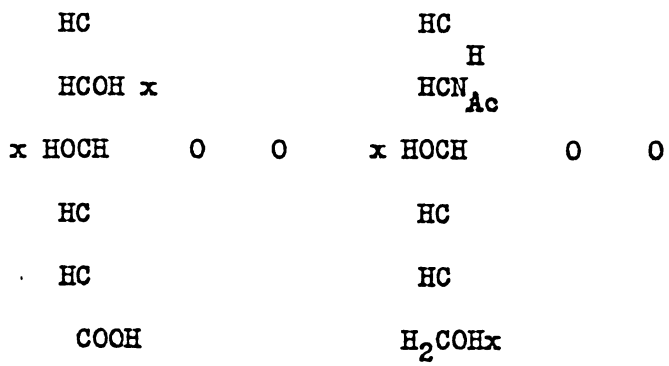
It was found that the polysaccharides obtained could be divided into two types: those like chondroitin and chondroitin sulfuric acid, and those like mucoitin and mucoitin sulfuric acid. Both the chondroitin and the mucoitin type carbohydrates are composed of glucuronic acid joined to an acetylhexosamine in equimolar proportions, with or without one or more attached sulfate groups. In chondroitin the hexosamine is galactosamine, in mucoitin it is glucosame.

The formula for chondroitin sulfuric acid is generally given (62) as:



chondroitin sulfuric acid

In the absence of knowledge of its linkage, the formula for mucoitin can be given only by extending hypothetical bonds between its constituent parts. If it is assumed that mucoitin has a structure similar to that of starch or cellulose, the formula may be represented (81) as:



mucoitin sulfuric acid

(the points marked x represent places where a sulfate group might be attached)

We are not here concerned with chondroitin, except as it may occur as an impurity, nor are we concerned with other polysaccharides not as yet found in sources similar to those from which mucoitin or hyaluronic acid may be extracted. Although the possibility exists that such substances as the various aldobionic acids may contaminate mucoitin preparations and especially preparations of hyaluronic acid from bacteria, such as those of Kendall (73), their presence does not seem to have been noticed, and the contamination, if any, is not serious. Other probable contaminants, because of their widespread distribution in animal tissues, are the "blood group A" substances, generally glucosamine dimannosides (53, 87, 149) or glucosamine galactoside (121).

Levene thought that all naturally occurring mucopolysaccharides were sulfated (84). This idea was reinforced by the consistency with which he and others were able to approach (though not to reach) theoretical values for sulfur in the preparations of chondroitin sulfuric acid. Preparations of mucoitin sulfuric acid, however, gave widely varying values for sulfur (82, 121) (Appendix I), almost all far below the value required by a formula in which sulfate stood in 1:1 ratio to glucosamine and glucuronic acid. In line with his belief that natural mucopolysaccharides were all sulfated, Levene (85) explained the varying sulfur values by postulating a degradation of the molecule in the course of preparation. Since some of the methods of preparation did not seem likely to effect this degradation, he was forced to postulate a highly labile sulfate linkage.

There appear to be at least two reasons for the erratic sulfur values obtained by workers during this period. One reason is the

probable existence, unsuspected by them, of a mixture of unsulfonated mucoitin and mono-, di-, and trisulfonated mucoitin in the tissues (70). Levene (85) recognized the existence of both mucoitin and mucoitin sulfuric acid in his preparations, but thought that this mixture was artificially created by the removal of sulfate during the process of preparation. To further complicate matters, sulfonated polysaccharides are said to dissolve out of tissue at a different rate from that of unsulfonated polysaccharide, and to a varying extent, dependent upon the "peptizing" value of the solvent (127, 136). Thus a change in the length of time during which a tissue was extracted, a slight change in the pH of the solvents, or the use of ammonia or urea would alter the sulfur content of the preparation and the product would appear now to be "degraded," now to be more "pure." Another reason for varying sulfur values, which perhaps accounts for the fact that Levene never prepared a sulfur-free mucoitin is that most of his preparations involved the use of hydrogen sulfide gas in order to remove barium or lead. Meyer (118) has found that barium sulfide is extremely difficult to wash out from mucopolysaccharide solutions or precipitates. The polysaccharide apparently acts as a protective colloid and holds the barium sulfide tenaciously. When the preparation is subsequently hydrolyzed with acid, the barium sulfide will appear as "acid hydrolyzable" sulfate.

This difficulty did not prevent a correct formulation of the constituents of mucoitin sulfuric acid, since the quantitative work was sufficiently accurate to establish the 1:1 ratio of the constituents, except for the varying values of sulfur.

Hyaluronic Acid

In 1934 Meyer (117) isolated a sulfur-free polysaccharide from vitreous humor, and named in hyaluronic acid from hyaloid (vitreous) plus uronic acid. Since that time hyaluronic acid has been found in streptococcus (73), in umbilical cord (118), in skin (131), in cornea (127), in tumor fluid (128), in edema fluid (124) and from similar sources (see General Introduction page 9 and Appendix I).

Meyer's initial isolation (151) was accomplished by precipitating aqueous extracts of an acetone powder of vitreous humor with neutral or acidified alcohol. Analysis of 4 samples showed 10.1, 4.0, 3.5, and 1.0% of ash, respectively, and those samples with a low ash contained only traces of sulfur, shown to be $CaSO_4$. The method of analysis for sulfur is not mentioned, though the demonstration of $CaSO_4$ would seem to indicate that sulfur analysis was done upon the ash. If this was the case it is possible that some sulfur was lost as volatile sulfur during the dry distillation and oxidation of the carbonaceous polysaccharide. In this paper (151) Meyer states, "It is evident that hyaluronic acid is not identical with what Levene and López-Súarez (81) considered a mucoitin sulfuric acid prepared from vitreous humor by alkaline treatment. Their material contained 3.6% sulfur, while our material contains only traces".

In later papers Meyer (119, 120, 127) found that a pneumococcus autolysate would hydrolyze hyaluronic acid but was without action upon a preparation from gastric mucosa which contained some mucoitin sulfuric acid.

Another difference between hyaluronic acid and mucoitin sulfuric acid is their degree of rotation of polarized light. For the rotation of hyaluronic acid various values have been given as follows, for $(a)_D^{25}$: -65; -51°(118); -51°(127); -67.5°, -74.9°(131); -57.2°, -65.0°, -68.8° (123); -76.8°, -70.3°, -.67.7°, -73.3°(128); -73°, -53°, -16°, -77°, -47°, -86° (73); whereas Meyer gives the rotation of a mucoitin sulfuric acid which he prepared (121) as -20.2°, to -22.2° for the acid and -35.7° for the neutralized salt.

Hyaluronic acid is thus seen to be distinguished from mucoitin sulfuric acid for three reasons: (a) the absence of sulfur in the molecule, (b) susceptibility to attack by pneumococcus enzyme and, (c) difference in specific rotation of sodium light. These reasons for believing hyaluronic acid to be different from mucoitin sulfuric acid are however not sufficient to distinguish it from mucoitin. Each reason will be examined in turn and its inadequacy shown.

(a) The lack of sulfur in hyaluronic acid is no bar to its identity with unsulfonated mucoitin.

(b) The failure of an enzyme system which attacked hyaluronic to attack a preparation containing some mucoitin sulfuric acid may be due to causes other than a fundamental difference in structure between the two polysaccharides. Firstly, the enzyme may not attack sulfonated hyaluronic acid, or may attack it only at a much slower rate. That this latter possibility is true has been shown by Meyer (127) who found that sulfonated hyaluronic acid is hydrolyzed at only 45% of the rate at which the unsulfonated polysaccharide is hydrolyzed. Secondly, Meyer

did not show that the pneumococcus enzyme, after incubation with the gastric mucosa preparation, still retained the ability to hydrolyze hyaluronic acid. In other words, he did not prove the absence of inhibitors, or of foreign polysaccharides which might bind the pneumococcus enzyme in such a fashion as to prevent its action upon the mucoitin sulfuric acid. The gastric mucosa preparation used was admittedly impure since galactose was obtained from it upon hydrolysis. In later papers Meyer (123, 131) states that gastric mucoitin sulfuric acid occurs partly as a disulfate, and in a subsequent investigation on gastric mucosa (121) he found that his early preparations contained various decomposition products and that the mucoitin sulfuric acid constituted only about 35% of the preparation. Since heparin, chondroitin sulfuric acid and gastric mucin have been found to bind hyaluronidase and to inhibit its activity (112), and since heparin is a polysulfuric mucoitin (70), the presence of an inhibitor in Meyer's gastric mucosa preparations must be presumed. Furthermore, in a later publication Meyer (129) found that hyaluronidase from Cl. Welchii would also attack mucoitin sulfuric acid. He ascribed this effect, however, to the possible existence of a second polysaccharose in the Welchii filtrate.

The specific rotation of hyaluronic acid is reported usually as -65°to -75°, the only report of the rotation of sulfated hyaluronic acid (127) gives it as -51° for the neutral salt. The same author gives a rotation of -35.7° for the neutral salt of mucoitin sulfuric acid. This would be a valid point of difference, if both preparations were pure and of the same degree of polymerization. However, no claim has

thus far been made to preparing either of these materials in greater than 90% purity.

As argument for the possible identity of the two substances, we have the fact that the solubility properties of mucoitin are such as to ensure its appearance in hyaluronic acid preparations if the presence of mucoitin is assumed in the vitreous humor used as a source material. Since Levine and Mori (87) have shown the presence of mucoitin sulfuric acid in vitreous humor, the presence of mucoitin is not unreasonable. Not only do hyaluronic acid and mucoitin have similar solubilities, but they also have similar distributions in nature and the same formulae, as far as is known. Hyaluronic acid is composed of equimolar quantities of glucosamine, glucuronic acid, and acetyl, as is mucoitin. Hyaluronosulfate has the same constituents as mucoitin sulfuric acid (127). Although Meyer's original preparation contained neither phosphorus nor sulfur (117), more recent authors have not refrained from designating as hyaluronic acid preparations which contain phosphorus (108, 113), and probably sulfur also.

The possibility remains, therefore, that hyaluronic acid may be identical with mucoitin. The two substances are certainly very similar.

History of the Preparation Described

Hyaluronic acid is not attacked by commercial trypsin (120). This fact was made use of by Robertson (150) who prepared hyaluronic acid from purified mucoprotein by digesting the protein with trypsin. Enzymic digestion of whole animal tissue has not been used in order to prepare hyaluronic acid, however, probably because most investigators

felt that the product would be too impure. The possibility that the product could be obtained easily in high yield, and in sufficient purity to serve as a substrate for bacterial growth, outweighed other considerations in the present case.

EXPERIMENTAL

Source Material

Umbilical cords were obtained as free from blood as practical and stored under acetone. The acetone-dried cords were ground in a meat grinder and the minced cords further dried by kneading in fresh acetone. The ground tissue was then spread thinly on manilla paper sheets and exposed to the air until part of the acetone had evaporated and the edges of the moist pieces of cord began to darken. This partially dried cord was used as the raw material for the preparation. Ground, partially dried cords representing about 150 cords weighed 1000 grams; the same material dried in a current of air at 48.5° C. for 18 hours weighed 500 grams; oven drying at 98° for 24 hours reduced the weight to 465 grams.

Tryptic Digestion

Five hundred grams of the partially dried cord were suspended in 6.5 liters of tap water, the pH adjusted to about 9.0 with 10% K_2CO_3 solution, and 40 grams of 1:100 trypsin (Coleman and Bell) stirred into the mixture. A liberal quantity of toluene was added as pre-

servative. Digestion was allowed to proceed at 45° to 50° C. for 24 to 48 hours, or until all the cord had been rendered fluid.

The fluid digest was siphoned from under a top layer of toluene, fat, and undigested blood particles and from over a bottom layer of undigested blood particles. The digest was dialyzed through cellophane sausage casing against running tap water for four days, with occasional rotation and inversion of the dialysis bags. A small amount of toluene was added to each dialysis bag as a preservative. After dialysis, the dialysis bags were suspended vertically from a horizontal rod and the toluene allowed to rise to the top. The bags were then tied off just below the upper layer of toluene, residual particles, and fat, and the dialyzed tryptic digest collected by puncturing the lower part of the casing and allowing the contents to drain into a cylinder or jar.

Filtration

The cloudy dialysate was filtered to a clear sparkling, amber colored fluid by mixing with "Supercel"* filter aid in the ratio of 20 grams of "Supercel" to 1 liter of dialysate and filtering under slight negative pressure through a thin layer of "Supercel" on a filter paper placed in a large Buchner funnel. Filtration by this procedure was easy and proceeded at a moderate rate. Fresh paper covered with a thin layer of "Supercel"was used for every 2 liters of dialysate.

* A product of Johns-Manville Company, consisting of partially vitrified diatomaceous earth.

Filtration of the viscous dialysate by other procedures tried was difficult, slow and tedious. Sharples centrifugation did not remove the cloudy material, even after several passages.

Precipitation of Potassium Hyaluronate

The polysaccharide was precipitated from the cleared, dialyzed tryptic digest of umbilical cord according to the procedure of McClean (114). Ordinary 95% ethyl alcohol was saturated with U.S.P. grade potassium acetate and 1.4 volumes of the saturated alcohol added slowly, with efficient stirring, to 1 volume of cleared dialysate. The mixture was then allowed to stand for several hours at 4° C. After the white, stringy precipitate had settled, the fluid layer was siphoned away and the precipitate concentrated by centrifugation. The supernatant after centrifugation was poured off and the precipitate washed successively with alcohol, acetone, ether, absolute alcohol and absolute ether. It was then dried *in vacuo* over P_2O_5.

Yield

The yield from 500 grams of partially dried, ground cord is between 10 - 14 grams. This is equivalent to a yield of 4.5 - 6 grams per 100 grams of oven dried cords.

Two 500 gram samples from the same batch of cord were processed, one by tryptic digestion and one by water extraction according to McClean (114). A yield of 13.3 grams of very white product was obtained by tryptic digestion while water extraction resulted in a yield of 2.6 grams of gray material. McClean reports yields of 2 - 3 grams by the extraction method.

Physical Characteristics

The product is a white, fluffy, stringy solid. It may be powdered easily. It is not conspicuously hygroscopic when stored in glass-stoppered containers, although it takes up water readily after it is wetted. It is only slightly sticky when pinched between the fingers.

A 0.2% solution in distilled water is neutral to indicators and has a viscosity of 30-75 times that of water, at 37° C.

Qualitative Analysis

A 0.2% solution gives negative Fehling's and Benedict's tests for reducing sugars, a negative iodine test for glycogen, and a negative biuret test. The viscosity of such a solution is quickly reduced to that of water upon addition of hyaluronidase from rabbit testicle, rattlesnake venom, or from culture filtrates of pneumococcus or Cl. welchii. This indicates that the high viscosity is caused entirely by hyaluronic acid and is not contributed to by any other component of the preparation.

Quantitative Analysis

The results of quantitative analysis of these preparations are given in Table I.

Ash was determined by carefully igniting the preparation first over a free flame, and then by maintaining at red heat in a muffle furnace for several hours. The crucible was cooled, the ash wet with a drop of concentrated sulfuric acid, and then reheated until all the sulfuric acid was driven off. The crucible was cooled once more and a little solid ammonium carbonate added to convert any $K_2S_2O_7$ into K_2SO_4.

Glucosamine was determined colorimetrically by the method of Morgan and Elson (43) against a thrice- recrystallized sample of glucosamine hydrochloride as standard. The standard was dried in vacuo for 20 hours at 40° C., over phosphorus pentoxide, and gave the same color as a sample of glucosamine-HCl obtained from Eastman Kodak Company. The glucosamine was liberated from the hyaluronic acid by hydrolyzing the preparations in sealed tubes with 4 N HCl for 18 hours at 100° C. This ensures complete hydrolysis and maximal values for glucosamine (144).

Nitrogen was determined by micro-Kjeldahl.

Sulfur was determined as hydrolyzable sulfate. The preparation was hydrolyzed in sealed tubes with 4 N HCl for 18 hours at 140° C. The hydrolyzed material was transferred to a volumetric flask, phosphorus precipitated as magnesium ammonium phosphate and sulfate determined according to the benzidine method as modified by Fiske (50).

Phosphorus was determined by the method of Fiske and Subba-Row (51).

TABLE I

Quantitative Analysis of Potassium Hyaluronate Preparations

Analysis for	Values obtained	Theoretical values*	Values obtained by other authors		
			McClean (121)	Meyer (122)	Kendall (123)
Analysis	in percent	in percent	in percent	in percent	in percent
Ash	10.8, 10.9, 12.6	8.65	-	2.08, 4.95 (free acid)	9.2 - 10.2**
Glucosamine	32.3 (av.)	39.7	12	23.5 - 41.7	-
Nitrogen	2.9-3.5	3.12	4.7	3.0-3.7	3.1 - 3.7
Sulfur	1.1, 1.4, 1.5	0	-	trace	-
Phosphorus	0.091	0	2	0	-

* Calculated on the basis of a unit formula = $KC_{14}H_{23}O_{13}N$

** Converted from Na to K

Fractionation of the Preparation

A gram of the once precipitated preparation was suspended in distilled water and dialyzed through cellulose sausage casing against repeated changes of distilled water. The dialysate, though originally clear, developed a noticeable turbidity on standing. The total dialysate was evaporated almost to dryness on the steam bath, then transferred to a small beaker and dried in the oven at 100° C. The yield was 0.2311 grams of dark brown (after oven drying), hygroscopic solid, or 23.1% of the original preparation. After weighing, the dialysate was hydrolyzed with 4 N HCl for 18 hours at 140° C. The hydrolysate was brown in color and contained small black flakes of solid. The sulfur content was 1.06%, or 17.5% of the total sulfur present.

To the residue in the dialysis bag there was added 5 cc. of a Cl. welchii filtrate containing 1400 V.R.U. of hyaluronidase per cc. and some NaCl as an activator. Dialysis was resumed as before. The total dialysate of the enzyme-hydrolyzed polysaccharide, after oven drying, weighed 0.4966 grams, which is 49.7% of the original preparation or 64.2% of the residue from dialysis of the unhydrolyzed polysaccharide. It contained 0.81% of acid-hydrolyzable sulfur, or 24.0% of the total sulfur present in the original preparation.

The total weight of the residue remaining in the bag after hydrolysis of the preparation with hyaluronidase was 0.2697 grams, or 27.07% of the original preparation. It contained 3.57% of sulfur, or 58.8% of the total sulfur.

These data are summarized in Table II.

TABLE II

Fractionation of the Once-Precipitated Preparation

Fraction	Weight in % of original weight	Sulphur % of original sulfur	Sulphur % of fraction analyzed
(a) once precipitated preparation	100	100	1.45
(b) Dialysate of (a)	23.1	17.5	1.06
(c) Residue from dialysis of (a)	76.9	82.5	1.79
(d) Dialysate from enzyme hydrolysis of (c)	49.7	24.0	0.81
(e) Residue from dialysis of (d)	27.0	58.8	3.57

A second dialysis was begun using 5 grams of the once precipitated potassium hyaluronate preparation in 500 cc. of water. The final volume in the bag, after dialyzing against 4-1500 ml. portions of distilled water, was 800 cc. The solids in the dialysate weighed gm., or % of the original preparation.

The 800 cc. of hyaluronic acid solution was shaken with 3 grams of barium acetate, ethyl alcohol then added to a final concentration of 20%, and the solution stored at 4° C. to precipitate as suggested by Meyer (131). After three days in the cold, the precipitate was centrifuged off and the hyaluronic acid recovered from the supernatant liquid by adding ethyl alcohol to a concentration of about 40%. Only the stringy material which separated at 40-50% alcohol was accepted as potassium hyaluronate. Further addition of alcohol brought down a relatively small amount of flocculant non-stringy material.

The yield of purified hyaluronic acid was 3.3 grams, or 66% of the original preparation. It contained % of sulfur and % of phosphorus, A 0.2% neutral solution had a viscosity of times the viscosity of water.

On dialysis, gms. of the purified preparation lost % of its weight. This loss is probably due to barium acetate, since addition of sodium sulfate to a solution of dialyzable material resulted in a fine precipitate, which, when dried, weighed, and calculated as barium acetate, accounted for % of the loss in weight on dialysis.

To the dialyzed purified preparation there was added ml. of Cl. perfringens hyaluronidase, containing V.R.U. of hyaluronidase per ml.; and dialysis was continued. A further % of the preparation was rendered dialyzable by the enzyme. The undialyzable unhydrolyzable residue in the bag weighed gms., or % of the purified preparation. It contained % sulfur. These data are summarized in Table

The purified preparation therefore contains:

% barium acetate (which may be removed by dialysis)

% hyaluronic acid and

% unknown

DISCUSSION

The results of fractionation show that the preparation is not homogeneous, and the results of analysis show that the preparation is not pure. The usefulness of the method therefore rests upon the relative ease with which maximal yields of greatly purified, protein

free, potassium hyaluronate may be obtained. McClean (113) has published a shorter method of preparation; Meyer (118, 124) has published a method of preparation which results in a purer product. McClean's method, however, gives only 1/5 to 1/3 the yield of a product which shows less than 1/3 the theoretical amount of glucosamine and consists, therefore, of more than 2/3 impurity. Meyer's method calls for repeated solution and precipitation, the removal of protein by repeated shaking with chloroform-amyl alcohol mixture, and repeated precipitations with zinc salts which are extremely difficult to wash out. The use of zinc necessitates precipitating in dilute solutions so as to avoid throwing down the zinc carbohydrate. The repeated precipitations lessen the yield and even this laborious method does not result in a pure product (124) since 0.47% of sulfur and a trace of phosphorus are still present.

The properties of chondroitin sulfuric acid are similar to those of hyaluronic acid and its presence in the tryptic digest preparation must be presumed from the fact that chondroitin sulfuric acid has been found in umbilical cord (118). Its presence is possibly also indicated by the fact that some of the organic material in the preparation is dialyzable from distilled water and this dialysate becomes turbid upon standing. These phenomena are consistent with the observation of Meyer (124) that chondroitin sulfuric acid is unstable in aqueous solution, as is the fact that part of the sulfur is dialyzable from the preparation. According to this view, the dialyzable sulfur would be derived from the breakdown of chondroitinsulfuric acid. A further indication of the presence of chondroitin sulfuric acid is the fact that 58.8% of the original sulfur is concentrated in the 27% of the preparation which is

not susceptible to attack by hyaluronidase, and remains within the dialysis bag.

The sulfur which becomes dialyzable after the action of hyaluronidase may be attributed to hyaluronosulfate (that is, mucoitin sulfuric acid), or to further decomposition of chondroitin sulfuric acid.

The phosphorus present is most probably derived from residual nucleic acid.

The amount of hyaluronic acid in the preparation must be taken to be somewhat greater than the amount of material which is rendered dialyzable by the action of hyaluronidase, in this case, 49.7%. This is because hyaluronidase does not effect 100% complete hydrolysis of hyaluronic acid to reducing sugars. Hydrolysis to varying extents have been reported: 96% (124); 65%, 78% (125); 87% (127); 44%, 55% (for hyaluronosulfuric acid) (127); 70% (120).

It is difficult to say to what degree this variation may be attributed to the use of preparations which did not themselves consist 100% of hyaluronic acid, to the use of enzyme preparations of various potencies, to the presence of some inhibitors, or to the "wearing out" of enzyme. The addition of fresh enzyme did not effect any further hydrolysis in at least one case (132). It would seem consdrvative to assume no more than 90% hydrolysis.

On the basis of the foregoing assumptions, the once precipitated tryptic digest preparation has approximately the composition which follows:

Total Hyaluronic salts		55.2%
Potassium Hyaluronate	48.3%	
Potassium Hyaluronosulfate	6.9%	
Total chondroitin sulfuric acid salts		18.8
"Dialyzable"	3.8%	
"Non-dialyzable"	15.0	
Nucleic acid (calculated as $C_{39}H_{59}N_{15}P_4O_{29}$).....		0.97
Potassium acetate and unknown dialyzable substances ..		19.3
Unknown non-dialyzable, non-hydrolyzable substances ..		6.5
Total percentage		100.77

The discrepance between the total percentage and 100% is due to the assumption of 90% hydrolysis by the enzyme, since the total hyaluronic salts were calculated as 100/90 times the total material rendered dialyzable by the action of hyaluronidase. The actual percentage hydrolysis is thus indicated to be 91%.

The small percentage of nucleic acid and the absence of glycogen are probably attributable to the action of nucleinase and amylase in the commercial trypsin preparation.

The viscosity of hyaluronic acid preparationsis of importance because this viscosity has biological significance (see introduction page 9) and because it is an indication of the state of polymerization of the molecule. According to Standinger (156) the high viscosity of dilute solutions indicates that the molecules of solute are arranged in

two long thread-like patterns. Preparations of hyaluronic acid of varying viscosity but similar chemical analysis may be taken to represent preparations with a greater or lesser degree of thread-like polymerization. Meyer (118) found that 0.25% hyaluronic acid solution had a viscosity relative to water of from 2 - 13, depending on the method of preparation. In other papers (124, 128) he found that although no viscous substances other than hyaluronic acid could be found in synovial fluid, nevertheless the hyaluronic acid actually isolated possessed only a small part of the original viscosity. The viscosity of a native pleural fluid, for example, was 147.6 times that of 0.9% NaCl, while the hyaluronic acid isolated from the pleural fluid gave, in corresponding concentration of 0.177%, a solution having a viscosity of only 1.54. It is apparent that Meyer's method of preparation results in a depolymerized or disaggregated hyaluronic acid, since the only other way to account for the viscosity is by assuming an impure preparation. This latter possibility is ruled out by the close correspondence of Meyer's analytic results to those required by theory. The highest viscosity reported for purified hyaluronic acid is that of Robertson, Ropes, and Bauer (150), who found that a 0.2% solution was 40 times as viscous as water, at 25. C.

The viscosity of once-precipitated tryptic digest preparation ranges from 30 - 75 times that of water for a 0.2% solution at 37° C. This viscosity is due entirely to the hyaluronidase-susceptible materials. Since such materials constitute only 55% of the preparation, it is evident that the hyaluronic acid is in a highly aggregated or polymerized condition. If the 45% of impurity were replaced by hyal-

uronic acid, the viscosity of solutions of the preparation would in all probability be equal to the viscosity in the native state, or over 147 times that of 0.9% NaCl (128). This is even more evident when one considers the fact that the rate of increase of viscosity of hyaluronic acid solutions by additional increment of solute rises steeply as the initial concentration is increases (see page

SUGGESTIONS FOR FUTURE WORK

Further purification of the preparation might be effected along these lines.

1. Simple dialysis, and precipitation of the non-dialyzable residue with alcohol or acetone. This should remove over 23% of impurity and therefore raise the percentage of hyaluronic salts in the preparation to over 70%.

2. Removal of the sulfated polysaccharides and nucleic acid. This could be done by precipitating them as barium or brucine salts, or as proteinates. Meyer (13) has reported a sharp separation of chondroitin sulfuric acid from hyaluronic acid or fractionation by alcohol in the presence of barium acetate. Jorpes (70, 70A) in connection with studies on heparin, has separated mucoitin sulfuric acid into five fractions, corresponding to mucoitin trisulfuric acid, mucoitin disulfuric (two fractions), mucoitin monosulfuric acid, and unsulfonated mucoitin by means of partial precipitation with barium. The unsulfonated mucoitin remained in water solution as the barium salt and was finally precipitated with alcohol. Levene (82) has removed nucleic acids from polysaccharide solutions with barium. Jorpes (70, 70A) has also fractionated

mucoitin by precipitating the sulfonated molecules as brucine salts. A similar separation ought to be possible by forming protein salts of the polysaccharides; Meyer (127) states that sulfuric esters of the polysaccharides form very stable protein salts which are water insoluble, while corresponding hyaluronic acid salts are water soluble.

Separation of the sulfated polysaccharides and nucleic acid from the dialyzed preparation would increase the concentration of hyaluronic acid to over 88%. Hyaluronosulfate would, of course, be removed.

3. Determination of the nature of the remaining impurities. These are the non-sulfonated, non-dialyzable, non-hyaluronidase susceptible materials which constituted 6.5% of the once precipitated preparation. Knowledge of their nature would be helpful in effecting their removal. Resolution and reprecipitation of the product from step 2 might be in order. Electrodialysis is also a possibility. Although Madinaveitia found that hyaluronic acid could not be electrodialyzed without precipitating out of solution, it would appear possible, perhaps, to alter conditions so as to prevent this; Jorpes (70) has electrodialyzed mucoitin sulfuric acids successfully.

The pure hyaluronic acid so obtained would provide the necessary pure substrate for study of the constants of the enzyme system hyaluronic acid-hyaluronidase, for biological and bacteriological work, and for a determination of the structure of this biologically important polysaccharide.

SUMMARY

A good yield (4.5 - 6 grams per 100 grans of dried cords) of white potassium hyaluronate of high viscosity (0.20% neutral solutions were 30-74 times as viscous as water) was prepared by digesting human umbilical cords with trypsin, dialyzing to remove amino acids and other small molecules, clearing by filtration under gentle suction with "Supercel" and reprecipitating with saturated alcoholic solution of potassium acetate. The preparation contains 1.45% of sulfur and 0.09% of phosphorus. On the assumption that hyaluronidase hydrolyzes 90% of the hyaluronic adid present to molecules of dialyzable dimensions, the preparation is considered as consisting of 55% potassium hyaluronate, 19% chondroitin sulfuric acid, 0.97% nucleic acid, 6.5% unknown high molecular weight substances, and about 19% of dialyzable salts.

The concentration of potassium hyaluronate may be raised to 70% by dialyzing the preparation, and to 88% by precipitating sulfonated polysaccharides and nucleic acid with barium.

III. THE DETERMINATION OF URONIC ACIDS

INTRODUCTION

The determination of the uronic acid content of polyuronic acids such as pectins, saponins, cellulosic materials, heparin, chondroitin sulfuric acid, and hyaluronic acid is at present a tedious gravimetric or titrimetric procedure involving a careful control of the rate of hydrolysis of the sample, the use of a purified stream of carrier gas, and calling for an elaborate reaction train similar to those used for the organic microanalysis for carbon. All the difficulties presented in the accurate measurement of micro quantities of carbon dioxide by gravimetric or titrimetric procedure are present in these methods, and standard conditions for the manipulation of adsorption tubes and very dilute standard alkali must be rigidly observed. Some appreciation of the many possible sources of error may be gained from the handbooks of Pregel (146), Weygand (168), Fredrich (54) or Boetuis (10) on quantitative determination of carbon in organic materials.

The method described below was developed in order to provide a shorter, easier micro-determination of uronic acid in hyaluronic acid preparations. It makes use of the standard Van Slyke-Neill manometric blood gases apparatus, a piece of equipment commonly found in biochemical and hospital laboratories, and with whose

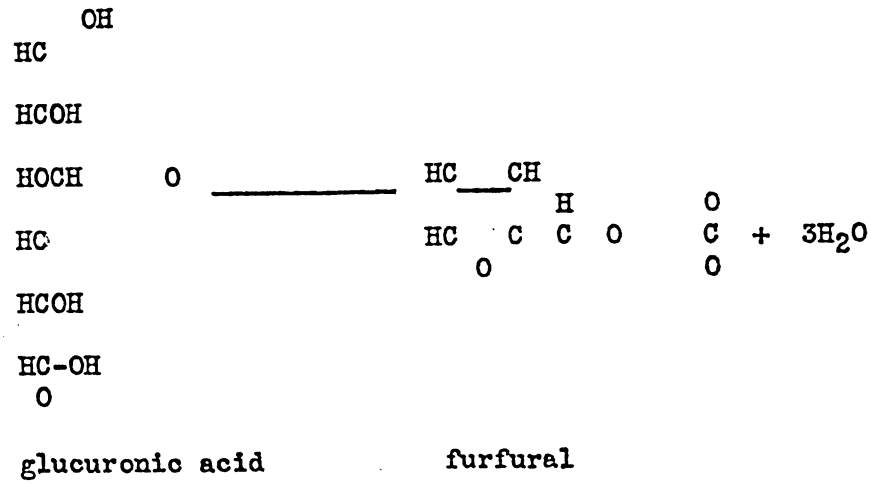
manipulation most hospital laboratory technicians are familiar. The pitfalls of gravimetric or titrimetric technique are thus avoided. Only micro samples are required. A large number of samples may be hydrolyzed at one time and analyzed when convenient, each analysis requiring only one-half hour to complete.

Although time was not available in which to apply the method to polyuronic acids, its applicability is nontheless evident (see discussion).

HISTORY

Methods in the Literature

The original "micro" method of Tollene and Lefevre (159) and subsequent modifications (14,15,26,27,58,74,115,143,159) are based on the fact that when a uronic acid is heated with hydrochloric acid, the formation of furfural and carbon dioxide takes place according to the equation:



glucuronic acid furfural

The yield of carbon dioxide is quantitative, although the yield of furfural is not (6). The samples required by the various gravimetric or titrimetric methods vary from 10 mgm to 1 gram, and the time of heating the sample, which is roughly equivalent to the time required to perform a determination after the blank value is known, varies from a minimum of at least two hours to six to eight hours. The concentration of hydrochloric acid used is 12%, as used originally by Tollens and Lefevre. The temperature of hydrolysis varies from about 120° to 140° C. The most usual temperatures are 130°-135° C. It is evident from the descriptions of these techniques that skill and considerable practice is required in order to obtain consistent results.

The Present Method

In 1939 Danielson and Hastings (25) described a method for the determination of CO_2 in tissues by the use of the regular Van-Slyke-Neill gasometric apparatus. The principle of the method was to place the tissue in a side tube connected to the apparatus, to release CO_2 from the tissue, and to conduct the CO_2 released into the chamber of the apparatus where it was absorbed by alkali. The CO_2 in the alkali was then determined in the usual manner. It was suggested (90) that uronic acids might be determined in a similar manner.

EXPERIMENTAL

Principle of Method

The principle of the method is to hydrolyze the uronic acid with

H_2SO_4 in a sealed thin-walled glass bomb and then to place the bomb in a special side tube connected to the Van Slyke apparatus. The bomb is subsequently crushed and the CO_2 which has been liberated by the hydrolysis is absorbed by alkali in the reaction chamber, from whence it is determined as usual.

Description of the Apparatus

The special side tube and its glass connection to the Van Slyke apparatus are the only unusual pieces of equipment. The side tube is a piece of thick walled Pyrex glass tubing about 18 X 75 mm. The upper end of the tube is slightly flared so as to fit a number 3 rubber stopper. The lower end of the tube is fitted into a short length (about 30 mm.) of moderately thick walled rubber tubing which is bound in place by several turns of cord, and shellacked. The rubber tubing is closed at its lower end by being pressed flat and cemented together with rubber cement. A brass clip prevents the rubber from regaining its shape. Figure 1 shows this side tube (A) attached to the Van Slyke apparatus, with a glass bomb (B) in place. It is connected to the side arm of the apparatus (C) through a rubber stopper (D) well greased with vacuum stopcock grease, a glass tube (E) and a short length of greased pressure tubing (F). The connecting glass tube (E) has an internal diameter equal to the diameter of the side arm of the Van Slyke apparatus and its end is in immediate contact with the free end of the side arm. The special tube is held against the water jacket by means of two rubber bands (G), and thus prevented from tearing away during the shaking.

The Determinations of Uronic Acids

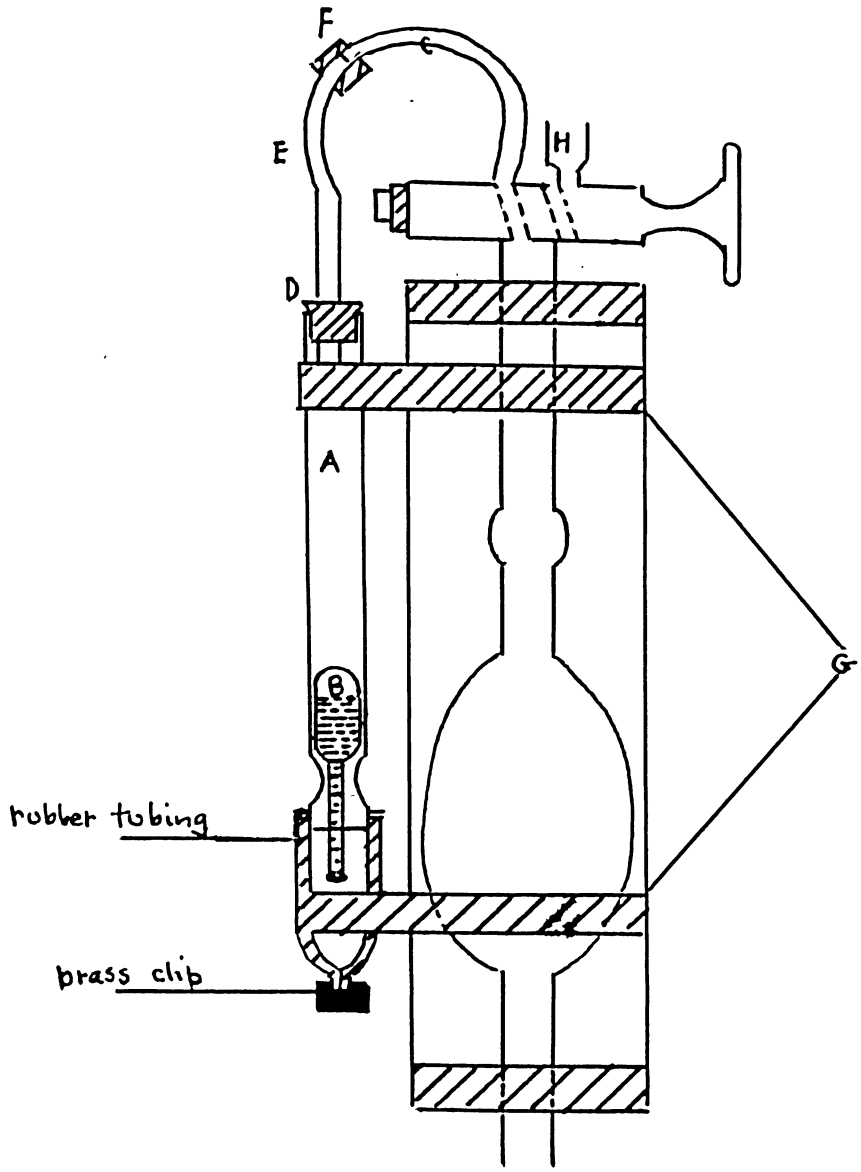


Figure 1-Side tube connected to the Van Slyke reaction chamber. Bomb in place.

Solutions required

1. approximately 0.1 N CO_2-free NaOH.
2. approximately 5 N NaOH.
3. approximately 1 N H_2SO_4.
4. approximately 1 N lactic acid.

Sulfuric acid is used to hydrolyze the sample because it is not volatile and so will not be transferred to the reaction chamber along with the CO_2 produced.

Hydrolysis of the Sample

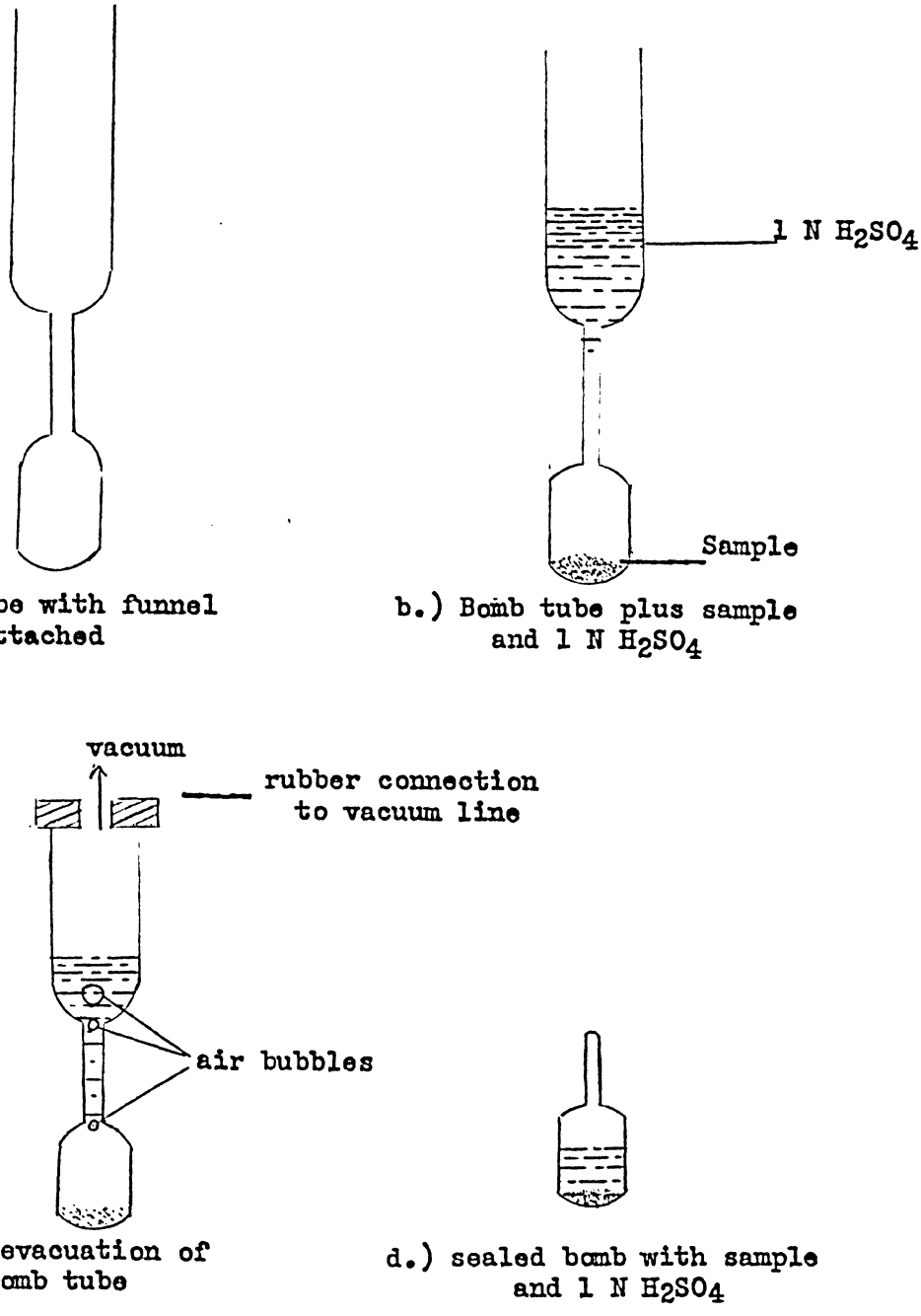
An amount of sample which will evolve about 0.025 millimoles of CO_2 is weighed into a bomb tube. The bomb tube is made by sealing one end of a piece of 10-mm. diameter Pyrex tubing and then necking the tubing down to a diameter of 1 to 2 mm. a short distance above the seal. The large open end is left on at this stage so as to act as a funnel. (Fig.2, part a) After the sample has been placed in the funnel of a bomb tube, the bomb is tapped lightly several times. The tapping will shake most of the dry sample to the bottom of the bomb. One-half cc. of 1 N H_2SO_4 is then placed in the funnel. (Fig.2, part b) It will remain in the funnel since surface tension prevents it from passing down the capillary. The mouth of the funnel is now placed momentarily against the open end of a piece of pressure tubing connected to the vacuum line. When two or three air bubbles have passed up through the sulfuric acid, the funnel mouth is withdrawn from the vacuum. Air pressure forces the sulfuric acid down into the bomb and its passage washes down any part of the sample which may have

adhered to the walls of the capillary. The bomb is now sealed off close to the funnel, and the sample is ready to be heated. (Fig. 2, part d) This sequence of manipulations is shown in Fig. 2, page

Hydrolysis is carried out for 3 hours at 130° C. Any number of samples may be hydrolyzed at the same time as there does not appear to be any appreciable increase in CO_2 when the hydrolyzed samples are stored at room temperature.

Transfer of the Carbon Dioxide

The bomb tube containing the hydrolyzed sample is placed in the special side tube, as shown in figure 1. The bulb of the bomb is uppermost, while the slender neck projects from the glass part of the special side tube into the rubber tubing. The stopper is greased with vacuum stopcock grease, and the special side tube is fitted over it. The air in the previously washed Van Slyke reaction chamber is now expelled through the cup, allowing the chamber to fill with mercury and forcing mercury into the stopcock capillary, so as to seal it. The stopcock is now given a half-turn, so that neither capillary connects with the chamber, and a vacuum is produced in the chamber by lowering the leveling bulb until the mercury has fallen to a level slightly above the 50 cc. mark. The reaction chamber and the side arm C are now connected by turning the stopcock a further half-turn. A part of the air in the special side tube is thus drawn into the reaction chamber. The stopcock is given a half-turn to return it to a neutral position and the leveling bulb is placed in the upper bracket. The air collected is again expelled through the cup H. This procedure is



a.) Bomb tube with funnel attached

b.) Bomb tube plus sample and 1 N H_2SO_4

c.) slight evacuation of bomb tube

d.) sealed bomb with sample and 1 N H_2SO_4

Fig. 2 Manipulation and filling of bomb tube

repeated 3 or 4 times until the special side tube A has been exhausted of air. The rubber tubing selected for the lower part should be of such wall thickness that it collapses when completely evacuated, but retains its shape under partial evacuation. This thickness of rubber is sufficient to prevent leakage of air into the apparatus, and the collapsed form forces the sample into the glass part of the special side arm where complete extraction of the CO_2 is facilitated.

After the last portion of air has been expelled from the reaction chamber, 2 cc. of 0.1N CO_2-free sodium hydroxide are drawn into the chamber from the cup H, and a mercury seal is made in the stopcock capillary between the cup and the chamber. The mercury leveling bulb is lowered until the level of the sodium hydroxide in the chamber is a little above the 50 cc. mark, and the lower stopcock, connecting the apparatus with the leveling bulb, is closed. The leveling bulb is returned to the lower bracket. The stopcock is turned so as to connect the reaction chamber and the side arm C. The capillary portion of the bomb tube B is now crushed by pressure upon it through the rubber tubing in the special side tube A. The hydrolyzed sample rushes out of the bomb and comes to rest on the collapsed portion of the rubber tubing, just below the glass part of the special side tube A.

The special tube is fastened to the water jacket with the rubber bands G and the machine allowed to shake for 3 minutes. At the end of the period of shaking the lower stopcock is opened, allowing the gas to contract somewhat. Care must be taken that the 0.1N NaOH does not rush over into the evacuated special side tube. By alternately raising and lowering the mercury leveling bulb the gases in the chamber and

the special tube are mixed and the CO_2 is pumped over into the 0.1N NaOH. Eight full excursions of the gases are made, that is, the mercury leveling bulb is raised eight times and lowered eight times. The level of the liquid in the chamber is returned to just above the 50 cc. mark, and the lower stopcock is closed. The entire procedure described in the above paragraph is repeated twice more.

During the excursions of gases, it sometimes occurs that the liquid sample will run back into the bulb of the bomb tube and momentarily appear to trap gases therein. This condition usually remedies itself in the course of the repeated excursions and, in any event, has not been found to produce any error. However, if this occurs, it may be remedied by removing the rubber bands which fasten the special side tube to the water jacket, and rotating the glass connection at its joint with the side arm C until the special side tube A is at right angles to the water jacket. When the special tube is in this position, lowering the mercury leveling bulb will force the sample out of the bomb tube, whence it will come to rest against the wall of the glass part of the special side tube. Further excursions will not then cause the liquid sample to re-enter the bomb. The special side tube must be replaced in its original position, of course, before shaking is begun again.

After the shaking and eight excursions of the gases have been performed 3 times, the upper stopcock is closed, so that neither capillary communicates with the chamber. The special side tube is removed from the rubber stopper D. The side arm C and the capillary in the stopper between C and the reaction chamber are sealed with

mercury by dipping rubber stopper D under a reservoir of mercury in a small cylinder and opening the upper stopcock for a moment. Rubber stopper D is fastened to the water jacket by means of one of the rubber bands, and the determination of the CO_2 transferred to the alkali is carried out in the usual way, after ejecting most of the air from the chamber through the cup H.

A correction is made for the CO_2 of the reagents by performing a blank determination in the same manner as the determination of the unknown. If care is observed, this connection should amount only to 0 to 5 mm. of mercury.

Results:

All the analyses were performed upon a sample of ∝-d- galacturonic acid, sample number B8303, received from the California Fruit Growers' Exchange. The purity of the sample was not checked by independent means. This was because originally it was intended to ascertain approximately the condition for hydrolysis which would yield correct values for CO_2, and then to perform a series of analyses with standard preparations and known mixtures of uronic acids. There was time, however, to complete only the first half of this plan.

The data on Table III show the results obtained when ∝-d-galacturonic acid is analyzed after 3 hours hydrolysis with 1N H_2SO_4 at 130° C.

Lesser concentrations of acid result in lower values, greater concentrations in higher values. Longer periods of hydrolysis result in higher values as do higher temperatures of hydrolysis. These

THE DETERMINATION OF URONIC ACIDS

TABLE III
Values Obtained for Galacturonic Acid

(3 hours' hydrolysis with 1N H_2SO_4 at 130° C)

Known weight of Sample	mM CO_2 found (x 10^{-3})	weight found	recovery in percent
4.66 mgm.	24.0	4.68 mgm.	100.4
4.61	23.6	4.60	99.8
5.51	28.2	5.50	99.8
4.70	24.0	4.68	99.6
4.70	24.0	4.68	99.6

TABLE IV
Variation of Recovery of CO_2 from Galacturonic Acid with Acidity and Hydrolysis Time

Average Recovery	Acid Concentration	Hydrolysis Time	Hydrolysis Temperature
in percent	in normality	in hours	in °C
85	0.05	3	120-140
95	0.5	3	130
100	1	3	130
102	0.5	5	130-140
106	0.1	18	130-140
110	1	5 1/2	130-140
115	1	15	142

changes are shown in Table IV. It is probable that combinations of acidity with hydrolysis time besides the combination chosen might be found which would also be satisfactory.

Discussion

Although the conditions for the analysis of pure galacturonic acid are the only ones which have been worked out, the possibility of the method comparing favorably with published micro methods is already evident, since the published methods are recommended only for pure uronic acids or their derivatives. This is because other carbohydrates and some organic acids produce small amounts of CO_2 when heated with 12% (approx. 3.5 N) HCl.

Suggestions for Future Work

An analytical survey of pure uronic acids and their derivatives should be made, in order to ascertain the conditions necessary for their determination. In this series there might be included glucuronic acid and borneol glucuronic acid, methyl galacturonide, chondroitin sulfuric acid, and known samples of pectins and alginic acid. Standard samples of the last two substances, of known uronic acid content, might be obtained from the research laboratories of the Department of Agriculture. The Van Slyke analysis on the survey series of known uronic acids should be checked by analysis according to some conventional method. When the proper conditions for these determinations have been worked out, the possibility of analyzing impure uronic acids should be explored. The Van Slyke method, where

hydrolysis is carried out under pressure, may permit conditions of acidity, hydrolysis time, and temperature to be arranged so as to obviate significant CO_2 production by foreign carbohydrates.

SUMMARY

A short convenient method for the determination of galacturonic acid (and presumably of other uronic acids) is to hydrolyze the sample for 3 hours at 130° C. with 1 N H_2SO_4 in a sealed bomb tube and subsequently to determine the quantitatively evolved CO_2 with the aid of a special side tube attached to the Van Slyke-Neill gasometric apparatus. Samples are chosen so as to evolve a convenient quantity (0.025 mM) of CO_2. Any number of samples may be hydrolyzed simultaneously stored at room temperature, and analyzed when convenient.

IV. ASSAY OF HYALURONIDASE

INTRODUCTION

Publications have appeared describing six different assays for hyaluronidase. These are reviewed below in chronological order, the reasons for the choice of methods used in the present work are discussed, and typical results obtained with these chosen methods are presented.

HISTORY

ASSAY BY SPREADING POWER

The ability of aqueous extracts of mammalian testicle to enlarge the area of skin attacked by vaccinia virus (28,30,104) was the first effect reported which was later to be ascribed to hyaluronidase. It was quickly found that testicle extracts would render the connective tissue more permeable to all manner of fluid and particulate agents (66), including staphylococcus toxin (29), diphtheria toxins (104), filterable viruses (67), various bacterial infections (147), india ink (46,158), hemoglobin (93), and so forth. In these circumstances, it was natural that the first method for the assay of hyaluronidase solutions should rest upon the determination of the relative permeability of the connective tissue to fluids containing more or less of the enzyme. Such methods were

published by Claude and Duran-Reynals (22), by Hoffman and Duran-Reynals (66), by McClean (104), by Boyland and McClean (11), by Madinaveitia (93,94), and by Bacharach, Chance and Middleton (158).

These assays depend upon the ability of diffusing factors to increase the area of skin penetrated by an indicator, usually dyes or hemoglobin, but in the earlier experiments, toxins or india ink. The mixture of indicator and diffusing factor is injected intracutaneously. Quantitative differences are brought out by dilution of the spreading factor before injection, as the area of spread does not increase proportionately to an increase in the concentration of the injected factor, (44), making direct assay impossible.

The drawbacks of this method are manifold: (a) it is non-specific, as it measures not only hyaluronidase but other possible diffusing factors as well, such as ascorbic acid, (108,150), and diazo compounds (98); (b) it is inaccurate, detecting a minimum of tenfold (93,94,44) or fourfold(4), differences in concentration; (c) it requires large numbers of white rabbits of definite age and breed which, once used, are no longer suitable for test; (d) time consuming, multiple injections and the services of an assistant are required for maximal accuracy; and (e) the results are interpretable only with the aid of statistical analysis (4).

Assay by Production of Reducing Substances

The determination of reducing sugar values on polysaccharide preparations after longer or shorter periods of hydrolysis by hyaluronidase has been used, principally by Meyer (64,119,132) as an

indication of the concentration of the enzyme. It is shown (119,132), for instance, that 1% of a sodium flavianate precipitate of hyaluronidase will produce 35% of the theoretical maximum of reducing sugars from a hyaluronic acid preparation after 44 hours of hydrolysis; 0.01% of enzyme will produce only 3% of reducing suger, while 0.05% and 0.1% of enzyme both produce 9% of sugar.

The principle a priori objection to this method is the possibility that the rate of generation of reducing sugars may not parallel the diffusing potency of the enzyme preparations (40). The diffusing potency is the hyaluronidase property of immediate interest because of its possible connection with infection. As a practical matter, the test can be used only with protein-free hyaluronic acid preparations, since proteolytic anzymes in bacterial filtrates would otherwise liberate reducing substances (63). The state of purity of the hyaluronic acid preparation used would markedly affect the reducing values obtained, and render comparisons difficult. In addition to the foregoing, there is also objection on the score of the length of time necessary to complete the test and the sources of error latent in whichever of the current quantitative methods for estimation of reducing sugar is selected.

Assay by Production of Acetylglucosamine

Measurement of the increase in glucosamine in solutions of hyaluronic acid acted upon by hyaluronidase has been suggested by Chain and Duthie (18). These authors incubated hyaluronic acid-substrate mixture for 16 hours, and then determined liberated

N acetylglucosamine by the method of Morgan and Elson (135).

This method has not yet been used for quantitative assay of the potency of hyaluronidase preparations, so that comparative data cannot be cited. The various objections to assay by determination of reducing sugars ought also to apply in the present case.

Assay by Viscosity Reduction

This is the method that has been used most frequently in the recent literature. It is based upon the fact that, under certain conditions, the rate of decrease of viscosity of solutions of hyaluronic acid is proportional to the concentration of hyaluronidase present. Demonstration of the viscosity-reducing power of hyaluronidase was first made by Chain and Duthie (17) and was confirmed by McClean and Hale (106,107). Essentially similar methods have been published by Madinaveitia and Quibell (99), by Chain and Duthie (18), and by McClean and Hale (108).

The method is rapid, requiring about 40 minutes to an hour for one determination if the concentration is completely unknown and about 30 minutes if the approximate concentration is known in advance of the determination. It is relatively the most accurate method described, Madinaveitia assigning it an accuracy of± 5% while Chain and Duthie find ±10%. Crude preparations of hyaluronic acid may be used as substrate.

Although viscosimetry has been the method of choice in the literature, Kass and Seastone (72) have objected to it on the grounds that it requires the full time of the investigator and an ample supply

of substrate. Other substances besides hyaluronidase have been reported to decrease the viscosity of hyaluronic acid solutions, although they do not give a typical hyaluronidase viscosity-reducing curve. These substances are ascorbic ancid and its oxidation products (96,108), certain diazo compounds (96,107), phenylhydrazine (96), and reducing substances such as thiolacetic acid, H_2S, hydroquinone, pyrogallol, sodium sulfite, and metol (108).

Most of the hyaluronidase assays reported in this thesis were obtained viscosimetrically, as described in this chapter under "Experimental," pages 59 to 68.

Assay by Inhibition of Mucoid Clot

A mixture of hyaluronic acid and protein forms a stringy precipitate or "clot" on the addition of dilute acetic acid (154). McClean (109) found that if increasing dilutions of hyaluronidase were inoculated with serum protein-hyaluronic acid mixture, the potency of the enzyme could be determined by ascertaining the highest dilution capable of destroying the clotting power of the substrate in a definite period of time. In a later paper (113) the method was more fully described. McClean states that 100% differences are easily detectable, and that 20% differences are titratable with special precautions.

This method appears especially suited to work with inhibiting agents such as anti-sera or enzyme inhibitors. In such cases extreme sensitivity in the method is unnecessary and may even be undesirable.

When used as a measure of hyaluronidase the mucin clot

prevention test does not give results to better than 50-100% of their true value, unless the test be repeated many times on a small group of dilutions which bracket the true end point. It is sensitive to change in the concentration of hyaluronic acid in the substrate, and to the salt concentration.

Part of the animal serum antihyaluronidase values and all of the human serum antihyaluronidase values were obtained by the use of the mucoprotein clot prevention test; the technique is described under "Experimental," page 91 to 94.

Assay by Turbidity Prevention

Recently, Kass and Seastone (72) have published a method for the assay of hyaluronidase which depends upon the ability of hyaluronic acid to yield a turbid solution when mixed with carefully buffered protein. The hyaluronidase is incubated with the hyaluronic acid for a definite period of time, after which the reaction mixture is poured into a buffered serum solution. The turbidity formed is compared with the turbidity produced by a standard solution, and the hyaluronidase potency calculated. The stated advantage of the method is that many analyses may be performed simultaneously and stored for several hours, until it is convenient to read the turbidities.

The turbidity method was not published until most of the work described in this paper had been completed, and was not considered when making a choice of methods. The reliance on a standard turbidity produced by a standard hyaluronic acid preparation appears to be a drawback, since all current hyaluronic acid preparations contain more

or less of other polysaccharides which will produce a non-specific turbidity. Another drawback is the necessity of striking a nice balance in the amount of digestion of the hyaluronic acid and the degree of acidity. If this balance is not attained the precipitate will be flocculent or stringy, instead of being turbid.

EXPERIMENTAL

Viscosimetric Assay

PRINCIPLE The time required for a hyaluronidase sample to reduce the viscosity of a substrate containing hyaluronic by an arbitrarily selected percent of the original viscosity is inversely proportional to the concentration of the enzyme, when certain conditions are maintained.

UNIT A viscosity reducing unit (V.R.U.) is defined as that concentration of hyaluronidase which, in 20 minutes, will cause a loss of viscosity of substrate equal to 1/2 of the maximum possible loss, under the conditions described below. The substrate-buffer-enzyme mixture is then said to be at the half viscosity level.

MAXIMUM VISCOSITY LOSS In all cases, the substrate is essentially a solution of hyaluronic acid in buffer fluid. The basic level, below which the viscosity cannot fall, is taken to be the viscosity of the buffer and is designated f_{∞}. The viscosity of the substrate-buffer mixture is the highest viscosity encountered, and is designated f_o. The maximum possible reduction in the viscosity of the substrate buffer mixture is a reduction to the viscosity of the buffer and is equal to $f_o - f_{\infty}$.

HALF VISCOSITY LEVEL When, by action of hyaluronidase, the substrate has undergone a reduction in viscosity equal to 1/2 of the maximum viscosity loss, it is said to be at the half viscosity level. The actual viscosity in seconds of the substrate at the half viscosity level is equal to the initial viscosity, f_o, minus 1/2 of the maximum possible loss in viscosity, or 1/2 $(f_o - f_{oo})$, where f_{oo} is the viscosity of the buffer solution. Thus,

$$\text{Half Viscosity Level} = f_o - 1/2\ (f_o - f_{oo})$$
$$= 1/2\ f_o + 1/2\ f_{oo}$$
$$= 1/2\ (f_o - f_{oo})$$

REACTION TIME The duration of the reaction is measured from the time at which the enzyme is mixed with the substrate. Measurement of viscosity in the Ostwald pipette, however, is not instantaneous but endures for the span necessary for the fluid to flow through the viscosimeter. While the viscosity measurement is being made, of course, the reaction continues. It therefore is necessary to make correction to the duration of the reaction at the start of a viscosity measurement in order to obtain the average reaction time (R.T.) which may properly associated with that viscosity. This is done by adding to the duration time at the beginning of a viscosity measurement 1/2 of the time required to make that measurement. Thus,

Reaction Time (R.T.) = (Duration of reaction until the beginning of viscosity measurement)
+ 1/2 (time necessary for obtaining that viscosity measurement)

REACTION TIMETO HALF VISCOSITY This is the reaction time associated with the half viscosity level (see page). It is designated

R.T. 1/2 and is found by plotting the viscosity measurements on each side of the half viscosity level against reaction time. The point at which the plot crosses the half viscosity level is taken as R.T. 1/2.

PROCEDURE Five ml. of substrate are placed in a small test tube and maintained at 36.95 ± 0.05° C. on a constant temperature bath. The sample of enzyme is appropriately diluted, with buffer and the diluted enzyme is maintained at bath temperature. It may be necessary to try several different dilutions of enzyme before a suitable dilution is found. One ml. of the diluted enzyme is run into the 5 ml. of substrate and mixed by inverting the tube, using a small square of waxed paper to cover the mouth of the tube. A clock started at the moment the enzyme is added to the substrate records the reaction time (R.T.). The six ml. of enzyme substrate mixture are poured from the small test tube into an Ostwald viscosimeter which previously has been placed in the bath. At appropriate intervals, the fluid is blown into the upper bulb of the Ostwald viscosimeter and viscosity readings are taken by timing the rate of flow of the fluid through the instrument by means of a stopwatch. Viscosity measurements are repeated until the flow time of the fluid is less than the value at the half viscosity level (see page

The viscosity readings on either side of the half viscosity level are then plotted against reaction time (R.T., see page 60) and the point at which the plot crosses the half viscosity level is taken as the reaction time to half viscosity level (R.T. 1/2) of the sample under test. The potency of the hyaluronidase sample is calculated from this value.

CALCULATION OF POTENCY OF SAMPLE A typical calculation of the activity of a Cl. welchii filtrate follows. Pages 59 to 60 should be read if a more detailed definition of the terms is desired.

Flow time (viscosity) of 5 ml. of potassium hyaluronate solution, plus 1 ml. of buffer = f_o = 266.0 seconds.

Flow time (viscosity) of 6 ml. of buffer = f_{oo} = 76.5 seconds.

Then, half viscosity level (see page) $= \frac{f_o - f_{oo}}{2}$

$$= \frac{266.0 - 76.5}{2}$$

$$= \frac{342.5}{2}$$

$$= 171.25 \text{ seconds}$$

The original Cl. welchii filtrate was diluted 1:1000 with buffer. The dilution factor (D) is therefore 1000.

One ml. of this diluted enzyme required 1276 seconds to reduce the viscosity of 5 cc. of substrate to the half viscosity level, 171.25 seconds. The reaction time to half viscosity (R.T. 1/2) is therefore 1276 seconds.

Then, Viscosity-Reducing Units (V.R.U.) $= \frac{20 \text{ minutes}}{\text{R.T. } 1/2}$

$$= \frac{1200 \text{ secs}}{1276 \text{ secs}}$$

$$= 0.94$$

The diluted enzyme solution therefore contains 0.94 V.R.U. in 1 ml. The original Cl. welchii filtrate contains 1000 times this concentration since it was diluted to yield the enzyme solution.

The original filtrate therefore contains 940 V.R.U. per ml. The foregoing calculation is repeated below, using symbols.

f_o = 266.0 seconds

f_{oo} = 76.5 seconds

$\frac{f_o - f_{oo}}{2} = \frac{342.5}{2}$ = 171.25 seconds = Half Level Viscosity

R.T. 1/2 = 1276 seconds

V.R.U. of original filtrate = $\frac{1200 \text{ seconds}}{1276 \text{ seconds}}$ D

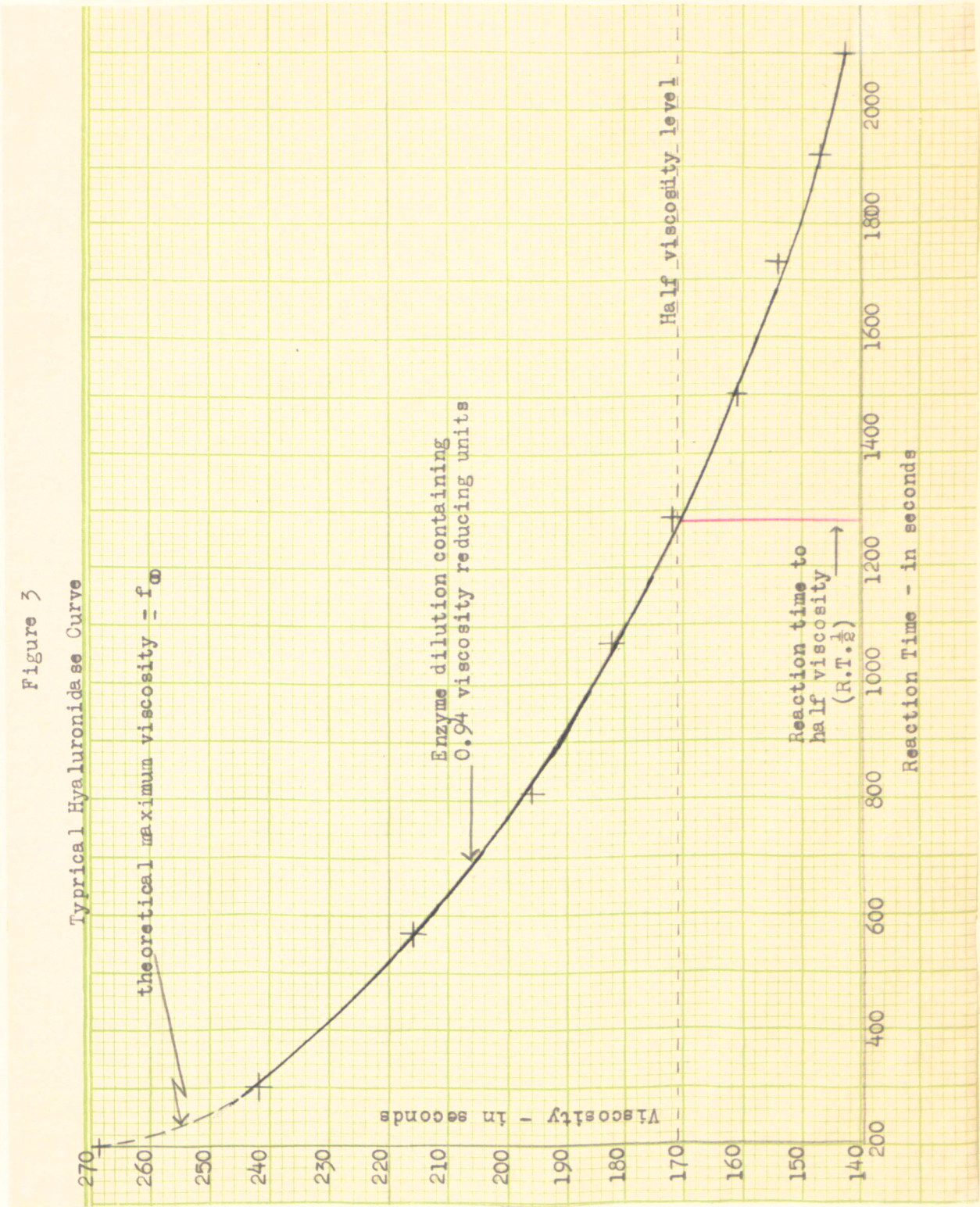
$= \frac{1200}{1276}$ 1000 = 940

The hyaluronidase viscosity reduction curve from which R.T. 1/2 was taken is given in Figure 3, page

SUBSTRATE The substrates used for the earlier assays were synovial fluid, water extracts of umbilical cord, or solutions of synovial mucin or umbilical mucin. These latter were prepared according to the directions of Chain and Duthie (18) and of McClean and Hale (108). Later assays constituting the greater part of the values reported were run on substrates of dialyzed, filtered, tryptic digest of umbilical cord or of solutions of potassium hyaluronate preparation. The production of these materials is described in the chapter on "Preparation of Potassium Hyaluronate" pages 24 to 26.

Although the V.R.U. values obtained for a given enzyme solution are independent of the concentration of substrate (99,108), it is practical to maintain the substrate concentration within certain limits. If the substrate concentration is too low, experimental error will be large; while if the substrate concentration is too great, individual viscosity determinations will require too long a time and the corrections to the reaction time will be excessively large. It has been

Figure 3


Typrical Hyaluronidase Curve
theoretical maximum viscosity = f_∞
Enzyme dilution containing
0.94 viscosity reducing units
Half viscosity level
Reaction time to
half viscosity
(R.T.$\frac{1}{2}$)
Viscosity - in seconds
Reaction Time - in seconds
270
260
250
240
230
220
210
200
190
180
170
160
150
140
200
400
600
800
1000
1200
1400
1600
1800
2000

found best to arrange the substrate concentration so that the initial viscosity f_o of the substrate-buffer mixture is from 2 to 4 times as great as the viscosity f_{oo} of the buffer alone. If potassium hyaluronate preparation (see Chapter II) is used as source of hyaluronic acid, a satisfactory concentration is 0.1 to 0.25%, depending upon the viscosity of the preparation. Synovial fluid or hyaluronic acid solutions may be diluted to the required viscosity, or water removed by evaporation *in vacuo* at 40 to 60° C., as the case requires. The viscosity of the substrate-buffer mixture is considerably less than would be the case were the substrate to be diluted to the same extent with distilled water. This is because hyaluronic acid is less viscous at the acid value for pH by the buffer, and because the NaCl present in the buffer also reduces the viscosity of the mixture.

BUFFER The M/3 citrate buffer was made by mixing two volumes of M NaOH with one volume of M citric acid; NaCl was then dissolved in this solution until the buffer was 1.2 M with respect to NaCl. The pH (glass electrode) of the citrate buffer *before* addition of NaCl was pH= 4.91. The final pH of the buffer *after* addition of the NaCl was pH = 4.25 - 4.30. The synovial fluid or other substrate possesses buffering power of its own, so that the pH of the final mixture of 5 cc. of substrate with 1 cc. of buffer or buffer-enzyme dilution has a pH = 5.1 or 5.2 (indicators).

Sodium chloride in appreciable concentration is a necessary addition to the buffer in order to minimize the possible effects of varying amounts of salts added with different enzyme preparations. These effects on the substrate and on the enzyme are discussed by other

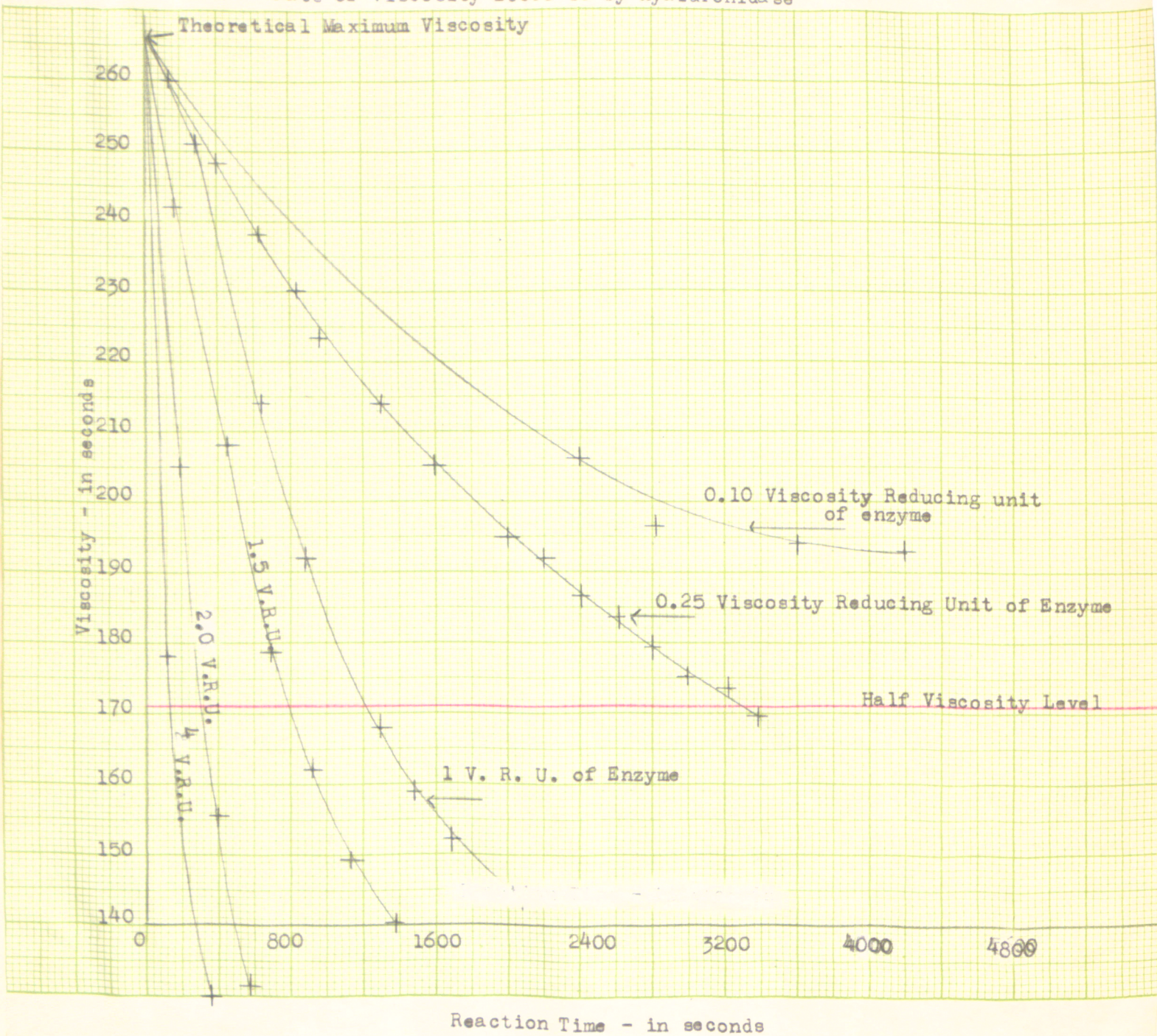
workers such as Madinaveitia and Quibell (99), McClean (113), and Robertson, Ropes, and Bauer (150).

LIMITS OF REACTION TIME In order that the rate of decrease of viscosity shall be proportional to the enzyme concentration, it is necessary to dilute the original sample so that the dilution of enzyme tested will have a reaction time to half viscosity level (R.T. 1/2) of between 800 and 1900 seconds. That is, the dilution actually run must contain between 1.5 and 0.63 V.R.U. If R.T. 1/2 is below 800 seconds, the values obtained are usually too high, and experimental error is large; if R.T. 1/2 is more than 1900 seconds, the values obtained are usually too low, and the determination is tedious and difficult to interpret graphically. The reason for these changes in the values obtained are apparent in the shape of the viscosity reduction curve. The initial drop is steep, while the final drop in viscosity is very gradual and the curve is quite flat. If the concentration of hyaluronidase is too high, the curve intersects the half viscosity level during the initial stage of rapid viscosity decrease, while if the concentration is too small the viscosity reduction curve nearly parallels the half viscosity level at the point of intersection, or viscosity reduction may practically cease, and the curve may intersect the half viscosity level only at infinity. These conditions are exemplified in figure 4, page 67 departure from proportionality at reaction times greater than 1900 seconds or less than 800 seconds are shown in figure 5, page 68.

ACCURACY If only those values are accepted which are calculated from reaction time to half viscosity falling between 800 and 1900 seconds, the accuracy of the determination is well within $\pm$ 10%.

Figure 4

Rate of Viscosity Decrease by Hyaluronidase



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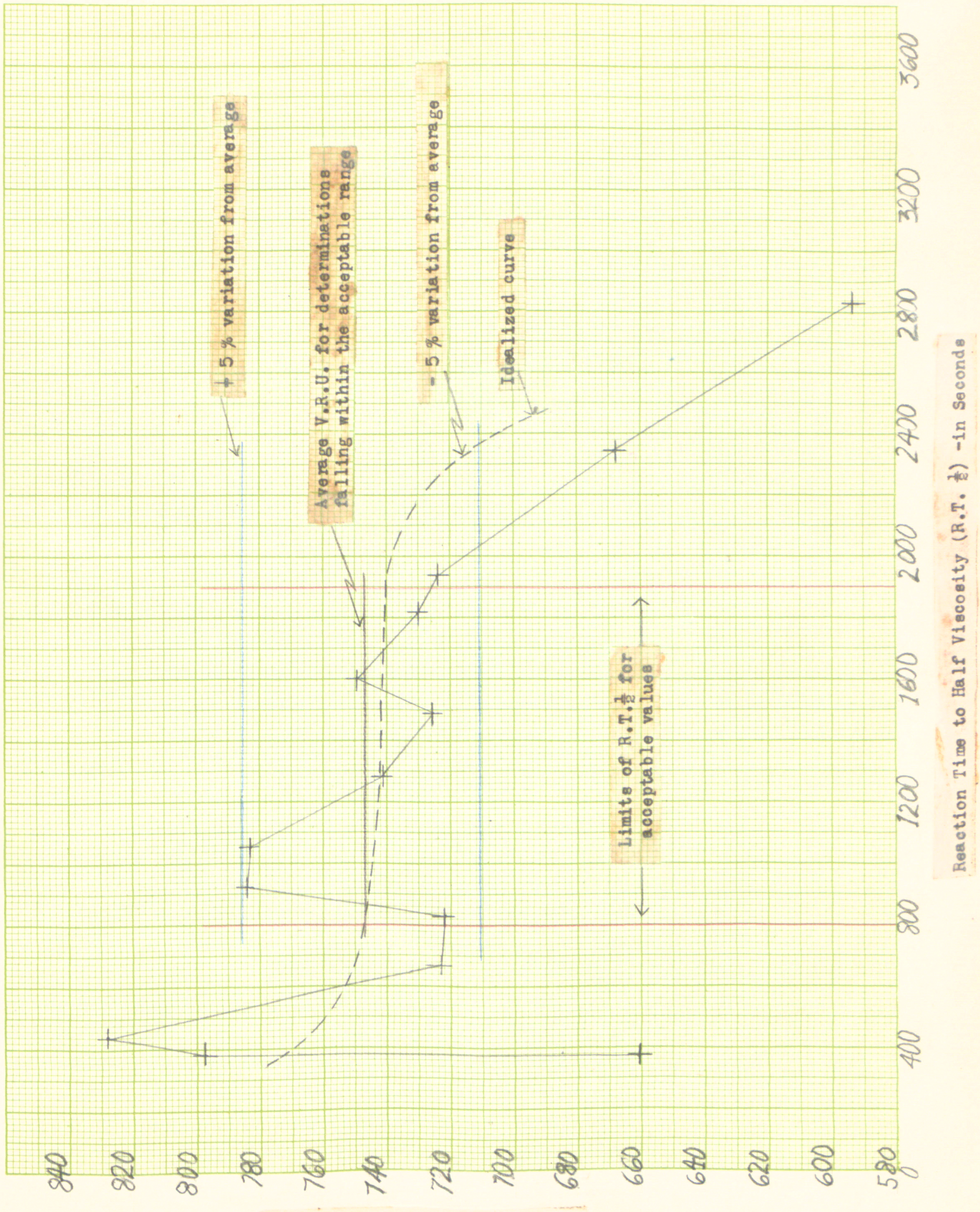


(plotted from data in Ta[illegible] V, Page_)

This is shown in figure 5, page 68, where dilutions of an anzyme sample, yielding various values for R.T. 1/2, are plotted against the V.R.U. found for the original sample. The data plotted are set forth in Table V page 69.

TABLE V

Accuracy of Viscosimetric Test
(Data plotted on page 68.)

Dilution of original sample	R.T. 1/2 in seconds	V.R.U. found	Average V.R.U. in acceptable range
1/1400	2828	594	
1/1300	2335	668	
1/1200	1948	724	
1/1100	1807	730	
1/1000	1603	749	
1/900	1490	725	
1/800	1296	741	747.7 or 748
1/700	1072	784	
1/600	920	784	
1/500	832	721	
1/400	665	722	
1/300	435	828	
1/250	380	797	
1/200	363	661	

ACCEPTABLE RANGE

OTHER GRAPHICAL TREATMENT OF VISCOSITY DATA The equation to the curve obtained by plotting change in viscosity against reaction time (a typical curve is shown on page 64) is unknown, and comparison is made between various enzyme solutions on the basis of the time necessary for the enzyme to effect 50% of the maximum loss of viscosity. If a function of the data could be found whose equation was known, and particularly if that function plotted as a straight line during the early part of the reaction, the viscosity reduction test would become more rapid and more accurate. In an effort to attain a straight line graph, various functions were plotted.

(a) Log enzyme concentrations against reaction time. This graph usually resembles a hyperbola or parabola (figure 6, page 71). The data from which the curve is plotted are the same as those used in figure 5, page 68, and in Table V, p. 69

(b) Enzyme concentration against reaction time. This also is not a straight line (figure 7, page 72). The data is the same as that plotted in figure 5, page 68 and figure 6, page 71.

(c) Reciprocal enzyme concentration against reaction time. The irregularities of measurement in the early stage of the reaction when viscosity decreases is rapid are evident here. The middle portion of this curve, on visual inspection appears to be more or less straight. The limits, however, are no wider than the limits used in the regular assay, so there is no advantage gained by the use of this function. The graph appears on page 73 as figure 8. Data is the same as that in the previous figures.

(d) Enzyme concentration against reciprocal reaction time. The remarks given under item (c) pertain here also. The graph appears on

Figure 6

Viscosity Curve: (a) Log. Enzyme

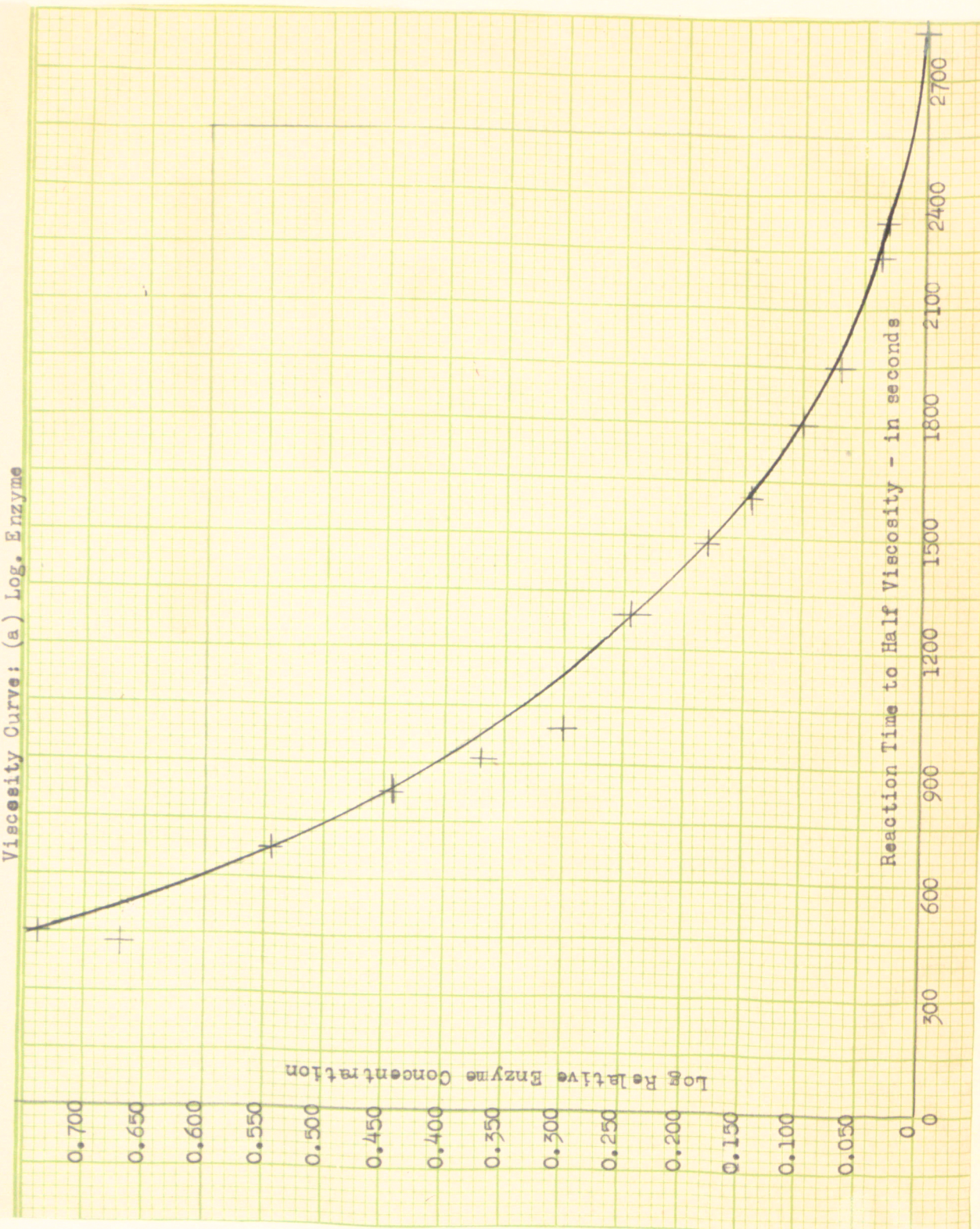


Figure 7

Viscosity Curve : Enzyme Concentration

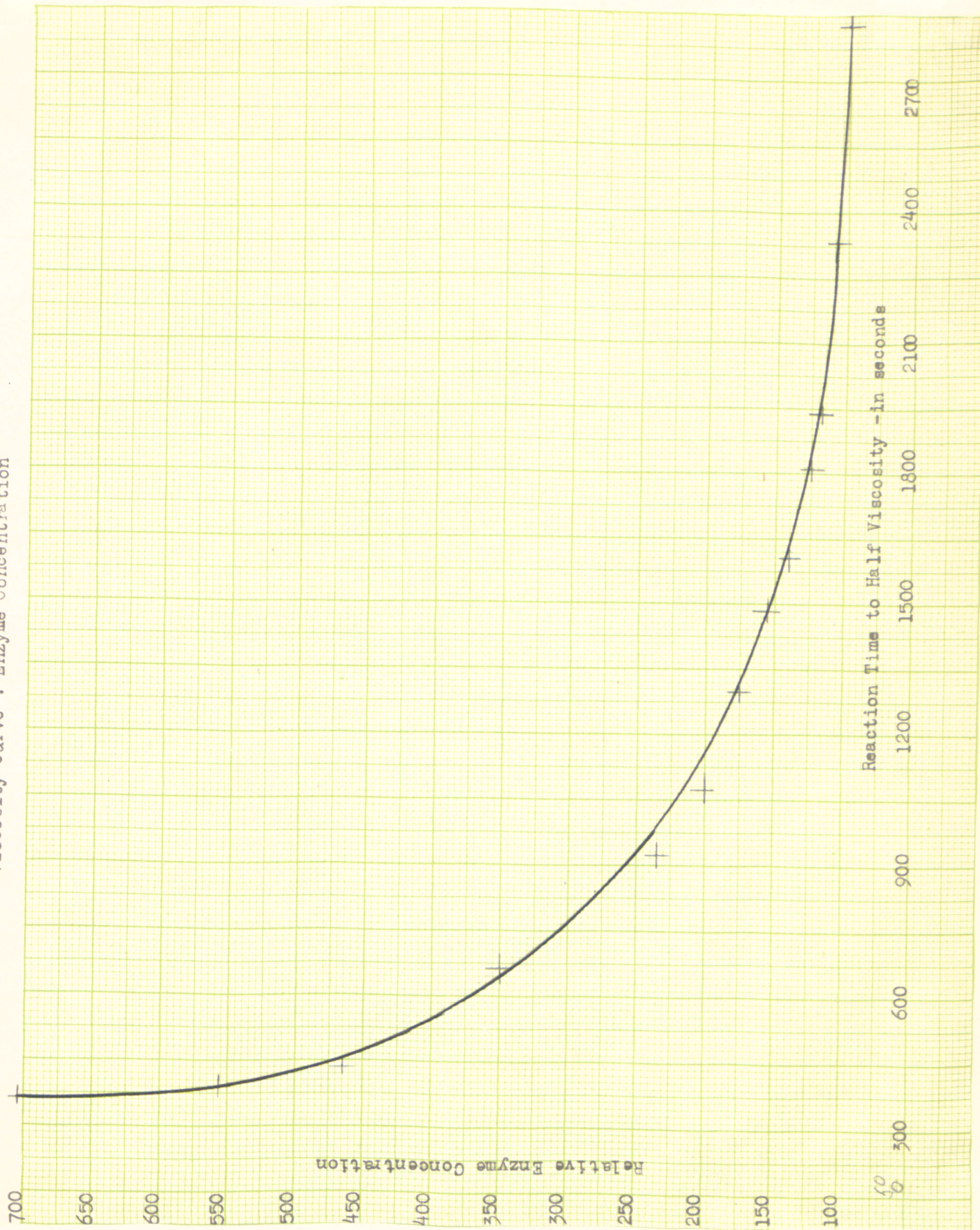


Figure 8

Viscosity Curve: Reciprocal Enzyme Concentration

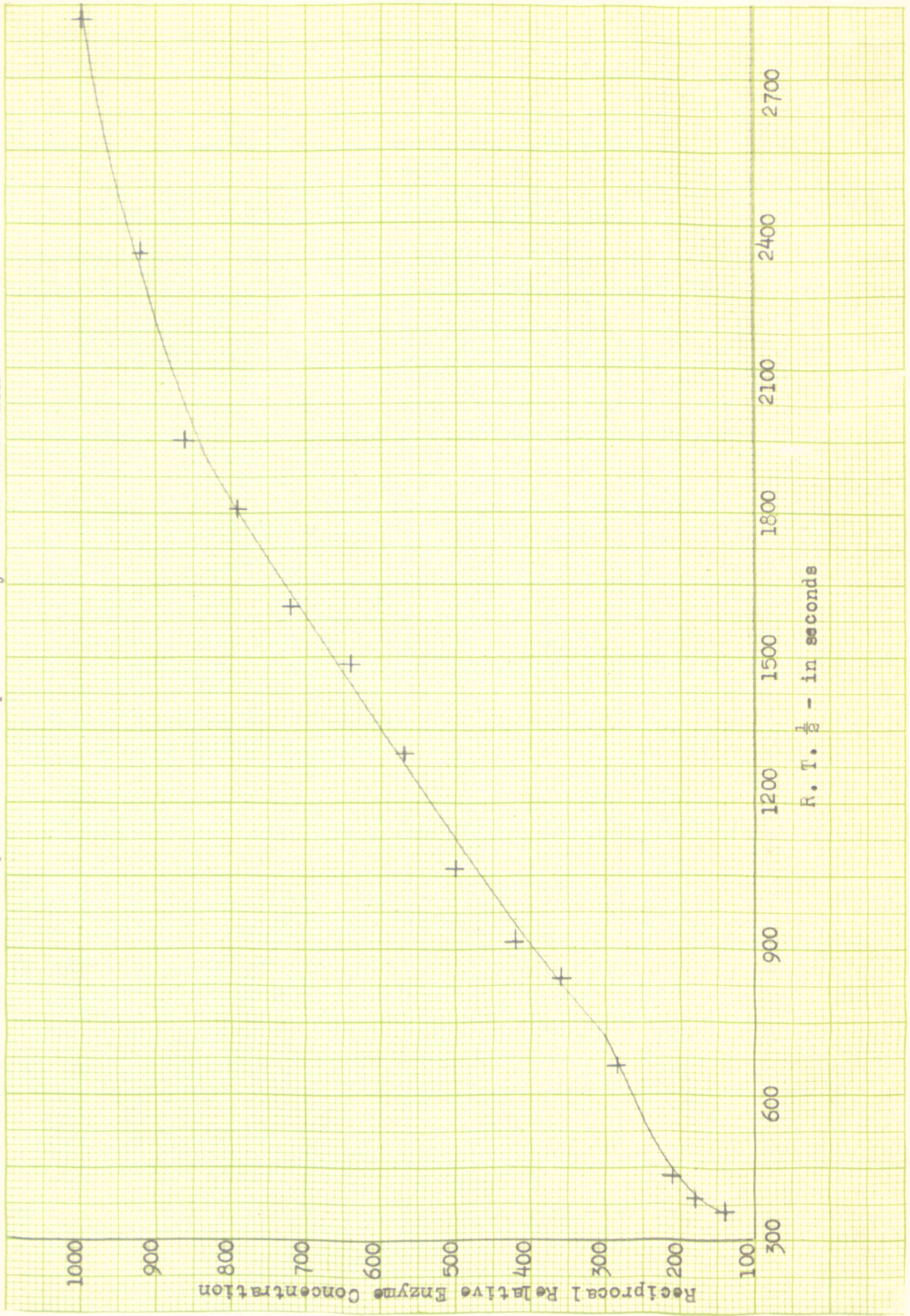


Figure 9

Viscosity Curve: Reciprocal R. T.

page 74 as figure 9.

(e) Many hydrolytic enzymes follow the Schutz-Borrissov Law (see Suggestions for Future Work) this chapter, page 95 which calls for a reaction rate which is proportional to the square root of the reaction time. Although loss of viscosity of hyaluronic acid solutions is not a direct measure of loss of substrate (see this chapter page the possibility of linear relationship to the square root of the reaction time was investigated by plotting various functions of the viscosity changes. Some parts of a number of these curves seemed to be linear, as determined by visual inspection, therefore the absolute slope of the plotted points on these curves was calculated algebraically. It was then seen that the linearity was more apparent than real, for the deviation from the mean of values of the absolute slope were frequently 14 to 30%. The graphs are actually shallow curves. A number of these curves, together with the data from which they were plotted and values for the absolute slopes, are given in figures 10 to 15 and Tables 6 to 11.

Tables XII-XV are calculated similarly to Tables VI-XI, but there are no accompanying graphs to Tables XII-XV. The series of Tables (VI-XV) represent over 40 similar calculations, which were made in attempting to verify the apparent linearity of the curves plotted from the functions indicated in the respective tables. In no case was an average deviation from the mean less than 16% encountered, and therefore there is no advantage in the use of these curves over the curve ordinarily used for the calculation of viscosity reducing units. (Figure 3, page 64.

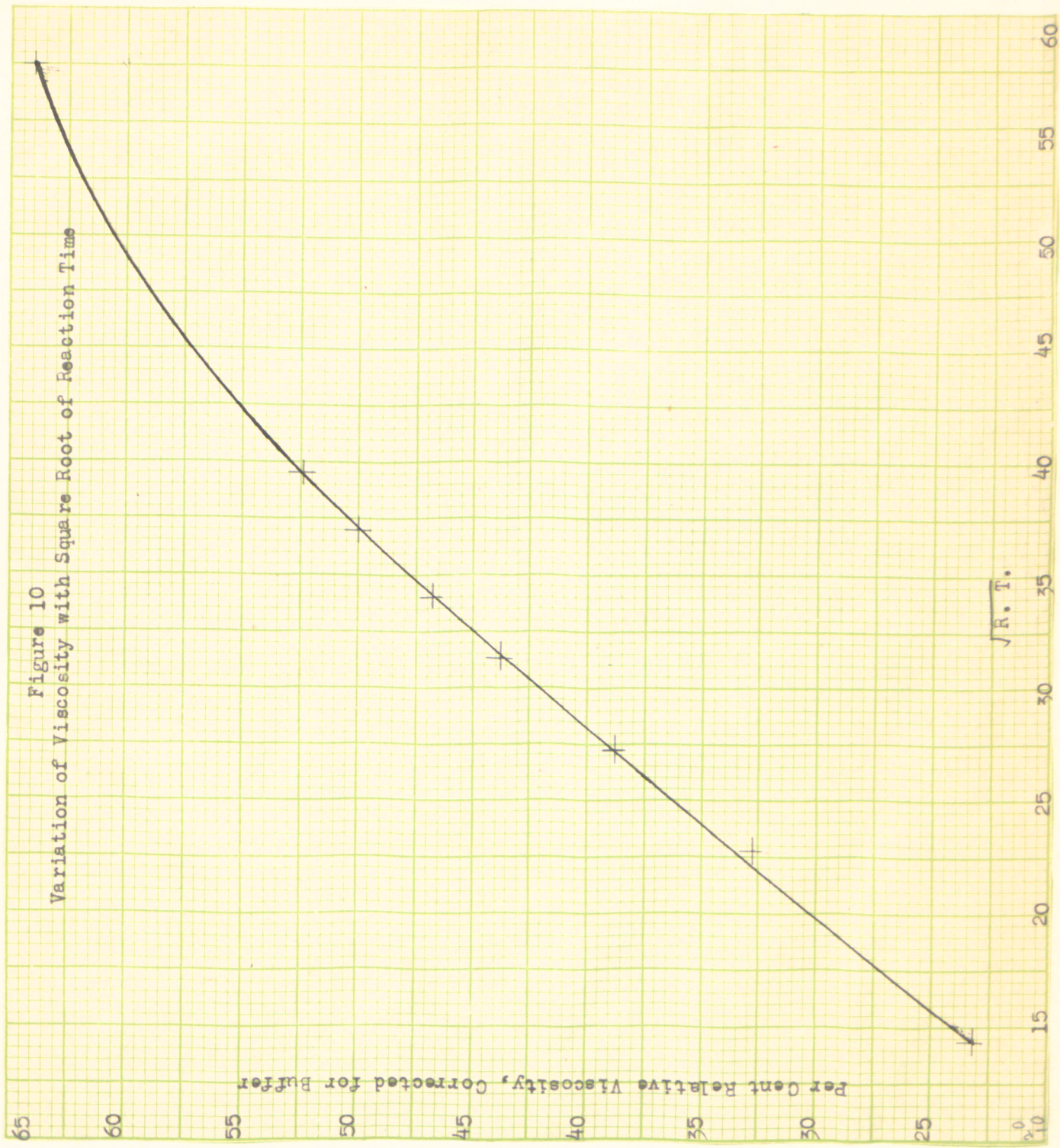
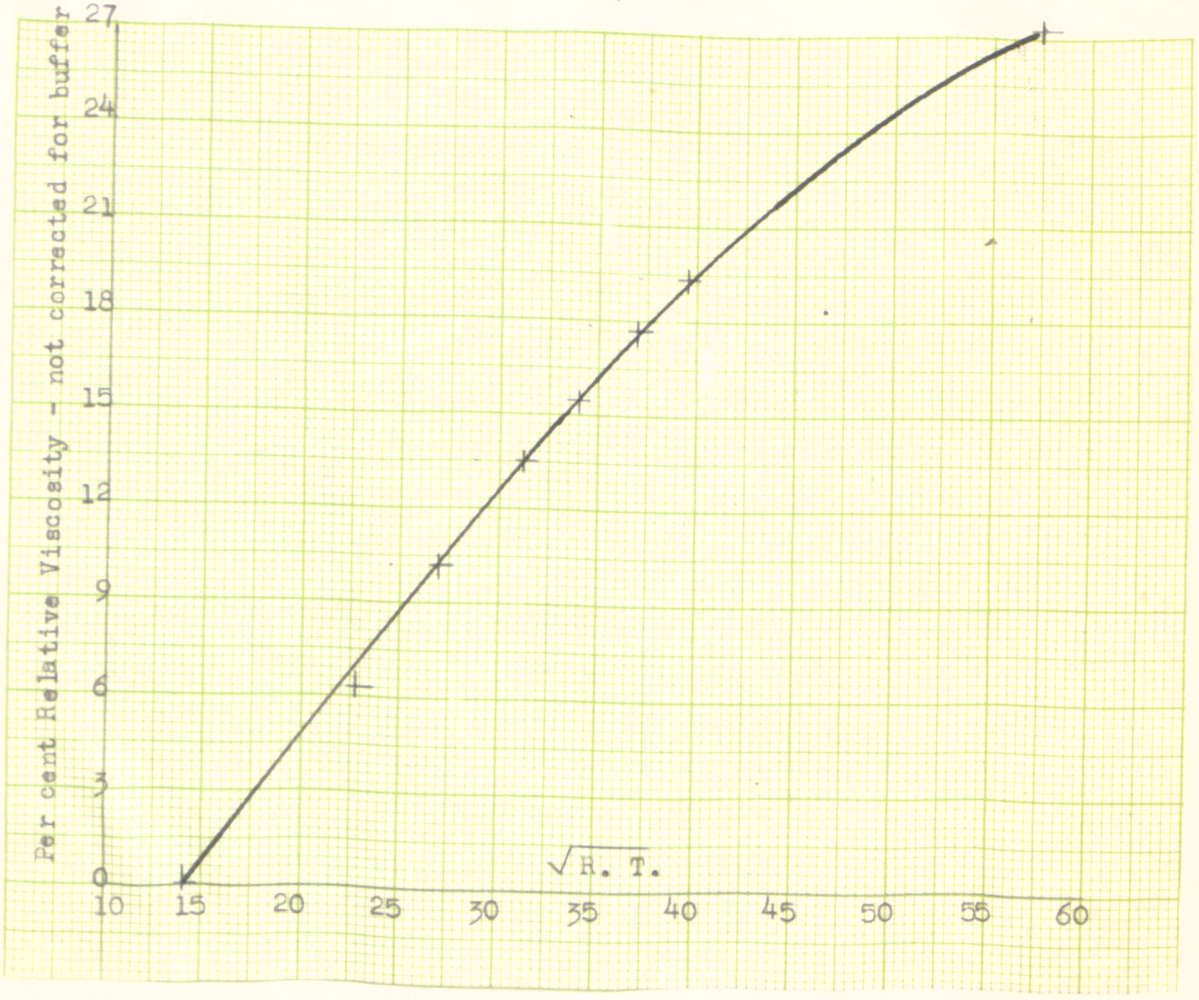


Figure 10
Variation of Viscosity with Square Root of Reaction Time

Figure 11

Variation of Viscosity with Square Root of Reaction Time



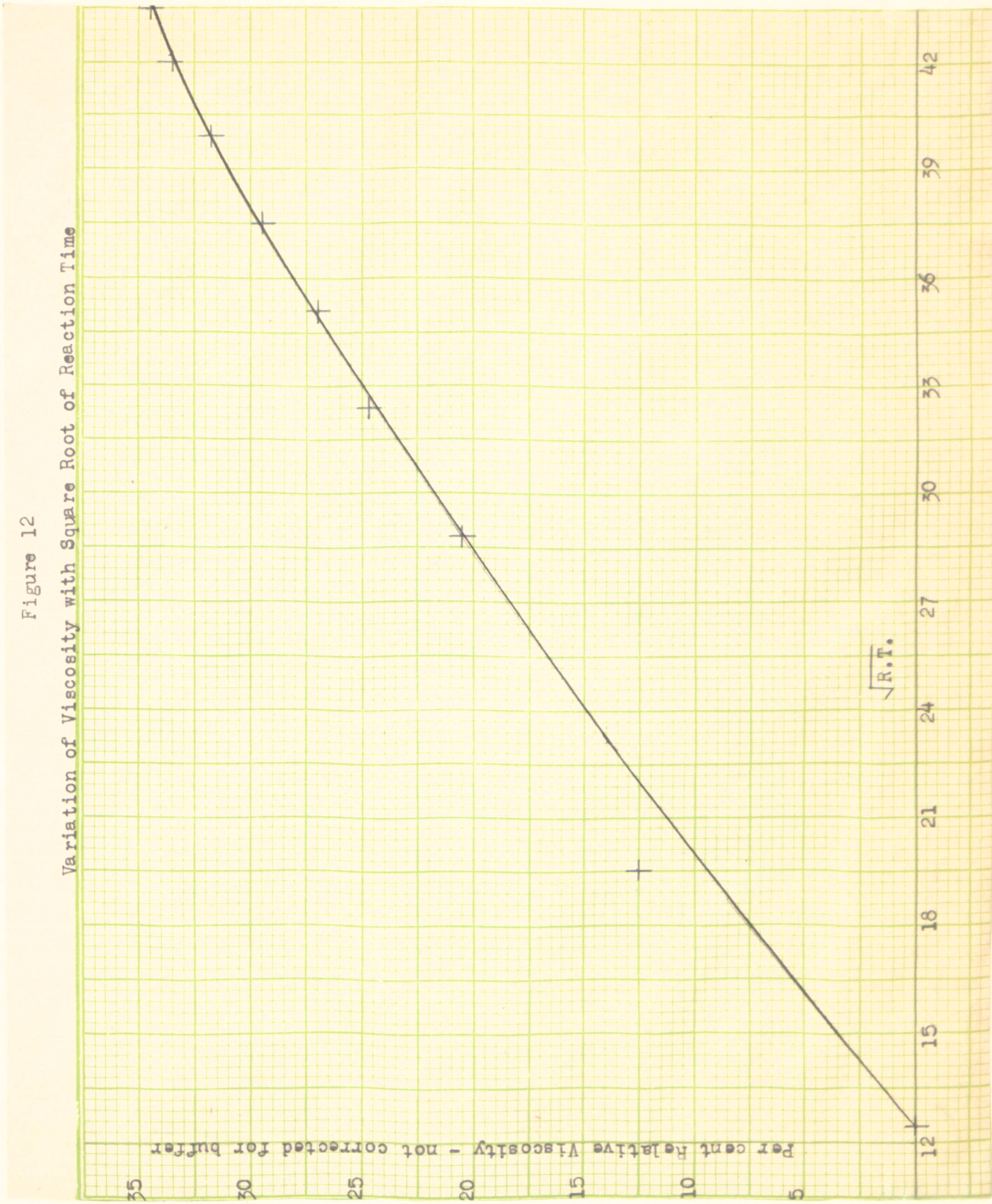


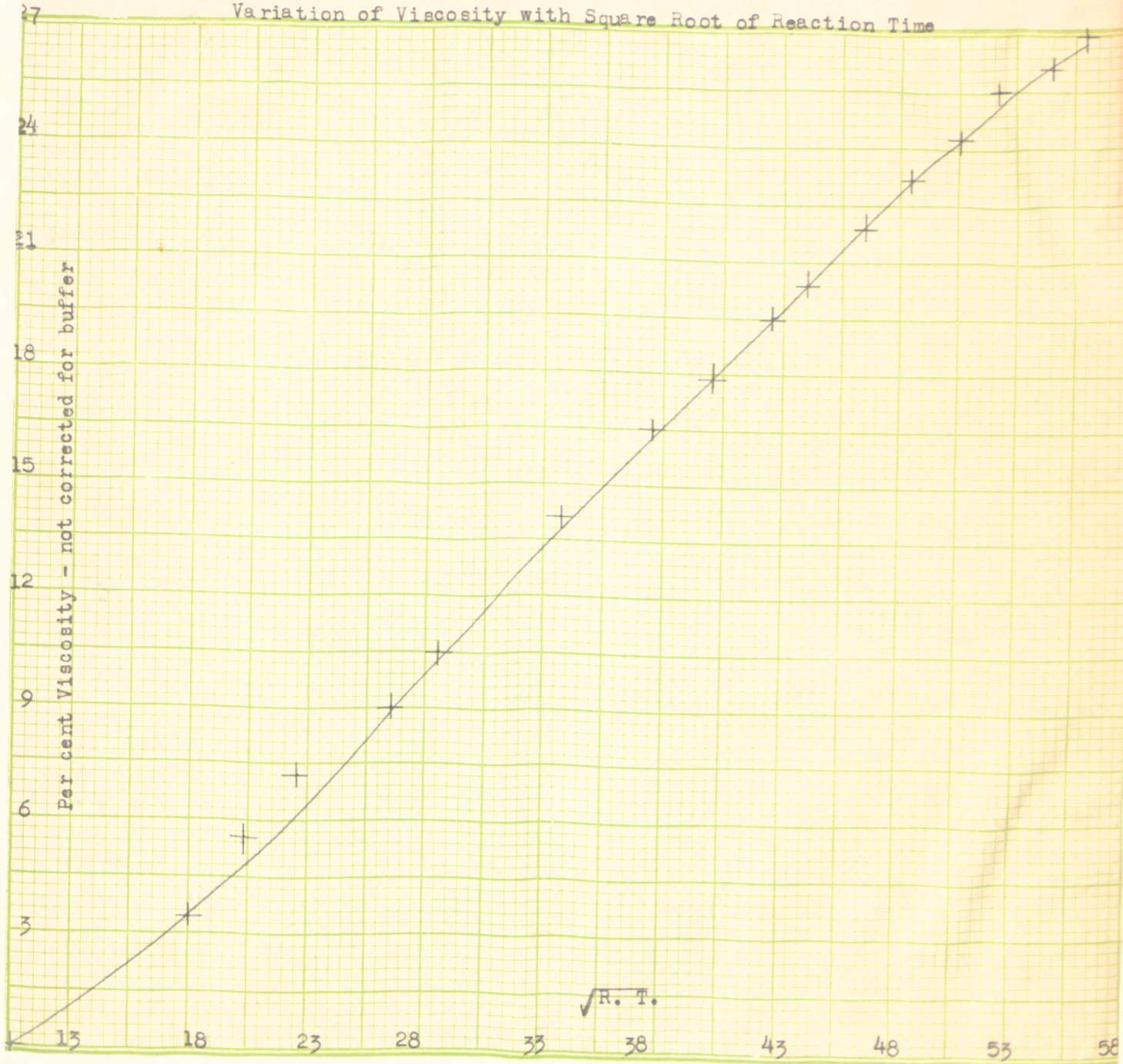
Figure 12

Variation of Viscosity with Square Root of Reaction Time

VARIATION OF VISCOSITY W SQUARE ROOT OF REACTION TIME

Figure 14

Variation of Viscosity with Square Root of Reaction Time



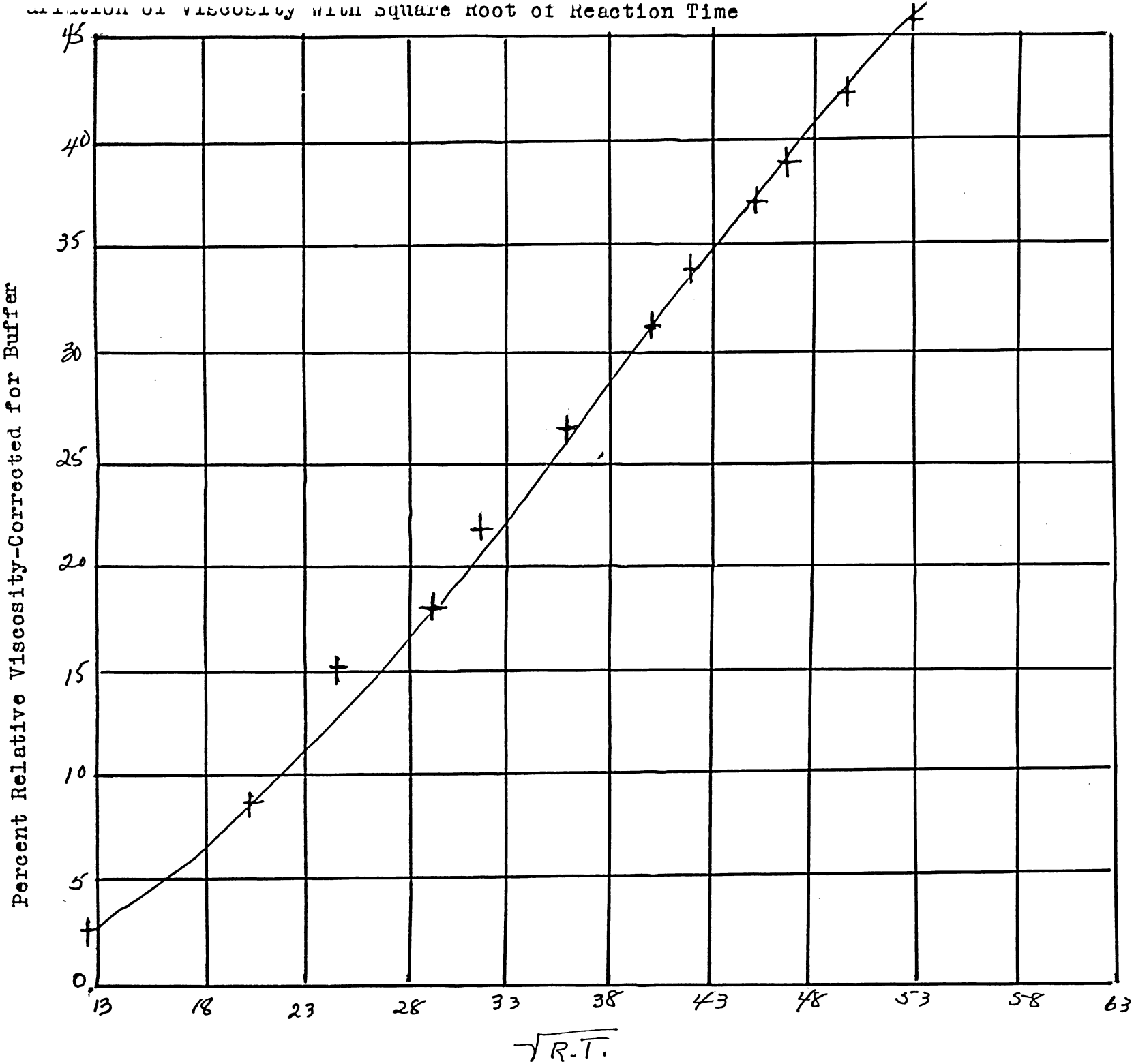

Addition of Viscosity with Square Root of Reaction Time
Percent Relative Viscosity-Corrected for Buffer
0
5
10
15
20
25
30
35
40
45
13
18
23
28
33
38
43
48
53
58
63
√R.T.

TABLE VI

Variation of Viscosity with Square Root of Reaction Time

(This data is plotted in Figure 10, page 76).

R.T.	V % relative viscosity, corrected for buffer Loss in viscosity	K K calculated from $K = \frac{V_2 - V_1}{R.T._2 - R.T._1}$	Deviation of K from the mean
14.3	23.2		
22.8	32.7	2.07	+0.86
27.0	38.6	1.40	+0.19
31.2	43.6	1.19	-0.02
34.1	46.8	1.10	-0.11
37.0	50.0	1.10	-0.11
39.4	52.3	0.96	-0.25
57.5	64.1	0.65	0.56

Mean value for K = 1.21

Average deviation from mean = 0.30

$\frac{\text{average deviation}}{\text{mean}} = \frac{0.30}{1.21} \times 100 = 23\%$

TABLE VII

Variation of Viscosity with Square Root of Reaction Time

(This data is plotted in Figure 11, page 77)

$\sqrt{VR.T.}$	V % relative viscosity, corrected for buffer Loss in viscosity	K calculated from $K = \frac{V_2 - V_1}{VR.T. - R.T.1}$	Deviation of K from the mean
14.3	0		
22.8	6.3	0.74	0.00
27.0	10.3	0.95	+0.21
31.2	13.6	0.79	+0.05
34.1	15.7	0.94	+0.02
37.0	17.8	0.72	-0.02
39.4	19.3	0.63	-0.11
57.5	27.2	0.43	-0.31

Mean value for K 0.74

Average deviation from mean 0.10

$\frac{\text{Average deviation}}{\text{Mean}} = \frac{0.10 \times 100}{0.74} = 14\%$

TABLE VIII

Variation of Viscosity with Square Root of Reaction Time

(This data is plotted in Figure 12, page 78.)

$\sqrt{R.T.}$	V % relative viscosity, corrected for buffer Loss in viscosity	K calculated from $K = \frac{V_2 - V_1}{VR.T._2 - R.T._1}$	deviation of K from the mean
12.5	0		
19.6	12.6	1.77	-0.77
28.8	22.07	0.88	-0.12
32.1	24.9	1.27	-0.63
34.8	27.1	0.81	-0.19
37.4	29.3	0.85	-0.15
39.8	31.7	1.00	0
42.2	33.5	0.75	-0.25
45.0	35.4	0.68	-0.32

Mean value for K 1.00

Average deviation from mean 0.30

$\frac{\text{Average deviation}}{\text{Mean}} \times 100 = \frac{0.30}{1.00} = 30\%$

TABLE IX

Variation of Viscosity with Square Root of Reaction Time

(This data is plotted in Figure 13, page 78.)

R.T.	V % relative viscosity, corrected for buffer Loss in viscosity	K calculated from $K = \frac{V_2 - V_1}{R.T._2 \; R.T._1}$	deviation of K from the mean
12.8	3.0		
19.9	9.0	0.85	-0.15
25.0	15.0	1.15	+0.15
29.0	18.5	0.88	-0.12
31.0	22.5	2.00	+1.00
36.0	26.7	0.85	-0.15
40.0	31.0	1.08	+0.08
42.2	33.5	0.11	-0.89
44.8	36.5	1.15	+0.15
47.0	38.0	0.68	-0.32
49.0	40.7	1.35	+0.35
51.1	42.5	0.86	-0.14
53.0	44.5	1.05	+0.05
54.7	46.5	1.18	+0.18
56.7	47.5	0.50	-0.50
58.3	49.5	1.25	+0.25
Mean value for K		1.00	

Average deviation from mean 0.30

$\frac{\text{Average deviation}}{\text{Mean}} = 0.30 \times 100 = 30\%$

TABLE X

Variation of Viscosity with Square Root of Reaction Time

(This data is plotted in Figure 14, page 80)

VR.T.	V % relative viscosity, corrected for buffer Loss in viscosity	K calculated from $K = \frac{V_2 - V_1}{\sqrt{R.T._2} - \sqrt{R.T._1}}$	deviation of K from the mean
12.8	0		
19.9	3.5	0.49	+0.19
25.0	7.0	0.69	+0.01
29.0	9.1	0.53	-0.15
31.0	11.4	1.15*	- - -
36.0	13.9	0.50	-0.18
40.0	16.4	0.63	-0.05
42.2	17.8	0.64	-0.04
44.8	19.6	0.69	+0.01
47.0	20.5	0.68	0
49.0	22.0	0.75	+0.07
51.1	23.1	0.53	-0.05
53.0	24.3	0.63	-0.05
54.7	25.4	0.65	-0.03
56.7	26.0	0.30	-0.38
58.3	27.2	0.80	+0.28

Mean value for K 0.68

Average deviation from mean 0.11

$\frac{\text{Average deviation}}{\text{Mean}} = \frac{0.11}{0.68} \times 100 = 16\%$

*Not included in the average

TABLE XI

Variation of Viscosity with Square Root of Reaction Time

(This data is plotted in Figure 15, page 81.)

$V\sqrt{R.T.}$	V % relative viscosity, corrected for buffer Loss in viscosity	K calculated from $K = \frac{V_2 - V_1}{V\sqrt{R.T._2} - V\sqrt{R.T._1}}$	deviation of K from the mean
12.8	3.1		
19.9	9.3	0.87	-0.19
25.0	15.5	1.24	+0.18
29.0	19.1	0.90	-0.16
31.0	23.2	1.55	+0.49
36.0	27.5	0.66	-0.40
40.0	31.9	0.85	-0.11
42.2	34.5	1.18	+0.12
44.8	27.6	1.19	+0.13
47.0	39.2	1.18	+0.12
49.0	42.0	1.40	+0.34
51.1	43.8	0.86	-0.20
53.0	45.9	1.11	+0.05
54.7	47.9	1.18	+0.12
56.7	49.0	0.55	+0.51
58.3	51.0	1.25	-0.19

Mean value for K 1.06

Average deviation from mean 0.22

$\frac{\text{Average deviation}}{\text{Mean}} = \frac{0.22}{1.66} \times 100 = 21\%$

TABLE XII

Variation of Viscosity with Square Root of Reaction Time

$V\sqrt{R.T.}$	V % relative viscosity, corrected for buffer Loss in viscosity	K calculated from $K = \frac{V_2 - V_1}{V\sqrt{R.T.}_2 - V\sqrt{R.T.}_1}$	deviation of K from the mean
12.5	0		
19.6	12.6	2.54	+0.93
28.8	20.7	1.50	-0.11
32.1	24.9	1.82	+0.21
24.8	27.1	1.69	+0.08
37.4	29.3	1.35	-0.26
39.8	31.7	1.67	+0.06
42.2	33.5	1.25	-0.36
45.0	35.4	1.07	-0.54

Mean value for K 1.61

Average deviation from mean 0.32

$\frac{\text{Average deviation}}{\text{Mean}} = \frac{0.32}{1.61} \times 100 = 20\%$

TABLE XIII

Variation of Viscosity with Square Root of Reaction Time

$\sqrt{R.T.}$	V % relative viscosity, corrected for buffer Loss in viscosity	K calculated from $K = \frac{V_2 - V_1}{\sqrt{R.T._2} - \sqrt{R.T._1}}$	deviation of K from the mean
13.7	0		
20.2	24.1	2.09	+0.70
24.7	35.6	1.98	+0.59
27.8	41.5	1.72	+0.33
30.5	45.9	1.46	+0.07
32.9	48.3	1.12	-0.27
34.9	50.3	0.35	-0.94
37.3	52.0	1.00	-0.39

Mean value for K 1.39

Average deviation from mean 0.47

$\frac{\text{Average deviation}}{\text{Mean}} = \frac{0.47}{1.39} \times 100 = 34\%$

TABLE XIV

Variation of Viscosity with Square Root of Reaction Time

$V\sqrt{R.T.}$	V % relative viscosity, corrected for buffer Loss in viscosity	K calculated from $K = \frac{V_2 - V_1}{V\sqrt{R.T._2} - V\sqrt{R.T._1}}$	deviation of K from the mean
13.8	0		
21.6	15.3	1.97	+0.59
26.9	22.7	1.40	+0.02
30.9	29.6	1.73	+0.35
37.5	38.7	1.38	0
40.1	41.7	1.15	-0.13
42.3	43.9	1.00	-0.38
46.3	47.9	1.00	-0.38

Mean value for K 1.38

Average deviation from mean 0.26

$\frac{\text{Average deviation}}{\text{Mean}} = \frac{0.26}{1.38} \times 100 = 19\%$

TABLE XV

Variation of Viscosity with Square Root of Reaction Time

$V\sqrt{R.T.}$	V % relative viscosity, corrected for buffer Loss in viscosity	K $K = \frac{V_2 - V_1}{V\sqrt{R.T._2} - V\sqrt{R.T._1}}$	deviation of K from the mean
16.9	0		
23.9	10.7	1.53	+0.20
28.7	19.0	1.75	+0.42
32.7	24.7	1.43	+0.10
35.9	29.3	1.44	+0.11
38.8	33.3	1.34	+0.01
41.6	36.4	1.11	-0.22
43.8	39.0	1.18	-0.15
45.8	40.7	0.85	-0.48

Mean value for K 1.33

Average deviation from mean 0.21%

$\frac{\text{Average deviation}}{\text{Mean}} = \frac{0.21}{1.33} \times 100 = 16\%$

VISCOSITY AND SUBSTRATE CONCENTRATION The viscosity of hyaluronic acid solutions increases at a greater rate than the rate at which the concentration increases. This finding of McClean (108) was confirmed. The rate of decrease of viscosity by hyaluronidase cannot, therefore, be taken to parallel the rate of disappearance of substrate. The viscosity of a water extract of umbilical cord at various concentrations is shown in Figure 16, page 92.

Mucoprotein Clot Prevention Test

This method of testing for hyaluronidase was developed by McClean (113).

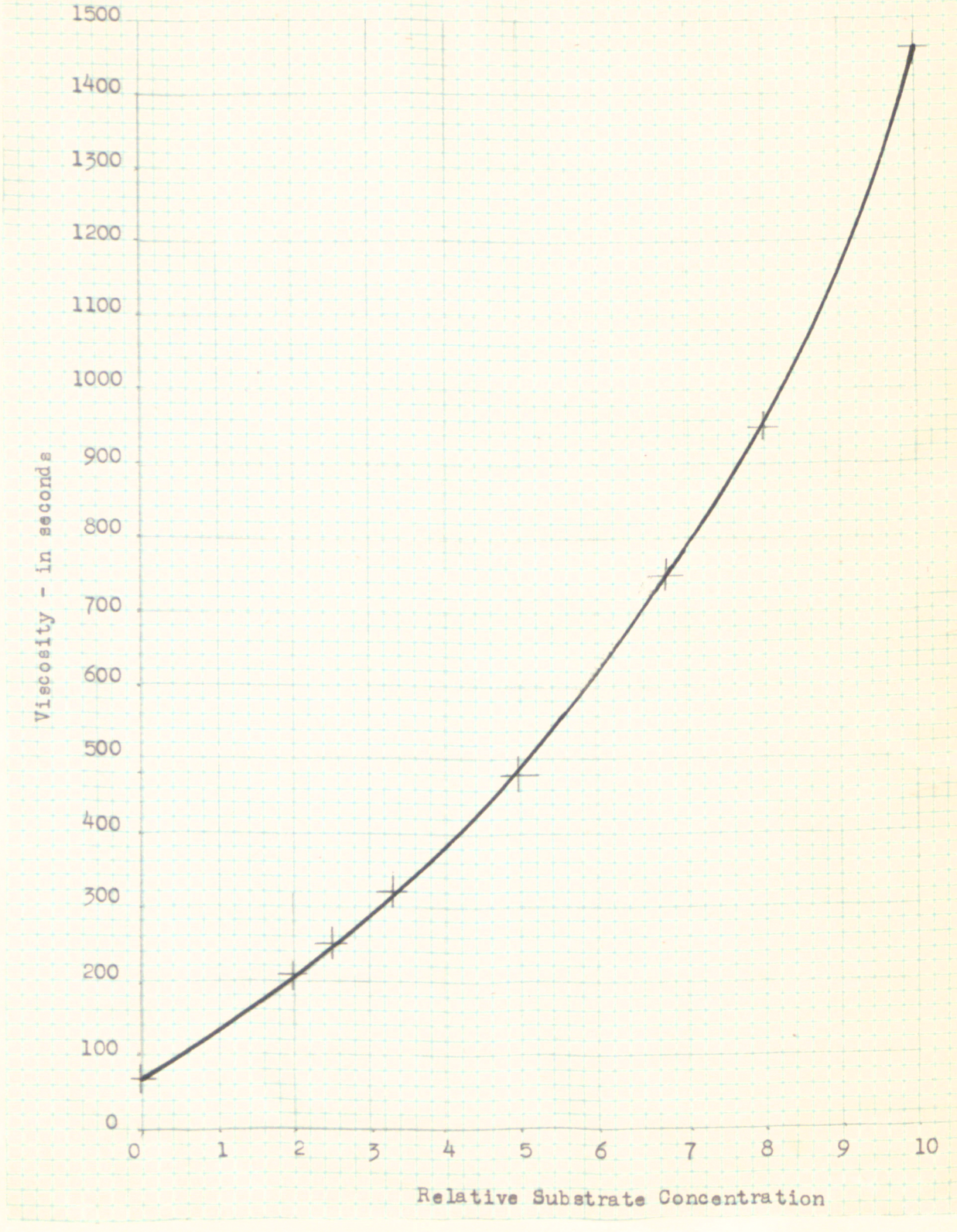
PRINCIPLE A mixture of hyaluronic acid and protein gives a stringy precipitate or "clot" in the presence of dilute acetic acid. If hyaluronidase has hydrolyzed the polysaccharide, the clot does not form.

UNIT A mucoprotein clot prevention unit (M.C.P. unit) is an amount of enzyme which, after acting upon the substrate for 20 minutes at 37°C, will just prevent the formation of a clot when acid is subsequently added.

PROCEDURE Appropriate dilutions of the sample to be tested are made in a final volume of 0.5 ml. of distilled water in small serological test tubes. One ml. of substrate is added to each tube and to a control. The viscous substrate is mixed by inverting the tubes, using a piece of waxed paper to cover the mouth of the tube. A fresh piece of paper is used for each series of tubes, the addition of substrate and mixing are started from the tube containing the highest dilution of enzyme. The enzyme-substrate mixture is incubated at 37° for 20 minutes. The

Figure 16

Relation of Viscosity to Substrate Concentration



tubes are then placed in ice-water for 5 minutes in order to stop enzyme action. After cooling, 0.5 cc. of 1 N acetic acid is added and the tubes shaken or inverted. A clot or stringy precipitate will appear in those tubes containing too little enzyme. Those with enzyme in excess will not contain any strings. The greatest dilution of enzyme still preventing the appearance of a clot is taken as the end point, and the original sample is said to contain a number of M.C.P. units equal to the number of times it was diluted in the end point tube.

SUBSTRATE The substrate is a mixture of hyaluronic acid and serum protein. The serum protein is furnished by addition of normal horse serum. The particular serum used must be tested to be sure that it neither inhibits nor enhances the hyaluronidase activity. Some samples of serum contain antibodies against bacterial hyaluronidase. More rarely, a serum with hyaluronidase activity will be found. Hyaluronic acid was supplied as a dialyzed tryptic digest of umbilical cord containing 0 .1% of potassium hyaluronate (see page) or as 0.1-0.2% solutions of potassium hyaluronate preparation (see page

The substrate-protein mixture is made in the following proportions:

1 volume hyaluronate solution.

1 volume 1:10 serum -0.9% NaCl solution.

2 volumes of distilled water.

RELATION OF M.C.P. UNIT TO V.R.U. McClean (113) gives a ratio of 0.16 V.R.U. to 1 M.C.P. unit for one sample. This relationship, however, is quite variable, depending upon the dilution range in which both measurements are being made, upon the particular substrate used, and so forth. For instance, in connection with antisera assays (page

it was found that a single enzyme sample gave ratios of 14, 7.9, and 0.8 M.C.P. unitsto 1 V.R.U. McClean's (113) ratio is 1 V.R.U. to 6.25 M.C.P., but he also cites ratios of 3.5 to as much as 44.5 for M.C.P. / V.R.U. The M.C.P. test units and viscosity reduction test units can only be related to each other in a general way. Possibly they may not measure exactly the same effects of the enzyme.

PRECISION OF THE M.C.P. TEST. Repeated tests on the same enzyme sample with the same substrate usually result in identical end-points when two-fold dilutions are used, that is, when each dilution differs from the preceding by 100%. Where 50% differences are used the end point may vary by plus or minus one tube, and closer titrations are more uncertain.

DISCUSSION

The technique of viscosity measurement in Ostwald type viscometers has been discussed by Cannon and Fenske (16). The magnitude of possible errors is not great. For example, the instrument must be tilted 2.5° from the vertical in order to cause an error of 0.1%. A deviation of 2° is said to be readily detectable by visual inspection. Other likely errors such as drainage errors and surface tension errors are much smaller. The technique of viscometry is about 100 times more accurate than the viscometric test for hyaluronidase.

Although tests for hyaluronidase activity are not of the highest order of accuracy, yet they appear to be sufficiently accurate for most biological purposes.

SUGGESTIONS FOR FUTURE WORK

The disappearance of viscosity during hyaluronidase action does not follow Schutz's law, but the actual disappearance of substrate may very well follow this rule. The amount of substrate required to produce a given fiscosity could be ascertained in solutions free of enzyme, so that the substrate remaining after any enzyme action for any given time might then be deduced from the original viscosity. A straight line function expressive of hyaluronidase action might thus be found.

Schutz's law is the result of observations by Emil Schutz to the effect that the quantity of albumin digested in a given time was proportional to the square root of the amount of pepsin employed, and by Borrissov and Samojloff, who showed that when the quantity of enzyme is constant the extent of the digestion was proportional to the square root of time. This subject has been examined by Moelwyn-Hughes, Pace, and Lewis (133) and by Langmuir (80). Haines (60) has found the law to apply to the reduction in the viscosity of gelatin by bacterial gelatinase. The Schutz-Borrissov equation may be written:

$$k = \frac{x}{\sqrt{E\,T}}$$

where k = a constant

x = the amount of substrate hydrolysed

E = the concentration of enzyme

and T = the reaction time

This rule has been found to hold for the action of pepsin on egg albumin, the action of trypsin on fibrin, of pancreatin on egg yolk, of yeast ferments on cane sugar, and for the action of steapsin on fats (for

references see 133). If the above equation is differentiated with respect to T, it becomes

$$\frac{dx}{dt} = \frac{k^2E}{2x}$$

Here it is seen that the rate of the reaction is directly proportional to the concentration of enzyme and independent of the concentration of substrate.

The possibility of the Schutz-Borrissov law holding for hyaluronidase action on hyaluronic acid is shown by the wide applicability of the law and by the fact that hyaluronidase action is directly proportional to the concentration of the enzyme and independent of the concentration of the substrate.

SUMMARY

The various tests for hyaluronic acid have been described and the viscosity reduction test and mucoprotein clot prevention test have been described in detail and explained. The possibility has been suggested that the Schutz-Borrissov law may hold for the action of hyaluronidase.

V. PNEUMOCOCCUS HYALURONIDASE

INTRODUCTION AND HISTORY

McClean and Hale (107) have reported that filtrates from cultures of a virulent type I pneumococcus grown on a complex medium exhibited hyaluronidase activity; Meyer, Dubos, and Smyth had earlier reported it in autolysates of a rough type II pneumococcus (120). Meyer, et al (132) also demonstrated the enzyme in preparations from a smooth strain of type II, from a type I strain in both the smooth and rough phases, from two strains of type VI, and one strain of type III. The cultures were grown on complex media.

In the present work the same enzyme is demonstrated to be in filtrates of a type III pneumococcus culture grown on a semi-synthetic medium free of protein, and the rate of its appearance with time of incubation is measured. The presence of the potassium salt of hyaluronic acid in the medium is shown to stimulate the production of hyaluronidase, and an increase in the rate of appearance of hyaluronidase in such a medium is found to begin at about the time that bacteriolysis, as evidenced by decrease in culture turbidity, becomes evident.

Duran-Reynals, in his review (39) restates Aylward's conclusion (3) that purified testicle hyaluronidase is not affected by X-rays. Apparently no other work on the effect of radiation upon hyaluronidase has been done. A determination of the susceptibility of pneumococcus

hyaluronidase to irradiation by soft X-rays is included here.

EXPERIMENTAL

Culture Methods*

A Christ Hospital strain of type II pneumococcus, fermenting inulin and soluble in bile, was maintained virulent by passage through mice twice weekly. The mouse heart blood was cultured into a veal phosphate broth made according to Park and Williams' (145) directions for making "Beef Heart Phosphate Broth for Pneumococcus," with veal substituted for beef heart, and Difco Proteose-Peptone used in place of Park Davis peptone. This stock culture was subcultured into 10 cc. of veal phosphate broth containing 0.2% glucose. After incubation for five hours at 34°, 5 cc. of the subculture was centrifuged and washed wish vitamin-free casein hydrolysate (S.M.A. Company, or hydrochloric acid hydrolysed material made in this laboratory) basal medium recentrifuged and resuspended in 5 cc. of the basal medium. One-tenth cc. of this washed suspension was used to inoculate 10 cc. of casein hydrolysate medium supplemented with growth factors (for individual growth factors see page 100) under "Supplement") and containing either glucose, postassium salt of hyaluronic acid, or both, as carbohydrate source.

The potassium hyaluronate was prepared according to Meyer, et al (124) (see Appendix I, preparation number 110) omitting the precipitation of protein with zinc acetate. Residual protein was hydrolysed by digestion with trypsin as suggested by Robertson, Ropes, and Bauer (150) as follows: after as much protein as possible had been removed from a neutral solution of the easily dissociable protein salt of hyaluronic

* Bacteriological work done by Elizabeth Badger.

acid by shaking with chloroform-ethyl alcohol mixture (2), 1 gram of trypsin (Coleman and Bell 1:100) was added per liter of saturated solution of mucoprotein, the pH adjusted to 9.0 with 0.5% K_2CO_3, and the mixture incubated at 40° for two days. Undigested protein was removed from the digest with chloroform-ethyl alcohol mixture. The resulting solution gave no precipitate with four volumes of 10% trichloracetic acid and no protein-gel interface film on further shaking with chloroform-ethyl alcohol mixture. The carbohydrate used in these experiments gave a positive biuret test.

Enzyme Preparation and Assay

After incubation at 34° for varying periods of time up to 132 hours, the culture was centrifuged and the supernatant fluid filtered through a Mandler (9-pound) filter. Since unfiltered cultures were never tested, it is not known whether any loss occurs on filtration. The enzyme potency was estimated by the viscosity reducing test (see "Tests for Hyaluronidase" pages to). The enzyme potency was expressed in V.R.U. (Viscosity Reducing Units) per ml. of original filtrate tested.

Growth Measurement

The relative amounts of bacterial growth were followed by measuring the turbidity of the culture, using the photo-electric turbidity comparator described by Krebs, Perkins, Tytell, and Kersten (76). In all cases the value obtained for turbidity, due to growth, was corrected by subtracting a figure corresponding to the turbidity produced by the inoculum alone. The turbidity due to the inoculum was read from a tube which was inoculated as usual and incubated concurrently with the

experimental tubes, but from which one of the essential growth factors (choline) was omitted.

Medium

The semi-synthetic medium for this strain of pneumococcus, was developed by Elizabeth Badger (5) and was made up as follows:

Basal medium - S.M.A. vitamin free, or Cincinnati vitamin free casein hydrolysate, hydrolysate from S.M.A. vitamin free casein =500 mg. of nitrogen

Cystine (S.M.A.)	25 mg.
Glutamic acid (Amino Acid Mfg. A.P.)	75 mg.
$MgSO_4$ ($7H_2O$)	200 mg.
KH_2PO_4	5 grams
Distilled water to make	1 liter

Supplement added to yield final concentration of:

Biotin (S.M.A. Crystalline or S.M.A. #5000)	0.02 ugm/cc
Pantothenic acid (Ca-d-)	1.0 ugm/cc
Choline (S.M.A.)	5.0 ugm/cc
Ascorbic acid (Lederle)	300.0 ugm/cc
Glutamine (S.M.A.)	150.0 ugm/cc

The pH was adjusted to 7.6 (indicators) with sodium hydroxide. Glucose was separately sterilized by placing the solution in a boiling water bath for 1/2 hour. After the medium was autoclaved, this heated solution of glucose was added when desired. Hyaluronic acid was autoclaved in the medium. This usually resulted in the formation of a

flocculent precipitate containing part of the hyaluronic acid.

Results

The values of the amount of growth and hyaluronidase production after various incubation times are stated in Table XVI, page 102 and graphed in figure 17, page 103.

The growth which took place when potassium hyaluronate was the sole carbohydrate source was only half as great as the growth which took place on glucose. This may be due to removal of part of the potassium hyaluronate by autoclaving (see "Suggestion for Future Work.") page

When potassium hyaluronate was the sole source of carbohydrate, production of hyaluronidase increased steadily throughout the period of incubation and finally rose to a value of 1730 V.R.U. per ml. This contrasts with a corresponding value of zero for a culture grown on glucose. Although a culture grown on glucose was devoid of hyaluronidase, an inoculated glucose tube, in which growth failed for lack of choline, showed 2.5 V.R.U. per ml., indicating that some hyaluronidase was carried over from the veal broth seed culture. Hyaluronidase is also produced on infusion broth-blood medium; a culture filtrate from this medium contained 50 V.R.U./ml., even though no carbohydrate was added.

A mixed carbohydrate source, half glucose and half potassium hyaluronate, produced only 30 V.R.U. per ml. in 108 hours and had a pH of 6.0 as contrasted with pH =6.7 for cultures grown on potassium hyaluronate alone and pH = 4.8 for cultures grown on glucose alone.

A lag period of over 12 hours is evident before growth on

TABLE XVI

GROWTH AND HYALURONIDASE PRODUCTION OF PNEUMOCOCCUS

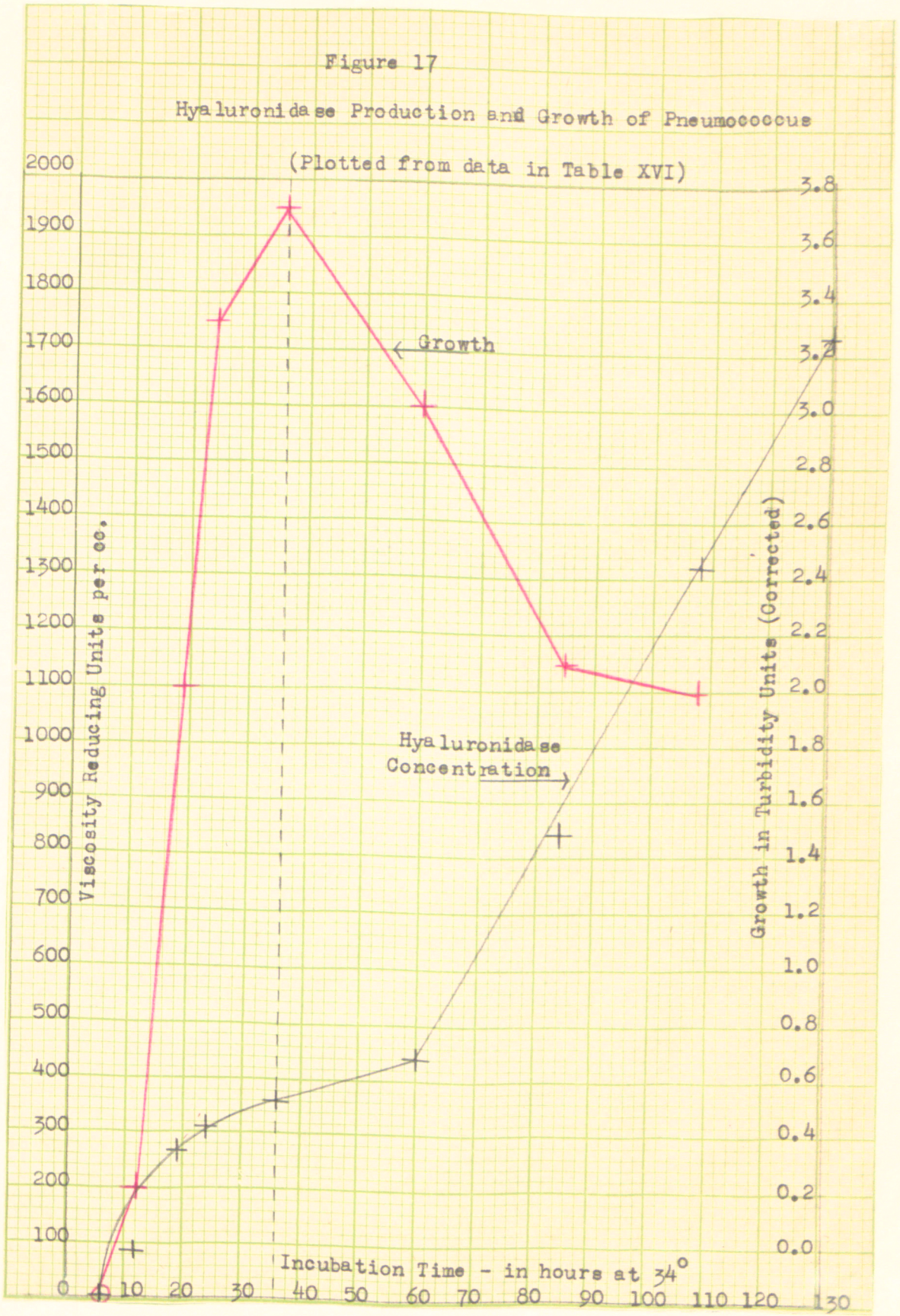
(part of this data is plotted in figure 17, page 103)

	Potassium Hyaluronate (5 mg/cc medium)			Potassium Hyaluronate 2.5 mg/cc medium; glucose 2.5 mg/cc medium			Glucose, 5 mg/cc medium		
Incubation Time	Growth in turbidity units	Hyaluronidase in V.R.U./cc	pH	Growth in turbidity units	Hyaluronidase in V.R.U./cc	pH	Growth in turbidity units	Hyaluronidase in V.R.U./cc	pH
6 1/2 hrs.	-2	6.5	8.0	29	0	-	12	-	-
12	2	85	8.0	39	6.4	6.1	62	0	5.8
19	20	267	7.0	43	20	6.0	71	-	-
24	33	318	7.0	25	21	6.1	74	trace	-
36	37	367	7.0	15	18	6.1	72	0	4.8
60	30	436	7.0	18	13	6.1	-	-	-
84	21	840	7.0	21	11	6.1	-	-	-
108	20	1320	6.8	23	30	6.0	-	-	-
132	33(?)	1730	6.7	-	-	-	69	0	4.8

Figure 17

Hyaluronidase Production and Growth of Pneumococcus

(Plotted from data in Table XVI)



potassium hyaluronate is well established. The corresponding lag period with glucose is less than 6 hours.

After 30-50 hours of incubation, the hyaluronidase concentration in culture subsisting on potassium hyaluronate begins to rise at a rapid rate. This rise in hyaluronidase is coincident with a clearing of the culture.

The Effect of Soft-X-rays on Pneumococcus Hyaluronidase

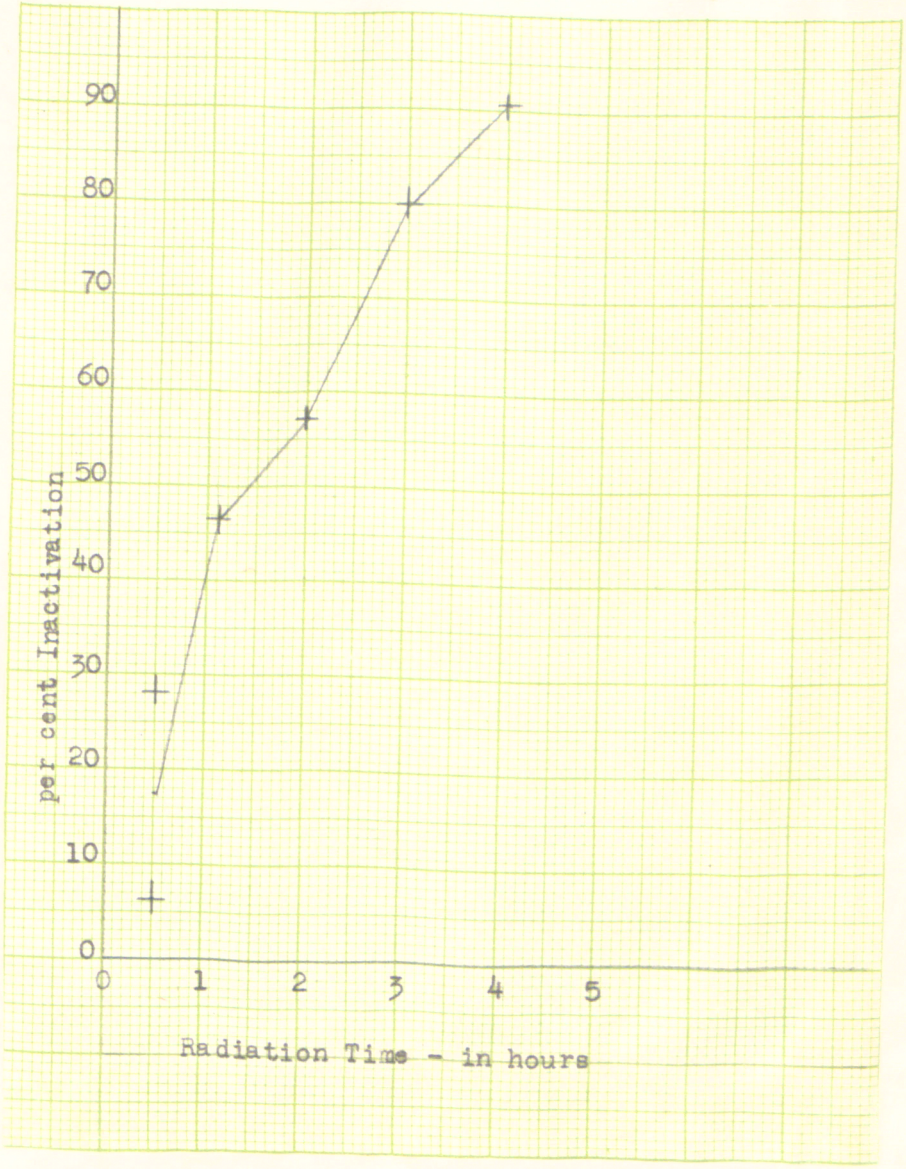
Fifteen cc. of pooled pneumococcus culture filtrate from cultures grown on potassium hyaluronate were diluted to 70 cc. with distilled water. Samples of this dilution, containing 33.5 V.R.U. per ml., were submitted to the action of soft x-rays of greatest intensity at the K alpha line of copper, or 1.54 Angstroms wave length and acting at a distance of 3 cm. from the focal spot of the copper target. Before and after irradiation, enzyme potency was estimated by the viscometric test (see pages 59 to 68). Unirradiated cultures kept at the same temperature as the irradiated samples showed no loss of potency. In the accompahying graph, figure 17-A, page 105, the per cent destruction of enzyme activity is plotted as ordinate against irradiation time as abscissa. A progressive disappearance of activity is evident as irradiation is continued for long periods.

DISCUSSION

The "adaptive" nature of hyaluronidase production by pneumococcus is evident, since no detectable enzyme is produced in the absence of the specific substrate. Complex media are not necessary to the production

Figure 17a

Effect of Soft X-Rays on Pneumococcus Hyaluronidase



of hyaluronidase, but the presence of some hyaluronic acid in such media may be inferred from the fact that a small amount of hyaluronidase is produced upon them.

A drop in culture turbidity shows that lysis, clumping, or other clearing phenomenon begins to take place at approximately the same time that hyaluronidase appearance assumes a rapid, regular rate. This favors the view of Meyer, et. al. (129) that hyaluronidase is an endogenous enzyme in pneumococcus, and is released by lysis of the cell body.

The inactivation of the hyaluronidase by soft X-rays is an indication of the relative purity of the enzyme, since foreign protein protects enzymes against such inactivation. Thishas been found to be the case for urease (160) and for Cl. welchii hyaluronidase (see pages 153 to 153). Aylward's (3) finding that testicular hyaluronidase was not inactivated may be explained either on the basis of an inherent resistance to X-ray or on the basis of the impurity of the "purified" sample used. This latter contention is supported by Aylward's finding that considerable protein could be removed from the purified testicular hyaluronidase without removing any of the activity.

SUGGESTION FOR FUTURE WORK

While working with Cl. welchii it was found that autoclaving of the medium with consequent precipitation of part of the potassium hyaluronate substrate, could be avoided. With this bacillus, simple steaming or heating of the medium to 100. C. is sufficient to ensure

growth of an unmixed culture. The cultures so grown produce more hyaluronidase than cultures grown on autoclaved medium, and growth is also superior. The reason for the superior results presumably lies in the complete availability of the substrate to the bacteria. The pneumococcus should also be grown in steamed media; it seems likely that a much improved yield of hyaluronidase would be attained, and that the growth on potassium hyaluronate might be comparable to that on glucose.

SUMMARY

1. Pneumococcus type III, C.H.A. strain is able to produce hyaluronidase on a semi-snythetic casein hydrolysate medium.

2. The hyaluronidase is produced as a strictly "adaptive" enzyme, none appearing unless the specific substrate, hyaluronic acid, is present.

3. The presence of glucose in a medium containing potassium hyaluronate reduces the production of hyaluronidase to less than one-fiftieth of the production on potassium hyaluronate alone.

4. Hyaluronidase introduced with the inoculum disappears in cultures growing on glucose.

5. The most rapid, regular appearance of hyaluronidase coincides with clearing of the culture.

6. Soft X-rays are able to inactivate pneumococcus hyaluronidase in diluted culture filtrates.

VI. THE PRODUCTION OF HIGH TITER PERFRINGENS HYALURONIDASE

INTRODUCTION

In the course of studies on the production of active immunity to infections by Clostridium perfringens, Type A, it was thought desirable to ascertain separately the protective value of each of two antigenic substances, Alpha-toxin (lecithinase) and a spreading factor (hyaluronidase), normally found in filtrates from cultures of many strains of Cl. perfringens, Type A. It was therefore necessary to prepare these antigens separately and in good titer. The Alpha-toxin may be obtained free from hyaluronidase, without resort to chemical separations which possibly may damage the antigen, by the use of a strain of Cl. perfringens, Type A., which produces Alpha-toxin but does not produce hyaluronidase, for instance strain "Corcoran" (45). The existence of a strain which regularly produces hyaluronidase but does not produce Alpha-toxin has not yet been reported. This chapter will describe methods whereby strains of Cl. perfringens, Type A which normally produce both Alpha-toxin and hyaluronidase may be cultured on protein-free media so as to produce high titer hyaluronidase in filtrates with little or no Alpha-toxin activity or Alpha-antitoxin combining power. The effect of certain factors on hyaluronidase production alone, without regard to Alpha-antigen, will also be discussed.

Characteristics of the Antigens

The Alpha-toxin is hemolytic and lethal (56); it produces an opacity with inactivated human serum and with egg yolk suspension (92, 142); and it is an enzyme which acts on lecithin preparations to release acid soluble phosphorus thought to be phosphorylcholine (91).

Hyaluronidase is an enzyme which hydrolyzes the complex polysaccharide hyaluronic acid, destroying its viscosity and preventing its precipitation by acid in the presence of protein. It has been characterized in pages to and pages

HISTORY

The occurrence of a spreading factor in Cl. perfringens filtrates was noticed by McClean (105) in 1936, and the presence of a mucinase was reported by Robertson, Ropes and Bauer (150). The production of hyaluronidase by Cl. welchii (Cl. perfringens, Type A)has been studied by Meyer (129) and by McClean and Hale (108). The latter authors have demonstrated its adaptive nature, showing that the inclusion of potassium hyaluronate as a source of carbohydrate in Evans peptone medium increases manyfold the concentration of hyaluronidase yielded by the culture. McClean and Hale make no mention of the Alpha-toxin content or Alpha-antitoxin combining power of these filtrates.

EXPERIMENTAL

It has been found possible to include a preparation of potassium hyaluronate in several simplified, reproducible media, and to adjust the culture conditions so that hyaluronidase yields of 1000-1500 V.R.U.

per ml. could be obtained, with Alpha-toxin yields of only 0-10 LD_{50} mouse I.V. per ml.; Alpha-antitoxin combining power of 0-0.5 per ml., measured against National Institute of Health standard antitoxin.

Materials and Methods

POSTASSIUM HYALURONATE A purified white powder, consisting of a mixture of complex polysaccharides containing 55% of hyaluronic salts was prepared from human umbilical cords as described in chapter II "Preparation of Potassium Hyaluronate", pages 24 through 27.

PANCREATIC DIGESTS OF VEAL OR BEEF HEART* Finely ground veal or beef heart was washed thoroughly in running water for several hours. The washed residue was covered with distilled water and boiled for 15-20 minutes, allowed to cool, filtered through paper, and the residue on the filter paper pressed dry in a clean cloth. The pressed residue was suspended in 10 volumes of distilled water and grams of ground fresh pancreas was added per kilogram of residue. The pancreas and beef residue were well mixed with each other and with a quantity of toluene adequate to prevent bacterial decomposition of the digestion mixture. Economy with regard to toluene may result in spoiled digests which are not generally suitable for culture media. The digestion mixture was adjusted to pH = 9 with 20% Na_2CO_3 solution, and placed in a 45° incubator. The digestion was allowed to proceed for 24 hours, with adjustment of pH back to 9 after 12-15 hours. The acidity was then adjusted to pH = 5 and the digest solution filtered through paper.

*These digests were made by Dr. M. A. Logan

The filtrate was adjusted to pH = 7.6 with 20% Na_2CO_3 and stored at 4° C. under a layer of toluene. Total solids in each digest were determined by drying at 110° C. for 20 hours and weighing the solid residue.

PAPAIN DIGEST OF BEEF HEART* One kilogram of beef heart grossly freed of fat, is ground, mixed with 4 liters of water, and the suspension adjusted to pH = 7.0 with NaOH. The suspension is maintained at a temperature of 60° C. and 0.5 gm. of papain powder (Merck) is added every 1/2 hour for 2 1/2 hours, the pH being kept at 7.0. The digest is then boiled for 5 minutes to destroy the papain, the pH adjusted to 5.5 with HCl, and filtered.

CASEIN HYDROLYSATE This was vitamin-free acid hydrolyzed casein obtained from Difco (Casamino acids) or S.M.A. Company, in which case it was used in the medium in a concentration of 1.0-1.5% solids, or it was prepared by acid hydrolysis of vitamin-free casein (Difco) according to the method of Mueller (217,218), in which case it was used in the medium in a concentration of 1000 to 1600 mg. of nitrogen per liter of medium. The higher figure for nitrogen content of the medium usually resulted in higher yields of hyaluronidase.

VITAMIN SALT SUPPLEMENT This supplement was made up double strength and added to veal or beef heart digests or to casein hydrolysate, in such quantity that the final concentration of salts and vitamins per liter of

*These digests were made by Dr. M. A. Logan

completed medium was as follows:

Na_2HPO_4 $12H_2O$	5.76 grams
KH_2PO_4	0.48 "
$MgSO_4$ 7 H_2O	0.04
Ca-d-pantothenate	0.001
nicotinic acid	0.001
thiamine	0.001
pyridoxine	0.001
pimelic acid	0.001
riboflavin	0.0001

MEDIUM The complete medium was prepared as follows:

Veal or beef heart digest, or casein hydrolysate, or Bacto-Tryptose, salt vitamin supplement, and distilled water were added in such proportions as to give a final concentration of 4 to 4.5% total digest solids or 1.0-1.5% casein hydrolysate. The mixture was heated to effect complete solution and the pH was adjusted to 7.8. As the sole source of carbohydrate, 0.5% (final concentration) of potassium hyaluronate preparation was dissolved in the complete medium by shaking at 34° C. The media were then sterilized by filtration through 8 lb. Mandler candles, or by steaming for 20 minutes at 1 pound pressure, or by heating in boiling water for a half hour. Media were then aseptically distributed to the appropriate tubes.

Uninoculated controls were incubated with each group of tubes incubated to assure control in sterility.

CULTURES Six strains of Cl. perfringens, type A (B. welchii) were studied: BP6K, BP364 (British) BP365 (British), WX (from Dr. Ried through

the U.S. Nat'l. Inst. of Health), 1508Ad (from Dr. Ivan C. Hall), and BG6 (from Eli Lilly Research Laboratories). These strains were kept as stock cultures on a routine casein hydrolysate-salt-vitamin medium supplemented with shreds of alcohol-ether extracted beef heart, and containing 0.6% dextrin.

INOCULUM Before use for the preparation of a seed culture, the stock culture was either passed through 300-400 gram pigeons, or subcultured twice on two succeeding days. The seed culture was made as follows: 1 cc. of the pigeon passed or subcultured stock was inoculated into casein hydrolysate-beef heart broth containing 0.6% dextrin and the seed culture allowed to grow for 6 hours at 38° C. The seed culture was centrifuged, washed twice with potassium hyaluronate medium, and then taken up in potassium hyaluronate medium equal to twice the original volume of seed culture. One-half ml. of this suspension was inoculated into the bottom of 40 cc. of potassium hyaluronate medium contained in a 200 X 25 mm. tube.

INCUBATION The cultures were incubated at 37° C. When a volume of medium equal to or less than 40 cc. was used, somewhat better yields were obtained by incubating under reduced air pressure.

CLEARING AND FILTRATION The cultures were cleared by centrifuging through 8 lb. Mandler candles or Seitz sterilizing pads. The loss of hyaluronidase titer on filtration is negligible.

SPECIFIC PERFRINGENS ANTISERA

1. Horse serum from hyperimmunized perfringens horses. Preserved with phenyl mercuric acetate (1:10,000) and 50% glycerol.

2. Refined perfringen antitoxin. Preserved with glycerol.

3. National Institute of Health Standard Perfringen Antitoxin.

STANDARDIZED PERFRINGENS ALPHA-TOXIN A perfringens Berkfeld filtrate preserved and stabilized by dialysis against C.P. glycerol for 18 hours at 4-6° C. The LD_{50} mouse IV titer was 660 per ml. The antitoxin combining power (L_B) was 7.7 units LD_{50} against National Institute of Health Standard Antitoxin.

HYALURONIDASE ASSAY Hyaluronidase concentration in the filtrates was measured by the viscosimetric and mucoprotein clot prevention tests (see chapter IV, pages to and pages

ASSAY FOR ALPHA-TOXIN AND ALPHA-ANTITOXIN COMBINING POWER*

The Alpha-toxin (lethal toxin) content of the culture filtrates was assayed by the following methods:

1. Intravenous and Intraperitoneal injections into17-22 gram mice.

2. By a turbidimetric modification of Van Heyningen's (162) lecitho-vitellin method.

3. By the assay of acid soluble phosphorus hydrolyzed from purified lecithin. (Unpublished method by Boyd and Logan).

Alpha-antitoxin combining power was determined by several methods as follows:

1. Modified method by Seal and Stewart (153) using egg-yolk-borate instead of inactivated human serum.

2. Lecithin acid soluble phosphorus method (unpublished method by Boyd and Logan).

3. Mouse assays (intravenous and interperitoneal injections).

*These assays were performed by Dr. A. A. Tytell

DEFFERRATION Iron was removed from beef heart and veal digests, or from casein hydrolysates, by precipitating $Ca_3(PO_4)_2$ in the solution. Two mM of $CaCl_2$ was added for each liter of the digest, the pH adjusted to 7.6-7.8, and the digest heated to 80° C. An equivalent amount of Na_2HPO_4 was then added and heating continued until a flocculent precipitate of $Ca_3(PO_4)_2$ formed. This precipitate adsorbed the iron, and was removed by filtration. The deferrated digests usually gave a barely detectable test for iron when 0.1 cc. of digest is used as sample in the $\alpha\,\alpha'$ dipyridyl test (49). If necessary, the deferration is repeated until the test shows only this minimal amount of iron. The limit of identification of iron with this test is 0.03 micrograms.

RESULTS

Incubation Time

The laboratory strain of Cl. perfringens BP6K was inoculated into the bottom of 250 cc. of medium contained in a large test tube. The nitrogenous components of the medium were furnished by including 4.5% of a tryptic digest of beef heart and the carbohydrate was supplied by 0.6% of potassium hyaluronate preparation. The tube was incubated at 37° C. From time to time 5 cc. samples were aseptically withdrawn from the top of the culture and assayed for hyaluronidase. These results are given in Table XVII , and graphed in figure 18, page 117.

TABLE XVII

Hyaluronidase Production on Tryptic Digest of Beef Heart

(This data is graphed in figure 18 page

Time of Incubation at 37° C. in hours	Hyaluronidase Concentration in V.R.U./ml. medium
20	686
48	1076
68	1315
90	1371
120	1389

Cl. welchii, strain BP6K, was inoculated into the bottom of 10 cc. of medium contained in ordinary test tubes. The nitrogen in the medium was supplied by Labco vitamin-free casein hydrolysate added so as to furnish 1625 mgm. of nitrogen per liter of medium. Potassium hyaluronage, 0.5%, was dissolved in the medium, and 0.1% glucose or dextrin were added to certain tubes. Duplicate tubes were inoculated, one of each pair being incubated for 18 hours, the other for 45 hours, at 37° C. The hyaluronidase concentrations attained are given in Table XVIII.

Figure 18

Hyaluronidase Production on 4.5% Tryptic Digest of Beef Heart Medium plus Potassium Hyaluronate

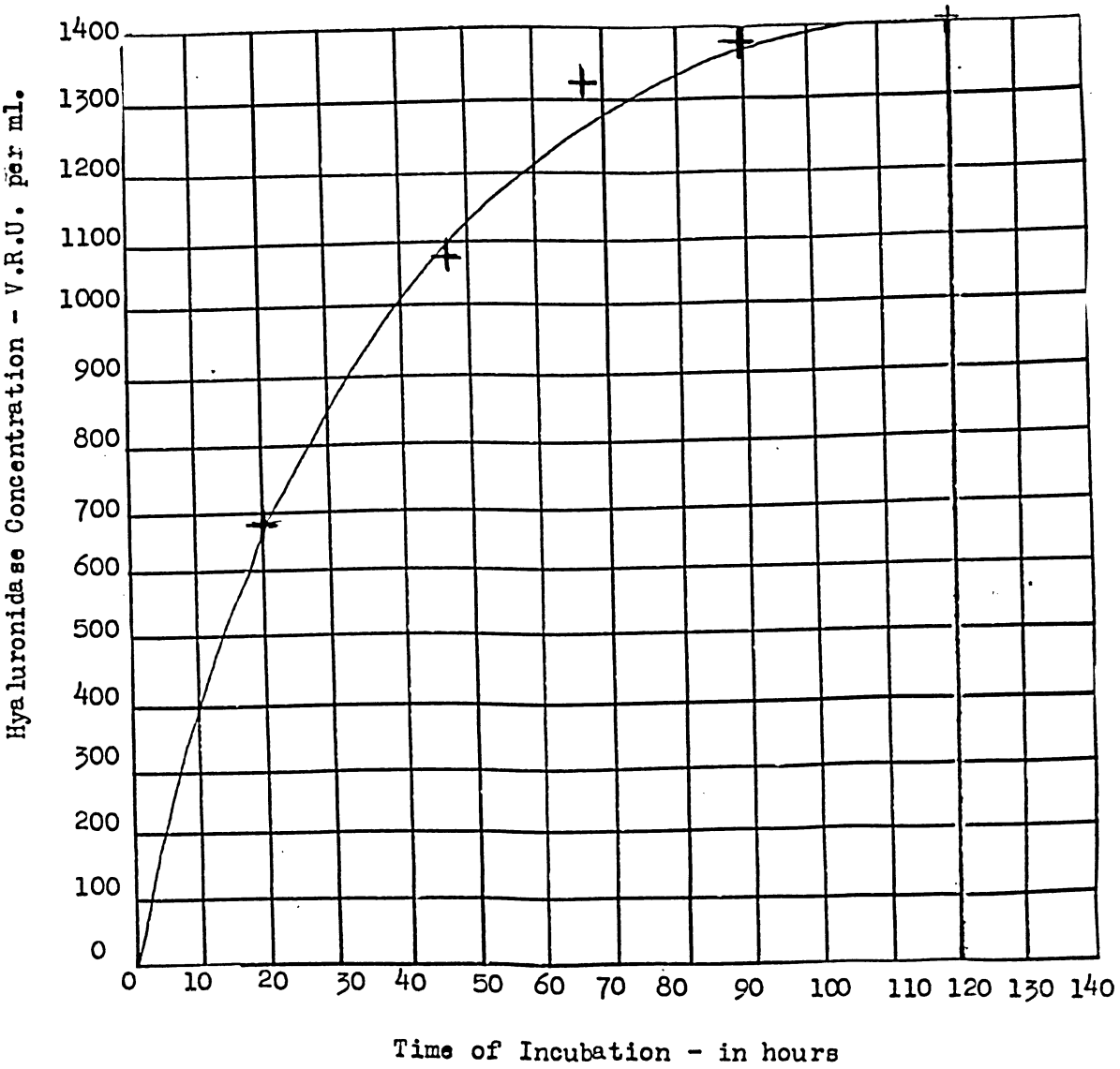


TABLE XVIII

Hyaluronidase Production on Casein Hydrolysate

	Carbohydrate added in percent	Incubation Time in hours	Hyaluronidase in V.R.U./ml medium
K hyaluronate	0,5	18	160
		45	388
K hyaluronate + glucose	0.5	18	188
	0.1	45	160
K hyaluronate + dextrin	0.5	18	223
	0.1	45	360

An experiment similar to those described above was run using samples of various tryptic digests of beef heart. Ten ml. of medium were used in each tube. $FeSO_4 \cdot 7\ H_2O$ was added to a concentration of 240 mgm. per 100 ml. of medium. The results are in Table XIX, page 118.

TABLE XIX

Hyaluronidase Production on Digest of Beef Heart with Added Iron

Digest Number	Incubation Time in hours	Hyaluronidase Concentration in V.R.U./ml. medium
1	14.5	280
	60	416
2	14.5	257
	60	366
3	14.5	300
	60	465
4	14.5	250
	60	316

Our laboratory strain of Cl. welchii PB6K was inoculated into the bottom of 4.5% veal digest medium containing 0.6% of potassium hyaluronate preparation, as before. The media was tubed in 40 cc. quantities. Inoculation was at 37°, under reduced air pressure. At appropriate intervals after the start of incubation a tube was removed and assayed for hyaluronidase. The values obtained are given in Table XX, page 119 and graphed in figure 19, page 120.

TABLE XX

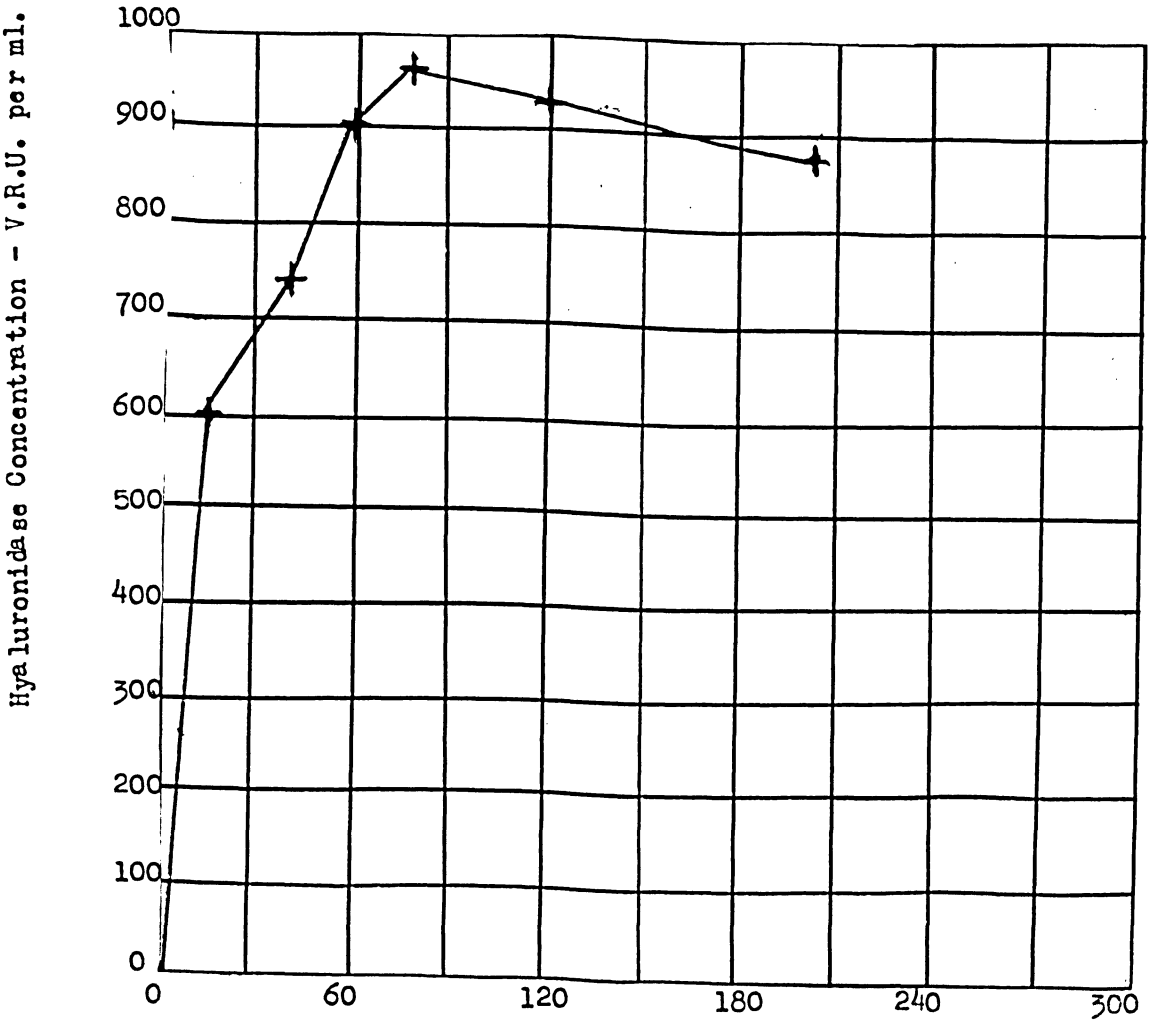
Hyaluronidase Production on Tryptic Digest of Veal

Time of incubation at 37° in vacuo	Hyaluronidase Concentration
in hours	in V.R.U. per ml. medium
17	600
44	740
60	900
80	972
120	931
200	880
300	727

The foregoing results indicate that hyaluronidase concentration in these cultures reaches a maximum after 60-100 hours of incubation. The enzyme is stable in the presence of the organisms and disappears from the culture only very slowly after the maximum concentration is reached. About two-thirds of the maximum concentration is attained within 20 hours.

Figure 19

Hyaluronidase Production on 4.5 % tryptic Digest of Veal, plus 0.6 % Potassium Hyaluronate



Incubation Temperature

Satisfactory yields of hyaluronidase may be obtained when the culture is incubated at 34 to 40° C. Other temperatures were not investigated. The yield at 42° is somewhat smaller than the yields at 34 or 37° C. No time curve was run at 42° C., but it is presumed from the more rapid growth at this temperature that the hyaluronidase concentration will reach an earlier peak. Comparative data are shown in Table XXI, page 122. The cultures were grown on casein hydrolysate medium containing 1600 mg. of nitrogen per liter and 0.5 % of potassium hyaluronate preparation. They were incubated for 30 hours at the temperature indicated.

Nitrogen Source

As shown in Table XXII, page 123, the best yields of hyaluronidase produced by our laboratory strains of Cl. perfringens BP6K were obtained on 4.5% beef heart and 4.5% veal tryptic digest media. Lower percentages of digest in the medium resulted in lower yields. Higher percentages of digests have not been tried. The usual per cent of casein hydrolysate used in the medium in from 1.5 - 2% as "casamino acids" (Difco). If more than 2% of casamino acids are used, growth is poor. The hyaluronidase yield is cut down in 3% casamino acids and is reduced to negligible traces in 4% casamino acids.

The poorer performance of cultures growing on casamino acids compared with that of cultures growing on meat digests is consistent and striking. A number of supplements were added to casein hydrolysate media in an attempt to supply a factor which would increase the yield

THE PRODUCTION OF HIGH TITER PERFRINGENS HYALURONIDASE

TABLE XXI

Hyaluronidase Concentration and Incubation Temperature

Incubation Temperature	$FeSO_4 \cdot 7H_2O$ added to medium	Hyaluronidase Concentration
in ° C.	in mgm/100 ml.	in V.R.U./ml. medium
34 ± 0.5	0.3	80
37 ± 0.5	0.3	82
42.5 ± 2	0.3	48
34 ± 0.5	0.6	112
37 ± 0.5	0.6	118
42.5 ± 2	0.6	84
34 ± 0.5	0.9	89
37 ± 0.5	0.9	109
42.5 ± 2	0.9	96

THE PRODUCTION OF HIGH TITER PERFRINGENS HYALURONIDASE

TABLE XXII

Hyaluronidase Yields in Different Media Containing Potassium Hyaluronate

Medium	Hyaluronidase Concentration in V.R.U. . ml. medium
Casein Hydrolysate,	200
1600 mgm. N, per liter	380
4% Bacto Tryptase	620
	680
3.8% papain digest	685
of beef heart	742
4.5% tryptic digest	1150
of beef heart	1389
4.5% tryptic digest	1380
of veal	1400

to the same level as that produced on meat digests, without success. The supplements tried were: iron (as $FeSO_4 \cdot 7H_2O$), reduced metallic iron, hemin, various alkaline extracts of hog brain, various fractions of tryptic digest of beef heart separated according to solubility in methyl alcohol-water mixtures and electrophoretically, chloroform extract of beef heart, and yeast. The supplements which most greatly increase the yield of hyaluronidase over the yield on unsupplemented medium were hemin and a filtrate from alum precipitation of alkaline extract of brain. Unheated tryptic digest of beef heart was not tried.

Effect of Iron

When $FeSO_4 \cdot 7 H_2O$ is added to these media in a concentration of about 3 mg. per 100 ml. of media, or greater, a cloudy white precipitate forms at the point of entry of the concentrated iron solution into the medium. It seems likely that this precipitate is ferrous phosphate, $Fe_3(PO_4)_2 \cdot 8 H_2O$, which is insoluble in water. As the culture grows, at least part of the iron is reduced to the metallic state and remains suspended in the medium either as black colloidal iron, or sulfide, or falls to the bottom as a fine black silt. Despite these changes, deferrated cultures grown on tryptic digest of meat media show no significant variation of hyaluronidase yield in the presence or absence of iron. Deferrated casein hydrolsate media, however, supports the growth of Cl. welchii BP6K on potassium hyaluronate only very poorly in the absence of iron and hyaluronidase is absent, or very nearly so in these cultures. This affect is shown in Table XXIII. The iron is not wholly replaceable by hemin.

THE PRODUCTION OF HIGH TITER PERFRINGENS HYALURONIDASE

TABLE XXIII

Effect of Iron on Hyaluronidase Production in Deferrated Casein Hydrolysate Media

Experiment No.	$FeSO_4 \cdot 7H_2O$ or other iron source added	Hyaluronidase Concentration
	in mgm. per 100 ml.	in V.R.U./ml.medium
I	0	10
8 cc of media in 13 X 200 mm. tubes 1600 mgm N./l.	0.3	42
	0.45	69
	0.60	63
	0.90	58
	1.8	53
II	0	0
1600 mgm N./l.	not deferrated	280
III	0	0
1.6% casamino acids	24.0	171
	solid reduced Fe hemin, 58 mgm	50
		100
IV		
1.6% casamino acids plus 0.02% dextrin in addition to the usual 0.5% potassium hyaluronate	0	20
	24.0	114
	solid reduced Fe hemin, 58 mg	87
		50

The ferrous sulfate crystals were dissolved in 0.1 N HCl in such concentration that the requisite quantity of the salt to be added to 40 cc. of medium was contained in a volume of 0.05 - 0.15 ml. This volume of 0.1 N acid does not significantly alter the pH of 40 ml. of medium. The iron solution was sterilized separately from the medium by autoclaving for 20 minutes at 20 pounds of steam pressure.

In one series of deferrated casein hydrolysate culture tubes without added iron, no growth had taken place after 38 hours of incubation. At this time $FeSO_4.7\ H_2O$ was added in a final concentration of 1.2 mgm/100 ml. of medium. After twelve hours, growth was fairly under way, and growth equal to that attained in culture tubes was reached in 18 hours.

Concentration of Potassium Hyaluronate

The limit of solubility of the potassium hyaluronate in the media used is about 0.6%. The cultures were usually grown on a concentration of 0.5% or 0.6%. The higher percentage is felt to result in higher yields, but no strict comparison has been made. The yield of hyaluronidase increases with increasing concentrations of potassium hyaluronate, up to the limit of solubility. This effect is illustrated in Table XXIV, page 127. The yields were obtained after 48 hours incubation on deferrated casein hydrolysate supplemented by various amounts of ferrous sulfate. The effect of carbohydrate in excess of the limit of solubility has not been tried.

TABLE XXIV

Effect of Potassium Hyaluronate on Hyaluronidase Production

(deferrated casein hydrolysate medium)

Potassium Hyaluronate Concentration	$FeSO_4 \cdot 7 H_2O$ added	Hyaluronidase Concentration
in per cent	in mgm per 100 ml.	in V.R.U. per ml. medium
0.1	0.6	25
0.25	0.6	61
0.50	0.6	150
0.1	1.2	44
0.25	1.2	68
0.50	1.2	134

Effect of Other Carbohydrates

The highest yields of hyaluronidase are obtained when hyaluronic acid is the sole source of carbohydrate in the medium. The presence of other carbohydrates along with potassium hyaluronate, especially of glucose, sharply reduces hyaluronidase production. Production is lowest in media from which hyaluronic acid has been omitted and some other carbohydrate has been included. Among these other carbohydrates, strain BP6K produces more hyaluronidase when utilizing polysaccharides than when it does utilizing simple sugars such as glucose. Some of the experimental data which led to these conclusions are included in Table XXV, page 128. Table XXVI, page 129, contains data on comparative production of hyaluronidase on

TABLE XXV

Effect of Different Carbohydrates on Hyaluronidase Production

Experiment No.	Medium	Carbohydrate Source in per cent	Hyaluronidase Concentration in V.R.U. per ml medium
1	Casein hydrolysate	0.5% K hyaluronate	388
		0.5% K hyaluronate +0.1% dextrin	360
		0.5% K hyaluronate +0.1% glucose	160
		0.5% glucose	0
2	Casein hydrolysate	0.5% K hyaluronate +0.06% glycogen	282
		0.5% K hyaluronate +0.25% glycogen	205
		0.5% K hyaluronate +0.62% dextrin	175
		0.5% K hyaluronate +0.25% dextrin	164
		0.5% K hyaluronate +0.52% glucose	136
		0.5% K hyaluronate +0.25% glucose	55
		0.25% glucose	1.4
3	Casein hydrolysate	0.6% dextrin	66
		0.6% glucose	7
		0.6% maltose	25
4	4% Tryptic digest of beef heart	0.6% dextrin	125
		0.6% maltose	45
		0.6% glucose	56
5	2% Tryptic digest of beef heart	0.9% dextrin + 0.006% K hyaluronate	88
		0.09% dextrin + 0.004% K hyaluronate	87
		0.9% dextrin + 0.001% K hyaluronate	74
		0.9% dextrin	67
6	Brewers thioglycollate medium	0.2% K hyaluronate	185
		0.2% glucose	3.4
		0.6% dextrin	100-300 (usual variation)
7	4.0% Tryptic digest of beef heart	0.6% K hyaluronate	800-1400

THE PRODUCTION OF HIGH TITER PERFRINGENS HYALURONIDASE

TABLE XXVI

Comparative Production of Hyaluronidase by Strains BP6K and BG6

Strain	Medium	Carbohydrate added in percent	Incubation Time at 34° in hours	Hyaluronidase Concentration in V.R.U. per ml. medium
BG6	shredded beef heart + casein hydrolysate	0.6% glucose	7.5	8
BP6K	"	"	"	7
BG6	"	"	16.5	11
BP6K	"	"	"	15
BG6	"	0.6% maltose	7.5	40
BP6K	"	"	"	25
BG6	"	"	16.5	48
BP6K	"	"	"	30

THE PRODUCTION OF HIGH TITER PERFRINGENS HYALURONIDASE

TABLE XXVI (Continued)

Comparative Production of Hyaluronidase by Strains BP6K and BG6

Strain	Medium	Carbohydrate added in percent	Incubation Time at 34° in hours	Hyaluronidase Concentration in V.R.U. per ml. medium
BG6	shredded beef heart + casein hydrolysate	0.6% corn dextrin	7.5	53
BP6K	"	"	7.5	66
BG6	tryptic digest of	0.6% glucose	16.5	45
BP6K	beef heart	"	"	56
BG6	"	0.6% maltose	"	69
BP6K	"	"	"	45
BG6	"	0.6% corn dextrin	"	83
BP6K	"	"	"	100
BG6	"	0.6% potatoe dextrin	"	113
BP6K	"	"	"	125

glucose, maltose, and dextrin.

Various Strains of Cl. Perfringens, Type A

Most of the work has been done with a laboratory variant of strain BP6K. The strain was selectively cultured for antigen production, those cultures which produced the highest concentration of alpha antigen in the medium being chosen as seed. In the course of three years, the productivityof the strain increased manyfold. There is a parallel between production of alpha antigen and production of hyaluronidase, and the hyaluronidase production of this strain has increased concurrently with the production of alpha antigen.

The other strains examined were BP364 (British), BP365 (British) WX (from Dr. Ried),1508 Ad (from Dr. Ivan C. Hall) and BG6 (from the Lilly Research laboratories). Strains WX and 1508 were not found to produce hyaluronidase on any of the media tried. Strains BP364 and BP365 did produce hyaluronidase, although in lower concentration than the laboratory strain of BP6K. Strain BG6 was found to produce approximately the same concentration of hyaluronidase as BP6K, on the media tried. The BG6 strain was not cultured on the media permitting the highest levels of hyaluronidase production. The comparison between BP6K and BG6, therefore, is probably made at suboptimal levels of hyaluronidase production.

Strain 1508 Ad was received in an agar shake. A portion of the agar was inoculated into 40 cc. of casein hydrolysate media, containing 1600 mgm of nitrogen per liter of medium. Carbohydrate was furnished by 0.6% of glucose and accessory factors by the addition

of 1 gram of shredded, fat-solvent extracted beef heart. The culture grew well at 38° C. This culture was subcultured into both casein hydrolysate and tryptic digest of beef heart media, containing glucose and dextrin, respectively. From these tubes, successive subcultures were made into casein hydrolysate and tryptic digest media containing ground fat-extracted umbilical cord, and hyaluronic acid-protein complex. After several subcultures, 1 1/2 ml. of culture was injected into the breast muscle of a 400 gram pigeon. In 24 hours, the pigeon died, and a portion of the breast muscle was cultured into similar media. Subcultures from this pigeon muscle culture had increased in virulence, since 0.5 cc. was now a fatal dose for a pigeon. Portions of muscle from the second pigeon passage were cultured into casein hydrolysate media containing potassium hyaluronate, and, after a period of storage, into 4.5% Bact-Tryptose medium containing potassium hyaluronate; altogether, 33 subcultures were examined. No hyaluronidase activity (by viscosity reduction test) was found in any culture. Although growth took place in media containing potassium hyaluronate or hyaluronic acid-protein, the viscosity conferred on the medium by the presence of hyaluronate was not destroyed. Since only a small fraction of a Viscosity Reducing Unit of hyaluronidase will destroy the viscosity of hyaluronic acid, it seems likely that strain 1508 Ad did not secrete hyaluronidase into the medium in any significant amount or at any stage of its development.

Strain WX, received from the U.S. National Institute of Public Health, also proved not to produce hyaluronidase. A smaller series of subcultures was examined in this case.

Comparative data for strains BP6K and BG6, obtained at a level

of hyaluronidase production less than the best levels known to be obtainable with BP6K, are included in Table XXVI, page 129. As may be seen from the table, the relative production of the two strains varies with the particular carbohydrate source used.

The hyaluronidase production of BG6 in media containing 0.6% potassium hyaluronate is recorded in Table XXVII, page 132. Incubation at 38° C. was continued for 60 hours.

There is some indication of an iron requirement on tryptic digest media with strain BG6 at this level of production.

TABLE XXVII

Production of Hyaluronidase by Cl. Welchii, Strain BG6

Carbohydrate source: 0.6% potassium hyaluronate

Medium	$FeSO_4 \cdot 7H_2O$ added in mg per 100 ml	Hyaluronidase Concentration in V.R.U.per ml. medium
Casein hydrolysate + 0.1% yeast extract	0	133
"	12.0	198
"	24.0	154
Tryptic digest of Beef Heart	0	270
	12	290
	24	353
	48	222

Table XXVIII, page 133, particularizes the comparative output of strains BP6K, BP365, BP364, BP1508, and BPWX on 4.5% Bacto Tryptose medium containing potassium hyaluronate. Table XXIX, page /33, compares BP6K and BP364 when grown on media not containing potassium hyaluronate.

TABLE XXVIII

Hyaluronidase Production by Various Strains of Cl. Welchii

Carbohydrate Source: 0.6% potassium hyaluronate

Medium	Strain	Hyaluronidase Concentration in V.R.U. per ml. medium
4.5% Bacto Tryptose	BP6K	680; 630; 620
	BP365	450; 390
	BP364	265; 190
	BP1508	0
	BPWX	0

TABLE XXIX

Comparative Hyaluronidase Production by BP6K and BP364

Incubation Time: 16 hours

Strain	Medium	Carbohydrate	Hyaluronidase Concentration in V.R.U. per ml. medium
BP6K	4% tryptic digest of beef heart	0.9% dextrin	193; 206
BP364		"	166; 184
BP364	4.7% tryptic digest of beef heart	0.6% glucose	133
BP6K	(not run concurrently - value taken from Table XXVI, page		56

Alpha Antigen Concentration

Cultures grown on potassium hyaluronate and incubated for 60 hours or longer contain practically no alpha toxin or alpha antitoxin combining power. Some alpha antigen (about 60 M.L.D. of toxin) arises in the first 8-16 hours of incubation, but this is destroyed before the hyaluronidase is harvested at 60-100 hours. Alpha antigens were determined as described in this chapter under "Materials and Methods" pages 113 to 114. The lowest titer of alpha toxin tested for by lecithovetellin turbidity method was 1 M.L.D.; the lowest titer tested by the mouse intravenous injection method was 1 M.L.D. The lowest titer of alpha antitoxin combining power (alpha toxoid) tested for by the lecithinase method (measuring the acid soluble phosphorus split from lecithin by an excess of indicator toxin) was 0.1 unit; the least titer tested by the mouse protection test was 0.05 unit. These results are given in Table XXX, page 135.

DISCUSSION

The invariable response of hyaluronidase-producing strains of Cl. perfringens, type A to being forced to subsist on hyaluronic acid as sole carbohydrate source is a greatly increased production of hyaluronidase. Glucose, glycogen, dextrin, and maltose are perhaps more "available" substrates than is potassium hyaluronate, since the response to hyaluronate largely disappears if these carbohydrates are included in the medium along with the hyaluronate.

Many of the cultural phenomena, such as the effect of iron, of size of the inocula, etc., have been studied principally under conditions of

TABLE XXX

The Antigen Content of Cl. Perfringens Filtrates

Carbohydrate: 0.6% potassium hyaluronate
Incubation: 60+ hours at 37° C.

Medium	Strain	Hyaluronidase Concentration in V.R.U. per ml.	Alpha Toxin Concentration		Alpha Antitoxin Combining Power	
			by lecitho-vitellin test	by Mouse IP best LD_{50}	Lecithinase test	Mouse IV test
Casein hydrolysate 1600 mgm N/1 medium	BP6K	160	<1.0 LD_{50}	<1	-	-
		200	<1.0	<2	<0.25	<0.18
4.5% Tryptic digest of veal	BP6K	1380	trace	<2	trace	<0.18
		1400	trace	<2	trace	<0.18
4.5% Tryptic digest of beef heart	BP6K	1371	<1.0	<1	<0.05	<0.05
		1389	<1.0	<1	<0.05	<0.05
3.8% Papain digest of beef heart	BP6K	685	<1.0	<1	<0.05	<0.05
		742	<1.0	<1	<0.5	<0.05
4.5% Bacto-Tryptose	BP6K	630	-	<1	trace	<0.18
		620	-	<2	0.5	<0.18
	BP365	450	0.1	<1	-	-
		390	0.1	<1	-	-
	BP364	265	trace	<1	-	-
		190	trace	<1	-	-

low hyaluronidase production. It is possible that restudy at higher levels of production might discover other effects.

The production of filtrates of high hyaluronidase concentration which are at the same time devoid of alpha antigen is a step towards the elucidation of the role each of these antigens plays in active immunization to Cl. perfringens type A infections. This production on protein-free media facilitates the purification of bacterial hyaluronidase.

SUGGESTIONS FOR FUTURE WORK

A supplement to casein hydrolysate media which will raise hyaluronidase production on this medium to the values produced on meat digests is still to be found. As a first step, a determination of the approximate amount of the supplementary material necessary should be made by adding graded amounts of meat digest to casein hydrolysate media.

The reason for difference in the iron requirement for optimum hyaluronidase production on casein hydrolysate and on meat digests should be investigated. It may be that the precipitation of ferrous phosphate in the casein hydrolysate favorable alters this medium, by removal of phosphate, or by adsorbtion of part of one or more constituents of the medium, or both.

The hyaluronidase yields reported in this chapter were obtained by using a preparation containing 55% of hyaluronic acid salt. It is easily possible to prepare a material containing over 88% hyaluronic acid salts (see "Preparation of Potassium Hyaluronate", pages
Use of the purer preparation might increase the yield of enzyme.

The high concentration of enzyme produced in a protein-free medium provides an excellent source of bacterial hyaluronidase for purification. Various physical and chemical means have been described for the purification of diffusing factors. Although most of the published work has been done with testicular hyaluronidase, it may well be that the procedures described will serve for the purification and concentration of bacterial enzyme as well. The factor can be precipitated with basic lead acetate (134), with acetone (23), with various heavy metal salts (47), or as a flavianate (129). Non-active material may be precipitated by neutral lead acetate (97) or with acetic acid (49,72). The factor may be salted out by addition of $(NH_4)_2SO_4$ and inactive fractions removed by lesser concentrations of $(NH_4)_2SO_4$ than are required to precipitate the enzyme (94,97). It may be adsorbed on charcoal at neutral pH and eluted at pH = 5 (102), adsorbed on ortho aluminum hydroxide (alumina C gamma) and eluted with dilute sodium carbonate or basic sodium phosphate (94), or adsorbed on kaolin, from which it is said to be selectively eluted by pyridine (94). It may be adsorbed upon calcium phosphate, from which it can be eluted by 1/5 saturated ammonium sulfate, similarly to the alpha toxin (this thesis, page 151). It may be adsorbed on Lloyd's reagent (this thesis, page 151) or on Fullers earth (97). It may be concentrated by evaporation at 25° C. (this thesis, page 150). Many inactive materials or precipitants can be removed by dialysis (105). Hyaluronidase can be electrodialysed (3).

The published properties of hyaluronidase make it seem likely that purification might be successfully effected by following a procedure similar to that of Straub (157) in his preparation of lactic dehydrogenase.

The activity and the reaction constants of purified hyaluronidase might shed some light on the question raised by Meyer (132) and by McClean and Hale (108) as to whether hyaluronidased activity cannot be divided into polysaccharase and oligosaccharase phases.

SUMMARY

1. The effect of such cultural conditions as incubation time and temperature, nitrogen and carbohydrate sources, added extracts, supplements, and iron, upon the production of hyaluronidase by several strains of Cl. perfringens type A growing on protein-free media, has been studied.

2. Cultural conditions have been defined under which fair to good yields of hyaluronidase (up to 1400 V.R.U. per ml. of medium) are produced in the absence of alpha antigen. The three principle conditions necessary to this production are: (a) growth on hyaluronic acid or its salts as sole source of carbohydrate; (b) incubation for upwards of 60 hours; and (c) the presence of an enhancing factor found in meat digests but not in casein hydrolysate. Four strains of Cl. perfringens type A produced hyaluronidase while two strains did not.

3. Suggestions as to methods for purification and concentration of the hyaluronidase have been made. The production of the enzyme on a protein-free medium should facilitate such purification; its production free from alpha antigen should facilitate immunization studies.

VII. THE PRODUCTION OF HYALURONIDASE BY OTHER MICROORGANISMS

INTRODUCTION

A broad survey of representative species of bacteria, yeasts, and molds was originally intended. Opportunity was found to complete only a small part of this intention. A complete survey of hyaluronidase production of various microorganisms would be useful in interpreting their benign or pathogenic behaviour, and mode of living.

HISTORY

Spreading factors have been found to be secreted by many species of bacteria, but these factors have not always been identified with hyaluronidase. For instance, Ungar and Bacharach (161), using the skin diffusion test, detected spreading factor in many different microorganisms, including an unknown strain of Saccharomyces cerevisiae and an unknown strain of Penicillium. Since the skin test is purported to be more sensitive than in vitro tests for the detection of hyaluronidase, it can be said that hyaluronidase was probably absent from their negative cultures, but not necessarily present in their positive cultures. McClean (105) has found hyaluronidase in Cl. septicum (Vibrion septique) cultures, and in cultures of some strains of Cl. novgi, but not in Cl. tetani cultures.

EXPERIMENTAL

Yeast

Saccharmyces cerevisiae, Gebruder-Mayer strain, was grown for 24 hours on Sabouraud agar and then a loopful was inoculated into a completely synthetic medium to which glucose or hyaluronic acid has been added. The medium was made up according to Snell, Eakin, and Williams (155) as follows:

l-aspartic acid	0.1 gram
$(NH_4)_2SO_4$	3 gm.
KH_2PO_4	2 gm.
$CaCl_2$	0.33 gm.
$MgSO_4 \cdot 7H_2O$	0.25 gm.
H_3BO_3, $ZnSO_4$, $MnCl_2$, $TlCl_3$	1 mgm.each
$FeCl_3$	0.5 mgm.
$CuSo_4 \cdot 5H_2O$	0.1 mgm.
KI	0.1 mgm.
Inositol	5 mgm.
beta alanine	0.5 mgm.
Thiamine chloride	20 µgm.
Pyridoxin	20 µgm.
Distilled water to make	1 liter

Dextrose or potassium hyaluronate were dissolved in the medium as indicated in Table XXXI and ten cc. volumes of the medium were placed in 100 cc. Erlenmeyer flasks plugged with cotton, and autoclaved at 10 lbs. steam pressure for 10 minutes. After inoculation, the flasks were placed in a shaker and incubated at room temperature for 23 hours, with

regular gentle shaking. The experimental data are given in Table XXXI, page

TABLE XXXI

Hyaluronidase Production by Gebruder Mayer Yeast

Flask No.	Carbohydrate added	Growth at 23 hours	Hyaluronidase concentration
1	2% dextrose	good	0
2	2% dextrose	good	0
3	2% dextrose	none	0
uninoculated control		none	0
4	0.5% K hyaluronate	fair to poor	0
5	0.25% K hyaluronate	fair to poor	0
6	+1% dextrose	good	0

The original viscosity still persisted in the potassium hyaluronate flasks, after incubation, indicating that no hyaluronidase had been produced at any time during the incubation.

Since Chain and Duthie (18) have reported that shaking inactivates testicular hyaluronidase, it was thought possible that the shaking might have inhibited hyaluronidase production. To check this point, the experiment was reported in 11 X 220 mm. tubes. When these were placed in the shaker, a gentle rotary motion of the medium took place. Growth was perceptible, but not as good as in the previous experiment. No hyaluronidase was produced in any tube.

The effect of a complex medium was tried, in order to test the possibility of some unknown factor being necessary for hyaluronidase production. To this end, Sabouraud's dextrose broth, maltose broth, and potassium hyaluronate broth were prepared by dissolving 5 grams of Bacto Peptone in 500 cc. of distilled water and then adding 4% of dextrose or maltose, or 0.5% of potassium hyaluronate, to portions of the peptone solution. Ten cc. quantities of medium were placed in 100 cc. Erlenmeyer flasks and incubated with a loopful of Gebrüder Mayer yeast from a 6 hour culture in maltose broth. The flasks were shaken at room temperature for 36 hours. A flask containing casein hydrolysate medium similar to that described in the chapter on "Production of Cl. welchii Hyaluronidase" was included in the run, as was a flask containing some yeast extract (Difco). The protocol constitutes Table XXXII, page 143.

Molds

A stock culture of an unknown species of the Penicillium glaucium was obtained from the Bacteriology Department of the University of Cincinnati. After 10 days of growth on Sabouraud's agar, 9 cc. of sterile water was introduced into the tube and a suspension of spores made by shaking slightly. One-tenth cc. of this suspension was inoculated into 5 cc. of medium contained in 25 cc. Florence flasks. Some of the cultures were incubated at room temperature in the dark, while others were incubated at 37° C. These latter cultures were shaded from direct sunlight. The following media were used: (a) Czapek-Dox medium consisting of glucose, 20 grams; KH_2PO_4, 1 gram; $MgSO_4$, 1 gram;

THE PRODUCTION OF HYALURONIDASE BY OTHER MICROORGANISMS

TABLE XXXII

Production of Hyaluronidase by Gebruder Mayer Yeast

Flask No.	Medium	Carbohydrates	Growth at 36 hours	Hyaluronidase Concentration
1.	dextrose broth	4% dextrose	good	0
2.	maltose broth	4% maltose	good	0
3.	hyaluronate broth	0.5% K hyaluronate	poor	0
4.	1/2 dextrose broth, 1/2 hyaluronate broth	2% dextrose -0.25% K hyaluronate	good	0
5.	1/2 maltose broth, 1/2 hyaluronate broth	2% maltose 0.25% K hyaluronate	good	0
6.	dextrose broth	uninoculated control	none	0
7.	casein hydrolysate	0.5% K hyaluronate	poor	0
8.	yeast extract	0.5% K hyaluronate	poor	0
9.	maltose broth	6 hour culture	fair	0
10.	dextrose broth	6 hour culture	fair	0

$MgSO_4 \cdot 7H_2O$, 0.5 gram; KCl, 0.5 gram; $NaNO_3$, 2.0 gram; and water to make one liter; (b) Czapek-Dox medium using 0.5% of potassium hyaluronate instead of glucose; (c) Czapek-Dox medium with glucose and the addition of 0.2% of peptone; (d) Shell, Eakin and Williams (238) yeast medium, as given on page 140, with the addition of 2% glucose; (e) Snell, Eakin and Williams medium with 0.5% potassium hyaluronate instead of glucose. The protocol of this experiment is given in Table XXXIII, page 145.

No hyaluronidase was found in any of the cultures, nor was the viscosity conferred by the added potassium hyaluronate destroyed.

Penicillium notatum was cultured on Czapek-Dox medium to which 0.6% of potassium hyaluronate had been added. Growth was subsurface and slow. The viscosity of the medium was not changed after incubation for one month and therefore no hyaluronidase was produced.

A culture of Trichophyton interdigitals failed to produce any hyaluronidase when grown Sabouraud dextrose broth at 34° C.

TABLE XXXIII

Production of Hyaluronidase by a Penicillium Glaucum Species

Medium	Time of Incubation	Growth	Hyaluronidase Production
Czapek-Dox + glucose	2 days	just perceptible	0
	9 days	fair	
	18 days	mature	
Czapek-Dox + K hyaluronate	2 days	None	0
	9 days	none	
	18 days	none	
Czapek-Dox + peptone + glucose	2 days	slight	
	9 days	good	
	18 days	mature	0
Snell and Williams + K hyaluronate	2 days	none	
	9 days	slight	
	18 days	slight	0

Bacteria

The production of hyaluronidase by pneumococcus and by Cl. perfringens has been discussed in separate chapters. Several other members of the gas gangrene group were examined for hyaluronidase production.

Clostridium sordelli, strain CS4 (obtained from Lederle Laboratories), was found not to produce hyaluronidase on casein hydrolysate media supplemented with shreds of beef heart, when glucose or dextrin was used as carbohydrate source.

Clostridium septicum (Vibrion septique) strain V.S. 23 lacks hyaluronidase when grown on casein hydrolysate-meat media containing glucose and no hyaluronic acid. When the glucose was limited to 0.1% and fat solvent treated umbilical cord was added to the casein hydrolysate medium, some hyaluronidase activity was apparent (about 0.5 V.R.U.

per ml.). When the glucose concentration in the medium is doubled, hyaluronidase is no longer detectable. Another strain of Cl. septicum (Cincinnati strain B) produced over 1 V.R.U. per ml. of medium when grown on 4% veal tryptic digest medium with added serum and 0.6% of glucose as carbohydrate. This same strain produced between 5 and 10 V.R.U. per ml. of medium when grown as proteose peptone plus veal infusion and where potassium hyaluronate acted as sole carbohydrate source.

Clostridium Novyi strain N21, from Lederle Laboratories, was repeatedly tested for hyaluronidase production without positive results. The cultures tested were highly toxigenic and had been repeatedly pigeon-passed. The cultures were grown in various media including casein hydrolysate supplemented with meat, beef heart infusion broth plus proteose-peptone, casein hydrolysate supplemented with defatted liver or kidney, tryptic digest of kidney, tryptic digest of umbilical cord, and Brewer's thioglycollate medium containing potassium hyaluronate but no glucose.

B. tularensis* was grown on a gelatin hydrolysate medium plus glycerol. It failed to grow on potassium hyaluronate in the same media. The culture was passed through a Berkfeld "N" filter; the filtrate tested negatively for hyaluronidase.

A B. tetani toxin from Parke Davis and Company, which was labeled as containing 10,000 mouse M.L.D. per ml., had no hyaluronidase activity.

* Bacteriological work done by S. Gibby

SUGGESTION FOR FUTURE WORK

The survey should be extended to include representative members of the different classes of bacteria and fungi, with special effort directed towards the production of hyaluronidase on synthetic media of known composition.

SUMMARY

1. Two strains of Cl. septicum have been found to produce small amounts of hyaluronidase on complex media where glucose is absent or in low concentration.

2. Gebrüder-Mayer yeast, an unknown species of Penicillium glaucum, Penicillium notatum, Trichophyton interdigitale, Cl. sordelli, Cl. novyi, and B. tularensis were found not to produce hyaluronidase under the conditions tried.

3. A sample of commercial tetanus toxin was found to contain no hyaluronidase activity.

VIII. PROPERTIES OF HYALURONIDASE

INTRODUCTION AND HISTORY

Some properties of bacterial hyaluronidase have been discussed in more or less casual fashion by authors cited in previous chapters, particularly Madinaveitia (95), Meyer et al (129), Chain and Duthie (18) and McClean and Hale (108). A much greater amount of work, though still by no means an exhaustive survey, has been done on the properties of testicular hyaluronidase. How many of the data apply to both the testicular and the bacterial enzymes is unknown, but both quantitative and qualitative differences between mammalian and bacterial enzymes and between the various bacterial enzymes themselves, are apparent from the work of the authors cited. In the data given below, the hyaluronidase used is Cl. perfringens, Type A, enzyme, unless another source is specified.

EXPERIMENTAL

Stability

Stability to Storage

Cl. perfringens hyaluronidase, prepared as described in Chapter II, is quite stable to storage. Of two early preparations made on casein

hydrolysate media with ground dried beef heart and separated from bacteria by filtration, one lost 8% of its activity after storage for 2 months at 4° C. while the other lost 10% after 6 months of storage. This loss is within the limits of error of the viscosity reduction method. A sample concentrated by adsorption on Ca_3PO_4 (see page151) and containing over 900 V.R.U. per ml. showed no loss on storage at room temperature for seven days. Several cultures grown on tryptic digest of veal were pooled, centrifuged, and the clear supernatant fluid preserved by addition of one part of phenyl mercuric acetate to 20,000 parts of medium. This sample, assaying 750 V.R.U. per ml., showed no loss of activity after storage at 4° C. for three months. The pH of these Cl. perfringens cultures varied between pH = 6.5 and pH = 7.2. Cultures stored at 4° C. showed no loss of activity for 5-7 days, even though no effort was made to remove the bacteria. Only a very slow loss of activity occurs in cultures kept at room temperature or even at 37° C. This is borne out by the variation of hyaluronidase concentration with time of incubation of the culture (see page 115). A filtrate preserved by dialysis against glycerol retained its hyaluronidase activity of 2400 M.C.P. units per ml. unchanged after 60 days storage at 4° C. This same glycerolized preparation was diluted to 1/800 of its original concentration with distilled water, kept for 5 hours at room temperature, then transferred to a 4° C. refrigerator and stored over night. The next morning the diluted preparation had all of its activity, and it still possessed all this of activity after a further 8 hours at room temperature.

High dilutions (1 V.R.U. per ml.) of hyaluronidase in M/5 acetate buffer plus 1.2M NaCl, as used in the viscosity reduction test (see page 65) have occasionally shown 20-30% loss of activity after remaining at room temperature for 3-5 hours.

Hyaluronidase will precipitate from culture media when the media are saturated with ammonium sulfate. The dried, powdered precipitate showed the same hyaluronidase activity when brought into solution after 6 months storage at 4° C. as it did when an equal quantity of the freshly dried precipitate was dissolved.

Stability to Heat

The hyaluronidase is inactivated after being placed in boiling water for 30 minutes or by heating to 60° C. after 4 hours. It is stable at room temperature and up to 38° C.

Stability to Ultrafiltration

A sample was concentrated by ultrafiltration through Visking cellophane sausage casing without appreciable loss.

Stability to Evaporation

Samples have been concentrated by evaporation *in vacuo* at 25° C. without loss, at the pH of the culture filtrate.

Stability to Filtration

Hyaluronidase may be filtered through Berkfeld or Mandler candles without loss, at the normal pH of culture filtrates. Either pressure or suction filtration is satisfactory.

Stability to Adsorption

Hyaluronidase may be adsorbed on $Ca_3(PO_4)_2$ by the method Van Heyningen (162) gives for the adsorption of alpha-toxin, and eluted by means of 1/5 saturated $(NH_4)_2SO_4$ without loss, or with little loss (for literature references to various adsorptions see page 151). This is in agreement with Robertson et al (150) who adsorbed Cl. welchii mucinase on calcium phosphate in 50% acetone and recovered the mucinase by dissolving the adsorbent with acetic acid. The enzyme is also adsorbed on Lloys's reagent.

Stability to Precipitation

The enzyme in Cl. welchii filtrates may be precipitated by cold alcohol* and recovered by resolution, in 30-85% yield, depending upon the conditions of precipitation. A very considerable purification, in terms of weight of nitrogen per V.R.U., is effected. A weight of 0.2 micrograms of protein nitrogen or less, may be associated with one V.R.U. Due to present conditions, detailed information on the technique of alcohol precipitation is restricted.

* These precipitations were carried out by Dr. Milan A. Logan

Saturation of culture filtrates with $(NH_4)_2SO_4$ precipitates some of the hyaluronidase. Only a small fraction of the total activity is recovered in the precipitate, possibly due to a partial denaturation.

Hyaluronidase-antigen is found in commercial alum-precipitated toxoids.

Stability to Formaldehyde

Several formalized Cl. perfringens type A toxin filtrates were found to contain only a very small part of the original hyaluronidase activity. After incubation for 3 weeks at 34° C. with 0.2% formaldehyde, two filtrates declined in activity as follows: (a) from 26 V.R.U./ml. to 1 V.R.U.;ml.; (b) from 50 V.R.U./ml. to less than 1 V.R.U./ml. Two other filtrates also declined in activity to less than 1 V.R.U./ml. Since hyaluronidase is stable to storage, formaldehyde was evidently able to inactivate the enzyme.

This finding is not in agreement with that of McClean (105), who found no loss in activity after treatment of a Cl. welchii toxin with formaldehyde. The discrepancy may perhaps be explained by assuming that foreign protein, present in the complex medium used by McClean, protected the hyaluronidase from being inactivated, while the protein-free medium used in the present study afforded no such protection.

A sample of commercial formalized fluid toxoid (Lederle Laboratories, #178 H12A) which was free of alpha toxin activity, still retained 64 M.C.P. units of hyaluronidase activity per ml.

The formaldehyde-inactivated hyaluronidase retains its antigenicity and, upon injection into humans and animals, gives rise to circulating antihyaluronidase (page 175).

Stability to X-Radiation

Although as much as 4 hours exposure to x-rays of greatest intensity at the K alpha line of copper, or wave length 1.54 Angstroms failed to inactivate hyaluronidase in filtrates from Cl. perfringens cultures grown on casein hydrolysate medium plus ground dried beef heart, the same exposure of an alcohol-precipitated sample resulted in loss of 89% of the original activity. This instance is similar to that found by Tytell and Kersten (160) for urease. Inactivation by x-radiation took place with purified samples, while impurities protected the enzymes.

Stability to Antibiotics

PHENYL MERCURIC ACETATE. A saturated solution of this germicide was made in the buffer ordinarily used for the viscosity reduction test (see page 65). The value obtained for hyaluronidase concentration of a Cl. welchii culture filtrate, by the use of the saturated phenyl mercuric acetate (PMA) buffer was compared with the value obtained when ordinary buffer was used, and also when buffer only 1/2 saturated with P.M.A. was used. Table XXXIV, page 154, shows that the saturated P.M.A. solution inhibited hyaluronidase action by about 25%, while the 1/2 saturated solution had no effect.

* Sample rayed by Dr. H. J. Kersten

TABLE XXXIV

Effect of P M A on Hyaluronidase

Concentration of P M A	Hyaluronidase Concentration found V.R.U. per ml.
0	45; 43
saturated	32
1/2 saturated	45

SULFONE DRUGS. Saturated solutions of each compound were made in citrate buffer, and the saturated buffer used as diluent in the viscosity reduction test. Comparison was made with the values for hyaluronidase obtained with ordinary buffer. No inhibitory effect was produced by sulfaguanidine, sodium sulfathiazole, acetyl sulfanilamide, or acetyl sulfathiazole. Sulfadiazine and sulfapyrazine produced a mild inhibition, the hyaluronidase activity being about 20% lower.

These results agree with those of McClean (110) who found that neither sulfanilamide nor sulfapyridine inhibited the *in vitro* activity of the enzyme.

MOLD PRODUCTS. Each mold product was dissolved in physiological salt solution in a concentration of 1 mgm. of antibiotic per ml. of solution. One ml. of *Cl. perfringens* type A culture filtrate was incubated with 1 ml. of the antibiotic solution under test for 30 minutes, and the mixture then assayed for hayluronidase activity. The magnesium salt of penicillin had no effect on hyaluronidase activity,

either alone or when glucose was added (on the theory that a glucose oxidase effect might be involved). Aspergillic acid (Aspergillus flavus compound) reduced the hyaluronidas activity from 67 V.R.U. per ml. to 45 V.R.U., or a reduction of about 30%.

Is Acid Produced During Early Hyaluronidase Action?

To 3 cc. of synovial fluid maintained at 37° C. there was added 0.1 cc. of enzyme solution containing 870 viscosity reducing units per cc. (V.R.U. per cc.). No change in the color of added phenol red or of brom thymol blue indicators was noted as compared to similar tubes to which boiled enzyme solution was added. Observation was discontinued after 8 hours.

Five cc. of a 0.25% solution of hyaluronic acid in dilute NaOH was placed in each of 6 test tubes, together with 2 drops of phenol red indicator. The pH was adjusted to 8.0 with dilute HCl and 0.1 cc. of a highly potent (870 V.R.U.) enzyme solution added to some tubes but not to others. No color difference was observed between the experimental and control tubes, even after 6 hours at 34°.

Six cc. of 0.25% solution of hyaluronic acid, plus 2 drops of phenol red, were placed in a viscosimeter at 37° and 0.1 cc. of enzyme solution added. There was no perceptible color change on addition of the enzyme, nor was there any change at the end of 20 minutes, when the fiscosity of the solution had reverted nearly to that of dixtilled water. At this time 0.3 cc. of 0.01 N HCl were added. A distinct color change was noted, indicating a change in pH to pH = 6.8-7.0.

Evidently significant amounts of free acid are not liberated during the early action of hyaluronidase.

Specificity

Action on Glycogen

A mixture of 3.5 cc. of a 5% solution of glycogen with 0.5 cc. of a *Cl. perfringens* filtrate containing 11 V.R.U. per ml. did not undergo any loss of viscosity after incubation for 100 minutes at 37°C. A mixture of 4 cc. of 10% glycogen in 0.85% NaCl with 0.5 cc. of the same enzyme solution was similarly unaffected.

Action on Dextrin

A slow decrease in viscosity, amounting to 5% after 40 minutes incubation at 37°, was observed when a *Cl. perfringens* filtrate containing 11 V.R.U. of hyaluronidase per ml. was mixed with 5 or 10% suspensions of dextrin in 0.85% NaCl. The amylase activity of *Cl. welchii* filtrates has been noted by McClean (108).

Action on Gastric Mucin

A *Cl. perfringens* culture filtrate and a culture filtrate of D. pneumoniae were tested for activity in reducing the viscosity of gastric mucin.

One gram of commercial gastric mucin (Armour and Company) was shaken with 100 cc. of distilled water, yielding a milky suspension. Five cc. of this suspension was mixed with 1 cc. of the bacterial filtrate under test or with 1 cc. of a 1/5 dilution of the filtrate in

citrate buffer and the mixture incubated at 37° C. Viscosity determinations were made from time to time. The results are expressed in Table XXXV, page 157. Heat inactivated (30 minutes at 100°C.) filtrates were used as controls.

TABLE XXXV

Action of Filtrates on the Viscosity of Gastric Mucin

Filtrate used	Duration of reaction in hours	Viscosity loss minus loss in control in per cent
Undiluted perfringens filtrate = 48 V.R.U.	26	16.9
Perfringens filtrate diluted 1/5 with citrate buffor = 9.6 V.R.U.	9.5	3.4
	22	2.6
Undiluted D. Pneumoniac filtrate = 380 V.R.U.	26	0

If the viscosity of water be taken as the limit of possible reduction in viscosity, then the gastric mucin lost about 1/2 of its viscosity after 26 hours of incubation with Cl. welchii (B. perfringens) enzyme. Although the pneumococcus filtrate contained more than 7 times the concentration of hyaluronidas present in the Perfringens filtrate, it failed to change the viscosity of the gastric mucin suspension. Hence it is impossible that the viscosity loss produced by the perfringens filtrate is due to hyaluronidase. This same perfringens fil-

trate effected 100% decrease in the viscosity of synovial fluid in 4 minutes of incubation.

Neutralization of Hyaluronidase by Antisera

Investigation of the neutralization of hyaluronidase is a necessary preliminary to a determination of the value of, and possibility of securing, antispreading sera. For this reason, and because of the statement of Weed et al (167) to the effect that reactions obtained with Cl. perfringens toxin may be prevented by high concentrations of heterologous sera, the effect of various antisera on hyaluronidase activity was studied. Hyaluronidase from both Cl. perfringens type A, BP6K strain and D. pneumoniae type III, C H A strain, was used.

Cl. perfringens Hyaluronidase

Cultures grown on casein hydrolysate medium supplemented by dried beef heart were centrifuged at 4° C., filtered through a Berkfeld candle, and the filtrates concentrated to 1/4 of the original volume by evaporation at reduced pressure and a temperature of 25-28° C. These concentrated filtrates were then used as stock source of enzyme in the viscosity reducing tests. The enzyme source for the mucoprotein clot prevention tests was either an ammonium sulfate precipitated dried toxin, or a glycerol-dialyzed filtrate from a culture grown on tryptic digest of beef heart.

The effect of a serum was determined as follows: 1/2 cc. of stock enzyme solution (for viscosity reduction tests) or of an

appropriate dilution of enzyme (in the M.C.P. tests) was mixed with 1/2 cc. of appropriate dilutions of antiserum and the mixture maintained at 37° C. for 30 minutes. At the end of this time the mixture was immediately tested for hyaluronidase activity. The activity found for the serum-enzyme was compared with the activity found for enzyme solutions similarly incubated without serum.

Table XXXVI, page 160 , and figures 20, 21, and 22, pages 161, 162 , 163, show that high concentrations of several heterologous sera inhibit Cl. perfringens hyaluronidase to a greater or lesser extent, but this non-specific inhibition quickly disappears upon diluting the serum. Welch specific antisera, however, retain their inhibitory power at high dilutions. The data shown in Table XXXVI and figures 20, 21 and 22 were obtained with the viscosity reduction test against a solution containing 48 V.R.U. per ml. The following sera did not inhibit Cl. perfringens hyaluronidase:

normal horse serum (2 samples)

antibrucella serum (Bacteriology Dept. U.C.)

antitularemia serum (Bacteriology Dept. U.C.)

hystolyticus antiserum (Lederle #4307)

" " (" #3983)

Tetanus unrefined (" #6799)

Meningococcus unrefined (" #A4340)

botulinus unrefined (" #123V)

rabbit antipneumococcus Type I (Lederle #286).

TABLE XXXVI

(This data is plotted in figures 20, 21 and 22)

Per cent Inhibition of Cl. Welchi Hyaluronidase by Antisera

Final dilution of serum in incubation tube	Ml. of serum used (decimal fraction of Col. 1)	Histolyticus anti-serum, Lederle #3603 50-200 u/cc.	Novyi antiserum Lederle #3335 150 + 200 - u/cc	Novyi - Sordelli antiserum Lederle #1571 Novyi 600 u/cc Sordelli 1400 u/cc	Tetanus Standard Flocculating serum Lederle #2864F 4800 u/cc	Vibrion Septique Antiserum Lederle #4223 800 + 900 - u/cc	Welchi antitoxin (Refined serum) Lederle #6688b 105 u/cc preserved with glycerol	Specific Welch serum 100 u/ml preserved with glycerol
1/2	0.5	99+	89+	99+	-	-	-	-
1/4	0.25	70	75	99	99+	99+	99+	100
1/20	0.05	0	0	45.5	99	99	-	-
1/40	0.025			0	-	-	66.7	99+
1/50	0.020				33	93	-	-
1/100	0.010				0	47	45	99
1/200	0.005					0	27	94
1/400	0.0025						?	50
1/600	0.00166							42.3
1/800	0.00125							18.7

The following sera did not inhibit in the highest concentration: Normal horse serum (one sample); anti-brucella horse serum; Bact. Dept. U.C. 5120 u/cc; anti-tularemia horse serum (flocculating serum); Bact. Dept. U.C.; histolyticus antiserum Lederle #4307, 50 - 200 u/cc; histolyticus anti-serum Lederle #3603, 50 - 200 u/cc; tetanus unrefined serum, Lederle #6799; meningococcus unrefined serum, Lederle #A4340; botulinus unrefined serum, Lederle #123 V; unrefined rabbitt pneumococcus type I serum, Lederle #286.

Figure 20

Inhibition of Cl. perfringens Hyaluronidase by Horse Antisera

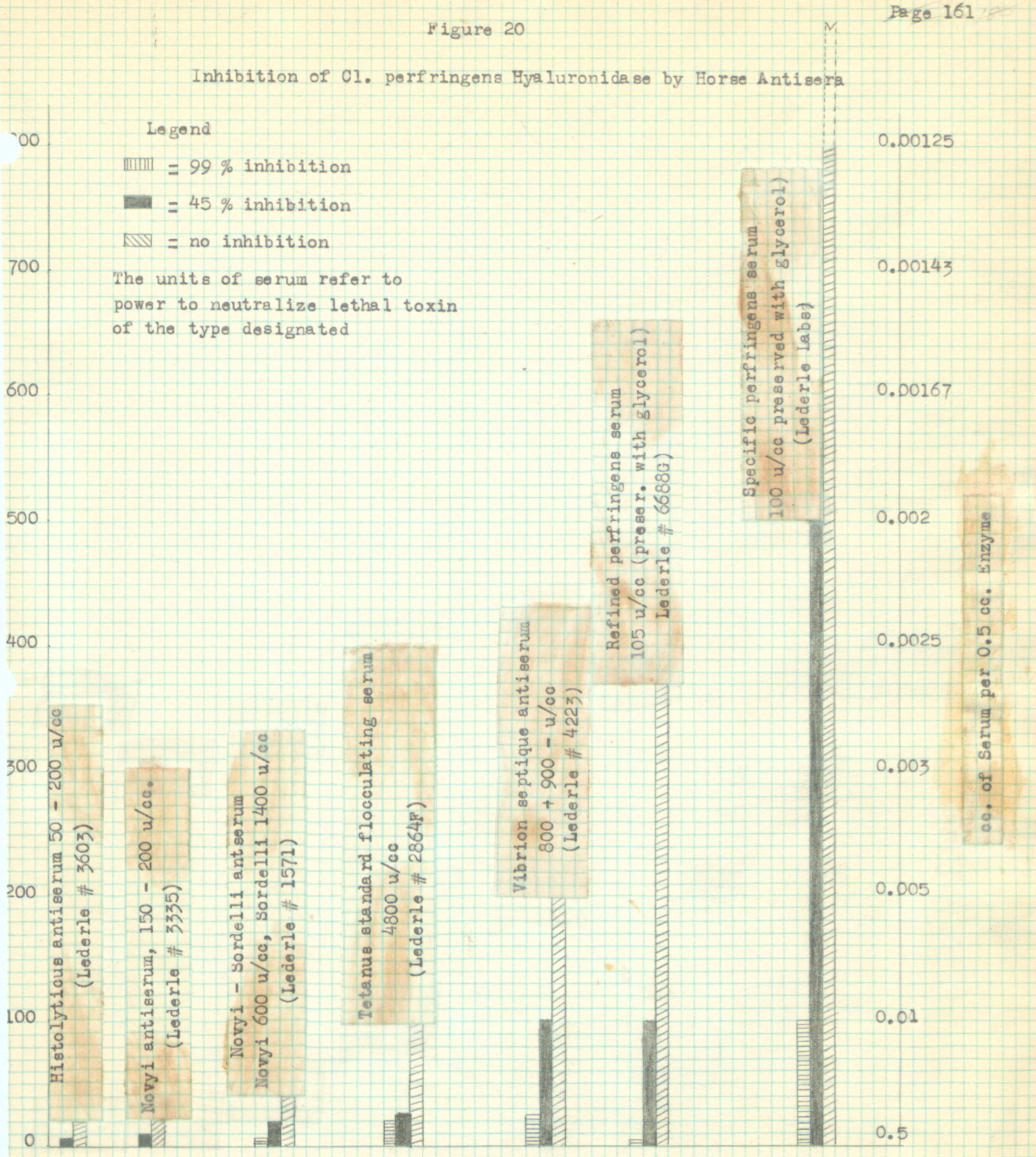


Figure 21

Inhibition of Perfringens Hyaluronidase by Antisera

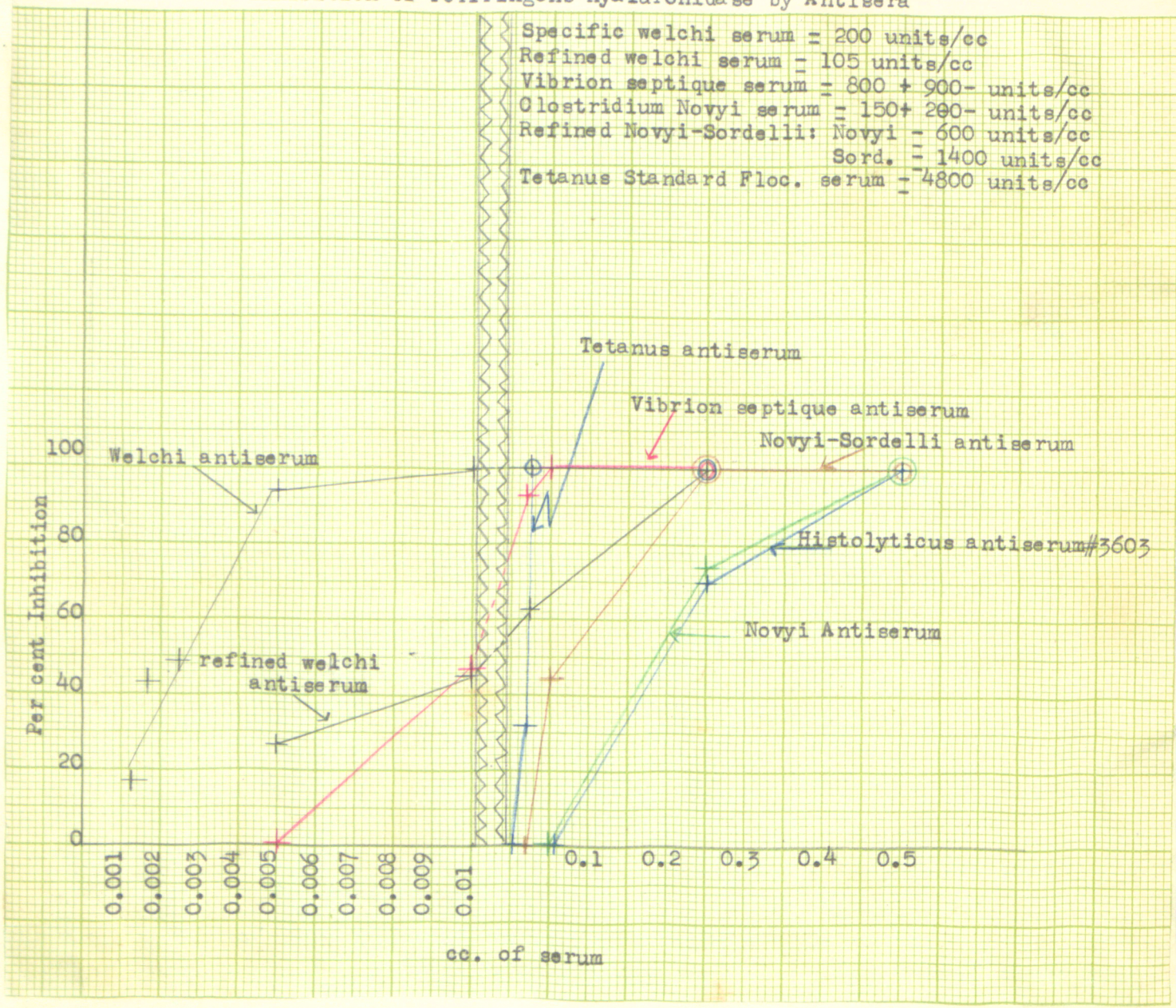
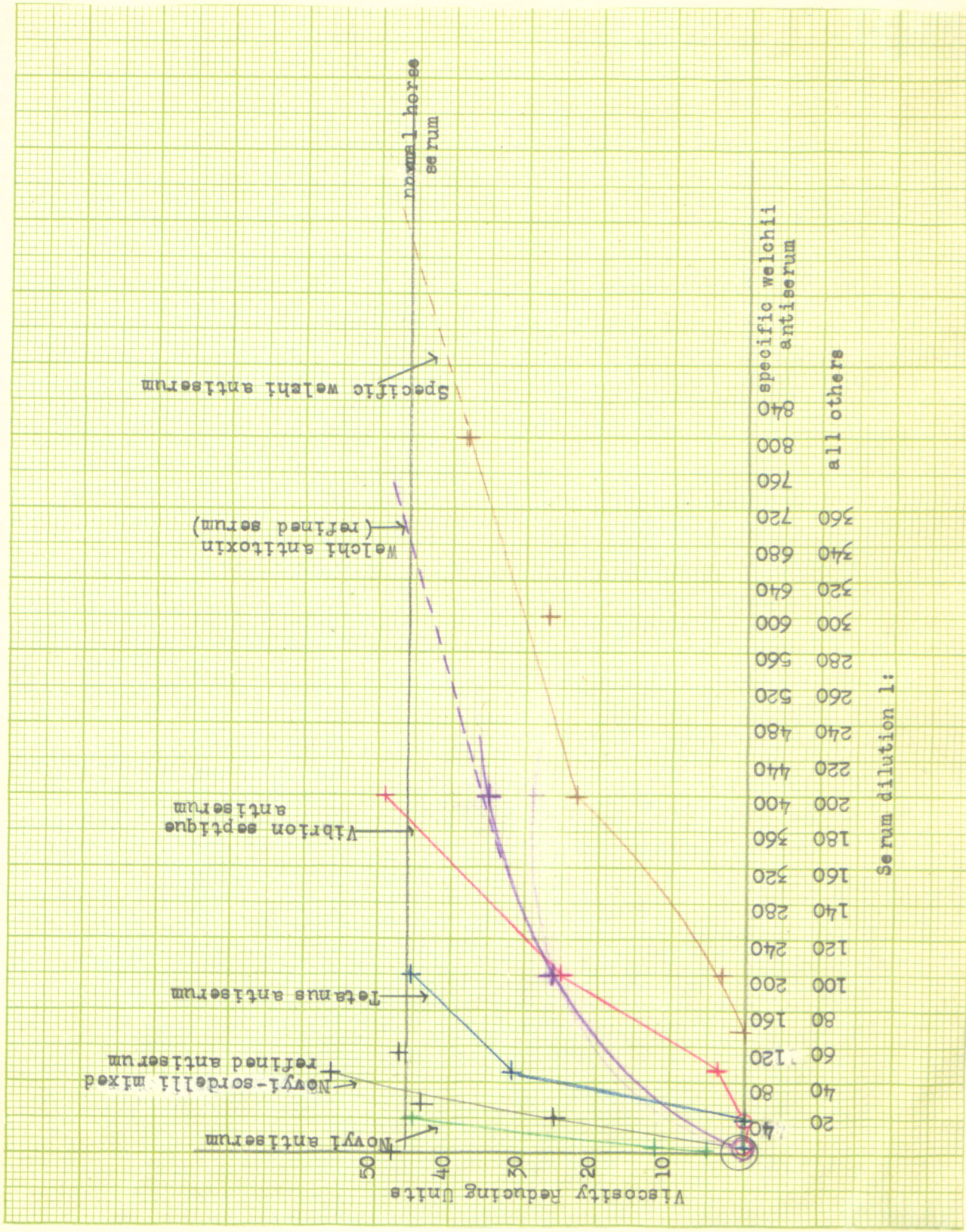


Figure 22

Effect of Sera on Welchi Viscosity Reducing Action



One-half ml. of serum from a rabbit which had been immunized with fluid formalized Cl. perfringens toxoid neutralized 69 V.R.U. of hyaluronidase contained in 1/2 cc. of a filtrate from a culture grown on infusion broth and hyaluronic acid. One cc. of sera from other immunized rabbits neutralized 160, and 148 V.R.U., respectively. Serum from a guinea pig immunized with alum precipitated neutralized 88 V.R.U. per ml. of serum.

Other data on neutralization of perfringens hyaluronidase with antisera were obtained by the use of the mucoprotein clot prevention test, in connection with antigenicity studies. A brief qualitative summary will be given here. Details may be found in the antigenicity section, page 175. The following horse sera were tested by the mucoprotein clot prevention test and found to be without any inhibition of the M.C.P. value of a standard glycerol preserved perfringens filtrate:

Botulinus unrefined	(Lederle #123V)
Tetanus "	" #6799
Meningococcus "	" #A4340
Histolyticus	" #3983
Novyi (pooled)	(Lederle Laboratories)

A 1:900 dilution of Lederle perfringens specific serum containing 160 anti-alpha units per cc., neutralized 4 M.C.P. units of enzyme. Another Lederle serum, #4838, preserved with 50% glycerol and containing 50 anti-alpha units per cc., failed to neutralize the same concentration of enzyme in dilution greater than 1:4.

Nineteen samples of normal dog blood, 10 samples of normal rabbit blood, 7 of normal mouse blood, and 6 of normal guinea pig blood did not show any antihyaluronidase in the serum.

Of 119 normal human sera tested, 12 had natural circulating antibodies against perfringens hyaluronidase.

After immunization with formalized toxoids or with hyaluronidase, many of the negative sera developed anti-hyaluronidase (see next section on antigenicity of hyaluronidase).

D. Pneumoniae, Type II, Hyaluronidase

The enzyme was produced as described in Chapter VI. The effect of a serum was determined in the same manner as with Cl. perfringens hyaluronidase (see page 158), using a sample of pooled, filtered culture diluted with distilled water so that it contained 30 V.R.U. per ml. Results are given in Table XXXVII and are depicted graphically in figures 23 and 24. No type specific antipneumococcus serum was available. The Lederle Tetanus Standard Flocculating Serum is by far the most potent inhibitor. Since this serum is a blend of many different horse sera, the possibility of the contribution of specific antibodies from the serum of a horse recovered from Pneumococcus type II infection is increased. The following sera did not inhibit in any degree:

normal horse serum (one sample)

refined perfringens serum (Lederle #66886)

TABLE XXXVII

(This data is graphed in figs. 23 and 24) Hyaluronidase

Percent Inhibition of Pneumococcus Type III, CHA, by Antisera

Final Dilution of Serum in Incubation Tube	cc. of Serum used per 0.5 cc. of enzyme (Decimal Fraction of Col.1)	Tetanus Standard Flocculating Serum Lederle #2864F 4800 u/cc	"Old" Normal Horse Serum	Unrefined Meningococcus Lederle #A4340	Vibrion Septique Antiserum Lederle #4225 800 + 900 - u/cc	Unrefined botulinus serum Lederle #123V	Histolyticus Antiserum 50-200 u/cc. Lederle #4307	Unrefined rabbit antipneumococcus serum Lederle #286 Type X	Tetanus unrefined Lederle #6799
1/2	0.5	99	65	38.5	54	54	27	43	40
1/4	0.25	99	48	--	--	--	23	43	
1/8	0.125	99	39	--	--	--	26	--	26
1/10	0.10	--	--	--	--	--	0	35	26
1/12	0.083	--	--	38.5	50	39.5	--	--	---
1/16	0.0625	98	32	--	--	--	--	--	--
1/20	0.05	--	--	--	--	--	--	0	--
1/24	0.047	--	--	26.0	18	23	0		0
1/32	0.032	98	23	--	--	?	0		
1/48	0.024	--	--	0	0				
1/64	0.0156	84	0						
1/128	0.0078	60							
1/256	0.0039	56							
1/512	0.00195	18							
1/1024	0.000975	0							

Figure 23
Inhibition Of Pneumococcus Hyaluronidase by Sera

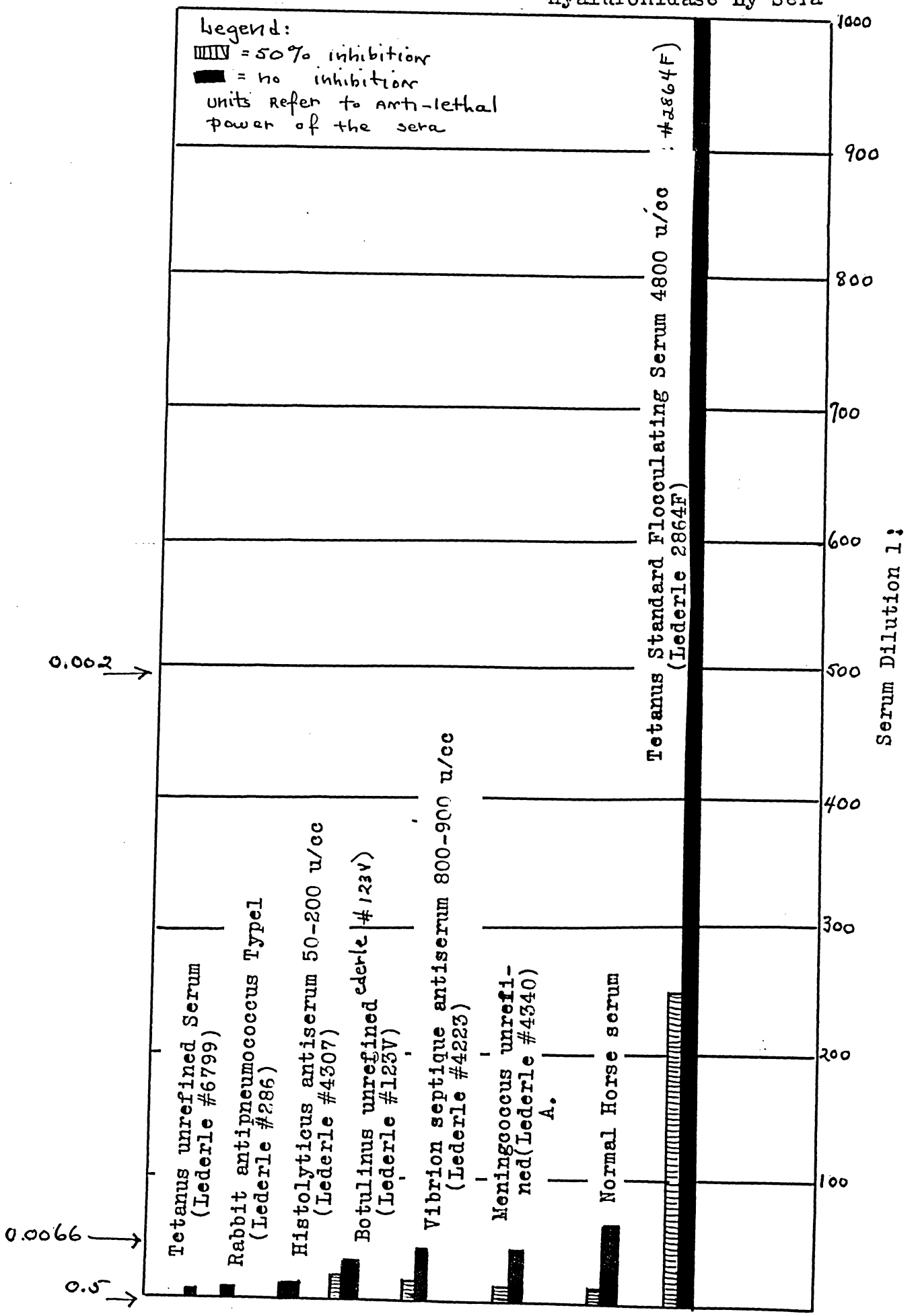
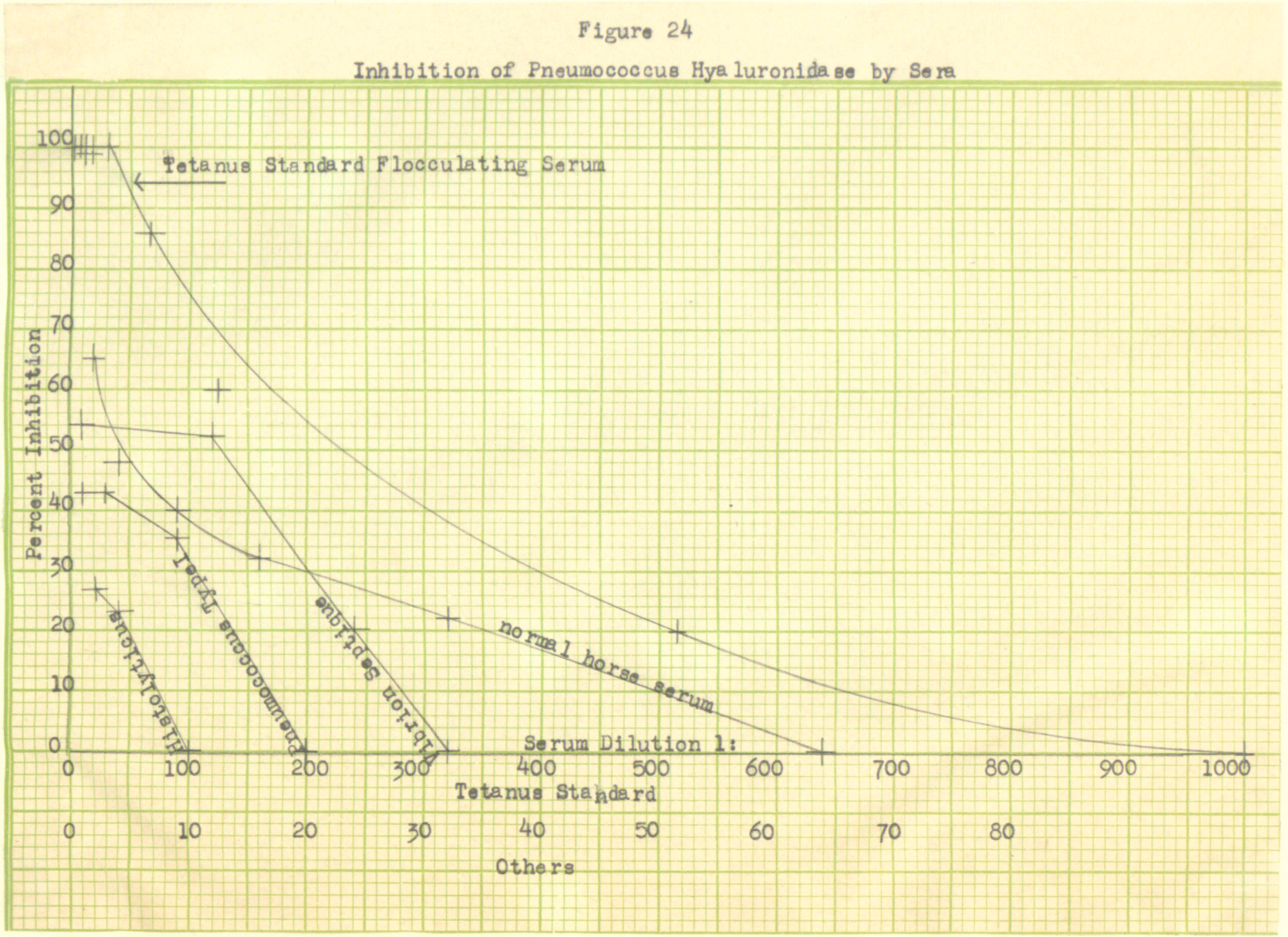


Figure 24

Inhibition of Pneumococcus Hyaluronidase by Sera



Discussion

Many heterologous horse sera inhibit hyaluronidase action when in high concentration. The inhibition may be distinguished from that of type specific sera by its failure to "titrate out," that is, to persist in dilution of the serum. Non-specific inhibition is encountered more frequently in commercial horse sera than among other species. This may perhaps be partly accounted for by the commercial practice of immunizing one animal with several antigens; many horse sera designated as "monovalent" therefore actually contain several different types of specific antibodies. Natural immunity probably also enters into the picture here.

Almost 9.5% of the human sera tested contained antibodies against perfringens hyaluronidase. The most likely source of the antigen seems to be the intestinal flora, which is often actively rich in Cl. perfringens, whose toxins sometimes pass the intestinal barrier (143A).

A perfringens antiserum which was efficacious in suppressing perfringens hyaluronidase had no effect on pneumococcus enzyme, thus indicating the specificity of the neutralization.

McClean (105, 108) and Hobby et al (64) have also found that hyaluronidase is inhibited by specific antisera, without cross neutralization.

Flocculation of Antisera by Hyaluronidase

The hyaluronidase containing filtrates produced particulation and flocculation with a refined specific perfringens horse antiserum, Lederle #2147F, containing 1500 units of anti alpha-toxin per cc. * The technique of flocculation was the Ramon, or B, procedure in which varying amounts of antiserum are added to a constant amount of antigen and the most rapidly flocculating mixture is taken to be the optimal proportioning point. The flocculation was carried out at 37-40° C. Table XXXVIII, page 171 , shows the optimal proportion points found for filtrates of different hyaluronidase concentrations. It will be seen that the ratio of hyaluronidase concentration to antiserum dilution is approximately constant and equal to about 0.0053 cc. of this serum per 400 Viscosity Reducing Units of hyaluronidase. These data are graphed in figure 25, page 172 . The protocol of a typical flocculation test is given in Table XXXIX, page 173; a graph on page 174.

The supernatant fluids from the flocculated tubes were completely devoid of hyaluronidase activity, indicating that flocculation too place at or near the neutralization point. The supernatants from tubes containing an insufficient quantity of serum showed hyaluronidase activity, which decreased progressively as more and more serum was added, until the end point was reached.

* This has been confirmed by Mr. Parsons of Lederle Laboratories, using 2 different specific antisera.

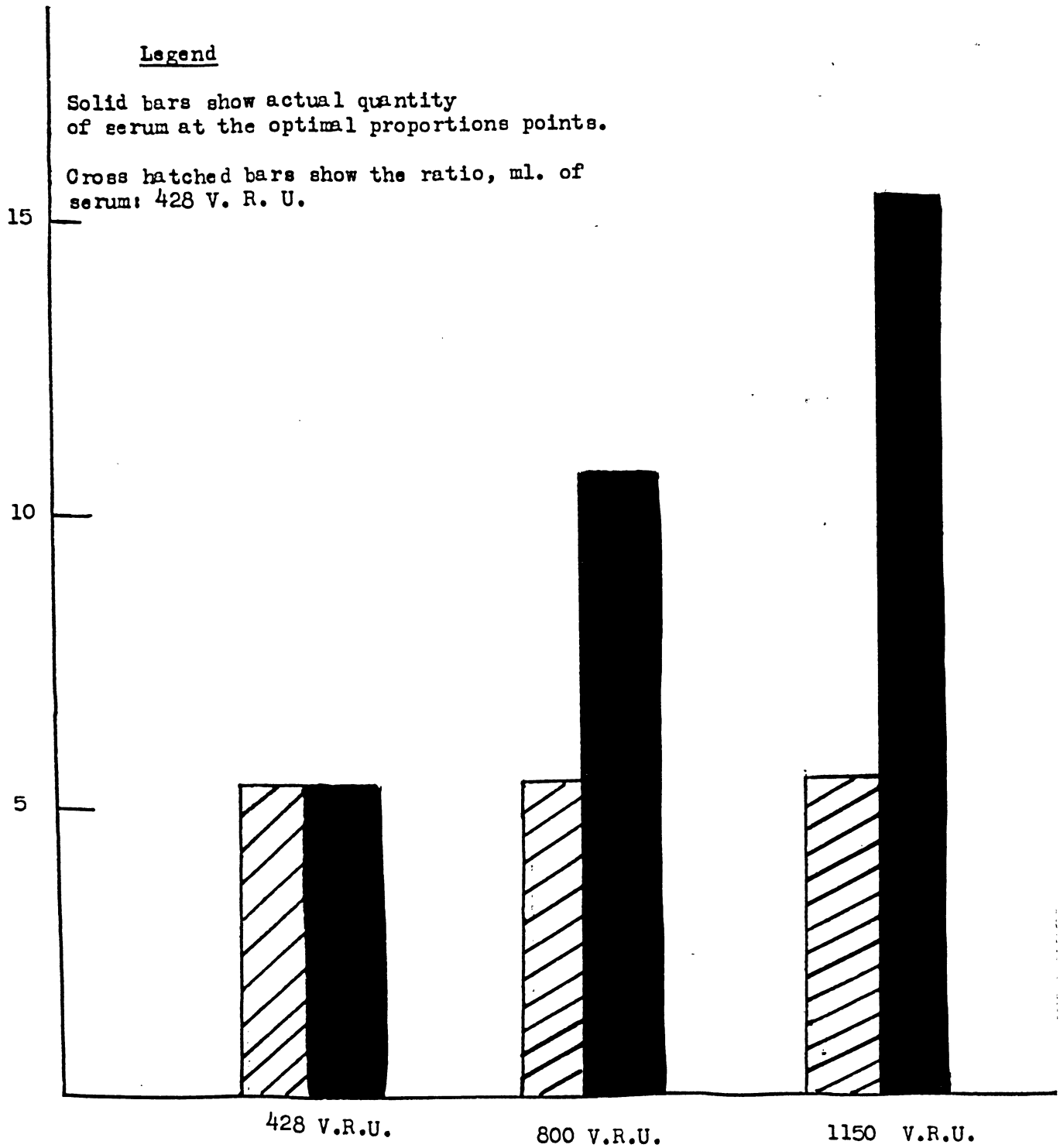
TABLE XXXVIII

Optimal Proportions Points of Hyaluronidase Filtrates with Lederle Serum #2147F

Hyaluronidase Concentration in the Filtrate	Ml. of Filtrate Used	Dilution of Serum	Ml. of Dil'd Serum at the Optimal Point	Ml. of Original Serum per 1 ml. of Filtrate	Ml. of Original Serum per 400 V.R.U.		Ratio of Values in Column 6
428 VRU/ml	2	1:75	0.7	0.00466	0.00466	=	0.8743
428	3	1:50	0.8	0.00533	0.00533	=	1
428	3	1:10	0.175	0.00583	0.00583	=	1.094
800	3	1:5	0.16	0.01066	0.00533	=	1
1150	3	1:5	0.22	0.0154	0.00536	=	1.006

Figure 25

Flocculation of Antisera by Hyaluronidase



PROPERTIES OF HYALURONIDASE

TABLE XXXIX

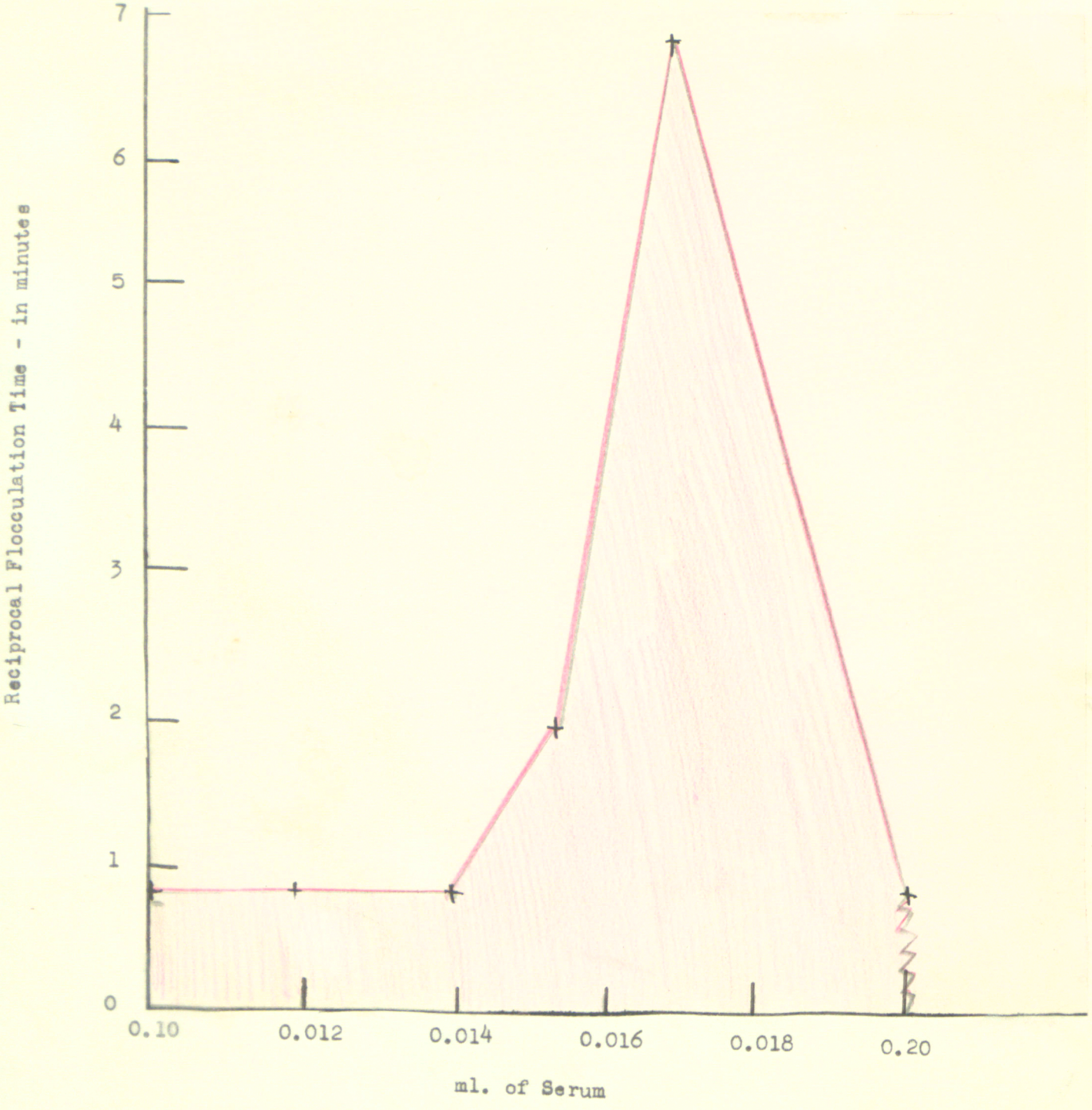
Protocol of Flocculation Test

Hyaluronidase concentration in filtrate= 428 V.R.U. Antiserum= Lederle refined perfringens antitoxin #2147F, preserved with glycerol, diluted 1:10

Tube No.	1	2	3	4	5	6	7	8	9
Ml. dil'd. Antiserum	0.1	0.120	0.140	0.160	0.175	0.2	-	0.2	0.2
Ml. Saline	-	-	-	-	-	-	0.2	3	
Ml. Filtrate	3	3	3	3	3	3	3	-	
Particulation at 15 min.				*					
Flocculation at 45 min.	-	-	-	+	+	-	-	-	
at 130 min.	+	+	+	+++	+++	++	-	-	
Hyaluronidase in the Supernatant Fluid	present	present	present	detectable	just detectable	none	none	none	445VRU

Figure 26

Flocculation of Serum by Hyaluronidase



Discussion

The formation of a precipitate when a soluble antibody and antigen are mixed is a phenomena observed with many different systems, and its occurrence in this case confirms the antigenic nature of hyaluronidase. Hobby et al (64) found that a preparation of pneumococcus hyaluronidase would give definite precipitates when incubated with homologous antisera. Rabbit antisera against testicle hyaluronidase will precipitate the homologous antigen (32).

The precipitin reaction of Cl. perfringens hyaluronidase is of immediate importance inasmuch as some commercial houses use a precipitin method for assaying the combining power of therapeutic sera against alpha toxin. Insofar as hyaluronidase contributes to this precipitation, the value assigned to the serum will be too high.

The Antigenicity of Hyaluronidase

Hyaluronidase and formalized hyaluronidase, when injected into animals and man, cause the production of antibodies in a large percentage of cases. These antibodies may be detected in the blood serum, where they confer upon the serum the capacity to inhibit and to neutralize the in vitro action of hyaluronidase of the same kind as that originally injected. This section is concerned with the capacity, extent, and duration of such response.

Materials and Methods

ANTIGENS. When hyaluronidase itself was injected, it was in the form of atoxic culture filtrates from Cl. perfringens cultures grown

on tryptic digest of veal or beef heart media, or on Bacto-tryptose broth (see chapter VI). Occasionally 1 volume of 10% potassium alum solution was mixed with 10 volumes of hyaluronidase. Such hyaluronidase is called alum-treated.

Formalized or "toxoided" hyaluronidase was made in this laboratory by Dr. A. A. Tytell or, according to his directions, by Lederle Laboratories. These toxoids were made by incubating toxic culture filtrates containing both alpha toxin and hyaluronidase with varying concentrations of formaldehyde up to 0.3%, usually at 34° C., until they contained less than 1 mouse M.L.D. per cc. and less than 1 guinea pig M.L.D. in 5 cc. The tests for lethality were made by intraperitoneal injection. Both fluid and alum precipitated toxoids were used.

The antigenicity of hyaluronidase or "toxoided" hyaluronidase was determined by injecting suitable quantities of antigen subcutaneously into humans or animals. Blood samples were taken before, during, and after each series of injections and the antihyaluronidase value of the serum assayed according to the mucoprotein clot prevention test.

ANTISERA ASSAY. The antihyaluronidase titre of a given serum was considered to be the highest saline dilution of that serum which was still capable of preventing the action of a fixed amount of welch hyaluronidase preparation upon a hyaluronic acid-protein substrate. One-half ml. of serum was added to 0.5 ml. of saline and serial dilution continued this way. Each of the serial dilutions were incubated for 1/2 hour at 37° C. with 0.5 ml. of a standard toxin dissolved in distilled water. The standard toxin in the earlier tests was a dried, ammonium sulfate precipitated preparation containing 60 M.C.P. units

per milligram. In later tests a glycerol preserved Cl. welchii filtrate containing 2400 M.C.P. per cc. was used as stock source of hyaluronidase. In each case, 4 M.C.P. units of enzyme was added to every dilution of the serum under test. The incubated mixture of serum and standard enzyme was then allowed to act upon 1 cc. of a hyaluronic acid-protein substrate for 20 minutes at 37° C. as in the Mucin Clot Prevention Test for hyaluronidase potency (114) (see pages 91 to 94). The first tube showing a mucin clot indicated an excess of antihyaluronidase and was taken to be the end point of the reaction. The antihyaluronidase value of a serum, therefore, represents the highest dilution of that serum which will still neutralize over 3 M.C.P. units of hyaluronidase in the standard enzyme preparation. Four M.C.P. units of enzyme were used as the standard strength of enzyme added to each dilution of serum as this quantity is sufficiently large to give a sharp end point, to override slight or non-specific inhibitors, and to ensure that a significant concentration of antihyaluronidase is being measured. Three controls were run concurrently with each assay: (1) a normal horse serum, to test for non-specific inhibition of the enzyme; normal serum showed no inhibitory effect; (2) a Mucin Clot Prevention assay on the enzyme preparation, to indicate possible variations in potency; (3) a standard anti-Welch horse serum titration, to reveal possible variations in the combining power of the enzyme preparation. This last control was supplemented by retitrating a previously assayed experimental serum from time to time. Only rarely did the second assay vary from the first, and then by only plus or minus one tube in

the series. This consistency was obtained only when twofold dilutions of sera were assayed. Closer titration gave a greater variation.

The hyaluronic acid-protein substrate was made as described on page 93.

Experimental

MICE. Seven mice were injected subcutaneously with 0.25 cc. of an atoxic culture filtrate containing 840 V.R.U. per ml. After 1 week they were injected with 0.25 cc. of fluid formalized toxoid; 20 days afterward they received a third injection of 0.25 cc. of hyaluronidase from a culture containing 400 V.R.U. per ml. The mice were decapitated in the 60th day following the last injection, and the serum from the pooled blood assayed. It contained 32 units of antihyaluronidase per ml., that is, a dilution of 1:32 would neutralize more than 3 M.C.P. units of standard enzyme.

Another group of mice received injections as follows:

at 0 days - 0.1 cc. of 800 V.R.U./ml. filtrate

at 2 days - 0.25 cc. of fluid toxoid

at 7 days - 0.25 cc. of 840 V.R.U./ml. filtrate

at 13 days - 0.25 cc. of 840 V.R.U./ml. filtrate and 0.25 cc. of fluid toxoid

at 26 days - 0.25 cc. of 400 V.R.U./ml. filtrate and 0.25 cc. of fluid toxoid

On the 60th day after the last injection, the pooled sera of this group assayed over 32 units antihyaluronidase per ml.

Unfortunately, the titration was carried no further than a dilution of 1/32 and the sample was insufficient for a re-titration, so that the exact titer of these pooled sera was not obtained.

Pooled sera from a group of 7 normal mice of the same age as the experimental animals had no antihyaluronidase action.

GUINEA PIGS. After an extensive course of injections, which included both fluid and alum toxoids and fluid hyaluronidase, 5 out of 6 guinea pigs treated had only 2 units of antihyaluronidase per ml. serum, while the 6th pig had a titer of zero.

RABBITS. Six rabbits, none of which had any serum antihyaluronidase, previous to treatment were divided into three groups and subjected to treatment as outlined in the following protocol:

Group 1, controls, received no injections. At 12 days 1 rabbit died. Death was preceded by diarrhea and emaciation, a condition which was prevalent in the rabbit colony at this time. At 16 days 10 cc. of blood was drawn from the remaining animal by heart puncture. The serum did not contain antihyaluronidase. At 23 days the other control died.

Group 2

at 0 days, received 1 cc. of filtrate containing 800 V.R.U.

at 3 days " " " " " " "

at 7 days " 1.5 cc. filtrate containing 800 V.R.U.

at 12 days, 1 rabbit died, similarly to control

at 16 days 10 cc. of blood was drawn by heart puncture from the remaining animal. The serum showed 8 units of hyaluronidase per ml.

Group 3 was managed similarly to group 2, but received alum treated hyaluronidase. At 16 days the sera of those rabbits contained 4 and 16 units of antihyaluronidase, respectively.

Another group of 8 rabbits showed no antihyaluronidase before treatment. Twenty-one days after the last of two injections of mixed Cl. perfringens-Cl. novyi toxoid, spaced 19 days apart, the pooled sera of this group assayed 8 units of antihyaluronidase per ml.

A pooled sample from a fresh group of 8 rabbits showed no hyaluronidase days after the last of injections of toxoid the antihyaluronidase titers of seven of these animals were 0, 0, 0, 8, 16, 16, and 16 units per ml., respectively.

DOGS. Sera from dogs which were put through a course of immunizing injections were received for assay from Dr. Dowdy at the University of Rochester. The protocols of these experiments are given in Tables XL, XLI, XLII and XLIII on pages 180 to 183 . The protocols show that out of a total of 24 dogs completing a course of toxoid injections, only 3 failed to respond by producing antihyaluronidase. Of the 21 dogs which responded to injection, three produced a maximum concentration of 2 units of antihyaluronidase per ml. of serum, three produced 4 units, three produced 8 units, three produced 16 units, five produced 32 units, two produced a maximum of 50 units per ml., and three produced 64 units. The largest titer measured was 80 units per ml., which occurred in three immunized animals which had recovered from experimental infection with Cl. perfringens culture. The response of the animals listed in Table XL, is depicted in figure 27, page 184.

TABLE XL

CANINE RESPONSE TO TOXOID

Dog No.	Weight Age Sex Kind	Toxoid Treatment	Time of: 1st blood sample 1st toxoid dose	Antihyaluronidase on first sample units per ml.	Time of: 2nd blood sample 2nd toxoid dose	Antihyaluronidase on second sample units per ml.	Time of 3rd blood sample	Antihyaluronidase on third sample units per ml.	Time of Challenging Infection	Time of: 4th blood sample	Antihyaluronidase on 4th sample
Tx1	14.5 kilos 3 years male Elkhound	alum precipitated 1 cc	0 days 0 days	0	10 days 14 days	64	24 days	32	28 days	87 days	80
Tx2	8.2 kilos 4 years female Airdale	alum precipitated 1 cc	0 days 0 days	0	10 days 14 days	,0	24 days	no sample	28 days	87 days	4
Tx3	15.7 kilos 3 years male Otter hound	Fluid, 1 cc-	0 days 0 days	0	10 days 14 days	16	24 days	50	28 days	87 days	80
Tx4	10.2 kilos 3 years male Shepherd	alum precipitated 1 cc	0 days 0 days	0	10 days 14 days	16	24 days	4	28 days	87 days	32
Tx5	15.7 kilos 5 years female hound	alum precipitated 1 cc	0 days 0 days	0	10 days 14 days	32	24 days	32	28 days	87 days	80
Tx6	8.2 kilos 2 years male Wire-Haired Terrier	fluid, 1 cc.-	0 days 0 days	0	10 days 14 days	4	24 days	4	28 days	87 days	32

TABLE XLI

CANINE RESPONSE TO TOXOID

Dog No.	Weight Age Sex Kind	Toxoid Treatment	Time of: 1st blood sample 1st toxoid dose	Antihyaluronidase on first sample units per ml.	Time of: 2nd blood sample 2nd toxoid dose	Antihyaluronidase on second sample units per ml.	Time of: 3rd blood sample 3rd toxoid dose	Antihyaluronidase on third sample units per ml.	Time of 4th blood sample	Antihyaluronidase on fourth sample units per ml.
Tx12	9.1 kilos 2 years male hound	alum precipitated, 1 cc	0 days 0 days	0	died on 16th day	-	-	-	-	-
Tx13	16 kilos 1 year male hound	alum precipitated, 1 cc	0 days 0 days	0	21 days 21 days	0	42 days no toxoid	8	no blood	-
Tx14	15 kilos 4 years male shepherd	alum precipitated, 1 cc	0 days 0 days	0	21 days 21 days	4	42 days no toxoid	2	no blood	-
Tx15	9.1 kilos 1 1/2 years male terrier	alum precipitated	0 days	0	21 days	16	42 days no toxoid	32	no blood	-
Tx16	13.2 kilos 1 1/2 years male terrier	Fluid, 1 cc	0 days 0 days	0	21 days 21 days	2	died at 26 days	-	-	-
Tx17	6.8 kilos 5 years female Beagle	Fluid, 1 cc	0 days 0 days	0	21 days 21 days	0	42 days 42 days	0	67 days	0
Tx18	14 kilos 5 years male Basset-hound	Fluid, 1 cc	0 days 0 days	0	21 days 21 days	16	42 days 42 days	32	67 days	50
Tx19	12 kilos 7 years male retriever	Fluid 1 cc	0 days 0 days	0	21 days 21 days	2	42 days 42 days	2	67 days	0

TABLE XLII

CANINE RESPONSE TO TOXOID

Dog No.	Weight Age Sex Kind	Toxoid Treatment	Time of: 1st blood sample 1st toxoid dose	Antihyaluronidase on first sample units per ml.	Time of: 2nd blood sample 2nd toxoid dose	Antihyaluronidase on second sample units per ml.	Time of: 3rd blood sample 3rd toxoid dose	Antihyaluronidase on third sample units per ml.	Time of 4th blood sample	Antihyaluronidase on fourth sample units per ml.
Tx20	13.2 kilos 1 year male hound	alum precipitated, 2 cc	0 days 0 days	0	21 days 21 days	0	42 days 42 days	0	no blood	-
Tx21	14.5 kilos 7 years male terrier	alum precipitated, 2 cc	0 days 0 days	0	21 days 21 days	16	42 days 42 days	32	no blood	-
Tx22	12.7 kilos 2 years female Airdale	alum precipitated, 2 cc	0 days 0 days	0	21 days 21 days	0	42 days 42 days	32	no blood	-
Tx23	14.5 kilos 2 years female Airdale	alum precipitated, 2 cc	0 days 0 days	0	21 days 21 days	16+	42 days 42 days	32	no blood	-
Tx24	14 kilos 5 years male Chow	Fluid, 2 cc	0 days 0 days	0	21 days 21 days	2	42 days 42 days	16	67 days	8
Tx25	14.5 kilos 2 years	Fluid, 2 cc	0 days 0 days	0	21 days 21 days	16+	42 days 42 days	64	67 days	50
Tx26	17.7 kilos 2 years male Doberman Pinscher	Fluid, 2 cc	0 days 0 days	0	21 days 21 days	0	42 days 42 days	0	67 days	8
Tx27	15.5 kilos 2 years male Collie	Fluid, 2 cc	0 days 0 days	0	21 days 21 days	0	42 days 42 days	2	67 days	0

TABLE XLIII

CANINE RESPONSE TO TOXOID

Dog No.	Toxoid Treatment	Time of: 1st blood sample 1st toxoid sample	Antihyaluronidase on first sample units per ml.	Time of: 2nd blood sample 2nd toxoid dose	Antihyaluronidase on second sample units per ml.	Time of third sample	Antihyaluronidase on third sample units per ml.
Tnx1	1:1Novyi-Welch alcohol concentrated alum precipitated, 2 cc	0 days 0 days	2	17 days 49 days	2	72 days	32
Tnx2	1: 1Novyi-Welch alcohol concentrated alum precipitated, 2 cc	0 days 0 days	0	17 days 49 days	2	72 days	64
Tnx3	1:1Novyi-Welch alcohol concentrated alum precipitated, 2 cc	0 days 0 days	2	17 days 49 days	4	72 days	8
Tnx4	1:1Novyi-Welch alcohol concentrated alum precipitated 2 cc	0 days 0 days	2	17 days 49 days	2	72 days	16

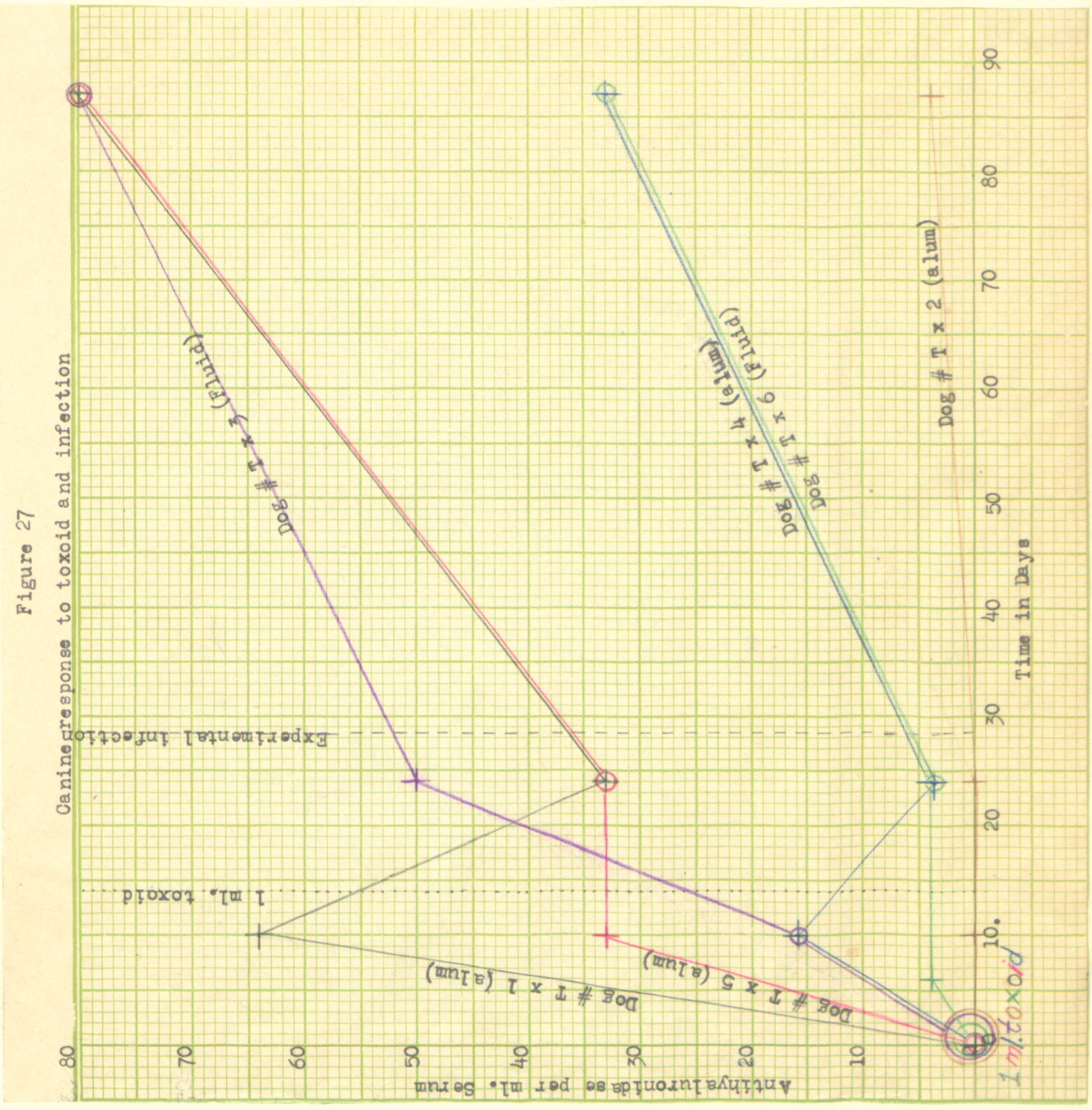

Canine response to toxoid and infection
Antihyaluronidase per ml. Serum
Time in Days
Experimental infection
1 ml. toxoid
1 ml toxoid
Dog # T x 2 (alum)
Dog # T x 4 (alum)
Dog # T x 5 (alum)
Dog # T x 6 (fluid)
Dog # T x 1 (alum)
Dog # T x 3 (fluid)
10.
20
30
40
50
60
70
80
90

Figure 27

HUMANS. A total of 119 human volunteers received courses of injections with either fluid or alum precipitated toxoid, 1 cc. being injected subcutaneously in the region of the deltoid muscle of the arm. Blood was drawn by venepuncture from time to time, and the serum assayed for antibodies against hyaluronidase. The protocols pertaining to these assays are given in Tables XLIV to LI, on pages 186 to 192 and some of the data is represented graphically in figures 28 and 29, pages 193 and 194.

A summary of the data is as follows:

Number of subjects	119 =	100%
Number of positive responses to toxoid	91 =	76%
Number of negative responses	28 =	24%
of this group, 14 received only 1 injection		
Number with natural circulating hyaluronidase previous to injection	11 =	9.2%

Of 64 subjects who received fluid toxoid 20, or 32% did not respond by production of antihyaluronidase. Of the number who failed to respond, 11 had received only 1 injection.

Of 53 subjects who received alum precipitated toxoid 12, or 23% did not respond by production of antihyaluronidase. Of this number, 3 received only a single injection.

The type of toxoid received by two subjects (M and F, Table XLIV) is unknown.

TABLE XLIV

HUMAN RESPONSE TO TOXOID

Volunteer	Toxoid Treatment	Time of: 1st blood sample 1st toxoid dose	Antihyaluronidase on first sample	Time of: 2nd blood sample 2nd toxoid dose	Antihyaluronidase on second sample	Time of: 3rd blood sample	Antihyaluronidase on third sample	Time of: 4th blood sample 4th toxoid dose	Antihyaluronidase on fourth sample	Time of: 5th blood sample	Antihyaluronidase on fifth sample	Time of: 6th blood sample 3rd toxoid dose	Antihyaluronidase on sixth sample	Time of 7th blood sample	Antihyaluronidase on seventh sample
			units per ml.		units per ml.		units per ml.		units per ml.		units per ml.		units per ml.		units per ml.
J.B.	alum precipitated	0 days 0 days	0	35 days 51 days	2	60 days	8	77 days no toxoid	8	86 days	2	110 days 110 days	0	130 days	0
S.B.	fluid	0 days 0 days	0	35 days 51 days	8	55 days	32	77 days no toxoid	50	86 days	80	112 days 112 days	10	130 days	8
M.L.	alum precipitated	0 days 0 days	0	35 days 51 days	0	52 days	0	77 days no toxoid	0	86 days		110 days 110 days	0	130 days	0
P.P.	alum precipitated	0 days 0 days	0	35 days 51 days	0	60 days	2	77 days no toxoid	0	86 days	0				
A.T.	fluid	0 days 0 days	0	35 days 51 days	4	60 days	16	no blood no toxoid	-	no blood		no blood			
M	?	0 days 0 days	2	30 days 57 days	4	57 days	16	99 days 99 days	4	121 days	4				
F	?	0 days 0 days	4	30 days 57 days	16	57 days	16	99 days 99 days	2	135 days					

PROPERTIES OF HYALURONIDASE

TABLE XLV

Human Response to Toxoid

This group received injections of 1 ml. of alum precipitated toxoid on the 1st, 28th and 49th day of the experiment.

Volunteer Number	Pre-immunization	Antihyaluronidase Titer at 28 days	at 49 days	at 69 days
		in units per ml. of serum		
15	0	0	0	0
16	0	0	0	2
17	0	16	32	
18	0	0	8	32
19	0	0	8	32
20	0	0	0	0
21	0	16	64	64
22	0	0	0	0
23	0	0	8	16
24	0	0	4	4
25	0	4	64	32
26	0	0	0	2
27	0	0	0	4
28	0	0	0	4
29	0	0	8	16
30	0	0	2	2
31	8	16	64	64
32	0	0	0	2
33	0	0	0	0
34	0	2	8	4
75	0	16	16	-
76	0	0	0	-
77	0	16	32	-
78	0	4	4	-
79	0	16	64	-

PROPERTIES OF HYALURONIDASE

TABLE XLVI

Human Response to Toxoid

This group received 1 cc. of fluid toxoid on the 1st, 21st, 44th and 51st days of the experiment.

Volunteer Number	Pre-immunization	Antihyaluronidase Titer at 21 days	at 44 days	at 51 days
		in units per ml. of serum		
82	0	4	8	8
83	0	0	0	16
84	0	0	0	0
85	0	16	64	100
86	0	4	32	80
87	2	16	64	128
88	0	8	32	128
89	2	16	64	-
90	0	0	0	8
91	0	16	16	32
92	0	0	0	-
93	0	0	2	8
94	4	16	64	-

PROPERTIES OF HYALURONIDASE

TABLE XLVII

Human Response to Toxoid

This group received 1 cc. of alum toxoid on the 1st, 28th and 66th day of the experiment.

Volunteer Number	Pre-immunization	Antihyaluronidase Titer at 28 days	at 66 days	at 73 days	at 94 days
		in units per ml. of serum			
47	0	2	2	2	2
48	0	0	4	8	4
49	0	0	0	16	16
50	0	0	0	0	4
51	0	0	0	0	0
53	0	8	8	4	8
54	0	16	16	16	16
55	0	4	8	16	-
56	2	4	8	8	4
58	0	4	32	32	16
59	0	0	0	0	2
60	0	8	4	8	8

PROPERTIES OF HYALURONIDASE

TABLE XLVIII

Human Response to Toxoid

This group received 1 cc. of fluid toxoid on the 1st, 21st and 42nd day of the experiment.

Volunteer Number	Pre-immunization	Antihyaluronidase Titer 21st day	42nd day	63rd day	93rd day
		in units per ml. of serum			
1	0	2	16	16	16
2	0	-	16	8	8
3	0	0	0	0	0
4	0	4	32	32	32
5	0	0	4	4	4
6	0	0	0	0	0
7	0	-	16	16	16
9	4	4	32	16	
10	0	0	32	16	
12	0	0	0	0	
35	0	0	2	16	
36	0	0	0	0	
37	0	0	0	0	
39	0	2	2	16	
40	0	0	0	4	
41	0	0	16	8	
42	0	0	0	8	
43	0	0	8	64	
45	0	0	0	4	
46	0	0	0	0	
95	0	4	8		
96	0	0	0		
97	0	0	0		
98	0	0			
99	0	0			
100	2	16			
101	0	4			
102	0	0			
104	2	2			
105	0	0			
106	0	0			
107	0	0			
108	0	0			
109	0	8			
110	0	0			
111	0	0			
112	0	0			
113	0	16			
123	0	2			
124	0	4			

PROPERTIES OF HYALURONIDASE

TABLE XLIX

Human Response to Toxoid

This group received 1 cc. of fluid toxoid on the 1st, 21st, 42nd and 49th day of the experiment.

Volunteer Number	Pre-immunization	Antihyaluronidase Titer 21st day	42nd day	49th day	63rd day
			units per ml. of serum		
61	0	2	0	0	-
62	0	4	32	32	80
63	0	4	16	8	100
64	0	0	0	0	0
65	0	0	4	-	16
66	0	0	2	0	-
81	0	0	-	-	64

TABLE L

Human Response to Toxoid

This group received 1 cc of alum precipitated toxid on the 1st and the 28th day of the experiment.

Volunteer No.	Pre-immunization	Antihyaluronidase Titer	
		28 days	49 days
		units per ml. of serum	
*112	4	16	
114	0	16	32
115	0	0	2
116	0	0	0
117	0	0	
119	0	0	
120	0	2	
121	0	0	

* single injection only

TABLE LI

HUMAN RESPONSE TO TOXOID

Volunteer Number	Toxoid Treatment	Time of: 1st blood sample 1st toxoid dose	Antihyaluronidase on first sample units per ml.	Time of: 2nd blood sample 2nd toxoid dose	Antihyaluronidase on second sample units per ml.	Time of: 3rd blood sample 3rd toxoid dose	Antihyaluronidase on third sample units per ml.	Time of: 4th blood sample 4th toxoid dose	Antihyaluronidase on fourth sample units per ml.	Time of: 5th blood sample	Antihyaluronidase on fifth sample units per ml.
14	Fluid, 1 cc	0 days 0 days	0	18 days 18 days	0	37 days 37 days	0	73 days no toxoid	0	81 days	0
80	Fluid, 1 cc	0 days 0 days	0	21 days 21 days	4	43 days 43 days	4	50 days 50 days	8		
57	alum precipitated, 1 cc	0 days 0 days	2	28 days 28 days	8	39 days 39 days	8	42 days no toxoid	16		
67	alum precipitated, 1 cc	0 days 0 days	0	28 days 28 days	4	35 days 35 days	8	42 days no toxoid	16		
68	alum precipitated, 1 cc	0 days 0 days	0	28 days 28 days	4	35 days 35 days	8	42 days no toxoid	8		
69	alum precipitated, 1 cc	0 days 0 days	0	28 days 28 days	2	35 days 35 days	4	42 days no toxoid	4		
72	alum precipitated, 1 cc	0 days 0 days	0	28 days 28 days	0	35 days	0	42 days no toxoid	0		

Figure 28

Human Response to Fluid Toxoid

Legend: The solid bars show the number of subjects with maximum response at the indicated levels.

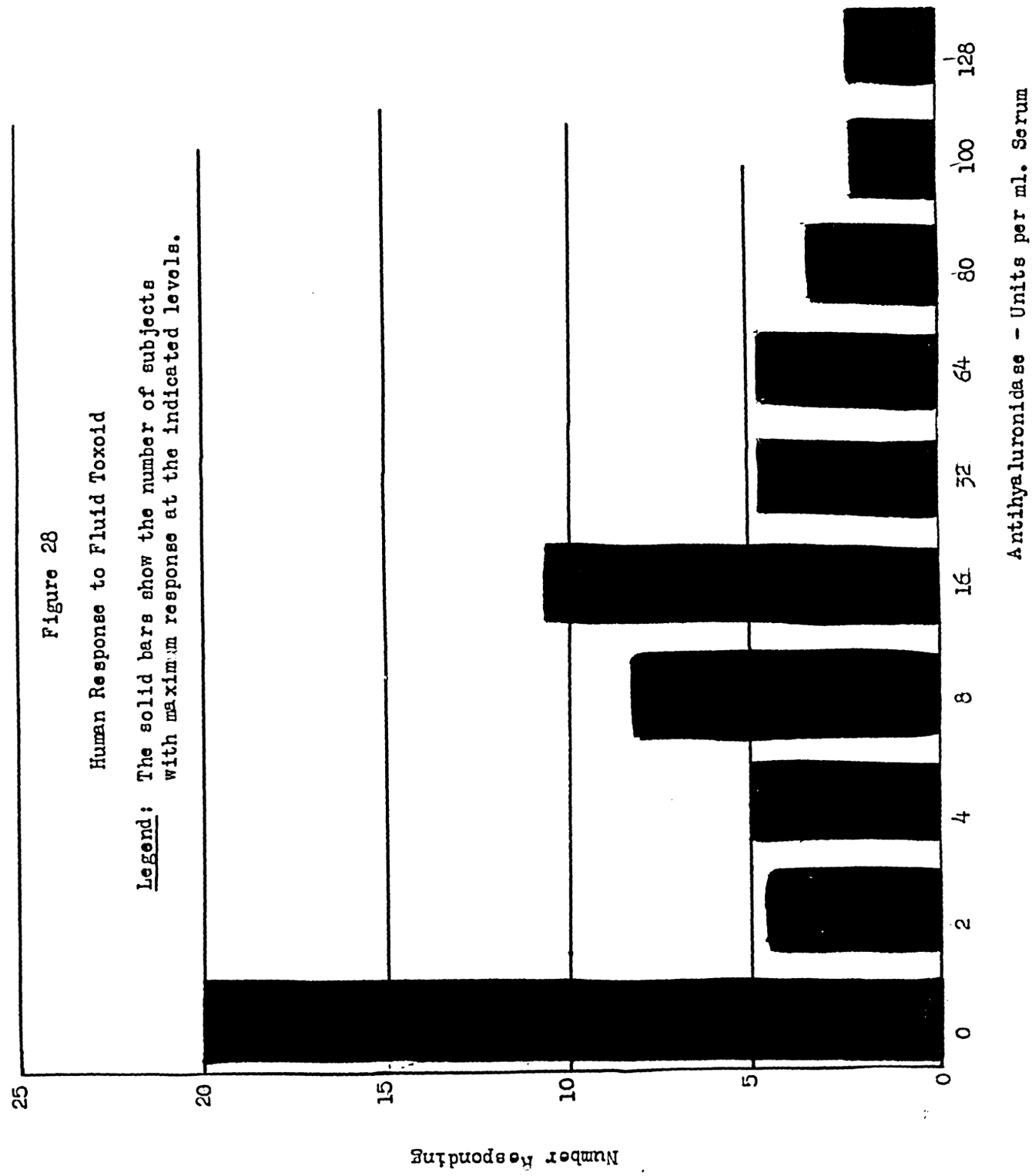
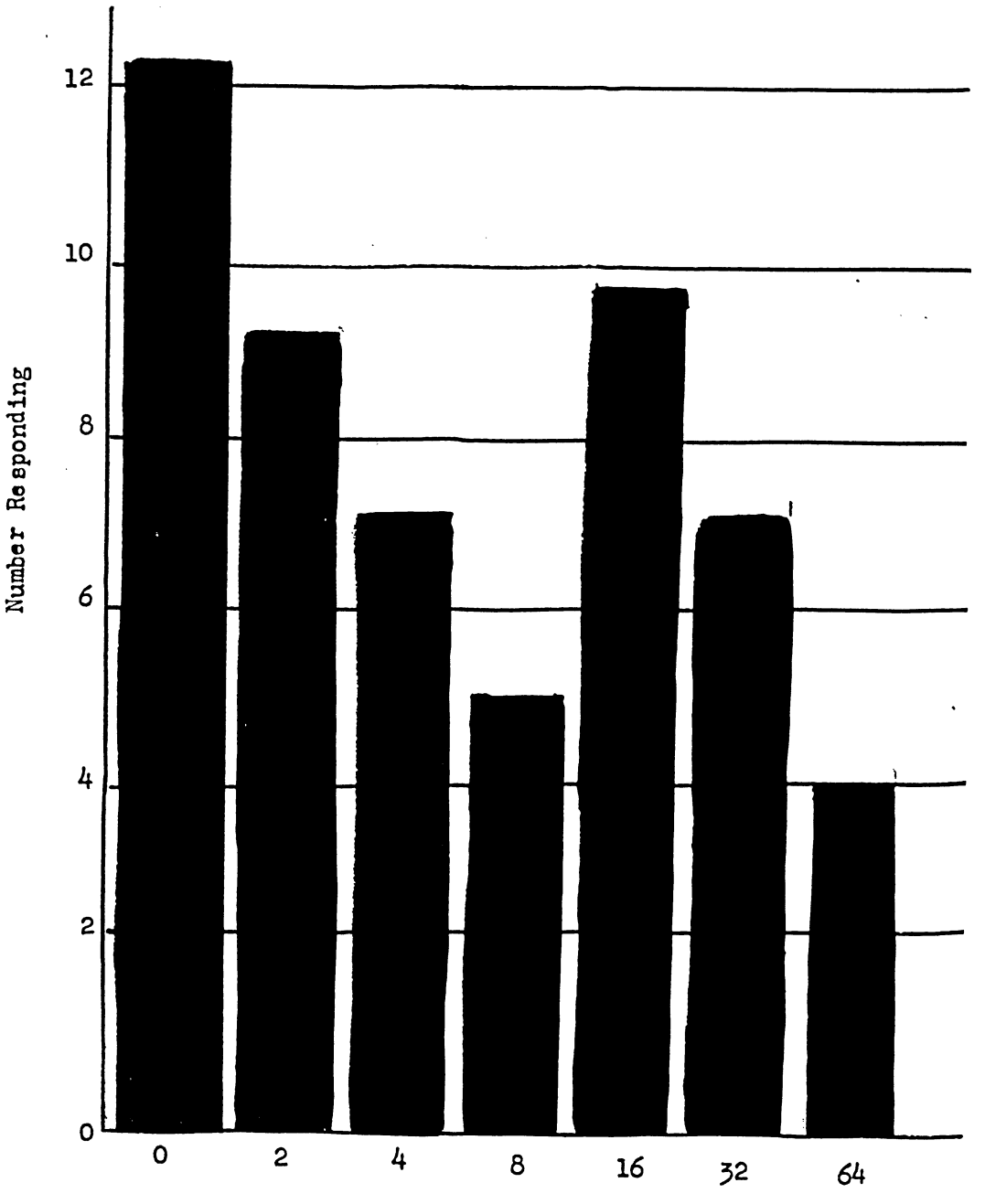


Figure 29

Human Response to Alum Toxoid

Legend

The solid bars show the number of subjects with maximum response at the indicated levels.



Discussion

All the species tested responded to the injection of formalized hyaluronidase or of hyaluronidase itself by the production of antibodies which would neutralize the in vitro activity of the enzyme. Some individuals of each species failed to produce antibodies while the output of the remainder varied widely. The highest concentration of circulating antibody was found in man, and the next highest in the dog. The response of the guinea pig was poorest. Since the mucoprotein clot prevention unit of hyaluronidase is accepted as being many times the minimal skin spreading dose, all the positive sera tested were capable of neutralizing many minimal spreading doses of enzyme. In preliminary experiments in this laboratory it has been found that the area of the lesion caused by intracutaneous injection of Cl. perfringens toxin is in fact greatly circumscribed in animals immunized with hyaluronidase as compared with unprotected controls.

These studies show no clear distinction as to the relative efficacy of fluid or alum precipitated formalized antigen. If either is used, it may be expected that about 75% of injected humans will show a detectable anti-hyaluronidase titer. It should be noted that the toxoids used were not prepared from filtrates especially high in hyaluronidase concentration; the toxoids were selected on the basis of alpha antigen content.

SUGGESTIONS FOR FUTURE WORK

Highly purified hyaluronic acid has been made (see chapter II); the relative stability, adsorption properties, and precipitability of hyaluronidase point the way to purification of this enzyme; since the components may be obtained in good purity, the characteristics of the enzyme system hyaluronic acid-hyaluronidase can profitably be investigated. Some of the determinations which might be made are:

temperature coefficient of reaction (Q10)
energy of activation (m/u value)
temperature optimum
activity - pH curve
velocity constant of reaction (K)
inhibitory and activating agents
synthetic reactions of the enzyme
competition, blocking, and specificity

The hyaluronidase-antihyaluronidase reaction seems a good one with which to carry out precipitin and other serological studies, since excess hyaluronidase in the fluid above the precipitate is readily detected and quantitatively determinable by means of the viscosity reduction test while excess antibody can be titrated with the M.C.P. test. Complete or partial inhibition, antigen or antibody excess, and equivalence points are thus susceptible to independent check.

The existence of such convenient tests for a bacterial toxin or enzyme is rare, the other outstanding instance being the alpha-

toxin of Cl. perfringens which is determined by its action on lecithovitellin (92), an action which also serves for assay of antibodies against this toxin. This coincidence should prove valuable in the study of the mechanism of invasion by Cl. perfringens. Hyaluronidase, through its power to attack substances in the skin, would appear to be important in invasion. The alpha-toxin vigorously attacks muscle tissue, and hence would seem to be the next "wave" in penetration of the bacteria into the body.

The protective value of active immunization with hyaluronidase has yet to be determined. Pilot experiments in this laboratory have shown that the area of infection is limited in animals immunized with hyaluronidase. Whether circulating antihyaluronidase supplements circulating anti-alpha antibodies in promoting resistance to infection, or whether antihyaluronidase is not very efficacious in this respect is a matter which can be determined by extensive, carefully planned and carefully executed experiments.

SUMMARY

1. Cl. perfringens hyaluronidase is stable to storage at room temperature or in the cold, with or without separation from the organisms.

2. Hyaluronidase solutions may be ultrafiltered through cellophane and filtered through Berkfeld or Mandler candles without loss of activity.

3. Hyaluronidase solutions may be evaporated at room temperature without loss of activity.

4. Hyaluronidase may be adsorbed on, and eluted from, calcium phosphate, without loss of activity.

5. Hyaluronidase may be precipitated from solution by cold alcohol and recovered by resolution, with little loss of activity.

6. Hyaluronidase in inactivated by 0.2% formaldehyde and such formalized hyaluronidase is antigenic. The failure of McClean to observe inactivation with formalin is probably due to the protective action of foreign protein.

7. Purified hyaluronidase was inactivated by x-radiation; impure hyaluronidase was not inactivated.

8. Hyaluronidase is not affected by ordinary concentrations of phenyl mercuric acetate, sulfaguanidine, sulfathiozole, sulfanilamide or penicillin. A mild inhibition was caused by sulfadiazine and sulfapyrazine, and aspergillic acid.

9. No significant quantity of acid is produced during early hyaluronidase action.

10. Culture filtrates containing hyaluronidase slowly attack dextrin and gastric mucin, but not in a manner similar to hyaluronidase action on hyaluronic acid. The action on dextrin and gastric mucin is probably due to the presence of enzymes other than hyaluronidase.

11. Hyaluronidase in neutralized by specific antisera; inhibition by non-specific sera disappears upon dilution, but specific sera retain their inhibition power and "titrate out."

12. Cl. perfringens hyaluronidase flocculates specific anti-sera. The supernatant fluid at the equivalence points is wholly or almost wholly free from hyaluronidase activity.

13. Hyaluronidase and formalized hyaluronidase, when injected subcutaneously into animals and man, cause the appearance of specific neutralizing antibodies in the serum of about 75% of those treated. The highest antibody titers occur in man.

14. Suggestions for further investigation of the properties hyaluronidase have been made.

APPENDIX I

PUBLISHED METHODS OF PREPARATION OF MUCINS AND THEIR CARBOHYDRATES
(In chronological order, and with an alphabetical index).

Index to Appendix I

1.

Author: E. Eichwald

Reference: Ann. d. Chem. u. Pharm. (1865), 134, 177; Eichwald refers to earlier authors, going back to 1840.

Name of Product: Mucin; pure mucin

Method of Preparation: Grind the snail, Helix pomatia in sand. Coagulate in boiling water. Filter, Precipitate the mucin with acetic acid. Settle the floculent precipitate. Decant. Wash the precipitate with dilute acetic acid. Filter, Wash the precipitate with dilute acetic acid, then, with water. Dissolve in lime water. Reprecipitate with acetic acid. Wash with acetic acid and water as described above. Wash with alcohol and ether.

Analysis of Product:

C: 48.9

H: 6.8

N: 8.5

Reducing Substances: After hydrolysis, sample reduced Fehlings solution.

2.

Author: E. A. Jernström

Reference: Uppsala Lakareforen, Forh 15, 434 (1880).

Name of Product: Funis Mucin; Funis Mucoid

Method of Preparation: Extract minced navel cord with water. Precipitate with acetic acid. Transfer the mucoid to very dilute alkali. Redissolve. Reprecipitate. Repeat the last two steps. Wash. Dry with ether and alcohol.

Analysis of Product:

C: 51.33

H; 6.63

O: 26.87

N: 14.13

S: 1.04

3.

Author: P. Giacosa

Reference: Zeit. f. Physiol. Chem. (1882) 7, 40

Name of Product: Mucin

Method of Preparation: Extract the outer covering of frogs' eggs with lime water for 24 hours. Decant. Filter, Precipitate with 10% acetic acid. Remove the precipitate. Wash with alcohol and ether. Numerous references to previous literature.

Analysis of Product:

C: 52.7

H: 7.1

N; 9.33

S: 1.32

4.

Author: H.A.Z. Landwehr

Reference: Zeit. f. Physiol. Chem. (1882), 6, 74

Name of Product: Animal Gum; achroglycogen

Method of Preparation: Remove the snail, Helix Pomatia, from its shell, and extract with water. Remove the protein with Brucke's Reagent (i.e. mercuric iodide, potassium iodide and HCl). Precipitate with acetic acid.

Analysis of Product:

S: 1.1 - 1.2 (According to Hammarsten)

Protein: Landwehr viewed this compound as a mixture of protein and animal gum.

5.

Author: C. Fr. W. Krukenberg

Reference: Zeit. f. Biol. (1884), 20, 307

Name of Product: Chondroitin sulfuric acid

C: 36.19

H: 5.22

N: 4.97

P: 7.41

S: 4.12

6.

Author: Loebisch, W.F.

Reference: Zeit. f. Physiol. Chem. (1886), 10, 40

Name of Product: Tendon Mucoid

Method of Preparation: For every gram of moist tendon, extract with 2 cc. of lime water. Precipitate preparation I with 1.5% acetic acid, and preparation II, with 0.1% - 0.2% HCl. Preparation III is precipitated with 1.5% acetic acid and purified by reprecipitation with an alkaline solution. It is redissolved in 0.5% Na_2CO_3, and finally, precipitated with acetic acid.

Analysis of Product:

	I	II	III
C:	48.24	48.34	48.32

H:	6.44	6.43	6.53
O:	32.71	32.59	32.74
S: (violatile)	0.82	0.80	0.82

7.

Author: H.A.Z. Landwehr

Reference: Pfluger's Arch. f. d. ges. Physiol. (1886), 39, 193

Aslo: ibid, (1887), 40, 21

Name of Product: Animal Gum

Method of Preparation: Extract the minced tissues of the snail, Helix pomatia with water. Heat in the autoclave for from 3 to 5 hours. Filter. Boil the filtrate. Add just enough acetic acid to complete the protein coagulation. Add FeCl in order to remove the remaining protein. Boil until the supernatant liquid is clear. Filter the reaction product. Add FeCl and $CaCO_3$ alternately and cautiously. The gum is in the precipitate of the ferric oxide. Dissolve the protein contained in the precipitate with HCl. Precipitate the carbohydrate with 3 to 4 volumes of alcohol. Repeat the ferric oxide treatment as many times as necessary. When a high degree of purity is reached, the alcohol will not precipitate the gum, therefore, add NaCl.

8.

Author: H.A.Z. Landwehr

Reference: Pfluger's Arch. f. d. ges. Physiol. (1886), 39, 193; also ibid, (1887), 40, 21

Name of Product: Animal Gum

Method of Preparation: Digest the minced organs or scraped mucous membranes on a water bath for 3 hours. Boil over a free flame to coagulate

the protein. Concentrate the filtrate on a water bath. Saturate with sodium sulfate and slightly acidulate with acetic acid. This should bring about the final removal of the major part of the protein. Precipitate with $CuSO_4$ and NaOH. The insoluble precipitate contains the gum, while the filtrate contains the protein. Wash the precipitate for three days in water in order to remove the remaining protein. Dry the precipitate on paper. Decompose with HCl. Precipitate the gum with 3 to 4 volumes of alcohol. Repeat for further purification.

9.

Author: O. Hammarsten

Reference: Zeit. f. Physiol. Chem. (1888), 12, 163

Name of Product: Submaxillary mucin

Method of Preparation: Extract minced organs with water. Strain dissected submaxillary gland in water. Centrifuge. Add 0.1 to 0.15% concentration of HCl. Further addition of HCl will cause the flocculation to disappear. Add 4 volumes of distilled water. Agitate the dilution with a glass rod to which the mucin will adhere. Transfer this mucin to 0.1 - 0.15% HCl. Dissolve and reprecipitate the mucin as described above. Repeat. Wash with water. Dry with alcohol and ether. A readily pulverizible substance will result.

Analysis of Product:

C: 48.8

H: 6.8

O: 31.20

N: 13.3

S: 0.84

10.

Author: C. Th. Morner

Reference: Skand. Archiv. f. Physiol. (1889), 1, 210

Name of Product: Chondromucoid

Method of Preparation: Digest trachea cartilage with water and thymol for 3 days. Filter. Add aqueous hydrogen chloride to the solution until a concentration of 2% is reached. Form a precipitate by heating. Filter. Dissolve in a little alkali. Reprecipitate. Repeat.

Analysis of Product:

C: 47.30

H: 6.42

O: 31.28

N: 12.58

S: 2.42

11.

Author: C. Th. Mörner

Reference: Skand. Archiv. (1889), 1, 210

Name of Product: Chondroitin sulfuric acid

Method of Preparation: The product was made from cartilage

Analysis of Product:

C: 35.28

H: 4.68

N: 3.15

S: 6.33

Equiv. wt.: base content 4-20%

12.

Author: O. Hammarsten

Reference: Hoppe Seyler's Zeit. f. Physiol. Chem. (1890), 15, page 202

Name of Product: Mucoid; mucinalbumose

Method of Preparation: Precipitate ascitic fluid with acetic acid.

Analysis of Product:

C: 51.40

H: 6.80

N: 13.01; 12.4

S: not accurately ascertainable

Haematin derivatives: 13.01

13.

Author: O. Schmiedeberg

Reference: Arch. f. Exp. Path. u. Pharm. (1891), 28, 355

Name of Product: Chondroitin sulfuric acid

Method of Preparation: Extract a peptic digest of nasal bone cartilage with an aqueous solution of KOH. Precipitate the K salt with 1 to 3 volumes of alcohol; the protein remains in the alcoholic solution. Repeat until there is a negative Biuret for the precipitate and the filtrate. Dissolve in water. Neutralize with HCl. Precipitate with alcohol. Wash the precipitate with alcohol until all the salt is gone. Wash the precipitate with increasing concentrations of alcohol.

Analysis of Product:

Salts: K +

Protein: Biuret

14.

Author: O. Schmiedeberg

Reference: Arch. f. Exp. Path. u. Pharm. (1891), 28, 355

Name of Product: Chondroitin sulfuric acid

Method of Preparation: Extract a peptic digest of nasal bone cartilage with water. Alternate cupric acetate with KOH until the solution takes on a violet color and a blue opalescence. Add alcohol to form a blue precipitate of copper and potassium, and a violet colored supernatant liquid. Redissolve the precipitate in HCl and repeat the first step until the violet color is gone. Dissolve the copper precipitate with HCl and a minute quantity of alcohol is added to form an incipient precipitation. This precipitate is removed by filtration. Repeat if necessary. Dissolve the precipitate in water and HCl. The solution is precipitated with alcohol.

Analysis of Product:

Salts: K +

Protein: Biuret -

15.

Author: C. Th. Mörner

Reference: Zeit. Physiol. Chem. (1894), 18, 233

Name of Product: Mucoid

Method of Preparation: The product was prepared by the acidification of diluted and filtered

Analysis of Product:

N; 12.25

S: 1.19; 1.14

16.

Author: C. Th. Mörner

Reference: Zeit. Physiol. Chem. (1894), 18, 233

Name of Product: Mucoid

Method of Preparation: Precipitate native vitreous humor with dilute acetic acid.

Analysis of Product:

N: 12.27%

S: 1.19%

17.

Author: C. Th. Mörner

Reference: Zeit. f. Physiol. Chem. (1894), 18, 213

Name of Product: Cornea Mucoid

Method of Preparation: Extract 10 cc. of dissected cornea with 0.02% KOH or 0.02% ammonium hydroxide. Let the solution stand for 2 to 3 days. Filter. Precipitate with acetic acid or HCl. Settle. Decant. Redissolve. in water. Add alkali. Reprecipitate. Dry with ether and alcohol.

Analysis of Product:

C: 49.96; 50.36

H: 6.94; 7.01

O: 28.01

N: 12.8; 12.8; 12.8; 12.6; 13.0

S: 2.02; 2.12

18.

Author: K. Mitjukoff

Reference: Centralblatt für die medicinischen wissenschaffen, (1895), 66, 737

Name of Product: Ovarial Mucoid

Analysis of Product:

C: 51.76

H: 7.76

O: 28.69

N: 10.70

S: 1.09

19.

Author: K.A.H. Mörner

Reference: Skand. Arch. f. Physiol. (1895), 6, 332

Name of Product: Urine mucoid

Method of Preparation: Let the sediment in human urine settle. Collect this sediment and syphon off the supernatant. Filter the precipitate. Put it in 95% alcohol. Add water. Add enough ammonia (aqueous) to give an alkaline reaction. Through suspension CO_2 is passed until the solution is rendered slightly acid. Allow the undissolved part to settle and filter off the supernatant liquid. Add acetic acid to the filtrate until a concentration of 0.4% is reached. The solution at this point is viscous and clear. Shake with chloroform. Centrifuge. Wash with water and 0.2% to 0.4% acetic acid. Saturate with chloroform water. Part of the mucoid may be extracted by wash waters. The insoluble fraction is dissolved in amonia water. Reprecipitate with acetic acid. Dry with

alcohol and ether.

Analysis of Product:

C: 49.40

N: 12.74

S: 2.30

20.

Author: Chittenden and J.W. Gies

Reference: J. Exp. Med. (1896), 1, 186

Name of Product: Tendon mucoid

Method of Preparation: Extract the ox tendon Achilles for 36 hours in 10% NaCl. Re-extract for 48 hours. Combine the precipitates. Precipitate with 0.2% HCl. Redissolve in 0.5% Na_2CO_3 . Reprecipitate with HCl.

Yield: 1% chemically pure mucoid

Analysis of Product:

C: 48.74

H: 6.46

O: 30.65

N: 11.80

S: 2.35

21.

Author: Chittenden and J.W. Gies

Reference: J. Exp. Med. (1896), 1, 186

Name of Product: Tendon Mucoid

Method of Preparation: Extract the ox tendon Achilles with 10% NaCl for

36 hours. Then extract with lime water for 60 hours. Re-extract with dilute lime water.

Yield: 1% Chemically pure mucoid

Analysis of Product:

C: 48.76%

H; 6.53

O: 31.43

N: 11.51

S; 2.31

22.

Author: Chittenden and J.W. Gies

Reference: J. Exp. Med. (1896), 1, 186

Name of Product: Tendon Mucoid

Method of Preparation: For every gram of ox tendon Achilles extract with 2 cc. of lime water. Continue the extraction for 48 hours. Re-extract for 48 hours. Combine the precipitates. Precipitate with 0.2% HCl. Re-dissolve in lime water. Reprecipitate with HCl.

Yield: 1% chemically pure mucoid.

Analysis of Product:

C: 49.29%

H: 6.63

O: 29.80

N: 11.94

S: 2.34

23.

Author: N.P. Krawkow

Reference: Archiv. für Exp. Path. and Pharm. (1897), 11, 195

Name of Product: Amyloid

Analysis of Product:

C: 49.44%

H: 6.79

O: 27.06

N: 13.92

S: 2.79

24.

Author: C.U. Zanetti

Reference: Jahresbericht über die Fortschritte der Thier Chemie, (1897), 27, 31

Name of Product: Serum Mucoid

Method of Preparation: 1200 cc. of ox blood was freed of protein with dilute HCl, filtered in vacuo, evaporated to small volume, and precipitated with alcohol.

Analysis of Product:

C: 47.60%; 48.75; 48.94

H: 7.10; 6.90

O: 29.99

N: 12.93; 12.65

S: 2.38; 2.20

25.

Author: N.P. Krawkow

Reference: Arch. f. Exp. Path. u. Pharm. (1898), 40, 195

Name of Product: Chondroitin sulfuric acid

Method of Preparation: Krawkow used Schmiedeberg's copper and alkali method (see page 212 #17) for preparation from "amyloid containing" organs.

26.

Author: Umber

Reference: Zeit. für Klinische Med. B'd. 48

Name of Product: Mucin

Method of Preparation: Precipitate ascitic fluid with acetic acid. Wash the precipitate. Purify with alcohol and ether.

Analysis of Product:

C: 51.35, 50.23%

H: 6.72, 6.87

N: 14.91, 14.37

P: 0, no nucleins

S: 1.32, 1.32

27.

Author: Salkowski

Reference: Virchows. Arch. B'd. 131

Method of Preparation: Product prepared from fluid of chronic "coxitis".

Name of Product: Mucin Substance

28.

Author: Paijkull, L.

Reference: Malys Jahresbericht uber die Fortschritte der Thierchemie, (1892(22,558; see also Zeit. f. Physiol. Chemie, 12, 196 (1888) for

another preparation.

Name of Product: Precipitate the ascitic fluid from fluids of inflammatory origin, that is, serous exudate, with acetic acid. Redissolve in excess acetic acid. (The precipitate can be dissolved in 0.2% HCl).

Yield: 0.2%

Analysis of Product

Extensive non-elemental anaylses are reported, for albumins, water, globulins, salts, etc.

29.

Author: T. Panzer

Reference: Zeit f. Physiol. Chem (1899), 28, 363

Name of Product: Colloid of ovarian cysts, or, paramucin

Method of Preparation: Digest ovarian cysts with pepsin HCl. Extract the undigested residue with alkali. Add 3 volumes of alcohol to form a white precipitate. Redissolve and reprecipitate until all the protein is gone from the precipitate.

Analysis of Product:

C: 47.27%

H: 5.86

N: 8.40

S: 0.79

P: 0.54

Ash: 6.43

Fehlings solution: + on hydrolysis

30.

Author: J.B. Leathes

Reference: Arch. f. Exp. Path. u. Pharm. (1900), 43, 245

Name of Product: Paramucin

Method of Preparation: Digest the colloid of ovarian cysts in pepsin HCl. The addition of copper acetate and alkali brings about the precipitation of a substance in the residue which is soluble in water and reduces Fehlings solution after hydrolysis. If it is rapidly washed, dissolved in acid, and reprecipitated with copper acetate and alkali, the substance will give a negative biuret test. Boil small portions for 3 to 4 minutes and pour into alcohol. The substance will settle out if the solution is allowed to stand.

Analysis of the Product:

It has the composition of hexosamino hexoside

Reducing substances: - Fehlings

Protein: - biuret

31.

Author: F. Müller

Reference: Zeit. f. Biol. (1901), 42, 468

Name of Product: Mucin

Method of Preparation: Precipitate with acetic acid. Wash sputum with 0.5% HCl. Grind the residue in dilute HCl. Wash the precipitate with water and alcohol until all the HCl is gone. Dissolve in dilute alkali. Filter. Centrifuge. Reprecipitate. Wash the precipitate free of the acetic acid with alcohol and ether. Dry.

Analysis of Product:

C: 48.26%

H: 6.91

O: 10.7

S: 1.4, 1.04

Carbohydrate: 36.7 (on basis of glucose)

Equivalent weight: 1 gm. required for the neutralization of 0.050 gm. NaOH.

32.

Author: F. Müller

Reference: Zeit. f. Biol. (1901), 42, 201

Name of Product: Mucous

Method of Preparation: The author isolated mucous from bronchial tubes.

33.

Author: Buerger and J.W. Gies

Reference: Am. J. Physiol. (1901), 6, 219

Method of Preparation: Disect 4600 grams of tissue from the main shaft of the Achilles tendon of an ox. For every gm. of moist tissue, extract with 2 cc. of lime water. Shake at regular intervals. Precipitate from the filtered extract with dilute 0.2% HCl.* Wash the precipitate with dilute HCl and water until acid-free. Redissolve in dilute alkali. Reprecipitate with dilute HCl. Wash again. Dehydrate. Purify with boiling alcohol and ether. Dry in vacuo. Weigh. (Powdered thymol will prevent bacterial action).

Yield: 1.361 gms., 1.420, 1,332, 1.220, 1.043, 1.228, 1.380.

*
0.2% is a high enough concetnration for the first extracts. In subsequent extracts, if 0.2% concentration does not bring about a precipitation, add 1.5% HCl in very small quantities. Follow the same procedure on re-precipitation.

34.

Author: P.B.Hawk and J.W.Gies

Reference: From, Am. J. Physiol. (1901), 6, 155

Name of Product: Osseomucoid

Analysis of Product:

C: 47.07%

H: 6.69

O: 31.85

N: 11.98

S: 2.41

35.

Author: P.B. Hawk and J.W. Gies

Reference: Am. J. Physiol., 5, (1901), 387

Name of Product: Osseomucoid

Method of Preparation: Remove the inorganic material from the salt free shavings of an ox rib or femur by treatment with HCl. Precipitate with excess dilute acid. Continue the extract for 48 hours. Treat the filtered extract with 0.2% HCl. Wash in water and HCl. Wash in plain water. Filter. Dissolve in 1/2 saturated lime water (2 to 5 grams of lime water for every gram of moist tissue). Wash in acidified water, plain water and alcohol. Repeat. Boil in anhydrous alcohol and ether. Remove the alcohol with ether. Dry in air.

Yield: 1700 grams of wet femur yields over 7 gms. of the product. 875 grams of rib shavings yields 3.5 gms. of the product.

Analysis of Product:

	RIB	FEMUR
N:	12.78, 12.99, 12.89% 12.91, 13.17	13.38, 13.41, 13.45% 13.77
S:	1.68, 1.75, 1.76 in SO_3: 0.98, 0.91	1.89, 1.87, 1.93 in SO_3: 1.04, 1.87
P:	0.086, 0.031, 0.013 ash P: 0.051, 0.039	0.108, 0.054, 0.022 ash P: 0.057, 0.061
Ash:	2.28, 2.19	2.62, 2.57

36.

Author: Vandergrift and J.W. Gies

Reference: Am. J. of Physiol. (1901), 5, 387

Name of Product: Mucin

Method of Preparation: Shake 100 gms. of Ligamentum Nuchae tissue in 250 to 300 cc. of half saturated lime water, for several days at room temperature. Precipitate the glucoproteid from the extract with 0.2% HCl. Filter, Dry at 110° C. Weigh.

Yield: (in %) 0.565, 0.429, 0.539, 0.510, 0.490, 0.514, 0.569.

37.

Author: W.G. Cutter and W.J. Gies

Reference: Am. J. of Physiol. (1902), 6, 155

Method of Preparation: The procedure is described on page 220, number 33.

Name of Product: Tendon Mucoid

Analysis of Product:

N: 13.25, 13.17, 13.33; 12.85, 12.94, 12.71, 12.83; 13.24,13.27, 13.25; 13.84, 13.88, 13.86

S: (Ba salt) 2.25, 2.26, 2.36, 2.25, 2.31; 1.81, 1.66, 1.74; 1.23, 1.41, 1.32

Ash: less than 1.78%

38.

Author: W.G. Cutter and W.J. Gies

Reference: Am. J. of Physiol., 6, (1902), 155

Name of Product: Tendon mucoid

Method of Preparation: The procedure is described on page , number 37.

Note: In this case 1900 gms. of the sheathes of the branches of ox Achilles tendon are used.

Analysis of Product:

N: 13.14, 12.96, 13.05; 12.41, 12.46, 12.43, 13.47, 13.70, 13.59%

S: 2.11, 2.34, 2.22; 2.67, 2.72, 2.70, 2.23 (Ba salt)

Amino sugar: glucosamine +

Glucuronic acid: +

39.

Author: W.G. Cutter and W.J. Gies

Reference: Am. J. of Physiol. (1902), 6, 155

Name of Product: Tendon Mucoid

Method of Preparation: The procedure is described on page 220 number 33,

Note: 6600 grams of the main shaft and branches of ox Achilles tendon are used. In purifying, redissolve with 0.5% Na_2CO_3.

Analysis of Product:

C: 47.47, 47.46, 47.80

H: 6.68, 6.56, 6.60

O: 31.07, 32.39, 31.09

N: 12.64, 12.64, 12.64; 12.68, 12.70, 12.69; 13.89, 13.92

13.91; 1406; 14.56

S: 2.70, 2.91, 2.80; 2.28, 2.39, 2.34; 2.47, 2.28, 2.38

Amino sugar: glucosamine +

Glucuronic acid: +

40.

Author: W.G. Cutter and W.J. Gies

Reference: Am. J. Physiol. (1902), 6, 155

Name of Product: Tendon mucoid

Method of Procedure: The procedure is described on page 220, number 33.

Note: 4200 grams of the sheath of ox Achilles tendon are used. In purifying, redissolve in 0.5% Na_2CO_3.

41.

Author: Richards and W.J. Gies

Reference: Am. J. Physiol. (1902), 7, 116

Name of Product: Mucoid

Method of Preparation: For every gram of moist tissue from ox ligamentum nuchae extract with 2 cc. saturated lime water, for 48 hours. Re-extract 48 hours. Combine the precipitates. Precipitate with 0.2% HCl. Redissolve in water and lime water. Reprecipitate in HCl.

Analysis of Product:

N: 12.80, 13.81, 12.90; 13.40, 13.64, 13.52; 13.90, 13.82, 13.86; 13.74, 13.66, 13.70; 13.27, 13.22, 13.25

S: 2.05, 2.09, 2.07; 1.77, 1.68, 1.73; 1.49, 1.49, 1.37, 1.27, 1.32; 1.45, 1.40, 1.42

in SO_3: 1.32, 1.17, 1.25; 1.02, 1.02; 0.90, 0.90

42.

Author: G. von Holst

Reference: Hoppe Seyler's Zeit. Physiol. Chem. (1904)

Name of Product: Serös Mucin

Method of Preparation: Precipitate ascitic fluid (fluid from cancer ventriculi and peritonei) with acetic acid. (The precipitate is slightly soluble in acetic acid). Dissolve the precipitate in water and a small quantity of alkali. Repeat twice. Purify by 3 precipitates with 4 to 5 volumes of alcohol.

Analysis of the Product:

	Acetic Acid Precipitate	Alcohol Precipitate
C:	51.41	51.45
H:	6.68	6.65
N:	13.31	13.23
P:	0	

43.

Author: G. von Holst

Reference: Hoppe Seyler's Zeit. Physiol. Chem. (1904), 43, 145

Name of Product: Synovial Mucin, Serous Mucin

Method of Preparation: Dilute 15 to 20 cc. of synovia from fresh cattle joints with 3 volumes of water. Add acetic acid up to a 1% solution in order to form a precipitate. Dissolve the precipitate in very dilute alkali. Add acetic acid to complete the precipitation. Purify with 3 precipitates of 4 to 5 volumes of alcohol. Dry with alcohol and ether at 110° C.

Analysis of Product:

C: 51.05

H: 6.53

N: 13.01

S: 1.34

P: 0

Ash: 0.58

Nuclein: 0

44.

Author: J. A. Mandel

Reference: Zeit. f. Physiol. Chem. (1905), 45, 386

Name of Product: A sulfuric ester of a carbohydrate.

Method of Preparation: The product may be prepared from mammary glands, kidney, pancreas or liver.

Analysis of Product:

	Mammary gland	Kidney	Pancreas	Liver
N:	3.18	4.99	4.65	4.93
S:	3.48	3.91	2.43	3.69

Salts: Ba, 9.81

45.

Author: J. A. Mandel

Reference: Biochem. Zeit. (1907), 4, 78

Name of Product: A sulfuric ester of a carbohydrate

Method of Preparation: Product is made from leucocytes that have been obtained from Pyothorax produced in horses. The horses are injected with aleuronate followed by an inoculation of staphpyrogenes aurius.

Analysis of the Product:

N: 4.66

S: 3.07

Pentoses: Orcin, +

Protein: Biuret, -

46.

Author: E.H.B. van Lier

Reference: Zeit. f. Physiol. Chem. (1909), 61, 177

Name of Product: Chondroitin sulfuric acid, a sulfuric ester of a carbohydrate.

Method of Preparation: The product is prepared from mammalian dermis by "the older method of Levene."

Analysis of Product:

H: 3-4%

S: 1.80-3.00

Pentoses: Furfural, +; Orcin; +

Reducing substances: glucosazone

47.

Author: P.A. Levene and Jacobs

Reference: J. Exp. Med. (1908), 10, 557; Biochem. Z. (1909), 16, 248

Name of Product: Mucoitin sulfuric acid.

Analysis of Product:

C: 27.29%

H: 3.64

N: 2.58

S: 4.85

Salts: Ba, 21.90

48.

Author: P.A. Levene

Reference: J. Biol. Chem. (1913), 15, 155; ibid (1914), 18, 123; ibid (1915), 20, 433

Name of Product: Condroitin sulfuric acid

Method of Preparation: Stand the disected nasal sputums of cattle in portions of 5 kilos for 2 days, in 0.2% KOH. Wash once with water. Strain. Repeat with the residue and 5 liters of KOH. Wash once with water. Acidify the extracts with acetic acid. Concentrate to one-half the volume on a sta bath in the presence of excess $BaCl_2$. Pour off the clear liquid and filter the residue. Both the clear liquid and the filtered residue are brought to 2 liters and acidified. Centrifuge to remove $BaCO_3$. Drop the liquid in 8 times its volume of glacial acetic acid. Agitate with a turbin in order to bring out the potassium salt. Filter the K salt by suction. Wash with glacial acetic acid, then alcohol and ether. (At this stage 200 gms. of the product gives a slight positive biuret test). Dissolve in 10 liters of water. Stir with a turbine. Add basic lead acetate until there is a complete precipitation. Grind the lead salt in a mortar with water and filter through a suction. Repeat this three times. Suspend in 5 liters of water, 100 gms. Ba acetate and 50 cc. acetic acid. Decompose with H_2S and stir. Stand 12 hours. Filter of PbS. Precipitate the barium salt by the addition of 1/3 the volume of 95% alcohol. Filter. Wash with 50% alcohol, then 95% absolute alcohol and ether. (A white

powder that gives a negative Biuret is formed). At this point the product is the Ba salt of chondroitin and chondroitin sulfuric acid.

To repurify 25 gms. of the Ba salt of chondroitin sulfuric acid: Dissolve in 2 liters of water plus 10 gms. Ba chloride. Add 1 liter of 95% alcohol to form a precipitate. Wash the precipitate Cl-free by the addition of 50% alcohol, absolute alcohol and ether. Dry in a vacuum.

To remove small amounts of the lead salt: Dissolve the lead salt in 10% HCl. Remove the filtrate from lead chloride by precipitation of the chondroitin sulfuric acid with glacial acetic acid. Wash with glacial acetic acid, alcohol and ether.

49.

Author: P.A. Levene and J. López-Suárez

Reference: Biochem. Zeit. (1913), 56, 170

Name of the Product: Mucoitin Sulfuric acid

Analysis of Product:

C: 43.29

H: 5.47

S: 5.37

Salts: Ba, 4.29

50.

Author: F. Alzona

Reference: Biochem. Zeit. (1914), 66, 408

Name of Product: Chondroitin sulfuric acid

Method of Preparation: Add 2% NAOH to the minced pig stomach, intestines, urinary bladder or parenchymatous organs. Stand 24 hours. Partially neutralize the solution by the addition of acetic acid.

Add picric* and acetic acid by turn until further addition ceases to form a precipitate. Filter. Add 5% $CaCl_2$ to the filtrate. Concentrate the filtrate from the calcium precipitation until the addition of acetic acid brings about a floculant precipitate. This reagent precipitates the conjugated sulfuric acid.

Analysis of Product:

C: 28.97%

H: 5.09

N: 3.61

S: 8.03

Protein: Orcin -

Glucuronic acid: Naphthoresorcin +

Pentose : Orcin, +

Equivalent weight: Na, 11.27

51.

Author: F. Alzona

Reference: Biochem. Zeit. (1914), 66, 408

Name of Product: A sulfuric ester of a carbohydrate

Method of Preparation: The product is made from human prostate.

* "The reason for the use of picric acid .... The carbohydrate component of mucoproteins have in common with picric acid the property of combining with protein at a certain hydrogen ion concentration which is attained by acidulation with acetic acid. When the protein is detached from its carbohydrate by means of alkali and when the solution is rendered acid with acetic acid a large part of the protein recombines with chondroitin or mucoitin sulfuric acid. The addition of picric acid aims to reduce this combination to a minimum."

Analysis of Product:

C: 28.88%

H: 3.71

N: 2.72

Pentoses: Orcin, -

Glucuronic Acid: nephthoresorein, -

Equivalent weight: Na, 16.24

52.

Author: Levene, P.A. and La Forge

Reference: J. Biochem. (1914), 18, 239

Name of Product: Mucoitin sulfuric acid

Analysis of Product:

C: 25.13%

H: 3.88

N: 2.11

S: 4.26 (Ba salt)

Salts: Ba, 18.35

53.

Author: P.A. Levene

Reference: J. Biochem. (1916), 28, 373

Name of Product: Mucosin

Method of Preparation: Stand blood-free minced umbilical cords for three days in 7.2% NaOH. Acidulate. Centrifuge to remove the precipitate. Concentrate the liquid with excess Barium carbonate on water bath for 24 hours. Centrifuge. Add barium carbonate to the remaining liquid. Stand in warm

water bath. Add water from time to time. Continue for 2 days. Centrifuge. Precipitate the clear solution with glacial acetic acid. Redissolve in water and add barium acetate. Reprecipitate with glacial acetic acid. Wash with alcohol. Dissolve in water. Neutralize with BaOH. Add 95% alcohol. Wash with increasing concentrations of alcohol up to 99.5%. Wash with ether. The product thus obtained is the barium salt of mucoitin sulfuric acid. Dissolve 4.5 gms. of this barium salt in 100 cc. 10% HCl. Heat in water bath for one-half hour. Concentrate to 3 cc. in a 45°C. water bath under diminished pressure. Pour into 200 cc. alcohol and 400 cc. dry ether in order to bring about a white flocculent precipitate. Stand for 8 to 12 hours. Filter and dry.

Yield: 1.5 gm.

Analysis of Product:

C: 38.25%

H: 5.8

Glucosamine: Acetyl -

Glucuronic acid: +

Protein: NH_2N, 3.24

Specific rotation: $(\ )_D^{20}$ - + 25.55

54.

Author: P.A. Levene and J. López-Suárez

Reference: J. Biochem. (1916), 26, 373

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Collect 75 umbilical cords in 95% alcohol. Wash wihh water until free from alcohol and place in 5 liters 3% NaOH for 3 days. Acidulate with acetic acid. Effect neutralization in part by barium hydrate and complete with $BaCO_3$. Filter. Precipitate the conjugated

sulfuric acid by adding basic lead acetate. Wash the precipitate 6 times by decantation with distilled water. Filter. Suspend the precipitate in water and repeat the process. Filter on suction funnel. Dissolve in 100 cc. glacial acetic acid. Add excess (2 liters) of acid to the solution in order to precipitate the conjugated sulfuric acid. Filter in a suction funnel. Wash with alcohol, then ether. Suspend in water. Free from the lead by passing water through the mixture while it is mechanically stirred. Separate the lead in the colloidal state. Add a slight excess of barium hydrate to make possible its removal. Boil until the coagulation of the sulfide is complete. Remove the sulfide by means of the centrifuge. Remove the excess barium with CO_2 gas. Remove CO_3 by centrifuging. Treat the clear solution with an equal volume of 98% alcohol to precipitate the conjugated sulfuric acid. Filter. Wash with 50% alcohol, then alcohol of increasing concentrations. Dissolve the substance in a small amount of water. Centrifuge the insoluble part. Precipitate the clear solution with alcohol in order to obtain a Biuret-free substance. Dry under diminished pressure over sulfuric acid at a temperature of water vapor.

Analysis of Product:

C: 32.65%

H: 4.61

N: 4.53, for the Kjeldahl test, 0.1228 gm. neutralized 3.97 cc. 1/10N acid.

S: 3.05 (Ba salt)

Salts: Ba, 2167

Specific Rotation: $(\alpha)_D^{25}$ initial, 84.82°; equilibrium, 71.94°

55.

Author: P.A. Levene and J. López-Suárez

Reference: J. Biolchem. (1916), 26, 373

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Collect 75 umbilical cords in 95% alcohol. Wash in water free from alcohol and place in 5 liters of 3% NaOH for 3 days. Acidulate the solution with acetic acid. Effect partial neutralization by the addition of barium hydrate, and complete the neutralization by the addition of barium carbonate. Filter. Add a slight excess amount of barium carbonate. Place for 5 hours in a boiling water bath. Cool. Centrifuge to remove the precipitate of carbonate and coagulated protein. Add glacial acetic acid to clear the solution, until a precipitate no longer forms. Filter through a suction funnel. Wash with acetic acid, and alcohol. Dissolve in water. Make the solution alkali with addition of barium hydrate. Precipitate the excess reagent by means of CO_2 . Centrifuge for the removal of carbonate. Treat the clear solution with 98% alcohol until a precipitate no longer forms. Dry under diminished pressure over H_2SO_4 at the temperature of water vapor.

The resulting product is a white amorphous powder.

Analysis of the Product:

C: 35.61%

H; 5.30

N: 3.57; Keldehl, 0.1371 gm. neutralized 3.50 cc. 0.1 N acid.

Protein: Biuret, -

Specific rotation: $(\alpha)^{25}_{D}$ initial, 84.82, equilibrium, 71.94°

Salts: Ba, 21.67

S: 2.35

56.

Author: P.A. Levene and J. López-Suárez

Reference: J. Biol. Chem. (1916), 25, No. 3

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Dissolve the mucous contents of fresh pig stomach in 3% NaOH. Stand for 3 days. Acidify with acetic acid. Add excess $BaCO_3$. Place in a water bath until the liquid is clear. Remove the precipitate with a centrifuge.

To precipitate with lead acetate: Suspend the lead salt in 10 parts of water. Add excess $BaCO_3$. Pass H_2S through the solution. Agitate with a mechanical stirrer. Keep at 95° C. Concentrate the filtrate under diminished pressure to 1/4 the volume. Add an equal amount of 95% alcohol to bring out the crude barium salt.

Or, to precipitate with glacial acetic acid, the precipitate should be dissolved in water and precipitated with an equal volume of alcohol.

To remove the nucleic acid: Dissolve the crude substance in water. Add excess BaOH. Remove the excess BaOH by passing a stream of carbonic acid gas through the solution. Centrifuge to get rid of the barium salt of the nucleic acid and the $BaCO_3$. Add an equal volume of alcohol to clear the filtrate. Redissolve the precipitate. Reprecipitate the solution. Continue until the final precipitate gives an absolutely clear solution. (80% of the crude material is lost). Dry under diminished pressure at the temperature of water vapor.

Analysis of Product:

C: 36.08%

H: 5.32

N: Kjeldahl, 1.38 gm. neutralized 3.5 cc. 0.1 HCl.

S: 1.80, 1.85 (Ba salt)

Amino sugars: glucosamine, 3.53, 2.89

Salts: Ba, 21.49, 14.38

Specific rotation: $(\alpha)^{20}_{D}$ initial, -90.10; equilibrium, - 70.63

57.

Author: P.A. Levene

Reference: J. Biolchem. (1918), 36, 128

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Stand blood serum mucoid for three days in 50% NaOH. Acidulate with acetic acid. Concentrate on water bath in excess $BaCO_3$. Convert the filtrate into the lead salt. Treat with glacial acetic acid. Dry with alcohol. Free from the lead. Reprecipitate with lead acetate. Wash the lead salt many times with glacial acetic acid. Dry.

Yield: 14 gms.

Analysis of Product:

N: 5.10%

58.

Author: P.A. Levene

Reference: J. Biolchem. (1918), 36, 128

Method of Preparation: Boil 12.5 liters of beef blood serum. Stand 3 days at 40°C. in 50% NaOH. Acidify with acetic acid. Concentrate on water bath with excess $BaCO_3$. Convert filtrate into Pd salt. Treat with glacial acetic acid. Dry with alcohol. Free from lead. Reprecipitate with lead acetate. Wash the lead salt many times with glacial acetic acid. Convert

the lead salt to a barium salt the following way: Suspend the lead salt in water. Add excess $BaCO_3$. Pass H_2S through the solution until the lead has separated out. Get rid of the H S by aeration.. Precipitate the solution with 99.5% alcohol. Dissolve the precipitate in a little water. Centrifuge to remove all the soluble barium salts. Repeat this a few times. Precipitate the solution with 99.5% alcohol. Dry.

Yield: 1.5 gm.

Analysis of Product:

C: 29.71%

H: 4.44

N: 5.25

S: 1.96 (Ba salt)

Salts: Ba, 29.14

59.

Author: P.A. Levene

Reference: J. Biochem. (1918), 36, 111

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Add barium hydroxide to the mucous from gastric wall until a concentration of 3% is reached. Stand for three days at room temperature. Add H_2SO_4 until the solution is acid to Congo Red. Centrifuge. Neutralize the supernatant liquid with Ba $(OH)_2$ until it is neutral to Congo Red, but, acid to litmus. Neutralize to litmus with $BaCO_3$. Boil for three hours. Filter. Add $BaCO_3$ to the filtrate. Place in a water bath for 2 to 3 days, until negative to the biuret test. Centrifuge. Precipitate the supernatant liquid with glacial acetic acid. Redissolve the precipitate in water. Reprecipitate with glacial acetic acid. Wash

the precipitate many times with 95% alcohol until almost all the glacial acetic acid is gone. Dissolve in a minimum amount of water. Make the solution exactly neutral with $Ba(OH)_2$. Precipitate the barium salt of mucoitin sulfuric acid with alcohol. Wash the crude salt repeatedly with a 50% solution of alcohol until the inorganic impurities are gone. Wash with alcohol of increasing concentrations. The product at this point is a salt composed of mucoitin sulfuric acid and nucleic acid. Take up the mixture in water and centrifuge in order to separate the two acids. Repeat. Pour the clear solution into excess alcohol to precipitate the barium salt of mucoitin sulfuric acid.

Analysis of Product:

C: 3.47%

S: 1.48; $BaSO_4$, 0.1500 gm. of the product gave 0.0162 gm $BaSO_4$.

Equivalent weight: 0.1000 gm. neutralizes 2.48 cc. 0.1 N acid

Specific rotation: $(\alpha)_D^{20}$, 22.54°

60.

Author: P.A. Levene

Reference: J. Biolchem. (1913), 36, 111

Name of Product: Mucosin

Method of Preparation: Prepare mucoitin sulfuric acid as described above (#59). Dissolve 14 gms. of the barium salt in 100 cc. water and 15 cc. HCl. Stand on boiling water bath for 20 minutes. Concentrate the solution under diminished pressure, in a 40° C. water bath, to 5 cc. Pour into 1 liter 95% alcohol and 1 liter ether. Filter the precipitate. Dissolve it in 3 cc. water. Precipitate with 400 cc. 95.5% alcohol. Add

an equal volume of ether to form a second precipitate.

Analysis of Product:

Protein: NH_2N, 2.12%; Van Slyke, 1 gm. gave 1.38 cc N. at 22 and 75.3 mm. pressure.

Specific rotation: $(\alpha)_D^{20}$ +25.75°

61.

Author: P.A. Levene

Reference: J. Biol. Chem. (1918), 36, 111

Name of Product: Chondroitin sulfuric acid

Method of Preparation: Stand, hashed, dissected aorta for 36 hours in 2% NaOH. Decant and extract for 36 hours. Strain combined solutions and neutralize them. Concentrate with excess $BaCO_3$. Filter. Wash the precipitate with glacial acetic acid. Wash with alcohol. Dry. Dissolve the remaining product after hydrolysis in water. Reprecipitate with glacial acetic acid. Wash the precipitate with glacial acetic acid and alcohol. Dry and dissolve in KOH. Add excess $BaCl_2$. Add an equal volume of 95% alcohol. Wash with 50% alcohol until free from $BaCl_2$. Continue with increasing strengths of alcohol, and ether. Dry.

Analysis of Product:

C: 28.7%

H: 3.35

N: 2.54

S: 2.4; $BaSO_4$, 2 gms. of the product gave .0354 gm. of $BaSO_4$ on fusion.

Equivalent weight: 0.2 gm. are required to neutralize 3.63 cc. cc. 0.1 N acid

Specific rotation: (α), + 42.0°

62

Author: P. A. Levene

Reference: J. Biolchem. (1918), 36, 111

Name of Product: Chondrosin

Method of preparation: Prepare chondroitin sulfuric acid as described above (#61). Hydrolyze chondroitin sulfuric acid for 1 hour in a boiling water bath with 60 cc. of 20% HCl. Filter the reaction product. Concentrate under diminished pressure. Precipitate with alcohol and ether.

Yield: 4 gms.

Analysis of Product:

N: 4.34%; NH_2N, 3.41

Specific rotation: + 42.0

Equivalent weight: 0.1 gm. are required to neutralize 3.1 cc. 0.1 N acid

Van Slyke micro-apparatus: 0.020 gm. gave 1.2 cc. N at 25° C. and 759.6 mm. pressure

63

Author: P. A. Levene

Reference: J. Biolchem. (1918), 36, 111

Name of Product: Chondroitin sulfuric acid

Method of Preparation: Extract the sclera of 1000 eyes in 20 liters of 3% NaOH. Continue the extraction for 3 days. Strain. Neutralize

with acetic acid. Add barium carbonate in excess. Concentrate to a small volume in a water bath. Filter the reaction product on a suction funnel. Convert into lead salt, and then into sodium salt.

Analysis:

C: 34.27%

H: 4.86

N: 5.66

S: 4.67 (Ba salt)

64

Author: P. A. Levene

Reference: J. Biolchem. (1918), 36, 111

Name of Product: Mucoitin sulfuric acid

Method of Production: Extract vitrous humor for 3 days in 50% NaOH. Add excess $BaCO_3$. Acidulate and concentrate in a water bath. Filter on suction. Add basic lead acetate to precipitate all the acid. Wash the lead acetate by decantation. Filter. Wash the precipitate with glacial acetic acid. Filter. Wash the precipitate with alcohol. Put in water. Add KoH until the solution is slightly alkaline. Decompose the lead salt with H_2S. Remove the H_2S by aeration. Pour the solution into 2 liters of alcohol. Wash the precipitate with ether and alcohol.

Analysis of Product:

O: 34.39%; 0.0958 Ba salt yielded 0.1208 gm. CO_2

H: 5.72

N: 4.96

S: 3.63; $BaSO_4$, 0.2 gm. of the product gave 0.0528 gm. $BaSO_4$

Moisture: 0.0958 Ba salt yielded 0.0490 gm. H_2O

Equivalent weight: 0.1 gm. neutralized 3.54 cc. 0.1 N acid

65

Author: P. A. Levene

Reference: J. Biolchem. (1918), 36, 111

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Extract the cornea of beef eyes in 3% NaOH. Strain. Acidulate with acetic acid. Add $BaCO_3$ and concentrate on a water bath. Filter ~~on a~~ WITH suction. Add lead carbonate to precipitate all the acid.

An alternative method is to acidulate the solution concentrated with barium carbonate. Pour the filtrate into excess glacial acetic acid. Wash many times with glacial acetic acid and alcohol. Redissolve the dry substance in water and KOH. Pour the solution into excess 99.5% alcohol. Dry and hydrolyse the potassium salt.

Analysis of the Product:

C: 37.85%; 0.0950 of the K salt yielded 0.1318 gm. CO_2

H: 6.32

N: 4.62

S: 14.74; in the first method 0.200 gm. of the product yielded 0.0330 gm. $BaSO_4$; in the second method 0.0452 gm. was yielded

Moisture: 0.0950 gm. K salt yielded 0.0528 gm. H_2O

Equivalent weight: 0.1000 gm. of the product neutralized 2.42 cc. 0.1 N acid, in the second method, 6.9 cc.

66

Author: P. A. Levene

Reference: J. Biolchem. (1925), 65, No. 3, 690

Name of Product: Mucoprotein

Method of Preparation: Remove the snail, Helix Aspersa from its shell and by rubbing the body with a glass rod, collect the mucous. Keep this mucous in alcohol until 500 grams are collected. Acidulate with acetic acid. Filter. Put the filtrate in fresh alcohol. Reflux for 2 to 3 hours. Pass the mucoprotein through a hydrolic press. Mince the dry cake that results. Suspend in large quantities of hot water and 1 to 2% acetic acid. Remove the lime salts by turbinating. Renew the water every 2 hours until incineration of the suspended material leaves only a small residue. Wash the suspended material with cold water. Filter. Pass through a hydrolic press.

67

Author: P. A. Levene

Reference: J. Biolchem. (1925), 65, No. 3, 689

Name of Product: Obtain mucoprotein from the snail, Helix aspersa according to directions given above (#66). Extract 500 gms. of the moist mucoprotein in 1000 cc. of 5% NaOH. Place in shaking machine for 48 hours. Put the viscous fluid in twice the volume of 95% alcohol. Centrifuge to remove the precipitate. Wash and repeat the process with 95% alcohol. Suspend the precipitate in 800 cc. 5% aqueous NaOH. Shake for 24 hours until the sodium salt appears. Transfer the mixture to twice its volume of 95% alcohol. Centrifuge to separate the precipitate from the fluid.

To remove the protein: Suspend in 5% NaOH. Place in shaking machine for 1 hour. Centrifuge to sediment the mucoitin sulfuric acid. Replace the alkali with distilled water. Place in a shaking machine. Repeat until a negative biuret is given.

To remove the calcium: Add HCl to the mucoitin sulfuric acid in water, until it is acid to Congo Red. Shake for 1/2 hour. Keep replacing HCl with fresh water until the chloride ions are gone. Dry with alcohol and ether.

Yield: 500 gms. of moist mucoprotein yeilds 15-18 gms. mucoitin sulfuric acid.

Analysis of Product:

N: 2.66%

S: 2.34; 0.2048 gms. of the product gave 0.350 gms. $BaSO_4$

Protein: Biuret, -

Equivalent weight: 0.1000 gms. of the product are required to neutralize 0.350 gm. $BaSO_4$.

68.

Author: P.A. Levene

Reference: J. Biol. Chem. (1925), 65, No. 3, 689

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Obtain mucoprotein from the snail, Helix aspersa as described above (# 66). Place the mucoprotein in twice its weight of NaOH. Place in water bath for 3 hours. Cool the clear solution. Neutralize with acetic acid. Centrifuge in order to remove the protein. Precipitate the mucoitin sulfuric acid from the supernatant liquid with

basic lead acetate and amonia. Centrifuge to free the precipitate from the supernatant liquid. Wash in water. Add sufficient HCl to convert all the lead into its chloride. Remove the lead chloride by centrifuging. Pour the supernatant fluid into excess glacial acetic acid to form the flocculant precipitate of mucoitin sulfuric acid. Free from the supernatant liquid by centrifuging. Add a small amount of water. Precipitate with alcohol.

Yield: 500 gms. of moist mucoprotein yields 10 gm. of mucoitin sulfuric acid.

Analysis of the Product:

C: 40.43%

H: 5.66%

S: 1.14; 0.1635 gm. of the product yielded 0.0136 gm. of $BaSO_4$

Protein: Biuret -

Equivalent weight: 0.1626 gm. of the product are required to neutralize 3 cc. 1 N acid.

69.

Author: P.A. Levene

Reference: J. Biol. Chem. (1925), 65, No. 3, 690

Name of Product: Mucoitin sulfuric acid.

Method of Preparation: Obtain mucoprotein as described above (#66). Obtain the lead salt of mucoitin sulfuric acid as described above (#68). Suspend the lead salt in water and barium acetate. Pass H_2S through the mixture. Concentrate the filtrate to a small volume. Pour in large excess glacial acetic acid. Add water. Reprecipitate with alcohol.

Analysis of Product:

N: 4.90%

S: 1.65; 0.2590 gm. of the product yields 0.0312 gm. $BaSO_4$.

Equivalent weight: 0.1000 gm. of the product are required to neutralize 3.50 cc. 1 N acid.

70.

Author: P.A. Levene

Reference: J. Biol. Chem. (1925), 65, No. 3, 689

Name of Product: Mucosin

Method of Preparation: Obtain mucoprotein as described above (# 66). Obtain mucosin sulfuric acid as described above (#s 67, 68 or 69).

To hydrolyze no further than mucosin formation: Place 10 gms. of mucoitin sulfuric acid in 200 cc. 20% oxalic acid. Reflux for 1 hour over a free flame. This results in the formation of the white precipitate of calcium oxalate. Centrifuge to free from sediment. Pour into 2 1/2 times its volume of ether. Dissolve 5 gms. of the product in 100 cc. 10% HCl. Reflux for 1/2 hour on a boiling water bath. Concentrate the solution under reduced pressure. Pour into a mixture of 300 cc. alcohol and 300 cc. ether. Dissolve in 50 cc. of 20% HCl and treat as before.

Yield: 1.2 gms.

Analysis of Product:

N: 4.69%; NH_2N, 4.02

Glucuronic acid: 50

71.

Author: P.A. Levene

Reference: J. Biol. Chem. (1925), 65? No. 3, 689

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Extract mucoprotein from minced snails foot. Centrifuge the solution. The mucoprotein will precipitate from the supernatant liquid after the addition of acetic acid. Redissolve and reprecipitate several times. Reflux the final precipitate in 95% alcohol for several hours. Add alkali. Treat as described above (# 68).

Analysis of Product:

C: 40.39%

H: 6.19

N: 3.58

S: 0.48

Glucuronic acid: strongly +

Protein: Biuret, -

72.

Author: P.A. Levene

Reference: J. Biol. Chem. (1925), 65, No. 3, 689

Name of Product: Mucoitin sulfuric acid; polysaccharide from "glucoprotein."

Method of Preparation: Prepare mucoprotein and mucoitin sulfuric acid as described above (# 71). Isolate the carbohydrate as described above (# 68). Precipitate out of solution with alcohol.

Analysis of Product:

C: 42.61%

H: 6.38%

N: 0.98

S: 0.67

Specific rotation: initial, $(\alpha)_D^{20}$ + 0.35, equilibrium, + 0.16

73.

Author: P.A. Levene

Reference: J. Biol. Chem. (1925), 65, 689

Method of Preparation: Extract snail bodies in twice their volume of boiling water for two hours. Add 3% KOH and 8% potassium iodide. Add alcohol until a precipitate is no longer produced. Centrifuge. Wash the precipitate several times with 50% alcohol and 8% potassium iodide. Dissolve the crude substance in HCl of a specific gravity of 1.19. Cool on ice and salt. Centrifuge. Pour the supernatant liquid into alcohol. Centrifuge the precipitate. Wash with alcohol to get rid of the free mineral acid. Suspend the precipitate in water. Reprecipitate with alcohol. At this point the product is a mixture of polysaccharide and mucoitin sulfuric acid.

To separate the two substances: Precipitate the centrifuge supernatant with alcohol. Purify part of the insoluble fractions with HCl. Redissolve the polysaccharide in water. Centrifuge. Precipitate the supermatant liquid in alcohol.

Name of Product : Polysaccharide

Analysis of Product:

C: 42.61

H: 6.38

O: 0.98

N: 15.69

S: 0.67

Equivalent weight: 0.0925 gm. of the product is required to neutralize 0.65 cc. of 0.1 N alkali.

74.

Author: P.A. Levene

Reference: J. Biol. Chem. (1925), 65, No. 3, 689

Name of Product: Mucoitin sulfuric acid

Method of Preparation: Prepare mucoitin sulfuric acid from the snail bodies of Helix pomatia as described above(#s 67,68).

Analysis of Product:

N: 2.32%

S: 5.37

Salts: Ba, 9.01

75.

Author: P.A. Levene

Reference: J. Biol. Chem. (1925), 65, No. 3, 689

Method of Preparation: The product is prepared from the foot of the snail, Helix pomatia, according to the procedure described in number 72.

Analysis of Product:

C: 36.88%

H: 5.09

N: 1.2

S: 0.8

Ash: 8

Specific rotation: $(\alpha)_D^{25}$, initial + 0.10°, equilibrium, + 10°

76.

Author: P.A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co., London, (1925), 135

Name of Product: Mucin

Method of Preparation: Take up submaxillary glands in ether. Mince and extract with water for 24 hours. Add chloroform to prevent bacterial growth. Decant supernatant fluid. Add ether and thoroughly shake the mixture. Draw off the clear lower layer continue according to Hammersten as described above (# 9).

77.

Author: P.A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co., London, Co., (1925), 137

Name of Product: Mucin

Method of Preparation: Mince the bodies of snails. Extract with 0.01% - 0.02% KOH. Centrifuge. To facilitate the removal of suspended particles Hammarsten liberal dilution of the extract, e.g. 60-70 snails into 9 to 12 liters of water. Precipitate with acetic acid. Continue according to Hammarsten as described above (# 8).

78.

Author: Willanen

Reference: From, Hexosamines and Mucoproteins, Longmans, Green and Co., P.A. Levene, (1925), 137

Name of Product: Ovomucoid

Method of Preparation: Add white of egg to 4 volumes of water. Strain. Make strongly acid with acetic acid and pour into 1 1/2 volumes of boiling water. Bring the mixture to a boil with constant stirring. Concentrate the filtrate from the coagulated protein to a small volume and pour into

5 volumes of alcohol. Repurify the mucoid by re-extraction with water and reprecipitate with alcohol.

79.

Author: Melesi

Reference: From, Hexosamines and Mucoproteins, Longmans, Green and Co., (1925), P.A. Levene, 137

Name of Product: Ovomucoid

Method of Preparation: Precipitate the total protein of egg white with 99 1/2% alcohol. Filter the precipitate and dry in vacuo at ordinary temperature. Extract with cold water and precipitate the mucin with alcohol.

80.

Author: P.A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co., (1925), 137

Name of Product: Serum Mucoid

Method of Preparation: Dilute 1200 cc. of blood serum with 2 liters of 1/2% salt solution. Remove the coagulable proteins from this solution by boiling and acidifying with acetic acid. Concentrate the clear filtrate in vacuo at 45° to a small volume and precipitate with alcohol. Filter the precipitate and wash with ether and alcohol until it is practically clear.

81.

Author: P. A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co., (1925), London, 139

Name of Product: Pseudomucin

Method of Preparation: Filter the contents of cysts and pour into 2 to 3 volumes of 95% alcohol. Remove the precipitate on a glass rod. Free in press from adhering liquid and transfer into alcohol. Remove alcohol with ether. Dry. Purify by dissolving in water and repeat process as before. Solubility lost on long standing in alcohol.

82.

Author: P. A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co. (1925), London, 139

Name of Product: Paramucin

Method of Preparation: To the solid material from the semisolid content of cysts (colloid) add dilute aqueous hydrogen chloride until the mixture just begins to react to Congo Red. Wash the solid material with alcohol containing very little hydrogen chloride until the wash alcohol is colorless. Carefully wash the substance with absolute alcohol and then with ether and dry.

83.

Author: P.A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co., London (1925), 139

Name of Product: Acetic acid soluble fraction of urine mucoid

Method of Preparation: Prepare urine mucoid as described above (#19)

Concentrate the solution obtained on the washing of the insoluble fraction. Precipitate with acidulated alcohol. Redissolve the precipitate in a little water. Free from mineral salts by dialysis and take up the solution in alcohol. The mucoid settles out in little floccules after the addition of very little salt. Filter and wash the substance with alcohol and ether.

84.

Author: P.A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co., London (1925), 106

Name of Product: Chondroitin sulfuric acid

Method of Preparation: Minced tissues from pig's stomach, intestines, bladder, or parenchymatous organs are taken up in a 2% solution of NaOH and the solution is allowed to stand 24 hours. Partially neutralize the solution with acetic acid and then treat alternately with picric acid* and acetic acid until further addition ceases to form a precipitate. Precipitate the chondroitin sulfuric acid from the filtrate with alcohol. The nucleic acid present in the case of parenchymatous organs can be removed by means of cupric chloride. Precipitate the conjugated sulfuric acid that remains in the filtrate with alcohol. If nucleic acid is still present, dissolve the mixture in water, add excess barium hydroxide, and precipitate the mixed barium salts with alcohol and glacial acetic acid. The two substances are separated by their difference in solubility in water. Repeat the operation if necessary.

*The reason for the use of picric acid is described above(#50).

Analysis of the Product:

N: 5.43%

S: 3

85.

Author: P.A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co., London (1925), 107

Name of Product: Hyaloidin

Method of Preparation: When ovarial fluid is hydrolyzed with strong alkali hyaloidin, protein fragments and melanoid substances are formed. Add H_2O_2 to destroy the melanoid substances and some of the protein fragments. Add a small amount of H_2O_2 and water twice a day for 2 to 3 days at the end of which time the blue color from copper disappears and the suspension acquires a yellow color. By the addition of acetic acid, the hyaloidin is precipitated with copper acetate and alcoholic potassium hydroxide solution and further treated as described above.

86.

Author: P.A. Levene

Reference: Hexosamines and Mucoproteins, Longmans, Green and Co., London, (1925), 113

Name of Product: Chondroitin sulfuric acid

Method of Preparation: Clean portions of 50 Achilles tendons from cattle. Pass through a hashing machine and stand over night with 20 liters of 2/3 saturated lime water. Strain off the liquid and repeat once again on the residue. Just acidify the combined filtrates with hydrochloric acid to

produce a flocculent precipitate of tendomucoid. Siphon off the supernatant liquid and add an equal volume of 95% alcohol. Filter off the mucoid. Agitate the moist product with 1.5 liters of a 2% KOH solution. Stand over night. Acidify the turbid brown solution with acetic acid. Filter off the separated protein. Neutralize the filtrate with NaOH. Precipitate the chondroitin sulfuric acid by a solution of basic lead acetate. Wash the lead precipitate by triturating on a mortar with distilled water and filtering with suction. Suspend the washed product in about 2 liters of water. Add 10 cc. of glacial acetic acid and 20 gm. of barium acetate and effect decomposition by passing in H_2S with constant stirring. Filter off the lead sulfide with suction. Concentrate the filtrate to about 350 cc. Precipitate the barium salt by the addition of about 250 cc. of alcohol. Filter with suction. Wash with 50%, 95% and absolute alcohol, and then with ether.

Yield: 12 to 15 gms.

Analysis of Product:

C: 35.8%

H: 6.0

N: 3.45

Specific rotation: $(\alpha)_D + 41.5°$

87.

Author: D.H. Kling

Reference: DEutsch. Arch. Klin. Med. (1931), 172, 165

Name of Product: Mucin substance

Method of Preparation: Centrifuge cattle synovial fluid until clear of suspended cells. Transfer to large wide-based centrifuge tubes. Gradually

add 1/2 volume of 3% acetic acid. Shake, Centrifuge at high speed for 10 minutes. Remove the skin with a glass rod. Decant the near clear fluid. Put the slimy precipitate into the original volume of 1/10% NaOH and shake. Reprecipitate. Centrifuge. Decant the supernatant fluid. Wash the precipitate in alcohol and water. Dry the residue over H_2SO_4.

Analysis of Product:

P: -

88.

Author: K. Meyer and J.W. Palmer

Reference: J. Biol. Chem. (1934), 107, 629

Name of Product: Hyaluronic acid

Method of Preparation: Place the vitreous humor of 100 fresh cattle eyes in acetone. Extract the resulting acetone powder 3 times with 200 cc. portions of 90% alcohol.and acetic acid. Wash the residue with alcohol until most of the acetic acid is gone. Suspend in water. Neutralize with N NaOH. Repeat the extraction with water on centrifuged tissue.

Yield: 0.7 to 1 gm. of the product from every 100 eyes.

Analysis of Product:

O: 13.5%

N; 5-6

Reducing substances: glucose, 30-40

Carbohydrates: Molisch, strongly + after hydrolysis

89.

Author: K. Meyer and J.W. Palmer

Reference: J. Biol. Chem. (1934), 107, 629

Name of Product: Mucoid

Method of Preparation: Vitreous humor from 100 fresh cattle eyes is stirred vigorously. Place in ice box over night. Filter by suction. Wash with acetone and ether. Dry in vacuo over P_2O_5.

Analysis of Product:

N: 7%

Ash: 40

Moisture: 11%

PH: over 10

90.

Author: K. Meyer and J.W. Palmer

Reference: J. Biol.Chem. (1934), 107, 629

Name of Product: Hyaluronic Acid

Method of Preparation: Pour the aqueous extracts of the acetone precipitate of vitreous humor into 6 times the volume of alcohol and 3 cc. of glacial acetic acid. Stand in ice box over night. Take up in a small volume of water. Pour into 15 times the volume of glacial acetic acid. Place the stringy substance in the ice box over night. Wash with alcohol acetone and ether. Dry in vacuo over P_2O_5. The inorganic material may be removed by dissolving the substance in 0.2 N HCl and reprecipitating with glacial acetic acid.

Yield: 100 eyes yielded 0.73 gm. or 30% of organic material.

Analysis of Product:

N: 4.77%, 5.16, 3.84

S: Traces that were shown to be $CaSO_4$

P: -

Acetyl: 20.5

Ash: 4.04, 3.48, 10.1, 1.01

Carbohydrate: Molisbh +

Glucuronic Acid: Tollen's naphthorisorcinal -: Tollen's phloroglucinol +; Bial +

Mucic acid formation: -

Protein: Biuret, -

Reducing substances: After precipitation of the neutralized hydrolysate with $Zn(OH)_2$, 49.4, 49.0, 52.6, 51.0; on the neutralized hydrolysit, 58.9, 61.2, 60.7, 59.4

Uronic acid: Hexuronic acid, 20.5

Equivalent weight: 460, 464, 446, 453

Isolectric points: electrometric titration value 507

Disocciation constant: 4.58×10^{-5} at 32°

91.

Author: K. Meyer

Reference: J. Ophthal. (1936), 19, 860

Name of Product: Hyaluronic acid

Method of Preparation: Precipitate vitreous humor with acetone, and extract the precipitate with water. Or, umbilical cord may be extracted with water. Shake the extract (from vitreous humor precipitate, or umbilical cord extraction) with chloroform and amyl alcohol. Redissolve. Acidify with HCl. Precipitate with glacial acetic acid. Wash away the acid with alcohol. Dry over P_2O_5.

Analysis of Product:

N: 3.11%

Amino sugar: 39.3

Reducing substances: 64.6

Uronic acid: 45.3

Equivalent weight: 424

92.

Author: K. Meyer

Reference: J. Ophthal. (1936), 19, 860

Name of Product: Hyaluronic acid

Method of Preparation: The product is prepared from 200 cattle eyes.

Yield: 7 mg. of acid

Analysis of Product:

Amino sugar: hexosamine, 23.5%

Reducing substance: 41.6

93.

Author: K. Meyer

Reference: J. of Ophthal. (1936), 19, 860

Name of Product: Hyaluronic acid

Method of Preparation: The product is prepared from aqueous humor punctates of rabbit, dog, or human eyes. According to a modification of Elson's method (J. Biol. Chem., (1933), 27, 1824) 0.1 to 0.2 cc. of the raw material are extracted in H_2SO_4 or HCl. The tube is sealed with the acid and kept in boiling water for 8 hours. The product is then neutralized with 2 volumes of 2 N NaOH.

Analysis of Product:

Amino sugar: hexosamine, dog = 1.8 mg. per 100 cc.

human = 1.4 - 1.7 mg. per 100 cc.

rabbit = 4 mg. per 100 cc.

94.

Author: K. Meyer

Reference: J. Biol. Chem. (1936), 114, 689

Name of Product: Hyaluronic acid

Method of Preparation: The product is prepared from bovine vitreous humor.

Analysis of Product: (calculated to ash free basis)

N: 3.1%

Acetyl: 11.7

Reducing substance: (after hydrolysis) 69.5

Uronic acid: 41.1

Equivalent weight: 424

Specific rotation: $(\ )_D = -51°$

95.

Author: K. Meyer

Reference: J. Biol. Chem. (1936), 114, 689

Name of Product: Hyaluronic acid

Method of Preparation: The product is prepared from human umbilical cord.

Analysis of Product:

N: 3.4%

Acetyl: 13.4

Reducing substance: 62 —

Uronic acid: 41.1

Equivalent weight: 391

Specific rotation: $(\alpha)_D = -65°$

96.

Author: O. Karlberg

Reference: Z. Physiol. Chem. (1936), 240, 55

Name of Product: Funis mucin

Method of Preparation: Free navel cords from blood. Mince. Extract with water in cold room. Discard the first bloody extraction. Precipitate the mucin with acetic acid. Wash with water. Bring into solution by stirring with K_2CO_3. Reprecipitate twice with acetic acid.

Analysis of Product:

S: 0.24 (split off by hydrolysis)

Amino sugar: 5.1

Mannose: 5.3

Reducing substance: 12.6

97.

Author: O. Karlberg

Reference: Z. Physiol. Chem. (1936), 240, 55

Name of Product: Vitreous humor mucoid

Method of Preparation: Dilute the vitreous humor from cattle with water. Precipitate with acetic acid. Continue according to the method described above (#96).

Analysis of Product:

S: 0.05% (split off by hydrolysis)

Amino sugar: 6.1

Mannose: 6.2

Reducing substance: 15.6

98.

Author: O. Karlberg

Reference: Z. Physiol. Chem. (1936), 240, 55

Name of Product: Cornea mucoid

Method of Preparation: Extract the cornea with water. Precipitate and reprecipitate with acetic acid.

Analysis of Product:

S: 0.30% (split off by hydrolysis)

Amino sugar: 7.7

Mannose: 8.0

Reducing substance: 14.5

99.

Author: O. Karlberg

Reference: Z. Physiol. Chem. (1936), 240, 55

Name of Product: Ovomucoid

Analysis of Product:

Amino sugar: 13.5%

Mannose: 10.2

100.

Author: Kendall, Heidelberger and Dawson

Reference: J. Biol. Chem. (1937), 118, 61

Name of Product: Hyaluronic acid

Method of Preparation: Prepare a 10-liter meat infusion, phosphate broth containing 0.15% glucose seeded with an 8-hour culture of hemolytic streptococcus, Group A, strain C-203 (Griffith Type 1 (10) or Type 3 or 1 cc (N.Y.5) in the mucoid phase. Incubate for 24 hours at 37°. Pass through Sharple's centrifuge. Sterilize by adding 1% phenol. Concentrate *in vacuo* to 0.1 volume at a temperature that is below 25°. Add 100 grams of crystalline Na acetate and 25 ml. of glacial acetic acid. Precipitate the polysaccharide with 1.25 volumes of alcohol. Discard the supernatant. Dissolve the solid in 200 ml. of water. Treat with iodine. When glycogen is present, remove it with saliva at pH 6.5. Add 10 grams Na acetate and 5 ml. glacial acetic acid. Shake mechanically with 5 ml. of chloroform and 5 ml. of butyl alcohol. Repeat the shaking with fresh portions of solvents until the solid mulsion layer leaves after centrifuging. Add 1.25 volumes of alcohol in order to precipitate the polysaccharide. Dissolve the precipitate in 100 ml. of 5% Na acetate plus 2.5 grams of glacial acetic acid. Reprecipitate as above until the product is phosphate-free. Dissolve the polysaccharide in 100 ml. of water and add 500 ml. of redistilled alcohol. Wash the precipitate several times with redistilled alcohol. Filter. Dry.

Yield: (as the neutral sodium salt)

Type 1: 60-100 mg. per liter

Type 3: 60-140 mg. per liter

Type 10: 50 mg. per liter

Analysis of Product:

	Type 1	Type 3	Type 10
N*	3.5%	3.8%	3.8%
NH_2N:	-	-	-
P:	-	-	-
S:	-	-	-
Acetyl*	11.0	10.9	
Ash	5.3	5.9	5.8
Reducing sugar: (after hydrolysis)	75	84	82
Uronic anhydride:	41.1	42.4	42.6
Equivalent weight: (calculated from ash)	411	367	374
Specific rotation:*	-73°	-77°	-86°
Viscosity **		3.3	2.0
Viscosity ***	25	57	15

*calculated to ash free basis except for agar growth on R strain.

** relative to 0.9% NaCl (of 0.1% solution in 0.9% NaCl)

*** viscosity of a 0.2% solution in water.

101.

Author: Kendall, Heidelberger and Dawson

Reference: J. Biol. Chem. (1937), 118, 61

Name of Product: Hyaluronic acid

Method of Preparation: Grow Hemolytic streptococcus, strain C-203, in the mucoid phase, on agar, for 12 hours. Kill the organisms by adding a mixture of saline solution with an equal volume of alcohol. Stand in the

cold room for 3 days. Continue as described above (#100), taking care to remove the agar fragments. Precipitate the polysaccharide twice from aqueous solution with 5 volumes of glacial acetic acid.

Yield: small

Analysis of Product:

N: 2.2%; amino N, -

P: -

S: -

Specific rotation: -53°

Reducing substance: 65 (after hydrolysis)

Uronic anhydride: +

102.

Author: Kendall, Heidelberger, and Dawson

Reference: J. Biol.Chem. (1937), 118, 61

Name of Product: Hyaluronic acid

Method of Preparation: Prepare a 24-hour broth culture of hemolytic streptococcus strain 203 in the R phase. Continue as described above (#100). Precipitate the polysaccharide with 1.75 volumes of alcohol, instead of 1.25 volumes, as indicated above.

Yield: 9 mgs. per liter

Analysis of Product:

N: 3.7%; free amini N, -

P: -

S: -

Reducing sugar: 52(after hydrolysis)

Specific rotation: -16°

Uronic anhydride: +

103.

Author: Kendall, Heidelberger, and Dawson

Reference: J. Biol. Chem. (1937), 118, 61

Name of Product: Hyaluronic acid

Method of Preparation: Obtain the sodium salt of Type 3 strain of Hemolytic streptococcus. Acidify the chilled aqueous solution to Congo Red with diluted HCl. Precipitate with 1.5 volumes of redistilled alcohol. Redissolve the precipitate in water. Reprecipitate twice with redistilled alcohol in the presence of a drop of 10% HCl. Filter the precipitate. Wash free from the chloride with redistilled alcohol. Dry in vacuo over $CaCl_2$ and NaOH pellets.

Analysis of Product:

N: 3.1%; amino N, -

P: -

S: -

Ash: -

Uronic anhydride: 40

Equivalent weight: 416 (not calculated from the ash content).

Specific rotation: -77°

104.

Author: Meyer, K., Smyth, E.M., and Palmer, J.W.

Reference: J. Biol. Chem. (1937), 119, 73

Name of Product: Acid polysaccharide

Method of Preparation: The method of Levene and López-Suárez (J. Biol. Chem. (1916) 25, 511) is used for alkaline hydrolysis of the gastric mucin from pig stomach scraping. Fractionate the hydrolysate with glacial acetic

acid, alcohol, and acetone ether mixtures in order to obtain the acid fractions.

Analysis of Product:

Amino sugar: glucosamine, +; hexosamine, 1020%

Uronic acid: 12-25%

Equivalent weight: 400-500

105.

Author: Meyer, K., Smyth, E.M. and Palmer, J.W.

Reference: J. Biol.Chem. (1937), 119, 73

Name of Product: Neutral Polysaccharide

Method of Preparation: Dissolve 20 grams of gastric mucin from pig stomach scraping with 500 cc. of warm water. Neutralize. Add 10 gm. Na_2CO_3. Keep at 70° for 15 minutes. Cool the solution. Acidify with glacial acetic acid to pH = 5. Stand in the cold over night. Centrifuge to remove the precipitate. Bring the supernatant to 5% H_2SO_4. Shake with 100 grams of Lloyd's reagent. Centrifuge. Extract the sodium precipitate once with 300 cc. water. Combine the supernatant solutions. Add 2 volumes of alcohol.* Wash the precipitate in alcohol. Dissolve in 200 cc. water. Add 2 grams of gelatin in 50 cc. of water. Place in ice box over night. Centrifuge. Wash the precipitate with a small amount of water. Combine the supernatant solutions. Pour into 2 volumes of alcohol. Wash the precipitate with alcohol. Dissolve in 500 cc. water. Add 50 cc. of 25% neutral lead acetate. Remove the precipitate. Add N NH_4OH to the supernatant until a pH of 9 is reached. Add 20 cc. basic 25% lead acetate. Wash precipitate in water. Suspend in 200 cc. water. Heat the suspension to 60°.

*Since the precipitates were of rubbery consistency, and changed to a hard bitter mass in increasing alcohol concentrations, it was necessary every time to wash by triturating with alcohol in a porcelin mortar.

Pass CO_2 through it. Remove lead carbonate. If the solution is still turbid add NH OH and treat with CO_2 . Acidify with glacial acetic acid. Add 3 volumes of alcohol. Wash the precipitate in alcohol. Dissolve in 10 times the weight of water. Add 12 times its volume of glacial acetic aicd, to form a precipitate. Wash the precipitate with alcohol, acetone and ether. Dry.

Yield: 3.00 grams

Analysis of Product:

N; 5.4%

Acetyl: 10.2

Amino sugar: 36.2

Ash: 4.2

Moisture: 3.8

Uronic acid: 1.1

106.

Author: Meyer, K., Smyth, E.M., and Palmer, J.W.

Reference: J. Biol. Chem. (1937), 119, 73

Name of Product: Neutral Polysaccharide

Method of Preparation: Prepare neutral polysaccharide as described above (#105). Further purify by the following method: Dissolve 2.92 gm. of the powder in 150 cc. H_2O. Add 30 cc. 8% Zn acetate. Adjust to maximum precipitation with N NaOH. Pour the supernatant into 3 volumes of alcohol. Wash the precipitate. Dissolve by warming in 20 cc. water. Add 20 cc. glacial acetic acid with vigorous shaking. Wash precipitate (A) and dry. To the supernatant above add 200 cc. glacial acetic acid. Wash the precipitate and dry. Dissolve in 200 cc. water. Add 200 cc.

glacial acetic acid in small portions. Wash the precipitate (B) and dry.

To the turbid supernatant above, add 200 cc. acetone and a few cc. of sodium acetate in alcohol. Wash precipitate (C) and dry.

Yield: A: 0.245 gm.

B: 1.550 gm.

C: 0.4896 gm.

Analysis of Product:

	A.	B.	C.	1.	2.	3.	4.	5.	6.	7.	8.
N:	5.4	5.4	5.0	4.8	6.2	5.2	5.1	5.2	3.9	3.3	5.3
S:	-	-	-	-	-	-	-	-	-	-	-
Acetyl:	10.0	10.8	10.9	10.7	10.8	11.1	14.8	10.9	16.8	9.2	9.8
Ash:	2.3	1.2	0	1.2	0.9	0	0.8	0.9	1.6	2.0	1.3
Equivalent weight (carboxyl)	0	0.5	0.9	2.4	2.9	0.4	0.8	0.4			0
Moisture	6.2	5.0	5.3	1.3	7.7	5.6	6.5	7.3	6.1	2.5	2.8
Specific rotation $(\alpha)_D$		-4.9	-8.0					+8.0		+8.7	-2.2
Uronic acid	1.2	0.5	1.1	6.1	2.7	1.3	3.3	2.0	0.3	1.0	0.4

107.

Author: Meyer, K., Smyth, E.M. and Palmer, J.W.

Reference: J. Biol. Chem. (1937), 119, 73

Name of Product: Acid polysaccharide

Method of Preparation: Extract 40 gms. a commercial sample of mucin from

pig stomach scraping, with 400 cc. N NaOH, for 2 days at room temperature. Precipitate by the addition of 2 volumes of alcohol. Wash the precipitate with alcohol. Dissolve in a small amount of water. Reprecipitate with 10 volumes of glacial acetic acid. Wash the precipitate with alcohol and acetone. Dry. Dissolve the product (3 gm.) in 150 cc. neutral water. Add 0.1 N HCl. Shake with 15 gm. Lloyd's reagent. Centrifuge to separate the supernatant. Wash the precipitate with 50 cc. water. Precipitate the supernatant solutions with 4 volume s of alcohol. Wash the precipitate with alcohol. Extract twice with 25 cc. water. Precipitate the wet extracts with 5 volumes of alcohol. Wash. Dry.

Yield: 1.09 gm.

Analysis of Product:

S: 4.7%, 1.0 *

N: 4.0, 1.9 *

Acetyl: 8.8, 1.4 *

Ash: 8.2

Amino sugar: 32.8, 1.2 *

Uronic acid: 39.1, 1.5 *

Equivalent weight: 659

Specific rotation: at 25° C. = 20.2°, at 24°C. = -35.7° (neutralized sample).

108.

Author: Meyer, K., Smyth, E.M., and Palmer, J.W.

Reference: J. Biol. Chem. (1937), 119, 773

*Equivalent per equivalent weight

Name of Product: Acid Polysaccharide

Method of Preparation: Extract 50 gms. of a commercial sample of gastric mucin from pig stomach scraping, for 4 days in 500 cc. of 0.5 N NaOH. After precipitating the acid as described above (#107), convert into the neutral lead salt and then into the acid barium salt.

Yield: 1.827 gm.

Analysis of Product:

N: 3.0, 1.4% *

S: 3.1, 0.6 *

Acetyl: 8.8, 1.4 *

Amino sugar: 3.1, 0.6 *

Ash: 9.7

Moisture: 2.3

Uronic acid: 27.6, 1.0 *

Equivalent weight: 672

Specific rotation: at 24°C. = 22.2°

109.

Author: K. Meyer

Reference: Science, (1938), 88, 129

Name of Product: Mucopolysaccharide of synovial fluid

Method of Preparation: The product is prepared from cattle or human synovial fluid by a modification of the method for isolation of chondroitin sulfuric acid from cartilage (Meyer, K., J. Biol. Chem (1937) 119, 507).

*Equivalent per equivalent weight

Yield: per liter of cattle synovial fluid = 200-250 mgm. per liter of human synovial fluid = 225 mgm.

Analysis of Product:

S: -

P: -

N:	1 equivalent	per	equivalent	weight	
Hexosamine:	1 equivalent	per	equivalent	weight	
	" "	"	"	"	
Acetyl:	" "	"	"	"	
Hexuronic acid:	" "	"	"	"	
Carbohydrate:	" "	"	"	"	

Viscosity: high

110.

Author: K. Meyer

Reference: J. Biol. Chem. (1939), 128, 319

Name of Product: Polysaccharide acid

Method of Preparation: Obtain the synovial fluid from the asragalotibial joints of 1-year old freshly killed steers. Dilute with 2.5 volumes of distilled water. To bring out the protein salt of the polysaccharide add 10% acetic acid to a concentration of 2%. Keep 0-5° C. for 18 hours. Remove the precipitate with a glass rod and by filtration. Wash several times with distilled water. Bring into solution with 10% $CaCl_2$. Keep at pH9 by adding NaOH. Stir mechanically until the material goes into solution. Remove the bulk of the protein by repeatedly shaking with chloroform amyl mixture. Treat with 1.5 volumes of alcohol until the solution is almost clear. Wash the centrifuged precipitate with alcohol. Dissolve in 5% sodium acetate solution.(If the solution is turbid treat with chloroform amyl mixture). Remove some more protein by adding zinc

acetate solution. Neutralize with N NaOH. (Work in the dilute solution ... 0.5% carbohydrate lest the polysaccharide precipitate as a zinc salt). Treat the centrifuged solution with alcohol acidified with acetic acid. Redissolve the precipitate in a minum amount of water. Reprecipitate with 5 volumes of glacial acetic acid, or alcohol acidified with glacial acetic acid. Remove the gel by centrifuging. Wash in a filter with alcohol, acetone and ether. Dry over P_2O_5.

The free acid is white, fibrous and of fair tensils strength.

Analysis of Product: (corrected for ash and moisture).

	61.	67.	69.	77A1	78II	82
N:	3.69%	3.68	3.59	3.36	3.21	3.00
N: *	1.19	1.34	1.22	0.96	1.12	1.11
S: (volatile)			-			
S: (in ash)			.47			
Acetyl:	9.4	10.6	10.3	10.5	10.5	10.3
Acetyl: *	1.00	1.26	1.15	0.98	1.20	1.24
Amino sugar:	30.2	41.8	43.7	34.5	36.0	40.3
Amino sugar: *	0.97	1.19	1.16	0.77	0.99	1.17
Ash:	3.97	2.08	4.95	4.14	2.50	2.90
Moisture:	3.28	3.73	5.60	3.50	5.61	5.10
Uronic acid:	41.3	44.1	50.7	38.4	35.9	44.5
Uronic acid: *	0.97	1.16	1.25	0.79	0.91	1.19
Acid equivalent weight $\frac{454}{-68.8°}$	511	477		401	492	520
Specific rotation:	-71.9	-73.8		-78.2	-69.9	-77.4

*Equivalent per equivalent weight

111

Author: K. Meyer

Reference: J. Biol. Chem. (1939), 128, 319

Name of Product: Polysaccharide from synovial fluid

Method of Preparation: Dehydrate the synovial fluid protein salt with alcohol. Reprecipitate the sample as described above (110). Add an equal volume of saturated ammonium sulfate solution. Make the solution alkali in reaction by the addition of strong ammonia water (pH9), the protein will precipitate out at this point. Remove the sulfate from the clear solution by adding barium acetate acidifying with a small amount of acetic acid. Treat the supernatant with 2 volumes of alcohol. Extract the precipitate with 5% sodium acetate solution. Clear the solution by means of precipitation with ZnOH. Treat with alcohol. Reprecipitate from the alcohol.

Analysis of the Product:

P: in traces

Amino sugar: 33.1%

The rest of the analysis is listed above (#110).

112

Author: C. V. Seastone

Reference: J. Exp. Med. (1939), 70, 361

Name of Product: Hyaluronic acid

Method of Preparation: (Seastone used the method of Savag, Biochem. Z. (1934), 273, 419 as applied by Meyer, K. and Palmer, J.W., J. Exp. Med. (1939), 70, 347).

Obtain 750 cc. vitreous humor from 60 beef eyes. Squeeze through fine gauze 3 times. Precipitate with 10 volumes of cold acetone. Wash with acetone and ether. Redissolve by grinding in 100 cc. of water gradually added. Reprecipitate with 5 volumes of alcohol and a few drops of 20% NaCl. Centrifuge. Redissolve by grinding with 150 cc. of water gradually added. Dissolve 1.5 gm. NaCl in this solution. Shake violently for 1/2 hour in the presence of 300 cc. chloroform and 3 cc. butyl alcohol. Centrifuge to separate the gel from the precipitate. Decant and save the supernatant. Extract the gel with 50 cc. water. Precipitate the extract with 5 volumes of alcohol plus a few drops of 20% saline solution. Dissolve in the supernatant of the first chloroform treatment. Shake with fresh portions of chloroform and butyl alcohol. Continue until only a fair precipitate appears at the chloroform-water interface after centrifuging. Bring to neutrality with HCl. Precipitate twice with alcohol and 20% NaCl.

Yield: 255 mgm.

Analysis of Product:

N: (MicroKjeldahl) 1.9%

P:

Amino sugar: 27

Reducing sugar: (Hagedorn-Jenson), 45; (orcinol), 13

Relative viscosity of a 0.1% solution measured in an Ostwald viscosimeter in an 0.85% salt solution at 21° C. compared with a salt solution containing no carbohydrate was 1.9%.

113.

Author: C. V. Seastone

Reference: J. Exp. Med. (1939), 70, 361

Method of Preparation: Inoculate 8-10 colonies of group C streptococcus into 10% horse serum and 1% dextrose digest, directly from a flood plate. After 12 hours of growth add to 1% digest dextrose broth, with occasional stirring. Add 50 cc. concentrated phenol. Stand for 15 minutes. Remove the organisms in a Sarple's centrifuge. Concentrate to 0.1 volume in vacuo at a temperature not over 35° C. Add octyl alcohol to prevent foaming. Add 100 grams sodium acetate plus 25 cc. of glacial acetic acid. Precipitate with 2 volumes of alcohol. Dissolve the precipitate in 200 cc. 5% sodium acetate plus 2.5% glacial acetic acid. Add 50 cc. chloroform and 5 cc. of butyl alcohol. Shake for 1/2 hour. Centrifuge. Discard the chloroform layer. Add fresh chloroform and butyl alcohol to the supernatant. Shake and centrifuge until only a faint ring of the precipitate appears at the chloroform water interface. Reprecipitate the polysaccharide from the 5% sodium acetate and 2.5% glacial acetic solution with 1.25 volumes of alcohol until it is phosphorus-free. Redissolve in 50 cc. water. Precipitate with 5 volumes of alcohol, plus a few drops of 1/2 saturated sodium acetate. Wash the precipitate with alcohol. Dry in vacuo over $CaCl_2$.

Yield: per liter, 0.106, 0.23, 0.069, 0.107, 0.040, 0.081, 0.056, 0.038.

Analysis of Product:

N: 4.1%, 3.9, 3.8, 3.5, 2.7, 4.2, 3.8, 3.2

Amino sugar: -, -, -, 33, 29, 33, -, -.

Mucoid carbohydrate: 45, 20, 30, 20, 0.8, 5, 1.5

Relative viscosity of 0.1% solution measured in Ostwald viscosimeter in 0.85% NaCl solution at 21° c. compared with the same salt solution without carbohydrate: 1.43, 1.03, 1.17, 1.33, 1.20, 1.00, 1.03, 1.01

114

Author: C. V. Seastone

Reference: J. Exp. Med. (1939), 70, 361

Name of Product: Mucoid polysaccharide

Method of Preparation: Suspend 8-10 colonies of group C streptococcus in 200 cc. horse serum plus 1% dextrose digest broth. Incubate for 12 hours. Transfer to 2 liters of 1% dextrose broth. Incubate for 3 hours with occasional stirring. Cool in ice bath. Centrifuge the organisms. Wash twice with 100 cc. 0.85% salt solution. Resuspend in 100 cc. salt solution. Add 1 cc. chloroform and stir. Incubate for 24 hours at 37° C. Remove organisms in the centrifuge. Incubate for 6 hours with 50 cc. fresh salt solution. Remove. To the combined supernatants (150 cc.) add 1.5 gm. NaCl, 2.5 cc. chloroform, and 2.5 cc. butyl alcohol. Shake mechanically for 1/2 hour. Centrifuge. Withdraw the supernatant. Add fresh portions of chloroform and butyl alcohol to the supernatant until the chloroform water interface is almost clear. Shake. Precipitate the carbohydrate with 1.25 volumes of cold alcohol. Dissolve the precipitate in 10 cc. water.

Weigh the precipitate and centrifuge with 3 volumes of alcohol plus 20% salt solution. Stand for 1 hour in ice bath. Throw down the precipitate in the centrifuge. Discard the supernatant. Dry in vacuo and weigh.

Yield: per liter, 0.0067%, -, 0.007, 0.012, 0.005, -, 0.0018, -

Analysis of Product:

N: 2.2%, -, 2.6, 2.1, 2.5, -, -, -

Amino sugar: 23, -, 23, 31, 28, -, 17, -

Mucoid carbohydrate: 42, -, 52, 58, 40, -, 30, -

Reducing substance (Orcinol) 10, -, 21, 11, 14, -, 16, -

Relative viscosity of 0.1% solution measured in an Ostwald viscosimeter in 0.85% NaCl solution at 21° C., compared with the same salt solution without carbohydrate: 2.70, -, 2.60, 3.80, 2.10, -, 1.7, -

115

Author: K. Meyer

Reference: Am. J. Ophthal. (1940), 23, No. 12, 1320

Name of Product: Corna polysaccharide

Method of Preparation: Add 10 volumes of 10% Ca $(OH)_2$ or N NaOH to disintigrated cornae. Acidify with glacial acetic acid. Fractionate with alcohol.

This method is similar to the method for the preparation of chondroitin sulfuric acid with cartilage. (Meyer, K., J. Biol. Chem. (1937), 119, 507).

Yield: 400 mgm.

116

Author: K. Meyer

Reference: Am. J. Ophthal. (1940) 23, No. 12, 1320

Name of Product: Cornea polysaccharide hyaluronic acid.

Method of Preparation: Mince the cornae from 200 dissected eyes. Add 50% urea and about 25 cc. NaOH to give a pH of 9. Stand in room temperature for 3 weeks. Centrifuge. Extract twice more with 50% urea. Dilute to 4 volumes with water. Acidify with 20% acetic acid. Wash the precipitate with water. Dissolve the protein salt in 10% $CaCl_2$ in a slightly alkaline solution. Shake with chloroform alcohol mixture (as in J. Biol. Chem. (1936), 240, 55). Precipitate the clear fluid with 2 volumes of alcohol. Dissolve in water and neutralize. Treat in 1 N. acetic acid and Lloyd's reagent. Precipitate with 2 volumes of alcohol. Extract the precipitate with small amounts of water. Precipitate the aqueous solution with glacial acetic acid. Wash with alcohol, acetone and ether. Dry in vacuo to a white fibrous material.

Yield: 150 mgm.

Analysis of Product:

N: 2.63%, 1.05*

S: 5.03, 0.88*

Ash: 10.8

Amino sugar: 31.8, 0.99*

Acetyl: 0.60, 1.25*

* Equiv. per equiv. weight.

Uronic acid: 35.0, 1.01*

Water: 5.06

Acid equivalent weight: 560

Specific rotation of neutralized substance: -51°; on the basis of an ash and sulfur-free compound, -65°, -75°

117

Author: K. Meyer

Reference: J. Biol. Chem. (1940), 133, 83

Name of Product: Hyaluronic acid

Method of Preparation: The product is obtained from the viscous pleural fluid of a malignant tumor of the pleura or peritoneum. The method is similar to the one for the isolation hyaluronic acid from synovial fluid (J. Biol. Chem. (1939), 128, 319, Meyer, Hobby, and Dawson) except that the final precipitation of the material in 10% glacial acetic acid by 2 volumes of alcohol was substituted for the glacial acetic acid precipitation previously used. This results in a mixture of the free acid and its calcium salt.

Analysis of the Product:

	I	II	III
N:	3.21%	3.00	2.95
N:*	1.43	1.23	1.33
Acetyl:	9.85	10.5	10.3
Ash:	4.04	2.16	1.44
Amino sugar:	39.6	40.8	40.0
Amino Sugar:*	1.38	1.31	1.43
Moisture:	1.27	6.67	3.15
Uronic acid:	44.9	46.6	46.7
Uronic acid:*	1.44	1.38	1.54
Specific rotation (neutralized solution)	-76.8°	-70.3°	-67.7°
Mobility:	the fastest component was -10×10^{-5}		

118

Author: Van B. Robertson

Reference: J. Biol. Chem. (1940), 133, 261

Name of Product: Synovial fluid mucin

Method of Preparation: Separate the synovial fluid from the astragalotibial joints of steers within 30 minutes after death. Centrifuge. Dilute with 5 volumes of water. Precipitate the mucin with acetic acid. Wash the ropy precipitate with water. Dissolve in 1/2 the original volume of 0.5 N secondary $NaPO_4$. After 2 hours add 2 volumes of alcohol to the solution. Mix well. Complete the precip-

* Equivalent per equivalent weight

itation by the addition of 1 volume of ether. Wash the precipitate. Redissolve in the same volume of M secondary $NaPO_4$. Pour into 4 volumes of 1% acetic acid. Wash the mucin precipitate. Dissolve in 0.5% Na_2CO_3. Dialyse for 2 weeks against cold water. Centrifuge sharply. Concentrate by evaporating from the surface of a cellophane sac in an ice box. Add 0.2M solution phosphate buffer. At pH 7.4 concentrate to 0.05M. Obtain a white ropy precipitate by the addition of dilute acetic acid.

Analysis of Product:

N: 12.5%

Amino sugar: 7% (upon hydrolysis).

119

Author: Van B. Robertson

Reference: J. Biol. Chem. (1940), 133, 261

Name of Product: Polysaccharide of synovial fluid mucin

Method of Preparation: Obtain 10 liters of synovial fluid from the astragalotibial joints of steers. Precipitate with acetic acid. Dissolve the precipitate in 2 liters of 0.5% Na_2CO_3. Reprecipitate with 2 volumes of alcohol acidified with acetic acid. Redissolve in 0.5% Na_2CO_3. Adjust the pH to 9. Add 5 grams of trypsin. Allow the mucin to digest for 36 hours at 38°. Precipitate the remaining protein by the addition of 10% trichloracetic acid. Centrifuge. Neutralize with KOH. Precipitate with 3 volumes of alcohol. Take up the flocculant precipitate in 600 cc. water. Reprecipitate with 5 volumes of alcohol. Wash well with alcohol. Acidify with acetic acid and ether. Dry in vacuo.

Yield: 3 gms. from 10 liters of fluid.

Analysis of the Product:

S: -

P: traces

Viscosity: 2% solution 40 times more viscous than water at 25°

120.

Author: George K. Hirst

Reference: J. Exp. Med. (1941), 73, 493

Name of Product: Capsular polysaccharide

Method of Preparation: Concentrate a beef heart infusion of 60 liters, in vacuo to 4 liters. Or, dissolve 600 gms. vagex paste in 4 liters of water. Dissolve 1500 gm. Pfansteihl peptone in the solution. Add 2.5 volumes of ethyl alcohol. Settle over night. Discard the precipitate. Remove the alcohol fromthe supernatant by distilling in vacuo. Dissolve in 60 liters of water. Adjust the pH to 7.4. Add NaCl. Bring the pH to 7.8. Filter through Chamberland filters, No. L5. Add 100 cc. young actively growing culture to each 4 liter flask. Incubate over night. Add 2% formalin or acetic acid to kill the organisms. Remove them by Sharple's supercentrifuge. Concentrate the supernatant in vacuo to 1/10 the volume. Add 1 volume of glacial acetic acid, 500 gms. sodium acetate, and 1.25 volumes of ethyl alcohol. Redissolve the precipitate in 2 liters of water plus acetic acid and sodium acetate. Precipitate with 1.25 volumes of ethyl alcohol. Dissolve in water. Shake with chloroform plus butyl alcohol until there is no precipitate at the interface. Precipitate

3 times with alcohol. Dissolve in water. Dialyse for 2 days against distilled water. Add 1 drop HCl to the carbohydrate solution. Precipitate in 10 volumes of cold acetone. Dry in vacuo.

Yield: 120 mgm. or less per liter

Analysis of Product:

O: 42.8%, 42.7, 44.7

N: 3.30, 3.55, 3.16

Ash: 1.80, 1.78, 1.58

Acetyl: 11.50, 12.12, 10.70

Specific rotation: -60.2°, -60.7°, -66.1

121.

Author: K. Meyer

Reference: J. Biol. Chem. (1941), 138, 491

Name of Product: A sulfuric acid containing fraction, hyaluronic acid

Method of Preparation: Incubate 2.23 kilos of fresh pig skin with 6 liters of NaOH at 37° for 28 hours. Neutralize the near liquid material with acetic acid. Filter with Hyflo-Supercel. Pour into 2volumes of alcohol. Stand overnight. Centrifuge. Wash the precipitate with alcohol. Take up in 500 cc. 10% $CaCl_2$, alkaline to phenolphthalein. Shake with 100 cc. 4 parts chloroform and 1 part amyl alcohol. Centrifuge. Filter the supernatant through Filter-Cel. Pour into 2 volumes of alcohol. Wash with alcohol. Dissolve in H_2O. Add 8 gms. Lloyd's reagent plus glacial acetic acid in order to obtain a 6% acetic acid solution. Centrifuge. Wash once with normal acetic acid. Pour the combined solutions into 2 volumes of alcohol. Wash the precipitate with alcohol and ether. Dry. Dissolve the residue in 100 cc.

1% sodium acetate solution. Add normal acetic acid until a pH of 6 is reached. Incubate for 6 hours with 2 cc. filtered saliva plus toluene to digest the glycogen. Pour the solution into 2 volumes of alcohol. Extract with 100 cc. barium acetate. Add alcohol up to a 20% concentration. Stand in the cold overnight. Remove the precipitate by centrifuging. Wash. Extract with water. Pour into 5 volumes of glacial acetic acid. Remove the precipitate. Wash with alcohol, acetone and ether. Dry.

To purify the sulfuric containing fraction: After precipitation as the barium salt (above) dry the precipitate in vacuo. Dissolve in water. Adjust the pH to 5.8. Add 5 mg. pneumococcus hyaluronidase. Incubate in the presence of toluene for 2 days. Remove the enzyme by adsorption on Lloyd's reagent in normal acetic acid. Precipitate the polysaccharide first from alcohol and then from 5 volumes of glacial acetic acid. Retest for hyaluronic acid by pneumococcus enzyme and by the routine procedure with 1% solution (Meyer, K., Hobby, Chaffee, E., and Dawson, J. Exp. Med. 71,137 (1940).

Yield: 1.85 gm. (of the unpurified polysaccharide)

Analysis of Product:	I	II	III	IV
N:	2.24	2.15	2.19	2.05
N:*	0.88	1.03	0.82	0.91
S:	5.98	5.12	5.22	5.07
S:*	1.02	1.10	0.83	0.98
Acetyl:	8.33	6.10	8.95	7.84
Acetyl:*	1.06	0.95	1.09	1.13
Amino sugar:	27.0	27.3	30.5	25.1
Amino sugar:*	0.85	1.02	0.89	0.87
Equivalent weight:	547	668	525	620
Specific rotation:	-53.9°, -28.4, -57.9, -55.1			

122

Author: K. Meyer

Reference: J. Biol. Chem. (1941), 138, 491

Name of Product: Hyaluronic acid fraction

Method of Preparation: Precipitate hyaluronic acid by bringing the 20% alcohol solution (#121) to a 77% concentration. Remove the stringy precipitate by centrifuging. Wash with alcohol. Extract with small portions of water. Pour the combined solutions into 5 volumes of glacial acetic acid. Remove the precipitate immediately. Wash dry.

Yield: 1.08 gm.

* Equivalent per equivalent weight

Analysis of Product:

	I	II
N:	2.99	2.78
N:*	1.02	0.91
Acetyl:	13.1	9.50
Acetyl:*	1.45	1.01
Amino sugar:	39.7	37.2
Amino sugar:*	1.05	0.95
Equivalent weight	475	455
Specific rotation:	-74.9°	-67.5 (in neutralized solutions)

123

Author: D. McClean, and C. W. Hale

Reference: The Biochem. J. (1941), 37, 159

Name of Product: Hyaluronic acid

Method of Preparation: Extract umbilical cords with water. Precipitate with 3 volumes of ice cold ethanol and a trace of sodium acetate.

Analysis of Product:

N: 7%

124

Author: A. Pirie

Reference: Brit. J. Exp. Path. (1942), 23, 277

Name of Product: Hyaluronic acid

Method of Preparation: Extract dried tumor tissue with 10% NaCl on a shaker for a few hours in order to remove the protein. Remove the

* Equivalent per equivalent weight

polysaccharide by tryptic digestion and alcoholic precipitation. Extract the residue over night with 10% NaOH. Filter. Neutralize. Add B.D.H. to the trypsin to 0.1% of the supernatant fluid. Incubate at 37° C. in toluene. Centrifuge. Precipitate the polysaccharide with 2 volumes of alcohol. Redissolve the precipitate in water. Centrifuge at 3000 r.p.m. for 1 hour. Remove the supernatant fluid. Repeat the alcohol precipitation and centrifuge until the solution is clear and colorless. Dialyze, freeze and dry.

Analysis of Product:

N: 3.7%, 3.0, 4.5

S: nil, nil, 0.1

Acetyl: 12.5, 14.8, 13.7

Amino sugar: 39.4, 31.3, 38.8

Reducing sugar: 60.0, 45.2, 67.3

125

Author: J. H. Humphrey

Reference: Bichem. J. (1943), 37, 460

Name of Product: Hyaluronic acid (K salt)

Method of Preparation: Repeatedly extract human umbilical cord with 90% phenol solution. Repeatedly precipitate from water solution at pH 9-10 by 1 1/4 volume of ethanol saturated with K acetate. Treat with P nitrobenzyl bromide and centrifuge in order to remove the insoluble haematin derivatives.

Analysis of Product:

Glucosamine: 75%

Haematin derivatives: slight

126

Author: D. McClean and C. W. Hale

Reference: The Biochem. J. (1943), 37, 169

Name of Product: Hyaluronic acid

Method of Preparation: Extract umbilical cords with water. Precipitate with 1.25 volumes of ice cold ethanol saturated with potassium acetate. Bring pH to 9-10 with KOH. Break up the stringy clot precipitate. Remove the residual potassium acetate and KOH by washing with alcohol. Wash with ether. Dry over P_2O_5 *in vacuo*.

Analysis of Product:

N: 4.8%

P: 2

Amino sugar: 12

no clot on addition of acetic acid in the aqueous solution.

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