A Biochemical Study of Streptococci

With Special Emphasis on the Determination of Their Chemical Composition

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University

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FRANCES KRASNOW, B.S., A.M.

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CONTENTS

I. Introduction
   1. General Statement
   2. Historical
   3. Object of Research

II. Character of Organisms
   1. Source
   2. Preparation of media for use in the determination of the biochemical reactions.
      (a) Sugar-free broth
      (b) Carbohydrate broth
      (c) Meat infusion agar
      (d) Meat infusion gelatin
      (e) Milk
      (f) Inulin—serum—water
      (g) Bile
   3. Preparation of the reagents for the determination of the hydrogen-ion concentration.
      (a) Standard solutions
      (b) Indicators
   4. Isolation
   5. Stock Cultures
   6. Seed Cultures
   7. Biology and Biochemistry of the organisms
      (a) Blood reactions
      (b) Immunity reactions
      (c) Other biochemical reactions
   8. Description of the test organisms

III. Preparation of the Mass Cultures
   1. Medium
      (a) Choice of the Infusion basis
         (1) Hemolyzed blood-veal-infusion agar
         (2) Huntoon agar
         (3) Hasting gelatin-agar
         (4) Beef liver agar
         (5) Beef brain agar
      (b) Concentration of agar—agar
      (c) Constancy in composition

2. Cultures
(a) Preparation of the cultures for mass growing
(b) Planting of cultures
(c) Removal of growth from agar
(1) Washing method
(2) Spatula method
(d) Drying of growth

IV. Analysis of the Bacterial Material
1. Description of methods employed
(a) Nitrogen
(b) Total sulphur and phosphorous
(c) Ash
(d) Moisture
(e) Lipins
(f) Blanks
2. Records of analysis
3. Summary of the data for the average percentage composition of the streptococci
4. Conclusions from the analytical data

V. Analysis of the Lipin Mixture
1. Outline of the procedure
2. Records of analysis
3. Summary of the data for the average percentages of phosphatide and cholesterol.
4. Conclusions from the data on the cholesterol and phosphatide content

VI. Effect of the Lipin Material on Hemolysis
1. Record of experiments
2. Conclusions from the data on hemolysis

VII. Summary of Conclusions

VIII. Bibliography.

I. INTRODUCTION

1. General Statement: The chief purposes pervading researches on the streptococci are two: (1) to get a sound basis for the classification of the different types and (2) to understand the role they play in the general processes of nature. All the results, thus far, are based on the changes which these organisms produce in their environment. Though this information is very valuable we must not forget that in order to comprehend function we must understand structure and composition. Discovery of the varieties of relation of the actual constituents of the bacterial bodies must lead to a better realization of the very things for which we are seeking. With this purpose in view, we have undertaken the work to be detailed here.

2. Historical: This is not the first study on bacterial composition. Thus Brieger as early as 1885 (1) analyzed pneumococcus cultures; Weyl (2) and Hammerschlag in 1891 (3), Hoffman in 1894 (4), De Schweinitz and Dorset in 1895 (5), 1896 (6), 1897 (7), 1898 (8), Ruppel in 1898 (9) and Levene in 1898 (10) and 1901 (11) investigated the chemistry of Baccilus Tuberculosis; Drymount in 1886 (12) and later Vaughan (13) and Wheeler (14) worked with Bacillus Anthrax. More recently Vaughan (15), Leach (16) and Dawson (17) analyzed Bacillus Coli cultures and Nicholle and Alilaire (18) and Bradley (19) used Bacillus Diphtheria as their material for analysis.

3. Object of Research: Each of these reports supply interesting additions to our knowledge of the chemical make-up of bacteria. It might, therefore, seem that our problem is a very simple one, differing from previous investigation only in respect to the organism, considered. Such a view cannot be more than superficial. Our aim, here, is not merely to gather data on the chemical composition of the streptococcus—it is to gather such data under standardized conditions. Practically nowhere in the literature is there mention of such an attempt, though it is precisely the want of standard methods that has made so many results in bacteriological work of little or no value. We have, therefore, made special effort to work out details. This will become apparent with a careful consideration of the methods of procedure described below:

II. CHARACTER OF ORGANISMS

1. Source: All the strains of streptococci were obtained from root canal infections in the teeth of man.*

2. Preparation of media for use in determining the biochemical reactions:

(a) *Sugar-free broth:* 75 grams of Bacto-veal in 1000 cc. of tap water were heated in the Arnold sterilizer for two hours and filtered through filter paper. This infusion was inoculated with B. coli communior (20), incubated at 37° C. for 18 hours and sterilized at "Arnold" temperature for 45 minutes. The hot bouillon easily dissolved the 10 grams of peptone and 5 grams NaCl that were added at this point. An aliquot part (10cc.) was cooled and adjusted with 0.05 M NaOH to the hydrogen-ion concentration, $P_H = 7.9$ (21). The calculated amount of M NaOH was added

*All the cultures were obtained from Dr. M. L. Rhein and Dr. J. M. Levy, New York City.

to the remainder and set in the Arnold sterilizer for 15 minutes. Such treatment usually results in an increased acidity, the PH changing to a value between 7.7 and 7.5. This preparation had a final PH = 7.7. The medium was filtered and sterilized in the "Arnold" for 30 minutes on three successive days.

(b) *Carbohydrate broth:* The chief point to guard against in this connection is that a sugar in slightly alkaline solution decomposes readily. This occurs to some extent even at room temperature (23° to 25° C.) but very marked decomposition sets in at the temperature of the Arnold sterilizer. It is best, therefore, to sterilize a concentrated (20 per cent) aqueous solution of the carbohydrate by boiling (10 to 15 minutes being sufficient) (20). This concentrated solution is then mixed with sterile sugar-free broth in amounts to make the desired dilution. The sterility of the mixtures was tested by incubating them for 18 hours at 37° C.

(c) *Meat infusion agar:* The basis for this medium was made by infusing 75 grams Bacto-veal in 500 cc. tap water in the "Arnold" for two hours. The steps in the procedure for obtaining the finished bouillon product parallelled those for sugar-free broth with the exception that there was no inoculation with B. coli. To this substrate was added an equal quantity of a 4 per cent solution of agar-agar, giving a final concentration equal to 2 per cent.

(d) *Meat infusion gelatin:* Hiss and Zinsser recipe (22).

(e) *Milk:* Hiss and Zinsser recipe (23).

(f) *Hiss inulin-serum-water:* Hiss and Zinsser recipe (24).

(g) *Bile:* Ox-bile obtained from the slaughter house was sterilized for 20 minutes on three successive days.

3. PREPARATION OF THE REAGENTS USED IN THE DETERMINATION OF HYDROGEN-ION CONCENTRATIONS: In determining hydrogen-ion concentrations the colorimetric method was chosen. The principles involved need not be reiterated here; they have been thoroughly described by Clark and Lubs (25). The chief sources of error, color of medium and tubidity of culture were obviated by diluting the medium (one part medium to two parts distilled water) and then compensating for the color by Walpole's comparator method of superimposing the color of the medium upon that of the indicator (26, 27).

(a) *Standard solutions used:* 1/15 M disodium hydrogen phosphate
1/15 M potassium dihydrogen phosphate
1/5 M sodium acetate
1/5 M acetic acid

The salts were recrystallized repeatedly until they satisfied Sörensen's purity standards (28). The acetic acid was obtained by redistilling C.P. glacial acetic acid. The phosphate mixtures were prepared by Sörensen's technique and the acetic acid-sodium acetate mixtures according to Walpole's directions (26). The accuracy of these standard mixtures was verified by the hydrogen electrode.* The actual values obtained practically duplicate those given by these authors. A study of the following table will make this evident.

*We are indebted to Dr. Hastings, of the Rockefeller Institute, New York City, for aid in this connection.

Author	Mixtures in c.c.		PH	
Walpole	CH_3COONa	CH_3COOH	Given	Obtained
	5.0	5.0	4.62	4.63
	5.0	7.0	4.27	4.34
Sörensen	Na_2HPO_4	KH_2PO_4		
	9.0	1.0	7.64	7.57
	5.0	5.0	6.81	6.78

These figures were chosen as examples because the test materials matched these most often in the colorimetric determinations.

(b) *Indicators used:* The indicators used were those recommended by Clark and Lubs and manufactured by Hynson, Westcott and Dunning, Baltimore. The following table shows the character of the indicator solutions and notes the amounts of the several varieties used:

Name	Concentration of Solution	Solvent	Amount used per 10 c.c.	Range of PH
Tetabromphenolsulphonephthalein (Brom phenol blue)	0.04%	95% alcohol	0.3 c.c.	2.8–4.6
Orthocarbobenzeneazodimethylanilin (Methyl red)	0.02%	water	0.5 c.c.	4.4–6.0
Dibromocresolsulphonephthalein (Brom cresol purple)	0.04%	95% alcohol	0.3 c.c.	5.2–6.8
Phenolsulphonephthalein (Phenol red)	0.02%	95% alcohol	0.3 c.c.	6.8–8.4

4. ISOLATION OF THE STRAINS: The fresh material taken from the root canal was planted on hemolyzed sheep blood-glucose-veal infusion agar slants (0.2 c.c. sterile hemolyzed defibrinated blood plus 0.5 c.c. 12% sterile glucose solution for each 6 c.c. medium) to which 0.1 c.c. sterile 1% glucose-beef-infusion broth had been added after solidification. After 18 to 20 hours incubation at 37° C., a loopful was streaked on several whole sheep-blood glucose infusion agar plates. Single colonies fished from these plates were inoculated into glucose infusion broth. If microscopic examination proved this a pure streptococcus culture, transplants were made to whole sheep blood infusion agar (0.5 c.c. blood plus 9.5 c.c. agar) and ox bile. The reactions obtained for 70 cultures used as stock strains are given in the following table:

Strain	Reaction to *Whole Blood*	*Bile*	Strain	Reaction to *Whole Blood*	*Bile*
852	I	O	981	I	O
853	I	O	1007	I	O
863	G	O	1009	I	O
874	I	O	1012	G	O
877	I	O	1015	I	O
882	I	O	1028	I	O
894	I	O	1041	I	O
904	H	O	1044	G	O
909	I	O	1074	G	O
911	I	O	1081	I	O
922	I	O	1094	I	O
948	I	O	1148	I	O
953	G	O	1151	G	O
956	I	O	1154	G	O
961	I	O	1155	I	O

Strain	Reaction to *Whole Blood*	*Bile*	Strain	Reaction to *Whole Blood*	*Bile*
1159	G	O	777	G	O
1161	I	O	802	I	O
1210	H	O	804	I	O
1220	G	O	830	I	O
1221	I	O	838	I	O
1232	I	O	898	I	O
1236	I	O	907	I	O
1239	I	O	910	I	O
1279	G	O	942	G	O
1286	I	O	947	I	O
1293	G	O	948	I	O
682	I	O	966	G	O
684	I	O	978	I	O
688	I	O	1004	I	O
703	G	O	1010	H	O
720	I	O	1011	H	O
725	I	O	1060	I	O
746	I	O	1097	G	O
748	I	O	3	I	O
770	I	O	4	I	O

O = No solution of organisms by bile.
I = Indifferent to blood cells.
H = Hemolysis of blood cells.
G = Green-producing action on blood cells.

5. Stock cultures: After isolation, each organism was planted in 2 c.c. defibrinated sheep blood, incubated for four hours at 37° C. and then stored in the ice-chest at 10° C. Some of the strains were used after several days isolation, others after several months of cultivation.

6. Seed culture: Two days before a specific culture had to be used, a loopful (platinum loop, 4mm. in diameter) from the stock blood culture was planted in 1% glucose broth (9.5 c.c. sugar-free broth plus 0.5 c.c. sterile 20 per cent glucose solution and incubated for 18 hours at 37° C. Then 0.05 c.c. of this subculture was inoculated into plain infusion broth (not made sugar-free) and incubated for the same period. This is designated as a seed culture.

In order to insure uniformity in the number of organisms transplanted from such seed cultures to the test media it was necessary to estimate the amount of growth. The cultures were well shaken and diluted to equal opacity by matching in a comparator block (the kind used in the colorimetric determination of hydrogen-ion concentration). Then the accuracy of this procedure was verified by the results obtained from plating definite amounts of the matched cultures and counting the colonies after 18 hours incubation at 37° C. For all comparative tests these matched cultures served as the seeds.

7. Biochemistry and biology:

(a) *Reaction on blood medium:* The significance of this reaction may be clearly brought out by a brief review of the literature on the classification of the streptococci. At the very outset investigators attempted to differentiate these organisms according to morphological characters. Thus arose the groups (1) Streptococcus longus and (2) Streptococcus brevis (29, 30). Such a distinction between types could be nothing more than tentative and therefore was short-lived.

Collateral with this grouping, but of more lasting influence, there existed another—that based on source of origin. We refer here to such groups as Streptococcus erysipelas (31, 32), Streptococcus equinus, Streptococcus pyrogenes, Streptococcus salivarans, etc. (33, 34, 35). This scheme too, could have but little value for there would be as many

groups of streptococci as there are places from which these organisms may be isolated.

Then Schottmüller (36) suggested a new classification into streptococcus erysipelas, Streptococcus viridans and Streptococcus mucosus, varieties recognized by certain distinctive appearances on blood media. While this method was gaining ground in Germany, English bacteriologists were studying the fermentation reactions of streptococci in order to ascertain whether essential differences could be discovered. In this connection the researches of Gordon (33) and Andrews and Horder (34) are significant.

Still another basis for classifying the streptococci has been considered—that involving the immunity reactions (35, 37-47). Most investigators think these reactions parallel the blood reactions (43, 44, 45), while Kligler (35) seems to be at variance with such a conclusion. From his experiments he finds a closer relationship between strains fermenting the same carbohydrates than between strains having the same hemolytic power.

More recently investigations have introduced nothing new so far as fundamental principles of classification are concerned. All of them treat with one, or with a combination of the bases outlined above. Various schemes have been proposed. Most attention has been given to those which make use of the blood reaction. We may mention here the work of Holman (48), Blake (49) and Smith and Brown (45). These authors recognize two main types—hemolytic and non-hemolytic, divided into subgroups which depend on the fermentative power of the organisms. And finally, the scheme which has superceded all of these and is now considered as the scheme by some of the most prominent bacteriologists is based primarily on the blood reaction and includes three main groups. Reference is made to the work of Lyall (50), Brown (51) and Park and Williams (52).

A thorough consideration of the various suggestions has led also us to conclude that the best basis for a preliminary grouping of the streptococci is that last named. The three types recognized are (1) that which hemolyzes the blood corpuscles; (2) that which produces a green coloration; (3) that which is indifferent to the presence of blood.

Detailed technique for differentiating the "blood types":—0.05 cc. culture from seed tube was dropped on the blood agar (0.5 cc. blood plus 9.5 cc. agar) in a petri dish and spread over the entire surface with a special smooth glass rod. The depth of the agar was 2 mm. Precaution was taken to have the blood evenly distributed. The results recorded were obtained from the examination of colonies widely separated after 24 hours incubation at 37° C.

In view of the fact that different laboratories use blood from different animal species we felt it necessary to find out whether the various strains reacted alike on the bloods most commonly used, human, rabbit and sheep. As might have been expected, the response of the organisms to the different kinds of erythrocytes was not the same. The actual experimental findings for 70 cultures are as follows:

Strain	Reaction to *Human Blood*			*Rabbit Blood*			*Sheep Blood*		
	H	I	G	H	I	G	H	I	G
852		+			+			+	
853		+		+				+	
863			+			+			+
877		+			+			+	
874		+			+			+	

Strain	Reaction to *Human Blood*			Reaction to *Rabbit Blood*			Reaction to *Sheep Blood*		
	H	I	G	H	I	G	H	I	G
882		+			+			+	
894		+			+			+	
904	+			+			+		
909		+			+			+	
911		+			+			+	
922		+			+			+	
948	+			+				+	
953			+			+			+
956	+			+				+	
961		+			+			+	
981		+			+			+	
1007		+			+			+	
1009		+			+			+	
1012	+			+					+
1015		+			+			+	
1028		+			+			+	
1041	+			+				+	
1044		+			+				+
1074			+			+			+
1081		+			+			+	
1094		+			+			+	
1148		+			+			+	
1151		+			+				+
1154	+			+					+
1155		+			+			+	
1159	+			+					+
1161	+			+				+	
1210	+			+			+		
1220			+			+			+
1221		+			+			+	
1232		+			+			+	
1236		+			+			+	
1239		+			+			+	
1279			+			+			+
1286		+			+			+	
1293		+			+				+
682		+			+			+	
684		+			+			+	
688		+			+			+	
703			+			+			+
720		+			+			+	
725		+			+			+	
746		+			+			+	
748	+			+				+	
770		+			+				+
777		+			+			+	
802		+			+			+	
804		+			+			+	
830		+			+			+	
838		+			+			+	
898		+			+			+	
907		+			+			+	
910		+			+			+	
942		+			+				+
947		+			+			+	
948		+			+			+	
966	+			+					+
978	+			+				+	
1004		+			+			+	
1010	+			+			+		
1011	+			+			+		
1060		+			+			+	
1097			+			+			+
3	+			+				+	
4	+			+				+	

Note: H = hemolysis. I = indifferent. G = green producing.

These results were checked by triplicate determinations.

The above detailed tabulation may be summarized as follows:—

Source of Blood	No. of Strains Producing Hemolysis	No. of Strains Producing Green Coloration	No. of Indifferent Strains
Man	16	7	47
Rabbit	17	7	46
Sheep	4	16	50
No. of "fast" strains	4	7	40

Note: A "fast" strain is one which reacts alike to the blood of all species.

This table indicates definitely the probable source for many discrepancies. It indicates, too, that a very decided advance in the systematic study of the streptococci may be made by instituting definite standards of procedure.

The group of organisms used for analytical material included two "fast" strains of each type.

(b) *Immunity reactions:* These reactions were studied in order to demonstrate that the chosen organisms were biologically distinct. To prove this point, the agglutinative capacity of each antiserum against each of the antigens was determined. The method of producing the immune sera and the technique for conducting the titrations was that used by Dochez, Avery and Lancefield (53).

The results obtained (as may be seen from the table) show no duplication in the strains used.

Organism	Immune Serum					
	904	1210	863	1074	688	720
904	1–500	1–100	0	0	0	0
1210	1–150	1–450	0	0	0	0
863	0	0	1–350	1–250	0	0
1074	0	0	1–200	1–650	0	0
688	1–50	0	1–50	1–30	1–200	1–100
720	0	1–10	1–20	1–20	1–50	1–550

Note: The numbers under "immune serum" designate the highest dilutions of the antiserum which showed definite specific clumping of the organisms.

(c) *Other biochemical reactions:* Although our choice of the experimental strains depended almost entirely on the blood reactions yet a complete characterization of the organisms must include the description of their ability to (1) ferment various carbohydrates and related substances (33, 34); (2) coagulate milk (33, 34); (3) coagulate inulin-serum-water (53, 54); (4) liquefy gelatin (34); (5) dissolve in bile (55).

The test substance (10 c.c.) in each series (except 5) was inoculated with 0.05 c.c. culture from the seed tubes. In series (5) 0.3 c.c. bile was mixed with 3 c.c. culture. All except series (3) were incubated at 37° C. for 24 hours. Series (3) was incubated for 60 hours. The following tabulation lists the reactions given by the experimental cultures:

Strain	Blood Reaction	Immunity Reaction	Hydrogen-ion Concentration, PH, in Broth										Reaction in			
			Plain	*Dextrose*	*Mannite*	*Galactose*	*Lactose*	*Maltose*	*Sucrose*	*Raffinose*	*Salicin*	*Inulin*	*Gelatin*	*Milk*	*Bile*	*Inulin-Serum-Water*
Control			7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7	o	o	o	o
904	Hemolysis	+	7.7	4.8	6.2	4.8	7.7	4.3	4.6	5.9	5.0	7.7	o	+	o	o
1210	Hemolysis	+	7.7	4.6	5.9	4.6	4.6	4.3	6.8	5.9	4.6	7.7	o	+	o	o
863	Green-producing	+	7.7	4.6	7.7	4.6	4.6	4.3	7.7	6.5	7.7	7.7	o	+	o	o
1074	Green-producing	+	7.7	4.3	6.5	4.6	5.3	4.3	5.3	6.2	4.6	7.7	o	+	o	o
688	Indifferent	+	7.7	4.6	5.6	4.6	4.6	5.0	7.7	6.2	4.3	7.7	o	+	o	o
720	Indifferent	+	7.7	4.3	6.5	4.6	4.6	4.3	4.8	6.5	4.3	7.7	o	+	o	o

Note: In the column under "immunity" the "+" sign designates that the culture is biologically distinct.

8. DESCRIPTION OF THE TEST ORGANISMS: Six test organisms were used: two hemolytic strains, two, green-producing and two, indifferent. The members of each pair were not duplicated individuals of the same type (see carbohydrate reactions). Moreover there was no duplication of strain in the entire series (see agglutination reactions).

III. PREPARATION OF MASS CULTURES

1. MEDIUM:

(a) *Choice of Infusion basis:* A comparative study of the different solid media available for streptococcus cultivation shows that the organisms accumulate more rapidly on some than on others. Five media were considered.

(1) Hemolyzed blood glucose veal infusion agar: The veal infusion was prepared as described above diluted with an equal volume of 3 per cent agar-agar solution and sterilized. When needed, the agar was melted and cooled slowly to 43°-45° C. Sterile hemolyzed defibrinated blood (1 volume blood to 2 volumes of distilled water) and sterile 12% glucose solution were then added. The proportion used was 0.1 c.c. of the diluted blood and 0.25 c.c. of the sugar solution to every 3.0 c.c. agar.

(2) Huntoon agar (56).

(3) Hasting gelatin-agar (57): The original directions are somewhat vague. This description is our interpretation: One pound beef meat in 1000 c.c. water was boiled for one hour, strained through a wire sieve, made up to the original volume, 10 grams peptone and 5 grams NaCl added and set in the Arnold sterilizer for 20 minutes. The infusion thus obtained was used as the solvent for the 15 grams agar-agar and 20 grams gelatin added at this point. Finally, enough NaOH was added to make the mixture neutral to phenolphthalein, the medium being kept at the boiling point during the titration.

(4) Beef liver agar: 500 grams fresh hashed beef liver were infused with 500 c.c. tap water for three hours at 55° C., strained through a wire sieve, 10 grams peptone and 5 grams NaCl added and set in the "Arnold" until solution was complete. The hydrogen-ion concentration was adjusted to a PH value = to 7.9. After being mixed with 500 c.c. 3 per cent agar-agar solution, the infusion was

sterilized in the autoclave for 15 minutes at 15 pounds pressure.

(5) Beef brain agar: This preparation was made exactly as the beef liver agar except that brain tissue was substituted for liver tissue.

Thus prepared, the five media were compared by this procedure: Each medium was tested with each experimental culture. We had, therefore, six series of tests—one for each strain. 0.05 c.c. of culture from the several seed tubes were evenly distributed over the surfaces of agar slants of the respective media. All the slants were formed by equal amounts of medium. Readings were made after 18 hours incubation at 37° C. The data tabulated below indicate the results obtained for one series. They are representative since each series showed the same gradations in the amounts of growth.

Medium	Estimated Amount of Growth	
Hemolyzed blood agar	Abundant	+++
Huntoon agar	Abundant	+++
Hasting gelatin-agar	Slight	++
Brain agar	Slight	++
Liver agar	Very abundant	++++

The results are necessarily only comparatively quantitive. They indicate, however, that the liver infusion furnished the optimum material for maximum growth. This may be explained by the fact that the liver normally contains a large store of glycogen, amino acids, various secretions, etc., all probably valuable to bacterial nutrition.

(b) *Concentration of agar-agar:* The more concentrated the agar-agar in a medium, the greater is the insurance against scratching the surface of a solidified layer in a petri dish when removing growth with a smooth spatula. But, on the other hand, too concentrated a preparation will practically inhibit bacterial growth. It therefore, became necessary to find that concentration which permitted the maximum development of colonies and at the same time insured against the possible breaking of the surface. Six series of tests were performed, one for each experimental culture. The infusion subtrate was the same for all; the concentration of the agar-agar was varied for each series. The results show definitely that for the organisms used 2 per cent is the optimum concentration.

Per cent. agar-agar	Growth for the Various Organisms						Streaking Character	Removal of Growth
	688	720	863	1074	904	1210		
1.0	++++	++++	++++	++++	++++	++++	Unfavorable	Impossible
1.5	++++	++++	++++	++++	++++	++++	Difficult	Difficult
2.0	++++	++++	++++	++++	++++	++++	Very favorable	Very favorable
2.5	+++	++++	+++	+++	++++	+++	Very favorable	Very favorable
3.0	++	++	+++	++	+++	++	Very favorable	Very favorable
3.5	++	++	++	++	+++	++	Very favorable	Very favorable
4.0	+	+	++	+	++	+	Very favorable	Very favorable
4.5	+	±	+	±	++	+	Very favorable	Very favorable
5.0	±	±	+	±	+	±	Very favorable	Very favorable

(c) *Constancy in composition of medium:* To insure absolute constancy in the composition of the medium a stock infusion was prepared from one large shipment of fresh hashed liver (200 pounds). This was estimated as the quantity large enough to cover the needs of the entire investigation.

2. CULTURES:

(a) *Preparation of cultures for mass growing:* Jablons liver peptone broth (58) was used for the cultivation of the test organisms preliminary to mass growing. This medium attracted our attention because it contained the same ingredients as the solid medium selected, thus favoring exceptional uniformity in the metabolic reactions of the bacteria throughout the entire experimental period. That the bouillon supported hardy development may be proven by the following experiments comparing it with other good liquid media: (1) Huntoon broth (56) and (2) Rosenow broth (59). The test media were inoculated with one loopful of culture from the seed tubes, kept at room temperature for 60 hours and then incubated for 24 hours at 37° C. Tests were made on 50 strains of streptococci. A comparison of the amounts of growth in the different preparations will give convincing proof that Jablons medium favors rapid growth of the organisms under consideration. The results tabulated represent the reactions given by all the organisms.

Medium	Amount of Growth	
	Estimate	Description
Jablons Broth	++++	Very abundant
Rosenow Broth	++++	Very abundant
Huntoon Broth	+++	Quite abundant

At regular intervals transplants to glucose blood agar were made, incubated for 24 hours at 37° C. and examined for growth. If growth was slight or doubtful, subcultures were made to confirm the results. By this procedure further evidence was obtained regarding the quality of the liver broth. A mere glance at the following summary shows that, in addition to supporting very rapid growth, Jablons medium also supports very prolonged viability.

Medium	No. of Cultures Dead in Specified No. of Weeks After Inoculation			Per cent. Viable After 10 Weeks
	4	6	10	
Jablons Broth	0	0	0	100
Rosenow Broth	0	1	2	96
Huntoon Broth	0	1	3	94

These results place each medium in the order of preference for the cultivation of streptococci, if longevity without transplant is taken as the criterion. With four weeks as the limiting period no differences are noticed; the three preparations match each other exactly. But, more prolonged viability necessarily infers less interference with normal metabolism. This makes Jablons medium the most efficient since it is so constructed as to give the organisms the greatest chance for continued vitality.

After growing the organisms in this broth for one month, 0.05 c.c. was transplanted to liver agar plates and grown at 37° C. for 18 hours. From this two successive subcultures to solid liver medium were made on two consecutive days. The last culture served as the seed culture for mass planting.

The mass cultures were grown for 16-18 hours at 37° C., thus producing cultures that contained a minimum of degenerated cells. Of course, this procedure did not supply the maximum amount of growth from each planting, but it did supply the optimum material for the chemical analysis of the normal organisms. This point seems to have been entirely overlooked in previous investigations on bacterial composition. In these, the chief interest was to procure the maximum amount of material from each planting. No attention was, therefore, paid to the metabolic plane of the organism. Thus Brieger (1) used growth four weeks old, Leach (60) worked with cultures one to two weeks old, and Vaughan (15), stating no definite period, gathered the growth when it had reached the maximum.

(b) *Planting of Cultures:* The agar was allowed to cool slowly to 43°-45° C., thus insuring a minimum volume of water of condensation in the petri dishes after solidification. This is a necessary precaution since in too moist a chamber the growth becomes saturated with moisture. Such a condition is very unfavorable for the purpose at hand, because (1) it is impossible to remove any significant part of the bacterial masses from the agar surface and (2) the small fraction that is obtained is contaminated with diffused substance from the medium.

The depth of the agar layer in the plates was 4mm.

Transplants were made from the special seed plates described above by means o a bent smooth glass rod.

(c) *Removal of the growth from the agar:* Two possible procedures suggested themselves. One method involves the detachment of the growth from the subjacent agar with bent glass rods, simplifying its removal by washing with physiological salt solution and pipetting off the bacterial suspension (15, 16). It is evident that a maximum amount of material may be obtained in this way but it is not optimum material for the present study. Much of the soluble substances in the medium are washed into the salt solution. Then, too, some substances diffuse from the cell bodies into the outer liquid. Such material may be removed by several washings and centrifugations, each time discarding the supernatant fluid. By doing so, however, the diffusible bacterial constituents are continually being drawn upon.

The other method, although not as economical regarding the collection of material, is far superior in providing a pure mass consisting only of "intact" bacterial structures. This process is simpler and involves fewer manipulations than the first. Thus, the smoothened end of a microscope slide is held lightly but firmly on the agar surface and rotated slowly clockwise. At the same time the dish is turned slowly counterclockwise. The growth is thereby gathered on the slide and very easily tapped off into a thoroughly dried shallow glass dish.

In order to determine definitely the method best suited for our purposes, tests were made on:

(1) The supernatant NaCl solution after centrifugation of the bacter-suspension, obtained by removing the growth from the agar with NaCl solution.

(2) The supernatant fluid after centrifugation of the bacterial suspension in distilled water, obtained as in (1) with distilled water substituted for the NaCl solution.

(3) The NaCl solution after being applied to a fresh agar surface and manipulated as though growth was actually being removed.

(4) The distilled water wash under the same conditions as in (3).

(5) The supernatant fluid from a bacterial suspension in a NaCl solution, the bacteria having been removed by the spatula method.

(6) The supernatant fluid from a bacterial suspension in distilled water, the growth having been removed as in (5).

(7) The NaCl solution used to wash a glass slide manipulated over a fresh agar surface as though growth was being removed.

(8) The distilled water wash obtained as in (7).

The tabulation below gives the results of the qualitative tests for phosphate, chloride, protein and protein split products, and dextrose. The numbers in parentheses refer to the several descriptions listed above.

Test Material	Tests Performed			
	Chloride	*Phosphate*	*Protein or Protein Split Products*	*Dextrose*
(1)	++++	++++	++++	o
(2)	++++	++++	++++	o
(3)	++++	++++	++++	o
(4)	++++	++++	++++	o
(5)	±	±	o	o
(6)	±	±	o	o
(7)	o	o	o	o
(8)	o	o	o	o

Note: With medium containing 1 per cent. dextrose (1) to (4) gave ++++ result for sugar.

These experiments show that:

(1) Substances are transferred from the medium to the wash solution.

(2) Bacteria suspended in distilled water or NaCl solution may give up some of their substance to the surrounding liquid.

(3) Nothing is removed from a medium by the mere application of a smooth spatula (70).

This evidence points definitely to the second method as the superior one.

(d) *Drying of growth:* The technic was that recommended by Shackell (61) with one exception: the bacteria were not frozen before drying. Layers 1 to 2 mm. thick in shallow plates dried to a scaly, porous mass in 24 to 36 hours. The temperature during the process varied between 21° and 22° C., the pressure between 0 and 10 mm. Hg.

The material was then pulverized with glass mortar and pestle to a very fine powder, spread in thin layers on glazed paper plates and dried to constant weight.

The dried substance was transferred to weighing bottles fitted with ground glass stoppers and stored in a dessiccator over concentrated H_2SO_4.

IV. ANALYSIS OF THE BACTERIAL SUBSTANCE

1. Methods:

(a) *Nitrogen:* Nitrogen was determined by the Kjeldahl process (Gunning-Arnold Dyer modification) (62). Digestion of the substance in concentrated sulphuric acid was completed with small quantities of metallic mercury. Before distillation, the mercury was precipitated with potassium sulphide. The titrations were made with 0.02 M NaOH.

(b) *Total Sulphur and Phosphorus:* Sulphur was determined by the method adopted by the the Association of Official Agricultural Chemists (63). The filtrate from the barium chloride precipitation was used to determine phosphorus (64).

(c) *Ash:* Inorganic matter was estimated by direct incineration of the dried substance in a platinum crucible over a very low flame.

(d) *Moisture:* The medium was cooled slowly to 43° C. The agar for the entire series of determinations was obtained from the same flask and the necessary number of petri dishes filled at the same time. The organisms were grown and collected as described above. About 0.1 gram of growth was rapidly transferred to a rubber stoppered glass vial weighing about 0.3 gram. Two sets of determinations of water content were made: (1) by drying in vacuo (0-10 mm. Hg and 22° C.) and (2) by drying in an air bath (100°-110°).

(e) *Lipins (material extracted with ether):* The substance was weighed in cone-shaped filter paper thimbles. The thimbles were suspended by means of wires to reflux condensers. These were fitted with flasks containing ether. After 24 hours of hot extraction the thimbles containing the extracted bacteria were dried in vacuo to constant weight. The difference in the weight of the thimble before and after extraction represents the weight of lipins.

(f) *Blanks:* Blank determinations on the reagents used were made in all cases. In the analyses for N the accuracy of the method for the small quantities obtained was checked by estimating the N recovered from known quantities of pure salt (ammonium sulphate).

2. Records of analysis:

(a) *Organism No. 688:*

Nitrogen: 0.0199 gram material gave 0.0019 gram N = 9.55 per cent N; 0.0217 gram material gave 0.0022 gram N = 10.13 per cent N.

Sulphur: 0.5336 gram material gave 0.0247 gram $BaSO_4$ = 0.63 per cent S; 0.5769 gram material gave .0259 gram $BaSO_4$ = 0.61 per cent S.

Phosphorus: 0.5336 gram material gave 0.0393 gram $Mg_2P_2O_7$ = 2.05 per cent P; 0.5769 gram material gave 0.0432 gram $Mg_2P_2O_7$ = 2.08 per cent P.

Ash: 0.5354 gram material gave 0.0526 gram Ash = 9.82 per cent Ash; 0.4694 gram material gave 0.0458 gram Ash = 9.75 per cent Ash.

Moisture: A. Determined by drying the bacterial material in vacuo: 0.0661 gram material gave 0.0485 gram H_2O = 73.37 per cent; 0.0866 gram material gave 0.0636 gram H_2O = 73.44 per cent. B. Determined by drying the bacterial material at 100° C: 0.0899 gram material gave 0.0671 gram H_2O = 74.63 per cent; 0.0769 gram material gave 0.0573 gram H_2O = 74.52 per cent.

Lipins: 0.3073 gram bacterial material gave 0.0047 gram Lipins = 1.53 per cent; 0.3343 gram bacterial material gave 0.0055 gram Lipins = 1.64 per cent.

(b) *Organism No. 720:*

Nitrogen: 0.0257 gram material gave 0.0026 gram N = 10.11 per cent N; 0.0243 gram material gave 0.0025 gram N = 10.28 per cent N.

Sulphur: 0.5741 gram material gave 0.0253 gram $BaSO_4$ = 0.60 per cent S; 0.5320 gram material gave 0.0234 gram $BaSO_4$ = 0.60 per cent S.

Phosphorus: 0.5741 gram material gave 0.0429 gram $Mg_2P_2O_7$ = 2.08 per cent P; 0.5320 gram material gave 0.0390 gram $Mg_2P_2O_7$ = 2.04 per cent P.

Ash: 0.2910 gram material gave 0.0301 gram Ash = 10.34 per cent Ash; 0.3011 gram material gave 0.0311 gram Ash = 10.33 per cent Ash.

Moisture: A. Determined by drying the bacterial material in vacuo: 0.0638 gram material gave 0.0452 gram H_2O = 70.85 per cent; 0.0864 gram material gave 0.0611 gram H_2O = 70.72 per cent. B. Determined by drying the substance at 100° C: 0.0781 gram material gave 0.0560 gram H_2O = 71.70 per cent; 0.0897 gram material gave 0.0643 gram H_2O = 71.68 per cent.

Lipins: 0.3377 gram bacterial material gave 0.0035 gram Lipins = 1.03 per cent; 0.3131 gram bacterial material gave 0.0033 gram Lipins = 1.05 per cent.

(c) *Organism No. 904:*

Nitrogen: 0.0235 gram material gave 0.0025 gram N = 10.64 per cent N; 0.0213 gram material gave 0.0022 gram N = 10.33 per cent N.

Sulphur: 0.6347 gram material gave 0.0265 gram $BaSO_4$ = 0.57 per cent S; 0.5096 gram material gave 0.0202 gram $BaSO_4$ = 0.54 per cent S.

Phosphorus: 0.6347 gram material gave 0.0494 gram $Mg_2P_2O_7$ = 2.17 per cent P; 0.5096 gram material gave 0.0405 gram $Mg_2P_2O_7$ = 2.21 per cent P.

Ash: 0.5208 gram material gave 0.0538 gram Ash = 10.33 per cent Ash; 0.4933 gram material gave 0.0508 gram Ash = 10.30 per cent Ash.

Moisture: A. Determined by drying the substance in vacuo: 0.0475 gram material gave 0.0347 gram H_2O = 73.05; 0.0620 gram material gave 0.0453 gram H_2O = 73.07 per cent. B. Determined by drying the bacterial material at 100°: 0.0530 gram material gave 0.0401 gram H_2O = 75.66 per cent; 0.0668 gram material gave 0.0506 gram H_2O = 75.75 per cent.

Lipins: 0.3390 gram bacterial material gave 0.0122 gram Lipins = 3.59 per cent; 0.2981 gram bacterial material gave 0.0108 gram Lipins = 3.62 per cent.

(d) *Organism No. 1210:*

Nitrogen: 0.0209 gram material gave 0.0020 gram N = 9.57 per cent N; 0.0238 gram material gave 0.0023 gram N = 9.66 per cent N.

Sulphur: 0.5301 gram material gave 0.0230 gram $BaSO_4$ = 0.60 per cent S; 0.5007 gram material gave 0.0217 gram $BaSO_4$ = 0.60 per cent S.

Phosphorus: 0.5301 gram material gave 0.0410 gram $Mg_2P_2O_7$ = 2.15 per cent P; 0.5007 gram material gave 0.0399 gram $Mg_2P_2O_7$ = 2.22 per cent P.

Ash: 0.2687 gram material gave 0.0266 gram Ash = 9.90 per cent Ash; 0.2087 gram material gave 0.0206 gram Ash = 9.87 per cent Ash.

Moisture: A. Determined by drying the bacterial material in vacuo: 0.0745 gram material gave 0.0544 gram $H_2O = 73.02$ per cent; 0.1033 gram material gave 0.0753 gram $H_2O = 72.89$ per cent. B. Determined by drying the bacterial material at 100°: 0.0466 gram material gave 0.0349 gram $H_2O = 74.90$ per cent; 0.0739 gram material gave 0.0554 gram $H_2O = 74.97$ per cent.

Lipins: 0.2051 gram bacterial material gave 0.0083 gram Lipins = 4.04 per cent; 0.2251 gram bacterial material gave 0.0086 gram Lipins = 3.82 per cent.

(e) *Organism No. 863:*

Nitrogen: 0.0265 gram material gave 0.0026 gram N = 9.81 per cent N; 0.0224 gram material gave 0.0023 gram N = 10.27 per cent N.

Sulphur: 0.6206 gram material gave 0.0254 gram $BaSO_4 = 0.56$ per cent S; 0.7039 gram material gave 0.0283 gram $BaSO_4 =$ 0.55 per cent S.

Phosphorus: 0.6206 gram material gave 0.0444 gram $Mg_2P_2O_7 =$ 1.99 per cent P; 0.7039 gram material gave 0.0498 gram $Mg_2P_2O_7 = 1.97$ per cent P.

Ash: 0.4887 gram material gave 0.0457 gram Ash = 9.35 per cent Ash; 0.5881 gram material gave 0.0552 gram Ash = 9.38 per cent Ash.

Moisture: A. Determined by drying the bacterial material in vacuo: 0.0665 gram material gave 0.0465 gram $H_2O = 69.93$ per cent; 0. 0743 gram material gave 0.0521 gram $H_2O = 70.12$ per cent. B. Determined by drying the substance at 100°: 0.0577 gram material gave 0.0432 gram $H_2O = 74.87$ per cent.

Lipins: 0.4098 gram bacterial material gave 0.0226 gram Lipins = 5.51 per cent; 0.3933 gram material gave 0.0208 gram Lipins = 5.29 per cent.

(f) *Organism No. 1074:*

Nitrogen: 0.0209 gram material gave 0.0021 gram N = 10.05 per cent N; 0.0198 gram material gave 0.0019 gram N = 9.59 per cent N.

Sulphur: 0.5037 gram material gave 0.0221 gram $BaSO_4 = 0.60$ per cent S; 0.4791 gram material gave 0.0199 gram $BaSO_4 =$ 0.57 per cent S.

Phosphorus: 0.5037 gram material gave 0.0358 gram $Mg_2P_2O_7$ = 1.98 per cent P; 0.4791 gram material gave 0.0338 gram $Mg_2P_2O_7 = 1.96$ per cent P.

Moisture: A. Determined by drying the bacterial material in vacuo: 0.1070 gram material gave 0.0765 gram $H_2O = 71.50$ per cent; 0.0896 gram material gave 0.0642 gram $H_2O = 71.65$ per cent. B. Determined by drying the substance at 100°: 0.0668 gram material gave 0.0506 gram $H_2O = 75.60$ per cent; 0.0552 gram material gave 0.0416 gram $H_2O = 75.36$ per cent.

Lipins: 0.3080 gram bacterial material gave 0.0166 gram Lipins = 5.39 per cent; 0.2995 gram bacterial material gave 0.0162 gram Lipins = 5.40 per cent.

Ash: 0.3024 gram material gave 0.0301 gram Ash = 9.95 per cent Ash; 0.4024 gram material gave 0.0398 gram Ash = 9.89 per cent Ash.

3. SUMMARY OF THE DATA FOR THE AVERAGE PERCENTAGE COMPOSITION OF THE STREPTOCOCCI.

Description of Organism		Composition						
Number	Type	N	S	P	Ash	Lipins	Moisture	
							Vacuo	100°-110°
688	Indifferent	9.8	0.62	2.06	9.78	1.58	73.40	74.57
720	Indifferent	10.1	0.60	2.06	10.33	1.04	70.78	71.69
904	Hemolytic	10.4	0.55	2.19	10.31	3.60	73.06	75.70
1210	Hemolytic	9.6	0.60	2.18	9.88	3.93	72.95	74.93
863	Green producing	10.0	0.55	1.98	9.36	5.40	70.02	74.87
1074	Green producing	9.8	0.58	1.97	9.92	5.39	71.57	75.48

4. CONCLUSIONS FROM THE ABOVE ANALYTICAL DATA: The above summary indicates the average results of the various analyses. It also brings into comparison the figures for the composition of the different streptococci and points out these facts:

(a) There are no marked graded differences in either the nitrogen or the sulphur content. It becomes apparent, therefore, that the total amounts of protein material in the different organisms varies but slightly, if at all.

(b) The phosphorus content is practically constant. This indicates no striking variations in the quantity of nucleoprotein (the phosphorus due to phosphatide being very small in amount).

(c) Regarding the results for the moisture determinations only slight deviations are noticed. The percentages obtained by drying the substance at 100°C. are higher than those obtained by drying the material in vacuo. Such increases in the values were expected because of the greater disintegration at the higher temperature.

(d) Most interesting, however, are the figures obtained for the amounts of Lipin material. These vary greatly for the different types of streptococci. The percentage of lipins is lowest in the indifferent type and highest in the green producing type, the hemolytic type taking a position between these two. The characteristic relations described above may be expressed by the ratio: 10 : 29 : 41 ; or briefly :

Indifferent: Hemolytic: Green-producing

1 : 3 : 4

These results separate the streptococci into three groups corresponding to those obtained when the reaction on blood media is used as the criterion.

Because of the apparent significance of this relationship, we repeated this part of the investigation. Fresh cultures were amassed and dried as described above. A series of nine organisms were used, three from each representative group, the strains 688, 720, 904, 1210, 863 and 1074 being included. The three new strains were chosen at random from our stock cultures of indifferent, green-producing and hemolytic streptococci.

These results were obtained:

Designation of Organism		
Number	*Type*	Average Percentage Lipins
688	Indifferent	1.46
720	Indifferent	1.54
725	Indifferent	1.16
904	Hemolytic	3.16
1210	Hemolytic	3.73
1012	Hemolytic	3.79
863	Green-producing	5.31
1074	Green-producing	5.34
953	Green-producing	5.68

A mere glance at the table brings to light the possible ratio:

1 : 3 : 4

This similates the data gathered from our first group of determinations.

V. ANALYSIS OF THE LIPIN MIXTURE

1. OUTLINE FOR THE WORK: The very striking regularity in the amounts of Lipin material and the fact that the groups of streptococci parallel those obtained by the blood media reaction suggested the possible relation between the nature of the Lipin mixture and the hemolyzing capacity of the organisms. In an investigation of this character the chief factors to be considered are (1) the phosphatide content, (2) the cholesterol content and (3) the ratio between the phosphatide and cholesterol.

A preliminary qualitative examination of the material showed that all the extracts contained both cholesterol and phosphatide. These substances were then determined quantitatively by nephelometric methods (65, 66, 67, 68).

2. RECORDS OF ANALYSIS OF THE LIPIN MIXTURE.

(a) *Organism No. 688:*

Phosphatide: 2.3560 gram bacterial material gave 0.0400 gram Lipins which yielded 0.0000125 gram H_3PO_4 = 0.25 per cent Phosphatide; 2.4960 gram bacterial material gave 0.0380 gram Lipins which yielded 0.0000125 gram H_3PO_4 = 0.25 per cent Phosphatide.

Cholesterol: 2.5360 gram bacterial material gave 0.0400 gram Lipins which yielded 0.00064 gram Cholesterol = 1.6 per cent Cholesterol; 2.4960 gram bacterial material gave 0.0380 gram Lipins which yielded 0.00064 gram cholesterol = 1.7 per cent cholesterol.

(b) *Organism No. 720:*

Phosphatide: 2.4411 gram bacterial material gave 0.0290 gram Lipins which yielded 0.0000087 gram H_3PO_4 = 0.24 per cent Phosphatide; 2.4301 gram bacterial material gave 0.0280 gram Lipins which yielded 0.0000087 gram H_3PO_4 = 0.24 per cent Phosphatide.

Cholesterol: 2.4411 gram bacterial material gave 0.0290 gram Lipins which yielded 0.00046 gram Cholesterol = 1.5 per cent 2.4302 gram bacterial material gave 0.0280 gram Lipins which yielded 0.00046 gram Cholesterol = 1.5 per cent.

(c) *Organism No. 904:*

Phosphatide: 1.7090 gram bacterial material gave 0.0710 gram Lipins which yielded 0.000006 gram H_3PO_4 = 0.064 per cent Phosphatide; 1.6980 gram bacterial material gave 0.0640 gram

Lipins which yielded 0.000006 gram H_3PO_4 = 0.072 per cent Phosphatide.

Cholesterol: 1.7090 gram bacterial material gave 0.0710 gram Lipins which yielded 0.00078 gram Cholesterol = 1.09 per cent; 1.6980 gram bacterial material gave 0.0640 gram Lipins which yielded 0.0007 gram Cholesterol = 1.08 per cent.

(d) *Organism No. 1210:*

Phosphatide: 1.7091 gram bacterial material gave 0.0700 gram Lipins which yielded 0.000006 gram H_3PO_4 = 0.064 per cent Phosphatide; 1.7100 gram bacterial material gave 0.0710 gram Lipins which yielded 0.000006 gram H_3PO_4 = 0.064 per cent Phosphatide.

Cholesterol: 1.7091 gram bacterial material gave 0.0700 gram Lipins which yielded 0.0007 gram Cholesterol = 1.0 per cent Cholesterol; 1.7100 gram bacterial material gave 0.0710 gram Lipins which yielded 0.00074 gram Cholesterol = 1.02 per cent.

(e) *Organism No. 863:*

Phosphatide: 2.5725 gram bacterial material gave 0.1366 gram Lipins which yielded 0.000006 gram H_3PO_4 = 0.035 per cent Phosphatide; 2.5728 gram bacterial material gave 0.1362 gram Lipins which yielded 0.000006 gram H_3PO_4 = 0.035 per cent Phosphatide.

Cholesterol: 2.5725 gram bacterial material gave 0.1366 gram Lipins which yielded 0.0010 gram Cholesterol = 0.73 per cent; 2.5728 gram bacterial material gave 0.1362 gram Lipins which yielded 0.0010 gram Cholesterol = 0.73 per cent.

(f) *Organism No. 1074:*

Phosphatide: 2.5361 gram bacterial material gave 0.1321 gram Lipins which yielded 0.000006 gram H_3PO_4 = 0.036 per cent Phosphatide; 2.5360 gram bacterial material gave 0.1325 gram Lipins which yielded 0.000006 gram H_3PO_4 = 0.036 per cent Phosphatide.

Cholesterol: 2.5361 gram bacterial material gave 0.1321 gram Lipins which yielded 0.0010 gram Cholesterol = 0.75 per cent; 2.5360 gram bacterial material gave 0.1325 gram Lipins which yielded 0.0010 gram Cholesterol = 0.75 per cent.

3. SUMMARY OF THE DATA FOR THE AVERAGE PERCENTAGES OF PHOSPHATIDE AND CHOLESTEROL.

Organism No.	688	720	904	1210	863	1074
Phosphatide	0.25	0.24	0.068	0.064	0.035	0.036
Cholesterol	1.65	1.50	1.08	1.01	0.73	0.75
Ratio C:P	6:1	6:1	16:1	16:1	21:1	21:1

4. CONCLUSIONS FROM THE DATA FOR THE CHOLESTEROL AND PHOSPHATIDE CONTENT.

(a) The content of phosphatide decreases in the different types in the order named: indifferent, hemolytic, green-producing.

(b) The same relation is noticed in the amounts of cholesterol contained in the different organisms.

(c) The ratio C:P increases when the types are listed in the same order.

VI. EFFECT OF THE LIPIN MATERIAL ON HEMOLYSIS

1. Record of Experiments: Since the ratios of cholesterol to phosphatide are different for the different types of streptococci it was interesting to investigate the effects which the lipin mixtures would have on the hemolytic action of saponin.* A 10 per cent washed rabbit cell suspension was used in the experiments. The method used was essentially that of Ranson (69). Preliminary titrations were made to obtain the dose of saponin that will hemolyze 1.0 c.c. of cell suspension in the presence and absence of cholesterol (the amount of cholesterol used approximated that in the lipin mixture). That amount of saponin which produced hemolysis in the absence of cholesterol but which failed to do so in those tubes which contained cholesterol was taken as the test dose. This dose was then added to graded amounts of lipin material dissolved in ether. The protocols below show in detail the experiments performed and the results obtained.

Experiment (a): To determine the largest amount of saponin that produces hemolysis in the presence of cholesterol.

Tube no.	1	2	3	4	5	6	7	8	9
Saponin in c.c. 0.2% in 0.9% NaCl	1.0	0.5	0.25	0.15	0.10	0.08	0.06	0.04	0.02
Cholesterol in c.c. 0.01% in Ether	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
0.9% NaCl in c. c.	1.90	2.40	2.65	2.75	2.80	2.82	2.84	2.86	2.88
Incubation at 25° C. for 15 Minutes									
Cell suspension in c.c.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Readings:									
8 minutes	++++	++++	++++	+++	+++	0	0	0	0
15 minutes	++++	++++	++++	+++	+++	+	+	0	0
60 minutes	++++	++++	++++	++++	++++	+++	++	0	0
12 hours	++++	++++	++++	++++	++++	++++	++++	++++	0

This experiment shows that in the presence of cholesterol 0.08 cc. of a 0.2% saponin solution is the largest amount that does not hemolyze 1.0 c.c. cell suspension in 8 minutes.

Experiment (b): To determine the smallest amount of saponin that produces hemolysis in the absence of cholesterol.

Tube no.	1	2	3	4	5	6	7	8	9
Saponin	1.0	0.5	0.25	0.15	0.1	0.08	0.06	0.04	0.02
Ether	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Salt Solution	1.90	2.40	2.65	2.75	2.80	2.82	2.84	2.8	2.88
Incubation at 25° C. for 15 Minutes									
Cell Suspension	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Readings:									
8 minutes	++++	++++	++++	++++	++++	++++	+	0	0
15 minutes	++++	++++	++++	++++	++++	++++	++	+	0
60 minutes	++++	++++	++++	++++	++++	++++	++++	++	0
12 hours	++++	++++	++++	++++	++++	++++	++++	++++	0

*The saponin was obtained from Eimer and Amend, New York City.

The results of this experiment indicate that in the absence of cholesterol 0.08 c.c. of a 0.2% saponin solution is the smallest amount that produces hemolysis with 1.0 c.c. cell suspension in 8 minutes. This is, therefore, the test dose and was used in determining the effect of the ether extract on the action of saponin.

The lipins extracted from the bacteria were dissolved in definite volumes of ether: three-fifths of this solution was evaporated to a volume of 3cc. and then emulsified with 27 cc. of 0.9% NaCl solution. Graded amounts of this emulsion were added to the various tubes.

Experiment (c): To determine the effect of the extract from organism no. 688 on the action of saponin.

Tube No.	1	2	3	4
Extract in c.c.	1.0	1.5	2.0	2.5
Saponin in c.c.	0.08	0.08	0.08	0.08
Salt solution in c.c.	1.92	1.42	0.92	0.42
Incubation at 25° for 15 Minutes				
Cell suspension in c.c.	1.0	1.0	1.0	1.0
Readings:				
10 minutes	0	0	0	0
20 minutes	0	0	0	0
1 hour	+	+	+	+
12 hours	+	+	+	+

Experiments (d), (e), (f), (g), (h) were performed respectively with extracts from organisms 720, 904, 1210, 863, 1074. The results parallelled exactly those of experiment (c).

As checks on the validity of the above experiments controls were run on the various ingredients:

No.	Combination	Hemolysis
1	Salt solution and cell suspension	0
2	Lecithin and cell suspension	0
3	Cholesterol and cell suspension	0
4	Saponin and cell suspension	++++
5	Extract 688 and cell suspension	0
6	Extract 720 and cell suspension	0
7	Extract 904 and cell suspension	0
8	Extract 1210 and cell suspension	0
9	Extract 863 and cell suspension	0
10	Extract 1074 and cell suspension	0

2. Conclusions from data on hemolysis.

(a) The lipin mixtures from the various organisms show the same effect on the action of saponin—they inhibit hemolysis.

(b) The differences, mentioned above, in the ratios of cholesterol to phosphatide cannot be correlated with differences in the action of the ether extracts on the hemolytic power of saponin.

VII. SUMMARY OF CONCLUSIONS.

1. There are no marked graded differences in either the N or S content. It becomes apparent, therefore, that the total amounts of protein material in the different organism varies but slightly.

2. The P content is practically constant. This indicates no striking variations in the quantity of nucleoprotein (the P from the phosphatide being very small in amount).

3. Regarding the results for the moisture determinations only slight deviations are noticed. The percentages obtained by drying the material at 100° C. are higher than those obtained by drying the substances in vacuo. Such increases in the values were expected because of the greater disintegration at the higher temperature.

4. Most interesting are the figures obtained for the amount of lipin material. These vary greatly for the different types of streptococci. The percentage of lipins is lowest in the indifferent type and highest in the green-producing type, the hemolytic type taking a position between these two. The characteristic relations may be expressed by the ratio: Indifferent: Hemolytic: Green Producing:: 1 : 3 : 4. These results seem to seperate the streptococci into three groups corresponding to those obtained when the reaction on blood is used as the criterion.

5. The content of phosphatide in the lipin mixture decreases for the different types in the order named: indifferent, hemolytic, green-producing.

6. The same relation is given by the amounts of cholesterol contained in the different organisms.

7. The ratio C:P increases when the types are listed as above.

8. The lipin mixtures from the various organisms show the same effect on the action of saponin: they inhibit hemolysis.

9. The above mentioned differences in the ratios of cholesterol to phosphatide cannot be correlated with differences in the action of the ether extracts on the hemolytic power of saponin.

BIBLIOGRAPHY

(1) Brieger, *Zt. f. phys. Chem.*, 1885, ix, 1.
(2) Weyl, *Deutsch. med. Wochschr.*, 1891, xvii, 256.
(3) Hammerschlag, *Centralbl, f. klin. Med.*, 1891, xii, 9.
(4) Hoffman, *Wien. klin. Wochschr.*, 1894, 712.
(5) De Schweinitz and Dorset, *J. Am. Chem. Soc.*, 1895, xvii, 605.
(6) ——*J. Am. Chem. Soc.*, 1896, xviii, 449.
(7) ——*J. Am. Chem. Soc.*, 1897, xix, 782.
(8) ——*J. Am. Chem. Soc.*, 1898, xx, 618.
(9) Ruppel, *Zt. f. phys. Chem.*, 1898, xxvi, 218.
(10) Levene, *Med. Record*, 1898, liv., 873.
(11) ——*J. Med. Research*, 1901, vi, 135.
(12) Drymount, *Arch. f. exp. Path. u. Pharm.*, 1886, xxi, 309.
(13) Vaughan, *Trans. Ass. Am. Phys.*, 1902, xvii, 313.
(14) Wheeler, *J. Am. Med. Ass.*, 1905, xliv, 1271.
(15) V. C. Vaughan, V. C. Vaughan, Jr., J. W. Vaughan, Protein Split Products in Relation to Immunity and Disease, 1913. (Lea & Febiger, Philadelphia and New York).
(16) *Leach, J. Biol. Chem.*, 1906, i, 463.
(17) Dawson, *J. Bact.*, 1919, iv, 133.
(18) Nicolle and Alilaire, *Ann. de. l'Institute Pasteur*, 1909, xxiii, 547.
(19) Bradley, *J. Biol. Chem.*, 1918, xxxiii, 525.
(20) Avery & Cullen, *J. Exp. Med.*, 1919, xxx, 359.
(21) ——*J. Exp. Med.*, 1919, xxix, 215.
(22) Hiss and Zinsser, Text Book of Bact., 1916, 126, (Appleton & Co., New York.)
(23) ——Text Book of Bact., 1916, 130.
(24) ——Text Book of Bact., 1916, 132.
(25) Clark and Lubs, *J. Bact.*, 1917, ii, 1, 109, 191.
(26) Walpole, *Biochem. Jour.*, 1914, viii, 628.
(27) Dernby and Avery, *J. Exp. Med.*, 1918, xxvili, 345.
(28) Sörensen, *Biochem. Zt.*, 1909, xxv, 131.
(29) Von Lingelsheim, *Zt. Hyg.*, 1891, x, 331.
(30) Kurth, Quoted by Kolle and von Wassermann, Handbuch der path. Microorg. 2″ Ed. 1912, 14, 456.
(31) Fehleisen, Die Aetiologie des Erysipels, Berlin, 1883.
(32) Rosenbach, Microorg. bei d. Wundinfections des Menchen, Weisboden, 1891.
(33) Gordon, *Lancet*, 1905, ii, 1400.
(34) Andrews and Horder, *Lancet*, 1906, ii, 708.
(35) Kligler, *J. Inf. Dis.*, 1915, xvi, 327.
(36) Schottmüller, *Munch. med. Wochschr.*, 1903, l, 849.
(37) Von de Welde, *Centr. Bakt.*, 1898, xxiii, 481.
(38) Baginsky and Sommerfeld, *Ber. klin. Wochschr.*, 1900, xxvii, 588.
(39) De Waele and Sugg, Archiv. internat. pharm. et thérap. 1904, xii, 205.
(40) Meyer, *Deutsch. med. Wochschr.*, 1902, xxviii, 751.
(41) Aronson, *Deutsch. med. Wochschr.*, 1903, xxix, 439.
(42) Reudiger, *J. Inf. Dis.*, 1906, iii, 663, 775.
(43) Reudiger, *Science*, 1912, xxxv, 223.
(44) Davis, *J. Inf. Dis.*, 1913, xii, 386.
(45) Smith and Brown, *J. Med. Res.*, 1915, xxxi, 445.
(46) Besredka and Dopter, *Ann. de. l'Institut Pasteur*, 1904, xviii, 37.
(47) Swift and Thro, *Arch. Int. Med.*, 1911, vii, 24.

(48) Holman, *J. Med. Res.*, 1916, xxxiv, 377.
(49) Blake, *J. Med. Res.*, 1917, xxxvi, 99.
(50) Lyall, *J. Med. Res.*, 1914, xxx, 487, 415.
(51) Brown, Monograph, Rock., Inst. Med. Res., no. 9, 1919.
(52) Park and Williams, Path. Microorg., 1920 (Lea & Febiger, Philadelphia and New York).
(53) Dochez, Avery and Lancefield, *J. Exp. Med.*, 1919, xxx, 179.
(54) Hiss, *J. Exp. Med.*, 1905, vi, 317.
(55) Neufeld, *Ztschr. f. Hyg.*, 1900, xxxiv, 454.
(56) Huntoon, *J. Inf. Dis.*, 1918, xxiii, 169.
(57) Dr. Hasting's formula obtained from Dr. M. L. Rhein, New York City.
(58) Jablons and Pease, *C. r. Soc. de biol.*, 1919, i, 205.
(59) Rosenow, *J. Dental Res.*, 1919, i, 205.
(60) Leach, *J. Biol. Chem.*, 1907, iii, 443.
(61) Shackell, *Am. J. Physiol.*, 1909, xxiv, 325.
(62) Sherman, Organic Analysis, 1919, 291 (Macmillan & Co., New York).
(63) Assoc. of Official Agricultural Chemists, U. S. Dept. Agr. Bur. Chem. Bul. 107 (Revised) p. 23.
(64) Koch, Hygienic Laboratory Bul. 103, p. 79.
(65) Bloor, *J. Biol. Chem.*, 1915, xxii, 133.
(66) ——*J. Biol. Chem.*, 1916, xxiv, 447.
(67) Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.
(68) Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.
(69) Ranson, *Deutsch. med. Wochschr.*, 1901, xxvii, 194.
(70) Nishimura, *Archiv. f. Hyg.*, 1893, xviii, 318.

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