Ph.D. Bacteriology

M. Jeanne Kahnke

STAPHYLOCOCCUS NUCLEOPROTEINS IN CONNECTION WITH IMMUNITY

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REVIEW OF THE LITERATURE OF BACTERIAL NUCLEOPROTEINS

Methods of Liberating Bacterial Nucleoproteins

The minute size of the bacterial cell and the fact that the bacteria is unicellular has made the problem of liberating the bacterial nucleoproteins difficult, and has led to numerous devices and methods for breaking the cells.

The methods for releasing bacterial intracellular proteins might be classified thus:

I Mechanical

II Sonic or Ultrasonic Vibration

III Freezing and Thawing

IV Chemical Treatments

I Mechanical Methods:

Buchner (1897) in his now classical fermentation experiments liberated enzymes from yeast cells by grinding the cells with quartz sand. He was able to show that the yeast cells were dead and that the solution of enzymes freed from the debris of yeast cells would promote alcoholic fermentation.

Lancefield (1925) subjected dry streptococci to grinding in a ball mill. Coghill (1931) and Heidelberger (1931) also used ball mill grinding to liberate protein from dried and defatted cells, while Topping (1934) and Hirst and Lancefield (1939) ground ether dried cells with a mortar and pestle. Mudd et al (1937) devised a low temperature ball mill which allowed the bacteria to freeze and dry before grinding occurred.

Wiggert et al (1940) ground cells with powdered glass in

a mortar and pestle and were able to obtain a cell free extract, while Kalnitsky (1945) improved this process by developing a mill for grinding a chilled suspension of bacteria with powdered glass.

Meltzer (1892) observed that vegetative cells mixed with sand, glass or steel and subjected to long and extensive shaking died readily. Curran and Evans (1942) used small glass beads to destroy spores of B. megatherium and cells of E. coli. The death of the spores and cells took place in a logarithmic fashion. The aforementioned authors stated that the shaking with beads produced protein from the bacteria which might be useful in preparing protein fractions for immunological studies.

II Sonic and Supersonic Vibration

Wood and Loomis (1927) originated investigations on the effects of high intensity sound waves on biological materials. Williams and Gaines (1930) were the first to use audible sound waves to kill bacteria. Chambers and Flosdorf (1936) described a sonic vibrator which gave 8,900 cycles per second. This vibrator was used to break up typhoid and streptococcus cells. Mudd, Czarnetzky, Lackman and Pettit (1938) and Czarnetzky, Mudd, Pettit and Lackman (1938) were able to prepare a highly labile surface antigen of streptococcus by using the sonic vibrator of Chambers and Flosdorf (1936).

Stumpf et al (1946) using a vibrator of 600 kilocycles and 2,000 volts with an output of 700 watts found no disintegration of bacteria in a thick bacterial paste. The authors

stated that disintegration of bacteria is independent of the frequency of the sound wave, and that the bacteria had to be in a relatively thin suspension before they were broken up by sound waves.

The supersonic vibrator of Stumpf et al (1946) was found to kill H. influenzae and Staphylococcus aureus, but not Sarcina lutea or Acetobacter suboxydans.

The Raython sonic vibrator, producing 9,000 cycles per second, was shown by Shropshire (1947) to rupture Staphylococcus albus.

III Freezing and Thawing:

Luyet and Gehenio (1940) reviewed extensively the effect of freezing on bacteria and Rahn (1945) discussed the mechanism of the freezing process on puncturing the bacterial cell. Gunnison (1934) ruptured the cells of C. parabotulinum by repeated freezing and thawing, but Boor and Miller (1934) found freezing and thawing had little effect on the cells of gonococci and meningococci.

IV Chemical Methods:

Levene (1901) extracted nucleoprotein from crushed and defatted tubercle bacillus cells with five percent sodium hydroxide. Albert et al (1902) first applied acetone and ether to yeast cells in order to remove zymase and other enzymes from the cells, and Buchner et al (1906) used this method to liberate lactase from Bacillus delbruckii. Lebedew (1911) dried bacterial cells with acetone and ether, resuspended them in water and noticed that the cells were disinte-

Johnson and Brown (1922) defatted the tubercle bacillus and dissolved the cells in 3 percent sodium hydroxide to extract nucleoprotein. Avery and Heidelberger (1923) dissolved pneumococci in bile to liberate the proteins, while Avery and Morgan (1925) suspended pneumococci in 0.002 N sodium hydroxide solution and subjected this suspension to repeated freezing and thawing in order to rupture the cells.

N/16 hydrochloric acid and boiling temperatures were used by Julianelle and Wieghard (1935) to rupture staphylococci. Boivin and Mesrobeanu (1935) isolated complex antigens from the Salmonella group of organisms by using ice cold 0.25 N trichloracetic acid. Morgan (1937) used diethylene glycol to disrupt acetone dried B. dysenteriae, while Walker (1940) used urea solutions to extract antigens from organisms of the Salmonella group. Henderson and Morgan (1938) used ethylene glycol on acetone dried typhoid bacilli, while Binkley, Goebel and Perlman (1945) used pyridine to disrupt dysentery bacilli. Phenol was used by Palmer and Gerlough (1940) to extract polysaccharide from typhoid bacilli.

Avery et al (1944) dissolved pneumococci in sodium desoxycholate; Thompson and Dubos (1938) used the bacterias' own autolytic enzymes to release nucleoprotein. They washed pneumococci with 0.1 M acetate buffer, resuspended the cells in 0.4 percent sodium chloride solution and incubated the cells at 37°C to secure a nucleoprotein solution.

Since bacteria do not usually excrete nucleoprotein, it might be said in summary that any process which destroys or disrupts the cell or its surface could be used to extract nucleoprotein: - provided the agent used had no deleterious effect on

ISOLATION, PURIFICATION AND PROPERTIES OF BACTERIAL NUCLEOPROTEINS

Since it is established that bacteria do contain protein and nucleic acid, it is logical to suppose that any way of disintegrating the cell would yield nucleoprotein. The work considered here includes only those protein fractions of bacteria which were specified by the original author to be nucleoproteins.

The first isolation of a nucleoprotein of a microorganism was reported by Stulzer (1882) who investigated the nucleoprotein of yeast. Levene in 1898 was the first to report that a pathogenic organism, the tubercle bacillus, contained nucleoprotein. In the same year Ruppel described a procedure for isolating nucleoproteins that was similar to modern methods. Ruppel was able to extract a protein fraction from disintegrated tubercle bacilli which was precipitated with acetic acid, was soluble in alkali, contained four percent phosphorus and showed a positive biuret test. This author isolated nucleic acid from the tuberculo-nucleoprotein and claimed that the nucleic acid was originally bound to the protein moiety.

Levene (1901) carried the experimental work on the nucleoprotein of the tubercle bacillus further by showing that five percent sodium hydroxide removed nucleic acid from the nucleoprotein.

Lustig et al (1913) outlined the method used at that time for the isolation of nucleoproteins using sodium hydroxide. Hammarsten (1920) stated that purification procedures using strong alkali changed the chemical properties of the protein fractions.

Toenniessen in 1919 suspended bacteria in strong hydrochloric acid, subjected the acid mixture to boiling temperatures, neutralized the mixture with alkali and succeeded in precipitating a protein from this mixture by acidification with acetic acid.

The first investigation of the pneumococcal nucleoproteins was described by Avery and Heidelberger (1923). Washed pneumococci were dissolved in bile, the undissolved cells removed by centrifugation, and the supernatant acidified with acetic acid. The precipitate which formed was washed with distilled water and dissolved in 0.1 N sodium hydroxide. The precipitation and solution was repeated two or three times before the final precipitate was washed with acetone and ether and dried. The purified product gave a positive biuret, Molisch, Hopkins Cole and Millon's reactions; after hydrolysis a positive test for purine was observed. The protein contained 16 percent nitrogen and 0.5 percent phosphorus.

Avery and Morgan (1925) suspended washed pneumococci in 0.002 N sodium hydroxide and subjected this suspension to repeated freezing and thawing to disrupt the cell bodies. The supernatant of the frozen and thawed pneumococci was diluted with saline and passed through a Berkefeld V candle. Purification of the nucleoprotein was accomplished by Avery's method (1923).

Thompson and Dubos (1938) liberated nucleoproteins from pneumococci by autolysis. The organisms were killed by incubation overnight at 37°C in 0.1 M pH 4 acetate buffer, then collected and resuspended in 0.4 percent sodium chloride and

the reaction of the suspension adjusted to pH 7 with sodium hydroxide. Further incubation of the neutral mixture caused the cells to become Gram negative, and nucleoprotein was found in the supernatant. The nucleoprotein solution was filtered through a Berkefeld V filter and acidified with acetic acid. At pH 4.2 a precipitate, "A", formed. When hydrochloric acid was added to the supernatant of precipitate "A", precipitate "B" separated from solution at pH 2.5.

The "A" fraction contained 14.15 percent to 15.50 percent nitrogen, while the phosphorus content varied from 3.79 to 4.40 percent. Therefore, the N/P ratio varied from 3.22 to 4.09. This product gave a positive biuret reaction, negative Millon's test, positive Bial's test for pentose and a positive reaction for purine bases. Lavin et al (1938) showed the "A" fraction to have an ultra violet absorption spectrum characteristic of nucleic acid.

The "B" fraction had 13 to 14 percent nitrogen and 7 to 7.5 percent phosphorus. Since "B" was attacked by ribonuclease, it was concluded that it contained yeast nucleic acid.

Thompson and Dubos (1938) also subjected pneumococci killed at pH 4 to successive incubations and purified the resulting protein solutions by acetic acid precipitation. Thompson and Dubos showed further that the supernatant of pneumococci which had been subjected to twenty-four hour incubation gave a product with an N/P ratio of 3.22 after purification by isoelectric precipitation. The same cells resuspended and reincubated an additional twenty-four hours gave a purified

product that had an N/P ratio of 3.65, while the third product, the result of a third resuspension and forty-eight hour incubation had on purification an N/P ratio of 22.65.

The nucleoproteins of pneumococci were extracted by Heidelberger (1947) with 0.01 N sodium hydroxide. The proteins were purified by precipitation with acetic acid.

Johnson and Brown (1922) and Johnson (1926) investigated the nucleoproteins of the tubercle bacillus. The organisms were defatted with toluene, ground in distilled water and extracted with sodium hydroxide. Sodium hydroxide was used in a 3 percent concentration to split protein from the nucleic acid; the nucleic acid was precipitated with alcohol and identified as a trinucleotide.

For the human strain of tubercle bacillus, extensive investigations were carried out by Heidelberger in 1933. The organisms were defatted and dried with acetone and ether and then ground in a ball mill. The cellular mash was extracted in the cold with buffers ranging from pH 4 to pH 11. Protein was isolated from each of the buffer extracts by precipitation with 50 percent acetic acid. The products after purification were shown to vary in their N/P ratios with the pH of the buffer used to extract the cell residue. The "D" fraction, extracted at pH 6.5, contained 15.7 percent nitrogen and 3.4 percent phosphorus; the "E" fraction extracted at pH 8.3 had an N/P ratio of 7.7/1; "F", extracted at pH 9, had an N/P ratio of 8.3/1; while "K", the fraction extracted with 0.1 to 0.5 percent sodium hydroxide had an N/P ratio of 40/1.

In 1938 Menzel and Heidelberger showed that the fractions

isolated from the tubercle bacillus could be subfractionated. The "D" fraction, for example could be separated into three fractions. "D" was precipitated by 33 percent sodium sulfate; glacial acetic acid added to the supernatant of "D", precipitated "D_2", while neutralized copper acetate caused the final fraction "D_3" to precipitate from the supernatant of "D_2". The other fractions could be subfractionated in the same manner. "D_1" had an N/P ratio of 19/1, "D_2" contained 1.7 percent phosphorus, while "D_3" had an N/P of 2.8/1. Heidelberger interposed some doubt as to the validity of calling "D" the nucleoprotein fraction, since purine nitrogen determinations carried out on this fraction of the tubercle bacillus were not as high as the phosphorus content would lead one to expect. Fraction "E", the pH 8.3 soluble fraction, was shown to have a subfraction "E_3"; the purine nitrogen content indicated 4.4 percent of the fraction to be nucleic acid; this was greater than the purine content of "D".

The first investigations of the streptococci were those of Lancefield (1924-1925). The streptococci were dried in vacuo, ground in a ball mill and finally extracted with N/100 sodium hydroxide. The dissolved protein was separated from the cellular debris by centrifugation, and the protein precipitated with acetic acid. The protein was further purified by Avery's method (1923).

In 1928 Lancefield showed that extraction of pulverized streptococci with N/100 sodium hydroxide yielded nucleoprotein, while 0.85 percent sodium chloride extraction yielded the type specific "M" protein.

Ando et al (1930) reported that a nucleoprotein would precipitate at pH 4 from Dick Toxin.

Hirst and Lancefield (1939) subjected four hour cultures of streptococci to boiling saline and then ground the organisms in a ball mill at room temperature. The pulp was mixed with N/10 hydrochloric acid and incubated 24 hours at 37°C, the mixture centrifuged, the precipitate resuspended in acid and reincubated a second and third time. The supernatants were pooled and sodium hydroxide was added until a precipitate formed at pH 4.5. The precipitate was redissolved in M/15 buffer pH 7.2 and reprecipitated with N hydrochloric acid.

Fifty to sixty percent of the nitrogen of a solution of this protein was precipitable with trichloroacetic acid. No polysaccharide could be detected in the purified extract. The nucleic acid content, calculated by comparison with spectrophotometric analysis of yeast nucleic acid, was thirty percent of the entire dry weight of the compound.

Heidelberger and Kendall (1931) investigated extensively the protein fractions of Streptococcus hemolyticus. The bacteria were washed with saline, dried with ether and acetone and ground in a ball mill at room temperature. The bacterial mash was extracted first with 0.2 N sodium acetate buffer, pH 4, then with M/15 phosphate buffer, pH 6.5. Glacial acetic acid was used to precipitate nucleoprotein or "D" fraction from the extract obtained at pH 6.5. The precipitate was dissolved in neutral sodium bicarbonate and reprecipitated with acetic acid. "D" was insoluble at pH 3.8 and had an

N/P ratio of 4:1.

"D" was easily degraded with 0.1 N sodium hydroxide. Allowing "D" to stand at 23° - 26°C for twenty-four hours in N/150 sodium hydroxide split nitrogen and phosphorus from the compound.

Heidelberger and Scherp (1939) published a fractionation scheme for "D" streptococcal nucleoprotein. If "D" was dissolved in sodium bicarbonate, "D_a" precipitated in twenty-seven percent ammonium sulfate, "D_b" precipitated from the supernatant of "D_a" after saturation with ammonium sulfate, "D_c" was obtained by addition of acetic acid to the supernatant of "D_b", while "D_d" was precipitated from the supernatant of "D_c" with hydrochloric acid. "D_a", had 2.4 percent phosphorus, "D_b" 3.35 percent phosphorus, "D_c" had 2.74 percent phosphorus, while "D_d" had 8.84 percent phosphorus; the nitrogen remained approximately constant in each case.

Sevag, Lackman and Smolens (1938) used sonic vibration to disintegrate streptococci. The protein was precipitated from the supernatant with 0.1N hydrochloric acid, redissolved at neutral pH and finally repricipitated from 50 percent alcoholic solution with .1N hydrochloric acid. This same method was used to precipitate and purify the nucleoprotein from streptococci that had been ground in a low temperature ball mill.

Chloroform and amyl alcohol were used by Sevag et al (1938) to remove protein from the hydrochloric acid precipitated nucleoprotein; the protein was previously split from its nucleic acid with heat and anhydrous sodium carbonate.

The nitrogen of the nucleic acid remaining after complete removal of the protein was 9.7 percent. The theoretical phosphorus content of yeast nucleic acid was calculated as 9.64 percent.

Zittle et al (1939) reported that the nucleoprotein (nucleoprotein agglutinogen or labile antigen) from sonic disintegrated streptococci gave a positive test for ribose and desoxyribose, and was digested by ribonuclease as well as by trypsin. Electrophoretic analysis showed a large amount of the nucleic acid of this preparation to be free rather than combined with the protein. Lancefield's type specific "M" protein, prepared by acid hydrolysis of labile antigen was shown to contain nucleic acid also.

Mudd and Lackman (1940) ruptured Group A streptococci with sonic energy; the nucleoprotein was isolated and purified by the procedure described above.

Sevag, Smolens and Lackman (1940) found that 2 to 6 percent of the total dry weight of streptococci was desoxyribonucleic acid. This constituted 10 to 30 percent of the total nucleic acid content of the organisms. The virulent strains of streptococci had a total of 19 to 22 percent nucleic acid, avirulent streptococci had 18 to 24 percent nucleic acid, while non-typable rough strains had only 14 to 16 percent.

Sevag and Smolens (1941) announced that exposure to hydrochloric acid was injurious to a naturally occurring nucleoprotein. Nucleoprotein liberated by sonic vibration and precipitated with ammonium sulfate had 0.72 percent phosphorus

and 0.72 percent purine nitrogen. Electrophoretic patterns of salt precipitated protein showed two components; the hydrochloric acid precipitated protein was less homogeneous. Sevag therefore precipitated nucleoprotein liberated from streptococcal cells by sonic energy with ammonium sulfate and dialyzed the precipitate against distilled water for further purification.

Gonococcus nucleoproteins were investigated by Boor and Miller (1934) and Stokinger et al (1944). The former extracted nucleoprotein from gonococci with cold water and enough N/100 sodium hydroxide to maintain the pH at 7.8. Dilute acetic acid was used to precipitate the nucleoprotein from the supernatant and sodium hydroxide employed for resolution.

Stokinger et al (1944) extracted gonococci in the cold with buffer of pH 9.2. The protein was precipitated with acetic acid and redissolved in sodium carbonate.

Only three preparations of nucleoproteins from staphylococci have been made. Boor and Miller (1934) treated staphylococci with cold water and enough N/100 sodium hydroxide to hold the pH at 7.8. The nucleoprotein was precipitated with acetic acid, redissolved in sodium hydroxide and reprecipitated with acetic acid. The final precipitate was dialyzed against distilled water and evaporated to dryness in an air current.

Julianelle and Wieghard (1935) dried a thin layer of staphylococci in a petri dish, washed the dried cells in alcohol and ether and ground them in a special grinding

apparatus. The cellular debris was dissolved in N/100 sodium hydroxide and the nucleoprotein precipitated with normal acetic acid.

Stevens and Jordani (1936) prepared nucleoproteins of the staphylococcus by using broth cultures of bacteria which were washed with acetone, dried, ground in a ball mill and extracted with distilled water made alkaline. The purification was the same as that of Boor and Miller (1934).

In summary, it may be stated that a variety of methods have been used to extract nucleoproteins from many organisms. The purification of the nucleoproteins depended on the fact that nucleoproteins are soluble in neutral or alkaline solutions and insoluble in acidic (pH 3 to pH 5) solutions or solutions of high salt concentrations. The chemical and physical properties of the nucleoprotein, on the other hand, depended on the method of isolation, the pH of isolation and the methods and agents of purification.

RESULTS OF IMMUNOLOGICAL INVESTIGATIONS OF NUCLEOPROTEIN

Avery and Heidelberger (1923) found that pneumococcus nucleoprotein was species specific and not type specific. Type II nucleoprotein precipitated type I, II and III pneumococcal antiserum but did not react with typhoid antiserum.

In 1925 Avery and Morgan showed that the pneumococcal nucleoprotein was antigenic. Antiserum against the nucleoprotein of Group IV pneumococci reacted with nucleoproteins from type I, type II, type III and Group IV pneumococci. The protein antisera, furthermore, was shown to lack antibodies to the type specific polysaccharides.

Heidelberger (1947) precipitated horse antipneumococcal serum with nucleoprotein from type I and type II pneumococci. The serum was prepared by intravenous injection of a horse with pleural exudate resulting from intratracheal injection of horses with type I pneumococci. The specific nitrogen precipitated by type I pneumococcal nucleoprotein from the horse serum was equal in amount to that precipitated by type II pneumococcal nucleoprotein.

The "D" and "K" fractions of the human tubercle bacillus were used by Heidelberger and Menzel (1934) to produce antisera in rabbits. The "D" fraction in a dilution of 1:4000 reacted strongly with anti "D" serum but only slightly with anti "K" serum. The authors concluded that "D" and "K" were chemically and immunologically distinct.

Quantitative immunological investigations of the fractions of human tubercle bacillus were carried out by

Menzel and Heidelberger (1938). The "K", "D" and "F" fractions were added to tubercle antiserum and the washed specific precipitates analyzed for N. "D" precipitated 0.37 milligrams of specific protein nitrogen, "F" precipitated 0.34 milligrams, while "K" removed only 0.1 milligrams. Since "K" removed little antibody, it was thought that the original extraction of "K" with sodium hydroxide had caused the reduction in activity; however, treatment of "D" and "F" with sodium hydroxide did not lower their ability to react with the antiserum.

Lancefield (1925) showed streptococcal nucleoprotein to be antigenic, and found that the sera reacted equally well with different nucleoproteins prepared from the same group of streptococci. This nucleoprotein antiserum precipitated with nucleoprotein from the pneumococcus and the staphylococcus but not with nucleoproteins from B. coli or B. diphtheria. Lancefield pointed out that the extent of cross reactions may have been due to chemical manipulation of the original cellular nucleoprotein.

In 1928 Lancefield showed that the nucleoprotein fraction "P", the somatic carbohydrate "C" and the type specific "M" protein were chemically and immunologically distinct substances.

Lancefield and Hirst's (1938) preparation of streptococcal nucleoprotein produced antisera in rabbits that gave both type specific and species specific antibodies. The authors concluded that the protein might have been a mixture and that the nucleoprotein may not have been the only antigen.

Antibacterial streptococcal serum was used to test the precipitin reactions of the protein fractions of Heidelberger and Kendall (1931). Qualitatively, the "D" fraction was shown to have a greater reaction with human sera than with rabbit sera. Antiserum to the whole organism when absorbed with "D" fraction still exhibited a strong reaction with "E" fraction, but absorption of the same sera with "E" or "F" removed all antibodies for "D" fraction. In agreement with Lancefield, "D" reacted to about the same extent with antipneumococcus horse serum (type I and type II) as it did with homologous streptococcal serum. Heidelberger stated that "D" corresponded to Lancefield's "P".

In 1939 Heidelberger showed that alkali degraded "D" was serologically the same as "D" although chemical analysis of degraded "D" resembled those of "F", "G" and "K" fractions. "D_a", the first of the subfractions of "D", reacted with type I and II antipneumococcus horse serum to a greater extent than did the original "D" fraction. Absorption of the antibacterial serum with "C" substance, then with "D" left antibodies for "E", "F" and "G" etc. Injection of "D" engendered anti-protein antibodies for both "D" and "K" fractions. Nucleic acid, isolated by alkaline degradation of "D" was used to absorb "D" serum, after absorption, the serum still reacted well with "D".

The labile antigen of Czarnetzky et al (1938) extracted from Group "A" streptococci gave precipitin reactions with antibacterial serum and serum produced against labile antigen. Antibacterial serum absorbed with labile antigen exhibited

no precipitin reaction with "M" protein. Labile antigen from Group "A" streptococci could be precipitated by sera against any type within Group "A".

Nucleic acid prepared by Zittle et al (1939) from streptococcal labile antigen absorbed antibodies from anti-pneumococcal horse serum.

Zittle, Lackman and Mudd (1939) showed that tryptic digestion of labile antigen (nucleoprotein agglutinogen) destroyed the serological behavior of the substance, but ribonuclease did not qualitatively affect the serological behavior of nucleoprotein agglutinogen.

Boor and Miller (1934) observed no difference in the ability of nucleoproteins of gonococci and the ability of whole gonococcal cells to engender immune substances, induce allergy or produce cutaneous reactions; they reported that gonococcal nucleoprotein had an antigenic factor in common with non-encapsulated pneumococci. Stokinger et al (1944) reported that rabbits immunized with gonococcal nucleoprotein produced a serum containing 0.2 to 0.3 milligrams of antibody nitrogen per cc of serum.

Boor and Miller (1934) reported that nucleoprotein of Staphylococcus aureus had an antigenic factor in common with type III pneumococcus.

Julianelle and Wieghard (1935) reported that staphylococcal nucleoprotein precipitated sera from homologous strains of staphylococci as well as heterologous strains; the nucleoprotein was devoid of type specificity.

EXPERIMENTAL

Introduction

The purpose of this work was to isolate from a staphylococcus a nucleoprotein, purify the protein and study its immunological reactions.

A Method of Producing Mass Cultures of Staphylococci

Large quantities of bacteria were required to produce a sufficient amount of bacterial nucleoprotein. It was considered advisable to use a medium devoid of protein and to reduce the contamination of the cells with media constituents to a minimum.

Dolman's (1938) media was used because it had a simple composition and gave as good a growth to the eye with staphylococcus #152 as did glucose broth. The composition of the media was as follows:

proteose peptone	20 gram.
sodium chloride	5 gram.
dipotassium hydrogen phosphate	1 gram.
dihydrogen potassium phosphate	1 gram.
magnesium sulfate	2/10 gram.
calcium chloride	1/10 gram.
dextrose	5 gram.

The volume was brought to one liter with distilled water and adjusted to pH 7.6 with sodium hydroxide.

Paper pulp was pressed into a petri dish in a mash, Dolman's liquid media added and cellophane put on top of the media after the method of Kisskalt (1943). When the petri

dishes were autoclaved, bubbles formed in the pulp; only a few areas of cellophane were in contact with the surface of the media so the yield was small.

A cellophane sack was next filled with blotting paper, soaked in Dolman's media and autoclaved in a closed glass dish. The exterior of this sack was inoculated with bacteria and gave a promising yield. Absorbent cotton wool was also tried but it did not maintain a firm surface. Finally, a cellophane sack was filled with a paper filter pulp, "All Rag Cellomas", sold by Reeve Angel and Co.; this base was most satisfactory because it maintained a firm surface after autoclaving.

The method used finally was essentially the same as that of Reed and McKercher (1949). Enamelware pans with fitted tops measuring 5" in depth, by $8\frac{1}{2}$" in width, by 13" in length were used as "petri plates". The "cellomas" pad was cut so that it fitted snugly into the pan. The pad was placed in the pan and thoroughly soaked with Dolman's media. The cellophane sheets (Canadian Industries Ltd., plain transparent #600) $11\frac{3}{4}$" by 13", were tucked underneath one side of the wet pad and then dropped across the pad. The wet cellophane was then stretched and smoothed across the top of the pad until all wrinkles were obliterated and then tucked underneath the other side of the pad. The top half of the pan was then fitted on and the unit was autoclaved. Water of condensation was removed by tipping the pan on its side.

The surface of the cellophane was inoculated with ten cc. of ten hour cultures, the inoculum was rubbed into the surface

with a sterile one cc. pipette held horizontally. The pipette was also used to flatten the cellophane back onto the surface of the pad in any place where it did not adhere for bacteria would not grow where there were air pockets.

After incubation, the culture was harvested with small sterile spatulas pushed in parallel strokes across the surface of the cellophane. Staphylococcus strain #152 grew well on a pad saturated with Dolman's synthetic medium to which 0.5 percent glucose had been added. The yield of bacterial nitrogen per pan as described was usually forty milligram.

Stability of Staphylococcus Strain #152

Staphylococcus strain #152 from the National Type Culture Collection was tested for stability under the conditions required for thorough washing with chilled merthiolated saline. The supernatant from a thrice washed fresh sixteen hour culture of staphylococcus strain #152 contained 0.01 milligram of nitrogen per cc. The culture was resuspended and allowed to stand overnight at 5°C. The fourth supernatant contained 0.019 milligram of nitrogen per cc. The 5°C incubations were repeated for two, seven and forty-two days. The nitrogen content of these supernatants were 0.038 milligram., 0.02 milligram and 0.04 milligram, respectively. Throughout this period the organisms remained Gram positive.

Further washing studies in relation to nitrogen excretion of staphylococcus #152 were carried out in a typical experi-

ment, equal volumes of a suspension of a sixteen hour culture were treated as follows: to the first and second was added enough merthiolate to make the concentration 1:10,000 and 1:5,000 respectively. The third was heated to 56°C for one hour; the fourth was dried with two treatments of acetone and resuspended in saline; and the fifth was used as a control. The mixtures were allowed to stand for one hour at 0°C. and then were washed four times in the cold with 10 cc. portions of chilled merthiolated (1:10,000) saline. The nitrogen contents, in mg./cc., of the supernatant are given in Table I.

All of the samples could be washed free of extraneous nitrogen. Heat killed organisms did not lyse appreciably after one overnight period; heat killed organisms gave off the least nitrogen into the fifth supernatant.

Heat killed organisms were subjected to further quantitative study. The organisms were killed by heat for one hour at 60°C. and suspended in five cc. aliquots of sterile chilled merthiolated saline. After the third washing, the suspension had 0.86 milligrams of bacterial nitrogen per cc. and the third supernatant contained 0.004 milligram of nitrogen per cc. After standing at 5°C. for one day, two days, one week and six weeks, the supernatants of the resuspended bacteria contained 0.008 milligram, 0.01 milligram, 0.009 milligram and 0.032 milligram of nitrogen.

Preparation of Vaccine and Production of Rabbit Antisera

Staphylococcus strain #152 was grown 16 hours on cellophane in pans as previously described, transferred to sterile

flasks and diluted with saline until the suspension contained approximately 0.5 milligram of nitrogen per cc. The flasks were placed in a 60°C water bath for one hour, the suspension tested for sterility, and, after three washes in chilled merthiolated saline were diluted to 0.22 milligram of nitrogen per cc. and used for immunization. The bottles were kept at 5°C and checked periodically with Gram's stain. Six weeks after the vaccines were made, the unused portions were discarded and a new lot prepared.

Rabbits were immunized by intravenous injections of the heat killed vaccines according to the schedule given below. The vaccine was given intravenously in four courses, each animal received a total of twenty-six milligrams of bacterial nitrogen. The first injection of each course subsequent to the first was given intraperitoneally. The schedule is given in Table II. The rabbits were bled from the heart ten days after the last intravenous injection. The blood was allowed to clot and the sera removed sterily. Merthiolate was added to the sera in an amount such that the final concentration would be 1/10,000; the merthiolated sera was stored at 5°C.

Rabbits were also immunized with alum precipitated nucleoprotein. The purified protein solution, which had been previously lyophylized was dissolved in saline so that each cc. contained 3.0 milligram of protein; 0.4 cc. of 1 percent sterile alum was added for each 13 cc. of solution followed by merthiolate to make the final concentration 1:10,000. Only one course of intravenous injections was given, the schedule

is in Table III. Thus each rabbit was given a total of 81 milligrams of purified protein. The rabbits were bled ten days after the last injection and the sera collected and preserved as above.

For formalized vaccines, the sixteen hour cultures were scraped off the cellophane directly into 0.5 percent formalized saline. The concentration of bacterial nitrogen per cc. was about 0.3 milligram. The organisms were allowed to stand two hours at room temperature and tested for sterility after five days in the ice box at 5°C. The vaccines were Gram's stained periodically and no lot was used for more than six weeks after the day of preparation.

Behavior of Staphylococcus Strain #152 when Subjected to Usual Methods of Cell Disruption.

In order to obtain somatic protein from the organisms, the following methods of protein liberation were studied in detail:

1) Freezing and Thawing

A thick bacterial suspension was frozen at -20°C. overnight. The suspension was then thawed in an ice bath and centrifuged at 0°C. The supernatant gave a negative test for protein with trichloroacetic acid.

2) Shaking with Broken Glass

A packed bacterial suspension was poured into a flask containing sharp pieces of broken glass. The flask was shaken on a Kahn shaker in the cold for seven hours. During the first few hours no protein was liberated, after the seventh hour some protein was detected in the supernatant,

but the yield was too small for the method to be practical.

3) Acetone-Ether Treatment

Ten volumes of ice cold acetone were added to a thick suspension of cells which were previously smeared over the walls of the tube. The mixture was centrifuged in the cold and the precipitate again treated with acetone, dried in vacuo and resuspended in chilled saline. The supernatant contained no protein. This process was varied unsuccessfully using ether in addition to acetone.

4) Sodium Hydroxide

Two percent sodium hydroxide was added to a washed culture of staphylococcus and the suspension allowed to stand at 0°C. for fifteen minutes. The supernatant gave a positive test for protein; however, Heidelberger's (1931) work on the alkali degradation of "D" fraction of streptococcus discouraged the use of this method.

5) Incubation

Another washed fresh bacterial culture was suspended in saline and allowed to incubate at 37°C. for two days. The supernatant then gave a protein; all the bacterial cells were Gram negative. Again, although protein was liberated by the action of the cells autolytic enzymes, it might have been a partially digested component of the bacterial cell.

6) Sonic Vibration

Sixteen hour washed cultures of Staphylococcus #152 were subjected to the sonic vibrations of the Raython sonic vibrator. The amount of nitrogen found in the supernatants was in direct proportion to the voltage output and the time of exposure.

The amount of total nitrogen found was relatively large but the amount of protein nitrogen produced was small. In a typical experiment, a saline suspended washed living culture containing 2.66 milligram of nitrogen per cc. was subjected to vibration. At regular intervals, samples were removed. To determine living populations, plate counts were done on each sample, and the supernatants of each sample were analyzed for total nitrogen and for trichloroacetic acid precipitable nitrogen. A control was allowed to stand at 23°C., the temperature of the contents of the vibrator cup. The results of this experiment are to be found in Table IV. Since these counts were plate counts of staphylococci which were diluted in the usual manner; i.e., one cc. of suspension added to nine cc. of sterile saline, it is doubtful that the increase in population was absolute; the vibration may have broken up the clumps of staphylococci. It is evident that although some protein nitrogen was liberated into the supernatant, little if any killing took place.

Because of the reported success of Shropshire (1947) in rupturing staphylococci with the Raython sonic vibrator, the experiments were continued. In an effort to see if volume had any effect on the process, ten cc. of bacterial suspension were used in the cup of the vibrator instead of the usual twenty-five cc. with the voltage output at 200 volts, it was possible to liberate 2.6, 5.2 and 8.5 percent of the bacterial nitrogen. The yield was not sufficient to encourage the use of this small volume of solution.

It was thought possible that most of the sound waves

were not striking the bacteria since it was calculated that 2×10^{12} organisms could occupy one cc. Therefore, a suspension was prepared in which 50 percent of the available space in the solution was occupied by bacteria. No protein nitrogen was liberated from this suspension in 1½ hours at 200 volts.

In addition, a supersonic vibrator emitting 500,000 vibrations per minute did not liberate protein from this strain of staphylococcus.

The Method of Liberating Nucleoprotein

Two methods were found satisfactory for rupturing staphylococci. The first method employed was a freezing and grinding process. The interior of a hollow ground glass plug was packed with dry ice, the organisms frozen on the ground surface of the plug and sand added to the surface as an abrasive. The plug was inserted into a matching tube shaped receptacle with ground glass surface and the plug rotated by hand. This device was satisfactory but was difficult to operate since the plug would often become frozen to the tube.

The second method was essentially that of Curran and Evans (1942) who shook bacteria with 60-80 mesh glass beads to kill the cells. Flint glass beads 3 millimeter in diameter were added to a flask of washed staphylococci and the flask shaken on a Kahn shaker in the ice box. The number of cells ruptured was in direct proportion to the time of shaking. In a controlled experiment twenty-five cc. of a washed sixteen hour culture of staphylococci were added to

a sterile 100 cc. screw capped bottle containing 50 grams of 3 millimeter glass beads, and the bottle attached to a standard Kahn shaker. The bottle was placed on its side so that there was maximum movement of the beads. The shaking process (at 5°C.) was continued thirteen hours; samples were removed at hourly intervals for testing. Gram's stains were made from an aliquot of the removed samples, plate counts made from another aliquot, while a third portion of the sample was centrifuged and the trichloroacetic acid precipitable nitrogen of the supernatant determined.

Figures I and II illustrate the results of this experiment; the actual data used in these figures is given in Table V.

After the first and second hour, most of the cells were Gram positive and intact, but after the third hour, much cellular debris was evident. The majority of the intact cells in the three hour sample were Gram negative. The five hour smear showed few intact cells, but gave a pink staining background, while the thirteen hour sample was an evenly staining Gram negative smear with an occasional Gram negative cell.

This method was used to produce staphylococcal somatic protein, sixteen hour cultures of staphylococci grown on cellophane were washed three times in 1:10,000 merthiolated saline so that each cc. of saline contained about 1.6 milligram of bacterial nitrogen. Twenty-five cc. of the suspension was added to a sterile one hundred cc. bottle containing

fifty grams of 3 millimeter glass beads; the bottle was shaken four hours on a Kahn shaker at 5°C. The cellular mash was diluted 1:1 with cold saline and centrifuged at 0°C. until the supernatant was clear. This supernatant was referred to as unpurified protein or bacterial extract.

Purification and Storage of the Protein

Preliminary tests on the bacterial extract indicated that the pH of maximum precipitation was below 4. A more exact quantitative estimation of the amount of nitrogen precipitated from pH 3.86 to 3.6 showed that the amounts precipitated were identical. N/10 hydrochloric acid was added to another sample of protein until a pH of 1.3 was reached, but none of the precipitate which formed at 3.8, dissolved in this process of acidification.

In order to remove a non-protein serologically active substance, and at the same time not change the chemical properties of the protein, numerous methods of purification were tried. The method finally was as follows: ten cc. of chilled protein were added to twenty cc. of chilled 0.1 M acetic acid buffer pH 3.7. The solution was centrifuged in the cold until the supernatant was water clear. The precipitate was dispersed, washed with 40 cc. of chilled 0.01 M acetic buffer pH 3.7; after the precipitate was again collected by centrifugation in the cold, it was redissolved in 10 cc. of 0.1 M chilled pH 7.8 borate buffer (Palitzch 1922). An aliquot of this solution was removed for total phosphorus and total nitrogen determinations. The protein was again

precipitated with chilled acetic buffer 0.1 M pH 3.7, the amount of buffer was corrected for the amount of protein removed for analysis. The process was repeated three times. The results of this experiment are given in Table VI.

When another sample of bacterial extract was used to compare the trichloroacetic acid precipitable nitrogen with the isoelectrically precipitable nitrogen, the amounts of nitrogen precipitated were identical.

It has thus been shown that all of the protein nitrogen in the bacterial extract was precipitated at pH 3.7, that the isoelectric point was not changed by purification, that no nitrogen was split off the protein by precipitation at pH 3.7 and re-solution at pH 7.8. It will be noted that almost one-third of the total phosphorus of the bacterial extract was left in the first supernatant.

In order to ascertain whether nucleic acid was split off from the compound, the ultraviolet absorption spectrum of this first supernatant was determined; the density readings were done on the Beckman spectrophotometer. The ultraviolet absorption spectrum was not characteristic of nucleic acid. The first supernatant of another lot of protein gave a slight absorption at 2600 A^{o}, but this amount was so small compared to the spectrum of the purified protein that it was considered negligible. The second and third supernatants of the 3.7 buffer precipitation were not analyzed because there was no phosphorus present.

A sample of protein was dialyzed against 0.1 M sodium

chloride in 0.05 M sodium acetate. The dialysis was continued in the cold until the dialysate contained no phosphorus. The N/P ratio of this dialyzed product was 4.08.

Thus it has been shown that by the methods used for testing, no change took place in the protein purification process.

The purification of large lots of protein was accomplished as follows: the clear supernatant of the cellular mash was subjected to 3.7 buffer precipitation and redissolved in borate buffer pH 7.8. After the third precipitation, the protein was washed with 0.01 M acetic buffer pH 3.7 and redissolved in a minimum amount of 0.1 M sodium bicarbonate. A drop of phenol red indicator was added so that the pH would be kept below neutrality; when dissolved, the protein was immediately shell frozen and dried in vacuo.

All processes described here were carried out as rapidly as possible at 0°C. since experiments showed that allowing the unpurified protein to stand for a few weeks at 5°C. caused the isoelectric point of the protein to change.

The phosphorus determinations were carried out by the method of King (1932). The nitrogen determinations were done by the Kjeldahl method using either a Markham (1942) still or a micro-Kjeldahl still depending upon the amounts of nitrogen in the sample.

0.210 milligram total phosphorus per cc. and 0.042 milligram of inorganic phosphorus per cc. If the sample of nucleic acid used for comparison had been pure, the proportion of the total phosphorus of the protein that was in the form of nucleic acid might be roughly estimated to be about fifty-six percent.

When the bacterial extract was kept at 5°C. for one month before isoelectric precipitation, it was found that the isoelectric range began at pH 3; however, the N/P ratio of this purified material was 4. The aged product precipitated in the form of fine floccules, while the fresh bacterial extract precipitated in long viscid strands.

The same solution of bacterial extract after ageing for four months at 5°C. yielded protein, which after purification had an N/P ratio of 2.86.

Results of the Investigation of Supernatant One

When the protein was purified by isoelectric precipitation, the first supernatant contained a material serologically active with anti-bacterial serum. The second an third supernatants of this purification process did not contain any serologically active material. Because the trichloroacetic test was negative, Millon's test was negative and the Mollisch test strongly positive , it may be considered probable that

this was the carbohydrate fraction described by Julianelle and Wieghard (1935).

Estimation of Agglutinin Nitrogen Content of Anti-Bacterial Staphylococcus Serum.

Before quantitative antibody estimations could be made, it was necessary to determine conditions under which no nitrogen was lysed from the bacteria and no nitrogen was absorbed by the bacteria from normal rabbit sera.

All operations described below were carried out at 0°C. unless otherwise specified. Formalized vaccines were washed three times with chilled merthiolated saline and then filtered through cotton in order to remove clumps of bacteria. To duplicate 1 cc. portions of vaccine were added two cc. of chilled M/15 phosphate buffer. The samples were mixed well and allowed to stand overnight in the ice box. The samples were centrifuged for 45 minutes at 2200 r.p.m., the supernatant decanted and the cells washed twice with 3 cc. portions of appropriately buffered saline as described by Heidelberger (1936). Since it was difficult to break up the lump of staphylococci which collected at the bottom of the tube it was found convenient to allow the tubes to stand half an hour in an ice bath, after which the pellet of organisms could easily be dispersed for further washing. The washed cells were transferred to flasks for nitrogen determination by the micro-Kjeldahl method. The results of a typical experiment are given in Table VII. It was concluded that in the pH range from 7.5 to 6.5 the organisms were stable under the conditions required for thorough

washing.

In order to ascertain if normal rabbit serum exerted a lytic effect on the cells, and if any non-specific nitrogen was absorbed from normal rabbit sera, the following experiments were done. Normal rabbit sera were heat inactivated for 45 minutes at 56°C. The sera were diluted 1:1 with M/15 phosphate buffer pH 7.5 for the most alkaline sample. In order to adjust the other samples to pH 7 and 6.5, a drop of phenol red was added to 2½ cc. aliquots of serum in a 5 cc. volumetric flask, then N/10 hydrochloric acid was added until the color was orange red for pH 7, and orange for 6.5. The appropriate M/15 buffer at pH 6.5 and pH 7 was then added to volume. The sera were allowed to stand overnight at 5°C. and centrifuged in the cold before they were used.

From Table VIII it was concluded that no non-specific nitrogen was absorbed from normal rabbit sera, and that the sera had no lytic effect on the vaccine.

For the determination of agglutinin nitrogen of anti-bacterial staphylococcus sera a pool was made of first course, second course and third course bleedings. Buffer at pH 6.8 was used and the experiments were conducted in the same fashion as the one previously described. All operations were carried out at 0°C. The pooled sera were diluted 1:1, as described previously with N/10 hydrochloric acid and M/15 phosphate buffer pH 6.8. Preliminary testing with trichloroacetic acid indicated that two washings with buffered saline were sufficient to remove all non-specific protein from the tubes. Since all of the antibody was not removed with one absorption for twelve hours at 0°C., the super-

natants of duplicate tubes were not pooled but were transferred to a second tube and a further charge of vaccine added. This process had to be repeated three times before all antibody was removed. The results of this experiment are given in Table IX.

The first course sera contained a total of 0.68 milligram of specific antibody nitrogen per cc., pool 2 contained 1.10 milligrams., while pool 3 contained 0.74 milligram of antibody nitrogen per cc.

The titers of these sera were also determined by the usual relative qualitative method before the sera was pooled. The sera were diluted in halving dilutions, and to 1/2 cc. of this diluted sera was added 1/2 cc. of a bacterial vaccine containing 0.2 milligrams of nitrogen. The tubes were incubated for four hours and read at the end of this time. The results are given in Table X.

Quantitative Immunological Studies on Nucleoprotein.

Pooled sera from the first course was used for a study of the quantitative relationships between purified nucleoprotein antigen and its homologous antibody in anti-bacterial serum. The antigen used was nucleoprotein purified as described above. Directly before use the lyophilized protein was dissolved in chilled physiological saline. Preliminary tests indicated that the reaction between the antigen and antibody was very slow. Therefore, the reactions were carried out at 37°C. for four hours followed by an overnight period at 5°C. Centrifugation and washing were carried out in the manner described by Heidelberger and Kendall (1929,

1933, 1935). The results of this experiment are given in Table XI.

The reaction between antigen and antibody was not complete as supernatants returned to the ice box for forty-eight hours contained a precipitate, when this was removed further precipitation occurred after an additional forty-eight hours. The supernatants were carefully centrifuged, tube 1, 2 and 3 became clear, but 4, 5, 6 and 7 remained cloudy and deposited further precipitate when they were centrifuged to test for excess antibody and antigen.

Another set of the duplicates of this experiment were kept at 0°C. for two weeks. Even after this length of time, tube No. 2, e.g. showed only a faint trace of precipitate.

In order to determine the chemical and immunological changes which took place in nucleoprotein antigen altered by heat, alkali, acid and aging, the following experiment was conducted. Purified lyophilized protein was dissolved in saline, and two 5 cc. aliquots containing 0.44 milligram N/cc. added to two 15 cc. volumetric flasks. A third flask received 4.3 cc. of protein and was used as a control. To one was added 5 cc. of 0.2 N hydrochloric acid, to the second was added 5 cc. of 0.1 N sodium hydroxide, the third received 4.3 cc. of saline. The flasks were allowed to stand 16 hours at room temperature, the contents neutralized and diluted to volume; saline was added to the control flask.

To determine the effects of aging, the protein in a bacterial extract which had stood four months at 5°C., was purified by three precipitations with acetic acid and re-

solutions in 0.1 M sodium bicarbonate. The N/P ratio of this aged product was 2.96 : 1.

Five cc. samples of the antigens subjected to acid and alkali and five cc. of the control sample were precipitated with 10 cc./of pH 3.6 (0.1 M) chilled acetate buffer and the supernatants were analyzed for total nitrogen, inorganic phosphorus and total phosphorus. The results are given in Table XII. The precipitates were re-dissolved in a volume of five cc. and aliquots taken for analysis of nitrogen and phosphorus. The results are given in Table XIII. It is thus seen that each treatment appreciably altered the chemical properties of the antigen. Alkali split the largest amount of nitrogen and phosphorus off the compound, yet room temperature for sixteen hours was sufficient to appreciably alter the chemical properties of the compound.

In order to investigate the changes in serological behavior, precipitin reactions were run with each antigen using pool 1 sera and the same conditions as for the study of the unaltered antigen. The room temperature control used above was heated to 60°C. for one hour before it was used. The results of this precipitation are given in Table XI. It is thus seen that any of the treatments described appreciably lowered the ability of the antigen to react with its homologous antibody.

Qualitative Serological Investigations of the Anti-Protein Sera.

Sera produced in rabbits by immunization with alum precipitated nucleoprotein were not strong. The serum of the

first course of injections was diluted in halving dilutions and to each 1/2 cc. of diluted sera was added 0.01 milligram of antigen nitrogen. Only the first tube of the precipitin reaction was positive. To a duplicate set of tubes an equal amount in milligrams of heat killed organisms were added. Agglutination was positive in the sample of serum diluted 1:32 after all-night incubation at 37°C.

Anti-bacterial sera completely absorbed with vaccine did not precipitate nucleoprotein antigen.

DISCUSSION

Staphylococcus strain #152 was grown successfully on cellophane. Three washings with chilled merthiolated saline were sufficient to remove all non-bacterial nitrogen.

The organisms could be disintegrated by shaking them with glass beads, and nucleoprotein was obtained from the supernatant of this bacterial mash.

The protein was purified in such a manner that no nucleic acid or trichloroacetic acid precipitable nitrogen was split off in the process; the N/P ratio of the purified compound was four, and this remained constant throughout the purification process. It was found that that substantially all the non-protein nitrogen and phosphorus in the bacterial extract could be removed by dialysis against physiological saline because the N/P ratio of the protein in this dialyzed extract was also four.

Qualitative agglutination studies indicated that the rabbit anti-staphylococcus serum were of low titre, yet quantitative nitrogen estimations showed the amount of antibody was not low.

The reaction between anti-bacterial serum and the nucleoprotein antigen was incomplete after four hours at 37°C. It was not possible to measure absolute amounts of precipitin nitrogen because all the antigen nitrogen added was not found in the precipitate, and supernatants gave tests for both antigen and antibody. Heidelberger (1947) however found the reaction between pneumococcal nucleoprotein and antipneumococcal serum to be complete in forty-eight to ninety-six hours at 0°C; this was not found to hold in the staphylococcus system. The serum Heidelberger used was not true anti-bacterial serum but consisted of serum which had been prepared by the injection of a

pleural exudate containing autolyzed pneumococci. Heidelberger stated that ordinary antipneumococcal horse serum contained little antinucleoprotein precipitin, but chiefly antibodies to the specific carbohydrate and to "C" substance.

Antigen altered by heat, alkali and acid exhibited a greatly decreased ability to react with anti-bacterial sera as did antigen altered by aging. It is interesting to note that heat treated and aged nucleoprotein produced similar results in the precipitin reaction.

The alkali treatment of the nucleoprotein produced a compound with an N/P ratio of 8.7/1; both phosphorus and nitrogen were split from the compound, but the alkali degraded fraction still produced a reaction with bacterial antiserum, as did the acid treated compound. The acid treated substance had an N/P ratio of 5.95/1, thus chemically it was not as drastically changed as the alkali treated compound.

The nucleoprotein was antigenic, but produced a weak antisera. Anti-bacterial sera when completely absorbed with the staphylococcal vaccine gave no precipitin reaction. Nucleoprotein antisera on the other hand gave a weak agglutination with staphylococcal vaccine.

SUMMARY

1. Staphylococcus strain #152 was successfully grown on cellophane supported by paper pulp which had been soaked in Dolman's media.
2. The culture could be washed free of all non-bacterial nitrogen. Heat killed bacteria gave off little nitrogen.
3. Shaking the bacteria with glass beads proved a successful method of disintegrating the bacteria and liberating nucleoprotein.
4. The protein in the supernatant of bacteria shaken five hours with beads was forty percent of the bacterial protein.
5. Purification of the protein was accomplished by precipitation with 0.1 M acetic acid buffer at pH 3.7 and re-solution in 0.1 M borate buffer at pH 7.8. No nucleic acid or trichloroacetic acid precipitable nitrogen was split off the compound by the purification.
6. The first supernatant of the purification contained a non-protein serologically active substance.
7. The N/P ratio of the purified protein was four. The compound exhibited an absorption spectrum characteristic of nucleic acid. The purified product was not heat stable.
8. The strongest pool of anti-bacterial rabbit serum contained over 1.0 milligrams of agglutinin nitrogen.
9. The precipitin reaction of purified antigen with anti-bacterial serum was incomplete when carried out at 37°C.

for four hours. It was not possible to measure absolute amounts of precipitin nitrogen because all the antigen nitrogen added was not found in the precipitate, and supernatants gave tests for both antigen and antibody.

10. Alkali treatment split both nitrogen and phosphorus off the nucleoprotein; the compound which precipitated at pH 3.7 after alkali treatment had an N/P ratio of 8.7/1. The acid treated nucleoprotein precipitated with 3.7 buffer had an N/P ratio of 5.95/1. A room temperature nucleoprotein control had an N/P ratio of 6.1/1 when precipitated at pH 3.7. Four months aging of the bacterial extract at 5°C. produced a compound with an N/P ratio of 2.87/1.
11. All altered antigens exhibited a decreased ability to react with anti-bacterial serum.
12. The aged nucleoprotein was serologically similar to the heated sample.
13. The nucleoprotein was a weak antigen.

TABLE I

Nitrogen analysis of the first, second, third and fourth saline supernatants of staphylococci which were treated with various agents.

Supernatant	1:10,000 Merthiolate Mg.N/cc.	1:5,000 Merthiolate Mg.N/cc.	56°C. Mg.N/cc.	Acetone Mg.N/cc.	Saline Control Mg.N/cc.
1	0.167	0.173	0.232	0.056	0.125
2	0.022	0.027	0.019	0.018	0.023
3	0.009	0.008	0.007	0.012	0.013
4	0.01	0.007	0.006	0.001	0.013
5*	0.029	0.024	0.0126	0.06	0.06

*The organisms were resuspended and allowed to stand overnight at 5°C.

TABLE II

The immunization schedule of rabbits injected with heat killed staphylococcal vaccine.

COURSE 1

Week 1	¼ cc.	¼ cc.	½ cc.	½ cc.
2	½ cc.	¾ cc.	¾ cc.	1 cc.
3	1 cc.	1 cc.	1½ cc.	1½ cc.
4	1½ cc.	2 cc.	2 cc.	2 cc.

COURSE 2

Week 1	½ cc.	¼ cc.	½ cc.	½ cc.
2	¾ cc.	¾ cc.	1 cc.	1 cc.
3	1 cc.	1½ cc.	1½ cc.	2 cc.
4	2 cc.	2½ cc.	2½ cc.	3 cc.

COURSE 3

Week 1	½ cc.	½ cc.	½ cc.	1 cc.
2	1 cc.	1½ cc.	1½ cc.	2 cc.
3	2 cc.	2½ cc.	2½ cc.	3 cc.
4	3 cc.	3½ cc.	4 cc.	4 cc.

COURSE 4

Week 1	½ cc.	1 cc.	1 cc.	1½ cc.
2	1½ cc.	2 cc.	2 cc.	3 cc.
3	3 cc.	3 cc.	4 cc.	4 cc.
4	5 cc.	5 cc.	5 cc.	5 cc.

TABLE III

The immunization schedule of rabbits injected with purified nucleoprotein.

Week 1	½ cc.	½ cc.	1 cc.	1 cc.
Week 2	1½ cc.	1½ cc.	2 cc.	2 cc.
Week 3	2½ cc.	2½ cc.	3 cc.	3 cc.
Week 4	3 cc.	3 cc.		

TABLE IV

The results of subjecting living cultures of staphylococci to the vibrations of the Raython Sonic Vibrator.

Time subjected to Vibration	Total N in mg/cc. of supernatant		Protein N in mg/cc of supernatant		Bacterial Count	
	Control	Vibrated	Control	Vibrated	Control	Vibrated
½ hr.	0.064	0.085	0.004	0.034	46×10^9	48×10^9
1 hr.	0.075	0.146	0.007	0.045	39×10^9	35×10^9
1½ hr.	0.088	0.190	0.023	0.09	90×10^{10}	347×10^{10}

TABLE V

The results of shaking viable cultures of staphylococci with glass beads.

Hours Shaken	Plate Counts		Mg. of Protein N/cc. in supernatant		percent yield protein nitrogen
	Control	Shaken	Control	Shaken	
0	$90x10^9$	$90x10^9$	0.01	0.01	
1		$193x10^8$		0.41	19.5
2		$102x10^8$		0.68	32.4
3	$40x10^9$	$73x10^8$	0.01	0.75	35.4
4					
5	$40x10^9$	$40x10^8$		0.86	39.2
13			0.017	1.01	49.[illegible]

TABLE VI

The results of repeated precipitation of the nucleoprotein with 0.1 M acetate buffer pH 3.7 and re-solution of the protein in 0.1 M borate buffer pH 7.8.

Precipitation	Mg. of N/cc in redissolved Protein	Mg. of P/cc in redissolved Protein	N/P	Total Mg.N/cc of Supernatant	Mg.Protein N/cc Supernatant	Mg.P/cc Supernatant
1	0.278	.075	3.94	0.054	0.0013	0.035
2	0.254	.061	4.15	0.003	-	-
3	0.236	.057	4.09	0.0026	-	-
4	0.218	.054	4.03	-	-	-

TABLE VII

The results of subjecting formalized vaccine to various pHs.

pH	Mg. of Nitrogen found
6.5	0.44
7.0	0.45
7.5	0.45
Original vaccine	0.45

TABLE VIII

The results of absorbing normal rabbit serum at various pHs with formalized vaccine.

pH	Serum cc	Buffer cc	Vaccine cc	Saline cc	Mg. of N found	Mg. of N absorbed
6.5	1	1	1	0	0.45	0
Vaccine	0	2	1	0	0.44	
Ser.blk.	1	1	0	1	0.01	
7.0	1	1	1	0	0.75	0
Vaccine	0	2	1	0	0.74	
Ser.blk.	1	1	0	1	0.01	
7.5	1	1	1	0	0.75	0
Vaccine	0	2	1	0	0.74	
Ser.blk.	1	1	0	1	0.01	

TABLE IX

Results of quantitative agglutinin nitrogen determinations at pH 6.8 with formalized vaccine and pooled rabbit sera diluted 1:1.

Pool 1 first absorption

	Serum cc	Vaccine cc	Buffer cc	Saline cc	Mg. N. found	Mg. Agglutinin N. by difference
	1	1.5	1	0	1.14	0.16
Vaccine control	0	1.5	2	0	0.97	
Serum control	1	0	1	1	0.01	
Pool 1 second absorption						
	total	1.5	0	0	1.08	0.12
Vaccine control	0	1.5	2	0	0.96	
Pool 1 third absorption						
	total	1.5	0	0	1.04	0.04
Vaccine control	0	1.5	2	0	1.00	
Pool 1 fourth absorption						
	total	1.5	0	0	1.00	0.02
Vaccine control	0	1.5	2	0	0.98	

Total antibody nitrogen per cc. = .34 x 2 = 0.68 mg.

Pool 2 first absorption

	1	1.5	1	0	1.19	0.21
Vaccine control	0	1.5	2	0	0.97	
Serum control	1	0	1	1	0.01	
Pool 2 second absorption						
	total	1.5	0	0	1.16	0.20
Vaccine control	0	1.5	2	0	0.96	
Pool 2 third absorption						
	total	1.5	0	0	1.08	0.085
Vaccine control	0	1.5	2	0	0.095	
Pool 2 fourth absorption						
	total	1.5	0	0	0.99	0.01
Vaccine control	0	1.5	2	0	0.98	

Total antibody nitrogen per cc. = .505 x 2 = 1.10. mg.

Pool 3 first absorption

	1	1.5	1	0	1.13	0.15
Vaccine control	0	1.5	2	0	0.97	
Serum control	1	0	1	1	0.01	
Pool 3 second absorption						
	total	1.5	0	0	1.11	0.15
Vaccine control	0	1.5	2	0	0.96	
Pool 3 third absorption						
	total	1.5	0	0	1.06	0.07
Vaccine control	0	1.5	2	0	1.00	
Pool 3 fourth absorption						
	total	1.5	0	0	0.99	0.01
Vaccine control	0	1.5	2	0	0.98	

trogen per cc = .37 x 2 = .74 mg.

TABLE X

The agglutination titers of the various sera comprising Pool 1, Pool 2, Pool 3, and Pool 4.

Serum No.	Course 1.	Course 2.	Course 3.	Course 4.
1539	1/512	1/512		
1541	1/512	1/512	1/1024	
1543	1/512			
1544	1/512	1/512	1/512	
1546	1/128	1/256	1/256	1/1024
1540	1/256			
1522	1/256	1/256		
1547		1/256	1/512	1/512
1576	1/256	1/512		
1545	1/256			
1542	1/512			

TABLE XI

The results of the quantitative precipitin study of unaltered antigen, and antigen altered by heat, acid, alkali and aging.

Antigen added	Serum cc.	Anti-gen	Mg. N. pptated	Mg. N. ppt/cc. Serum	Tests on Supernatant + Anti-gen	+ Anti-body	Super-natant
Unaltered	4.0	0.098	0.152	0.032	+++	+	+
"	2.0	0.098	0.095	0.041	+++	−	+
"	2.0	0.196	0.136	0.059	+++	+	+
"	2.0	0.588	0.256	0.118	++	++	+++
"	1.0	0.98	0.190	0.174	+	+++	+++
"	1.0	1.96	0.208	0.182	+	+++	+++
"	0.5	1.96	0.105	0.162			
Serum blank	4.0		0.021				
Antig. "		1.96	0.021				
Antig. "		.196	0.008				
Acid treated	2	0.088	0.032	0.016			
Acid "	1	0.88	0.027	0.027			
Acid " blk.		0.088	0.006				
Acid " "		0.88	0.009				
Alkali "	2	0.088		-.003			
Alkali "	1	0.88	0.030	0.030			
Alkali " blk.		0.088	0.003				
Alkali " "		0.88	0.005				
Heat "	2	0.070	0.012	0.006			
Heat "	1	0.70	0.060	0.060			
Heat " blk.		0.07	0.002				
Heat " blk.		0.70	0.002				
Aged	2	0.048	0.016	0.008			
Aged	1	0.48	0.041	0.041			
Aged blank		0.048	0.003				
Aged "		0.48	0.005				

The volume of each sample was brought to 5 cc. with saline.

TABLE XII

The results of the nitrogen and phosphorus analysis of the supernatants of altered antigens precipitated with pH 3.6 acetate buffer.

Supernatant of	Total Mg.N/cc.	Inorganic P/cc.	Total P in Mg/cc
Acid treated	0.017	θ	0.0085
Alkali treated	0.040	θ	0.019
Control	0.019	θ	0.0085

TABLE XIII

Results of the analysis of the precipitate which formed on addition of 3.6 acetate buffer 0.1 M to antigen altered by alkali, acid, and 20°C. for 16 hours (i.e. control).

Sample	Total N/cc in Mg.	Total P/cc in Mg.	N/P ratio of precipitate
Acid	0.37	0.062	5.95/1
Alkali	0.322	0.037	8.7/1
Control	0.292	0.048	6.1/1

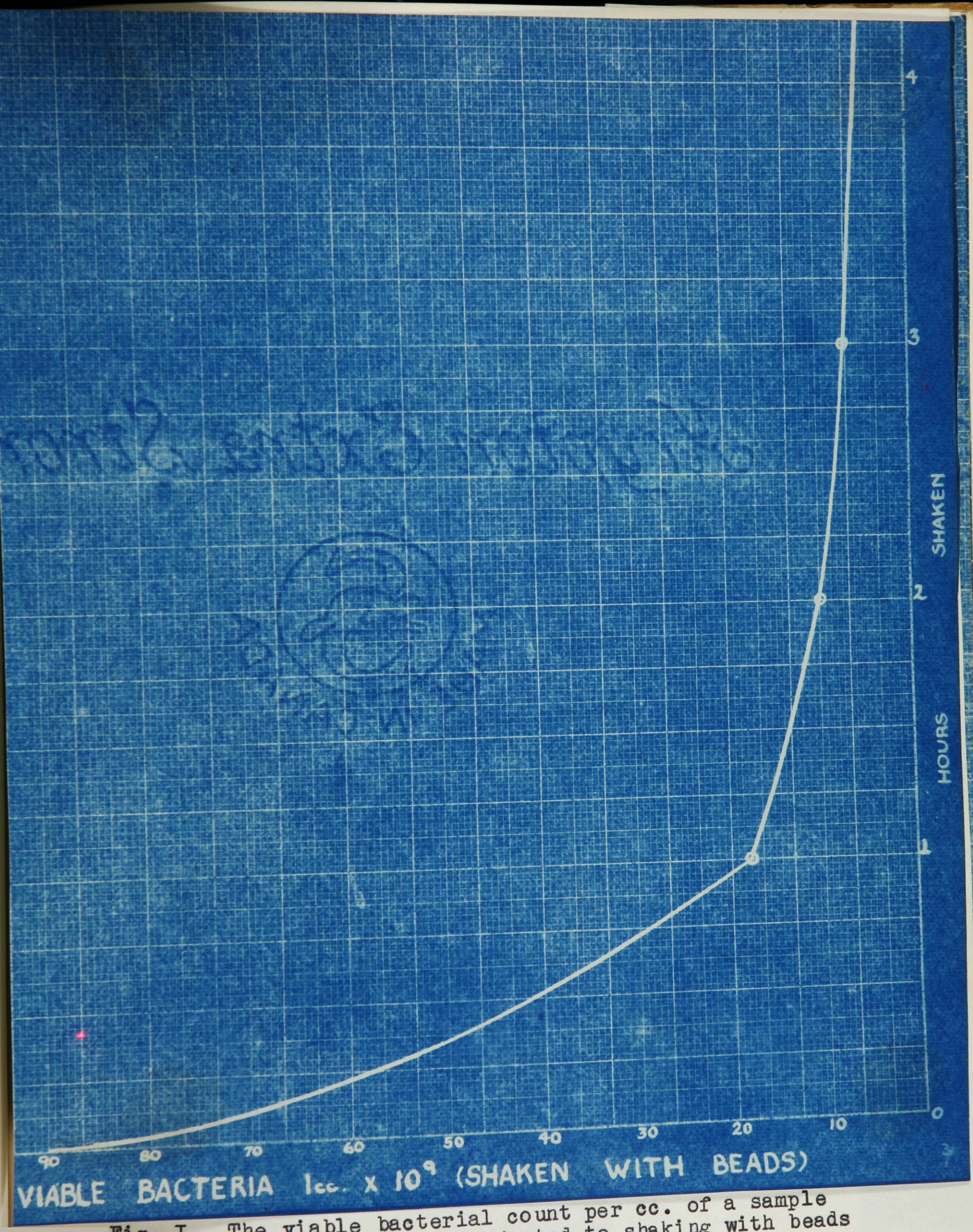

Fig. I. The viable bacterial count per cc. of a sample of staphylococci subjected to shaking with beads for varying intervals of time.

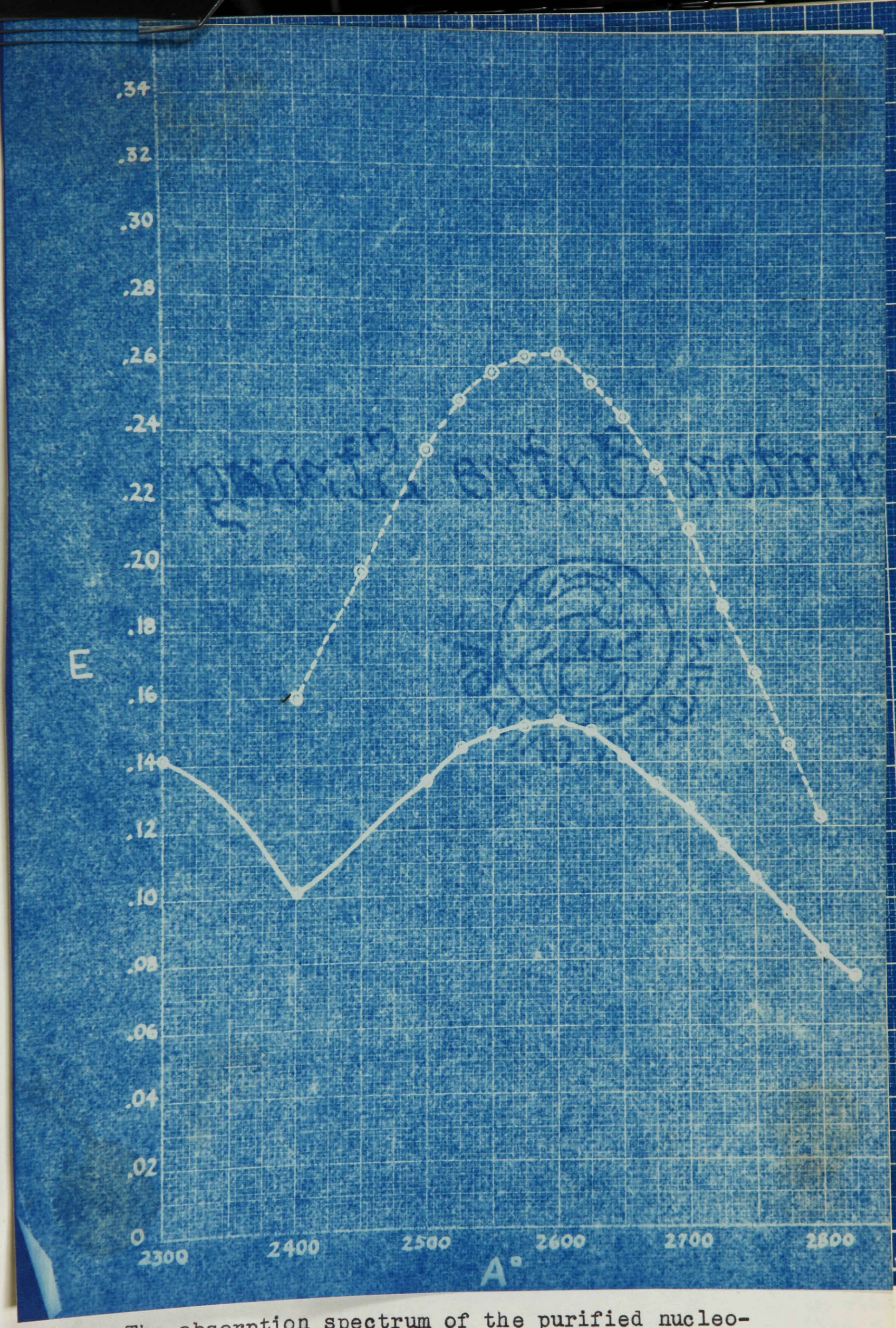

Fig. III. The absorption spectrum of the purified nucleoprotein and of a sample of commercial yeast nucleic acid. The solid line represents the protein absorption curve; the dotted line the yeast nucleic acid.

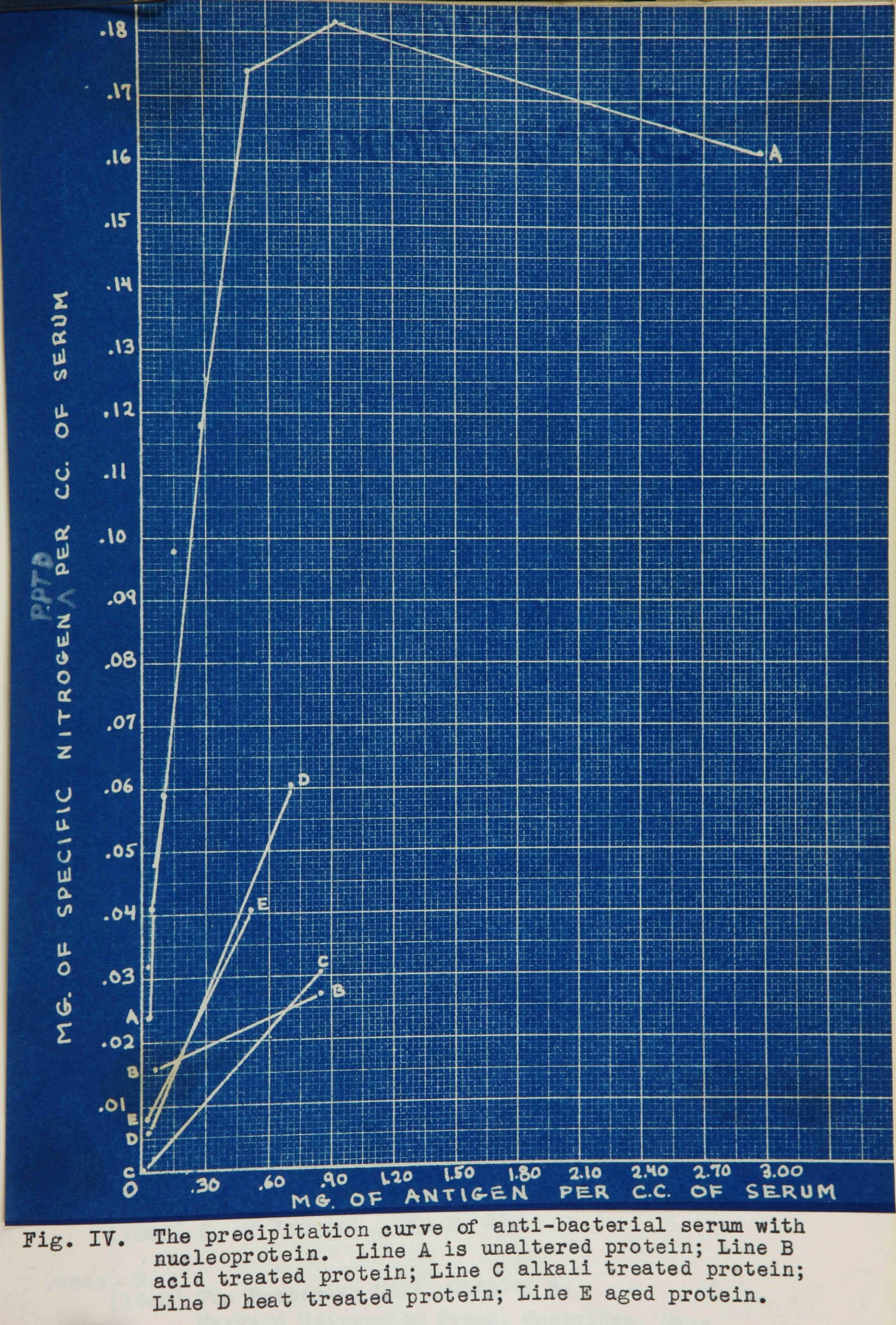

Fig. IV. The precipitation curve of anti-bacterial serum with nucleoprotein. Line A is unaltered protein; Line B acid treated protein; Line C alkali treated protein; Line D heat treated protein; Line E aged protein.

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CLAIM TO ORIGINALITY

Conditions for the measurement of the agglutinin nitrogen content of anti-bacterial staphylococcus rabbit serum were defined.

It was found that the staphylococcus could be disintegrated by shaking the organisms with glass beads.

The supernatant of the disintegrated organisms contained a somatic protein which was isolated and purified. The ultraviolet absorption spectrum of this protein showed that it was a nucleoprotein.

Quantitative immunological relationships between this nucleoprotein and its homologous antibody found in staphylococcus serum were studied.

The effects of strong acid and alkali, heat and aging on the chemical and immunological properties of the nucleoprotein were determined.