A STABLE HEMOLYSIN-LEUCOCIDIN AND ITS CRYSTALLINE DERIVATIVE ISOLATED FROM BETA HEMOLYTIC STREPTOCOCCI*

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The hemolysis produced by various strains of streptococci is a characteristic of such importance that it serves as a basis for classification. Of the large amount of work on the hemolysins of the true or β hemolytic streptococci, the greater part has been done with filtrates of broth cultures. Thus the active hemolysin was contained in a mixture composed of the known constituents of the broth, and the unknown products of cellular metabolism, and products of their interactions. In general the hemolysins have fallen into two classes, those which are stable, and those which are labile to oxygen. The hemolysin to be reported here is a pure substance, free of the extraneous constituents of the culture fluid. This hemolysin is stable to oxygen, as well as to heat and alterations in pH within the physiological range. It will be referred to as the stable hemolysin (S. H.).

Among the earlier reports on oxygen-labile hemolysins may be mentioned that of McLeod (1) who in 1912 reported a hemolysin from streptococcal filtrates which was labile to heat as well as to oxygen. Neill and Mallory (2) in 1926 reported a reversibly oxidizable streptolysin. Later Todd (3) demonstrated that hemolysins which were oxygen-labile, antigenic and neutralizable by immune sera occurred in streptococci of Lancefield's group A only. Oxygen-stable hemolysins on the other hand were not confined to group A strains but were produced by strains of animal as well as of human origin, falling into groups A, B, C, D, and E. The oxygen-stable hemolysin of Todd was not neutralizable by antistreptolysin and appeared to be non-antigenic.

Since the earliest recognition of bacterial leucocidins, many fragmentary reports

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have appeared. As with the hemolysins, there is an apparent lack of parallelism between the various leucocidins reported, perhaps due to the fact that they were present in a complex mixture of broth culture filtrate. Comparisons of such properties as heat and oxygen lability are of doubtful significance. Much of the discussion centers around the identity of the hemolysin and leucocidin of a given strain of streptococci.

Weld (4) showed that under certain conditions serum extracts of hemolytic streptococci are markedly hemolytic and are toxic for mice when injected intravenously, causing hemoglobinuria, anemia and death. Further studies showed that the principal pathologic finding in mice was marked degeneration of the renal tubular epithelium.

Channon and McLeod (5) reported a thermolabile toxin from serum broth cultures of hemolytic streptococci. This toxin showed a marked lytic activity on leucocytes, and they believed it to be the same as the hemolysin.

Nakayama (6) tested for leucocidin from streptococcal broth filtrates by two methods: (a) by the loss of ameboid motion of leucocytes when mixed with leucocidin, and (b) by the bioscopic method of Neisser and Wechsberg (7); that is, the effect of the leucocidin in inhibiting the reduction of methylene blue by leucocytes. A modification of the latter method has been used in the experiments reported here.

An extensive review by Evans (8) brought evidence for the non-identity of the streptococcal leucocidin (heat-stable) and the hemotoxin (heat-labile) which were obtained from hemolytic streptococcal broth culture filtrates. Gay and Oram (9) largely confirmed the work of Evans and described the action of streptococcal leucocidin on cells other than the polymorphonuclear neutrophils.

The stable hemolysin-leucocidin (S. H.) reported here was obtained in pure, that is, homomolecular form. Thus the activity of the S. H. was due to a single species of molecule acting as hemolysin and as leucocidin. The parallel activity of S. H. as hemolysin and as leucocidin will be brought out, dealing with the material in its "native" form and in the crystalline derivative of this. The crystalline derivative will be referred to as C. S. H.

Description of the Strains of Organisms Used

Streptococcus hemolyticus. Lancefield group A.

Strain 1896. Falls into none of Griffith's original types. Original source lobar pneumonia. Colony form mucoid.

Strain 1685. Griffith type 1. Original source erysipelas. Colony form mucoid.

Strain 1685G. Smooth (glossy) variant of the above. Injection of 0.1 ml. of culture not fatal to mice.

Strain S43G.

S. hemolyticus. Lancefield group B, strain O90R.

S. hemolyticus. Lancefield group C, strain F132.

S. hemolyticus. Lancefield group G, strain H46C.

Strains Used for Purposes of Control.—

S. viridans. Strain P25. Laboratory strain.

Staphylococcus albus. Colony form rough.

Bacterium typhosum. Strain O901. Old laboratory strain described by Felix.

S. pneumoniae. Type I. Colony form mucoid.

Fuller description of the strains of streptococci used will be found in a preceding paper (10).

Preparation of the Stable Hemolysin

The stable hemolysin can be prepared either (a) from the lyophile-dried whole bacteria, or (b) from lyophile-dried plate washings as starting material. The yield of S. H. in either case is less than 1 per cent. (a) The organisms are grown in neopeptone broth in sufficient amount to yield about 1 gm. of bacteria after drying by the lyophile process. (b) Saline washings from neopeptone blood-agar plates are centrifuged and the supernatant fluid is filtered through a Berkefeld N candle. The clear solution which results is dried by the lyophile process; 1 gm. of the dried material is used for the preparation of the S. H. fraction.

The dried microorganisms or plate washings are extracted in a Soxhlet apparatus for 2 days with moist ethyl ether (5 parts water + 95 parts absolute ether; *i.e.*, a two-phase system, so that in the collecting flask a layer of water underlies the ether which refluxes over the dried organisms). This crude two-phase extract is then placed in a boiling water bath until all traces of the ether have been removed. From the remaining watery extract the stable hemolysin may be readily obtained by adding absolute acetone (about 8 volumes) until a brown precipitate of rubbery consistency comes down. The acetone is decanted, the precipitate dried and dissolved in physiological saline solution. This solution contains the S. H. in crude form.

When a purified preparation is desired, the watery extract obtained as described above is added to 1 liter of saline and centrifuged to remove extraneous matter. The supernatant fluid is distilled to dryness *in vacuo* over a boiling water bath, and taken up in 50 ml. of 50 per cent acetone, centrifuged, and the supernatant precipitated with 15 volumes of dry ethyl ether followed by 5 volumes of absolute acetone. The precipitate is removed and dried at 100°C. until all odor of acetone has disappeared. Further purification may be effected by repetition of the above process. All samples used for chemical analysis were carried through the purification process until they gave a constant nitrogen-phosphorus ratio on analysis. This required 6 to 8 purifications. Evidence for the purity of the samples studied will be brought out later in this paper.

Crystalline Derivative of the Stable Hemolysin

A crystalline derivative of the stable hemolysin (C. S. H.) was obtained by the following procedure.

100 mg. of purified S. H. of known homomolecularity were dissolved in 25 ml. of distilled water and alkalified with 1 ml. of 40 per cent NaOH. The mixture was heated on a boiling water bath for 10 minutes, and then cooled rapidly under the tap. On standing in the refrigerator overnight, in a tightly sealed flask, small peg-shaped crystals separated. They were recovered by centrifugation, and dissolved in a minimal amount of hot 95 per cent alcohol. On cooling, similar crystals separated. After three additional alcohol and water recrystallizations, the material was found to consist of a trisodium salt, a derivative of the S. H.

Serological Specificity of the Stable Hemolysin

Injections of 0.01 ml. of a 1:1000 dilution of S. H. three times per week for 4 weeks failed to produce precipitins against the S. H. fraction. S. H. from both cells and the plate washings were prepared from strains 1685 and 1896 and each preparation was injected into three rabbits.

However, the S. H. is precipitable by antisera prepared by the injection of whole organisms, and is therefore a hapten. The specificity of the S. H. is shown in Table I. The results show that the S. H. is species-specific, in that any of the S. H. preparations are precipitated by any of the sera against β hemolytic streptococci, but not by antisera prepared against hemolytic staphylococci, pneumococcus Type I, *B. typhosum* or the broth used in the growing of the organisms. Moist ether extracts of all the strains of β hemolytic streptococci tested yield S. H. with the above properties, whereas moist ether extracts of *S. viridans*, hemolytic staphylococci, *B. typhosum* and pneumococcus Type I, do not have similar chemical properties, are not active hemolytically, nor are they precipitated by any of the antisera against these organisms or against β hemolytic streptococci.

Thus, the lack of antigenicity of S. H., its species specificity and its presence only in β hemolytic streptococci indicate its similarity to Todd's (3) oxy-stable hemolysin.

The C. S. H. is not serologically active; it neither precipitates antisera prepared against streptococci, nor inhibits the precipitation of S. H. by such antisera.

TABLE I
The Specificity of the Stable Hemolysin as Determined by Precipitation Tests

Group	Type	Rabbit antiserum versus	Stable hemolysin derived from							
			1896 cells	1896 wash- ings	1685 cells	1685 wash- ings	1685G cells	O90R cells	F132 cells	H46C cells
A	1	1685	0344	0333	0333	0333	0333	0333	0333	0333
A	1	1685G	0344	0333	0331	0333	0333	0333	0333	0333
A	1	1685G*	0334	0333	0333	0333	0333	0333	0333	0333
A	6	1048	0344	0333	0333	0333	0333	0333	0333	0333
A	14	1850	0344	0333	0332	0333	0333	0333	0333	0333
A	18	2084	0344	0333	0333	0333	0333	0333	0333	0333
A	—	2169	0344	0333	0333	0333	0333	0333	0333	0333
A	—	1793	0344	0333	0333	0333	0333	0333	0333	0333
A	—	1896	0344	0333	0333	0333	0333	0333	0333	0333
B	—	O90R	0333	0333	0333	0333	0333	0333	0333	0333
C	—	F132	0111	0111	0111	0111	0111	0111	0221	0222
D	—	H69	0333	0333	0333	0333	0333	0333	0333	0333
F	—	H60R	0333	0333	0333	0333	0333	0333	0222	0333
G	—	H46C	0333	0333	0333	0333	0333	0333	0333	0333
H	—	F90A	0333	0333	0333	0333	0333	0333	0333	0333
		Stable hemolysin	0000	0000	0000	0000	0000	0000	0000	0000
		Staphylococcus	0000	0000	0000	0000	0000	0000	0000	0000
		Pneumococcus	0000	0000	0000	0000	0000	0000	0000	0000
		<i>B. typhosum</i>	0000	0000	0000	0000	0000	0000	0000	0000
		Broth	0000	0000	0000	0000	0000	0000	0000	0000
		Normal horse serum	0000	0000	0000	0000	0000	0000	0000	0000
		Normal rabbit serum	0000	0000	0000	0000	0000	0000	0000	0000

The four figures under each antigen indicate the amount of precipitate in the saline control, and the antigen diluted 10^{-3} , 10^{-4} and 10^{-5} , respectively.

All antisera, with the exception of 1685G*, were prepared by the injection of living organisms. 1685G* antiserum was prepared by the injection of heat-killed organisms.

The precipitation tests were carried out with 0.5 ml. volumes of antigen and antibody. The tubes were incubated for 15 minutes at 37°C ., placed in the refrigerator overnight, and read after centrifugation at high speed. 0 = no precipitate; 1 = small granules; 2 = small floccules; 3 = large floccules; 4 = very heavy precipitate.

Hemolytic Activity of the Stable Hemolysin

Hemolytic Test System.—Preliminary tests for the activity of the stable hemolysin on erythrocytes of various species indicated that the rate of hemolysis (using dilutions of hemolysin from 1:2000 to

1:20,000) was in the following order: rabbit (shortest time for complete hemolysis), horse and sheep. For this reason rabbit erythrocytes were used in the following experiments. The blood was freshly drawn (never more than 3 days old) from normal rabbits. The cells were washed three times with physiological saline solution before use.

Unless otherwise indicated the test system consisted of 0.5 ml. of 5 per cent rabbit erythrocytes plus 0.5 ml. of the hemolysin dilution to be tested. Rapid mixture was carried out in a horizontal tube (Fig. 1), in which holes along one side allowed for introduction of each substance separately. A hollow in the wall of the tube opposite each hole held the fluid until mixing was desired. In order to mix the constituents, the tube was quickly raised to the vertical position, and was shaken continuously with a gentle motion. The amount of fluid left in the cups or adhering to the walls of the vessel above the surface of the mixture was negligible. The end of the tube was constricted to a bore of 0.7 cm. for greater ease in

• Fig. 1

reading. Hemolysis was read at room temperature by transmitted daylight. Time for complete hemolysis was taken as the end point, although this was not entirely satisfactory, especially in the higher dilutions of hemolysin. However, the reproducibility of results, with the use of this apparatus, seemed to justify the method.

Activity of Highly Purified Stable Hemolysin.—It can be seen from Fig. 2 that complete hemolysis was brought about in a dilution of 1:128,000. (A separate pipette was used for each dilution.) Although partial hemolysis occurred at the next dilution (1:256,000), it was never complete. The stable hemolysin kept in a sterile condition for a period of a year retained its activity. There was no diminution in its activity after 30 minutes in a boiling water bath. After exposure to acid or alkali within what might be called a physiological range (with subsequent neutralization), the activity was not impaired. A comparison of the activity of S. H. which had not been treated, and S. H. which had been exposed to heat and to changes in pH is shown in Fig. 2. A time-dilution curve may be drawn through the points representing the same preparation of S. H. treated in various ways; all of the points fall within the limit of error of the method used.

The crystalline derivative retains hemolytic activity. This activity parallels the activity of S. H., as shown in Table II. We feel that the indicated difference in activity of S. H. and C. S. H. may be due to difficulties in weighing the solid material in order to make solutions of

FIG. 2. Stability of the stable hemolysin from β hemolytic streptococci.

known concentrations rather than to an actual difference in activity. The parallel activity of the C. S. H. and the S. H., together with the fact that only the S. H. is serologically active, seems to indicate that those groups in the molecule responsible for serological activity are not those concerned in hemolysis.

In order to determine the nature of the hemolytic reaction, a number of observations were made with the use of the Evelyn colorimeter (11). The progress of the reaction was followed by a decrease in transmissibility of light through the mixture. The transmitted light falls on a photoelectric cell; readings were made on a millivoltmeter. The time-millivolt curve (indicative of time-hemolysis relationship) was S-shaped. That is, after the initial delay, hemolysis proceeded rapidly. The rate then decreased again near the end point of the reaction. For example, when a dilution of 1:150,000 of S. H. acted on 15 ml. of 1 per cent rabbit erythrocytes, there was a lag phase of 2 minutes. Hemolysis then proceeded rapidly, reaching almost complete hemolysis in 10 minutes. The rate then decreased, coming to a constant (complete hemolysis) in 40 minutes. With higher concentrations of stable hemolysin the lag was shorter, as was the time for complete hemolysis. With higher dilutions, the lag was longer, the maximum rate of reaction was less and complete hemolysis was never reached.

These observations show that the stable hemolysin can be classed with the simple hemolysins for which the S-shaped reaction curve is characteristic. The kinetics of simple hemolysins which give such curves have been discussed by Ponder (12).

The Lack of Specific Neutralization of Stable Hemolysin by Immune Sera.—When S. H. is allowed to remain in contact for 30 minutes at 20°C. with immune serum *versus* the homologous or heterologous type of β hemolytic streptococcus, with concentrated antistreptolysin,¹ or with normal rabbit serum, some diminution in the titer of the hemolysin is observed. However, this inhibition of hemolysis was no greater with the immune sera than with the normal serum. After this length of contact no precipitation by the immune serum is visible, and it might well be questioned whether the hapten-antibody reaction had occurred in this time. Therefore, a hemolysis test was performed after the second stage of the precipitation reaction had set in; that is, the precipitation test was carried out in the usual way: dilutions of S. H. were set up with homologous antiserum (in duplicate), with antiserum to a heterologous type, with normal rabbit serum, and with saline. After 15 minutes in the 37°C. water

¹ Obtained from Dr. Homer F. Swift of The Rockefeller Institute.

bath, the mixtures were allowed to stand overnight in the refrigerator. After centrifugation, a marked precipitate was visible with S. H. plus immune serum (heterologous as well as homologous) and not with normal serum nor with saline. A subsequent hemolysis test showed no more inhibition by the immune sera (in the presence of the precipitate) than by normal serum. In the duplicate precipitin test with the homologous serum, the supernatant in each tube was removed to a corresponding tube in another series, and the remaining precipitate was washed once with saline. In a hemolysis test carried out on these two series, the supernatant showed the same degree of hemolysis as the corresponding precipitate plus supernatant; the precipitate alone showed no hemolysis. In summary, anti- β hemolytic streptococcal serum (whether of the homologous or heterologous type) does not inhibit hemolysis by S.H. to any greater extent than normal serum (whether the hapten-antibody reaction has proceeded to the first or second stage). This is in contrast to the very effective neutralization of the oxy-labile hemolysin by immune sera (3, 13).

Leucocidic Activity of the Stable Hemolysin and Its Crystalline Derivative

As it is known that intact cells of streptococci are often leucocidic⁷ the stable hemolysin and its crystalline derivative were tested for their action on leucocytes. The bioscopic method of Neisser and Wechsberg (7) was used. This method depends on the ability of normal leucocytes to reduce methylene blue.

Methods and Materials.—A leucocyte suspension was obtained according to the method of Mudd, Lucké, McCutcheon and Strumia (14) by introducing 200 ml. of sterile saline into the peritoneal cavity of a normal rabbit. 4 hours later the suspension of leucocytes which had accumulated was drawn out through a large needle. It was found that a suspension of leucocytes of at least 10,000 per c. mm. was necessary to bring about the reduction of methylene blue in an appropriately short time. After longer time intervals, the cells settled so that reduction was not uniform throughout the tubes. The cells were left in the peritoneal fluid to keep them in suspension. When it was necessary to concentrate the suspension it was centrifuged for 30 seconds, and enough of the comparatively clear supernatant fluid was removed to make the concentration desired. A control was included in each series, using clear supernatant fluid (peritoneal fluid after intense centrifugation) to determine the effect of this substance on methylene blue. Other controls

are enumerated below. The special methylene blue suggested by Gay and Oram (9) was used throughout these experiments.

Into a series of test tubes with a bore of 0.7 cm. were placed 0.5 ml. serial dilutions of S. H. in saline; 2.0 ml. of leucocyte suspension were added to each tube and allowed to remain in contact for 30 minutes at 37°C. Then 1 drop of 0.1 per cent special methylene blue was added to each tube. The contents of each tube were thoroughly mixed by repeated inverting of the tubes, and covered with a layer of paraffin oil about 2 cm. deep. The paraffin oil had been recently heated in a boiling water bath and cooled in order to reduce the amount of oxygen contained in it. The tubes were then incubated at 37°C., and readings of the amount of reduction of methylene blue were made at intervals of 1 hour, 2 hours and overnight. Controls included leucocytes plus saline (positive control), saline plus leucocytes heated at 56°C. for 30 minutes, hemolysin alone, and the supernatant fluid from a centrifuged suspension of leucocytes in peritoneal fluid (negative controls which invariably failed to reduce methylene blue).

In choosing 30 minutes as the time for contact between leucocytes and stable hemolysin, there were two factors to be considered: (a) that the leucocidin has sufficient time to produce its toxic effect on the leucocytes, and (b) that the leucocytes remain fairly well suspended. After contact for 15 minutes, no further leucocidic activity was noted. The longer period of time was chosen to allow for a margin of safety.

Table II shows the results of a comparison of the leucocidic activity of the native form of stable hemolysin and its crystallizable derivative. At the overnight reading the S. H. prevented the reduction of methylene blue up to a dilution of 1:800, whereas C. S. H. prevented the reduction of methylene blue by leucocytes only up to 1:400. The activity is of the same order. The apparent difference in activity may be due to difficulty in measuring the dry weight of both substances, which exist as gummy masses. (The C. S. H. exists in the crystalline state only as the sodium salt. As it was desired to test the leucocidic activity of the uncombined material, it was necessary to convert it into the free acid, which does not exist in the crystalline state. The free acid is so slightly ionized that it produces no appreciable acidity in solution.)

The leucocidic activity of the crystalline derivative of the stable hemolysin is not altered by 30 minutes' exposure in a boiling water bath. A comparison of the hemolytic activity of the same two preparations used for testing leucocidic activity is also shown in Table II. This particular preparation of S. H. hemolyzed rabbit red blood

cells up to a dilution of 1:25,600, whereas the C. S. H. from the same preparation hemolyzed up to a dilution of 1:6400.

It is evident that the same material acts both as an hemolysin and as a leucocidin. This is true of the native form and of its derivative. The activities of both substances as hemolysins and leucocidins are parallel, and such activity is not destroyed by heat.

TABLE II
Comparison of the Hemolytic and Leucocidic Activity of Stable Hemolysin and Crystalline Stable Hemolysin

Final dilutions of S. H. and C. S. H.	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:25,600	1:51,200	
	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>		
As hemolysins									
S. H.	4	5	9	19	44	88	332	nc	
C. S. H.	11	24	26	97	135	nc	nc	nc	
As leucocidins									Leucocytes alone
S. H.	—	—	++	++	++	++	++	++	++
C. S. H.	—	++	++	++	++	++	++	++	++

Numbers under dilutions refer to minutes required for complete hemolysis.

nc = hemolysis not complete.

— = no reduction of methylene blue (inhibition by S. H. or C. S. H.).

++ = maximal reduction.

Test systems described in text.

Chemical Composition of the Stable Hemolysin and Crystalline Stable Hemolysin

Highly purified samples of the stable hemolysin and its crystalline derivative (strain 1685) consist in each case of a single molecular species. This was determined by observing no change in the per cent composition after repeated recrystallizations of C. S. H., and repeated reprecipitations of S. H. There was also no change in the boiling point of saturated solutions upon the addition of more material, which is a further indication of a very high degree of purity. Measurements were made directly on S. H., but C. S. H. was first converted to the free acid.

The boiling point elevation of a solution of S. H. (100 gm. per 1000 ml.) was found to be between 0.023° and 0.025°C. , indicating a molecular weight of the order of 2000; that of C. S. H., 0.075°C. , indicating a molecular weight of 720. The S. H. fraction contains phosphorus in the form of phosphoric acid to the extent of 1.37 per cent (colorimetric), and nitrogen (Kjeldahl) to the extent of 1.87 per cent. Thus the nitrogen-phosphorus atomic ratio is 3:1. The minimum molecular weight based on phosphorus is 2260. As boiling point measurements have shown the molecular weight to be of the order of 2000, the actual

TABLE III
Nitrogen and Phosphorus Content of Stable Hemolysin and Molecular Weights Based on Phosphorus

S. H. derived from Strain No.	Phosphorus	Nitrogen	N/P atomic ratio	Molecular weight
	<i>per cent</i>	<i>per cent</i>		
1685 cells	1.34	1.88	3.11	2314
1685 washings	1.40	1.92	3.04	2215
1896 cells	1.35	1.84	3.02	2297
1896 washings	1.40	1.87	2.96	2215
S43G cells	1.32	1.87	3.14	2349
1685G cells	1.36	1.84	3.00	2280
O90R cells	1.40	1.84	2.91	2215
F132 cells	1.41	1.89	2.97	2200
		Averages.....	3.02	2260

Cells = S. H. isolated from whole streptococci.

Washings = S. H. isolated from plate washings of mucoid strains of streptococci, from which the organisms had been removed.

A substance of similar solubility could not be isolated from lyophile-dried broth.

molecular weight must be 2260. As there are 3 atoms of nitrogen to 1 of phosphorus, the actual molecular weight based on nitrogen is again the same within the accuracy of the analyses (minimum molecular weight based on nitrogen = $749 \times 3 = 2247$).

The definite ratio of 3 nitrogen atoms to 1 phosphorus atom is further evidence of the high degree of purity of S. H. The phosphorus and nitrogen content of S. H. from eight different sources, and the molecular weight based on these analyses are given in Table III. The

nitrogen-phosphorus ratio is about 3:1 in each case, and indicates that the eight different preparations of S. H. are probably identical, regardless of the strain from which they were isolated.

The stable hemolysin also contains carbon 39.87 per cent, hydrogen 6.54 per cent and oxygen. Its tentative molecular formula obtained by per cent analysis is $C_{75}H_{148}O_{71}N_3P$.

In the formation of C. S. H., which is accomplished by heating with NaOH, the nitrogen and phosphorus are lost, leaving only C, H and O.

FIG. 3. Crystalline stable hemolysin photographed through crossed Nicol prisms. $\times 150$.

The nitrogen is given off in the form of ammonia, and is therefore probably acid amide nitrogen. It is evident that during the loss of the N and P, the S. H. has been split into three smaller molecules which are identical (constant composition of C. S. H. after repeated recrystallizations), as three times the molecular weight of C. S. H. plus three times the atomic weight of nitrogen, plus the atomic weight of phosphorus approximates the molecular weight of S. H. ($720 \times 3 + 42 + 31 = 2233$). This molecular weight was confirmed,² using a

² Confirmation by Dr. Charles G. Grosscup.

modification of the thermoelectric vapor pressure apparatus as introduced by Hill (15); a molecular weight of 2070 ± 300 was reported.

Further work is in progress on the chemical structure of the two compounds.

C. S. H. is one of the few toxic bacterial substances which have been obtained in a crystalline form (16). A photomicrograph of C. S. H. taken through crossed Nicol prisms is shown in Fig. 3. Characteristic crystals are peg-shaped.

Other Properties of Stable Hemolysin and Crystalline Stable Hemolysin

Saturation of homologous antiserum with S. H. does not remove phagocytosis-promoting nor agglutinating antibodies (10).

C. S. H. has been shown to be extremely toxic for mice and rabbits. Further work on the toxicity of and pathological changes produced by C. S. H. and S. H. in animals is being carried out.

SUMMARY

1. A chemically pure hemolysin-leucocidin has been isolated from β hemolytic streptococci, but not from other species of bacteria studied.
2. It does not give rise to antibodies, but precipitates immune sera against hemolytic streptococci, and is therefore a hapten.
3. A highly purified sample of S. H. up to a dilution of 1:128,000 hemolyzes red blood cells. Its hemolytic activity is not specifically neutralized by antiserum *versus* β hemolytic streptococci. It is leucocidic in that it inhibits the reduction of methylene blue by leucocytes.
4. The hemolysin-leucocidin is stable to oxygen, to heat and to moderate changes in hydrogen ion concentration. Its chemical structure has been determined in part. Its molecular weight is 2260.
5. A crystalline derivative has been isolated as the sodium salt from the hemolysin-leucocidin. As the free acid it has a molecular weight of 720. Its hemolytic and leucocidic activity parallels that of S. H., although it is not serologically active. It possesses a high degree of toxicity for mice and rabbits.

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