There was no significant change in the number of basophils, which varied from 0 to 1%.

*Summary and Conclusions.* After the injection of the water-soluble potassium salt of dicarboxy-benzanthracene into Swiss mice there is a marked decrease in the number of polymorphonuclear neutrophils, a slower, and moderate decrease in the number of monocytes, with but slight changes in the number of lymphocytes, eosinophils, and basophils. Recovery is complete 7 weeks after the last injection. Data on the blood of normal Swiss mice are given.

11263

## Relation Between Growth of Pneumococcus III and Concentration of Capsular Polysaccharide Appearing in Culture Filtrates.*‡

SAMUEL CHARLES BUKANTZ.† (Introduced by Jesse G. M. Bullowa.)

*From the Littauer Pneumonia Research Fund, New York University College of Medicine, and the Medical Service, Harlem Hospital, Department of Hospitals, New York City.*

A method of adapting the photronreflectometer[1] to the quantitative determination of pneumococcal capsular polysaccharide in solutions of unknown concentration has been described[2] In the present investigation, the method was employed to determine the concentrations of SSS III appearing in Seitz filtrates of blood broth cultures, following inoculation with varying amounts of a standardized strain of Pneumococcus III; these concentrations were correlated with the phase and amount of growth of the organisms.

A strain of Pneumococcus III obtained from the sputum of a pneumonia patient was brought to constant virulence after the suggestions of Schmidt and Hilles.[3] $5 \times 10^{-4}$ ml of a 12-hour blood-

* These studies received financial support from the Metropolitan Life Insurance Company, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch, and Mrs. H. Robert Samstag.

† Littauer Fellow in Pneumonia Research.

‡ Technical assistance in this work was rendered by Miss Anita Cooper.

1 Libby, R. L., *J. Immunol.*, 1938, **34**, 71, 268.

2 Bukantz, S. C., and Bullowa, J. G. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 418.

3 Schmidt, L. H., and Hilles, C., *J. Inf. Dis.*, 1939, **65**, 273.

broth culture of the strain was injected intraperitoneally into an 18 g mouse. Twelve hours after the inoculation the mouse was sacrificed and a standard loop of heart's blood of the freshly killed animal was inoculated into 10 ml of blood broth. This cycle was repeated daily and used routinely for the preservation of the strain. After the eleventh and twelfth passages, the M.L.D. of the strain was found to be the same—8 organisms.§ Subsequent determinations have indicated that the virulence has not changed, even after the 50th passage. The 12-hour cultures contain a fairly constant number of organisms, usually between $50 \times 10^6$ and $100 \times 10^6$ per ml.

In the following experiments, all bacterial counts were made by the plating method with dilutions in broth. Separate pipettes were used for each dilution which was mixed 25 times before transfer. One ml of diluted culture, containing the desired inoculum, was seeded into each of a series of tubes containing 9 ml of 4% horse-blood-broth. A colony count of the original culture was performed, at the same time, in order to determine the size of inoculum used. All inoculated tubes were then incubated at 37°C for the duration of the experiment.‖ At 2, 4, 6, 8, 10, 12, 24, 28, 32, and 48-hour intervals, one tube was removed, a colony count performed and the contents of the tube filtered through the Swinney Seitz Filter Adapter.¶ All filtrates were sterile, and the pH, determined with nitrazine paper, was at least 7.0, even after a 48-hour period of incubation.** The concentration of capsular polysaccharide in each filtrate was then determined by titration of 1 ml of varying dilutions of each filtrate with 1 ml of a standardized serum in the zone of antibody excess; photronreflectometric measurements were made after incubation of each mixture in refraction cells for 20 minutes at 37°C. Dilutions of the filtrates were made in broth and the concentrations determined by reference to a standard curve prepared by titrating varying dilutions of the polysaccharide in broth, with the standard serum. Filtration control studies indicated that filtration of SSS in broth was not quantitative until adsorption by the filter had been completed. This adsorption occurred most rapidly in the presence of large numbers of bacteria (100,000,000 per ml) and the higher concentrations of SSS

§ According to the modification of the Park-Cooper technic employed by The Massachusetts Antitoxin and Vaccine Laboratory (White, *Biology of the Pneumococcus*, page 653).

‖ In preliminary experiments 4 tubes separately inoculated with the same number of pneumococci grew out an identical number of organisms in the same time.

¶ Becton-Dickinson, manufacturers, Rutherford, N. J.

** The original broth contained no glucose.

TABLE I.

ion of Various Filtrates from Experiment II (9,000 Organisms per ml Inoculum) with Standard Serum III. Sensitivity of Photronreflectometer at 15.

| | Dilution of Filtrate in Broth. | | | | | | | | | | | Avg SSS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Und. | 1-5 | 1-10 | 1-20 | 1-30 | 1-35 | 1-40 | 1-50 | 1-60 | 1-80 | 1-100 | 1-150 | per ml |
| Corrected Photron Rdg.[1] | | | | 5.0 | 3.0 | | 1.5 | 1.0 | | | | | .013 |
| SSS Equiv. of Dilution[2] | | | | .00075 | .0004 | | Ind.[5] | Ind. | | | | | |
| SSS Conc. in Orig. Filt.[3] | | | | .015 | .012 | | | | | | | | |
| C. P. R. | | | | | 8.5 | | | 5.0 | | 3.0 | 2.0 | | .035 |
| SSS Eq. Dil.[2] | | | | | .00125 | | | .00075 | | .0004 | | | |
| SSS Or. Fil.[3] | | | | | .037 | | | .037 | | .032 | | | |
| C. P. R. | | | | | | | 20.0 | 16.0 | | 8.0 | 7.0 | | .12 |
| SSS Eq. Dil.[2] | | | | | | | .0032 | .0025 | | .0012 | .001 | | |
| SSS Or. Fil.[3] | | | | | | | .13 | .13 | | .10 | .10 | | |
| C. P. R. | | | 17.5 | 24.0 | 24.0 | | 21.0 | | 15.5 | 12.5 | 8.0 | | .13 |
| SSS Eq. Dil.[2] | | | E.A.[4] | E.A. | | | .0033 | | .0023 | .0019 | .0012 | | |
| SSS Or. Fil.[3] | | | | | | | .13 | | .14 | .15 | .12 | | |
| C. P. R. | | | 14.0 | 24.0 | 23.0 | 20.5 | 21.0 | | 13.0 | 10.0 | 6.5 | | .12 |
| SSS Eq. Dil.[2] | | | E.A. | | | | .0033 | | .00195 | .0015 | .001 | | |
| SSS Or. Fil.[3] | | | | | | | .13 | | .12 | .12 | .10 | | |
| C. P. R. | | | 7.5 | 19.0 | 23.0 | 23.5 | 23.5 | 23.0 | | 15.0 | 12.0 | 8.0 | .18 |
| SSS Eq. Dil.[2] | | | E.A. | E.A. | E.A. | | | .0034 | | .0022 | .0018 | .0012 | |
| SSS Or. Fil.[3] | | | | | | | | .17 | | .18 | .18 | .18 | |

orrected Photronreflectometer Readings—Obtained by subtracting the dry blank reading of the cell from the final turbidity g.

SS (Capsular Polysaccharide) Equivalent of Dilution—Obtained by referencee to a previously determined standard curve. This expresses the actual amount of SSS present in the 1 ml sample of the dilution titrated.

SS Concentration in the Original Filtrate—Obtained by multiplying SSS Equiv. of Dil. by the Dilution [*i.e.*, For 10 hr $\times$ 20 = .015] expressed as milligrams per milliliter.

.A.—Excess of antigen.

ıd.—Indeterminate, because of inaccuracy in calculations of points on lowermost extrapolations of the standard curve.

(10-20 mg %). However, following a very large initial inoculum (900,000 per ml, Experiment III), low concentrations of SSS were obtained in early filtrates despite the presence of large numbers of bacteria (63,000,000 per ml), while later filtrates, obtained from cultures having approximately the same number of bacteria, yielded considerably higher concentrations of SSS. The influence of autolysis upon the increase of concentration of SSS has not yet been adequately investigated because of the difficulty in defining a technic for quantitative determination of the degree of autolysis. Judging from the rate of decrease of viable organisms, however, there is at least one period of significant increase in concentration of SSS in filtrates which appears to be unrelated to autolysis (13th to 24th hour, Experiment II) and more probably represents an alteration in the physiologic activity of the organisms.

A small quantity of capsular polysaccharide (0.5 mg %) was found after 48 hours, following an initial inoculum of 165 organisms per ml (Experiment I). A much larger quantity of polysaccharide (18 mg %) was found after an initial inoculum of 9,000 organisms per ml (Experiment II). Table I summarizes the titrations of some of the filtrates obtained following the inoculation of 9,000 organisms per ml. The remaining filtrates were titrated in the same way. Table II summarizes the results of this experiment in relation to the

TABLE II.

Experiment II. Growth of highly virulent pneumococcus III in 4% horse blood broth following an initial inoculum of 9,000 organisms per ml. Concentrations of SSS determined with the photronreflectometer.

| Hrs. | Colony count | Log. of count | *No. of generations | †Generation time (min) | SSS concentration mg/100 ml |
|---|---|---|---|---|---|
| 2 | 18,200 | 4.26 | 1.0 | 120 | 0 |
| 4'30" | 384,500 | 5.59 | 4.4 | 34 | 0 |
| 6'40" | 5,100,000 | 6.71 | 3.7 | 35 | .18 |
| 8 | 24,060,000 | 7.38 | 2.2 | 36 | .70 |
| 10 | 138,000,000 | 8.14 | 2.5 | 48 | 1.30 |
| 12'50" | 170,000,000 | 8.23 | 0.3 | 566 | 3.5 |
| 24 | 105,000,000 | 8.02 | — | — | 12.0 |
| 28 | 75,000,000 | 7.88 | — | — | 13.0 |
| 32 | 47,500,000 | 7.68 | — | — | 12.0 |
| 48 | 40,000 | 4.60 | — | — | 18.0 |

* From formula for law of geometric progression

$$N = \frac{\text{Log. of Final Count} - \text{Log. of Initial Count}}{\text{Log. 2}}$$

† $\frac{\text{Time in minutes}}{\text{Number of generations}}$

After Chesney.[7]

[7] Chesney, A. M., *J. Exp. Med.*, 1916, **24**, 387.

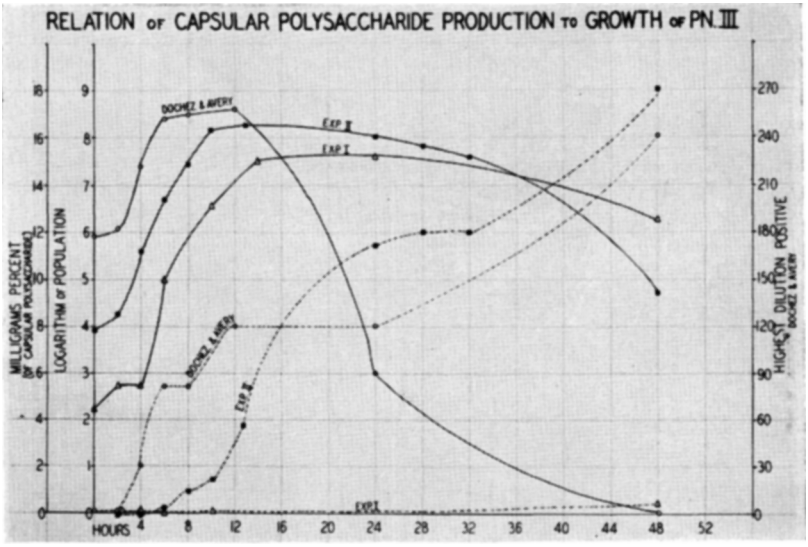


FIG. 1.

The relationship of increases in concentration of capsular polysaccharide III in culture filtrates to the phase of growth of Pneumococcus III in blood-broth; two different sizes of inoculation of an organism having the same virulence in each experiment.

Solid lines ———— Logarithm of populations.
Broken lines - - - - - - - - - Experiments I and II, Concentration of SSS in mg per 100 ml (mg%).
Broken lines - - - - - - - - - Dochez and Avery, highest dilution of SSS giving a positive reaction with the solution of antibody used by them.
Experiment I—Inoculum of 165 organisms per ml.
Experiment II—Inoculum of 9,000 organisms per ml.

growth-rate of the organisms. In Fig. 1 the logarithms of colony counts and mg % of polysaccharide produced are plotted, as ordinates, with time as abscissa.

Dochez and Avery[4] concluded, from their studies, that the largest quantity of capsular polysaccharide was found in those pneumococcal culture filtrates obtained during the phase of maximal growth of the organism. The present observations indicate that the greatest quantity of capsular polysaccharide is found in filtrates of blood-broth cultures obtained after the maximal growth phase is completed, *i. e.*, after the completion of the logarithmic phase of growth. In this regard it was interesting to plot the logarithms of the population and the polysaccharide production described in Dochez and Avery's protocol. (Polysaccharide concentration was plotted as the highest dilution giving a positive reaction, since a dilution method was em-

[4] Dochez, A. R., and Avery, O. T., (a) PROC. SOC. EXP. BIOL. AND MED., 1917, **14**, 126; (b) *J. Exp. Med.*, 1917, **26**, 477.

ployed by them in estimating polysaccharide produced.) These are also represented in Figure I; it is apparent that the major portion of polysaccharide in their experiment was found after completion of the logarithmic phase of growth. Of additional interest are the recent observations of Hoogerheide,[5] that the size of the capsule surrounding *Klebsiella pneumoniae (Bact. Friedlander)* begins to increase only after the completion of the logarithmic phase of growth, and the earlier observations of Cowan[6] that hemolysin appears in pneumococcal broth cultures only after completion of the logarithmic phase of growth.

*Summary.* The concentration of capsular polysaccharide appearing in blood-broth culture filtrates following the growth of a highly virulent, standardized strain of Pneumococcus III, was determined photronreflectometrically and correlated with the growth rate of the organism. An initial inoculum of 165 organisms per ml yielded a very small quantity of polysaccharide (0.5 mg %). An inoculum of 9,000 organisms per ml yielded 18 mg % of capsular polysaccharide in 48 hours. The greatest increases in concentration of polysaccharide occurred in two stages after the completion of the logarithmic phase of growth.

11264

## Effect of Intravenous Injections of Yeast Extract on Spontaneous Breast Adenocarcinomas in Mice.

R. LEWISOHN, C. LEUCHTENBERGER, R. LEUCHTENBERGER AND D. LASZLO. (Introduced by Gregory Shwartzman.)

*From the Laboratories of the Mount Sinai Hospital, New York City.*

In this laboratory, studies on the action of different spleen extracts on transplanted and on spontaneous tumors have been carried out for several years. It was shown in previous publications[1, 2] that the complete retrogression of transplanted Sarcoma 180 could be accomplished in 60% of the treated animals following subcutaneous injections of biologically[2] or chemically concentrated spleen

[5] Hoogerheide, J. C., *J. Bact.*, 1939, **38**, 367.

[6] Cowan, S. T., *J. Path. and Bact.*, 1934, **38**, 61.

[1] Lewisohn, R., *Surg., Gyn. and Obst.*, 1938, **66**, 563.

[2] Lewisohn, R., Leuchtenberger, R., and Laszlo, D., Internat. Cancer Congress, Atlantic City, Sept., 1939.