activated. Naphthol sulfonate indophenol also brings about this irreversible inhibition, particularly at 37°, but in addition it can inhibit fermentation in such a way that making the experiment anaerobic and adding ascorbic acid and hexose-diphosphate as Lipmann reports restores the fermentation. This inhibition is, according to our experiments, due to the removal of the diphosphate or the normal intermediates that result from it and the restoration is due to the addition of more diphosphate and not to the reduction of the dye. The addition of sufficient diphosphate at the beginning diminishes this inhibition. The reduction of the dye after it has inhibited fermentation does not restore the process, but the addition of diphosphate without reducing the dye does restore it.

8279 C

Experiments on Purification of Bacteriophage, and a Respiratory Pigment in Escherichia Coli Communis.

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During a study of the bacteriolytic enzyme, lysozyme,[1] we wished to compare its properties with those of bacteriophage. The method used for the purification of lysozyme was not applicable to bacteriophage because of the extreme sensitivity of phage to the common organic solvents.

Previous efforts at purification of bacteriophage have usually employed physico-chemical methods: ultrafiltration through membranes of graded porosity,[2] adsorption,[3] and more lately centrifugation.[4] In the present work the removal of undesired culture medium and bacterial components was attempted by chemical methods and the behavior of phage toward some chemical agents was observed.

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[1] Meyer, K., Thompson, R., Palmer, J. W., and Khorazo, D., *Science*, 1934, **79**, 61; complete report in press.

[2] Elford, W. J., and Andrews, C. H., *Brit. J. Exp. Path.*, 1932, **13**, 446.

[3] Gildemeister, E., and Herzberg, K., *Zentralbl. Bakt.*, 1924, **91**, 228; **93**, 402; Kligler, I. J., and Olitzki, L., *Brit. J. Exp. Path.*, 1931, **12**, 172; Clifton, C. E., PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 32.

[4] Schlesinger, M., *Z. Hygiene*, 1932, **114**, 161.

The phage and coli strains here used were obtained from Dr. M. Holden, of the Department of Bacteriology. The organism gives the sugar reactions of *Escherichia coli Communis*. The phage was isolated from a normal stool several years ago and has been propagated since on the coli strain.

In obtaining phage for concentration an 18-hour culture was inoculated into a liter of beef heart infusion broth (pH 7.4-7.6) and incubated at 37° until clouding was plainly visible. A small amount of the phage broth was added and the flask was incubated another 18 hours. Complete clearing usually occurred and the broth was filtered through a Berkefeld N filter.

In titrating the various phage preparations 0.5 cc. of progressive dilutions were placed in sterile Wassermann tubes and 4.5 cc. portions of a 3 to 4-hour culture of the organism were added. A control tube of 0.5 cc. of sterile broth with the culture was used with each series. After 18 hours at 37° readings were made, the highest dilution giving complete clearing being designated as the end point.

In preparing the concentrates of phage, 500-1000 cc. of phage broth were made slightly alkaline (just pink to phenolphthalein paper) and commercial colloidal iron (60-65 cc. per liter of broth) was slowly added with vigorous stirring. The bulky precipitate, which carried down only a small amount of phage, was removed. A 4% aqueous solution of naphthol yellow S (sodium flavianate) was added, and the solution was brought to a pH of about 4 with HCl. A negligible amount of phage was carried down, except in a few unexplained instances, where it was apparently adsorbed on some polypeptide since it could be washed out with acidified water. The acid solution was treated with solid $BaCl_2$ and neutralized in steps with NaOH until a pH of about 7.6 was reached. The resulting precipitates were centrifuged off and discarded. The pH was then brought from about 7.6 to 8.4. This last precipitate contained the phage in very good yield. After the precipitate was washed several times with water (whereby no phage passed into the washings), it was suspended in about 50 cc. of water acidified with a small amount of H_2SO_4 and the rest of the barium removed with Na_2SO_4. The mixture was left overnight in the cold. The supernatant fluid after centrifugation contained the phage. The solutions at this stage had a slight yellow color from the flavianic acid used.

The phage was inactivated by precipitation with alcohol and to a lesser degree with acetone. Evaporation *in vacuo* over P_2O_5 while the material was in a frozen state destroyed most of the activity of the phage. In a beam of strong light (from a slit lamp) there

was only a faint Tyndall effect. One preparation contained only 5.5% nitrogen on an ash-free basis, and was active in a dilution greater than $1:10^9$ (end point not reached). In a 1% solution the xanthoproteic and Greenberg phenol reactions were positive, the Molisch weakly positive, and biuret, Millon, glyoxylic acid, nitroprusside, and alkaline lead reactions were negative.

The following example indicates that the precipitation of the phage by barium is not due to adsorption, a fact also suggested by the failure of colloidal iron to precipitate phage at the same pH. Phage broth was carried through the procedure outlined above as far as the barium precipitation. The final barium precipitate was centrifuged off, washed with alkaline water, suspended in water, and made neutral. The supernatant fluid was freed of barium with Na_2SO_4, and found to be active in $1:10^2$ dilution. The remaining barium precipitate was suspended in water, acidified with H_2SO_4, and barium was removed with Na_2SO_4. The solution (50 cc.), active to $1:10^7$, contained per cc. 0.628 mg. organic matter with 9.17% nitrogen. Obviously the bulk of the phage was not removed from the barium precipitate until the suspending medium was acid enough to liberate weak acids from their barium salts.

Another similar preparation was active to $1:10^6$ (end point not reached). After dialyzing in a collodion membrane against distilled water it was active to $1:10^5$. After evaporation of 15 cc. while frozen, 10 mg. of a very soluble material of slightly yellow color was obtained, which when dissolved in 5 cc. of water was active to $1:10^2$.

The nature of bacteriophage is still unknown. The precipitate described, probably still quite impure, indicates that the phage is of an acidic nature, forming an insoluble barium salt.

A similar procedure was used by Hosoya, Nagase, and Yoshizumi,[5] for the purification of toxins and of phage. They found phage completely precipitated by zinc hydroxide. The zinc precipitate was redissolved in acid, the solution dialyzed and again precipitated by zinc acetate and ammonia. The lysin did not dialyze and gave negative biuret, Millon, xanthoproteic, diazo, Molisch, and ninhydrin reactions.

Recently Schüler,[6] working with phage prepared by Schlesinger,[4] failed to find any metabolism and concluded it was not a living organism. He found a phosphatase associated with the phage, but could not show that the phage and phosphatase were identical. Our

[5] Hosoya, S., Nagase, K., and Yoshizumi, T., *Jap. J. Exp. Med.*, 1932, **10**, 101.

[6] Schüler, H., *Biochem. Z.*, 1935, **276**, 254.

phage concentrates also failed to show either oxygen uptake or glycolysis.

The results of our observations are also hardly compatible with the theory of a living organism. It seems probable that further experiments on the particle size of a purified preparation of phage should yield helpful information.

Note on a Respiratory Pigment in Escherichia coli Communis.—During the work with acetone-dried coli a pigment was observed on fractionation. The dried bacteria were extracted with 200 cc. of N/200 NaOH per gram. After centrifuging, the colorless supernatant liquid was acidified with HCl, the precipitate removed, and the solution precipitated with flavianic acid. To the supernatant solution 4% phosphotungstic acid was added. After standing cold over night the flesh colored precipitate was centrifuged off, washed, and dissolved in a small amount of alkaline water, giving a colorless solution. This solution was brought to 2N H_2SO_4 (the color was restored upon acidification) and extracted with a mixture of equal parts of amyl alcohol and ether; the red organic solution was washed 5 times with water until the washings no longer had a yellow color (flavianic and phosphotungstic acids being removed) and then evaporated *in vacuo*. The residue was free from phosphotungstic acid. It had a violet-red color, was insoluble in acid and alkaline water (in contrast to the substance before treatment with phosphotungstic acid), chloroform, petroleum ether, and acidified ether, and was soluble in amyl alcohol and pyridine.

The pyridine solution had an orange-red color and a greenish-brown fluorescence. The color of the solution disappeared on addition of bisulfite and was restored by peroxide. In a grating spectroscope the pyridine solution showed 2 absorption bands which were not very sharp. The larger was at 543 mμ, and the smaller at 497 mμ. For comparison, a pyridine solution of prodigiosin, which showed no fluorescence, had a larger absorption band at 532.5 mμ, with a smaller at 494 mμ.*

This pigment, while somewhat resembling prodigiosin, is distinctly different from it. It does not seem likely that this pigment is derived from cytochrome since its reduced form is colorless. Apparently in the bacteria it is bound on protein and present in the reduced state.

The pigment seems to be characteristic of this organism, since its preparation was repeated a number of times with the same re-

* We thank Dr. C. B. Coulter of the Institute of Public Health for the spectroscopic observations.

sults and by the same procedure no such pigment could be obtained from sarcinae or from *B. coli Communior.*

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Agglutination and Precipitation Between Hemolytic Streptococci of Various Groups and Sera of Rheumatoid Arthritis Patients.

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It has been established[1, 2, 3] that hemolytic streptococci are agglutinated by sera of patients with rheumatoid arthritis. It has also been shown[4, 5] that the C substance of hemolytic streptococci[6] gives precipitation with sera of patients with this and other diseases. Since only strains of hemolytic streptococci isolated from human sources had been used in the investigations quoted, the present study was undertaken to observe the results obtained when antigens for agglutination and precipitation were prepared from hemolytic streptococci of Lancefield's groups A, B, C, D, E, F, and G.[7] Strains of groups B through G were kindly provided by Dr. R. C. Lancefield.

Agglutination tests were done with strains of groups A through E only. Eighteen-hour living broth cultures in 0.5 cc. quantities were mixed with 0.5 cc. of the various dilutions of serum obtained from patients with rheumatoid arthritis. Tests were read after 2 hours at 56°C. and 20 hours in the ice box. The results (Table I) indicate that, while the strongest agglutination usually occurred with strains of Group A, definite reactions were obtained also with those of other groups.

It seemed probable that the cross reactions were due to non-group-specific fractions common to all the strains, and to test this the work

1 Nicholls, E. E., and Stainsby, W. J., *J. Clin. Invest.*, 1931, **10**, 323.

2 Dawson, M. H., Olmstead, M., and Boots, R. H., *J. Immunol.*, 1932, **23**, 187.

3 Keefer, C. S., Myers, W. K., and Appel, T. W., *J. Clin. Invest.*, 1933, **12**, 267.

4 Seegal, D., Heidelberger, M., Jost, E. L., and Lyttle, J. D., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 582.

5 Dawson, M. H., Olmstead, M., and Jost, E. L., *J. Immunol.*, 1934, **27**, 355.

6 Lancefield, R. C., *J. Exp. Med.*, 1928, **47**, 481.

7 Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571.