By our results in I and II, we have shown that apparently the length of time out of the body has a definite influence on the virulence of the organisms. That the virulence may diminish in the course of the disease is suggested by a single observation: A mouse was injected with the sputum of a case of influenza on the first day of the disease; it died 6 hours later with positive cultures of *B. influenzæ* in the peritoneal exudate and heart's blood; on the second day of disease, some more sputum, smears of which showed numerous *B. influenzæ*, from this patient was injected into a mouse; this mouse, however, did not die for 48 hours, and at autopsy cultures from the heart's blood and peritoneum were negative.

CONCLUSIONS.

1. The virulence of *B. influenza* is very variable and this may have some bearing on the epidemiology of the disease influenza in man.

2. A few observations are present that may throw some light on the results of bacteriological studies of this disease.

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The formol titration of bacteriological media.

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The formol titration devised by Malfatti[1] (1908), Sörensen (1907[2], 1908[3]), and by Henriques and Sörensen[4] (1909) for the titration of the ammonia and amino acids of urine has been more or less modified by bacteriologists for the titration of media and cultures. Ammonium chloride reacts with formaldehyde to produce hexamethylentetramine and hydrochloric acid. Amino acids and polypeptides react with formaldehyde to produce acid methylene derivatives which are stronger acids than the amino acids

[1] Malfatti, H., *Z. f. anal. Chem.*, 1908, xlvii, 273.

[2] Sörensen, S. P. L., *Comptes-rendus des Travaux du Lab. de Carlsberg*, 1907, vii, I.

[3] Sörensen, S. P. L., *Biochem. Zeitschr*, 1908, vii, 45.

[4] Henriques, V., and Sörensen, S. P. L., *Z. f. physiol. Chem.*, 1909, lxiii, 27.

from which they are derived. The increase in acidity may be titrated against a standard alkali solution and serve as a measure of the amino acids and ammonia present in the sample.

Sörensen (1907, 1908) pointed out that the reaction of amino acids with formaldehyde is a reversible one. A considerable excess of formaldehyde is required to effect complete conversion of the amino acids into their methylene derivatives, and, since water is a product of the reaction, the presence of too much water serves to throw the reaction back in the opposite direction. Sörensen found it necessary to add as much as 10 c.c. of formalin (40 per cent. formaldehyde) to 20 c.c. of amino acid solution.

The amino acids are ampholytes. If a pure monocarboxylic amino acid is dissolved in distilled water the solution will be found to have a hydrogen ion concentration at or near the isoelectric point of the amino acid, a fact also noted by Eckweiler, Noyes and Falk[5] (1921). If it is titrated with alkali and the titration curve plotted with amounts of alkali added as abscissae and hydrogen ion exponents as ordinates the curve will be seen to drop almost vertically toward the alkaline side as the first drops of alkali are added. This represents a portion of the "isoelectric zone" (Michaelis[6], 1914, p. 40). As more alkali is added the curve assumes a more nearly horizontal position and if the curve is continued it again assumes a nearly vertical position as the point of complete neutralization of the amino acid is approached (at about P_H 11.3 in the case of glycine). This latter vertical portion of the curve may be called the "zone of neutralization." When formalin is added the amino acid solution becomes more acid not because the carboxyl groups are increased but because the amino groups are destroyed. The titratable acidity of the acid methylene derivative is the same as that of the amino acid but the point of neutralization of the methylene derivative lies at a higher hydrogen ion concentration (at about P_H 8.0 in the case of methylenglycine) than does that of the amino acid. For the formol titration of an amino acid the solution should be reduced to a hydrogen ion concentration within its isoelectric zone and after the addition of formalin the titration

[5] Eckweiler, H., Noyes, H. M., and Falk, K. C., *J. Gen. Physiol.*, 1921; iii, 291.

[6] Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914.

should end at a hydrogen ion concentration within the zone of neutralization of the methylene derivative. To determine the location of these zones we have plotted the titration curves of a number of representative amino acids and ammonium salts before and after the addition of formalin[7]. The following pure substances were titrated: glycine, alanine, phenylalanine, tryosine, asparagine, aspartic acid, glutamic acid, ammonium chloride, ammonium lactate, ammonium phosphate, ammonium carbonate. Excluding ammonium phosphate and ammonium carbonate which present a special problem it is found that the isoelectric zone of each of these substances extends beyond P_H 6.0 and as far as P_H 7.0, some as far as P_H 8.0[8]. After the addition of formalin the zone of neutralization for each substance was found to begin on the acid side at about the hydrogen ion concentration here listed; glycine P_H 6.8, alanine P_H 8.0, phenylalanine P_H 7.6, tryosine P_H 7.6, asparagine P_H 6.0, aspartic acid P_H 8.0, glutamic acid P_H 8.0, ammonium chloride P_H 6.0, ammonium lactate P_H 6.0, ammonium phosphate P_H 8.0, ammonium carbonate P_H 7.0. For the formol titration of most amino acids or of mixtures of amino acids, therefore, their solutions should be reduced to a hydrogen ion concentration of not less than P_H 7.0 and after the addition of formalin the titration should end at a hydrogen ion concentration not greater than P_H 8.3[9]: Theoretically P_H 8.4 is a better end point than P_H 8.0 but we have found the buffer effect of the formalin so great at P_H 8.4 that the end point of the titration judged colorimetrically is very poor, and have obtained more accurate results at P_H 8.0.

Sörensen recognized phosphates and carbonates as disturbing elements in the formol titration of urine. In fact any substance which exerts a buffer effect between P_H 7.0 and P_H 8.0 is a serious source of error. To overcome this difficulty Henriques[10] (1909) and Henriques and Sörensen (1909) described methods for the

[7] A correction was made for the buffer effect of the formalin itself.

[8] Of the substances titrated, tyrosine showed the greatest degree of dissociation at P_H 7.0 and was about 3.8 per cent. dissociated at this hydrogen ion concentration. Glycine, leucine and alanine are less than 2 per cent. dissociated at P_H 8.0.

[9] Henriques and Sörensen (1909, 1910) recommend titration from P_H 6.8 to about P_H 8.4 or beyond. Northrop (1921) started his titration at P_H 7.0.

[10] Henriques, V., *Z. f. physiol. Chem.*, 1909, lx. 1.

removal of phosphates and carbonates by precipitation with barium and filtration. These methods may be adopted by the bacteriologist, but are time-consuming. If many cultures are to be titrated the element of time is very important. Of almost equal importance to the bacteriologist is the ability to make determinations with small samples of material. Bacterial cultures present a special problem in some respects. Their color and turbidity are not easily matched by artificial controls. They may contain other buffer substances than phosphates and carbonates. We have tried to reduce the formol titration of bacterial cultures to its simplest terms. Color and turbidity may be overcome and much greater accuracy in judging a colorimetric end point may be attained by the use of a comparator block and Clark and Lubs' indicator solutions. The error due to the presence of buffer substances may be eliminated by starting and ending the titration at the same hydrogen ion concentration. If the isoelectric zone of an amino acid or ammonium salt overlaps the zone of neutralization of its methylene derivative this may be done with perfect results. This is the case with glycine, alanine, asparagine, ammonium chloride, ammonium lactate and probably with others not studied. In the case of the other amino acids titrated there is no actual overlapping of the zones, but there are certain hydrogen ion concentrations at which the zones approach each other closely. The overlapping of the zones or their points of nearest approach are not at the same hydrogen ion concentration for all the substances titrated. It was determined empirically in the case of standard bouillon and bouillon cultures of various bacteria that maximum formol titrations were obtained when the reaction of the sample was reduced to P_H 8.0, formalin added, and the mixture immediately titrated back to P_H 8.0. The results so obtained were reasonably close (within about 6 per cent.) to those obtained by titration from P_H 7.0 to P_H 8.0 after removal of phosphates and carbonates by precipitation with barium and filtration. The details of the method recommended are in press.

[11] Henriques, V., and Sörensen, S. P. L., *Z. f. physiol. Chem.*, 1910, lxiv, 120.