A STUDY OF PNEUMOCOCCUS AUTOLYSIS.

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Although it has long been recognized that one of the distinguishing characters of Pneumococcus is the ease with which the cell undergoes autolysis, relatively little is known of the chemical changes accompanying this process.

Rosenow (1) measured small increases in amino nitrogen in pneumococcus autolysates and found that the appearance and subsequent disappearance of toxic substances in saline suspensions of pneumococci and other bacteria, when kept at 37°C., are associated with proteolysis. Jobling (2) and his collaborators, in their studies on ferment action, concluded from the results of determinations of the amino nitrogen content of pneumococcus autolysates, that the solution of bacteria is not to be confused with proteolysis. They observed also that leucoprotease acting on pneumococci results in lower cleavage products than those obtained with the autolytic enzymes of Pneumococcus itself; and that the products of protease digestion are less toxic than the autolytic products, presumably because, in the former instance, proteolysis is carried beyond the toxic stage.

Neufeld (3) was the first to show that Pneumococcus is soluble in bile and that the salts of cholic acid have the same action as bile itself. The solvent action of bile Neufeld attributes to the marked swelling and precipitation of the cell membrane whereby it no longer affords the bacterial body the same protection as does the normal membrane.

Lord and Nye (4) concluded that solution of Pneumococcus results from the action of the intracellular enzymes, and that this action is facilitated by the addition of bile. More recently Atkin (5), investgating the relationship of autolysis to bile solubility, states that "the solution of the pneumococcus in bile consists in an acceleration by this substance of the normal autolytic process."

Avery and Cullen (6), studying the action of the intracellular enzymes of Pneumococcus on foreign substrates, found that bacterial extracts containing these active agents brought about cleavage of various carbohydrates, esters, and protein substances.

The present investigation includes the study of the enzymes of Pneumococcus not on foreign substrates, but on the native substances

of the cell itself. The experiments fall into two groups: (1) those which have to do with the chemical changes accompanying autolysis as it occurs spontaneously in suspensions and bile solutions of living cells; (2) those which deal with the action of these same enzymes when, freed from the cell, they are allowed to act upon suspensions of heat-killed pneumococci, or upon emulsions of the alcohol-soluble lipoids extracted from them. These experiments were undertaken not only to acquire additional information concerning the nature of the autolytic process, but also with hope that an analysis of the chemical changes taking place during autolysis might furnish some clue to the nature of change, which, following cell lysis, results in the diminution or loss of the type-specific antigenicity of the dissolved material. For, as has been pointed out in preceding papers, cell disintegration, whether brought about by physical means (freezing and thawing), by chemical agents (bile or alkali), or by enzyme action (autolysis), is generally accompanied by partial or complete loss of the capacity on the part of the dissolved material to stimulate in the animal body the type-specific antibodies which characterize the immune response to the intact cell.

Previous studies have dealt in detail with the chemical nature and immunological significance of the so called soluble specific substance of Pneumococcus upon which the type specificity of the cell has been found to depend (7). It need only be pointed out here, that chemically these type-specific substances fall into the class of carbohydrates and that immunologically they belong to that important group of specifically reactive but non-antigenic substances which Landsteiner has named "haptens." In the protein-free form, they all possess in common the properties of polysaccharides, but individually they exhibit differences in chemical and immunological reactions which are specific and constant for each of the three types of Pneumococcus. When isolated from the cell in purified form, these complex sugars (haptens) retain the property of specific precipitation in antibacterial sera of the homologous type, but lose completely the antigenic function of inciting antibody formation. The fact that, in the natural state in which they occur in the bacterial cell, these carbohydrate substances are not only highly type-specific but extraordinarily effective as antigens, and the additional fact that they lose their antigenic properties

to a greater or less extent whenever cell dissolution occurs, afford strong evidence that the encapsulated cell contains a complex antigen in which the type-specific carbohydrate (hapten) is combined with some other constituent, possibly protein, to form the intact antigen. The nature of the substance which confers antigenicity upon the hapten, the character of the antigenic union, and the conditions determining antigenic dissociation *in vitro* and *in vivo* are questions as yet unsolved. A direct study of the chemistry of this complex antigen, and the solution of the problems relating to it involve technical difficulties which are not easily overcome. However, it seemed possible that a detailed investigation of the chemical changes accompanying autolysis might indirectly at least provide a lead, since the processes of cell autolysis and antigenic dissociation are intimately associated if not causally related.

EXPERIMENTAL.

1. Proteolysis as Measured by the Increase of Amino and Non-Coagulable Nitrogen in Pneumococcus Autolysates.

The growth from 1.5 liters of broth cultures of pneumococci was collected by centrifugation and washed once with M/150 phosphate buffer of the desired hydrogen ion concentration. After recentrifugation the bacteria were suspended in 30 cc. of the same buffer. The suspension was divided into two equal portions. One part, which served as the control, was immediately inactivated by heating at 100°C. for 10 minutes. The other part containing the active cells was placed at 37°C. to autolyze. After 48 hours incubation, complete disintegration of the bacteria occurred as shown by the fact that in stained preparations only Gram-negative detritis remained. In these experiments, aseptic technique was employed throughout, and no preservatives were added to the bacterial deposits.

The heated control material (Tube C) and the active autolysate (Tube A) were each analyzed for amino and total nitrogen. Duplicate determinations were made in each instance. The method of Van Slyke (8) was employed for determination of amino nitrogen, 5 cc. of bacterial suspension or autolysate being used for each analysis. Total nitrogen was estimated by the Pregl (9) micro Kjeldahl method, 2 cc. of material being used for analysis. Non-coagulable nitrogen was estimated by the method described below. A summary of these experiments is given in Tables I and II. The experiments summarized in Table I were made on pneumococci of different types with buffer solutions of different hydrogen ion concentrations. In Experiments 4, 9, and 11, Type I pneumococcus was employed. The bacteria were washed once in M/150 buffer before resuspending them in the same buffer for autolysis. Tube C, the control tube, was heated 10 minutes

TABLE I.

Proteolysis Accompanying Autolysis of Pneumococcus as Measured by the Increase in Amino Nitrogen.

Exp. No.	Pneumococcus type	Tube	pH	Total nitrogen	Amino nitrogen	N in amino form	Increase in amino nitrogen
				mg. per cc.	*mg. per cc.*	*per cent*	*per cent*
4	I	C*	7.5	0.42	0.042	10.0	—
		A		0.43	0.060	13.9	39.0
9	I	C	7.5	0.47	0.050	10.6	—
		A		0.44	0.065	14.7	38.7
11	I	C	5.5†	0.29	0.030	10.4	—
		A		0.34	0.044	12.9	24.1
22	III	C	7.5	0.31	0.027	8.7	—
		A		0.30	0.035	11.6	33.3
6	III	C	6.5	0.58	0.064	11.0	—
		A		0.56	0.080	14.3	30.0
7	III	C	5.5	0.43	0.049	11.4	—
		A		0.41	0.059	14.4	26.2
8	III	C	NaCl‡	0.47	0.053	11.3	—
		A		0.56	0.067	11.9	5.3
15	II	C	7.5	0.49	0.029	5.9	—
		A		0.49	0.037	7.5	27.1
20	II	C	7.5	0.52	0.059	11.3	—
		A		0.50	0.068	13.6	20.3
16	II	C	6.5	0.51	0.053	10.4	—
		A		0.51	0.061	12.0	15.4
17	II	C	6.5	0.53	0.052	9.8	—
		A		0.51	0.060	11.8	20.4
19	II	C	NaCl	0.51	0.059	11.5	—
		A		0.51	0.073	14.3	24.4
18	II	C	NaCl	0.45	0.047	10.4	—
		A		0.45	0.064	14.2	36.5

* C = control, heat-inactivated. A = active, autolysate.

† Acetic acid-sodium acetate buffer.

‡ Isotonic salt solution.

TABLE I—*Concluded.*

Exp. No.	Pneumococcus type	Tube	pH	Total nitrogen	Amino nitrogen	N in amino form	Increase in amino nitrogen
				mg. per cc.	*mg. per cc.*	*per cent*	*per cent*
28	II	C	7.5	0.25	0.019	7.6	—
		A		0.28	0.026	9.3	22.4
31	II	C	7.5	0.48	0.045	9.4	—
		A		0.48	0.054	11.3	20.2
29	II	C	6.5	0.38	0.030	7.9	—
		A		0.38	0.039	10.3	30.4
30	II	C	6.5	0.31	0.028	9.0	—
		A		0.31	0.035	11.3	25.5
32	II	C	5.5	0.36	0.026	7.2	—
		A		0.36	0.028	7.8	8.3
33	II	C	5.5	0.31	0.020	6.5	—
		A		0.31	0.022	7.1	9.2
27	II	C	NaCl	0.20	0.014	7.0	—
		A		0.21	0.018	8.6	22.9

at 100°C., and was placed on ice. Tube A, the autolysate, was incubated for 48 hours at 37°C. In Experiments 22, 6, 7, and 8 the Type III organism was used in buffer solutions of the same molar concentration. In Experiments 15 to 18, however, the Type II organism and M/15 buffer were used. In Experiments 28 to 27 the bacteria were washed three times with M/15 buffer before incubating at 37°C.

A careful consideration of the data in Tables I and II reveals that the autolysis of pneumococci is accompanied by an increase in the amino and non-coagulable nitrogen of some substance in the bacterial suspension. Although various experiments were carried out with pneumococci of different types, and buffer solutions of different molar and hydrogen ion concentrations, in each experiment, regardless of these variations, a definite increase was always observed. At first it was thought that this increase in amino and non-coagulable nitrogen might be due to the action of the intracellular proteolytic enzymes on peptone adsorbed by the organism from the meat infusion broth, but experiments on pneumococci washed as often as three times before

autolysis showed an increase of the same order of magnitude as that observed when the cells were washed only once. Furthermore, when a substrate of heat-killed pneumococci is treated with a potent pneumococcus extract containing the active intracellular enzymes, the cells undergo lysis (10), and there occurs an increase in amino and non-coagulable nitrogen of the same order of magnitude as that observed in true autolysis (see Table II, Experiment K).

TABLE II.

Proteolysis Accompanying Autolysis of Pneumococcus as Measured by the Increase in Amino and Non-Coagulable Nitrogen.

Exp. No.	Pneumococcus type	Tube	pH	Total nitrogen	Non-coagulable N	Amino nitrogen	N in amino form	N in non-coagulable form	Increase in amino N	Increase in non-coagulable nitrogen
				mg. per cc.	*mg. per cc.*	*mg. per cc.*	*per cent*	*per cent*	*per cent*	*per cent*
40	II	C	6.5	0.44	0.17	0.042	9.5	38.6	—	—
		A	6.5	0.39	0.23	0.045	11.5	59.0	21.1	52.9
41	II	C	6.5	0.40	0.15	0.042	10.5	37.5	—	—
		A	6.5	0.36	0.20	0.045	12.5	55.4	19.1	47.8
42	II	C	NaCl	0.20	0.05	0.024	12.0	25.0	—	—
		A	NaCl	0.16	0.07	0.023	14.4	43.7	20.0	75.0
K*	III	C	7.5	0.74	0.21	0.098	13.2	28.4	—	—
		A	7.5	0.74	0.36	0.122	16.5	48.7	25.0	71.5

* Heat-killed pneumococci to which inactive (Tube C) and active (Tube A) bacteriolytic enzymes were added and both tubes incubated 48 hours at 37°C.

During autolysis of Pneumococcus, about 20 per cent of the total nitrogen is changed from the coagulable to the non-coagulable form, and about 2 per cent of total nitrogen from the non-amino to the amino form. It seems, therefore, that this proteolysis is of the nature of a peptic digestion in which the protein is broken down to the albumose stage, rather than of a tryptic digestion in which the ratio of amino to total nitrogen is invariably greater than that found in pneumococcus autolysates.

Proteolysis is apparently due not to the action of the bacterial

enzymes on a foreign substrate, but to their action on the parent substances of the cell itself. It seems difficult to explain the great fluctuations in the increase of amino and non-coagulable nitrogen, when conditions of experimentation were apparently kept constant. It must be remembered, however, that bacterial suspensions behave not as solutions of inert chemical substances, but as aggregates of living cells where variations of every sort may be encountered. Even a single disturbance, such as a slight change in the proportion of substrate to the content of cellular enzyme could account for these fluctuations.

2. Proteolysis in Pneumococcus Solutions Containing Sodium Desoxycholate.

The growth from 3 liters of broth culture of Pneumococcus was collected as described above. The washed bacteria were suspended in 100 cc. of M/15 phosphate buffer solution and the suspension was divided into four equal parts. The first part, which served as a control, was immediately heated for 10 minutes at 100°C. The second part was allowed to autolyze at 37°C. for 48 hours, during which time complete disintegration of the bacterial cells occurred. The third and fourth parts were cooled to 0°C., and to each were added 1.5 cc of 10 per cent sodium desoxycholate. After complete solution of the bacteria in the cold, one portion was immediately heated for 10 minutes at 100°C. to inactivate the autolytic ferments; the other portion, unheated, was placed in the incubator at 37°C. for 48 hours. The contents of all four tubes were then analyzed for amino, non-coagulable, and total nitrogen. Three experiments were carried out in this manner.

Non-coagulable nitrogen was determined as follows: 7 cc. of bacterial suspension were placed in a centrifuge tube and 7 cc. of 10 per cent trichloracetic acid were added. After standing for 1 hour the tube was centrifuged for 4 minutes at high speed. The supernatant liquid was filtered through quantitative filter paper. 6 cc. of the filtrate were analyzed for non-coagulable nitrogen by the Pregl micro Kjeldahl method.

The results of the analytical findings are given in Table III.

From the data presented in Table III it becomes evident that proteolysis is inhibited in solutions of pneumococci when the living cells are dissolved with an excess of sodium desoxycholate. The relation of this enzyme inhibition to the concentration of the bile salt in solutions of pneumococci is brought out in the following experiments.

3. Proteolysis in Solutions of Pneumococcus Containing Decreasing Amounts of Sodium Desoxycholate.

The preceding experiments showed that proteolysis is inhibited in solutions of pneumococci containing high concentrations of sodium

TABLE III.

Proteolysis in Sodium Desoxycholate Solutions of Pneumococcus (Type III) as Measured by the Increase in Amino and Non-Coagulable Nitrogen.

Exp. No.	Tube No.	pH	Total nitrogen	Non-coagulable nitrogen	Amino nitrogen	Nitrogen in amino form	Nitrogen in non-coagulable form	Increase in amino nitrogen	Increase in non-coagulable nitrogen
			mg. per cc.	*mg. per cc.*	*mg. per cc.*	*per cent*	*per cent*	*per cent*	*per cent*
A	1	6.5	0.69	0.29	0.107	15.5	42.1	—	—
	2	6.5	0.69	0.35	0.118	17.1	50.6	10.3	20.2
	3	6.5	0.67	0.28	0.103	15.4	41.8	0.0	—
	4	6.5	0.68	0.29	0.108	15.9	42.6	2.6	1.2
B	1	7.5	0.65	0.22	0.092	14.1	33.9	—	—
	2	7.5	0.65	0.32	0.109	16.8	49.3	19.2	45.5
	3	7.5	0.66	0.23	0.092	13.9	34.8	0.0	2.6
	4	7.5	0.66	0.22	0.092	13.9	33.3	0.0	—
C	1	7.5	0.57	0.23	0.099	17.4	40.4	—	—
	2	7.5	0.53	0.36	0.114	21.5	67.9	23.5	68.2
	3	7.5	0.56	0.23	0.095	17.0	41.1	0.0	1.7
	4	7.5	0.54	0.22	0.094	17.4	40.8	0.0	1.0

Tube 1. Control tube, 25 cc. of bacterial suspension. Heated 10 minutes at 100°C. to destroy active cellular enzymes.

Tube 2. 25 cc. of bacterial suspension to which was added 0.5 cc. of chloroform. Autolyzed 48 hours at 37°C.

Tube 3. 25 cc. of bacterial suspension; cooled to 0°C., then 1.5 cc. of 10 per cent desoxycholate added. After solution immediately heated at 100°C. for 10 minutes to destroy active cellular enzymes.

Tube 4. Same as Tube 3, but unheated. Autolyzed 48 hours at 37°C.

desoxycholate. In order to ascertain the minimum concentration of this reagent which would dissolve the cells and at the same time permit proteolysis to take place, experiments were carried out with decreasing concentrations of desoxycholate.

The bacteria from 3 liters of broth culture were collected as before. They were suspended in 100 cc. of isotonic salt solution, divided into four equal parts, cooled

TABLE IV.

Proteolysis in Sodium Desoxycholate Solutions of Pneumococcus (Type III) with Decreasing Concentrations of Desoxycholate, as Measured by the Increase in Amino and Non-Coagulable Nitrogen.

Exp. No.	Tube	pH	Total nitrogen	Non-coagulable nitrogen	Amino nitrogen	Nitrogen in amino form	Nitrogen in non-coagulable form	Increase in amino nitrogen	Increase in non-coagulable nitrogen	Remarks	Concentration of desoxycholate
			mg. per cc.	*mg. per cc.*	*mg. per cc.*	*per cent*	*per cent*	*per cent*	*per cent*		
G	1	NaCl	0.44	0.14	0.065	14.8	31.8	—	—	25 cc. suspension + 1 cc. 10 per cent sodium desoxycholate	1:250
	2	NaCl	0.43	0.14	0.064	14.9	32.5	0.7	2.2	25 cc. suspension + 0.5 cc. 10 per cent sodium desoxycholate	1:500
	3	NaCl	0.44	0.14	0.065	14.8	31.8	0.0	0.0	25 cc. suspension + 0.25 cc. 10 per cent sodium desoxycholate	1:1000
	4	NaCl	0.44	0.20	0.073	16.6	45.5	12.2	43.1	25 cc. suspension + 0.10 cc. 10 per cent sodium desoxycholate. All tubes incubated for 4 days at 37°C.	1:2500
H	1	NaCl	0.69	0.26	0.093	13.5	37.7	—	—	Same as experiment G	1:250
	2	NaCl	0.69	0.26	0.093	13.5	37.7	0.0	0.0		1:500
	3	NaCl	0.69	0.27	0.095	13.7	39.2	1.5	4.0		1:1000
	4	NaCl	0.69	0.35	0.114	16.5	50.8	22.2	34.8		1:2500

to 0°C., and to each part was added, respectively, 1.0, 0.5, 0.25, and 0.1 cc. of 10 per cent solution of sodium desoxycholate. The tubes were placed in the incubator at 37°C. for 48 hours. An analysis of the amino, non-coagulable, and total nitrogen was made, after heating for 10 minutes at 100°C. The analytical data are summarized in Table IV.

It is seen from the results of the experiments presented in Table IV that if the concentration of sodium desoxycholate in suspensions of pneumococci is diminished to 1 part in 2500, proteolysis progresses in the usual manner. It appears, therefore, that sodium desoxycholate in a concentration greater than 1:2500 inhibits the activity of the proteolytic enzyme of Pneumococcus.

4. Proteolysis Accompanying Autolysis of Pneumococcus under Aerobic and Anaerobic Conditions.

A comparison of proteolysis during autolysis under aerobic and anaerobic conditions is made in the following experiment.

For this purpose the washed bacteria were suspended in isotonic salt solution as in the preceding experiment. The bacterial suspension was divided into two equal portions. In order to have the bacteria in the control tube in soluble form similar to that of the autolysate, the former suspension was cooled to 0°C., and the bacteria dissolved by adding 2 cc. of 10 per cent solution of sodium desoxycholate. After complete solution of the organisms had taken place, the tube was immediately heated for 10 minutes at 100°C. to inactivate the cellular enzymes. 2 cc. of isotonic salt solution were added to the second portion, and the tube was sealed with 10 cc. of vaseline and incubated at 37°C. for 48 hours. The contents of both tubes were then analyzed for amino and non-coagulable nitrogen (Experiment 34).

A second experiment (Experiment 35) was carried out in a similar manner. 4 equal portions of a bacterial suspension were treated as follows: The first portion was cooled to 0°C. and 1 cc. of 10 per cent solution of sodium desoxycholate was added. After solution of the cells had taken place the tube was immediately heated for 10 minutes at 100°C. The second portion was also treated with desoxycholate but was not heated. This tube, containing the active enzymes, was incubated for 48 hours at 37°C. The third portion, consisting of the living formed cells, untreated with sodium desoxycholate was sealed with 10 cc. of vaseline and incubated at 37°C. for 48 hours. The fourth portion was treated precisely as the third except that it was incubated without vaseline seal. All four samples were then analyzed for amino and non-coagulable nitrogen. The results of both these experiments are summarized in Table V.

From the experiments presented in Table V it is seen that proteolysis progresses under anaerobic as well as under aerobic conditions and that, as pointed out in the preceding experiment (Table IV), solu-

TABLE V.

Proteolysis Accompanying Autolysis of Pneumococcus (Type III) under Aerobic and Anaerobic Conditions.

Exp. No.	Tube No.	pH	Total nitrogen	Non-coagulable nitrogen	Amino nitrogen	Nitrogen in amino form	Nitrogen in non-coagulable form	Increase in amino nitrogen	Increase in non-coagulable nitrogen
			mg. per cc.	*mg. per cc.*	*mg. per cc.*	*per cent*	*per cent*	*per cent*	*per cent*
34	1	NaCl	0.29	0.07	0.034	11.8	24.1	—	—
	2	NaCl	0.31	0.13	0.043	13.9	42.0	17.8	74.3
35	1	NaCl	0.235	0.055	0.022	9.4	23.4	—	—
	2	NaCl	0.235	0.055	0.023	9.8	23.4	4.3	0.0
	3	NaCl	0.235	0.075	0.027	11.5	31.9	22.3	36.3
	4	NaCl	0.235	0.09	0.027	11.5	38.3	22.3	63.8

Experiment 34.—Tube 1. The control tube: 35 cc. of bacterial suspension + 2 cc. of 10 per cent sodium desoxycholate at 0°C.; after solution heated immediately for 10 minutes at 100°C. to inactivate the enzymes.

Tube 2. The autolysate: 35 cc. of bacterial suspension, 2 cc. of saline, 1 cc. of chloroform, sealed with 10 cc. of sterile vaseline and incubated 48 hours at 37°C.

Experiment 35.—Tube 1. The control tube: 25 cc. of bacterial suspension, 1 cc. of 10 per cent desoxycholate at 0°C.; then after solution heated immediately for 10 minutes at 100°C.

Tube 2. Same as Tube 1, but unheated, and incubated for 48 hours at 37°C.

Tube 3. 25 cc. of bacterial suspension, active, no sodium desoxycholate added, sealed with 10 cc. of sterile vaseline, and incubated for 48 hours at 37°C.

Tube 4. 25 cc. of bacterial suspension, active, without seal, incubated 48 hours at 37°C.

tions of living bacterial cells made by dissolving the organisms with an *excess* of sodium desoxycholate, undergo no proteolysis.

5. Lipolysis in Pneumococcus Autolysates.

From the foregoing experiments it is evident that autolysis is accompanied by definite proteolysis. It is known that the Pneumo-

coccus contains an active lipase. It was thought that if this enzyme were active during the process of cell digestion, a demonstrable in-

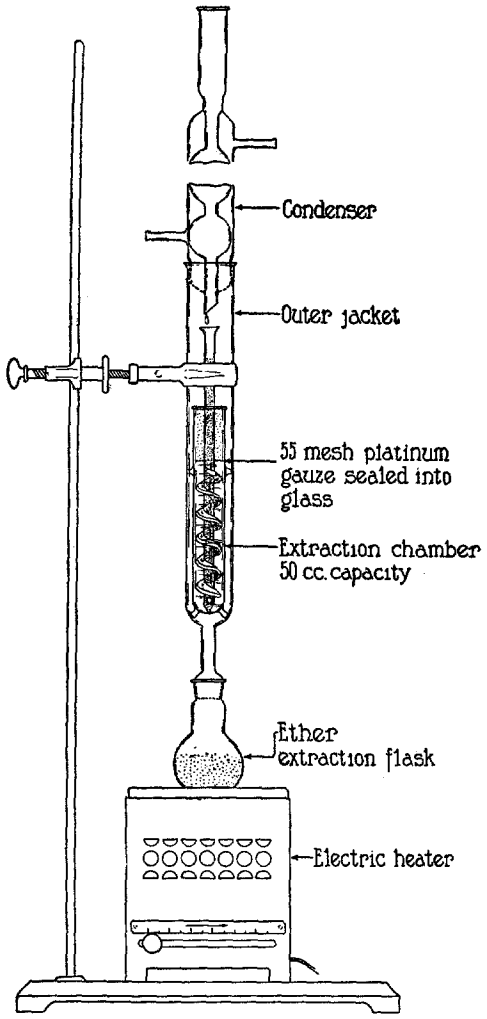

FIG. 1. Liquid extraction apparatus for estimating bacterial fatty acids.

crease in fatty acids should be found in pneumococcus autolysates when compared with the heated control of the same suspension, in which autolysis has been prevented by inactivation of the enzymes.

For the purpose of estimating fatty acids in bacterial suspensions and in autolysates, a small liquid extraction apparatus[1] was devised (Fig. 1).

A measured amount of bacterial substance was placed in the extraction chamber, 5 cc. of N/1 sulfuric acid were added, and the mixture was extracted for 24 hours, 75 cc. of purified ethyl ether[2] being used as a solvent. At the end of 24 hours extraction, the flask containing the ether and dissolved bacterial fatty acids was removed. The ether was evaporated over a warm water bath.

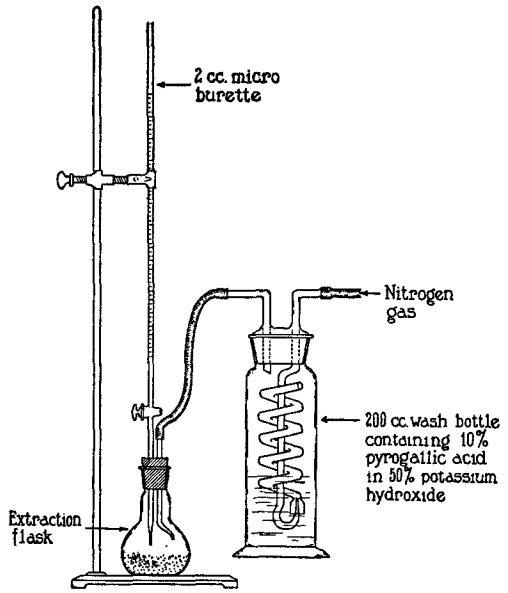

FIG. 2. Apparatus for titrating bacterial fatty acids in an atmosphere of nitrogen.

The flask was now fitted with a 3-hole rubber stopper containing an intake tube for nitrogen gas, an opening for insertion of the micro burette, and a third opening

[1] The authors wish to express their thanks to Dr. P. A. Levene who suggested the principle of liquid extraction for the estimation of fatty acids.

[2] Ethyl ether was shaken in a separatory funnel with a saturated solution of calcium chloride to remove alcohol, then with alkaline potassium permanganate to remove aldehydes and acids, and finally with water. After drying over calcium chloride, the ether was twice distilled. 75 cc. of this ether, when evaporated to dryness, left a residue, which when dissolved in the purified alcohol described in Foot-note 2, neutralized 0.08 cc. N/100 sodium hydroxide.

for the introduction of the solvent. The flask was then filled with nitrogen gas which had been washed free from oxygen and carbon dioxide by bubbling through a 10 per cent solution of pyrogallic acid in 50 per cent potassium hydroxide. 5 cc. of purified ethyl alcohol[3] and 1 drop of 0.5 per cent phenolphthalein solution were introduced. The fatty acids in solution were then titrated with N/100 sodium hydroxide, by means of a special microcapillary burette of 2 cc. capacity, graduated in 1/100 cc. The first faint pink was taken as the end-point. Since

TABLE VI.

Lipolysis Accompanying Autolysis of Pneumococcus (Type III).

Exp. No.	Tube	pH	Bacterial nitrogen in sample extracted	N/100 NaOH to neutralize bacterial fatty acid	N/100 NaOH per mg. of bacterial nitrogen	Increase
			mg.	*cc.*	*cc.*	*per cent*
2	C*	NaCl	6.91	1.480	0.214	—
	A†	NaCl	5.70	1.580	0.276	28.9
3	C	NaCl	4.00	1.192	0.298	—
	A	NaCl	3.20	1.209	0.378	26.9
4	C	6.5	8.40	3.780	0.450	—
	A	6.5	7.60	6.740	0.887	97.2
5	C	6.5	10.00	3.181	0.318	—
	A	6.5	8.60	4.559	0.530	66.7
6‡	C	NaCl	—	2.134	—	—
	A	NaCl	—	3.461	—	62.2

* C = control tube.

† A = autolysate.

‡ Alcoholic extract of pneumococci, suspended in salt solution, and to which had been added pneumococcus extract containing active enzymes (Tube A) and heat-inactivated enzymes (Tube C).

phenolphthalein is sensitive to carbon dioxide, the titration was carried out in an atmosphere of nitrogen. In this manner a most accurate titration and lasting end-point were obtained. Three blank determinations, with 2.84 mg. of crystalline stearic acid, gave titrations of 0.990, 0.995, and 1.000 cc. N/100 sodium hydroxide, after subtracting a blank of 0.08 cc. N/100 sodium hydroxide for the ether and alcohol. A diagram of the titration apparatus is given in Fig. 2.

The results of four experiments are given in Table VI.

[3] Absolute ethyl alcohol was twice distilled over sticks of potassium hydroxide, and then once without potassium hydroxide.

6. Lipolysis in Solutions of Pneumococcus Containing Sodium Desoxycholate.

It has been pointed out above that sodium desoxycholate inhibits the action of the proteolytic enzyme of Pneumococcus. In order to ascertain whether the lipase is still active in solutions of Pneumococcus containing sodium desoxycholate, the following experiments were undertaken.

The growth from 3 liters of broth culture of Type III pneumococcus was obtained as in previous experiments. The living bacteria were taken up in 50 cc.

TABLE VII.

Lipolysis in Sodium Desoxycholate Solutions of Pneumococcus (Type III).

Exp. No.	Tube	pH	Bacterial nitrogen in sample extracted	N/100 NaOH to neutralize the extract	Increment of increase in terms of cc. of N/100 NaOH
			mg.	*cc.*	
F	C*	7.5	12.8	12.13	—
	A†		12.8	14.60	2.47
H	C	7.5	7.32	14.30	—
	A		7.32	16.00	1.70
I	C	7.5	7.30	11.95	—
	A		7.30	14.11	2.16
J	C	7.5	7.32	11.85	—
	A		7.32	13.67	1.82

* C = control tube.

† A = autolysate.

of M/15 phosphate buffer at pH 7.5, the suspension was cooled in ice to 0°C., and to it were added 2 cc. of a 10 per cent solution of sodium desoxycholate. After complete solution of the bacterial cells had occurred in the cold, 25 cc. of the mucoid-like material were inactivated by heating for 10 minutes at 100°C. as control; and the remaining 25 cc. were allowed to autolyze at 37°C. for 48 hours. 0.5 cc. of chloroform was added as a preservative.

After incubation, and subsequent evaporation of the chloroform, 20 cc. of these solutions were placed in the liquid extraction apparatus and each extracted for 24 hours as described above. The extracts were then titrated.

The results of these experiments are given in Table VII.

In order to determine whether lipolysis accompanies the solution of pneumococci by bile at 0°C., the following experiments were carried out.

The growth from 3 liters of Pneumococcus Type III was obtained as in 1. The bacteria were suspended in 60 cc. of isotonic salt solution. Two portions of 25 cc. each of living organisms were quantitatively measured into two tubes. The first tube, as control, was immediately heated for 10 minutes at 100°C., then cooled, and to the suspension of heat-killed bacteria exactly 2 cc. of a 10 per cent solution of sodium desoxycholate were added. The second tube, unheated, was kept at 0°C. and to it were added exactly 2 cc. of the same solution of desoxycholate.

TABLE VIII.

The Absence of Lipolysis during Solution of Pneumococci (Type III) by Sodium Desoxycholate at 0°C.

Exp. No.	Tube	pH	Bacterial nitrogen in sample extracted	N/100 NaOH to neutralize the extract	Increment of increase in terms of cc. of N/100 NaOH
			mg.	*cc.*	
1	a*	NaCl	6.32	20.12	—
	b†	NaCl	6.32	20.14	0.02
2	a	NaCl	5.93	18.63	—
	b	NaCl	5.91	18.64	0.01

* a = Heated control.

† b = Sodium desoxycholate solutions of living pneumococci.

After complete solution of the organisms in the cold, this tube was plunged directly into boiling water for 10 minutes to inactivate the enzymes.

20 cc. of each of the two samples were placed in the liquid extractor, extracted for 24 hours, and the extract titrated with N/100 sodium hydroxide.

The results are given in Table VIII.

In Tables VI to VIII are found the results of experiments on the occurrence of lipolysis during pneumococcus autolysis. It is seen that the lipase is active not only during normal autolysis, as measured by the increase in ether-soluble fatty acids, but that after solution of the cells by sodium desoxycholate, it retains its activity even in the presence of relatively high concentrations of the bile salt. When, however, pneumococci are dissolved at 0°C. with sodium desoxycholate,

and the solution immediately heated, no increase in fatty acid is found to have occurred. In other words, the mere solution of pneumococci by sodium desoxycholate at 0°C., a temperature at which enzyme action is retarded, is not accompanied by lipolysis.

DISCUSSION.

The phenomenon of pneumococcus autolysis is accompanied by two distinct chemical changes: first, enzymatic degradation of some protein or proteins of the cell into less complex molecules; second, a liberation of free fatty acids, probably of high molecular weight, by the action of pneumococcus lipase on certain cellular esters. On the other hand, when bile (sodium desoxycholate) solutions of pneumococci are incubated, only lipolysis and no proteolysis takes place when an excess of the bile salt is used.

Throughout the literature there are statements to the effect that the solution of pneumococci in bile is due to an acceleration of normal autolytic processes; in the present study it has been found that proteolysis does not occur when pneumococci are dissolved in the presence of an excess of sodium desoxycholate. Furthermore, pneumococci can be brought rapidly into solution by sodium desoxycholate at temperatures (0°C.) where the rate of enzyme action is retarded, and where one would scarcely expect either proteolysis, lipolysis, or any other enzyme action to play an important rôle. Under these conditions it seems difficult, therefore, to conceive of the solution of pneumococci by bile as an acceleration of the normal autolytic process, since the chemical changes which accompany *true autolysis* are not associated with *solution* of the organisms by bile.

Julianelle and Reimann (11) have shown that extracts of pneumococci contain a toxic principle capable of producing experimental purpura in animals. They pointed out that bile solutions of pneumococci do not yield the purpura-producing substance. They concluded that: "it appears probable, therefore, that the purpura-producing property is due not to a constituent of the living pneumococcus cell, but to a degradation product of pneumococcus." Mair (12), on the other hand, has found that bile and sodium desoxycholate solutions of pneumococci produce purpura in mice. He attributes the differences between his results and those of Julianelle and Reimann to differences

in technique. The analytical data in the present investigation show at least that proteolysis does not accompany solution of pneumococci, if the concentration of sodium desoxycholate is greater than 1 part in 2500.

It has been pointed out that in autolysates and in bile solutions of pneumococci, the type-specific antigen undergoes a change whereby it loses in greater part the capacity to stimulate type-specific antibodies. On the other hand, the cell proteins in soluble form retain their antigenic properties and induce only the species-specific antibodies; that is, the dissolved substances yield a serum containing a high titer of antiprotein antibodies. The immunological evidence, therefore, supports the view that during autolysis and dissolution of the cell, particularly under conditions which release the intracellular enzymes in an active state, the complex antigen is broken down into its component parts, one of which, at least, consists of the antigenically inert hapten,—the type-specific carbohydrate. While the existing information emphasizes the importance of the principle of antigenic dissociation, it is, as yet, too meager to admit of any decision as to the nature of the chemical union or the factors which determine the rate and extent of dissociation of the conjoined antigen in the test-tube and in the animal body.

Although earlier investigators have isolated carbohydrate material in the form of bacterial gums from a variety of microorganisms, the relation of these polysaccharides to the immunological specificity and antigenic properties of the cell has only recently been recognized. While reasoning by analogy is often fallacious, the relation of the carbohydrate radicle to the conjugated protein of "animal gums" is at least suggestive that a similar relationship may exist in the carbohydrate-protein complex of Pneumococcus. In the group of conjugated proteins of animal origin, Levene (13) has pointed out that the carbohydrate radicle is bound to the protein by a quite simple and comparatively labile union in the nature of an ester linkage. If similar relations prove to exist in the case of Pneumococcus, an antigenic linkage of this nature would be susceptible to most chemical agents and to action of the cell's own esterase, which, as the present study shows, is active during autolysis and in the presence of bile salts. Under these conditions, the differences observed in the

rate and degree of antigenic dissociation of the three fixed types of Pneumococcus would be referable to the particular form of linkage, dependent upon known differences in the chemical structure of the type-specific polysaccharides. For example, the complex sugar of Type I contains nitrogen partly in amino form, while the corresponding carbohydrates of both Type II and Type III are each nitrogen-free. From the chemical point of view, therefore, the greater stability of the Type I antigen might be conceived of as due to a more resistant linkage or one of a different order than that found in the other two types. Similarly, from an immunological point of view, the most efficient of these complex antigens would be the one least easily dissociable; that is, in terms of type specificity, the antigenic potency of any given type of Pneumococcus would be inversely proportional to the rate and extent of antigenic dissociation.

The results of the present study of autolysis, as well as the earlier observations on the differences in antibody response of animals to the different types of Pneumococcus, suggest the possible relationship of enzyme action to antigenic dissociation *in vitro* and *in vivo*.

CONCLUSIONS.

1. Autolysis of Pneumococcus is accompanied by proteolysis, which results in an increase in amino and non-coagulable nitrogen.

2. Autolysis of Pneumococcus is accompanied by lipolysis during which there is a liberation of ether-soluble fatty acids.

3. When extracts containing the active intracellular enzymes are added to heat-killed pneumococci, lysis of the cells occurs and there is an increase in the non-coagulable and amino nitrogen, comparable to the changes accompanying spontaneous autolysis.

4. When extracts containing the active intracellular enzymes are added to emulsions of the alcohol-soluble lipoids extracted from pneumococci, an increase in the ether-soluble fatty acid occurs.

5. Sodium desoxycholate in excess inhibits the action of pneumococcus protease; it does not inhibit the action of pneumococcus lipase.

6. When suspensions of pneumococci are cooled to 0°C., a temperature at which the rate of enzyme action is greatly retarded, the organisms go into solution rapidly when sodium desoxycholate is added, but this process is not accompanied by lipolysis or proteolysis. It

does not seem probable, therefore, that the "bile" solution of pneumococci is identical with the phenomenon of autolysis as ordinarily understood and measured.

7. The relation of enzyme action to antigenic dissociation is discussed.

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