THE RÔLE OF THE SOLUBLE SPECIFIC SUBSTANCE IN ORAL IMMUNIZATION AGAINST PNEUMOCOCCUS TYPE I

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(Received for publication, July 29, 1931)

Experiments previously reported have shown that the oral administration of dead pneumococci (Type I) produces in rats an increased resistance to intraperitoneal and subcutaneous injection of the virulent organism. It has also been found that the use of mechanically disrupted cells or of the bile salt-dissolved bacteria produces a similar immunity (1). These observations indicate that the effect may be ascribed to the action of one or more constituents of the cell rather than to the whole organism. When a degraded, avirulent form of Type I was fed, very little increased resistance was developed. Precipitin tests done on such cultures and on extracts from large quantities of the centrifuged organisms failed to demonstrate the presence of the soluble specific substance. The marked difference in the effect elicited by feeding the degraded and virulent forms, and the corresponding difference in the type-specific polysaccharide content, suggested that this material might be of importance in the process of oral immunization. There was also other evidence in favor of this view.

In a preliminary report (2) it has been shown that the soluble specific substance, when fed to white rats, produces an immunity which resembles in several respects that obtained when the intact or dissolved pneumococci are used. It is with the purpose of giving the data there briefly referred to, as well as the results of experiments bearing on the

* The author wishes to record his thanks to Mrs. Lawrance Harriman who very kindly provided funds in aid of this work. He is also indebted to the Harriman Research Fund for a grant.

question of whether this substance may be considered the only constituent which is immunologically active when the pneumococcus is fed, that the present report is made. These data are as follows: (1) the similarities between the effects of feeding the pneumococcus and the polysaccharide to rats, (2) comparison of results of feeding the pneumococcus and polysaccharide to mice, (3) the influence of certain treatment of the bile salt-dissolved pneumococcus and of the whole cell, on the immunity created by feeding these materials.

Although principally concerned with the effect of oral administration of the specific substance, experiments are included in which the polysaccharide was injected into mice, because of the bearing these have on the problem as a whole. All the experiments were done with Type I specific substance and Type I pneumococcus.

The Soluble Specific Substance.—Two samples of carefully prepared polysaccharide obtained through the courtesy of Dr. Harry Sobotka,¹ and which will be referred to as Lots 1 and 2, were employed. There was too little of Lot 1 to do extensive chemical tests. The xanthoproteic and Millon tests were negative for both. The biuret gave a faintly positive result when done on relatively large quantities of dry substance of Lot 2. Both samples could be detected in a dilution of 1 part in 3 million of water by the use of antiserum. Analyses² of Lot 2 yielded the following data: nitrogen, 5.1 per cent; carbon, 41.7 per cent; hydrogen, 5.6 per cent; ash, 0.4 per cent; reducing substances in terms of glucose (after hydrolysis), 24.7 per cent. These figures are calculated on the dry basis. There was a loss of 16 per cent on drying at 100°C. The reducing substances were determined by the Shaffer-Hartmann-Somogyi method, after boiling several milligrams with 1 cc. of 1.4 normal nitric acid under a reflux condenser, and neutralizing the mixture. These figures agree fairly well with those of Heideberger, Goebel and Avery (3).

Resemblance of the Effects of Feeding the Soluble Specific Substance to Those Obtained When the Pneumococcus was Orally Administered

The immunizing effect of feeding the pneumococcus has been largely dealt with in earlier papers. Table III in the present report illustrates

¹ He also furnished the Type II and III preparations used in experiments described in the paper, "The rôle of the soluble specific substance in oral immunization against *Pneumococcus* Types II and III," in the following number.

² Analyses for C, H, N, ash and loss on heating, were kindly performed by Dr. Frances Krasnow, of the College of Physicians and Surgeons of Columbia University.

the features which characterize the protection so obtained. These are (1) a single feeding of the bacteria contained in 5 cc. growth is sufficient; (2) the increased resistance appears within 48 hours; (3) in extent it is equivalent to 1000 to 10,000 fatal doses;³ (4) it lasts for about 2 weeks at which time a new feeding causes it to reappear. This can be repeated as often as one wishes. The duration of 2 weeks refers to the experiments in which 1 or more feedings of the bacteria from 5 cc. growth are given. By increasing the size of each dose the immunity can be made to last for longer periods of time.⁴

Number of Feedings Required. Degree and Time of Appearance of Immunity.—In the first experiment each animal received 0.5 mg. (Lot 1) on each of 3 days. The material was dissolved in N/15 HCl. The solution was mixed with cracker meal and each rat was fed individually. All tests for measuring the resistance to pneumococcus in this and subsequent experiments were done by injecting the required dose intraperitoneally in a volume of 0.2 cc. It will be seen from Part 1 of Table I that the rats which were fed the specific polysaccharide possess an increased resistance to the virulent organism. Among the controls, 10^{-8} and 10^{-7} cc. killed, in each case, 2 out of 3 rats. Among the treated rats, 2 injected with 10^{-8} cc., 2 with 10^{-7} cc. and 1 with 10^{-6} cc. survived. The one injected with 10^{-6} cc. died.

In the next experiment the number of feedings (Lot 1) was reduced to 2. The procedure was the same as described above, except that a slight insoluble residue was separated from the solution by centrifugation (Part 2, Table I). Although the response is not quite uniform, some rats are protected against 1000 fatal doses. 1 of 2 survived 1 fatal dose, 1 of 2 lived after receiving 10 fatal doses, 2 of 3 survived 100 and 1000 fatal doses, respectively.

The number of feedings was then further reduced to 1 (Lot 1), keeping the conditions the same as they were in the preceding experiment. The result is shown in Part 3 of Table I. Although the extent of resistance to the pneumococcus reaches 1000 fatal doses in some instances, a smaller proportion of animals was protected than when 2 or 3 doses were administered.

In Part 1 of Table II are given the results of an experiment in which the Type I specific substance of Lot 2 was fed. Two solutions were prepared, one in which the material was dissolved in N/15 HCl; in the other the solvent was a phosphate solution of pH 7.7. The slight insoluble residue in both was removed. The HCl solution was fed to one lot of rats and the phosphate solution to another, with the object of learning whether the solvent exerted any influence. There were 2 feedings. The results indicate that the two solvents gave about the same general

³ Less in the case of Types II and III.

⁴ See, for example, Tables IV and V in Reference 1.

TABLE I

Resistance of Rats to Pneumococcus Following Feeding of Soluble Specific Substance

Dose	Part 1		Part 2		Part 3	
	C	E	C	E	C	E
cc.						
10 ⁻⁹	S	8*	S.S**		S.S	
10 ⁻⁸	S.3.2	S.S	2.3	S.4°	3.2	2.3†S
10 ⁻⁷	S.3.4	S.S	2.3	S.4	3.3	S.2.2.2†
10 ⁻⁶	2	3.	4	S.S.2	2.2	2.3.S
10 ⁻⁵	2	S.	2	S.S.2	S	S.2.S
10 ⁻⁴				2.		
	Each E rat received 0.5 mg. on each of 3 successive days. Test was done 4 days after last feeding		Each E rat was fed 0.5 mg. S.S.S. on each of 2 successive days. The test was done 48 hrs. after 2nd feeding		Each E rat was fed 0.5 mg. S.S.S. Test was done 48 hrs. later.	

C = control. E = treated rat. S = survived. Number = died, — days.

* Heart blood sterile. ** Sick but recovered.

° Ill before injection; pneumococcus in heart blood. † Tested after about 44 hours.

TABLE II

Resistance of Rats to Pneumococcus Following Feeding of Soluble Specific Substance

Dose	Part 1			Part 2	
	C	E ¹	E ²	C	E
cc.					
10 ⁻⁹	S*. S°			S.S	
10 ⁻⁸	3°. 4*	2.3	3.2	S.7	S.S
10 ⁻⁷	3°. 4*	S.2.2	2.2	6.6	S.S
10 ⁻⁶	5*. 6°	S.5.3	S.3.S.	2.4	2.4
10 ⁻⁵	3*	S.2	2.2	2	S.4.2
10 ⁻⁴		2.2	S		2.2
10 ⁻³					2.2
	Each E rat was fed 0.5 mg. S.S.S. (Lot 2) on each of 2 successive days; dissolved in N/15 HCl for E ¹ rats, and in phosphate solution for E ² rats. C rats marked* were fed HCl alone, those marked ° were fed phosphate alone. Test was done 72 hrs. after feeding			Each E rat was fed 0.25 mg. S.S.S. (Lot 2) on each of 3 successive days. The S.S.S. was dissolved in phosphate solution, pH. 7.73; centrifuged and filtered through a Berkefeld V candle. Test was done 72 hrs. after last feeding	

effect. The effect of feeding 3 doses of 0.25 mg. each per rat (Lot 2) is shown in Part 2, Table II.

The five experiments described illustrate the resemblance between the immunity obtained by administering the specific polysaccharide, with respect to the time of its appearance and the need for but 1 feeding. The response is in general, however, more uniform when 1 feeding of whole organisms or of the Berkefeld filtrate of bile salt-dissolved cells is used. Also the degree of resistance is more often larger.⁵

Duration of the Protection Afforded by Feeding the Soluble Specific Substance as Compared with That Obtained by Oral Administration of the Acid-Killed Intact Cell.—After it had been found that feeding the

TABLE III
Duration and Renewal of Immunity Produced by Feeding a Single Dose of HCl-Killed Pneumococcus (Organisms from 5 cc. Growth)

Dose	48 hrs. after feeding		67 hrs. after feeding		10 days after feeding		48 hrs. after new feeding		72 hrs. after new feeding	
	C	E	C	E	C	E	C	E	C	E
cc.										
10 ⁻⁹	3*		2*		2*		S		S.S.	
10 ⁻⁸	5.3		S		3	S	2		S.S. 3	S
10 ⁻⁷	2.2	S.S	2	S.S	2	3	2	S	2.3	2
10 ⁻⁶	2	S.S	2	S.S	2	2	2	2	3.3	S
10 ⁻⁵		S.S		S.S		2		4		S
10 ⁻⁴		4.2		2.S		2		2.2		2
10 ⁻³		S		3						

* Pneumococcus in heart blood.

polysaccharide to rats produces an immunity to the pneumococcus, experiments were planned to learn whether the presence of this substance in the cell is sufficient to explain all the effects observed when the latter is orally administered to rats. It has previously been shown that the immunity conferred on rats by feeding them the tissues of

⁵ Table IV in the paper, "Fate of orally administered soluble specific substance of Pneumococcus Types I, II, and III," in the following number, will show that a single feeding of S.S.S., Type I, recovered from the feces of rats to which it was orally administered, produced a very uniform immunity. Several additional experiments are recorded there.

animals killed by the pneumococcus lasts for at least 4 months, at which time it is almost, if not quite as high as it is directly after the feeding (4). Although the protection against the pneumococcus which follows the oral administration of 1 dose of acid-killed organisms from 5 cc. growth is quantitatively as high as is obtained when repeated doses of infected tissue are fed, its duration appears to be considerably less.⁶ It is the similarity between the duration obtained in the former kind of experiment and that which follows feeding of the soluble

TABLE IV
Duration and Renewal of Immunity Produced by Feeding Soluble Specific Substance

Dose	48 hrs. after 2nd of 2 feedings		10 days after 2nd of 2 feedings		72 hrs. after a new feeding	
	C	E ^a	C	E ^b	C	E ^c
cc.						
10 ⁻⁹	S. S. S. 2		S. S.	S. S.	7*	
10 ⁻⁸	2. 2. 5. 4	S. 2	S. S.	S. S. 2	8*. 2	S. S. S.
10 ⁻⁷	2. 2	3. 3. S	S. S.	3. 3. 2	2. 3	S. S. S. 4*
10 ⁻⁶	2.	3. 2. 3	2. 5	2. 2	5. 3	S. S. 7†
10 ⁻⁵	3.	S. S. 2	2.	2. 2	2.	S. 2. 4
10 ⁻⁴		2. 2		2		2. 2

E^a and E^b rats received 0.5 mg. S.S.S. (Lot 2) on each of 2 successive days. S.S.S. was dissolved in N/15 HCl, centrifuged and filtered through filter paper. E^c rats received same 2 feedings and in addition 0.5 mg. dissolved in N/15 HCl, centrifuged but not filtered, 12 days after 2nd feeding.

* Contaminant in heart blood. † Pneumococcus in heart blood.

° Did not receive new feeding; had a litter after 2nd feeding.

specific substance, that is brought out by the following observations. Table III shows what happens when 1 dose of acid-killed pneumococci is given.

Two experiments with the specific polysaccharide were done.

The results of the first are given in Table IV. Each animal received 0.5 mg. (Lot 2) on each of 2 successive days. 2 days after the 2nd dose some of the rats were tested and it was found that, of 13 rats, 4 had survived quantities of pneumococci equivalent to from 1 to 1000 fatal doses. It seemed that the low percentage of survivors might make it difficult to determine the disappearance of the protection, but judging by the data of Column 2 of the same table, (13 additional rats of the original group) it is evident that most, if not all of the increased resistance, was lost 10 days after the feedings. For some reason the controls survived somewhat

⁶ See Tables IV and V, Reference 1.

larger doses than the experimental rats.⁷ The remaining animals of the original group were now fed 1 additional dose of 0.5 mg. of specific substance (Lot 2) and were tested 3 days later, with the result shown in the third column of this table. It is evident that the protection is renewed. More uniform results were obtained than after the first feedings.

The experiment was repeated, using Lot 1 of the soluble specific substance. The data appear in Table V. The immunity which followed the ingestion of 2 doses of polysaccharide appeared in 48 hours and was as regular and as high as that which follows the ingestion of the intact acid-killed pneumococcus or the bile salt-

TABLE V
Duration and Renewal of Immunity Produced by Feeding Soluble Specific Substance

Dose	48 hrs. after 2nd of 2 feedings		10 days after 2nd of 2 feedings		72 hrs. after a new feeding	
	C ^a	E ^a	C ^b	E ^b	C ^c	E ^c
cc.						
10 ⁻⁹	3*.2		S.2*	S.S	S.S	
10 ⁻⁸	S.2	S.S	2.2	S.2	2*.3	2 ¹
10 ⁻⁷	2.2	S.S.S	2.2	S.S.2	3.3	2 ⁶ .S ⁹
10 ⁻⁶	2.1	S.S.4	2.2	2.2	3.2	S ⁴ .3 ⁸ .S ²
10 ⁻⁵	2.	S.S.S	2	2.2	3	2 ⁸ .S ⁷ .2 ³

The E^a and E^b rats were fed 0.5 mg. S.S.S. (Lot 1) on each of 2 successive days. The S.S.S. was dissolved in N/15 HCl, centrifuged and the supernatant fluid was mixed with cracker meal. The E^c rats received the same 2 feedings and in addition 0.5 mg. S.S.S. similarly prepared, 19 days after the 2nd feeding. C^a and C^b were fed N/15 HCl alone in cracker meal.

The exponents in the E^c column identify the rats in Table II, Experiment 1, in the paper "Fate of orally administered soluble specific substance of *Pneumococcus* Types I, II and III" in the following number.

* *Pneumococcus* in heart blood.

dissolved cell. 10 days after the 2nd dose some additional rats were tested. A smaller percentage of animals was protected and the general appearance of the results is like that which is found after a similar interval of time when 2 doses of the dead bacteria are fed. The remaining untested animals were fed 0.5 mg. each of the polysaccharide and were injected with the pneumococcus 72 hours later. The original immunity reappears but, as in most of the other experiments with the soluble specific substance, a single feeding seems to be insufficient to give the regularity of protection that a single ingestion of the organisms yields.

The immunity created by the oral administration of the specific substance to white rats is thus seen to bear a marked resemblance to

⁷ The C and E rats came from different sources. However, this has rarely been found to be correlated with a difference in the minimum fatal dose.

that obtained by feeding small quantities of the organism with respect to duration as well. The data below show the possibility that there is a second active substance within the pneumococcus cell.

Comparison of Effects of Feeding the Pneumococcus and the Soluble Specific Substance, Using Mice as Experimental Animals.

Injection of the Soluble Specific Substance

The following experiments were done with the object of learning whether the specific substance plays as important a part in the oral immunization of mice as it does in the rat. It has been stated in an earlier communication (5) that mice are not well immunized by feeding the pneumococcus. A smaller percentage of animals is protected than when rats are used, and in some experiments no evidence of any success is obtained. This made it apparent from the start that the rôle of the polysaccharide in the oral immunization of this animal would be more difficult to determine than in the case of rats. Aside from this, however, if mice could be protected against the pneumococcus by feeding the soluble specific substance with approximately the same degree of success as when the bacteria are used, there would be evidence that it is equally important in the oral immunization of both animals. On the other hand a failure to protect by feeding the polysaccharide might mean that a constituent other than this is active when mice are fed the intact or dissolved cocci.

Feeding the Intact Pneumococcus and the Bile Salt-Dissolved Cell to Mice.—In Table VI are given the data of an experiment in which acid-killed pneumococci were administered. Of 7 controls injected with 10^{-8} cc., 1 lived. Among the treated mice, 1 of 3 survived 10^{-8} cc., 5 of 10 survived 10^{-7} cc. and 5 of 8 survived 10^{-6} cc. The response is inferior to that obtained in rats. No difference in results which might be ascribed to the difference in initial weights of the mice (11 to 25 gm.) was observed.

Table VII gives the data for a similar experiment in which acid-killed and heat-killed pneumococci were used. The moderate degree of protection that was found in the preceding test was not obtained. Counting only the mice which were fed the HCl-killed cells,⁸ 2 survived 1 fatal dose, 1 lived after receiving 10 and 200 fatal

⁸ Since heating at 80°C. for 2 hours has been found to largely destroy the immunizing action for rats, the same would probably be expected for mice. The presence of a little acid from the culture doubtless played a part in the loss which followed such heating.

doses, respectively. This, however, takes no account of the mice in the last column of the table where treated animals succumbed to doses of 10^{-8} and 10^{-7} cc., whereas 1 control injected with 10^{-8} cc. lived, and 1 of 2 injected with 10^{-7} cc. also sur-

TABLE VI
Resistance of Mice to Pneumococcus Following Feeding of Acid-Killed Organisms

Dose	C	E 15/2	C	E 17/1	C	E 17/2	C	E 17/7	C	E 17/9	C	E 17/12
cc.												
10^{-9}			S						S			
10^{-8}	2	S	2	4	3	2	2.2		2		S	
10^{-7}	2	S	2	S	2	2	2	S.S	2	2.2	2	S.3.2
10^{-6}	2	2	2	S	1	2	2	S.S	2	3	2	S.S
10^{-5}										2		

C = control. E = treated mouse. S = survived. Number = died, — days.

The figures at the head of the E columns give the number of feedings, followed by the interval in days between the last feeding and the day of the test. Growth was killed by 2 hours contact with N/15 HCl at room temperature. Mice weighed between 11 and 25 gm. at the beginning of the experiment.

TABLE VII
Resistance of Mice to Pneumococcus Following Feeding of Acid- and Heat-Killed Organisms

Dose	C	$\frac{3}{E^h E^a}$		C	$\frac{4}{E^h E^a}$		C	$\frac{6}{E^h E^a}$		C	$\frac{11}{E^h E^a}$		C	$\frac{14}{E^h E^a}$		C	$\frac{18}{E^h E^a}$	
cc.																		
10^{-9}	S			S						S			S					
10^{-8}	S	S	3	2	2	S	2	S	3	S	S	2	2	3	S	S	2	4.2
$2 \cdot 10^{-7}$	2	2	6															
10^{-7}				3	2	S	2	2	2	2	2	2	2	2.3	2.2	S.2	2.2.2	2.2.2
$2 \cdot 10^{-6}$							2											
10^{-6}				2	2	2				2	2	2	2	2.2	2.2	2	6	2.2
10^{-5}													2	2	2			

E^h and E^a mice fed heat-killed and acid-killed organisms respectively. The figure above E^h and E^a equals the interval in days between the last of 16 feedings (over a period of 25 days) and the day of test. For killing by acid, 2 hours contact with N/15 HCl was used. For killing by heat, concentrated suspensions of pneumococci were heated 2 hours at 80°C . Mice weighed 20 gm. or more at beginning of experiment.

vived. Also in the tests done 3 and 11 days after feeding, the controls survived 10^{-8} cc. while the rats fed the acid-killed bacteria succumbed.

Because the vaccine employed had been stored (and used as needed) it seemed that the indecisive results might be owing to a destruction of the antigen. Hence the test was repeated and the bacteria were grown daily. Three forms of pneumo-

cocci were employed: living, acid-killed and heat-killed (80°C. for 2 hours). The mice in each of these three groups were subdivided into those weighing between 7 and 10 gm. and those whose weights were between 18 and 30 gm. The data are given in Table VIII. Again excluding from the count those animals fed the heat-

TABLE VIII
Resistance of Mice to Pneumococcus Following Feeding of Living, Acid-Killed and Heat-Killed Organisms

Dose	C	7			C	11			C	13
		E ¹	E ^a	E ^b		E ¹	E ^a	E ^b		E ¹
cc.										
10 ⁻⁹	S				S				S	
10 ⁻⁸	2	2.2	2	2	2.5	2.S.S.S.S.	2.2.S.	S.S.	S	2.2.S.
10 ⁻⁷	2	4		2	2	S.2.2.2.5.4.	2.2.S.	2.2.2.	2	3.2.2
10 ⁻⁶	2	2.3	2	2	2	2.2.2.3.	2.1.2	2.2.2.	2	S.2

E¹, E^a and E^b = mice fed living, acid-killed and heat-killed organisms respectively.

Figures above these letters = intervals in days between last of 17 feedings of living organisms (over a period of 28 days), and day of test, and interval between last of 12 feedings of heat- or acid-killed (over a period of 25 days) and day of test.

TABLE IX
Resistance of Mice to Pneumococcus Following Feeding of Acid-Killed Organisms

Dose	C	E ^a	C	E ^b	C	E ^c
cc.						
10 ⁻⁹			S.S.S.		6.2*	
10 ⁻⁸	2.2	3.S	2.2.2.	S.S	2.2	2
10 ⁻⁷	2.2	4.S	2.2	5.3.4.	2.2	3.3
10 ⁻⁶	2.2	2.S	2.2	S.3.2.	2.2	2.S
10 ⁻⁵				5		

E^a and E^b mice each received bacteria from 50 cc. growth each of 5 days (over a period of 6 days). E^c mice received 3 feedings (organisms from 30, 30 and 50 cc. on 3 successive days). The first feeding in all cases was preceded by a 24 or 48 hour fast. Test took place 7 days after last dose for E^a and E^b mice, and 11 days after last dose for E^c mice.

* Pneumococcus in heart blood.

killed organisms, we find among those given either the living⁹ or acid-killed pneumococci, the following: of 11 injected with 10⁻⁸ cc., on days when this was a fatal dose for controls, 5 lived; of 10 injected with 10⁻⁷ cc., 2 lived. The only other larger dose used, 10⁻⁶ cc., killed all the treated animals. This does not take

⁹ The feeding of living pneumococci caused many deaths among the mice but none among the rats.

TABLE X
Resistance of Mice to *Pneumococcus* Following Feeding of Organisms Grown in Milk
or Dissolved in Sodium Taurocholate

Weight	Dose	Result	Feedings and interval	Remarks	Weight	Dose	Result	Feedings and interval	Remarks
gm.	cc.				gm.	cc.			
C 20	10 ⁻⁸	S		n.d.	C 21	10 ⁻⁸	3		n.d.
C 22	10 ⁻⁷	2		n.d.	C 20	10 ⁻⁸	2		s.t.
C 23	10 ⁻⁸	3		n.d.	C 20	10 ⁻⁷	2		s.t.
E 18	10 ⁻⁸	4	18/1	Milk	C 23	10 ⁻⁸	2		s.t.
E 19	10 ⁻⁷	2	18/1	Milk	E 19	10 ⁻⁸	6	18/1	T.I
					E 20	10 ⁻⁷	3	18/1	T.I
C 20	10 ⁻⁸	2		n.d.	E 20	10 ⁻⁸	2	18/1	T.I
C 22	10 ⁻⁷	2		n.d.	E 18	10 ⁻⁸	2	9/1	Milk
C 22	10 ⁻⁸	2		n.d.	E 21	10 ⁻⁷	2	18/1	Milk
E 22	10 ⁻⁸	2	19/2	Milk	E 23	10 ⁻⁸	2	9/1	Milk
E 17	10 ⁻⁸	S	19/2	Milk					
E 22	10 ⁻⁷	2	19/2	Milk	C 19	10 ⁻⁸	2		n.d.
E 22	10 ⁻⁷	2	19/2	Milk	C 20	10 ⁻⁷	2		n.d.
E 22	10 ⁻⁸	2	19/2	Milk	C 23	10 ⁻⁸	2		n.d.
E 25	10 ⁻⁸	2	19/2	Milk	E 19	10 ⁻⁸	2	19/2	T.I
					E 22	10 ⁻⁷	S	19/2	T.I
C 21	10 ⁻⁷	2		n.d.	E 23	10 ⁻⁸	S	19/2	T.I
C 22	10 ⁻⁸	2		n.d.	E 20	10 ⁻⁸	S	10/2	Milk
C 19	10 ⁻⁷	2		s.t.	E 21	10 ⁻⁷	2	19/2	Milk
C 20	10 ⁻⁸	2		s.t.	E 23	10 ⁻⁸	3	10/2	Milk
E 19	10 ⁻⁷	S	13/1	T.I					
E 20	10 ⁻⁸	4	13/1	T.I	C 22	10 ⁻⁸	2		n.d.
E 20	10 ⁻⁷	S	16/1	Milk	C 23	10 ⁻⁷	2		s.t.
E 21	10 ⁻⁸	2	16/1	Milk	C 27	10 ⁻⁸	2		n.d.
					E 23	10 ⁻⁷	3	19/3	T.I
C 20	10 ⁻⁸	3		n.d.	E 26	10 ⁻⁸	S	19/3	T.I
C 20	10 ⁻⁷	S		n.d.	E 23	10 ⁻⁸	S	19/3	Milk
C 21	10 ⁻⁸	4		n.d.	E 23	10 ⁻⁷	4	19/3	Milk
C 20	10 ⁻⁸	2		s.t.	E 24	10 ⁻⁸	2	19/3	Milk
C 21	10 ⁻⁷	3		s.t.					
C 21	10 ⁻⁸	2		s.t.	C 21	10 ⁻⁸	3		n.d.
E 20	10 ⁻⁸	S	17/1	T.I	C 24	10 ⁻⁷	2		n.d.
E 20	10 ⁻⁷	4	17/1	T.I	C 24	10 ⁻⁸	2		n.d.
E 19	10 ⁻⁸	3	17/1	T.I	*E 24	10 ⁻⁷	1	19/4	T.I
E 22	10 ⁻⁸	S	17/1	Milk	E 25	10 ⁻⁷	S	19/4	T.I
E 21	10 ⁻⁷	2	17/1	Milk	E 19	10 ⁻⁸	S	10/4	Milk
E 21	10 ⁻⁸	2	17/1	Milk	E 24	10 ⁻⁷	2	19/4	Milk
					E 26	10 ⁻⁸	2	19/4	Milk
C 19	10 ⁻⁸	2		n.d.	E 28	10 ⁻⁸	2	10/4	Milk
C 21	10 ⁻⁷	3		n.d.					

The pneumococci were centrifuged and removed with a small quantity of water, rubbed about until evenly distributed, sodium taurocholate added, and left for about 1 hour to dissolve the organisms. Microscopic examination of stained smears were made daily to observe the solvent effect; only an occasional intact cell was observed. The mice were not fed individually, but the dissolved pneumococci, soaked up in bread, and the cultured milk, were put in dishes into the cages. In the case of the bile salt-dissolved organisms, the amount fed was equivalent to 20 cc., per mouse (average). The quantity of sodium taurocholate employed was equivalent to 2 to 3 mg. per mouse per day, and the controls received this as well. The cultured milk was fed in quantities averaging 2 cc. per mouse daily.

Milk = fed cultured milk. s.t. = fed sodium taurocholate alone. n.d. = nothing done. T.I = fed sodium taurocholate-dissolved *Pneumococcus* Type I.

* Sick before injection.

account of the last day's test in which 10^{-7} cc. was the fatal dose for a control, whereas 2 of 3 treated mice succumbed to 10^{-8} cc., all 3 injected with 10^{-7} cc., and 1 or 2 injected with 10^{-6} cc. died.

In Table IX are listed the data of an experiment in which the ingestion of acid-killed pneumococci by mice was preceded by a period of fasting and accompanied

TABLE XI
Resistance to Pneumococcus of Mice Kept on an Exclusive Milk or Meat Diet and Fed Berkefeld Filtrate of Sodium Glycocholate-Dissolved Organisms

Dose	C	5 days after last dose		7 days after last dose		
		E ^a	E ^b	C	E ^a	E ^b
cc.						
10^{-9}	S.S			5.S		
10^{-8}	2.3	4.S	2.2	2.2	2.2	2
10^{-7}	2.2	7.S	3.2	2.2	S.2.2	2.2.2
10^{-6}	2.2	2.S	2.3	2.2	5.2.2	2.2.2
10^{-5}			2.2			

E^a mice kept on milk diet. E^b mice kept on meat diet. Each E mouse received the bacteria from 2, 5 and 5 cc. on 3 days (over a period of 5 days).

TABLE XII
Resistance of Mice to Pneumococcus Following Feeding of HCl-Killed Mechanically Disrupted Organisms

Dose	C	5 E	C	6 E	C	9 E	C	11 E	C	12 E	C	15 E
cc.												
10^{-8}	2	2	3	3	2	2.3	2	S	2	S	3	3
10^{-7}	2	3	3	8	2	3.2	2	3.2	3	2	3	3.3
10^{-6}	2	2	2	2	3	2.2	2	3.3.3	2	2	2	3.3

The figure above E equals the interval in days between last dose and test. The E mice were fed pneumococcus, grown in glucose meat extract broth, pH 7.6, killed by HCl and desiccated in partial vacuum. The pulverized bacteria were mixed with milk and fed by medicine dropper. A fast of 4 hours preceded and followed each feeding. There were 15 feedings (over a period of 17 days). The bacteria from 30 cc. growth comprised a single feeding for a mouse.

by a restricted diet. Out of a total of 20 treated animals, 3 survived 1 fatal dose, 1 survived 10, 2 lived after injection with 100, and 1 after 1000 fatal doses.

The use of bile salt-dissolved organisms and of pneumococci grown in milk, having been found effective for rats, was tried with mice. The number of feedings varied between 9 and 19, and the individual dose from 2 cc. in the case

of the organisms cultured in milk, to 20 cc. for the dissolved cells. A summary of Table X shows that of 31 control mice, 2 survived; of 15 mice fed the dissolved pneumococci, 6 survived, and of 26 fed the cultured milk, 6 lived.

An additional experiment (Table XI) was done with bile salt-dissolved cells, keeping one lot of mice on an exclusive milk, and another on an exclusive meat diet. Of the 14 mice on the milk diet, 1 survived a single fatal dose, 2 survived 10, and 1 lived after 100 fatal doses. None among the meat-fed animals lived.

The ingestion of the desiccated mechanically disrupted pneumococci protected only 2 mice against 1 fatal dose, out of a total of 26 animals (Table XII).

The irregularity of these results with mice, combined with the relatively low percentage of animals protected, indicated in advance that feeding the specific substance would probably yield at best an even smaller proportion of immunized animals.

TABLE XIII
Resistance of Mice to Pneumococcus Following Feeding of Soluble Specific Substance

Dose	Controls	0.20 mg. S.S.S. per mouse on each of 2 days	0.01 mg. S.S.S. per mouse on each of 2 days	0.001 mg. S.S.S. per mouse on each of 2 days
cc.				
10 ⁻⁹	S. S. S. 2	S. S. S. 2	S. S. 2. 2.	S. S. S. 2
10 ⁻⁸	S. 2. 2. 3	2. 2. 2. 2	S. S. 2. 2.	2. 2. 2. 2
10 ⁻⁷	2. 2. 2. 2	2. 2. 2. 2	2. 2. 2	2. 2. 2
10 ⁻⁶	2. 2. 2	2. 2. 2	2. 2. 2	2. 2. 2

The S.S.S. (Lot 2) was dissolved in HCl, mixed with cracker meal and fed on 2 successive days. Test was done 48 hours after 2nd feeding.

Feeding the Soluble Specific Substance to Mice.—Three experiments were done. In the first, the mice were divided into three groups. Each of the members of one of these received 0.20 mg. (Lot 2) on each of 2 successive days. Those in the second group were fed 0.01 mg. and those in the third, 0.001 mg. each, on each of 2 successive days. Table XIII gives the results. There is no evidence for any immunity under the conditions employed.

Another experiment was done in order to see whether better results might follow the use of intervals between the feedings. The data obtained contain no evidence of any immunity (Table XIV).

It was then decided to use a more prolonged period of feeding. The mice were given 12 doses, each equal to 0.10 mg. per animal. Out of a total of 17 mice so treated, and injected with 1 or more fatal doses, 2 survived; 1 of these had received 1, and the other 10 fatal doses of pneumococci (Table XV).

It appears from these three experiments that no definite immunity against the pneumococcus followed the ingestion of the soluble specific substance. This is in contrast to the protective effect produced in rats by this means, and would seem to suggest that there is a second substance within the cell responsible for the protection created in some mice as a result of feeding the pneumococcus. This possibility will be

TABLE XIV

Resistance of Mice to Pneumococcus Following Feeding of Soluble Specific Substance

Dose	Controls	E ^a	E ^b	E ^c
cc.				
10 ⁻⁹	S.S.S.2	S.S.1	S.2.2	S.S.S
10 ⁻⁸	S.2.2.3	S.S.2	2.2.2	S.2.3
10 ⁻⁷	2.2.2.2	2.2.2	2.2.3	2.2.2
10 ⁻⁶	2.2	2.2.2	2.2.1	2.2.2
10 ⁻⁵		1.2.2	2.2.1	1.2

There were 3 feedings of S.S.S. (Lot 2) dissolved in HCl. Interval between 1st and 2nd feedings, 3 days; between 2nd and 3rd, 4 days. Test took place 3 days after last dose.

Quantities fed:—E^a, 0.001, 0.01 and 0.02 mg. E^b, 0.01, 0.01 and 0.02 mg. E^c, 0.10, 0.10 and 0.10 mg.

TABLE XV

Resistance of Mice to Pneumococcus Following Feeding of Soluble Specific Substance

Dose	Controls	E
cc.		
10 ⁻⁹	S	S.S.S
10 ⁻⁸	S.S.S	S.S.S.S.
10 ⁻⁷	2.2	S.2.2.2.2.2
10 ⁻⁶	2.2	S.2.2.2.2.2
10 ⁻⁵		2.2.2.2.2

There were 12 feedings (over a period of 16 days). S.S.S. (Lot 2) was dissolved in HCl. Each feeding = 0.1 mg. per mouse. Test took place 2 days after last feeding.

discussed later. The failure to immunize cannot be owing to a destruction of the soluble specific substance in the intestine, because a similar effect would be expected to follow the ingestion of the whole or dissolved organism. Furthermore, a large porportion of orally administered polysaccharide can be recovered from the feces. Since the bile salt-dissolved pneumococcus protected 6 out of 15 mice, the need

for an intact cell is obviously ruled out. Insufficient dosage does not seem to be the cause since 1.2 mg. was fed to each mouse in the final feeding experiment, and this amount is probably greater than is contained in the bacteria fed to those mice which were immunized by this means.¹⁰

Injection of the Soluble Specific Substance.—In order to see whether the soluble specific substance would produce an immunity in mice when given parenterally, several experiments were done in which the animals were injected either subcu-

TABLE XVI
Resistance of Mice to Pneumococcus Following Subcutaneous or Intraperitoneal Injection of Soluble Specific Substance

Dose	Part 1			Part 2	
	C	Subcutaneous injection		C	Intraperitoneal injection
		0.1 mg. per mouse	0.01 mg. per mouse		0.1 mg. per mouse on each of 3 successive days
cc.					
10 ⁻⁹	S. 2	S. S. 3	S. 2. 2	S. S. 3*	S. S. S. S
10 ⁻⁸	2.	2. 4. 2	2. 2. 2	2. 2. 3	2. 2. 3. 2
10 ⁻⁷	2. 2	2. 2. 2	2. 2. 2	2. 2	S. 2. 2. 3
10 ⁻⁶	2	2. 2. 2	2. 2. 2	2. 2	S. 2. 2. 2
10 ⁻⁵		2. 2. 2	2. 2. 2		2
10 ⁻⁴		2. 2. 2	2. 2. 2		
10 ⁻³		2. 2. 2	2. 2. 2		

S.S.S. (Lot 2) dissolved in phosphate solution pH 7.73 and filtered through a Berkefeld V candle. 0.20 cc. injected for the 0.1 mg. doses. For mice getting 0.01 mg., the solution was diluted ten times with phosphate solution. Test was done 72 hours after the final injection of S.S.S. The 2 experiments were done at different times.

* Contaminant in heart blood.

taneously or intraperitoneally. The results are given in Tables XVI and XVII. In Table XVI, Part 1, the data of an experiment in which mice were subcutaneously injected with a single dose of the polysaccharide, appear (Lot 2). Each animal received either 0.01 or 0.10 mg. and 3 days later was tested. There is no sign of immunity among the treated animals.

A similar test in which the polysaccharide (Lot 2) was injected intraperitoneally, is summarized in Part 2 of Table XVI. Each mouse received 3 equal doses.

¹⁰ Judging by the yield of 2.75 mg. of S.S.S. recovered from the growth from 1 l. of Type II culture obtained by Heidelberger and Avery (6).

There were 2 survivors; 1 had been injected with 10, the other with 100 fatal doses.

To see whether starting with a small dose and increasing its size would give better results, mice were injected subcutaneously with 0.001, 0.02 and 0.10 mg., the intervals being 3 days between the 1st and 2nd, and 4 days between the 2nd and 3rd injections. Another group received the same doses, at the same time, intraperitoneally. The control mice were injected with phosphate solution alone.¹¹ The data are shown in Table XVII, Part 1. Considering 10^{-8} cc. as a minimum fatal dose, 1 of 6 controls and 4 of 8 treated mice survived 1 such dose, 1 treated animal survived 10, 100 and 1000 fatal doses respectively.

TABLE XVII

Resistance of Mice to Pneumococcus Following Subcutaneous or Intraperitoneal Injection of Soluble Specific Substance

Dose cc.	Part 1			Part 2		
	Controls	S.S.S. Subcut.	S.S.S. i.p.	Controls	S.S.S. Subcut.	S.S.S. i.p.
10^{-9}	S.S.S.S.S.2	S.2.2	S.S.S.S.	S.3.7	S.S.S.S.	S.S.2.2.7
10^{-8}	2.4.2.S.3.2	S.S.S.2	S.2.2.4	S.2.2.2	S.S.2.2	S.2.2.2.2.2
10^{-7}	2.2.2.2	2.2.2	S.2.3.2	2.2.2.1	2.2.2.2	2.1.5.2.2
10^{-6}	2.2	2.2.2	S.2.2.2	2.2.2.2	S.2.2.2.2	2.2.2.2.3.2
10^{-5}		S.1.2	2.2.2		2.2.2.2	2.2.2.2

S.S.S. (Lot 1) was dissolved in phosphate solution, pH 7.5 and filtered through Berkefeld V candle. Dilutions of this were made with more phosphate solution. 1st dose = 0.001 mg., 2nd = 0.02 mg., 3rd = 0.10. Test was done 3 days after last dose. Half the controls received 3 injections of filtered phosphate solution alone, subcutaneously, the other half, intraperitoneally. The experiments (Parts 1 and 2) were done at different times.

The entire experiment was repeated in an identical manner, with the results shown in Part 2 of Table XVII. There is no indication, this time, of any increased resistance to the pneumococcus.

It would seem from these experiments that the injected purified specific substance produced at best only a doubtful immunity in mice. This result further eliminates the destruction of the soluble specific substance in the gastrointestinal tract as the cause of failure to immunize when it is administered to mice orally.

In addition to the possibility that a constituent of the cell other

¹¹ It is interesting to note that the injection of phosphate solution or of weak HCl solution produced an increased resistance to Type I in some rats. Even water did this occasionally.

than the polysaccharide is the active one when the bacteria are fed to mice, it is also conceivable that during isolation and purification, the specific substance undergoes an intramolecular change which does not affect its precipitin reaction but which does influence its antigenic activity. Schiemann and Casper (7) immunized mice by injecting them with bile salt solutions of pneumococci from which all protein had been removed, and which gave a good precipitin reaction for the soluble specific substance. They believed the effect was due to this substance. One may suppose that in such a solution the specific polysaccharide had not been altered in the suggested manner.

These two alternatives, the presence of a second constituent, and a change in the intramolecular arrangement of the polysaccharide, also suggest themselves as possible explanations for the decline in immunizing power for rats, which a bile salt solution of pneumococcus was observed to undergo on standing. Since this experiment is of importance in the consideration of whether a second substance is immunologically active when the pneumococcus is ingested, the data are given here.

Decline in Immunizing Action of a Bile Salt Solution of Pneumococcus on Standing

10 l. of pneumococcus culture were prepared in beef heart broth, pH 7.6 to 7.7. The centrifuged bacteria were dissolved in 500 mg. of sodium glycocholate, and water was added to make 40 cc. After standing and centrifugation, the supernatant was removed and the sediment extracted with 50 cc. water. The combined liquids were passed through a Berkefeld candle. The filtrate, the pH of which was 6.6, was diluted with ten volumes water. The pH was now 6.8.¹² 2 per cent CHCl_3 was added to 1 portion and other portions were heated either at 60°C. for 1 hour or at 90°C. for 10 minutes. All were left at room temperature and exposed to light. At the end of 2½, 3½, 4½ and 12 months tests were made on rats with the part of the solution to which chloroform had been added.¹³ No test was done directly after preparing the mixture because of the fairly uniform results obtained in earlier experiments. These had shown that the equivalent of 5 cc. growth was sufficient to protect a rat against approximately 1000 to 10,000 fatal doses, 72 hours after ingestion. However, a change had been made in the method of preparation of the solution and significance was attached to this only after the first examination after

¹² The pH values of corresponding solutions of sodium glycocholate alone were 5.8 and 6.0.

¹³ After 4½ months the pH was slightly less than 6.2 and after a year it was 5.7.

TABLE XVIII

Immunizing Effect of Feeding Bile Salt Solutions of Pneumococcus after Standing in Slightly Acid Solution at Room Temperature and Exposed to Light

Directly after preparing			After 2½ mos.			After 3½ mos.			After 4½ mos.			After 12 mos.					
Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result	Weight	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.		gm.	cc.		gm.	cc.		gm.	cc.	
C86	10 ⁻⁸	S	C58	10 ⁻⁸	S	C78	10 ⁻⁸	S	C101	10 ⁻⁸	S	C74	10 ⁻⁸	S	C68	10 ⁻⁸	S
*C83	10 ⁻⁸	D3	C68	10 ⁻⁷	S	C80	10 ⁻⁸	D2	C127	10 ⁻⁸	S	C84	10 ⁻⁸	D2	C74	10 ⁻⁸	S
C86	10 ⁻⁸	D2	C88	10 ⁻⁸	D2	C85	10 ⁻⁷	D3	C116	10 ⁻⁸	S	C92	10 ⁻⁸	D2	C77	10 ⁻⁸	D2
C86	10 ⁻⁸	D2				C87	10 ⁻⁷	D2	C141	10 ⁻⁸	D5	C90	10 ⁻⁸	D2	C75	10 ⁻⁸	D2
C97	10 ⁻⁷	D3				C91	10 ⁻⁸	D2	C129	10 ⁻⁸	D3	C93	10 ⁻⁷	D2	C78	10 ⁻⁷	D2
C88	10 ⁻⁷	D2				C95	10 ⁻⁸	D2	C146	10 ⁻⁷	D2	C94	10 ⁻⁷	D2	C98	10 ⁻⁷	D2
C103	10 ⁻⁸	D2							C150	10 ⁻⁷	D3	C95	10 ⁻⁸	D2	C120	10 ⁻⁸	D2
C118	10 ⁻⁸	D4							C155	10 ⁻⁸	D3	C133	10 ⁻⁸	D2	C123	10 ⁻⁸	D2
									C160	10 ⁻⁸	D3	C138	10 ⁻⁸	D2	C125	10 ⁻⁸	D1

Each rat fed equivalent of 5 cc.

E91	10 ⁻⁸	S	E61	10 ⁻⁷	S	E73	10 ⁻⁷	S	E90	10 ⁻⁷	D5	E85	10 ⁻⁸	D2	*E164	10 ⁻⁸	D4
E91	10 ⁻⁸	S	E67	10 ⁻⁸	D3	E80	10 ⁻⁸	S	E58	10 ⁻⁷	D2	E85	10 ⁻⁸	D6	E72	10 ⁻⁸	D2
E96	10 ⁻⁷	S	E69	10 ⁻⁸	S	E78	10 ⁻⁸	S	E67	10 ⁻⁸	D2	E91	10 ⁻⁷	D2	E91	10 ⁻⁷	D2
E100	10 ⁻⁷	S	E76	10 ⁻⁴	S	E89	10 ⁻⁸	D3	E51	10 ⁻⁸	S	E88	10 ⁻⁷	D2	E98	10 ⁻⁸	D2
E100	10 ⁻⁸	S	E79	10 ⁻⁸	D2	E82	10 ⁻⁸	S	E72	10 ⁻⁸	D2	E101	10 ⁻⁸	D2	E106	10 ⁻⁸	S
E101	10 ⁻⁸	S				E92	10 ⁻⁴	D4	E70	10 ⁻⁸	D2	E93	10 ⁻⁸	D2	E107	10 ⁻⁸	D1
E105	10 ⁻⁸	D4				E95	10 ⁻⁴	D4	E95	10 ⁻⁴	S	E104	10 ⁻⁸	D2	E115	10 ⁻⁸	D3
E108	10 ⁻⁸	D2				E99	10 ⁻⁸	S	E90	10 ⁻⁴	D2	E109	10 ⁻⁸	S			
E110	10 ⁻⁴	D2															
E111	10 ⁻⁸	D2															

Each rat fed equivalent of 1 cc.

			E63	10 ⁻⁸	S	E79	10 ⁻⁸	S	E69	10 ⁻⁸	S						
			E65	10 ⁻⁷	D3	E80	10 ⁻⁷	D2	E59	10 ⁻⁸	S						
			E61	10 ⁻⁸	S	E84	10 ⁻⁷	D2	E67	10 ⁻⁷	D5						
			E67	10 ⁻⁸	S	E87	10 ⁻⁸	D3	E80	10 ⁻⁷	D3						
			E74	10 ⁻⁴	D2	E91	10 ⁻⁸	D3	E86	10 ⁻⁸	D2						
			E75	10 ⁻⁸	D2	E92	10 ⁻⁸	S	E80	10 ⁻⁸	D2						
						E100	10 ⁻⁸	D2	E90	10 ⁻⁸	D2						
						E104	10 ⁻⁴	D3									
Test done 72 hrs. after feeding			Test done 48 hrs. after feeding			Test done 72 hrs. after feeding			Test done 48 hrs. after feeding			Test done 48 hrs. after feeding			Test done 96 hrs. after feeding		

* Pneumococcus in heart blood.

2½ months. In all earlier experiments the bile salt-treated bacteria from 5 cc. culture were dissolved in 0.5 cc. water, whereas in the present one approximately 0.05 cc. was used, and only after passing through the Berkefeld candle was this made up to 0.5 cc. The same quantity of bile salt was employed. Although practically all the cells were opened by both procedures, it seemed that the active material might

not have completely dissolved in the smaller volume of water. A new solution of pneumococci, prepared at this time in the same manner as the original, was therefore tested. In Table XVIII appear the data for this new preparation and for the tests done at the end of $2\frac{1}{2}$, $3\frac{1}{2}$, $4\frac{1}{2}$ and 12 months on the original preparation. In all instances a volume equivalent to 5 cc. growth was fed, and in some a volume equivalent to 1 cc. as well. These quantities were used because it has been found that the minimum dose for approximately maximum degree of protection lies between them.

It will be seen that the freshly prepared solution gave a uniform protection, though in degree, it was less than was obtained when 0.5 cc. water was used to dissolve the bile salt-opened cells from 5 cc. growth, being equivalent to between 100 and 1000 fatal doses.¹⁴ At the end of $2\frac{1}{2}$ and $3\frac{1}{2}$ months there is no definite loss of activity in the original solution as compared with these results. However, after $4\frac{1}{2}$ months there is a decline which becomes more pronounced at the end of a year. The results at the expiration of the latter period are in marked contrast to those obtained with a freshly prepared solution.

Since hydrolysis of the polysaccharide would account for the loss in antigenic activity on standing, an attempt was made to learn whether this took place.

A comparison of the intensities of the precipitin reactions in the solutions referred to above, after they had stood in the laboratory exposed to light for 2 years, and in a freshly prepared solution, was made. The new solution was made in as nearly an identical manner with the original as possible and was found to give precipitates so nearly like those given by the original preparations that little or no significant difference could be observed.¹⁵ It must be realized, however, that there is a considerable error involved in such a determination, particularly in view of the fact that the number of organisms may have been unequal in the 2 cultures compared. It may be that hydrolysis to the extent of 50 per cent of the original quantity of the specific polysaccharide took place, without being detected. At this time the original solution to which CHCl_3 had been added was again tested, together with a part of the solution to which nothing was done. The equivalent of 10 cc. instead of 5 cc. growth, however, was fed, and it was found that the rats were approximately as well protected as they were by feeding the smaller quantity of the

¹⁴ See Tables X, XI and XIII, in Reference 1.

¹⁵ Comparisons were made between solutions (1) to which nothing was done, (2) to which chloroform was added, (3) heated at 60°C. for 1 hour, (4) heated at 95°C. for 10 minutes. 1:10 and 1:100 dilutions of the Berkefeld filtrates to which ten volumes of water had previously been added, were used.

freshly made solution (Table XIX). This shows that the loss of activity, even after 2 years standing probably did not exceed 50 per cent, and since this is within the limits of error of the precipitin tests, it appears that the hydrolysis of the specific polysaccharide as an explanation for the loss, is not ruled out. The small number of rats protected by the solution which had stood for $4\frac{1}{2}$ and 12 months gives the impression that a loss greater than 50 per cent took place. This may be because the quantity fed (equivalent to 5 cc.) is near the lower limit of the amount which immunizes a large percentage of the animals, and when one uses less than this the proportion of animals protected drops sharply.

TABLE XIX
Immunizing Effect of Feeding 2 Year Old Bile Salt Solution of Pneumococcus
The equivalent of 10 cc. growth was fed to each rat

Dose	Controls		Fed solution to which CHCl ₃ was added		Fed plain solution	
	Weight	Result	Weight	Result	Weight	Result
cc.	gm.		gm.		gm.	
10^{-8}	72	S	65	S	55	S
	73*	D 4	62	S	63	S
			65	S	60	S
10^{-7}	77	S	72	D 6	64	S
	73	D 2	67	S	65	S
			66	S	65	S
10^{-6}	78	D 2	77	S	72	S
	80	D 2	80	S	76	D 5
			73	S	73	S
10^{-5}			84	S	77	S
			96	S	84	D 10

D = died, — days. S = survived.

* Pneumococcus in heart blood.

Assuming that the estimation of the specific polysaccharide in the 2 year old solution was correct, however, and that there was actually no loss of this substance, the fall in activity of the solution would suggest that the immunizing action of the specific substance was supplemented, in the fresh preparation, by that of another substance, and that it was this other constituent which had been partially or wholly destroyed on standing. According to this explanation the initial content of specific substance in 5 cc. culture would be considered too low to produce the observed immunity by itself, and hence even if it had not been partly destroyed on standing, would continue to remain too low.¹⁶ On the other hand, in view of the direct demonstration that the purified soluble specific substance does immunize

¹⁶ The minimum quantity of purified S.S.S. necessary to immunize rats regularly has not been determined.

rats, the previously mentioned alternative regarding a change in the intramolecular structure of this substance as an explanation for the loss of activity which the solution suffered on standing, seems more significant.

From a practical point of view the use of solutions of pneumococci for oral immunization would appear to be, for the present, limited to relatively freshly made solutions. In distinction to this it has been found that the procedure used in most of the experiments for killing, namely contact with hydrochloric acid, does not appear to cause any loss in activity¹⁷ and that desiccation, a convenient means of keeping the vaccine, is also without effect. These experiments are reported here because the results are what one would expect in view of the demonstration of the important rôle of the specific polysaccharide, and because they supplement the other indirect evidence which points to the fact that this substance is active when rats are immunized by feeding the whole or the dissolved cell. These data show that the degree of protection and the percentage of animals immunized, closely resemble those obtained when freshly centrifuged living pneumococci are fed, and that therefore little or no loss is sustained by the process of killing and drying.

Although previous data had demonstrated that contact for $2\frac{1}{2}$ to 3 hours at room temperature with $N/12$ HCl, as well as desiccation, left the bacteria in a condition capable of immunizing rats, the tests involved the use of such large quantities of organisms that partial destruction of the active material could not readily be observed. In the experiments described below, a single feeding of the bacteria from 5 cc. was employed (in one case 1 cc. as well), since, as mentioned above, this is the smallest quantity which has been found to give uniform immunity equivalent to 1000 to 10,000 fatal doses of pneumococci, 48 to 72 hours after feeding.

Influence of Contact with $N/12$ HCl Solution and Desiccation.—The pneumococcus was grown in glucose meat extract broth, pH 7.6 to 7.8. After the culture had reached room temperature, normal HCl solution was added to make $N/12$ and the whole left for $2\frac{1}{2}$ hours. After centrifugation, a portion of the bacterial sediment was put in the ice box for use 2 days later. The remainder was spread in a thin layer, dried over $CaCl_2$ in an evacuated desiccator and ground up in a mortar.

¹⁷ At least, with respect to degree and time of appearance of the immunity, as well as to the percentage of animals protected.

Table XX gives the data and shows that treatment with acid, and desiccation, did not affect the degree of immunity obtained nor the percentage of animals protected. They are about the same as result from the ingestion of untreated organisms.

TABLE XX

Immunizing Effect of a Single Feeding of Desiccated Acid-Killed Organisms. (Rats)

Dose	Moist bacteria kept 2 days in ice box. 48 hrs. after feeding			Desiccated bacteria					
	C rats	E rats fed bacteria from		48 hrs. after feeding			72 hrs. after feeding		
		5 cc.	1 cc.	C rats	E rats fed bacteria from		C rats	E rats fed bacteria from	
					5 cc.*	5 cc.°		5 cc.*	5 cc.°
cc.									
10 ⁻⁹	S. 2			S.			S.		
10 ⁻⁸	2. 2		3. 3	S. 3			3. 3		
10 ⁻⁷	2	S. S.	S. 2	5.	S. S.	S. S.	2. 6	S. S.	S. S.
10 ⁻⁶	3	S. S.	S. 2	3.	S. S.	S. 4.	2. 5	S. 3.	S. 9.
10 ⁻⁵		S. 2	S.	4.	S. S.	S. 4.	6	S. S.	S. S.
10 ⁻⁴		S. 2			3. 6	S. 4		S. 3	S. 2
10 ⁻³		2							

* Suspension of bacteria prepared directly before feeding.

° The bacteria were ground up with water in a mortar and left to soak overnight before feeding.

TABLE XXI

Immunizing Effect of a Single Feeding of Pneumococci Kept in Contact with N/12 HCl for 24 Hours at Room Temperature and Then Dried

Dose	24 hrs. after feeding		48 hrs. after feeding		72 hrs. after feeding	
	C	E	C	E	C	E
cc.						
10 ⁻⁹	S. S	S	S. S		2. 2	
10 ⁻⁸	2. 2	S. 4	2. 2		3. 2	
10 ⁻⁷	3	3. 2	2	3	3	S
10 ⁻⁶	2	2	1	S. 2	2	S. 3
10 ⁻⁵		2		S. 3		S. 3
10 ⁻⁴				S		2
10 ⁻³				2		3

Each E rat was fed the bacteria from 5 cc. growth.

In another experiment (Table XXI) the culture in glucose meat extract broth was treated with normal HCl to make N/12 and left for 24 hours at room temperature. After centrifugation, the bacterial sediment was left in the desiccator over CaCl₂ at atmospheric pressure for 15 hours, at which time most of it was still wet.

It was then dried in a current of air from an electric fan and powdered. The results, 48 hours after feeding, are somewhat poorer than in the preceding experiment, but 72 hours following the ingestion of the bacteria there is protection against 1000 to 10,000 fatal doses.¹⁸ Little, if any loss resulted from this relatively severe treatment.

The absence of destructive action by the acid on the immunizing substance is in accord with the known resistant behavior of the polysaccharide toward acid at room temperature. Desiccation proves to be, as one would expect, also without effect.

DISCUSSION

Whether or not the soluble specific substance is the only constituent of the Pneumococcus Type I, which is responsible for the immunity which follows the oral administration of this organism to rats, is at present unknown. However, the feeding experiments with this substance illustrate the important rôle which it doubtless plays. A single feeding of either the specific polysaccharide, or of the organisms, elicits the increased resistance. In some of the experiments with the former, the degree of protection and the percentage of animals immunized are so like those obtained when the entire cell is fed, as to be almost indistinguishable from them. There is a marked similarity with regard to the duration of the protection as well. Moreover, when the effect of ingesting the specific substance has worn off, an additional feeding once more brings it out. This is exactly what happens when the pneumococci are administered.

Resistance to the intestinal enzymes became an obvious property of the active constituent of the pneumococcus when immunity was obtained by feeding this organism. Other characteristics assigned to the substance were (a) its solubility in and resistance to, bile salt solutions, (b) resistance to heating at 60°C. for 1 hour in the presence of small amounts of acid, (c) type specificity, (d) considerable destruction when heated at 80°C. for 2 hours in the presence of the acid of the culture,¹⁹ (e) ready passage through the Berkefeld filter, (f) resistance to N/12 HCl at room temperature for 3 hours and (g) resistance to

¹⁸ It has been shown elsewhere that an occasional rat is protected 24 hours after feeding and vaccine.

¹⁹ The pH of the glucose-meat extract culture is approximately 4.5.

desiccation. The last two have been examined more carefully in the present work and have been confirmed. These properties are consistent with the known properties of the specific substance.

The failure to immunize mice by feeding the soluble specific substance, as opposed to the fair amount of success obtained with the whole or dissolved organism, may be considered as evidence for the presence of a second substance which is active. The partial loss of immunizing action sustained by the sodium glycocholate solution of pneumococcus on standing, may possibly be interpreted in the same way. An alternative explanation which would account for both of these observations, requires a change in the intramolecular configuration of the specific substance during its isolation and purification, and on standing in solution, accompanied by the retention of the precipitin reaction and a diminution in immunizing activity. The new form would supposedly immunize rats less well than the unaltered polysaccharide contained in the intact or freshly dissolved cell, and in the case of mice which are protected less regularly than rats by either of the latter means, would confer no protection. The absence of such a change in the whole organisms treated with N/12 HCl for 24 hours at room temperature could be ascribed to the fact that the rearrangement takes place more readily when the specific substance is separated from the cell. Such an assumption can be tested by determining the optical activity of a solution of purified Type I polysaccharide (presumably already partly changed) at intervals, and correlating it with the minimum quantity necessary to produce immunity when fed to rats, since a change in optical rotation is generally ascribed to a change in internal configuration of the molecule. It is interesting to note that Heidelberger, Goebel and Avery (3) have found that a sample of the specific substance of Type III pneumococcus with an initial $[\alpha]_D$ of -34.1° showed a drop in alkaline solution to -21.2° with a return to -36.0° on acidification.

SUMMARY

1. Feeding the purified soluble specific substance of Type I pneumococcus protects rats against an intraperitoneal injection of the virulent organism.

2. This increased resistance resembles that obtained when the intact

(dead) or dissolved bacteria are fed, as follows: (a) one feeding is sufficient, (b) the interval between the feeding and the appearance of the immunity is the same, (c) the duration is approximately the same, (d) when the immunity is exhausted it can be renewed by a new feeding, (e) the immunizing action is type-specific.²⁰

3. The differences between the effects of feeding the purified specific substance and the intact or dissolved organism to rats, appear to be quantitative rather than qualitative, the proportion of animals protected and the height of the immunity being generally, though not always, less in the case of the former.

4. In contrast to the immunizing action which the soluble specific substance possesses when administered to rats, feeding it to mice failed to protect them. Neither were mice definitely immunized by parenteral administration.

5. A sodium glycocholate solution of *Pneumococcus* Type I lost part of its immunizing activity on standing for 1 year.

6. The failure to immunize mice and the loss of activity of the bile salt solution of pneumococcus, on standing, are discussed in terms of (a) the possible presence of a second cell constituent which is active by mouth, and (b) a possible intramolecular change in the type-specific polysaccharide associated with a loss of immunizing action while retaining the precipitin reaction.

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²⁰ In the paper, "The rôle of the soluble specific substance in oral immunization against *Pneumococcus* Types II and III," in the next number, it is shown that feeding of Type I specific substance does not protect against Types II or III pneumococcus.