A STUDY OF THE THERAPEUTIC MECHANISM OF ANTI-PNEUMOCOCCIC SERUM ON THE EXPERIMENTAL DERMAL PNEUMOCOCCUS INFECTION IN RABBITS

II. A Comparison of the Therapeutic Effect of Unrefined, Antipneumococcic Serum with That of Its Various Protein Fractions: the Rôle of the Non-Antibacterial Factor

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In a preceding communication (1) it was shown that antipneumococcic serum contains, in addition to the antibodies against the various bacterial constituents, a so called non-antibacterial factor which apparently is capable of playing an important rôle in pneumococcus infection. The therapeutic effectiveness of this factor was demonstrated on the experimental, dermal pneumococcus infection of rabbits in serum from which the antibacterial bodies were removed by absorption with the homologous, virulent, heat-killed pneumococci. The immunological nature and the mode of action of the non-antibacterial factor are still obscure.

Since fractionation of antipneumococcic serum by various methods showed that almost all of its mouse protective potency was associated with the water-insoluble globulin, refined preparations, which are at present used and recommended by many for the treatment of certain types of pneumococcus pneumonia in man, contain this fraction only. On the other hand, it is known that diphtheria and tetanus antitoxin, for example, are for the most part associated with the water-soluble globulin, and that in the refined and concentrated preparations of antiscarlatinal serum, both the water-soluble and water-insoluble globulins are included. The purpose of the investigations reported in this communication was first, to determine the distribution of the

non-antibacterial factor of antipneumococcic serum among its various protein fractions, partly as an aid in establishing its nature by correlation with the distribution of the known immune bodies; and second, to observe and compare the effect of the different fractions and of the whole serum on the experimental dermal pneumococcus infection in rabbits.

EXPERIMENTAL

The details and procedures of the dermal pneumococcus infection as used in these experiments, were described in the first communication. The same virulent strain of Type I Pneumococcus was employed and the same qualifications and acknowledgments obtain here. Two additional problems presented themselves in the following experiments: One dealt with the manner of determining the concentration of the non-antibacterial factor, and the other with a suitable and reliable method of titrating the therapeutic effectiveness of a preparation.

Although it was previously shown that a certain number of rabbits are protected when treated with the non-antibacterial factor alone, this effect could not be used as a criterion on account of its obvious irregularity. The complementary effect of this factor when administered with a certain, constant, definitely subeffective dose of serum, appeared to be the more suitable, available criterion. Although the virulence of the culture was such that with the number of organisms injected none of the untreated rabbits survived, the resistance of different rabbits appears to vary to such an extent that some when treated are protected by a dose which is ineffective for most, whereas certain others are not protected by a dose effective for most. The term subeffective is used to designate a dose which contains an adequate amount of antibacterial bodies, expressed in units of mouse protective antibody, but an insufficient concentration of the non-antibacterial factor, with the result that the majority of rabbits do not survive. The standard subeffective dose selected for this work contained 300 mouse protective units in 0.3 cc. of serum, and of various groups of rabbits treated with this dose 60 to 80 per cent died as instanced by the fact that of groups of five rabbits tested with this dose on various occasions, three always died and sometimes four. For titrating the concentration of the non-antibacterial factor, varying amounts of the absorbed preparations were injected along with the subeffective dose; a survival of at least three of five rabbits was required to indicate an effect of the added substance. It is realized that an absorbed serum or preparation does not necessarily contain all of the non-antibacterial factor that may be present in the original, for during the process of precipitation and agglutination some may conceivably be carried down and removed with the organisms. However, by employing the same method on all the preparations, the relative values may, nevertheless, be comparable.

For the determination of a minimal effective dose of a serum, Goodner (2) used a single rabbit for each dose, and certain criteria with regard to temperature, lesion,

and bacteremia to determine its efficacy. In the course of the present work, it was observed, however, that many rabbits which do not fulfill those criteria in the specified time or manner, nevertheless survive. If rabbits of uniform susceptibility or resistance could be gotten, it is not improbable that results obtained with single rabbits might be reproducible; but among the rabbits which were available for these experiments such uniformity did not obtain. To overcome largely this variation in resistance, groups of five rabbits were used for each dose; and for a dose to be considered effective at least three of the five rabbits had to survive for 10 days.

Distribution of the Non-Antibacterial Factor among the Protein Fractions of Antipneumococcic Serum

In the fractionation of horse antipneumococcic serum with $(NH_4)_2SO_4$, Banzhaf (3) showed that the protein precipitated at 30 per cent saturation, contained relatively little mouse protective antibody (approximately 10 per cent of the total), whereas that which is precipitated when the concentration of $(NH_4)_2SO_4$ is increased to 50 per cent saturation contains all the rest; and in agreement with Felton's (4) observations, he further showed that the mouse protective antibody in the latter fraction (*i.e.* 30 → 50 per cent saturation with $(NH_4)_2SO_4$) was associated with its water-insoluble protein. It is essential to recall here that in the fractionation of antitoxic sera, most of the antitoxin is found with the water-soluble portion of the same globulin.

With these facts in mind, the distribution of the non-antibacterial factor was studied in two monovalent, Type I antipneumococcic sera.

The following fractions of Serum 54 were isolated for testing: (*a*) that precipitated at 30 per cent saturation with $(NH_4)_2SO_4$, and which, for convenience, will henceforth be called 30 per cent $(NH_4)_2SO_4$ globulin; (*b*) that precipitated by increasing the concentration of $(NH_4)_2SO_4$ to 50 per cent saturation, and to be called the 30 → 50 per cent $(NH_4)_2SO_4$ globulin; this fraction was further subdivided into water-soluble (soluble at a N/100 NaCl concentration) and water-insoluble (insoluble at N/100 NaCl) portions; (*c*) that precipitated by increasing the $(NH_4)_2SO_4$ concentration from 50 per cent to complete saturation; this fraction is commonly called albumen. (The (*a*) and (*b*) fractions were deliberately not designated as euglobulin and pseudoglobulin, because these terms, in their present state of definition as regards serum proteins, convey neither the same meaning nor the same constitution to all people.) The *acid fraction* (*i.e.* insoluble at pH 5.0 and N/20 salt concentration) was removed from the 30 → 50 per cent $(NH_4)_2SO_4$ globulin. Felton (5) first observed that this acid fraction contained very little antibody, and that by removing it from his refined and concentrated preparations the incidence

of "chills and hyperpyrexia" was decreased; Sabin and Wallace (6) in a controlled, experimental study on the nature of the chill-producing principle, confirmed this observation and showed that the chill-producing principle is actually associated to a much greater extent with this acid fraction than with any of the other protein fractions. After isolation, the various globulin fractions of Serum 54 were dissolved each in one-tenth the volume of serum from which they were derived; *i.e.*, they were concentrated ten times by volume.

In testing for the content of non-antibacterial factor, all the fractions, except the albumen, were completely absorbed with concentrated suspensions of heat-killed, virulent, Type I pneumococci; the albumen had no demonstrable agglutinins. Varying amounts of each absorbed fraction and of the absorbed original serum were administered intravenously along with the standard subeffective dose (0.3 cc. of Serum 624), 6 hours after the intracutaneous injection of the culture; as a rule, five rabbits were injected with each dose. On account of the large number of rabbits required for such a test, the titration is not as fine as might be desired.

The data presented in Table I were obtained in four separate tests. 2.0 cc. of absorbed Serum 54 contained sufficient of the non-antibacterial factor to produce a typical effect in conjunction with the subeffective dose. An equivalent dose of 10.0 cc. of the absorbed water-soluble as well as water-insoluble fractions of the 30 → 50 per cent $(NH_4)_2SO_4$ globulin was necessary to produce an effect similar to that exerted by 2.0 cc. of the original serum. With equivalent doses of 5.0 cc. of the 30 per cent $(NH_4)_2SO_4$ globulin and 4.0 cc. of albumin, doubtful effects were exerted; it is not improbable that larger doses would have been effective. Thus the results obtained with the various protein fractions suggest that the non-antibacterial factor is probably distributed among all of them; particularly significant, however, is the fact that there does not appear to be any difference between the quantity present in the water-soluble and water-insoluble fractions. The concentration of the factor in each individual fraction is, relatively, considerably less than in the original. Although the association of the factor with all the protein fractions would necessarily reduce its effective concentration in each one, it does not completely explain the extent of the observed diminution.

In absorbed Serum 348, the activity of the non-antibacterial factor is well demonstrated, 0.5 cc. of it being an effective dose. Three fractions of Serum 348 were tested; (1) The total globulin precipitated by 50 per cent saturation with $(NH_4)_2SO_4$, (2) the 30 per cent $(NH_4)_2SO_4$

TABLE I

Distribution of Non-Antibacterial Factor among Protein Fractions of Antipneumococcic Serum

Fraction added to subeffective dose—0.3 cc. Serum 624	Dose	Equivalent to volume of whole serum	Effect on rabbits	Action of added non-antibacterial factor
	cc.	*cc.*		
Absorbed Serum 54	2.0	2.0	S S S S D_2	Positive
Absorbed 30 per cent $(NH_4)_2SO_4$ Globulin 54	0.5	5.0	D_9? S S D_6 D_6	Doubtful
Absorbed water-insoluble 30 → 50 per cent $(NH_4)_2SO_4$ Globulin 54	0.3	3.0	S D_6 D_6 D > 1 D > 1 } excluded	Negative
	1.0	10.0	S S S S D_8?	Positive
Absorbed water-soluble 30 → 50 per cent $(NH_4)_2SO_4$ Globulin 54	0.3	3.0	S D_4 D_6 D_6 D_8	Negative
	0.75	7.5	S S D_5 D_6	Doubtful
	1.0	10.0	S S S D_8? D_6	Positive

S = survived. D_4 = dead on 4th day. D? = death considered as probably not being due to pneumococcus infection.

TABLE I—*Continued*

Fraction added to subeffective dose—0.3 cc. Serum 624	Dose	Equivalent to volume of whole serum	Effect on rabbits	Action of added non-antibacterial factor
	cc.	*cc.*		
Albumin of Serum 54	4.0	4.0	S S D_6 D_7	Doubtful
Absorbed Serum 348	0.2	0.2	S S D_7 D_9 D_{10}	Negative
	0.5	0.5	S S S D_{11}? D_3	Positive
	1.0	1.0	S S S S S	Positive
Absorbed 50 per cent $(NH_4)_2SO_4$ total Globulin 348	0.5	0.5	S S D_4 D_5 D_7	Negative
	1.0	1.0	S D_3 D_5 D_7 D_{10}	Negative
Absorbed 30 per cent $(NH_4)_2SO_4$ Globulin 348	1.0	1.0	S D_3 D_5 D_7 D_{10}	Negative

TABLE I—*Concluded*

Fraction added to subeffective dose—0.3 cc. Serum 624	Dose	Equivalent to volume of whole serum	Effect on rabbits	Action of added non-antibacterial factor
	cc.	*cc.*		
Absorbed Serum 348	0.5	0.5	S S S S D_8	Positive
Absorbed 50 per cent $(NH_4)_2SO_4$ total Globulin 348	2.5	2.5	S S S S D_7	Positive
Albumin of No. 348	1.0	1.0	S S S D_4 D_5	Positive
	5.0	5.0	S S S D_7 D_9	Positive
None	0	0	S S S D_5 D_6 D_6 D_6 D_7 D_9 D_{10}	

globulin, and (3) the albumen. The fractions were dissolved in the same volume of 1 per cent NaCl as that of the serum from which they were derived. Of the total globulin, which represented approximately 65 per cent of the total protein nitrogen of the serum, twice the minimal effective dose of the original serum failed to produce the typical

effect of the non-antibacterial factor, whereas five times that dose did. The albumen which was added without preliminary absorption (because it contained no agglutinins) also increased the effectiveness of the subeffective dose. However, the method used for determining the concentration of the non-antibacterial factor is probably inadequate; the chief source of probable error may perhaps be associated with the necessary absorption procedure, which may conceivably remove certain, varying amounts of the factor in the precipitate which forms on the bacteria. For this reason the results of this experiment are regarded primarily from a qualitative point of view; as such they show the association of the non-antibacterial factor with protein fractions which contain little or none of the mouse protective antibody. This distribution of the factor in no way corresponds to that of the known antitoxins.

The Relationship between the Antibacterial and Non-Antibacterial Factors in the Therapeutic Effect Exerted by Whole Antipneumococcic Serum and Some of Its Globulin Fractions

The results of the preceding experiment showed that the non-antibacterial factor apparently is associated with almost all the protein fractions of the serum, and with the method used for its determination it appeared as though there might be no marked difference in its quantitative distribution. Since, however, approximately 90 per cent of the antibacterial bodies are associated with the water-insoluble fraction of the 30 → 50 per cent $(NH_4)_2SO_4$ globulin, the ratio of the antibacterial to the non-antibacterial factor would be expected to vary in the different fractions. Thus if the therapeutic effect of a given dose were to depend primarily upon its content of antibacterial bodies, one would expect the amounts of the different fractions required for a minimal effective dose (M.E.D.) to be roughly proportional to the number of mouse protective units; on the other hand, if the therapeutic effect depended largely on the non-antibacterial factor, the amounts required for 1 M.E.D. should be approximately the same for the different fractions.

The monovalent, Type I Serum 54 and the globulin fractions used in the preceding experiment were tested for their content of mouse

protective antibody and for the minimal dose effective against the experimental, dermal pneumococcus infection in rabbits.

In the mouse protection test at least 5 to 10 mice were used for each dose and the unit represents ten times the smallest amount of serum required to protect a majority of the mice for at least 96 hours, the serum being injected intraperitoneally simultaneously with 100,000 M.L.D.'s of a fully virulent culture. In determining the minimal effective dose (M.E.D.) on rabbits, the serum was administered intravenously 6 to 7 hours after the intracutaneous injection of the organisms. Although the severity of the lesions, and the course of the temperature and bacteremia were observed, they served merely as a qualitative index, the final criterion of effectiveness being a survival of a majority of the rabbits for at least 10 days. When only three rabbits were used for each dose, as in a preliminary test, all three had to survive; with five rabbits per dose, the survival of at least three also constituted the result of an effective dose.

The data are presented in Table II. The M.E.D. of the original serum was approximately 0.6 cc., this dose containing 600, < 900 m. p. u. (mouse protective units). The M.E.D. of the water-insoluble fraction of the 30 → 50 per cent $(NH_4)_2SO_4$ globulin was not more than 0.2 cc., containing 800, < 2,000 m. p. u.; the M.E.D. of the water-soluble fraction of the same globulin was 1.0 cc., containing 40, < 60 m. p. u.; and the M.E.D. of the 30 per cent $(NH_4)_2SO_4$ globulin was approximately 0.25 cc., containing 200, < 300 m. p. u. In comparing the doses of the original serum with those of the globulin fractions, it should be recalled that the latter were dissolved in one-tenth the original volume. An analysis of the data reveals that the amounts required for 1 M.E.D., as well as the m. p. u. content of 1 M.E.D., vary markedly for the different globulin fractions and the original serum. These differences are striking, even if one should allow 100 per cent experimental variation, and are best observed between the water-soluble and water-insoluble fractions of the 30 → 50 per cent $(NH_4)_2SO_4$ globulin. 200, < 500 m. p. u. in 0.05 cc. of the water-insoluble fraction is definitely ineffective, whereas 40, < 60 m. p. u. in 1.0 cc. of the water-soluble fraction is fully effective. Similarly 1 M.E.D. of the 30 per cent $(NH_4)_2SO_4$ globulin has a m. p. u. content which is in the ineffective range of both the water-insoluble globulin and the original serum. It is thus quite obvious that even in a comparison of antipneumococcic serum and the protein fractions derived from it, the therapeutic effectiveness

TABLE II

Rôle of Antibacterial and Non-Antibacterial Factors of Antipneumococcic Serum

Preparation	Experiment	Dose		Rabbit No.	Dermal lesion	Result	Postmortem cultures	
							Heart's blood	Brain
		cc.	*m.p.u.*					
Original Serum 54 Type I		0.45	450, < 675	1	Slight	S		
				2	Moderate	D_9	Sterile	Sterile
				3	Marked	S		
				4	Marked	D_7	Sterile	Sterile
				5	Marked	D_7	Pneumococcus	Sterile
		0.60	600, < 900	6	Slight	S		
				7	Moderate	S		
				8	Marked	S		
				9	Moderate	D_4	Pneumococcus	Sterile
				10	Marked	D_8	Sterile	Sterile
Globulin precipitated at 30 → 50 per cent saturation with $(NH_4)_2SO_4$		0.20	800, < 2,000	11	Very slight	S		
				12	Very slight	S		
				13	Moderate	S		
Water-insoluble fraction dissolved in 1/10 the original volume (without acid fraction)	*b*	0.40	1600, < 4,000	14	Very slight	(D_3)?	Sterile	Sterile
				15	Very slight	S		
				16	Marked	D_4	Pneumococcus	Sterile
		0.60	2400, < 6,000	17	Marked	D_7	Pneumococcus	Sterile
				18	Very marked	D_6	Pneumococcus	Pneumococcus
				19	Very marked	S		

Globulin precipitated at 30 → 50 per cent saturation with $(NH_4)_2SO_4$		0.05	200, < 500	20	Moderate	D_9	Pneumococcus	Sterile
				21	Marked	D_9	Pneumococcus	Sterile
				22	Very slight	S		
Water-insoluble fraction dissolved in 1/10 the original volume (without acid fraction)				23	Marked	D_9	Pneumococcus	Sterile
				24	Moderate	D_7	Sterile	Sterile
		0.20	800, < 1,600	25	Very slight	S		
				26	Slight	S		
	c			27	Moderate	S		
				28	Moderate	S		
				29	Moderate	D_8	Sterile	Sterile
		0.60	2400, < 6,000	30	Slight	S		
				31	Moderate	D_6	Pneumococcus	Pneumococcus
				32	Marked	S		
				33	Marked	S		
				34	Marked	S		
Globulin precipitated at 30 → 50 per cent saturation with $(NH_4)_2SO_4$		0.75	30, < 45	35	Moderate	D	Pneumococcus	Pneumococcus
				36	Very slight	(D_5)?	Sterile	Sterile
				37	Marked	D	Sterile	Sterile
Water-soluble fraction dissolved in 1/10 the original volume		1.50	60, < 90	38	Very slight	S		
	b			39	Moderate	S		
				40	Very slight	(D_6)?	Sterile	Sterile
		3.00	120, < 180	41	Very slight	S		
				42	Moderate	S		
				43	Very slight	S		

m. p. u. = mouse protective units. S = survived. D_3 = dead on 3rd day. (D) = death considered as not being due to pneumococcus.

TABLE II—*Concluded*

Preparation	Experiment	Dose		Rabbit No.	Dermal lesion	Result	Postmortem cultures	
							Heart's blood	Brain
		cc.	*m.p.u.*					
Globulin precipitated at 30 → 50 per cent saturation with $(NH_4)_2SO_4$ Water-soluble fraction dissolved in 1/10 the original volume	*c*	1.00	40, < 60	44	Marked	S	Pneumococcus	Sterile
				45	Marked	D_6		
				46	Marked	S		
				47	Marked	S		
				48	Marked	S		
Globulin precipitated at 30 per cent saturation with $(NH_4)_2SO_4$ dissolved in 1/10 the original volume	*c*	0.25	200, < 300	49	Moderate	S	Pneumococcus	Pneumococcus
				50	Slight	D_6		
				51	Moderate	D_2	Pneumococcus	Sterile
				52	Slight	S		
				53	Slight	S		
		0.50	400, < 600	54	Moderate	S		
				55	Very slight	S		
				56	Moderate	S		
				57	Very slight	(D_2)?	Sterile	Sterile
				58	Very slight	S		

does not depend solely upon the concentration of mouse protective antibody, an observation which is fully in agreement with those made in the first communication. When the volumes and the m. p. u. content of 1 M.E.D. are correlated, one finds that the lower m. p. u. values are associated with almost proportionately higher volumes, and *vice versa*. Of the globulin fractions, which contain relatively little mouse protective antibody, like the water-soluble or 30 per cent $(NH_4)_2SO_4$, the volume of 1 M.E.D. is greater than that of the water-insoluble protein but the m. p. u. content, instead of being about the same, is almost proportionately less. It can be stated, therefore, that when a given dose contains a relatively large amount of one factor (antibacterial or non-antibacterial), a relatively smaller amount of the other is required and *vice versa*. Thus, the therapeutic effectiveness of antipneumococcic serum or its protein fractions is found to depend not upon the absolute concentration of either the antibacterial or the non-antibacterial bodies, but upon the relative concentration of the two, an abundance of one apparently making up for the scarcity of the other.

Effect of Whole Antipneumococcic Serum and of Various of Its Protein Fractions on the Experimental Dermal Pneumococcus Infection in Rabbits

In the treatment of Types I and II pneumococcus pneumonia in man, the use of refined and concentrated preparations of antipneumococcic serum has been quite generally accepted, although Cole (7) of the Hospital of The Rockefeller Institute for Medical Research still advocates the use of whole unrefined serum for various reasons which he has set down. Chief among the methods of refining and concentrating antipneumococcic serum are those of Felton and of Banzhaf. Whichever of Felton's methods is used, the final preparation contains only the water-insoluble globulin. Banzhaf (3) early attempted to achieve further purification by excluding also the globulin precipitated by 30 per cent saturation with $(NH_4)_2SO_4$ or by 12.5 per cent Na_2SO_4. The exclusion of this fraction by Banzhaf and of the albumen and total water-soluble globulin in all the other methods, is based upon the observation that these fractions contain very little or none of the so called protective antibody as determined by the mouse protection test.

In the preceding communication, as well as in the preceding experiments of the present communication, it has been shown that the therapeutic action of antipneumococcic serum is dependent not only upon this mouse protective antibody but also upon other factors which are apparently non-antibacterial. This consideration brings up certain questions which have a direct bearing upon the nature of the preparations to be used in the therapy of pneumococcus pneumonia in man. One question, naturally, is whether by excluding all the proteins, except the water-insoluble globulin, from the refined preparations one does not also exclude factors which may play an important part in overcoming the infection. Experiments thus far show that as regards the non-antibacterial factor, although it is associated with the water-insoluble globulin, it is also associated, and apparently to the same degree, with the water-soluble globulin and probably with the albumen as well.

It thus appeared desirable to reinvestigate the fractionation of antipneumococcic horse serum, determining the therapeutic value of the various fractions by testing against the experimental dermal pneumococcus infection of rabbits. From a practical point of view the immediate question was whether or not a concentrated, refined preparation, containing the water-insoluble globulin only, was therapeutically as efficacious as potent whole serum. In a comparison of such a preparation with an unrefined serum of approximately similar mouse protective potency, the former appeared to be not only as good but even better than the latter. It thus appears that the water-insoluble globulin may contain all the factors of the unrefined serum, in so far at least as one may judge from their effects on the dermal infection of rabbits; the result, furthermore, is not as paradoxical as it may seem when one considers the possibility that different sera may vary in their content of non-antibacterial factor. However, to determine what loss, if any, results from the exclusion of the water-soluble globulin, the 30 per cent $(NH_4)_2SO_4$ globulin, and the albumen, from refined serum preparations, it is necessary to study the effect of the various fractions separately and to compare them with the original, unrefined serum from which they were derived. Such a study was undertaken with Serum 348, a monovalent Type I serum, containing at least 2,000 to 3,000 mouse protective units per cc.

The serum was fractionated in three different ways: (1) Standard fractionation with $(NH_4)_2SO_4$. (2) Preliminary dialysis and removal of acid fraction (Banzhaf-Klein technique (8)), with subsequent precipitation by $(NH_4)_2SO_4$. (3) Preliminary removal of acid fraction as in (2) and subsequent precipitation with sodium-magnesium sulfate.

1. Standard Fractionation with $(NH_4)_2SO_4$.—(*a*) One portion of Serum 348 was precipitated by 50 per cent saturation with $(NH_4)_2SO_4$. From half of the resulting total globulin, after dialysis, the total water-insoluble globulin was obtained by dilution with distilled water. (*b*) Another portion of Serum 348 was precipitated by 30 per cent saturation with $(NH_4)_2SO_4$; to the filtrate sufficient $(NH_4)_2SO_4$ was added to increase the saturation to 50 per cent (30 → 50 per cent $(NH_4)_2SO_4$ globulin). After dialysis, these globulin fractions were dissolved in the same volume of 1 per cent NaCl as the volume of serum from which they were derived. From half the 30 → 50 per cent $(NH_4)_2SO_4$ globulin, the water-insoluble fraction was obtained by dilution with fifteen volumes of distilled water; after centrifugation the supernatant liquid was poured off and precipitated by 50 per cent saturation with $(NH_4)_2SO_4$, the precipitate containing the water-soluble globulin.

2. Preliminary Removal of Acid Fraction; Subsequent Fractionation with $(NH_4)_2SO_4$.—The acid fraction has already been referred to as that globulin fraction, which is insoluble at pH 5.0 and a N/20 salt concentration, and which has been found to have associated with it most of the chill-producing principle, when that principle is present in the original serum. In the Banzhaf-Klein technique this fraction is removed by dialysis of the original serum against running water for 3 to 4 days, and by adjusting the dialyzed solution to a salt concentration of N/20 and a pH 5.0. After thus removing the acid fraction from a quantity of Serum 348, we did not follow their technique further; the remaining solution was divided in two lots. One lot was then precipitated with $(NH_4)_2SO_4$ as in Paragraph 1 (*a*); the other was saved for Experiment 3.

3. Fractionation with 47.5 Per Cent Sodium-Magnesium Sulfate.—For the fractionation of antityphoid serum, Reiner and Shwartzman (9) used 47.5 per cent sodium-magnesium sulfate as the equivalent of saturated $(NH_4)_2SO_4$ and found the antibody, that they were studying, in what corresponds to the fraction obtained with 30 → 50 per cent $(NH_4)_2SO_4$ saturation. They preferred the use of the sodium-magnesium sulfate to that of $(NH_4)_2SO_4$, because of ease with which nitrogen determinations could be made, and employed it instead of Na_2SO_4, because it could be used at room temperature.

To the lot of serum saved as described in Paragraph 2, sufficient of the 47.5 per cent sodium-magnesium sulfate solution was added for a final concentration of 14 per cent of the salt by weight, which should correspond approximately to 30 per cent saturation with $(NH_4)_2SO_4$. To the filtrate more of the sodium-magnesium sulfate was added to increase its concentration to 24 per cent, or what should correspond to 50 per cent saturation with $(NH_4)_2SO_4$.

The volumes of all fractions are comparable with that of the original serum. As a rule, five rabbits were used for each dose. In several instances when the outcome appeared doubtful the test was repeated. The same procedure, previously outlined for the rabbit test, was followed. On account of the large volume of data, Table III indicates only the survival or death of the rabbits used.

Original Serum 348.—The original serum proved to be effective in a dose as low as 0.075 cc. With this dose, although all the dermal lesions were very severe, three of the five rabbits survived with a drop in temperature by crisis between the 2nd and 4th days and rather slow resolution of the lesions. In the rabbits that died the temperature remained high until the end, one dying with, and the other without a bacteremia. Excepting the death of an occasional rabbit, with a resistance apparently lower than that of the average, there was no indication of a zone phenomenon in the higher doses.

50 Per Cent $(NH_4)_2SO_4$ Globulin.—This fraction represents the total globulin of the serum. Paradoxical as it may seem, this fraction, in equivalent volume, in spite of a certain unavoidable loss incurred by the necessary manipulations of the process, actually proved to be more potent than the original. Although an end-point was not reached, the lowest dose tested, 0.05 cc., gave the same result as 0.075 cc. of the original serum. With 0.075 cc. of the total globulin, the rabbits responded decidedly better than with the same dose of the original serum, the dermal lesions being very slight and the temperatures dropping to normal within 1 day.

50 Per Cent $(NH_4)_2SO_4$ Globulin: Water-Insoluble Fraction.—This fraction represents the entire water-insoluble globulin and differs from the total globulin, just described, by the exclusion of the total water-soluble portion. It is interesting to note that the water-insoluble globulin alone appears to be as potent as the original serum, although when compared with the total globulin it is decidedly less effective both quantitatively and qualitatively, the lesion and temperature being much better controlled with the latter.

30 → 50 Per Cent $(NH_4)_2SO_4$ Globulin: Total.—The purpose of testing this fraction was to determine the effect of excluding the globulin, which is precipitated by 30 per cent saturation with $(NH_4)_2SO_4$. The potency of this fraction is definitely less than either the original

serum or the total globulin, its M.E.D. probably being about 0.1 cc. A peculiar zone phenomenon presented itself. In the first test, the 0.2 and 0.15 cc. doses were definitely ineffective, whereas the 0.1 cc. dose was fully effective; not only did fewer rabbits survive with those doses, but the dermal lesions and the course of the disease were considerably worse. The test was repeated with 0.3 and 0.1 cc. The rabbits receiving the higher dose were again definitely worse. This fraction was then isolated from another portion of Serum 348, and after pressing and absorbing away most of the excess $(NH_4)_2SO_4$, it was dissolved by the remaining imbibed salt and the addition of distilled water, instead of by 1 per cent NaCl after dialysis of the $(NH_4)_2SO_4$. Tests performed with 0.2 and 0.1 cc., showed the former to be the M.E.D.; no zone phenomenon was now observed.

30→50 Per Cent $(NH_4)_2SO_4$ Globulin: (a) Water-Insoluble Fraction.— This fraction excludes both the 30 per cent $(NH_4)_2SO_4$ globulin and the water-soluble portion of the 30 → 50 per cent $(NH_4)_2SO_4$ globulin. It is the globulin which constitutes the refined preparations made according to Banzhaf's technique. The results of the rabbit tests obtained with this water-insoluble fraction are almost identical with those of the total 30 → 50 per cent $(NH_4)_2SO_4$ globulin; it has the same M.E.D. and the same zone phenomenon.

This zone phenomenon apparently differs from the prozone sometimes observed in agglutination and other immunological, *in vitro*, reactions, as well as in the mouse protection test, in that the effective zone is so narrowly limited. Another peculiarity requiring explanation is the fact that the original serum, the total globulin, and the total water-insoluble globulin did not exhibit this phenomenon in the same range of dosage; it appears as though the removal of the 30 per cent $(NH_4)_2SO_4$ fraction had upset some equilibrium which prevented this zone phenomenon. An examination of the results obtained with the 30 → 50 per cent $(NH_4)_2SO_4$ fraction of Serum 54 (see Table II) suggests the operation of a similar phenomenon there, for the lesions and course of the rabbits treated with 0.6 cc. were definitely worse than in those treated with 0.2 cc. It cannot be stated how common this zone phenomenon may be, but it is obvious that at least in this instance, the 30 → 50 per cent $(NH_4)_2SO_4$ globulin, either total or water-insoluble, would be quite undesirable for therapeutic purposes.

TABLE III

Effect of Whole Antipneumococcic Serum and of Various of Its Protein Fractions on the Experimental Dermal Pneumococcus Infection in Rabbits

Preparation	Dose in cc.										
	1.0	0.75	0.60	0.50	0.40	0.30	0.20	0.15	0.10	0.075	0.05
Original Serum 348 Type I			S			S		S	S S	Dermal lesions marked: S	S
			S			S		S	S S	Dermal lesions marked: S	S
			S			S		S	$S(D_{12})$ S	Dermal lesions marked: S	D_6
			S			S		D_5	D_3 S	Dermal lesions marked: D_5	D_6
			(D_1)?			D_5		D_5	D_8 D_6	Dermal lesions marked: D_8	D_7
50 per cent $(NH_4)_2SO_4$ globulin; total							S	S	S	Dermal lesions very slight: S	Dermal lesions marked: S
							S	S	S	Dermal lesions very slight: S	Dermal lesions marked: S
							S	S	S	Dermal lesions very slight: S	Dermal lesions marked: S
							S	S	S	Dermal lesions very slight: S	Dermal lesions marked: D_5
							S	S	$S(D_{12})$	Dermal lesions very slight: $S(D_{11})$	Dermal lesions marked: D_7
50 per cent $(NH_4)_2SO_4$ globulin; water-insoluble fraction							S	S	S	Dermal lesions marked: S	S
							S	S	S	Dermal lesions marked: S	D_{10}
							S	S	S	Dermal lesions marked: S	D_7
							$S(D_{12})$	D_{10}	S	Dermal lesions marked: D_4	D_4
							D_5	D_7	S	Dermal lesions marked: D_5	D_4
30 → 50 per cent $(NH_4)_2SO_4$ globulin; total						S*	S† S‡	S†	S† S* S‡	S	
						S	S S	S	S S S	S	
						D_2	D_4 S	D_3	S S D_4	D_{10}	
						D_6	D_4 S	D_5	S D_6 D_7	D_7	
							D_6 D_6	D_6	D_4 D_8	D_6	

30 → 50 per cent $(NH_4)_2SO_4$ globulin; water-insoluble fraction						S D_2 D_4	S S D_4 D_4 D_5	S S D_3 D_5 D_8	S S S S S S D_5 D_6 D_9	S D_{10} D_7 D_5	
30 → 50 per cent $(NH_4)_2SO_4$ globulin; water-soluble fraction	S S S D_4 D_9	S S S S D_8		S S S D_{10} D_7		S D_5 D_9					
30 per cent $(NH_4)_2SO_4$ globulin; total			S S S D_{10} D_6			S S S D_7					
50 per cent $(NH_4)_2SO_4$ globulin without acid fraction			S S S S S S D_4 D_4 D_5 (D_9)?			S S S S S S S D_4 D_{10} D_7		$S(D_{12})$ S D_4 S D_4 D_5 D_7 D_6 D_8 (D_4)?			
50 per cent $(NH_4)_2SO_4$ water-insoluble globulin without acid fraction			S S S S S D_7 D_2 D_4 D_2 D_7			S S S D_4 S D_9 S (D_4)? D_4 (D_1)?		S S S S S S S S D_8 D_9			

S = survived. D_3 = dead on 3rd day. (D)? = death considered as not being due to Pneumococcus.

No more than five rabbits per dose were used in a single test; where more than one column of results appears for a dose, it indicates a repetition of the test.

*,†,‡ These signs are to indicate the groups of rabbits which were tested simultaneously.

TABLE III—*Concluded*

Preparation	Dose in cc.										
	1.0	0.75	0.60	0.50	0.40	0.30	0.20	0.15	0.10	0.075	0.05
14 per cent sodium-magnesium sulfate globulin			D_3			S		S			
			D_3			S		S			
			D_5			S		S			
			D_7			S		D_4			
			D_7			(D_8)?		D_7			
14 → 24 per cent sodium-magnesium sulfate globulin					S		S		S		
					$S(D_{11})$		$S(D_{12})$		S		
					D_3		D_6		D_5		
					D_6		D_6		D_6		
					D_7		(D_5)?		D_8		

It is interesting to note that agglutination tests with the fraction of Serum 348 (Table IV), all performed at the same time with the same antigen, showed neither a zone phenomenon, nor any parallelism with the therapeutic results obtained on the rabbits. The agglutination tests were better correlated with the mouse protection tests, which also showed no zone phenomenon; in the mouse protection test, as contrasted with the results obtained in the rabbits, the 30 → 50 per cent $(NH_4)_2SO_4$ globulin was more potent than the total globulin.

TABLE IV

Agglutination Test with Protein Fractions of Serum 348

Preparation	Dilution of preparation before addition of antigen								
	Undiluted	1:2	1:5	1:10	1:20	1:40	1:60	1:80	1:100
Original Serum 348.....	++++		++++	++++	++++	++++	+	−	−
50 per cent $(NH_4)_2SO_4$ globulin; total.....	++++		++++	++++	+++	±	−	−	−
50 per cent $(NH_4)_2SO_4$ globulin; water-insoluble.....		+++		++++	+++	−	−	−	−
30 → 50 per cent $(NH_4)_2SO_4$ globulin; total.....	++++		++++	++++	++++	+++	+	−	−
30 → 50 per cent $(NH_4)_2SO_4$ globulin; water-insoluble..		++++		++++	++++	+	±	−	−
30 → 50 per cent $(NH_4)_2SO_4$ globulin; water-soluble....		+++	−	−	−	−	−	−	−
30 per cent $(NH_4)_2SO_4$ globulin.	++++	++	±	−	−	−	−	−	−

++++ = complete agglutination. + = very slight agglutination. ± = doubtful agglutination.

30 → 50 Per Cent $(NH_4)_2SO_4$ Globulin: (b) Water-Soluble Fraction.— It is important to observe that this fraction, which is not the total water-soluble globulin, and which has very little of the antibacterial bodies associated with it, contains approximately 10 to 15 per cent of the potency of the original serum. It is also essential to recall that in the processes of refining and concentrating serum at present in use, this fraction would be discarded, although it is, by itself, quite as efficacious as some sera which would be considered good, and suitable for therapeutic purposes (see Serum 54, Table II).

30 Per Cent $(NH_4)_2SO_4$ Globulin.—Although an end-point was not reached in the test on this fraction, it is apparent that at least 25 per cent of the potency of the original serum is associated with it.

Removal of Acid Fraction from Whole Serum, Preliminary to Salt Fractionation.—Since the removal of the acid fraction is desirable in certain instances, it was essential to observe the effect which various ways of eliminating it might have on the final preparation. In the preceding experiment (Table II) it was removed from the 30 → 50 per cent $(NH_4)_2SO_4$ globulin of Serum 54; but since this globulin fraction has proved to be less desirable for therapeutic purposes than the total globulin, the effect of eliminating the acid fraction from the latter had to be tested. Using the Banzhaf-Klein technique, it was eliminated from Serum 348. The amount of acid fraction that was precipitated was rather small; part of the remaining protein was fractionated with $(NH_4)_2SO_4$ and part with sodium-magnesium sulfate.

50 Per Cent $(NH_4)_2SO_4$ Globulin.—The M.E.D. appears to be as much as 0.3 cc. as compared with a M.E.D. of at least 0.05 cc. for the total globulin, isolated from the same serum which was not subjected to this preliminary process.

50 Per Cent $(NH_4)_2SO_4$; Water-Insoluble Fraction.—This fraction was derived from the preceding one by dilution with distilled water; the total water-soluble globulin is thus excluded. No end-point was reached in this test, but a seeming paradox is again evident here, in that the part appears to be more effective than the whole. The rabbits treated with the higher doses appear to have had a worse course and fewer survived.

It is difficult to account for the marked reduction in the potency and effectiveness of the fractions obtained with $(NH_4)_2SO_4$ simply by the removal of the small amount of acid precipitate; it seems more likely that the prolonged dialysis to which the serum was subjected prior to the fractionation with $(NH_4)_2SO_4$, had somehow influenced the subsequent precipitation of the antibody.

14 Per Cent Sodium-Magnesium Sulfate Globulin.—This fraction which should have corresponded to the 30 per cent $(NH_4)_2SO_4$ globulin, proved to be considerably more potent. The zone phenomenon was strikingly evident in this test; all the rabbits treated with 0.6 cc. died,

whereas 0.15 cc. was an effective dose. This fraction had 4.5 mg. of nitrogen per cc., and almost all the agglutinins were associated with it.

14 → 24 Per Cent Sodium-Magnesium Sulfate Globulin.—This fraction, which should have corresponded to the 30 → 50 per cent $(NH_4)_2SO_4$ globulin, had 3.0 mg. of nitrogen per cc. and was ineffective in the three doses tested.

Whether or not it be the effect of the preliminary dialysis of the serum, the 47.5 per cent sodium-magnesium sulfate solution certainly did not behave like the equivalent of saturated $(NH_4)_2SO_4$ with regard to the antibodies in antipneumococcic serum.

DISCUSSION AND SUMMARY

The experiments of the preceding communication showed that the therapeutic action of antipneumococcic serum depends to a considerable extent upon a certain non-antibacterial factor. The experiments reported in the present communication had two main objects; the first was to determine the distribution of the non-antibacterial factor among the various protein fractions of the serum, and incidentally to correlate this property of the factor with that of certain known antibodies, as well as to learn whether or not the protein fractions commonly excluded from refined preparations of antipneumococcic serum, have any therapeutic value; the second was to determine the rôle of the non-antibacterial factor in the therapy of pneumococcus infection, as exemplified by the experimental, dermal, pneumococcus infection in rabbits.

To determine the distribution of the non-antibacterial factor, Type I antipneumococcic serum was fractionated with $(NH_4)_2SO_4$, and the antibacterial bodies were absorbed by concentrated suspensions of heat-killed pneumococci. The activity of the non-antibacterial factor in the different protein fractions was then tested for by adding varying amounts of the absorbed supernatant liquids to a certain constant, subeffective dose of serum. Even though the method of titration has certain inherent faults it was possible to ascertain that the non-antibacterial factor was apparently associated with all the globulin fractions to a similar degree, and to a certain extent with the albumen as well. In this respect the non-antibacterial factor resem-

bles neither the antibacterial bodies of antipneumococcic serum, nor, for example, the known diphtheria or tetanus antitoxins.

The determination of the relative importance of the antibacterial and non-antibacterial factors in the therapy of pneumococcus infection in rabbits, was rendered possible by the fact that the various globulin fractions differ in their relative content of the two factors. Thus whereas the water-insoluble fraction of the globulin, precipitated when the saturation of $(NH_4)_2SO_4$ is raised from 30 per cent to 50 per cent, contains about 90 per cent of the antibacterial (mouse protective) bodies, it apparently contains no more of the non-antibacterial factor than the water-soluble fraction. The minimal effective doses as well as the mouse protective unit content of the different fractions were determined. Before the tests were performed, it seemed that if the therapeutic effect of a given dose were to depend chiefly upon its content of mouse protective antibody, the amounts required for 1 M.E.D. would be determined by the number of mouse protective units it contained; on the other hand, if it depended primarily on the non-antibacterial factor, one would expect the amounts required for 1 M.E.D. to be of approximately the same volume. Actually the therapeutic effect was found not to depend entirely upon either factor, alone; the M.E.D.'s varied both in volume and m.p.u. content, the absolute concentration of either factor being inconstant and almost proportional to the relative concentration of the other. Thus a relative abundance of non-antibacterial factor made up for a scarcity of mouse protective antibody (as in the water-soluble globulin), and *vice versa* (as in the water-insoluble globulin). It appears, therefore, that when a relatively larger amount of antibacterial bodies are acting, less of the non-antibacterial factor is necessary; conversely, when more of the non-antibacterial factor is available, less of the antibacterial bodies are necessary.

The last experiment dealt with the practical question as to whether any protein fraction or combination of fractions is as good a therapeutic agent as the whole, unrefined serum from which it was derived. Two considerations were in mind in making the comparison; one had to do with the quantitative recovery of potency, the other with the qualitative effect of the various fractions as regards the lesion, course, and duration of the experimental disease. The results may be sum-

marized as follows: (*a*) the total globulin was not only as good, but in this instance, definitely better than the original serum; (*b*) the total water-insoluble globulin, although almost as potent as the original serum, was qualitatively and quantitatively less effective than the total globulin; (*c*) the removal of the 30 per cent $(NH_4)_2SO_4$ fraction not only diminished the potency of the remaining globulin to a considerable extent (at least 25 per cent), but also seems to have disturbed a certain equilibrium, which resulted in an undesirable zone phenomenon. As a result of this zone phenomenon, which, it is important to note, was not observed in the agglutination or mouse protection tests on the same fractions, the range of effective dosage is very narrowly limited. Although the results of agglutination tests correlated well with those of the mouse protection tests, both of these showed no parallelism with the therapeutic effects of the various fractions on the experimental, dermal, pneumococcus infection in rabbits.

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