STUDIES ON MENINGOCOCCAL INFECTION

XII. Immunochemical Studies on Meningococcus Type II

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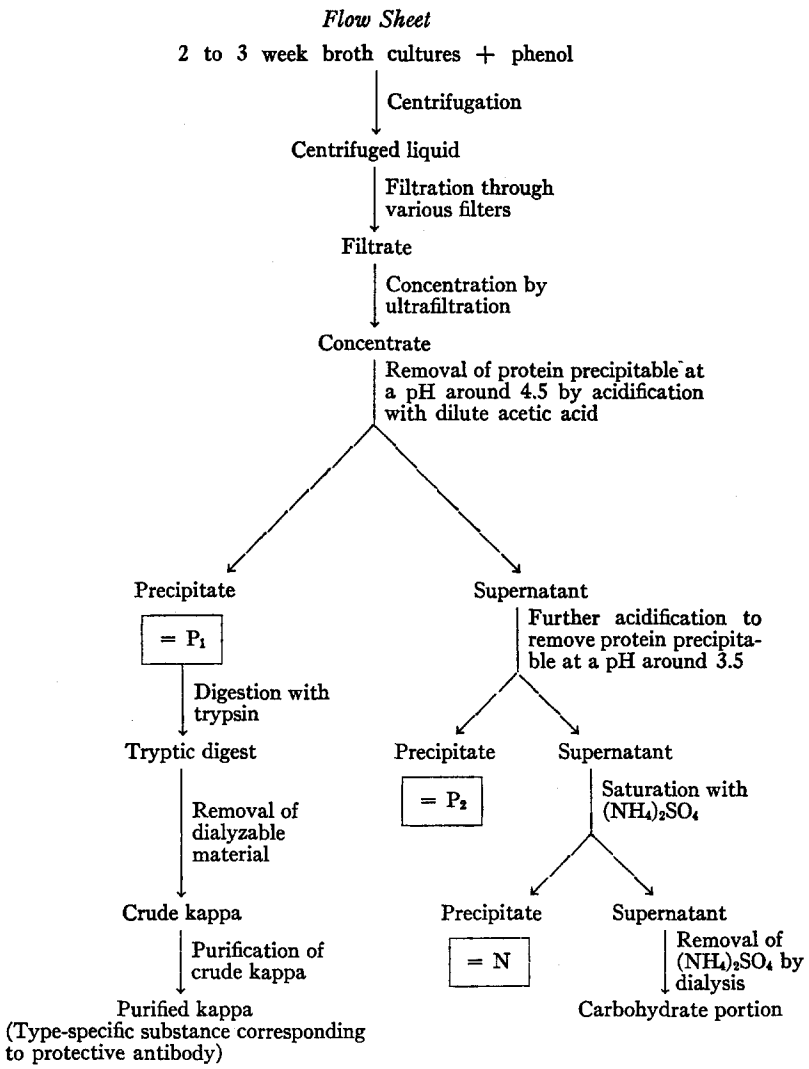
Rake and Scherp (1, 2) have isolated from broth autolysates of meningococcus group I-III three principal fractions, one of protein nature and two of carbohydrate nature. They have demonstrated that the former carries group-specific properties while of the latter, one has type-specific and the other group-specific properties. Little was known about the immunochemistry of Type II meningococcus when the present work was initiated. Rake and Scherp (1, 2) had isolated from this type a group-specific protein component and demonstrated that type-specific properties were connected with a certain fraction, which gave both carbohydrate and protein reactions.

The present work was undertaken to further our knowledge of Type II meningococcus; it was carried out for the most part on a virulent Type II strain (Herrington), and comprised: (*a*) studies on autolysates from cultures grown in hormone broth and (*b*) studies on young cultures grown on blood agar.

Studies of Broth Autolysates

Broth autolysates were obtained as described previously (1, 2). After phenol to 0.5 per cent had been added they were centrifuged at 30,000 to 35,000 R.P.M. in a Sharples supercentrifuge, and the supernates filtered through various filters to clarify and to remove bacterial debris. Final filtration was always carried out through Chamberland L5 filters. The filtrates were concentrated by ultrafiltration through Bronfenbrenner alundum candles (3) coated with 7 per cent collodion in glacial acetic acid. The concentrates were "washed" on the membrane with distilled water until the filtrates were Molisch-negative. The filtrates were discarded, as they were found to be serologically inactive; 5 filtrates of the 7 different preparations having been tested.

The dark brown colored concentrates may be fractionated as shown in the flow sheet. The P_1 precipitates were further purified by repeated solution in slightly alkalized water, followed by precipitation with dilute acetic acid, until the supernates had become Molisch-negative. These steps were carried out in the cold. The water-washed precipitates were either dried from frozen state



on a cryochem apparatus (4) or further washed with acetone, filtered, and dried *in vacuo*.

Chemical properties of P_1 fractions obtained from 7 different preparations are summarized in Table I. These substances are gray or brown, insoluble in distilled water or saline, but soluble on addition of minimum amounts of dilute NaOH to brown solutions, which flocculate with dilute acetic acid or Tanret's reagent and give positive biuret, Millon, and Sakaguchi reactions.

In one instance a similar substance was obtained by acidifying the super-

centrifugate directly. This latter method was not used more frequently, however, as ultrafiltration removed broth constituents which otherwise might precipitate together with P_1.

As exemplified in the flow sheet, further portions of protein nature (Tanret and biuret-positive) can be separated from the supernates of P_1. Type specificity was found to be present not only in P_1, but also in both P_2 and N. Data concerning P_2 and N are included in Tables II and V. These data, which will be clarified in connection with the experiments described for component P_1, show that P_1 was considerably richer in type-specific substance than P_2 and somewhat richer than N (Table V), N being antigenically less homogeneous than P_1 (Table II).

The deproteinized mother liquors contain portions of carbohydrate nature. Only very weak type-specific serological reactions were given by these polysac-

TABLE I

Extreme and Mean Values of Chemical Properties of P_1 Fractions Isolated from Various Preparations of the Herrington Strain of Type II Meningococci

	Yield per liter of broth	pH of maximum precipitability	$[\alpha]_D$	N	P	Carbohydrate*	Ash as Ca
	mg.			*per cent*	*per cent*	*per cent*	*per cent*
Highest value	234.2	3.9	−58	14.6	0.9	3.2	0.24
Lowest value	93.0	4.7	−38	13.1	Trace	1.8	0.15
Mean	162	4.4	−46	13.7	0.4	2.8	0.2

* The method of Heidelberger and Kendall (5) was applied and the colors, read in a photoelectric colorimeter, evaluated from equations calculated from galactose standards.

charides when present in high concentrations. Carbohydrate fractions were, however, reactive with polyvalent antimeningococcus horse sera, known to contain antibodies for the group-specific C fraction, and they also showed some reactivity with polyvalent Type 4-8 antipneumococcus horse sera. Such fractions contained about 35 to 37 per cent hexosamine. As peptone was a constituent of the medium it seems reasonable to assume that such portions contain chiefly blood group A substance (from peptone (6)) together with meningococcus C substance and mere traces of type-specific material.

For these reasons P_2, N, and carbohydrate components were studied less thoroughly.

Serological Studies

For serological studies the ring test technique was applied throughout; the readings recorded in the tables are those taken after 2 hours at 37°.

The following antimeningococcus Type II sera were used: (1) A pool from several

bleedings of two rabbits designated 13-15; these animals had been immunized with whole cultures of Type II meningococcus and the mouse protective value of this pool was 67 units. (2) Another pool, also from several bleedings of two rabbits and designated 3185-3192, exhibited 180 mouse protective units. For certain tests the latter serum was exhaustively absorbed with young Type I organisms grown on blood agar, and was then designated "3185-3192 absorbed." (3) Three more sera (M18, M22, and M37) were prepared by immunizing rabbits with 6 hour cultures of Type II meningococci. The mouse protective values of these three sera were 16, 47, and 27 units respectively. (4) Similarly, anti-P_1 sera were prepared by immunizing rabbits with 1:1000 saline dilutions of a P_1 fraction, starting usually with 0.1 ml. and increasing the dose gradually to 1.0 ml. P_1 proved to be quite toxic, as evidenced by temperature reactions and by several fatalities. Anti-P_1 sera gave strong precipitin reactions with meningococcal proteins (*cf.* Tables V and X), but were of only low mouse protective value; the highest potency observed was 9 units. They reacted also with acid-precipitable protein derived from Type I (Table V) but exhibited, on the other hand, a certain degree of type specificity in the agglutination reaction.

In serological respects, P_1 exhibited two important properties: (1) It reacted strongly with both antiprotein serum and (absorbed) antimeningococcus Type II serum 3185-3192, as will be seen from data recorded in Table V. During the course of experiments attempting to separate the type-specific fraction from the protein it was found that the reactivity with antiprotein serum was lost after digestion with proteolytic enzymes while the reactivity with serum 3185-3192 was retained fully or even increased. These experiments will be described in detail later (*cf.* page 443, and Table V). (2) It removed from protective serum (13-15) all protective antibody. Thus samples of serum 13-15 were absorbed with various P_1 fractions at the antigen excess end of the equivalence zones; the absorptions were carried out by keeping the mixtures in the cold and shaking occasionally for 4 days. All the P_1 fractions from Type II removed all protective antibody; a protein component derived from Type I and comparable to P_1 in so far as it was acid-precipitable and prepared in an analogous manner, failed to do so. The data of these experiments are recorded in Table II.

As mentioned above, we have reason to believe that P_1 is richer in type-specific substance than either P_2 or N. Accordingly, attempts were made to separate the type-specific principle from P_1 protein. However, contrary to the experiences with Type I meningococcus (1, 2, 7), we could not separate the type-specific substance from protein by repeated acid precipitation. The following methods also failed to separate type specificity from "accompanying" protein: (*a*) treatment with $CHCl_3$ and butyl alcohol, according to Sevag (8), at neutrality and at slight alkalinity; (*b*) heating P_1 in diluted Na_2CO_3 solution at 50 to 55°C., followed by neutralization and repeated treatment with $CHCl_3$ and butyl alcohol, according to Sevag, Lackman, and Smolens (9); (*c*) subfractionation with the aid of $(NH_4)_2SO_4$; (*d*) electrophoresis in a 7 cell electrophoresis apparatus (10).

a. Saline solutions of a P_1 preparation (BII^5P_1) were treated with $CHCl_3$ and butyl alcohol. The aqueous phases were tested serologically. Protein removal was paralleled by loss of all serological reactivity.

TABLE II

Precipitin Reaction of Various Fractions with Serum 13-15 (67 Mouse Protective Units) and Mouse Protective Units Remaining in the Serum after Absorption

	Reactivity of absorbed serum with fraction used for absorption and with unabsorbed serum*											
		BII^6P_1‡	13-15	BII^7P_1‡	13-15	BII^8P_1‡	13-15	BII^6N§	13-15	BII^2P_{II}‖	13-15
Serum absorbed with equal volume of saline dilution 1:	100	±	+++±	−	+++±	−	++	++	(∓)		
	250	+	++±	(±)	++	(∓)	±	++	(∓)	−	+±
	500	+±	+	+	+±	(∓)	(±)	++±	(∓)	(∓)	±
	750	++	(±)	+±	+(±)	±	(∓)			(∓)	(±)
	1000	++±	(∓)	+±	+	+±	−			(±)	(∓)
Serum absorbed with equal volume of dilution 1:		200		300		200		100		600	
Mouse protective units in absorbed serum		<5**		<10**		<4**		<5**		56	

* In this and the following tables the following denotations are used:

(∓) questionable reactions. +

(±) very weak but still definite reactions. +±

± weak but definite reactions. ++

++± = increasing amounts of precipitate.

+++

+++±

++++

‡ Laboratory designation of P_1 fraction used.

§ Laboratory designation of N fraction.

‖ Laboratory designation of acid-precipitable protein component prepared from Type I; the dilutions used in this instance were 1:300, 600, 900, and 1200 respectively.

** Least value tested.

b. About 100 mg. of a P_1 preparation ($BII^{10}P_1$) were treated under the conditions described by Sevag, Lackman, and Smolens (9). Aliquots were withdrawn before and in the course of $CHCl_3$ treatment and tested serologically, as recorded in Table III. From the combined $CHCl_3$ layers a substance was isolated by addition of redistilled acetone. This substance was of protein nature, as evidenced by its preparation and by its being biuret-positive. It was tested before and after tryptic treatment with antiprotein serum and with

TABLE III

P_1 Treated According to Sevag, Lackman, and Smolens (9). Parallelism of Protein Removal with Decrease of Serological Reactivity

Conditions	No. of treatments	*Vs.* antiprotein serum M2-1.5.39			*Vs.* 3185-3192 absorbed		
		1T	5T	10T	1T	5T	10T
Untreated		++++			+++		
Heated at 50 to 55°C. in diluted Na_2CO_3 solution	0	+++±			+++		
	2	+++			+		
	4	++			(±)		
	6	+±			(∓)		
	8	+			−		
	10	+			−		
	12	±			−		
	14	±			±		
Tryptic digested after 14 treatments*	14	(±)			±		
Substance isolated from $CHCl_3$ phase							
Before trypsin		+++±	+++	++	+	±	−
After trypsin		±	−	−	+++	++	+±

* Dilution 1:2.5T.

TABLE IV

Cell Contents after 21 Hours Electrophoresis of P_1 and Serological Tests on Them

Cell No.	1 (Cathodic)	2	3	4		5	6		7	
Appearance	Water-clear colorless	Water-clear colorless	Water-clear colorless	Water-clear supernate over flocculent precipitate		Water-clear colorless	Slightly turbid		Slight flocculent precipitate	
				Supernate	Redissolved precipitate		Supernate	Redissolved precipitate	Supernate	Redissolved precipitate
Biuret	−	−	−	±	+++	−	−	−	−	+
pH	9.6	8.0	6.8	6.2		5.9	5.5		3.6	
Vs. antiprotein serum M2–12.14.38	−	−	−	−	+++± (±)*	−	−	−	−	++ −*
Vs. serum 3185-3192 absorbed	−	−	−	−	+± +++*	−	−	−	−	(±) +*

* After digestion with trypsin.

absorbed 3185-3192 serum, as recorded in Table III. This experiment shows again that the active principle is firmly bound to protein, as it disappeared gradually from the aqueous phase and could be found in the protein portion isolated from the $CHCl_3$ layer.

c. 820 mg. of a P_1 preparation (BII^5P_1) were fractionated with $(NH_4)_2SO_4$. Three fractions of protein nature were obtained, precipitable at 23 per cent, 45 per cent, and 100 per cent saturation in yields of 317, 143, and 130 mg. respectively. These fractions gave positive biuret and Sakaguchi reactions. Only the portion precipitable at 23 per cent saturation gave a distinct Millon test. All three were reactive with both antiprotein serum and with absorbed 3185-3192 serum, and all three removed nearly all protective antibody from serum 13-15 (67 units). From the final $(NH_4)_2SO_4$ supernates a small, serologically inactive, biuret-negative, and strongly Molisch-positive fraction (64 mg.) was obtained. This experiment also shows that the active principle is connected with protein.

d. A dialyzed solution of 40 mg. of a P_1 preparation (BII^9P_1) in 20 ml. distilled water was subjected to electrophoresis in a 7 cell apparatus (10) at 2,000 volts. The substance was placed in the middle cell, the other cells being filled with distilled water. After electrophoresis for 21 hours, the picture, including biuret and serological tests, was as recorded in Table IV. This experiment shows that, on electrophoresis, partial migration and separation take place, but that serological reactivity again is only connected with those portions which also are biuret-positive; *i.e.*, only with components of protein nature. Two more electrophoresis experiments on another P_1 fraction ($BII^{10}P_1$) and on a P_2 fraction (see flow sheet) led to the same conclusions.

Action of Enzymes on P_1—Kappa Substance

Under the influence of proteolytic enzymes P_1 loses its reactivity with anti-P_1 serum, while its reactivity with (absorbed) antimeningococcus Type II serum is fully retained or even intensified, as exemplified by the following experiment:

Two 1.0 ml. samples of an aqueous solution of a P_1 fraction (BII^7P_1) containing 159 mg. per 15 ml. were pipetted into Wassermann tubes. To each sample 1.0 ml. M/5 phosphate buffer pH 7.0 and 2 drops of toluene were added. To one of the tubes 0.25 ml. of a trypsin solution (250 mg./10 ml.), which had been roughly purified by removing acid-precipitable material, was added. The tubes were capped and placed into a water bath at 37° for 4 days. Saline dilutions corresponding to 1:1000, 1:5000, and 1:10,000 of the substance originally used, were made up and tested against anti-P_1 serum and against absorbed 3185-3192 serum.

All of the P_1 fractions were tested according to this general scheme. The mean results are recorded in Table V. Trypsin controls, which had been incu-

bated in absence of P_1, reacted neither with anti-P_1 serum nor with absorbed 3185-3192 serum.

The results of numerous other enzymatic experiments (Table V) may be summarized as follows: (*a*) Serial addition of trypsin up to 2 ml. trypsin (*i.e.* 50 mg.) per 10 mg. P_1 and incubation up to 10 days did not alter the results in any way. (*b*) Identical results were obtained when tryptic digestion was

TABLE V

Reactivity of Meningococcus-Protein Fractions with Antiprotein Serum and with Absorbed Antimeningococcus Type II Serum

Laboratory designation of fraction	Enzyme	*Vs.* antiprotein serum						*Vs.* anti-Type II serum, absorbed					
		Before enzyme			After enzyme			Before enzyme			After enzyme		
		1T	5T	10T	1T	5T	10T	1T	5T	10T	1T	5T	10T
P_1 (Mean of 7 preparations)*	Commercial trypsin‡	+++±	++±	++(±)	+(±)	±	−	++±	+(±)	+	+++±	++(±)	+±
BII^7P_1	Activated papain at neutral reaction§				++	+±	+				+++±		+±
$BII^{11}P_1$	Crystalline pepsin at pH 3.0‖				±	−	−				+++±	++±	++
BI^2P_1**	Commercial trypsin	++±		+±	(±)		−	−		−	−		−
		500	5T	10T	500	5T	10T	500	5T	10T	500	5T	10T
BII^6P_1	Commercial trypsin	+++	++		+	±		++	−		++±	−	
BII^6N	Commercial trypsin	++±	±	±	++	±	±	+++±	+	±	+++±	+	±

* Similar results on P_1 from a culture grown on a semisynthetic medium, containing casein-hydrolysate and yeast extract as principal ingredients.

‡ Similar results when digested at pH 7.6, 8.5, or in N/4 Na_2CO_3. Similar results with crystalline trypsin, crystalline chymotrypsin, or mixtures of both at neutral reaction.

§ Similar results on three other fractions digested at pH 5.2.

‖ Similar results when digested in N/10 HCl.

** Acid-precipitable protein component prepared from Type I.

carried out in saline solution or at pH values of 7.6 or 8.5 or in N/4 Na_2CO_3 solution. (*c*) Activated papain at neutral reaction and at pH 5.2 caused essentially the same effect, although the destruction of anti-P_1 serum reactivity was less marked. (*d*) Crystalline pepsin acted at pH 3.0 and in N/10 HCl solutions essentially the same way as trypsin at the conditions described.

It appeared evident that the type-specific principle could not be removed from protein by mild, simple, and but slightly destructive means. As, however, this principle was found in enzymatic digests of P_1, we attempted to iso-

late this substance, termed kappa substance, from tryptic digests. By purifying tryptic digests with charcoal, followed by dialysis,[1] fairly colorless kappa was obtained (*cf.* Table VI); as, however, by repeated charcoal treatment eventually all serological reactivity was lost, the following method of purification was chosen:

P_1 from 4 different preparations was pooled and digested with trypsin in presence of phosphate buffer pH 7.5. The digest was freed from phosphate by dialysis and brought to dryness on a cryochem apparatus. 1.8 gm. of crude kappa substance were obtained from 9.4 gm. P_1. On the basis of the precipitin

TABLE VI

Chemical and Serological Properties of Crude Kappa, Purified Kappa, and of Component Separated from Crude Kappa by $(NH_4)_2SO_4$ Fractionation

	$[\alpha]_D$	N	P	Carbohydrate	Serological reactivity with						
					Anti-protein serum			3185-3192, absorbed			
					1T	10T	100T	1T	10T	100T	1M
		per cent	*per cent*	*per cent*							
Crude kappa.....		9.0	1.5	8.9	+	(±)	−	++++	+++	+	
Purified kappa		9.6	1.4	12.3	(∓)	−	−		++++±	+±	(∓)
		9.6		12.9	(±)	−			++++±	+±	−
Main component of the $(NH_4)_2SO_4$ supernate	+7.5	8.7	1.7	7.8	−	−	−	+±	(∓)	−	
	+13.5	8.1		7.0	(∓)	−	−	+	−	−	
	+14.1	6.8			−	−		+	−		
Kappa obtained by charcoal treatment	+2	5.2			+±	−		+++±	++±	±	

test there was approximately a tenfold increase in activity from P_1 to crude kappa.

Such crude kappa substance was further purified by removal of a small portion, insoluble in chilled 0.2 M acetate buffer pH 4.0, followed by $(NH_4)_2SO_4$ fractionation.[2] At 62.5 per cent $(NH_4)_2SO_4$ concentration, the bulk of active material flocculated out. Material isolated from the supernate showed very

[1] 250 mg. trypsin autodigested in presence of phosphate buffer and dialyzed against distilled water yielded after drying from frozen state a residue too small to be collected. This small residue dissolved only partially in 0.2 M acetate buffer at pH 4.0, the clear centrifuged supernate rendering only a faint opalescence on saturation to 62.5 per cent with $(NH_4)_2SO_4$. Trypsin may, therefore, be excluded as tangible impurity in any of our kappa preparations.

[2] $(NH_4)_2SO_4$ being removed subsequently by dialysis.

little serological reactivity. The portion precipitable at 62.5 per cent $(NH_4)_2SO_4$ was repeatedly treated with $CHCl_3$ and butyl alcohol, according to Sevag (8), when only a small amount of material was removed into the $CHCl_3$ phase. The aqueous phase was brought to dryness on a cryochem apparatus. The yield was 172 mg. purified kappa from 727 mg. crude kappa. In addition, 169 mg. "almost inactive" material from the $(NH_4)_2SO_4$ supernates, and (active) minor fractions, aggregating to 93 mg., were isolated; the $CHCl_3$ phase rendered 10 mg. of (active) material. In another experiment, 177 mg. purified kappa were obtained from 545 mg. crude kappa. On the basis of the precipitin test, there was only little increase in serological activity from crude kappa to purified kappa.

Purified kappa consists of a grayish brown powder, readily soluble in distilled water or saline to give a somewhat opalescent solution, which clears up on addition of minimal amounts of diluted NaOH. It is Tanret, Sakaguchi, and ninhydrin-positive but biuret-negative. Chemical and serological properties are recorded in Table VI.

When tested in the precipitin reaction with three different anti-Type I sera, kappa was found non-reactive with one of these sera, while weak reactions (++ and + respectively in 1:1T, and + and ± in 1:10T) were observed in the other two sera.[3] It is not known whether these weak reactions are due to small amounts of an impurity or to the "existence of antigenic determinants of diverse specificity" in the kappa molecule, in the sense of Hooker and Boyd's observation on systems of crystalline duck and egg albumin and their antibodies (11).

Kappa removes practically all protective antibody from Type II protective antisera, as shown in Table VII. Quantitative nitrogen determinations performed according to the method of Heidelberger and Kendall (12–14) on the precipitates of these experiments, indicate a correlation between total precipitated nitrogen and mouse protective value.

Electrophoresis experiments carried out both in the Tiselius apparatus,[4,5] and in a 7 cell apparatus (10) did not free kappa from colored matter. Part of the color at least seems, nevertheless, not to be an integral part of the molecule, as supernates of serum M22 pooled-1, absorbed with kappa at the equivalence point, were distinctly brown colored, but did not react with unabsorbed serum. Possibly electrophoresis in the Tiselius apparatus at other pH values might have led to separation of colored matter from kappa. Lack of material

[3] Weak heterologous reactions of the same order of magnitude had also been observed with the mother substance of kappa; *i.e.*, P_1.

[4] We wish to express our thanks to Dr. D. Moore, of the Presbyterian Hospital in New York, and Dr. B. Chow, of this Institute, for carrying out these tests.

[5] Kappa being dissolved in 0.02 M phosphate buffer pH 7.45 and pH 7.47 respectively.

has prevented us from such studies. As the color of kappa interfered in the Tiselius apparatus with observation and photographic recording the question about the electrophoretic homogeneity of kappa remains still unanswered.

In the experiment conducted by Dr. Chow, small quantities of a fast moving colored portion (*a*) and a slow moving, slightly colored portion (*b*) were collected and isolated.[6] Both were equally reactive with absorbed serum 3185-3192. By further absorption of this serum with (*a*) all reactivity with (*b*) and with kappa had disappeared. When kappa was subjected to electrophoresis

TABLE VII

Equivalence Zone Determination of Various Anti-Type II Sera with Kappa and Mouse Protective Units Remaining after Absorption at Antigen Excess End of Equivalent Zone

Serum absorbed		M18-pooled-1 (16MPU)		M22-pooled-1 (47MPU)		M37-11.22.40 (27MPU)	
		Reactivity of absorbed serum with kappa or with unabsorbed serum					
		Kappa	M18-pooled-1	Kappa	M22-pooled-1	Kappa	M37-11.22.40
Serum absorbed with equal volume of saline dilution 1:	320			(∓)	++		
	500					−	+++
	640			±	−		
	1T			+++±	−	−	+±
	1.5T	(±)	+±			−	±
	2.0T	(±)	±			(±)	−
	2.5T	±	−			(±)	−
2.0 ml. serum absorbed with 1.0 ml. saline dilution of kappa 1:		750		239		750	
Mouse protective units remaining in absorbed serum		4		4		3	
Mg. total precipitated N_2 per ml. serum		0.175		0.533		0.278	

from the cathodic end-cell of the 7 cell apparatus (10) migration took place through the whole system into the anodic end-cell. After 21 hours the main portions of kappa had accumulated in the cathodic and anodic end-cells, as indicated by the color and as confirmed by weight determinations on the isolated cell contents. No serological cross reactions occurred, when samples of absorbed serum 3185-3192 were further absorbed with these main portions of kappa which had been subjected to electrophoresis, and were cross tested in

[6] We must leave the question open whether kappa separated under the experimental conditions into 2 (or more) portions, or whether the appearance of a colored, fast moving, and a but slightly colored, slow moving portion was due to the superimposed effect of colored matter.

the precipitin reaction. The fact that electrophoresis effected no separation into serologically different fractions indicates a certain degree of purity for kappa. Neither the experiments in the Tiselius apparatus nor those in the 7 cell apparatus gave any indication of separation of serologically active carbohydrate from kappa. Carbohydrate appears to be rather in chemical linkage to the rest of the molecule. This point is supported by the fact that serum-kappa precipitates are always of the finely granular type, which usually is interpreted as a protein-antiprotein reaction; compact discs, which are considered as indicative of carbohydrate-anticarbohydrate, were never observed. Considering the various data, kappa itself appears as a protein split-product, carrying some colored matter as an impurity which can be separated only with difficulty. The protein nature of kappa is manifested by positive ninhydrin and Sakaguchi tests and is indicated by the electrophoretic mobility in the Tiselius apparatus which is in the order of $10^{-5} \frac{\text{cm./sec.}}{\text{volt/cm.}}$ Besides amino acids, there appears to be carbohydrate present as part of the molecule. The bulk of kappa, therefore, can be considered to be a carbohydrate-containing polypeptide.

Studies on Young Cells

As we had isolated this kappa substance from broth autolysates, we were interested in ascertaining whether we could identify the protein from broth autolysates with protein from young meningococci Type II cultures. Five to 6 hour blood agar cultures were fractionated according to the method developed by Heidelberger and Kendall (15) on a scarlatinal strain of hemolytic streptococcus and later applied by Heidelberger and Menzel (16) in their studies on tubercle bacilli. Save for slight, unessential modifications in the procedure, methods and designations are the same in the present work. The properties of the main fractions (D and K) obtained in two different preparations are compiled in Table VIII.

These quantitatively most abundant fractions were used to absorb protective serum 13-15 in the same manner as described above. This serum contained originally 67 units. The results are recorded in Table IX.

Furthermore, the various fractions were subjected to tryptic digestion and serological tests performed as described above. The results upon D and K are recorded in Table X.

The absorption experiments show that specificity corresponding to protective antibody is predominantly connected with fraction K, although this specificity is encountered to a considerably lesser degree also in fraction D; the experiments on tryptic digested fractions show clearly a basic difference between D and K. The serological, as well as the chemical properties, of the several intermediate fractions lie broadly between those of D and K. It

TABLE VIII

Chemical and Immunological Properties of Protein Fractions D and K of Type II Meningococci

Preparation	Fraction	Yield	pH of extraction	pH at which precipitated	pH of solution used for $[\alpha]_D$ determination	$[\alpha]_D$	N	P	Carbohydrate	Basic ash as Ca
		gm.					*per cent*	*per cent*	*per cent*	*per cent*
BII[6]	D	1.966	7.0	3.7–3.9	6.7	+12.3	15.7	3.1	10.0	0.1
	K	0.731	$\frac{N}{10}$ NaOH	4.5–5.3	6.7	−45.7	13.3	0.5	2.1	0.0
BII[6A]	D	1.358	6.8	3.6–4.0	7.5	+27.0	16.0	3.7	11.1	0.3
	K	1.256	$\frac{N}{10}$ NaOH	4.6–4.8	6.8	−41.1	13.8	0.8	2.2	0.2

TABLE IX

Equivalence Point Determination of Various Fractions from Whole Cells, with Serum 13-15, and Mouse Protective Units Remaining after Absorption at Equivalence Point

		Reactivity of absorbed serum with fraction used for absorption and with unabsorbed serum, respectively							
		BII[6]D	13-15	BII[6A]D	13-15	BII[6]K	13-15	BII[6A] K	13-15
Serum absorbed with equal volume of saline dilution 1:	100	+	+++	−	++(±)	−	+++		
	250	+++	+±	++±	++	−	+++	−	+++
	500	++++±	+	++++±	(±)	−	++	−	++
	750	++++	±	++++±	−	+	+	−	+
	1000	++++	±			+	±	(±)	±
	1250	++++	(±)			+	−	±	(±)
	2500	++++	−			++	−	+	−
Serum absorbed with equal volume of saline dilution 1:		100		100		500		750	
Mouse protective units in absorbed serum		57		24		<5*		<4*	

* Least value tested.

TABLE X

Reactivity of Various Fractions Derived from Young Mass Cultures before and after Tryptic Digestion

Fraction	*Vs.* antiprotein serum						*Vs.* anti-Type II serum absorbed					
	Incubated control			After trypsin			Incubated control			After trypsin		
	1T	5T	10T	1T	5T	10T	1T	5T	10T	1T	5T	10T
BII[6]D	++±	+±	+	+	−	−	++	+	(±)	±	−	−
BII[6A]D	+++	++	+	(∓)	−	−	+++	±	−	±	−	−
BII[6]K	++++±	+++±	+++±	++	+	+	++++±	+++±	+±	++++±	++	+±
BII[6A]K	++++±	+++±	++	++	+	±	++++±	+++±	+±	++++	+++±	++

is, therefore, possible that those intermediate fractions are mixtures of "ideal" D and K. No indication of the presence of type-specific substance was found in the acid extracts of the meningococci in the precipitin test with absorbed 3185-3192 serum, the final conclusion being that specificity corresponding to protective antibody in young cells is also intimately connected with protein, especially that protein portion (K) which requires the highest alkali concentration to be brought into solution.

It seemed conceivable that by immunizing rabbits with K, sera of high protective values could be obtained. This was not the case. The highest potency ever observed in 3 animals was 8 units. Precipitins against meningococcal proteins were, however, obtained in high titer.

DISCUSSION

The preceding experiments show that in meningococcus Type II Herrington strain, type specificity corresponding to protective antibody is intimately connected with protein. In this respect meningococcus Type II differs strikingly from Type I and also differs from the various strains of pneumococci.

Rake and Scherp (1, 2) have isolated from Type I broth autolysates a protein component, which on account of its acid precipitability might be compared with P_1 of the present studies. Both components occur uniformly in the step of acid precipitation, although neither is believed to be a chemically pure entity. P_1 removes from protective anti-Type II serum all protective antibody, it is furthermore reactive with absorbed 3185-3192 serum before and after tryptic digestion; the acid-precipitable component from Type I, on the other hand, lacks all these qualities (Tables II and V).

It appears that type specificity of Type II, as manifested by correspondence to the protective antibody, is firmly connected with protein. Also in young cells type specificity occurs in connection with protein constituents, predominantly with that protein portion which requires the highest alkali concentration to effectuate dissolution.

In broth autolysates this type-specific substance appears to be connected with various components of protein nature. We have used P_1 as an easily accessible starting material for isolation of this substance, although possibly other protein portions (P_2 or N of the flow sheet) could be used to advantage for this purpose. The kappa substance could be split from P_1 by tryptic digestion and further purified by methods described above. Although this substance has not as yet been obtained in absolutely pure state, we feel that the purity arrived at indicates that the bulk of kappa is composed of a carbohydrate-containing polypeptide. The chemical linkage of the phosphorus still remains to be ascertained.

The term "type-specific substance," is usually connected with the idea of polysaccharides, although a few examples of type specificity in connection with protein or protein-like substances are known. Such instances are well estab-

lished in Group A hemolytic streptococci (17–19, 15) and in Type A staphylococcus (20). Type specificity is completely destroyed by proteolytic enzymes in the cases of Lancefield's M substance (18, 19) and Verwey's staphylococcal protein (20). In the instance of meningococcal protein, however, this is not so. Kappa substance indeed behaves as if it constitutes an enzyme-resistant prosthetic group to meningococcal protein. For this reason, we believe that we are dealing with a new kind of type-specific substance, different from the type-specific polysaccharides and also different from the above discussed type-specific proteins.

Polysaccharidic antigens, which also contain some polypeptide as part of their molecule, are known; for instance the specific antigen obtained from *B. dysenteriae* (21). Therefore, kappa would seem to belong in the middle of a sequence of type-specific substances varying from proteins to polysaccharides as follows: (1) proteins, all specificity destroyed by enzymes; (2) proteins, type specificity connected with an enzyme-resistant prosthetic group, comprising polypeptides plus some carbohydrate; (3) polysaccharides containing some polypeptide; (4) polysaccharides, polypeptide-free. Such a scheme would find further confirmation in the fact that results somewhat analogous to ours have been obtained by Henriksen and Heidelberger by the tryptic digestion of protein fractions of the hemolytic streptococcus (22).

Our findings differ from the results of Miller and Boor (23). These investigators are of the opinion that the type-specific substance of Type II meningococcus is of lipocarbohydrate nature, comparable to the substances isolated by Boivin and Mesrobeanu (24) from *B. aertrycke* and other microorganisms. This discrepancy, however, may find an explanation, in the fact that Miller and Boor have worked with strains of meningococcus Type II different from our strain.

Experiments carried out by us on strains other than the Herrington strain, namely the Albany strain 36 and two strains of Miller and Boor's, designated 44 and 45, indicated that only the Albany strain 36 resembled closely the Herrington strain, in that from it an acid-precipitable P_1 could be obtained which chemically and serologically resembled P_1 from the Herrington strain. Autolysate concentrates of strains 44 and 45 were reactive with both antiprotein serum and with absorbed anti-Type II serum, losing the former but retaining the latter reactivity after digestion with trypsin. Acid-precipitable portions could not be obtained from such autolysate concentrates, but on saturation with $(NH_4)_2SO_4$ to 33.3 per cent precipitates resulted, which serologically, although not chemically, resembled the P_1 fractions obtainable from Herrington strain preparations. These facts certainly indicate the presence of kappa substance also in strains 44 and 45.

CONCLUSIONS

From meningococcus Type II broth autolysates, a substance, corresponding to the protective antibody has been isolated. This substance, termed kappa

substance, is firmly connected with protein but can be separated by the action of proteolytic enzymes. Methods of preparation and purification are given, together with chemical and serological data. In whole cells from young cultures, this substance is also connected with protein, especially with one protein fraction which requires highest alkali concentration to effect solution.

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