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Polyvinylalcohol Sulfonate as an Anticomplementary Substance

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SUMMARY

An anticoagulant, polyvinylalcohol sulfonate (PVS), was found to be anticomplementary. The anticomplementary action was dose-dependent in the sense of Klein (1956b) and proved to be reversible.

The point of attack of the complement system by PVS was demonstrated to be C'_1. The C'_M postulated by Klein (1956a) was found to be a doubtful entity.

INTRODUCTION

In the quantitative studies on immune bacteriolysis previously reported (Inoue *et al.*, 1959), bacterial cells of *Escherichia coli* B were changed to hardly stainable cell-wall like rods by an immune bacteriolytic system lacking serum lysozyme. However they were converted to serum spheroplasts by a system containing lysozyme. The former type of bacteriolysis was enhanced by the addition of a platelet extract from rabbits (Inoue *et al.*, unpublished). From this fact it was assumed that the former type of the bacteriolysis would be caused by some platelet factor liberated during blood clotting in addition to antibody plus four complement components. To prove the assumption, attempts were made to find an inhibitor of the hypothetical platelet factor. Meanwhile, Kato *et al.* (unpublished) in this laboratory found that the anthracidal activity of a platelet extracts from rabbits was inhibited by a low concentration of polyvinylalcohol sulfonate (PVS) which had previously been shown to be a powerful inhibitor of ribonuclease by Nomura *et al.* (1958). Thus PVS was examined for its anticomplementary action in immune hemolysis. It was found to be anticomplementary at a very low concentration. The mechanism of the anticomplementary action of PVS was further studied and results are presented in this report.

The following abbreviations are used in this paper: polyvinylalcohol sulfonate, PVS; veronal buffered saline containing 1.5×10^{-4} M $CaCl_2$ and 5×10^{-4} M $MgCl_2$, VBS; minimum hemolytic unit, H. U.; complement, C'; midpiece, M; endpiece, E; ammonia-treated C', N; zymosan-treated C', Z; formalin-treated C', F; 1st to 4th component of complement, C'_1 to C'_4; sensitized red blood cells, EA; complexes of EA with C'_1, C'_1 and C'_4 or C'_1, C'_4 and C'_2, EAC'_1, $EAC'_{1,4}$ or $EAC'_{1,4,2}$; complex of EA and C' inhibited by PVS, EAC'_p; a component in midpiece postulated by Klein (1956a), C'_M.

MATERIALS AND METHODS

Veronal buffered saline: Veronal buffered saline containing 1.5×10^{-4}M $CaCl_2$ and 5×10^{-4} M $MgCl_2$ (VBS) was prepared according to the description of Mayer *et al.* (1948). It was used for diluting and washing. When the effect of Ca^{++} and Mg^{++} was studied, VBS without added $CaCl_2$ and $MgCl_2$ was used. When $EAC'_{1,4}$ was prepard, VBS without added $CaCl_2$ and $MgCl_2$ was passed through an IRC50 Na^+-column according to the method of Levine *et al.* (1953).

Red blood cells: Citrated bovine blood was stored in a refrigerator after the addition of an equal volume of the Alsever's solution. Before use, the red blood cells were washed four times with VBS and resuspended in VBS to give an optical density of hemoglobin of 0.6 in the laked blood prepared by adding 14 times volume of distilled water. The optical density was measured with a Coleman junior spectrophotometer using a 6-310B cuvette. To the red blood cell suspension an equal volume of diluted hemolysin containing 10 H.U. per ml. was added and the mixture was incubated at 37°C. for 10 minutes and then kept in an ice-bath.

Hemolysin: Bovine erythrocytes antiserum of rabbits was kindly supplied by Dr. G. Ohmori of the Osaka Municipal Hygienic Laboratory. Its hemolysin content was 1,000 H.U. per ml.

Complement (C'): Fresh sera of more than 20 guinea pigs were pooled, sealed in ampules and kept frozen on dry ice.

Reagent of complement components: The heated complement (H), ammonia-treated complement (N) and zymosan-treated complement (Z) were prepared according to the method of Kabat and Mayer (1948). Midpiece (M) and endpiece (E) were prepared by the dilution method (Kabat and Mayer, 1948). Formalin-treated complement (F) was prepared according to Rapp (1958). These reagents were shown to contain the required component(s) before use and employed at a concentration which evoked no anticomplementary action.

EAC'_1: 3.0 ml. of N were added to 20.0 ml. of EA, and incubated at 37°C. for 20 minutes and subsequently centrifuged. The precipitates were washed twice and resuspended in 20.0 ml. of VBS.

$EAC'_{1,4}$: The method by Levine et al. (1954) was applied.

$EAC'_{1,4,2}$: Rapp's method (1958) was adopted using F.

EAC'_P: The complex of EA and complement inhibited by PVS was prepared as follows: To a mixture of 41.4 ml. of VBS, 1.2 ml. of C' and 2.4 ml. of PVS solution (containing 1.0 mg. per ml.) 15 ml. of EA were added. The mixture was incubated at 37°C. for 20 minutes and centrifuged. The precipitates were washed twice with VBS and resuspended in 15.0 ml.

Polyvinylacohol sulfonate (PVS): Kato of this laboratory synthesized PVS by the method of Nomura *et al.* (1958). The saline solution containing 10.0 mg. per ml. was stored in a refrigerator. The pH of the solution was approximately 7.2. Before use, the stock solution was diluted with VBS.

Egg albumin: The preparation of Merck Co. was purified by ammonium sulfate treatment.

Assay of immune hemolysis: Immune hemolysis was carried out by the method of Kabat and Mayer (1948). The total volumes of the reaction mixtures were usually 4.0 ml. The EA or its equivalent was added last. Except for the time course studies, the reaction mixtures were incubated at 37°C. for 45 minutes and then the tubes were immediately placed in an ice-bath. To each of these tubes were added 3.0 ml. of chilled VBS and the tubes were centrifuged in the cold. The hemoglobin contents were measured in the supernatants at 550 mμ. in a Coleman junior spectrophotometer using a 6-310B cuvette. As a 100 per cent control of hemolysis a tube containing 1.0 ml. of EA or its equivalent and 6.0 ml. of distilled water was also incubated and was subsequently treated as the test solutions. As a blank control a tube containing 1.0 ml. of EA or its equivalent and 6.0 ml. of VBS was also included.

RESULTS

Anticomplementary action of PVS:

As shown in Table 1, the hemolytic activity was inhibited by PVS.

Table 1. Inhibition of complement activity by PVS.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Aq. dest.	—	6.0	—	—	—	—	—	—	—	—	—	—	—	—
VBS	6.0	—	2.0	2.2	2.4	2.6	2.7	2.8	0.2	0.6	1.0	1.4	1.8	2.2
1 : 100 C′	—	—	1.0	0.8	0.6	0.4	0.3	0.2	0.4	0.4	0.4	0.4	0.4	0.4
10μg/ml. PVS	—	—	—	—	—	—	—	—	2.4	2.0	1.6	1.2	0.8	0.4
EA	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

After 45 min. incubation at 37°C., the tube were placed in an ice-bath and 3.0 ml. of chilled VBS were added to all except tubes No. 1 and 2. After immediate centrifugation, the hemoglobin content of supernatant was estimated at 550 mμ.

Optical density of supernatant	0	.580	.580	.558	.515	.383	.250	.099	.000	.000	.000	.002	.000	.003

The minimum concentration of PVS required to completely inhibit the complement added varies according to the amount of complement added (Fig. 1A).

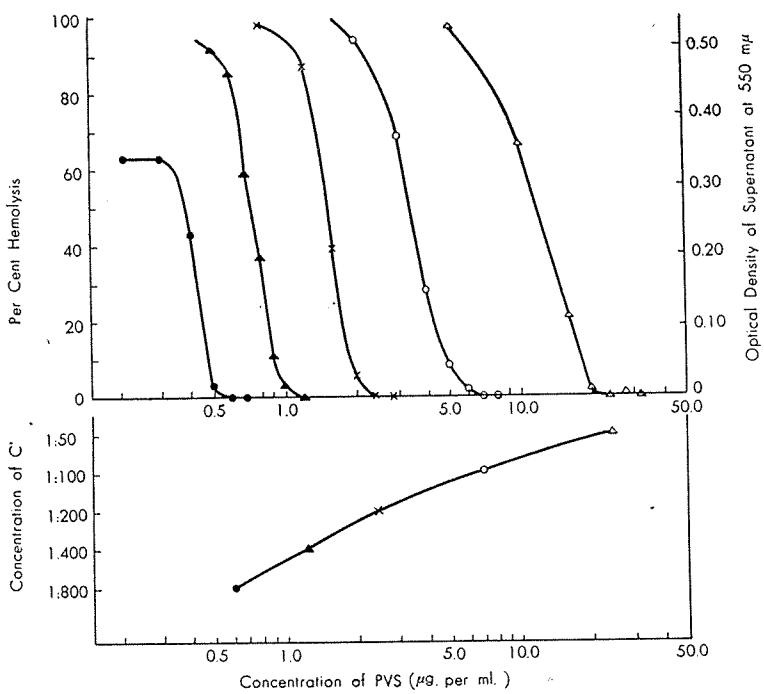

Fig. 1 A: The effect of PVS upon immune hemolysis with varying concentrations of C'
B: Relationship between the concentration of C' and the minimum PVS concentration for complete inhibition

As can be seen from Fig. 1B, a linear correlationship was found between the amount of complement added and the minimum concentration of PVS required to inhibit the complement.

Since Ca^{++} and Mg^{++} are required for complement hemolysis, the inhibitory action of PVS was first presumed to be due to the chelating action of PVS. No table is given but the inhibition was found not to be caused by the chelating action.

Reversible inhibition of complement by PVS:

As the anticomplementary action of polyanethol sulfonate (liquoid) was shown to be reversible by sequently adding a nonspecific protein, that of PVS was examined. To examine the reversible reactivation of the complement egg albumin was added to the C′-EA system inhibited with previously added PVS.

To each of 5 test tubes containing 0.5 ml. of VBS and 10.0 μg. of PVS dissolved in 1.0 ml. of VBS was added 1.0 ml. of 1:100 diluted C′ and 1.0 ml. of EA. As controls 5 more tubes with the same content were also included. The tubes were incubated at 37°C. for 15 minutes. 0.5 ml. of 1.0 per cent egg albumin were added to each of the 5 test tubes. To each of the 5 control tubes were added 0.5 ml. of VBS. At 15 minute intervals, to one tube from the test and control series 3.0 ml. of chilled VBS was added. The tubes were centrifuged in the cold and assayed for hemolysis. The results are presented in Fig. 2.

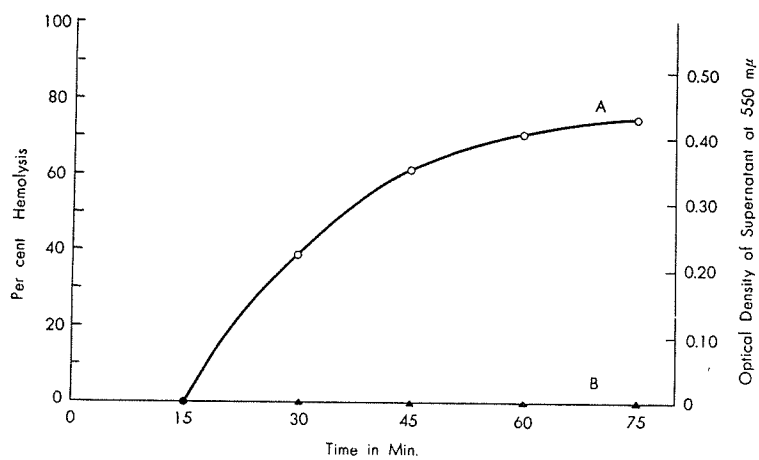

Fig. 2. Reversible nonspecific reactivation of PVS-inhibited complement by egg albumin
Curve A: egg albumin
Curve B: VBS control

As can be seen in the figure, complement activity was reactivated after the addition of egg albumin and in the control no hemolysis was found.

Complement components inhibited by PVS:

Klein (1956a) reported that liquoid inhibited his component C'_M and combined with $EAC'_{1,4}$ before C'_2. As PVS exhibited the similar character to liquoid during reversible reactivation of complement, attempts were made to identify the complement components inhibited by PVS.

EAC′$_p$ was prepared by incubating EA with complement inhibited by PVS. Suitable mixtures of complement components were further incubated with EAC′$_p$. The contents of the tubes and results are shown in Table 2. The experimental procedures are described above.

Table 2. Lyses of EAC′$_p$ and EAC′$_1$

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Aq. dest.	—	6.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
VBS	6.0	—	2.0	2.0	2.5	2.0	2.5	1.0	1.5	2.0	2.5	1.0	1.5	2.0	2.5	2.0	2.5
1:100 C′	—	—	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1:25 H	—	—	—	1.0	0.5	—	—	1.0	1.0	—	—	1.0	1.0	—	—	—	—
1:25 M	—	—	—	—	—	1.0	0.5	1.0	0.5	—	—	—	—	—	—	—	—
1:25 E	—	—	—	—	—	—	—	—	—	1.0	0.5	1.0	0.5	—	—	—	—
1:25 N	—	—	—	—	—	—	—	—	—	—	—	—	—	1.0	0.5	—	—
1:25 Z	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.0	0.5
EAC′$_p$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
As in the experiment shown in Table 1																	
Optical density of supernatant		.548	.547	.008	.002	.003	.001	.005	.001	.335	.180	.438	.295	.015	.008	.005	.001
Per cent hemolysis			100	1.5	0.4	0.5	0.2	0.9	0.2	61.1	32.8	79.8	53.8	3.7	1.5	0.9	0.2

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Aq. dest.	—	6.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
VBS	6.0	—	2.0	2.0	2.5	2.0	2.5	1.0	1.5	2.0	2.5	1.0	1.5	2.0	2.5	2.0	2.5
1:100 C′	—	—	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1:25 H	—	—	—	1.0	0.5	—	—	1.0	1.0	—	—	1.0	1.0	—	—	—	—
1:25 M	—	—	—	—	—	1.0	0.5	1.0	0.5	—	—	—	—	—	—	—	—
1:25 E	—	—	—	—	—	—	—	—	—	1.0	0.5	1.0	0.5	—	—	—	—
1:25 N	—	—	—	—	—	—	—	—	—	—	—	—	—	1.0	0.5	—	—
1:25 Z	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.0	0.5
EAC′$_1$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
As in the experiment shown in Table 1																	
Optical density of supernatant		.510	.510	.010	.002	.002	.002	.003	.002	.510	.419	.510	.425	.005	.002	.007	.004
Per cent hemolysis			100	2.0	0.4	0.4	0.4	0.6	0.4	100	82.1	100	83.3	1.0	0.4	3.4	0.8

As can be seen from the table, EAC′$_p$ was lyzed in tubes containing E or E and H. The E used in this experiment was shown to contain C′$_2$, C′$_3$ and C′$_4$. H contained C′$_3$ and C′$_4$. The behavior of EAC′$_p$ was the same as that of EAC′$_1$ as also shown in the table. Therefore EAC′$_p$ corresponds to EAC′$_1$.

Table 3. Lysis of EAC'_1 in the presence of PVS

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Aq. dest.	—	6.0	—	—	—	—	—	—	—	—	—	—	—	—
VBS	6.0	—	2.0	2.0	2.0	1.0	2.0	1.0	1.0	1.0	—	1.0	1.0	—
1:100 C′	—	—	1.0	—	—	—	—	—	1.0	—	—	1.0	—	—
1:25 H	—	—	—	1.0	—	1.0	—	1.0	—	—	1.0	—	—	1.0
1:25 M	—	—	—	—	1.0	1.0	—	—	—	—	—	—	—	—
1:25 E	—	—	—	—	—	—	1.0	1.0	—	1.0	1.0	—	1.0	1.0
10γ/ml. PVS	—	—	—	—	—	—	—	—	1.0	1.0	1.0	—	—	—
40γ/ml. PVS	—	—	—	—	—	—	—	—	—	—	—	1.0	1.0	1.0
EAC'_1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
As in the experiment shown in Table 1														
Optical density of supernatant	.470	.470	.006	.002	.007	.470	.470	.007	.032	.405	.005	.015	.082	

Table 4. Lysis of $EAC'_{1,4}$ in the presence of PVS

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Aq. dest.	—	6.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
VBS	6.0	—	2.0	2.0	2.0	1.0	2.0	1.0	2.0	2.0	1.0	1.0	—	1.0	1.0	1.0	—	1.0
1:100 C′	—	—	1.0	—	—	—	—	—	—	—	1.0	—	—	—	1.0	—	—	—
1:25 H	—	—	—	1.0	—	1.0	—	1.0	—	—	—	—	1.0	—	—	—	1.0	—
1:25 M	—	—	—	—	1.0	1.0	—	—	—	—	—	—	—	—	—	—	—	—
1:25 E	—	—	—	—	—	—	1.0	1.0	—	—	—	1.0	1.0	—	—	1.0	1.0	—
1:25 N	—	—	—	—	—	—	—	—	1.0	—	—	—	—	1.0	—	—	—	1.0
1:25 Z	—	—	—	—	—	—	—	—	—	1.0	—	—	—	—	—	—	—	—
10γ/ml. PVS	—	—	—	—	—	—	—	—	—	—	1.0	1.0	1.0	1.0	—	—	—	—
40γ/ml. PVS	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.0	1.0	1.0	1.0
$EAC'_{1,4}$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
As in the experiment shown in Table 1																		
Optical density of supernatant	.455	.455	.010	.007	.020	.460	.463	.450	.002	.010	.220	.465	.245	.003	.020	.085	.023	

To confirm this, the hemolysis of EAC'_1, $EAC'_{1,4}$ and $EAC'_{1,4,2}$ were compared on the addition of PVS with various complement reagents. The contents of the tubes and the results are shown in Tables 3, 4 and 5. These experiments were all performed on the same day with the same materials.

Table 6 summarizes the results of these tables. As can be seen from Table 6, C'_3 was unaffected because $EAC'_{1,4,2}$ was lyzed in the presence of PVS. On comparison of EAC'_1 and $EAC'_{1,4}$, it is quite clear that C'_4 was the most sensitive of the components, because $EAC'_{1,4}$ was appreciably lyzed in the presence of 2.5 μg. per ml. of PVS. However, when they were compared using more dilute

Table 5. Lysis of $EAC'_{1,4,2}$ in the presence of PVS

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Aq. dest.	—	6.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
VBS	6.0	—	2.0	2.0	2.0	2.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1:100 C′	—	—	1.0	—	—	—	—	1.0	—	—	—	—	1.0	—	—	—	—
1:25 H	—	—	—	1.0	—	—	—	—	1.0	—	—	—	—	1.0	—	—	—
1:25 M	—	—	—	—	1.0	—	—	—	—	1.0	—	—	—	—	1.0	—	—
1:25 E	—	—	—	—	—	1.0	—	—	—	—	1.0	—	—	—	—	1.0	—
1:25 N	—	—	—	—	—	—	1.0	—	—	—	—	1.0	—	—	—	—	1.0
10γ/ml. PVS	—	—	—	—	—	—	—	1.0	1.0	1.0	1.0	1.0	—	—	—	—	—
40γ/ml. PVS	—	—	—	—	—	—	—	—	—	—	—	—	1.0	1.0	1.0	1.0	1.0
$EAC'_{1,4,2}$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
As in the experiment shown in Table 1																	
Optical density of supernatant	.480	.480	.470	.475	.470	.480	.380	.480	.304	.470	.418	.370	.030	.025	.425	.284	

Table 6. Lyses of EAC'_1, $EAC'_{1,4}$ and $EAC'_{1,4,2}$ in the presence of PVS

	EAC'_1		$EAC'_{1,4}$		$EAC'_{1,4,2}$	
PVS	2.5γ/ml.	10γ/ml.	2.5γ/ml.	10γ/ml.	2.5γ/ml.	10γ/ml.
	%	%	%	%	%	%
1:400 C′	1.5	1.1	2.2	0.7	79.2	77.2
1:100 H	—	—	—	—	100	6.3
1:100 M	—	—	—	—	63.3	5.2
1:100 E	6.8	3.2	48.4	4.4	97.9	88.5
1:100 H	86.3	17.4	102.1	18.7	—	—
1:100 N	—	—	53.8	5.1	82.1	59.2

complements, neither EAC'_1 nor $EAC'_{1,4}$ were lyzed. Therefore C'_2 could be inhibited after the inhibition of C'_4 when PVS was present in relative excess.

Therefore C'_4 was the most sensitive to inhibition by PVS, and C'_2 was sensitive secondarily if PVS was in relative excess.

DISCUSSION

It is well known that anticoagulants such as heparin, liquoid and germanin are anticomplementary. The mode of the anticomplementary action of the anticoagulants has been extensively studied by Klein *et al.* (Klein and Wecker, 1955; Wecker and Klein, 1955; Klein and Lange, 1956; Klein 1956a, 1956b). They differentiated two types of the action; the one is the type dependent on the dose and the other is dependent to the concentration. The anticomplementary action of the anticoagulant, PVS, presented in this paper was dose-dependent and hence

PVS is very similar to heparin and liquoid in its action.

Heparin and liquoid were reported (1956b) to inhibit a newly postulated complement component C'_M by Klein. However this was not so in our experiments on the point of attack of PVS. EAC'_p was demonstrated to be the same as EAC'_1. EAC'_p was hemolyzed by the addition of E containing C'_2, C'_3 and C'_4. If EAC'_p were the same as the EAK_L of Klein (1956a), it should be hemolyzed by N containing C'_1, C'_2 and C'_3. However EAC'_p was not lyzed by N. Klein's EAK_L was lyzed by N and not by E. If Klein's hypothesis were correct, EAC'_p should not be lyzed by the addition of E and should require further addition of M to be hemolyzed. As liquoid was not available, the point of action of liquoid could not be confirmed. The authors are inclined to think that a new component C'_M need not be postulated in the midpiece fraction of the complement.

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