acid was correspondingly decreased. 2. In contrast, the supernatant carried out anaerobic and aerobic glucolysis, but not respiration of glucose. The submicroscopic particle fraction did not carry out the reactions of either system.

1. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, 1948, v172, 389.
2. Green, D. E., *Science*, 1952, v115, 3.
3. Harman, J. W., *Exp. Cell Res.*, 1950, v1, 382.
4. Judah, J. D., *Biochem. J.*, 1951, v49, 271.
5. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, 1948, v172, 847.
6. ———, *J. Biol. Chem.*, 1949, v179, 957.
7. Leuthardt, F., and Mauron, J., *Helv. Physiol. Acta*, 1950, v8, 386.
8. Millerd, A., Bonner, J., Axelrod, B., and Bandurski, R., *Proc. Nat. Acad. Sci.*, 1951, v37, 855.
9. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1949, v177, 893.
10. Showacre, J. L., *J. Nat. Cancer Inst.*, 1953, v13, 829.
11. Adler, E., von Euler, H., and Günther, G., *Naturwissenschaften*, 1937, v25, 282.
12. Colowick, S. P., Kalckar, H. M., and Cori, C. F., *J. Biol. Chem.*, 1941, v137, 343.
13. Geiger, A., *Biochem. J.*, 1940, v34, 465.
14. Ochoa, S., *J. Biol. Chem.*, 1941, v141, 245.
15. Utter, M. F., Wood, H. G., and Reiner, J. M., *J. Biol. Chem.*, 1945, v161, 197.
16. Hogeboom, G. H., *Fed. Proc.*, 1951, v10, 640.
17. LePage, G. A., and Schneider, W. C., *J. Biol. Chem.*, 1948, v176, 1021.
18. Schneider, W. C., and Hogeboom, G. H., *Cancer Res.*, 1951, v11, 1.
19. duBuy, H. G., Woods, M. W., and Showacre, J. L., *Pigment Cell Growth*, New York, Academic Press, 1953, pp. 335-351.
20. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, v138, 535.

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Nature of Growth Inhibitor in Some Mammalian Sera For Pleuropneumonia-Like Organisms of Human Origin. (20267)

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The inhibitory effect of some mammalian sera, when used in a concentration greater than 10%, on the growth of pleuropneumonia-like organisms (PPLO) has been noted by several investigators. Rabbit, sheep, and horse sera are satisfactory supplements but guinea pig, steer, and cow sera are inferior for the cultivation of the bovine pleuropneumonia organism(1). Growth of this organism was partially inhibited by 20% and completely inhibited by 50% horse serum. Edward(2), likewise, found that 17 strains of pleuropneumonia organisms of various origins grew poorly when bovine serum was used as a supplement. Morton, Smith and Leberman (3) compared various mammalian sera as supplements for the cultivation of human strains of PPLO. Bovine serum and citrated human plasma added to the basal medium in concentrations exceeding 10% were definitely inhibitory. Separation of the active growth factor in bovine serum eliminated this growth inhibitor(4). Since the fractionation of serum to obtain the growth factor removed the globulins, it was speculated that the inhibitor might be associated with antibody against PPLO. This report shows that the inhibitory component of bovine plasma is associated with alpha-globulin and the inhibitory action is enhanced by the presence of complement.

Materials and methods. The method employed for measuring the inhibitory activity of various fractions was essentially the same as previously reported(4). The preparations to be tested were incorporated into Bacto-PPLO agar with added 1% fraction A derived from bovine serum. Fraction A is the serum fraction containing the protein growth factor required by some strains of PPLO of human origin and has been described previously(4).

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Duplicate plates for each preparation were inoculated with 0.05 ml of the supernates after grinding agar blocks containing the PPLO colonies in Bacto-heart infusion broth. The inoculum for each strain in any one experiment was derived from the same supernate. The inocula were spread evenly over the plates with a bent glass rod. Two-day-old stock cultures of 4 representative human strains, 07, 09, 48 and 60, were used for preparing the inocula. The stock cultures were maintained on Bacto-PPLO agar containing 1% fraction A. Incubation was carried out aerobically for 72 hours at 37°C. The diameters of ten randomly selected colonies on each plate were measured by means of an ocular micrometer under the low power of the microscope. The *amounts* of the various bovine serum or plasma fractions added to the test medium were such as to give a final concentration equivalent to the amount of the test fraction found in 30% whole serum. A 30% final concentration was selected because with this amount definite inhibition of growth was easily detectable. Bacto-PPLO agar plates containing 30% final concentration of whole bovine serum and containing 1% fraction A were set up as controls. The agar concentration in all cases was kept at 1.2% so as to eliminate the effect of agar concentration on the size of the developing colonies. *Four fractions* of bovine serum were prepared by the ammonium sulfate precipitation method of Cohn *et al.*(5). These fractions consisted of the proteins separating between the following percentage saturation with ammonium sulfate (Merck reagent): 0-34%; 34-40%; 40-50%; and 50-60%. The precipitates were washed in the corresponding solutions of ammonium sulfate, redissolved in distilled water and dialyzed against frequent changes of distilled water for 72 hours in the refrigerator. Whole bovine serum was dialyzed in the same manner. Sterilization of all fractions was accomplished by filtration through Selas 02 unglazed porcelain filters. The *role of complement* in the inhibition of the growth of PPLO by whole serum and its globulin components was studied. Whole bovine serum inactivated by heating at 56°C for 30 minutes, inactivated bovine serum plus guinea pig complement (Lyovac), alpha globulin (bovine plasma fraction IV, Armour) plus complement, beta globulin (bovine plasma fraction III-1, Armour) plus complement, and gamma globulin (bovine plasma fraction II) plus complement were added to portions of the basal medium. The final concentration in the medium of alpha-globulin was 0.28 g %, of beta-globulin 0.26 g %, and of gamma-globulin 0.22 g %. Complement was added to give a 1:30 final dilution. The complement was the last component added to the medium. The plates were poured immediately and were inoculated as soon as possible in order to minimize inactivation. One per cent fraction A was added to all of the test media except those containing whole serum since the various globulin fractions will not support growth of PPLO. Normal bovine serum, complement plus fraction A, and fraction A were added to the basal medium as controls. *Complement fixation tests* were attempted using the various serum components as antibody in order to substantiate serologically the role of complement in the inhibition of growth of PPLO. Antigens were prepared for only two strains, 07 and 60, by sedimenting the organisms from 2-day-old 150 ml broth cultures. The medium employed was Bacto-heart infusion broth with added 1% fraction A derived from bovine serum. The sedimented cells were washed in saline solution and resuspended in 10 ml of a 1:30 dilution of guinea-pig complement (Lyovac). The *fraction of bovine serum* separating at 34% saturation with ammonium sulfate and used in the concentration found in the original serum served as antibody in one case. Solutions of bovine alpha-, beta-, and gamma-globulins were made in saline solution in concentrations corresponding to that found in the original serum. These concentrations are as follows: alpha-globulin 0.84 g %, beta-globulin 0.78 g %, and gamma-globulin 0.66 g %. These various serum components employed as antibody were heated at 56°C for 30 minutes. The *hemolytic system* consisted of a 1.5% suspension of washed sheep red blood cells which were sensitized by rabbit anti-sheep red blood cell serum. To 0.5 ml *amounts of varying dilutions* of the serum

TABLE I. Inhibitory Effect on Growth of Pleuropneumonia-Like Organisms of Various Ammonium Sulfate Fractions of Bovine Serum without Added Complement.

Serum fraction	Mean colony diameters in μ — Strain 07	09	48	60
0- 34% saturation	0*	0	0	0
34- 40% "	0	0	0	0
40- 50% "	37.9	23.3	0	0
50- 60% "	43.8	63.0	23.4	15.8
60-100% " (Fraction A)	105.4	74.0	107.5	121.2
Dialyzed serum	23.9	26.0	0	0
Normal "	25.4	15.8	0	0

* Indicates that no growth occurred.

fractions ranging from 1:6 to 1:40 were added 0.5 ml of the antigens suspended in complement. After mixing and allowing to stand overnight in the refrigerator, the sensitized red blood cells were added. The tubes were read after 30 minutes incubation in a 37°C water bath. Hemolytic, red blood cell, antigen, and anticomplementary serum controls were also run.

Results. The inhibitory effect, measured as decrease in mean colony diameters, of the various ammonium sulfate fractions of bovine serum on the four test strains is shown in Table I. It can be seen that the inhibiting component separates with the globulin fractions when precipitated with ammonium sulfate. The inhibitor appears to be scattered throughout the globulin fractions. Dialysis does not remove the inhibitor. To further substantiate the protein nature of the inhibitory component in bovine serum the fraction separating upon 34% saturation with ammonium sulfate was subjected to heat, acid, alkali, and enzyme hydrolysis. Portions of this fraction containing a high concentration of growth inhibitor were heated in a boiling water bath for 10 minutes in N HCl, N NaOH, and at pH 7.0. The neutralized hydrolysates exhibited no inhibitory activity against the 4 strains of PPLO while the unheated control remained inhibitory. Digestion with pepsin and papain under optimal conditions for two hours altered the inhibitor enough that only slight inhibition was noted for the 2 less susceptible strains. The 2 highly susceptible strains were completely inhibited by the 2 enzymatic digests.

Further definition of the serum component inhibitory for PPLO was attempted with the ethanol fractions of bovine plasma containing as their major components the alpha-, beta-, and gamma-globulins, respectively. The addition of amounts of these various globulins equivalent to that found in a final concentration in the basal medium of 30% whole serum, as described previously, failed to inhibit the growth of PPLO. The higher degree of purification of the ethanol globulin fractions as compared with the crude ammonium sulfate fractions had apparently eliminated either the inhibitory component or some factor necessary for the inhibition. In a further attempt to associate the inhibitory activity of whole bovine serum to one of its globulin fractions, guinea pig complement (Lyovac, Sharp and Dohme) was added to the basal medium as an adjuvant to the various globulin fractions.

The effect of the addition of complement to the various preparations is shown in Table II. Inactivated bovine serum exhibited no inhibitory activity for strains 07 and 09 but partially inhibited the growth of the 2 highly susceptible strains, 48 and 60. The addition of complement to the inactivated serum restored the inhibitory activity to some extent. The 3 globulin fractions alone do not inhibit growth but, in the presence of complement, alpha-globulin markedly inhibits growth of PPLO. The inhibitory component is concentrated in

TABLE II. Inhibitory Effect on Growth of Pleuropneumonia-Like Organisms of Various Fractions of Bovine Plasma in Presence of Complement.

Plasma fraction	Mean colony diameters in μ — Strain 07	09	48	60
Normal serum	26.0	19.2	0*	0
Inactivated serum	138.2	112.2	30.5	47.9
Inactivated serum + complement	80.7	46.6	17.8	0
α-globulin + complement	28.8	20.6	0	0
β-globulin + complement	64.4	58.8	57.5	47.9
γ-globulin + complement	64.4	58.8	56.2	39.7
Complement	63.0	54.8	39.7	35.6
Fraction A	131.8	117.9	71.2	71.2

* Indicates that no growth occurred.

TABLE III. Complement-Fixation Titers of Various Components of Bovine Plasma in Presence of Human Strains of PPLO.

Antigen	Plasma component			
	0–34	α-globulin	β-globulin	γ-globulin
07	1:5*	1:6	0	0
60	1:10	1:6	0	0

* Titer represents 50% fixation of complement.

the alpha-globulin fraction since the inhibition of growth caused by the beta- and gamma-globulins was not significantly different from that caused by complement alone. Complement in the form of normal guinea pig serum is inhibitory. As was mentioned above, guinea pig serum is one of the mammalian sera which are known to inhibit the growth of PPLO.

The presence of the growth inhibitor in the globulin fractions and its lability to heat, acid, alkali, and enzymes suggests an antibody-like nature. Experiments were run to determine if the inhibitor would fix complement in the presence of the organisms.

Table III lists the complement-fixation titers of the various bovine serum components in the presence of human strains of PPLO. The 34% ammonium sulfate fraction and alpha-globulin fixed complement in the presence of PPLO but the beta- and gamma-globulins failed to do so. Titration of the fractions used as antibody failed to reveal any anti-complementary activity. Hemolytic and antigen controls likewise exhibited complete hemolysis.

Since the alpha-globulin in the presence of complement causes inhibition of growth and in the presence of cells of PPLO fixes complement, it appears that the inhibitor found in whole serum is antibody-like in nature and is associated with the alpha-globulin fraction. That the ammonium sulfate fractions will inhibit growth without the addition of complement may be explained by the possibility that some complement or fraction of complement may be sedimented along with the other proteins during the crude fractionation.

Discussion. The antibody-like nature of the growth inhibitor for PPLO found in certain mammalian sera is evident from its protein nature, the requirement of complement for the initiation of growth inhibition, and the fixation of complement in low titer in the presence of the organisms. The partial destruction of the inhibitory component by enzymatic digest is in accord with the action of proteolytic enzymes on antibody for short periods of time. The inhibition of growth by the alpha-globulin in the presence of complement is possibly brought about by lysis of many of the organisms in the inoculum as well as those being produced during colony formation. There may possibly be a component of complement or of the guinea pig serum which is exerting an additive effect.

Beveridge, Campbell, and Lind(6) found a high percentage of normal human sera fixed complement in the presence of human strains of PPLO. It appears that only the sera of species presently known to be susceptible to infections with PPLO are inhibitory. Increasing the amount of toxic serum in the medium brings about greater inhibition of growth. It is probable then that a low antibody titer exists in most susceptible individuals and is indicative of previous exposure to PPLO. From the information obtained from these experiments, complement fixation would appear to warrant further investigation as a method for the diagnosis of infections caused by PPLO. The detection of circulating antibody-like substances in individuals might supply important information on the prevalence of these organisms.

Summary. Bovine alpha-globulin (bovine plasma fraction IV, Armour) in the presence of guinea pig complement inhibits the growth of the pleuropneumonia-like organisms. Heat-inactivated bovine serum is slightly inhibitory but the alpha-, beta-, and gamma-globulins are non-inhibitory in the absence of complement. Complement by itself does not inhibit growth as extensively as when in the presence of globulin. Alpha-globulin in the presence of the organisms fixes complement in low titer.

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1. Tang, F. F., Wei, H., McWhirter, D. L., and Edgar, J., *J. Path. Bact.*, 1935, v40, 391.

2. Edward, D. G. ff., *J. Gen. Microbiol.*, 1950, v4, 311.

3. Morton, H. E., Smith, P. F., and Leberman,

P. R., *Am. J. Syph., Gonorrh. and Ven. Dis.*, 1951, v35, 361.

4. Smith, P. F., and Morton, H. E., *J. Bact.*, 1951, v61, 395.

5. Cohn, E. G., McMeekin, T. L., Oncley, J. L., Newell, J. M., and Hughes, W. L., *J.A.C.S.*, 1940, v62, 3386.

6. Beveridge, W. I. B., Campbell, A. D., and Lind, P. E., *Med. J. Aust.*, 1946, v1, 179.

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Plasma Thromboplastin Component (PTC) Potency of Plasma Fractions.* (20268)

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In plasma thromboplastin component (PTC) deficiency disease, little or no shortening of the prolonged blood coagulation time or improvement in deficient prothrombin utilization follows the *in vivo* or *in vitro* addition of fraction I of Cohn(1,2). Other differential characteristics of the anti-hemophilic factor (AHF) and PTC are shown in Table I. A purified preparation of PTC, free of AHF,

TABLE I. Differential Characteristics of Plasma Thromboplastin Component (PTC) and Anti-Hemophilic Factor (AHF).

Source of material	PTC	AHF
Normal serum	P*	A*
Hemophilic plasma	P	A
PTC deficient plasma	A	P
45-50% saturated $(NH_4)_2SO_4$ fraction of normal plasma	P	A
0-33% saturated $(NH_4)_2SO_4$ fraction of normal plasma	A	P
Pseudoglobulin-albumin fraction of normal serum†	P	A
$BaSO_4$ adsorbed normal plasma	A	P
Citrate eluate from $BaSO_4$ which was mixed with normal plasma or serum	P	A

* P = Present; A = Absent.
† Supernate after removal of euglobulin by dilution and acidification.

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TABLE II. *In Vitro* Effect of Plasma Fractions on Residual Serum Prothrombin in PTC Deficiency.

Substance added (0.1 cc to 2. cc of patient's blood)	Concentration, mg/cc*	Residual serum prothrombin† (units/cc)
0.32% sodium citrate in 0.9% NaCl		180
Normal plasma		<10
Fraction I	3.80	105
II	5.10	255
II + III	18.50	<10
IV	9.52	15
IV-1	4.09	<10
IV-4	5.43	100
V	29.8	200

* Each fraction was reconstituted to its original plasma concentration and dialysed against 0.32% sodium citrate in 0.9% NaCl overnight. The dialysis fluid was then changed and the fractions dialysed another 4 hr.
† Two-stage method of Ware and Seegers(4), after incubation of blood for 1 hr at 37°C.

prothrombin and prothrombin conversion accelerators has been made by acidification of normal serum, adsorption of the serum with barium sulfate, and elution of the PTC from the barium sulfate with sodium citrate(2). This preparation is highly potent *in vitro*, but is unsuitable for intravenous administration in the human subject. The present investigation was conducted in an effort to find a potent PTC preparation suitable for intravenous administration among the fractions precipitated from plasma by varying the alcohol concentration and pH at low temperatures according to Cohn's methods(3).

Results. The PTC potency of the various fractions was determined by testing their