however, and complete digestion of the coagulum is now commonly avoided by the successive addition of fresh layers of plasma, as they become necessary. Since this procedure is laborious and requires large amounts of plasma, it was hoped that the problem might be solved by the use of antitryptic agents. The present experiments, involving more than 150 cultures, would seem to eliminate egg-white antitrypsin as a possible antiproteolytic agent for use in tissue cultures.

The failure of egg-white antitrypsin to prevent plasma digestion in tissue cultures is in marked contrast to its ability to prevent the proteolytic action of trypsin upon either casein or fibrin substrates. However, soybean trypsin-inhibitor has been found effective both in tissue culture and in test-tube experiments(1). These divergent results may possibly be attributed to a difference in the degree of specificity of the two antiproteolytic agents. Egg-white antitrypsin has been shown(9,10) to be almost entirely specific for trypsin, whereas soybean antitrypsin has been found to be a general inhibitor of proteolytic enzymes(10). The general antiproteolytic activity of the soybean trypsin-inhibitor might account for its interference with normal plasma coagulation, whereas egg-white antitrypsin, which is active only against trypsin, does not interfere.

Digestion of the plasma coagulum by cells cultivated *in vitro* has been attributed to the secretion of proteolytic enzymes(8,11) or to the elaboration of a substance that activates profibrinolysin to fibrinolysin(12,13). The present findings do not furnish evidence in support of either of these hypotheses, but they do render it unlikely that plasma digestion is caused by *trypsin.*

Summary. Egg-white antitrypsin not only fails to prevent but actually stimulates the digestion of plasma by cells cultivated *in vitro.* The presence of even low concentrations of antitrypsin inhibits tissue-culture growth but does not interfere with normal plasma coagulation. The antitrypsin preparations shown to be ineffective in tissue cultures strongly inhibit the proteolytic activity of crystalline trypsin in test-tube experiments.

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Antibacterial Activity of Hydrolyzed Red Blood Cells *in Vitro.** (17902)

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In our recent studies(1) the presence of an antibacterial factor in ticks was interpreted mainly as a biological phenomenon which may have bearing on the natural transmission of arthropod-borne infections. The higher antibacterial potency of blood engorged ticks as compared with unfed specimens was a significant indication of a possible growth inhibitory role of blood *per se.* Preliminary tests gave evidence that the tryptic digest of guinea pig blood exhibits a marked growth inhibition of *Bacillus subtilis*(1). This observation stimulated the present inquiry as to whether the blood of various animals may serve as a source of antibacterial substances. The possible relationship of the following antibacterial substances of animal origin have been considered in the present study: Flem-

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1. Anigstein, L., Whitney, D. M. and Micks, D. W., *Tex. Rep. Biol. and Med.*, 1950, v8, 86.

ing's lysozyme; the antibacterial peptides isolated by Bloom, Watson, *et al.*(2) from the cells of various animal tissues; bacteriostatic extracts from rabbit liver and human placenta reported by Konikova, *et al.*(3); and "erythrin" originating from red blood cells and quoted by Waksman(4).

Recent developments in the field of antibiotics of animal origin are reflected by the series of papers of Pavan(5) in Italy. Extracts from tissues of helminths, arthropods, molluscs, fish, amphibia and reptiles were shown by this author to possess antibacterial properties.

Material and methods. Blood samples from man, cattle, dog, rabbit and chicken were allowed to coagulate, the clot separated, triturated in distilled water and lyophilized. The desiccated material (0.1 g) was suspended in 5.0 ml of 0.1 M phosphate buffer (pH 7.8) containing 0.1 ml of 5% trypsin (Pfanstiel 1:110) and the mixture incubated for 5 hours at 37°C. This was followed by heating in 65°C waterbath for 30 min. The clear, dark-brown tryptic digest was tested for antibacterial activity by occasional assays with streak plates, but mainly by the spot dilution method on tryptone glucose agar plates previously flooded with bacterial cultures. This technic, previously described(1) for antimicrobial assays, proved very convenient, serving the double purpose of qualitative and quantitative evaluation. In the latter case, the gradual diminishing diameter of the circular inhibition zones in serial dilutions of the material placed in a standard drop (gauge 27) on the flooded plate can be measured and compared with circular spots produced by a conventional antibiotic of known potency. The same technic as applied to blood was used later for the hydrolysis of bovine hemoglobin powder (Armour Laboratories). The following modification was also used: before adding trypsin hemoglobin was denatured by heating at 65°C for 30 min in N/10 NaOH followed by adjustment to pH 7.8 with N HCl.

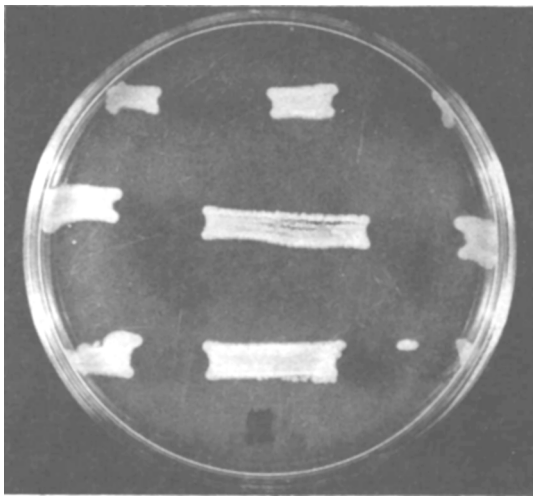

FIG. 1.
Growth inhibition of *Bacillus subtilis* as shown by interrupted streaks on agar plate in areas of contact with trypsin digested human red blood cells (dilution 1:50).

Experiments and results. A complete growth inhibition of *Bacillus subtilis* on streak agar plates was shown in contact areas with processed blood of human, cattle, dog and rabbit origin (Fig. 1) corroborating the previously observed phenomenon with guinea pig blood(1). Since this observation was made with washed and digested red cells, it was concluded that red cells or hemoglobin liberate the active principle inasmuch as lyophilized serum and washed, dried fibrin (Armour) after hydrolysis showed no action against *B. subtilis*. Crude extracts of blood of man, cattle, dog and chicken were tested against a series of gram-positive and gram-negative bacteria. The qualitative tests with mammalian blood indicated a general uniformity in the degree of growth inhibition (Table I) as compared with the lower activity of chicken blood. Of the three mammals tested, cattle blood showed the greatest activity. The highest sensitivity was shown by the spore-bearing bacilli, *Sarcina lutea* and *Staphylococcus aureus*, but inhibition of growth was also recorded among some gram-negative organisms (Table I).

2. Bloom, W. L., Watson, D. W., Cromartie, W. J. and Freed, M. *Jour. Inf. Dis.*, 1947, v80, 41.

3. Konikova, A. S., Urasova, A. P. and Asarkh, *Acad. Sc. U.S.S.R.*, 1945, v47, 565.

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5. Pavan, M., *La Ricerca Scient.*, 1949, v19, 1011.

TABLE I.

Antibacterial Activity *in Vitro* of Hydrolyzed Blood as Established by Complete + or Partial ± Inhibition of Growth.

Spot dilution method on flooded agar plates. All dilutions 1:50 containing 20 mg of the raw dehydrated material per 1.0 ml or 0.4 mg per drop.

Organism	Blood origin			
	Human	Cattle	Dog	Chicken
Staphylococcus albus	±	+	±	±
" *aureus*	+	+	+	—
Sarcina lutea	+	+	+	—
Bacillus subtilis	+	+	+	+
" *anthracis*	+	+	+	—
" *pumilus*	+	+	+	±
" *megatherium*	+	+	+	+
Salmonella typhimurium	±	+	±	±
Escherichia coli	±	+	±	+

Thus, by enzymatic hydrolysis of red blood cells substances were liberated which exerted an inhibitory action *in vitro* on various organisms. The degree of growth inhibition of *B. subtilis* as a test organism was estimated in serial dilutions noting the endpoint of activity and measuring the diameter of the circular zones produced by the active material. Under these constant conditions the radius of activity varied somewhat with the blood donor. For example, the average diameter for zones produced by human blood was approximately equal to 17-19 mm, for cattle blood 15-18 mm, for dog blood 14-16 mm, whereas the range for chicken blood was from 11 to 15 mm, all measurements being taken at the same initial dilution of 1:100 (Fig. 2). Thus it became evident that the antibacterial activity of human and cattle blood cells was relatively higher than that of dog or chicken, not only in the size of the inhibition zones, but also by the endpoint titers in serial dilutions.

Attempts to localize the source of the antibacterial products of blood gave evidence that the tryptic digest of hemoglobin powder is at least equally as inhibitory to *B. subtilis* as the blood products. This was followed by quantitative evaluation on a wide spectrum of various organisms (Table II). Whereas *B. subtilis* and other spore-bearing bacilli show the highest sensitivity, the inhibitory effect on gram-positive cocci and on some gram-negative bacilli is of interest. In this regard, the considerable sensitivity of *Streptococcus viridans* may prove of practical importance. It may be noted that the usual resistance of *Pseudomonas aeruginosa* to antibiotics is lowered by the hemoglobin hydrolysates. For reasons of comparison with conventional antibiotics, Bacitracin was chosen in view of its similar spectrum of activity. Using the spot dilution technic with *B. subtilis* circular zones of 15 mm diameter were produced by a standard drop of Bacitracin in a concentration of 10 mg/ml. This

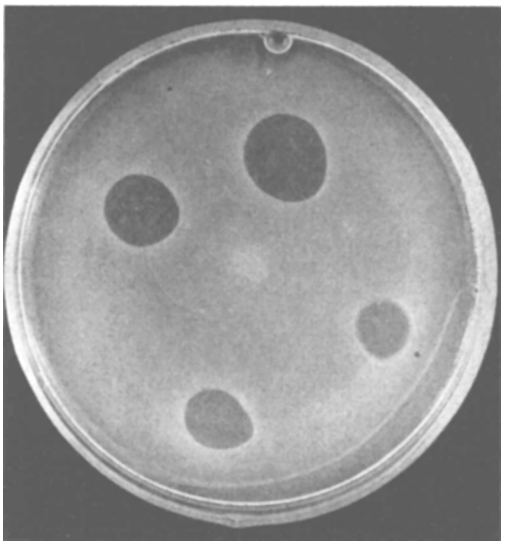

FIG. 2.

Growth inhibition of *Bacillus subtilis* on flooded agar plate as shown by circular zones produced by digested rabbit blood cells. Spot dilution method beginning with 1:100 in the largest circle and ending with 1:1,600 in the smallest.

TABLE II.
Antibacterial Activity *in Vitro* of Hydrolyzed Bovine Hemoglobin.
Spot dilution method on flooded agar plates.

Test organism	Endpoint		Inhibition
	mg/ml	Dilution	
Staphylococcus albus	2.5	1:400	Complete
" *aureus*	10.0	1:100	"
Sarcina lutea	10.0	1:100	"
Streptococcus viridans	1.25	1:800	"
Bacillus subtilis	0.12	1:9600	"
" *anthracis*	0.24	1:4800	"
" *pumilus*	1.25	1:800	"
" *megatherium*	0.24	1:4800	"
Salmonella oranienburg	10.0	1:100	"
Escherichia coli	2.5	1:400	"
Pseudomonas aeruginosa	2.5	1:400	"

diameter corresponded to that produced by an identical drop of bovine hemoglobin hydrolysate in a concentration of 0.2 mg/ml. In other words, the potency of our product in respect to *B. subtilis* compares well with that of a recognized antibiotic. However, this comparison of a crude product with a purified antibiotic is only of relative value.

Initial toxicity tests showed that daily intra-abdominal injection of 20 mg of the blood hydrolysate per guinea pig (450-600 g) over a period of 5 days had no untoward effect. Similar tests on mice inoculated intra-abdominally with 1.0 ml of the crude hydrolysate containing 20 mg of the dry material showed that these animals were unaffected after a total of 160 mg were administered over 8 days.

The physical properties of the antibacterial substance or substances derived from red blood cells are characterized by a significant thermostability resisting exposure to 121°C for 15 minutes in a liquid medium at a pH range from 7.8 to 9.0. In contrast to the unchanged appearance of the autoclaved tryptic digest within this pH range accompanied by a moderate loss in antibacterial activities against *B. subtilis*, autoclaving of the blood hydrolysates at pH 5.5 or lower causes a heavy precipitate and complete loss of antibacterial activities of the supernate. Similar properties were shown by extracts of blood engorged ticks as reported in our recent study (1). The activity of the clear, dark brown liquid of blood hydrolysates indicates solubility of the active principle in aqueous solutions of phosphate buffer or in saline, with optimum activity in the range of pH 7.0 to 8.0.

Discussion. The continuity in the transmission of infectious agents in nature by biological vectors, the bacterial sterility of the blood sucking arthropods, their remarkable resistance to specific pathogenic agents, and above all, the observations on the bacteriostatic factors in blood engorged ticks, have prompted the present study. Of these phenomena, the resistance or the so-called natural immunity of the arthropod vector to organisms pathogenic to higher animals dominates the host-parasite problem. The survival of the resistant host may be due to previous contacts with the pathogenic agent or perhaps to the production of metabolic substances, some of which may neutralize or suppress the activities of the parasite. In this regard, the isolation of an anthracidal polypeptide from leukocytes and other animal cells by Watson, Cromartie, *et al.*(6) indicates that such substances as antibacterial peptides of animal origin may be vital contributors to host resistance. Another and earlier example is Fleming's lysozyme as a typical representative of an antimicrobial substance of animal origin. In view of its wide prevalence in animal tissues, lysozyme and its possible relationship to our products was considered.

For this purpose, crystalline egg white

6. Watson, D. W., Cromartie, W. J., Bloom, W. L. *et al., J. Inf. Dis.*, 1947, v80, 121.

lysozyme (Armour Company) was submitted to conditions under which blood hydrolysates exhibit their optimal antibacterial activities with the result that contrary to the pH range of 7.8 to 9.0 in which our products resist autoclaving, lysozyme activities were destroyed regardless of pH (5.4-9.0). Furthermore, the stability of lysozyme in acid solution and its inactivation in alkaline solutions is contrary to the characteristics of our product. Although the specific identity of our antibacterial substance or substances has not been determined as yet, it is plausible that the ultimate products obtained from hemoproteins by hydrolysis are peptides or amino acids. In its present crude form, the material is most probably a hydrolysate complex rather than an entity. As to the antibacterial substances of animal origin reported by others and previously mentioned by us, the bacteriostatic preparation from liver obtained by Konikova *et al.*(3) by acid precipitation is water-insoluble and apparently differs from our product inasmuch as they obtained no bacteriostatic fractions from processed blood. A sample of "erythrin" made available to us by Dr. Waksman was highly acid and insoluble in water.

It becomes apparent from the present study that the bacterial growth inhibition *in vitro* compares with the phenomenon induced by antibiotics. This similarity refers particularly to the selective antibacterial action of the substances released from red blood cells, affecting primarily the gram-positive organisms. Considering this selectivity and the biological source of origin of our products, their inclusion into a broader concept of antibiotics seems to be justified. This would be also in line with the recognition of antibiotic products from higher plants(7).

Summary. Red blood cells of various animals were submitted to enzymatic hydrolysis in alkaline medium, the resulting products exhibiting *in vitro* marked antibacterial activities. The latter covered a relatively wide spectrum of most gram-positive and a few gram-negative organisms. The highest activity was displayed by hydrolysates of human and bovine hemoproteins. Hydrolyzed bovine hemoglobin powder likewise exhibited antibacterial properties. These antibacterial products are heat-resistant and water-soluble. Their optimum activity lies in the pH range of 7.0 to 8.0. It is assumed that the active principle of the crude substance is a peptide-amino acid complex. This complex proved to be nontoxic to guinea pigs and albino mice. Its relation to other antibiotics is discussed.

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Antigenic Identity of *Shigella alkalescens* Type I and Kauffmann's Escherichia O Group 1. (17903)

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Antigenic relationships between the coliform bacteria and members of the genus Shigella have been studied by many workers (1-5). The sharing of minor antigenic components by members of the two groups has been noted frequently, but complete antigenic identity has been demonstrated in only a few instances. The closest relationships to the coliform group have been found in *Shig-*

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