TABLE I.
Relative Effects of Cobaltous Chloride ($CoCl_2 \cdot 6H_2O$) on Production of Erythrocytes and Hemoglobin in Rats when Administered Orally and by Subcutaneous Injection.

No. animals	Daily dose mg/kg Oral	Daily dose mg/kg Subcut.	Erythrocytes in millions/cu mm. Time in weeks 0	2	4	6	8	Hemoglobin in g per 100 ml Time in weeks 0	2	4	6	8
4	2.5		8.84	9.07	8.90	9.56	8.64	16.2	16.2	17.0	17.0	17.2
4		2.5	8.65	9.44	10.33	11.38	11.65	16.1	16.4	19.4	21.8	22.5
4	10.0		9.03	9.76	10.28	10.58	10.40	16.2	16.6	18.2	20.0	20.0
4		10.0	9.36	10.07	12.59	11.98	11.73	16.4	18.2	22.2	22.9	22.4
4	40.0		8.83	9.25	10.05	11.17	11.42	15.9	17.0	18.6	20.8	21.7
4*		40.0	8.84	10.01	—	—	—	15.8	17.8	—	—	—
6	Controls		8.72	9.01	9.51	8.82	9.06	16.0	16.5	16.7	16.8	16.2

* None of these 4 animals survived beyond 8 days due to the toxicity of cobalt in such massive doses. Erythrocyte and hemoglobin determinations were made at the end of 6 days rather than at 2 weeks. The animals receiving the same dose orally survived.

similar effect, but required an additional 2 weeks.

Rats which received 2.5 mg/kg of cobaltous chloride subcutaneously or 40 mg/kg by mouth did not manifest significant toxic effects. Their body weight remained essentially constant, as did that of the controls. Rats which received 10 mg/kg of cobaltous chloride subcutaneously lost an average of 24% of their body weight by the end of 6 weeks. Those animals which received 40 mg/kg daily, subcutaneously, survived 5, 7, 8, and 8 days, respectively.

Summary. The relative effect of parenteral versus oral administration of cobaltous chloride on erythrocyte and hemoglobin formation in rats has been determined. Daily subcutaneous injection of 2.5 mg/kg of body weight, over a period of 6 weeks, resulted in an increase of more than 30% in the number of erythrocytes per cu mm and in grams of hemoglobin per 100 ml of blood. To produce a similar effect from oral administration of cobaltous chloride, within the same period of time, 40 mg/kg of body weight was required. These dosages are without significant toxic effects. Rats which received 10 mg cobaltous chloride/kg daily, subcutaneously, averaged 24% weight loss by the end of 6 weeks. Rats which received 40 mg/kg body weight, subcutaneously, did not survive beyond 8 days.

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Inhibition of the Hirst Haemagglutination Reaction by Pneumococcal Extract, Normal Serums, and Blood Cell Esterases.*

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During the course of other studies it was observed that culture filtrates of Type I and Type V pneumococci would inhibit the agglutination of chicken red cells by influenza virus. Stone[1,2] found that lecithinase would

* While the final draft of this paper was being prepared several studies[3-5] were reported before the Society of American Bacteriologists, in Philadelphia, dealing with the nature of agglutinin-inhibiting substances.

1 Stone, J. D., *Australian J. Exp. Biol. and Med. Sci.*, 1946, **24**, 191.

2 Stone, J. D., *Australian J. Exp. Biol. and Med. Sci.*, 1946, **24**, 197.

TABLE I.

Substrate	Cu mm CO_2 evolved in 30 minutes							
	Cholinesterase from human A cells	Cholinesterase from human AB cells	Type I pneumococcus autolysate	Type V pneumococcus Autolysate	Type V pneumococcus Filtrate	Normal rabbit serum	Normal G.P. serum	Normal human serum
Tributyrin .03 M	0	0	38	43	14	200	256	184
Acetyl choline .003 M	40	34	—	—	—	—	—	—

inhibit the hemagglutinating action of vaccinia and ectromelia viruses but did not affect the agglutination of red cells by influenza virus.

The discovery[6] of an esterase in pneumococcus cultures, subsequently identified as cholinesterase[7] suggested to us that the latter enzyme might bear a causal relationship to the inhibition of influenzal hemagglutination. While some of our results do not fit smoothly into such an explanation, they are not altogether contradictory and the data obtained seem of sufficient importance to warrant publication.

Materials and Methods. Pneumococcal esterase was prepared from Type I and Type V cultures grown in a beef heart infusion hormone broth. In one method of preparation, after 18-24 hours incubation the cultures were adjusted to pH 7 with N/1 NaOH, were refrigerated overnight (for increased autolysis) and then centrifuged. The supernatant was Seitz-filtered. A second preparation was obtained by repeated freezing and thawing (6 cycles) of saline suspensions of once-washed 18-hour cultures. In the following discussion the first preparation is designated as pneumococcal filtrate, the second as pneumococcal autolysate.

For comparative purposes a partially purified specific cholinesterase was prepared from human red cells by the method of Mendel and Rudney.[8]

The inhibitory substances were titrated by a modification of the Hirst agglutinin-inhibition technic in which successive 2-fold dilutions of the inhibiting substance were substituted for serum dilutions. In all such tests 10 agglutinating units of PR8 influenza virus were employed per tube and a 0.75% chicken red-cell suspension was used instead of the heavier suspension recommended by Hirst. Naked-eye readings are facilitated by the use of the lighter cell suspensions. Inhibition titers are 2- to 4-fold higher than when 1.5% suspensions are used.

Experimental. Table I records the esterase activity of the above preparations and also of normal rabbit, guinea pig, and human sera. Schachter's modification[9] of the Ammon-Warburg technic was used with tributyrin as the substrate. Since the esterase of blood cells is a specific cholinesterase, acetyl choline was used in determining its activity.

In Table II are recorded the results of agglutinin-inhibition tests using normal human and animal sera as inhibiting agents as well as the esterases prepared from pneumococci and from human red cells.

To determine whether the effect of the pneumococcal inhibitory substance was due to action upon the virus, an unconcentrated allantoic fluid having a CCA titer of 1/1280 was mixed in equal proportions with Type

[3] Friedewald, W., Miller, E. W., and Whatley, L. R., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 62.

[4] Liebmann, A. J., Perlstein, D., and Snyder, G. A., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 63.

[5] Woolley, D. W., and Green, R. H., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 63.

[6] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, **29**, 215; 1920, **32**, 547, 571, 583; 1923, **38**, 199.

[7] Schaller, K. Z., *Physiol. Chem.*, 1942, **276**, 271.

[8] Mendel, B., and Rudney, H., *Biochem J.*, 1943, **37**, 59.

[9] Schachter, R. J., *Am. J. Physiol.*, 1945, **143**, 552.

TABLE II.

Source of inhibiting material	Type I pneumococcus filtrate	Type I pneumococcus autolysate	Type V pneumococcus filtrate	Type V pneumococcus autolysate	Cholinesterase from human A cells	Cholinesterase from human AB cells	Normal G.P. serum	Normal rabbit serum	Normal human serum (Group O)	Normal human serum (Group AB)
Agglutinin-inhibition titer	1/16	1/128	1/256	1/512	1/32	1/64	1/512	1/2048	1/4096	1/512

V pneumococcal filtrate diluted 1/5 and the mixture was incubated at room temperature for 1⅛ hours. The virus thus treated was then diluted 1/128 with physiological saline to make a final virus concentration of 10 agglutinating units per cc and a final dilution of the filtrate of 1/640 (beyond the concentration at which it could inhibit agglutination). When a red cell suspension was added typical agglutination occurred. Virus similarly treated with red-cell cholinesterase or with guinea pig serum was also still capable of agglutinating chicken red cells.

The pneumococcal filtrate, diluted 1/5 was then incubated at room temperature with an equal volume of 7.5% chicken red-cell suspension for 1½ hours after which the cells were washed twice, resuspended in 0.75% concentration and tested for agglutinability. They were found to be no longer agglutinated by PR8 virus. Cholinesterase from human red cells had no such effect upon the agglutinability of chicken red cells.

According to Avery and Cullen[6] pneumococcal esterase suffers progressive loss of activity at temperatures above 50° C and becomes completely inactive within 10 minutes at 70° C. McCrea has reported[10] similar progressive destruction between 56° C and 65° C of the substance in normal rabbit serum which inhibits influenzal hemagglutination and complete inactivation when such serum was heated for 30 minutes at any higher temperatures. Had we been able to confirm McCrea's observations, a striking analogy could have been established between the inhibiting agent and the cholinesterases studied. As Table III shows, however, the inhibitory power of normal guinea pig and rabbit sera were found to be either unaffected or were somewhat enhanced by temperatures between 60° C and 70° C. Type V pneumococcal filtrate and human red-cell cholinesterase, on the other hand, were entirely inactive after being heated to 60° C for half an hour.

It is interesting to note (Table IV) that when the inhibition test is performed at room temperature agglutination may occur at first

10 McCrea, J. F., *Australian J. Exp. Biol and Med. Sci.*, 1946, **24**, 283.

in tubes in which with further incubation the clumps will be dispersed under the influence of the inhibiting agent. Whether the agent is a serum or a pneumococcal preparation, it appears to be slower in its action at this temperature than the agglutinating factor but in time is capable within its effective concentration of counteracting it completely.

Discussion. Tables I to IV, inclusive, reveal significant similarities in the action of pneumococcal filtrates, human red-cell extracts, and sera of 3 species of non-immunized animals. Since chicken red cells exposed to the Type V pneumococcal preparation rendered them refractory to agglutination by PR8 virus whereas the human red-cell extract failed to produce any such effect, some doubt is aroused regarding the relationship between specific cholinesterase (which the human cell extract contains) and the inhibitory phenomenon.

When these discordant findings have been considered, however, the fact remains that all of the agents employed in this study were capable of inhibiting influenzal haemagglutination and all contained either specific or non-specific cholinesterase.

Filtrates of a number of other organisms were tested for the inhibitory effect, but all were found to be inactive. The organisms tried included *Staph. aureus, Strept pyogenes, Strept. mitior, C. diphtheriae, C. hoffmanni, E. coli, B. subtilis,* and *Prot. vulgaris.* All of these organisms have been reported[11] to contain no cholinesterase.

TABLE III.
Agglutination-inhibition Titers.

Inhibiting agents	Treatment			
	Unheated	60°C ½ hr	65°C* ½ hr	70°C* ½ hr
Type V pneumococcus filtrate	1/256	<1/4		
Red-cell cholinesterase	1/32	<1/4		
Guinea pig serum	1/320	1/1280	1/1280	1/1280
Rabbit serum	1/320	1/320	1/1280	1/2560

* Sera were diluted 1/10 with physiological saline prior to heating.

Summary. 1. Filtrates and autolysates of Type I and Type V pneumococcus cultures inhibited the agglutination of chicken red cells by influenza virus. 2. Chicken red cells treated with such a filtrate (or autolysate) could not then be agglutinated by the virus. 3. Human red-cell extracts and normal sera of man, guinea pigs, and rabbits were also shown to be capable of inhibiting agglutination of chicken red cells by influenza virus, although some differences of behavior were noted. 4. The presence of a cholinesterase in each of these agents was shown and their possible significance in agglutinin inhibition was discussed.

11 Vincent, D., and de Trat, J., *Compt. Rend. Soc. Biol.*, 1945, **139**, 1148.

TABLE IV.
Inhibition Titers.

Incubation time	G.P. serum vs PR8 (Type A)	G.P. serum vs Lee (Type B)	Rabbit serum vs PR8 (Type A)	Rabbit serum vs Lee (Type B)	Type V pneumo filtrate vs PR8
25 min	<1/10	1/10	0	1/20	1/10
90 min	1/1280	1/2560	1/640	1/80	1/80
16 hr*	1/2560	1/2560	1/1280	1/1280	1/160

* Placed in 4°C refrigerator after 90 minutes at indicated temperature.