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Composition of Antigen-precipitin Precipitate.

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Practically nothing is known about the composition of the antigen-precipitin precipitate beyond the fact that the amount of the precipitate produced may be much more than the amount of the antigen used.[1] Just how much of the antigen is present in the precipitate does not seem to have been determined. This is due to the fact that there exists no chemical method of determining the antigen (protein) and the precipitin (protein) separately.

One of us has worked out a method for the determination of minute amounts of hemoglobin.[2] The method is based on the benzidine reaction, and as little as 0.02 mg. hemoglobin suffices for a quantitative determination. When hemoglobin is used as the antigen its amount in the antigen-precipitin precipitate can be determined directly by means of that method. The amount of the precipitin in the precipitate can be determined by the difference between the total protein and the hemoglobin. The assumption is here made that the precipitate contains only the antigen and the precipitin and no other protein.

The word hemoglobin is used here in the generic sense, including oxyhemoglobin and its immediate derivatives. Oxyhemoglobin on standing is slowly converted into methemoglobin. This is, however, a matter of no consequence since oxyhemoglobin and methemoglobin have the same immunological properties[3] as well as the same chemical properties utilized for their quantitative determination in the above mentioned method.

Sheep oxyhemoglobin solution was prepared from washed cells by laking with 4 volumes of water and shaking with 2.5% suspension of freshly prepared aluminium hydroxide. The mixture was centrifuged and the clear supernatant liquid was filtered through a Seitz filter. Dog's oxyhemoglobin solution was prepared from crystals obtained by electrodialysis according to the method of Stadie.[4] Alumina cream was also used. The final solutions contained about 5% hemoglobin.

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[1] Wells, H. G., "The Chemical Aspects of Immunity," The Chemical Catalog Co., New York, 146.

[2] Wu, H., *J. Biochem.* (Japan), 1923, ii, 189.

[3] Heidelberger, M., and Landsteiner, K., *J. Exp. Med.*, 1923, xxxviii, 561.

[4] Stadie, W. C., *J. Biol. Chem.*, 1926, lxviii, 229.

For the immunization of rabbits, we have tried different procedures, but none has been found to be thoroughly satisfactory. Only 15 out of about 50 rabbits gave usable sera. This is undoubtedly due to the generally recognized weak antigenic property of hemoglobin. The last few rabbits were immunized by giving 10 or more subcutaneous injections at intervals of 3 days. Each dose was 4 cc. of 5% hemoglobin solution. The sera were tested on the 4th day after the last injection.

After a preliminary precipitin reading, suitable amounts of serum were measured into graduated centrifuge tubes and equal volumes of hemoglobin solution of different concentrations were added. The solutions were mixed by gentle rotation and left in the cold room for 24 hours. The tubes were then centrifuged and the supernatant liquid carefully decanted. The precipitate was washed with 0.9% NaCl, stirring gently with a slender glass rod, centrifuged and the supernatant liquid decanted. The washing was repeated once more. The precipitate was then dissolved in 2-3 cc. distilled water with the addition of 1 to 2 drops of 1-N Na_2CO_3. The solution was then diluted to such volume that its tint matched approximately that of a standard hemoglobin solution containing 0.025 mg. per cc. Weaker standard solutions of hemoglobin were sometimes used. 1 cc. portions of the solution were used for hemoglobin determination and suitable amounts were taken for total N determination. This latter determination was made by a modification of the Folin-Wu micro-method. Potassium persulphate was used to aid the digestion and the ammonia was aspirated into 1 cc. of 1/10 HCl solution before Nesslerization. The N content of the stock hemoglobin solution from which dilute solutions were prepared was also determined. The results of the last 5 experiments are shown in the accompanying table.

It will be noted that the percentage of the hemoglobin N in the total N remains approximately constant whether the concentration of the hemoglobin was high or low and whether the serum is strong or weak. The average value is about 10. In some experiments there was a tendency for the percentage to decrease when the concentration of the hemoglobin was low, but in such cases the precipitates produced were so small that analytical errors became appreciable.

It seemed very probable, therefore, that the antigen-precipitin precipitate has a constant composition and that the reaction between the antigen and the precipitin is, therefore, chemical and not merely physical or colloidal.

The assumption that the antigen-precipitin precipitate contains only the antigen and precipitin (protein) and no other protein

TABLE I.

Analyses of precipitates produced by the reaction of hemoglobin with anti-hemoglobin sera.

Rabbit No.	Antigen	Amt. Serum cc.	Amt. Hb-soln. cc.	Conc. of Hb-soln. Mg. N per cc.	Total Hb-N used mg.	Hb-N in ppt., mg.		Total N in ppt.,mg.		Hb-N in total-N
						Total	per cc. serum	Total	per cc. serum	%
741	Sheep Hb.	3	3	0.189	0.567	0.029	0.010	0.305	0.102	9.64
		3	3	0.126	0.378	0.058	0.019	0.553	0.184	10.45
		3	3	0.095	0.285	0.056	0.019	0.480	0.160	11.71
		2	2	0.063	0.126	0.023	0.012	0.237	0.119	9.83
		4	4	0.021	0.084	0.042	0.011	0.418	0.105	10.05
		5	5	0.007	0.035	0.024	0.005	0.301	0.060	8.10
783	Dog Hb.	5	5	0.180	0.898	0.088	0.018	0.844	0.169	10.41
		3	3	0.120	0.359	0.068	0.023	0.666	0.222	10.13
		3	3	0.060	0.180	0.120	0.040	1.167	0.389	10.31
		5	5	0.020	0.100	0.071	0.016	0.953	0.191	8.54
		6	6	0.007	0.040	0.026	0.004	0.393	0.066	6.61
778	Dog Hb.	4	4	0.190	0.760	0.026	0.007	0.220	0.055	11.78
		3	3	0.127	0.381	0.032	0.011	0.307	0.102	10.38
		3	3	0.064	0.191	0.026	0.009	0.255	0.085	10.23
780	Dog Hb.	5	5	0.064	0.318	0.046	0.009	0.418	0.084	10.90
		5	5	0.021	0.106	0.028	0.006	0.309	0.062	9.00
		6	6	0.007	0.043	0.013	0.002	0.193	0.039	6.85
782	Dog Hb.	4	4	0.127	0.507	0.099	0.025	0.836	0.209	11.85
		4	4	0.064	0.254	0.079	0.020	0.795	0.199	9.93
		5	5	0.021	0.106	0.037	0.008	0.336	0.067	11.12

seems also justified in the light of our findings. If this were not the case, it would be difficult to explain the approximate constancy of the hemoglobin content of the precipitate.