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Electrophoresis and Antibody Nitrogen Determinations of a Cold Hemagglutinin.*

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We have recently reported a case of gangrene of the tips of the fingers and toes in a patient whose blood contained extremely potent cold hemagglutinins.[1] During the investigation of this case, studies relative to electrophoresis and cold hemagglutinin antibody nitrogen determinations were carried out. They are reported because such details have not been published hitherto.

That cold hemagglutinins are in the globulin fraction of plasma proteins was suggested by Landsteiner[2] in his careful study of this antibody. Clough and Richter[3] and Koepplin,[4,5] using salt precipitation procedures, came to similar conclusions.

Methods and Results. 1. Electrophoresis. 50 ml of venous blood were clotted firmly for several hours at 37°C. Using aseptic technic, the serum was separated from the clot and divided into equal portions. One was stored at 4°C. The other portion was subjected to 6 absorptions at 4°C using homologous "erythrocyte ghosts."†

The agglutinated erythrocyte ghosts were separated from the serum by centrifugation at 4°-8°C. By this method the serum agglutinins were partially removed. The cold hemagglutinin titer of the unabsorbed serum was 1/2560 at 4°C; whereas the titer of the absorbed serum was 1/320 at 4°C. Further exhaustion of the absorbed serum was not attempted because of the prolonged centrifugation which would have been required to separate the weakly agglutinated ghosts from the serum. The multiple absorptions had not altered the color of the serum. (The absorptions were carried out under aseptic conditions).

The two samples of serum were then diluted separately, with diethylbarbituric acid-sodium hydroxide buffer of pH 8.63 and ionic concentration 0.10, to reduce their protein content to 1.5 g per 100 ml. Each sample was then placed in a cellophane bag and dialyzed against the buffer at 4°C for 3 days until salt equilibrium had taken place. (In this part of the experiment asepsis was not maintained).

At the conclusion of the dialysis, electrophoretic patterns of each serum were made and are reproduced in Fig. 1 and 2.

The electrophoretic curves were obtained by the method described by Longsworth, Shedlovsky and MacInnes.[6] Planimetric measurements are indicated beneath each of the figures. There is a 16% decrease in gamma globulin concentration after absorption, the areas of the other components maintaining the same ratio to the area of the albumin component. We interpret this to mean that a portion of the gamma globulin

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1 Stats, D., and Bullowa, J. G. M., *Arch. Int. Med.*, to be published.

2 Landsteiner, K., *Muenchen. med. Wchnschr.*, 1903, **50**, 1812.

3 Clough, M. C., and Richter, I. M., *Bull. Johns Hopkins Hosp.*, 1918, **29**, 86.

4 Koepplin, F., *Z. f. klin. Med.*, 1936, **129**, 512.

5 Koepplin, F., *Z. f. klin. Med.*, 1936, **130**, 784.

† Erythrocyte ghosts were prepared by hemolyzing packed saline-washed red blood cells with 10 volumes of distilled water. 18% sodium chloride solution was added to raise the tonicity to 0.9% and the hemolyzed cells were then centrifuged. After decanting the supernatant hemoglobin solution, the procedure of hemolysis by water and removal of hemoglobin was repeated 7 or 8 times to secure the ghosts almost free of hemoglobin. Before use in absorption tests, they were washed 4 times with 0.85% sodium chloride solution.

6 Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, **70**, 399.

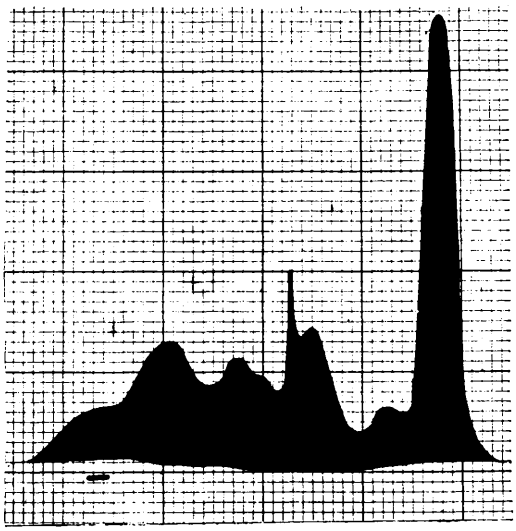

FIGURE 1. Descending electrophoretic pattern of unabsorbed serum.
Area under albumin = 170 (arbitrary planimeter units)
Area under γ globulin = 87.5 (arbitrary planimeter units)

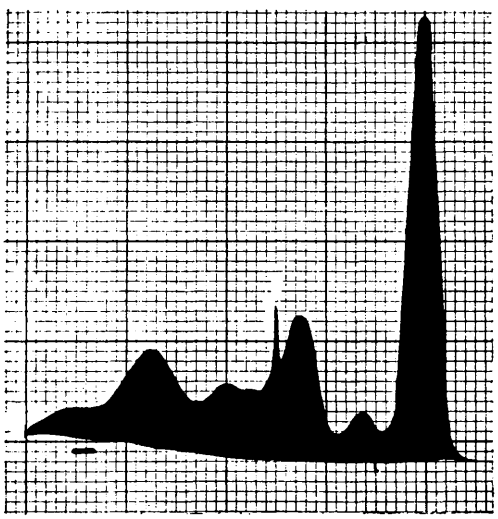

FIGURE 2. Descending electrophoretic pattern of the absorbed serum. Note: The changes in the $\alpha 1$, $\alpha 2$ and β fractions were not significant.
Area under albumin = 150 (arbitrary planimeter units)
Area under γ globulin = 64.5 (arbitrary planimeter units)

of the absorbed serum had been removed and that the cold hemagglutinins have the same mobility as gamma globulin.

2. Cold hemagglutinin antibody nitrogen. 1 ml of packed erythrocyte ghosts, almost free of hemoglobin, prepared as in the previous experiment was mixed with 5 ml of serum obtained from blood clotted at 37°C. The mixture was placed at 4°C for 6 hours. The ghosts were tightly agglutinated on the surface of the serum. After removal of the serum, 5 ml of ice cold 0.85% saline solution was added to the ghosts. The temperature was maintained at 4°C. This saline (1st washing) was replaced with 5 ml of fresh cold saline (2nd washing), etc., until the agglutinated ghosts had been washed 4 times. Throughout all of these procedures, the temperature did not rise above 6°C and was maintained as close to 4°C as possible.

The packed agglutinated washed ghosts were resuspended in 2 ml of 0.85% saline solution and placed in the water bath at 37°C. In a few minutes the agglutination broke up and the ghosts were freely dispersed. After 30 minutes, during which time the tube was shaken several times, the tube was centrifuged at room temperature. The supernatant clear saline solution was removed (resuspended antibody).

One ml of packed washed normal ghosts was mixed with 2 ml of 0.85% saline solution and kept at 4°C for one hour. The temperature was then increased to 37°C for one hour and the tube centrifuged at 22°C.

Cold hemagglutinin titrations of the original serum, the 4 washings and the resuspended antibody were performed according to the technic previously described.[1] Total nitrogen determinations of the washings, resuspended antibody, saline solution and saline solution which had been in contact with normal ghosts were performed by a micro-Kjeldahl technic.

Chart I summarizes these results.

CHART I.

Specimen	Titer cold hemagglutinins	Total nitrogen mg/ml
1st washing	1/40	0.614, 0.722
2nd "	1/20	0.153, 0.146
3rd "	1/10	0.059, 0.060
4th "	1/10	0.035, 0.028
Resuspended antibody	1/2560	1.483, 1.464
Original serum	1/5120	
Saline solution	—	0.009, 0.003
Saline solution in contact with normal ghosts	—	0.004, 0.004
Blank for reagents	—	0.027, 0.034
(Blank has been deducted)		

The high nitrogen values for the washings are due to non-antibody protein of the serum. The progressive reduction in the values indicates the removal of this protein by successive dilution. The possibility that non-antibody protein was non-specifically taken up in the lattice of agglutinated ghosts cannot be excluded. Therefore a portion of the nitrogen in the resuspended antibody may not represent antibody nitrogen.

Summary. Study of a human cold hemagglutinin revealed:

1. The cold hemagglutinin had the electrophoretic mobility of gamma globulin. 2. A cold hemagglutinin titer of 1/2560 at 4°C was equivalent to 1.473 mg per ml of antibody nitrogen.

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Metabolism of Sulfapyridine in the Dog.

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The therapeutic efficacy of the sulfonamides has stimulated an interest in the possible changes these compounds may undergo in the animal body. It is well known that the sulfonamides are acetylated in the human body and in this form they are relatively inactive. However, it is possible that other forms of conjugation or alteration in structure may occur which might modify their toxicity or activity.

Scudi[1] reported the isolation from dog urine of an hydroxysulfapyridine conjugated with glucuronic acid following the administration of sulfapyridine. No details were given on the isolation of this compound nor data concerning the probable position of the hydroxy group. Thorpe, Williams, and Shelswell[2] have suggested the possibility that the hydroxyl group might be introduced in the animal body in either the 2 or 3 position of the benzene ring of the sulfonamide.

Our interest in this problem was aroused when we were unable to isolate from the urine sulfapyridine as the reduction product of 2-(p-nitrobenzenesulfonamido) pyridine when the latter compound was administered orally to dogs. The same difficulty was encountered when sulfapyridine was administered. Sulfapyridine added to urine can be isolated readily by prolonged ether extraction. We therefore, decided to attempt the isolation of the diazo reacting substances which are excreted in dog urine when sulfapyridine is administered orally. The values given for diazo reacting substances in the urine are those determined by the Bratton and Marshall[3] method, using sulfapyridine as a standard.

Four dogs were given orally 5 g of sulfapyridine daily for 4 days and the urines were collected daily for 6 days, and preserved with chloroform. The combined urines gave a total sulfapyridine value of 67 g and no increase in color was obtained after acid hydrolysis indicative of the fact that the dog apparently does not conjugate any of the sulfapyridine as an acetyl derivative. Prolonged ether extraction in a continuous extractor for 9 hr resulted in the extraction of 9 g of diazotizable material as determined colorimetrically. When small quantities were extracted with ether in a more efficient extractor, 16.5% of the total diazotizable material was extracted by ether and 13.8% was extracted in the first 3 hours. This value of 16.5% probably represents the total amount of sulfapyridine which is excreted unconjugated. The gummy material extracted with

[1] Scudi, J. V. *Science*, 1940, **91**, 486.

[2] Thorpe, W. V., Williams, R. T., and Shelswell, *Biochem. J.*, 1941, **35**, 52.

[3] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.