1964, v116, 167.
6. ———, Nature, 1964, v201, 1338.
7. Paul, J., in Cell and Tissue Culture, Williams & Wilkins, Baltimore, 1965, 3rd ed., p82.
8. Darken, M. A., Pharmacol. Rev., 1964, v16, 223.
9. Nathans, D., Proc. Nat. Acad. Sci., 1964, v51, 585.
10. Riggs, T. R., Walker, L. M., J. Biol. Chem., 1963, v238, 2663.
11. Martin, G. M., Exp. Mol. Path., 1964, v3, 622.
12. Oyama, Z. I., Eagle, H., Proc. Soc. Exp. Biol. and Med., 1956, v91, 305.
13. Benditt, E. P., Martin, G. M., Platter, H., Sympos. of the Int. Soc. for Cell Biol., C. P. Leblond and K. B. Warren, Eds., Academic Press, New York, 1965, v4, 65.

Received June 9, 1966. P.S.E.B.M., 1966, v123.

Anti-Staphylococcal β-Hemolysin Antibodies in Humans with Neurological Disease. (31425)

C. T. UYEDA, B. GERSTL, J. K. SMITH, AND W. T. CARR
Laboratory Service, Veterans Administration Hospital, Palo Alto, Calif., Department of Pathology, Stanford University School of Medicine, Stanford, Calif., and Veterans Administration Hospital, Martinez, Calif.

The hot-cold β-hemolysin is one of many diffusible substances produced by *Staphylococcus aureus*. It is called "hot-cold" because hemolysis of red blood cells (RBC) is strongest when the system is incubated at 37°C followed by refrigeration. β-hemolysin acts strongly on sheep red cells while α-hemolysin acts on both sheep and rabbit red cells, and delta hemolysin on sheep, rabbit and human RBC. The β-hemolysin bears no antigenic relationship to the other two hemolysins(1). Reports on chemical and physical characteristics of β-hemolysin are available(2-6).

Doery *et al*(2) reported that β-hemolysin-producing strains of *Staphylococcus aureus* hydrolyze sphingomyelin. This phospholipase C-like activity was confirmed in this laboratory and was shown to be specific for sphingomyelin (Eng *et al*, personal communication). Thus, the possibility was entertained that β-hemolysin might be implicated in the pathogenesis of neurological diseases in which destruction of sphingomyelin is a prominent feature. Individuals subjected to repeated exposure to β-hemolysin-producing staphylococci and incapable of producing adequate antibodies against the hemolysin, could be assumed to develop such conditions. Consequently, a study of anti-β hemolysin antibodies in patients with multiple sclerosis and other neurologic disease was undertaken.

Methods. A beef heart infusion broth was employed in the preparation of the β-hemolysin filtrate(7). *Staphylococcus aureus* strain No. 31, which exhibits strong hot-cold hemolysis on sheep blood agar and weak action on human and rabbit blood agar plates or strain J32,* which produces only β-hemolysin was used as inocula. The inoculated medium was incubated for 96 hours at 37°C in a 25-30% carbon dioxide environment. The culture was centrifuged at 2,000 × *g* for 30 minutes and the supernatant Seitz filtered. The filtrates were stored at —4°C until use.

β-Hemolysin titration. Bacterial filtrates were heat treated according to Smith and Price(5) and 2-fold serial dilutions made in phosphate-buffered saline(4). To each tube was added 1.5 ml of a 1% suspension of thrice-washed sheep red cells. A 50% hemolysis control was prepared by adding one drop of a 1% aqueous solution of saponin to 0.75 ml of the RBC suspension in 2.25 ml of diluent. Appropriate controls were included. The mixed contents of all tubes were incubated for 80 minutes at 37°C followed by 30 minutes in an ice-bath(3). Remaining intact red cells were settled by centrifuging at 2,000 × *g* for 5 minutes and 2 ml of supernatant were pipetted into Coleman cuvettes.

* Generously provided by Dr. C. E. Dolman, Univ. of British Columbia, Vancouver, Canada.

A Coleman Junior Spectrophotometer was adjusted to 50% transmission at 545 mμ wavelength using the 50% hemolysis control and the degree of hemolysis in the other tubes recorded in % transmission. The titer was defined as the highest dilution showing complete hemolysis of sheep RBC and was expressed in reciprocal "units" of that dilution. Similar titrations using human and rabbit RBC were also performed.

Anti-staphylococcal β-hemolysin antibody titration. Serial 2-fold dilutions from 1:2 to 1:1024 or higher, of inactivated (56°C for 30 minutes) human serum were made in buffered-saline in a final volume of 1.5 ml. To each tube was added 0.3 ml of heat treated β-hemolysin filtrate diluted to 8 to 16 "units" per ml. A toxin control tube containing 0.3 ml of the same diluted filtrate and 1.5 ml of diluent and a 50% hemolysis control tube as described in the method for β-hemolysin titration were included. The mixed contents of the tubes were incubated for 2 hours in a 37°C water bath, permitting neutralization of toxin by any anti-β hemolysin antibodies present in the serum. Then 1.5 ml of a 1% suspension of washed sheep RBC were added to each tube except the 50% hemolysis control tube which received only 0.75 ml. The tubes were reincubated at 37°C for 80 minutes followed by 30 minutes in an ice-bath, and the degree of hemolysis recorded as in the hemolysin titration described above. The titers recorded were based on the highest dilution of serum showing a transmission 6-8% higher than that of the toxin control. A control serum with an anti-β hemolysin antibody titer of 1:512 was used during these studies. If the titer of this serum was lower or higher than 1:512 in a series of titrations it was adjusted to 1:512 and the other titrations in the same series correspondingly aligned.

Results. The fact that only sheep and not human and rabbit RBC were lysed by the heated filtrates indicates that β-hemolysin alone was retained by the filtrates(1). The results of anti-β hemolysin antibody titrations of the sera are presented in Fig. 1. The 165 individuals were grouped as follows: 31 normals (primarily new employees who had never worked in a hospital), 43 patients with multiple sclerosis (MS) (in various stages of the disease), 41 patients with other neurological disorders (brain tumors, convulsive disorders, cerebral vascular accidents), 26 patients with *Staphylococcus aureus* infections and 24 patients with other diagnoses. Titrations of sera obtained from same individuals at different times varied no more than a 2-fold dilution and their titers were identical in most cases. The data were submitted for statistical evaluation, the results of which are shown in Table I. The hypothesis tested was that there were just as many cases in each category above and below the combined median of both categories. There were significant differences between the neurological disease group and MS patients, and between the staphylococcal infected group and MS patients.

TABLE I. Statistical Analysis of Anti-β Hemolysin Antibody Titrations.

Group comparisons	Corrected chi-square*	Significance
Normal and MS	1.72	No $p \cong .15$
Other neurological diseases and MS	4.71	Yes $p < .05$
Staphylococcal infection and MS	4.02	Yes $p < .05$
Miscellaneous and MS	<1	No

* The chi-square median test was used with Yates correction. A chi-square of 3.84 is significant at the 0.05 probability level.

Discussion. Our results showing that all of 165 individuals had demonstrable anti-β hemolysin activity were unexpected in view of a report(8) that it is present in only 59% of normal individuals. The controls employed in these investigations corrected for differences in hemolytic activity that may occur with different preparations and, therefore, the observation of a 100% incidence of anti-β hemolysin antibodies appears to be valid.

The data presented in Fig. 1 and evaluated in Table I show that there were statistically significant differences in the titers between the group of MS patients and those with other neurological diseases, and between the staphylococcal infected group and MS patients. The higher anti-β hemolysin antibody titer in the staphylococcal infected group can be explained on the basis of greater opportunity for antigenic encounter. The neurological disease

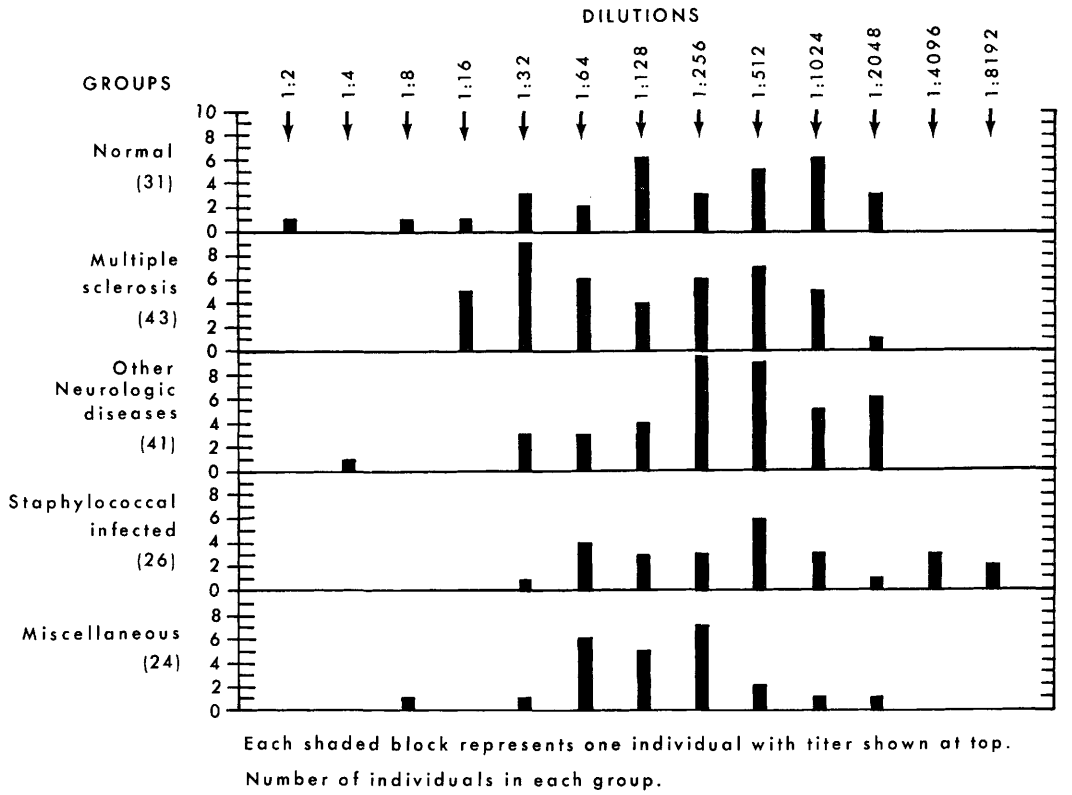

FIG. 1. Results on anti-β hemolysin antibody titrations.

and MS groups were exposed to similar microbial environments while hospitalized and, therefore, factors other than exposure could supposedly be responsible.

If one assumes that the higher incidence of low antibody titers in MS patients is a consequence of the neurologic disease, other antibodies should also be decreased. The sera of 15 MS patients in the group studied were also titrated for heterophile, anti-typhoid O and anti-streptolysin O antibodies and found to possess levels similar to control sera. Ross *et al*(9), Sibley and Foley(10) and Wright *et al*(11) also found no evidence for a general immunological incompetence in MS patients.

An alternative explanation would be the inability of more MS patients to react specifically and adequately to β-hemolysin, *i.e.*, partial immunologic incompetence. One type of this phenomenon described by Owen(12) implies complete absence of a specific antibody while in the tolerant state. Since all our MS patients possessed some anti-β hemolysin antibody, this explanation appears to be inapplicable. Another type, "immunological paralysis"(13), which in reality may be "immunological masking"(14) requires the presence of large amounts of antigen and would be difficult to relate to our studies. Thus, at present there is no adequate immunological explanation for the lower anti-β hemolysin antibodies found in the MS patients. Wiseman(6) reported that β-hemolysin was sensitive to trypsin and to mild reducing agents such as ascorbic acid and cysteine. Consequently, the possibility of a non-antibody mechanism responsible for the results obtained should be considered.

Summary. Anti-staphylococcal β-hemolysin levels in patients with multiple sclerosis and other diseases and in controls were studied. Statistically significant differences in titers were found between the neurologic disease group and MS patients, and between the staphylococcal infected group and MS patients. Possible explanation for these dif-

ferences are presented.

The assistance of Dr. Bernard Hanes, Western Research Support Center, Veterans Admin. Hospital, Sepulveda, Calif., for statistical analysis of the data presented here is gratefully acknowledged. The technical assistance of Sylvia Minor is greatly appreciated.

1. Elek, S. D., Staphylococcus Pyrogenes and its Relation to Disease, E. & S. Livingstone, Ltd., Edinburgh & London, 1959, p219.

2. Doery, H. M., Magnusson, B. J., Cheyne, I. M., Gulasekharam, J., Nature, 1963, v198, 1091.

3. Haque, R., Baldwin, J. N., J. Bact., 1964, v88, 1304.

4. Jackson, A. W., Mayman, D., Can. J. Microbiol., 1958, v4, 477.

5. Smith, M. L., Price, S. A., J. Path. Bact., 1938, v47, 361.

6. Wiseman, G. M., ibid., 1965, v89, 187.

7. Elek, S. D., Levy, E., ibid., 1950, v62, 541.

8. Ramon, G., Richou, R., Kourilsky, R., Richou, H., C. R. Acad. Sci., (Paris), 1953, v237, 8.

9. Ross, C. A. C., Lenman, J. A. R., Rutter, C., Brit. Med. J., 1965, v1, 226.

10. Sibley, W. A., Foley, J. M., Ann. N. Y. Acad. Sci., 1965, v122, 457.

11. Wright, R., Morton, J. A., Taylor, K. B., Brit. Med. J., 1965, v1, 491.

12. Owen, R. D., Science, 1945, v102, 400.

13. Felton, L. D., Ottinger, B., J. Bact., 1942, v43, 94.

14. Stark, O. K., in Mechanisms of Hypersensitivity, Shaffer, J. H., LaGrippo, G. A., Chase, M. W., eds., Little, Brown & Co., Boston, 1957, 519.

Received June 9, 1966. P.S.E.B.M., 1966, v123.

Manganese and Hydralazine as Studied by Neutron Activation and Radioactive Mn. (31426)

A. SAKAMOTO (Introduced by K. N. Prasad)
Veterans Administration Hospital, Northport, Long Island, New York

Hydralazine produces a "Lupus-like" syndrome in a few patients. This syndrome has been reproduced experimentally in the laboratory, and, circumstantially, a Mn-deficiency seemed implicated. The results of a dietary deficiency of Mn were compared to the effects of hydralazine administration. The alterations produced by dietary Mn found in mice were not duplicated by hydralazine.

Studies relating hydralazine ("Apresoline," CIBA) to manganese (Mn) metabolism have suggested that a Mn deficiency might be produced by long term administration of this drug(1). Because hydralazine can induce a syndrome similar to Systemic Lupus Erythematosus (SLE) in patients, it seemed possible that Mn deficiency might play a role in the etiology of this disease.

As neutron activation analysis (NAA) has proven to be a specific and accurate technique to measure tissue concentrations of Mn, it was used to determine whether hydralazine administration might lower Mn in tissues. Although tracer techniques are not a perfect answer to the study of physiologic disposition and metabolism of a biological material, the apparent identity of a compound and its radioisotope from a physiological point of view is widely accepted. Thus, kinetic studies utilizing ^{54}Mn were performed to determine whether hydralazine might induce functional deficiency of this trace metal.

The results of hydralazine administration were compared with the alterations of Mn induced by dietary manipulations of Mn. Tissue concentrations of Mn, whole body turnover rates of ^{54}Mn, and organ partition studies of ^{54}Mn responded readily to dietary alterations of Mn but not to hydralazine administration.

Male Swiss albino mice (BNL strain) were used throughout these studies. Effects of hydralazine on tissue levels of Mn were compared with effects of Mn-deficiency-inducing diet, as described by Hurley and associates (2). This diet contained 40 μg/g of Mn (unpublished data). ^{54}Mn kinetics were studied in mice fed a low Mn diet as well as in mice on a high Mn diet. Low Mn diets* contained

* Low Mn diet supplied by Nutritional Biochem-