the intraintestinal flora sufficient to overwhelm the normal detoxifying potential, and so induces irreversible shock. 7) These observations are in conformity with prior evidence that irreversibility to transfusion in hemorrhagic shock is due to endotoxins derived from the intestinal flora, and suggest further that deficient flow through the intestine accounts for the invasion of circulation by these endotoxins in sufficient quantity to produce irreversibility.

1. Jacob, S., Weizel, H., Gordon, E., Korman, H., Schweinburg, F., Frank, H., Fine, J., *Am. J. Physiol.*, 1954, v179, 523.

2. Schweinburg, F. B., Shapiro, P. B., Frank, E. D., Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 646.

3. Smiddy, F. G., Fine, J., *ibid.*, in press.

4. Schweinburg, F. B., Frank, H. A., Fine, J., *Am. J. Physiol.*, 1954, v179, 532.

5. Schweinburg, F. B., Yashar, Y., Aprahamian, H. A., Davidoff, D., Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 587.

6. Schweinburg, F. B., Fine, J., *ibid.*, 1955, v88, 589.

7. Rutenburg, S. H., Fine, J., *ibid.*, 1956, v91, 217.

8. ———, *ibid.*, 1956, v93, 484.

9. Frank, E. D., Fine, J., Pillemer, L., *ibid.*, 1955, v89, 223.

10. Ravin, H. A., Rutenburg, S. H., Fine, J., *ibid.*, in press.

11. Jacob, S., Friedman, E. W., Levenson, S., Glotzer, P., Frank, H. A., Fine, J., *Am. J. Physiol.*, 1956, v186, 79.

12. Smiddy, F. G., Segel, D., Fine, J., PROC. SOC. EXP. BIOL. AND MED., in press.

13. Thomas, L., *J. Exp. Med.*, 1956, v104, 865.

14. Frank, H. A., Seligman, A. M., Fine, J., *J. Clin. Invest.*, 1946, v25, 22.

15. Lillehei, R. C., *Surgical Forums, Am. Coll. Surg.*, 1956, v7, 6.

Received September 9, 1957. P.S.E.B.M., 1958, v97.

Bactericidal Activity of Normal Serum Against Bacterial Cultures. I. Activity Against *Salmonella typhi* Strains. (23748)

LOUIS H. MUSCHEL, RICHARD H. CHAMBERLIN, ETSUKO OSAWA
(Introduced by O. Felsenfeld)
Division of Immunology, Walter Reed Army Institute of Research, Washington, D.C.

The bactericidal action of normal serum, long recognized as a factor in the total complex of "non-specific" resistance to infection, is still of uncertain significance. Smooth forms of the Gram-negative bacilli are generally relatively virulent and resistant to the bactericidal action of normal mammalian serum; rough forms are avirulent and more susceptible. On the other hand, it has been reported that *Escherichia coli* is more resistant to normal serum than pathogenic microorganisms of the Enterobacteriaceae(1). This report requires modification since it is now recognized that certain serotypes of *E. coli* may be pathogenic and that different strains of a bacterial species may show marked differences in their susceptibility to the bactericidal action of normal serum. Also, in the case of anthrax, the serum of the naturally immune dog has little effect against the anthrax bacillus, but serum of the susceptible rabbit is bactericidal(2). Moreover, different mechanisms may be involved in serum bactericidal action. Serum substances, active against some Gram-positive organisms, are non-specific and heat stable. Complement is probably not involved in the activity of these substances(3). In contrast, the action exerted upon most Gram-negative organisms is mediated by natural antibody and complement and recently the properdin system has been implicated(4). Previous investigators have concluded that bactericidal antibodies in normal serum active against Gram-negative organisms are species specific(5,6); others have considered them non-specific or of limited specificity(7,8).

The present study was undertaken to reinvestigate some aspects of the bactericidal action of normal serum with the quantitative

spectrophotometric growth assay method(9). *S. typhi* strains of varying antigenic constituents were tested for their resistance to normal serum. In addition, the resistance of the strains to phagocytin(10), another substance implicated in natural defense mechanisms, and to chloramphenicol, the antibiotic of choice in typhoid fever therapy, was determined.

Materials and methods. S. typhi strains. Cultures were received from Mr. Arthur Abrams from the stock collection of this laboratory, and were maintained on meat extract agar. Strains Ty6S, ViI, Ty2, Watson, H901, and O901 have been described by Felix and Pitt(11). *S. typhi* strain 47 was received from Lt. Col. Oscar Felsenfeld. It was isolated recently from the holy water in the Temple of Karat in central Thailand where it was implicated in the etiology of a serious outbreak of typhoid fever. Strain 63 had been isolated posthumously from the meninges of a typhoid fever patient in 1935. Strain 58 had been isolated in Panama from the feces of a "chronic carrier" of long standing and is used for production of typhoid vaccine in the U.S. The antigenic constituents of various strains are given in Table I. *Sera.* The human serum pool was obtained from four individuals who had no history of typhoid fever or of immunization and whose sera lacked detectable *S. typhi* agglutinins; the rabbit serum was a pool from three animals; the guinea pig material was the lyophilized commercial product of Swanson Biological Laboratories and was reconstituted with distilled water to its original volume. Sera were stored at about –30°C; all testing was performed within 3 weeks of drawing of the blood or of reconstitution of the lyophilized serum. *Bactericidal tests.* Tests for measuring bactericidal activity of normal serum were performed by a modification of the spectrophotometric growth assay technic developed for the titration of bactericidal antibody(9). In brief, the test involved a series of test tubes which contained variable amounts of the test serum, and duplicate control tubes without serum. Foreign complement was not added; the test serum itself served as the complement source. All tubes were brought to constant volume with the saline diluent containing 0.85% NaCl and 0.063% $MgCl_2 \cdot 6H_2O$. The appropriate culture, prepared by suspension of the growth from an overnight slope culture (maintenance meat extract agar) in broth was optically standardized and then added in constant amounts (approximately 2 x 10^7 organisms) to each tube. The tubes were incubated in a water bath at 37°C for one hour when the bactericidal reaction occurred. Determination of the relative number of surviving organisms was then made by addition of 2.5 volumes of broth which terminated the reaction and practically eliminated any contribution of residual non-viable organisms to the final turbidity. When the set of tubes reached a suitable reading range with the control tubes still in the log-growth phase, the tubes were chilled in ice water to check further growth and the optical densities of the tubes were read with a broth blank in a Coleman Universal Spectrophotometer. The percentages of growth, which approximate the percentage survival of the organisms, were obtained by dividing the optical density for each experimental tube by the average of the optical densities of the control tubes, and multiplying by 100. Linear representations of the data were obtained by plotting the logarithm of the serum amount against the probit of the percentage growth. From the line, a 50% endpoint by interpolation, or if required, by extrapolation, and the slope of the response line was estimated. The bactericidal action of phagocytin prepared as outlined in reference 10 and of chloramphenicol were also determined similarly with the substitution of graded amounts of these substances in place of serum. The diluent for the tests with phagocytin was the intracellular salt solution proposed for use with that substance(10). *Agglutination tests.* These tests were performed according to the procedures given by Felix and Pitt(11). *Vi content of the strains.* HCl extracts of the acetone-dried organisms (3% of the dried organisms in 1% concentrated HCl) were prepared(12). The highest dilution or smallest amount of the neutralized extract required to inhibit the agglutination of maximally sensitized sheep red blood cells coated with purified Vi antigen(13), obtained

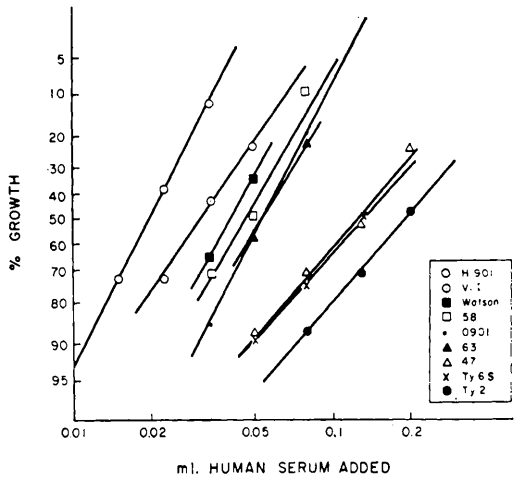

FIG. 1. Growth inhibition of *S. typhi* strains by human serum.

from Dr. Marion Webster, by an anti-Vi (*Paracolobactrum ballerup*) rabbit serum was determined.

Results. Bactericidal tests. The results obtained in testing the bactericidal activity of normal sera against the *S. typhi* strains are given in Fig. 1, 2, 3. Generally the strains were considerably more susceptible to human serum than to guinea pig or rabbit sera. Strains Ty2 and 47 were the most resistant to the bactericidal action of the normal serum of all 3 species. Strain 6S, rather resistant to human and rabbit sera, was more susceptible to guinea pig serum. This unexpected species difference was confirmed with an additional pool of guinea pig serum. At the other extreme, strain H901 was the most susceptible to the bactericidal action of the sera of all species. The other strains intermediate in their order of activity varied slightly in their relative susceptibility to the sera of the 3 species. In addition, the slope response of the bactericidal reaction was low where the 50% end-points of the serum were high and conversely the slopes were high when the 50% end-points of the serum were low. *O-inagglutinability.* O-inagglutinability of the strains was determined by comparison of their agglutinability in the killed and living state by anti-O (strain O901) rabbit serum. The results (Table II) indicate that the O-inagglutinability of the strains containing both O and Vi antigens, in descending order, is as follows: Ty2, 47, 63, Watson and 58. *Vi content.* The results of the hemagglutination-inhibition tests with the extracts of the different strains are given in Table II. The procedure was found to be sensitive to about 2 μg/ml of purified Vi substance. Calculation indicated that the content of Vi substance in strain Ty2 was about 0.4% if complete extraction were assumed. For strains 47 and ViI. it would be approximately half that value. The results have indicated that the Vi content and O-inagglutinability in strains possessing both antigens show a marked correlation. The living cultures were also tested against an anti-Vi (*P. ballerup*) rabbit serum. Strains that show the strongest agglutination with this serum are those poorest in Vi antigen. Decreased agglutinability by increased amounts of Vi antigen has been observed also by Felix and Pitt(11). The agglutination of living cultures of strains H901 and O901 by anti-Vi (*P. ballerup*) was unexpected and may be attributed to the presence of antigenic factors of those organisms, other than Vi, cross-reactive with *P. ballerup.*

Incubation of strain Ty2 cultures at temperatures other than 37°C. Nicolle, Jude, and Diverneau(14) reported that *S. typhi* grown at 18°C or 41.5°C fails to produce Vi antigen and is immune to Vi phage. At these temperatures, enzymes responsible for synthesis of

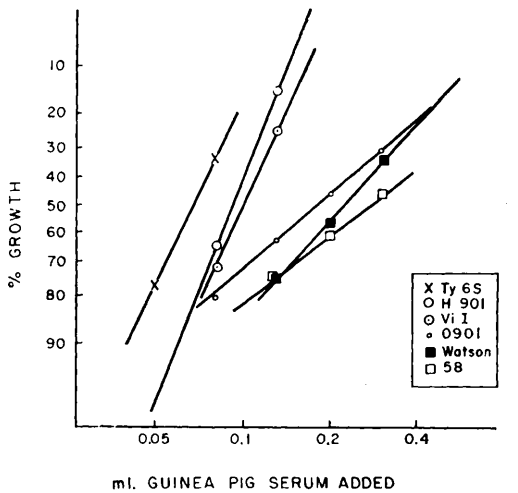

FIG. 2. Growth inhibition of *S. typhi* strains by guinea pig serum. Strains Ty2, 47, and 63 were too resistant for titration (50% point greater than .3 ml of serum).

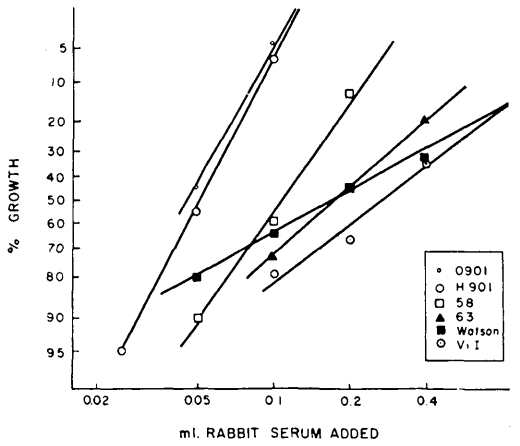

FIG. 3. Growth inhibition of *S. typhi* strains by rabbit serum. Strains Ty2, 47, and 6S were too resistant for titration (50% point greater than .4 ml of serum).

Vi antigen exist in the cells for a number of generations despite the absence of the antigen. Three cultures of strain Ty2 were accordingly grown on meat extract agar at 3 different temperatures: one at 41.5°C for 16 hours, another at 18°C for almost 3 days to provide adequate growth, and a third at 37°C for 16 hours. The living cultures were then tested simultaneously for agglutinability in anti-O and for susceptibility to the bactericidal action of normal serum; the acetone dried cells were tested for their Vi content by the hemagglutination-inhibition procedure. The results of these tests (Table III) indicate that the partial loss of Vi antigen by growth at temperatures above or below 37°C, reflected by a 4-fold drop in hemagglutination-inhibition, was accompanied by a 5-fold increase in susceptibility to the bactericidal action of normal human serum. However, the concomitant 16-fold loss in O-inagglutinability in the 41.5°C culture and the 64-fold loss in the 18°C culture indicates the marked sensitivity of O-inagglutinability as an index of the Vi content of a culture. Reincubation of the cultures at 37°C for 16 hours resulted in almost complete restoration of O-inagglutinability and resistance to normal serum.

TABLE I. Antigens Present in *S. typhi* Strains.

Strain	Antigens Vi	O	H
Ty6S*	+	—	tr
ViI*	+	tr	tr
Ty2	+	+	+
47	+	+	+
63	+	+	+
Watson	+	+	+
58	+	+	+
H901	—	+	+
O901	—	+	—

tr = trace amt; + = present; — = absent.
* Strains serologically rough.

Specificity of bactericidal action of normal serum. A normal human serum was divided into 3 aliquots; one of these was absorbed at 4°C with saline washed heat-killed organisms of strain O901, another aliquot was absorbed with organisms of strain Ty6S, and a third aliquot was untreated and served as a control. The aliquot absorbed with strain O901 showed a 4-fold loss of bactericidal activity against strains V58, Ty2, and O901, which contain all the *S. typhi* O antigens, an approximate 2-fold loss in activity against *Salmonella paratyphi* A and *Salmonella strasbourg*, organisms which possess one of the O antigens of *S. typhi*, and no loss against *S. typhi* Ty6S, *Salmonella newington*, and *Salmonella oranienburg*, organisms which do not possess the *S. typhi* O antigens. On the other hand, absorption of normal serum with unwashed organisms of strain Ty6S which retained its Vi antibody combining capacity effected an 8-fold loss of activity against the homologous strain and

TABLE II. *O and Vi Antigenic Analysis of S. typhi* Strains.

Test strain	Anti-O titers* Living cultures	Killed cultures	Vi hemagglutination inhibition titer†	Anti-Vi titer* (living cultures)
Ty6S	Neg. at 1/20	Neg. at 1/20	128 or greater	40
ViI	"	"	16	80
Ty2	20	5120	32	80
47	80	"	16	320
63	160	"	8	320
Watson	640	"	8	640
58	1280	"	4	1280
H901	2560	2560	No inhibition	160
O901	"	"	"	80

* Titer represents reciprocal of highest reacting dilution.
† Titer represents highest dilution inhibiting agglutination.

TABLE III. O-inagglutinability, Relative Vi Content of Extracts, and Resistance to Bactericidal Action of Normal Human Serum of Strain Ty2 Cultivated at 3 Different Temperatures. These cultures were later all reincubated overnight at 37°C and retested.

Temp of growth, °C	Anti-O titer with living cultures	Hemagglutination inhibition of extracts in Vi system	Bactericidal tests: ml normal human serum for 50% endpoint	Slope
37	40	32	.24	2.7
18	2560	8	.047	3.8
41.5	640	8	.046	3.6
After reincubation at 37°C				
37	40	Not done	.26	3.0
18	80	"	.15	2.5
41.5	80	"	.15	3.1

ViI, but no loss with strains Ty2, 58 and O901.

Phagocytin. The bactericidal activity of phagocytin was determined against the *S. typhi* strains (Table IV). Strains Ty2, 47, and 6S, the most resistant to normal serum constituents, were among the most susceptible to phagocytin. *Chloramphenicol.* The typhoid strains were tested against varying amounts of antibiotic in saline solution and 50% endpoints were obtained (Table IV). The relatively uniform resistance of the strains to chloramphenicol (mean of the 50% endpoints, 1.05 μg, with average deviation equal to 0.17 μg) is in contrast to their greater variability to phagocytin (mean of 0.033 ml with average deviation equal to 0.013) and to normal human serum (mean 0.078 ml with average deviation equal to 0.048).

Discussion. Although typhoid fever is a human disease, normal human serum possesses considerable greater bactericidal activity against *S. typhi* than does rabbit or guinea pig serum. Obviously the resistance of a species to a particular parasite cannot be defined simply by the bactericidal action of its serum. Strain Ty2, of maximum mouse virulence and of proven virulence for chimpanzees(15) was the most resistant of all strains tested, followed by strain 47, responsible for a recent severe human outbreak. Generally, resistance to normal serum was positively correlated with the Vi content and O-inagglutinability of the strains. The experiments in which strain Ty2 was cultivated at temperatures other than 37°C with loss of resistance to normal serum also demonstrated this association. An abnormal temperature, however, may not be of advantage to the host. Pasteur found that chilling reduced the resistance of birds to anthrax. Subnormal body temperatures also lower the resistance of rabbits to *Treponema pallidum*. The relatively high resistance of strain 6S to rabbit and human serum was another interesting finding. The large amount of Vi antigen in this strain may prevent access of serum substances to the susceptible R antigen of the cell(16). Also noteworthy was the relatively greater susceptibility of strain H901, compared to O901, a variant of H901 lacking the H antigen. This finding supports the belief that the H antigen plays no part in the virulence of *S. typhi*. In addition to the endpoints of the response, the slopes point to a greater protective effect against a strain such as 58 compared to Ty2. Greater slopes were obtained against those strains requiring smaller amounts of serum for a 50% endpoint (Fig. 1,2,3). If the slopes are relatively low, it may be difficult to attain killing as high as 95% with the amount of available serum, although 50% endpoints are within reach. The significance of this point depends upon whether almost all of the organisms must be removed by the bactericidal reaction for effective resistance to infection.

The absorption experiments were concerned with the question of the specificity of natural bactericidal antibodies. Our findings have

TABLE IV. Bactericidal Action of Phagocytin and Chloramphenicol against *S. typhi* Strains.

Strain	Phagocytin: 50% endpoint, ml	Phagocytin: Order of resistance	Chloramphenicol: 50% endpoint, μg	Chloramphenicol: Order of resistance
6S	.017	8	.9	9
ViI	.019	7	1.3	3.5
Ty2	.034	6	.98	8
47	.04	4	1.4	1.5
63	.044	2.5	1.3	3.5
Watson	.050	1	1.0	7
58	.044	2.5	1.4	1.5
H901	.011	9	1.1	5.5
O901	.040	5	1.1	5.5

indicated a marked specificity. Absorption with one organism resulted in a loss of activity only against other bacterial species which possess a cross-reactive antigen. Our results suggest that the natural bactericidal antibodies active against strains Ty2, 58, and O901 are specific for the O antigen since absorption of serum with strain O901 organisms resulted in almost complete loss of activity against those strains. Absorption with the Vi rich, but serologically rough strain Ty6S resulted in a loss of activity against the homologous strain and the semirough strain, ViI, but not against Vi antibody susceptible strains, Ty2 and V58(16). The results imply that the R antigen, in addition to the O antigen, is the susceptible target for bactericidal activity of normal serum against strains containing these antigens. Natural antibodies specifically oriented against the Vi antigen are either not present or their concentration is too low to exert a bactericidal effect. The relationship of these findings to the bactericidal action of the nonspecific properdin system(4) is of interest. Our results indicate that natural bactericidal antibody of marked specificity plays the major role. A recent report has indicated also that the properdin content of normal animal sera of different species did not correlate with differences in the bactericidal activity of the blood of the animals against *Pasteurella tularensis*(17). That the natural antibodies are specific does not necessarily favor any particular theory concerning their origin. It merely means that they react with certain organisms, and not with others, because of a chemical correspondence of certain specific combining groups.

It was considered of interest to determine relative resistance of the typhoid strains to phagocytin, an extract of rabbit polymorphonuclear leucocytes, since phagocytin may constitute another factor in natural resistance. No correlation was shown to exist between the resistance of the various strains to phagocytin and to normal serum. Strains Ty2 and 47 were not particularly resistant to phagocytin, and one is tempted to believe that serum may be of greater significance than phagocytin in protection against typhoid organisms. In contrast to the variability of the strains to the bactericidal action of serum and phagocytin, the uniform resistance of the different strains to chloramphenicol indicates that the action of the antibiotic is directed against a process or target, possibly protein synthesis (18), common to all the strains. It is not too unexpected that, as the data suggest, the bactericidal actions of normal serum, phagocytin, and chloramphenical involve different mechanisms.

Summary. The resistance of strains of *S. typhi* to the bactericidal action of normal guinea pig, rabbit, and human serum was determined. The order of resistance of the strains to sera of different species was in close agreement, but human sera exerted a greater bactericidal effect than rabbit or guinea pig sera. The resistance of the strains containing both O and Vi antigens was associated with the O-inagglutinability or Vi content of the organisms. The natural bactericidal antibodies against *S. typhi* were of marked specificity and directed against either the O or R antigens. The resistance of the strains to phagocytin or to chloramphenicol did not correlate with their resistance to normal serum components.

1. Georgevitch, A., *Compt. rend. Soc. de Biol.*, 1926, v95, 954.

2. Muir, R., in *A System of Bacteriology in Relation to Medicine*, v6, His Majesty's Stationery Office, London, 1931, p285.

3. Myrvik, Q. N., *Ann. New York Acad. Sci.*, 1956, v66, 391.

4. Wardlaw, A. C., Pillemer, L., *J. Exp. Med.*, v103, 553.

5. Mackie, T. J., Finkelstein, M. H., *J. Hyg.*, 1932, v32, 1 and previous papers.

6. Adler, F. L., *J. Immunol.*, 1953, v70, 69.

7. Gordon, J., Carter, H. S., *J. Path. and Bact.*, 1932, v35, 549.

8. Gordon, J., Johnstone, K. I., *ibid.*, 1940, v50, 483.

9. Muschel, L. H., Treffers, H. P., *J. Immunol.*, 1956, v76, 1.

10. Hirsch, J. G., *J. Exp. Med.*, 1956, v103, 589.

11. Felix, A., Pitt, R. M., *J. Hyg.*, 1951, v49, 92.

12. Luippold, G., *Am. J. Pub. Health*, 1946, v36, 15.

13. Landy, M., Lamb, E., PROC. SOC. EXP. BIOL.

AND MED., 1953, v82, 593.
14. Nicelle, P., Jude, A., Diverneau, G., *Ann. Inst. Pasteur*, 1953, v84, 27.
15. Gaines, A., Landy, M., Edsall, G., Trapani, R., *Fed. Proc.*, 1956, v15, 588.
16. Nagington, J., *Brit. J. Exp. Path.*, 1956, v37, 397.
17. Stanziale, W. G., *J. Immunol.*, 1957, v78, 156.
18. Hahn, F. E., Schaechter, M., *Bact. Proc.*, 1957, p.138.

Received October 9, 1957. P.S.E.B.M., 1958, v97.

Studies on *in vivo* Stability of an Iron Chelate. (23749)

MARVIN J. SEVEN* AND RALPH E. PETERSON
National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service

N - hydroxyethylethylenediamine triacetic acid is a chelating agent similar to ethylenediamine tetraacetic acid, but with one acetic acid group replaced by an ethanol group. This alters the chelating properties for iron and other heavy metals(1). This compound has been studied in hemochromatosis and found to be effective in the removal of iron from the tissues when given intravenously(2). Since the stability constant of the iron chelate formed *in vitro* is high, it might be expected to hold iron firmly in the body. However, experimental studies have shown that when ferric chelate is injected intramuscularly in iron-deficient mice, hemoglobin regeneration occurs, and degree of hemoglobin response is slightly greater than with the corresponding chelate of ethylenediamine tetraacetic acid.† The studies here reported were carried out in 2 iron deficient males. One received Fe^{59} tagged ferric disodium N-hydroxethylethylenediamine triacetate intramuscularly, and the second received the tagged chelate orally, and by duodenal tube.

Methods. The Fe^{59} tagged ferric chelate of N-hydroxyethylethylenediamine triacetic acid was prepared from the disodium salt of this chelating agent by simultaneous addition of a ferric chloride solution and dilute sodium hydroxide within a pH range of 6.0-7.5. This preparation was evaporated to a small volume and sterile filtered, resulting in a solution stable for 3 weeks. The first subject, T.R., a 71-year-old male, showed depletion of body iron stores with hypochromic anemia after repeated phlebotomies for study purposes. At the time of the study, fasting serum iron levels varied between 19 and 24 μg% with unsaturated iron binding capacity (UIBC) between 262 and 286. Hemoglobin values varied between 9.4 and 9.7 g, hematocrit between 32 and 35%, RBC between 4.14 and 4.33 million, reticulocytes between 0.1 and 1.6%, and indices showed MCV 75-85, MCH 22-23, MCHC 27-29. He was given 2.75 ml of iron chelate by intramuscular injection in the left hip, representing 50 mg elemental iron and 10 μc Fe^{59}. Surface counting was carried out over the injection site and other body areas at regular intervals with a scintillation detector. Blood and plasma samples were analyzed for radioactivity in a "well-type" scintillation detector. Fractional urine collections were obtained on the day of injection and at daily intervals thereafter. These were evaporated to a small volume before counting in three 4 ml aliquots. Feces collected over a 4 day period were passed directly into a Waring Blendor, blending was carried out for 20 minutes with a small amount of water, and three 4 ml aliquots were taken directly from this slurry to be counted in the same "well" counter. After 12 days, studies were undertaken to determine the effect of therapeutic doses of iron chelate without radioactive iron on the serum iron and UIBC levels within the hours immediately following injection. Serum iron and UIBC values were obtained by a modification of the method of Schade(3) using Nitroso R as the

* Present address: Department of Medicine Hahnemann Medical College, Philadelphia, Pa.

† Rubin, M., personal communication.