Complement fixation in tuberculosis / by A.M. Stimson.

TREASURY DEPARTMENT
UNITED STATES PUBLIC HEALTH SERVICE

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AUGUST, 1915

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CONTENTS.

ILLUSTRATION.

COMPLEMENT FIXATION IN TUBERCULOSIS.[1]

By A. M. Stimson, *Surgeon, United States Public Health Service, Washington, D. C.*

Complement fixation has been investigated in its relations to tuberculous infections from several points of view; from the standpoint of its bearing on clinical medicine to discover whether it was of value in the diagnosis, prognosis, or in controlling the specific therapy, of tuberculosis in man; from the standpoint of pure pathology to determine what light it might throw upon the essential nature of the disease and immunity thereto; and from the position of the veterinary official who is in search of diagnostic methods less susceptible of evasion than those commonly employed.

Undoubtedly the chief interest has centered around the diagnostic and prognostic possibilities of the reaction. In the interest of the individual patient, as well as of public health, early diagnosis is essential, and any test which will aid in this direction, or assist in estimating the progress of the battle between the patient and his disorder would be a welcome addition to our resources. The procedures now commonly employed leave much to be desired, there being little doubt that many cases of tuberculous infection escape detection by them until significant or irreparable damage has been done. Moreover some of them, notably the von Pirquet and some other tuberculin tests, are rendered valueless except in restricted groups of persons by the very reason of their delicacy, and their persistance after the subsidence of activity and the establishment of cure.

The earlier work with complement fixation in connection with tuberculosis gave such contradictory results that little hope was promised of practicable employment of the method. The more recent work, however, tends to approach unanimity as to the significance of the reaction. In the following table the work of the past few years is briefly reviewed, the arrangement being such as to permit a comparison of the methods employed and the results obtained.

[1] Manuscript submitted for publication Apr. 15, 1915.

Authority and references.	Methods.	Hemolytic system.	Antigens.	Materials examined.	Results.
1. Ruppel and Rickmann. Zeit. f. Immunitatsfg., 1910, VI, 344.	Wassermann, Bruck, and Citron.	Sheep cells; rabbit amboceptor; guinea pig complement.	Old tuberculin.....	Herbivores, normal and tuberculous, with and without tuberculin treatment.	Complete fixation only in tuberculous animals and those treated; no fixation in some tuberculous animals.
2. Laub..... Ibid., 1911, IX, 126.	.....do.....	.....do.....	.....do.....	Guinea pigs, rabbits, and goats.	No fixation with normal animals nor with those treated first with killed tubercle bacilli and then with tuberculin. In tuberculous animals, injections of tuberculin produced fixing bodies, as they did also in the normal horse.
3. Schultz..... Idem., 709.	Reduced volumes.....	.....do.....	Old tuberculin and bacillen emulsion.	Tuberculous and healthy persons.	Old tuberculin gave strong fixation in 15 of 77 tuberculous cases, and in 3 of 162 nontuberculous cases. Bacillen emulsion gave strong fixation in 5 of 34 tuberculous cases, and none of 115 nontuberculous. Milder grades of fixation given by each antigen in both classes, but less often in the nontuberculous.
4. Borissjak, Sieber and Metalnikow. Ibid., XII, 65.	.....	.....do.....	Various products of tubercle bacilli and lecithin.	Animals treated with these antigens.	Tubercle bacillus wax, fat-free tubercle bacilli, and lecithin caused the greatest production of antibodies. Tuberculin does not cause antibody formation, but hinders it. The antibodies are not specific for the antigens administered.
5. Porter..... Jour. Hygiene, 1911, XI, 112.	Tests secondary to precipitin test; large amount of rabbit serum.	.....do.....	Filtrate from milled and shaken tubercle bacilli, human and bovine.	Tuberculous and healthy cows.	With bovine antigen 88 per cent tuberculous animals and 18 per cent normal gave positive reaction. With human antigen 72 per cent of tuberculous and 19 per cent of normal animals were positive.
6. Caulfield..... Arch. Int. Med., 1911, VIII, 440. Caulfield and Beatty. J. Med. Res., 1911, 24, 122. Caulfield. Ibid., 128.	Wassermann.....	.....do.....	Bacillen emulsion; old tuberculin; alcohol-ether extract of tubercle bacilli.	Human tuberculosis..	Antigen. Cases, Turban classification. I. II. III. B. E. (positive). 33 per cent. 70 per cent. 62 per cent. O. T. (positive). 10 per cent. 48 per cent. 44 per cent. No fixation in clinically normal cases. Usually positive in more unfavorable type of tuberculous cases. Negative reactions may be due to the presence of inhibiting substances which can overcome large anticomplementary doses of antigen, causing hemolysis. Believes that whether the reaction is of fixation, inhibitory, or indifferent (negative) is of prognostic value.
7. Debre and Paraf.... Compt. Rend. Soc. Biol., 1911, 228 et seq.	Using known antituberculous serum to demonstrate antigen.	.....do.....	Sought for in pathological fluids; pleuritic, ascitic, and urinary.	Tuberculous and normal subjects.	Found high degree of specificity in the test, especially in the urine of renal tuberculosis. The test is for the presence of antigen, not antibody.

8. Dellmann. Zeit. f. Immunitatsfg., 1911, X, 421.	Not detailed.		Carbolized emulsions of T. B. and other acid fasts; tuberculin; partial antigens.	Human sera.	Findings in accord with those of Much (15). Group fixation of acid fasts with quantitative differences. Fixation in the clinically nontuberculous. Fixation indicates only a previous contact with the virus.
9. Laird. Jour. Med. Res., 1912, XXVII, 163.	Wassermann.	Sheep cells; rabbit amboceptor; guinea pig complement.	Watery emulsion of tubercle bacilli.	Tuberculous patients.	Fixation in only four of 34 cases. Article contains valuable suggestions.
10. Mollers. Cent. Bact. ref., 1912, LIV, Beiheft, 202.					Fixation reactions permit no diagnostic or prognostic conclusions. In human serum they indicate merely previous contact with the virus of tuberculosis.
11. Hammer. Deutch. Tierarzt. Woch., 1912, XX, 593.	v. Dungern, modified by room temperature incubation.	Ox cells.	1, old tuberculin; 2, extract of tuberculous nodules; 3, mixture of 1 and 2.	48 tuberculous cows. 48 nontuberculous cows.	Fixation in all, autopsy control. Fixation in 2; probably small foci overlooked.
12. Hammer. Munch, med. Woch., 1912, LIX, 1750.	do.	do.	do.	46 tuberculous persons.	43 strongly positive, 2 weakly positive, 1 negative, perhaps a healed case.
13. Zweig. Berl. klin. Woch., 1912, XLIX, 1845.	Original. Active serum in two series, one with and one without antigen; colorimetric reading of hemolysis.	Complement in patient's serum; sheep cells; 5 units of amboceptor.	Bacillen emulsion.	Tuberculous and nontuberculous persons, a small series.	In general fixation is proportional to severity. No fixation in 2 rapid cases and 1 healed gland case. Fixation in 2 scarlatina cases. Bovine emulsion sometimes positive where human is negative.
14. Calmette and Massol. Compt. Rend. Soc. Biol., 1912, LXXIII, 120. Calmette and Mazie. Ibid., 1913.	Bordet-Gengou.	Sheep cells; rabbit amboceptor; guinea pig complement.	Preparations from tubercle bacilli; A, dialysible exobacillary antigen; B1, water-soluble endobacillary antigen: B2, water-insoluble endobacillary antigen. (Peptone-soluble.)	134 cases human tuberculosis.	Fixation in 5.96 per cent cases with antigen A, B1, and B2. Fixation in 40.28 per cent cases with antigen B1, and B2. Fixation in 46.25 per cent cases with antigen B2. Fixation in 92.49 per cent cases altogether. The reaction is of diagnostic value. Antibodies shown by B2 appear early, those by B1 in late stages. Disappearance of B1 antibodies indicates aggravation of the disease.
15. Much. Munch. med. Woch., 1912, LIX, 685.			Various acid-fast bacteria and partial antigens prepared from them.	Tuberculous and healthy persons.	Various antigens gave fixation with tuberculous serum, the other acid-fasts differing only quantatively from the tubercle bacilli. Healthy persons gave fixation with tuberculin and tubercle bacilli emulsions in 77 per cent of cases. The specific antigens of tubercle bacilli are probably contained in both the fatty acids and proteins.

Tabulated review of recent literature—Continued.

Authority and references.	Methods.	Hemolytic system.	Antigens.	Materials examined.	Results.
16. Calmette and Massol. Compt. Rend. Soc. Biol. 1912, LXXIII, 122.	Bordet-Gengou.....	Sheep cells; rabbit amboceptor; guinea pig complement.	1, killed and concentrated liquid cultures of tubercle bacilli; 2, same, minus bacteria; 3, watery extract filtered tubercle bacilli; 4, peptone extract of tubercle bacilli.	Serum of tuberculous persons treated with these antigens.	The greatest augmentation of antibodies followed the use of No. 3, and persisted 1 or 2 months after treatment.
17. Meyer, Kurt..... Zeit. f. Immunitatsfg. 1912, XIV, 359.	.....	.....	Various derived portions of tubercle bacilli.	Hoechst antituberculous serum.	The fixing properties of tubercle bacilli for this serum are contained in 2 fractions, probably phosphatid derivatives. Fat, fatty acid, and waxlike fractions have slight or no fixing properties for this serum.
18. Letulle..... These Fac. Med. Paris. 1912.	Bordet-Gengou.....	Sheep cells; rabbit amboceptor; guinea pig complement.	Calmettes: 1, water-soluble; 2, water-insoluble antigens.	Tuberculous and nontuberculous persons.	89 per cent of tuberculous cases gave fixation. In general patients with resistance give fixation. Calmette's inhibiants verified.
19. Aoki..... Zeit. f. Immunitatsfg. 1912, XIII, 192.	Modified Wassermann.	Sheep cells.....	Not described.....	Tuberculous and treated rabbits.	Complement fixing bodies and also spontaneous hemolysis restraining properties of serum increase in definite curves, the relation of which is significant. In infection they are parallel, but in immunizing experiments they are at times opposite in direction.
20. Fraser..... Ibid., 1913, XX, 291.	Wassermann..... In some tests removed human antisheep amboceptor.	Sheep cells; rabbit amboceptor; guinea pig complement.	Great variety.....	Tuberculous and nontuberculous persons.	The best antigen is an emulsion of living tubercle bacilli. Removing the human antisheep amboceptor increases the positive results. Syphilitic serum may give a positive reaction. Otherwise nontuberculous serum gives negative results from the test.
21. Dudgeon, Meek and Weir. Lancet, 1913, CLXXXIV, 19.	Wassermann.....	.....do.....	.....do.....	Tuberculous persons.	1. Patients not given specific treatment, 85 per cent positive. 2. Patients specifically treated, O. T. or B. E., 100 per cent positive. 3. Arrested cases, 74 per cent positive. Killed tubercle bacilli emulsion the best antigen. Consecutive tests on the same patient are informative.
22. Harris and Lanford. Jour. Inf. Dis., 1913, XIII, 301.	Not detailed.....	.....do.....	Various acid-fast bacilli, whole or as Besredka antigens.	Serum of rabbits treated with these antigens.	Besredka antigens produced the most powerful sera. Group fixing bodies shown, not specific for the strains employed.

23. Bang and Andersen. Cent. Bact. orig. 1913, LXIX, 517.	Modified Wassermann.	Goat cells; rabbit amboceptor; guinea pig complement.	Emulsion of killed tubercle bacilli preserved with phenol; tuberculin.	Cattle, with and without tuberculosis and other acid-fast infections.	Strong reactions have diagnostic significance. In general they are proportionate to the severity of infection. Other acid-fast infections (Johne's disease) give the same reaction as tuberculosis. An emulsion of tubercle bacilli is a good, and tuberculin is a poor antigen.
24. Armand Delille, Rist, and Vaucher. Compt. Rend. Soc. Biol. 1913, LXXIV, 791.	.....	.....	Crude tuberculin; Calmette's endobacillary antigens.	Human tuberculosis, 100 cases.	With crude tuberculin, 4 per cent gave a definite positive and 30 per cent a partial reaction. With the water-soluble antigen 20 per cent were definite and 25 per cent partial. With the peptone-soluble antigen 92 per cent gave a definite reaction.
25. Wyschellesky..... Zeit. f. Tuberk. 1913, XIX, 209.	Wasserman.....	.....	Phymatin; emulsion of tubercle bacilli; solution of tubercle bacilli in 2 per cent lactic acid.	Cattle, tuberculous and nontuberculous.....	Phymatin the best antigen. Fixation in 18.1 per cent of tuberculous and 9.7 per cent of healthy cattle.
26. Bundschuh..... Zeit. Hyg. 1913, LXXIII, 427.	.....	.....	.....	.....	Found that antibody formation (fixation) was induced in healthy as well as in tuberculous animals following the injection of tuberculous antigens.
27. Francescelli..... Zeit. f. Immunitatsfg. 1913, XX, 309.	.....	.....	.....	.....	Contrasted to Bundschuh, found that antibody formation was induced only in tuberculous animals.
28. Kinghorn and Twitchell. Zeit. f. Tuberk. 1913, XX, 11.	Wassermann, one-half classical amounts.	Sheep cells; rabbit amboceptor; guinea-pig complement.	Bacillen emulsion.....	Tuberculous and healthy persons.	Of 7 healthy persons, fixation in none. Of 8 incipient tuberculous cases, fixation in 37.5 per cent. Of 14 advanced tuberculous cases, fixation in 93.33 per cent. Of 3 healed cases, fixation in 100 per cent. They attach no prognostic value to the test.
29. Calmette and Massol Compt. Rend. Soc. Biol. 1913, no. 28, 160.	(See 14, 16).....	.....	.....	.....	Inhibiting bodies which prevent fixation in some sera, are removable by diluting, heating to 57° C., and passing CO_2 gas through the serum.
30. Rothe and Bierbaum Deutch. med. Woch. 1913, no. 14, 644.	No details.....	.....	Watery extract of tubercle bacilli, preserved in glycerin and phenol.	Cattle, healthy and tuberculous; before and after treatment.	No strong fixation before treatment except in a small percentage of tuberculous cattle. Killed, whole tubercle bacilli, intravenously, produced the most antibody, and did so in healthy and tuberculous cattle.
31. Momose..... Ibid. no. 22, 1029.	Few details; active serum.	.....	Bodies of tubercle bacilli, after extraction of fat.	Persons.....	Fixation was obtained in all phthisical persons, and in nearly all exposed by association and suspected persons. Of sick persons not suspected of tuberculosis one-half reacted. One-third of apparently well persons reacted.
32. Besredka..... Zeit. f. Immunitatsfg. 1914, XXI, 77.	.....	.....	Author's special egg medium antigen.	Tuberculous and nontuberculous persons, rabbits, and guinea pigs.	1, human: In the first stage of tuberculosis the reaction is always positive; in the second stage nearly always; in the third often partial or negative. 2, animal: The antibodies in infected animals follow curves which vary with the type and method of infection.

Tabulated review of recent literature—Continued.

Authority and references.	Methods.	Hemolytic system.	Antigens.	Materials examined.	Results.
33. Wwedensky, rev. by Wulffius. Ibid. referat., 1914, VIII, 931.	Wassermann		Various tuberculins, suspensions, and extracts.	Tuberculous and nontuberculous persons and animals.	In 62 tuberculous cases 82.8 per cent were positive and 16.2 per cent doubtful. In 20 suspected cases 55 per cent doubtful, 45 per cent negative. All of 10 nontuberculous cases were negative. In 24 lupus cases 41.6 per cent were positive, 58.4 per cent negative. Of 82 tuberculous animals 52.4 per cent were positive, 40.2 per cent doubtful, and 7.4 per cent negative. Of 77 nontuberculous animals all were negative. Incipient and healed cases give weak reactions; pronounced cases give more and stronger reactions. At least 5 different antigens should be used. Best results from suspensions and extracts.
34. Debains and Jupille. Comp. Rend. Soc. Biol., 1914, LXXVI, 199.	Constant amounts of antigen and serum; varied amounts of complement.		Besredka's egg medium antigen.	About 600 persons, tuberculous and nontuberculous.	Reaction very sensitive in all forms of tuberculosis except miliary and meningeal. Absent in the healthy. Advanced grave cases and those on the road to recovery frequently give partial or negative reactions. Syphilitic antibodies also react to this antigen.
35. Kuss, Leredde and Rubinstein. Ibid., 244.	2 different methods multaneously.	Sheep cells; rabbit amboceptor; guinea pig complement.	do	Tuberculous and nontuberculous persons.	Well-developed cases of pulmonary tuberculosis are positive in 89 per cent. Mild, nonprogressive cases positive in two-thirds. Nontuberculous cases are negative, except those giving positive Wassermann reaction for syphillis.
36. Inman. Ibid., 251.	Amounts of serum varied; other reagents constant.	Sheep cells	do	do	In 100 cases of pulmonary tuberculosis 95 per cent were positive. In 50 suspected cases 60 per cent were positive. In 100 patients in hospital for nontuberculous disease 24 per cent were positive, but not in high dilutions. A negative reaction indicates either the absence of tuberculosis or the arrest of a former lesion. A positive reaction indicates (especially in high dilutions) an active tuberculous lesion.
37. Bierbaum and Berdel. Zeit. f. Immunitatsfg., 1914, 21, 1–5, 249.	Hammer's; room temperature incubation.	Sheep cells; guinea pig complement.	Bovine old tuberculin; extracts of tuberculous tissue.	Serum of 120 slaughtered cattle.	Autopsy findings and serological findings agreed in only 65 per cent of cases.
38. Calmette and Massol. Annal. d. l'Inst. Past., 1914, no. 4, 339.	(See 14, 16)				The "inhibitrice," which may be present in a serum and prevents fixation by the antibody, acts on the antigen, but may be overcome by an excess of the latter.

39. Davidovics. Deutch. med. Woch., 1914, XL, 1, 21.	Not detailed	Ox cells; rabbit amboceptor; guinea pig complement.	Old tuberculin	Tuberculous cases	The immune body causing fixation with O. T. is thermolabile and not shown in inactivated serum.
40. McIntosh and Fildes Lancet, 1914, II, no. 8, 485.	Modified Wassermann.	Sheep and ox cells; guinea-pig complement.	Fresh, living tubercle bacilli emulsion.	Tuberculous and nontuberculous persons.	In pulmonary tuberculosis 76.7 per cent of cases were positive. In surgical tuberculosis, not glands, 80.7 per cent positive. In proven cases of gland tuberculosis, 37.5 per cent positive. In less well-established cases smaller percentages. In controls there were no positive results except in lepra and Addison's disease. Syphilitics did not react. A positive reaction indicates active tuberculosis.
41. Radcliffe. Ibid., 488.	do	do	do	do	Cases with T. B. in sputum. Turban classification: I. 88.6 per cent positive. II. 89.6 per cent positive. III. 79.3 per cent positive. Controls negative. In suspected cases a positive finding is of value in diagnosis.
42. Dudgeon. Jour. Hyg., 1914, XIV, no. 1, 52.	3 units of amboceptor	Sheep cells; guinea pig complement.	Alcoholic extract of tubercle bacilli, and various other antigens.	Experimental animals.	Rabbits treated with killed T. B. developed strong antibodies, but while still retaining them, succumbed to virulent tuberculous infection quicker than controls did. Tubercle bacilli sensitized with serum did not evoke antibodies. Killed tubercle bacilli injected after virulent inoculation, reduced the antibodies. The best antigen is an alcoholic extract of living tubercle bacilli.
43. Dudgeon, Meek, and Weir. Ibid., 72	do	do	do	Tuberculous cases	In 234 cases of pulmonary tuberculosis, 89 per cent were positive, but some only on repeated examination. Of 33 cases of surgical tuberculosis 20 were positive and 13 negative. A negative reaction is of little value in diagnosis. Variations in the same patient are noted. Improvement of the patient with reduction of the antibodies is sometimes noted.
44. Meek. Ibid., 76.	do	do	do	Pulmonary tuberculosis.	The greatest amount of antibody is found in severe cases with extensive lesions. Cases similar clinically may give very different reactions. No constant relation exists between acute exacerbations or improvement or retrogression and the antibody content of the serum.
45. Bronfenbrenner. Arch. Int. Med., 1914, no. 6, 786. Also Zeit. f. Immunitatsfg., 1914, XXIII, 2, 221.	Resembling Noguchi's	Human cells	Besredka's egg medium antigen.	Human tuberculosis and great variety of other diseases.	In active tuberculosis 93.84 per cent of cases gave a positive reaction. Besredka's antigen is specific. Syphilitic and tuberculous antibodies occurring in the same patient's serum are distinct and separable. 43 per cent of syphilitics gave a positive reaction with Besredka's antigen, indicating an undue pervalence of tuberculosis in this class of patients.

In considering the trend of the work here tabulated, interest must center chiefly around the antigens employed. We are not justified in assuming that because an antigen gives positive fixation in a comparatively small proportion of established cases of tuberculosis it is necessarily valueless. Such an antigen might conceivably have the greatest value in prognosis. But, for the present, greater interest in the diagnostic possibilities of the test will be assumed. Here the antigen giving the greatest number of positive reactions in tuberculous cases, while at the same time giving negative reactions with other diseases and with normal subjects, will obviously be of the greatest value. Considered in this light and giving preponderance to those observations which have been confirmed by the largest numbers of workers, it appears that the claims for greatest diagnostic usefulness must be adjudged among three or four antigens. The most recent of these is Besredka's egg-medium antigen, and observers are unanimous thus far in finding that it gives positive reactions in a very high percentage of tuberculous cases, at least at certain stages of the disease. The objection has been adverted to by several authors that syphilitic antibodies were capable of causing fixation with this antigen, a circumstance which would, of course, impair its usefulness to a great degree. This luetic fixation has been attributed to the lipoid content of the antigen derived from the eggs used in making the medium. The researches of Bronfenbrenner are reassuring in this connection, and it is to be hoped that they will be confirmed in both their theoretical and practical aspects. It is possible that another objection may develop as other workers take up the use of this antigen, namely, difficulty in preparing the antigen. The description of its preparation given by Besredka is lacking in definiteness, and attempts by my assistants in this laboratory to duplicate his product have not been successful. I believe that thus far the work with this antigen reported by various observers has been done, like my own, with antigen supplied through the courtesy of Dr. Besredka.

Next in order must be considered antigens composed of suspensions of tubercle bacilli in salt solution. A fair number of observers have had favorable results with antigens of this class, using both killed and living tubercle bacilli, and have found, moreover, a high degree of specificity, except in veterinary work, where other acid-fast infections may cause confusion. Luetic fixation with these antigens does not appear to enter in as an objection. Their use is more troublesome than that of Besredka antigen, since the suspensions must frequently be made up fresh and standardized.

The antigens elaborated by Calmette and his coworkers, taken collectively, and the peptone-soluble antigen by itself, make serious claim for consideration; for not only was that author able to secure fixation in 92 per cent of cases using all three antigens, but another

worker (24) obtained an equally high percentage of positives with the peptone antigen alone. Moreover, some prognostic significance was attached to variations in the activity of the sera with the different antigens.

Unfortunately, not all authors have described their antigens in sufficient detail. Some of the most promising results have been reported from the use of antigens briefly described as alcohol or alcohol-ether extracts. Another antigen which has some promise—from an experimental standpoint, at least—is that prepared by precipitation with alcohol from suspensions of tubercle bacilli, after the method of Besredka as modified by Gay and others.

The antigens which were so much used in the early work, old tuberculin, commercial "Bacillen emulsionen," extracts of tuberculous tissues, and various watery extracts, have failed in the hands of most workers to give hopeful results as diagnostic aids.

The so-called split products of the tubercle bacillus, the fats, fatty acids, proteins, "toxins," etc., which have been derived by physical and chemical means, have thus far had no successful application to the diagnostics of tuberculosis. Much, who with his students has written most extensively on these substances in connection with tuberculosis, does not detail his methods, but from the fact that he reports fixation in a large majority of the clinically nontuberculous, with tuberculin and tubercle bacilli emulsions, one is led to infer that his methods differ so radically from those commonly employed that comparison is impracticable. It is also a question whether by "splitting off" the substances we do not reduce them below the state of combination where they have any essential relation to the tubercle bacillus.

Considering next the methods employed by the various observers, it is noted that the Wasserman type of technic has been most extensively employed, with the use of sheep cells, rabbit hemolytic amboceptor, and guinea-pig complement. In a few instances ox or goat corpuscles have been used, and in one the native antisheep amboceptor of human serum has been removed. The original Bordet-Gengou technic has had several adherents, and one observer has employed a technic resembling that of Noguchi for syphilis. Special technics have been devised by several of the authors in attempts to solve peculiar problems—as, for example, that of the supposed thermolability of certain antibodies. A discussion of technical considerations will be given later. Suffice it to remark that the greater proportion of the work in the last few years appears to have been performed with understanding of basic principles and to be at least as worthy of confidence as a similar amount of syphilitic fixation work.

Most of this work has been primarily addressed to the practical application of the tests to human tuberculosis, a number of workers

have been interested particularly in the application of the method to the detection of cattle tuberculosis, and another group have endeavored by animal experimentation to illuminate the essentials of the serological processes. Special reference must be made to the work of Debre and Paraf and others who have attempted to demonstrate tuberculous *antigens* for diagnostic purposes in such pathological fluids as urine and milk, using as a test fluid a serum known to contain tuberculous antibodies. This work, while of promise, especially in the diagnosis of genito-urinary tuberculosis, has not proceeded far enough for definite evaluation. An attempt to summarize the other work, especially as regards its practical application to diagnosis and prognosis in human tuberculosis, will be found in the general conclusions at the end of the article.

Original work.—The *serum* examined has come from patients in a tuberculosis sanatorium at a long distance from the laboratory; from a tuberculosis hospital convenient to the laboratory; from healthy persons exhibiting no evidence and giving no history of tuberculous infection; and from subjects of an industrial-hygiene investigation. The specimens were obtained in all cases by excellent technic from an arm vein, were adequate in amount, and showed no evidence of deterioration on inspection at the time of examination. Except where otherwise stated the serum was inactivated for 30 minutes at 55° C.

The technic employed closely follows the Wassermann. Sheep *corpuscles* were used. After repeated washings in large bulks of saline they were made up to the original bulk of the defibrinated blood from which they were derived, and a 5 per cent suspension of this was used. This suspension included also 1 unit of hemolytic amboceptor per c. c., the cells being thus sensitized before application to the test. The unit of *amboceptor* was constant and so chosen after several preliminary tests of the hemolytic system that its reinforcement by at least an equal amount of native amboceptor would be unlikely to increase the hemolysis, on the one hand, or enter on the Neisser-Wechsberg phase,[1] on the other. In other words, it was selected from the relatively flat portion of the hemolysis curve. The same supply of hemolytic serum was used throughout the series, and, since it was a relatively stable and potent specimen, deterioration was slow and negligible in extent. *Complement* was that of guinea-pig serum. In much of the work it was preserved by salting, being restored to normal tonicity before use by the addition of correct amounts of water, further dilutions being made with saline. The unit of each specimen was determined by titration before each test

[1] With constant amounts of complement and cells increasing amounts of amboceptor cause increasing degrees of hemolysis up to a certain point, beyond which excess of amboceptor "deviates" complement, and hemolysis is lessened.

and varied from 0.03 to 0.05 c. c. of the undiluted serum. Two units were used in the tests.

It was, unfortunately, impossible to test all of the sera against all of the *antigens* which were used during the course of the work. The antigens used were: (1) Calmette's peptone-soluble endobacillary antigen, prepared at the laboratory from both fresh and dried human tubercle bacilli by heating 1 part by weight of bacilli after thorough trituration with 20 parts of 10 per cent peptone solution at 65° C. for 48 hours and filtering through a Berkefeld; (2) suspensions of human tubercle bacilli, both living and killed by heat, made by simple trituration in salt solution; (3) Besredka's egg medium antigen, kindly furnished by Dr. Besredka; (4) an antigen prepared by precipitating a suspension of living tubercle bacilli in salt solution with an equal bulk of absolute alcohol, sedimenting, decanting, drying the residue over H_2SO_4 *in vacuo*, pulverizing together with sufficient NaCl, so that when the emulsion resulting from trituration with water contained 1 per cent of the bacterial powder, the salt solution would be of physiological concentration. These antigens were all titrated before making tests, and the unit adopted for each test did not exceed one-half of the maximum nonself-binding dose.

The methods of handling these reagents involved careful measurements of all ingredients with graduated pipettes; a total bulk, made up with saline, of 3 c. c. for the first-phase incubation and of 4 c. c. for the second; incubation in water bath at 37° for 1 hour for the first phase, and the same supplemented by standing in the cold room over night for the second; controls of double amounts of the serum and of the antigens; and controls on the hemolytic system and corpuscle suspension. Instead of expressing the results by the usual methods of ++++, +, −, etc., or in terms like "strong positive," "weak positive," etc., readings were made of the tube contents on a colorimeter, using the hemolytic system control tube as the 100 per cent standard. The amount of fixation was taken as being 100 minus the reading for the tube contents considered. This method, although not giving a strictly proportionate measure, has the advantage of permitting the striking of averages for groups of tests for comparative purposes. As experience with these tests was gained the impression developed that 20 per cent or more of fixation in well-controlled, careful tests was certainly of significance but that below this experimental error might be operative. Eighty per cent fixation would be called a strong positive or 4+ by the majority of observers. Several authors have mentioned that the antibodies in tuberculosis are not as strong as in syphilis and that consequently the reactions are not as definite. While this is possibly true, one nevertheless frequently sees as sharp and positive results as could be desired.

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As regards the different technical procedures, judgment must be withheld as to which is the best and what one of the present ones, if any, is suitable for adoption as the standard. For the present it seems better that each worker should employ the method in which he has the most experience, provided that certain fundamentals are observed. These I should briefly summarize as the sacrifice of ease and speed to thoroughness and reliability; accurate measurements; controls sufficient to detect spurious evidences of fixation, especially those due to summation of anticomplementary effects; the use of at least two complement units to avoid the results of the deterioration of diluted complement at water-bath temperature; the avoidance of too great discrepancies between the total bulk in the first and second phase incubations. The criticism of the use of sheep cells on the ground of their being subject to the action of native amboceptor in human serum is not altogether well founded. It is granted that a fair proportion of human sera have this amboceptor, but these same sera may give excellent fixation, because if the complement has been fixed in the first phase, the mere increment of amboceptor will not cause hemolysis in the second. In fact it is quite possible that this reinforcement of hemolytic amboceptor may in certain quantitative relations reduce the hemolysis through the operation of the Neisser-Wechsberg phenomenon, and give a spurious appearance of fixation, a contingency which has not been sufficiently emphasized. However, I believe that the positive results in a series of tests will be somewhat increased by the use of human cells, or by removing the native antisheep amboceptor from the human serum.

The results of examination of 72 sanatorium patients is given in Table I. Of these, 37 had received no specific treatment, 21 had been treated for a considerable time with a watery extract of tubercle bacilli, and 14 had been treated with a so-called vaccine of avirulent acid-fast bacilli. It is realized that the numbers are too small in some of the groups to warrant any conclusions as to the significance of the figures, but considering the groups having eight or more cases, it would appear that the positive results with peptone extract reach their maximum in the moderately advanced stage, being reduced in the far advanced and very significantly small in arrested or cured cases. With the suspension of killed bacilli the maximum would appear, as Calmette states, to be deferred to the later stages. From this table it appears that the administration of the specific treatment referred to, resulted in a somewhat higher percentage, and greater intensity of positive reactions. In this connection the results given in Table II become of interest.

TABLE I.—*Summary of fixation reactions in the first series.*

[Serum sent from a distance, several days old when examined.]

Class of cases.	Sanatorium treatment only.		Treated with a watery extract of tubercle bacilli.		Treated with a vaccine of avirulent acid-fasts.	
	Antigens.		Antigens.		Antigens.	
	Peptone extract tubercle bacilli.	Suspension-killed tubercle bacilli.	Peptone extract tubercle bacilli.	Suspension-killed tubercle bacilli.	Peptone extract tubercle bacilli.	Suspension-killed tubercle bacilli.
Far-advanced cases:						
Number examined	20	16	18	11	13	10
Average fixation	36%	21%	53%	34%	44%	35%
Cases in group giving 20 per cent or more fixation	50%	37%	83%	45%	54%	50%
Moderately advanced cases:						
Number examined	8	4	2	1	1	1
Average fixation	45%	2%	75%	75%	53%	3%
Cases in group giving 20 per cent or more fixation	63%	0%	100%	50%	100%	0%
Incipient cases:						
Number examined	1	1	1	1	0	0
Fixation	13%	2%	51%	0%	.....	.....
Cases in group giving 20 per cent or more fixation	0%	0%	100%	0%	.....	.....
Arrested or cured cases:						
Number examined	8	7	0	0	0	0
Average fixation	11%	2%	.....	.....	.....	.....
Cases in group giving 20 per cent or more fixation	13%	0%	.....	.....	.....	.....

NOTE.—In this and subsequent tables the words "average fixation" mean the arithmetic average of the fixations shown by all the sera in the group considered. The fixation shown by a serum is 100 per cent minu the percentage of hemolysis in the tube determined by calorimetric comparison with a completely hemos lyzed control tube.

In Table II are shown the results as regards the clinical progress of the patients from six months to a year after the taking of the specimens reported in Table I. It should be remarked that at the institution in question (the Public Health Service sanatorium at Fort Stanton, N. Mex.) the manner of taking and recording physical examinations is rigorous and the determinations are as reliable as the nature of the case permits.

TABLE II.—*Clinical results in 68 patients whose serum examinations are summarized in Table I, 6 to 12 months after examination.*

(Peptone extract antigen.)

Condition of patient.	Sanatorium treatment.		Watery extract treatment.		Avirulent vaccine treatment.		Total group.
	Number of cases.	Group.	Number of cases.	Group.	Number of cases.	Group.	
Gave fixation 20 per cent or more:		*Per cent.*		*Per cent.*		*Per cent.*	*Per cent.*
Same	4	33.3	5	27.7	3	37.5	31.6
Better	4	33.3	4	22.2	2	25.0	26.3
		66.6		49.9		62.5	57.9
Worse	3	25.0	7	38.8	3	37.5	34.2
Died	1	8.3	2	11.1	0	0.0	7.9
		33.3		49.9		37.5	42.1
		99.9		99.8		100.0	100.0
No fixation, i. e., under 20 per cent:							
Same	8	36.3	1	33.3	0	0.0	33.4
Better	7	31.8	1	33.3	0	0.0	23.3
		68.1		66.6		0.0	56.7
Worse	3	13.6	1	33.3	1	20.0	16.6
Died	4	18.1	0	0.0	4	80.0	26.6
		31.7		33.3		100.0	43.2
		99.8		99.9		100.0	99.9

The most significant finding indicated in this table is that although, as shown in Table I, a greater proportion of persons specifically treated with the preparations named had complement-fixing bodies in their serum, the subsequent course of their disease did not show that this was of any advantage to them. In fact, the evidence is rather that patients who responded to the stimulus of these preparations by the production of antibodies detected by this antigen, progressed less favorably than those who failed to respond and those who did not receive the injections. That no significant difference among the sanatorium cases is apparent, depending upon whether they had or had not shown antibodies, may perhaps be explained by a balancing action of the hopeless and of the arrested cases which, although representing opposite extremes from a clinical standpoint, still give on the average the same reaction, namely, a negative one. That this is so is indicated by the following figures, which give the average percentage of fixation for the various groups in the sanatorium cases of the above table:

Condition.	Same.	Better.	Worse.	Died.
Average fixation of group, per cent	18.7	26.1	41.1	14.6

The "Same" group has a low index for the reason that it includes the arrested cases in which retrogression did not, and marked improvement could not, occur. Eliminating these cases and averaging the active cases which remain the index for this group becomes 26.9 per cent.

It is evident, therefore, that the significance of the reaction as regards prognosis depends on the associated clinical findings. In the active but as yet indecisive period of the battle between patient and disease, a positive reaction is rather of bad than of favorable import. One might assume from it that the patient would be worse before he would be better. A negative reaction with failing powers of the patient is almost confirmatory of the fact that the battle has been lost, while negative reactions associated with clinical improvement are hopeful harbingers of an arrest or cure. While no such definite conclusions could be drawn from a single test in an individual patient, nevertheless the above seems to be the general trend of the prognostic import of the reaction as indicated by this series, with Calmette's peptone-soluble antigen.

Another group of sanatorium cases in which the serum was examined shortly after it was secured, by reason of the convenience to the laboratory, gave no greater incidence of positive reactions than the one just reported. Of 27 patients but 11 showed significant fixation with the peptone-soluble antigen (40.7 per cent). These cases were for the most part far advanced, and it is probable that many were beyond the stage when antibodies are to be expected. Comparing the results in this series with those in the former one, it may be inferred that the antibodies demonstrated by this antigen are not so labile as to be destroyed by the conditions of transportation operating for several days.

In a third group from this same institution there is opportunity for comparing the action of different antigens. In all, 47 cases are represented. Classifying these so that the comparisons of antigens are referable to the same sera the results may be thus tabulated:

TABLE III.—*Comparison of results with three antigens on the same sera from tuberculous cases.*

(No specific treatment.)

Number of sera for comparison.	Suspension of living tubercle bacilli.		Calmette's peptone antigen.		Alcoholic precipitate antigen.	
	Fixation in—	Average fixation.	Fixation in—	Average fixation.	Fixation in—	Average fixation.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
18	66.6	44.0	72.2	27.9	.....	.....
12	66.6	44.1	75.0	33.3	58.3	43.1
33	.....	.....	55.8	27.3	55.8	39.0

From these results it may be assumed that the Calmette peptone antigen gives a reaction in a greater proportion of well-developed cases such as these were, but that these reactions were less well marked than those with the other antigens. The other two antigens were approximately equivalent as regards the strength of the reaction given by them, but the suspension had somewhat the advantage in the number of cases giving a positive reaction.

Tables IV and V represent still another group of cases from the near-by institution, and indicate that the antigens used reach their maximum proportion of positives in the second stage of pulmonary tuberculosis, and in patients who are progressing unfavorably, but that the reduction of this proportion in the last stage and in favorable cases is relatively greater in the case of the peptone antigen while the strength of the reaction given by the peptone antigen is in general inferior to that of the alcohol precipitate.

In all of these series controls consisting of the serum of one or more healthy persons not suspected of being tuberculous have been run with each test. In none of them has fixation ever been demonstrated.

TABLE IV.—*Comparison of results with two antigens in the three stages of pulmonary tuberculosis (Turban).*

(No specific treatment.)

	Stage I.		Stage II.		Stage III.	
	Peptone extract.	Alcohol precipitate.	Peptone extract.	Alcohol precipitate.	Peptone extract.	Alcohol precipitate.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Fixation in	43	25	66.6	57.8	36.3	50
Average fixation	23	18	32.5	45.2	12.5	38

TABLE V.—*Comparison of results with two antigens, according to whether at the time of examination the patient was progressing favorably or not.*

(No specific treatment.)

	Favorably.		Unfavorably.	
	Peptone extract.	Alcohol precipitate.	Peptone extract.	Alcohol precipitate.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*
Fixation in	31.2	38.4	62.5	46.1
Average fixation	19.0	32.5	28.2	30.1

In addition tests were made with the peptone antigen and Besredka's egg medium antigen upon 52 sera from the subjects of an investigation into the physical condition of workers in the garment industries in New York. The subjects were mostly those in whom a

history of cough or of loss of weight or the general appearance had suggested the possibility of tuberculous infection, although definite symptoms and signs were lacking. In two of these cases fixation was obtained with Besredka's antigen, and in one with the peptone antigen. The patient giving fixation with both antigens had definite signs and symptoms of pulmonary tuberculosis. The patient giving fixation only with the Besredka antigen was a frank case of secondary syphilis.

It has been suggested by several authors that some, at least, of the tuberculous antibodies were thermolabile, and consequently escaped detection in the ordinary tests with inactivated serum. I made some tests to determine this, using a method much like that of Zweig (13). Two series of tubes were set up, each containing the active serum in decreasing amounts. To one of the series was added a dose of antigen, the same amount for each tube. After bringing the volume up to a uniform amount with saline, and incubating for an hour, sensitized cells were added. The series without antigen was virtually a test for complement in the serum, and by comparing the hemolysis in the tubes of this series with that of corresponding tubes in the series with antigen, the occurrence and degree of fixation could be determined. Due to fluctuations in the amount of complement in different sera, the maximum amount of fixation occurred with varying amounts of serum, so that there was no basis for comparison of the antibody strength, except to accept this maximum. These same sera were then inactivated and a test made in the usual way. Fukuhara,[1] has shown that peptone solutions will cause fixation with normal active serum, although not with inactivated serum. It was not surprising, therefore, that with the peptone extract some fixation was shown in all the active sera examined, including the normal controls. The other antigens (tubercle bacilli suspension, and alcohol precipitate) appeared to be more specific, giving only minimum amounts of fixation with normal sera. Comparisons of the results with active and inactivated serum in 21 cases showed in general a very significant reduction of antibody strength as indicated by the peptone extract tests, but with the other antigens there were but three significant discrepancies—two cases in which the reaction was negative in the inactivated serum while positive in the active, and one in which the converse occurred, which was readily accounted for by the absence of complement from this specimen of uninactivated serum. The antibodies of tuberculosis (reacting to these antigens) are therefore quantitatively reduced by heating, as indeed are those of syphilis, and it is quite possible that when present in the active serum in small quantity they may be reduced by heating below the point of definite determination.

[1] Fukuhara. Zeit. f. Immunitätsforschung, vol. 12, 1911, p. 183.

The question of the artificial stimulation of antibodies is important. There can be no doubt that by the injection of certain products of the tubercle bacillus the formation of antibodies which will fix complement with some at least of the tuberculous antigens can be induced or increased. It is not demonstrated, however, that this artificial increase is desirable from the standpoint of improving the condition of the patient. Neither is it demonstrated that the presence of antibodies is an indication of established immunity. In fact, we do not find these antibodies in the serum of healthy persons who have reached middle age without developing recognizable tuberculous infections, and who therefore may be assumed to possess at least a relative immunity or high resistance. Again, the evidence is strong that after recovery from tuberculosis the antibody content of the serum tends to diminish and disappear. Since of all the forms of acquired immunity the strongest and most permanent is assumed to be that afforded by an actual attack of the disease in question followed by recovery, it would seem that in the relative absence of antibodies from the serum of patients of this class we find a strong argument against their playing an important part in immunity, and against the assumption of improvement in the condition of the patient by their artificial production. It is apparent that antibodies do not check or reduce the *production* of the antigen liberated from a tuberculous focus. Their action takes place after its liberation, if indeed at all intra-vitam, and we are almost wholly in the dark as to what effect this fixation would have as regards the deleterious effects of the antigens upon the body. It could conceivably be harmful. We do know that patients with fever and the other manifestations of toxemia may yet show strong complement-fixing bodies in their serum. In this connection an experiment which I performed may be of interest. Five active sera were tested against a suspension of *living* tubercle bacilli as antigen. Three of these gave strong fixation and two a negligible degree, one of the latter being from a clinically normal person and all the rest from tuberculous persons. The tube contents were then injected into guinea pigs with results as shown:

Serum from—	Fixation.	Pig died in—	Findings.
	Per cent.	*Days.*	
Tuberculous case	82	35	Tuberculosis of lymph nodes, spleen, and liver.
Do	98	55	Tuberculosis of lymph nodes, spleen, liver, and lungs.
Do	87	62	Do.
Do	10	66	Do.
Normal person	8	67	Do.

While an isolated experiment of this kind has no conclusive significance, the evidence, as far as it goes, is that the presence of complement-fixing bodies is not necessarily associated with an effect on tubercle bacilli in the direction of reducing their virulence, or toxic

effects. In fact the animals receiving the tube contents in which fixation had occurred all died earlier than those in which no, or little, fixation was shown. Baldwin has found that animals inoculated with tubercle bacilli which have been exposed to the action of serum containing antibodies, succumb quicker than those injected with bacilli acted upon by normal serum. This fact he was inclined to ascribe to the agglutinins which accompany the other antibodies, and which by clumping the bacilli render them less susceptible to phagocytosis.

I may perhaps illustrate the difficulties of ascribing the proper rôle to complement-fixing antibodies in tuberculosis by drawing a parallel with a very different disease, namely, furunculosis. Many persons in spite of exposure do not have furunculosis. They are immune. Others are continually suffering from the disease. In them the introduction of the causative cocci is followed by heat, redness, swelling, suppuration. How shall we describe this inflammatory reaction? We can not call it an immune reaction—immune persons do not have it. If we call it a defensive reaction we must admit that as long as it persists the result is dubious, and that meanwhile the patient suffers. We are at least safe in calling it a reaction and thereby we imply the activity of the invader. So in tuberculosis, the antibodies do not imply immunity; if they are present they indicate a doubtful battle, during which the patient suffers, and we should remain more closely within the bounds of our knowledge if we described them as reaction bodies rather than as immune bodies, protective bodies, or even as antibodies.

The finding of complement fixation in a tuberculous patient appears to be dependent upon the interplay of two, and possibly three, fundamental factors. First, the *antigens* liberated in the body as a result of invasion of the tubercle bacillus. The exact nature of these is still unknown. They may conceivably consist of the bacillus itself, of products of the disintegration of the bacillus, of endotoxins, or of products of the disintegration of the tissues evolved during the tuberculous process. We must remember also the antigens which may be artificially introduced. Second, the *antibodies*, which may be produced in response to the stimulus exerted by these antigens. Third, we must consider the rôle possibly played by the *inhibiting substances* which are stated by observers, notably Calmette and his coworkers, and Caulfield, to occur in certain tuberculous sera, and which have the effect of producing negative reactions in sera which contain antibodies. The interaction of these factors in various quantitative relations may give rise to various hypothetical contingencies in the test reactions of a serum, the most important of which appear to be the following:

Case 1.—No antigen is or has been present in the body, therefore we get a negative reaction since there is also no antibody.

Case 2.—Antigen is present but for some reason there is no antibody formation—negative reaction.

Case 3.—Antigen is present, but the antibody is quantitatively insufficient to more than saturate the antigen; it therefore remains undetected, and the reaction is negative.

Case 4.—Antigen and antibody are present, the latter in such excess that it can be detected, and a positive reaction results.

Case 5.—Both are present, but there is also an inhibiting substance which interferes with the reaction of fixation, which therefore results negatively.

Case 6.—The antigen formerly present has disappeared and no more is being liberated, but the antibody evoked by it still remains, being demonstrable in decreasing amounts on successive examinations.

Considered in the light of these hypothetical possibilities the observed discrepancies in the results of examining a series of patients become more reasonable, and susceptible of interpretation.

Cases 2, 3, and 5 are those in which confusing disagreement between the serum and clinical findings would be expected. In case 2 the presence of antigen means that there is active tuberculosis. Certain individuals and certain experimental animals are unable because of some physiological peculiarity to respond to the stimulus of antigen by the production of antibodies. Human tuberculous cases in this category will not be detected by complement-fixation tests unless these tests are developed in the direction of demonstrating circulating antigen rather than antibody. In the same case are those patients in advanced stages with lost "resistance" and also lost reactive ability, but here the clinical picture will be sufficiently distinct to prevent confusion as to the real condition. Under case 3 would come those cases of moderately advanced tuberculosis in which, owing to an even balancing of antigen and antibody, neither would be demonstrable in vitro in case we assume intra vitam fixation of complement. In this class of cases repeated tests might be valuable. In case 5 the operation of inhibiting bodies is assumed. Calmette and his coworkers have devoted their attention principally to devising means of avoiding the operation of these bodies in tests—of detecting antibody despite their presence. My own attempts to remove supposed inhibiting bodies from the serum of active cases which failed to show fixation, by the method used by these authors (dilution, heating, passing through CO_2 gas and restoring tonicity) have been but partially confirmatory, although they have as yet been too few to be conclusive. I have not thus far been able to convince myself that the changes produced by this treatment of serum are related to specific fixation, since I have seen such treatment result in the *appearance* of fixation in the serum of persons not having any suspicion of active tuberculosis.

Caulfield, on the other hand, instead of attempting to avoid the obscuring effect on antibodies of these inhibiting bodies, has found significance in their presence. In a general way they seem to be of

favorable import, while the complement fixation is indicative of a dubious or stormy course of the disease with many ups and downs. Both observers are at one in attributing the action of the inhibiting bodies to their influence upon the antigen. These bodies are selective as regards the particular antigens which they affect. That they do not operate upon all antigens is shown by the fact that observers are obtaining positive reactions of fixation in over 90 per cent of cases of tuberculosis with certain antigens.

In summing up the present status of complement fixation tests in relation to tuberculosis as indicated by a review of the recent literature and in the light of my own work, the following generalizations appear to be justified. Depending upon the antigen and the technic employed, the proportion of tuberculous cases in which positive fixation will be demonstrated will vary from a maximum of some 95 per cent down to a much lower figure. While those antigens and technics giving the higher percentages of positive results are more valuable in confirming suspected and detecting unsuspected cases, they tend to approach such tests as that of von Pirquet in failing to afford much information as to the stage, extent, and activity of the tuberculous process. Nevertheless, the continued presence of reactive bodies in the serum of a given patient on repeated examination is, I believe, strong presumptive evidence of continued or recent activity of the lesions, when no antigens have been artificially administered.

Those antigens and technics affording a smaller percentage of positive reactions may still be valuable in confirming clinical observations as to the activity of the disease. Repeated observations would appear to have particular value in this connection. Thus in a patient who from a clinical standpoint had reached the condition of arrest or cure, repeated negative reactions would be confirmatory, while as long as positive results cropped up we should be justified in suspecting continued activity and expecting perturbations. Only in the terminal stages does a negative reaction have sinister import, and here it may be regarded as part of the general loss of vital reactivity. Positive reactions are not indicative of immunity and the reaction should not be described as an immune reaction. The artificial increase of complement-fixing bodies by the therapeutic administration of products derived from the tubercle bacillus can not be assumed to indicate improvement in the condition of the patient. That it may prove to have such significance in the case of certain products can not be denied, but proof must be demanded in each case. It appears from my own work that the administration of certain products derived from the tubercle bacillus may result in the increase of complement-fixing bodies, but that this is not necessarily associated with improvement in the condition of the patient—in fact, the evidence is rather in the opposite direction.

The complement-fixation reaction with inactivated serum and with the antigens now more generally employed appears to be highly specific. Inasmuch as a number of recent workers have obtained high percentages of positive results with Besredka's new antigen, it is likely that this preparation will be the subject of considerable investigation in the near future. If Bronfenbrenner's conception of the nonlipotropic nature of the reaction of syphilitic serum with this antigen proves to be correct (and his opinion appears to be well grounded), one objection to the employment of the latter will be removed. At the same time serological support will be given to the idea that syphilis greatly predisposes to tuberculous infection, and the venereal disease will assume even greater importance from a public health standpoint.

It is difficult to forecast the rôle which complement fixation tests are destined to play as applied to practical tuberculosis work. Now that work along these lines is tending toward greater unanimity, and we are able to summarize the significance of a positive result as reactive power against recently liberated antigen, some promise is given that tests of this nature may become valuable aids in the diagnosis, prognosis, and management of tuberculous cases. Especially will this be the case when practicable methods are developed for estimating not only the antibody, but also the antigen content of serum, a phase of the subject which is already receiving attention.

In its present condition this test can be of little assistance to the private practitioner. In institutional practice, however, I believe that the introduction of these tests as a routine, parallel with the lues fixation tests, would be advantageous. This I believe because the tests are highly specific and because when positive they indicate present or at least recent activity. The fact that negative reactions have no value in excluding tuberculous infection must, however, be fully appreciated. In every large general hospital will be found cases in which physical examination has elicited obscure signs of tuberculous lesions, but in which, perhaps after a single negative sputum examination, no further attention has been paid to the tuberculous element. Some of these patients undoubtedly have active lesions, and yet the excellent opportunity afforded by the hospital conditions for salutary information, advice, and training is often lost, to the detriment of both the patient and the public. Any test therefore which will serve to emphasize the presence of tuberculous complications must ultimately be of benefit, even though it may not operate at all stages nor in every patient.

I desire to make grateful acknowledgment of the courtesy of Dr. W. D. Tewksbury, of the Washington Tuberculosis Hospital, and his assistants, and of Passed Asst. Surg. F. C. Smith, Public Health Service, in securing or permitting the taking of the specimens of serum examined in the original work here reported.

II.—REPORT OF AN INVESTIGATION OF DIPHTHERIA CARRIERS.[1]

By Joseph Goldberger, *Surgeon*, C. L. Williams, *Assistant Surgeon*, and F. W. Hachtel, *Bacteriologist*, *United States Public Health Service*.

INTRODUCTION.

During the winter of 1913–14 an opportunity occurred for study of the distribution of diphtheria carriers among the population of the city of Detroit, Mich. This was undertaken as a part of an extensive investigation of diphtheria from a public-health standpoint. Unfortunately this work was interrupted by the detailing of both the senior writers of this report on epidemic duty. However, the investigation of carriers in Detroit forms a fairly complete unit, and we feel that the results are of sufficient importance to be presented at this time.

ACKNOWLEDGMENTS.

We are indebted to Asst. Surg. Joseph Bolten and Temporary Bacteriologist Aubrey Straus for valuable assistance in the examination of smears. It is also a pleasure to acknowledge the very helpful cooperation extended to us by Dr. W. H. Price, health officer of Detroit, and many courtesies shown by Dr. E. M. Houghton, director of the research laboratory of Parke, Davis & Co.

OBJECT.

For nearly a year prior to the commencement of this investigation diphtheria had been unusually prevalent in Detroit, and it was originally our object to determine the percentage of carriers present during a time when the disease was of frequent occurrence. This object was modified, however, due to the marked decrease in the number of cases and deaths about three weeks before starting the collection of cultures, so that it resolved itself into a determination of the percentage of diphtheria carriers in the population at large shortly after a time of greatly increased prevalence of the disease.

In reviewing the literature on the subject of diphtheria carriers one is struck by the fact that in most instances the subjects of examina-

[1] Manuscript submitted for publication Mar. 15, 1915.

tion are inmates of institutions or are restricted to one or two particular classes of persons, as school children, medical students, patients at clinics, etc. The reason for this is probably the more ready accessibility and less expense involved when the examination is confined to members of more or less well-defined readily accessible groups. A fairly comprehensive survey of the literature has failed to show any previous study where the incidence of diphtheria carriers was determined by cultures taken indiscriminately from healthy persons of all classes in the community.

During the progress of this investigation opportunity was found for the following coincident studies:

1. Comparison of the relative values of cultures from different sources.
2. Tests of virulence and acid production.
3. Observations of Hoffman's bacillus.

LITERATURE.

We regret that the pressure of other duties has prevented the preparation of a résumé of the pertinent literature. The reader is referred to Nuttall and Graham Smith's excellent monograph, The Bacteriology of Diphtheria, and to the article by Neisser and Gins,[8] in Kolle & Wasserman's Handbook (2d ed.) for a complete bibliography.

COLLECTION OF CULTURES.

Our search for diphtheria carriers was inaugurated December 22, 1913, and concluded March 4, 1914. In that time a total of 4,093 apparently healthy persons and 95 persons suffering with clinical symptoms of diphtheria were examined. A total of 9,489 cultures were taken from these and examined.

In the hope of securing a representative portion of the population as a whole, a house-to-house visitation was carried out in representative sections of the city. In addition some cultures were taken from children visiting the daily clinics at the board of health, principally the dental clinic, and on a few occasions from children at school and workers in factories. In going from house to house, cultures were taken so far as possible from all persons found at home.

TABLE 1.—*Showing number of healthy persons examined, grouped according to age.*

	Age in years.										Unknown (adult females).
	0–1	2–5	6–10	11–15	16–25	26–35	36–50	51–65	66–80	80	
Number of persons in each group	158	510	599	673	699	633	450	176	82	3	110

An attempt was made to get our cultures so distributed as to be representative of the age and sex distribution of the general population. Table 1 shows the age distribution of the healthy individuals cultured. As will be seen, we succeeded in part only since, as a matter of fact, we have a disproportionately large number of females and of children between the ages of 5 and 15 with consequently smaller numbers of males and persons over 15 years of age. Among the 4,093 apparently well persons examined there were 1,600, or 39 per cent, males, and 2,493, or 61 per cent, females. In the total population of the city of 465,776 (Census of 1910) there were 240,354, or 51.7 per cent, males, and 225,412, or 48.3 per cent, females. Among persons examined the age groups 0–5 years; 6–15 years, and 16 years and over constitute 16.3 per cent, 31 per cent, and 52.7 per cent, respectively, while among the total population these groups comprise 12.3 per cent, 16.4 per cent (approximately), and 71.3 per cent (approximately).

The occupations of the persons furnishing our cultures were in a general way as follows: School, 1,974; in business and various occupations mostly engaged in by men, 693; housework, 1,182; unknown or none, 244.

The cultures were made on Löffler's blood serum exclusively. This was prepared for us by Parke, Davis & Co., in whose excellently equipped laboratory our work while in Detroit was done, space for this purpose being rented from the firm. The medium was controlled at short intervals by cultures from clinical cases of diphtheria and test cultures of known B. diphtheriæ. It was found to support a good growth of this organism at all times.

Cultures were made from the throat, from the nose, or from both, in each case on a single slant. From some individuals only a single culture inoculated from the nose and throat was taken. From others three cultures were secured at one time; one from the throat, one from the nose, and the third from both nose and throat as above. When three cultures were taken at one time the procedure was to make the throat culture first and to reenter the throat with the same swab for material for the combination culture. Next the nose culture was made with a fresh swab, reentering both nostrils with the same swab for material to complete the combination culture previously inoculated with the throat swab. In this way only two swabs were necessary for making the three cultures. When only a single culture, inoculated from both the nose and throat, was made, two swabs were always used, one for the throat and the other for the nose.

EXAMINATION OF CULTURES.

Cultures were made during the day between the hours of 9 a. m. and 5 p. m. They were taken to the laboratory and placed in the incubator at 5.30 p. m., and removed the next morning at 8.30, remaining in the incubator at 37° for 15 hours. The making of smears was commenced at 8.30 and finished as a rule by 10 a. m. The average number of smears per day was 158, much less at first, but rising to 300 or more toward the end of our work. Smears were stained with Löffler's alkaline methylene blue for 30 seconds and examined under oil immersion lens. Where doubtful organisms were encountered additional smears were made and stained with either Neisser (old) or Ljubinsky's stain, or both.

The routine procedure in the examination of smears was to view at least 30 fields before declaring a smear negative. At first every slide was examined by two persons, each keeping a separate record and comparing notes after all were examined. Discrepancies were checked by reexamination of the same or additional smears. When after examination of about 2,000 cultures the daily average was raised to 300, it was found impracticable for two men to see every slide, so that a system was adopted whereby the smears from the different cultures from each individual were examined by different workers. Thus, one man would see the nose smear, another the smear from the throat, etc. Whenever a positive smear was encountered by one man it was checked by one or more others, and the smears of the other cultures from the same individual were given additional scrutiny before considering them negative. However, in those instances where only a single culture was taken from an individual the smear was always examined by two persons. In doubtful cases the culture was reincubated and reexamined the next morning while coincident isolation of the suspicious organism was attempted, final decision being based on the results obtained with these procedures.

In order to standardize our method, Wesbrook's Types (1) were used as a criterion, only organisms corresponding to the A. C. D. and A′ or C′ types being declared positive, including, however, the "Sheath" type as described by Eyre (2). As has been noted by other workers, we found organisms that, strictly speaking, did not belong to any of these types. Atypical forms were classed with the types which they most closely resembled. In only four or five cases, however, was there any doubt as to the nature of the organisms not corresponding strictly to the types. In these instances reincubation of the culture or isolation of the organisms showed them to be, at least morphologically, not B. diphtheriae. As a help toward close

observation, a note was made of the different forms of bacteria seen in each smear. Wesbrook's types were used to designate diphtheriod bacilli, types E^2 and D^2 being regarded as Hoffman's bacillus.

An attempt was invariably made to isolate the organisms that we recognized as morphologically diphtheria. For this purpose Löffler's serum media plates were used. A loopful of an emulsion of the culture in bouillon was streaked on a series of three plates. After 20 hours incubation colonies were picked and smears examined. In the search, at least 30 colonies were examined, if necessary. This method of isolation was not satisfactory when the organisms sought for were few in number in the original smear; in most of such cases the plates failed to show diphtheria colonies. In one or two cases where the organisms were numerous in the original smears they were lost in the plates, because of the presence of a "spreader" in the culture. When isolated, the culture was always replated in order to insure purity, two cultures, each representing a separate colony, being preserved for further study.

The routine procedure for the preservation of cultures was to grow them on serum at 37° until good growth was obtained, one to three days being required, and to keep thereafter in cold room at 15° C., transfers to fresh media at first being made every two weeks. This time was soon shortened to one week after three cultures from carriers had been lost through failure to grow on transplanting. After three months of this treatment most of the cultures were transferred to plain agar, kept at room temperature and replanted monthly. A few still required serum and fortnightly transfer for preservation; all of the latter proved nonvirulent. The same technique was used for the isolation and preservation of diphtheroids, including Hoffman's bacillus.

All cultures were taken to the Hygienic Laboratory, Washington, D. C., for virulence and acid-production tests.

FREQUENCY OF CARRIERS.

During the course of this investigation a total of 4,098 individuals, all apparently healthy, were examined. Two of these very shortly after cultures were taken developed mild clinical symptoms of diphtheria (sore throat, slight temperature, and general malaise) and gave positive virulent cultures; while three others, with positive cultures, gave definite history of contact with cases very suspicious of, or positive, for diphtheria. The culture from one of these, a contact with one of the mild cases cited above, was virulent; that from the second was nonvirulent; the third we failed to isolate. Excluding these five we have for consideration 4,093 apparently healthy indi-

viduals without history of direct contact. These furnished us with a total of 8,758 cultures, as shown in Table 2:

TABLE 2.

Type of culture or cultures.	Number of individuals.	Total cultures of different types.		
		Nose.	Throat.	Combined.
Nose culture only	5	5	.....	.....
Throat culture only	320	.....	320	.....
Combined nose and throat only	1,422	.....	.....	1,422
A nose and a throat only	26	26	26	.....
A throat and a "combined" only	1	.....	1	1
A nose, a throat, and a combined only	2,319	2,319	2,319	2,319
Total	4,093	2,350	2,666	3,742

These cultures showed a total of 38 carriers or about 1 (0.928) per cent of the population examined. Since these cultures were taken from a large number of individuals indiscriminately selected from the population at large in various parts of the city, the results obtained will probably serve as a fair index of the proportion of carriers among the whole population. They indicate that persons harboring organisms in the throat or nose morphologically identical with B. diphtheriae constituted about 1 per cent of the population of Detroit, or in other words, there were in that city about five to six thousand diphtheria bacilli carriers.

These results are low as compared with those obtained by the majority of other workers in this field. In reviewing the literature results above 2 per cent are more often found than results below this figure. In 1902 the committee of the Association of Massachusetts Boards of Health (4) reported on the examination of 3,096 healthy persons in the East among whom were found 43 carriers, or 1.39 per cent, and of 1,154 persons in Minnesota among whom were found 80 carriers, or 6.93 per cent. In 1907 von Sholly (5) reported on the examination of 1,000 persons, most of them children. Throat cultures only were taken. Among these 56 positives were found, or 5.6 per cent. The bacilli were all isolated in pure culture and tested for virulence, 18, or 1.8 per cent, of the persons examined being found virulent. In 1913 Moss (6) reported on the examination of 1,217 school children in Baltimore, Md., and of 1,290 inmates of faculty members' homes and students' boarding houses of Johns Hopkins University Medical School. Throat cultures only were taken. Among the school children were found 44 carriers, or 3.61 per cent. The organisms were isolated from 33 and tested for virulence, 6 being found virulent. In the other group of 1,290 persons were found 45 carriers, or 3.48 per cent. The examination of these latter persons

seems to have been conducted at the time of an epidemic of diphtheria at the Johns Hopkins Medical School and Hospital, reported by Ford (7) in 1911.

Table 3, taken from Nuttall and Graham Smith (3), gives the findings of some other workers:

TABLE 3.—*Observations on prevalence of carriers.*

Observer.	Number nonexposed persons examined.	Persons harboring virulent bacillus diphtheriae.	Persons harboring nonvirulent bacillus diphtheriae
Parke & Beebe (1895)	274	2	23
Kober (1899)	588	0	3
Denny (1900)	235	0	1
Cobbett (1901)	43	0	0
Cobbett (unpublished)	90	0	0
Pugh (1902)	415	0	17
Graham Smith (1903)	362	0	1
Pennington (1907)	125	2	11
Total	2,132	[1] 4	[2] 56

[1] 0.18 per cent. [2] 2.62 per cent.

RELATIVE VALUE OF DIFFERENT TYPES OF CULTURE.

The question of how many and what cultures to take in searching for the bacillus diphtheriae for purposes of "release" from quarantine or in the search for carriers is one of considerable importance. The increase in expense and trouble in taking multiple cultures is a decided argument against this procedure, and one sufficient to justify the use of any single culture that in practice will give an approximation to the ideal. Having this consideration in mind we planned our work with a view of obtaining data that might be helpful in estimating the relative value of different types of culture. In Table 4 we give a statement of the number of the different types of cultures furnished by the 4,093 individuals studied and also the number of carriers disclosed by each method of culture. This shows at a glance the relative value of each.

TABLE 4.

Type of culture.	Number of cultures.	Total number of individuals furnishing cultures.	Number of carriers.	Per cent carriers.
Throat	2,666	2,666	14	0.525
Nose	2,350	2,350	16	.680
Combined nose and throat	3,742	3,742	26	.694
2 cultures, 1 from nose and 1 from throat	4,690	2,345	24	1.023
3 cultures, 1 from nose, 1 from throat, and 1 from both throat and nose	6,957	2,319	26	1.121

The low rate from the throat culture is of obvious import. This is the culture most commonly taken in board of health work, and it is evident that if it is used alone a considerable percentage of carriers will be missed. The slightly higher result obtained by the use of a single combined nose and throat culture and decidedly higher result obtained by employing two cultures, one from the throat and the other from the nose, suggests the greater value of these methods for detecting the presence of carriers. The combined result of the three simultaneous cultures, as might be expected, shows the greatest percentage of positives, but the lead over separate nose and throat cultures considered together is slight and is evidently due to the greater chance of striking the organisms sought for when the larger number of cultures is taken.

It would appear that in a search for carriers (or contacts) where the taking of a single culture only from each person is practicable the combined throat and nose culture should be used. Its use requires a comparatively slight increase of expense over single throat or single nose cultures, a double quantity of swabs being necessary. Where more than one culture from each individual is practicable, from the standpoint of cost and laboratory facilities, separate throat and nose cultures should be made. All extra cultures besides these two increase the chance of getting positive results where the bacilli sought for are few in number.

Data similar to the foregoing in regard to cultures from clinical cases were obtained by taking from each of a number of such cases one nose culture, one throat culture, and one "combined" culture at the same time. Positive results were obtained in one or more of the three cultures from each of 42 cases. Comparing the results from different cultures, we find that of the 42 throat cultures, 34, or 80.1 per cent, were positive; of the 42 nose cultures, 21, or 50 per cent, were positive; and of the 42 combined cultures, 32, or 76.2 per cent, were positive.

Further examination of the results shows that in 18 cases the throat culture was positive while the nose culture was negative, while in only 5 cases was the reverse true. In 3 cases only the "combined" culture was positive, while the throat culture and the nose culture were both negative. These results indicate that no single culture in clinical cases gives 100 per cent results but that, as might be anticipated, a throat culture comes nearer to this than any other single culture.

VIRULENCE TESTS.

Technique.—Virulence tests of cultures isolated were made as follows: The two strains of each culture were planted in Smith's sugar-free bouillon and transferred through five generations or more, until a good growth was obtained in 24 hours. Daily transfer was

made when there was good growth, but when the growth was poor transfers were made at 48 or even 72 hour intervals until organisms were sufficiently habituated to allow daily transplants. Ordinary culture tubes were used to contain the bouillon and were slanted in the incubator to allow more surface exposure of the media. When good growth was obtained 2 c. c. of a 72-hour culture were injected beneath the skin of the belly wall of a 250-gram guinea pig. If the guinea pig remained alive 10 days or more and no paralysis developed within four weeks, the organism was considered nonvirulent. All animals dying were autopsied, and if signs of diphtheria were not found the inoculation was repeated. None of our cultures, other than those proving virulent, caused the death of any animals on repeating the test, though in the first test the injection of two of what proved to be nonvirulent cultures was followed by death within six days without, however, causing any appreciable macroscopic lesions of diphtheria. None of our animals developed paralysis. When an animal died within 10 days with signs of diphtheria at autopsy, a set of three guinea pigs, 250 grams weight, were next inoculated with 1 c. c., 0.1 c. c., and 0.01 c. c., respectively, of a seven days' culture of the same organism. Under these conditions all of the cultures that we considered virulent killed within six days in 1 c. c. and 0.1 c. c. doses. None of them killed in 0.01 c. c. dose. A final test was made by inoculating one guinea pig with 0.2 c. c. of a seven days' culture, that is, twice the fatal dose, while a control received 2 c. c., 20 times the fatal dose plus 250 units of antitoxin. For a positive result it was required that the unprotected animal die within six days with signs of diphtheria, while protected animals survived. In all cases the amount of fluid injected was 2 to 2½ c. c., salt solution 0.9 per cent being used as a diluent.

Results.—We isolated the organism in pure culture from 19 of our 38 carrier cases, an even 50 per cent. All of these were tested and 2 found virulent. On this basis it may be assumed that 4 of our 38 carriers harbored virulent bacilli. This would give a ratio of 4 in 4,093 persons, or 0.097 per cent who were carriers of virulent bacilli. This result is somewhat lower than the rate compiled by Graham-Smith and shown in Table 2, namely, 0.18 per cent.

It is interesting to note that our two carriers with virulent culture were school girls, 7 and 11 years of age, respectively.

ACID PRODUCTION.

Technique.—All of the cultures isolated were tested for acid production. The organisms were planted on sugar-free bouillon to which had been added 1 per cent dextrose, instead of the 0.1 per cent used for toxin production. These cultures were allowed to grow for eight days, and then titrated against N/20 sodium hydrate. Nearly all of

the cultures grew well in this medium without education; a few had to be educated by transfer every other day through three or four generations.

The cultures were titrated according to the standard methods for titrating media. The cultures were always planted in a media with a reaction of +0.5. Two blank tubes of the same media were incubated with every set of cultures and titrated along with them in order to determine the increase of acidity due to evaporation, etc.

Results.—All cultures isolated from carriers were tested for acid production with results as shown in Tables 5 and 6.

TABLE 5.—*Showing results of test for acid production by 2 virulent cultures from carriers, 2 strains of each culture being tested.*

(Result after 8 days' incubation.)

Culture No.	Reaction.		Net increase acidity.	Culture No.	Reaction.		Net increase acidity.
	Culture.	Blank.			Culture.	Blank.	
4149	+5.2	+1.1	4.1	7646	+5.7	+1.1	4.6
4149a	+4.8	+1.1	3.7	7646a	+5.7	+1.1	4.6

TABLE 6.—*Showing results of acid production by 17 nonvirulent cultures morphologically diphtheria from carriers, 2 strains of each culture being tested.*

(Result after 8 days' incubation.)

Culture No.	Reaction.		Net increase acidity.	Culture No.	Reaction.		Net increase acidity.
	Culture.	Blank.			Culture.	Blank.	
1392	+6.5	+1.9	4.6	3473a	+6.4	+2.0	4.4
1392a	+6.1	+1.9	4.2	3992	+5.8	+1.6	4.2
1839	+5.5	+1.9	3.6	3992a	+5.8	+1.6	4.2
1839a	+5.9	+1.9	4.0	4013	+3.8	+1.6	2.2
2177	+4.0	+1.9	2.1	4013a	+3.6	+1.6	2.0
2177a	+5.0	+1.9	3.1	4080	+4.7	+1.1	3.6
2248	+3.5	+1.9	1.6	4080a	+4.7	+1.1	3.6
2248a	+3.2	+1.9	1.3	4208	+1.7	+1.1	.6
2650	+4.5	+1.5	3.0	4208a	+1.8	+1.1	.7
2650a	+4.5	+1.5	3.0	5398	+4.0	+1.1	2.9
2944	+6.4	+2.0	4.4	5398a	+2.6	+1.1	1.5
2944a	+6.8	+2.0	4.8	6507	+5.3	+1.1	4.2
2983	+6.2	+2.0	4.2	6507a	+5.7	+1.1	4.6
2983a	+5.8	+2.0	3.8	9009	+4.6	+1.1	3.5
3020	+3.5	+2.0	1.5	9009a	+4.9	+1.1	3.8
3020a	+3.7	+2.0	1.7	9337	+5.4	+1.1	4.3
3473	+6.2	+2.0	4.2	9337a	+5.6	+1.1	4.5

It will be seen that all are acid producers, only one nonvirulent culture (two strains) causing an increase of less than one.

AGES AND SEX OF CARRIERS.

It is of interest to note the wide range of ages among persons found with positive cultures. The youngest among the carriers found was 4 years of age, the oldest 72. Four carriers were 60 years old or over and 8 under 15, the others ranging between these ages.

Table 7 shows the distribution of carriers in the different age groups.

In regard to sex it is found that among the 1,600 males examined there were detected 12 carriers, or 0.75 per cent, and that among the 2,493 females examined there were found 26 carriers, or 1.09 per cent.

TABLE 7.

Age.	Number positives.	Number in group.	Per-centage positive.	Age.	Number positives.	Number in group.	Per-centage positive.
0–1 years	.....	158	.....	36–50 years	3	450	0.67
2–5 years	1	510	2	51–65 years	3	176	1.73
6–10 years	4	599	67	66–80 years	2	82	2.5
11–15 years	7	673	1.05	80 years	.....	3	.....
16–25 years	8	699	1.15	Unknown (all adult females)	4	110	3.77
26–35 years	6	633	.95				

SUPPLEMENTARY STUDIES.

VIRULENCE AND ACID TESTS ON B. DIPHTHERIÆ—DIPHTHEROID BACILLI AND HOFFMAN'S BACILLUS.

Virulence and acid production tests were made on 30 cultures morphologically diphtheria, isolated from clinical cases. We also tested 47 cultures morphologically Hoffman's bacillus and 12 cultures of short barred (D′ and E′ type) organisms that were isolated, some from clinical diphtheria and some from healthy individuals.

In no case was there found a virulent culture of Hoffman's bacillus or of the short barred forms. Among those morphologically B. diphtheriæ, all isolated from clinical cases, 29 were found virulent and 1 nonvirulent. The single nonvirulent organism was secured from the throat of a patient while in the active stage of the disease. This same patient became on convalescence a carrier, virulent B. diphtheriæ being isolated from a throat culture taken over a month after all clinical signs had disappeared. This would suggest that this individual harbored both nonvirulent and virulent organisms, the former being isolated at the first and the latter at the second test.

Among the organisms morphologically B. diphtheriæ, acid production ranged from an increase of 2.4 as the lowest figure to 4.7 as the highest. The single nonvirulent culture caused an increase of 4.3.

Among the barred bacilli, acid production ranged from 2.4 increase to 3.8 increase, with one exceptional 5.5 increase.

Among the cultures morphologically Hoffman's bacillus the range was from a decrease of 1.9 to an increase of 1.6. Among the 47 cultures, however, only 6 showed an increase, all of the others causing a decrease in acidity.

PREVALENCE OF HOFFMAN'S BACILLUS.

The method of recording our findings in the smears examined for carriers has given us some interesting data with respect to the frequency of occurrence of Hoffman's bacillus. In the 4,093 individuals examined we found 1,327, or 32.4 per cent, with this bacillus. In a special group of 2,319 of these individuals examined by the "three cultures" method we found it in 971, or 41.9 per cent. The latter finding we believe to be the more nearly representative of actual conditions. Throat cultures from 2,666 individuals showed its presence in 113, or 4.2 per cent, whereas nose cultures from 2,350 individuals showed it in 790, or 33.7 per cent. These findings, in harmony with those of previous workers, point very clearly to the nose as the normal habitat of this organism.

SUMMARY.

1. Cultures from 4,093 healthy people in the city of Detroit were examined for diphtheria bacilli.

2. Thirty-eight (0.928 per cent) were found to harbor B. diphtheriæ (morphological).

3. Of 19 cultures isolated from 19 of the carriers, 2 were found to be virulent, suggesting a possible 0.097 per cent of the individuals examined as carriers of virulent bacilli.

4. Comparison of the value of different types of single cultures for the detection of carriers shows that the throat culture gives the lowest and the combined nose and throat the highest results. The latter, however, is but slightly higher than that from the nose alone.

5. The combined results of a throat and of a nose culture give markedly better findings than those of any single culture.

6. The combined results of a throat, a nose, and of a combined nose and throat gave us the highest findings, but this does not appear to be greatly superior to the two-culture finding.

7. All carrier cultures islolated, 19 in all, were found to be acid producers.

8. Twenty-nine of 30 cultures of B. diphtheriæ from clinical cases were virulent and all 30 were acid producers.

9. Of 47 cultures morphologically Hoffman's bacillus, 6 showed some acid production; 41 were alkali producers. All 47 cultures were avirulent.

10. Hoffman's bacillus was present in at least 41.9 per cent of 2,319 individuals.

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III.—THE EXCRETION OF THYMOL IN THE URINE.[1]

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It has been shown in a previous paper[2] from this laboratory that only insignificant amounts of ingested thymol are excreted in the feces. This would indicate that thymol is almost completely absorbed from the alimentary tract and must therefore be oxidized in the body or excreted in the urine. That the latter process undoubtedly takes place to some extent, at least, was shown a number of years ago by Blum,[3] who isolated thymol glycuronate in the form of the dichlor compound from the urine of patients who had received thymol. Although the quantitative recovery of thymol has apparently not been attempted, experiments with phenol have been made. Thus Tauber[4] showed that of various amounts of phenol administered to a dog, less than 50 per cent was recovered from the urine and only traces from the feces. Similar results were also obtained by Schaffer[6] and the additional fact established that no increase in excreted oxalic acid occurs after phenol. An experiment made upon himself by de Jonge[5] showed that, of a total of 0.100 gm. phenol taken in three doses during some seven days, approximately 78 per cent was recovered in the urine.

The method used by Blum for the isolation of the thymol glycuronate is not susceptible of quantitative application, and no experiments have so far been made which show what proportion of ingested thymol is excreted in the urine. The results of Tauber and of Schaffer indicate that only 31 to 62 per cent of phenol is recovered according to the amount given; the higher percentages being in the cases af the larger doses. It was believed that similar data concerning the fate of thymol in the body would be of value in showing the mechanism of the action of thymol upon hook worms and consequently in indicating the path to be followed in developing drugs of greater potency and safety than thymol.

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[1] Manuscript submitted for publication Jan. 16, 1915.

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[6] de Jonge. Ztschr. f. physiol. Chem., *3*, 181 (1897).

The method which was developed for the determination of thymol in urine was similar to that used in the work upon feces, and consisted in steam distillation from strongly acid solution, redistillation of the nearly neutral distillate and titration of the second distillate by the bromine-hydrobromic acid method.[1] The apparatus was composed of two round-bottom distillation flasks of about 300 c. c. capacity each, arranged in series and tilted back in such a manner that mechanical carrying over of the liquid from one to the next flask was prevented to the greatest possible extent. A steam generator was provided in front of the first flask and an efficient condenser followed the second.

The preliminary experiments upon normal urine were designed to show the most successful means of preventing substances which might interfere with the titration, from coming over with the thymol. This was necessary since the method for the titration of the thymol requires that the solution be neutral, and contain no other compounds than thymol, which liberate hydrobromic acid upon bromination. The earlier experiments indicated that a previous treatment of the sample with aqueous copper sulphate and dilute sodium hydroxide solutions, followed by removal of the precipitate by centrifugation and decantation, was efficient in eliminating a considerable portion of the interfering substances. Such a treatment was, however, not found necessary in cases where the urine contained large amounts of thymol and a relatively small volume of it could therefore be used for a determination. Since the hydrolysis of the thymol glycuronate, which is the form of combination in which the thymol occurs in the urine, can only be effected by heating with strong mineral acids, provision must naturally be made for retaining the volatile acids, which distill over from the strongly acidified solution, and would therefore interfere with the titration of the thymol. The means by which this was finally accomplished consisted in adding solid magnesium oxide to rather strong copper sulphate solution contained in the second flask of the apparatus, thus yielding a suspension of cupric hydroxide and magnesium oxide in aqueous copper sulphate solution. By this arrangement, the excess of strong acid added to the urine sample in the first flask retains the volatile ammonium compounds, and the faintly alkaline medium in the second flask retains a portion, at least, of the volatile acids which are set free from the urine sample. The copper sulphate solution also performs the further function of retaining hydrogen sulphide and similar compounds occasionally yielded by certain samples of urine. It was found that a steam distillation of the urine samples in the double flask apparatus as above described yielded in practically all cases a distillate neutral to litmus paper. In order, however, to be more certain of obtaining a distillate as free as

[1] Seidell. Am. Chem. J., 47, 508–26 (1912).

possible from substances which might interfere with the titration of the thymol, the procedure was extended to include the redistillation of the neutral distillate. This was distilled from flask 1 of the apparatus, and fresh quantities of the reagents, copper sulphate and magnesium oxide, placed in flask 2. By this double distillation method it was found that blank determinations upon normal dog urine required per 100 c. c. of urine, from 1.0 to 3.0 c. c. of standard 0.05 normal sodium thiosulphate for volatile constituents which accompany the thymol. These quantities of thiosulphate correspond, respectively, to 0.0037 and 0.0113 gram thymol.

One other point which arose was as to the furfural which, according to the Tollens method for the estimation of pentoses, pentosans, and glycuronic acid, would be set free from urine containing thymol glycuronate, during the acid distillation. Tests of the distillates with aniline acetate were therefore made in many instances, but negative results for furfural were obtained in all cases. In one experiment, however, upon a sample of urine containing a large amount of thymol glycuronate and using hydrochloric acid according to the details prescribed in the Tollens method, positive results for furfural were obtained, even up to over 200 c. c. of distillate. It was concluded, therefore, that under the conditions of the distillation as selected for the thymol determination, negligible amounts of furfural accompany the thymol.

Of the several details of the method which required refinement, that of the amount of acid to use for liberation of the thymol from its combination with glycuronic acid was given early attention. A sample of urine amounting to several hundred cubic centimeters was obtained from a dog to which a rather large dose of thymol had been given. Aliquot portions of this were distilled with the addition of different amounts of concentrated sulphuric acid and diluted with water in each case to a volume of about 200 c. c. The following results were obtained:

Aqueous mixture in distilling flask No. 1.			Thymol found.
Urine.	Concentrated H_2SO_4.	H_2O.	
c. c.	*c. c.*	*c. c.*	*Gram.*
100.....	10	90	0.099
200.....	30	.....	.220÷2=0.11
100.....	20	80	.125
100.....	30	70	.135
100.....	40	60	.119

Similar determinations were also made on two other samples of urine, and results in agreement with the above were obtained. From these it was concluded that a satisfactory concentration of sulphuric

acid to use is 10 per cent by volume, that is, 20 c. c. of concentrated sulphuric acid added to 80 c. c. of water and this mixed with 100 c. c. of urine.

The mixture in the second flask consists of about 5 grams of copper sulphate dissolved in about 100 c. c. of water to which is added about 1 to 2 grams of magnesium oxide.

During a distillation, the acidified urine sample in the first flask gradually becomes darker as the heating progresses. The thymol begins to distill over as soon as the boiling point of the contents of the flask is reached. The approximate quantity of thymol is indicated by the amount of beading noted in the condenser and the opalescence of, or floating globules on, the surface of the distillate. Practically all the thymol comes over with the first 100 c. c. of distillate; but, to be certain that none remains behind, a second 150 c. c. portion of distillate is collected in a separate receiver. Glass-stoppered bottles of about 300 c. c. capacity are convenient receptacles for the distillates since the titration of the thymol can be made directly in them.

The titration of the distillate is made as follows: About 1–2 c. c. of carbon tetrachloride are added to the bottle and bromine vapor poured in intermittently with active shaking, until a permanent marked yellow color of the carbon tetrachloride layer is obtained. The bottle is then allowed to stand in a dark place for about one-half an hour. Approximately 6 c. c. of carbon bisulphide and 5 c. c. of 5 per cent potassium iodide are added and the liberated iodine titrated with standard, approximately 0.05 normal thiosulphate solution. Five c. c. of 2 per cent potassium iodate solution are then added and the liberated iodine corresponding to the free hydrobromic acid, is titrated with the same standard thiosulphate. The difference between the first and second readings of the thiosulphate solution, corresponds to the hydrobromic acid formed in the bromination of the thymol. The calculation is made on the basis of 2 molecules of HBr per 1 of thymol; 1 c. c. 0.05 normal thiosulphate is, therefore, equal to 0.003753 gram thymol.

The experiments on dogs were made as follows: A cage provided with a false bottom of woven wire above a metal covered bottom, arranged to convey the urine to a receptacle, was used. The dog was usually kept in the cage for a day previous to administration of the thymol in order to obtain a sample of normal urine. The thymol was administered in a gelatin capsule which was always placed inside of a larger capsule to guard against possible loss while inducing the dog to swallow it. After the administration of the thymol, the urine receptacle was examined from time to time and whenever a sample was present it was removed and a fresh bottle placed under

the cage. The exact time at which each sample of urine was voided was not known, but only the time of the visits at which the samples were found. The following table contains a summary of an experiment made with dog A:

Date.	Amount thymol administered.	Urine samples.		Thymol found.	Per cent thymol recovered.
		Time after thymol.	Volume of sample.		
1914.	*Gram.*	*Hours.*	c. c.	*Gram.*	
July 22–23	0.50	6–23	155	0.18	44
		23–24	152	.04	
July 26–30	.61	3½–5½	145	.16	44
		5½–23	420	.11	
		29½–30½	48	.01	
		30½–48	300	.01	
July 30–31	.77	6–22½	570	.38	50
		26½–29½	250	.02	

From this experiment it is apparent that less than one-half of the thymol absorbed from the alimentary tract is excreted in the urine. That which is excreted, comes through fairly promptly; probably within the first few hours after its administration.

Three mongrel dogs were used for the experiments. Dog A was a female bull-fox terrier of 23½ pounds weight. Dog C was a cur, of approximately 20 pounds weight. This dog had been used previously for hookworm experiments and was several times infected with worms and treated with thymol and other hookworm remedies. Dog D was a male Scotch terrier of 18½ pounds weight and had not been previously kept in a urine collecting cage. He therefore showed considerable restlessness at first accompanied by retention of urine, and it will be noted that this condition apparently affected the results obtained in the first experiment made with him, October 8–12.

Although previous work had shown that only inconsiderable amounts of thymol come through with the feces, several determinations were made in the present series of experiments upon samples of feces collected simultaneously with the urine samples. The results confirmed the previous conclusion as to the practical absence of thymol from the feces.

Many experiments were made, to ascertain whether the method as devised was giving the total amount of thymol present in the sample. As already shown, more acid for the hydrolysis was not advantageous. It was also found that increase of the amount of distillate did not augment the yield. Blank determinations made with mixtures of normal urine and thymol showed that no appreciable loss due to reagents or manipulation was experienced. Blank distillations of normal urine did show however, as already mentioned, that small varying amounts of substances which react in the titration just as

thymol, were obtained. The corrections due to these were of course found to be roughly proportional to the volume of the sample of urine. It was therefore advisable, in making determinations upon urines containing thymol, to use as small an aliquot as would yield enough thymol for accurate titration. In this connection, however, it should be noted that, during the first acid distillation and sometimes even in the second neutral distillation, a darkening occurred which probably indicated a destruction of some of the thymol. This would therefore constitute a compensating negative error, balancing to a certain extent the plus error, due to accompanying substances which react as thymol during the titration. Taking these points into consideration, and the fact that very great accuracy is not required, it did not appear expedient to attempt to determine correction factors to apply to the results of the titrations. The uncorrected results, as reported in the tables, are believed to show certainly the maximum amounts of thymol which are excreted in the urine in combination with glycuronic acid.

Among the several other points which were investigated during the development of the method, was the stability of the thymol glycuronate. A sample of urine, which was found by analysis to contain a rather large amount of thymol, was subjected to steam distillation without the addition of the acid required for hydrolysis. No thymol was found in the distillate. On adding the necessary amount of acid and again distilling, the same amount of thymol was found as in an original direct determination. A number of experiments upon samples of urine containing thymol glycuronate, and kept in the laboratory for rather long periods, showed that no appreciable reduction in the amount of thymol obtainable from them occurred. It was therefore apparent that no necessity existed for the very prompt determination of thymol in samples of urine.

In the case of the experiments upon human patients, recorded in the accompanying table, the samples were collected by Acting Asst. Surg. William S. Keister at the Wilmington (N. C.) Hospital of the Public Health Service. Two patients, B. B. and T. C. B., boys, 13 and 9 years old, respectively, were each treated for hookworms by being given the usual 5 grain capsule of thymol at 7, 8, and 9 a. m. The urine passed between 7 a. m. and 12 noon constituted the first sample, that between noon and 6 p. m. the second, and that passed between 6 p. m. and 6 a. m. the third. These were forwarded to the Hygienic Laboratory and the thymol determinations made as described above for the samples of dog urine.

TABLE I.—*Showing the thymol content of urine from dogs and human patients.*

DOG URINE.

Dog.	Date.	Dose administered.		Urine samples.		Thymol found.	Per cent thymol recovered.
		Thymol.	Olive oil.	Time after thymol.	Volume of sample.		
	1914.	*Gram.*	*Gram.*	*Hours.*	*c. c.*	*Gram.*	
C	Aug. 25–27	0.67	9.37	0–19½	85	0.23	46
				19½–22	95	.07	
				42–44½	116	.02	
A	Aug. 31–Sept 1	.60		2¾–3¾	65	.085	41
				5¼–22	480	.16	
				22–29	83	Trace.	
C	Sept. 9–11	1.046		0–1	74	0.01	38.5
				7–24	71	.346	
				30–48	109	.045	
A	Sept. 15–17	.338		1–18	75	.126	46
				24–42	74	.030	
C	Sept. 21–23	.303		2–19	81	.105	41
				25½–44½	117	.019	
A	Sept. 23–26	1.277		2–19	127	.376	36
				25–46	192	.073	
				46–70	88	.011	
C	Sept. 29–Oct 1	1.856		3½–21	211	.51	31
				28–49½	149	.07	
				49½–52	50	.01	
D	Oct. 8–12	.81		26½–44	136	.104	29
				44–46	92	.061	
				51–69	292	.072	
D	Oct. 27–29	1.317		0–3	140	.077	35
				6–23	125	.335	
				23–25½	60	.031	
				30–48½	85	.022	
D	Oct. 29–31	1.238	14.24	5½–23	218	.424	36
				29–47	81	.030	

HUMAN URINE.

Patient.	Date.	Dose administered.		Urine samples.		Thymol found.	Per cent thymol recovered.
		Thymol.	Time.	Time after thymol.	Volume of sample.		
	1914.	*Gram.*	*a. m.*	*Hours.*	*c. c.*	*Gram.*	
B. B	Oct. 13–14	0.32	7	0–5	280	0.244	35
		.32	8	5–11	85	.046	
		.32	9	11–23	395	.046	
T. C. B	do	.32	7	0–5	175	.264	33
		.32	8	5–11	30	.018	
		.32	9	11–23	490	.039	

The analytical results are recorded in Table I in the order in which the experiments were made. Two points were particularly in mind in selecting the dose of thymol to be given; first to ascertain the effect of varying amounts of thymol upon the percentage of it excreted in the urine, and, second, to ascertain what effect, if any, the simultaneous administration of an oil would have upon the excretion of the thymol.

It is seen that, although the results agree in general with those of Tauber upon phenol, they show a higher percentage of recovery

from the smaller than from larger doses. Thus in the case of doses of 0.3 to 0.7 gram thymol, the amount recovered varied from 41 to 46 per cent; while with doses of 1.2 to 1.8 grams, only 31 to 36 per cent was recovered.

In regard to the effect of the simultaneous administration of oil, the two experiments which were made show that very slight, if any, effect was produced.

One other point, which was shown by the experiments, is that no significant difference in the ratio of recovered thymol is noted between initial and subsequent doses. Of the three dogs one, C, had been previously used for many experiments and had received large doses of thymol; one, A, had been given three doses of thymol in a preliminary experiment; and one, D, had not previously received thymol. In spite of this, with one exception which was probably due to another cause (Dog D, Oct. 8–12), the apparent fate of thymol in the three dogs is approximately the same. In regard to the two human patients, it is seen that both with a dose of thymol proportionally equivalent to the smallest given to the dogs, excreted approximately the same percentage of thymol that the dogs excreted after a proportionally much larger dose.

As to what becomes of the thymol that is not excreted in the urine as the glycuronate, nothing positive can be said at present. Surmises as to its fate are of little value; nevertheless, one point should be mentioned and that is, the possibility of its temporary fixation by the tissues, or elimination by volatilization with the expired air. According to a review of the literature by C. Neuberg,[1] the experiments of several investigators have failed to show that the glycuronic pairing occurs in the liver. It is therefore probable that thymol is present in the blood stream passing through the lungs, in which event a certain amount would no doubt be lost by volatilization even in spite of the slight alkalinity of the blood. An effort, however, to detect thymol by its odor in the expired breath of a dog after giving a large dose was unsuccessful.

In this connection, it is generally known that, in fatal cases of phenol poisoning, death eventually occurs from asphyxia. Autopsies made by Langerhans[2] showed that marked bronchitis and laryngitis had been produced, not by direct aspiration of the phenol, but by indirect action after absorption from the intestinal tract. It has been observed in fatal thymol poisoning that marked congestion and even consolidation of the lungs has occurred (Cushny).

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[1] Ergebn. d. Physiol. (3) *1*, 447 (1904).

[2] Deutsche med. Wchnschr., *19*, 269, 1256 (2893).

Although a method for the accurate determination of phenol in the urine has been developed[1] and many determinations made by various investigators upon normal and pathological urines of both man and animals, a careful search of the literature has failed to show that any experiments other than the old ones referred to above have been made with the urine of animals to which known amounts of phenol have been administered. The observation of Tauber and Schaffer, that about one-half of the phenol can not be recovered from either the feces or urine, appears not to have attracted particular attention.

SUMMARY.

A method for the determination of thymol in urine has been developed. Applying this method to samples of urine obtained from dogs to which thymol was given, it was found that 41 to 46 per cent was recovered when doses of 0.3 to 0.7 gram were given and 31 to 36 per cent when doses of 1.2 to 1.8 grams were given. The simultaneous administration of olive oil apparently caused very slight if any effect upon the percentage of excreted thymol. It is a question, therefore, whether oils really increase the amount of absorption of thymol or only the rate. Determinations upon the urines of two human patients who received the thymol treatment for hookworms gave results agreeing in general with those obtained upon samples of urine from dogs. These results, in connection with previous experiments upon the determination of thymol in feces, show that of the thymol administered, from one-half to two-thirds is apparently destroyed or temporarily fixed in the body. These observations are in accord with the work done by Tauber and Schaffer many years ago upon phenol. As yet no satisfactory explanation of this apparent disappearance of administered phenols has been found.

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[1] Neuberg, C., Der Harn, 1911, 1. Teil, p. 476.

IV. THE STERILIZATION OF DENTAL INSTRUMENTS.[1]

By H. E. Hasseltine, *Passed Assistant Surgeon, United States Public Health Service.*

The possibility of the transmission of disease from one person to another through the medium of instruments and appliances used in dental operations has long been recognized. Reports of such occurrences are frequently heard from the laity, but authentic reports in scientific publications are practically never published. Few men will deny that such cases do occur occasionally; their frequency, however, is not determinable.

Anyone familiar with asepsis has probably noticed errors in the surgical technique of a dentist while receiving dental treatment. Among these errors may be mentioned the placing of sterilized instruments on a swinging tray or glass plate which has not been sterilized, the cleaning of burrs on a scratch wheel which has not been sterilized since the burrs used on the previous patient have been cleaned thereon, and the frequent handling of the cable of the dental engine, which receives contamination from one patient through the operator's hands and in turn returns a portion of this contamination to the operator's hands when used on subsequent patients. These are some of the errors of technique seen personally, and are mentioned here to emphasize the necessity of having everything which comes in contact with the instruments or hands of the operator free from organisms obtained from previous patients in order to prevent transmission of infection from one patient to another.

A sterile towel over the tray, a scratch wheel which can be removed and sterilized with the instruments, and a simple sterile linen covering to put over the cord of the engine would make the operation many times more acceptable to the critic, provided, of course, a fresh towel and covering were used for each patient and the scratch wheel sterilized when each patient vacates the chair.

For improvement of the technique of the future dentists we should look to the dental schools. They all have courses in bacteriology, which, however, in many schools are largely theoretical instead of practical. The morphology and cultural characteristics of various organisms are considered, but their resistance to disinfectants is passed over by noting what certain books say regarding this phase

[1] Manuscript submitted for publication Apr. 16, 1915.

of the subject. Little actual laboratory work is done to show the relative value of the sterilizing agents applicable in dentistry and how their efficiency is tested. As a result the student forms the opinion that any sterilizing agent is effective, and does not learn to check his sterilization by bacteriological tests. For the coming dentists the improvement should come through the schools. For those already in practice it must come through reading and exchange of ideas among the men of the dental and medical professions.

Realizing the necessity of improvement, some members of the dental profession are striving to bring about better sanitary conditions in the offices of dentists at large; also among their patients, by teaching them oral hygiene. This work is most commendable, and to this end the officers of some dental societies have asked the Surgeon General of the Public Health Service to work out a detailed method for sterilizing dental instruments and appliances, keeping in mind the important factors, simplicity, efficiency, and duration of the process of sterilization. As a result of this request the writer was designated by the Director of the Hygienic Laboratory to consider the matter, make experiments, collect data, and make a report thereon.

The progress of the work has been interrupted frequently by exigencies of the service and the report much delayed as a result.

THE STERILIZATION OF DENTAL INSTRUMENTS.

In considering this subject the writer has endeavored to keep within practical bounds rather than to pose as an idealist.

In testing the efficiency of various methods of sterilization of dental instruments, the consideration of spore-forming organisms has been disregarded. A high degree of heat for a prolonged period is required to kill the spores of spore-bearing pathogenic organisms. Fortunately, the number of such organisms is comparatively few. The vegetating forms of these organisms are killed as easily as are the nonspore bearers. The spore represents a defensive element of the organism against unfavorable influences; that is, though capable of becoming alive, it is apparently in a lifeless phase. Since it remains in the spore stage while in conditions unfavorable for its growth, the spore is easily moved from place to place by mechanical means, thereby increasing the chance of finding a more suitable environment.

By reason of this fact mechanical means of removal of spores from instruments is relied upon. Mechanical cleansing of instruments by a brush or sponge, preferably in running water, will reduce the number of spores so that the number remaining will be too small to consider. For experimental data on the efficiency of mechanical cleansing in the removal of spores, the reader is referred to Francis (1).

Such organisms as the pus-producing cocci, the bacillus of typhoid fever, the bacillus of diphtheria, the bacillus of tuberculosis, the *Treponema pallidum* (syphilis), the bacillus of influenza, the amœbæ, and the thrush fungus, are known organisms that should be considered. To this list may be added the virus of diseases of unknown or uncertain origin, such as that of measles, scarlet fever, mumps, infantile paralysis, and smallpox, all of which seem to be caused by nonspore-bearing organisms.

Of the nonspore-bearing organisms, the staphylococci are reported to be the most resistant to heat, our most efficient disinfecting agent. On this account various strains of staphylococci were used as the test organisms in the experiments made in connection with this investigation.

TABLE I.—*Showing thermal death point of various organisms according to different authorities.*

Authority.	Influenza.	Staphylococcus aureus.	Streptococcus.	Pneumococcus.
Sternberg (2)	60°, 5 minutes	Moist, 58°, 10 minutes. Dry, 100°	54°, 10 minutes	52°, 10 minutes.
McFarland (3)		62°, 10 minutes 80°, 1½ minutes		
Muir and Ritchie (4)		80°, 30 minutes		
Park and Williams (5)	60°, 5 minutes	80°, 5 minutes	54°, 10 minutes	
Hiss and Zinsser (6)	60°, few minutes	58°, 10 minutes Dry, 90 to 100°	54°, 10 minutes	52°, 10 minutes.
Jordan (7)		80°, 30 minutes		
Abbott (8)		62°, 10 minutes	54°, 10 minutes	52°, 10 minutes.
Rosenau (9) (10)	60°, 10 minutes		54°, 10 minutes	52°, 10 minutes.

Authority.	Meningococcus.	Typhoid.	Diphtheria.	Tubercle bacillus.
Sternberg (2)		56°, 10 minutes	58°, 10 minutes	70°, 10 minutes.
McFarland (3)		60°, 10 minutes	58°, 10 minutes	60°, 20 minutes.
Muir and Ritchie (4)		60° 30 minutes	60°, 10 minutes	70°, 1 hour.
Park and Williams (5)	Readily	60°, 1 minute	60°, 10 minutes	80°, 1 minute.
Hiss and Zinsser (6)	Extremely sensitive to heat.	56°, 10 minutes	58°, 10 minutes	60°, 20 minutes. 80°, 5 minutes.
Jordan (7)			55°, 45 minutes	60°, 20 minutes.
Abbott (8)		60°, 10 minutes	58°, 10 minutes	80°, 5 minutes. 65°, 15 minutes.
Rosenau (9) (10)		60°, 20 minutes	Less than typhoid.	60°, 20 minutes.

Table I shows the thermal death point of various organisms, according to different authorities on bacteriology. It was found that the stock laboratory cultures of staphylococci were much less resistant than strains of the same organism obtained from human beings. For this purpose smears from cases of suspected diphtheria or from cases of tonsillitis were used, thereby getting organisms similar to those encountered in the mouth. Mixed cultures were used to simulate the conditions found in nature. In a few cases, *Bacillus subtilis*, a nonpathogenic spore-forming organism was encountered.

The resistance of the staphylococci was also tested, when dried on instruments prior to sterilization and when not dried. The resistance

was always greater when the contaminated instruments were dry. Consequently the dried infectious material was used in most of the tests.

The physical processes involved in sterilization have been the subject of much research and discussion. It is not necessary to consider here to any extent the theories. They can be stated briefly as the coagulation and hydrolytic theories. The first strives to show that the cell substance of the organism is coagulated by the disinfecting agent, just as the white of an egg is coagulated by heat when cooked. The second theory claims that the death of the organism is brought about by the introduction of water into the cell substance, the consequent hydrolysis being accelerated by the disinfecting agent.

The process of sterilization has been described by Phelps (11) as an increase of the death rate of organisms. But in determining the result of this process there are three factors to be considered, viz: (*a*) The velocity of the death rate of the organisms; (*b*) the time through which (*a*) is allowed to proceed; (*c*) the number of organisms present. The product of these three factors gives the result. Sterilization is, therefore, a relative result from a mathematical viewpoint and is usually arbitrarily recorded as perfect when the number of bacteria in the volume tested is less than one. On this death rate of organisms, the number present, and the time allowed, depend the efficiency of a sterilizing agent.

However, the exact mode of killing the organisms does not interest the practicing dentist and surgeon so much as does the assurance that all will be killed, in some manner, under certain conditions.

The methods of sterilization employed at present in most dental offices may be classified as follows:

Thermal	Boiling in water.	Chemical	Carbolic acid, followed by alcohol.
	Use of boiling water.		Other coal-tar products, followed by alcohol.
	Passing an instrument through a free flame.		Formaldehyde vapor in a tight chamber.
	Moist heat in a closed or open chamber.		Other chemicals, such as gasoline, solution of biniodide of mercury.
	Dry heat in a closed chamber.		

The efficiency of most of these methods was tested, and the advantages and disadvantages of each considered.

BOILING IN WATER.

Results of experiments.—Most authorities state that nonspore-bearing organisms are killed by boiling for 10 minutes. A few have reported organisms of this class that have resisted boiling for 30 to 60 minutes, but such are of too infrequent occurrence to require consid-

eration. Of the various strains of staphylococci in mixed culture, which I have tested, I have found none that survived 3 minutes' boiling. This means exposure to water heated until noticeable ebullition takes place before the organisms are placed therein, the time being taken by the watch. Undried instruments were sterilized in 2 minutes. Sterility tests were made by dropping the sterilized instruments into tubes of nutrient bouillon and incubating these for 48 hours at 37°. Though my experiments show sterilization in a shorter time, a period of 10 minutes' boiling is recommended, to provide an ample margin on the side of safety. A small amount of alkali, such as sodium carbonate, or sodium hydroxide, should be added to the water to prevent rusting of instruments.

The advantages of this method are: (*a*) It is the most rapid and most efficient; (*b*) it is always available where fire and water can be obtained; (*c*) the procedure is simple and can be carried out by any one; (*d*) the expense of sterilization by this method is small.

The disadvantages are: (*a*) Dulling of the sharp edge of cutting instruments, such as knives, lancets, etc.; (*b*) increase of effect of action of any chemical impurity in the water upon the instruments, a factor that will vary according to local conditions.

USE OF BOILING WATER.

Results of experiments.—If used immediately after the heat is cut off, and in quantity sufficient to guard against a rapid cooling, this method is nearly the equivalent of boiling, so far as practical results are concerned. The use of 1 gallon of water, boiled until the moment before the instruments are submerged in it, sterilizes as rapidly as does boiling, at least during the first five minutes of exposure. If care be taken to carry out this procedure fully, the method is efficient, the advantages and the disadvantages being the same as with boiling, save that the rapid cooling of the water will necessitate reheating to the boiling point to insure proper results.

The use of water of too low temperature or for too short a period of time makes possible incomplete sterilization. As the temperature is lowered the period of exposure must be lengthened; but, as the loss of heat is uncertain, the required length of exposure will necessarily be uncertain. On account of the uncertainty of the temperature of the water, except it be boiled immediately prior to use on each occasion, this method is not as good as boiling or the use of a *known* temperature at a degree lower than the boiling point.

PASSING AN INSTRUMENT THROUGH A FREE FLAME.

This method is effective, since, if properly carried out, it virtually amounts to incineration of the organisms. The degree of heat to which the instrument is subjected is much higher than the boiling

point, and on this account only a few instruments can be sterilized by this method without interfering with cutting qualities, temper of steel, or plating of instruments. It is applicable to certain small instruments whose cost is not high, but is not available for general use.

MOIST HEAT IN AN OPEN OR CLOSED CHAMBER.

This is applied by the use of steam in an open chamber (Arnold) or in a tight chamber under pressure (autoclave). Its use for instruments is not very extensive, as the same results can be obtained by direct boiling and some time saved, since boiling a small amount of water requires much less time than heating up a large apparatus. It is of greatest value for sterilizing linens, gowns, towels, etc., and the steam under pressure is preferable, on account of its greater penetration.

DRY HEAT IN A CLOSED CHAMBER.

This method is not used for instruments, on account of the high temperature required and the long time required to carry it out. It can be used for glassware or for sterilizing linen, if the temperature be not too high.

USE OF CARBOLIC ACID FOLLOWED BY ALCOHOL.

This method consists of submersion of the instruments in phenol of varying strength. The solubility of phenol in water is about 1 part in 20, giving a 5 per cent solution. This solution is more effective than the pure phenol. After submitting the instruments to this treatment they are immersed in alcohol, to remove the excess of phenol, and may then be rinsed in water, or used without rinsing.

Results of experiments.—With many strains, 10 minutes' exposure to 5 per cent phenol, followed by one minute in alcohol, sufficed to sterilize. Some strains of staphylococci, however, were more resistant and uniformly gave growth after 15 minutes; in a portion of the tubes even after 30 and 45 minutes' exposure. No strain used survived one hour in 5 per cent phenol. The same resistant strain was uniformly killed by boiling for three minutes. It was also killed by three minutes' exposure in an 80° water bath.

Its advantages are: (a) The cost is moderate; (b) it can be applied to practically every instrument and appliance without damage resulting therefrom.

Among its disadvantages are: (a) It requires a longer period of time to sterilize; (b) it requires more care in the application of the method (care must be taken to see that all surfaces of instruments are exposed to the solution); (c) instruments must be removed from phenol by forceps, as the solution has a bad effect on the skin if the

hands are used in it frequently; (d) it requires a much better trained assistant, or the attention of the dentist himself, to use this method properly; (e) the odor of phenol is, to some, objectionable.

The use of other coal-tar products resembles that of phenol in application and efficiency. The advantages and disadvantages mentioned under phenol apply also to these products, except as follows: (a) Some have a higher coefficient than phenol; that is, they are more germicidal than phenol; (b) some have a soapy composition, giving them the cleansing property of soap when mixed with water; (c) their cost, however, is in most cases considerably greater.

FORMALDEHYDE GAS IN AN AIR-TIGHT CHAMBER.

This method has, of late years, been very popular among dentists and is efficient, provided a sufficient length of time is allowed for action.

Results of experiments.—Exposure of contaminated instruments to formaldehyde vapor in an air-tight glass jar for periods varying from 10 minutes to 1 hour gave growth more or less constantly. Some strains of staphylococci were always killed by 30 minutes' exposure; the more resistant strains survived for 60 minutes. An exposure of one and one-half to two hours killed the most resistant strain used. These results were obtained with an excess of formalin in a small glass jar closed by ground-glass joints sealed with vaselin, so the air was filled to saturation with the formaldehyde vapor.

The advantage of this method lies solely in its simplicity and ease of application.

Its disadvantages are: (a) The time required to sterilize is too long for a busy dentist; (b) formaldehyde causes quite a marked rusting of unplated steel instruments; (c) inefficiency is likely to result from the lessening of the density of formaldehyde vapor, unless special care be taken to replenish the supply of formalin solution at frequent intervals; (d) inability to remove a single instrument from the air-tight chamber without interfering decidedly with the process of sterilization, because of the lessening of the density of the formaldehyde vapor in the chamber; (e) the disagreeable odor and irritating effect on mucous membranes will be experienced more or less by both operator and patient.

OTHER CHEMICAL METHODS.

Disks containing mercuric iodide with some alkaline salt are theoretically practicable. The alkalinity is supposed to guard against the corrosive action of the mercury on the metal instruments. However, this brings a rather delicately balanced chemical reaction into use, and unless the application of the method be carried out by the

dentist himself, or an assistant who has some knowledge of chemistry, bad results may be obtained. For instance, the chemical impurities of the water used may vary from day to day, and thus disturb the proper degree of alkalinity. No experiments were made with these disks, as it is not conceivable that their use is an improvement on some of the simpler and less technical methods.

The action of various chemical substances, such as gasoline, petroleum-ether, etc., was tried by the writer. Their efficiency is doubtful and uncertain. No sterilization was obtained except on prolonged exposure (24 hours or more).

In addition to these methods the writer tried the application of moist heat at a constant degree, lower than the boiling point; 80° was the temperature used. This was tried in order to get a method that would interfere less with the edge of cutting instruments than does boiling; also, a method that could be applied to certain instruments that could not be boiled without marked deleterious effect on them, such as dental mirrors.

A jacketed water bath, designed by Mr. W. F. Wells, which was equipped with an apparatus for maintaining a constant level of water in the jacket, was used for this purpose. The inner chamber contains a thermoregulator set at 80°. In this bath the contaminated instruments were placed for varying periods.

To alkalinize the water, 0.25 per cent sodium hydroxide was added. When once raised to 80°, the amount of gas or electric current required to maintain a bath containing 1 to 2 gallons is very small, indeed. In fact, it can be maintained at that temperature for the whole 24 hours cheaper than it can be extinguished and reheated each day.

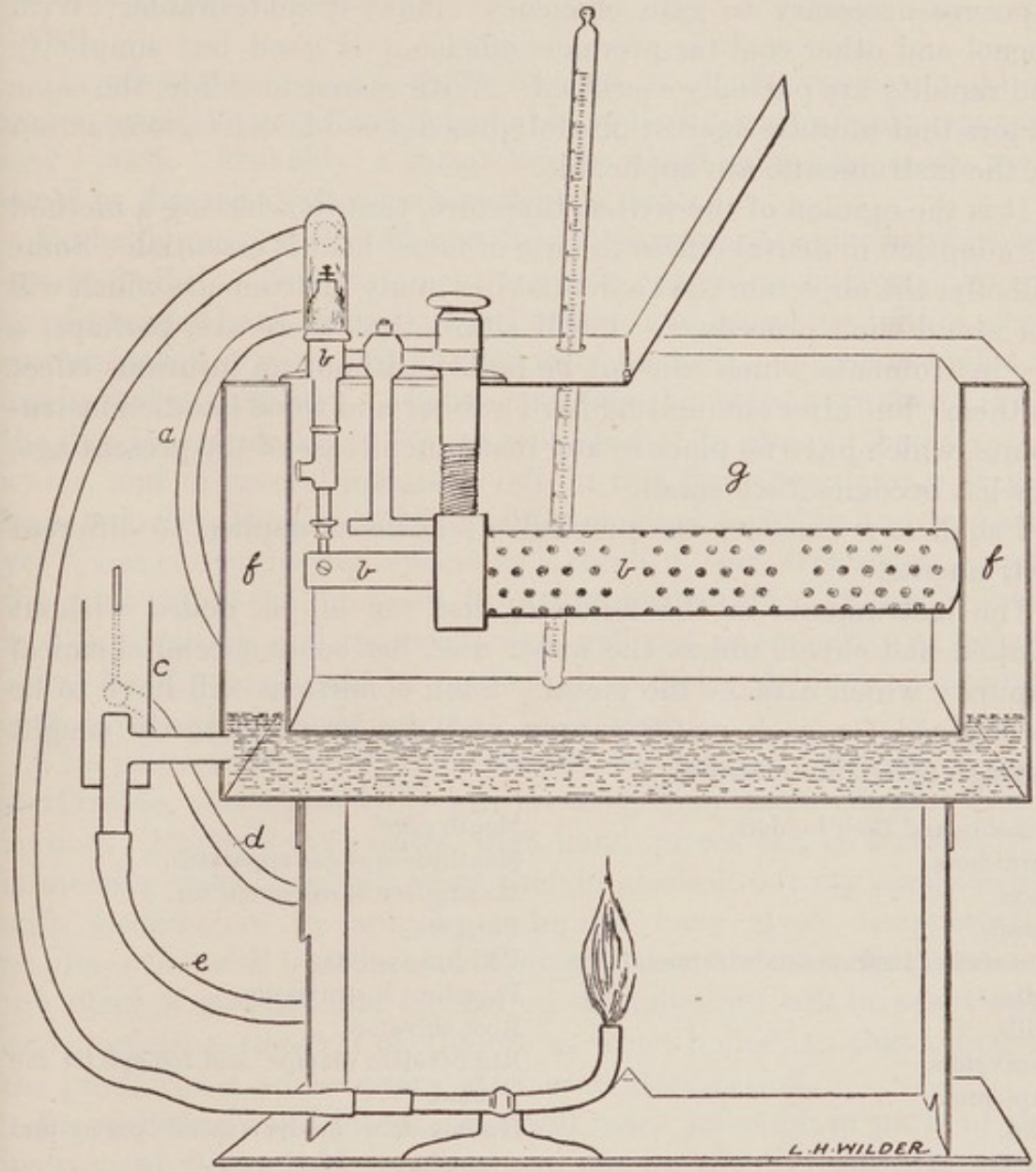
The use of this degree of heat had no deleterious effect on metal instruments or dental mirrors after prolonged and repeated exposure. Sterilization was always obtained in three minutes, even with the most resistant strains of staphylococci used. It is thus seen that this is practically as effective as boiling, but the temperature is 20° lower. It is simple to operate when once installed and is very efficient and rapid.

As stated above, no bad effects were noted on dental mirrors or instruments. However, there was a bad effect on instruments with wooden or hard-rubber handles. In the present era, however, no instrument that is not entirely of metal has any place in the armamentarium of surgeons or dentists. There are a few exceptions to this rule, where flexibility or elasticity is a factor in the use of the instrument.

From the above experiments and considerations the methods depending upon moist heat were found to be by far the most rapid and efficient.

In order of merit the writer places the methods depending upon heat as the active disinfecting agent as follows:

1. Boiling for at least 10 minutes in 0.25 per cent sodium hydroxide.
2. Use of water bath at 80° for at least 10 minutes.



WATER BATH USED FOR STERILIZING INSTRUMENTS AT 80°.
a = GAS SUPPLY. b = THERMO REGULATOR. c = CONSTANT WATER-LEVEL REGULATOR. d = WATER SUPPLY. e = OVER-FLOW PIPE. f = JACKET CHAMBER. g = STERILIZING CHAMBER.

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3. Use of moist heat in free chamber (Arnold sterilizer) for at least 10 minutes after thermometer reaches 100°.
4. Submersion in boiling water for at least 10 minutes, the source of heat being removed immediately prior to submersion of the instrument.
5. Application of dry heat by passing instrument through a free flame.
6. Dry heat in closed chamber.

The arrangement of the chemical methods in order of merit is much more difficult; in none are simplicity, efficiency, and rapidity combined as in some of the methods dependent on heat.

Using formaldehyde, the simplicity is ideal; but the length of exposure necessary to gain efficiency makes it undesirable. With phenol and other coal-tar products efficiency is good but simplicity and rapidity are partially sacrificed. With mercuric iodide, the same factors that militate against phenol, plus a possible deleterious action on the instruments, are applicable.

It is the opinion of the writer, therefore, that in selecting a method for adoption in dental offices the use of moist heat is essential. Some will offer the objection that a dentist has many instruments which will not stand such procedure. I will admit that there are, perhaps, a few instruments which can not be boiled without an injurious effect on them; but after eliminating hard rubber and wood handled instruments, which have no place in any instrument case of the present age, this list becomes very small.

I shall now consider the method of choice as applied to different instruments.

The instruments in the following list can all be boiled without marked bad effects unless the water used has some special chemical impurity which attacks the metal. Such conditions will have to be determined for each water supply and the proper remedy sought through scientific channels..

Broaches and their holders.
Burnishers.
Burrs.
Chisels.
Cone-socket instruments with metal handles.
Drills.
Excavators.
Explorers.
Files.
Forceps, extracting.
Forceps, foil.
Forceps, tongue-holding.
Impression trays.
Knives and lancets.
Mallets, automatic and hand.
Mixing slabs.
Mouth gags.
Mouthpiece of gas apparatus.
Mouthpiece of saliva ejector.
Pluggers.
Polishing points.
Pyorrhœa instruments.
Root elevators.
Rubber-dam clamps, and forceps for the same.
Rubber-dam holder (metal parts) and weights.
Saws.
Scissors.
Scalers.
Spatulas, metal.
Syringes, water.
Syringes, hypodermic.

The latter should be of glass or metal without washers; they can then be boiled repeatedly without impairing their efficiency in any way. An inferior hypodermic syringe is always unsatisfactory, whether it be sterilized or not.

To the above list I will add the following as instruments that will stand boiling:

Chip blower.
Masks for giving nitrous oxide gas.
Wire scratch wheel for head of dental engine.
Engine hand pieces.

The wire scratch wheel on the head of the dental engine should be made entirely of metal and detachable, so that it can be easily removed and boiled. Probably a cheap bristle wheel might be made that could be discarded after each patient is treated.

Probably many will challenge the statement that engine hand pieces can be boiled. This statement is made only after having procured a right-angled hand piece, boiled the same, and tested its sterility, then contaminated it with a fluid culture of bacteria, and again boiled it and tested its sterility. After completing the experiments with it for the day it was placed in 95 per cent alcohol to remove the excess of water, and in several instances left in this fluid overnight. It has been boiled repeatedly and frequently exposed to 80° in a water bath, yet it has shown no bad effects as a result of such treatment, except the removal of the lubricant from the mechanism. It can be oiled in a few seconds and its mechanism will run as smoothly as before. These hand pieces could be made with oil holes at the proper points so that the oiling would be much facilitated.

This instrument is one which dentists have usually considered non-sterilizable, consequently it has received very little efficient sterilization. Marshall (12) states that hand pieces can be sterilized by immersing them in gasoline and then in alcohol; but my experiments with sterilization by anhydrous liquids have given disappointing results, even with instruments of simple construction. Furthermore the effect of gasoline and alcohol on the lubricant will be practically as complete a removal of the oil as when boiled, so that oiling of the piece would be necessary after each sterilization.

In sterilizing hand pieces, heat will reach infection in parts of the instrument where cold solutions can not be effective, on account of the oil present, and where gas does not penetrate. With heat, the metal itself is raised to the temperature of the surrounding medium, so that the organisms are attacked from all sides; while with solutions or gases more or less of their surface is protected.

In seeking a method of sterilization for hand pieces, several methods were tried, designed to obtain sterilization without the introduction of water into the instrument. As stated above, the gasoline method did not sterilize rapidly and completely. Immersing in a fixed oil, liquid vaseline, was then tried, placing this in a steam chamber and raising the temperature to 100° for one hour. This proved inefficient,

because the heat is only the equivalent of 100° of dry heat, the instrument being in an anhydrous fluid. As a last resort I turned to moist heat by the method of boiling, or an 80° water bath, in 0.25 per cent sodium hydroxide and then removed the excess of water by absorbing it with alcohol. This gave the desired result.

All of the above mentioned instruments may be submitted to 80° in a water bath without any injurious effect on the instruments. Mouth mirrors may also be sterilized by this method without injuring them. This was determined by obtaining several new mirrors, contaminating them, and then placing them in 0.25 per cent sodium hydroxide solution at 80°. Sterilization was accomplished in 3 mintes, though the mirrors were frequently left in the bath for a much longer period, 10 to 60 minutes. The test of the effects of this treatment was made by handing the sterilized mirrors, together with a new mirror of the same lot that had not been sterilized, to other persons and asking them to pick out the one that had not been sterilized, Everyone acknowledged his inability to determine any difference.

Boiling, or heating to 80°, the rubber bulbs of chip blowers, and the face piece of the gas inhaler, will shorten the life of these articles. It is believed, however, that the increased expense due to these rubber fittings will not be great enough to work any hardship on the dentist. It certainly will not be as great as the cost of rubber gloves used by the average surgeon; and at present nearly every surgeon uses rubber gloves and insists on their sterilization by boiling. The dental surgeon should not be content with a lower grade of work, nor deterred by the small sum necessary to replace articles which have been worn out a little sooner by reason of improved methods in his practice.

The instruments considered above, which can be sterilized by boiling, include the greater portion of those used by the dentist; except those whose cost is so slight, or whose usefulness so impaired after using once, that they can be thrown away.

This list includes:

Polishing points and disks.
Rubber dam.
Nerve broaches (these may be sterilized by heat if desired).
Bristle brushes.

Mounted stones must be treated according to their construction and composition. Carborundum disks, wheels, and stones, which are bonded with porcelain, can be boiled without injury. Those made with rubber as the adhesive base can not be heated. Their sterilization can be accomplished by use of a phenol solution followed by alcohol. Corundum stones can not be boiled, so the phenol solution should also be used on these. Stones mounted on a mandrel by a cement or shellac can not be subjected to heat, while those

mounted by metal devices can be boiled, provided their composition allows such treatment. Inasmuch as many of the stones and disks must be sterilized without heat, it is permissible to sterilize all stones by immersing in 5 per cent phenol solution for one hour, to be followed by immersion in alcohol to remove the excess of phenol solution.

Tortoise shell, and instruments of similar composition, which might be injured by heat, should be immersed in phenol solution, as are mounted stones. Mouth mirrors may also be sterilized in this manner, if not subjected to 80°.

The life of a mouth mirror, however, depends entirely on the efficiency of its construction in keeping fluids of any kind from the amalgam surface of the glass. As long as this can be kept dry the mirror is clear. When it becomes wet by any fluid, either water or saliva, it becomes cloudy, consequently a well-constructed mirror is the cheapest in the end.

This method may also be applied to instruments whose composition must be other than metal, on account of the discoloring effect of metal on cement, or for other good reason.

However, barring the few instruments whose chemical composition is such that a serious change in character would result from heating, all instruments should be sterilized by heat, and no instrument made of material that is destroyed by heat should be allowed in the instrument case, if the same instrument can be made of thermostabile matter.

I have seen some dental instruments with rubber handles which have been boiled repeatedly, with no bad effect except discoloration of the rubber. These, however, had a central metal shaft supporting the rubber, to prevent bending when heated. While this construction permits the boiling of rubber-handled instruments, it can not be said that rubber handles are as acceptable from an aseptic viewpoint as are all-metal instruments. Of course, instruments whose cost is so slight that they are used once and then discarded do not require sterilization to prevent transfer of infection from patient to patient.

OTHER SANITARY MEASURES.

The following suggestions relating to dental asepsis, while not strictly within the subject of sterilization of instruments, are given as contributory factors in improved office sanitation.

Cuspidors are a necessity in a dental office, and the problem of keeping them in sanitary condition is a difficult one. The installation of the fountain cuspidor has been a great improvement over the old metal or earthen bowls which were so hard to keep clean. To those dentists who still have the old type of cuspidor, on account of lack

of running water or for other reason, I would recommend at least daily cleaning, with a broom or stiff brush and water. This is to be followed by placing the cuspidor in a bucket, covering it with boiling water, and leaving it there for at least one hour. It would be well to have several of these, so that a clean one can be put in use for each patient while the others are being cleaned by assistants or charwomen. In offices having the single-bowl fountain cuspidor, with bowl that can not be removed, flushing with hot water and antiseptic solutions is our main effort in sterilization. All fountain cuspidors should be fitted with both hot and cold water, so that the hot water can be used for cleaning the bowls after each patient is dismissed. Those having bowls that can be removed can have the bowls immersed in antiseptic solutions, or may even be boiled in a large container. However, absolute sterility of cuspidors is not of vital importance, provided a high degree of cleanliness is maintained. It is well, however, to have these articles of equipment installed so that complete sterilization can be carried out in case an unusual infection, such as diphtheria, is encountered.

The possibility of the operator transferring infection to the cable of the dental engine above the hand piece, and then later from the cable to another patient, has been mentioned. If the cables have the power-transmitting mechanism inside a covering, this can be avoided by a simple sterile or clean cotton sleeve, with a draw string in each end, which can be slipped over the cable and tied after the hand piece is in place, so that the whole cable is covered down to the sterile hand piece. This allows the operator to handle the cable at will during his operation, and, when a second patient is treated, a second clean sleeve is put on in place of the first one. It is also probable that a flexible metal covering, of tubing such as is used for conducting gas to lamps, might be constructed so that this could be removed and sterilized by heat. This is not applicable to engines which transmit the power from engine head to hand piece by open cord belt.

The use of foot levers for turning on the flow of hot and cold water in the washbowl is a great convenience to a busy operator who desires to keep his hands sterile. The use of a clean washable covering for the operating chair is a step in the direction of asepsis and cleanliness. The headrest should have a clean linen cover, renewed for each patient.

There should be toilet facilities for patients and, in addition to the usual lavatory equipment, a dental bowl, like that seen on Pullman cars, so that a patient may brush his teeth without doing so over the washbowl.

To place the patient's mouth in as clean condition as possible, each patient should brush his teeth, preferably immediately prior to

coming to the chair. This should be followed by a thorough rinsing of the mouth with some good mouth wash. This procedure is a mechanical cleansing, aimed primarily at the removal of spores from the field of operation, though many other organisms are removed at the same time.

In order to do this the dentist should have a supply of tooth-brushes on hand and, if necessary, supply a new brush to each patient. This may be the patient's first encounter with the oral hygiene educational movement, but I believe it will be productive of good. I believe that the dentist should add the cost of the brush to the patient's bill, unless brushes can be obtained at so low a figure as to make their free distribution practicable. A supply of mouth wash should be on hand in a convenient place for use by patients.

Paper drinking cups and paper, or small individual, towels for patients should be furnished. The common drinking cup and towel are abolished on all interstate carriers, and in hotels and public places in many States. It is, therefore, fitting that the members of the medical and dental profession should apply the spirit of these laws to their own establishments, even though the letter of the law does not require it.

Clean linen direct from the laundry is not sterile, but offers only the remotest chance for transmission of infection through it. Sterile linen is desirable and for a busy dentist, apparatus for its sterilization will be a great addition to office equipment. Steam pressure sterilizers are preferable for this, though other types may be used.

SUMMARY.

As a result of the experiments and investigations detailed above the writer reaches the following conclusions and recommends the following methods for sterilization of dental instruments:

CONCLUSIONS.

1. Moist heat is our best disinfecting agent for the sterilization of all metal instruments.

2. For the destruction of nonspore-bearing bacteria, moist heat at 80° is nearly as efficient as boiling, and for practical purposes can be used in place of boiling.

3. Instruments constructed of metal, whose complicated mechanism has heretofore caused them to be considered as nonsterilizable, can be sterilized by moist heat, provided the water is removed from them by immersing in alcohol subsequent to sterilization.

4. Instruments, whose construction does not permit of boiling, can be sterilized by chemical disinfectants.

5. There is need for more practical instruction in dental schools and clinics in the methods of sterilization, and the subsequent testing of the same by bacteriological methods.

6. Dentistry, which is a highly specialized branch of surgery, should use the two factors, asepsis and anæsthesia, which have made possible the wonders of modern surgery, with skill and precision equal to that of surgeons.

RECOMMENDATIONS.

1. That all instruments and appliances be rendered mechanically clean by washing in water with a brush or sponge.

2. That the following instruments and appliances be boiled or submitted to 80° in a slightly alkaline solution (0.25 per cent sodium hydroxide):

Artificial teeth used in matching and measuring.
Broaches and their holders.
Burnishers.
Burrs.
Chip blowers.
Chisels.
Drills.
Excavators.
Explorers.
Files.
Forceps, extracting.
Forceps, foil.
Hand pieces for engine.
Impression trays.
Knives and lancets.
Mallets, hand and automatic.
Mixing slabs.
Mouth gags.
Mouthpiece of saliva ejector.
Pliers.
Pluggers.
Pyorrhœa instruments.
Polishing points and brushes (if not discarded after using once).
Reamers.
Root elevators.
Rubber dam clamps and forceps for same.
Rubber dam weights and metal parts of holder.
Saws.
Scalers.
Scissors.
Scratch wheel on head of engine.
Spatulas, metal.
Syringes, hypodermic.
Syringes, water.
Tongue-holding forceps.
Mirrors (if 80° bath be used, but not to be boiled).

3. That instruments in the above list whose mechanical construction makes it difficult to remove the excess of water are to be placed in 95 per cent alcohol for 10 minutes to remove water, then removed and allowed to dry.

4. That only instruments with metal handles be used by dentists desiring to follow this method.

5. That the following instruments be sterilized by immersion in 5 per cent solution of phenol for at least 60 minutes:

Mounted stones.
Tortoise-shell instruments.
Mirrors (when 80° bath is not used).
Other instruments not of metallic nature and which can not be replaced by metallic instruments.

6. That instruments, after using, be placed in a fluid medium, preferably clean water, to avoid drying of infectious material and to facilitate their mechanical cleansing.

7. That no instrument or appliance, used on a patient directly or indirectly, be used on any other patient until recommendations 1 and 2, or 1 and 5, as the case may be, have been complied with.

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V.—A MODIFICATION OF ROSE'S METHOD FOR THE ESTIMATION OF PEPSIN.[1]

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In spite of all the work that has been done to isolate pepsin in a pure condition the results have been negative; for this reason it is as yet impossible to offer a method for its quantitative determination. As a matter of fact, while a number of so-called quantitative methods have been devised, the results obtained are only of value from a comparative standpoint. Such comparisons, however, are of decided importance and any means which will further their approach to a standard basis are worthy of consideration. It is with this view that a modification of Rose's method is here offered.

It is not necessary to review all of the many methods proposed for the estimation of pepsin. Suffice it to say that those of Mett (1), Jacoby-Solms (2), and Rose (3) are most used. Each has certain advantages at once apparent to a worker, but the rapidity and ease of the latter method, with the modification here offered, would seem to warrant its use. The Mett method has been in use so long that it is not necessary to describe the procedure. That of Jacoby depends on the digestion of a 0.5 per cent solution of ricin in 5 per cent sodium chloride, at 37° C. for three hours, with varying amounts of a $\frac{1}{100}$ diluted gastric juice. Rose's method demands the digestion of a 0.25 per cent solution of pea globulin in 10 per cent sodium chloride, at 37° C. for one hour or at 50° to 52° C. for 15 minutes, with varying amounts of a previously neutralized gastric juice usually diluted five times.

Against the use of both the Mett and the Jacoby methods can be offered at once the consumption of time, not to mention other factors. It is not always possible to get ricin and this led us to use the Rose method. It does not seem justifiable, however, to neutralize the gastric contents as Rose does. He lays much stress upon this point and says the failure to neutralize is a serious objection to the Jacoby method. He thinks the final acidity in each tube should be the same. In so neutralizing the method is rendered unreliable. Evidence is offered in the present paper sustaining this proposition and a correction is made.

[1] Manuscript submitted for publication Apr. 19, 1915.

Rose's complete method follows:

0.25 gram globulin of the pea prepared as described * * * is dissolved in 100 c. c. of 10 per cent sodium chloride (by warming slightly if necessary) and filtered. Portions of the clear filtrate of 1 c. c. each are introduced into a series of 11 small test tubes about 1 cm. in diameter. To each tube is added 1 c. c. of 0.6 per cent hydrochloric acid, and about five minutes are allowed for the development of the turbidity. A measured volume of the stomach contents is then exactly neutralized to litmus paper with dilute alkali. If a precipitate of acid protein forms, this is filtered off, and the clear neutral solution is diluted a known number of times (usually five) with distilled water, allowance being made for the dilution of neutralization. A portion of the diluted juice is boiled, and amounts decreasing by 0.1 c. c. added to the tubes of turbid protein; to the first, 1.0 c. c.; to the second, 0.9 c. c.; to the third, 0.8 c. c.; and so on to the eleventh, to which none is added. The unboiled juice is then rapidly added in increasing amounts, as follows: To the first, none; to the second, 0.1 c. c.; to the third, 0.2 c. c.; to the fourth, 0.3 c. c.; to the fifth, 0.4 c. c.; to the sixth, 0.5 c. c.; to the seventh, 0.6 c. c.; to the eighth, 0.7 c. c.; to the ninth, 0.8 c. c.; to the tenth; 0.9 c. c.; and to the eleventh, 1.0 c. c. Each of the tubes thus has a total volume of 3.0 c. c.; and a total acidity of 0.2 per cent of hydrochloric acid. * * *

The measurements of the solutions may be easily and accurately made with a pipette of 1 c. c. capacity, graduated to 0.01 c. c. The tubes are well shaken and allowed to stand in a thermostat or water bath for 15 minutes at a temperature of 50° to 52° C. Exactly the same endpoint is obtained by keeping the tubes at a temperature of 35° to 36° C. for one hour. At the end of the digestion time that tube in the series is selected which contains the least amount of gastric juice and which exhibits no turbidity. The peptic activity is calculated on the basis of the amount of gastric juice in this tube. The enzyme content is expressed by the number of cubic centimeters of the 0.25 per cent globulin that would be digested by 1 c. c. of the undiluted gastric juice under examination, if the activity were exerted for a period of one hour at 35° to 36° C., or for 15 minutes at 50° to 52° C. For example, if 0.5 c. c. of a gastric juice diluted five times clears up 1 cubic centimeter of the 0.25 per cent globulin solution in 15 minutes at the given temperature, the activity of the solution would be expressed:

$$\text{Peptic activity} = (1 \div 0.5) \times 5 = 10.$$

For clinical purposes it suffices to use the scale of pepsin units here proposed, which gives figures about one tenth of those expressed on the Jacoby-Solms scale.

Rose further states:

In the above method the conditions are constant in every trial, in respect to acidity, volume, protein content, and temperature.

This statement is true enough, but in attaining this point it is necessary first to neutralize, and in so doing a grave error is made. The effect upon pepsin of alkalis, even in dilution, is a question about which there is no dissention. Even very dilute alkalis will inhibit, if not destory, the action of pepsin. The following experiment brings out the point: A 0.1 per cent solution of scale pepsin was made containing 77.0 c. c. N/14 hydrochloric acid. 1 c. c. of this solution was exactly neutralized with 0.77 c. c. N/14 sodium hydroxide and diluted to 10. Another 1 c. c. portion, without neutralization, was diluted to 10. Both were now subjected to digestion at 50° to 52° C. for 15 minutes, as follows:

Pepsin solution.

	Neutralized tubes.[1]					Unneutralized tubes.[2]				
	1	2	3	4	5	1	2	3	4	5
0.25 per cent pea globulin	1	1	1	1	1	1	1	1	1	1
0.6 per cent HCl	1	1	1	1	1	1	1	1	1	1
Distilled water	0.9	0.7	0.5	0.2	0.0	0.9	0.7	0.5	0.2	0.0
10×diluted pepsin	0.1	0.3	0.5	0.8	1.0	0.1	0.3	0.5	0.8	1.0

[1] No perceptible digestion in any.
[2] Complete digestion in 3, 4, and 5. Pepsin=(1+0.5)×10=20.
Controls: Boiled pepsin+globulin+acid=negative. Water+globulin+acid=negative.

In the above case it is evident that the greatest part of the pepsin has been destroyed or inhibited. All of the pepsin has not necessarily been destroyed but evidently enough to prevent digestion within the limit of time and temperature. If more pepsin and less acid were present, then after neutralization one would expect to find some pepsin. It seems that an enzyme is not destroyed immediately, in the way an acid solution can be neutralized. In neutralization of weak acid solutions with alkali as dilute as N/14 sodium hydroxide, there is no question but that, for the moment, there is an alkaline reaction at the point of contact with the alkali. Since this is true, if we partially neutralize a weak acid solution containing pepsin, there ought to be a reduction of pepsin. About the effect of alkalis on pepsin there can be no question; hence the above assumption is supported by the following experiment: Gastric contents were removed from two normal individuals, after an Ewald test meal, and these determinations made:

	Total acidity.	Free acidity.	Rose exact.	Proposed method.	Jacoby exact.	Jacoby neutralized.	Neutralized one-half, then Rose.
R	55	42	Trace.	41	100	Trace.	16
M	60	45	Trace.	41	100	Trace.	16

Goodman (4), working with the Jacoby method, concluded from a limited number of cases that a uniform acidity was not necessary. Rose takes exception to this and demands for his method a final uniform acidity. He states—

> It will be seen that the enzyme content is expressed by much larger numbers when the total acidity is 0.05 per cent of hydrochloric acid than when it is 0.2 per cent. This is not due to the greater activity of the pepsin under such conditions, but to the decreased turbidity of the globulin solution, resulting from the decreased acidity.

In part I can agree with him, but I do not see that this justifies the neutralization of the stomach contents under examination. His statement is true if the final acidity is low, but if 1 c. c. of 0.6 per cent

hydrochloric acid be added, then the additional acidity, present by virtue of that contained in the gastric contents, can have no influence on the turbidity, and hence is not misleading as to enzymolysis. The following typical experiment illustrates the point:

	Tubes.												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1 c. c. of per cent HCl....	0.6	0.3	0.15	0.075	0.0375	0.0187	0.0093	0.0046	0.0023	0.0012	0.0006	0.0003	H_2O
1 c. c. of 0.25 per cent pea globulin.....	1	1	1	1	1	1	1	1	1	1	1	1	1
Final total acidity, per cent.....	0.3	0.15	0.075	0.0375	0.0187	0.0093	0.0046	0.0023	0.0012	0.0006	0.0003	0.0001	0.0

These tubes were allowed to stand 15 minutes, and at the end of that time tubes 1, 2, 3, 4, and 5 showed the same maximum turbidity, so far as perceptible to the naked eye. Tubes 6, 7, and 8 showed no maximum and progressively less turbidity. Tubes 9 to 13 were all clear, not even showing an opalescence. The same result was obtained at the end of an hour. To another series, with the above result at the end of 15 minutes, was added 1 c. c. of 0.6 per cent hydrochloric acid; after 10 minutes, all the tubes showed a maximum turbidity, with no perceptible difference between them. That is to say, if the final total acidity is only 0.2 per cent, or increased to even 0.4 per cent, there is no apparent difference in the turbidity of the tubes. This certainly shows, from the standpoint of turbidity, that it is unnecessary first to neutralize the gastric contents. Even were the total acidity of a gastric juice under examination 100 to 120, the final total acidity of the tube containing the greatest amount of diluted gastric juice would never reach 0.4 per cent.

In a series of 24 observations (Table I) on normal and pellagrous individuals, there are only two instances in which a value has been obtained by following exactly Rose's method. Strict attention has been paid to neutralization. Titrations have first been made with alizarin red and phenolphthalein. The first tinge of brown with alizarin red reacted neutral to litmus. In other instances the contents have been titrated to the first perceptible tinge of pink with phenolphthalein, and then, for the Rose method, another sample was made neutral to litmus by stopping short of this point. In no case has the reaction been alkaline.

TABLE I.

Case.	Total acidity.	Free acidity.	Pepsin number.		Remarks.
			Proposed method.	Rose method.	
1	91	70	50	Trace.	Normal.
2	60	45	41	Trace.	Do.
3	44	21	31	Trace.	Do.
4	61	44	41	12	Do.
5	83	67	41	Trace.	Do.
6	84	69	41	Trace.	Do.
7	61	46	25	Trace.	Do.
8	55	42	41	Trace.	Do.
9	71	49	41	Trace.	Do.
10	89	70	40	Trace.	Do.
11	69	49	42	Trace.	Do.
12	51	26	42	Trace.	Do.
13	58	42	42	Trace.	Do.
14	71	53	40	Trace.	Do.
15	78	64	33	12	Pellagra.
16	25	12	33	Trace.	Do.
17	76	57	41	Trace.	Do.
18	57	38	40	Trace.	Do.
19	45	25	25	Trace.	Do.
20	84	64	31	Trace.	Do.
21	56	31	21	Trace.	Do.
22	21	0	Trace.	0	Do.
23	35	7	12.5	0	Do.
24	14	0	Trace.	0	Do.

No attempt is made in this paper to take up the question of gastric digestion in pellagra.

That neutralization in the Jacoby method will in a like manner destroy or inhibit some of the pepsin, and consequently influence the results appears from the following:

Unneutralized juice.		Neutralized juice.		Remarks.
Proposed method.	Jacoby method.	Rose method.	Jacoby method.	
41	100	Trace.	Trace.+	Normal.
41	100	Trace.	Trace.+	Do.
41	100	Trace.	Trace.+	Do.
41	83	Trace.	Trace.+	Pellagra.

The following method has been adopted in this laboratory.[1] The gastric contents are strained through cheesecloth. Two c. c. are measured by means of an Ostwald pipette into a 25 c. c. stoppered volumetric cylinder, and diluted to the mark with distilled water. Into each of seven small test tubes (1×10 cm.) is measured, with an Ostwald pipette, 1 c. c. of a 0.25 per cent filtered pea globulin in 10 per cent sodium chloride solution. To each tube is added 1 c. c. of 0.6 per cent hydrochloride acid, also by means of an Ostwald pipette. The tubes are allowed to stand about 5 minutes, until the maximum turbidity develops. To the first five, distilled water is added as fol-

[1] I take pleasure in here thanking Dr. R. C. Lewis of the staff for the preparation of pea globulin used in these experiments.

lows: To the first, 0.9 c. c.; to the second, 0.8 c. c.; to the third, 0.7 c. c.; to the fourth, 0.6 c. c.; and to the fifth, 0.2 c. c.; to the sixth and seventh, none. Then there are rapidly added to each test tube the following amounts of the 1/12.5 gastric juice; to the first, 0.1 c. c.; to the second, 0.2 c. c.; to the third, 0.3 c. c.; to the fourth, 0.5 c. c.; to the fifth, 0.8 c. c.; to the sixth, 1.0 c. c.; and to the seventh, 1.0 c. c. of the diluted juice boiled. These measurements can be accurately made with a 1 c. c. pipette graduated in 0.01 c. c. All tubes are then immersed for 15 minutes in a water bath at 50° to 52° C. At the end of this time, the tube is selected which is clear and contains the least amount of diluted gastric juice. Upon this basis, the peptic activity is calculated as the number of c. c. of 0.25 per cent globulin digested by 1 c. c. of undiluted gastric juice. For example, if tube 2 containing 0.3 c. c. of a 12.5 times diluted juice be clear, then the result would be expressed:

$$\text{Peptic activity} = (1 \div 0.3) \times 12.5 = 41.2.$$

Ordinarily this scheme of seven tubes is used, though it is not a rule. If the free acidity be high, sometimes a dilution of 1/25 is made. The number of tubes used will depend upon the accuracy desired.

	Tubes.						
	1	2	3	4	5	6	7
0.25 per cent pea globulin c. c.	1	1	1	1	1	1	1
0.6 per cent HCl c. c.	1	1	1	1	1	1	1
Distilled water, c. c.	0.9	0.8	0.7	0.5	0.2	0.0	0.0
Diluted gastric juice	0.1	0.2	0.3	0.5	0.8	1.0	0.0
Boiled gastric juice	0	0	0	0	0	0	1.0
Final volume, c. c.	3	3	3	3	3	3	3
Peptic activity	125	62.5	41	25	17	12.5	0

The gastric contents are never filtered, but strained through cheesecloth, as it is believed in this way less enzyme is adsorbed. The dilution has been kept at ten times, or greater, and water substituted for boiled juice to make up the final volume. This has been done in line with Nierenstein and Schiff's[7] procedure, in order to lessen the amount of disturbing factors, be they proteoses, peptones, or antiferments. At the same time it serves in keeping the final total acidity nearer 0.2 per cent than if boiled unneutralized juice were added.

The pea globulin is made according to Rose's description from the ordinary garden pea, *Pisum sativum:*

The finely ground peas, freed as much as possible from the outer coating, are repeatedly extracted with large quantities of 10 per cent sodium chloride solution, the extracts combined, strained through fine bolting cloth, and allowed to stand overnight in large cylinders to deposit insoluble matter. The supernatant fluid is siphoned

off and saturated with ammonium sulphate. The precipitate of albumins and globulins is filtered off, suspended in a little water, and dialyzed in running water for three days, until the salt has been removed and the albumins have been dissolved. The globulins are filtered off and washed two or three times with water, to remove the last trace of albumins. To purify further, the precipitate is extracted with 10 per cent sodium chloride solution and filtered until perfectly clear. The resulting solution is exactly neutralized to litmus paper, by the cautious addition of dilute sodium hydroxide, and again dialyzed in running water for three days to remove the salts completely. The precipitated globulins are then filtered off and dried on a water bath at 40° C. During the complete process of separation, the proteins should be preserved with a mixture of alcoholic thymol and toluene. The globulins so prepared dissolve, practically completely, in 10 per cent sodium chloride solution, and after slight acidification with hydrochloric acid yield a turbid solution which does not settle out on standing.

I have found this solution of globulin to settle out slightly in the case of controls, though as a rule it remains in suspension and certainly is much better than the precipitated ricin, which will settle out and rise to the top of the tubes. If preserved, as Rose directs, with toluene, it can be kept for a month or more. On the assumption that exact neutralization only inactivated the pepsin, and that it might be reactivated at once, though this is not possible according to Tichomirow (5), the following experiment was tried: Some gastric juice was neutralized exactly to litmus with N/14 sodium hydroxide and at once the previously determined acidity was attained by adding N/14 hydrochloric acid. This juice was then subjected to the determination and no quantitative amount of pepsin found, while with the unneutralized juice there was a peptic value of 40. The pepsin can be determined in 25 minutes from the time the gastric contents are received, provided there are on hand stock solutions of pea globulin and 0.6 per cent hydrochloric acid. Using a burette to deliver the globulin solution, the 0.6 per cent hydrochloric acid, and the water, and graduated pipettes for the diluted juice, six determinations can be completed easily in an hour and a half.

The present method is recommended because it is believed to be as accurate as any and less time consuming. That it will check itself is shown by the following analyses on the same normal individual on different days:

Total acidity.	Free acidity.	Pepsin number.	Contents removed after—
			Minutes.
61	46	25	30
46	21	31	45
61	44	41	60
55	42	41	60
71	49	41	60
89	70	40	60
83	67	41	75
84	69	41	90

The low value (25) found above I believe is due to the fact that only 30 minutes elapsed after the giving of the test meal, as Hawk (6) and his collaborators have found that the height of acid and pepsin secretion is not reached in that time. The high value (50) found (see Table I) in a normal individual may be due to a hypersecretion of pepsin, called forth by the high acidity (90). On the following day the same case with a total acidity of 60 showed a peptic value of 41. The limit for normal individuals found so far extends between 25 and 50. The following table gives a comparison of the results obtained on the same contents, by the proposed method and by that of Jacoby:

Case.	Total acidity.	Free acidity.	Pepsin number.		Remarks.
			Proposed method.	Jacoby method.	
1	51	26	42	100–	Normal.
2	55	42	41	100–	Do.
3	58	42	42	100–	Do.
4	60	45	41	100–	Do.
5	69	49	42	100–	Do.
6	71	49	41	100–	Do.
7	71	53	40	100–	Do.
8	89	70	40	100–	Do.
9	56	31	21	60–80	Pellagra.
10	57	38	40	100–	Do.
11	69	53	33	100–	Do.
12	84	64	31	100–	Do.

By "100–" is meant that the solution is almost clear, only a trace of turbidity being left, the tube containing the next higher concentration of gastric content being clear; therefore it is more exact to say "100–." From this table it is seen that the values obtained by my method correspond to about four-tenths of those of Jacoby-Solms.

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HYGIENIC LABORATORY BULLETINS OF THE PUBLIC HEALTH SERVICE.

The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building, located in Washington, was authorized by act of Congress March 3, 1901.

The following *bulletins* [Bulls. Nos. 1–7, 1900 to 1902, Hyg. Lab., U. S. Mar.-Hosp. Serv., Wash.] have been issued:

*No. 1.—Preliminary note on the viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 2.—Formalin disinfection of baggage without apparatus. By M. J. Rosenau.

*No. 3.—Sulphur dioxid as a germicidal agent. By H. D. Geddings.

*No. 4.—Viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 5.—An investigation of a pathogenic microbe (*B. typhi murium* Danyz) applied to the destruction of rats. By M. J. Rosenau.

*No. 6.—Disinfection against mosquitoes with formaldehyde and sulphur dioxid. By M. J. Rosenau.

†No. 7.—Laboratory technique: Ring test for indol, by S. B. Grubbs and Edward Francis; Collodium sacs, by S. B. Grubbs and Edward Francis; Microphotography with simple apparatus, by H. B. Parker.

By act of Congress approved July 1, 1902, the name of the "United States Marine-Hospital Service" was changed to the "Public Health and Marine-Hospital Service of the United States," and three new divisions were added to the Hygienic Laboratory.

Since the change of name of the service the bulletins of the Hygienic Laboratory have been continued in the same numerical order, as follows:

*No. 8.—Laboratory course in pathology and bacteriology. By M. J. Rosenau. (Revised edition, March, 1904.)

†No. 9.—Presence of tetanus in commercial gelatin. By John F. Anderson.

*No. 10.—Report upon the prevalence and geographic distribution of hookworm disease (uncinariasis or anchylostomiasis) in the United States. By Ch. Wardell Stiles.

*No. 11.—An experimental investigation of *Trypanosoma lewisi*. By Edward Francis.

*No. 12.—The bacteriological impurities of vaccine virus; an experimental study. By M. J. Rosenau.

*No. 13.—A statistical study of the intestinal parasites of 500 white male patients at the United States Government Hospital for the Insane; by Philip E. Garrison, Brayton H. Ransom, and Earle C. Stevenson. A parasitic roundworm (*Agamomermis culicis* n. g., n. sp.) in American mosquitoes (*Culex sollicitans*); by Ch. Wardell Stiles. The type species of the cestode genus *Hymenolepis*, by Ch. Wardell Stiles.

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*No. 17.—Illustrated key to the trematode parasites of man. By Ch. Wardell Stiles.

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