I. The cause of death from subdural injection of serum / by Worth Hale ; II. Some new cholera selective media / by Joseph Goldberger.

Contributors

Hale, Worth, 1876-
Goldberger, Joseph, 1874-1929. Some new cholera selective media.
Hygienic Laboratory (U.S.)

Publication/Creation

Washington, D.C. : Government Printing Office, 1914.

Persistent URL

https://wellcomecollection.org/works/ycwm5m9d

TREASURY DEPARTMENT
UNITED STATES PUBLIC HEALTH SERVICE

HYGIENIC LABORATORY—BULLETIN No. 91
DECEMBER, 1913

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By WORTH HALE

2. SOME NEW CHOLERA SELECTIVE MEDIA

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United States Public Health Service.

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1.—THE CAUSE OF DEATH FROM SUBDURAL INJECTIONS OF SERUM.[1]

By Worth Hale.

[From the Hygienic Laboratory, United States Public Health Service.]

The treatment of cerebrospinal fever by a specific antiserum was introduced as a therapeutic measure in 1906 and 1907 subsequent to certain experiments on animals inoculated with the *Diplococcus intracellularis* Weichselbaum. In these experiments it was shown that the animals were not only protected from the disease but also that the treatment was without danger to the animal, although introduced directly into the subarachnoid space of the spinal cord.

As had been demonstrated by these animal experiments, the possibility of reducing the mortality rate in this disease by the use of this serum was soon verified by the successful treatment of many cases. Statistics covering the ground have been compiled which show the results of both the older methods of treatment and the specific serum treatment during the same epidemics. It is clear from the data thus gathered that the antiserum has been particularly efficacious when contrasted with the results where other treatment was resorted to, since in the latter cases the death rate has been from two-thirds to three-fourths greater than among the cases which were given the antiserum treatment.

The serum treatment of cerebrospinal fever appears, however, to entail certain dangers to the patient and recently there have appeared a number of reports describing alarming symptoms and, in a number of instances, death. These untoward results occurred in such close relation to the administration of the treatment that the treatment, rather than the disease, seemed to be responsible.

Serious symptoms were observed by Sophian[2] and Litterer[3] and a number of cases of sudden death are reported by Kramer,[4] who also calls attention to certain cases of death which appeared in Parmlee's[5] report. This occasional undesirable effect of the serum treatment should not, however, be considered as militating against its regular and invariable use in all cases of the disease, for the dangers which may arise from the treatment are not to be feared in

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[1] Manuscript submitted for publication Nov. 1, 1913.
[2] Sophian. Journ. Am. Med. Assn., 1912, vol. 58, p. 843.
[3] Litterer. Idem, 1912, vol. 59, p. 531.
[4] Kramer. Idem, 1913, vol. 60, p. 1348.
[5] Parmlee. Idem, 1913, vol. 60, p. 659.

any such way as is the disease itself. The risk, as compared with that from the disease itself, is very small; but it nevertheless becomes of great importance to discover to what agency these accidents may be ascribed and from this knowledge to determine some procedure by which they may be reduced in number or entirely avoided.

A number of theories have been advanced to account for these accidents during the administration of the serum. Thus, at various times it has been claimed that these deaths were due to rapid lysis of the meningococcus and the consequent liberation of a toxic amount of bacterial toxin; to the production, through the introduction of large amounts of a foreign proteid, of anaphylactic shock; to increased intracranial tension, from the too rapid or too free use of the antiserum, and the consequent interference with the vital centers; to the presence in the serum of poisonous preservatives which were carried directly to the centers of respiration, with a resulting paralysis and death.

One of these possible sources of danger from the administration of the serum seems to have been early recognized. Thus, Flexner and Jobling [1] published certain rules to govern the therapeutic use of the serum, among which occurs the following: "The quantity of antiserum should not exceed, for the present, 30 cubic centimeters. It is desirable, although it would not appear to be essential, to withdraw from the spinal canal at least as much fluid as the amount of the antiserum to be injected. The injection should be made slowly and carefully to avoid the symptoms due to *increased pressure*. This precaution should be exercised especially when the quantity of cerebrospinal fluid withdrawn is less than the amount of antiserum injected."

More recently Koplik [2] seems to have been influenced by a like consideration of danger in the sudden increase in intracranial tension from the subarachnoid introduction of serum. He says: "The pressure exerted by a syringe at all times against the respiratory and vascular pressure is certainly not as gentle a mode of introduction of serum or as safe as allowing the serum to flow into the canal." He accordingly favors a gravity method of introducing the serum rather than by means of a syringe.

Sophian [3] likewise, apparently with the purpose of avoiding too great increase in intracranial tension, attempted to control his injections of serum by measuring the pressure of the fluid in the subarachnoid space. He believed that as soon as the pressure returned to the normal point, as shown by the initial reading preceding the withdrawal of cerebrospinal fluid, the inflow of serum should be stopped. However, he observed in certain cases, in spite

[1] Flexner and Jobling. Journ. Exper. Med., 1908, vol. 10, p. 141.
[2] Koplik. Med. Record, 1908, vol. 74, p. 557.
[3] Sophian. Journ. Am. Med. Assn., 1912, vol. 58, p. 843.

of these precautions, severe and even alarming symptoms, although the pressure readings were still lower than the intracranial tension had been at the beginning.

More recently Kramer [1] has advanced the hypothesis that the cases of sudden death reported by him were due to the presence in the serum of a poisonous preservative—tricresol. Kramer attempted to show in an experimental way that tricresol was particularly toxic when introduced by a direct path into the region of the fourth ventricle. In his first experiments he introduced antimeningitis serum and 0.5 per cent tricresol solution cephalically into the vertebral artery, his purpose being to deliver the injected substance by way of the blood stream directly to the medullary centers. The results, both with the serum, which contained a preservative, and with the tricresol solution were the same, respiratory activity being checked temporarily; but death did not follow. Two other experiments which he reported seemed to prove that the same results could be produced by subarachnoid injections of the serum or of the tricresol solution. A dose of 2 c.c. of a 0.5 per cent tricresol solution caused a stoppage of respiratory movements which threatened to become permanent; but artificial respiration having been maintained for 10 minutes, breathing was again resumed. In the other experiment, 2 c. c. of antimeningitis serum checked respiratory activity which, however, was again resumed after a half minute of artificial respiration; but the dog died five minutes later with respiratory symptoms.

From these results Kramer definitely implicated the tricresol used as a serum preservative as the cause of a number of deaths which occurred at the Cincinnati hospital. From his observations in these cases and from an earlier series of experiments [2] he believed that toxic substances injected subdurally were carried directly to the medullary centers by way of the central canal of the cord, which he assumes remains patent in a certain number of cases, particularly in children. Further, he assumes that the ciliated epithelium lining the canal is active in carrying the toxic solution to the fourth ventricle.

Kramer's hypothesis as to the cause of sudden death from the serum has been opposed by Flexner [3] who, in a recent paper, enters into a theoretical discussion of the problem of sudden death following the treatment of cerebrospinal fever.

Flexner's argument is that Kramer's experiments are not vital to the problem; that sudden deaths have been reported in France where a serum is used which contains no preservative; that serum preserved with tricresol has been introduced directly into the third ventricle and is borne quite as well as are injections into the lumbar

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[1] Kramer. Journ. Am. Med. Assn., 1913, vol. 60, p. 1348.
[2] N. Y. Med. Journ , 1912, vol. 95, p. 532.
[3] Flexner. Journ. Am. Med. Assn., 1913, vol. 60, p. 1937.

meninges, and yet the path is much more direct from the lateral ventricles to the medullary centers than from the lumbar region, by way of the central canal of the lumbar cord, to the fourth ventricle.

Flexner appears, therefore, to be opposed to the belief that the tricresol used in preserving the serum can ever be at fault. He appears to believe, rather, that the deaths reported by Kramer and others were due to intracranial tension, thus reaffirming his early opinion[1] that increased pressure should be avoided in administering antimeningitis serum. He assumes that too large doses of serum were injected and that the method of introduction should have been by the gravity method advocated by Quincke, Koplik, and Sophian. Under such careful administration he believes that deaths would not have occurred.

In support of this view, Sophian's results of 1,500 administrations of serum without a single death are given. Sophian has shown, however, that symptoms entirely like those appearing in the cases reported by Kramer may occur even under the most exact technique. That Sophian's patients recovered apparently depended less on the care with which he observed precautions to prevent increased intracranial pressure and more on another criterion of danger which he had introduced upon the discovery that pressure observations were worthless in preventing accidents. Had Sophian not been warned of approaching danger by the rapidly falling blood pressure he might easily have killed his patient, who resumed breathing only after vigorous stimulant treatment. Sophian's excellent results then were independent of any especial care which he exercised to prevent increased intracranial tension, but were dependent upon his custom of making routine blood-pressure observations during the time the serum was being administered.

Likewise, an observation made by Cushing[2] is taken to support the belief that increased tension is responsible for the alarming symptoms and cases of sudden death which have been reported. Cushing's work demonstrated that with increased intracranial tension the respiratory center suffers most acutely and is the first of the vital nervous centers to fail, the heart beating for some time afterwards; in one case under artificial respiration 23 hours intervening before cardiac death. This fact does not seem applicable, however, in explaining the cases of sudden death on the grounds of increased intracranial tension, at least not applicable if one considers Sophian's[3] observations that the blood pressure is lowered during the administration of serum and that the rate of fall is much accelerated with

[1] Flexner, and Jobling. Journ. Exper. Med., 1908, vol. 10, p. 141.
[2] Cushing. Am. Journ. Med. Sci., 1902, vol. 124, p. 375.
[3] Sophian. Journ. Am. Med. Assn., 1912, vol. 58, p. 843.

larger quantities of serum or with smaller amounts introduced too rapidly. Cushing showed that—

> The usual consequence of this condition (increased intracranial tension) is not death, as commonly stated, but a stimulation of the vasomotor center, which occasions a *rise in blood pressure* sufficient to overcome the high intracranial tension. * * * respiration which may have ceased is resumed * * *.

In some instances a pressure of 250 mm. mercury, 3,400 mm. water pressure, was used without evidence of vasomotor failure, and always back of the respiratory failure was the failure of the vasomotor center to respond to the enormous pressure used. Cushing's work does not, therefore, lend itself to the support of the theory advanced by Flexner. At least it would be very difficult to believe, even when the injections of serum are being made very rapidly, that such enormous pressure as was shown by Cushing to be fatal could result. Likewise the high blood pressures observed under conditions of increased intracranial tension do not accord with Sophian's observation that the administration of serum always caused a fall of pressure, not a rise.

The cause of such sudden deaths as are reported by Kramer does not, therefore, appear to be due to increased intracranial tension. On the other hand, there is some reason for assuming that the tricresol preservative is responsible for the sudden failure of respiration and for the lower blood pressure which have been noted in these unexpected accidents and deaths. At least Kramer seems to have adopted this hypothesis only after submitting experimental evidence, however inadequate it may have been.

The problem suggested by Kramer of the essential toxicity of the tricresol preservative used in antimeningitis serum, due to its being brought directly to the fourth ventricle by a patent central canal of the spinal cord, and the issue raised by Flexner make the experimental observation of the points at issue of the greatest importance. If the tricresol so universally used as a serum preservative actually possesses marked toxic properties under the conditions in which it is introduced into the body, the facts should be known and search should be made for a less poisonous substitute. To this end an experimental investigation was undertaken not only to determine in what degree Kramer's conclusions agreed with experimental evidence, but also to secure if possible an explanation of the symptoms of lowered blood pressure and weakened respiration, as noted by Sophian.

Most of the experiments were carried out on dogs, but some experiments were made on cats. In all cases the anesthetic used was the same. The dogs were anesthetized by the administration of morphine sulphate, 0.005 gram per kilogram of body weight, given subcutaneously, and by a solution of ethyl carbamate, 0.045 gram,

and chloral hydrate 0.018 gram per kilogram, given by the stomach. In all cases the cats were anesthetized with the solution of ethyl carbamate and chloral hydrate given by the stomach in the same dosage as used for the dogs, but morphine was not used. Usually a depth of anesthesia was secured in this way to permit of painless operative procedures, but in an occasional instance the anesthetic was supplemented by giving ether vapor.

Blood-pressure tracings were secured from the carotid artery, using a mercury manometer. A number of different methods were tried for securing a graphic record of the respiratory movements and finally the method of using a canula inserted into the pleural space just above the diaphragm was adopted, the respiratory movements being recorded through a tambour connected by rubber tubing to this canula.

In the first experiments the vertebral artery was dissected out and injections of 0.5 and 1 per cent tricresol were made in the direction of the blood stream through a fine hypodermic needle. The effects of such injections were similar to those observed by Kramer. In one typical experiment carried out on a dog weighing 8.2 kilograms, the injection of 5 c. c. of normal serum was without effect. Following the injection of 2 c. c. of 0.5 per cent tricresol solution the blood pressure fell from a normal of 166 to 140 mm. The respiratory movements from a normal of 30 per minute were increased to 66 and were momentarily much deeper. Respiratory effort then ceased for a half minute, but was again resumed without resort to stimulation or artificial means. The rate subsequently rose to 30 per minute as at the beginning, but the respiratory movements were much more shallow, thus indicating marked respiratory derangement.

These experiments were soon discontinued, since it was apparent that the results so obtained would give but little information regarding the problem. These experiments served to show, however, that a tricresol solution introduced by the blood stream without much dilution into the region of the medulla was particularly toxic. Thus, in certain experiments, in which 1 per cent tricresol in normal horse serum and in Ringer's solution was injected intravenously into the saphenous vein, the effect was much less marked, indicating very clearly that tricresol, if brought by a direct route into contact with the medulla, possesses a greatly augmented toxic effect.

Further experimentation was confined to injections into the subarachnoid space, which, when carried out in the lumbar region, corresponds closely with the procedure prevailing during the administration of antimeningitis serum in therapeutics. From this point the problem was readily separated into two main parts: To determine the effects produced by the introduction into the subarachnoid space of serum free from preservative, together with an effort to produce

severe symptoms or death from the pressure action of the serum on the vital centers; to determine the effects produced by similar injections of serum to which, however, tricresol had been added, with the purpose of determining whether the symptoms resulting would be the same, or if the symptoms differed from those observed in the first instance to discover in what way they differed.

THE EFFECT OF INCREASED TENSION.

A number of experiments were undertaken to determine whether dangerous symptoms or death could be produced from increased intracranial tension arising from the introduction of serum free from preservative, or Locke's solution, into the subarachnoid space either in the cerebellar or in the lumbar region.

Death resulted in these experiments in only a very few instances out of a large number of attempts. The experimental procedure was as follows:

After preparing the animal for blood pressure and respiratory tracings, the syringe needle was introduced through the skin and muscles into the spinal canal and was considered to be in the subarachnoid space when cerebrospinal fluid escaped. As the symptoms of increased tension were somewhat irregular in appearing under this technique, in the later experiments laminectomy was performed and the syringe needle was introduced carefully below the exposed dura. The results were more uniform by this method, and it seems probable that in the first experiments the needle, although permitting the escape of cerebrospinal fluid, was displaced, permitting the injections to escape into the epidural tissues.

The symptoms noted in these experiments, in the nonfatal cases and those preceding death in the fatal cases, were similar to those which Cushing [1] and Eyster [2] have so fully described as occurring from increased intracranial tension.

As a rule, there was a marked rise in blood pressure, which rose progressively with the increase in tension. In many instances, however, the heart rate became very slow from the excessive vagus stimulation, which usually came on after each injection, and in consequence the blood pressure was occasionally lower than normal although usually, even with a very slow heart rate, the blood pressure was higher than before the injection. Further, and most important, the blood pressure always rose to a height much greater than normal when death was imminent. If the vagi were cut or were paralyzed by atropine, a marked rise in pressure always followed each injection.

Coincidently with, or a few seconds later than the rise in blood pressure there was a complete cessation of respiratory activity; but

[1] Cushing. Am. Journ. Med. Sci., 1902, vol. 124, p. 375.
[2] Eyster. Journ. Exper. Med., 1909, vol. 11, p. 489.

in the nonfatal cases respiration was resumed at some point during the elevation of the blood pressure, after which the blood pressure began to fall to its original height. In the fatal cases the blood pressure continued to rise and after reaching a very high level (in the fatal cases noted in these experiments to a height of more than twice the normal) suddenly began to fall rapidly to zero, apparently from the failure of the vasomotor center to respond to the excessive demands placed upon it.

A tracing is given in figure 1 to show the effect of a fatal increase of intracranial tension on the vasomotor and respiratory centers.

EFFECT OF TRICRESOL SERUM.

In these experiments tricresol was added to antimeningitis serum in amounts varying from 0.3 to 1 per cent. About an hour before making the injections the tricresol was measured into the serum and the resulting precipitate thoroughly mixed with the serum. Just preceding the beginning of an experiment the serum was filtered through cotton wool to remove all large precipitated particles and the opalescent filtrate was then placed in a water bath at 39° C. so as to make possible the injection of the phenolized serum at approximately body temperature.

In some of the early experiments, in which the serum was introduced through a hypodermic needle without operation, the results were irregular, as they had been in the experiments with the serum without preservative. In some cases, although cerebrospinal fluid escaped from the needle, a large amount of phenolized serum could frequently be injected without the appearance of any noticeable effect on blood pressure or on the respiratory movements. Or after a large amount of serum had been thus introduced the blood pressure became progressively lower, the heart rate more rapid, and the respiratory movements more and more shallow, finally very slow and shallow, and death resulted apparently from a progressive poisoning similar to that occuring in the experiments in which the tricresol serum had been introduced intravenously.

In many of these experiments, however, death came on suddenly with respiratory failure. The blood pressure was not raised, as in the instances with serum free from preservative where increased intracranial tension resulted, or if raised at all only to a slight degree and then followed a slow fall in pressure to zero, the heart ceasing to beat some two or three minutes after respiratory movements had ceased. In other cases when the early injections had produced no apparent results, the reintroduction of the needle was often followed by death of the animal at the first injection of not more than 2 c.c. of serum.

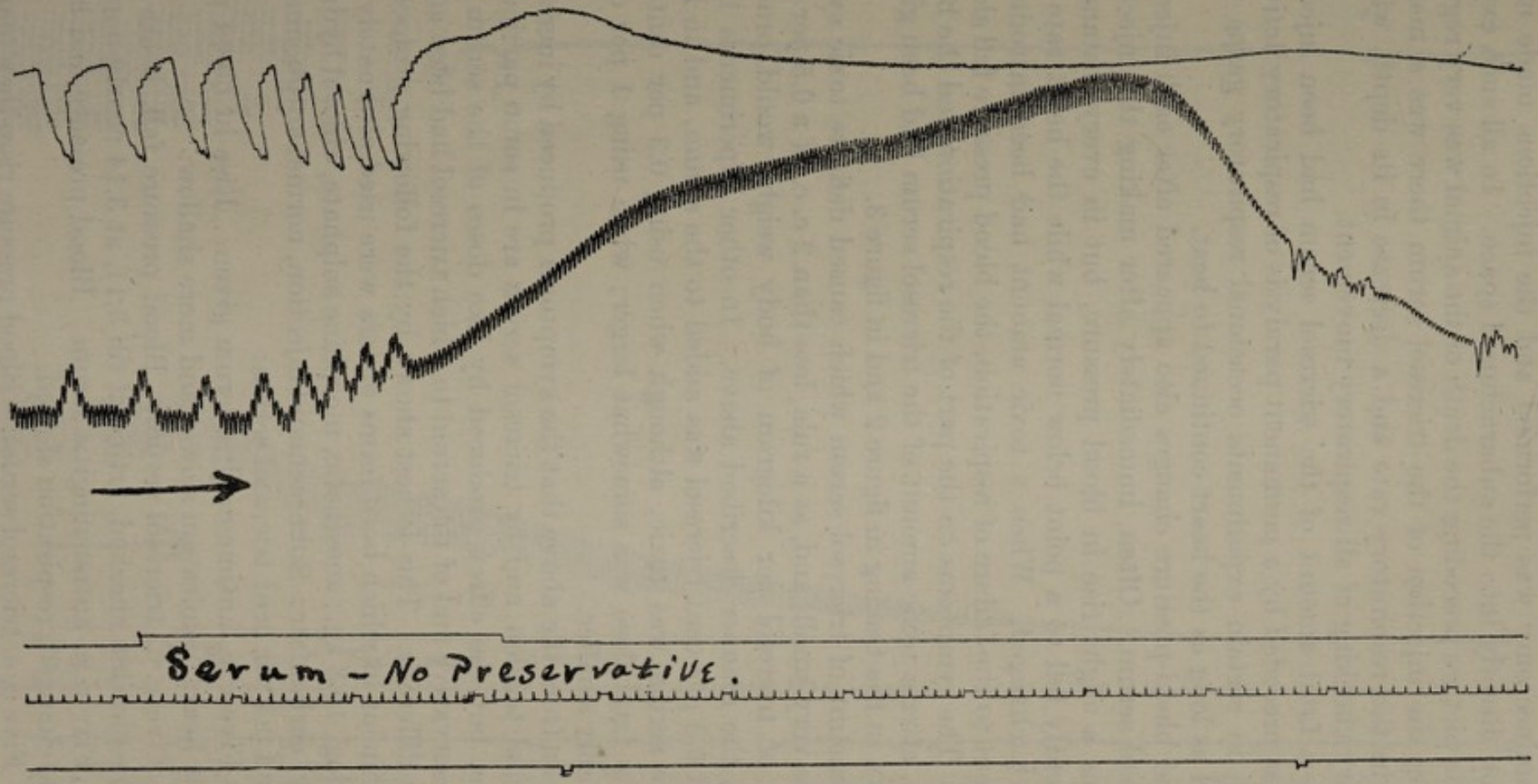

Fig. 1.—Upper tracing, respiratory; lower, blood pressure. Tracing shows manner of death following the seventh injection, each of 5 c. c., serum free from preservative. Dog weight, 7.8 kg.

In all of the later experiments as with the serum free from preservative, laminectomy was performed and the injections made under inspection directly into the subarachnoid space. In all such experiments the picture preceding the death of the animal was very regular. Following the injection of the tricresol serum there was a marked slowing in the respiratory rate and a decrease in its depth, with a temporary checking of all respiratory movement.

When a fatal amount of the tricresol serum had been injected, death was preceded by a permanent paralysis of respiratory activity, although in certain experiments occasional respiratory gasps were observed as long as the heart continued to beat.

Marked blood-pressure changes also appeared after each injection of tricresol serum. Often, immediately after making the injection, there was a slight rise in blood pressure, but in every instance it subsequently fell to a point below normal while the heart rate was not much changed. When a toxic amount had been introduced, subsequent to the failure of respiration, the blood pressure fell slowly to zero. The symptoms on the part of the respiration and the blood pressure, after a toxic amount of the tricresol serum had been given, are shown in the tracing in figure 2 and in figure 3.

The amount of tricresol serum which caused definite toxic symptoms was very small, and, as a rule, less than 2 c. c. of a 0.5 per cent solution of tricresol per kilogram of body weight would produce death in the manner described above. In other experiments 1 per cent and 0.3 per cent tricresol was added to the serum, and in both cases the serum was toxic, although when using 0.3 per cent tricresol the fatal dose was somewhat larger; when using 1 per cent, considerably smaller.

It is seen from the above that the symptoms produced by increased intracranial tension and by tricresol serum are in some particulars the same, but the effect produced by like doses of the serum free from preservative and of the serum to which tricresol had been added are very different. This is best shown by the following protocol of an experiment in which both forms of sera were used alternately.

Male dog, 10.8 kg.; anesthetic, morphine sulphate, chloral hydrate, and ethyl carbamate: Subarachnoid injection, normal antimeningitis serum and 0.3 per cent tricresol serum:

3.03. Five c. c. antimeningitis serum given. Rise in blood pressure, slow heart, respiration slower and more shallow.

3.08. Five c. c. tricresol serum. Blood pressure fall, heart rate slower; respiration checked, artificial till 3.11, at 3.14 nearly normal.

3.21. Five c. c. antimeningitis serum. Blood pressure and heart rate little changed, respiration slowed.

3.29. Five c. c. tricresol serum. Blood pressure rise, then marked fall; respiration checked 50 seconds.

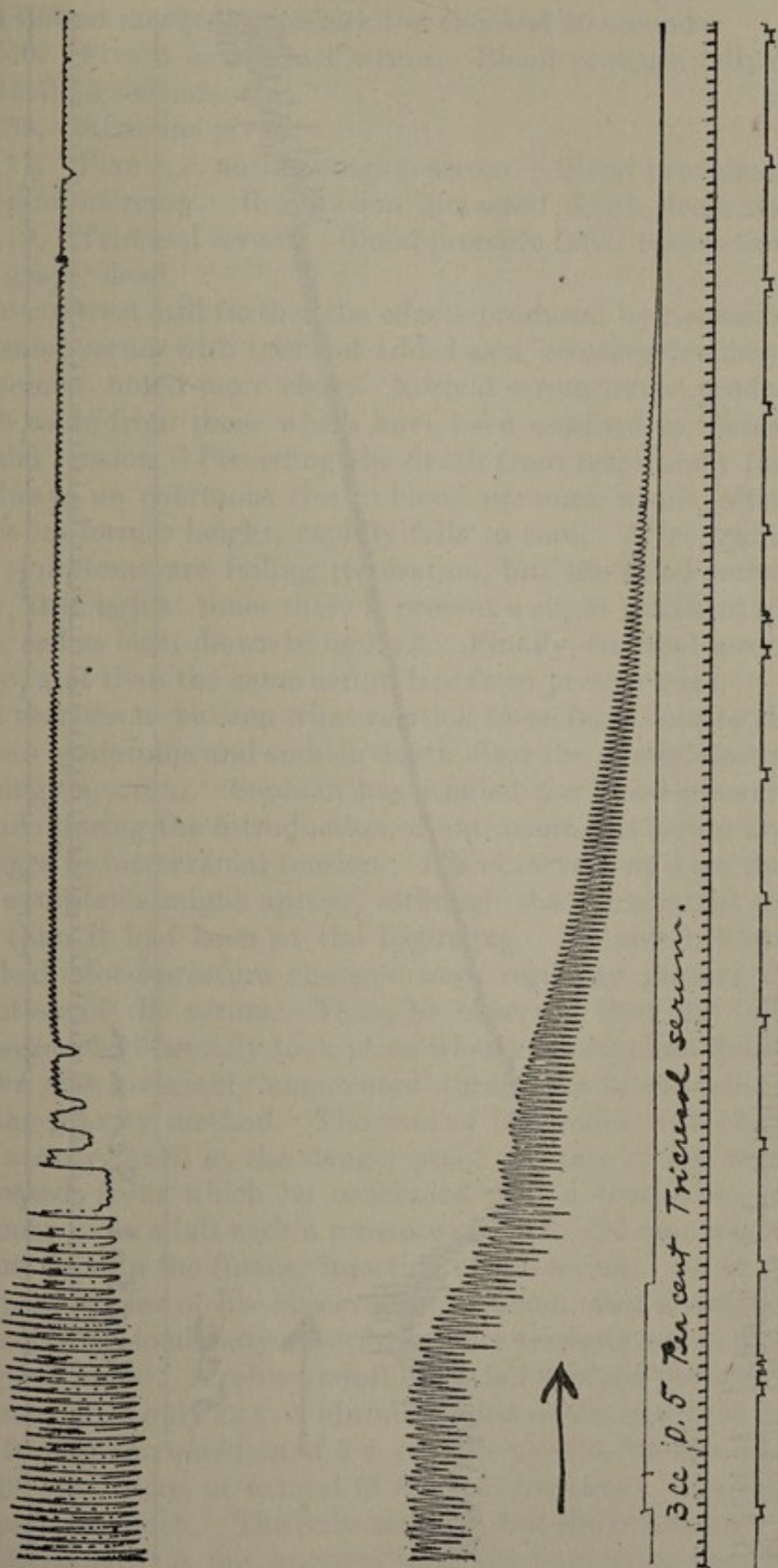

Fig. 2.—Upper tracing, respiratory; lower, blood pressure. Fatal amount of tricresol serum injected —; 10 c. c. of 0.5 per cent tricresol had been injected. This injection, 3 c. c.; dog weight, 8.9 kg.

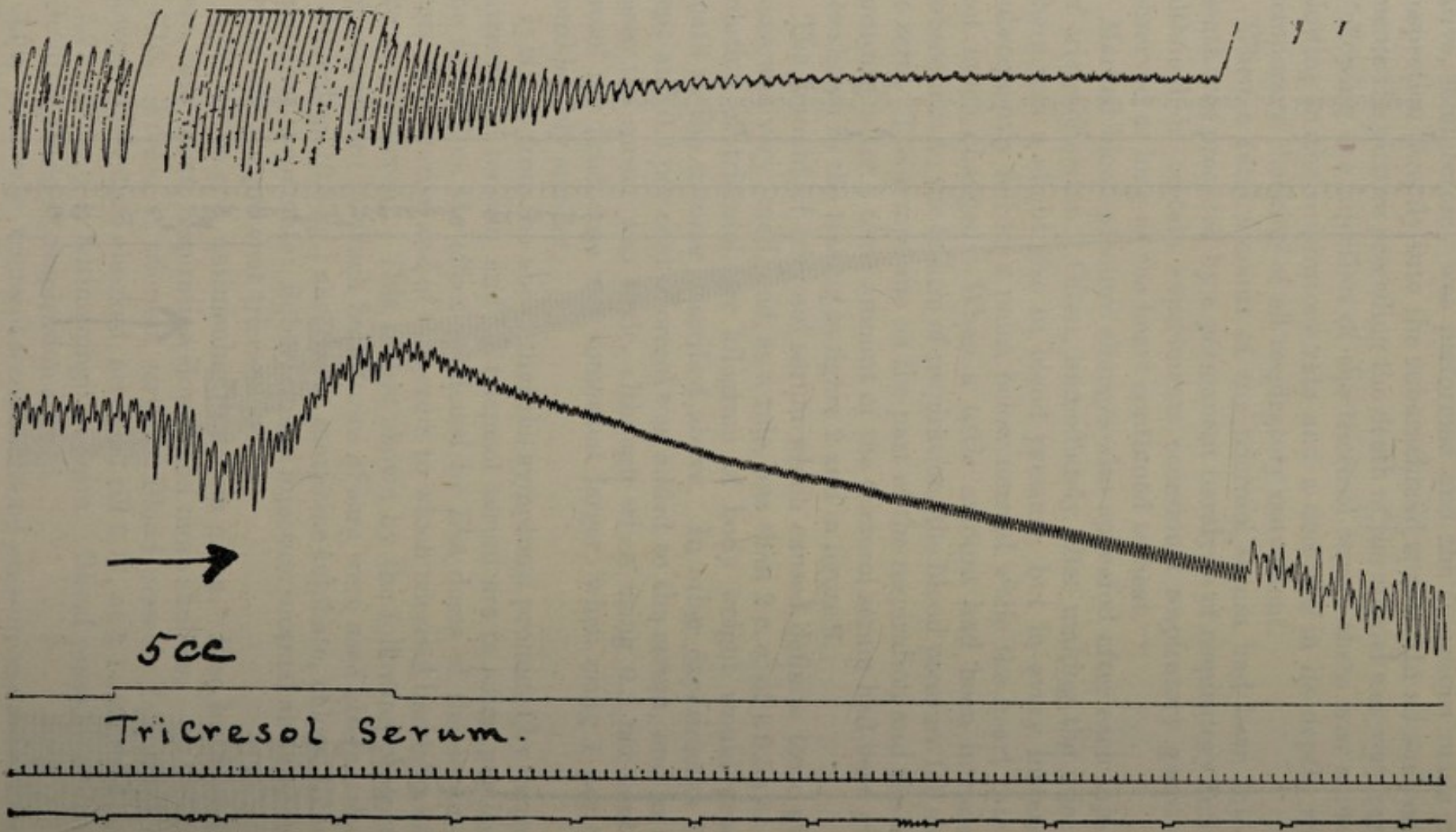

Fig. 3.—Upper tracing, respiratory; lower, blood pressure. This tracing shows the occasional rise of blood pressure after tricresol serum. Death from a total injection of 15 c. c. of 0.3 per cent tricresol. Dog weight, 6.5 kg.

3.38. Five c. c. antimeningitis serum. Blood pressure rise, heart rate slowed markedly; respiration checked 20 seconds.

3.49. Five c. c. tricresol serum. Blood pressure fall; respiration checked 65 seconds.

3.58. Atropine given.

4.11. Five c. c. antimeningitis serum. Blood pressure rise, 120 to 280 mm. mercury. Respiration increased depth, transient slowing.

4.19. Tricresol serum. Blood pressure falls; respiration checked; few gasps, dead.

To contrast still further the effects produced by normal serum and the same serum with tricresol added as a preservative may make the differences noted more clear. Normal serum never produced symptoms aside from those which have been ascribed to increased intracranial tension. Preceding the death from respiratory failure there is always an enormous rise in blood pressure, which, after rising to twice its former height, rapidly falls to zero. After tricresol serum the symptoms are failing respiration, but the *blood pressure always falls*, although at times there is present a slight transient rise in pressure, as has been shown in figure 3. Finally, tricresol serum is vastly more fatal than the same serum free from preservative.

It remains to be seen what relation these facts bear to the cases of serious symptoms and sudden death after the administration of antimeningitis serum. Sophian has studied the blood-pressure changes in man during the introduction of antimeningitis serum and also the changes in intracranial tension. His observations were that dangerous symptoms might appear, although the intracranial tension was less than it had been at the beginning. He noted, however, that marked blood-pressure changes were regularly present during the injection of the serum. Thus, he observed that the fall in blood pressure which usually took place when cerebrospinal fluid was withdrawn was invariably augmented during the introduction of serum by the gravity method. The rate of fall under such circumstances was steady, until at the danger point the rate of fall was suddenly increased; from which he concluded that a total drop of 20 mm. mercury in an adult with a pressure of 110 to 120 mm. was a safe indication to stop the further injection of the serum. As an example of the possibilities of his observation, Sophian cites a case with symptoms like those already described under tricresol serum in the protocol given above. A robust adult had a fall in blood pressure of 30 mm. mercury after only 12 c. c. antimeningitis serum had been introduced. The further introduction of 3 c. c. more caused the blood pressure to fall 30 mm. more, or a total of 60 mm. mercury. Clinical signs did not indicate shock. The pulse was fair, but the breathing was shallow and irregular. A few minutes later the patient stopped breathing, but immediate active treatment was followed by immediate response.

Here, then, is a case presenting symptoms similar to those noted in experiments with tricresol serum, but with blood-pressure changes entirely unlike those seen following the introduction of serum free from preservative. It seems evident therefore, accepting Sophian's observations in this case and on the changes in blood pressure, that tricresol is responsible for the cases which present alarming symptoms, and even death, and this conclusion seems all the more correct when one considers the difficulty with which alarming symptoms and death can be produced by an increase in the intracranial tension following the administration of serum free from preservative.

The experiments, therefore, substantiate Kramer's contention that tricresol is a dangerous preservative for sera which are to be introduced into the subarachnoid space, or, for that matter, in any way whereby they will be brought directly into contact with the vital nervous centers.

It would appear accordingly that death from the introduction of antimeningitis serum may result either from an increase in intracranial tension or from the presence in such serum of tricresol. The danger from tricresol, however, seems much greater and more certain than that which may possibly arise from increased pressure. And on that account an effort should be made to discover a serum preservative which would not have the peculiarly toxic action of tricresol on the central nervous system. Failing in that, antimeningitis serum should be dispensed in sterile containers free from any preservative.

In either event it would seem, from these experiments, that blood-pressure observations should be made in every case where cerebrospinal fluid is withdrawn or serum is introduced into the meninges. By this procedure warning will be given of any possible danger either from increased intracranial tension or from the poisonous action of any of the serum preservatives. Further, also, it would seem much wiser to discard the syringe as a method of introducing serum in favor of the gravity method, which permits the use of much greater care and gentleness in making the administration. Finally, it should be stated that too much emphasis can not be laid upon the advisability and importance of the early and free use of antimeningitis serum in all cases of epidemic cerebrospinal meningitis, even though occasionally its administration may be followed by untoward effects.

Work in developing a suitable preservative for serum will be carried out, the author making toxicological experiments. Dr. Anderson, Director of the Hygienic Laboratory, will investigate the effect of these substances in relation to their action on the potency of the serum.

My thanks are due to Surg. John F. Anderson, Director of the Hygienic Laboratory, who suggested that the author should make an experimental study of this subject.

2.—SOME NEW CHOLERA SELECTIVE MEDIA.[1]

By Joseph Goldberger, *Surgeon, United States Public Health Service.*

SELECTIVE PLATING MEDIA.

The publication by Dieudonné in 1909 of an alkaline blood agar as a selective plating medium for cholera has given rise to a considerable amount of work in this direction.

As originally described by Dieudonné, supplemented by Huntemüller, the medium is to be prepared as follows: Mix equal volumes of bovine blood and a normal solution of potassium hydrate and steam one-half hour. Mix 3 volumes of this alkaline blood with 7 volumes of neutral (to litmus) meat-broth agar and pour plates. Dry plates at 60° for half an hour, then keep at room temperature for at least 24 hours before using in order to free them from ammonia, the evolution of which during this time would prevent the growth of cholera itself.

This medium has been tested by a great many workers, both with artificial and with natural cholera stools, and while it is by no means as sharply selective as was at first thought, it has proved itself a very valuable auxiliary in the isolation of the cholera vibrio. Practically its chief defect is that plates are not immediately serviceable, so that the medium is not available in "first cases." Other, subordinate, defects are that blood is not always readily available and that the medium is opaque. To overcome these drawbacks various modifications and substitutes have been proposed.

With the view of making the medium immediately available for use, Neufeld and Woithe (1910) suggested adding 2 c. c. of a 10 per cent lactic-acid solution to each 100 c. c. of the fluid medium. After this addition and thorough admixture the plates are poured, allowed to set, and then dried for a few minutes at 60°. After this the plates are ready for use.

Thus prepared, however, the medium was found not to retain its selectivity for over 24 to 48 hours.

With the same object in view, Esch (1910) devised an alkaline-hemoglobin agar, which he prepares as follows: Dissolve 5 gms. horse hemoglobin (Merck) in 30 c. c. of a half-normal sodium-hydrate solution. Steam for half an hour. Mix 15 c. c. of this alkaline hemoglobin solution with 85 c. c. of neutral (to litmus) meat-broth agar, pour plates, and dry at room temperature with covers off. The plates are ready to use as soon as dry—a matter of about an hour.

[1] Manuscript submitted for publication Dec. 2, 1913.

In the same paper Esch describes a second modification in which he replaces the alkaline blood of the Dieudonné medium with an alkaline meat solution, which he prepares by dissolving with the aid of heat 500 gms. of beef in 250 gms. of normal sodium-hydrate solution. The resulting medium, however, like the original Dieudonné, is not ready for use for 24 hours after plates are poured. From the point of view of availability, therefore, this alkaline-meat agar has only the advantage that meat is more readily obtainable than blood.

To avoid the difficulty in the isolation of cholera, especially in the Tropics, caused by the frequent concomitant presence of the *B. pyocyaneus*, Crendiropoulo and Panayotatou (1912) devised an alkaline-peptone agar. This they prepare as follows: Five gms. of peptone (of Witte or of Chapoteau) are dissolved in 190 c. c. of distilled water, to which are added 8 c. c. of a 10 per cent caustic soda solution (if Witte's peptone has been used, or 10 c. c. if that of Chapoteau has been employed) and then heated for 3 to 5 minutes. After cooling this is filtered through paper and steamed for half an hour. The alkalinity of this solution should be between 0.28 per cent and 0.4 per cent, calculated in soda.

Of the alkaline-peptone solution, 4 parts are mixed with 6 parts of peptone agar (agar 3, peptone 1, salt 0.5, water 100) and plates poured. The medium is colorless, transparent, and ready to use at once.

Like the workers already cited, Pilon (1911) tried to devise a medium which would have the good points of Dieudonné's but be serviceable without delay. He believes that this object is attained by substituting a 12 per cent solution of crystalline sodium carbonate for the caustic potash solution in the preparation of the alkaline blood solution of the original Dieudonné. Of this (unheated) alkaline-blood solution Pilon mixes 3 volumes with 7 of melted neutral (to litmus) 4 per cent agar, and pours plates which are allowed to set with covers off. In half to three-quarters of an hour the plates are ready for seeding.

The drawback to the Dieudonné medium, caused by the lack of ready availability of the necessary blood, led Krumwiede, Pratt, and Grund (1912) to try egg as a substitute, with the result that they devised the following medium: Shake thoroughly equal volumes of egg (whole) and water. Then mix equal volumes of this egg water and a 12 per cent solution of crystalline sodium carbonate and filter through a thin layer of cotton. Steam the alkaline egg solution for 20 minutes.

Three volumes of the alkaline egg solution are mixed with 7 volumes of a 3 per cent peptone agar (salt 0.5, peptone 1, agar 3, water 100) and plates poured. Allow to set and dry with covers off for 20 to 30 minutes. They are then ready for seeding.

Of the foregoing media, the original Dieudonné, the modification suggested by Neufeld and Woithe, that of Esch, and that of Pilon were subjected to a very thorough comparative study by Haendel and Baerthelein (1912), who also included in this study a modification suggested by Hoffmann and Kutscher and one by Moldavan (1912).

Hoffmann and Kutscher avoid the delay in the preparation of Dieudonné plates by drying the medium and keeping it for use in the powdered form. For use, 8 gms. of the powder are dissolved in 100 c. c. of water and this solution steamed for half an hour. Plates are then poured, and are ready for seeding after drying at 60° for a short time.

Moldavan's modification consists in a reduction in the proportions of alkaline blood to agar from 3:7 to 1:4. A change that is claimed to make the plates available for use at the end of 6 instead of 18 to 24 hours.

As a result of their study Haendel and Baerthelein conclude that the original Dieudonné proved itself the most selective and the most reliable. The modification of Neufeld and Woithe, as regards selectiveness, they found to be close if not superior to the original, but was unreliable, in that cholera itself at times failed to develop.

Pilon's medium and that of Esch they regard as about equivalent and consider that these may be serviceable in "first cases," but regard the modification of Hoffmann and Kutscher as preferable for this purpose. For other than first cases they consider that the original Dieudonné is to be preferred because of its greater selectivity. With respect to Moldavan's modification, they found that this medium could not be depended on to permit cholera to grow in all instances, and on this account was, for practical purposes, to be classed with the medium of Neufeld and Woithe.

Shortly after the publication of Dieudonné's paper the writer began a study of the medium described by him, but the pressure of other matters prevented its continuance until last winter. Meanwhile a series of studies, some of the more important of which have been referred to above, appeared, that covered the ground which it had been planned to go over. It seemed desirable, nevertheless, to make a comparison of some of the media that had been proposed, particularly as none of the published comparative studies so far have included either the alkaline peptone agar of Crendiropoulo and Panayotatou, or the alkaline egg agar of Krumwiede, Pratt, and Grund. It is the desire of the writer to record at this time some of the results of this work.

The writer has worked with Dieudonné's medium, with the hemoglobin agar of Esch, and the modification proposed by Pilon, and so far as these are concerned his experience confirms that of Haendel and Baerthelein. In this work the media of Esch and Pilon were

prepared in strict accordance with the original directions. In the preparation of Dieudonné some minor modifications suggested by Neufeld and Woithe were adopted; thus, in preparing the alkaline blood solution the potassium and sodium hydrates were used interchangeably. After the plates hardened, strips of filter or blotting paper were inserted between dish and cover, the whole inverted and so placed in the incubator at 37° and kept there to dry and ripen[1] for 15 to 18 hours. After this they were kept at room temperature for a few hours before using or stored in the cold (15°) room. Pergola (1911) has suggested replacing the neutral (to litmus) meat broth agar with a plain peptone agar as simplifying the preparation of Dieudonné's medium. The writer has tested this and found that on the resulting medium the cholera vibrio grows much less luxuriantly than on the original. Instead of the simple peptone agar suggested by Pergola the writer has substituted a meat-extract agar (see p. 25) with the production of a medium practically identical with that of the original Dieudonné.

THE ALKALINE-PEPTONE AGAR OF CRENDIROPOULO AND PANAYOTATOU.

By reason of the extreme simplicity of its preparation (see p. 20) and the ready availability of the ingredients, this medium invited immediate attention, particularly as Crendiropoulo (1913) reports that he has employed this medium with very satisfactory results. The results obtained with it by the writer, however, were extremely disappointing. While cholera grew on it fairly well, its restraining action on the concomitant fecal bacteria appeared to be very slight. This rather surprising result is perhaps attributable to the quality of the peptone, an explanation that is made probable by the statement of Crendiropoulo and Panayotatou that not all brands of peptone are suitable, although they found Witte's (the peptone employed by the writer) to be satisfactory. If this explanation is correct, each batch of peptone must be tested to determine its suitability, in which event it constitutes a fatal objection to the general availability of the medium. On this account it was dropped from further practical consideration.

THE ALKALINE-EGG PEPTONE AGAR OF KRUMWIEDE, PRATT, AND GRUND.

A medium that is practically as easy to prepare (see p. 20) and the ingredients of which are as readily available as that of Crendiropoulo and Panayotatou is that devised by Krumwiede, Pratt, and Grund. This medium is described as being somewhat less selective than Dieudonné's, restraining the common fecal bacteria somewhat less

[1] The earlier workers believed that this process consisted of the dissipation of free ammonia; Pilon seems to have shown, however, that it is rather a process of conversion of the free caustic alkali into a carbonate.

than the latter. On the other hand, the cholera colonies on the egg medium are more distinctive, having, by transmitted light, a peculiar hazy look and appearing to be deep in the agar.

The results of the writer's tests of this medium were almost as disappointing as those of the medium of Crendiropoulo and Panayotatou. The egg medium was found to restrain not only the ordinary fecal bacteria, but also to markedly inhibit the growth of cholera itself. It was found, however, that the character given to the medium by the egg was such that when the cholera did grow the colony was, as originally described, decidedly characteristic. It seemed desirable, therefore, if possible, to so adjust or modify its ingredients or their relative proportions as to retain this highly desirable quality and yet allow cholera to grow well.

After a long series of tests made with these objects in view the following modification proved to be the most satisfactory:

(*a*) *Alkaline-egg solution.*—First, prepare an egg water by beating up a whole egg (or any multiple) with an equal volume of distilled (or good quality of tap) water. Then mix one volume of this with an equal volume of a 6.5 per cent solution of *anhydrous* sodium carbonate and steam for half to one hour.

(*b*) *Meat extract glucose agar.*—This is prepared as follows: Meat extract (Liebig's) 3, peptone (Witte) 10, sodium chloride (c. p.) 5, glucose 1, agar 30, distilled (or good quality of tap) water 1,000. Steam for three hours to bring the agar into thorough solution and decant. Distribute in flasks in convenient quantities and sterilize by steaming for an hour and a half. Store and use as needed.

For use, 1 volume of the alkaline-egg solution (*a*) is well mixed with 5 of the hot, freshly melted meat extract glucose agar (*b*) and plates poured.

The plates, if poured in a quiet room free from dust, may be left to set and dry with the covers off for half to three-quarters of an hour, when they are ready for seeding. If such a room is not available or if the plates are not for immediate use, it is much better to allow the plates to cool and set with the covers on and to get rid of the condensed moisture and dry the plates in the incubator at 37°. This is conveniently done by sliding the dish partly over the edge of the cover in an inverted position. In this way the chances of contamination with molds or air cocci is greatly reduced. In passing, it may be noted that this applies equally to the original Dieudonné or to any of its modifications. Many a valuable plate has been ruined by failure to observe this precaution.

The resulting medium is translucent and of a pale straw color.

The alkaline-egg solution undergoes, more or less rapidly on standing, a series of changes. Its original straw yellow first changes to a greenish or olive hue with the formation of a dark-green sedi-

ment on the bottom and sides of the containing flask. In the course of a few more days (at 15°) it recovers its original appearance and the sediment develops a creamy tint. These changes, however, in no appreciable degree affect the serviceability of the solution. Such a solution tested 83 days after it was prepared seemed in no way inferior to one that was freshly made. Plates kept 10 days at 15°, when compared with those freshly prepared, seemed to differ in no appreciable degree from the latter.

On this medium cholera and some noncholera vibrios grow luxuriantly, while the ordinary fecal organisms are markedly restrained. The vibrio colony retains the distinctive characters described by Krumwiede, Pratt, and Grund.

In the earlier tests various strains of colon, typhoid, dysentery, and pyocyaneus were employed. It was found, however, that the colon, typhoid, dysentery strains were always more markedly restrained than the *B. pyocyaneus* and the latter more readily than the *B. fecalis alkaligenes*. The later tests and comparisons were, therefore, made chiefly with cultures of *B. pyocyaneus* and *B. fecalis alkaligenes* in pure culture or mixed with feces with or without cholera. The cholera cultures used were chiefly the following: New York, 1,132; New York, 1,189; Naples, 152; Naples, 159; and Naples, 202; isolated, respectively, at the New York quarantine and in Naples in 1910, and now part of the Hygienic Laboratory collection.

Repeated comparative tests with Dieudonné's medium showed that on this alkaline-egg glucose-agar, as it will be referred to, cholera develops more luxuriantly and more distinctively, while its restraining action is but little less marked, the difference in the latter respect being mainly due to its being somewhat less restraining for cocci than is Dieudonné's medium.

As for noncholera vibrios, some of these grow well on this medium, as they do also on Dieudonné's; but there is not always a parallelism in this regard, some growing well on one and not on the other. An illustrative test will be given further on.

An objection that arises to this medium, as to that of Dieudonné's, relates to its lack of transparency. Although of relatively slight practical importance, an attempt was made to devise a medium that would possess this advantage in addition to the desirable qualities of the alkaline-egg glucose-agar or of Dieudonné's alkaline-blood agar. The writer believes that this is attained in the medium that will now be described.

AN ALKALINE MEAT-INFUSION AGAR.

To prepare this medium there must first be prepared an alkaline meat infusion and a 3 per cent meat-extract agar as follows:

(a) *Alkaline meat infusion.*—Put 500 gms. of finely chopped lean beef (round steak) in 500 c. c. of distilled (or of a good quality of tap)

water, mixing thoroughly, and let stand in cold (15°) room for 3 hours, stirring thoroughly two or three times during this period. At the end of this time squeeze out the juice, using the meat press to complete the extraction. Having done this, filter through cotton. Now adjust the reaction of the filtered infusion to the litmus neutral point by adding enough of a normal solution of sodium carbonate (=5.3 per cent of the *anhydrous* carbonate), so that the blue paper is no longer affected, while the red just begins to show the faintest tint of blue. In each 100 c. c. of this neutral meat infusion dissolve 2.5 gms. of *anhydrous* sodium carbonate, steam for half to one hour and pass through a paper filter. No special precuations with respect to asepsis need be taken in filtering. This is now ready for use. A specimen of such alkaline meat infusion, kept in the cold room 123 days, appeared to be as serviceable at the end of this time as one freshly prepared.

(b) *Three per cent meat-extract agar.*—This is prepared in the ordinary way, using the following ingredients: Meat extract (Liebig's) 10, peptone (Witte) 10, sodium chloride (c. p.) 5, agar 30, water 1,000. The reaction of this agar is allowed to stand without adjustment; it varies only slightly from +2 to phenolphthalein. It is preferable, though not necessary, to clear it in the ordinary way, after which it may be distributed in convenient quantities in Erlenmeyer flasks, sterilized by steaming for an hour and a half, and stored for use as required.

To prepare plates, mix one volume of the alkaline meat infusion (*a*) with three of the 3 per cent meat-extract agar (*b*) while hot and pour plates. In pouring plates the same precautions as those referred to above in connection with the alkaline-egg glucose-agar should be observed. The plates are ready for seeding as soon as dry.

The plated medium is transparent and has a slight brownish tint. On it cholera grows well, while the ordinary fecal organisms are markedly restrained. The vibrio colony on this medium is clear, circular, sharply outlined, and by transmitted light shows a slightly brownish tinted center. Its appearance reminds one strongly of that on the ordinary alkaline agar, the chief difference being the lack of the bluish opalescence so characteristic of the vibrio on agar and the possession of the faintly brownish tinted center. Another difference relates to its consistency, in which regard it approximates that of the vibrio colony on the egg-glucose agar or on the alkaline-blood agar of Dieudonné, being somewhat more firm or waxy in consistency, lacking the diffluent quality (to touch with the needle) of the colony on ordinary agar.

With respect to the degree of luxuriance of growth of the cholera colony, as also with respect to its restraining action for other bacteria, this medium appears to be very close to, if not identical with, that

of Dieudonné and of course differs in these respects from the egg-glucose agar in substantially the same degree as does Dieudonné's medium.

To determine these points, numerous and varied tests were made with pure cultures and with feces. In the following experiment cited as an illustration we have a comparison between the alkaline-egg glucose agar, the alkaline meat-infusion agar, and Dieudonné's medium:

Experiment No. 1.

A comparison of alkaline-egg agar, alkaline meat-infusion agar, and Dieudonné's medium: The cholera and noncholera vibrios were 4-hour peptone cultures, one loopful of each being streaked on a corresponding set of two plates, in series, in order to get well-separated colonies. The pyocyaneus, alkaligenes, and fecal cultures were 48-hour peptone cultures, one loopful of each being smeared with a bent glass rod on each of a corresponding pair of plates. The results, shown in the following table, were recorded after 18 hours at 37° and show what has been stated above, (1) that cholera grows about equally well on the meat-infusion agar and Dieudonné's medium but distinctly more luxuriantly on the egg-glucose agar, (2) that the noncholera vibrios grow about equally well on all three media, and (3) that so far as restraint for other bacteria is concerned the three media are close together.

TABLE 1.

Culture.	Media.		
	Dieudonné's.	Alkaline meat-infusion agar.	Alkaline-egg glucose agar.
Cholera:			
N. Y. 1132	29[1]	29[1]	34.[1]
N. Y. 1189	27	27	35.
Nap 152	27	31	35.
Nap 159	28	28	28.
Nap 202	26	26	30.
Noncholera:			
Nassik	15	17	17.
Metchnikovi	17	7	6.
Finkler-Prior	26	29	24.
B. pyocyaneus: Surface of agar control completely covered by a moist film of growth.	A patch of dustlike ($^2/_{17}$ mm.) colonies perceptible.	No perceptible growth.	No perceptible growth.
B. fec. alkalig.: Agar control is overgrown.	Abundant colonies $^8/_{17}$ mm..	Patches of dustlike ($^2/_{17}$ mm.) colonies present.	Like Dieudonné's.
Feces: Confluent growth on agar control.	Abundant growth colonies up to $^9/_{17}$ mm.	Sparse growth colonies up to $^{14}/_{17}$ mm.	Very scanty growth, colonies up to $^{15}/_{17}$ mm.

[1] These numbers indicate the diameter of representative colonies in seventeenths of a millimeter.

Varied tests were also made with mixtures of cholera and feces with results that were entirely in harmony with those of the experiment just cited.

The agglutinability of the cholera vibrio is not appreciably affected by its growth on either this or the alkaline-egg glucose agar so far as may be judged in performing the provisional macroscopic agglutination test on the slide. This test the writer has been in the habit of doing in the following way: A drop of a $\frac{1}{100}$ dilution of an agglutinating serum having a titer of about $\frac{1}{4000}$ is placed on a slide near its distal end. Close to this is placed a drop of a $\frac{1}{100}$ dilution of homologous normal serum. Now a little of the colony to be tested is picked up with the needle and carefully rubbed up in the drop of normal serum, which should show nothing but a uniform clouding. Having assured oneself of this, the drop of normal serum—now a bacterial suspension—is carefully mixed with the drop of agglutinating serum. By observing carefully, a beginning "curdling" of the suspension may be noted before the mixing has been completed and becomes *progressively* more marked.

The comparative merits of the two media above described may be summarized as follows:

Availability of ingredients.—There is very little difference in this respect; if any, it is in favor of the alkaline-egg glucose agar.

Simplicity and ease of preparation.—In this respect the alkaline-egg glucose agar has a slight advantage.

Restraining action.—Cholera grows more luxuriantly on the egg medium, but the ordinary fecal bacteria are somewhat more markedly restrained by the meat-infusion medium.

Appearance of vibrio colony.—The vibrio colony has a more distinctive appearance on the egg medium.

Appearance of plates.—The egg plate is translucent, the meat infusion is transparent.

Cost.—The egg medium is distinctly cheaper, and in work on a large scale this must be a consideration.

All things considered, the choice for practical work must fall upon the alkaline-egg glucose-agar medium. In comparison with Dieudonné's the alkaline-egg glucose agar permits of a more luxuriant growth of the vibrio colony; the vibrio colony is more distinctive in appearance; it exercises but little if any less restraint for the common fecal bacteria; its ingredients are more generally available, and, most notably, plates may be used at once.

SELECTIVE ENRICHMENT SOLUTIONS.

The use of alkaline proteid solutions in the preparation of selective plating media naturally suggests the possibility of their application in the preparation of a selective enrichment solution. This possibility did not escape Dieudonné, who mixed his alkaline-blood solution with peptone solution and with bouillon, but reported that these had no advantage over the ordinary peptone solution.

Hachla and Holobut (1909) confirmed Dieudonné's finding that the liquid medium has no advantage.

Neufeld and Woithe (1910) made a similar application. They treated peptone solutions with various proportions of blood alkali. but they found that such solutions had no advantage over the usual alkaline-peptone solution.

Pergola (1910) also found, as did Dieudonné, that the mixture of blood alkali and bouillon or neutral peptone were not especially advantageous. This worker reported better results from enrichment in a fluid blood-alkali gelatin from which he was able to recover the cholera vibrio even when in very small numbers. The author does not give definite directions for preparing this medium, but the inference is that after mixing the medium is not ready for use for 24 hours.

In a later publication (Pergola, 1911) he returns to this subject and gives the following directions: Make a 10 to 15 per cent gelatin solution in peptone water and bring to neutral litmus point; then to 70 parts of this gelatin peptone add 30 parts of the alkaline-blood solution and sterilize. No indication is given as to how soon after preparation this medium is ready for use. This medium, the author states, gives a specific enrichment, inasmuch as the growth of the cholera vibrio is not hindered, whereas it holds back the numerous other bacteria. Compared with the ordinary alkaline-peptone solution and with a gelatin-peptone solution, also suggested by this author, he found that they gave substantially identical results. Having found, however, that at times, although rarely, one of these solutions would give a positive result while the other two would be negative, he recommends the synchronous use of all three.

The problem of a selective enrichment solution along the lines under review was also studied by Kraus, Zeki Zia, and Zubrizciky (1911), and after various preliminary tests concluded that by the addition of alkaline blood to neutral (to litmus) broth a selective enrichment solution could be obtained. They give the following directions for the preparation of this solution:

Add 25 c. c. alkaline blood (prepared as for Dieudonné's medium) to 100 c.c. nutrient broth, neutral to litmus. This is first kept in an unstoppered flask for 3 hours at 50° and then for 24 hours at 37° C. It is then distributed in quantities of 5 c. c. in tubes ready for use.

It is to be noted, however, that these authors state that it is necessary always to make a preliminary test of each batch of alkaline-blood bouillon, for they found that the proportion of alkaline blood to bouillon may have to be varied with each batch of alkaline blood.

The advantage claimed for this over the usual peptone solution is its power to suppress the ordinary fecal bacteria while permitting the enrichment of the cholera vibrio.

The medium proposed by Kraus, Zeki Zia, and Zubrizciky was examined by Haendel and Baerthelein (1912). From the results of their tests they conclude that this medium gives markedly more favorable results than the peptone solution. They add, however, that in two instances it failed altogether and they emphasize what Kraus, Zeki Zia, and Zubrizciky had already suggested, that this medium may inhibit not only the ordinary fecal bacteria but also the cholera vibrio, and that therefore it will be necessary to test each batch of alkaline blood before making practical use of it. They point out, moreover, that as this test as well as the preparation of the medium takes time, the medium can hardly serve in first cases. In later cases, as also in "carriers," they believe this medium is decidedly to be recommended for general use because of its advantage over the peptone solution.

A medium for cholera enrichment of a somewhat different nature has been proposed by Ottolenghi (1911). This investigator recommends an alkaline bile, which he prepares as follows: Fresh ox bile is filtered through paper and to the filtrate there is added 3 per cent of a 10 per cent solution of crystalline sodium carbonate and one-tenth per cent of sodium nitrate. This is distributed in 5 c. c. quantities in tubes which are sterilized by exposure for 15 to 20 minutes in the autoclave under a pressure of half an atmosphere.

The advantages claimed for this medium are: (1) That in it the ordinary fecal bacteria either develop very sparsely or not at all; (2) that even when the cholera vibrios are present in but small numbers in the feces favorable enrichment takes place in a few hours; (3) that by its means the isolation of cholera is much hastened; (4) that it permits of planting of a fairly large amount, as much as 0.1 c. c., of feces.

Weiszkopf (1911) examined this medium and concluded that the alkaline bile was more selective than the peptone solution and should be used, as recommended by Ottolenghi, in conjunction with peptone solution.

Like Weiszkopf, Bochia (1911) also concluded that the alkaline bile gave excellent practical results, although he noted, as had Ottolenghi himself, that certain cholera strains multiplied more rapidly in the peptone solution than in the bile.

Similarly favorable results are reported by Haendel and Baerthelein (1912), who subjected Ottolenghi's medium to a variety of tests. They found that the bile gave better results in some cases than the peptone solution, but that it did not have sufficient advantage over the latter to justify replacing the peptone with the bile, for in some cases the peptone gave results when the bile failed.

In practice, they thought, it would be better to use both side by side, as they seem to supplement each other.

Finally, Ottolenghi's medium was subjected to an exhaustive study by Krombholz and Kulka (1912).

As a result of their very painstaking study they decide unfavorably on the claims for the alkaline bile, for they found that although this was an unfavorable medium for certain of the concomitant fecal bacteria, it was at the same time a much less favorable medium for cholera, at least of laboratory strains, than the peptone solution.

The divergence of their results from those of other workers they explain, and correctly so, the writer believes, to a difference in methods of testing, the technic employed by them being better calculated to furnish precise data for accurate appraisal of the media tested.

Moldavan tested Ottolenghi's bile medium side by side with the peptone solution on actual cholera stools and found that it had no practical advantage over the latter; indeed, in some cases it failed when the peptone solution was still positive.

In the course of the work with the selective plating media already described it occurred to the writer that fluid media having the same selective properties as the solid media might be obtained by simply omitting the agar from among the ingredients. A few preliminary tests soon showed, however, that the problem is not quite so simple as that. It was found that with the solid medium, that is in the presence of the agar, a very much greater proportion of alkaline-egg or alkaline-meat infusion was permissible—in fact desirable—than with the agar omitted. Continuing these tests, it was found that by combining certain proportions of an alkaline-egg solution with Dunham's peptone water or of the alkaline-meat infusion with Dunham's solution, media were obtained that markedly restrained the multiplication of the common fecal bacteria, but permitted the cholera vibrio to grow quite freely.

The technique of the preparation of these media is as follows:

ALKALINE-EGG-PEPTONE SOLUTION.

(*a*) Prepare an alkaline-egg solution by first shaking up or beating up an egg with an equal volume of water and then adding to this egg water an equal volume of a 5 per cent solution of *anhydrous* sodium carbonate. Steam three-fourths to one hour. (*b*) Prepare Dunham's solution: Peptone 10, salt 5, water 1,000.

For use mix (*a*) and (*b*) in proportion of 1:9. Run through paper filter; distribute in 10 c. c. quantities in tubes and sterilize by steaming 1½ hours, after which they are ready for use. This solution is of a pale-straw color. It is opaque, and a slight precipitate settles to the bottom of the tube on standing, but this interferes in no way with its serviceability. It will keep at least a week.

ALKALINE-MEAT-INFUSION-PEPTONE SOLUTION.

(*a*) Prepare an alkaline-meat infusion exactly as for the plating medium. (See p. 24.)

(*b*) Prepare Dunham's solution.

For use mix (*a*) and (*b*) in proportion of 1:9; filter through paper and sterilize by steaming 1½ hours.

This medium has a brownish tint and when fresh is slightly turbid. On standing a slight precipitate forms and the medium clears. The formation of the precipitate does not affect the serviceability of the solution. Like the egg-peptone, it will keep at least a week, and probably much longer, though a longer period was not tested.

Both solutions, as is evident, are easily and quickly prepared, but especially the alkaline-egg peptone.

The cholera vibrio multiplies with about the same freedom in both but distinctly less luxuriantly than in the alkaline-cholera-peptone solution. This is shown in experiment No. 2.

Experiment No. 2.

Dilutions of cholera suspensions in sterile tap water were made with the strains, Naples 152, Naples 159, and Naples 202. One c. c. of each was planted in a corresponding flask containing 50 c. c. of the enrichment solution to be tested. After incubating 7 hours plates were prepared from each flask and counts made with the following results:

Medium.	Culture.		
	N.159.	N.152.	N.202.
	Per c. c.	*Per c. c.*	*Per c. c.*
Alkaline peptone	15,000	12,000	44,000
Alkaline-egg peptone	2,700	1,650	10,800
Alkaline-meat-infusion peptone	2,000	3,400	12,100

The colon bacillus is markedly restrained and in about equal degrees by both solutions. The following experiments illustrate this:

Experiment No. 3.

Uniform volumes of a very dilute suspension of a 24-hour agar culture of a colon strain were planted in flasks containing 50 c. c. of the corresponding solutions. After seven hours incubation at 37° C. plates were prepared and counts made with the following results: Alkaline peptone, 950,000 per c. c.; alkaline-egg peptone, less than 10 per c. c.; alkaline-meat-infusion peptone, less than 10 per c. c.

In both solutions *B. pyocyaneus* and *B. fecalis alkaligenes* are distinctly inhibited, the former more markedly than the latter. The

degree of restraint exercised by the two solutions is substantially identical.

The following experiment (No. 4) illustrates these points.

Experiment No. 4.

A dilute emulsion of *B. pyocyaneus* and one of *B. fecalis alkaligenes* were prepared and 1 c. c. of each was planted into corresponding flasks of 50 c. c. of medium. After incubating seven hours at 37° plates were prepared and the following counts obtained:

Medium.	Cultures.	
	B. pyocyaneus "A."	B. fecalis alkaligenes "LL."
Alkaline peptone	4,700	60,000
Alkaline-egg peptone	60	20,000
Alkaline-meat-infusion peptone	95	17,000

The results of tests with feces alone or with mixtures of feces and cholera are in harmony with the foregoing tests, which were made with pure cultures.

In both solutions the bacteria of normal feces are markedly restrained, as is evident from the following experiment:

Experiment No. 5.

A suspension of normal feces was made in ordinary tap water, centrifugalized to throw down coarse particles, then run through a paper filter to remove clumps. Then 1 c. c., representing 21,000 bacteria, was planted in each flask containing 50 c. c. of medium, and after incubating at 37° for 7 hours plates were prepared and counts made with the following results: Alkaline peptone, 100,000,000 per c. c.; alkaline-egg peptone, 100,000 per c. c.; alkaline-meat-infusion peptone, 50,000 per c. c.

The restraining action of the solutions on the ordinary fecal bacteria is made manifest also by the greater ease in detecting cholera in mixtures with feces when plated on ordinary agar. This is shown in experiment No. 6.

Experiment No. 6.

A suspension of normal feces was prepared in ordinary tap water and centrifugalized to remove coarse particles. Then there was added to it some cholera culture (Naples 152), and this mixture run through a paper filter to remove clumps. Of this mixture $\frac{1}{10}$ c. c., representing $\frac{1}{10}$ c. c. of fecal emulsion and $\frac{1}{10,000}$ of a loop of the cholera (agar) culture, was planted in corresponding tubes of solution containing 10 c. c. each.

Plating was done at the end of 3 hours, one loopful being smeared on each of a pair of alkaline-agar plates and these incubated at 37° for 20 hours.

Results.—Cholera indistinguishable on the plates from the *alkaline-peptone* tube. On the plates from the *alkaline-egg-peptone* tube cholera distinguishable and fished with ease.

On the plates from the *alkaline-meat-infusion-peptone* tube there is to be noted little of anything but cholera.

It is evident from the foregoing that although cholera does not multiply as luxuriantly in the two special solutions as in the ordinary alkaline peptone, yet the multiplication is rapid enough to furnish practically satisfactory enrichment, at least, when cholera is present in moderate amounts. These points are perhaps more clearly brought out in the following experiment, in which the alkaline-egg-peptone solution only is compared with the ordinary alkaline-peptone solution.

Experiment No. 7.

A fecal emulsion was prepared and divided in three parts, to which cholera (Naples 152) was added in graded dilutions, so that the final mixtures represented in 1 c. c. of emulsion $\frac{1}{100.000}$, $\frac{1}{10.000.000}$, and $\frac{1}{1.000.000.000}$, respectively, of a loop of the cholera agar culture. One c. c. of each emulsion was planted in a corresponding tube (10 c. c.) of enrichment solution. At the end of 5 and of 9 hours enrichment a loopful was smeared on each of a corresponding pair of selective plates and the number of cholera colonies developing on these recorded.

The results are given in the following table:

TABLE No. 2.

Fecal emulsion.	5 hours (No. cholera colonies).	9 hours (No. cholera colonies).
+Cholera $\frac{1}{100.000}$ loop):		
Peptone	87	[1] 1,000
Alkaline-egg	68	232
+Cholera $\frac{1}{10.000.000}$ loop):		
Peptone	5	[1] 700
Egg	12	93
+Cholera $\frac{1}{1.000.000.000}$ loop):		
Peptone	28	[1] 500
Egg	2	39

[1] About.

For the purpose of further comparing the two solutions plates were prepared at the end of 24, 48, 72, and 96 hours from the tubes in which the emulsion with the smallest amount of cholera was planted. The following notes were made of the results:

Twenty-four hours.—Abundance of cholera on the plates from both solutions, but apparently in somewhat greater numbers from the peptone.

Forty-eight hours.—The plates show very few cholera colonies from the peptone solutions; large numbers are shown from the alkaline-egg peptone. It is evident that cholera is disappearing from the peptone tube.

Seventy-two hours.—Peptone tube, no cholera; alkaline-egg-peptone tube, cholera plentiful.

Ninety-six hours.—Same as 72 hours.

This clearly shows that although multiplication of cholera is more rapid in the peptone solution the cholera maintains itself decidedly longer in the special solution. The difference in this regard is more decidedly brought out in the following experiment:

Experiment No. 8.

Prepared a fecal emulsion in plain tap water to which a suspension of cholera (Naples 152) was added in such a way that 1 c. c. of the emulsion represented $\frac{1}{1000000}$ of a loop of the cholera (agar) culture. One c. c. of this emulsion was then planted in a corresponding tube containing 10 c. c. of solution. Plates were made daily from these tubes, with the following results:

	Day.						
	First.	Second.	Third.	Fourth.	Seventh.	Fourteenth.	Twenty-first.
Alkaline peptone	+	+	0	0	0	0	0
Alkaline-egg peptone	+	+	+	+	+	+	+
Alkaline meat-infusion peptone	+	+	+	+	+	+	+

As the table shows, cholera could no longer be recovered from the peptone tube at the end of 72 hours, while from the tube of alkaline-egg peptone and from that of alkaline meat-infusion peptone cholera was still present when the experiment was discontinued, namely, after 21 days' incubation.

It is evident from what has been presented that although cholera does not multiply as rapidly in the special solutions as in the ordinary peptone, yet the former clearly present a more favorable environment for the cholera when it is in the presence of large amounts of feces. This fact appeared to the writer to have important practical bearings.

It suggested the idea that by the use of these solutions when working with feces from convalescents or carriers in which the cholera vibrio might be present in minimal amounts it might be possible to recover cholera when the alkaline peptone failed. In the first experiment designed to determine this point there was planted with a constant amount of cholera a graded amount of feces, as follows:

Experiment No. 9.

A fecal emulsion was prepared with ordinary tap water. One c. c. of this was found to represent 3,500,000 bacteria. A suspension of cholera (Naples 152) was prepared in saline, such that 1 c. c. represented 120,000 vibrios.

Of the fecal emulsion 1 c. c., 4 c. c., and 9 c. c., respectively, were planted in corresponding flasks of 50 c. c. of alkaline peptone and of alkaline-egg peptone. Then to each flask was added 1 c. c. of the cholera emulsion, the whole well shaken, and then put in the incubator.

For the purpose of ready identification the flasks were designated as follows:

Peptone.

Feces 1 c. c.+cholera 1 c. c. is flask A.
Feces 4 c. c.+cholera 1 c. c. is flask B.
Feces 9 c. c.+cholera 1 c. c. is flask C.

Alkaline-egg peptone.

Feces 1 c. c.+cholera 1 c. c. is flask X.
Feces 4 c. c.+cholera 1 c. c. is flask Y.
Feces 9 c. c.+cholera 1 c. c. is flask Z.

At the end of 4½ and of 8 hours the flasks were well shaken and one loopful smeared on each of a corresponding pair of selective plates; the cholera colonies developing on these were counted, with the results shown in the following table:

TABLE No. 3.

Flask.	4½ hours.	8 hours.
	Colonies.	*Colonies.*
A	18	700
X	0	65
B	10	105
Y	1	142
C	0	0
Z	0	85

The results recorded in the foregoing table clearly indicate that as the proportion of fecal bacteria to cholera rises the alkaline peptone gives less and the alkaline-egg peptone relatively more favorable results. Beyond a certain point cholera, though still recoverable from the special solution, can no longer be recovered from the alkaline peptone even if the enrichment is prolonged, as is shown by the fact that plates made from flasks C and Z, after 24 hours' incubation, show no cholera in flask C. These results were confirmed in a second

experiment, in which the solutions were tested by planting a constant quantity of feces and a graded amount of cholera, as follows:

Experiment No. 10.

Normal feces known to contain *B. pyocyaneus* were emulsified in tap water and filtered through cheesecloth. Nine c. c. were then measured out in each of three tubes. To one there was then added 1 c. c. of a cholera suspension representing about 700 vibrios, to the second 1 c. c. representing about 70 vibrios, and to the third 1 c. c. representing about 7 vibrios. After thorough mixing there was then planted 1 c. c. of each fecal cholera mixture in each of a corresponding set of 3 tubes (of 10 c. c. of solution each) of alkaline peptone and of alkaline-egg peptone, respectively, and after mixing these thoroughly they were placed in the incubator. The seeding was made in triplicate in order to minimize the error that might arise from working with such dilute cholera suspensions.

After 7 hours' and again after 24 hours' enrichment one loopful of the surface growth from each tube was smeared on each of a corresponding pair of selective plates on which the cholera colonies were enumerated, with the results shown in the following table:

TABLE No. 4.

Feces containing per c. c.—		Tube.	Alkaline peptone.	Alkaline-egg peptone.
After 7 hours	0.7 vibrios	a	0	0
		b	0	0
		c	0	0
	7 vibrios	a	0	2
		b	3	4
		c	6	1
	70 vibrios	a	8	14
		b	39	18
		c	111	30
After 24 hours	0.7 vibrios	a	0	40
		b	0	0
		c	0	10
	7 vibrios	a	3	[1] 150
		b	34	[1] 75
		c	44	[1] 250
	70 vibrios	a	92	103
		b	56	36
		c	[1] 157	[1] 250

[1] About.

Comparative tests similar to the foregoing have been made also with the alkaline meat infusion with substantially identical results. The conclusion appears justified, therefore, that by means of these special enrichment solutions it is possible to recover cholera from feces when present in smaller amounts than by means of the ordinary alkaline peptone solution.

Attempts were made to isolate cholera from sewage, but here the special solutions proved to be markedly inferior to the alkaline peptone.

We may now summarize the properties of the special solutions hereinabove discussed with a view to estimating their value and their place in the diagnosis of cholera:

1. Both special solutions are easily and quickly prepared.

2. In these two solutions the cholera vibrio multiplies with about equal freedom but distinctly less luxuriantly than in the ordinary peptone solutions.

3. The multiplication of the ordinary fecal bacteria is markedly restrained in these solutions, notably that of the colon bacillus.

4. Although cholera does not multiply as luxuriantly in the two special solutions as in ordinary alkaline peptone, yet for practical purposes the multiplication is rapid enough to furnish, within the time usually allowed, satisfactory enrichment, provided cholera is present in moderate amounts.

5. In the presence of considerable or very large numbers of fecal bacteria the cholera organism maintains itself decidedly longer in the special solutions than in the ordinary peptone.

6. By means of the special solutions cholera may be recovered from feces when present in smaller amounts than by means of the ordinary peptone enrichment.

7. Certain sewage forms (*Proteus*) overgrow cholera in the special solutions so that these are quite unsuited for the purpose of isolating cholera from sewage and probably water.

Briefly, then, the advantage of these special solutions over the ordinary peptone lies in their furnishing a more favorable environment for cholera in the presence of the ordinary fecal bacteria. As a consequence of this they permit of the recovery of cholera from feces under circumstances when the ordinary peptone enrichment fails.

It is evident that they present no advantage where the vibrio is present in large or even moderate numbers. Under these circumstances, the method of enrichment in peptone solution and plating on ordinary agar is highly efficient. Difficulties arise, however, when dealing with cases in which the vibrio is sparse, although even these have been materially reduced by the introduction of the special selective plating media. It is felt, however, that they have not yet been altogether removed, and it is believed that it is in this field that the special enrichment solutions will be of service. In convalescents and healthy carriers, which furnish the special conditions referred to, the special solutions will permit of the enrichment of a much larger specimen of feces than has heretofore been permissible.

For routine purposes the writer would suggest the synchronous use of a tube of ordinary peptone solution and one of 10 c. c. of one of the special solutions, the former to be seeded with about 0.1 c. c., the latter with 1 c. c., of feces or fecal emulsion. Where it is

desired to examine larger amounts one may plant 5 c. c. in a flask of 50 c. c. of the special solution.

As the writer has been unable to determine any material difference in the usefulness between the alkaline-egg peptone and the alkaline-meat-infusion peptone, in choosing between them he would be guided entirely by the considerations of ease and simplicity of preparation and cost. On this basis he would choose and recommend for use the alkaline-egg-peptone solution.

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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 hookworn 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.

*No. 14.—Spotted fever (tick fever) of the Rocky Mountains; a new disease. By John F. Anderson.

*No. 15.—Inefficiency of ferrous sulphate as an antiseptic and germicide. By Allen J. McLaughlin.

*No. 16.—The antiseptic and germicidal properties of glycerin. By M. J. Rosenau.

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

*No. 18.—An account of the tapeworms of the genus *Hymenolepis* parasitic in man, including reports of several new cases of the dwarf tapeworm (*H. nana*) in the United States. By Brayton H. Ransom.

*No. 19.—A method for inoculating animals with precise amounts. By M. J. Rosenau.

*No. 20.—A zoological investigation into the cause, transmission, and source of Rocky Mountain "spotted fever." By Ch. Wardell Stiles.

*No. 21.—The immunity unit for standardizing diphtheria antitoxin (based on Ehrlich's normal serum). Official standard prepared under the act approved July 1, 1902. By M. J. Rosenau.

*No. 22.—Chloride of zinc as a deodorant, antiseptic, and germicide. By T. B. McClintic.

*No. 23.—Changes in the Pharmacopœia of the United States of America. Eighth Decennial Revision. By Reid Hunt and Murray Galt Motter.

No. 24.—The International Code of Zoological Nomenclature as applied to medicine. By Ch. Wardell Stiles.

*No. 25.—Illustrated key to the cestode parasites of man. By Ch. Wardell Stiles.

*No. 26.—On the stability of the oxidases and their conduct toward various reagents. The conduct of phenolphthalein in the animal organism. A test for saccharin, and a simple method of distinguishing between cumarin and vanillin. The toxicity of ozone and other oxidizing agents to lipase. The influence of chemical constitution on the lipolytic hydrolysis of ethereal salts. By J. H. Kastle.

*No. 27.—The limitations of formaldehyde gas as a disinfectant with special reference to car sanitation. By Thomas B. McClintic.

*No. 28.—A statistical study of the prevalence of intestinal worms in man. By Ch. Wardell Stiles and Philip E. Garrison.

*No. 29.—A study of the cause of sudden death following the injection of horse serum. By M. J. Rosenau and John F. Anderson.

†No. 30.—I. Maternal transmission of immunity to diphtheria toxine. II. Maternal transmission of immunity to diphtheria toxine and hypersusceptibility to horse serum in the same animal. By John F. Anderson.

†No. 31.—Variations in the peroxidase activity of the blood in health and disease. By Joseph H. Kastle and Harold L. Amoss.

†No. 32.—A stomach lesion in guinea pigs caused by diphtheria toxine and its bearing upon experimental gastric ulcer. By M. J. Rosenau and John F. Anderson.

*No. 33.—Studies in experimental alcoholism. By Reid Hunt.

†No. 34.—I. *Agamofilaria georgiana* n. sp., an apparently new roundworm parasite from the ankle of a negress. II. The zoological characters of the roundworm genus *Filaria* Mueller, 1787. III. Three new American cases of infection of man with horsehair worms (species *Paragordius varius*), with summary of all cases reported to date. By Ch. Wardell Stiles.

†No. 35.—Report on the origin and prevalence of typhoid fever in the District of Columbia. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle. (Including articles contributed by Ch. Wardell Stiles, Joseph Goldberger, and A. M. Stimson.)

†No. 36.—Further studies upon hypersusceptibility and immunity. By M. J. Rosenau and John F. Anderson.

†No. 37.—Index-catalogue of medical and veterinary zoology. Subjects: Trematoda and trematode diseases. By Ch. Wardell Stiles and Albert Hassall.

No. 38.—The influence of antitoxin upon post-diphtheritic paralysis. By M. J. Rosenau and John F. Anderson.

†No. 39.—The antiseptic and germicidal properties of solutions of formaldehyde and their actions upon toxines. By John F. Anderson.

†No. 40.—1. The occurrence of a proliferating cestode larva (*Sparganum proliferum*) in man in Florida, by Ch. Wardell Stiles. 2. A reexamination of the type specimen of *Filaria restiformis* Leidy, 1880=*Agamomermis restiformis*, by Ch. Wardell Stiles. 3. Observations on two new parasitic trematode worms: *Homalogaster philippinensis* n. sp., *Agamodistomum nanus* n. sp., by Ch. Wardell Stiles and Joseph Goldberger. 4. A reexamination of the original specimen of *Tænia saginata abietina* (Weinland, 1858), by Ch. Wardell Stiles and Joseph Goldberger.

†No. 41.—Milk and its relation to the public health. By various authors.

†No. 42.—The thermal death points of pathogenic microorganisms in milk. By M. J. Rosenau.

†No. 43.—The standardization of tetanus antitoxin (an American unit established under authority of the act of July 1, 1902). By M. J. Rosenau and John F. Anderson.

No. 44.—Report No. 2 on the origin and prevalence of typhoid fever in the District of Columbia, 1907. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle.

†No. 45.—Further studies upon anaphylaxis. By M. J. Rosenau and John F. Anderson.

No. 46.—*Hepatozoon perniciosum* (n. g., n. sp.); a hæmogregarine pathogenic for white rats; with a description of the sexual cycle in the intermediate host, a mite (*Ielaps echidnimus*). By W. W. Miller.

No. 47.—Studies on Thyroid: I. The relation of iodine to the physiological activity of thyroid preparations. By Reid Hunt and Atherton Seidell.

No. 48.—The physiological standardization of digitalis. By Charles Wallis Edmunds and Worth Hale.

No. 49.—Digest of comments on the United States Pharmacopœia. Eighth decennial revision for the period ending December 31, 1905. By Murray Galt Motter and Martin I. Wilbert.

No. 50.—Further studies upon the phenomenon of anaphylaxis. By M. J. Rosenau and John F. Anderson.

No. 51.—Chemical tests for blood. By Joseph H. Kastle.

No. 52.—Report No. 3 on the origin and prevalance of typhoid fever in the District of Columbia (1908). By M. J. Rosenau, Leslie L. Lumdsen, and Joseph H. Kastle.

No. 53.—The influence of certain drugs upon the toxicity of acetanilide and antipyrine. By Worth Hale.

No. 54.—The fixing power of alkaloids on volatile acids and its application to the estimation of alkaloids with the aid of phenolphthalein or by the Volhard method. By Elias Elvove.

No. 55.—Quantitative pharmacological studies; adrenalin and adrenalinlike bodies. By W. H. Schultz.

No. 56.—Milk and its relation to the public health. (Revised edition of Bulletin No. 41.) By various authors.

No. 57.—I. The presence of tubercle bacilli in the circulating blood in clinical and experimental tuberculosis. By John F. Anderson. II. The viability of the tubercle bacillus. By M. J. Rosenau.

No. 58.—Digest of comments on the Pharmacopœia of the United States of America (eighth decennial revision) and the National Formulary for the period ending December 31, 1906. By Murray Galt Motter and Martin I. Wilbert.

No. 59.—The oxidases and other oxygen catalysts concerned in biological oxidations. By Joseph Hoeing Kastle.

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