TRIMETHYLAMINE AS A NORMAL CONSTITUENT OF HUMAN BLOOD, URINE AND CEREBROSPINAL FLUID

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In the course of some experiments undertaken with a view to ascertain the variation of trimethylamine in the urine in cases of nervous disease, we have modified the methods for the estimation of trimethylamine in urine in such a way as greatly to accelerate the process. The following paper contains an account of this method, together with observations on blood and cerebrospinal fluid, which go to prove that trimethylamine is normally present in these fluids.

The method of de Filippi¹ for the estimation of trimethylamine in urine, which has subsequently been employed by Bauer,² Caccio,³ and one of us,⁴ depends upon the collection of the total volatile alkali from the urine by distillation with strong caustic potash, the evaporation of the mixed ammonium chlorides in the distillate, and the repeated extraction of these with alcohol, which removes the hydrochlorides of the methylamines and leaves the bulk of the ammonium chloride behind. The final alcoholic extract is then evaporated to dryness, the residue dissolved in water, and treated in a special all-glass distillation apparatus with sodium hypobromite. By careful preliminary experiments, which were confirmed by Bauer, de Filippi proved that all the bases present, except trimethylamine, are decomposed by this reagent. Before distillation, however, it is necessary to remove the excess of hypobromite, and this is done by acidifying and distilling off the bromine liberated. The liquid remaining in the flask is then again made alkaline, and the trimethylamine distilled off and collected in standard acid. These processes are extremely tedious, some two and a half days being required for each estimation, and this delay militates against the use of the

1. Filippo de Filippi, *Zeitschr. physiol. Ch.*, 433, 1900.
2. Bauer, *Beitrage*, 11, 502.
3. Caccio, *International Congress of Applied Chemistry*, London, 1909.
4. Golla, *Quarterly Journal of Medicine*, II, 231, 1909.

method for experiments on the metabolic variations of the trimethylamine output in the urine under varying conditions of diet.

We therefore modified the method in several important directions, which render the process much more expeditious and at least as accurate as the original one. Our modifications consist in :—(a) The distillation of the urine in a vacuum at a low temperature, so that only the free ammonia and the trimethylamine are obtained in the distillate. The troublesome process of repeated extraction of the evaporated distillate with alcohol to separate the methylamines from the enormous quantity of ammonium chloride obtained by the original method, is now replaced by a single extraction, as comparatively little ammonium chloride has to be removed. (b) After treatment of the mixed ammonium and methylamine hydrochlorides with sodium hypobromite, the excess of hypobromite is destroyed by the addition of sodium arsenite solution. This reagent reacts instantly, and avoids the troublesome distillation of free bromine in a special all-glass apparatus as in de Filippi's process.

The procedure followed by us may be detailed as follows :—

Taking advantage of the fact shown by Schlösing that urine, heated *in vacuo* with baryta water at a temperature below 40° C. gives off only its free ammonia, and having satisfied ourselves that small quantities of trimethylamine could be determined in a similar way, we place one and a half to three litres of urine in a strong five-litre bolthead flask, and add milk of baryta till the reaction is permanently alkaline. A few pieces of pumice, to ensure even boiling, are thrown in, and about 5 c.c. of purified paraffin oil added to prevent the liquid frothing. We found the so-called odourless paraffin very effective for the purpose. Some of the paraffin distils over, but its presence in the distillate causes no trouble. The bolthead flask is connected with a long condenser, the end of which dips into a large receiver flask. The latter is connected with two smaller wash-bottles containing hydrochloric acid, then to a trap bottle, and then to a powerful water pump. The absorption apparatus is placed in a box and surrounded with a good freezing mixture. The urine is distilled at a temperature of from 25° to 30° C. and under a pressure of about 20 mm. for two to three hours. From one and a half litres of urine we obtain about 500 c.c. of distillate, which is washed out and evaporated to dryness. To economise hypobromite it is advantageous to extract the residue with alcohol, filter, evaporate the extract to dryness, and again extract with a little alcohol. The aqueous solution is then placed in a small distillation flask, and connected with a condenser dipping into a receiver

which is fitted with a side U-tube containing a little strong hydrochloric acid. The bulk of the distillate solution need not be large. Sodium hypobromite is added through a dropping funnel till a permanent golden colour remains. Air is now drawn through the whole apparatus, being admitted through the tap of the dropping funnel, and the traces of trimethylamine liberated by the alkali of the hypobromite solution are thus collected in the acid of the U-tube. The excess of hypobromite is now removed by the addition of an alkaline solution of sodium arsenite, and the distillation is completed in the usual way, the amount of trimethylamine being estimated by titration of the neutralised acid, methyl orange being used as an indicator. To ensure good results the following points are worthy of note:—(1) During the vacuum distillation the receiver should be thoroughly cooled. (2) The distillate should be reduced to small bulk before treatment with the hypobromite solution. (3) The hypobromite should be fresh. It is most important that in its preparation the temperature should be kept down to zero during the addition of the bromine to the sodium hydrate solution. (4) If any doubt exists as to the presence of ammonia in the final distillate a few drops of this may be tested with Nessler's reagent, which gives no colour or precipitate with a dilute solution of trimethylamine.¹

An estimation of the trimethylamine in 1,500 to 2,000 c.c. of urine can be carried out by this process in about four hours, but good results can be obtained with a litre of urine in considerably less time. The free ammonia of the urine can be determined simultaneously by using a known excess of acid in the first distillation process. Thus 1,500 c.c. of fresh normal urine gave total volatile alkali equivalent to 365 c.c. decinormal sodium hydrate solution. Of this the trimethylamine was found to be equivalent to 3.7 c.c., so that the free ammonia was equivalent to 361.3 c.c. These figures give for 1,500 c.c. of urine a free ammonia content of 0.6142 gram (0.0409 per cent.), and trimethylamine 0.02183 gram—numbers which are in close agreement with those found by other observers and by ourselves, for normal urine.

In order to compare the results obtained by the modified method with that of the original de Filippi process, we made the following parallel

1 See Delépine, C.R. CXXII, 1064, 1172, 1896, who has shown that pure di- and trimethylamines give white precipitates with Nessler's reagent which disappear on the addition of water. This we can confirm. Bauer (*loc. cit.*), however, speaks of a brown coloration with trimethylamine. Monomethylamine gives a yellow precipitate which does not disappear on dilution.

experiments. A large sample of fresh normal urine was divided into equal parts for each comparison.

(1) One and a half litres of urine gave by the above method 0.020 gram of trimethylamine; by that of de Filippi 0.018 gram.

(2) Two litres of urine gave by the above method 0.025 gram of trimethylamine; by that of de Filippi 0.020 gram.

(3) One and a half litres of urine gave by the above method 0.017 gram of trimethylamine; by that of de Filippi 0.015 gram.

The results are sufficiently close, and, if anything, point to the fact that less loss occurs in our process than in the more lengthy one of de Filippi.

REACTIONS OF AND TESTS FOR TRIMETHYLAMINE

The discovery of Mott and Halliburton¹ that choline occurred in the cerebrospinal fluid of patients suffering from degenerative nervous diseases led to the investigation of various tests for the detection of minute quantities of that substance. Rosenheim,² who worked the matter out carefully, recommends three reagents for this purpose, which have since been extensively employed. These are (a) Potassium periodide (Florence's test); (b) alloxan—a colour test; (c) Bismuth periodide (Dragendorff's reagent as modified by Kraut³). The macro- and microchemical reactions of pure choline and pure neurine have been tabulated by Gulewitsch,⁴ who has also stated the limits of sensitiveness of the above reagents, and also those of phosphotungstic and phosphomolybdic acids, picric acid, etc.

In order to be in a position to detect and differentiate minute quantities of trimethylamine such as might conceivably be obtained from cerebrospinal fluids, we made an examination of the action of the above-mentioned reagents on trimethylamine. The pure hydrochloride obtained from Kahlbaum was employed. Its solution gave no coloration with Nessler's reagent. For most experiments it was, moreover, purified by treatment with sodium hypobromite and distillation into pure hydrochloric acid. As a result of the experiments made by us it was found that trimethylamine in very small quantities behaves with the reagents

1. Mott and Halliburton, *Phil. Trans.*, 4, 1899

2. Rosenheim, *J. Physiol.*, XXXIII, 221.

3. Kraut, *Ann. d. Chem.*, CCX, 310.

4. *Zeit. physiol. Chem.*, XXIV, 514; XXVI, 175.

employed almost exactly as does choline, and that whereas slight differences manifest themselves when comparatively strong solutions are employed, with very dilute solutions there is practically no difference in the reactions of the two substances. It is thus rendered possible that some of the 'choline' previously found in various fluids and tissues may have consisted of trimethylamine.

Our observations are as follows :—

1. *The Alloxan Test.* Parallel tests with purified hydrochlorides of choline and trimethylamine were made, using a strong solution of alloxan. The rose-red colour turning to deep blue-violet on addition of potassium hydrate was given by both substances, and it was not found possible to differentiate them in this way.

2. *The Bismuth Iodide Test.* The solution used was prepared according to the directions of Kraut, as this was found by Rosenheim to be more sensitive towards choline than the original Dragendorff's reagent. We made it, however, half as strong again as required in Kraut's formula, and for microscopic tests with minute amounts of substances we found it best to add the reagent directly to the solid residue to be tested. The presence of water seems to disturb the equilibrium of the reagent, and is apt to modify the formation of the crystals. 0.1 to 0.05 c.c. of solutions of choline and trimethylamine of known strengths were evaporated to dryness at a very gentle heat on a microscopic slide, and the dried residue covered with a slip and treated with a drop of the reagent. 0.02 milligram of trimethylamine hydrochloride thus deposited on a slide gave a distinct smell of trimethylamine on treatment with one drop of potassium hydrate solution, and the same weight treated with Kraut's solution gave a well-marked yellowish-red colour visible to the naked eye, whilst under the microscope short six-sided prisms and column-shaped crystals were observed which were indistinguishable from those given by an equal amount of choline. The macroscopic appearance of the choline precipitate, however, is a bright red as distinguished from the yellowish-red of trimethylamine. Crystallisation of the choline precipitate also takes place less readily than in the case of trimethylamine and the frequency with which an amorphous precipitate is obtained with choline led us at first to hope that the test would constitute a distinction between the two substances. This hope, however, proved to be vain, as when crystallisation occurs the two precipitates are indistinguishable.

3. *The Potassium Periodide Test (Florence's Test).* The proportions required for this reagent are given by Florence as Potassium iodide: iodine: water, as 5:8:100 (i.e. approximately the proportions required by the formula KI_3); whilst Rosenheim, who used it extensively as a test for choline, gives the proportions 6:2:100. We found the proportion 4:4:100 most convenient. This test is extremely sensitive, both for choline and trimethylamine. We found after careful comparison using the dry slide method, that crystals formed by choline periodide do not vary in appearance, whether there was much or little choline present. Choline always showed the characteristic isolated rectangular needles, shaped like haemin crystals, which are figured by Rosenheim.¹ On adding a drop of the reagent to the dry choline salt innumerable droplets are observed, which rapidly crystallise. The crystals are, however, more stable than those which appear to have been obtained by Rosenheim, and when once they have resolved into drops we did not succeed in reforming them. When a solution of trimethylamine hydrochloride containing as much as 0.02 milligram of the salt is evaporated on a slide and treated with a drop of the reagent, an immediate formation of black oily drops takes place which rapidly turns to crystals visible to the naked eye. Under a low power (fig. 1) these are seen to consist of dagger-shaped crystals, which group themselves in a very characteristic cross shape. When smaller amounts of trimethylamine, such as about 0.001 milligram are used, a cloud of black drops is immediately observed on the addition of the reagent, and these more or less quickly crystallise, forming minute rod-like crystals (fig. 2), which very closely resemble those of choline (fig. 4). It will be seen from the photographs that in the highly dilute solutions used it is practically impossible to differentiate between choline and trimethylamine by this reaction.

COMPOSITION AND PROPERTIES OF TRIMETHYLAMINE PERIODIDE

The remarkable ease with which the crystals of trimethylamine periodide are formed, even in very dilute solution, led us to examine them more closely with a view to ascertaining whether their formation could not be employed as a method for the estimation of small quantities of trimethylamine. Although a great deal of information has been collected with regard to the solid organic polyiodides,² the data refer to

1. *J. Physiol.*, XXXIII, 222.

2. Abegg, *Zeit. anorgan. Chem.*, L, 403 (1906); Geuther, *Annalen*, CCXL, 66.

iodides of the type $\text{NR}_4\text{I.I}$, and the characteristic crystalline derivative found in Florence's test does not seem to have been examined or its crystalline nature recognised by physiological workers.¹ Staněk² states that choline periodide has the formula $\text{C}_5\text{H}_{14}\text{NOI.I}_8$, agreeing in this respect with the opinion of Abegg,³ that all these organic iodides yield a periodide of the formula R.I_9 . This choline enneaiodide described by Staněk is formed quantitatively, and is permanent, so that it can be washed, dried, and the nitrogen content determined by Kjeldahl's process. The periodide of trimethylamine did not prove so satisfactory. Trimethylamine periodide was apparently first described by J. Weiss, and in a communication by Schmidt⁴ some analytical data are given which point to a formula $\text{NMe}_3\text{HI.I}_4$. The substance was also prepared by Delépine,⁵ who stated that he obtained figures which supported this conclusion. We have not, however, been able to find any very precise account of this body, and of the conditions under which it was formed. It was important, for example, to ascertain whether the composition of the crystals varies with the iodine concentration of the reagent; whether, seeing that they are generally prepared from the hydrochloride, they were free from chlorine, and also under what conditions; if any, they were stable.

We have investigated these points, and have arrived at the following conclusions:—On the addition of a strong solution of trimethylamine hydrochloride to an excess of the reagent a brown precipitate forms which rapidly resolves itself into a deposit of large flake-like crystals showing a blue-green dichroic effect. These can be filtered off through glass wool on the pump, and washed with a little ice-cold water to remove excess of the reagent. In the moist state, however, water continues to abstract iodine from them, and it is not possible to obtain a wash water free from iodine, though the loss, when very cold water is used, is very small. The crystals were allowed to dry on a tile and found to be comparatively stable in the air, a slight loss of iodine taking place in warm weather. All the usual organic solvents extract iodine from them though very slowly. Benzene appears to act most rapidly as a solvent, whereas pentane of b.p. 60°C . is almost without solvent action. The crystals,

1. Cf. Staněk, *Zeit. physiol. Chem.*, XLVII, 83, 1906.
2. Staněk, *ibid.*, XLVI, 280, 1905; XLVII, 83; XLVIII, 334, 1906.
3. *Loc. cit.*, 405.
4. Schmidt, *Annalen*, CCLXVII, 258, 1892.
5. Delépine, *Annales de Chim. et de Phys.*, vii, VIII, 439, 1896.

if pressed with a glass rod partially liquefy, forming a semi-solid mass, but harden and crystallize again on being left. When slowly heated they fall together to a black pasty fluid at about 66° C., and decompose between 150° and 180° C. On ignition no residue is left, so that the compound contains no potassium, and, as will be seen below, it is free from chlorine. An analysis by Carius' method of a sample washed with a little ice water, dried and washed with pentane, gave the following result:—

0.4837 gave 0.8059 AgI—Iodine found 90.2 per cent.

$\text{NMe}_3\text{HI}\cdot\text{I}_3$ requires 89.4 per cent, $\text{NMe}_3\text{HI}\cdot\text{I}_4$ requires 91.34 per cent, and $\text{NMe}_3\text{HI}\cdot\text{I}_5$ requires 92.72 per cent of iodine.

To obviate the uncertainty of drying the crystals for analysis the ratio $\text{NMe}_3:\text{I}$ in the freshly-formed product was determined in the following way. The crystals were washed with small quantities of ice water till the filtrate was very pale. They were then put into a large flask connected with a condenser and receiving apparatus charged with standard acid for collecting the trimethylamine. Pure potash solution was then run into the flask, and the solution gently warmed till the crystals had dissolved to a clear solution. The trimethylamine was distilled off and estimated as usual.

The liquid remaining in the flask contained iodide and iodate of potassium. It was made up to a known volume, acidified, and a portion titrated with sodium thiosulphate, no difference in the value being obtained if potassium iodide was previously added.

Another measure of the liquid after acidification was extracted with chloroform to remove the iodine, and as it was found that the solution still contained potassium iodide, the iodine in this was estimated by treatment with hydrogen peroxide, followed by extraction with ether. The sum of these two values gave the total iodine. The liquid left after the removal of the iodine by ether gave no precipitate with silver nitrate, so that chlorine was absent. Some of the results obtained are as follow:—

Specimen 1. Made from reagent 4 KI : 4 : 100. Crystals washed four times with ice water.

Specimen 2. Reagent 6 KI : 2 : 100.

Specimen 3. Reagent 4 KI : 1.6 : 100. (Proportions given in grams, not gram molecules).

Found		NMe_3 in grams		Iodine in grams		Atoms of Iodine combined with NMe_3
1.	...	0.18821	...	2.1209	...	5.22
2.	...	0.13570	...	1.4927	...	5.26
3.	...	0.08251	...	0.90598	...	5.04

It thus appears that there are five atoms of iodine present combined with each trimethylamine molecule. To ascertain whether the body was of the type NMe_3I_5 , or whether it was of the type $\text{NMe}_3\text{HI.I}_4$, the following experiments were made:—

Preparation A. A strong reagent (4:4:100) was made and the crystals formed were washed with a little ice water and dried over sulphuric acid. They dissolved very slowly in 20 per cent. potassium iodide solution, but on the addition of benzene formed a greasy mass which slowly went into solution.

0.3164 required 17.89 c.c. N/10 thiosulphate. Iodine found 71.8 per cent.

Preparation B. Same method and treatment.

0.7781 required 43.78 c.c. N/10 thiosulphate. Iodine found 71.5 per cent.

Preparation C. made from weak reagent (4 KI:1.6:100). 0.4308 required 27.36 N/10 thiosulphate. Iodine found 72.3 per cent.

$\text{NMe}_3\text{HI.I}_5$ requires 67.08, $\text{NMe}_3\text{HI.I}_4$ 73.09 and $\text{NMe}_3\text{HI.I}_5$ 77.4 per cent. of iodine.

These results are in agreement with the constitution $\text{NMe}_3\text{HI.I}_4$. We prepared the same body from trimethylamine hydriodide, so that when the hydrochloride is used the potassium iodide must convert the hydrochloride to the hydriodide in the first instance. It would then appear that variations in the method of preparation do not affect the composition of the crystals.

As the crystals seemed to have a definite composition under varying conditions of preparation, we next carried out a number of experiments to ascertain whether their formation could not be utilised as a method for the estimation of trimethylamine. As it was not possible to collect and weigh them, and as the Staněk process used for choline required larger quantities of the base than were likely to be contained in a litre of urine, we endeavoured to measure the absorption of iodine from the reagent on the addition of known quantities of trimethylamine.

For this purpose a solution of trimethylamine hydrochloride containing 7.3 grams per litre was used. Quantities of 25 c.c. of the periodide reagent were accurately measured out into a series of test-tubes, and 0, 1, 2, 3, &c., cubic centimetres of the trimethylamine were introduced. After standing, the crystals formed were removed by filtration through glass wool, and a measured quantity of the filtrate was titrated with thiosulphate, and the absorption determined by comparison with the blank experiment.

(a) Reagent KI:I:Aq. as 4:3:100 grams.

With this strength the absorption was always variable, and increased with the number of cubic centimetres of trimethylamine solution added.

Thus, on calculating the formula of the periodide from the absorption of iodine observed, it was found that with 2 c.c. of hydrochloride solution the formula indicated would contain I_7 , while with 3 c.c. the formula would contain I_8 . On analysis of the crystals separated, however, they gave the normal result NMe_3HI_5 . These results pointed to the precipitation of iodine by the aqueous solution employed, but no better results were obtained when the requisite volume of the hydrochloride was first evaporated to dryness before addition of the reagent.

(b) Reagent KI:I:Aq. as 4:1·5:100 grams.

With this strength precipitation was not complete. The absorption pointed to a formula containing only I_2 , whereas the crystals obtained had the normal composition. The filtrate on treatment with a stronger reagent gave a further precipitation of crystals. In the course of a large number of experiments we were unable to discover the reason for the variations observed, and were consequently obliged to abandon the method.

Trimethylamine in Blood

Dessaigues¹ in 1856 demonstrated the presence of trimethylamine in calf's blood. The blood, which showed no signs of decomposition, was acidified, and twelve hours after its removal the clot was separated by pressing out the liquor. The serum obtained was distilled with lime. From the distillate of twelve litres of blood three kinds of platinum crystals were obtained, one of which Dessaigues proved to be trimethylamine platinochloride. One of us² first demonstrated the presence of trimethylamine as a normal constituent of human blood, and, using the method of de Filippi, found that in the healthy subject the amount per cent. varies between 0·0018 and 0·0011. It was found that in cases of uraemia there was a marked increase in the percentage of trimethylamine, and that this body is most probably responsible for the urinous smell of the breath of uraemic patients. In all these experiments the blood was treated in the same drastic method that was used by Filippi in his urinary determinations, and it was by no means certain that the trimethylamine might not have originated from the decomposition of lecithin phosphatides by means of the strong alkali added to the blood.

1. Dessaigues, *J. Phar.* (3) XXXII, 43.

2. Golla, *loc. cit.*

In 1906 Rzentowski attempted to determine the potential alkalinity of the blood by an estimation of the ammonia given up on boiling the blood in vacuo together with an ammonium salt. Though this failed as a method for determining alkalinity, his results showed that the potential alkalinity of the blood is abundantly sufficient to expel all the trimethylamine that the use of Filippi's method had shown us to be likely to be contained in the blood. It is, therefore, only necessary to boil the blood or serum in a vacuum at room temperature, and collect the distillate by passing it through a flask containing hydrochloric acid, which is surrounded by a freezing mixture. The trimethylamine and the ammonia are expelled by the potential alkalinity of the blood, and the distillation is proceeded with until the blood has been evaporated to dryness. Inasmuch as nothing is added to the blood, and it is never raised above the temperature of 20° C., this procedure cannot be held to have brought about the decomposition of lecithin or choline.¹ This method, though excellent for the identification of trimethylamine from the distillation of such small amounts of blood as 20 c.c., offers insuperable difficulties to the quantitative estimation of trimethylamine in larger amounts of blood, inasmuch as the frothing cannot be controlled. The proof that trimethylamine is given off under these conditions rests on microchemical tests, the validity of which we have already discussed, and the invariable production of the smell of trimethylamine on treatment of the alcoholic extract of the evaporated distillate with alkali. In our procedure the hydrochloric acid from the receiving flask is evaporated to dryness on a water bath, and the dry distillate again taken up with alcohol and evaporated. The residue is then examined by Florence's test, with Kraut's reagent, and another portion treated with alkali, and the characteristic smell of trimethylamine recognised. Using this method we have never failed to demonstrate the presence of trimethylamine in samples of fresh human blood and serum, of which over twenty have been examined. We have also shown the presence of trimethylamine in the blood of the cat, guinea-pig, ox, and the serum of the horse.

An attempt to estimate the trimethylamine of blood by a modification of this method was made. A litre of ox blood was treated with three times its volume of absolute alcohol, and filtered from the coagulated albumen. The filtrate was acidified with hydrochloric acid, so as just to redden litmus, and was evaporated to dryness at 37° C. The residue was

1. See further, p. 321.

taken up with alcohol, and the alcoholic extract again evaporated to dryness. The residue was then dissolved in 25 c.c. of water, to which 5 c.c. of decinormal sodium carbonate were added, and treated by the modification of de Filippi's method with sodium hypobromite already described. The acid neutralised by the distillate was titrated, and the results from three such experiments gave values of 0·001, 0·0015, and 0·0013 per cent. These results are in rough agreement with those published by one of us using the Filippi method for normal human blood.

An attempt was made to separate sufficient trimethylamine platinochloride from blood to allow of an analysis. For this purpose fifteen litres of ox blood were treated with alcohol, and distilled in the way described. The distillation, which was done in quantities of two litres of blood at a time, proved extremely tedious, and the amount of platinochloride obtained is in no way to be regarded as giving a quantitative estimation of the trimethylamine originally present in the blood. The platinochloride crystals purified by repeated extraction with alcohol gave on analysis the following figures:—

0·2590 grams of platinochloride gave 0·1345 CO₂ and 0·1050 H₂O.

Found C=14·1; H=4·5 per cent.

2 NMe₃HCl.PtCl₄ requires C=13·7; H=3·8 per cent.

The Presence of Trimethylamine in the Cerebrospinal Fluid

Through the kindness of Mr. McGavin, we have had exceptional opportunities for the examination of normal cerebrospinal fluid. Most previous observations on the chemistry of the cerebrospinal fluid have been conducted on fluid obtained by lumbar puncture from cases suffering from nervous disorders, or on post-mortem fluid, as the operation of lumbar puncture in the sound subject would have been unjustifiable. Mr. McGavin, however, allowed us to have the cerebrospinal fluid withdrawn from his numerous cases of lumbar anaesthesia, the custom being to withdraw a few cubic centimetres of fluid before introducing the stovaine into the lumbosacral puncture. By saving up this fluid, which was all obtained from cases suffering only from surgical disorders, relatively large amounts of pooled fluid were obtained, and a much larger material afforded to work upon than has been used by most other observers. Dr. Firth, from the Victoria Hospital for Children, and Dr. Eve, of Hull, also very kindly supplied us with fluid from cases of meningitis. With the fluid obtained from the clinique of Mr. McGavin we have been able

to make some thirty experiments with quantities of fluid varying from ten to fifty cubic centimetres. In every case examined we have found trimethylamine to be present and choline to be absent. Trimethylamine must then be regarded as a normal constituent of cerebrospinal fluid.

Method of Experiment:—The method employed was again that of distillation in a vacuum, but to avoid as far as possible the objection that any trimethylamine found in the distillate might have resulted from the decomposition of lecithin or choline—a point which will be discussed in the sequel—the distillation was conducted in a vacuum at a room temperature which varied between 15° and 20° C. The normal fluid being alkaline, nothing whatever was added to it, and the distillation was continued till only the dry residue was left in the flask. In a number of cases the proteid was first precipitated with carefully neutralised absolute alcohol, but in the majority the crude fluid was distilled. The volatile alkali was collected in well-cooled hydrochloric acid, which was afterwards washed out, evaporated to dryness at a gentle heat, the residue extracted with alcohol, and drops of the concentrated alcoholic extract allowed to evaporate on a microscopic slide. The dry residue was tested with the periodide reagent as previously described. When only small quantities of trimethylamine are present the recognition of the crystals requires considerable skill and practice, and it is, of course, highly desirable so to manipulate the alcoholic extract that the whole extracted matter is contained in the last one or two drops finally evaporated on the slide. If any trimethylamine is present a cloud of minute black globules is at once seen under the microscope, apparently streaming from the dry crystalline masses treated with the reagent. After a time these globules gradually coalesce, become elongated and crystalline. The neighbourhood of air bubbles under the cover-slip should be carefully examined as these seem to collect the droplets and crystals. The crystals formed seemed to be of a more permanent character than those described by Rosenheim with choline, and do not very readily re-form droplets.

Among experiments carried out in this way the following may be cited:—

(1) Jan. 17th, 1910. 47 c.c. of normal cerebrospinal fluid were received from Mr. McGavin. This was distilled in a vacuum at 20° C. and when two-thirds had passed over the distillate was evaporated, extracted with alcohol, and the alcohol extract converted to the platinum salt. Portions of this examined under the microscope showed on treatment with the periodide reagent clouds of droplets streaming out of the crystalline masses. These gradually elongated and crystallised in minute rod-like crystals exactly like the crystals figured by Rosenheim as choline periodide. Some of the larger droplets, however, showed a tendency to crystallise into

irregular, somewhat hexagonal formed crystals. On continuing the distillation of the fluid remaining in the flask to dryness and collecting the distillate in fresh acid, a considerable quantity of the platinum salt was obtained, each portion of which gave, with the periodide, a large number of minute rod-like crystals which are shown in the photograph (Fig. 3). The alcoholic extract of the residue remaining in the flask was repeatedly tested with the periodide reagent, but no indication of droplets or crystals could be observed.

(2) Jan. 1st, 1910. 35 c.c. of normal cerebrospinal fluid was distilled to dryness at 20° C. The evaporated distillate was taken up with alcohol, the extract allowed to dry up on a slide, and the residue treated with periodide reagent. In each case minute needles were seen under the microscope. The residue in the flask was extracted with alcohol, but the extraction on evaporation and treatment with periodide gave no crystals.

(3) Dec. 18th, 1909. 11 c.c. of normal cerebrospinal fluid treated in the same way showed many droplets some of which took on an elongated form, but no definite crystals were observed. This and similar experiments with small quantities would seem to show that this quantity of fluid was too small to give definite results.

Considering the numerous experiments that have been carried out with cerebrospinal fluid with a view to demonstrating the presence or absence of choline in it, it is a little surprising that with the exception of Gumprecht¹ no previous observers have recorded the presence of any base giving reactions similar to choline in fluids obtained from the normal animal, or in those suffering from diseases other than nervous. Halliburton² regards the base found by Gumprecht as choline, but Kaufmann,³ who worked up a litre of cerebrospinal fluid, obtained chiefly from cases of paralysis, isolated a base which he regarded as certainly not choline. His method of experiment and conclusions have, however, been called in question by Donath,⁴ who, with Halliburton, regards the presence of choline in cerebrospinal fluid as an accompaniment of degenerative nervous disease.

The explanation probably lies in the method of treatment of the fluid used by nearly all observers. Following the original method of Mott and Halliburton, the fluid is precipitated with alcohol and evaporated to dryness at 40° C. The alkalinity present would in these circumstances be sufficient to drive off any trimethylamine present, more especially if the process is repeated several times. The following experiment illustrates this:—

(4) 48 c.c. of normal cerebrospinal fluid was divided into two equal parts. One was made just acid with hydrochloric acid, and evaporated to dryness. The residue gave crystals with the periodide reagent. The other half evaporated by itself gave no crystals.

1. Gumprecht, *Verh. d. Congress f. innere Med.*, Wiesbaden, 320, 1900.
2. Croonian Lectures, p. 20, 1901.
3. Kaufmann, *Neurol. Centralblatt*, VI, 260 (1908).
4. Donath, *Neurol. Centralblatt*, 964, (1908).

Kaufmann is the only worker who acidified the fluid before evaporation, and, in our opinion, he must have obtained trimethylamine hydrochloride. Relying upon the smell of the residue when treated with alkali he obtained a peculiar 'spermageruch,' and says that this could not be trimethylamine. But the smell of trimethylamine, when very much diluted, agrees with Kaufmann's description. We were invariably able to smell trimethylamine in our own residues, for example:—

(5) 20 c.c. of normal fluid was treated as described by Kaufmann. Portions treated on a slide with a drop of potash solution were stated by three observers to smell of trimethylamine. The bulk of the residue was extracted with alcohol, and converted to a platinum salt, and this, with periodide gave irregular crystals as well as needles.

According to our experience, laboratory alcohol is frequently acid, and sometimes fairly strongly so, and it seems not at all improbable that in some experiments in which choline was found by other observers sufficient acid was present in the alcohol to neutralise the alkalinity of the fluid,¹ and to have permitted crystals of trimethylamine periodide to be obtained which would be described as choline. This may account for the discrepancies in the observations of various writers with regard to the presence or absence of choline in the cerebrospinal fluid of epileptics.

We have, in addition, found that if the alcoholic extract of the evaporated fluid be examined directly with the periodide reagent as recommended by Rosenheim, the sensitiveness of the reaction is very greatly diminished, and a large amount of trimethylamine or choline must be present in order to obtain crystals. The black droplets always form, but crystallisation is inhibited by the sodium, potassium and ammonium salts probably present.² This is well illustrated by the following experiments:—

Two mixtures A and B were prepared containing respectively per 100 c.c. 1 gram of salts (NaCl and NH_4Cl in equal proportions), and of trimethylamine hydrochloride (A) contained 0.02 gram and (B) 0.002 gram. 1/20 c.c. of each of these was evaporated at a low temperature on a slide, and the results obtained on treatment with the reagent compared with similar slides containing the same amount of pure trimethylamine.

1. Especially when we consider the small amounts of fluid used by some observers. Thus Kajiura (*Quart. Jour. of Exper. Physiol.*, Vol. I, p. 293), in one set of experiments employed 2.5, 3, 5, and 10 c.c. of fluid respectively.

2. Bertheaume, C.R. (1910). CL, 1065, however, says that the solubility of the crystals is lowered by the presence of salts especially by ammonium chloride.

Weight of NMe ₃ HCl mgms.			Weight of salts mgms.			Periodide reaction
I.	(a)	0.01	...	—	...	Large crystals typical cross-like feathery form—visible to the naked eye.
	(b)	0.01	...	5	...	Immediate formation of droplets. In 20 minutes no crystals; large droplets. In 30 minutes a few small isolated rod-like crystals—large droplets.
II.	(a)	0.001	...	—	...	Numerous rod-like crystals easily obtained in a few minutes.
	(b)	0.001	...	5	...	Many small black drops; after a long time a few isolated crystals were seen.

These results explain our own early failures when large amounts of cerebrospinal fluid (up to 50 c.c.), acidified with hydrochloric acid, were simply evaporated to dryness, and the alcoholic extract of the dry residue tested directly.

The Possibility of the Trimethylamine obtained in the Physiological Fluids arising from the Decomposition of Lecithin or Choline

Cerebrospinal Fluid:—Our results have been unfortunately confined to normal fluids, and we hope that our method of detecting trimethylamine will be applied to fluids from various degenerative nervous diseases. In spite of the low temperature at which we worked, and the fact that we added nothing to the cerebrospinal fluid distilled, the objection that the trimethylamine found might originate from the decomposition of lecithin or choline requires attention. With regard to lecithin, what little evidence we possess goes to show that lecithin itself is not present in cerebrospinal fluid. Zdarek,¹ who examined 300 c.c. of fluid from a meningocele, was not able to detect any. We ourselves made one experiment in this direction. 47 c.c. of fresh normal cerebrospinal fluid were mixed with alcohol, and allowed to dry up in a vacuum. The dry residue was then ground up, and repeatedly extracted with a mixture of ether and alcohol. This extract on evaporation yielded a small quantity of greasy residue, which was boiled with strong nitric acid and the clear solution tested with a very sensitive ammonium molybdate solution. No indication of the presence of a phosphate could be observed. Although lecithin when treated with acids and alkalis² is fairly easily decomposed with formation of choline, yet the proportion of volatile nitrogen found by Maclean³ as the result of treating lecithin cadmium chloride with such a

1. Zdarek, *Zeit. physiol. Chem.*, XXXV, 201 (1902).

2. *Zeit. physiol. Chem.*, LV, 352, 1908.

3. Maclean, *Zeit. physiol. Chem.*, LV, 365, 1908.

powerful reagent as alcoholic baryta solution was extremely small. The possibility of choline yielding trimethylamine under the conditions employed by us is very unlikely on a consideration of the results of Gulewitsch,¹ who has shown that choline is comparatively stable when treated with dilute acids and alkalies. In order to ascertain more exactly the effect of our working conditions on choline, we carried out the following experiment:—

(a) Preparation of choline hydrochloride free from trimethylamine hydrochloride—200 c.c. of a 2 per cent. solution of Kahlbaum's choline hydrochloride were mixed with 50 c.c. of a N/10 sodium hydrate solution and the liquid distilled in a vacuum as usual. After one hour at 35° C. the distillation was stopped. The distillate, evaporated and treated in the ordinary manner, gave crystals of trimethylamine periodide and the characteristic smell on the addition of potash. The liquid remaining in the flask was further distilled, and the distillate collected in fresh hydrochloric acid. But in this case no crystals were obtained. It appears probable that the trimethylamine found existed as an impurity in the choline, and was not formed by decomposition of the choline under the conditions employed. The liquid whose volume was now about 80 c.c. was exactly neutralised with hydrochloric acid, and made up to 100 c.c.

(b) 2 c.c. of the above choline hydrochloride solution were mixed with 8 c.c. of a solution containing 0.08 gram of sodium chloride and 0.08 gram of ammonium chloride, and 10 c.c. of N/10 sodium carbonate solution were added. This artificial alkaline cerebrospinal fluid gave on distillation in the usual way no reactions indicative of trimethylamine.

(c) In another experiment 15 c.c. of normal cerebrospinal fluid were distilled to dryness at room temperature. The distillate yielded crystals of trimethylamine periodide. To the dry residue in the flask was now added 2 c.c. of the above-mentioned pure choline solution, and 13 c.c. of distilled water. The distillate obtained from this mixture gave none of the tests for trimethylamine.

It would appear, then, as a result of these observations that our methods could be employed to detect trimethylamine in cerebrospinal fluid even in the presence of choline.

CONCLUSION

It would appear from the evidence obtained that trimethylamine is to be regarded as a normal constituent of the blood and the cerebrospinal

1. *Zeit. physiol. Chem.*, XXXIV, 514.

fluid. The evidence is not so conclusive as regards urine, since here the employment of an alkali other than that normally present in blood and cerebrospinal fluid might be held to cause a decomposition of some precursor of a phosphatide nature.¹ It seems equally clear that choline is not present as a normal constituent of the blood and cerebrospinal fluid, and, in common with other observers, we have failed to detect it in urine.

The results obtained with microchemical tests for trimethylamine make it obvious that no method of testing for choline can be valid when the trimethylamine is not taken into account. In the methods of Mott and Halliburton the presence of alkali must have eliminated all the trimethylamine during the process of evaporation, and these observers now admit that they fail to find choline in cerebrospinal fluid taken from the living subject, even in cases of general paralysis.² The evaporation of an extract of blood or cerebrospinal fluid as practised by nearly all the other searchers after choline would tend to drive off the trimethylamine more or less completely. The fact that laboratory alcohol is frequently sufficiently acid to more than neutralise the small amount of cerebrospinal fluid used is, we suspect, a reason for the discordant results recorded in the observations of those who have looked for choline in physiological fluids, and also explains the invariable finding of choline in blood treated with acid by Claude and Blanchetière.³ It is to be noted that a further source of error in attempts to bring about the periodide reaction with very small amounts of blood or cerebrospinal fluid lies in the diminution of the sensitiveness of the reagent due to the presence of neutral salts, a source of error obviated by our method of distillation.

1. See Erdmann, *J. Biol. Chem.*, VIII, 57-60, 1910.

2. *Croonian Lectures*, 1910.

3. *Journ. de Physiologie*, Vol. II, 1909.

FIG. 1.—Crystals of Trimethylamine periodide, $\times 40$, from 0.2 milligram of the hydrochloride.

FIG. 2.—Crystals of Trimethylamine periodide, $\times 200$, from less than 0.01 milligram of hydrochloride.

FIG. 3.—Crystals of Periodide, $\times 200$, obtained from the distillate from normal cerebrospinal fluid.

FIG. 4.—Crystals of Choline periodide, $\times 200$.