THE PYRIDINE TEST AS A QUANTITATIVE METHOD FOR THE ESTIMATION OF MINUTE AMOUNTS OF CHLOROFORM.*

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(Received for publication, October 20, 1926.)

I.

In spite of many investigations concerning the distribution and fate of anesthetic and lethal doses of chloroform in animal fluids, tissues, and organs,¹ there have been relatively few accurate quantitative determinations of the amounts of chloroform in various parts of the animal body following chloroform poisoning. The most reliable data are evidently those reported by Buckmaster and Gardner (1906, 1907) for blood, and by Nicloux and Yovanovitch (1924, 1925) for nervous tissues and blood. The obvious reason for the absence of such determinations is the lack of any simple quantitative method whereby minute amounts of chloroform can easily be measured. This lack also explains why satisfactory studies on the permeability of animal and plant membranes to chloroform have not been made. Although there are several qualitative tests for chloroform, such as the phenylisocyanide, resorcinol, naphthol, cyanide, and reduction tests (see Autenrieth and Warren, 1921, pp. 36–38), none of these is delicate enough to determine minute amounts, and some of them are too elaborate to be of much practical value. The important quantitative tests are those which involve the decomposition of chloroform and the subsequent determination of chlorine, either by gravi-

* A part of the expense of this investigation was defrayed by a grant from the American Association for the Advancement of Science.

¹ Some of the more important studies include those by Grehant and Quinquand, 1883; Luedeking, 1886; Pohl, 1890–91; Angiolani, 1891; Nicloux, 1906, 1907; Nicloux and Yovanovitch, 1924; Buckmaster, 1917–18; Buckmaster and Gardner, 1906, 1907; all of which are listed in the Bibliography of this paper.
metric or volumetric methods, as in those of Ludwig-Fisher, Nicloux (1906, 1924), Gibson and Laidlaw (1922), Buckmaster and Gardner (1906, 1907), and others; all of these are laborious without being very delicate. Several years ago, however, Fujiwara (1914, 1917) described the pyridine reaction with chloroform and related substances in the presence of strong alkali, stating that 1 part of chloroform in a million parts of water could easily be detected. Later, Ross (1923-24) reported the same test, indicating the independent discovery that substances containing the R-C-halogen group (chloroform, bromoform, chloretone, chloral, iodoform, etc.) give a pink or red color to pyridine when the mixture is heated in the presence of sodium hydroxide. Fujiwara applied the test to extracts and distillates of animal fluids and tissues, attempting to estimate the amount of chloroform present by the depth of the color produced. These experiments suggested to the writer the possibility of developing the pyridine reaction quantitatively, so that accurate determinations of the amounts of chloroform and related substances present in solutions might be made. The value of such a simple and delicate test for studies on the permeability of membranes to chloroform, on the distribution of chloroform in the tissues of animals anesthetized or killed by chloroform, as well as for the determinations of chloroform poisoning in medicolegal practice, is obvious.

II.

For quantitative estimations of chloroform, the procedure has been standardized and found satisfactory for measuring the amounts between 0.1 and 0.0001 per cent, or 0.01256 and 0.00001256 molar concentrations, when the specific gravity is 1.5. Future experiments will deal with the test as applied to the other substances containing the R-C-halogen group. The procedure in the case of chloroform is as follows. Into a narrow test-tube of about 10 cc. capacity are measured 2 cc. of 20 per cent NaOH from a long burette graduated to 0.1 cc. To this are added 1 cc. of chemically pure pyridine (colorless) from a similar burette, and 1 cc. of the solution to be tested, measured by a 1 cc. pipette. The test-tube is loosely corked to prevent undue evaporation of

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See Autenrieth and Warren, 1921, p. 38.
the pyridine, and, with constant shaking, is immersed well above
the liquid level for 1 minute in water at 100°C. The tube is trans-
ferred to cold water until the temperature of the mixture has fallen
to about 20°C. During the cooling, the pyridine and test solution,
colored pink or red if chloroform is present, rise above the alkali,
and are removed by a 1 cc. pipette to a color comparison tube.
The solution may then be compared with color standards pre-
viously made up to match the colors obtained from known con-
centrations of chloroform. For quantitative work it is necessary
that the amounts of pyridine and test solution be measured
accurately, since the color produced is a function of the volume
over which it is distributed. Moreover, since pyridine is volatile
(b. p. about 115°C.), the heating should be done in long loosely
corked tubes, so as to keep the volume of pyridine as constant as
possible. The color comparison tubes must be of the same diame-
ter and of the same kind of glass as those used for the color stand-
ards. When all directions are followed accurately, the color from
any one concentration of chloroform may be duplicated regularly.

Since the colored pyridine compound is unstable and gradually
loses its color, it cannot be used as the standard. It has been found
that basic fuchsin (certified by the Commission on Standardization
of Biological Stains) may be used to match the colors obtained from
all the higher concentrations of chloroform with considerable
accuracy. For the more dilute solutions it does not match exactly,
but comes nearer than any other dye so far found. The basic
fuchsin standards were made up with 50 per cent alcohol containing
about 0.01 per cent HCl, and enough dye to match the desired
color.

A 0.1 per cent stock solution of chloroform was made by dis-
solving 1 cc. of Squibb’s chemically pure chloroform, sp. gr. 1.500,
at 15°C., measured by a certified pipette graduated in 0.1 and 0.01
cc., in distilled water and making up to 1000 cc. in a volumetric
flask at 15°C. The other solutions were made by diluting the
stock solution with varying amounts of distilled water, using
large volumes to insure greater accuracy. All of these solutions
were kept in ground glass-stoppered bottles whose volumes were

The firm of Hynson, Westcott and Dunning of Baltimore kindly made
up an experimental set of color standards, for which the author expresses
his thanks.
176 Chloroform Estimation with Pyridine

Only slightly in excess of the solution they contained. In making the stock chloroform solutions to be used as standards in matching the fuchsin color solutions, it is highly important that all measurements of volume be as accurate as possible, and that the temperature of all fluids be 15°C. Only certified pipettes, volumetric flasks, and thermometers should be used. These precautions are necessary because of the volatility of chloroform and the delicacy of the test. It was found that a nearly saturated solution of basic fuchsin in acidified alcohol matched the color obtained from the pyridine test on a 0.1 per cent chloroform solution. For the other color standards (0.05, 0.025, 0.01, 0.005, 0.003, 0.001, 0.00078, 0.0005, 0.0002, and 0.0001 per cent, corresponding to molar concentrations of 0.00628, 0.00314, 0.001256, 0.000628, 0.0003768, 0.0001256, 0.0000979, 0.0000628, 0.00002512, and 0.00001256) this stock fuchsin solution was diluted until it matched as closely as possible the colors obtained from known concentrations of chloroform. Each color standard was checked frequently and corrected whenever necessary.

The colored pyridine solution obtained from a test on a chloroform solution in water is only slightly turbid and may be compared directly with the color standards for concentrations of 0.1, 0.05, and 0.025 per cent. Below the latter concentration the turbidity interferes with the matching of colors, and two sets of tubes must be used in the comparator. One set consists of distilled water and the pyridine colored solution from the test; while the other consists of the pyridine mixture from a test on a solution containing no chloroform and the color standard tube. When extracts of tissues are used the double set of tubes is always necessary, an extract from tissues containing no chloroform being used in the control tube.

III.

Preliminary experiments were concerned with qualitative tests on extracts of animal organs and tissues in various solvents. The following were found satisfactory for making tests: physiological salt solution, Ringer’s solution, acidified distilled water (about 0.01 per cent HCl), alcohol, ether, and tartaric acid-alcohol (70 per cent alcohol with 1 per cent tartaric acid). The only solvent, however, which will allow the detection of chloroform in the small-
est amounts, is acidified distilled water (cf. Fujiwara, 1914, p. 3). This seems to be due to the facts that distilled water extracts from the tissues a larger percentage of the chloroform than the other solvents, and that the pyridine reaction is more delicate in water than in alcohol or ether. Slight acidity prevents any decomposition of the chloroform which might occur in an alkaline medium. Toluene may safely be used in small amounts as a preservative. On the other hand, it is perfectly clear that blood seriously interferes with the test. If considerable blood is present in the extract the pink color of the test will be hidden by an orange-brown turbidity. A moderate amount of blood in the test mixture produces an orange color which rapidly becomes orange-red, then brown, green-brown, and finally light green. In general, less than 5 minutes are required for the completion of this color series, the actual time depending upon the amount of blood present. If an extract contains only enough blood to give it a barely perceptible pink tint, the test may be made without interference, since control tests on such extracts yield a colorless pyridine solution in the absence of chloroform.

The following procedure was used in preparing the extracts. The organ is dissected out and submerged immediately in a mortar with a small amount of the solvent to be used. It is then cut up into small pieces by scissors, care being taken not to expose the tissue to the air. With a pestle the material is ground until it is thoroughly minced or shredded, and then the whole mass is placed in a rubber-stoppered flask with the desired amount of solvent. This amount is determined by the quantity of chloroform expected to be found. The extract should stand at room temperature for at least 1 hour, with occasional shaking, so that all the chloroform may be extracted. The pyridine tests are made within the next few minutes, since the longer the mixture stands after complete extraction the less accurate will be the estimation, due to the unavoidable loss of chloroform vapor. When enough blood is present to hinder the test, distillation is necessary. A small distilling flask (25 cc.) with a long neck, and a side arm surrounded by running cold water, has been used. The heating was done in a sand or an oil bath. The distillate is conducted into from 3 to 5 cc. of acidified distilled water in a small test-tube loosely corked. At the end of distillation, the distillate solution is tested. This
method has not yet been improved for reliable quantitative estimations of chloroform in the original extract.

The qualitative tests were made on extracts (1) from the tissues of animals killed by chloroform inhalation; (2) from tissues of animals killed by ether, illuminating gas, a blow on the head, electricity, and natural causes; as well as on (3) solutions containing chloroform which had diffused through frog skin. In the first and third groups positive tests were always found, indicating the presence of chloroform; while in the second group the tests were always negative. It was further found that slight decay of the tissues does not seriously interfere with the test. 200 tests were made on extracts from the tissues of frogs, mice, guinea pigs, rats, cats, dogs, and human beings, and in no case did the method fail to indicate chloroform when the latter was known to be present. It was concluded that the pyridine reaction is a reliable test for chloroform under the conditions stated.

IV.

For quantitative tests the concentrations of chloroform mentioned (p. 176) were carefully prepared by the writer, and the bottles were given non-successive arbitrary numbers by a disinterested person. Each solution was then tested three times by the pyridine reaction, and the estimated concentration of chloroform was recorded under the corresponding number of the bottle. Although this scheme was used many times there was less than 1 per cent discrepancy, and all of this was restricted to the last three most dilute solutions. It appears that the pyridine test is a reliable quantitative method for estimating minute amounts of chloroform in aqueous solutions.

As a further check on the method, extracts of the brains of rats killed by illuminating gas were prepared in the described manner, and to the different extracts the following amounts of chloroform were added: 0.1, 0.05, 0.025, 0.001, and 0.0001 per cent. In one series the chloroform was introduced previous to maceration of the brain, and in another series subsequently. In each of the twenty experiments the correct amount of chloroform, i.e. the percentage which had been added, was indicated by the tests.

A third series consisted in soaking twenty-three brains from rats, killed by illuminating gas, in pure chloroform for at least 24 hours.
The brains were then washed either successively in running water, alcohol, and ether, or in running water alone, for varying periods of time. In any one group of brains which had been washed in the same way for the same length of time, closely concordant results were obtained. The amounts of chloroform present could be calculated as cc. per gm. of brain tissue, since the weight of the brain and the volume of the extract were known. For example, in Group 2, where washing in water alone was continued for 2 minutes, the amounts of chloroform were: 0.2097, 0.2059, 0.2105, and 0.2083 cc. per gm.; in Group 1, where running water was used for 10 minutes: 0.1703, 0.1721, 0.1754; and in Group 5, where water, alcohol, and ether were each used for 1 minute: 0.0978, 0.09422, 0.0912, and 0.09174 cc. per gm. It is to be expected that considerable variation in the absolute amount of chloroform found in such extracts will exist, due to the differences in (1) the absorbing surfaces of the brains; (2) the method of washing; and (3) the length of time of washing. The last two causes are the most significant, and it is believed that if the volume of running water or other washing agent used, as well as the time of washing, were precisely measured and constant, the absolute amount of chloroform absorbed by a rat brain in 24 hours could be accurately determined. As a check on the reliability of the method, these experiments are satisfactory and appear to indicate that the pyridine test may safely be used to detect small amounts of chloroform in watery extracts of brains.

SUMMARY.

The pyridine reaction with chloroform and other substances containing the R-C-halogen, group in the presence of strong NaOH has been standardized as a quantitative colorimetric method for the estimation of small amounts of chloroform (0.1 down to 0.0001 per cent, corresponding to 0.01256 and 0.00001256 molar), in pure water and in aqueous extracts of animal tissues. Detailed directions for using the test are given, and several checks on the reliability of the method are described.
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