CLXXXI. A MICRO-METHOD FOR ACCURATE DETERMINATION OF D_2O IN WATER.

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Deuterium oxide can be utilized as a tool for the study of certain chemical and biological problems, including the constitution of complex organic compounds *in vitro* and *in vivo*, and in a number of such cases it is essential to be able to determine very small quantities, available, say, from the combustion of tissue fragments or expensive compounds.

The method here described is a modification of one worked out by Barbour & Hamilton [1926] for specific gravity determination of blood and tissue fluids and also applied [Vogt & Hamilton, 1935] to D_2O. It consists essentially in measuring the rate at which a drop of water will sink through an immiscible fluid of slightly lower specific gravity. We have succeeded in increasing the accuracy to the sixth decimal place in specific gravity corresponding to 0·001 % D_2O in H_2O.

The important points in the method are:

(1) A very rigorous purification of the water to be tested, so as to exclude the presence of any substance save D_2O and H_2O.

(2) The production of drops of exactly the same volume by means of a special pipette.

(3) Temperature control of a water bath to within 0·001°.

(4) The measurement of time intervals of 15–30 sec. to within $\pm$0·02 sec.

(1) The water to be determined can sometimes be taken directly from an experiment, but more often it is obtained by distillation or even by combustion. When distilling off the water from organs we use a small oven which is heated to a constant temperature of 105° and evacuated by means of a filter pump connected up at intervals so as to keep the pressure below 10 mm. Hg. The water given off is condensed by means of solid CO_2 in alcohol giving a temperature of about $-70°$.

It is important that as much water as possible is driven off and condensed, because H_2O being slightly more volatile than D_2O a certain fractionation takes place.

The residues are pulverized and finally dried at about 108° in an electric oven or *in vacuo* below 100°, thereby losing from 2 to 10 % water.

Protein solutions are evaporated *in vacuo* from a flask to which minute quantities of octyl alcohol can be added (Fig. 1). Most of the water is condensed by means of ice and the rest by means of solid CO_2.

The D and H in organic combination are liberated by combustion. 0·5–2 g. dry powder is usually mixed with copper oxide-quartz and burned in a quartz tube in a current of dry air. That part of the tube containing the organic material is heated slowly and cautiously but the vapours must pass through copper oxide at 900°. The air is dried by $CaCl_2$ and P_2O_5 and supplied at the rate of about 50 ml. per minute.

In a few cases water distilled off, say from urine, must undergo an initial combustion to facilitate the final purification.

This is done by blowing the vapour through a small combustion tube with copper oxide heated to about 900°.

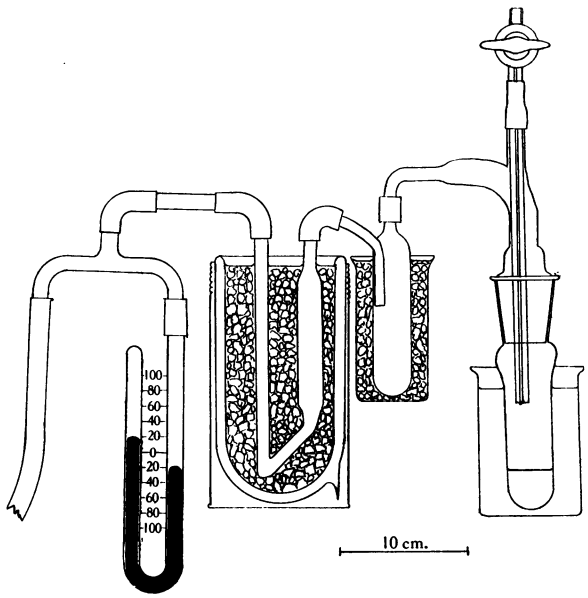

Fig. 1.

The routine purification which is sufficient in almost all cases is performed as follows:

The water sample of 0·5–2 ml. is heated in a sealed ampoule of 4 ml. capacity with about 25 mg. of permanganate and 1 mg. of sodium peroxide to about 150°, for not less than 1 hour. We use a small autoclave shown in Fig. 2 for the purpose and allow about 20 min. for raising the temperature, whilst the cooling to room temperature requires about 1 hour. Each ampoule is wrapped in filter-paper and a total number of 8 can be placed in a small porcelain dish. Almost all organic substances are destroyed by this treatment. It is of course essential that no substance should be present which can give rise to the liberation of D_2O.

The content of each ampoule is put into a pyrex distilling apparatus shown in Fig. 3 and distillations to dryness are repeated until the specific gravity becomes constant. Usually four distillations are required. Heating is done by means of a small flame which is moved by hand.

(2) The micro-pipette is shown in vertical section in Fig. 4. It is filled with mercury and the measuring device is the steel piston working from an adjustable stop (*A*) to a fixed one. The glass tube (*B*) is fixed in the nut (*C*) by de Khotinsky cement. The mercury column can be moved also by means of the screw in the top *D* and be made to fill the pipette completely. This device is used to wash out the pipette whenever a fresh fluid is to be measured. Consecutive deliveries of water from this pipette agree in weight at least to within 0·01–0·02 mg. The size used by us is 45 μl., but the pipette can be adjusted to any volume between 10 and 100 μl.

(3) The water-bath is a battery jar 480 mm. high, 240 mm. broad and 440 mm. long. The water is kept thoroughly mixed while it is cooled by tap water in a coil

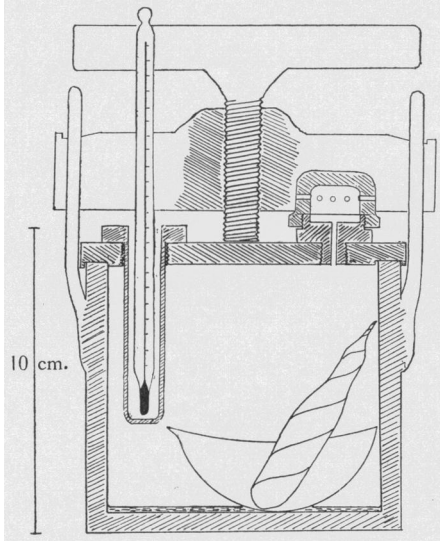

Fig. 2.

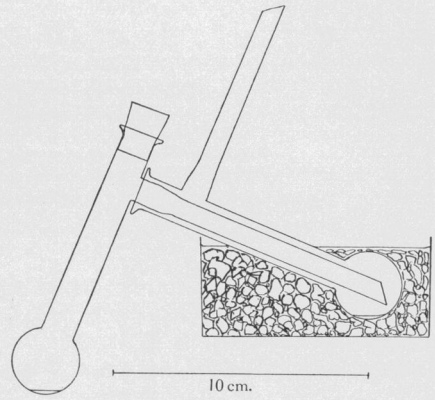

Fig. 3.

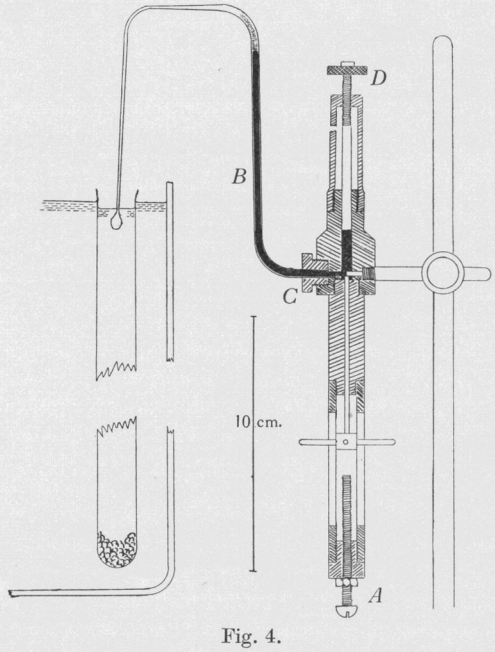

Fig. 4.

of lead tubing and heated by an electric bulb switched on and off by a thermostat arrangement. The variations in temperature should not exceed 0·001°. Once every few months the mercury contact should be cleaned with nitric acid to

maintain maximum sensitivity. It is essential that the temperature is maintained slightly (about 1°) below that of the room. The water-bath should be sheltered against sunlight, strong radiant heat and draughts.

In the bath is a stand of 10 stoppered glass tubes 450 mm. long and 16 mm. in diameter. Each contains a different mixture of bromobenzol with xylene. In the lightest mixture drops of pure H_2O delivered by the pipette will fall 9 cm. in about 30 sec. The others are adjusted to give nearly the same rate of fall for 0·5, 1, 1·5, 2, 2·5 and 3 % D_2O respectively.

We have not found it possible to give definite instructions for the preparation of these mixtures. They are kept in stoppered bottles and adjusted by trial in the tubes with solutions of known specific gravity. When not quite right they are poured back into the bottle and a small amount of either bromobenzol or xylene is added. About 4 g. dry Na_2SO_4 are placed at the bottom of each tube to take up the water.

The measurement is made as follows.

The pipette is washed out and filled with the solution. The tip is placed just below the surface in the tube which is expected to correspond to the solution and the piston raised. If the drop is slightly warmer than the fluid the heat given off will not cause any disturbance by convection further down whilst even a slight difference in the opposite direction completely spoils the determinations. The drop is released by lifting the tip just clear of the surface and allowed to fall through 18 cm. before any measurement is made. We have arranged a stand with 3 microscopes with a magnification of 3–4 times at vertical distances of 90 mm. and use two stop-watches reading to 0·01 second. It is a very useful control on the temperature constancy and the absence of convection currents that the two readings on the same drop should agree within $\pm 0{\cdot}02$ sec.

(4) We have found it a little difficult to obtain stop-watches of sufficient accuracy and reliability and a chronograph would certainly be preferable. On the other hand the reading by means of microscopes is scarcely necessary. If the tubes were provided with circular marks at the appropriate distances reading by means of a simple lens could be made just as accurate.

All determinations are made as comparisons with solutions of known specific gravity, the falling rates of which are measured before and after that of the solution under test.

As standard solutions we use dilutions of concentrated D_2O with freshly distilled H_2O prepared by means of syringe pipettes [Krogh, 1935].

The calculations are made as shown in the following example:

Drop of 2·460 % D_2O in tube No. 6 falls 90 mm. in 28·30, 28·30, 28·28, 28·30 sec.—mean 28·295.

Drop of 2·952 % D_2O, 18·28, 18·26, 18·28, 18·30—mean 18·28.

The difference of 10·015 sec. corresponds to 492 units or 1 unit to 0·02036 sec. When the fluid under test takes 23·00 sec. to fall its concentration will be $2460+\frac{23{\cdot}00-18{\cdot}28}{0{\cdot}02036}=2645$ units.

Accuracy and sources of error.

The really dangerous source of error lies in the purification of the water samples for determination. The standard dilutions should be redistilled at least once a week, and whenever there is any doubt the experimental solutions are purified over again. To facilitate this samples should not be too small, less than 1 ml. being inconvenient. It is often necessary to dilute water obtained by combustion to obtain a sufficient quantity and the acuracy is of course reduced accordingly.

One important point is that D_2O solutions will enter into exchange with water vapour. They must therefore be carefully protected. When they are distilled in the way here recommended we have observed no change in standard water kept for a year and redistilled repeatedly.

The primary standard solution has a specific gravity of 1·1049, 99·6% D_2O as bought from the Norsk Hydro. We have compared the standard solutions originally prepared and determined by Hofer over a year ago and redistilled repeatedly with fresh dilutions made up by weight from the 99·6% D_2O. Assuming these latter to be correct, the water assumed to be 496 was found to be 498 and the water 2975 found to be 2973.

The syringe pipettes used for making up the dilutions of the standard are accurate generally to 1 part in 10,000 and the errors in the dilutions are therefore negligible. The errors on the size of the drop as delivered by the automatic pipette are also, we believe, too small to have any influence, being of the order of 1 in 5000 or less. The main source of error in the determination proper is in the rate of fall. We have been unable to get beyond 0·02 sec. on a distance of 90 mm. The difference in time corresponding to about 500 units is about 10 sec. so that the error corresponds to about 1 unit. We do not know whether the variations are real and due to temperature variations in the tubes or whether they are caused mainly by a personal error in the observer or by imperfections in the stop-watches employed, but we suspect these latter to be mainly responsible.

We give the following examples of results obtained:

1. Dilutions of D_2O 492 units

	Calculated	Found
$9H_2O + 1D_2O$	49·2	49
$8H_2O + 2D_2O$	98·4	97
$7H_2O + 3D_2O$	147·6	147
$6H_2O + 4D_2O$	196·8	195
$5H_2O + 5D_2O$	246·0	248
$4H_2O + 6D_2O$	297·2	294
$3H_2O + 7D_2O$	344·4	341
$2H_2O + 8D_2O$	393·6	393
$1H_2O + 9D_2O$	442·8	440

There is in this case a positive systematic difference of 1·3 units and accidental variations of about ± 2 units.

2. A similar series of dilutions of D_2O 2975 with D_2O 2483 gave

	Calculated	Found
$2 \times 2483 + 8 \times 2975$	2876·6	2875
$4 \times 2483 + 6 \times 2975$	2778·2	2780
$6 \times 2483 + 4 \times 2975$	2679·8	2679
$8 \times 2483 + 2 \times 2975$	2581·4	2581

3. A sample of egg albumin dissolved in about 3% D_2O was evaporated in a vacuum. The main portion of distillate was found to have a concentration of 3076 units. The final 1 ml. gave 3081. The increase observed can be taken as due to fractionation.

4. The egg albumin (dried completely at 108°) was burned with dry air in a quartz tube. The water produced was determined after dilution.

On one dilution of 0·1824–1·186 g. the concentration was determined as 190·6 units or for the undiluted sample 1240 units.

A second combustion yielding 0·2066 g. diluted to 1·2103 gave 208·4 units or for the undiluted sample 1240 units.

5. Rat urine distilled off through a combustion tube and diluted gave 400 units or for the undiluted 2550 units. The same urine purified by combustion but not diluted gave 2560 units.

6. Water which had been in contact for hours with human skin proved exceptionally difficult to purify. One such sample carefully purified in the usual way gave 2895 units whilst the same sample after combustion gave 2894 units showing that in this case the normal treatment was sufficient.

Results on the exchange of hydrogen atoms between the water and the tissues of organisms will be published shortly in the *Skandinavisches Archiv für Physiologie*.

Summary.

Methods are described for obtaining water samples of 0·2–2 ml. by distillation or combustion, for purifying such samples without contamination, for measuring with a precision micro-pipette drops of any size between 0·01 and 0·1 ml. and for determining the D_2O content of such drops by their rate of fall in an immiscible fluid.

The accuracy is 1–2 in the sixth decimal place of the specific gravity or about 0·001 % of D_2O.

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