A new, rapid and precise method for measuring the intra-cellular water content by gas chromatography and ^{3}H-sucrose

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Summary. A new rapid method for measuring intra-cellular water content by gas chromatography and an isotopic (^{3}H-sucrose) technique is described. Water content of circulating red cells was revealed to be 71.26±0.31%. Intra-cellular water content increased to 102.9% of its original value following addition of 3 volumes of acid-citrate-dextrose (ACD) to 20 volumes of blood.

Intra-cellular water content is assumed to change during maturation, aging and cellular activity. Therefore, a versatile and precise method for measuring the cellular water content is required in many investigations. The conventional method is a time-consuming, troublesome drying and weighing procedure[1-5], combined with a radioisotopic technique using ^{131}I-albumin to correct for the inter-cellular space[6,7].

Kageyama[8] developed a new method for measuring the water content of red cells by GLC correcting the trapped water with sucrose, which distributes homogeneously in plasma, while not penetrating the red cell membrane[9]. In this method, the water and sucrose content of the packed red cells containing trapped plasma were measured by GLC. We developed a more rapid and simple method using GLC and ^{3}H-sucrose, available in a laboratory equipped with facilities for radioisotopic techniques. This paper deals with the analytical procedure for this new method and its application for determining the effect of an acid-citratedextrose (ACD) mixture on the water content of red cells.

Materials and methods. Heparinized venous blood was obtained from healthy male volunteers ranging in age from 22 to 45 years. 10 µl of physiological saline containing 2 µCi ^{3}H-sucrose (Radiochemical Center, Amersham) and 0.1 mg sucrose was added to 1 ml of the blood. This was put into a hematocrit (Wintrobe tube) and centrifuged at 3000 rpm for 30 min. The 50 µl of supernatant was taken with a microliter syringe and injected into a sample bottle[8-10] containing 1.95 ml of 95.9% methanol +4.1% n-butanol (internal standard, IS) and 2 glass beads. The sample bottle was shaken well and allowed to stand. Next, the remaining plasma was completely removed from the hematocrit and the packed red cells were sucked up with a capillary pipette.

The Pressure-Lok Liquid Syringe (Precision Sampling Corp. Baton Rouge) shown in figure 1 was used as follows: a) Both plungers A and B were pulled out from the enlarged upper barrel. b) The packed red cells sucked up with a capillary pipette were transferred into the enlarged upper barrel (see fig. 1). c) Plunger A was pressed down until the teflon tip sealed within the calibrated bore of the smaller barrel. d) Plunger B was pressed down as far as it

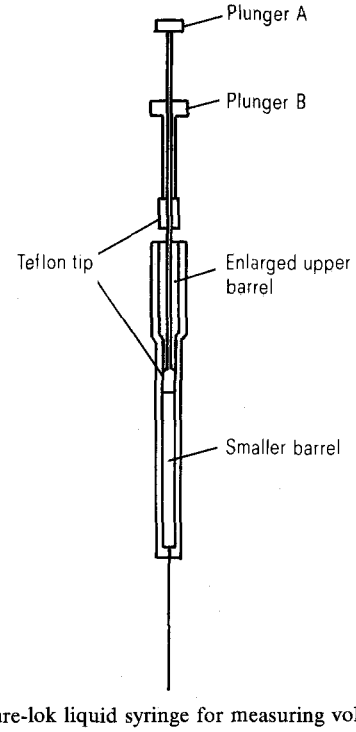

Fig. 1. Pressure-lok liquid syringe for measuring volume of packed red cells.

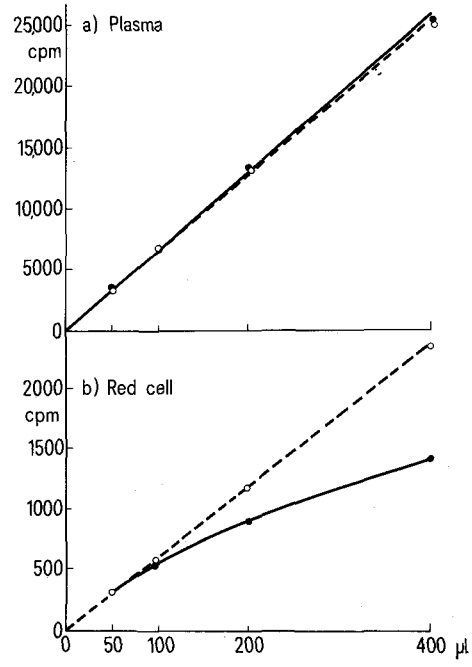

Fig. 2. Relation between volume and radioactivity of supernatant mixtures of plasma and packed red cells: plasma sample (A) and packed red cell sample (B), —●— supernatant mixture; ---○--- control. The examination was carried out as follows: 1. Supernatant mixtures from plasma and packed red cells were prepared from blood without addition of ^{3}H-sucrose. 2. ^{3}H-sucrose was added to the plasma sample in a concentration of 0.1 µCi/ml and to the packed red cell sample in a concentration of 0.01 µCi/ml, which was estimated from radioactivity of ^{3}H-sucrose used to determine trapped water. 3. Each control sample consisted of methanol with the same concentration of ^{3}H-sucrose. 4. Radioactivities in 50 µl–400 µl of the experimental and the control samples were measured as described in 'Materials and methods' section.

Effect of ACD mixture on water content in red cells

Mixture (µl) added to 1 ml blood	Water content (%) ± SE Subject 1	Subject 2	Subject 3	Subject 4	Mean
Control	71.41 ± 0.35	71.13 ± 0.24	71.03 ± 0.15	71.49 ± 0.03	71.27 ± 0.22
ACD 50	71.65 ± 0.21	71.59 ± 0.37	71.32 ± 0.56	71.98 ± 0.15	71.64 ± 0.27
100	72.15 ± 0.17	72.10 ± 0.14	71.77 ± 0.46	72.41 ± 0.36	72.11 ± 0.26
150	73.21 ± 0.16	73.49 ± 0.44	73.06 ± 0.29	73.69 ± 0.32	73.36 ± 0.28
Saline 150	70.41 ± 0.43	70.28 ± 0.21	70.11 ± 0.52	70.79 ± 0.26	70.40 ± 0.29

would go. e) Plunger A was pulled back until the tip was within the enlarged upper barrel. f) A fraction of the packed red cells in the upper barrel was transferred into the smaller barrel by high pressure. g) Plunger A was moved to the 50 µl calibration mark to adjust the volume of the packed red cells exactly. h) The blood sample was injected into the sample bottle containing the solvent mixture described above. The sample bottle was shaken rigorously as soon as possible to avoid clot-formation and left to stand. The remaining cell sample in the enlarged upper barrel could be pushed out by repeating the procedures as described above to produce 3 or 4 specimens, needed for testing reproducibility. The protein component in the plasma or red cells was precipitated following coagulation by methanol. The sample bottles were centrifugated at 1400 rpm for 5 min. The coagulated sample was sedimented, and water contained in the plasma or red cells was extracted in the supernatant by the dehydrating effect of methanol.

A calibration curve was obtained as follows: 1) 3 control sample bottles containing only the original solvent without any additions, and 4 standard sample bottles consisting of solvent and 20 µl, 30 µl, 40 µl and 50 µl of water instead of plasma or packed red cells, were prepared. 2) 2 µl of the supernatant taken from each of the sample bottles was analyzed by a gas chromatograph (Shimadzu Model GC-4BPT, Japan) connected with a thermal conductivity detector. The 3 m × 3 mm inner diameter stainless column was filled with 15% PEG-6000 on Shimalite F (fluororesin solid support, 30–60 mesh). The column temperature was kept at 120 °C and the helium flow rate at 60 ml/min. A ratio of response of water to n-butanol (IS) was printed with a integrator (Shimadzu Model I-1A) connected to the gas chromatograph. A small amount of water contained originally in the methanol and n-butanol was subtracted from the value of the standard or the experimental sample. A calibration curve was obtained by plotting the values for the standard samples, and used to determine the water content of the experimental samples.

Determination of ^{3}H-sucrose content to measure intercellular space was carried out as follows: 50 µl of the remaining supernatant mixture in the sample bottle containing plasma or packed red cells was poured on to a glass microfibre paper (Whatman Ltd, England), which was put into a vial. The solvent was dried, scintillation mixture was added and the radioactivity of the sample was measured in a liquid scintillation counter (Packard Instruments, Downers Grove). The water content in the red cells was calculated from the following equation:

$$\frac{W - S_2/S_1 \times W_1}{V - S_2/S_1 \times V} \times 100\,(\%)$$

where W and W_1 denote the water content (µl) in the packed red cells and in the supernatant (plasma) respectively:

S_1 and S_2 represent cpm of ^{3}H-sucrose in the supernatant and trapped fluid respectively; V denotes the volume of the packed red cells or supernatant; $S_2/S_1 \times W_1$ and $V - S_2/S_1 \times V$ represent the volume of trapped water and red cells respectively.

The water content of the red cells was evaluated from the average value in triplicate determinations of samples obtained from the same source.

The effect of ACD on the water content of red cells was investigated as follows. 50 µl, 100 µl and 150 µl ACD (Nihon Seiyaku Co., Tokyo) were added to 1 ml blood respectively. The water content of each mixture was measured as described above.

Results. The inability of sucrose to penetrate into red cells was checked beforehand. The radioactivity of red cells washed with isotonic phosphate buffered saline after an addition of ^{3}H-sucrose to blood or ACD mixed blood was almost equal to background activity. This finding revealed that sucrose was not penetrating into red cells, as previously reported[9]. Quenching effects of blood samples on the radioactivity of ^{3}H-sucrose are shown in Fig. 2. The curve of cpm of the plasma sample (having a slight yellow color and rather higher radioactivity) agrees well with the control curve and indicates that there is no quenching. On the other hand, the curve of cpm of the packed red cell sample (containing some yellow colored materials and less ^{3}H-sucrose) revealed a depression with increasing sample volumes. In general, 50 µl of the supernatant mixture was used to determine intercellular space, because this volume gave hardly any quenching effect (98.4 ± 2.1% of the control value).

A chromatogram of water in packed red cells is shown in figure 3. Good separation of methanol (solvent), water and

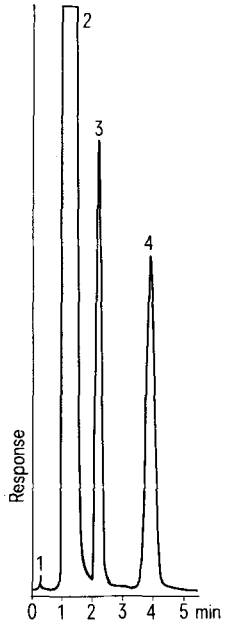

Fig. 3. Gas-liquid chromatogram of water in packed red cells: *1* air, *2* methanol, *3* water, *4* n-butanol (internal standard).

n-butanol (IS) was obtained within 5 min. The chromatogram of the water in the plasma has a pattern similar to that of figure 3. The calibration curve for water is linear[10]. The average value and standard error (SE) of the 6 mean water contents in red cells obtained from one individual in triplicate determinations are 71.39 ± 0.28%. The mean SE of the values determined in triplicate is 0.25%. Thus, the data show excellent reproducibility.

The mean water content in fresh red cells of 14 adults, who ranged in age from 22 to 45 years, was 71.26 ± 0.31%. The water content of red cells obtained from blood supplemented with ACD is shown in the table. It has a tendency to increase as the amount of ACD increases. The water content of red cells with the addition of 150 µl ACD to 1 ml blood increased from 71.22 ± 0.22% (original content) to 73.36 ± 0.28%.

A similar tendency is shown in the content of trapped water. The volume increased 6.8 ± 0.5 to 7.4 ± 0.4% in the packed red cells before and after the addition of 3 volumes ACD to 20 volumes of the blood.

In contrast, physiological saline in the same ratio did not increase the intra and inter-cellular water content.

Discussion. The previous data on water content in red cells according to the weighing method are as follows: 72.2% (v/v) reported by Hald et al.[1], 71.7% (v/v) by Savitz et al.[4], 71.0% (v/v) by Murphy[5] and 66% [(w/w) about 70% (v/v)] by Kuroda[3]. The values are considered to differ from each other as a result of the use of nonstandardized equipment different temperatures and times for the drying procedures, and neglect of other volatile components in the red cell.

Our result (71.26%) agrees well with the value found by Savitz et al. and that found by Murphy, and also 71.73% found previously by Kageyama[8].

The average increase in the water content of red cells following the addition of ACD is 2.93% of the original content when the ratio of blood to ACD was 20:3 (as used to store blood for transfusion), in spite of the fact that the pH (7.4) and osmotic pressure (291 mosmol) of the mixture did not change. The increase is not due to dilution of the blood supplemented with the ACD, because the same concentration of physiological saline does not cause an increase in water content. The mechanism of the phenomenon cannot yet be clarified but is now being studied.

We have also developed a method for measuring water content of brain tissue by GLC (Yamagata et al., unpublished). The new rapid and precise method for measuring water content will be useful for many studies, using various tissues or cells.

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Electrocorticographic activity induced by gamma hydroxybutyrate in the rat during ontogenesis

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Summary. GHB at a dose of 200 mg · kg^{-1} i.p. elicited groups of slow waves with a frequency of 4–5 Hz in both frontal and occipital ECoG leads in adult rats. In 25- and 18-day-old rats similar slow wave activity became continuous and exhibited a clear-cut maximum in the frontal regions. In 15- and 12-day-old animals slow wave activity was also registered in the frontal region but it was organized into short groups of unstable frequency. No ECoG effects of GHB could be found in 9-day-old rats.

Gammahydroxybutyrate (GHB) which was originally proposed to be a hypnotic and anaesthetic agent[1] has been shown to be a useful drug in experimental epilepsy. Godschalk and collaborators[2,3] found that GHB at a subhypnotic dose of 200 mg · kg^{-1} injected i.p. elicited 'hypersynchronous activity' in rats. The bursts of large slow waves with a frequency of 3–5 Hz were accompanied by a sudden arrest of ongoing motor activity; this immobility lasted for the duration of the burst. These bursts could be suppressed selectively by anticonvulsants effective against petit mal epilepsy[2]. Because this type of epilepsy is predominant in children of the preschool and school age[4], we studied the ECoG effects of GHB during ontogenesis[5].

Methods. Rats aged 90 (i.e. adult animals), 25, 18, 15, 12 and 9 days were used. Surgical preparation (trephine openings, tracheal cannula) was performed under ether anaesthesia, then the wounds were carefully infiltrated with procaine, the anaesthesia was disrupted and the animals were immobilized by d-tubocurarine. Artificial ventilation as well as body temperature were maintained.

Electrocorticograms were recorded by means of silver ball electrodes from sensorimotor (frontal) and visual (occipital) areas of both hemispheres, whereas an indifferent electrode was placed on the nasal bone. The electrocardiogram served for monitoring the state of the animals. At the beginning of the experiment, a 5-min period of spontaneous ECoG was recorded, then gammahydroxybutyrate sodium was administered i.p. at a dose of 200 mg · kg^{-1} and ECoG was continuously recorded for at least 30 min. In adult, 25- and 15-days-old rats driving was also studied. 15-sec periods of rhythmic photostimulation with frequencies of 3 and 5 Hz were applied before and 30 min after the injection of GHB.

Results. Adult rats (n = 8, fig. 1): Development of 5-6,5 Hz rhythm was visible after the 2nd to 3rd min after GHB administration; during the next minutes the incidence of