Micromethods for the simultaneous measurement of intracellular potassium and corticosterone output of isolated adrenal cortical cells

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Summary

1. A technique is described for the measurement of potassium and water spaces in less than 1 μl of packed cells.

2. The total potassium content of cell pellets is measured in a perchloric and nitric acid extract by flame spectrophotometry. The potassium in trapped medium is estimated from the distribution space of hydroxy[14C]methylinulin and subtracted from the total potassium content to give intracellular potassium content. Corticosterone output was measured by radioimmunoassay.

3. The simultaneous measurement of total water from the [3H]water space allows calculation of the intracellular water space.

4. Values obtained for intracellular potassium content, intracellular water space and calculated intracellular potassium concentration are presented for different preparations of isolated adrenal cortical cells.

Key words: adrenal cortex, intracellular electrolytes, isolated cells, potassium.

Introduction

The use of dispersed cell suspensions for the measurement of intracellular electrolytes offers several advantages over the use of intact tissues, with which there are difficulties in defining the extent and composition of the extracellular space (Mannery, 1954).

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Methods

Rats

Female Sprague–Dawley rats weighing between 180 and 200 g were obtained from Carworth Europe, Huntingdon, U.K. The animals were fed on a diet containing Na+ 250 mmol/kg dry weight and K+ 210 mmol/kg dry weight.
Media

The 'standard medium' used was Krebs-Ringer bicarbonate containing glucose, 11.1 mmol/l, and bovine serum albumin (Pentex fraction V from Miles Laboratories Inc., U.S.A.), 40 g/l, with the following ionic composition (mmol/l): Na⁺ 145, K⁺ 3.79, Ca²⁺ 2.54, Mg²⁺ 1.18, HCO₃⁻ 24.9, Cl⁻ 128, phosphate 1.18, SO₄²⁻ 1.18. High-potassium medium was similar but with potassium concentration 13.4 mmol/l and sodium concentration 135.4 mmol/l.

Preparation of adrenal cells suspensions

Batches of adrenal glands from sixteen to twenty-four rats were pooled to prepare the cells. Isolated cell suspensions were prepared from adrenal capsular stripplings or decapsulated glands with collagenase (batch CLS 3BC from Worthington Biochemicals Corp., New Jersey) digestion as previously described for zona glomerulosa cells (Tait, Tait & Bradley, 1972) or zona fasciculata–reticularis cells (Haning et al., 1974). The cells were suspended in standard medium so that 50 pl of cell suspension contained the cells derived from one adrenal (approximately 1 × 10⁹ cells). In two series of experiments, zona glomerulosa cells were purified by unit gravity sedimentation (Tait et al., 1974) and used in similar incubations. Cell counts were performed at 400 × magnification with an improved Neubauer haemocytometer.

Incubations

Incubations were performed in 2 ml conical polypropylene tubes under an atmosphere of O₂ + CO₂ (95:5, v/v), in a shaking incubator. The mixtures contained 50 pl of cell suspension, 60 pl of standard or high-potassium medium and 10 pl of sodium chloride (154 mmol/l).

The mixtures were incubated at 37°C for 90 min and then for a further 30 min after addition of isotopes as described below.

Water spaces

Water spaces were determined by using the [³H]water space for total water and the hydroxy[¹⁴C]methylinulin space for extracellular water. The method is similar in principle to those described for isolated fat cells (Perry & Hales, 1969; Gliemann, Osterlind, Vinten & Gammeltoft, 1972). The hydroxy[¹⁴C]methylinulin (11.4 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was purified before use by gel-filtration chromatography on a column (60 cm × 1 cm) of Sephadex G-25 (fine grade). Elution was performed with distilled water while fractions of 2-0 ml were collected. ¹⁴C radioactivity was found as a single peak at an elution volume of 18–22 ml, which corresponded to the void volume of the column as determined with Blue Dextran 2000 (Pharmacia Chemical Co., Sweden).

The first two fractions comprising the peak were pooled, divided into aliquots, each of approximately 4 μCi, freeze-dried, stored at 4°C and dissolved in 0.2 ml of Krebs-Ringer bicarbonate medium immediately before use.

Hydroxy[¹⁴C]methylinulin (10 μl; 20 μCi/ml, in standard medium) and tritiated water (150 μCi/ml) were then added to each tube. The medium used to carry the tritiated water was standard or high-potassium medium in order to readjust the final [K⁺](') of the incubation mixtures to 3.6 or 8.4 mmol/l respectively. To one tube, only hydroxy[¹⁴C]methylinulin was added, and to another tube, only tritiated water; these tubes served as ¹⁴C and ³H standards (see below). The tubes were gassed with O₂ + CO₂ (95:5), capped and incubation was continued for a further 30 min at 37°C in order to allow the water space markers to equilibrate with the cell suspensions.

Corticosterone output

Six replicate incubations were prepared for each experimental manipulation. To two of the replicates, the isotopic water space markers, hydroxy[¹⁴C]methylinulin and tritiated water were omitted; these tubes were also incubated for a total duration of 2 h and were then used to determine corticosterone output, radioimmunoassay being used as described by Tait et al. (1972). The remaining four tubes were used for determination of water spaces and potassium by the following methods.

Separation of cells and media

The incubation mixtures were aspirated into silicone-treated glass capillary tubes (70 mm × 0.31–
Adrenal cortical potassium

0.34 mm o.d.) provided with an upper reservoir of volume 0.5 ml (Fig. 1). A single column of air, approximately 30 mm long, was introduced below the cell suspension and the capillary tip flame-sealed.

The tubes were centrifuged in polythene holders at 1400 rev./min (500 g) for 5 min at 4°C in an MSE Mistral 4L centrifuge. The tubes were removed and the overlying medium was aspirated to within 1 mm of the cell pellet which lay in the capillary tube above the air column. After breakage of the tip of the capillary tube, the cell pellets were discharged into 100 µl of distilled water and disrupted by repeated aspiration and expulsion from the tubes.

Preparation of extracts of cells and media

After standing at room temperature for 1 h, the lysed cells were deproteinized by the addition of 100 µl of perchloric acid (0.5 mol/l) and the extract was then centrifuged at 1400 rev./min at 25°C. A portion (100 µl) of the resulting supernatant solutions was withdrawn and added to counting vials containing 2 ml of the scintillator mixture: Triton-X 100, 300 ml (Koch-Light Laboratories Ltd, Bucks., U.K.), and Permaflour 1 (Packard Instrument Co., Ill., U.S.A.), 40 ml made up to 11 with toluene. To the remaining 100 µl of cell extract, 10 µl of concentrated HNO₃ was added to ensure complete extraction of potassium, and the extract was allowed to stand for 14 h at 22°C. The mixture was centrifuged again at 1400 rev./min for 30 min at 35°C and 50 µl of the supernatant solution diluted to 1.0 ml with sodium chloride (3 mmol/l) for potassium measurements.

Samples of the media were treated in a similar manner for radioactivity and measurements of potassium concentration; 50 µl of medium was diluted to 1.0 ml with perchloric acid (0.5 mmol/l) and the mixture centrifuged at 1400 rev./min for 30 min at 25°C. A portion (100 µl) of the resulting supernatant solution was placed in counting vials with 2 ml of the scintillator mixture. An additional 50 µl sample of the untreated medium was diluted to 5.0 ml with NaCl (3 mmol/l) for potassium measurement.

¹⁴C and ³H radioactivity in the extracts was measured in a Packard Tri-Carb liquid-scintillation counter (model 3320) equipped with a model 543 tape perforator. Sufficient radioactivity counts were accumulated to keep the counting error below 1%. Count rates in the ³H and ¹⁴C channels were corrected for background and for overlap with the values obtained with the ³H and ¹⁴C standard incubations for both cells and media. Quenching of scintillations was monitored for each vial by the channels-ratio method. Recovery of ³H and ¹⁴C was measured in both the cells and media in nine experiments.

The hydroxy[¹⁴C]methylxaminulin (extracellular water) and the [³H]water (total water) spaces were calculated for each cell pellet. The difference between the two spaces was used to measure the intracellular water space.

Potassium measurements

Potassium concentration in the cell extracts and diluted media were determined with a Unicam SP. 90A series 2 Atomic Absorption Spectrophotometer, in emission mode at 766.5 nm wavelength (method 1). An SP.92 Automatic Sample Changer was used to aspirate 0.6 ml of the sample over 16 s. A single-slot burner was used at 2 cm flame height with gas flows of acetylene 1 l/min and compressed air 5 l/min. Output from the instrument was recorded with a Servoscribe chart recorder. With this arrangement, and using the amplifier at the 'high gain' setting, reproducible measurements were obtained with potassium concentrations as low as 1–2 µmol/l. However, particular care was necessary to prevent contamination of samples from dust particles, perspiration from fingers, or K⁺ leaching from glassware. Satisfactory results were obtained when disposable plastic containers were used for all handling and dilution of cell extracts. In addition, it was found that presence of sodium in the samples affected potassium flame-emission intensity and to render this effect constant all samples and standards were diluted in NaCl (3 mmol/l).

For comparison, in some cases potassium concentration was also measured with the Unicam
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instrument in atomic absorption mode, by employing a K⁺ hollow-cathode lamp at 766.5 nm (method 2).

In addition, potassium and sodium concentrations were also measured in some samples with a separate flame photometer designed for use with microsamples (method 3). Details of the construction and operation of this instrument have been described elsewhere (Bosher & Warren, 1968). In brief, samples of approximately 200 nl of cell extract were applied to a platinum filament, dried in a stream of hot air, and placed in the burner assembly. Potassium was volatilized by electrically heating the filament and carried into the hydrogen-air flame. Emission output from the flame at the appropriate wavelength was integrated to yield a signal proportional to the total K⁺ in the sample. Methods 2 and 3 were used only to establish the validity of results obtained with method 1, which was used in all experiments.

The total potassium content of the cell extract was corrected for the contribution from trapped medium, the measured values for the extracellular water hydroxy[¹⁴C]methylinulin space) and potassium concentration in the medium, in each experiment, being used to calculate the intracellular K⁺ content, (K⁺)ᵢ.

Fig. 2 is a flow diagram for the handling of cells and extracts for measurement of water spaces, corticosterone output and intracellular K⁺.

Additional methods

Comparison of different procedures for extracting intracellular K⁺. In order to evaluate whether the routine extraction procedure released all of the intracellular K⁺, cell extracts were prepared by different methods and their [K⁺] was measured. Replicate samples of zona fasciculata cells, each containing cells equivalent to 0.5 adrenal or 0.61 × 10⁵ cells, were centrifuged in capillary tubes and the resulting

Fig. 2. Flow diagram of micromethods used to measure corticosterone output, water spaces and intracellular potassium in dispersed adrenal cortical cells.

\[
\text{Dispersed cell suspension} \quad \xrightarrow{\text{incubate: 90 min, under } O_2 + CO_2 (95:5, v/v)} \quad \text{Polypropylene tubes}
\]
\[
[\text{³H}] \text{Water} \quad [\text{¹⁴C}] \text{inulin}
\]
\[
\text{Two replicates} \quad \text{Steroid assay} \quad \xrightarrow{\text{incubate: 30 min, 37°C}} \quad \text{Four replicates}
\]
\[
\text{Water space and } K^+ \quad \xrightarrow{\text{Centrifuge: 500g, 5 min}} \quad \text{Medium}
\]
\[
\text{Isotopes} \quad [K^+]
\]
\[
\text{Isotopes} \quad [K^+]
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\text{Cell pellet}
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cell pellets lysed in 0.1 ml of distilled water and deproteinized with 0.1 ml of perchloric acid (0.5 mol/l). Four groups, each of four samples, were then treated as follows: (a) no further additions; (b) 10 μl of concentrated HNO₃ for 5 min at 22°C; (c) 10 μl of HNO₃ for 14 h at 22°C (routine method); (d) 50 μl of HNO₃ for 14 h at 60°C. The samples were centrifuged and [K⁺] was measured as described above.

**Human erythrocytes.** These were used to test the validity of the methods for intracellular water and K⁺; 10 ml of blood was withdrawn from the antecubital vein and anticoagulated with 1.0 ml of sodium citrate (38 g/l). After centrifugation at 2500 rev./min (1500 g) for 30 min at 4°C the plasma was withdrawn and the cells were resuspended in isotonic sodium chloride and centrifuged again. The packed cell volume of the suspension was determined by centrifugation at 1500 g for 30 min. The cells were finally resuspended in standard medium, so that 50 μl of the suspension contained exactly 1 μl of packed erythrocytes. This suspension was used in place of adrenal cells in the methods described.

**Sodium concentration.** This was measured in some of the adrenal cell extracts to provide an independent measure of extracellular water in the cell pellets. In these experiments the cell extracts were diluted in distilled water and sodium was measured with method 3.

**Sodium and potassium status of the animals.** This was assessed by analysis of the diet and by 24 h urinary Na⁺ and K⁺ excretion. Twelve animals were placed in separate metabolic cages and three consecutive 24 h urine samples collected. The diet pellets (approximately 30 g) were digested in 60 ml of concentrated HNO₃ for 14 h, the extracts were diluted with distilled water, filtered and made up to 250 ml. Na⁺ and K⁺ were measured in the diet extracts and in urine according to the Pye Unicam Methods Manual (1969) for the Unicam SP.90 Atomic Absorption Spectrophotometer.

**Statistical methods.** Linear regression analysis was used to assess the relationships between water space or potassium measurements performed by independent methods on the same samples. The significance of gradients and intercepts of the regression lines were tested, where appropriate, by t-tests with estimates of the standard errors of the regression parameters obtained from the residual mean square calculated by analysis of variance (Armitage, 1971).

**Results**

**Expression of potassium results**

Intracellular K⁺ contents were expressed as nmol per 'adrenal equivalent', which is the yield of cells obtained from one adrenal gland in the particular experiment. Intracellular K⁺ contents are designated [K⁺] in the results. Intracellular K⁺ concentration (designated [K⁺]i, mmol/l of intracellular water) was also calculated for each incubation by dividing the intracellular K⁺ content by the intracellular water volume. However, measurements of intracellular water volume showed a greater variance than those of K⁺ content and the calculated K⁺ concentrations therefore showed a wider scatter than of K⁺ contents. For this reason most of the results are presented as K⁺ contents. Where there were sufficient measurements to make meaningful comparisons, K⁺ concentrations are also presented and are given as [K⁺]i.

**Validation of water space methods**

**Purity of hydroxy[¹⁴C]methylinulin.** Initial experiments with unpurified hydroxy[¹⁴C]methylinulin gave variable results, with low values for intracellular water space with some batches of inulin. This suggested that the preparation might contain some low-molecular-weight materials capable of penetrating cell membranes. For this reason the inulin was always purified before use by gel-filtration chromatography. The elution pattern of hydroxy[¹⁴C]methylinulin from the Sephadex G-25 column showed a single peak of radioactivity at the void volume (determined with Dextran Blue 2000) of the column.

Pooling the first three fractions from the radioactivity peak followed by rechromatography gave an essentially identical elution pattern. However, intracellular water space measured on replicate samples (packed volume 25 μl) of human erythrocytes with the unpurified inulin gave a value of 15.71 μl (SEM 0.26, n = 7), which was significantly lower than that obtained simultaneously with the purified material of 17.14 μl (SEM 0.30, n = 7, P < 0.01). Although the differences between these values is small when cell volumes of this magnitude are involved, the errors of over-estimating the extracellular volume are more serious when cell volumes of less than 0.5 μl are used.

**Comparison of sodium content of cell extracts and...**
extracellular water measured from the inulin space. Since Na\(^+\) is predominantly an extracellular cation and its intracellular concentration relatively constant between samples of the same cell suspension, the variation in total Na\(^+\) in replicate samples of cells should be accurately predictable from their extracellular water spaces.

When used to measure extracellular water, the Na\(^+\) space is usually larger than the inulin space (Goodford & Leach, 1966), probably because some Na\(^+\) can enter the cells. However, in this experiment equal numbers of cells were present in each pellet and therefore the small intracellular component of Na\(^+\), \((\text{Na}^+)_i\), should be constant; analysis of the data by linear regression essentially removes this constant \((\text{Na}^+)_i\) component, which should appear as the \(y\) intercept so that the gradient of the line reflects only extracellular Na\(^+\).

The relationship between the sodium content as measured by microflame emission spectrophotometry (method 3) and the extracellular water space measured with the hydroxy\([^{14}\text{C}]\)methylinulin method was determined for nine different extracts of zona glomerulosa cells. The calculated regression line between sodium content \((y)\) and intracellular water \((x)\) was: \(y = 17.4 \text{ (SEM 19.0)} + 153.4 \text{ (SEM 21.2)} \times \), with a correlation coefficient of \(0.940 \text{ (P<0.001)}\). The value for the slope of the line is not significantly different from the sodium concentration of the medium of \(146 \text{ mmol/l}\). The agreement of this gradient with the Na\(^+\) concentration of the medium supports the quantitative validity of the extracellular water space measured with hydroxy\([^{14}\text{C}]\)methylinulin.

Dependence of intracellular water space on cell number. The relationship between intracellular water space and the number of aliquots (each containing 0.5 adrenal equivalent or \(5 \times 10^4\) cells) of the cell suspension used for each measurement, was determined with zona glomerulosa cells. There was a significant linear relationship between intracellular water \((y)\) and the number of cells \((x)\): \(y = 0.027 \text{ (SEM 0.049)} + 0.143 \text{ (SEM 0.015)} \times \text{ (r = 0.9599, } P<0.005)\). The intercept on the \(y\) axis is not significantly different from zero \((t = 0.55, P>0.6)\), indicating that there was no systematic bias in the intracellular water space measurements.

The total water space of these cell pellets varied widely and was not related to cell number; this is not surprising since the intracellular water space represented only 25% of the total water space. By subtracting the extracellular component of the total water space a significant linear relationship between intracellular water and cell number is revealed. This relationship is therefore a test of the validity of the measurements of both total water and extracellular water.

**Intracellular water space of human erythrocytes.** A sample of fresh human erythrocytes was diluted in standard medium to obtain a suspension containing exactly 1 µl of packed cells in 50 µl of suspension. Intracellular water space measured by the technique described was 0.705 µl \((\text{SEM 0.018, } n=10)\), a value in good agreement with published values of intracellular water of 0.70–0.75 ml of intracellular water/ ml of total cell volume (Altman, 1961).

**Effect of duration of incubation on intracellular water space.** The values obtained for intracellular water when zona glomerulosa cells were incubated for various times with the isotope-labelled water space markers is shown in Fig. 3. The values were not significantly different for the times at 15 min, 30 min and 60 min but had fallen at 2 h, suggesting that some hydroxy\([^{14}\text{C}]\)methylinulin had penetrated the cells in this longest period. From these results, 30 min of incubation with the isotopes was used in all experiments.

**Variability of measurements of intracellular and extracellular water.** The total water space of the cell pellet obtained in these studies was in the range 0.8–1.5 µl, of which 75–80% was extracellular. Variability of intracellular water measurements in triplicate samples was dependent on the ratio of
extracellular water to total water spaces. When the extracellular water was 54% of the total water the coefficient of variation of measurements of intracellular water (mean value of 0.075 μl) was 8.0%. For extracellular water/total water ratios of 72% and 89% the coefficients of variation of measurements of intracellular water (mean values 0.454 and 0.0746 μl) were 22% and 34% respectively. Generally, the extracellular water/total water ratio in the glomerulosa cell pellets was 75–80% and the coefficients of variation of intracellular water measurements 12–25%. The mean coefficient of variation of intracellular water measurements on fasciculata cells was 11–9% (SD 6.6, n = 25).

Recovery of both isotopes was measured in both the cells and media. The total recovery of 3H2O in cells and medium was 97.4% (SD 8.3, n = 8), of which 9.5% was in the cell pellet. Corresponding values for hydroxy[14C]methylulinulin were 106.7% (SD 7.2, n = 9), of which 9.5% was in the cell pellet.

Validation of potassium measurement

Correlation of different techniques. The relation between potassium measurements performed on twenty-nine different extracts of zona glomerulosa cells was investigated by flame emission with the Unicam instrument (method 1) and by flame spectrophotometry on an independent instrument (method 3). The calculated regression line relating the two measurements was y = 0.663 (SEM 1.029) + 1.030 (SEM 0.113) x with a correlation coefficient of 0.8687 (P < 0.001). The intercept on the y axis is not significantly different from zero and the gradient is not significantly different from 1.0, indicating that there is no systematic difference between the methods and that the results obtained from each are in good quantitative agreement. Similarly, the relationship between potassium measurements on sixteen different extracts of glomerulosa cells was measured both by flame-emission (method 1) and atomic-absorption spectrophotometry (method 2) with the Unicam instrument. The calculated regression line was y = 0.359 (SEM 0.377) + 1.315 (SEM 0.089) x with a correlation coefficient of 0.959 (P < 0.001). Again, the intercept on the y axis is not significantly different from zero (t = 0.953, P > 0.3) and the gradient is not significantly different from 1.0 (t = 1.503, P > 0.1).

Recovery of added K+ and extraction of endogenous K+. The recovery of 1.5 nmol of K+ added to extracts of zona fasciculata–reticularis cells containing an initial mean value of 10.4 nmol was 84% (SEM 22, n = 9). When 10 nmol of K+ was added to the extracts the recovery was 100.8% (SEM 2.2, n = 7).

These results exclude the possibility that substances in the cell extracts interfere with the measurements of K+ and also indicate that the extraction of soluble, endogenous K+ in the cell extracts is likely to be complete.

When extracts of cells were prepared with different volumes of nitric acid and different durations of extraction, the values obtained for [K+] in the extracts were similar; for groups (a) to (d) (see under 'Additional methods'), the values were respectively (μmol/l) 8.59 (SEM 0.56, n = 4), 8.86 (SEM 0.34), 8.45 (SEM 0.34) and 8.98 (SEM 0.63). These values show no significant differences between groups, which suggests that the mildest extraction procedure (lysis with distilled water and perchloric acid) is sufficient to liberate the maximum amount of intracellular K+.

K+ content of glomerulosa cells as a function of duration of incubation. Intracellular potassium content, ([K+]), was determined on replicate aliquots of zona glomerulosa cells, each of two adrenal equivalents or 2.8 × 10⁶ cells, after different times of incubation. Values (nmol of K+) obtained were 35.3 (SD 3.8, n = 4) at 15 min, 36.4 (SD 8.0) at 30 min, 44.6 (SD 2.4) at 60 min and 36.6 (SD 5.8) at 120 min.

These results indicate that the intracellular K+ of the cells was in a steady state during the incubation and suggests (a) that the cells had not lost K+ during the preparation procedure (in which case (K+), would be expected to rise during the incubation) and (b) that there is not an appreciable population of damaged cells (in which case (K+), might fall during incubation).

Relation between intracellular K+ content and cell number. The relation between intracellular K+ content and the number of aliquots (each containing cells equivalent to 0.5 adrenal or 5.1 × 10⁶ cells) of the suspension used for each determination was obtained with zona glomerulosa cells. The equation for the regression line relating intracellular K+(y) and the number of cell aliquots (x) was: y = 4.80 (SEM 3.07) + 10.96 (SEM 0.92) x, with a correlation coefficient of 0.973 (P < 0.01). The intercept on the y axis is not significantly different from zero (t = 1.57, P > 0.1).

This relationship suggests that the methods used provide a valid measure of intracellular K+.
**Intracellular potassium concentration of human erythrocytes.** Human erythrocytes were chosen as a convenient cell to test the methods since published studies (Spector, 1956; Altman, 1961) have established their (K+), at 89–101 mmol/l of cell volume and [K+]i at 136 (SD 12) mmol/l of cell water. With 1 μl of packed erythrocytes, the (K+), was found to be 98.0 mmol/l of cell volume (SEM 1.5, n = 10) and [K+]i was 140.8 mmol/l of cell water (SEM 4.0, n = 10).

These results suggest that both the water space and the (K+), measurements provide results which are in quantitative agreement with those determined by conventional methods performed on a macroscale.

**Variability of potassium measurements.** Potassium concentration in the extracts of the cells used for emission spectrophotometry was in the range 4–8 μmol/l, which is equivalent to total K+ contents in the glomerulosa cell pellets of 18–36 nmol. Extracellular potassium accounted for 14–22% of the total content. The mean coefficient of variation of triplicate measurements of intracellular K+ in glomerulosa cells was 8.0% (SD 7.1, n = 44), and for fasciculata cells was 6.9% (SD 3.6, n = 23).

**Observations on isolated adrenal cells**

Sodium and potassium status of the animals was assessed by measurements of 24 h urinary Na+ and K+ excretion. For twelve animals fed on the standard diet (containing Na+, 247 mmol/kg dry weight and K+, 214 mmol/kg dry weight) the results of three consecutive 24 h urine collections were as follows: urinary volume was 11.9 ml/24 h (SEM 0.83, n = 36); urinary Na+ excretion was 2.41 mmol/24 h (SEM 0.13, n = 36); urinary K+ excretion was 1.70 mmol/24 h (SEM 0.09, n = 36). By comparison with published values (Möhring & Möhring, 1972) these results indicate that the animals were replete in Na+ and K+.

The following results present the values of (K+),, intracellular water spaces and calculated [K+]i, found in various preparations of adrenal cells. Corticosterone outputs were determined in parallel incubations and are presented in the next paper (Mendelsohn & Mackie, 1975).

**Unpurified zona glomerulosa cells.** Examination of cell suspensions prepared by collagenase dispersion of adrenal capsular strippings by phase-contrast microscopy revealed approximately 95% zona glomerulosa cells (9.6 μm diameter, range 8–10 μm), with 2–5% contamination by zona fasciculata cells (19.6 μm diameter, range 13–22 μm), and small numbers of other cell types (Tait et al., 1974).

The yield of zona glomerulosa cells was 1.71 × 10⁸ cells per adrenal (SEM 0.22, n = 11). The cells derived from one adrenal were used in each incubation and are referred to as one ‘adrenal equivalent’ for expression of (K+)i and intracellular water spaces.

The (K+), of these cells was 20.04 nmol/adrenal equivalent (SEM 2.94, n = 11) and intracellular water space was 216 nl/adrenal equivalent (SEM 56, n = 11).

An estimate of the mean [K+]i may be obtained by dividing (K+), by the intracellular water space. However, this treatment involves various assumptions (see the Discussion section). The value of [K+]i, so calculated for the unpurified glomerulosa cells was 103.4 mmol/l of intracellular water (SEM 19.8, n = 11).

In a separate series of experiments, which were performed at a different time, the yield of glomerulosa cells was 0.81 × 10⁸ per adrenal (SEM 0.11, n = 5). The value for (K+),, in this second series was 15.63 nmol/adrenal equivalent (SEM 0.69, n = 5) and of intracellular water space 162 nl/adrenal equivalent (SEM 19, n = 5). The calculated [K+]i, was 98.8 mmol/l of intracellular water (SEM 8.2, n = 5), which is similar to that obtained in the first series of experiments.

**Zona glomerulosa cells purified by unit gravity sedimentation.** The absolute values of (K+),, and intracellular water spaces obtained with unpurified cells may not accurately reflect the glomerulosa cell population since the unpurified suspensions are contaminated by zona fasciculata cells (see the Discussion section). For this reason three experiments were performed with glomerulosa cells purified by unit gravity sedimentation: 1.52 × 10⁸ cells were used in each incubation in the first experiment and 1.36 × 10⁸ and 1.12 × 10⁹ cells in the second and third experiments. The (K+), values were found to be 10.7, 12.3 and 13.0 nmol respectively and the calculated [K+]i, values were 143 mmol/l of intracellular water (SEM 21, n = 4), 77 mmol/l (SEM 5, n = 4) and 68 mmol/l (SEM 6, n = 4) respectively.

**Zona fasciculata–reticularis cells.** Phase-contrast microscopy of suspensions of cells prepared from decapsulated glands showed a predominance of large (16–19 μm diameter), lipid-laden cells whose appearance was typical of zona fasciculata cells.
TABLE 1. Effects of cold storage and unit gravity sedimentation on fasciculata cells

Results are shown as mean values ± SEM, n = 9.

<table>
<thead>
<tr>
<th>Zona fasciculata cells preparation</th>
<th>Fasciculata cell count</th>
<th>Total intracellular water (nl)</th>
<th>Total intracellular K+ content (nmol)</th>
<th>[K+]i (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpurified (immediate)</td>
<td>1.78 x 10^5</td>
<td>684 ± 25</td>
<td>72.7 ± 1.7</td>
<td>107 ± 4</td>
</tr>
<tr>
<td>Unpurified (stored 4°C, 4 h)</td>
<td>1.78 x 10^5</td>
<td>613 ± 13</td>
<td>74.7 ± 1.4</td>
<td>121 ± 3</td>
</tr>
<tr>
<td>Purified</td>
<td>1.69 x 10^5</td>
<td>386 ± 21</td>
<td>40.2 ± 1.8</td>
<td>107 ± 7</td>
</tr>
</tbody>
</table>

(Haning et al., 1970). The yield of zona fasciculata cells was 1.10 x 10^5 per adrenal (SEM 0.17, n = 4).

(K+)_i was 58.82 nmol/adrenal equivalent (SEM 3.13, n = 4) and intracellular water space 518 nl/adrenal equivalent (SEM 57, n = 4). The calculated [K+]i was 125.9 mmol/l of intracellular water (SEM 26, n = 4).

Discussion

The present techniques appear to provide suitable methods to follow changes in intracellular K+ content, (K+)_i, of isolated adrenal cortical cells during incubations in vitro. The use of water space markers allows correction of the total K+ content of cell pellets for the contribution of K+ from trapped medium in order to determine intracellular K+. In addition the intracellular water space may be calculated in order to assess if changes of cell volume could account for observed changes in intracellular K+.

It is also of interest to estimate intracellular K+ concentration, [K+]i, in terms of intracellular water. However, there are some difficulties in the interpretation of the absolute values of [K+]i obtained with different cell preparations.

First, the measurement of intracellular water space, which is necessary to calculate [K+]i, is critically dependent on the assumption that the extracellular space marker does not penetrate cells. For some preparations of radioactively labelled inulin this assumption is probably invalid (Levi, 1969; Dryden & Mannery, 1970), although for other preparations of inulin good agreement has been shown with the spaces of distribution of extracellular water space markers such as [14C]sorbitol (Dryden & Mannery, 1970) or [14C]sucrose (Williams &
Woodbury, 1971), provided that the duration of exposure of the marker to cells was not prolonged. Hydroxy[\(^{14}\)C]methylinulin, the extracellular space marker used in these studies, was shown to behave similarly to unlabelled inulin in its physical properties and during experiments in vivo (Marlow & Sheppard, 1970).

Small errors in the measurement of the extracellular space may critically affect the value obtained for intracellular water space and hence the calculated \([K^+]_i\) in terms of cell water. However, the correction of total K\(^+\) content of the cell pellet for the contribution of trapped medium in order to calculate \((K^+)_{ii}\) (nmol/adrenal equivalent) is not so critically dependent on the extracellular water space since in the present experiments the total correction was of the order of 18% and small errors in this correction would have a negligible effect on the final \((K^+)_{ii}\).

The second problem in interpretation of \([K^+]_i\) is that the value of K\(^+\) content divided by intracellular water space will give a representative value of \([K^+]_i\) only for a homogeneous population of cells. For the unpurified glomerulosa cell suspensions, even a small percentage contamination by fasciculata cells would be expected to lead to a large contribution in terms of cell volume because of the larger relative volume of fasciculata cells (approximately eight times that of glomerulosa). An attempt at a semiquantitative assessment of the effects of the fasciculata contamination on the results with unpurified glomerulosa cells is presented below. Similarly, the possibility that the glomerulosa cells purified by unit gravity sedimentation would not be homogeneous as far as their potassium contents is discussed below.

However, it does not appear that these problems affect the interpretation of changes in intracellular K\(^+\) content when samples of the same cell suspensions are used, which is presented in the next paper (Mendelsohn & Mackie, 1975).

Significance of intracellular \(K^+\) concentrations of glomerulosa cells

There was a marked discrepancy between the values for \([K^+]_i\), found in zona glomerulosa cells purified by unit gravity sedimentation in the first experiment when compared with the latter two experiments; this difference is due to a lower intracellular water space in the first experiment, but the reason for this difference could not be determined. In view of the theoretical problems of measurement of intracellular water, which are discussed above, the absolute values of \([K^+]_i\) presented here need to be interpreted with caution.

The value obtained for \([K^+]_i\) in purified zona glomerulosa cells in the latter two experiments is lower than for a variety of other isolated mammalian cells whose \([K^+]_i\), has been reported, in the range of approximately 120–160 mmol/l (McDonald & DeHaan, 1973; Perry & Hales, 1969; Solomon, 1962).

It is conceivable that this value represents the true \([K^+]_i\) of glomerulosa cells in vivo. This possibility is difficult to assess because of the lack of published data on intracellular K\(^+\) in glomerulosa cells. Unfortunately, it is not possible to measure \([K^+]_i\) in fresh adrenals by methods analogous to those described here for dispersed cells, because of the difficulties in measuring the composition and extent of the extracellular space. It is possible that techniques such as electron micro-probe X-ray fluorescence (Bacsy, Szaly, Panto & Nagy, 1973) may provide useful information in this area. At present it is only possible to offer the following indirect observation on the possible significance of the \([K^+]_i\) value found for glomerulosa cells.

It is unlikely that the value of \([K^+]_i\) for glomerulosa is an artifact of the methods of measurement in view of the accepted value of 141 mmol/l found for erythrocytes and the value of 126 mmol/l found for fasciculata cells with the same techniques.

It is possible that glomerulosa cells could lose K\(^+\) during the technique of purification since this involves sedimentation at 4°C for 24 h, followed by centrifugation at 4°C and resuspension of the cells (Tait et al., 1974). Loss of K\(^+\) after exposure to cold has been reported for a variety of cells (e.g. Page & Storm, 1965; Glitsch, 1969), although viable cells usually regain K\(^+\) after incubation at 37°C. It is therefore of interest to compare \([K^+]_i\), in fractionated and unfractionated glomerulosa cells. The value of \([K^+]_i\), obtained for unfractionated cells was 103 mmol/l. However, examination of these cell suspensions by phase-contrast microscopy in a haemocytometer revealed an average of 3% contamination by zona fasciculata cells, whose volume is approximately eight times that of the glomerulosa cell. From the value of \([K^+]_i\), (126 mmol/l) found for fasciculata cells, the percentage contamination of the unfractionated glomerulosa cells and the relative volumes of the two cell types, it is possible to correct the \([K^+]_i\), observed for the mixed (unfractionated)
cell suspension to estimate the [K+]i of pure glomerulosa cells before fractionation. The value obtained is 76 mmol/l, which is close to the mean value of 72 mmol/l observed for fractionated glomerulosa cells in the latter two experiments. The observed fall in mean [K+]i after fractionation is therefore consistent with removal of fasciculata cells and need not imply K+ loss by glomerulosa cells during fractionation.

In one experiment where [K+]i was measured in fasciculata cells before and after purification by unit gravity sedimentation no fall in the concentration value was found. This experiment was designed indirectly to assess the behaviour of glomerulosa cells during purification by assuming that the fasciculata cells provide a suitable model. This approach may be partly justified by the finding that fasciculata cells showed a similar fall in (K+)i after treatment with ouabain and a similar increase in (K+)i in [K+]i 8·4 mmol medium as did glomerulosa cells (Mendelsohn & Mackie, 1975), suggesting that both cell types have similar membrane permeability to K+. [K+]i of incubated fasciculata cells did not alter with unit gravity sedimentation and it seems likely that the same conclusion might be true for glomerulosa cells.

If it were accepted that the [K+]i of glomerulosa cells is unchanged by unit gravity sedimentation, then the following observations on the (K+)i of the unfractonated glomerulosa cells may be relevant:

(i) (K+)i did not alter during 2 h of incubation at 37°C. The significance of this finding is discussed earlier and is not consistent with a predominant population of damaged cells, which would not be expected to maintain a steady (K+)i.

(ii) The following observations of changes in (K+)i of the unpurified glomerulosa cells are presented in detail in the next paper (Mendelsohn & Mackie, 1975), but are mentioned here because of their relevance to the state of active K+ pumping of the cells. The increase of (K+)i in cells exposed to [K+]i 8·4 mmol/l medium was 3·41 nmol/adrenal equivalent higher than those in [K+]i 3·6 mmol/l medium and is equivalent to an increase of [K+]i of 12·23 mmol/l (SEM 0·22, n = 7), which is more than three times the change in [K+]i in the medium (P < 0·02). This indicates that the change in (K+)i in this situation is due to stimulation of active inward K+ transport rather than a passively induced fall in net K+ efflux; this phenomenon suggests cells with active, intact potassium-conserving mechanisms rather than passively equilibrating sacs.

(iii) Treatment with ouabain (5 × 10−4 mol/l) caused a 70% fall in (K+)i of cells in [K+]i 3·6 mmol/l medium and 62% fall in cells in [K+]i 8·4 mmol/l medium, suggesting that at least these percentages of (K+)i were initially maintained by active inward transport of K+ before ouabain treatment.

(iv) The steroidogenic response of the isolated cells is probably equivalent to that observed for intact zona glomerulosa tissue in vitro and in vivo (see the discussion by Haning et al., 1970). However, the basal output of corticosterone from isolated cells is lower than that from intact tissue.

There are two likely possibilities consistent with these observations. First, that the [K+]i value represents the true value for zona glomerulosa cells in intact functioning tissue and that these cells are not typical of other mammalian cells with higher [K+]i. Alternatively, it is possible that there are two populations of glomerulosa cells in these preparations; one group has a high, typical [K+]i, which is actively maintained, sensitive to ouabain and is steroidogenically active. The second population is in some way damaged, is potassium-deficient but might still exclude inulin and therefore contribute to the total intracellular water space; such cells would lower the mean [K+]i, but not contribute significantly to changes in (K+)i, and may be steroidogenically inert.

The present data do not appear to permit a firm decision about these two alternatives, although indirect arguments support the second hypothesis. Fortunately, neither possibility impairs the validity of the measurements of changes in (K+)i accompanying steroidogenesis, for if the latter alternative were true it should not affect the percentage changes in (K+)i, occurring in the active population of glomerulosa cells.

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