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Rates of Efflux and Intracellular Concentrations of Potassium, Sodium and Chloride Ions in Isolated Fat-Cells from the Rat

By M. C. PERRY* and C. N. HALES
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge

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1. The metabolism of K^+, Na^+ and Cl^- has been investigated in isolated fat-cells prepared from the epididymal adipose tissue of rats. 2. Methods are described for measuring the intracellular water space, the rates of loss of intracellular $^{42}K^+$, $^{22}Na^+$ and $^{36}Cl^-$ and the intracellular concentrations of K^+, Na^+ and Cl^- in isolated fat-cells. 3. The intracellular water space, measured as the [^{3}H]water space minus the [*carboxylic acid*-^{14}C]inulin space, was $3{\cdot}93 \pm 0{\cdot}38$ µl./100mg. cell dry wt. 4. The first-order rate constants for radioisotope effluxes from isolated fat-cells were 0·029min.$^{-1}$ for $^{42}K^+$, 0·245min.$^{-1}$ for $^{22}Na^+$ and 0·158min.$^{-1}$ for $^{36}Cl^-$. 5. The intracellular concentrations of K^+, Na^+ and Cl^- were 146m-equiv./l., $18{\cdot}6 \pm 2{\cdot}9$m-equiv./l. and $43 \pm 2{\cdot}4$m-equiv./l. respectively. 6. The total intracellular K^+ content of isolated fat-cells was determined by atomic-absorption spectrophotometry to confirm the value obtained from the radioisotope-efflux data. 7. The ion effluxes from isolated fat-cells were: K^+, 1·5pmoles/cm.^{2}/sec., Na^+, 1·6pmoles/cm.^{2}/sec., and Cl^-, 2·4pmoles/cm.^{2}/sec. 8. The membrane potential of isolated fat-cells calculated from the Cl^- distribution ratio was −28·7mv.

The metabolism of isolated fat-cells from the rat (Rodbell, 1964) is strongly influenced by the ionic composition of the incubation medium in which the cells are suspended. Changes in the cationic composition have been shown to affect glucose uptake and metabolism of isolated fat-cells in the presence and absence of insulin (Rodbell, 1965; Letarte & Renold, 1967; Fain, 1968) and the lipolytic response to a variety of hormones (Ho, Jeanrenaud & Renold, 1966; Kypson, Triner & Nahas, 1968). Changes in the anionic composition also affect both basal and hormone-stimulated metabolism in adipose tissue (Hales, 1967; Hales, Chalmers, Perry & Wade, 1968). The interpretation of these experiments, however, is limited by the lack of an adequate description of the behaviour of univalent cations and anions in isolated fat-cells.

The present paper describes methods for the measurement of the intracellular water space of isolated fat-cells and the rates of efflux and intracellular concentrations of the radioisotopes ^{42}K, ^{22}Na and ^{36}Cl in isolated fat-cells incubated in Krebs–Ringer bicarbonate buffer.

MATERIALS

Animals. Male albino Wistar rats weighing 120–140g. were used in all experiments. They were bred in the Department of Biochemistry, Cambridge, or purchased from Ralph Tuck Ltd., Rayleigh, Essex. The latter animals were allowed an acclimatization period of 3 days before use. All animals were fed *ad libitum*.

Chemicals. Naphthalene was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex; toluene and 2-methoxyethanol from May and Baker Ltd., Dagenham, Essex; 1,4-dioxan from Koch–Light Ltd., Colnbrook, Bucks.; Aristar nitric acid and Triton X-100 from British Drug Houses Ltd., Poole, Dorset; and 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene from Thorn Electronics Ltd., Tolworth, Surrey. Other chemicals were AnalaR or the purest grade available from Hopkin and Williams Ltd. or British Drug Houses Ltd. Collagenase was obtained from Worthington Biochemicals Corp., Freehold, N.J., U.S.A. Bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

Other materials. Visking seamless dialysis tubing (18/32in.) was obtained from Hudes Merchandising Corp. Ltd., London W.1. Capped polythene specimen tubes (75mm. × 24mm.) were obtained from A. Gallenkamp, London E.C.2; LP3 polystyrene tubes were obtained from Luckham Ltd., London S.W.20.

Distilled water was deionized by passage through a mark II portable Deminrolit unit (Permutit Co. Ltd., London W.4). All solutions were made with deionized water.

Radiochemicals. All radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks. $^{42}K^+$ was obtained as ^{42}KCl in iso-osmotic solution and was used to replace the KCl component of Krebs–Ringer bicarbonate medium, to give a final specific radioactivity of 0·3–2 µc/ml. $^{22}Na^+$ was obtained as $^{22}NaCl$ in aqueous solution with specific radioactivity 33mc/mg. of Na^+, and was added to

* Present address: Department of Biochemistry, Royal Free Hospital School of Medicine, 8 Hunter Street, London W.C.1.

Krebs–Ringer bicarbonate medium in this form to give a final specific radioactivity of 2 μc/ml. $^{36}Cl^-$ was obtained as $Na^{36}Cl$ in aqueous solution with specific radioactivity 858 or 322 μc/g. of Cl^- and was used to replace the NaCl component of Krebs–Ringer bicarbonate medium to give a final specific radioactivity of 2 or 1·2 μc/ml. [*carboxylis acid*-^{14}C]Inulin was prepared by The Radiochemical Centre by condensation of [^{14}C]cyanide with inulin to yield a product with similar gel-filtration characteristics to inulin. The specific radioactivity was 1·35 μc/mg. (6·75 mc/ m-mole).

Glassware. All glassware coming into contact with the isolated fat-cells was silicone-treated by immersion for 5 min. in an aq. 1% (v/v) solution of Siliclad (Clay–Adams Inc., New York, N.Y., U.S.A.), followed by thorough rinsing and drying at 70°. All glassware was silicone-treated before each experiment.

METHODS

The standard incubation medium was Krebs–Ringer bicarbonate medium, containing half the stated amount of Ca^{2+} (1·3 mM) (Cohen, 1957). In the K^+-free medium, K^+ was replaced with equimolar Na^+. Unless otherwise stated the standard medium contained 4% (w/v) bovine serum albumin. The albumin was made up as a 30% (w/v) solution in the appropriate medium and dialysed overnight at 4° against a tenfold excess of the medium before dilution to the final concentration. All media were gassed with $O_2 + CO_2$ (15:5) and maintained at 37°.

Isolated fat-cells were prepared by the method of Rodbell (1964). The cells were washed with 3 × 10 ml. of the standard medium and finally suspended in this medium. Isolated fat-cells were handled with plastic or silicone-treated glass vessels and pipetted with silicone-treated glass wide-bore pipettes.

Cell dry weight. The dry weight of cells was used as a basis for the expression of results. Samples (1 ml.) of the cell suspension and the suspending medium were dried overnight at 70° on preweighed planchets. The cell dry weight was determined from the difference between the mean dry weight of the samples of cell suspension and the mean dry weight of the samples of medium. Because of the volume of the cells present, 1 ml. of cell suspension contained less than 1 ml. of medium. The cell volume was determined by flotation in a 1 ml. graduated pipette. The dry weight of an equal volume of medium added to the difference previously determined gave the dry weight of cells/ml. of cell suspension. This correction was always less than 10% of the original weight difference.

Intracellular water space of isolated fat-cells. The intracellular water space of isolated fat-cells was determined as the [^{3}H]water space minus the [*carboxylic acid*-^{14}C]inulin space.

Samples (1 ml.) of an isolated-fat-cell suspension were incubated in the standard medium containing 0·135 μc of [*carboxylic acid*-^{14}C]inulin/ml. and 4 μc of [^{3}H]water/ml. in capped polythene specimen tubes. The cells were equilibrated with the radioisotopes for 30 min. at 37° by shaking at 60 cyc./min. in a water bath. Further 1 ml. samples were incubated under identical conditions with either 0·135 μc of [*carboxylic acid*-^{14}C]inulin or 4 μc of [^{3}H]water/ml. to prepare ^{14}C and ^{3}H standards.

After equilibration the contents of each incubation vessel were transferred to a polystyrene tube and centrifuged at 300*g* for 1 min. The infranatants from the experimental samples were removed and pooled. Those from the standards were transferred to separate polystyrene tubes. Throughout all subsequent procedures all tubes were capped except when being sampled, to prevent [^{3}H]water loss by evaporation.

Each cell cake was suspended in 0·3 ml. of deionized water and deproteinized with 1·5 ml. of 0·5 M-$HClO_4$. The precipitated protein was removed by centrifugation. Of the supernatant beneath a floating fat layer, 1 ml. was transferred to a polystyrene tube and neutralized with M-KOH. The precipitate of $KClO_4$ was removed by centrifugation at room temperature. No cooling was used to remove the $KClO_4$ because of the possibility of precipitation of inulin. Samples (6 × 0·3 ml.) of the pooled infranatants and the infranatants from the ^{14}C and ^{3}H standards were similarly deproteinized and neutralized.

The ^{14}C and ^{3}H contents of the deproteinized extracts were determined in a Nuclear–Chicago type L725 liquid-scintillation counter by using a Triton–toluene scintillation fluid, prepared by mixing 2 parts of toluene containing 2,5-diphenyloxazole (4 g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0·1 g./l.) with 1 part of Triton X-100. Of the extracts, 0·25 ml. was added to 3 ml. of scintillation fluid. The overlap of counts from each radioisotope into the channel used for counting the other was determined separately for the samples of cells and medium by using the standards. The ^{14}C and ^{3}H counts recorded were corrected for background and for the overlap of counts from the other radioisotope to give the ^{14}C and ^{3}H contents of the cell extracts and samples of the infranatants. Sufficient counts were recorded to decrease the counting error to less than 2%. The [*carboxylic acid*-^{14}C]inulin and the [^{3}H]water spaces were calculated. The difference between the two spaces was corrected for the weight of cells present to give the intracellular water space in μl./100 mg. dry weight.

Determination of the rate of efflux of $^{42}K^+$, $^{22}Na^+$ *and* $^{36}Cl^-$ *from isolated fat-cells.* Isolated fat-cells were loaded with radioisotope by incubation for 60–90 min. in 3 or 10 ml. of standard medium containing the radioisotope at a specific radioactivity of up to 2 μc/ml. in a capped polythene specimen tube or in a silicone-treated 50 ml. Erlenmeyer flask. The cell suspension was centrifuged at 300*g* for 1 min. in a polythene specimen tube and the infranatant removed and retained for the assay of radioactivity.

The washing medium, unless otherwise stated, was the standard medium containing 2% (w/v) albumin. The use of 2% instead of 4% albumin facilitated the removal of samples for counting of radioactivity from a small volume after deproteinization with trichloroacetic acid. This decrease in the albumin concentration had no detectable effect on the rate of loss of intracellular radioisotopes.

The cells were washed once by gentle suspension in 24 ml. of washing medium. The suspension was centrifuged at 300*g* for 1 min. and the infranatant removed and discarded. This wash took 2 min. The determination of the rate of radioisotope efflux began at this stage. For wash 2, 22 ml. of washing medium was added to the cell cake from wash 1, the cells were suspended and three 1 ml. samples removed to preweighed polystyrene tubes. These samples and the remaining bulk cell suspension were centrifuged in parallel at 300*g* for 1 min. The cell cake and the infranatant of the 1 ml. cell samples were separated as completely as possible and both fractions retained for analysis. The infranatant of

the bulk cell suspension was removed and discarded. Medium for wash 3 was added and the above procedure repeated for each successive wash. At each wash the volume of washing medium added to the cell cake from the previous wash was decreased to take account of the volume of cell suspension removed and thus to keep the cell/wash-medium ratio constant throughout the determination of the rate of radioisotope efflux. Experiments comprised either six washes, after each of which the intracellular radioisotope content was measured (washes 2–7) for $^{42}K^+$, or three washes (washes 2–4) for $^{22}Na^+$ and $^{36}Cl^-$. After the final wash the cell dry weight was determined on samples (3×1 ml.) of the cell suspension. From wash 2 onwards a time-interval of 5 min. was set between the removal of the infranatants of the cell samples at one wash and the next, and this schedule was rigidly followed in all experiments.

Each polystyrene tube was reweighed to determine the weight of cells plus residual washing medium in the cell samples. The cells were lysed with 0·5 ml. of 10% (w/v) trichloroacetic acid and the precipitated protein was removed by centrifugation. Of the supernatant, 0·3 ml. was assayed for its radioactivity content in 2 ml. of scintillation fluid containing dioxan (1 l.), 2-methoxyethanol (200 ml.), naphthalene (80 g.), 2,5-diphenyloxazole (3 g.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0·2 g.) by using a Nuclear-Chicago bench-top liquid-scintillation counter (Unilux model). Of the infranatant corresponding to each cell sample, 0·5 ml. was deproteinized with 0·5 ml. of 10% (w/v) trichloroacetic acid, and the radioactivity determined as described above.

Calculation of intracellular radioactivity. All results were expressed in terms of dry weight and no correction was made for the small intracellular water content. The error thus introduced into the measurement of the intracellular radioisotope content was always less than 2%.

The extracellular radioactivity was calculated from the radioactivity recorded in the infranatant and the volume of residual washing medium, calculated from the difference between the weight of the cell sample + residual wash medium and the dry weight of cells present. The extracellular radioactivity subtracted from the total radioactivity in the cell lysate gave the radioactivity associated with the cells at that wash. Determinations of radioactivity were corrected for background and, in the case of $^{42}K^+$, for radioisotope decay, and expressed per 100 mg. cell dry wt. The intracellular radioisotope content (C_1) at each wash was the mean of three determinations.

A plot of $\log(C_1/100\text{ mg. cell dry wt.})$ against time gave a straight line. The linear regression was calculated by the method of least squares and the regression coefficient $b \pm$ the standard error of b was determined (Davies, 1957).

Measurements of the efflux of radioisotopes from the cells into the standard medium containing 2% (w/v) albumin were analysed to determine the permeability characteristics of isolated fat-cells to K^+, Na^+ and Cl^-.

The first-order rate constant, k, for radioisotope efflux was related to the regression coefficient, b, by the equation:

$$k = 2{\cdot}303b/5\text{ min.}^{-1}$$

Intracellular contents of $^{42}K^+$, $^{22}Na^+$ and $^{36}Cl^-$ of isolated fat-cells incubated in the standard medium. The intracellular radioisotope content at time zero was calculated from the linear regression of $\log(C_1/100\text{ mg. cell dry wt.})$ against time, and from this the apparent intracellular ion content was derived. The validity of this method for determining the intracellular concentrations of the ions is discussed below.

Recoveries of radioisotope added were not determined as a routine. However, control recoveries of the three radioisotopes added to a suspension of isolated fat-cells were $103{\cdot}8 \pm 0{\cdot}5\%$ for $^{42}K^+$, $100{\cdot}0 \pm 1{\cdot}3\%$ for $^{22}Na^+$ and $92{\cdot}2 \pm 2{\cdot}2\%$ for $^{36}Cl^-$. Each value was the mean ± S.E.M. of six determinations. No correction was made to the results for these recoveries.

Quenching of the radioisotopes by trichloroacetic acid in the scintillation fluid was slight, but as a control all radioactivity counting was performed in the presence of trichloroacetic acid.

Total K^+ content of isolated fat-cells. This was determined by atomic-absorption flame-photometric analysis of a HNO_3 digest of fat-cells by using a Unicam SP. 90 atomic-absorption spectrophotometer and a K lamp (Unicam Instruments Ltd., Cambridge). All glassware and polystyrene tubes used in this section were soaked overnight in deionized water. Samples (1 ml.) of a cell suspension in the standard medium were centrifuged at 300g for 1 min. in a polystyrene tube and the infranatants discarded. The cell cakes were dried overnight at 70° and digested with 0·5 ml. of conc. HNO_3 with gentle warming. No attempt was made to remove triglyceride to decrease possible contamination with Na^+ and K^+. Deionized water (1·5 ml.) was added and any precipitate removed by centrifugation. The Na^+ and K^+ contents of the samples were determined, after correction for deionized-water blanks, by comparison with standards that contained the same concentrations of HNO_3 and both Na^+ and K^+ in approximately the same ratio as in the samples. To calculate the intracellular K^+ content, the Na^+ present was used as a measure of the residual extracellular medium. The validity of this approach is demonstrated below.

Recoveries of known amounts of Na^+ and K^+ taken through the HNO_3 digestion and assay for ions were $99{\cdot}3 \pm 0{\cdot}5\%$ for K^+ and $99{\cdot}2 \pm 0{\cdot}9\%$ for Na^+. Each percentage recovery was the mean ± S.E.M. of six determinations.

Cell number and surface area. An isolated-fat-cell suspension in the standard medium was assayed for the number of cells/100 mg. cell dry wt. and for the surface area/100 mg. cell dry wt. Of the diluted samples of the cell suspension 5 μl. was placed, by using a silicone-treated 'Microcap' (Drummond Scientific Co., Broomall, Pa., U.S.A.), on to a cover-slip inverted over a cavity slide. The isolated fat-cells floated against the cover-slip and were counted by using a light microscope. Cells were distinguished from fat droplets by their different refractive index and the presence of a nucleus. In this way 200–250 cells were counted/5 μl.

With the same cell preparation the mean cell diameter was determined. A sample of the suspension was spread over a haemocytometer grid (Hawksley, Lancing, Sussex) and the diameter of 100 of the cells measured by comparison with the lines of the grid.

RESULTS

Intracellular water space of isolated fat-cells. The intracellular water space of isolated fat-cells prepared from the epididymal adipose tissue of rats weighing 120–140 g., measured as the [^{3}H]water space minus the [*carboxylic acid*-^{14}C]inulin space,

Table 1. $^{42}K^+$, $^{22}Na^+$ *and* $^{36}Cl^-$ *efflux from isolated fat-cells in Krebs–Ringer bicarbonate buffer*

Efflux rates are those of $^{42}K^+$, $^{22}Na^+$ and $^{36}Cl^-$ from isolated fat-cells loaded with the isotopes in Krebs–Ringer bicarbonate buffer containing 4% (w/v) bovine serum albumin and washed in Krebs–Ringer bicarbonate buffer containing 2% (w/v) bovine serum albumin. Results were calculated as described in the text, and expressed as the mean regression coefficient $b \pm$ S.E.M. (numbers of determinations in parentheses), the first-order rate constant k (min.$^{-1}$) and the half-time of the radioisotopes in the cells (in min.).

Radio-isotope	Regression coefficient b	Rate constant k (min.$^{-1}$)	Half-time (min.)
$^{42}K^+$	0·063 ± 0·003 (26)	0·029	23·9
$^{22}Na^+$	0·532 ± 0·034 (5)	0·245	2·8
$^{36}Cl^-$	0·344 ± 0·007 (30)	0·158	4·4

was 3·93 ± 0·38 µl./100mg. cell dry wt. (mean ± S.E.M. of 12 determinations varying from 2·2 to 6·0 µl./100mg. on the same fat-cell suspension).

Perchloric acid was used to deproteinize the samples in preference to the method of Somogyi (1945), since the latter method resulted in precipitation of the [*carboxylic acid*-^{14}C]inulin. Control experiments showed that this precipitation did not occur with perchloric acid.

Rate of efflux of $^{42}K^+$, $^{22}Na^+$ *and* $^{36}Cl^-$ *from isolated fat-cells in Krebs–Ringer bicarbonate medium.* Table 1 shows for $^{42}K^+$, $^{22}Na^+$ and $^{36}Cl^-$ the mean regression coefficient ± S.E.M., the first-order rate constant derived from the mean regression coefficient and the half-time of the radioisotope in the cells.

The plots of log(C_i/100mg. cell dry wt.) against number of washes or time were linear for $^{42}K^+$ over a six-wash experiment and for $^{36}Cl^-$ over a three-wash experiment. Hence first-order kinetics were applied to $^{42}K^+$ and $^{36}Cl^-$ efflux. In a few experiments the semi-logarithmic plots for $^{22}Na^+$ efflux were not linear over three washes but showed a decrease in rate when the intracellular radioactivity had fallen to values only 2–4 times background. However, this discrepancy was usually slight and could have been explained by the counting error. For comparison with $^{42}K^+$ and $^{36}Cl^-$, the $^{22}Na^+$ efflux was analysed similarly by first-order kinetics.

The half-times of the radioisotopes in the isolated fat-cells were calculated from the first-order rate constants (k) by the equation:

$$t_{\frac{1}{2}} = 0{\cdot}693/k \text{ min.}$$

Intracellular K^+, Na^+ *and* Cl^- *contents of isolated fat-cells in Krebs–Ringer bicarbonate medium.* Extrapolation of the semi-logarithmic plots of radioisotope effluxes to time zero gave the apparent intracellular ion contents of isolated fat-cells under the conditions of incubation with the radioisotope. From the specific radioactivity of the radioisotope in the incubation medium and the intracellular water space determined previously, the results were expressed in terms of the apparent intracellular concentrations of K^+, Na^+ and Cl^- (Table 2).

Table 2. *Apparent intracellular ionic concentrations of isolated fat-cells incubated in Krebs–Ringer bicarbonate medium*

Intracellular ionic concentrations are calculated from radioisotope-efflux data after incubation of isolated fat-cells in radioisotope-containing standard medium for 60 or 90min. Results are expressed in m-equiv./l. of intracellular water (mean ± S.E.M. of the number of determinations in parentheses). Intracellular water space was 3·93 µl./100mg. cell dry wt.

Ion	Time of incubation (min.)	Intracellular concn. (m-equiv./l.)
K^+	60	100·8 ± 8·5 (9)
K^+	90	134·0 ± 10·5 (15)
Na^+	60	18·6 ± 2·9 (7)
Cl^-	60	43·0 ± 2·4 (45)

These determinations depend on the following assumptions: (*a*) that the radioactive ions behave identically with the non-radioactive isotope; (*b*) that during the period of the incubation the radioisotope equilibrates across the cell membrane and its intracellular and extracellular specific radioactivities were equal; (*c*) that cells in the standard medium are in ionic equilibrium and not undergoing net ionic changes; and (*d*) that the radioisotope efflux over the first wash is the same as that measured over the rest of the experiment. A fraction of the intracellular radioisotope exchanging within 2min. would not be detected with this method, which would thus underestimate the intracellular radioisotope content. Under these conditions the half-time for the efflux of an ion would equal the half-time for its influx. $^{22}Na^+$ with its half-time in the cell only 2·8min. and $^{36}Cl^-$ with a half-time of 4·4min. would, after incubation for 60min., be virtually completely equilibrated across the cell membrane. $^{42}K^+$, however, would not have reached equilibrium, being 81% equilibrated after 60min. and 92% after 90min. This conclusion is borne out by the lower apparent intracellular K^+ concentration determined by the radioisotopic-efflux method after incubation for 60min. (100·8 ± 8·5m-equiv./l.) than after 90min. (134·0 ± 10·5m-equiv./l.). It can therefore be calculated that the true intracellular concentration of K^+ was 146m-equiv./l. The intracellular ion concentrations of isolated fat-cells incubated in standard medium were therefore: K^+, 146m-equiv./l., Na^+, 19m-equiv./l., and Cl^-, 43m-equiv./l.

Total K^+ content of isolated fat-cells. The total K^+ content of isolated fat-cells incubated for 1hr. in standard medium was determined by using the amount of Na^+ present as a measure of the extracellular space. In two experiments the total K^+ content was 629 ± 13 and 487 ± 18nmoles/100mg. cell dry wt. These results were the means $\pm$ S.E.M. of 12 determinations on two separate preparations of isolated fat-cells. If an allowance is made for the amount of Na^+ calculated to be within the cells from the $^{22}Na^+$-efflux data, these results are little changed, becoming 632 ± 13 and 491 ± 18nmoles/100mg. respectively. The amount of Na^+ therefore served as an accurate measure of the extracellular space. From these results, and by using the intracellular water space of 3·93μl./100mg. cell dry wt., the concentration of intracellular K^+ was calculated to be 160mM and 124mM respectively.

Cell number and surface area/100mg. dry weight. The cells in 30 samples (5μl.) of an isolated-fat-cell suspension were counted. The cell number was $2{\cdot}6 \times 10^6$ cells/100mg. dry wt. The cell diameter was determined on the same preparation and the mean diameter $\pm$ S.E.M. of 100 cells was 48·2 $\pm$ 1·6μm., with a range of 20–80μm. The surface area of a single cell, assuming it to be a perfect sphere, was 7300μm.^{2}, and the total cell surface area/100mg. cell dry wt. was 190cm.^{2}.

Effect of inhibition of active transport of Na^+ on the intracellular $^{22}Na^+$ concentration and ^{22}Na efflux from isolated fat-cells. To investigate the effects of a K^+-free medium (the standard medium with K^+ replaced by equimolar Na^+) and ouabain, an inhibitor of active transport of Na^+ (Schatzmann, 1953; Glynn, 1957) isolated fat-cells were washed after the digestion with collagenase, once with standard medium and twice with the K^+-free medium, and incubated in 3ml. of the K^+-free medium containing 2μc of $^{22}Na^+$/ml. in the presence and absence of ouabain. The incubation was performed at 37° with gentle shaking for 1hr., and the $^{22}Na^+$ efflux from the cells and the intracellular $^{22}Na^+$ content determined as described above. The mean intracellular Na^+ concentrations ($\pm$ S.E.M.) of fat-cells incubated in the K^+-free medium in the presence and absence of ouabain are shown in Table 3. The $^{22}Na^+$ efflux was determined in various washing media.

Incubation of the cells in the K^+-free medium increased the apparent intracellular Na^+ content to 52·5m-equiv./l. and the presence of ouabain increased it further to 110·6m-equiv./l. In these calculations no account was taken of possible changes in the intracellular water space in these media, since the space was not determined under these conditions. Hence these apparent concentrations represent the intracellular Na^+ contents on the basis of the intracellular water space measured in standard medium.

Table 3. *Effect of K^+-free Krebs–Ringer bicarbonate buffer and ouabain on the apparent intracellular Na^+ concentration of isolated fat-cells*

Isolated fat-cells were incubated for 1hr. in the K^+-free medium in the presence or absence of 0·1mM-ouabain and washed in various washing media (see the text). Na^+ replaced K^+ in the K^+-free medium. Intracellular water space was 3·93μl./100mg. The results are the mean apparent intracellular Na^+ concentrations $\pm$ S.E.M., the number of determinations being shown in parentheses.

Incubation medium	Apparent intracellular $[Na^+]$ (m-equiv./l.)
Standard	18·6±2·9 (7)
K^+-free	52·5±7·1 (6)
K^+-free + ouabain	110·6±4·3 (5)

They do, however, demonstrate that the intracellular Na^+ content increased on incubation of the cells in the K^+-free medium and in the K^+-free-medium + 0·1mM-ouabain, both conditions that would be expected to inhibit active transport of Na^+ out of the fat-cell. The intracellular Na^+ concentration in the K^+-free medium was lower in the absence of ouabain that in its presence. K^+ loss from the cells during incubation may have raised the K^+ concentration in the initially K^+-free medium, resulting in only partial inhibition of active transport of Na^+.

Further evidence for a mechanism of active Na^+ transport in isolated fat-cells came from a study of the effect of ouabain on $^{22}Na^+$ efflux from the cells. Isolated fat-cells were incubated in K^+-free medium containing 2μc of $^{22}Na^+$/ml. to increase the intracellular $^{22}Na^+$ content and hence facilitate measurement of $^{22}Na^+$ efflux. The cells were washed with the standard medium containing 2% (w/v) albumin in the presence and absence of ouabain. Ouabain (0·1mM) decreased the rate constant for the control $^{22}Na^+$ efflux ($-0{\cdot}245 \pm 0{\cdot}016$min.$^{-1}$; mean $\pm$ S.E.M. of five determinations) by 43% to $-0{\cdot}140 \pm 0{\cdot}011$min.$^{-1}$ (mean $\pm$ S.E.M. of seven determinations). This difference was highly significant ($P < 0{\cdot}001$ by Student's t test).

DISCUSSION

The value of 3·93μl./100mg. dry weight of cells for the intracellular water space of isolated fat-cells obtained in these experiments is not inconsistent with that of 5·9μl./100mg. reported by Crofford, Stauffacher, Jeanrenaud & Renold (1966). Very accurate determinations of the intracellular water are not possible, owing to the fact that the space represents a very small fraction of the total water content of the preparation. Similar difficulties have occurred in the determination of the intracellular water space of epididymal adipose tissue of the rat,

which has been reported as 4·2 μl. (Crofford & Renold, 1965) and 1·4 μl. (Denton, Yorke & Randle, 1966) per 100 mg. wet wt. Goldrick (1967) calculated that the cytoplasm of isolated fat-cells, as a percentage of the cell volume, decreased from 8% in cells from 80 g. rats to 0·8% in cells from 300 g. rats, over which weight range the triglyceride content increased markedly. An increase in the triglyceride content would decrease the intracellular water space/unit weight by decreasing the intracellular water space of the individual cells and by decreasing the number of cells/unit weight. This may explain the small intracellular water space observed by Denton *et al.* (1966), who used rats up to 250 g. in weight. In view of these considerations, the value derived for the intracellular water content of fat-cells prepared from the epididymal fat-pad of rats in the weight range 120–140 g. is consistent with previous reports. The variability of the proportion of cytoplasm in rat fat-cells with total body weight emphasizes the importance of using rats within a narrow weight range in studies of this type.

The rate constants for the efflux of $^{42}K^+$, $^{22}Na^+$ and $^{36}Cl^-$ from the isolated fat-cells are higher than those reported for other tissues of the rat. For $^{42}K^+$ efflux the rate constant has been reported to be 0·008 min.$^{-1}$ for the erythrocyte (Weller & Taylor, 1951), 0·0045 min.$^{-1}$ (Zierler, 1960) to 0·0136 min.$^{-1}$ (Gourley & Jones, 1954) for skeletal muscle, 0·019 min.$^{-1}$ for diaphragm (Creese, 1954) and 0·017 min.$^{-1}$ for kidney cortex (Wiggins, 1965). For $^{22}Na^+$ the efflux rate constant was 0·124 min.$^{-1}$ for diaphragm (Creese, 1968), 0·150 min.$^{-1}$ for kidney cortex (Wiggins, 1965) and 0·082 min.$^{-1}$ for uterus (Daniel & Robinson, 1963). The comparatively high rate of loss of intracellular radioisotopes from isolated fat-cells raises the possibility that the preparative technique introduced an artificially high membrane permeability or increased fragility of the cells. A consideration of the geometry of the fat-cell suggests an alternative explanation for the high rate of radioisotope efflux from isolated fat-cells. The rate constant for the efflux is the fraction of the intracellular radioisotope content lost from the cells per unit time. In the fat-cell, the intracellular content of a radioisotope is decreased by the high content of triglyceride, which is probably ion-free. Thus the rate constant appears high since a greater fraction of the intracellular radioisotope is lost per unit time, even though the permeability of the cell membrane is not abnormally high. If this postulate is correct the ion flux, the absolute amount of ion leaving the cell/unit area of cell membrane/unit time, should not be abnormally large. The ion fluxes have been calculated from the intracellular ion concentrations and the rate constants for the radioisotope efflux and are shown in Table 4.

Table 4. *Ion effluxes from isolated fat-cells in Krebs–Ringer bicarbonate buffer*

Ion effluxes from isolated fat-cells were calculated from the intracellular ion concentrations and the radioisotope-efflux rate constants and expressed as nmoles/100 mg./min. and, by using the surface area/100 mg. cell dry wt. (see the text), as pmoles/cm.^{2}/sec.

Ion	Efflux (nmoles/100 mg./min.)	Efflux (pmoles/cm.^{2}/sec.)
K^+	16·6	1·5
Na^+	17·9	1·6
Cl^-	26·7	2·4

In rat diaphragm the K^+ efflux has been measured at 21 pmoles/cm.^{2}/sec. (Creese, 1954), in rat skeletal muscle 2·5 pmoles/cm.^{2}/sec. (McLennan, 1955) and in rat uterus 9 pmoles/cm.^{2}/sec. (Daniel, 1963). Na^+ efflux has been measured at 28 pmoles/cm.^{2}/sec. in rat diaphragm (Creese, 1954) and 300 pmoles/cm.^{2}/sec. in guinea-pig *Taenia coli* (Goodford & Hermansen, 1961). K^+ and Na^+ effluxes from isolated fat-cells were slower than these values reported for muscular tissues. Hence it seems probable that the preparation of the isolated fat-cells did not introduce an abnormally large ion permeability to the cells and that the ionic fluxes studied were not the result of gross cell damage. However, no definite conclusion can be drawn until the ion fluxes in fat-cells in intact adipose tissue have been measured.

On the assumption that Cl^- was distributed passively across the fat-cell membrane according to the prevailing membrane potential, the Cl^- distribution ratio was used to calculate the membrane potential of isolated fat-cells from the Nernst equation:

$$E_{Cl} = \frac{-RT}{F} \ln \left(\frac{[Cl]_0}{[Cl]_i} \right)$$

where E_{Cl} is the Cl^- diffusion potential and $[Cl]_0$ and $[Cl]_i$ are the extracellular and intracellular Cl^- concentrations respectively. With the intracellular Cl^- concentration of 43 m-equiv./l., E_{Cl} was $-28·7$ mv. E_K and E_{Na} calculated similarly were -85 mv and $+56$ mv. This value for the fat-cell membrane potential in standard medium calculated from the Cl^- distribution ratio is in agreement with the membrane potentials of 15–40 mv recorded by Miller, Schlosser & Beigelman (1966) from microelectrode penetrations of isolated fat-cells suspended in Krebs–Ringer bicarbonate medium containing 1% agar.

The intracellular concentrations of K^+, Na^+ and Cl^- in isolated fat-cells obtained in this investigation fall within the range of concentrations previ-

ously determined in rat tissues (Perry, 1968). As a further control on the intracellular K^+ concentration calculated from the radioisotope-efflux data, the total intracellular K^+ content was determined by atomic-absorption spectrophotometric analysis. It was calculated, from the intracellular water space (3·93 μl./100mg.) and the intracellular K^+ concentration derived from the isotope-efflux data (146m-equiv./l.), that the K^+ content was 574nmoles/100mg. cell dry wt. Two separate determinations of the total intracellular K^+ gave values of 629 and 487nmoles/100mg. respectively, both values being close to the postulated total intracellular K^+ content. It therefore appears that the extracellular $^{42}K^+$ was equilibrating with most, if not all, of the intracellular K^+, and the whole intracellular K^+ content was determined by the radioisotopic method. The results reported here are consistent with the existence in the fat-cell membrane of an active-transport system for Na^+. Although Bonting, Simon & Hawkins (1961) could not detect a ($Na^+ + K^+$)-activated adenosine triphosphatase in cat adipose tissue, Modolell & Moore (1967) demonstrated both a Mg^{2+}-activated adenosine triphosphatase and a ($Na^+ + K^+$)-activated Mg^{2+}-requiring adenosine triphosphatase in rat epididymal adipose tissue. Much evidence has accumulated that the ($Na^+ + K^+$)-activated enzyme forms an integral part of the sodium pump, the system by which Na^+ is actively transported out of cells (Skou, 1965). Clausen, Rodbell & Dunand (1969) have recently demonstrated the existence of a Na^+-linked, energy-dependent and ouabain-sensitive mechanism for K^+ accumulation in isolated fat-cell 'ghosts'.

The results reported here suggest that isolated fat-cells in Krebs–Ringer bicarbonate medium show no gross abnormalities in their ionic metabolism. In view of their known responsiveness to many hormones, it appears that the cells represent a suitable system for investigation of the hormonal control of ion metabolism and the interrelations between inorganic ions and hormones in the control of adipose-cell metabolism.

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