not change after recrystallization from acetone and did not depress the m.p. of authentic glycerol tri-*p*-nitrobenzoate. Jacquemain & Muscovits (1936) give m.p. 192°.

Neutral 'glycerides' from *P. chartarum* 2 (2.007 g.) yielded 0.127 g. of glycerol as determined by the periodate method in the aqueous layer obtained after saponification and extraction of lipids. The amount of the recovered fatty acids with a mean molecular weight of 274.0 was 1.333 g. and the content of α -monoglycerides in the original material 1.5 %. Thus about 83 % of the total fatty acids was apparently present in the form of triglycerides. It would seem that the remainder of the fatty acids was combined with unidentified alcohols, which formed a considerable portion of unsaponifiable matter as evidenced by its acetyl value of 111.5, and to a lesser extent (approximately 3 % of the total acids) with sterols.

SUMMARY

1. The fatty acid composition (molar percentages) of the lipids from *Stemphylium dendriticum*, family Dematiaceae, consisted of 52 % of linoleic, 19 % of palmitic and 17 % of oleic acid as major components, thereby resembling that of the fungal mats and spores of *Pithomyces chartarum*, another member of the same family. In contrast, the fatty acid composition of *Cylindrocarpon radiculicola*, from the related Tuberculariaceae family, resembled more that of other fungi hitherto examined in containing 29 % of linoleic, 24 % of palmitic, 27 % of oleic and 10 % of linolenic acid.

2. The amounts of total sterols in the 'glyceride' fraction of *Pithomyces chartarum* and *Cylindrocarpon radiculicola* were found to be from 8.3 to 9.6 % and of free sterols from 5.4 to 8.4 %, whereas the corre-

sponding amounts of *Stemphylium dendriticum* were 15.6 and 14.3 %.

3. Glycerol has been isolated from the 'glycerides' of *Pithomyces chartarum*, their saponifiable fraction consisting predominantly of triglycerides.

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Further Experiments on the Potassium Uptake by *Alcaligenes faecalis*

By R. HEMS AND H. A. KREBS

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry,
University of Oxford

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Krebs, Whittam & Hems (1957) have shown that *Alcaligenes faecalis* readily loses its intracellular potassium on washing if the potassium concentration of the washing medium is low, and it recovers the lost ion when placed in dilute solutions of potassium salts. The recovery of potassium was

found to be due partly to active transport depending on respiration, but even without a supply of energy considerable amounts of potassium were taken up against concentration gradients. Measurements of the simultaneous movements of K^+ , Na^+ and Cl^- ions failed to explain how electroneutrality

Table 1. *Uptake of potassium by four strains of Alcaligenes faecalis in the absence of substrate*

To samples (4 ml.) of water-washed cells was added 0.1 ml. of 1.15% KCl. Incubation was for 30 min. at 20° in air. (For further details, see Krebs, Whittam & Hems, 1957, Table 4.)

Strain no.	...	...	...	...	323	324	325	326
K in washed cells initially ($\mu\text{g./ml.}$)					627	489	182	283
K in supernatant after incubation ($\mu\text{g./ml.}$)					132	124	131	140
K in cells after incubation ($\mu\text{g./ml.}$)					1133	1012	626	766
Ratio: (intracellular K)/(extracellular K)					8.6	8.2	4.8	5.5
Rise of intracellular K ($\mu\text{g./ml.}$)					506	523	444	483

Table 2. *Anaerobic uptake of potassium by washed cells at 0°*

Each cup contained 1.0 ml. of washed cells (22.8 mg. dry wt.) and 4 ml. of 0.029% KCl in the main compartment; 0.5 ml. of ^{42}KCl solution was in the side arm and yellow phosphorus in centre well; gas phase, N_2 . Period of incubation stated includes time required for separation of cells by centrifuging. The initial intracellular potassium concentration was 6.77 $\mu\text{moles/ml.}$ (For further details of procedure, see text and Krebs *et al.* 1957, Table 11.)

Cup no.	...	...	...	...	...	1	2	3	4	5
Period of incubation (min.)						0.88	5.2	10.2	20.7	60.3
K in supernatant ($\mu\text{moles/ml.}$)						4.00	3.92	4.10	4.15	4.15
K in cells ($\mu\text{moles/ml.}$)						31.7	33.6	34.1	33.1	32.8
Counts in supernatant/ $\mu\text{l.}$						138.3	140.1	141.3	144.6	140.8
Counts in cells/ $\mu\text{l.}$						825	935	935	964	918
Sp. activity in supernatant (counts/min./ $\mu\text{mole of K}$)						34 500	35 700	34 500	34 800	34 100
Sp. activity in cells (counts/min./ $\mu\text{mole of K}$)						26 000	27 700	27 400	29 000	27 900
Ratio: $\frac{\text{Sp. activity in cells}}{\text{Sp. activity in supernatant}}$						0.752	0.776	0.795	0.834	0.818

was maintained when K^+ ions migrate from the medium to the cell. This paper is concerned with a further study of the accumulation of potassium in the absence of an energy source.

MATERIALS AND METHODS

Growth conditions. The basal medium contained 1% (w/v) of Difco peptone, 1% (w/v) of casein hydrolysate (tryptone) and 0.5% of Difco yeast extract; it was adjusted to pH 7.0 with NaOH. The inoculum consisted of a 5 ml. subculture of the same medium. The cells were grown at 30° for 18–24 hr. in a 2 l. conical flask containing 1 l. of medium and fitted with a sintered-glass distributor through which sterile air was bubbled. The yield was about 0.46 g. dry wt. of cells (4.25 g. fresh wt. of packed cells). Before use the cells were usually washed three times either with water or with 0.11 M-sucrose. This sucrose concentration was approximately iso-osmotic with the growth medium after the cells had been harvested. Cells which were not required at once were suspended in a small volume of water or sucrose solution and stored at 2°.

Organisms. Five strains of *A. faecalis* were used. They were strain 1347 of the National Collection of Type Cultures, and strains 323, 324, 325 and 326, described and kindly supplied by Dr R. C. Cleverdon (see Denault, Cleverdon & Kulp, 1953). Unless stated otherwise, strain 1347 was used.

Distribution of potassium. The distribution of potassium between medium and cells was determined as described by Krebs *et al.* (1957).

Measurement of pH. A glass electrode and the Electronic Instruments Ltd. model 23A pH meter, calibrated according to British Standard 1647:1950, was used. With this

instrument readings were accurate to ± 0.02 pH unit; there was no detectable drift.

Analyses. Magnesium was determined according to Mann & Yoe (1956), and calcium by flame photometry according to MacIntyre & Davidsson (1958). The determinations were carried out on material ashed in a muffle oven at 400°. Phosphate was determined according to Berenblum & Chain (1938). Ammonia was separated in Conway units and determined spectrophotometrically after nesslerization.

EXPERIMENTAL AND RESULTS

Uptake of potassium by four further strains of Alcaligenes faecalis. The four strains of *A. faecalis* obtained from Dr R. C. Cleverdon showed the same characteristic loss of potassium on washing with water, and its recovery against a gradient on addition of potassium, as reported for the three strains examined previously (Table 1). The amounts of potassium left in the cells after washing varied from strain to strain, whereas the amounts taken up on incubation with a medium containing potassium were fairly constant. In consequence, the $(\text{intracellular K}^+)/(\text{extracellular K}^+)$ after incubation was higher in cells which retained more potassium on washing (strains 323 and 324).

Anaerobic exchange of extra- and intra-cellular potassium at 0°. In previous experiments with ^{42}K (Krebs *et al.* 1957; Table 11) 66% of the intracellular potassium exchanged anaerobically at 30° in 5 min. with the potassium of the medium. After 30 min., 94% had exchanged. Table 2 shows that

Table 3. *Capacity of the Alcaligenes faecalis cell to lose and regain intracellular potassium repeatedly on changing the medium*

Sample no.	1	2	3	4	5	6
Treatment of cells	Once-washed		Twice-washed		Thrice-washed	
Suspension medium	Water	KCl	Water	KCl	Water	KCl
K in supernatant (μ moles/ml.)	0.51	4.71	0.23	4.92	0.13	5.18
K in cells (μ moles/ml.)	11.49	23.49	7.85	16.10	4.31	13.46
Increase in K in cells (μ moles/ml.)	—	12.00	—	8.18	—	9.13
Ratio: $\frac{\text{K in cells}}{\text{K in supernatant}}$	5		3.3		2.6	

the exchange is also very rapid at 0°. In the experiment recorded in this Table, washed cells were preincubated in manometer cups anaerobically for 30 min. with 0.023 % KCl solution and ⁴²KCl was added from the side arm after 30 min. At various intervals after this addition, manometers were removed from the bath for analysis; samples (3 ml.) of the suspension were transferred as quickly as possible to cold centrifuge tubes and centrifuged at 24 000g in a refrigerated centrifuge. The times of incubation stated in Table 2 refer to the period between the addition of the isotope and the attainment of the full gravitational field in the centrifuge. Measurement of the sedimentation rates showed that most of the cells were sedimented in 50 sec. and that the pellet was packed firmly in 75 sec. The data given in Table 2 indicate that 75 % of the intracellular potassium had exchanged during the time taken to manipulate the first sample (about 53 sec.). There was a slow further rise of the specific activity of the intracellular potassium but the ratio (sp.act. of intracellular K⁺)/(sp.act. of extracellular K⁺) did not exceed 0.83 even after 60 min. It follows that a fraction of the intracellular potassium did not readily exchange at 0°. The amount of potassium which had remained in the cells after washing (6.77 μ moles/ml.) was about 20 % of the potassium in the cells after incubation. Thus the fractions of the intracellular potassium which could not be removed by washing and which did not readily exchange with the isotope were of the same order.

Capacity of Alcaligenes to lose and regain potassium repeatedly. If a soluble cation, such as NH₄⁺ ion or a basic amino acid, or an amine played a role in the intracellular binding of potassium by exchanging with potassium, this cation would be lost when potassium is taken up and the capacity for binding would therefore be depleted when the potassium is repeatedly eluted. The capacity of the cells to lose and regain potassium by placing them alternatively in water and potassium solutions was therefore tested. Freshly harvested cells (1.7 g. wet wt.) were washed three times with water and suspended in a volume of 50 ml. A sample (8 ml.) was

Table 4. *Swelling of cells in water and potassium chloride solutions*

Cells centrifuged from a 24 hr. culture were resuspended in growth medium and divided into four equal portions. Each was centrifuged and the cells were washed once with various media and finally suspended in the medium used for washing. The packed-cell volume was determined in the haematocrit by centrifuging to constant volume. The osmolarity of the growth medium at the end of the incubation, as determined by freezing-point depression in the apparatus of Fiske (Fiske Associates Inc., Boston, Mass.), was 110 m-osmoles/kg., i.e. the same as 0.11 M-sucrose or 0.055 M-KCl.

Medium	Vol. of packed cells (μ l./ml. of suspension)	Change in volume (%)
Growth medium	25.6	—
Water	51.8	+ 102
4 mM-KCl	49.8	+ 94
0.155 M-KCl	23.0	- 10

removed for analysis and sufficient 0.1 M-KCl was added to the remaining suspension to give a final concentration of about 5 mM-KCl. After 20 min. at 20°, another sample (8 ml.) was removed for analysis. The remaining suspension was washed three times with water on the centrifuge and resuspended in the original volume of water. A sample was put aside and KCl was added to a concentration of 5 mM. After 20 min. a further sample was collected for analysis. The process was repeated three times in all so that three samples of water-washed and water-suspended cells and three samples of cells suspended in KCl solution were obtained. The potassium content of cells and supernatant was determined in each sample. As shown in Table 3, the potassium taken up by washed cells was readily removed again by washing with water and reabsorbed on suspension in KCl solution. The amounts of potassium remaining in the cells after washing decreased somewhat with each washing, as did the amounts taken up (from 12.0 to 9.13 μ moles/ml.), but it is difficult to visualize, on the basis of these figures, how intracellular soluble cations could play a major part in the binding of

Table 5. *Comparison of uptake of potassium and respiratory activity of cells washed in water and in iso-osmotic sucrose*

Side arms of cups 1 and 2 contained 0.5 ml. of water-washed cells; cups 3 and 4, 0.5 ml. of sucrose-washed cells; the cell numbers were equal in both sets. The main compartments contained 0.04 ml. of M-KCl. Incubation was at 30° in air for 130 min.

Cup no.	1	2	3	4
Main compartment	Water (3.5 ml.)	Water (3.3 ml.) 0.1 M-Sodium acetate (0.2 ml.)	0.11 M-Sucrose (3.5 ml.)	0.11 M-Sucrose (3.3 ml.) 0.1 M-Sodium acetate (0.2 ml.)
O ₂ used during second hour (μl.)	18	88	22	388
Packed-cell volume of cells after incubation (μl.)	81.6	81.6	53	53
Initial K in cells (μmole/cup)	0.144	0.144	0.107	0.107
(μmoles/ml. of cells)	1.77	1.77	2.03	2.03
Final K in cells after incubation (μmoles/cup)	2.38	2.18	1.87	2.05
(μmoles/ml. of cells)	29.2	26.8	35.4	38.9
K in medium after incubation (μmoles/ml.)	8.85	8.92	9.38	9.38

Table 6. *Effect of hypo-osmoticity of the washing medium on respiration*

Cells from the same batch were washed in water and 0.11 M-sucrose respectively. Respiration in air at 30° was then measured with 3 mg. (dry wt.) of cells in 4 ml. of suspension.

Additions	Sucrose-washed cells			Water-washed cells		
	None	Sodium glutamate (5 mm)	Sodium glutamate (5 mm)-KCl (0.01 M)	None	Sodium glutamate (5 mm)	Sodium glutamate (5 mm)-KCl (0.01 M)
Q _{O₂} (first 30 min.)	0	41	137	8	19	53
Q _{O₂} (second 30 min.)	0	67	191	9	32	114

potassium, by exchanging with the potassium of the medium.

Swelling of bacterial cells in water. When cells are centrifuged from the growth medium they form a pink, tightly packed pellet. On washing in water the cells swell and much of the pink disappears, leaving a buff colour; at the same time the volume occupied by the pellet increases. Spectroscopic examination of the pigments by Dr June Lascelles showed that the pink colour is mainly due to a reduced iron-porphyrin derivative similar to cytochrome *b*; 1 mg. dry wt. of cells contained 0.9 μmole (expressed as haemin), an exceptionally high concentration. The buff colour is the oxidized form. On prolonged centrifuging of cells suspended in water two layers appear, the lower being similar in texture to the unwashed cells whereas the larger upper one is gelatinous. The extent of swelling in water is shown in Table 4. In water or in 4 mM-KCl the volume increase was of the order of 100 %, whereas there was a slight decrease when 0.155 M-KCl (which was hyperosmotic) was used for washing.

Effect of iso-osmotic sucrose solution on potassium loss and potassium uptake. In order to test whether the loss of potassium was due to the hypo-osmotic

city of the washing medium, a freshly harvested cell suspension in the growth medium was divided into two parts which were washed respectively with water and a sucrose solution iso-osmolar with the growth medium (0.11 M). As seen from the initial potassium content of the cells (Table 5), washing with water and sucrose lowered the potassium concentration almost identically. The increases in the potassium content of the cells on aerobic incubation were also approximately equal. The packed-cell volume after incubation was appreciably smaller in the presence of sucrose and the concentration of the intracellular potassium was therefore higher in the sucrose-washed cells.

Comparison of respiratory activity in water-washed and sucrose-washed cells. The oxygen consumption of sucrose-washed cells, in the presence of sodium acetate, was more than four times as fast as that of water-washed cells (Table 5). This observation suggests that washing with water removes cell constituents other than potassium essential for respiration, which are retained on washing with sucrose. This was borne out by a further experiment in which glutamate was the substrate (Table 6). The initial rate of respiration was again much lower in the water-washed cells, but, owing

to a more rapid increase of the rate with time, the differences decreased on prolonged incubation. The magnitude of the potassium effect was not decreased by the addition of sucrose to the washing medium.

Effect of octan-2-ol on the potassium uptake. Anaerobically the addition of octanol reduced the potassium content of cells by about 20% but the increase in the potassium content which occurred in the presence of an oxidizable substrate and of oxygen was almost completely abolished by octan-2-ol (Table 7). Thus octan-2-ol prevents the active but not the passive transport of potassium. In the presence of octan-2-ol the packed-cell volume decreased by one-third. Whether this is due to tighter packing or to actual shrinking of the cells has not yet been examined.

Movement of ions other than potassium. It was reported in the previous paper that the movement of potassium is not accompanied by parallel movements of Cl^- ions or opposite movements of Na^+ or nitrogenous cations. These findings have been confirmed in many further experiments, carried out under a variety of conditions on water-washed and sucrose-washed cells. The discharge of nitrogenous cations was determined by ammonia estimation after wet incineration. In an experiment in which 3 μmoles of potassium were taken up less than 0.25 μmole of nitrogen appeared in the supernatant. Changes in the sodium content were likewise negligible. $^{42}\text{K}^{36}\text{Cl}$ was used to confirm directly the separation of potassium and chloride when potassium is taken up by the cell. Owing to the great difference in the half-lives of ^{42}K (12.5 hr.) and ^{36}Cl (3.1×10^5 years), the relative quantities of ^{42}K and ^{36}Cl in cells and medium can be calculated from the change of counts with time, without chemical analyses.

Solutions of about 0.2 M- $^{42}\text{K}^{36}\text{Cl}$ were prepared by neutralizing a solution of H^{36}Cl with a solution of $^{42}\text{K}_2\text{CO}_3$. Initially the counts due to ^{42}K were approximately three times those due to ^{36}Cl . The relatively low specific activity of the available H^{36}Cl made it necessary to use rather high concentrations of KCl in the medium where the concentration gradients between cells and medium are less marked than at lower concentrations. Sucrose-

washed cells were placed in the side arms of manometer vessels and mixed in an atmosphere of N_2 with the isotopic KCl solution placed in the main compartment. After incubation for 30 min. 3 ml. was removed and centrifuged for 5 min. at 20 000g. The supernatant was drained off and the tubes were dried with filter paper. The pellet was weighed and resuspended in water to make a total volume of 10 ml. Samples of the supernatant and resuspended cells were counted in a liquid M6-type tube of 20th Century Electronics and the counts were corrected to an arbitrary zero counting time. After about 19 hr. the material was recounted and the counts were corrected to a time representing 1.5 times the half-life of ^{42}K (18.75 hr.). Controls showed that the chloride count was the same in a cell suspension and in water. If a was the initial count rate and b the count rate after 18.75 hr., the count rate due to ^{42}K at zero time was $(a-b)100/64.7$ and the count rate due to ^{36}Cl was $a - [(a-b)100/64.7]$ (64.7 being the percentage decay after 1.5 half-lives). As shown in Table 8 the ratio (counts due to ^{42}K)/(counts due to ^{36}Cl), which initially was about 3, remained the same in the supernatant. It increased to 22.5 in the pellet in vessel 2, where the potassium concentration was lowest. Addition of non-isotopic KCl (vessel 3) led to a decrease of the ratio. This is expected because the gradients decrease when the potassium concentration increases. It should be pointed out that the counts in the pellet must have been partly due to contaminating supernatant. They are therefore maximum values. About 0.5% contamination would account for all the counts due to ^{36}Cl , but for no more than 15–30% of the counts of ^{42}K . These findings prove conclusively that the movement of potassium into the cells is not accompanied by an appreciable movement of chloride.

Potassium exchange for hydrogen ions. According to Conway & O'Malley (1946) baker's yeast can, under certain conditions, take up K^+ ions in exchange for H^+ ions. Krebs *et al.* (1957) reported that no major pH changes occurred in the suspension when KCl was added to *Alcaligenes*. Further measurements with improved techniques employing the 23A pH meter (Electronic Instruments Ltd.) showed that small, but definite, pH changes

Table 7. *Effect of octan-2-ol on the uptake of potassium by Alcaligenes*

Each cup contained, in the main compartment, 0.25 ml. of 0.1 M-KCl, 0.5 ml. of 0.1 M-sodium acetate, 0.1 ml. of 0.1 M-sodium fumarate and 3.65 ml. of water. Side arm: 0.5 ml. of washed cell suspension containing 83 $\mu\text{l.}$ of packed cells. Initial concn. of K: 4.26 $\mu\text{moles/ml.}$ of packed cells. Where octan-2-ol was added, 1 drop was placed in the side arm and in the main compartment.

Cup no.	...	...	...	...	1	2	3	4
Gas	...	...	...	...	O_2	O_2	N_2	N_2
Octan-2-ol	...	...	...	...	—	+	—	+
K in supernatant ($\mu\text{moles/ml.}$)	...	...	...	...	4.69	4.87	5.15	5.15
K in cells ($\mu\text{moles/ml.}$)	...	...	...	...	26.5	16.0	19.4	15.1

can in fact be observed under favourable conditions, i.e. when the volume of cells is not too small in relation to that of the medium. A suspension containing 85.5 mg. dry wt. of cells (0.470 ml. of packed wet cells) in 5 ml. of water was incubated at 22° in air whilst the recording glass electrode was in the suspension. On addition of 2.23 μ l. of M-KCl the pH changed from 6.16 to 5.68 within seconds and then remained constant. This was accompanied by a rise of the K⁺ ion concentrations in the cell from 69 to 121 μ moles/ml. of packed cells. As the cells of 5 ml. of suspension, containing 0.47 ml. of packed cells, took up to 24.4 μ moles of potassium, a complete displacement of K⁺ ions by H⁺ ions in the medium would have resulted in a discharge of 24.4 μ equiv. of H⁺ ions in 4.53 ml., i.e. in a fall of pH to about 2.3 (assuming the buffering capacity of the medium was zero, which was virtually the case). It is true that the buffering capacity of the cells is considerable, but any binding of H⁺ ions by the intracellular buffers does not satisfy the requirements of electroneutrality in the suspension medium. The interaction of H⁺ ions with intracellular buffers would release an equivalent amount of base, which must appear in the medium in exchange for K⁺ ions, and as already shown no equivalent amounts of base were detected in the medium.

As pH measurements in unbuffered solutions may not be regarded as very accurate, further experiments were carried out in which dilute buffer

solutions served as the suspending medium. Cells (0.896 ml. packed volume) washed with and suspended in 0.11M-sucrose (4 ml.) were mixed with an equal volume of medium containing 0.01M-phosphate buffer (made from equimolar amounts of primary and secondary salt), 0.02M-KCl and 0.011M-sucrose. The mixture was allowed to stand for 30 min. at 20°. Before addition of the cells the pH of the medium was 6.89. It fell to 6.43 after the addition of the cells. A sample of the mixture was centrifuged and potassium and phosphate were determined in the supernatant and cells. Control analyses were carried out on the original cell suspension. The cells contained in the 8 ml. of suspension took up 29.5 μ moles of potassium and 11.2 μ moles of phosphate. After incubation the concentration of potassium was 33.9 μ moles/ml. of packed cells, and 18.1 μ moles/ml. of medium. From the change of pH and the concentration of the buffer it can be calculated that 17.2 μ moles of H⁺ ions had been discharged into the medium by the cells. On the assumption that the intracellular pH was the same as the extracellular pH, about 75 % of the intracellular phosphate was present as H₂PO₄⁻ ions and 25 % as HPO₄²⁻ ions. Thus 11.2 μ moles of phosphate were equivalent to 14.9 μ moles of potassium. The sum of H⁺ ions discharged and phosphate taken up is therefore equivalent to 32.2 μ moles, whereas 29.5 μ moles of potassium were taken up. In other words, a little more than half of the uptake of potassium was balanced by a

Table 8. *Distribution of ⁴²K³⁶Cl between cells and medium*

A suspension of sucrose-washed cells (1 ml.) was incubated at 30° for 30 min. with various additions. Vessels 1 and 3 contained 1 ml. and vessel 2 contained 0.5 ml. of 0.2M-⁴²K³⁶Cl solution. Vessel 3 also contained 1 ml. of 0.1M-KCl (non-labelled). The total volume was made up with water to 4 ml. Gas space N₂. For full details see text.

Vessel no.	1	2	3
Supernatant, after incubation			
Total counts/min./ml. at zero time (a)	20 352	10 125	20 598
Total counts/min./ml. after 1.5 potassium half-lives (b)	10 153	5 173	10 366
Counts/min./ml. due to ⁴² K at zero time	15 764	7 654	15 815
Counts/min./ml. due to ³⁶ Cl at zero time	4 589	2 741	4 784
Pellet, after incubation			
Wt. from 3 ml. of suspension (mg.)	142	154	133
Total counts/min. in pellet at zero time (a)	4 084	2 725	3 439
Total counts/min. in pellet after 1.5 potassium half-lives (b)	1 593	1 037	1 413
Counts due to ⁴² K at zero time	3 850	2 609	3 131
Counts due to ³⁶ Cl at zero time	234	116	308
Ratio: $\frac{\text{Counts due to } ^{42}\text{K in medium}}{\text{Counts due to } ^{36}\text{Cl in medium}}$	3.43	3.09	3.30
Ratio: $\frac{\text{Counts due to } ^{42}\text{K in pellet}}{\text{Counts due to } ^{36}\text{Cl in pellet}}$	16.4	22.5	10.2

discharge of H^+ ions and a little less than half by an uptake of an equivalent amount of phosphate.

Whilst there are some uncertainties in the assumptions, e.g. the intracellular pH, the errors would not affect the general conclusions. A notable feature of the experiment is the increase in the intracellular concentration of phosphate (12.5 μ -moles/ml. of packed cells) on incubation with phosphate. The phosphate concentration of the medium was initially 5 μ -moles/ml. and 3.7 μ -moles/ml. after incubation. Thus phosphate was taken up against a concentration gradient.

Another procedure, with a bicarbonate buffer, makes the potentiometric measurement of pH changes unnecessary. In this system an acid formed in the medium causes an evolution of an equivalent amount of CO_2 , which is measured manometrically. As diffusible buffer cations other than K^+ ions would complicate the issue, the anion-exchange resin (HCO_3^- form) was used. The commercial chloride form of Amberlite IRA-410 (cross-linked polystyrene with quaternary ammonium groups) was washed in a column with excess of NaOH solution to replace Cl^- by OH^- ion, then with water saturated with CO_2 at atmospheric pressure and finally with m-NaHCO₃ solution saturated with CO_2 . For 10 g. of resin about 1 l. of the washing fluid was used. The 'resin-bicarbonate' yielded 1.41 μ -moles of CO_2 /mg. of moist resin on acidification.

Three conical Warburg vessels were filled as indicated in Table 9. No. 1 contained no potassium but resin-bicarbonate. In mixing the contents of side arm and main compartment, any preformed acid discharged by the cells was measured by the CO_2

evolution. A control contained the same reagents plus KHCO₃. The CO_2 evolved in this cup on mixing was due to preformed acid plus acid discharged as a result of the uptake of potassium. In no. 2 the cells were neutralized with resin-bicarbonate before the addition of potassium and the pressure observed on mixing was due solely to the uptake of potassium.

The vessels were shaken in a water bath at 20° until equilibrium was reached. The contents of the side arm and main compartment were then mixed and shaken until the gas evolution ceased (about 20 min.). As seen from the data given in Table 9, the gas evolution in no. 2 was, as expected, approximately equal to the difference between the control and no. 1.

After completion of the manometric measurements the suspension was decanted from the resin and centrifuged in the presence of 5% CO_2 . Potassium and bicarbonate were determined in the medium and in the packed cells. Control analyses were carried out on the non-incubated suspension. Full details are given in Table 9; the decisive figures are given in bold face. The uptake of potassium on addition of KHCO₃ was 9.43 μ -moles. This was accompanied by an uptake of 5.39 μ -moles of bicarbonate and a discharge of 4.13 μ -moles of H^+ ions, as measured by the CO_2 evolution.

The electrochemical balance was thus very satisfactory and similar to that of the phosphate experiments. About 44% of the potassium uptake was balanced by the discharge of H^+ ions and 56% by an uptake of bicarbonate. The calculation of the balance was simpler than in the phosphate experiments as no assumptions about the intracellular

Table 9. Gas evolution associated with uptake of potassium by a suspension of *Alcaligenes faecalis* in bicarbonate buffer

For details see text. Gas phase, $CO_2 + N_2$ (5:95); temperature, 20°. A control vessel containing the same reagents as vessel 1, except that 3 ml. of water was replaced by 3 ml. of 0.033N-KHCO₃, evolved on mixing 5.44 μ -moles of gas.

Vessel no.	...	...	...	...	1	2
Side arm					1 ml. of washed cells in 0.11M-sucrose (156 μ l. of packed cells)	1 ml. of 0.1N-KHCO ₃
Main compartment					Resin-bicarbonate (100 mg.), 3 ml. of water	Resin-bicarbonate (100 mg.), 1 ml. of washed cells in 0.11M-sucrose (156 μ l. of packed cells), 2 ml. of water
Gas evolved on mixing (μ -moles)					1.07	4.13
K in 156 μ l. of packed cells (μ -moles)					2.23	11.66
K increase in cells (μ -moles)					—	9.43
K (μ -moles/ml. of packed cells)					14.5	74.5
K (μ -moles/ml. of medium)					0.97	22.8
HCO ₃ ⁻ ion in 156 μ l. of packed cells (μ -moles)					2.86	8.25
HCO ₃ ⁻ ion increase in cells (μ -moles)					—	5.39
HCO ₃ ⁻ ion (μ -moles/ml. of medium)					0.36	27.0
HCO ₃ ⁻ ion (μ -moles/ml. of packed cells)					—	52.0

pH were involved. It is possible that some of the intracellular bound CO_2 was carbamate rather than bicarbonate, but this would not be relevant to the present issue.

Uptake of phosphate and bicarbonate. The uptake of bicarbonate and phosphate against concentration gradients was confirmed in other experiments and in all cases where the balance of cation and anion movements was measured the requirements of electroneutrality were met. Table 10 shows experiments on water-washed and sucrose-washed cells to which various potassium salts were added. Although the sucrose-washed cells had a higher initial potassium concentration the increase on addition of 2 mM-potassium salt was about the same as in water-washed cells. The uptake of potassium was greater with KHCO_3 than with KCl and KH_2PO_4 . After incubation the intracellular bicarbonate concentration was six to seven times as high as that of the medium but it must be borne in mind that the bound CO_2 measured as bicarbonate may in part have been carbamate attached to the amino groups of protein, in which case the true concentration gradients would be smaller. The concentration gradient of phosphate was about 4 in the water-washed and about 2 in the sucrose-washed cells.

Binding of potassium by lipids in Alcaligenes. It has been suggested by Christensen & Hastings (1940) and Baer, Buchnea & Newcombe (1956) that lipids may play a part in the binding of intracellular potassium. Experiments were therefore carried out to examine the association of potassium with the lipid fraction of the bacterial cells. Respiring cells were incubated in a medium containing ^{42}K and the radioactivity of the total potassium and the lipid-bound potassium was measured after incubation for various periods.

Vessels were removed from the bath in pairs and samples (3 ml.) of the contents were centrifuged immediately at 0° and $24\,000g$ for 10 min. The supernatants were decanted and sample 1 was treated with 0.5 ml. of conc. HNO_3 , as previously described for the determination of total potassium. Sample 2 was freeze-dried and extracted with hot chloroform-methanol (2:1, v/v), as described by Solomon, Lionetti & Curran (1956). The combined extracts were made up to 10 ml. and a sample (2 ml.) was evaporated to dryness in a 5 ml. beaker under a current of warm air. The small amount of dry material which remained was re-extracted at room temperature with eight 1 ml. portions of dry chloroform and combined extracts were made up to 10 ml. for counting in a liquid counter tube. As shown in Table 11, there was a very rapid uptake and exchange of potassium by the cells, the specific activity of the intracellular potassium reaching 94 % of that of the extracellular potassium within 3.5 min. In contrast, the increase of radioactivity in the lipid fraction was very slow and reached only 4.3 % of the total radioactivity after 45 min. Thus the chloroform-methanol-extractable cell constituents are not major binding sites for potassium within the cells.

Binding of potassium by crushed cells. In order to test the importance of the cell structure and of membranes to the potassium-binding mechanism, cells washed in water were suspended in approximately 2 vol. of water and crushed in a Hughes press. The resulting material was cloudy and so viscous that it could not be pipetted. Addition of a deoxyribonuclease preparation (Streptodornase, Lederle Laboratories 1 mg. for 10 ml.) substantially reduced the viscosity and cloudiness. On centrifuging the crushed and Streptodornase-treated material no appreciable sediment was obtained.

Table 10. *Uptake of potassium and anions by cells washed in water or iso-osmolar sucrose on addition of dilute salt solutions*

Stock suspension in water or 0.11 M-sucrose (1 ml. containing about $150\mu\text{l}$. of packed cells) was mixed with 1 ml. of potassium salt solution containing 0.01 equiv. of potassium/l. and 3 ml. of water or 0.11 M-sucrose. Incubation was for 30 min. at 30° . Suspensions were set up in pairs and potassium was determined in the pellet of one suspension and the anion in the pellet of the other. Gas phase, air.

Salt added	Concn. of potassium (mm)					Concn. of anion (mm)				
	Cells			Medium		Cells			Medium	
	Initial	Final	Change	Initial	Final	Initial	Final	Change	Initial	Final
Water-washed cells										
KCl	1.0	6.35	5.35	2.08	1.22	—	—	—	—	—
KHCO ₃	1.0	10.0	9.0	2.08	1.16	0	10.0	+ 10	2.0	1.38
KH ₂ PO ₄	1.0	5.95	4.85	2.08	1.22	2.36	8.70	+ 6.3	2.28	1.90
Sucrose-washed cells										
KCl	10.7	16.9	6.2	2.41	1.47	—	—	—	—	—
KHCO ₃	10.7	21.0	10.3	2.41	1.39	0	10.0	+ 10.0	2.0	1.52
KH ₂ PO ₄	10.7	16.2	5.5	2.41	1.57	3.9	5.87	+ 2.0	2.50	2.46

Viable cells counted in the crushed material showed that 89.2% of cells had been disrupted. A portion (1 ml.) of the crushed and Streptodornase-treated material was placed in a Visking dialysis tube (13 mm. diam.) which was tied at both ends. Several such sacs were placed in 100 ml. of 2 mM-KCl solution and stirred magnetically. At intervals a sac was removed; its contents and samples of the outside medium were analysed for potassium. A parallel experiment was carried out on a sample of the original cell suspension that had not been crushed.

As shown in Table 12, the concentration of potassium in the suspension containing intact cells reached, within 1 hr., more than twice that of the outside medium and then remained fairly constant (second column). These values refer to the suspension as a whole. The potassium concentration within the cells was over five times that of the

medium (third column). Disruption did not reduce the capacity of the suspension to accumulate potassium (fourth column).

A similar experiment was carried out with sonically disrupted cells. A suspension containing about 20% of cells cooled in ice was placed for 4 min. in a MSE ultrasonic disintegrator (60 w at 18–20 kcy.). This converted the cell suspension into an opalescent solution which on centrifuging at 10 000g for 10 min. did not yield an appreciable sediment. The material which, unlike that obtained with the Hughes press, was not viscous, was pipetted into dialysing tubes in 1 ml. portions and several such sacs were placed in the 200 ml. of 2 mM-KCl solution as described. A parallel experiment was carried out on the original intact cell suspension. As shown in Table 12 the ultrasonically disrupted cells behaved similarly to the suspension of intact cells. The sac containing the ultrasonically disrupted

Table 11. *Test for binding of potassium by lipids in Alcaligenes*

The main compartments of six Warburg vessels contained 4.5 ml. of the following mixture: 6 ml. of ^{42}KCl solution (1 mc in 100 mg. of K_2CO_3 neutralized with HCl and dissolved in 50 ml. of water), 0.8 ml. of 0.2M-sodium fumarate, 0.2 ml. of 10M-sodium acetate and 23 ml. of 0.03% KCl. The side arms contained 1.0 ml. of a suspension of washed cells. Centre well: 0.2 ml. of 2N-NaOH. Gas phase, air. Temperature, 30°. Cells were mixed after temperature equilibration. The first sample was removed immediately after mixing but 3.5 min. was required before the samples were in a gravitational field of 24 000g. For further details see text.

Incubation time (min.)	...	...	...	...	...	3.5	15	45
K in supernatant ($\mu\text{moles/ml.}$)						5.69	5.64	5.84
K in cells ($\mu\text{moles/ml.}$)						25.6	25.2	23.3
Counts/min./ $\mu\text{l.}$ of supernatant						254.0	253.8	299.0
Counts/min./ $\mu\text{l.}$ of cells						117.6	137.6	154.0
Sp. activity of K in supernatant (counts/min./ $\mu\text{mole of K}$)						44 620	49 100	51 480
Sp. activity of K in cells (counts/min./ $\mu\text{mole of K}$)						42 040	46 250	47 270
Ratio: $\frac{\text{Sp. activity of K in cells}}{\text{Sp. activity of K in supernatant}} \times 100$						94.2	94.2	91.8
Counts in lipid extract						470	5 015	6 555
Counts in lipid extract (% of total counts in cells)						0.4	3.6	4.3

Table 12. *Potassium binding by ultrasonically disrupted cells and cells crushed in a Hughes press*

Disrupted cells were placed in dialysis sacs and shaken in 2 mM-KCl solution. For details see text. The controls contained intact cells. Potassium in cells in sacs was calculated on the assumption that the concentration of potassium of the suspension medium within the sacs was identical with that of the outside medium.

Incubation time (hr.)	Total K in sac (μmoles)	K in cell in sac ($\mu\text{moles/ml.}$)	K outside sac ($\mu\text{moles/ml.}$)	Total K in sac (μmoles)	K outside sac ($\mu\text{moles/ml.}$)
	Control (intact cells)			Ultrasonically disrupted cells	
0	1.17	5.92	2.13	1.18	2.18
1	4.41	13.5	2.13	5.08	2.18
3	4.92	16.2	2.13	5.84	2.18
6	4.67	14.9	2.13	6.31	2.18
	Control (intact cells)			Crushed cells	
0	2.15	6.89	2.10	2.13	2.13
1	4.82	10.8	2.10	4.90	2.13
3	4.62	10.1	2.10	5.13	2.13
7	5.18	11.9	2.10	5.74	2.13

Table 13. *Effect of external potassium concentration on the uptake of potassium by dialysis sacs containing ultrasonically disrupted cells*

Portions (1 ml.) of ultrasonically treated cells (20% suspension; 4 min.) were placed in dialysis sacs and suspended in portions (100 ml.) of KCl solutions of varying concentrations. Incubation was for 3 hr. at 20°. The preparation initially contained 1.61 μ moles of K/ml. For further details see text.

External concn. of K (μ moles/ml.)	Concn. of K in sac after incubation (μ moles/ml.)	Excess of internal concn. of K over external concn. of K (μ moles/ml.)
1.0	3.69	2.69
1.95	5.33	3.38
4.28	8.41	4.15
7.79	13.1	5.15
20.3	28.4	8.15

cells bound even a little more potassium than the sac with the intact cells. After 6 hr. the concentration of potassium within the sacs containing the ultrasonically disrupted cells was three times as high as that of the external medium. The effect of the external potassium concentration on the concentration attained within the sac is shown in Table 13. The difference between the external and internal concentrations increased with potassium concentration but the movement became smaller with increasing potassium concentrations.

These experiments show that the intactness of the cell structure is not essential for the binding of potassium. Colloidal cell constituents rather than specific structures appear to effect the binding.

DISCUSSION

The uptake of potassium by *A. faecalis* against concentration gradients presents two main problems. The first concerns the mechanism of the uptake in the absence of a source of energy; the second the mechanism of the additional uptake which occurs when the cells respire. Both problems are presumably interconnected in that the 'passive' uptake in non-respiring cells is probably the basic transport phenomenon upon which active transport is superimposed. The present work is mainly concerned with a study of the 'passive' transport which occurs in the absence of oxidizable substrates or of oxygen, or in the presence of inhibitors such as octan-2-ol preventing the utilization of energy sources, or at low temperatures (0°), when the rate of energy supply is negligible. Active, energy-dependent transport can greatly increase the concentration gradients which are reached by passive transport.

One of the main aims of the present work was to establish the manner in which electroneutrality is

maintained when potassium migrates into the cells. This aim has not been fully achieved. Electro-neutrality must be preserved in the medium as well as in the cell. The study of the medium presents the simpler problem, especially in the absence of substrates when the added potassium salt is the only initial solute. In this case the disappearance of potassium from the medium must either be accompanied by the removal of an equivalent anion or it must be compensated by the appearance of an equivalent cation. When the added salt was potassium bicarbonate or potassium phosphate these requirements were met. About half the K^+ ions were replaced by H^+ ions and the remainder migrated together with the cation. When the added salt was potassium chloride a discrepancy in the ionic balance remained. In this case relatively small quantities of H^+ ions and titratable nitrogenous bases appeared in the medium, but these cations together accounted for rather less than 20% of the potassium removed and no migration of chloride or sodium could be detected. Many checks on the accuracy of the determination of K^+ , Na^+ , Cl^- , H^+ ions and titratable nitrogenous cations confirmed these conclusions and discount the suggestion that the discrepancy is due to analytical errors.

In some respects the bacterial cells behave like a cation-exchange resin with a special affinity for potassium. As with exchange resins the movements of potassium are very rapid, even at 0°. But as the cells also accumulate anions passively the substance or substances effecting the passive uptake are not simple cation-exchangers. With regard to the exchange of K^+ for H^+ ions, the situation is similar to that described by Conway & O'Malley (1946) for yeast cells but there are major differences between the two organisms. Yeast does not lose potassium on washing, and the uptake of potassium can only be studied on special cells depleted of potassium. To obtain these it is necessary to grow them on a potassium-low medium. The amounts of free hydrochloric acid which can arise in yeast cells appear to be much greater than in *Alcaligenes*, a fact which may be related to the much greater resistance of yeast cells to acid. Yeast cells can respire and even grow in a medium of pH 2, whereas *Alcaligenes* fails to grow when pH falls below 6.0.

The demonstration of a potassium-binding colloid in solutions obtained by crushing or ultrasonically disrupting cells opens up investigations into the chemical characterization of the potassium-binding material.

SUMMARY

1. Cells of four strains of *Alcaligenes faecalis* showed the same loss of potassium on washing with water and its recovery against a concentration

gradient on the addition of potassium to the suspension medium as reported for three strains examined previously.

2. When cells were alternately exposed to water and potassium chloride solutions they lost and gained potassium repeatedly though the amounts taken up decreased slightly on repeated washing. Thus intracellular soluble cations do not seem to play a major part in the binding of potassium by exchanging with the potassium of the medium.

3. Cells suspended in hypo-osmotic solutions swelled to about twice the original volume but the loss of potassium was independent of swelling and hypo-osmoticity; it also occurred in sucrose solution iso-osmolar with the growth medium. The aerobic uptake of potassium was approximately the same from hypo-osmotic media and from iso-osmolar sucrose solutions.

4. Octan-2-ol prevented the active transport of potassium but had no major effect on the anaerobic uptake.

5. Electrotitration of the medium showed that small quantities of nitrogenous bases were discharged when potassium was taken up. These were equivalent to less than 10% of the potassium uptake.

6. The uptake of potassium from potassium chloride solutions was accompanied by a discharge of H^+ ions, causing the pH of the medium to fall by about 0.5 unit. The release of H^+ ions was equivalent to no more than a small fraction of the potassium uptake.

7. When cells were suspended in dilute potassium phosphate buffer the uptake of potassium was accompanied by a fall of pH of 0.46 unit and an

uptake of phosphate against a gradient. Calculations show that in this case electroneutrality was partly maintained by an exchange of K^+ for H^+ ions, partly by the movement of phosphate, each factor contributing about 50%. Analogous results were obtained when potassium bicarbonate was added and the bicarbonate form of a cation-exchange resin was used as a buffer.

8. Chloroform-methanol-extractable cell lipids did not bind significant amounts of potassium.

9. Cells disrupted by the Hughes press or ultrasonically when placed in a dialysing sac were still able to bind potassium. It is concluded that a colloidal cell constituent rather than specific structures effects the binding.

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Enzymic Determination of D(–)- β -Hydroxybutyric Acid and Acetoacetic Acid in Blood

By D. H. WILLIAMSON, JANE MELLANBY AND H. A. KREBS

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

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The current methods for the estimation of β -hydroxybutyrate (e.g. Greenberg & Lester, 1944; Thin & Robertson, 1952; Bloom, 1958; Bessman & Anderson, 1957) all involve its oxidation to acetone and the estimation of the latter. These methods are laborious and have several unsatisfactory features. They are unspecific in that other compounds also yield acetone on oxidation and they do not differ-

entiate between the optical isomers. Moreover, the conversion of β -hydroxybutyrate into acetone is not quantitative and the yield varies with the conditions. The method described in this paper is in principle more specific and more rapid than the older procedures. It is analogous to the enzymic determination of many other metabolites. It depends on the spectrophotometric measurement of