Induced and Spontaneous Movements of Potassium Ions into Mitochondria

By E. J. HARRIS

Department of Biophysics, University College London, Gower Street, London, W.C. 1

AND R. COCKRELL AND B. C. PRESSMAN

Johnson Foundation, University of Pennsylvania, Philadelphia, Pa., U.S.A.

(Received 13 September 1965)

1. Net movements of K^+ into metabolizing liver mitochondria before and after the addition of valinomycin have been measured by using selective glass electrodes. The advantage of using an automatic titrator to hold the K^+ concentration constant is demonstrated. 2. According to the energy source provided the induced movement after the addition of valinomycin can be either in or out. 3. Uptakes and rates of movement are higher in media containing acetate (20mM) than in media containing chloride (20mM). In each mixture comparisons were made at three pH values; at pH 6.36 the induced rates are less than at pH 7.0 or 7.8 but the final uptakes attained are increased. 4. The rate of uptake is increased by inorganic phosphate. 5. The presence of Mg^{2+} slightly decreases the induced uptake and rate of movement; Ca^{2+} can cause the induced movement of K^+ to be outward. 6. The rate of induced K^+ movement is related to the rate of extra oxygen consumption but with different factors in acetate (24 K^+ ions/oxygen molecule) and chloride media (10 K^+ ions/oxygen molecule). 7. The amount of K^+ gained is proportional to the loss of fluorescence of the suspension. 8. When K^+ moves there is a contrary movement of H^+ but the ratio depends on the conditions. At pH 6.36 in chloride media the K^+/H^+ ratio exceeded 10:1 and in no case did it fall to unity. 9. When K^+ is taken up there is a proportional diminution of light-scattering; it is inferred that swelling takes place along with K^+ accumulation. 10. It is shown by the use of tracer $^{42}K^+$ that turnover of the ion in mitochondria is increased by valinomycin. 11. It is concluded that valinomycin both increases the permeability to K^+ and also, given an adequate energy supply, stimulates the K^+ -accumulating mechanism.

There have been many speculations whether mitochondria might not be the mediators of active ion movements into cells (see, e.g., Bartley & Davies, 1954). On this account ion movements into the isolated particles have attracted attention. The net gain of K^+ by particles suspended in media either capable of supporting oxidative phosphorylation or containing ATP has been demonstrated (Share, 1958; Gamble, 1962; Christie, Ahmed, McLean & Judah, 1965; Rottenberg & Solomon, 1965). That the uptake is not due to occlusion or formation of an undissociated compound is inferred from measurements of tracer $^{42}K^+$ uptake though, indeed, a part of the K^+ exchanges slowly (Gamble, 1957; Amoore, 1960).

The accumulation of K^+ can be induced by the addition of valinomycin, a toxic antibiotic, to metabolizing suspensions (Moore & Pressman, 1964). This uptake is accompanied by the ejection

of H^+ and an increased metabolism, as also noted by Azzi & Azzone (1965).

The present experiments were made with the object of defining the effects of some variables on K^+ movement and its associated changes both without and with the addition of valinomycin. Instead of relying on terminal analyses of a pellet or filtered sediment we have obtained continuous records of the movements of ions between the solution and the particles by using selective glass electrodes (Moore & Pressman, 1964; Pressman, 1965). The results so obtained show that K^+ uptakes occur faster and in greater amount than were disclosed by analytical methods. Simultaneous observations of oxygen consumption, volume, fluorescence and pH were made in media containing either chloride or acetate together with substrate anion at different pH values.

Supplementary experiments were made with

$^{42}\text{K}^+$ to show that valinomycin increases K^+ turn-over besides causing accumulation.

METHODS

The activities of H^+ and K^+ in the suspension medium were monitored respectively by an A. H. Thomas 4848-L15 combination electrode and a Beckman 39047 electrode, as used by Pressman (1965). Movements of the two ions between the mitochondria and the solution cause activity changes that were either recorded after detection and suitable amplification or used to operate an automatic titrimeter (Radiometer; this is referred to below as the 'Stat'). In most experiments the 'Stat' was used to hold the K^+ activity constant because adequate buffer could be provided to keep the pH excursion within reasonable limits.

In addition to monitoring the ion concentrations, records were made of the oxygen concentration in the solution measured by a Clark-type oxygen electrode (Radiometer E5044), the fluorescence and the relative light-scattering. Fluorescence was excited by a mercury discharge lamp with peak emission from the line at $366\text{m}\mu$ and was measured through an interference filter peaking at $450\text{m}\mu$ by means of a photomultiplier (E.M.I. type 9524B). The beam of exciting radiation was directed on to one face of the square glass reaction vessel at an angle of about 25° to the normal and the emitted fluorescence emerging from the same face was collected on to the photomultiplier by means of a lens; the optical axis of the detector made an angle also about 25° to the normal to the face of cuvette so that there was 50° between incident and emitted beams. The light-scattering was measured from the opposite face of the reaction vessel; light from a lamp with a filter peaking at $546\text{m}\mu$ was fed into the vessel by means of a tapered Perspex light-guide having its curved surface wrapped in foil. The narrow end of the truncated cone of the light-guide was in contact with the glass; the axis of the guide was at about 25° with respect to the normal. Light scattered from the suspended mitochondria in the reaction vessel was collected by means of a lens on to a photomultiplier directed towards the area illuminated by the light-guide. By careful positioning of the detector it could be made relatively insensitive to light reflected from the internal surface of the face of the reaction vessel. The response of the light-scattering arrangement was not strictly linear and it was necessary to calibrate by making a series of equal additions of a yeast suspension. The same calibration run served to confirm that the fluorescence signal received was nearly linear with respect to concentration of fluorescent material. However, to put the fluorescence in quantitative terms requires knowledge of the enhancement brought about by combination of reduced nicotinamide nucleotides with protein. Such combination may conceivably not remain constant when large volume changes occur.

The mitochondria were prepared from rat liver, except those for the experiments in Fig. 13, which were prepared from pigeon heart muscle. The liver was minced into 0.25M-sucrose containing EDTA (1mM) at 0° and homogenized. The cellular debris was separated by centrifuging at $800g$ for 20 min. The mitochondria were then separated by centrifugation at $8000g$ for 20 min.; two washes by resuspension in 0.25M-sucrose at 0° were given. After the final separation the pellet was resuspended in sufficient

0.25M-sucrose to provide about 50 mg. of protein/ml. This is called the 'concentrated suspension'. In a number of experiments ethylene glycol bis-(β -aminoethyl ether) NN' -tetra-acetate (0.1mM) was added to the sucrose used for washing and for making up the final concentrated suspension but this had no effect on the results. The maximum capacity of the mitochondria for K^+ accumulation diminished with time so that after the lapse of 16 hr. at 0° it was, for example, half the original value.

The experiments were made by using 0.4–0.7 ml. of the concentrated suspension added to sufficient diluent to make 10 ml. of dilute suspension in the reaction vessel. The diluent was made up with 0.25M-sucrose containing tris chloride or acetate at the concentration (often 20mM) and pH specified in the text together with NaCl (2mM), tris glutamate (3mM) plus malate (3mM) and a specified concentration of KCl additional to the endogenous concentration. The use of malate plus glutamate provides a 'sink' for amino groups and prevents NH_4^+ formation, which would affect the K^+ -sensitive electrode. The reactions were run at ambient temperature (26 – 29°). The mixture was stirred by a magnetic stirrer driven from beneath the vessel.

Analyses for K^+ and Mg^{2+} were made with a Techtron atomic absorption spectrophotometer. Protein was measured by the biuret method (Layne, 1957).

Radioactivity measurements on the $^{42}\text{K}^+$ were made in a Tri-Carb scintillation counter (Packard Instrument Co.). The dried Millipore pads were immersed so as to lie flat on the base of the scintillation vials; they become translucent when the scintillation fluid is added. The results in Fig. 15 were obtained with at least 3000 counts collected per sample and those in Fig. 16 with at least 600 counts collected per sample. Count rates were corrected for decay to a standard time.

RESULTS

We wished to examine the dependence of net K^+ movements on the concentration. A lower limit to the concentration range is set by the dissociation of bound K^+ from the mitochondria either before or after the concentrated suspension (defined in the Methods section) is diluted. Suspensions having 20–25 mg. of protein/ml. were examined by layering samples on sucrose solution (sp.gr. 1.1) in centrifuge tubes followed by centrifugation at $8000g$ for 10 min. The particle-free upper layer was analysed for K^+ : concentrations found were 1.0 and 1.8 mM. The same technique applied to suspensions after 20 min. incubation at 37° gave the values 1.6 and 2.2 mM. The K^+ concentration in the suspension after dilution to 2–3 mg. of protein/ml. was measured by filtration through Millipore pads (0.45μ pore size). The concentrations so found were 0.2–0.5 mM. An additional resuspension during the preparation of the particles did not lead to a decrease in the external K^+ concentration in the final suspension, which is evidently maintained by loss of the internal K^+ . When the concentrated suspension was mixed with K^+ -free diluent in the reaction vessel the K^+ -sensitive electrode showed that a leakage of K^+ from mitochondria into the

medium occurred. The movement amounted to a few $\mu\text{equiv./g.}$ of protein/min.

If valinomycin ($0.05 \mu\text{g./ml.}$ or $0.01\text{--}0.02 \mu\text{g./mg.}$ of protein) was added under these conditions the response depended on what energy source had been provided (Table 1). The most potent energy source of those tested was the glutamate-plus-malate mixture; in its presence valinomycin reversed the direction of movement so that despite the low ambient concentration K^+ moved from the medium into the particles. On the other hand, with 2 mM-ATP in the presence of rotenone to block oxidation of endogenous substrate the addition of valinomycin accelerated K^+ loss. This difference in behaviour suggests a double action of valinomycin: namely, to increase permeability to K^+ and also, when energy at sufficient potential is provided, to bring about K^+ uptake. In the presence of ATP

but without rotenone the addition of valinomycin led to an uptake of K^+ even from the low- K^+ medium, presumably by using endogenous substrate as energy source. The addition of oligomycin ($1 \mu\text{g./ml.}$) had no effect whatever on the uptake of K^+ when glutamate-plus-malate substrate was used. It did, however, block uptake when ATP was provided as energy source. Hence it is inferred that ion accumulation occurs with the intervention of an intermediate of oxidative phosphorylation which leads to ATP production and which in the absence of oligomycin can be replenished from ATP.

The withdrawal of K^+ from a medium having initially only $0.2\text{--}0.5 \text{ mM-K}^+$ can so decrease the concentration that the movement is affected. The amount of K^+ that moves in these conditions will depend on the concentration of mitochondria used.

Table 1. *Rates of spontaneous and valinomycin-induced K^+ movements with different energy sources and with low K^+ concentrations at $26\text{--}28^\circ$*

Experimental details are given in the text. Expt. 1 shows that, with ATP as energy source and provided that the use of endogenous substrate is blocked, valinomycin ($0.05 \mu\text{g./ml.}$) hastens K^+ loss in low K^+ concentrations. Expt. 2 shows that, with sufficient malonate, found by trial, the K^+ accumulation which can be brought about by succinate is prevented. Expt. 3 shows that, with glutamate plus malate, the energy source used in most of the experiments described in this paper, the direction of K^+ movement is reversed by valinomycin.

	Energy source	Concn. of K^+ (mM)	Rate of K^+ movement ($\mu\text{equiv./g./min.}$)	
			Spontaneous	Induced
Expt. 1	ATP (2 mM) with rotenone ($0.5 \mu\text{g./ml.}$)	0.23	-1.4	-55
		1.33	0	30
Expt. 2	Succinate (7.5 mM) with malonate (10 mM)	2.3	0	0
Expt. 3	Glutamate (3 mM) + malate (3 mM)	0.23	-4	38

Table 2. *Comparison of uptakes of K^+ induced by valinomycin when the concentration in the medium falls during movement ('Free') and when it is held by the automatic titrimeter ('Stat')*

Experimental details are given in the text. The medium included tris acetate, pH 7.15 (40 mM). The dilute suspension had 3 mg. of mitochondrial protein/ml.

Initial concn. of K^+ (mM)	Rate of K^+ uptake ($\mu\text{equiv./g./min.}$)		Total quantity of K^+ ($\mu\text{equiv./g.}$)	
	'Free'	'Stat'	'Free'	'Stat'
0.42	21	24	42	106
0.52	36	43	56	133
0.72	63	58	100	173
1.02	140	99	154	224
1.42	166	133 (153*)	240	280
1.92	203	186 (276*)	270	321
4.17	270	—	416	—
7.92	416	—	483	—

* Rates measured during the short interval of 'free' run interposed just after the addition of valinomycin ($0.05 \mu\text{g./ml.}$).

For example, with 2mg. of protein/ml. and an initial K^+ concentration of 0.18mm an uptake of $45 \mu\text{equiv./g.}$ of protein will halve the concentration. Two comparisons in parallel runs were made; in

one of each pair the K^+ concentration was maintained by the 'Stat' and in the other it was not. The uptakes from the maintained K^+ concentrations were twice those found when the concentration

Fig. 1. Illustration of methods used and results obtained in the present work. Two experiments are shown in which spontaneous and induced K^+ uptakes are compared in systems supported by (a) succinate and (b) glutamate plus malate. The media contained: sucrose (0.25M), tris acetate, pH 7.4 (40mm), NaCl (2mm), KCl (10mm) and either (a) tris succinate (6mm) or (b) tris glutamate (3mm) plus malate (3mm); the total mitochondrial protein was 23.5mg. in 10ml. Valinomycin (0.05 $\mu\text{g./ml.}$) was added where indicated. A and A', K^+ added by 'Stat'; B and B', fluorescence; C and C', K^+ -electrode trace; D and D', H^+ -electrode trace; E and E', O_2 -electrode trace; F and F', light-scattering. Experimental details are given in the text. The following points may be noted. (1) The titrimeter addition of KCl solution (A and A') maintains the K^+ concentration during spontaneous uptake before the addition of valinomycin. Uptake is greater from the glutamate-plus-malate mixture. The K^+ -sensitive electrode shows a nearly constant concentration. (2) When valinomycin was added the 'Stat' was turned off for 40 and 20sec. respectively to obtain from the K^+ -electrode records (C and C') measures of the induced rate. The 'Stat' was then reset to restore the original K^+ concentration in the solution. However, the K^+ uptake soon ceased and the ions moved back into the solution (C and C'). (3) The change from K^+ uptake to loss occurred at the time the fluorescence had fallen nearly to zero (B and B'). (4) The pH traces (D and D') show a slow production of acid with (in D) a small additional amount appearing as induced K^+ accumulation proceeds. (5) The oxygen consumption (E and E') is increased during the phase of rapid K^+ accumulation and slows again when the ions are released. (6) Both spontaneous and induced K^+ uptakes are accompanied by a diminution of light-scattering; this is temporarily reversed when the ions are subsequently released.

was free to decline. In the absence of the 'Stat' after the induced uptake had ended the addition of K^+ to the reaction mixture led to a renewed uptake. Even when relatively high external K^+ concentrations had been provided the amount moved could, in suitable conditions, be so great that an appreciable fractional concentration change was caused. Absorption of $700 \mu\text{equiv.}$ of K^+ /g. of protein by a suspension having 2mg. of protein/ml. decreases the concentration of K^+ from 10 to 8.6 mM. Table 2 compares some movements measured at constant and shifting K^+ concentration in sets of experiments on the same mitochondrial preparation. As expected, the differences are most marked at the lower concentrations. If the data are expressed in a double-reciprocal plot, the differences, especially at low concentrations, result in different K_m values.

Although the use of the 'Stat' is desirable it is equally important to monitor the concentration of the K^+ because cessation of net uptake can be followed by a net loss. One would be unaware of the latter phase if the 'Stat' alone were used. When rapid uptakes were encountered it was more accurate to offset temporarily the balance position of the 'Stat' so that the dynamic record from the potassium electrode registered the rate of movement from the solution to the mitochondria. The interval allowed for the shift of concentration was brief and after it the K^+ concentration in the solution was restored to the initial value by means of the 'Stat'. Total movements are best obtained from the 'Stat' record showing the volume of standard potassium salt solution that was added to the reaction vessel.

A pair of experiments in which succinate and glutamate-plus-malate substrates were compared are illustrated in Fig. 1. For these experiments the 'Stat' was used along with recording of K^+ concentration as just described. The other parameters recorded simultaneously are described in the caption and the changes are discussed below.

The total quantities moved are less sensitive to K^+ concentration than are the initial rates; indeed, since they presumably represent time integrals of the difference between an influx and an increasing efflux this result would be expected. In media made up with tris chloride (20 mM) the quantity of K^+ taken up soon approaches saturation (Fig. 2 and curve A in Fig. 5). In some conditions the amount passes through a maximum as the external K^+ concentration is increased. The requirement for a preliminary K^+ depletion by warming at 37° for 20 min. (Christie *et al.* 1965) before the net accumulation of K^+ could be demonstrated led us to compare the uptakes by depleted and untreated mitochondria. The net movements have been plotted against the K^+ concentration (held constant by the 'Stat') in Fig. 2. The results show that

Fig. 2. Uptakes of K^+ induced by the addition of valinomycin ($0.05 \mu\text{g./ml.}$) to mitochondrial suspensions in media having different K^+ concentrations. ○, Results with mitochondria from the normal preparations; ●, results with mitochondria taken from the same preparations but additionally kept at 37° for 20 min. before use. The incubation leads to a leakage of K^+ from the particles into the solution so that the minimum K^+ concentration for the subsequent uptake experiments is somewhat higher than that in the controls. The medium contained: sucrose (0.25 M), tris chloride, pH 7.4 (20 mM), NaCl (2 mM), tris glutamate (3 mM) plus malate (3 mM) and KCl to give the specified concentration. Incubation was at 26° .

under our conditions the net gain depends on the external K^+ concentration but is relatively independent of the pretreatment.

Since counter movements of H^+ are usually found to accompany K^+ movements, as noted by the writers quoted in the introduction, it seemed likely that pH would affect the quantity and rate of K^+ movement. Sets of measurements were made at different pH values and over a range of K^+ concentrations. Fig. 3 compares results obtained in media buffered with 20 mM-tris chloride at three pH values. The media had measured concentrations of 0.05 mM-Mg^{2+} and $0.12 \text{ mM-inorganic phosphate}$, which had been carried in by the concentrated suspension. The results show that the spontaneous movement changes from a net efflux to a net influx as the K^+ concentration is raised, and the induced net uptake is greater, though the rate is less, at the lower pH. The combination of greater net uptake

Fig. 3. Rates of spontaneous and valinomycin-induced K⁺ uptakes and total K⁺ uptakes as functions of the external K⁺ concentration, which was maintained automatically; runs were carried out at (a) pH 6.35, (b) pH 6.95 and (c) pH 7.82. The medium contained: tris chloride (20 mM), sucrose (0.25 M), NaCl (2 mM) and tris glutamate (3 mM) plus malate (3 mM). Incubation was at 28°. ●, Spontaneous uptake rate (right-hand scales); ○, induced uptake rate (left-hand scales); ◐, total K⁺ uptake at its maximum (left-hand scales).

Fig. 4. Acceleration of the rate of K⁺ uptake after the addition of valinomycin (0.05 μg./ml.) in the presence of 1 mM-inorganic phosphate. Curve A, controls having endogenous phosphate at 0.06 mM; curve B, results obtained after supplementation with phosphate. Details of the medium were as given in Figs. 2 and 3. Incubation was at 29°.

Fig. 5. Uptakes of K⁺ as a function of the applied concentration from chloride and acetate media. Curve A, mixtures containing tris chloride, pH 7.3 (20 mM), and sucrose (0.25 M); curve B, mixtures containing tris acetate, pH 7.3 (20 mM), and sucrose (0.25 M). Incubation was at 28°.

with a lower rate points to a double action of valinomycin: an enhancement of K⁺-accumulating capacity is brought about by the acid conditions whereas the rate of K⁺ movement is less increased in acid than in alkaline conditions. One possible

Fig. 6. Rates of spontaneous and valinomycin-induced K⁺ uptakes and the total ion uptakes as functions of the external K⁺ concentration, which was maintained automatically; runs were carried out at (a) pH 6.36, (b) pH 7.0 and (c) pH 7.8. The media contained: tris acetate (20 mM), sucrose (0.25 M), NaCl (2 mM), tris glutamate (3 mM) plus malate (3 mM) and KCl to give the specified concentration. Incubation was at 26°. ●, Spontaneous uptake rate before valinomycin (0.05 μg./ml.) was added (right-hand scale); ○, induced uptake rate (left-hand scale); ◐, total K⁺ uptake at its maximum (left-hand scale).

explanation is that the ability to accumulate K⁺ depends on the presence of a labile intermediate of which the concentration can be permanently decreased if it is utilized at an excessive rate.

When inorganic phosphate is added to the chloride medium the uptake of K⁺ is accelerated (Pressman, 1965). This effect is illustrated in Fig. 4. There is, however, another effect when all but the lowest concentrations of K⁺ are present; the uptake stops sharply after ½–1 min. and a loss sets in. The change resembles that seen if the system becomes anoxic or when dinitrophenol is added. It presumably reflects the consequence of an excessive energy demand on the metabolic system.

If the chloride in the medium is replaced by acetate the net K⁺ uptakes from concentrations exceeding about 2 mM are more than those found in the chloride media (Fig. 5). Experiments at K⁺ concentrations up to 4.5 mM at three pH values were carried out in 20 mM-acetate medium (Fig. 6). Comparisons between Figs. 5 and 6 show that even within the restricted range of concentrations used the rates and quantities moved were as much as fourfold greater in the presence of acetate than in its absence. These observations pose the question why some anions favour K⁺ movement. There may be two distinct properties involved: one peculiar to phosphate (and perhaps arsenate) owing to cyclic participation of the anion in metabolism and the other a matter of provision of a permeable anion to

maintain electroneutrality. It is suggested that less energy is required to move K⁺ when acetate is provided than when the major anion is chloride. In the latter case it is probably the substrate anions that accompany that part of the K⁺ movement not balanced by ejection of H⁺. Gamble (1965) has demonstrated that malate penetrates mitochondria and that, when available, K⁺ enters at the same time. Experiments made with tracer chloride and acetate show that only the latter anion is accumulated along with K⁺ (B. C. Pressman, unpublished work).

It seemed possible that the undissociated form of a weak acid might penetrate as such and subsequently lose H⁺ in exchange for K⁺. However, a test of a series of acids having differing pK values and so providing various proportions of the un-ionized form showed no correlation between pK and K⁺ movement (Table 3). The comparison was made with ATP as energy source and with oxidation blocked with antimycin. Under oxidizing conditions both lactate and pyruvate will energize ion accumulation. Lactate added as the tris salt at 20 mM led to a net gain of 150 μequiv. of K⁺/g. of protein from a medium 4 mM with respect to K⁺.

When acetate is used as anion, its important property seems to be that large K⁺ uptakes can be supported without there being an excessive energy demand. In chloride media a raised K⁺ concentration, particularly when inorganic phosphate has

been added, leads to a short-lived rapid uptake followed by a net loss. Critical conditions can be established (Pressman, 1965) in which K^+ moves successively in and out in a series of damped oscillations.

A question arising when K^+ uptake is induced in a medium favouring spontaneous entry is the influence of the spontaneous movement on subsequent induced accumulation. More or less spontaneous uptake occurs in the minimum time necessary between adding the mitochondria and adding valinomycin. Table 4 suggests that the two uptakes are roughly additive. Other experiments, however, in which K^+ was not retained by the mitochondria after the induced uptake, showed that the time of addition would then alter the total achieved. The more the spontaneous uptake has been the slower is the induced uptake. This is to be expected if valinomycin increases permeability; the gross influx it causes will be opposed by a greater efflux from a particle having a raised K^+ content than from one with a low K^+ content.

However, mere increase of permeability will not account for a change from a negative or zero spontaneous uptake to a positive induced uptake.

Effects of ouabain and Mg^{2+} on spontaneous and induced uptakes of K^+ . The question whether the same basic mechanism and compartment is shared by the K^+ which enters spontaneously and that which enters after valinomycin addition can be examined by adding inhibitors or competitors.

Ouabain (strophanthin-G) is a well-known inhibitor of active transport at the cellular level; it also inhibits adenosine-triphosphatase activity. It is not particularly effective when applied to mitochondria, although an effect has been noted (Christie *et al.* 1965). We tested the substance at 0.5 mM; with an external K^+ concentration of 2.25 mM the spontaneous rate and the total induced uptake were decreased to 40 and 80% of the control values respectively, but the induced rate was not changed. If valinomycin opens additional pores it is unlikely that the competition between ouabain and K^+ would remain the same.

The presence of Mg^{2+} is required for the activation of many adenosine-triphosphatase systems, and Christie *et al.* (1965) state that it is a necessary cofactor for the ATP-energized uptake of K^+ by depleted mitochondria. We tested Mg^{2+} additions to the system energized with glutamate plus malate by starting at the endogenous concentration (0.06 mM) and making additions up to 9 mM. Fig. 7 shows that the spontaneous rate of K^+ uptake was unaffected over this range, whereas both the induced rate and the total uptake after the addition of valinomycin were decreased. This result shows that Mg^{2+} interferes with the change of permeability to K^+ caused by the antibiotic. Rasmussen, Fisher & Arnaud (1964) have noted that Mg^{2+} decreases K^+ turnover in both normal and parathyroid-hormone-treated mitochondria.

Addition of Ca^{2+} to the reaction mixture had a

Table 3. Comparison of K^+ uptakes from media containing different tris salts, pH 7.3 (10 mM)

Experimental details are given in the text. The process was energized by 2 mM-ATP; respiration was inhibited by 1 μ g. of antimycin/ml. The K^+ concentration was held automatically at 4 mM.

Anion	Induced K^+ uptake (μ equiv./g. of protein)	pK of acid at 25°
Acetate	155	4.75
Chloroacetate	155	2.89
Dichloroacetate	115	1.37
Pyruvate	155	2.49
Lactate	89	3.86

Table 4. Effect of allowing less or more spontaneous K^+ uptake to occur before adding valinomycin on the rate of induced uptake and on the total uptake of K^+ from a mixture with a K^+ concentration of 4 mM at 26°

Experimental details are given in the text.

Medium	Spontaneous rate (μ equiv./g./ min.)	Spontaneous uptake before adding valinomycin (μ equiv./g.)	Induced rate (μ equiv./g./ min.)	Total uptake (μ equiv./g.)
Chloride, pH 7.8	15	56	130	137
		5	150	112
Chloride, pH 7.8	19	68	110	167
		5	136	162
Acetate, pH 7.3	50	38	176	240
		122	148	266
		222	120	276

Fig. 7. Effect of Mg^{2+} concentration on the K^+ movements before and after the addition of valinomycin ($0.05 \mu g./ml.$). The medium contained tris chloride, pH 7.3 (20mM), and sucrose (0.25M). Incubation was at 26° . ●, Spontaneous uptake rate (right-hand scale); ○, induced uptake rate (left-hand scale); ◐, total K^+ uptake at its maximum (left-hand scale).

relatively greater inhibitory effect than Mg^{2+} . When $1.5 \mu moles$ of calcium salt were added to 10ml. of mixture containing 20mg. of mitochondrial protein the subsequent valinomycin-induced K^+ uptake was halved. With prior addition of $3 \mu moles$ of calcium salt the further addition of valinomycin led to K^+ loss at the rate of $18 \mu equiv./g.$ of protein/min. These tests were made on the same preparation as was used for the experiments plotted in Fig. 6. If calcium salt ($1.5 \mu moles$) was added to the reaction mixture after there had been a spontaneous uptake of K^+ from tris acetate mixture there was a release of K^+ ($0.45 \mu equiv.$) and H^+ ($0.8 \mu equiv.$). At the time of addition of Ca^{2+} an extra consumption of oxygen occurred (ratio of Ca^{2+} added to additional oxygen was 5.3) and the mitochondria swelled. When inorganic phosphate was added (final concn. 1mM) there was a brief shrinkage followed by swelling and K^+ release (Fig. 8).

Oxygen consumption and ratios for K^+ moved/oxygen used. If we are justified in deducing that one reason for an acceleration of K^+ uptake by valinomycin is an increased permeability there are some consequences that can be tested. To retain K^+ that has been accumulated the mitochondrion must continue to expend energy. It is therefore pertinent to examine the oxygen consumption and the state of oxidation of the nicotinamide nucleotides. The qualitative results for oxygen (Pressman, 1965) can be summarized as follows: there is little change in oxygen consumption associated with uptakes from low K^+ concentrations but as the

Fig. 8. Effect of the addition of Ca^{2+} to the suspension medium after there has been a spontaneous K^+ uptake by the mitochondria. The addition of $1.5 \mu moles$ of calcium salt causes the release of $0.45 \mu equiv.$ of K^+ and $0.8 \mu equiv.$ of H^+ from mitochondria containing 20mg. of protein in 10ml. of the sucrose-tris acetate (20mM) mixture, pH 7.4, made 1mM with respect to KCl. A, K^+ -electrode trace; B, H^+ -electrode trace; C, O_2 -electrode trace; D, light-scattering. The addition of Ca^{2+} leads to an increment in oxygen consumption (trace C) and decrease in light-scattering (trace D) interpreted as swelling. The further addition of inorganic phosphate (final concn. 1mM) leads after a brief delay to release of K^+ , increased oxygen consumption and a temporary shrinkage followed by swelling.

concentration is increased there is a marked stimulation of oxygen consumption associated with K^+ accumulation. Under conditions in which the K^+ is subsequently lost the rate of oxygen consumption falls as the K^+ content diminishes. Fig. 9 shows that with a given medium there is a correlation between the initial rate of K^+ influx and the increment in oxygen consumption above the basal level (which was $13\text{--}20 \mu moles/min./g.$ of protein). An important pointer to the reason for the difference between chloride and acetate as anions is the observation that for a given rate of uptake of K^+ less additional oxygen is required in acetate than in chloride media. In Fig. 9 the lines drawn correspond to movement of 10 and $24 K^+$ ions/molecule of oxygen, respectively.

During the course of the larger induced uptakes (several hundred $\mu equiv.$ of $K^+/g.$ of protein) from acetate media or smaller ($50\text{--}150 \mu equiv./g.$) uptakes from chloride media the oxygen rate increased. Hence it appears that a greater demand for energy is made and met up to some critical limit as the mitochondria acquire more K^+ . Confirmation of the greater energy flux, contingent on the maintenance of a high K^+ content, is provided by the

correlation between the diminished fluorescence and the induced K^+ uptake (Fig. 10). The fall in fluorescence is taken as an indication of the oxidation of reduced nicotinamide nucleotides. When there is a spontaneous K^+ uptake a slow loss of

Fig. 9. Collected results showing the relation between the initial rate of the K^+ uptake induced by valinomycin ($0.05 \mu\text{g./ml.}$) and the increment in oxygen consumption. $\circ$, Results obtained with the sucrose-tris chloride (20 mM) mixture, pH 7.4 (see the Methods section); $\bullet$, results obtained with sucrose-tris acetate (20 mM or 40 mM) mixtures, pH 7.4. Incubation was at 26° .

Fig. 10. Relation between the induced K^+ uptake and the diminution of fluorescence ($366\text{m}\mu$ excitation, $450\text{m}\mu$ measurement). The differently marked points are from different mitochondrial preparations. All results were obtained with sucrose-tris acetate media, pH 7.4 (see the Methods section). Incubation was at 26° .

fluorescence occurs instead of the brisk fall associated with the addition of valinomycin.

Adenosine-triphosphatase activity. Experiments were carried out in acetate medium (20 mM-tris acetate) with rotenone ($1 \mu\text{g./ml.}$) added to block oxidation of endogenous substrate and ATP (2 mM) added as energy source. The purpose of these was to complement the results for K^+ moved/oxygen used with values for K^+ moved/ATP split. Fig. 11 shows that as the external K^+ concentration was raised the adenosine-triphosphatase activity was stimulated, and particularly so after uptake had been increased by valinomycin. It is clear that one has in ADP production a basis for the observed stimulation of oxygen consumption in the oxidative

Fig. 11. Rate of production of inorganic phosphate from added ATP before ($\circ$) and after ($\bullet$) the addition of valinomycin ($0.05 \mu\text{g./ml.}$) as a function of K^+ concentration (left-hand scale). All results were obtained with sucrose-tris acetate (20 mM) mixture, pH 7.0. Incubation was at 26° . $\circ$, Initial rate of K^+ uptake (right-hand scale). Rotenone ($1 \mu\text{g./ml.}$) was added to block respiration. It is suggested that the increment in the rate of inorganic phosphate production (given by the value at $\bullet$ minus the value at $\circ$) is related to the rate of K^+ movement.

Fig. 12. Relation between K^+ and H^+ movements after the addition of valinomycin ($0.05 \mu\text{g./ml.}$) to a suspension that included ATP (2 mM) and rotenone ($1 \mu\text{g./ml.}$). Uptakes of K^+ are shown in the positive direction and are associated with outputs of H^+ . Results were obtained over a range of K^+ concentrations in sucrose-tris chloride (20 mM) mixture, pH 7.0 ($\bullet$), and in sucrose-tris acetate (20 mM) mixture, pH 7.0 ($\circ$). Incubation was at 26° .

system. If we are correct in relating the rate of K^+ uptake in the ATP-energized system to the increment in the rate of ATP hydrolysis the ratio K^+ moved/ATP split comes to between 4 and 6.

Release of H^+ . When sufficient K^+ moves either spontaneously or after the addition of valinomycin there is a contrary movement of H^+ (Moore & Pressman, 1964; Christie *et al.* 1965). Movements of H^+ and K^+ sometimes take place in equivalent amounts, but we do not find this to be so in general. To make this point results obtained under various conditions are presented.

The points in Fig. 12 relate the contrary movements of H^+ and K^+ occurring in a system energized with ATP and having respiration blocked with rotenone. In most experiments at least 2 K^+ ions are accumulated/ H^+ ion released. At the higher K^+ concentrations with valinomycin added there was a sustained stimulation of adenosine-triphosphatase activity that continued to produce H^+ after the fall in pH associated with the K^+ uptake. If a deduction of the H^+ equivalent (at a ratio 0.7 H^+ ion/ P_i molecule) to the inorganic phosphate liberated is applied the ratio between K^+ accumulated and H^+ liberated is brought up to between 7 and 10. The ratio is then in the range found with substrate oxidation as energy source.

The points in Fig. 13 relate movements of H^+ and K^+ in experiments with glutamate plus malate as energy source at three pH values in the chloride mixture. The ratio K^+ accumulated/ H^+ liberated is highest (about 10) in acid conditions and it falls to about 2 at pH 7.8.

From Fig. 3 the K^+ uptake at low pH is greater, though slower, than at higher pH. It may be inferred that a rapid K^+ - H^+ exchange is favoured by high pH but that the capacity of the exchange is limited. On the other hand, a slower movement of K^+ with an anion is favoured by low pH and can lead to a greater final accumulation.

Fig. 13. Relation between K^+ and H^+ movements after the addition of valinomycin ($0.05 \mu\text{g./ml.}$) to a suspension of mitochondria with glutamate plus malate as energy source in sucrose-tris chloride (20 mM) mixture (see the Methods section). Results were obtained over a range of K^+ concentrations at pH 7.8 (■), pH 7.0 (●) and pH 6.36 (○). Incubation was at 26° .

Volume changes associated with accumulation of K^+ . When the suspended mitochondria take up K^+ , either spontaneously or after the addition of valinomycin, they swell. Uptake from the low endogenous K^+ concentration is insufficient to lead to a significant volume change. The volume changes in these experiments were monitored by the light-scattering and there is some doubt about the correct way to deduce the volume from the readings. Qualitatively there is a correlation between the amount of K^+ that has moved and the decrease in scattered light received (Fig. 14).

Tracer experiments with $^{42}K^+$. The results so far presented show that valinomycin induces a rapid net movement of K^+ and that, depending on the conditions, the direction can be either in or out. It seemed worth testing with $^{42}K^+$ the inference that there is a permeability increase in addition to the stimulation of K^+ -accumulating capacity. The experiments were run in the measuring apparatus as before but with the addition of $^{42}K^+$ (after the valinomycin when that substance was added). At selected times a portion of the reaction mixture was withdrawn and either filtered through a Millipore pad that was then given two washes with ice-cold 0.25 M-sucrose, or added to a threefold excess of ice-cold sucrose and filtered through a Millipore pad without further washing. Both methods led to similar results and so they have not been distinguished in Fig. 15, which shows both the K^+ content and the fraction exchanged (for $^{42}K^+$) in a trichloroacetic acid extract of the filter pad. There are several sources of uncertainty in the procedure because, for example, the pad will retain

Fig. 14. Relation between the K^+ uptake and the percentage diminution in the light-scattering. Points are collected from a number of experiments made on different preparations. All results were obtained with tris acetate (20 mM) or tris chloride (20 mM) medium, pH 7.4. Incubation was at 26° . The K^+ uptakes were obtained either from the amount of the ion fed into the reaction vessel by the 'Stat' to maintain a constant concentration or from the K^+ -sensitive glass-electrode record.

Fig. 15. Uptake of $^{42}\text{K}^+$ by liver mitochondria without or with the addition of valinomycin ($0.05 \mu\text{g./ml.}$). Experiments were run either in the sucrose-tris acetate (20 mM) mixture, pH 7.4, with KCl (0.6 mM) added or in the sucrose-tris chloride (20 mM) mixture, pH 7.4, with KCl (1.2 mM) added (for other solutes see the Methods section). Incubation was at 26° . Samples were withdrawn for filtration at various times after adding the ^{42}KCl solution and the contents of $^{42}\text{K}^+$ and total K^+ were measured either after a wash on the filter or alternatively by dilution before the filtration. In the absence of valinomycin: $\circ$, K^+ exchange; $\bullet$, K^+ content. In the presence of valinomycin: $\circ$, K^+ exchange; $\bullet$, K^+ content. The results show that valinomycin leads to the $^{42}\text{K}^+$ content's being increased about fourfold.

Fig. 16. Uptake of $^{42}\text{K}^+$ by pigeon-heart mitochondria with and without the addition of valinomycin ($0.05 \mu\text{g./ml.}$). Experiments were run in the sucrose-tris chloride (20 mM) medium, pH 7.4 (see the Methods section), with K^+ concentrations between 0.12 mM and 0.46 mM. Incubation was at 26° . Samples were withdrawn for filtration at various intervals after the ^{42}KCl addition and the contents of $^{42}\text{K}^+$ and total K^+ were measured after washing on the filter. In the absence of valinomycin: $\circ$, K^+ exchange; $\bullet$, K^+ content. In the presence of valinomycin: $\circ$, K^+ exchange; $\bullet$, K^+ content. The results show that valinomycin leads to an increased exchange without a consistent effect on the total content of K^+ .

some $^{42}\text{K}^+$ and the apparent exchange will be too high on this account. It may be questioned how well the particles retain their K^+ , and especially that which has just been absorbed, when subjected to filtration. However, the pattern of the results obtained with liver mitochondria is clear. In the absence of added valinomycin the filtered particles (plus pad) have a fairly constant K^+ content and of this only a part is exchanged in the maximum possible time given. Exposure was limited by the rate of consumption of the dissolved oxygen. When valinomycin had been added both the total K^+ and the amount exchanged showed large increases. The $^{42}\text{K}^+$ was added after there had been an uptake of K^+ induced by valinomycin, so that the increased content of $^{42}\text{K}^+$ must represent the consequence of an accelerated turnover.

It was considered worth repeating the experiment with pigeon-heart mitochondria (see Fig. 16). Valinomycin caused little change in the K^+ content of the pad bearing these particles but it did lead to a marked increase of exchange. Only low external K^+ concentrations were used, which may explain

the absence of a net uptake. This observation is important because it shows that in some circumstances the effect of valinomycin is manifest only on the turnover of K^+ , so that use of the tracer method is essential.

In the work with liver mitochondria and $^{42}\text{K}^+$ we also tried some additions to a suspension in a chloride medium having 1.2 mM- K^+ . Dinitrophenol (5 μM) had little effect on either K^+ content or exchange. Inorganic phosphate (2.7 mM) also caused no significant effect; parathyroid hormone (1.68 mg./ml.) led to a doubling of the exchange without a significant increase of K^+ content. This last result agrees with an observation of Rasmussen *et al.* (1964); these authors stated that a net gain is only induced by the hormone from acetate media having more than 2 mM- K^+ .

According to Fig. 15 more than 100 $\mu\text{equiv.}$ of K^+ /g. of protein exchanges within 1 min. of adding valinomycin if the system contains either 1.2 mM- K^+ and 20 mM-chloride and 3 mM of each substrate or

0.6mM-K⁺ and 20mM-acetate at pH 7.3. The net uptake rates for these respective conditions (Figs. 3 and 6) were rather more than 100 μ equiv. of K⁺/g./min., so that there is reason to suppose that the initial value of the net rate after the addition of valinomycin is not far short of the true influx.

On the other hand, the exchange in the absence of valinomycin (20 μ equiv. of K⁺/g./min.; Fig. 12) must represent the consequence of turnover of a labile fraction. In common with Amoore (1960) we find that though some 40% of the K⁺ is exchanged within 1 min. this proportion does not then increase over 20 min. Amoore (1960) did obtain further exchange in 40 min. and still more in 60 min., but at these times there had been appreciable losses of K⁺. It is important to maintain constant conditions during exposure to the tracer ⁴²K⁺. A factor that has to be watched is the dissolved oxygen concentration; we found that long exposures (more than 15 min.) were often associated with decreased K⁺ contents and suppose that the suspension became anoxic during filtration.

DISCUSSION

These results show that metabolizing mitochondria will take up K⁺ and water from media with K⁺ concentrations less than 1 mM even without a preliminary treatment at 37°. It is of course questionable how much K⁺ has been lost during the preliminary isolation in sucrose and it may well be that the spontaneous uptake we observe represents a return towards the composition the particles had before isolation. Under favourable conditions such as are provided by high (40 mM) acetate concentration and 10 mM-K⁺ an uptake of 380 μ equiv. of K⁺/g. of protein at an initial rate of 100 μ equiv./g./min. can be obtained. Valinomycin increases both these quantities: an uptake of 800 μ equiv. of K⁺/g. with initial rate 1000 μ equiv./g./min. has been measured. A parallel effect on K⁺ has been described by Rasmussen, Fischer & Arnaud (1964), who induced K⁺ uptakes with parathyroid hormone from media containing more than 2 mM-K⁺ in the presence of acetate. Our results show that uptakes are less in chloride media than in acetate; Rasmussen, Chance & Ogata (1965) obtained no uptake in the absence of acetate. It seems probable that the possibility of uptake depends on the presence of a penetrating anion. Gamble (1963) observed that there was little uptake of ³⁶Cl⁻ in 15 min., amounting to an exchange of about one-quarter of the Cl⁻ content; on the other hand, Gamble (1965) has also shown that malate (one of our substrate anions) penetrates. The uptakes from the chloride mixture we observed may have been largely of the potassium salts of the substrate anions (and phosphate when this anion was added) rather than of potassium

chloride. Chappell & Crofts (1965) found that phosphate was essential for the induction by gramicidin of swelling and extra respiration in media containing alkali-metal salts.

The extra oxygen consumption associated with K⁺ accumulation is less when acetate is present than in its absence (Fig. 9) and this supports the idea that the energy demand reflects the ease of movement of the ions making up a potassium salt. A rapid rate of movement is associated with a high energy demand as shown by the oxygen rate or, in an ATP-energized system, by the adenosine-triphosphatase activity (Fig. 11). Where a high rate is induced in the absence of acetate the supply of energy may fall short of the demand. If some critical limit is exceeded the forces leading to K⁺ accumulation vanish and the accumulated ions are released. With carefully chosen concentrations several successive cycles of uptake and loss can occur (Pressman, 1965). The loss to which excessive K⁺ accumulation can give place presumably has a similar origin to the losses of K⁺ brought about by the addition of dinitrophenol or by anoxia; some vital intermediate is lacking. Following Moore & Pressman (1964) and in agreement with other authors cited in the introduction, we conclude from the lack of inhibition of respiration-coupled K⁺ uptake by oligomycin that the intermediate that supplies energy for accumulation is not phosphorylated.

A volume increase is associated with K⁺ uptake; this has also been noted by Rasmussen *et al.* (1964) in relation to parathyroid-hormone-induced K⁺ movement and by Chappell & Crofts (1965) for gramicidin-induced cation uptakes. The interrelation leads to the question at what concentration is K⁺ accumulated. It is necessary here to distinguish between movements into particles in suspension and those residual changes that can still be measured after a separation procedure. We were not able to isolate material with a K⁺ content exceeding about 170 μ equiv./g. of protein and suppose that the highly swollen K⁺-rich particles are extremely labile.

Fig. 6 shows that the scattered light is decreased to about half when the K⁺ uptake is 300 μ equiv./g. The problem is to relate the light-signal to the volume of the particles in the suspension. Tedeschi & Harris (1955) give graphical relations between the inverse of the extinction and the change in osmotically active volume. Tedeschi & Harris (1958) also state that the light scattered varies as the extinction. With these relations it appears that halving of the extinction corresponds to a three-fold increase of osmotically active volume. Values for the water associated with 1 g. dry wt. of matter in presumably unswollen particles are 2.2–2.3 ml./g. (Fonnesu & Davies, 1956; Christie *et al.* 1965).

If an initial 2.2 ml. of water increases to 6.6 ml. when the scattered light falls to half then a total of 600 μ osmoles of the ions making up the potassium salt will be associated with 4.4 ml. of water. This makes the osmolarity of the additional volume 140 m-osmolar in comparison with 250 m-osmolar sucrose (plus the 20 mm-tris salt) in the medium. It is accordingly not possible with these values to support the concept of purely osmotic behaviour. A number of authors have, in fact, shown that metabolism plays an important role in determining the volume (e.g. Price, Fonnesu & Davies, 1956; Disabato & Fonnesu, 1959). The response of the light-scattering, and presumably of the volume, to small quantities of Ca^{2+} and phosphate also speaks against a purely osmotic behaviour.

It is usual when presenting data that show a saturation trend like those indicated in Figs. 2, 3, 5 and 6 to seek to relate the variable by means of a double-reciprocal plot to an absorption isotherm, i.e. to obtain a Michaelis constant. The net gain of K^+ according to the present and earlier results is the consequence of a disparity between an influx and an efflux. The influx indeed may reasonably be determined by a function of the external concentration but it is more likely that the efflux depends primarily on the internal concentration. There is then no justification for applying the double-reciprocal plot to the net movements found with various K^+ concentrations. One may, however, try using the initial rates of uptake so long as the efflux initially may be neglected in comparison with the influx. When this procedure is applied to the induced rates of Fig. 3, K_m values are found to fall as pH is raised: at pH 6.36, K_m 3.2 mm; at pH 7.0, K_m 1.6 mm; at pH 7.8, K_m 0.64 mm. Similar treatment of the results obtained in acetate media (Fig. 6) indicated that the maximum K_m occurred nearer neutrality; the values were: at pH 6.36, K_m 1.3 mm; at pH 6.95, K_m 3.1 mm; at pH 7.8, K_m 0.9 mm.

The dependence of the rate of K^+ movement (or rate of swelling) on the external K^+ concentration differs according to the anion in the system, as noted by Azzi & Azzone (1965). It also depends on the energy source. The low values just noted for the glutamate-plus-malate substrate give place to higher concentrations when movements are energized by ATP in the presence of rotenone; at pH 7.0, for example, K_m was close to 10 mm.

One can seek to relate the proportion of mitochondria in various cells to the rapidity of ion movement in the whole cell. About 20% of the

weight of liver can be recovered as mitochondria; liver slices reaccumulate K^+ from a solution 10 mm with respect to K^+ at about 10 μ equiv./g./min. (Judah & Ahmed, 1962), which is about one-tenth the rate obtained with mitochondria in acetate solution.

The mechanism of K^+ accumulation remains obscure. H^+ - K^+ exchange can account for those movements that persist after separation or filtration. This process presumably differs in origin from the movements of K^+ and water that proceed between the suspended mitochondria and the medium.

This investigation was supported by U.S. Public Health Service Research Grant GM 12202. Individuals were supported by a Wellcome Travel Grant (E. J. H.), U.S.P.H.S. Training Grant ST 1-GM-694-05 (R. C.) and U.S.P.H.S. Research Career Program Award (B. C. P.).

REFERENCES

- Amoore, J. E. (1960). *Biochem. J.* **76**, 438.
 Azzi, A. & Azzone, G. F. (1965). *Biochem. J.* **96**, 1c.
 Bartley, W. & Davies, R. E. (1954). *Biochem. J.* **57**, 37.
 Chappell, J. B. & Crofts, R. C. (1965). *Biochem. J.* **95**, 393.
 Christie, G. S., Ahmed, K., McLean, A. & Judah, J. D. (1965). *Biochim. biophys. Acta*, **94**, 425.
 Disabato, G. & Fonnesu, A. (1959). *Biochim. biophys. Acta*, **35**, 358.
 Fonnesu, A. & Davies, R. E. (1956). *Biochem. J.* **64**, 769.
 Gamble, J. L. (1957). *J. biol. Chem.* **228**, 955.
 Gamble, J. L. (1962). *Amer. J. Physiol.* **203**, 886.
 Gamble, J. L. (1963). *Biochim. biophys. Acta*, **66**, 158.
 Gamble, J. L. (1965). *J. biol. Chem.* **240**, 2668.
 Judah, J. D. & Ahmed, K. (1962). *Biochim. biophys. Acta*, **57**, 245.
 Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 450. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Moore, C. & Pressman, B. C. (1964). *Biochem. biophys. Res. Commun.* **15**, 562.
 Pressman, B. C. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1076.
 Price, C. A., Fonnesu, A. & Davies, R. E. (1956). *Biochem. J.* **64**, 754.
 Rasmussen, H., Chance, B. & Ogata, E. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1069.
 Rasmussen, H., Fischer, J. & Arnaud, C. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 1198.
 Rottenberg, H. & Solomon, A. K. (1965). *Biochem. biophys. Res. Commun.* **20**, 85.
 Share, L. (1958). *Amer. J. Physiol.* **194**, 47.
 Tedeschi, H. & Harris, D. L. (1955). *Arch. Biochem. Biophys.* **58**, 52.
 Tedeschi, H. & Harris, D. L. (1958). *Biochim. biophys. Acta*, **28**, 392.