FURTHER STUDIES ON THE POTASSIUM REQUIREMENTS OF MITOCHONDRIA*

by

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It has been previously reported[1,2] that the presence of the microsomal fraction or an acetone extract thereof greatly enhances the response of respiring mitochondria to added K^+. Identification of the active components of the extract as free fatty acids will be the subject of a subsequent paper. It is proposed to consider here further aspects of the induced sensitivity to K^+, particularly with regard to the processes of oxidative phosphorylation.

The rate-limiting reactions in the basic systems commonly employed in studying the respiration of mitochondria are associated with the obligatory esterification of inorganic phosphate. Circumventing these rate-limiting reactions enhances mitochondrial respiration to a level which is limited by other reactions, presumably more closely related to the oxidative processes themselves, and, in theory, independent of the means used to effect this circumvention[3,4,5]. The fact that the agents which enhance the sensitivity of mitochondria to K^+ also stimulate mitochondrial respiration suggested studying this relationship with a variety of agents differing in their detailed mechanism of action. The respiration-stimulating substances studied include the glucose-hexokinase system, dinitrophenol, microsomes, and fatty acids. For comparison with the fatty acids, deoxycholic acid has also been used.

METHODS

Particulate fractions were prepared from rat liver in 0.25 *M* sucrose according to the procedure of SCHNEIDER[6]. The acetone extract of the microsome fraction (A_p)*** was prepared as described elsewhere[1]. ATP was obtained from the Pabst Laboratories (Milwaukee) as the disodium salt, cytochrome *c* from the Sigma Chemical Company (St. Louis), and the α-ketoglutaric acid was a commercial product recrystallized from acetone-benzene before use.

Oxygen consumption was measured by the conventional Warburg technique. Flasks routinely contained 10^{-5} *M* cytochrome *c*, 0.0017 *M* ATP, 0.005 *M* $MgCl_2$, 0.01 *M* α-ketoglutarate and 0.0133 *M* phosphate. The ATP, α-ketoglutarate and phosphate were adjusted to pH 7.4 with NaOH. When indicated, alkali cations were added in the form of the chloride at a concentration of 0.025 *M*. Flask contents were brought to a final volume of 3.0 ml with 0.25 *M* sucrose resulting

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*** The following abbreviations have been used: M_{w3}, thrice washed mitochondria; P_w, washed microsomes; A_p, a water emulsion of an acetone extract of P_w; DNP, 2.4-dinitrophenol; DCA, deoxycholic acid; ATP, adenosine triphosphate.

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in a final sucrose concentration of 0.18 *M*. The gas phase was air, the temperature 30° and NaOH was present in the center well.

Inorganic phosphorus was determined by the method of LOWRY AND LOPEZ[7] and nitrogen analyses were made by a direct nesslerization procedure. Phosphorylation efficiencies, *i.e.*, P:O ratios, were determined by the method described by LARDY AND WELLMAN[5].

RESULTS

Dinitrophenol (DNP) at levels usually found sufficient to produce optimal respiratory stimulation ($3 \cdot 10^{-5}$ *M*) did not significantly enhance the response of mitochondria to added K^+. In Table I a significant enhancement of K^+ sensitivity over that of the control appears only after the third hour. This effect could be ascribed to better maintenance of respiration in the presence of K^+. The figures for per cent stimulation by K^+ are based on a comparison between equi-osmolar systems wherein 0.025 *M* NaCl was replaced by 0.025 *M* KCl. The influence of simple substitution of one ionic species for another, however, fails to establish whether the resultant effects are due to the removal of one species or the addition of the other. On the other hand, substitution of an ionic species by an osmolar equivalent of sucrose does not necessarily leave unaltered those properties of mitochondria sensitive to the osmolarity of the medium[8]. Since respiration was not affected when the 0.025 *M* NaCl was replaced by the osmi-equivalent 0.040 *M* sucrose, it can be concluded that the effects of KCl addition truly represent a stimulation specific for the K^+ ion.

TABLE I

EFFECT OF DPN ON THE RESPONSE OF MITOCHONDRIA TO K^+

Additions to basal media	Q_{O_2} (*N*)								
	Control				$3.3 \cdot 10^{-5}$ *M DNP*				
	hour				*hour*				
	1st	*2nd*	*3rd*	*4th*	*½*	*2nd ½*	*2nd*	*3rd*	*4th*
Sucrose (0.18 *M*)	103	113	92	83	250	161	108	71	43
NaCl (0.025 *M*) + Sucrose (0.14 *M*)	90	112	94	90	285	175	110	70	41
KCl (0.025 *M*) + Sucrose (0.14 *M*)	99	134	112	81	327	212	116	113	115
Per cent stimulation of respiration by K^+	10	20	19	—10	15	21	16	61	180

1.3 mg Mitochondrial nitrogen were present in each flask. The substrate was 0.01 *M* α-ketoglutarate. The constituents of the virtually K^+-free media are described under METHODS.

Another aspect of the effects of DNP is brought out in Table II. A concentration of $3 \cdot 10^{-5}$ *M* DNP again failed to induce a K^+ effect on respiration during the first hour. However, when the DNP was lowered to $6.6 \cdot 10^{-6}$ *M*, inducing only a submaximal stimulation of respiration, an immediate effect of K^+ was obtained.

The detergent DCA was found to produce a similar pattern of results. Table II, Expt. C indicates K^+ effects at a level of 0.1 mg/flask ($8 \cdot 10^{-5}$ *M*) which disappeared when the concentration was increased to 0.3 mg/flask. The actual differences in responses to the two levels of DCA were initially much greater than would be indicated from the table since there was a rapid progressive falling off of respiration at the higher level. During the first ten minutes 0.3 mg DCA produced virtually the same

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TABLE II

EFFECTS OF VARIOUS AGENTS ON THE K^+ SENSITIVITY OF MITOCHONDRIA

Expt.	Added agent	Added cation	1st half-hour Q_{O_2} (N)	1st half-hour Per cent stimulation by K	2nd half-hour Q_{O_2} (N)	2nd half-hour Per cent stimulation by K
A	None	Na^+	74		64	
		K^+	72	−3	70	9
B	DNP, $6.6 \cdot 10^{-6}$ M	Na^+	156		114	
		K^+	180	15	148	30
	DNP, $3.3 \cdot 10^{-5}$ M	Na^+	250		138	
		K^+	248	−1	146	6
C	DCA, 0.1 mg	Na^+	128		106	
		K^+	160	25	172	62
	DCA, 0.3 mg	Na^+	172		64	
		K^+	174	1	68	6
D	P_W, 0.3 mg N	Na^+	172		116	
		K^+	168	−2	128	10
	P_W, 0.9 mg N	Na^+	204		70	
		K^+	296	45	241	244
E	A_P, 0.3 mg dry wt	Na^+	118		56	
		K^+	100	−15	58	4
	A_P, 0.6 mg dry wt	Na^+	85		48	
		K^+	235	176	112	134

0.8 mg Mitochondrial nitrogen were present in each flask. The indicated cations were added as chlorides at a final concentration of 0.025 M.

TABLE III

EFFECTS OF VARYING LEVELS OF ACETONE EXTRACT OF MICROSOMES ON OXIDATIVE PHOSPHORYLATION AND K^+ SENSITIVITY OF MITOCHONDRIA

A. Phosphorylation efficiency

Acetone extract of microsomes	0.0 mg		0.2 mg		0.4 mg		0.6 mg		$3 \cdot 10^{-5}$ M DNP : K^+
Added cation (0.025 M)	Na^+	K^+	Na^+	K^+	Na^+	K^+	Na^+	K^+	
μ Liters O_2:									
0–10 minutes	23	22	42	42	66	65	58	88	97
20–30 minutes	22	24	30	31	34	42	25	55	56
30–40 minutes (after addition of hexokinase)	49	74	45	72	47	81	59	79	
Net P uptake (μ moles)	14	21	14	20	10	20	4	19	
P:O	2.7	2.8	2.7	2.5	2.0	2.4	0.7	2.3	

B. Per cent K^+ stimulation of respiration

0–10 minutes	−5	0	1	52
20–30 minutes	9	3	24	120
30–40 minutes (after addition of hexokinase)	51	60	66	34

stimulation of respiration as $3 \cdot 10^{-5}$ *M* DNP, double that produced by 0.1 mg DCA, without bringing about any marked K^+ responses.

When the stimulation of mitochondrial respiration was induced by P_w or A_p, the response to added K^+ appeared to increase progressively even to the point of maximal stimulation of respiration. In Table II, Expts. D and E, low levels of these agents inducing suboptimal stimulation of mitochondrial respiration effected no increased K^+ sensitivity. When these agents were employed at higher concentrations capable of bringing about maximal respiratory rates, striking responses to added K^+ were elicited.

This point is illustrated in greater detail in Table III-A for the case of the A_p. An immediate respiratory response was obtained at all levels of A_p employed but an initial response to K^+ appeared only at the highest level of A_p tested. After twenty minutes the response to K^+ was further increased and even appeared at the next lowest level of A_p.

At the end of thirty minutes, phosphorylation efficiencies were assayed by tipping into the vessels the hexokinase-glucose phosphate acceptor system. The respiration of the subsequent ten minute interval became K^+ sensitive in all cases, even in the complete absence of added A_r. This effect, which can only be ascribed to the hexokinase-glucose system itself, constitutes an independent means of inducing a K^+ sensitivity in mitochondrial respiration.

The principal objective of the above experiment was to ascertain whether the phosphorylation processes accompanying mitochondrial respiration are the seat of the K^+ sensitive reactions. The respiratory records obtained before tipping in the hexokinase-glucose system allowed some appraisal to be made of the influence of K^+ and A_p before they could be obscured by the effects accompanying the addition of the phosphate acceptor system.

In the absence of added A_p, K^+ increases both respiration and phosphorylation in such proportions that the P:O ratio remains unchanged. At the level of 0.4 mg A_p, a slight depression of the P:O ratio appeared in the absence of K^+. This depression was much greater when the level of A_p reached 0.6 mg and is largely reversed by added K^+. It appears that added K^+ is definitely required for the preservation of the reactions involved in the phosphorylation processes. Attention should be called to the fact that even in the absence of other agents, added K^+ is required for maximal stimulation of respiration by the hexokinase-glucose system.

TABLE IV

EFFECTS OF MICROSOMAL PARTICLES AND OF ACETONE EXTRACT THEREFROM ON OXIDATIVE PHOSPHORYLATION AND K^+ SENSITIVITY OF MITOCHONDRIA

Expt.	*mg Mitochondrial N*	*Additions*	*Added cation*		*Per cent stimulation of respiration by K+ before addition of hexokinase*
			Na+	*K+*	
			P:O	*P:O*	
A	1.25	None	3.4	3.7	
		P_w, 1.25 mg N	2.7	2.7	100
B	1.2	None	2.6	3.3	
		A_p, 0.8 mg dry wt	0.0	1.8	150
C	1.0	None	3.0	2.6	
		A_p, 0.6 mg dry wt	1.6	2.0	100

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Data of several other experiments with both P_w and A_p preparations have been compiled in Table IV. These experiments also demonstrate that free fatty acids obtained from microsomes can cause oxidative phosphorylation to become dependent on added K^+. In one instance, experiment B, net esterification of inorganic phosphate exhibited an absolute requirement for added K^+.

DISCUSSION

Either the loss of bound K^+ initially available for those enzymic processes dependent on it, or changes in the intrinsic K^+ requirements could be considered sufficient to induce the mitochondria to respond to added K^+ by increasing the respiratory rate or the phosphorylating efficiency. Experiments conducted in this laboratory[9] and elsewhere[10, 11] have shown that several of the agents here employed are capable of causing mitochondria to release bound K^+. But unlike DNP and DCA which act directly on the mitochondria it seems less likely that the hexokinase-glucose system could effect a similar release of mitochondrially bound K^+ corresponding to the instantaneous induction of K^+ sensitivity. Conversely the higher levels of DCA and DNP, which bring about an even greater release of bound K^+, fail to produce an immediate K^+ effect. Thus, although the liberation of bound K^+ undoubtedly plays a role in the induction of K^+ sensitivity by DCA, DNP, the A_p extract, and prolonged incubation[1], it appears to be neither a requirement nor a guarantee for obtaining a response to K^+ under all conditions.

A conclusion which is consistent with all the facts assembled here is that the K^+ sensitivity resides in reactions closely allied to the esterification of inorganic phosphate. In the absence of agents which accelerate the esterification of phosphate beyond that necessary to maintain the slow rate of turnover occurring in the basic system, the quantity of K^+ initially bound to the mitochondria is sufficient to meet the demand of the processes of phosphorylation. When the rate of phosphate esterification is elevated by addition of either a phosphate acceptor system or agents which accelerate the turnover of esterified phosphate, the bound K^+ no longer satisfies the mitochondrial requirements and the availability of K^+ then becomes the rate-limiting factor for the over-all process of coupled oxidative phosphorylation. The disappearance of the K^+ effects at the higher levels of DCA and DNP supports the contention that the primary point of K^+ sensitivity is not directly associated with the processes of oxidation. The most probable explanation for this is that these agents effect a complete divorcement of phosphorylation from the oxidative processes, with which they are normally associated.

The known necessity of Mg^{++}[12] and the evidence furnished here relative to K^+ requirements for mitochondrial phosphorylation reactions are not without precedent amongst the requirements known for enzymic phosphorylations[13]. The enzyme pyruvic phosphopherase, originally demonstrated to exhibit a K^+ requirement for full activity[14], has now been demonstrated to have an absolute requirement for K^+[15]. Fructokinase of rat liver has been shown to be stimulated by K^+ as well as Mg^{++}[16] and a purified preparation of this enzyme from beef liver[17], has been found by Dr. ROBERT PARKS (unpublished) to have an absolute requirement for these ions. The K^+ activation of acetylation reactions[18] is explainable in terms of the K^+ requirement[19, 20] of each of the two known pathways for acetate activation[21] by ATP.

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SUMMARY

Added K^+ is necessary for maximum rates of respiration and phosphorylation by rat liver mitochondria when a phosphate accepting system such as glucose and hexokinase is present. K^+ is also necessary for maximum rates of respiration and phosphorylation when microsomes, or an acetone-soluble fraction therefrom, are present.

When respiration is maximally stimulated by 2,4-dinitrophenol, added K^+ does not enhance respiration except that in the 3rd and 4th hour of the experiment it prevents deterioration of the mitochondria.

It is concluded that K^+ participates in transphosphorylation reactions but does not *directly* influence the oxidative reactions.

RÉSUMÉ

Il est nécessaire d'ajouter des ions K^+ aux mitochondries de foie de rat pour obtenir les vitesses maximum de respiration et de phosphorylation, lorsque un système accepteur des phosphates, tel que glucose et hexokinase, est présent. K^+ est également nécessaire à l'obtention des vitesses maximum de respiration et de phosphorylation quand des microsomes, ou une fraction des microsomes soluble dans l'acétone, sont présents.

Quand la respiration est stimulée au maximum par le 2,4-dinitrophénol, l'addition d'ions K^+ n'accélère pas la respiration, mais dans les 3ème et 4ème heures d'expérience elle prévient la dégradation des mitochondries.

Les auteurs concluent que K^+ participe aux réactions de transphosphorylations mais n'a pas d'influence *directe* sur les réactions oxydatives.

ZUSAMMENFASSUNG

Hinzugefügtes K^+ ist für maximale Atmungs- und Phosphorylierungsgeschwindigkeiten der Rattenlebermitochondrien notwendig, falls ein Phosphat aufnehmendes System, wie Glukose und Hexokinase, gegenwärtig ist. K^+ ist gleichfalls für höchste Atmungs- und Phosphorylierungsgeschwindigkeiten notwendig, wenn Mikrosomen oder deren azetonlösliche Fraktion gegenwärtig sind.

Wenn die Atmung durch 2,4-Dinitrophenol auf ein Höchstmass gesteigert wurde, entsteht durch hinzugefügtes K^+ keine weitere Steigerung; es wird jedoch dadurch während der dritten und vierten Stunde des Versuches die Zerstörung der Mitochondrien verhindert.

Es wird daraus gefolgert, dass K^+ in Transphosphorylierungsreaktionen teilnimmt, ohne auf oxydative Reaktionen einen *unmittelbaren* Einfluss auszuüben.

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