Biochem. 7, 472.
Mauro, A. (1962), *Biophys. J. 2*, 180.
Mitchell, P. (1961), *Nature 191*, 144.
Mitchell, P. (1966), *in* Regulation of Mitochondrial Metabolism, Tager, J. M., Papa S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 65.
Moore, C., and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562.
Müller, P., and Rudin, D. (1967), *Biochem. Biophys. Res. Commun. 26*, 398.
Mustafa, M. G., Utsumi, K., and Packer, L. E. (1966), *Biochem. Biophys. Res. Commun. 24*, 381.
Pardee, A. B., and Potter, V. R. (1948), *J. Biol. Chem. 176*, 1085.
Pressman, B. C. (1965a), *Proc. Natl. Acad. Sci. U. S. 53*, 1076.
Pressman, B. C. (1965b), *Federation Proc. 24*, 425.
Pressman, B. C. (1967), *Methods Enzymol. 10*, 714.
Racker, E. (1965), Bioenergetics, New York, N. Y., Academic.
Rasmussen, H., Chance, B., and Ogata, E. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1069.
Rasmussen, H., and Ogata, E. (1966), *Biochemistry 5*, 733.
Schneider, W. S. (1948), *J. Biol. Chem. 176*, 259.
Szent-Györgyi, A. (1957), Bioenergetics, New York, N. Y., Academic.
Tyler, D. (1955), *J. Biol. Chem. 216*, 395.
Wenner, C. (1965), *Federation Proc. 24*, 545.
Williams, G. R. (1960), *J. Biol. Chem. 235*, 1192.
Wiskich, J. T., and Bonner, W. D. (1963), *Plant Physiol. 38*, 594.

Effect of Transport-Inducing Antibiotics and Other Agents on Potassium Flux in Mitochondria*

Eric J. Harris,† Graham Catlin, and Berton C. Pressman‡

ABSTRACT: A filtration technique which gives reliable results over a wide range of K^+ fluxes is described for measurement of the uptake of ^{42}K by mitochondria. It has been employed to determine the excess of gross over net influx of K^+ into rat liver mitochondria, as well as the dependence of gross transport on pH and on the concentrations of K^+ and transport-inducing antibiotics such as valinomycin. The steady-state flux of K^+ is also mildly stimulated by EDTA, purified histone, and parathyroid hormone, but not by albumin. On a molar basis the stimulatory activity of parathyroid hormone is four orders of magnitude lower than that of valinomycin or gramicidin. K^+ influx is inhibited when mitochondrial energy production is stopped by rotenone or antimycin, or when the energy is diverted away from transport for the support of oxidative phosphorylation, or dissipated by uncoupling with dinitrophenol or oleate. The concentration at which K^+ accumulates within the mitochondria in the presence of valinomycin is estimated to be 60–80 mM by three independent procedures. This is markedly lower than the osmotic equivalent of the reaction media (170 mM).

The experimental results have been discussed with regard to the mechanism and energetics of mitochondrial ion transport.

Our investigations of the rapid uptake of monovalent ions by mitochondria, which is induced by valinomycin and other antibiotics (Pressman, 1965), have focused attention on the gross K^+ flux across the mitochondrial membrane under various conditions of net K^+ influx. The following are specific questions which have arisen. (1) To what extent are intrinsic changes in the permeability of the mitochondrial membrane implicated in the mechanism of action of the various agents which influence mitochondrial ion transport, *e.g.*, the transport-inducing antibiotics, uncoupling agents, etc.? (2) When the maximal entry of induced uptake of K^+ has been achieved, *i.e.*, the *rate* of uptake falls to zero, does this represent a balance between influx and efflux, or does the transport process halt? (3) Would a dynamic steady state of ion flux, with an energy-requiring influx and passive exergonic efflux, account for the dissipation of energy manifested as an increased respiratory or ATPase activity? (4) Do certain anions which increase the

* From the Department of Biophysics and Physical Biochemistry, Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania. *Received December 16, 1966.* This investigation was supported by a U. S. Public Health Service grant (GM 12202) from the Institute of General Medical Sciences, U. S. Public Health Service.

† Permanent address: Department of Biophysics, University College, London, England.

‡ Recipient of a U. S. Public Health career development award (K3-GM-3626).

rate of K^+ movement into mitochondria, such as acetate or P_i, operate *via* a direct effect on membrane permeability, or by providing companion ions for the transported K^+, thereby holding down the buildup of a diffusion potential opposing ion movement? These questions can be best resolved by tracer methodology.

Previous studies of the uptake of ^{42}K by mitochondria have utilized centrifugal techniques for separating mitochondria from an isotope-containing reaction medium (Amoore and Bartley, 1958; Gamble, 1962). We have found that centrifugation can cause drastic changes in the K^+ content of mitochondria, particularly after treatment with valinomycin. More recently filtration on Celite-covered planchets (Ogata and Rasmussen, 1966) or Millipore filters has been employed (Harris *et al.*, 1966) to effect a rapid separation of the mitochondria from the reaction medium. However, under these conditions we also observe some release of bound constituents from the mitochondria. In the present study we have used an adaptation of the filtration method, which avoids washing the filtered mitochondria so that any ^{42}K released by the mitochondria while they are being sucked dry remains trapped in the filter pad and is eventually counted along with the activity remaining within the mitochondria.

Methods

Tracer measurements were carried out by removing 1-ml samples of the incubation mixture (specified in the respective figure and table legends), which were rapidly mixed with 4.0 ml of ice-cold 0.25 M sucrose in a test tube and immediately poured on a moist Millipore filter (45-mm diameter; 0.45-μ pore size) mounted on a suction flask under vacuum. The standard fitting above the fritted disk supporting the filter was cut down to a height of 2 cm to facilitate the pouring operation. The dilution with cold sucrose before filtration held down the background ^{42}K contributed by the medium remaining in the moist Millipore filter. Attempts to reduce this background further by washing the filter with fresh sucrose led to inconsistent results, particularly when valinomycin was present, due to the release of intramitochondrial ^{42}K. This might be ascribed to the low oxygen tension and low residual substrate concentrations within the filtered mitochondrial pad which lead to a failure of the energy supply, thereby permitting the reversal of the K^+ movement. The mechanical pressure forces developed during filtration could also have contributed to the loss of ^{42}K. The time required for the filtration operation was critically dependent upon the mitochondrial concentration. When the mitochondria were treated with valinomycin, the time required for draining the filter was about 5 sec when the level in the medium was about 2.5 mg of mitochondrial protein/ml. For the slower ^{42}K incorporation in the absence of valinomycin, longer filtration times could be tolerated, and the mitochondrial protein level was increased to about 5

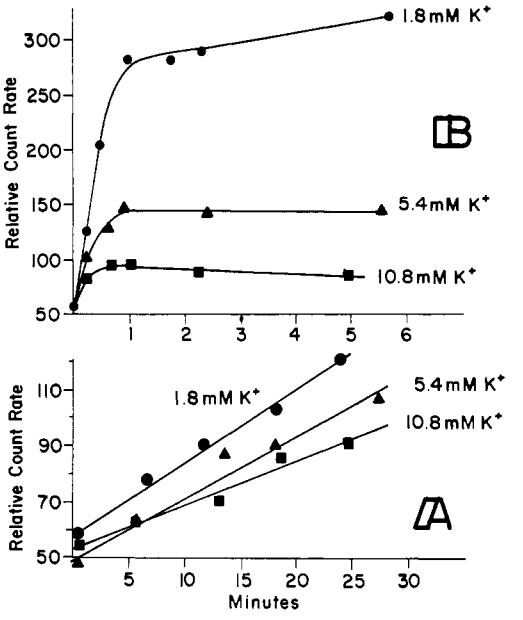

FIGURE 1: Total ^{42}K recovered from Millipore filters after filtration of mitochondria. The reaction medium contained Tris-glutamate (3 mM), Tris-malate (3 mM), Tris-Cl (20 mM), and sucrose (250 mM) at pH 7.2, temperature 10°, and final volume 14 ml. Mitochondrial protein was 5 mg/ml for series A (valinomycin absent) and 2.5 mg/ml for series B (30 μg of valinomycin/g of protein).

mg/ml. After removal of the drained filters from their mounts, they were extracted by macerating with 10 ml of 1% nitric acid, and the extract was clarified by centrifugation. The ^{42}K was measured by transference of aliquots to scintillation vials and counting of the ^{42}K-induced Cerenkov radiation (Haberer, 1965). The total K^+ present in the extracts was subsequently assayed by atomic absorption spectrometry (Technicon Model AA3) following a delay of several days to permit radioactive decay.

The considerable ^{42}K from the medium absorbed in the moist drained filter could not be determined by simple weighing of the Millipore filter, since part of the water held (about 30%) appeared to be bound and did not equilibrate with the ^{42}K in the medium. It was corrected for by taking a series of samples from a control experiment under conditions of slow ^{42}K incorporation and extrapolating the results back to zero time to obtain a blank. The validity of this operation is demonstrated in Figure 1. Identical amounts of ^{42}K were added to each experimental series, and the levels of KCl added were varied as indicated for each curve. In series A, illustrating the relatively slow spontaneous K^+ movements at all levels of K^+, ^{42}K uptakes extrapolate to the same value at zero time, which is taken to be the measure of ^{42}K from the medium trapped within the filter. The same extrapolated value appears to hold for the more rapid ^{42}K uptakes

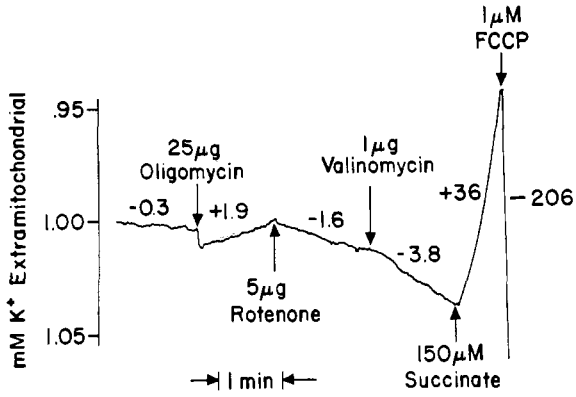

FIGURE 2: Effect of energy supply and valinomycin on net movement of K^+ into mitochondria. The reaction medium contained sodium β-hydroxybutyrate (6 mM), Tris-acetate (20 mM), KCl (1 mM), and sucrose (250 mM) at pH 6.7 and a temperature of 16.5°, and mitochondrial protein (4.8 mg/ml).

in series B in the presence of valinomycin (note the difference in time scales), although simple linear extrapolation back to zero time is more difficult. Note also the initial linearity of series B at 1.8 mM K^+, indicative of the precision with which initial ^{42}K rates could be estimated. Routinely in subsequent experiments, samples were taken somewhat more rapidly (initially four per minute) when determining the rapid ^{42}K uptake rates in the presence of transport-inducing antibiotics.

The rat liver mitochondria were prepared by the method of Schneider (1948). Respiration was measured by rotating platinum electrode polarograph (Hagihara, 1961). The capacity of the vessel was 4.0 ml and the protein level used was between 1 and 4 mg/ml. When investigating the effect of extramitochondrial P_i and K^+ on the ^{42}K uptake we corrected for the quantities of these ions arising by leakage from the mitochondria. Ion movements were recorded along with oxygen consumption, light scattering, and pH in the apparatus described by Pressman (1967). The ion movements were calibrated by adding known amounts of standard solutions to the fluid in the reaction vessel. The incubations were made at 10–11° in order to limit the rate of ion movements.

The following special reagents were kindly provided by the following investigators: highly purified parathyroid hormone, G. D. Aurbach; histone fraction f2a, A. Schwarz; dianemycin and nigericin, H. A. Lardy; oligomycin, F. M. Strong; gramicidin, purified fraction B, L. C. Craig; and FCCP,[1] P. Heytler. Valinomycin was prepared by means of a streptomyces culture donated by J. C. McDonald according to a modification of the procedure of the donor (McDonald, 1960).

[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphates; DPN, diphosphopyridine nucleotide; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone.

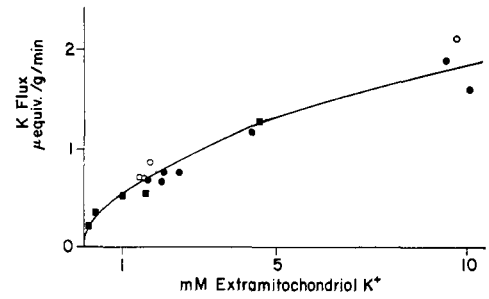

FIGURE 3: Effect of concentration of K^+ in medium on influx of K^+ into mitochondria. The reaction conditions and medium were the same as in Figure 1 (series A) for points marked ●. Points marked ○ and ■ indicate supplements of 2.5 mM P_i and 20 mM acetate, respectively. The temperature was 10°.

Results

The permeability of mitochondria to K^+ in the resting state (state 4) would be expected to be low, since this ion is retained during repeated washings, an operation which takes place in a metabolically quiescent state. The rapid uptakes or releases of K^+, which have been reported in earlier works with ^{42}K, would now appear to reflect movements of K^+ in and out of a superficial space which can be permeated by ions as large as inulin (Share, 1960). We will confine our attention to movements into a more restricted space, possibly the mitochondrial matrix, to which access can be controlled by a membrane barrier with selective ion permeability.

It had been suggested that valinomycin and other antibiotics which promote the uptake of monovalent ions by mitochondria act by increasing the permeability of mitochondria to these ions (Chappell and Crofts, 1965). Harris *et al.* (1966), however, demonstrated that valinomycin can reverse the direction of K^+ movement in mitochondria and concluded that, in addition to any permeability effect this antibiotic produces, it also affects the carrier or pump. The influence of valinomycin on K^+ in the absence of an energy supply, which illustrates its nonenergy-dependent permeability effects, is shown in Figure 2. At the prevailing K^+ concentration gradient and energy supply

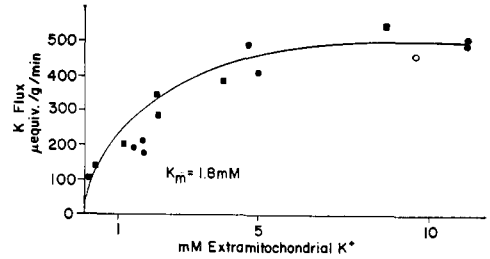

FIGURE 4: Same as Figure 3 in the presence of 60 μg of valinomycin/g of mitochondrial protein.

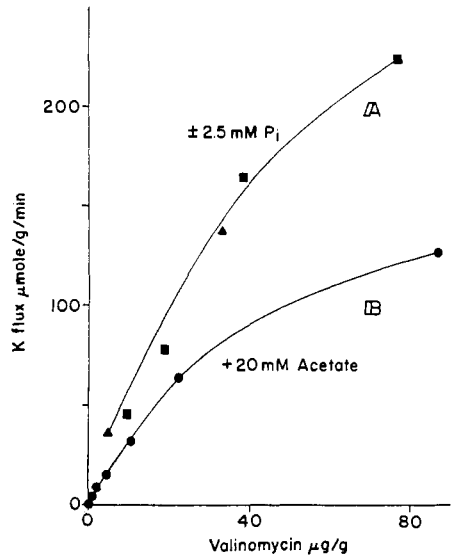

FIGURE 5: Effect of valinomycin concentration on influx of K^+ into mitochondria. Reaction conditions were the same as in Figure 1 except for the indicated levels of valinomycin; the $[K^+]$ was held constant at 2 mM. The upper curve includes points obtained in the absence of permeant anion (■) and with 2.5 mM P_i (▲). In the lower curve (●) 20 mM acetate was present.

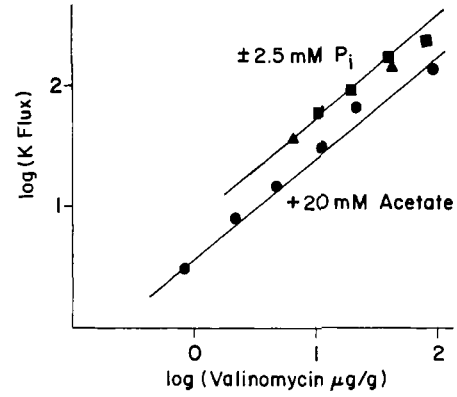

FIGURE 6: Log–log plot of the data of Figure 6. The K^+ flux is measured in micromoles per gram per minute.

provided, the preparation exhibited a very slight loss of K^+ in state 4. Addition of oligomycin (to render endogenously bound ATP unavailable as energy source) reversed the direction of K^+ movement and led to a net uptake, presumably by blocking the loss of energy from the mitochondria *via* ATPase. Addition of rotenone, which removes the remaining energy supply by blocking DPN-linked respiration, led to a net loss of K^+. When valinomycin was added to these energy-deprived mitochondria, the resultant increase of K^+ loss can best be explained as due to an enhanced permeability toward K^+ of the mitochondrial membrane. In the absence of oligomycin, the loss of K^+ produced by valinomycin addition was measurably less due to energy supplied by the endogenous ATP. When oligomycin was added subsequent to the valinomycin, an increased loss of K^+ resulted from the cutting off of the energy which would otherwise be available from the endogenous ATP. Restoration of energy to the mitochondria by initiating electron transport by a small addition of succinate causes a rapid uptake of K^+. The accumulated K^+ is subsequently released when the energy is dissipated by the addition of the uncoupling agent, FCCP.

Tracer Experiments. The effect of valinomycin in increasing mitochondrial permeability can be demonstrated by means of tracer techniques. The influxes of K^+, as a function of extramitochondrial K^+ in the absence of added agents, are indicated in Figure 1 (series A). The practical upper concentration of K^+, which can be used, is limited, since the ratio of mitochondrial K^+ to the K^+ trapped in the drained Millipore filter becomes lower as the extramitochondrial $[K^+]$ is raised. The influxes of K^+ calculated for Figure 1 (series A) corrected for the retention of radioactive medium in the Millipore filter are 1.2, 1.5, and 2.4 μmoles of K^+/g of mitochondrial protein for extramitochondrial $[K^+]$ of 1.8, 5.4, and 10.8 mM, respectively. A trend toward saturation is apparent. Results obtained in a number of runs with different mitochondrial preparations are given in Figure 3. The apparent K_m of saturation for ^{42}K influx appears to be about 5.5 mM. In the approximately steady-state situation prevailing in these experiments, the K^+ turnover is not greatly affected by the anions P_i and acetate, which facilitate the net K^+ uptake induced by valinomycin (Pressman, 1965; Ogata and Rasmussen, 1966; Harris *et al.*, 1966).

Effect of Valinomycin on ^{42}K Influx. Unequivocal demonstration that the uncoupling activity of the transport-inducing antibiotics does in fact involve

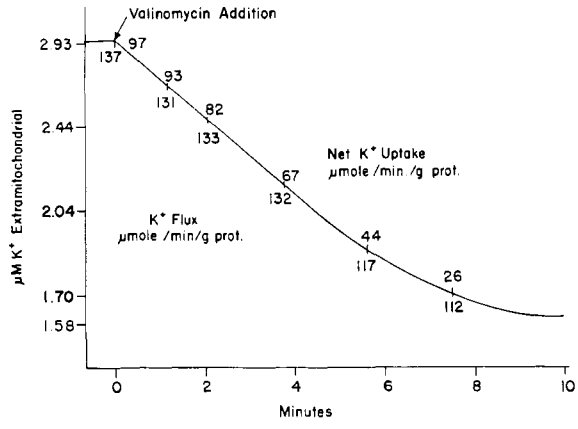

FIGURE 7: Comparison between gross and net influx of K^+ into mitochondria. Reaction conditions were the same as in Figure 1 (series B); experimental details in text.

the rapid steady-state turnover of ions is evident in Figure 1 (series B) from the ^{42}K uptake in the steady state which follows the valinomycin-induced net uptake of K^+. The initial rates of ^{42}K turnover can be determined with good precision, provided samples are taken rapidly enough. A composite of several experiments employing valinomycin-treated mitochondria is given in Figure 4. As in Figure 3, the trend to saturation is also evident, and from it an apparent K_m of 1.8 mM can be estimated, a value somewhat lower than is obtained for the spontaneous ^{42}K turnover.

Figure 5 shows the effect on the K^+ flux of varying the valinomycin concentration while holding the $[K^+]$ constant at 2 mM. The initial fluxes are not altered by the addition of 2.5 mM P_i, and are somewhat lower with 20 mM acetate; both permeant ions help sustain the initial net K^+ influx and lead to higher net K^+ uptake (Harris *et al.*, 1966). Replotting the data of Figure 5 on a log–log basis (Figure 6) results in a straight line, the slope of which indicates that the ^{42}K flux is proportional to $[\text{valinomycin}]^{0.8}$. This relationship offers a means of comparing systems under the influence of different levels of valinomycin, and the exponent derived from the slope indicates that the stimulation of K^+ flux is close to first order with respect to valinomycin. The upper line of Figure 6 was obtained with the basic system with and without added P_i. In the presence of acetate (lower line) the same slope is obtained, but the equivalent fluxes at a given valinomycin concentration happen to be lower.

In Figure 7 we have compared the net rates of valinomycin-induced K^+ uptakes with the corresponding rates of ^{42}K influx. A master experiment was first run with the glass electrode to trace out the course of K^+ uptake. In each of the subsequent experiments of this series the valinomycin-induced K^+ uptake was permitted to progress for a predetermined time interval before the addition of ^{42}K. Following the ^{42}K addition, six to eight samples were removed in each experiment at 10–20-sec intervals, and the rate of ^{42}K uptake was extrapolated to the instant of ^{42}K addition. From the slope of the K^+ uptake trace the rates of net K^+ uptake were also determined, corresponding to the times of ^{42}K addition. It can be seen that, as the K^+ uptake approaches completion, the rate of ^{42}K influx decreases somewhat but remains appreciable. In addition the gross K^+ efflux can be obtained from the difference between the gross and net K^+ uptake values. The data in Figure 7 indicate that the gross K^+ efflux increases as the mitochondria progressively gain K^+. In all experiments other than that shown in Figure 7, the net influx following the addition of agents which induced the rapid uptake of K^+ was permitted to fall to zero before addition of ^{42}K, and the resultant ^{42}K uptake rates represent true steady-state turnovers.

Effect of pH on K^+ Flux. The rate of net K^+ uptake by mitochondria induced by valinomycin increases as the pH is raised, although the maximal amount moved falls (Harris *et al.*, 1966). The K^+ uptake observed could be rate limited by either the permeability of the mitochondrial membrane or the availability of energy to drive transport. Tracer experiments (Table IA) reveal that the uptake of ^{42}K is increased as the pH is raised, even in the absence of valinomycin, and proportionately this increase exceeds that seen in the presence of valinomycin. By comparison with the rates of ^{42}K uptake in the presence of valinomycin, the corresponding rates in the absence of the antibiotic require only about 1% of the mitochondrial energy supply potentially available for transport. The pH dependence of ^{42}K uptake accordingly appears more closely related to the ease with which K^+ traverses the mitochondrial membrane than to the availability of energy.

Effect of Histone and Parathyroid Hormones. Parathyroid hormone has been reported (Sallis *et al.*, 1963; Rasmussen *et al.*, 1964) to cause the uptake or discharge of Mg^{2+} and K^+ by mitochondria. We observe that histone also releases mitochondrial Mg^{2+}, 4 mg/g of mitochondrial protein causing the almost complete discharge of mitochondrially bound Mg^{2+}. The data of Table IB show that histone can more than double the ^{42}K flux, while parathyroid hormone leads to a sixfold increase. Bovine serum albumin, employed as a control, does not augment ^{42}K turnover at all. It should be pointed out that these effects are small compared to those produced by valinomycin, particularly when the large amounts of hormonal protein are taken into consideration. We have also observed an inhibition of mitochondrial K^+ uptake by Mg^{2+}, which suggests that histone and parathyroid hormone function by permitting the release from the mitochondrial membrane of Mg^{2+}, which impedes the permeation by other cations. This view has also been considered by Azzone and Azzi (1966), who observed that EDTA, which is an avid complexer of Mg^{2+}, also induces a modest net K^+ accumulation. According to Table IB, EDTA also stimulates ^{42}K turnover.

Effect of Agents Imposing an Energy Drain. Substances which compete with the ion-translocating system for the common mitochondrial energy pool would be expected to reduce the influx of K^+. In Table IC, we see that removing energy by activating ATP synthesis with the addition of ADP produces just this effect. Experiments in the companion paper (Harris *et al.*, 1967) also show that ATP synthesis successfully competes with the energy supply for net transport of K^+ stimulated by valinomycin.

DNP, representative of the uncoupling agents which presumably discharge mitochondrial energy, also reduces the spontaneous uptake of ^{42}K, as does rotenone, which inhibits DPN-linked electron transport rather than interfering with the subsequent energy-transfer processes (Ernster *et al.*, 1963). Since DNP and rotenone could both be presumed to inhibit ^{42}K turnover by parallel mechanisms, *i.e.*, preventing the buildup of energized intermediates, it was somewhat unexpected that their effects would be additive and reduce the spontaneous flux to unmeasurable

TABLE I: Effect of Various Agents on ^{42}K Influx into Mitochondria.[a]

	pH	$[K^+]$	[Valinomycin] (μg/g of protein)	Flux (μmoles of ^{42}K/g of Protein)	
				Valinomycin	
				−	+
		A			
	6.3	3.0	30	0.44	68
	6.8	3.0	30	0.54	90
	7.4	3.0	30	0.79	100
	7.8	3.0	30	1.14	112
		B (20 mM acetate)			
Control	7.0	2.0	60	0.75	300
Histone (13 μg/ml)	7.0	2.0		1.60	
(33 μg/ml)	7.0	2.0		1.81	
Albumin (1 mg/ml)	7.0	2.0	60	0.67	250
Control	7.0	1.6	60	0.56	128
Parathydroid hormone (30 μg/ml)	7.0	1.6	Nil	3.2	
(55 μg/ml)	7.0	1.6	Nil	3.2	
EDTA (0.1 mM)	7.0	1.6	Nil	1.0	
		C (2.7 mM phosphate)			
Control	7.4	1.4	33	0.72	138
ADP (0.7 mM)	7.4	1.4	Nil	0.49	
Control	7.4	0.8	18	0.87	116
ADP (0.7 mM)	7.4	0.8	Nil		76
		D (40 mM acetate)			
Control	7.0	2.2	50	0.45	175
DNP (100 μM)	7.0	2.2	Nil	0.23	60
Rotenone (1 μg/ml)	7.0	2.2	Nil	0.10	60
DNP + rotenone (1 μg/ml)	7.0	2.2	Nil	0.00	6
Control	6.5	1.8	15.6	0.40	65
DNP (100 μM)	6.5	1.8	15.6		39
Oligomycin (14 μg/ml) + antimycin A (14 μg/ml)	6.5	1.8	15.6		18
Control	7.2	2.0	55	0.69	215
Tris-oleate (27 μM)	7.2	2.0	55	0.43	392
(80 μM)	7.2	2.0	55	0.19	196
(160 μM)	7.2	2.0	55	0.18	154
		E			
Control	7.2	4.6	90	0.52	295
Gramicidin B (10 μg/ml)	7.2	4.6	Nil	280	
Gramicidin B + NaCl (10 mM)	7.2	4.6	Nil	186	
Dianemycin (14 μg/ml)	7.2	4.6	90	0.00	295

[a] In addition to the components listed in the table, the basic medium contained Tris-glutamate (3 mM), Tris-malate (3 mM), Tris-Cl (20 mM), and sucrose (250 mM) at a temperature of 10°. The ^{42}K-uptake rates were measured on the steady state after net K movement has been completed *except* when DNP or dianemycin were tested, in which cases ^{42}K was added at the same time as the agent.

levels. Qualitatively the same synergistic effects are obtained at higher ^{42}K fluxes when these agents are added in the presence of valinomycin. Oleate, presumably by virtue of its surface activity, is another agent which leads to the inhibition of phosphorylation (Pressman and Lardy, 1955) as well as the loss of mitochondrially bound K^+ (Berger, 1957). According to Table ID, this agent suppressed the spontaneous turnover of K^+. In the presence of valinomycin, 27 μM oleate actually stimulates K^+ turnover, possibly by potentiating the effect of valinomycin on mitochondrial permeability, although higher levels are inhibitory.

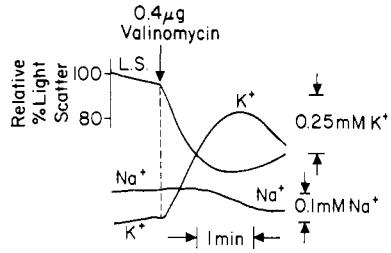

FIGURE 8: Effect of uptake of K^+ and water on extramitochondrial concentration of Na^+. An upward deflection of the Na and K electrode traces represents lowering of the concentration of the respective ion in the medium. The medium contained Tris-glutamate (3 mM), Tris-succinate (1.5 mM), Tris-acetate (20 mM), KCl (5 mM), NaCl (10 mM), and sucrose (250 mM), at pH 7.4, and mitochondrial protein (4.6 mg/ml). The temperature was 22°.

The transport-inducing antibiotic gramicidin is known to display less discrimination between Na^+ and K^+ than does the highly ion-selective valinomycin (Pressman, 1965; Chappell and Crofts, 1965). In Table IE, gramicidin stimulated the turnover of K^+ by mitochondria to much the same degree as valinomycin, although a valid quantitative comparison of the effectiveness of these antibiotics would require measurements at several levels. Addition of a low level of Na^+ to the gramicidin system reduces the stimulation of K^+ turnover, an effect not observed with valinomycin systems. This inhibition could be explained either by mutual competition between Na^+ and K^+ for entry into the mitochondria, or by the turnover of Na^+ dissipating energy that would otherwise have been available to drive the turnover of K^+.

Dianemycin (along with nigericin) belongs to another class of antibiotics which promotes the discharge of K^+ from mitochondria and effectively reverses valinomycin-induced net uptake of K^+ by mitochondria (Graven *et al.*, 1966). In Table IE, dianemycin added 1.5 min prior to ^{42}K completely inhibits the spontaneous turnover of K^+, but fails to inhibit the valinomycin-stimulated turnover. At the temperatures employed, the release from the mitochondria of K^+, previously accumulated in the presence of valinomycin, takes place slowly relative to the time scale of the ^{42}K sampling, and does not unduly complicate measurement of the initial ^{42}K turnover rate. As the K^+ release progresses (monitored by the glass electrode) the ^{42}K-uptake rate falls rapidly, and eventually there is a net ^{42}K release. The value included in Table I, showing no inhibition in ^{42}K uptake by dianemycin added to mitochondria loaded with K^+ in the presence of valinomycin, was derived from measurements made during the initial 30 sec following the rapid sequential addition of dianemycin and ^{42}K. The effects of dianemycin are reminiscent of those obtained with mercury compounds by Gamble (1962), which also cause the loss of mitochondrially bound K^+ while stimulating the turnover of ^{42}K.

Concentration of K^+ within the Mitochondria. Comparison of the K^+ content of the Millipore filtrate and the sucrose-diluted reaction system prior to filtration offers a means of independently corroborating the uptake of K^+ by mitochondria as deduced from K^+-sensitive glass electrode tracings. The values obtained in this fashion would not be expected to be highly accurate, since they are derived from a small difference between relatively large numbers. In a typical experiment using glutamate–succinate as substrate in the presence of 1.6 mM extramitochondrial K^+, the calculated net uptake of K^+ induced by valinomycin was 675 μmoles/g of protein, a figure consistent with the values obtained with the glass electrode under comparable conditions. In the same manner it was calculated that the original K^+ content of the mitochondria was 120 μmoles/g of protein, which agrees with the range previously reported (Harris *et al.*, 1966).

It was of interest to compare this latter figure with the concentration at which K^+ accumulates within mitochondria treated with valinomycin. If an extensive uptake of K^+ is initiated by valinomycin without the mitochondria becoming excessively permeable to Na^+, the concomitant entry of water into the mitochondria would concentrate the extramitochondrial Na^+. Such an effect can be measured by a Na^+-sensitive glass electrode as shown in Figure 8. The response of the Na^+-sensitive electrode lags behind those of the K^+ electrode and the light scattering, the latter indicative of the uptake of water by the mitochondria. The apparent lag can be accounted for by the intrinsic response of the Na^+-sensitive electrode being slower than that of the K^+-sensitive electrode and the transient response which this electrode exhibits toward K^+ (*cf.* Pressman, 1967). From the uptake of K^+ indicated by the K^+ electrode tracing, and the uptake of water as calculated from the Na^+ electrode tracing, in five experiments values of 50, 58, 60, 62, and 80 mM were found for the intramitochondrial concentration at which K^+ accumulates under the influence of valinomycin. These figures do not necessarily represent the total average intramitochondrial K^+ concentrations, since no allowance has been made for the mitochondrial content of water and K^+ prior to the addition of valinomycin.

Comparable ranges for the concentration of K^+ accumulated under the influence of valinomycin have been obtained by an independent method. Over the range 7.5–75 mM K^+, the light-scatter changes which accompany the valinomycin-induced uptake of K^+ are similar, which infers that the amount of water taken up by the mitochondria concomitantly with K^+ is reasonably constant. A typical experimental record of this series is given in Figure 9. The apparent K^+ uptake indicated by the glass electrode, however, decreases and extrapolates to zero at about 80 mM extramitochondrial K^+ (Figure 10). This can be explained by taking into account the fact that, as the mitochondria swell, the passage of K^+ through the

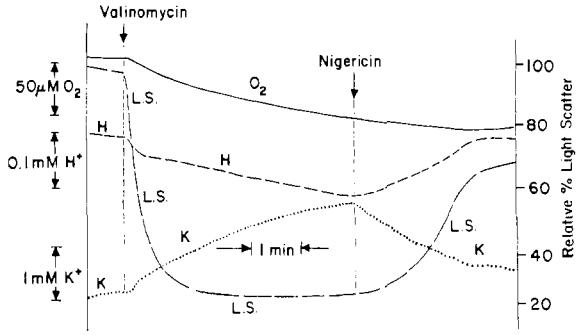

FIGURE 9: Multiparameter recording of events accompanying the uptake of K^+ induced by valinomycin (100 μg/g of protein) and its subsequent release after addition of nigericin (100 μg/g of protein). Upward deflections of the H^+ and K^+ traces recorded from the respective glass electrodes represent lowering of the ionic concentrations in the medium. Although the recordings are of logarithmic functions, they are evaluated in absolute terms from the calibrations because departure from linearity over the range being used is not serious. The medium contained Tris-glutamate (3 mM), Tris-malate (3 mM), Tris-acetate (20 mM), KCl (45 mM), and sucrose (165 mM), at pH 6.7, and mitochondrial protein (5.4 mg/ml). The temperature was 16.5°.

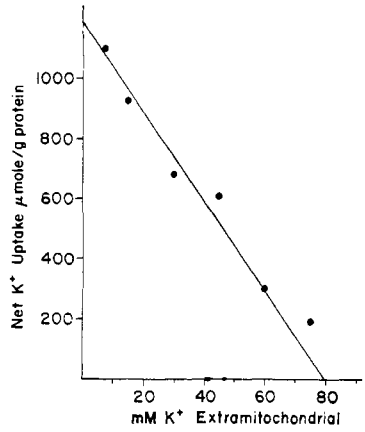

FIGURE 10: Apparent net uptake of K^+ as sensed by the glass electrode. The indicated concentration of K^+ for each experimental point was obtained under conditions of approximately constant osmolarity by substituting the required volume of 150 mM KCl for an equivalent volume of 250 mM surose. All other components and reaction conditions were as indicated for Figure 9.

mitochondrial membrane at the same concentration as exists extramitochondrially would have no effect on the K^+ concentration sensed by the K^+-sensitive electrode. According to Figure 10, this condition is achieved when the K^+ and water which enter mitochondria following valinomycin addition correspond to a concentration of about 80 mM, which agrees with the concentration determined above with the Na^+ exclusion technique. The above consideration indicates that a factor previously ignored should be applied to the apparent K^+ uptake to correct for the amount of extramitochondrial K^+ which is displaced by the expanding mitochondrion, *i.e.*

$$\Delta[K^+] = \frac{\Delta[K^+_{app}][K^+_{entering}]}{[K^+_{entering}] - [K^+_{external}]}$$

In previous data published from this laboratory, the $[K^+_{external}]$ was usually quite low, *ca.* 5 mM. If the K^+ were concentrating at 80 mM from a $[K^+_{external}]$ of 5 mM, the correction would be +7%. As the $[K^+_{external}]$ approaches the $[K^+_{entering}]$, however, the correction factor approaches infinity.

Discussion

The kinetics previously obtained with ion-specific electrodes for cation transport in mitochondria induced by antibiotics pertain exclusively to net influx and efflux. Tracer techniques permit measurement of the gross influx and efflux of ions as well, including the exchange flux which continues after net ion movement ceases.

A direct comparison between gross and net K^+ influxes into mitochondria (Figure 6) shows that the initial gross influx of K^+ exceeds the net influx by about 40%. The fact that the gross flux falls progressively as the mitochondria gain K^+ and a steady state is attained, *i.e.*, the net influx approaches zero, provides a potentially valuable diagnostic characteristic of the transport process.

For the purposes of this discussion, mechanisms for the active transport of K^+ into mitochondria may be divided into two categories, depending on whether the K^+ is moved across a semipermeable membrane barrier by an electrostatic field or an energy-driven carrier. Both alternative proposals could explain the reduction of gross influx and increase of gross efflux with progressive K^+ accumulation, since the entry of K^+ would either be encouraged less by a progressive reduction of intramitochondrial negative charge or opposed more by the progressive buildup of positive charge. Converse effects would be expected on the efflux. The course of the oxygen tracing of Figure 9 favors the alternative that the mitochondrial interior goes positive during K^+ transport, since, as the mitochondria gain K^+ in the presence of acetate and gross influx falls, respiration also decreases. According to the model proposed by Chappell and Crofts (1965), as the intramitochondrial negative charge decreases, the proton pump ought to be stimulated, thereby increasing the expenditure of mitochondrial energy, which in turn should stimulate mitochondrial respiration.

Chappell and Crofts (1965) have proposed that K^+ is drawn through a selective pore in the mitochondrial

membrane by an electrostatic force arising from a proton pump metabolically activated by the chemiosmotic mechanism of Mitchell (1961, 1966). The transport-inducing antibiotics are considered to act by increasing the rate-limiting permeability to K^+ of the mitochondrial membrane, thus permitting the proton pump to increase its activity from a low stand-by rate to its full capacity in order to supply enough charge separation for the observed rapid-induced transport of K^+. The chemiosmotic hypothesis as proposed, however, fails to explain how the hydrolysis of a single equivalent of ATP can lead to the translocation of as many as seven K^+ into the mitochondria (Cockrell *et al.*, 1966), or possibly more if we correct for the gross *vs.* net influx of K^+, *via* the pumping of a like number of proton equivalents. The concept of a proton pump supplying the primary driving force for K^+ transport is further weakened by the extreme variability observed for the H^+:K^+ transport ratios which we observe, ranging from almost unity in the absence of permeant ions at higher pH (*cf.* Moore and Pressman, 1964) to less than 0.01 at lower pH in the presence of high concentrations of permeant anions. Under the conditions of Figure 9, it can be seen that measurable expulsion of H^+ occurs only during the first 20% of the total K^+ transport. The subsequent slow decrease of extramitochondrial pH apparent in the tracing can be accounted for on the basis of metabolic CO_2 production, since in other experiments using succinate and rotenone as energy source it was absent.

While the available facts do not rule out the possibility of an energy-driven proton pump, they are not at all unfavorable for the existence of a cation pump which gives rise to secondary proton translocations. During cation transport the charge balance is preserved by the concerted movement of permeant anions including acetate, P_i, and oxidizable substrate, unless compensated for by the discharge of H^+ from unidentified sources. Alternatively all mitochondrial ion transport may involve the translocation of electrically neutral ion pairs, so that the extramitochondrial pH drop observed would result from the simultaneous uptake of K^+ and OH^- rather than a K^+ for H^+ exchange as has usually been assumed.

We have regarded the action of the transport-inducing antibiotics as twofold. Since the several species of transport-inducing antibiotics increase the cation permeability of model membranes devoid of energy transduction capacity with ion specificities that parallel those observed with mitochondria (Mueller and Rudin, 1967), they obviously should be capable of increasing the intrinsic permeability of the latter as well. However, as pointed out previously (Cockrell *et al.*, 1966), valinomycin also appears to increase the diversion of mitochondrial energy into the transport process. The statement attributed to our group that "valinomycin acts to promote K^+ uptake by increasing the synthesis of a phosphoprotein specifically involved in ion transport" (Ogata and Rasmussen, 1966), either in the reference cited by these authors (Moore and Pressman, 1964) or any place else, is incorrect.

Our previous calculations of the energetics of antibiotic-induced mitochondrial K^+ transport were based on the net K^+ influx as measured with the K^+-sensitive glass electrode (Cockrell *et al.*, 1966). If the relationship between gross and net K^+ influx, under the conditions employed, was similar to those observed in Figure 7, the energy of ATP hydrolysis actually harnessed to do transport work could have been appreciably higher than assumed previously. On the other hand, a portion of the excess of gross transport over net transport could have been due to diffusion exchange, which would not require the expenditure or dissipation of energy (*cf.* Anderson and Ussing, 1960). Since, at the values employed for the K^+ flux and K^+ gradient, the calculated efficiency for conversion of the free energy of ATP hydrolysis into a K^+ gradient was 80% (Cockrell *et al.*, 1966), any appreciable upward correction for the free energy conserved as ion transport approaches theoretical limitations.

In this connection it is of interest to examine the contention of Chappell and Crofts (1965), as well as Rasmussen and Ogata (1966), that K^+ is accumulated within mitochondria along with an osmotic equivalent of water. Our previous calculation of the energetics of transport employed a figure of 50 mM for the intramitochondrial [K^+]. This concentration was based on interpretation of light-scattering measurements according to the analysis of Koch (1961), who concluded that mitochondrial volume changes should be inversely proportional to the three-halves power of the relative light-scattering changes. More recent measurements in our laboratory of the light scattering by mitochondria as a function of osmotic pressure have indicated to us that the first-power relationship, as empirically determined by Tedeschi and Harris (1958), gives a better experimental fit of the data. If the first power relationship, therefore, is the more appropriate one to use, this would have the effect of raising the effective concentration at which K^+ accumulates within mitochondria to about 70 mM, rather than the 50 mM figure used in our previous calculations. In the present paper we have provided two additional means of measuring this value, based on Na^+ exclusion from mitochondria, and the responses of the K^+-sensitive electrode at high extramitochondrial [K^+]. The resultant estimates of the intramitochondrial concentration of accumulated K^+ agree with the above-amended conclusions derived from light scattering, and in the case of Figures 8 and 10 are considerably below the [K^+] osmotically in balance with the medium, namely, 170 mM. If K^+ would have concentrated isoosmotically at 170 mM in the experiments of Cockrell *et al.* (1966), utilizing ATP as an energy source, the energy requirement for transport at the resultant gradient (an increase of about 20%), would have exceeded the maximum theoretically obtainable. Thus three independent means of arriving at the intramitochondrial [K^+], as well as analysis of energy utilization, militate against the view that the volume changes accompany-

ing energy-dependent ion accumulation in mitochondria are a simple osmotic phenomenon.

Rasmussen and Ogata (1966) have stated that the fact that both swelling and contraction of mitochondria can be evoked by valinomycin in the absence of an energy source, depending on the extramitochondrial [K^+], is incompatible with hypoosmotic K^+ transport. We do indeed accept that passive movements of water and ions in and out of the mitochondria could be strictly osmotically related, since valinomycin affects the intrinsic permeability of biological and synthetic membranes devoid of an energy source (Mueller and Rudin, 1967). But our attention has centered on energy-driven transport, which is apparently another situation altogether.

Although in many systems close correlation between ion uptake and mitochondrial swelling appear qualitatively compatible with a simple osmotic swelling mechanism, special attention should be drawn to Figure 9, where K^+ uptake continues well beyond the point at which mitochondrial volume, as indicated by light scattering, ceases to change. Reversal of net K movement by the addition of nigericin also precedes by a considerable time the recontraction of the mitochondria.

We concur with the suggestions offered by Rasmussen and Ogata (1966) and by Azzone and Azzi (1966) that the effects of both low molecular weight basic proteins, parathyroid hormone, and histone on mitochondrial K^+ transport are indirect consequences of their ability to remove Mg^{2+} which hinders the transport of monovalent cations by blocking strategic sites on the mitochondrial membrane. It is relevant to point out that valinomycin is about 150,000 times more potent than the hormone on a weight basis, or 16,000 times more potent on a mole for mole basis. Competition between the transport by mitochondria of Mg^{2+} and another monovalent cation, guanidinium, has already been described (Pressman and Park, 1963). We have failed to detect any appreciable movements of Mg^{2+} induced by valinomycin.

Although mitochondrial systems offer several unique experimental approaches to the determination of mechanism of ion transport, at present we lack the means to measure the potential across the mitochondrial membrane. This measurement, which has been obtained in many cellular systems by the intracellular insertion of microelectrodes (*cf.* Lev, 1964), would definitely establish whether K^+ is transported into mitochondria *via* a carrier mechanism with the mitochondrial interior becoming positive, or by means of an electrostatic field with mitochondrial interior becoming negative with respect to the exterior. It is to be hoped that suitable techniques can be developed for obtaining equivalent information from mitochondria.

Acknowledgments

We wish to thank Mr. John Armor, Miss Jacqueline Wilson, and Miss Marjory Wilson who have rendered valuable technical assistance during various phases of the experimental work.

References

Amoore, J. E., and Bartley, W. (1958), *Biochem. J. 69*, 223.

Anderson, B., and Ussing, H. H. (1960), *Comp. Biochem. 2*, 371.

Azzone, G. F., and Azzi, A. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 332.

Berger, M. (1957), *Biochim. Biophys. Acta 97*, 107.

Chappell, J. B., and Crofts, A. R. (1965), *Biochem. J. 95*, 393.

Chappell, J. B., and Crofts, A. R. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 332.

Cockrell, R. S., Harris, E. J., and Pressman, B. C. (1966), *Biochemistry 5*, 2326.

Ernster, L., Dallner, G., and Azzone, G. F. (1963), *J. Biol. Chem. 238*, 1124.

Gamble, J. L. (1962), *Am. J. Physiol. 203*, 886.

Graven, S. N., Estrada-O, S., and Lardy, H. A. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 654.

Haberer, K. (1965), *Atomwirtschaft 10*, 36.

Hagihara, B. (1961), *Biochim. Biophys. Acta 46*, 134.

Harris, E. J., Cockrell, R., and Pressman, B. C. (1966), *Biochem. J. 99*, 200.

Harris, E. J., Höfer, M., and Pressman, B. C. (1967), *Biochemistry 6*, 1348 (this issue; preceding paper).

Koch, A. L. (1961), *Biochim. Biophys. Acta 51*, 429.

Lev, A. A. (1964), *Nature 201*, 1132.

McDonald, J. C. (1960), *Can. J. Microbiol. 6*, 27.

Mitchell, P. (1961), *Nature 191*, 144.

Mitchell, P. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Amsterdam, Elsevier, p 65.

Moore, C., and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562.

Mueller, P., and Rudin, D. O. (1967), *Biochem. Biophys. Res. Commun. 26*, 398.

Ogata, E., and Rasmussen, H. (1966), *Biochemistry 5*, 57.

Pressman, B. C. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1076.

Pressman, B. C. (1967), *Methods Enzymol.* (in press).

Pressman, B. C., and Lardy, H. A. (1955), *Biochim. Biophys. Acta 18*, 482.

Pressman, B. C., and Park, J. K. (1963), *Biochem. Biophys. Res. Commun. 11*, 182.

Rasmussen, H., Fischer, J., and Arnaud, C. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1198.

Rasmussen, H., and Ogata, E. (1966), *Biochemistry 5*, 733.

Sallis, J. D., DeLuca, H. F., and Rasmussen, H. (1963), *J. Biol. Chem. 238*, 4098.

Schneider, W. C. (1948), *J. Biol. Chem. 176*, 259.
Share, L. (1960), *Biochim. Biophys. Acta 38*, 154.
Tedeschi, H., and Harris, D. L. (1958), *Biochim. Biophys. Acta 28*, 392.

Diphosphopyridine Nucleotide Specific Isocitric Dehydrogenase of Mammalian Mitochondria. I. On the Roles of Pyridine Nucleotide Transhydrogenase and the Isocitric Dehydrogenases in the Respiration of Mitochondria of Normal and Neoplastic Tissues*

Abraham M. Stein, Jeanne H. Stein, and Sandra K. Kirkman

ABSTRACT: The respiration of pyridine nucleotide depleted mitochondria in the presence of isocitrate has been measured. Evidence has been obtained for the oxidation of isocitrate by both the triphosphopyridine nucleotide specific isocitric dehydrogenase–transhydrogenase (I) and the diphosphopyridine nucleotide specific isocitric dehydrogenase (II) pathways. Pathway II is independent of triphosphopyridine nucleotide, requires isocitrate and diphosphopyridine nucleotide concentrations higher than those of I, is stimulated by adenosine diphosphate, and is inhibited by 2,4-dinitrophenol in a manner closely similar to the behavior of the soluble enzyme. The respiration pattern is tissue specific: I predominates in heart, II in brain, and Ehrlich ascites mitochondria; liver mitochondria are intermediate with about equal capacity for I and II. Distribution of the two isocitric dehydrogenases in extracts of various mitochondria has been measured. Evidence for pyridine nucleotide transhydrogenase in Ehrlich ascites carcinoma has been obtained by pyridine nucleotide analog assay and respiration studies.

In the course of a reinvestigation into the problem of the occurrence of pyridine nucleotide transhydrogenase in mitochondria of neoplastic tissues we have identified in extracts of mitochondria of the Ehrlich ascites carcinoma an active DPN-specific isocitric dehydrogenase[1] which appears to be responsible for a major part of the respiration coupled to the oxidation of isocitric acid in this system. Properties of DICDH have been examined in recent studies from several laboratories. Chen and Plaut (1963) have reinvestigated the purified beef heart enzyme and have described an activating effect of ADP on the reaction. Hathaway and Atkinson (1963) and Atkinson *et al.* (1965) have shown a sigmoid dependence of activity on isocitrate concentration for the yeast enzyme and investigated the kinetic properties of the response of activity to the effectors citrate and AMP. Sanwal *et al.* (1963) have shown a parabolic dependence of the reciprocal rate on the reciprocal of isocitrate concentration in the reaction catalyzed by the *Neurospora crassa* enzyme and have discussed (Sanwal *et al.*, 1964) the enzyme in terms of the model proposed by Monod *et al.* (1963). Goebell and Klingenberg (1964) and Klingenberg *et al.* (1965) have investigated the pH dependence and effect of isocitrate and DPN^+ concentration on the DICDH activity of high-speed supernatants of disrupted mitochondria of some mammalian tissues and of locust flight muscle. Studies on the kinetic behavior of the enzyme from the various sources have suggested to all authors a role for DICDH in the regulation of citric acid cycle activity.

The present studies were prompted in part by the failure of Hawtrey and Silk (1961) to detect pyridine nucleotide transhydrogenase activity in their preparations (Hawtrey and Silk, 1960) of mitochondria of the Ehrlich ascites carcinoma and their finding in this system of significant DICDH activity dependent on unusually high DPN^+ concentration. It will be shown that assay of the Hawtrey and Silk preparation by the

* From the John Harrison Laboratory of Chemistry, The University of Pennsylvania, Philadelphia, Pennsylvania. *Received July 5, 1966.* Supported by grants from the U. S. Public Health Service (CA-05117) and the American Cancer Society (P-334A).

[1] Abbreviations used: DPN^+ and DPNH, diphosphopyridine nucleotide, oxidized and reduced, respectively; TPN^+ and TPNH, triphosphopyridine nucleotide, oxidized and reduced, respectively; APDPN and APTPN, the acetylpyridine analogs of DPN^+ and TPN^+, respectively; TNDPN, the thionicotinamide analog of DPN^+; ADP, adenosine diphosphate; AMP, adenosine monophosphate; DICDH and TICDH, the DPN^+- and TPN^+-specific isocitric dehydrogenases.