surface charge density and surface potential of the lipid (Dawson, 1969). The varying L:CL ratio in this study represents model lipid membranes of varying charge density and as this increases more protein is bound and, according to the proposed model, the protein simply becomes more densely packed on the lipid layer. The variation in CL:insulin ratio (Figure 1) suggests that once the charge density reaches a high enough value (approximately where L:CL = 87:13), the CL:insulin ratio stays close to five indicating the binding of approximately five cardiolipin charges to each insulin molecule. Since +5 is approximately the number of charges on the insulin molecule at pH 3.0 (Tanford and Epstein, 1954), this suggests that when the cardiolipin molecules are densely enough packed on the surface of the lipid bilayer, cardiolipin and insulin bind stoichiometrically at an equivalent ratio of one. Nevertheless, the lipid:protein ratios show no such stoichiometric combination. This suggests that varying amounts of a stoichiometric cardiolipin–insulin complex, analogous to an insulin "site" of a membrane, can be accommodated into and form a portion of the lipid bilayer.

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Effect of Cations and Protons on the Kinetics of Substrate Uptake in Rat Liver Mitochondria†

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ABSTRACT: In a low ionic strength sucrose medium, the addition of a variety of cations such as Na^+, K^+, $Tris^+$, Mg^{2+}, and La^{3+} to rat liver mitochondria incubated at 2–10° stimulate the initial rate of uptake of [^{14}C]succinate, [^{14}C]malonate, [^{32}P]phosphate, as well as the impermeable dicarboxylate [^{14}C]phenylsuccinate. The concentration of cation necessary for half-maximal stimulation of succinate uptake depends upon the number of charges associated with the cation, decreasing from 3 to 4 mM (K^+) to 0.1 to 0.2 mM (Mg^{2+} or Ca^{2+}) to 0.025 to 0.030 mM (La^{3+} or Pr^{3+}). Cations exhibit a competitive effect on the activation of substrate uptake. For example, while the V_{max} for succinate uptake is not significantly affected by increasing the charge from 1 (K^+) to 3 (La^{3+}), the K_m is decreased from 0.74 mM (K^+) to 0.33 mM (Mg^{2+}) to 0.15 mM (La^{3+}). In the absence of cations, decreasing the pH from 7.6 to 5.9 lowers the K_m of malonate from 0.84 to 0.22 mM, without affecting the V_{max}. This effect of pH could be partially masked by adding 20 mM K^+ to the medium. Preincubation at 2° with 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, an uncoupling agent, nearly completely abolishes the uptake of malonate at all pH values tested. The metal complexing agent bathophenanthrolinesulfonate (20 μM) competitively increases the K_m of malonate from 0.2 to 0.4 mM, and further addition of 1 mM Mg^{2+} or lowering the pH from 7.6 to 6.2 does not eliminate this effect. It is concluded that the initial rate of substrate uptake is controlled primarily by the energized state of the mitochondria, and secondarily by the positive charge density on the surface of the mitochondria.

The initial observation by Gamble (1965) that mitochondria take up substrates in an energy-dependent fashion has stimulated many experiments that provide support for the contention that the rate of respiration in intact mitochondria is controlled by the intramitochondrial level of substrates (Harris *et al.*, 1967a,b; Palmieri *et al.*, 1967; Quagliariello and Palmieri, 1968). The distribution of several mitochondrial substrate anions has been shown to depend upon

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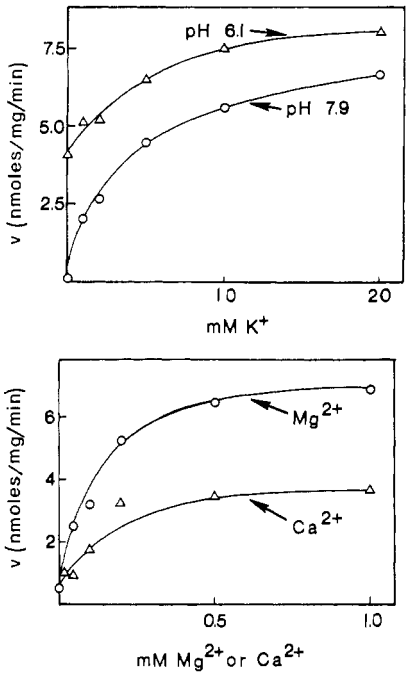

FIGURE 1: Effect of K^+ (A) and Mg^{2+} and Ca^{2+} (B) on the uptake of malonate. Mitochondria (2.2 mg in A, 3.5 mg in B) were incubated for one minute at 2° in 0.2 M sucrose, 2 mM buffer, 5 μg of oligomycin, 5 μg of rotenone, and cations at the concentrations indicated. The uptake was initiated with 0.1 mM [^{14}C]malonate, and stopped at 6 sec with 10 mM benzylmalonate (A) or at 5 sec with 5 mM butylmalonate (B). The final pH was 6.1 or 7.9 (A) or 7.8 (B).

the pH differential across the membrane (Palmieri and Quagliariello, 1969; Quagliariello and Palmieri, 1970; Palmieri *et al.*, 1970). By using specific inhibitors of the dicarboxylic acid uptake, kinetic evidence for the specificity and properties of the dicarboxylic acid carrier has been obtained (Quagliariello *et al.*, 1969; Palmieri *et al.*, 1971). It was found that the uptake of such dicarboxylic acids as succinate, malate, and malonate differ only in the K_m, but not in the V_{max}, and the conclusion was drawn that the maximal rate of translocation is a function of the carrier activity, while the K_m is mainly dependent upon the binding equilibrium of the carrier with the particular substrate.

Studies with the adenine nucleotide carrier (Meisner, 1971) has shown that the rate of translocation of ADP and ATP, as well as the binding of the impermeable anion atractyloside, is dependent upon the external cation concentration and pH. The present experiments describe the effects of pH and cations on the kinetics of substrate uptake in rat liver mitochondria. The data show that the initial uptake of various dicarboxylic acids as well as inorganic phosphate is dependent upon a number of mono-, di-, and trivalent cations, as well as on pH, which affect the K_m but not the V_{max} of the substrate uptake. A preliminary report on this work has been presented elsewhere (Meisner *et al.*, 1971).

Methods

Rat liver mitochondria were isolated in 0.25 M sucrose, 20 mM triethanolamine (pH 7.2), and 1 mM EDTA, and washed twice in 0.3 M sucrose adjusted to pH 7 with $Tris^+$. Protein was determined by the biuret method, using KCN to account for turbidity due to phospholipids (Kroger and Klingenberg, 1966).

The kinetics of the uptake of different anionic substrates were studied by using the "inhibitor stop" method (Pfaff *et al.*, 1969; Palmieri *et al.*, 1971). In this procedure, mitochondria are incubated in 1.0 ml of medium for 1 min, under conditions specified in the legends, at 2–10°. The uptake is initiated by adding the labeled substrate, stopped at 5–10 sec with either butylmalonate or 2-benzylmalonate ([^{14}C]malonate), or phenylsuccinate ([^{14}C]succinate), which are inhibitors of the dicarboxylate uptake (Chappell and Robinson, 1968). Phosphate uptake is inhibited by mersalyl (Fonyo, 1968; Tyler, 1969), which immediately blocks the exchange of P_i (A. Loebell, J. D. McGiven, and M. Klingenberg, unpublished data), and therefore can be used to measure the initial rate of uptake of substrate into the mitochondria. After rapidly centrifuging in an Eppendorf microcentrifuge for 1 min at 0°, the supernatant was carefully removed, 20% perchloric acid was added, and the radioactivity in the acid-soluble fraction of the pellet measured in a scintillation counter. Controls were incubated with the inhibitor present before the labeled substrate was added, and the amount taken up subtracted from the experimental samples, in order to arrive at the substrate incorporated into the matrix space (Quagliariello *et al.*, 1969; Palmieri *et al.*, 1971). Tritiated water ([^{3}H]H_2O) was added to all samples to correct for variations in the total water space.

The double-labeled samples were counted for radioactivity in a Nuclear-Chicago scintillation counter, and the final rate of substrate uptake in nanomoles per milligram per minute arrived at from a computer program.

Unless specifically noted, all solutions were neutralized with H^+ or $Tris^+$, and cations were added as the chloride salt.

Materials

Radioactive substrates [1,4-^{14}C]succinic acid, [1-^{14}C]malonic acid, [^{32}P]phosphoric acid, and [^{3}H]H_2O were obtained from either the Radiochemical Center (Amersham, England), or New England Nuclear Corp. (Boston, Mass.). Antimycin A and oligomycin were purchased from Sigma; rotenone was a gift from F. P. Penick and Co., N. Y., the lanthanide earths and 2-phenylsuccinate were purchased from K & K Laboratories, Plainview, N. Y. [^{14}C]Phenylsuccinic acid was prepared according to the method of Allen and Johnson (1950), using [^{14}C]KCN.

Results

The dependence of the uptake of malonate at pH 6.1 and 7.9 on the monovalent cation K^+ is shown in Figure 1A. At pH 7.9, the addition of K^+ increases the uptake of malonate from 0.1 to 6.5 nmoles per mg per min. At pH 6.1, although the rate of uptake in the absence of K^+ (4.0 nmoles/mg per min) is much greater than at pH 7.9, K^+ yields a smaller increase to 8.1 nmoles/mg per min. The stimulatory effect of the divalent cations Mg^{2+} and Ca^{2+} on malonate exchange is shown in Figure 1B. At a pH of 7.8, the exchange of 0.1 mM malonate was increased from 0.5 to 3.8 and 7.0 nmoles per mg per min by 1 mM Ca^{2+} and Mg^{2+}, respectively. From these experiments, the concentration of K^+ required for half-maximal stimulation is about 3.5 mM, and that of Mg^{2+} and Ca^{2+} between 0.1 and 0.2 mM.

There is a similar effect of Mg^{2+} and the trivalent cation Pr^{3+} on the uptake of 0.5 mM Tris-succinate. At a pH of 6.5 and at 10°, the K_m for the stimulation of the succinate exchange by Mg^{2+} is 0.12 mM and for Pr^{3+} is 0.024 mM, as

TABLE I: Effect of Cation Charge on the K_m and V_{max} of Malonate and Succinate Exchange.[a]

Additions	K_m (mM)	V_{max} (nmoles/mg per min)
A. None	0.66	8
20 mM K^+	0.12	8
B. 0.02 mM La^{3+}	5.0	22
0.1 mM La^{3+}	0.15	22
20 mM K^+	0.74	28
1 mM Mg^{2+}	0.33	22

[a] Mitochondria were incubated at 2° (A) or 10° (B) for one minute in 0.2 M sucrose, 2 mM Tris-Hepes, pH 7.6 (A), or 2 mM Tris-Tes, pH 6.5 (B), 2.5 μg of oligomycin and antimycin A, and the cation as specified. The reaction was started by the addition of malonate (expt A) or succinate (expt B), and stopped at 5 sec with 5 mM benzylmalonate (A) or at 6 sec with 10 mM phenylsuccinate (B). The values for K_m and V_{max} have been calculated from linear Lineweaver–Burk plots.

TABLE II: Effect of Monovalent and Divalent Cations on Malonate Uptake.[a]

	Additions	v (nmoles/mg per min)
Experiment 1	None	0.5
	2 mM Ca^{2+}	3.4
	2 mM Mg^{2+}	7.2
	2 mM Ba^{2+}	3.9
	2 mM Sr^{2+}	2.9
	2 mM Mn^{2+}	6.8
	2 mM Na^+	1.3
	2 mM K^+	1.5
	0.2 mM Mg^{2+}	5.3
	0.2 mM Mg^{2+} + 2 mM K^+	4.8
	20 mM K^+	3.9
	20 mM K^+ + 2 mM Mg^{2+}	6.3
Experiment 2	None	2.4
	10 mM K^+	5.7
	10 mM Na^+	4.8
	10 mM Cs^+	5.8
	10 mM Li^+	5.6
	10 mM $Tris^+$	3.6
	10 mM Na^+ (acetate)	6.0

[a] Mitochondria (3.5 mg, expt 1; 1.8 mg, expt 2) were incubated under conditions identical with Figure 1B. Final pH, 7.8.

revealed by linear $1/v$ *vs.* 1/cation plots. Taken as a whole, the results presented above and from other experiments show that the effectiveness of the cation in enhancing the exchange of succinate or malonate depends upon the number of charges associated with the cation. Thus, the K_m of the cation for increasing the uptake of 0.5 mM succinate, for example, decreases from 3–4 mM (K^+) to 0.1–0.2 mM (Ca^{2+}) to 0.025–0.030 mM (La^{3+} or Pr^{3+}), or approximately a 10-fold greater substrate binding with each additional positive charge possessed by the cation.

Table I shows that there is a competitive effect of 20 mM K^+ on the uptake of [^{14}C]malonate at pH 7.6 and 2°. In expt A, K^+ does not change the V_{max} (8 nmoles/mg per min), but lowers the K_m of malonate from 0.66 to 0.12 mM.

The competitive nature of another dicarboxylic acid, succinate, on the cation activation is shown in part B of Table I, where the effect of increasing cation charge on the K_m and V_{max} is summarized. These experiments, which were run at pH 6.5 and 10°, show that raising the La^{3+} concentration from 0.01 to 0.2 mM reduced the K_m of succinate from 5.0 to 0.15 mM, without affecting the V_{max}. Omission of La^{3+} from the medium, or for that matter omission of any cation except the 2 mM Tris buffer, gave no uptake of succinate except at concentrations above 1–2 mM. The uptake of succinate thus shows a higher dependence on the presence of cations than does malonate, but this may be at least partially explained by the higher K_m of succinate (Palmieri *et al.*, 1971). Table I reveals that although the V_{max} is not significantly affected by increasing the charge from 1 (K^+) to 3 (La^{3+}), the K_m of succinate is steadily decreased from 0.74 mM (K^+) to 0.33 mM (Mg^{2+}) to 0.15 mM (La^{3+}). The affinity of the succinate anion for the dicarboxylate carrier, and not the V_{max} of succinate uptake, is therefore dependent upon the number of charges possessed by the activating cation.

The general nature of the mono- and divalent cation stimulation of substrate uptake is revealed in Table II. All the alkaline earth as well as the monovalent cations tested stimulate the uptake of malonate. In expt 1, significantly, Mn^{2+} gave nearly the same degree of stimulation as Mg^{2+}, again pointing out that the charge possessed by the cation is more important than the chemical grouping. The stimulation by Na acetate points out that the anion may be changed without altering the cation effect. The $Tris^+$ cation, because of its bulkier size, is not as effective as the other smaller monovalent cations in stimulating the malonate exchange. Further, Table II shows that there appears to be little additive effect of a mono- and divalent cation. For example, 2 mM K^+ actually decreased the malonate uptake in the presence of 0.2 mM Mg^{2+} from 5.3 to 4.8 nmoles per mg per min. Likewise, 2 mM Mg^{2+} increased the uptake of malonate plus 20 mM K^+ from 3.9 to 6.3 nmoles per mg per min, which was below that obtained with 2 mM Mg^{2+} alone (7.2 nmoles/mg per min).

Figure 2 shows that the stimulation of substrate uptake by cations, which is due to a decrease in the apparent K_m, is accomplished by a lowering of the energy of activation. In this case, 20 mM K^+ reduces the E_a of malonate from 20.2 to 12.3 kcal per mole.

The activation of substrate uptake is not limited to cations, but can also be brought about by protons, as can be seen in Figure 3. In the absence of cations other than the 2 mM Tris buffer, raising the proton concentration 40-fold, from pH 7.6 to 5.9, lowers the K_m of malonate from 0.84 to 0.22 mM, but, significantly, does not affect the V_{max}. This confirms the competitive nature of pH on the dicarboxylate carrier as found by Quagliariello *et al.* (1969), who employed in addition to the $Tris^+$ buffer, 80 mM KCl in their reaction medium.

Figure 4 demonstrates the effect of changing both the H^+ and K^+ concentration on the uptake of malonate. Between pH 6 and 8, the uptake of 0.2 mM malonate is little affected if 20 mM K^+ is included in the medium, but exhibits a more pronounced decrease if K^+ is omitted. At pH 6, the stimula-

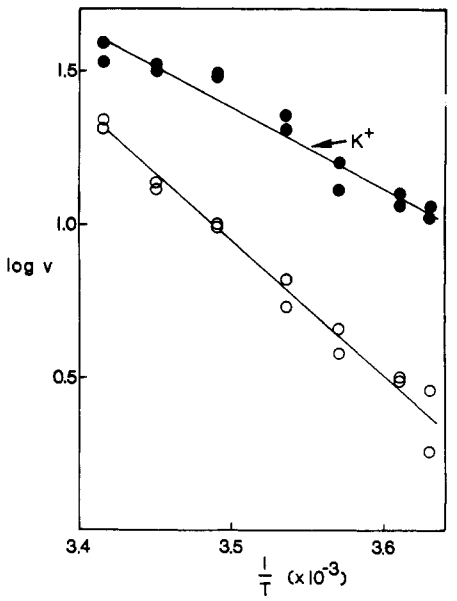

FIGURE 2: Effect of K^+ on the temperature dependence of the rate of malonate uptake. Mitochondria (2.0 mg) were incubated in 0.2 M sucrose, 2 mM Tris-Tes (pH 7.4), and 2.5 μg of oligomycin and rotenone. The reaction was started with 0.1 mM [^{14}C]malonate, and stopped at 5 sec with 5 mM benzylmalonate.

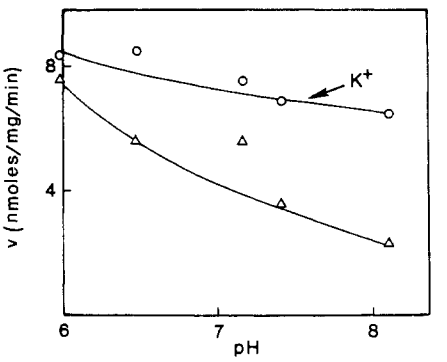

FIGURE 4: Effect of K^+ on the pH dependence of the malonate exchange. Mitochondria (1.6 mg) were incubated at 5° with 2.5 μg of oligomycin and rotenone, 0.2 M sucrose, 2 mM buffer, and 20 mM KCl where indicated. The reaction was initiated with 0.2 mM [^{14}C]malonate and stopped at 6 sec with 5 mM butylmalonate. The pH represents the values obtained with mitochondria present.

tory effect of K^+ becomes minimal. These results indicate the rationale behind the greater effect of K^+ at pH 7.9 than at 6.1 reported in Figure 1A of this paper.

By using low concentrations of the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to collapse the pH differential across the membrane, Figure 5 compares the importance of the H^+ concentration in the external medium to the H_0^+:H_i^+ ratio on the uptake of malonate. In this experiment, the H_0^+:H_i^+ ratio was brought toward unity by adding mitochondria to the reaction medium containing carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone at 2°, and after 2 min measuring the rate of uptake. Figure 5 shows that preincubation with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone at 2° markedly reduced the rate of uptake of malonate, and virtually abolished the pH dependence, confirming the primary role of the ΔpH in promoting substrate uptake. The uptake of malonate in the absence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone shows a maximum at pH 6.4 (compare to Figure 4), and may be due to the different incubation conditions employed. Meisner and Wenner (1970) have observed a sharp pH optima of 6.4 for gramicidin-mediated light-scattering changes induced by succinate.

It has been shown that rat liver mitochondria possess a phosphate carrier that catalyzes a P_i^-/OH^- antiport (influx of P_i^- and efflux of OH^-) or a P_i^-/H^+ symport (influx of P_i^- and H^+) (Chappell and Haarhoff, 1966; Mitchell and Moyle, 1969a; Papa *et al.*, 1969; Palmieri *et al.*, 1970; McGiven and Klingenberg, 1971). By employing 2 mM benzylmalonate to inhibit the uptake of P_i *via* the dicarboxylate carrier, and stopping the reaction with 0.6 mM mersalyl, the effect of cations on the rate of the P_i uptake *via* the P_i carrier was studied. Table III reveals that all the cations tested with the apparent exception of Pr^{3+} stimulate the exchange of P_i, Ca^{2+} being the most effective. In the case of Pr^{3+}, the binding of P_i to the mitochondria was enhanced approximately 10-fold, but mersalyl was ineffective in preventing the uptake of P_i. This effect may tentatively be ascribed to the phosphate anion-attracting ability of Pr^{3+}, which correspondingly increases the K_i of mersalyl.

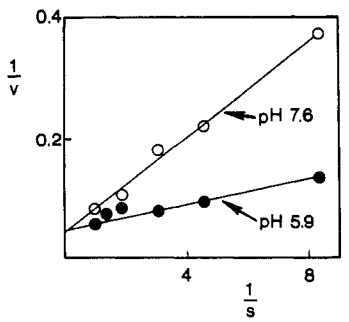

FIGURE 3: The competitive nature of the activation of malonate uptake by pH. Mitochondria (2.7 mg) were incubated 1 min at 2.3° with 0.2 M sucrose, 2 mM Tris-Mops (pH 5.9) or Tris-Hepes (pH 7.6), and 2.5 μg each of oligomycin and rotenone. The reaction was initiated with [^{14}C]malonate at the concentration indicated, and stopped at 5 sec with 5 mM benzylmalonate, pH 6 or 7.6.

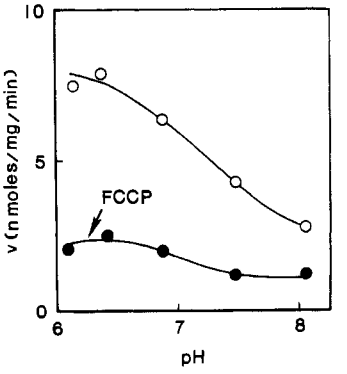

FIGURE 5: Effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on the uptake of malonate at different pH values. Mitochondria (3.0 mg) were incubated at 2° for 2 min in 0.2 M sucrose, 2 mM Tris-Mops buffer, 2.5 μg each of oligomycin and rotenone, and where indicated, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The pH represents the values obtained in parallel samples taken at 2 min, 2°. The reaction was started with 0.12 mM [^{14}C]malonate, and stopped at 6 sec with 5 mM benzylmalonate.

TABLE III: Effect of Cations on the Uptake of Phosphate *via* the Phosphate Carrier.[a]

Additions	Cpm −Mersalyl	Cpm +Mersalyl	v (nmoles/mg per min)
None	1,810	555	8.7
1 mM Mg^{2+}	2,900	585	15.5
1 mM Ca^{2+}	4,050	585	22.4
20 mM K^+	2,810	620	14.0
20 mM Li^+	2,620	620	13.1
0.1 mM Pr^{3+}	16,100	16,040	0

[a] Mitochondria (2.3 mg) were incubated at 2° for 1 min with 0.2 M sucrose, 2 mM benzylmalonate (pH 6.7), 2.5 μg each of rotenone and oligomycin, and cations as indicated. The reaction was started with 0.1 mM Tris-[^{32}P]P_i, and stopped at 6 sec with 0.6 mM mersalyl.

TABLE IV: Effect of Mg^{2+} and pH on the Inhibition of Malonate Uptake by Bathophenanthroline.[a]

Bathophenanthroline (μM)	v (nmoles/mg per min) pH 6.0 −Mg^{2+}	pH 6.0 +Mg^{2+}	pH 7.6 −Mg^{2+}	pH 7.6 +Mg^{2+}
0	5.0	12.8	2.7	7.2
20	3.1	6.9	2.2	5.5
50	1.4	1.9	1.4	1.7

[a] Mitochondria (2.6 mg) were incubated at 2° for 1 min in 0.2 M sucrose, 2 mM Tris-Mops (pH 6.2), or Tris-Hepes (pH 7.6), and when indicated, 1 mM Mg^{2+}, and 20 or 50 μM bathophenanthrolinesulfonate. The reaction was started with 0.12 mM [^{14}C]malonate, and stopped at 5 sec with 5 mM benzylmalonate.

It has been found by Tyler and Newton (1970) that metal complexing agents such as bathophenanthrolinesulfonate competitively inhibit the oxidation of succinate in intact mitochondria, and have proposed that a metal ion located at a site near the carrier enzyme is involved in the mechanism of transport of dicarboxylates. More direct measurements of the competitive inhibition by this metal complexing agent on the malonate exchange in the absence of cations at pH 6.2 show that 20 μM bathophenanthroline competitively increases the K_m of malonate from 0.20 to 0.41 mM, without changing the V_{max}, and yields an apparent K_i of 0.027 mM, which is similar to the values obtained by Quagliariello and Palmieri (1972) for the inhibition of citrate and α-ketoglutarate exchange. In Table IV, the effect of exogenous Mg^{2+} and H^+ on the malonate exchange inhibited with 20 and 50 μM bathophenanthrolinesulfonate is shown. While the uptake of malonate is inhibited under all conditions, the activation of substrate uptake by Mg^{2+} or pH is always present. Thus, the positive charges exert their effects by acting, at least partially, at sites other than the endogenous metal-carrier binding site.

Further evidence that the addition of cations directly affect the binding of the substrate anion to the membrane is shown in Table V. Here, the effect of various cations is compared at pH 6.5 and 7.6 on the binding of [^{14}C]phenylsuccinate, an impermeable dicarboxylate anion (Chappell and Robinson, 1968). All the cations tested, particularly Ca^{2+}, enhanced the binding of phenylsuccinate. The greater effect of cations at 0.5 mM phenylsuccinate is due to the higher real velocity at this concentration; the per cent activation by cations is actually smaller (see Table I). Although not brought out in the table, the decrease in pH from 7.6 to 6.5 increased the binding of phenylsuccinate by 0.15 (0.1 mM) and 0.42 (0.5 mM) nmoles per mg per min.

Discussion

Effect of Protons and Cations on K_m and V_{max} of Substrate Uptake. Palmieri *et al.* (1971) have shown that although the K_m of succinate, malonate, and malate uptake differ, the V_{max} of all three dicarboxylic acids, measured under identical conditions, is approximately the same, and have used this as evidence for the existence of one carrier for all the dicarboxylic acids. This paper confirms the fact that the V_{max}, which represents a property inherent in the carrier itself, is not significantly different, and shows conclusively that $1/K_m$, or the apparent binding affinity of any particular substrate for the carrier, is dependent upon the pH and the cation present in the surrounding medium. Thus, for example, by changing the K^+ concentration and pH, the K_m of malonate uptake can vary from approximately 0.1 to 0.8 mM, and would be expected to be considerably lower if measured at pH 6 plus a divalent cation or trivalent cation such as La^{3+} or Pr^{3+}.

In this context, it should be pointed out that binding affinities of various substrates measured by direct or indirect means have been reported in the literature, often with dissimilar results (Mitchell and Moyle, 1967; Robinson and Chappell, 1967; Haslam and Griffiths, 1968; Kraayenhof *et al.*,1969; Quagliarello *et al.*, 1969; Lofrumento *et al.*, 1970; Robinson and Williams, 1970; Palmieri *et al.*, 1971). For example, the K_m of malonate has been reported to be as high as 1.4 mM (Kraayenhof *et al.*, 1969), 1.0 mM (Mitchell and Moyle, 1967), and as low as 0.37 mM (Palmieri *et al.*, 1971) or 0.12 mM (this paper). Although the methods employed may be partially

TABLE V: Dependence of Phenylsuccinate Binding on pH and Cations.[a]

Cation	pH 6.5 0.1 mM	pH 6.5 0.5 mM	pH 7.6 0.1 mM	pH 7.6 0.5 mM
1 mM Mg^{2+}	0.49	0.82	0.36	0.77
1 mM Ca^{2+}	0.98	2.90	0.38	1.16
10 mM K^+	0.21	0.38	0.16	0.72
10 mM Li^+	0.19	0.40	0.20	0.55
0.1 mM Pr^{3+}	0.44	1.00	0.15	0.34

[a] Mitochondria (2.5 mg) were incubated at 10° for 1 min in 0.2 M sucrose, 2 mM Tris-Mops (pH 6.5), or Tris-Hepes (pH 7.6), 2.5 μg each of oligomycin and antimycin A, and cation as indicated. The reaction was started with 0.1 or 0.5 mM [^{14}C]phenylsuccinate (specific activity 500 cpm/nmole), and stopped at 20 sec by centrifugation in a microcentrifuge for 1 min at 0°. The values represent the difference between phenylsuccinate uptake in the presence and absence of cations.

responsible for these discrepancies, there can be little doubt that different conditions of ionic strength and pH are so important as to render comparison of K_m values meaningless unless determined from initial velocities and under identical conditions.

Mechanism of Action of Cations on Substrate Uptake. It is probable that cations affect the binding, and in this manner, the rate of uptake of substrates by combining electrostatically with amphoteric groups having a pK between 6.0 and 8.0 located on the outer surface of the mitochondrial membrane. It is assumed that these amphoteric groups are predominantly negatively charged at physiological pH, and normally hinder the binding of the substrate anions to their specific carriers. In this manner, the competitive nature of the activation of anion uptake by substrates may be explained. An increase in proton concentration (H_0^+) should likewise be expected to stimulate substrate uptake by protonating amphoteric groups on the membrane, but this effect is masked by (a) the predominance of the ΔpH in determining the rate of substrate uptake, and (b) the high concentration of cations (*i.e.*, 2×10^{-3} M Tris), compared to protons (10^{-6} to 10^{-8} M H^+) in media of low ionic strength. The marked dependence of the dicarboxylate uptake upon the number of charges possessed by the cation can be explained by the higher charge density of the multivalent cations, which enables them to have stronger electric fields (Lettoin *et al.*, 1964; Van Breemen and de Weer, 1970; Diamond and Wright, 1969). Thus, although Li^+, Mg^{2+}, Ca^{2+}, and La^{3+} all have about the same hydrated radii of 3 Å (Lettoin *et al.*, 1964), the greater electrostatic attraction of La^{3+} than Mg^{2+}, or Mg^{2+} than Li^+, for example, for negatively charged groups on the membrane will enable these cations to be bound more strongly, and likewise will more strongly attract the substrate anion to the membrane surface. The weaker stimulation of malonate uptake by $Tris^+$, a large monovalent cation, also supports these arguments.

Finally, the stimulation of malonate exchange by Mg^{2+} and H^+ in the presence of bathophenanthrolinesulfonate, which competitively inhibits the rate of dicarboxylate uptake (see Results) by complexing with a metal located at (Quagliariello and Palmieri, 1972) or near the carrier site (Tyler and Newton, 1970), suggests that the added cations do not act at an identical site as bathophenanthroline on the mitochondrial membrane. Most likely, the cations act rather indiscriminantly on negatively charged groups on the membrane.

Relationship between the Effect of pH and Cations on the Kinetics of Substrate Uptake and the Final Distribution across the Membrane. The importance of the pH differential across the mitochondrial membrane in driving the uptake of substrate has been demonstrated (Palmieri and Quagliariello, 1969; Quagliariello and Palmieri, 1970; Palmieri *et al.*, 1970). The ΔpH in rat liver mitochondria incubated in a sucrose–KCl medium is between 0.4 and 1.1 (Addanki *et al.*, 1968; Mitchell and Moyle, 1969; Quagliariello and Palmieri, 1970). In respiring mirochondria, it is of physiological importance that there is an outward driven proton translocation, which by enlarging the ΔpH, serves to exert a positive feedback on substrate uptake (Quagliariello *et al.*, 1971; McGiven and Klingenberg, 1971). Data presented in this paper have shown that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which abolishes the ΔpH, strongly inhibits the rate of malonate uptake, regardless of the external H^+ concentration, emphasizing the primary nature of the H_0^+:H_i^+ ratio as opposed to the proton concentration, in bringing about the uptake of substrates. Prezioso *et al.* (1971) have also shown that uncoupling concentrations of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone added before the labeled substrate, but not when added simultaneously, inhibit the initial rate of substrate uptake in mitochondria suspended in a KCl-containing medium. The fact that the latter experiments were carried out in the presence of KCl make it unlikely that the loss of K^+ ions from the mitochondria is responsible for the inhibition of substrate uptake observed here.

Quite separate from the effect of the H_0^+:H_i^+ ratio, there is a stimulation of the rate of substrate uptake by cations, as the result of a greater electrostatic attraction. This effect is not due to a change in the ΔpH caused by cations that are taken up by exchange-diffusion processes in respiring mitochondria (Mitchell, 1966), because of the low temperature employed, and the presence of the respiratory inhibitors rotenone, oligomycin, or antimycin A. Under such conditions, the ΔpH and the final substrate distribution are unaffected in either a sucrose- or KCl-containing medium (Palmieri and Quagliariello, 1969; Harris and Manger, 1968).

Thus, in energized mitochondria it is proposed that the initial rate of substrate uptake is controlled by the positive charge density on the surface of the mitochondria. The final distribution of substrates across the membrane, however, is dependent only upon the maintenance of a pH differential, or energized state.

General Nature of Cation Stimulation of Substrate Uptake. Studies on the effect of cations on the adenine nucleotide carrier by Meisner (1971) emphasize the general nature of the stimulation of anion translocation by cations. As was shown, the rate of ADP and ATP exchange in rat liver and heart mitochondria can be greatly affected by a variety of cations, provided that the concentration of ADP or ATP is low. Taken together with the experiments presented here that the exchange of dicarboxylates and P_i by their respective carriers can be influenced by cations, the data emphasize the possible role of cations in controlling the phosphorylation and respiration rates *in vivo*.

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Spectral Studies of the Binding of *O,O*-Diethyl *p*-Nitrophenylphosphorothionate (Parathion) to Cytochrome P-450†

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ABSTRACT: Parathion has previously been shown to be metabolized by a microsomal mixed-function oxidase system to paraoxon and to diethylphosphorothionate plus *p*-nitrophenol. The present study shows that parathion binds to cytochrome P-450 causing a type I difference spectrum. At low concentrations of parathion the minimum absorption is shifted to lower wavelengths and a shoulder in the type I spectra is evident. These changes in the type I spectra may be explained by the presence of a type II spectrum of parathion superimposed on the type I spectrum. The inhibition of the type II spectra of aniline by parathion further suggests the presence of a type II binding component of parathion. Hexobarbital diminishes the type I spectra of parathion seen with hepatic microsomes of untreated rabbits approximately 30% and that seen with hepatic microsomes from phenobarbital-treated rabbits approximately 50%. Similarly parathion decreases the type I spectra of hexobarbital seen with microsomes from untreated and phenobarbital-treated rabbits by approximately 30 and 50%, respectively. When hexobarbital and parathion are added to the same cuvet, the resulting spectral change is equal to 70–80% of the sum of the individual type I spectral components. These data indicate there are at least three separate type I spectral binding sites for these two substrates, a hexobarbital binding site, a parathion binding site, and a binding site with affinity for both hexobarbital and parathion. Phenobarbital, aminopyrone, ethylmorphine, and benzphetamine also diminish the type I spectra of parathion. When hexobarbital was added at a concentration that gave a maximal decrease along with either phenobarbital, aminopyrone, or ethylmorphine, the total decrease was essentially the same as that of hexobarbital alone. This seems to indicate that these drugs have a common binding site on cytochrome P-450. Benzphetamine, however, completely eliminates the type I spectra of parathion. The decrease of the type I spectra of parathion by benzphetamine is biphasic in character. These data also suggest that parathion may bind to more than one type I binding site.

Cytochrome P-450 has been shown to be the terminal enzyme of a liver microsomal enzyme system which can transfer oxygen to a variety of lipophilic compounds (Cooper *et al.*, 1965). This enzyme system has been named the microsomal mixed-function oxidase enzyme system. Substrates for this enzyme system may be categorized into two groups

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