Biochemistry

Volume 11, Number 6 March 14, 1972

Structural Studies by X-Ray Diffraction of Model Phospholipid–Insulin Membranes†

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ABSTRACT: X-Ray diffraction studies were made on precipitates formed at pH 3.0, between insulin and mixtures of lecithin (L) and the acidic phospholipid cardiolipin (CL) of mole ratios L:CL from 97:3 to 50:50. The precipitates are single lamellar phases, made up of alternating layers of lipid–protein and water, whose repeat distance d is constant at approximately 69 Å. For L:CL from 97:3 to 50:50 the composition of the precipitates varies systematically, showing no stoichiometry, the weight per cent lipid being 51% and the protein increasing from 8 to 37% as the water decreases from 41 to 15%. The CL:insulin ratio increases however to a constant value of aproximately 5. Molecular packing, based on partial thicknesses of the lipid, protein, and water, indicates that the phospholipid bilayer that exists without bound protein is unperturbed when insulin is electrostatically bound to it and no subsequent apolar interactions occur, *i.e.*, insulin does not penetrate into the lipid bilayer. As more insulin is bound to the lipid it simply replaces water in the interbilayer space.

Intact biological membranes simultaneously carry out a number of specific transport functions, each presumably related to a specific structure in the membrane, the whole array of sites being arranged tangentially in the plane of the membrane. Some of these sites must be in a dynamic state, their structure changing with fluctuations in the condition of the intra- or extracellular environment. In an attempt to circumvent the difficulties of resolving the structure of such a heterogeneous mosaic, model systems, made up of a few of the membrane components, have been investigated with the hope that these mimic the properties of specific sites. Our concern has been with the structural interactions that occur between membrane phospholipids and proteins using the technique of X-ray diffraction (Rand, 1971). Similar studies by Gulik-Krzywicki *et al.* (1969) have shown that many different structures and bonds can form between lipids and proteins depending on both the specific lipids and proteins involved and the conditions (pH, ionic strength, temperature, etc.) of their aqueous environment. This report deals with the interactions between lecithin–cardiolipin mixtures and the protein insulin.

Phospholipids alone in water form an enormous variety of structures (Luzzati, 1968) and although this variety is no less in lipid–protein systems one of the most widely occurring structures is the lamellar phase formed by repeating units of parallel planar layers. Lipids alone form classical bimolecular sheets, parallel and equidistant, separated by layers of water. On adding protein to the system, low resolution of the molecular arrangements within the sheets gives an indication of the molecular interactions, including the degree of hydrophobic bonding, between the lipids and protein. An equally interesting and important aspect of these studies is the interlayer interactions which are relevant to the problem of cell contact.

It appears that the basic mechanism by which insulin causes a reduction in blood sugar is not known and such diverse effects as changing protein synthesis, (*e.g.*, Wool and Krahl, 1959), redirecting glucose and lipid metabolism (*e.g.*, Rodbell and Jones 1966), and increasing the permeability of cell membranes to sugars and amino acids (Levine, 1961) have been demonstrated to be caused by insulin. These diverse effects may well be the manifestation of a chain of events in the cell's highly coordinated metabolism resulting from the primary effect of insulin (Villar-Palasi and Larner, 1970), even involving the ubiquitous 3′,5′-cAMP and adenylyl cyclase (*e.g.*, Jungas, 1966), but the primary effect is obscure. Nevertheless, one first step in the action of the hormone must be its interaction with the cell membrane and indeed it has been shown (Cuatrecasas, 1969) that insulin can cause some of its effects by interacting only with the cell membrane from the outside while not being allowed to enter the cell. This report is a preliminary attempt to study the interaction between insulin and membrane phospholipids, the interaction being made, on

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TABLE I: Values of d_l, the Thickness of the Lipid Bimolecular Layer in Lamellar Phases Formed by Mixtures of Lecithin (L) and Cardiolipin (CL), at the Indicated L:CL Mole Ratios, in 0.05 M Sodium Citrate Buffer (pH 3.0), at the Dry Weight Concentrations Shown.[a]

L:CL	$\sigma_1 \times 10^{-3}$ Charge/Å^2	Å^2/Charge	Concn (Dry Wt %) 0.66	0.70	0.73	0.77	0.80	0.90
100:0	0		39.1					
90:10	1.66	600				41.1		
87:13	2.17	460		38.6			40.6	43.0
75:25	4.16	240			39.0			
47:53	8.82	113			40.0			

[a] σ_1 and Å^2 per charge are the surface charge density, and its inverse, calculated on the basis of one phospholipid molecule occupying 60 Å^2 on the lipid surface.

initial contact between the components, by electrostatic binding between negatively charged lipid and positively charged protein.

Materials and Methods

Cardiolipin and lecithin, extracted from beef heart and pig liver, respectively, were obtained from Serdary Research Laboratories (Canada). The cardiolipin had, as determined by atomic absorption spectrophotometry, 2–4 mole per cent Ca^{2+} and Mg^{2+} was undetectable. Further indication that the phosphate groups of the cardiolipin were available for ionic binding was that (i) cardiolipin alone swelled indefinitely in water and (ii) cardiolipin–Ca^{2+} and cardiolipin–Mg^{2+} complexes are hexagonal structures, not lamellar (Rand and SenGupta, 1971). The lipid was assayed by thin-layer chromatography before and, with random checks, after the X-ray experiments. Crystalline insulin (Sigma) was used without further purification.

The method of preparation and the chemical analysis of the X-ray samples, and the derivation of the structural data from their X-ray diffraction patterns, are similar to those described in detail in a previous publication (Rand, 1971). They will be summarized here. Pure cardiolipin or lecithin:cardiolipin (L:CL)[1] mixtures of desired mole ratio were suspended (1 mg/ml) in 0.05 M soldium citrate buffer (pH 3.0) by stirring or occasionally sonicating briefly over an ice bath. This suspension, when added dropwise to a 1-mg/ml insulin solution in the same buffer and with constant stirring, resulted in a heavy precipitation. Unless otherwise noted this precipitation was always done so that not all of the protein was bound, *i.e.*, the precipitate was in equilibrium with dissolved protein. The precipitates were washed in the same buffer and dried to various extents under a stream of nitrogen, and their lipid, protein, and water contents determined as previously described (Rand, 1971). As controls, X-ray samples of the pure phospholipids were prepared by weighing the dry lipid and adding to it various amounts of buffer.

The structural analysis by X-ray diffraction of these sorts of systems has been described before in detail (Luzzati, 1968). It will be described briefly here as it applies to this study. The X-ray camera is of the Guinier type operating *in vacuo* and using a bent quartz crystal monochromator which isolates the Cu $K\alpha_1$ line (λ = 1.540 Å). The X-ray samples are sealed between mica windows approximately 1 mm apart and their temperature in the camera can be controlled. The only structure of interest in the present study is the lamellar phase which gives two to five X-ray reflections, all integral orders of the single repeat distance d of the one-dimensional crystal. The X-ray diagrams give otherwise only a broad band at 4.5 Å, typical of the liquid paraffin chains of the phospholipid molecules.

Within the planar lamellae of thickness d must be packed the lipid (l), protein (p), and water (w) in proportion to their respective relative volume concentrations, ϕ_l, ϕ_p, and ϕ_w, these latter being determined by the weight concentrations of the components c_l, c_p, c_w ($c_l + c_p + c_w = 1$) and their partial specific volumes, ν_l, ν_p, ν_w. As a first hypothesis, *if the lipid, protein, and water each formed separate layers* then the partial thicknesses d_l, d_p, and d_w of these layers can be found. Thus $d_l = \phi_l d$, where $\phi_l = c_l\bar{\nu}_l/(c_l\bar{\nu}_l + c_p\bar{\nu}_p + c_w\bar{\nu}_w)$, etc., and $d = d_l + d_p + d_w$.

The average area, A, available to each insulin molecule on the surface of the leaflet is given by: $A = V_p/d_p$ where V_p is the volume of one insulin molecule, taken to be 7000 Å^3 (Cohn and Edsall, 1965).

In order to determine the water content, c_w, of the lamellar structure of the precipitates, which were formed in excess buffer, the precipitates were dried with a stream of nitrogen until the repeat distance, d, started to decrease. Only at that point was the water concentration determined, since only at that point was the drying removing water from the structure and not from pools of excess buffer. In this way c_w of the sample was found and by extrapolating to the d value of the original undried precipitate, d_w of that precipitate was determined.

For the purposes of the results, the molecular weight of cardiolipin is taken as 740, one-half that as determined by the phosphorus analysis. This is the weight of each part of the whole molecule that contains one phosphate group, the acidic group that carries one of the molecule's two negative charges, and two fatty acyl chains, and therefore, is comparable in size and weight to one lecithin molecule. Hence the L:CL ratio as represented here gives the ratio of zwitterions to charges in the lipid mixture. However, it is clear that the cardiolipin molecule is two 740 units covalently bonded at the polar end and that, therefore, the charged groups on the surface of the lipid bilayer occur in pairs.

The partial specific volumes ν_l, ν_w, and ν_p used in all calculations are 1.00, 1.00, and 0.741 (Cohn and Edsall, 1965) cm^3/g, respectively, and the molecular weight of insulin is taken as 5733.

Results

Control Lipids. The control lipids (without protein) form a lamellar phase when mixed with buffer. Assuming, as has been proved a number of times (see, for example, Rand and Luzzati, 1968), that the lameller structure is formed by alternating layers of water and lipid (the latter being a bilayer of phospholipid formed by the fatty acyl chains forming a hydrocarbon layer with the polar groups occupying the interface between this layer and the water layer), the thickness of the lipid layer d_l can be calculated. Table I gives the values of d_l

[1] Abbreviations used are: L, lecithin; CL, cardiolipin.

TABLE II: Structural and Chemical Analyses of Insulin–Lecithin–Cardiolipin Precipitates. Each Column Is Discussed in the Results.

L:CL	σ_l ($\times 10^{-8}$)	Å²/ Charge	d (Å)	c_l	c_p	c_w	ϕ_l	ϕ_p	ϕ_w	CL: Insulin	d_l (Å)	d_p (Å)	d_w (Å)	$d_p + d_w$ (Å)	Area/ Insulin (Å)²
100:0	0		59.5	66.0	0	34.0	66.0		34.0		39.1	0	20.4		
97:3	0.50	2000	69.9	51.2	7.8	41.0	52.3	5.9	41.8	1.53	36.6	4.1	29.2	33.3	2409
95:5	0.83	1205	67.7	51.3	9.0	39.7	52.5	6.9	40.6	2.21	35.5	4.7	27.5	32.2	1499
93:7	1.16	862	72.8	50.0	10.1	39.9	51.3	7.7	41.0	2.68	37.3	5.6	29.9	35.5	1248
90:10	1.66	602	70.0	49.4	10.0	40.6	50.7	7.6	41.7	3.83	35.5	5.3	29.2	34.5	1316
87:13	2.16	463	68.6	52.5	11.4	36.1	54.1	8.7	37.2	4.63	37.1	6.0	25.5	31.5	1173
85:15	2.49	402	70.9	53.8	20.0	26.2	56.7	15.6	27.6	3.13	40.2	11.1	19.6	30.6	633
75:25	4.15	241	69.0	51.7	24.0	24.3	55.1	19.0	25.9	4.17	38.0	13.1	17.9	31.0	534
70:30	4.98	201	69.2	51.6	25.6	22.8	55.2	20.3	24.4	4.68	38.2	14.0	17.0	31.0	498
65:35	5.81	172	68.5	50.5	28.4	21.1	54.5	22.7	22.8	4.82	37.3	15.6	15.6	31.2	450
60:40	6.64	151	68.3	50.7	32.0	17.3	55.3	25.8	18.9	4.91	37.8	17.6	12.9	30.5	397
50:50	8.30	120	68.1	48.1	37.0	14.9	53.1	30.4	16.5	5.03	37.2	20.7	11.2	31.9	338

for a sampling of L:CL ratios and for a number of lipid concentrations (dry weight per cent). As with most other systems of natural phospholipids the thickness of the bimolecular layer is approximately 38–41 Å, increasing slightly in thickness at higher concentrations. The surface area available to each phospholipid molecule then is approximately 60 Å² and consequently, given the L:CL ratio, the surface-charge density, σ_l, and the area per charge, Å²/charge, are given in Table I.

Lipid–Protein Precipitates. The conditions, pH 3.0, were chosen so that a clear interaction between lipid and protein would occur, and whenever cardiolipin was present in the lipid an electrostatic interaction of the negatively charged lipids and positively charged protein resulted in heavy precipitation.

Table II and Figure 1 are summaries of the results of the chemical and structural analyses of the lecithin–cardiolipin–insulin precipitates. The results are based on five different experiments, covering a range of L:CL ratios, using three different batches of lecithin and two different batches of cardiolipin and insulin. Small consistent differences existed from experiment to experiment but within one experiment systematic changes of the various parameters, as those reported below, could be detected as the L:CL ratio was varied. The data of Table II and Figure 1 are pooled data from the five different experiments. Referring to Table II these results and their interpretation are discussed column by column in the following. L:CL represents the mole ratio of lecithin to cardiolipin and, as in the control lipids above, determines the surface charge density, σ_l, of the phospholipid *before* interaction with the protein. Both σ_l and Å²/charge are given in the table and the series represents model membranes of increasing acidic phospholipid content. d represents the repeat distance d, in ångstroms, of the lamellar phase of the precipitate. With no acidic lipid, *i.e.*, with lecithin alone, no insulin is bound and only the maximally swelled lecithin lamellar phase is observed where d = 59.5 Å. For L:CL from 97:3 to 50:50 a pure lamellar phase, characterized by two to five sharp reflections, is formed whose repeat distance d is constant at about 69 Å as the proportion of CL in the lipid increases (Figure 1). c_l, c_p, c_w, and ϕ_l, ϕ_p, ϕ_w represent the weight and volume concentrations of the three components of the precipitate. As the cardiolipin content increases the proportion of lipid in the precipitate remains constant but proportionally more protein is bound and less water is present. CL:insulin represents the mole ratio of cardiolipin to insulin in the precipitates or rather the number of negative charges in the lipid mixture per insulin molecule. As σ_l increases both more protein is bound and the CL:insulin ratio increases to about 5 (Figure 1). This value agrees with the value of approximately +5 for the charge on the insulin molecule at pH 3.0 (Tanford and Epstein, 1954). d_l, d_p, d_w represent the partial thicknesses of lipid, protein and water layers *if* these three components form three separate layers containing only, and all of, the one component. Consideration of d_l alone shows both that it is the same as d_l of the control lipid without protein and that it remains approximately constant over the whole range of L:CL (Figure 1). Therefore unless it is assumed that the phospholipid molecules, or a portion of them, change shape from what they are in the control lipid bilayer, the constancy of d_l means that the structure of the bilayer does not change, even in thickness, when insulin, even in widely varying amounts, is electrostatically bound to it under the conditions of this experiment. If, for example, it is assumed that some of the protein did penetrate into the lipid layer then it would have to be assumed as well that the lipid molecules must change shape, becoming longer on the average, and that as the amount of protein increases the elongation of the lipid molecules increases. Although this is a possibility, without further evidence it is rejected only because it is an unnecessarily complicated way of explaining the results. We therefore interpret the constancy of d_l as meaning that the insulin simply "coats" the normal lipid bilayer.

Consideration of d_p and d_w shows that these systematically increase and decrease, respectively, as the proportion of CL in the lipid increases (Figure 1). However, considering the low values of d_p and the fact that the insulin molecule, if spherical, would have a diameter of 24 Å, it is possible to conclude that the protein and water do not form separate layers. Both must occupy the same plane at least to some extent, and in fact ($d_p + d_w$) (Table II) is approximately constant at 31 Å except for a slight increase at the higher L:CL ratios. From Table II, as the CL content goes up and more protein is bound, the weight per cent of protein in this combined layer goes from 7.8 to 37.0, while the water goes from 41.0 to 14.9, *i.e.*, protein "replaces" water.

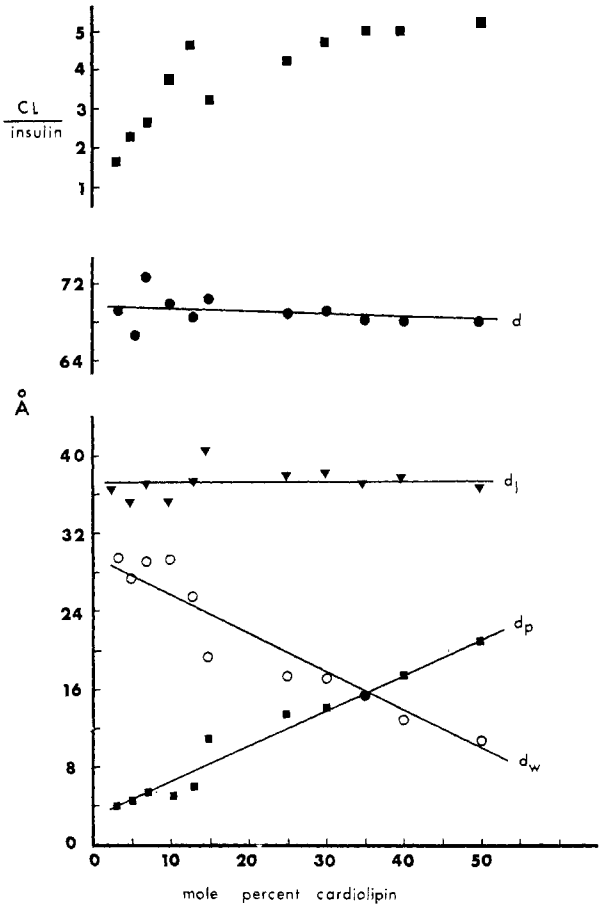

FIGURE 1: Plots of the structural parameters, given in Table II, of the phospholipid–insulin lamellar precipitate. The total repeat d and the partial thicknesses of lipid, protein, and water, d_l, d_p, and d_w, are given as a function of the mole percent of the charged lipid, cardiolipin, in the original phospholipid mixture. The cardiolipin:insulin ratio approaches a value of approximately 5 as more protein is bound to the lipid.

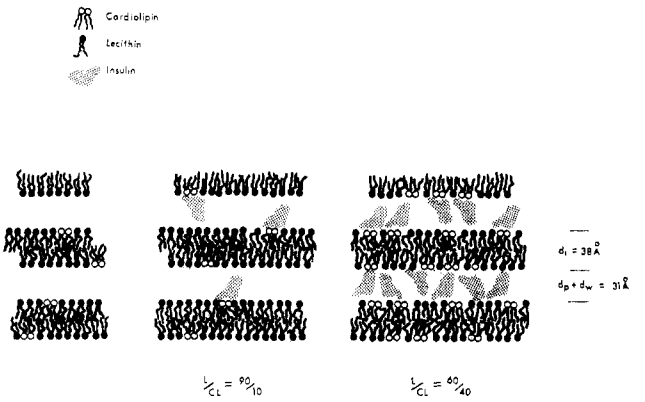

FIGURE 2: Left: the molecular arrangement of the cardiolipin–lecithin mixtures in water. Center and right: the molecular arrangement of lecithin–cardiolipin–insulin lamellar precipitates at two lecithin–cardiolipin ratios 90:10 and 60:40. When protein is bound to the phospholipid it does not change the thickness of the bimolecular lipid layer, therefore interacting only with the polar groups of the lipid molecules, and as more protein is bound to the lipid it simply replaces water in the interbilayer space, becoming more densely packed on the lipid surface.

Therefore these results are interpreted as showing that as more protein is bound even to the point where little more can be accommodated (see below), it displaces water between the phospholipid bilayers without changing the thickness of either the lipid or the protein–water layers, and the final lipid–protein interaction is with the polar groups only.

Area/insulin gives the area on the surface of the lamellar sheet that is available to each protein molecule. As more protein is bound its surface density increases to a value where at L:CL = 50:50 there is 338 Å^2 available to a volume of protein equal to the volume of one insulin molecule. A spherical insulin molecule would have a cross-sectional area of 435 Å^2. Therefore although the insulin molecules cannot be spherical when L:CL = 50:50, without some other measure of protein conformation, the degree of nonsphericity, or of unfolding, or of whether the molecular shape changes with the changing CL content, cannot be estimated. (The invariance of $(d_p + d_w)$ makes the latter possibility unlikely.) The low values of $(d_p + d_w)$ show that the protein does not form two continuous separate layers, within the protein–water layer, each attached to a layer of polar groups of the phospholipids. The thickness of each such layer would be $0.5d_p$.

Figure 2 represents a scale drawing of the molecular arrangement within the lipid–protein structure, incorporating the points discussed above. The protein molecules are neither spherical nor very largely unfolded to form two layers but are attached to the polar groups of the lipid molecules on one side or the other of the protein–water layer (but attachment to both sides by one molecule is not precluded).

Discussion

It is expected that under the conditions of this experiment wherein the lipid and protein have opposite charges, electrostatic interactions would predominate. Although this type of interaction constitutes the initial contact between the components, this does not preclude subsequent interactions from occurring, such as hydrophobic interactions between the protein and hydrocarbon chains of the phospholipids, as has been observed with other proteins (Gulik-Krzywicki *et al.*, 1969; Rand, 1971). However, even though insulin does have hydrophobic properties that lead to its self-association (Rees and Singer, 1956; Jeffrey and Coates, 1966), the only interaction with lipid that appears in this study is with the polar groups of the lipid molecules. Hence the insulin–lecithin–cardiolipin lamellar complex forms the classical Davson–Danelli model of membranes where the protein, through polar bonding, coats the lipid bilayer. In support of strictly electrostatic binding, preliminary observations indicate that Ca^{2+} can displace protein from the preformed complex.

Since the kinds of interaction that can occur between lipids and proteins depend on the specific components and on the conditions of combination, the interactions that might occur between insulin and other phospholipids and under different conditions of pH, etc., cannot be predicted and it is impossible at this stage to conclude that insulin makes only electrostatic interactions with phospholipid. However it is likely that electrostatic interactions may be required to get the lipid and protein components close enough for other shorter range interactions to occur (lecithin binds no insulin in this study). At physiological pH's where both the insulin and the lipid have net negative charges it may well be that divalent cations are required to form the initial contact; such a system is presently under study.

It has been amply demonstrated that the degree of lipid–protein interaction depends upon those factors that affect the

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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This work was supported by Public Health Service Research Grant No. CA11032 from the National Institutes of Health, and by the Consiglio Nazionale della Ricerche.