Biochemistry 10, 2606.
Gibson, D. M., and Dixon, G. H. (1969), *Nature (London) 222*, 753.
Gross, J. (1958), *J. Exp. Med. 107*, 247.
Hartley, B. S. (1964), *Nature (London) 201*, 1284.
Hummel, B. C. (1959), *Can. J. Biochem. Physiol. 37*, 1393.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 143*, 265.
Markland, F. S., and Smith, E. L. (1967), *J. Biol. Chem. 242*, 5198.
Moore, S. (1963), *J. Biol. Chem. 138*, 235.
Nagai, Y., Gross, J., and Piez, K. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 494.
Nagai, Y., Lapiere, C. M., and Gross, J. (1966), *Biochemistry 5, 3123*.
Neurath, H., Bradshaw, R. A., and Arnon, R. (1970), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., New York, N. Y., Academic Press, p 113.
Neurath, H., Walsh, K. A., and Winter, W. P. (1967), *Science 158*, 1638.
Ouchterlony, O. (1958), *Progr. Allergy 5*, 2.
Pétra, P. H., and Neurath, H. (1969), *Biochemistry 8*, 2466.
Prahl, J. W., and Neurath, H. (1966), *Biochemistry 5*, 2131.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.
Smith, E. L., Markland, F. S., and Glazer, A. N. (1970), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., New York, N. Y., Academic Press, p 160.
Smith, R. L., and Shaw, E. (1969), *J. Biol. Chem. 244*, 4704.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Tokoro, Y., Eisen, A. Z., and Jeffrey, J. J. (1972), *Biochim. Biophys. Acta 258*, 289.
Walsh, K., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247*, 3242.
Winter, W. P., and Neurath, H. (1970), *Biochemistry 9*, 4673.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zwilling, R., Pfleiderer, G., Sonneborn, H. H., Kraft, V., and Stucky, I. (1969), *Comp. Biochem. Physiol. 28*, 1275.

A Complex of Cytochrome *c* and Mixed Mitochondrial Phospholipids†

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ABSTRACT: The interaction of cytochrome *c* with mixed heart mitochondrial phospholipids has been studied as a model for cytochrome *c in vivo*. Under the conditions of our experiments, cytochrome *c* forms a complex with mixed phospholipids which is insoluble in water but can be solubilized with sodium deoxycholate. There is no preferential binding of any component of the phospholipid mixture by cytochrome *c*. The complex has an uncorrected sedimentation coefficient of 0.1 S in deoxycholate and is essentially homogeneous on ultracentrifugation and electrophoresis. Phosphorus analysis of the complex indicates that there are approximately 42 molecules of phospholipid per molecule of cytochrome *c*. The 695-nm absorbance band of ferricytochrome *c* is retaine in the complex indicating the integrity of the methionine-80–heme iron bond. Compared to cytochrome *c* the complex is stabilized against thermal denaturation in deoxycholate solution, monitored by the 695-nm absorbance band. The complex and cytochrome *c* display similar susceptibility to denaturation under conditions of high pH. There is a close correlation between solvents which extract cytochrome *c* from mitochondria and those which solubilize the complex. These results indicate the applicability of the cytochrome *c*–phospholipid complex as a model for cytochrome *c* in the mitochondrion.

Cytochrome *c* performs its function of biological electron transport in the mitochondrion where it is presumably complexed to phospholipids (Ambe and Crane, 1959; Machinist *et al.*, 1962), or to mitochondrial structural protein (Edwards and Criddle, 1966) and to cytochrome oxidase (Nicholls *et al.*, 1969).

In contrast to the other cytochrome components of the respiratory chain, cytochrome *c* is readily extractible from mitochondria with 0.15 M KCl (Jacobs and Sanadi, 1960). There has been much speculation concerning the existence of lipid–cytochrome *c* interactions in the mitochondrion and the role of lipid in the mechanism of action of cytochrome *c* (Ambe and Crane, 1959; Machinist *et al.*, 1962).

A number of attempts have been made to obtain an understanding of the environment of cytochrome *c in vivo* by studying complexes of phospholipid and cytochrome *c in vitro* (Reich and Wainio, 1961; Das and Crane, 1964; Das *et al.*, 1965; Kimelberg *et al.*, 1970; Sun and Crane, 1969; Quinn and Dawson, 1969). The majority of these studies have, however, involved complexes formed with a single phospholipid (*e.g.*, phosphatidylethanolamine) isolated from a source such as egg yolk which is not a site of action of

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cytochrome *c* (Reich and Wainio, 1961) and where the degree of saturation of the fatty acids differs markedly from that of mitochondrial phospholipids (Sorland, 1963). The phospholipid components of mammalian heart mitochondria, a frequent source of cytochrome *c* for such studies, comprise mainly phosphatidylethanolamine, phosphatidylcholine, and cardiolipin (Fleischer and Rouser, 1965), and very few studies have been made on the complex between a mixture of these phospholipids and cytochrome *c* (Das and Crane, 1964; Sun and Crane, 1969). An investigation of the properties of cytochrome *c* complexed to a mixture of phospholipids which are of identical composition with those present in the mitochondrion should provide a valuable insight into the behavior of the protein in an environment which may be a model for the native environment of the protein. Such a study appeared to be of value particularly in view of our earlier observation that lipids stabilize the cytochrome *c* molecule against denaturation by hydrophobic reagents (Kaminsky *et al.*, 1972b). In this paper we report on the preparation, characterization, and some physicochemical properties of a complex of cytochrome *c* with mixed heart mitochondrial phospholipids and in a subsequent paper (Kaminsky *et al.*,[1] 1973) we shall report on the functional properties of this complex.

Experimental Procedure

Materials. Total mitochondrial phospholipid was extracted from beef heart according to the procedure of Marinetti *et al.* (1958) with the following modifications. The cubed beef heart (1 kg) was homogenized in 3 l. of 0.25 M sucrose for 80 sec at high speed in a Waring Blendor. The isolated mitochondria were homogenized in 400 ml of 0.15 M potassium chloride for 6 min also at high speed. Repeated ether extraction and acetone precipitation were used to separate and purify the phospholipid fraction (Hanahan *et al.*, 1957). The phospholipids were stored under acetone at −25° under 1 atm of nitrogen. Cytochrome *c* (grade I, 95% pure, 0.425% Fe) was purchased from Miles-Servac, Maidenhead, England. It was oxidized with potassium ferricyanide or reduced with sodium dithionite or ascorbic acid and chromatographed on Sephadex G-25 to remove oxidant or reductant. The concentration of cytochrome *c* was determined *via* the pyridine hemochrome, $E^{1\text{ cm}}_{550\text{ nm}} = 2.91 \times 10^4$ (Norton, 1958), or by the absorbance of the fully oxidized protein at 530 nm, $E^{1\text{ cm}}_{530\text{ nm}} = 1.12 \times 10^4$ (Margoliash and Frohwirt, 1959). Sodium deoxycholate (for bacteriology) was purchased from Merck. Water was distilled and deionized.

Methods. The complex of cytochrome *c* and total mitochondrial phospholipid was formed by mixing a solution of 10 mg of oxidized cytochrome *c* with a sonicated suspension of 15 mg of purified phospholipid each in approximately 5 ml of water at pH 5. Complex formation was indicated by a red flocculent precipitate, which was collected by centrifugation at 1300*g* for 5 min. Excess lipid and protein were removed by washing the precipitate twice with dilute HCl, pH 5.0. The freshly prepared complex was routinely solubilized in 0.025 M sodium deoxycholate (Reich and Wainio, 1961), pH 8.2–8.4, and the resulting solution was filtered through a 0.45-μ Millipore filter.

Thin layer chromatography of phospholipids, before and after complex formation, was performed on Merck nonfluorescent silica gel plates (20 cm × 20 cm × 0.25 mm). The plates were heated at 120–130° for 20 min and then cooled for 30 min; the samples were applied and the chromatography was begun immediately. Approximately 100 μg of phospholipid or 150 μg of complex was applied. Chromatography was performed in one of the following solvents: A, chloroform–acetone–methanol–acetic acid–water (10:4:2:2:1, v/v); B, chloroform–methanol–28% aqueous ammonia (65:35:5, v/v) (Fleischer *et al.*, 1967); C, chloroform–methanol–water (65:25:4, v/v); or D, *n*-butyl alcohol–acetic acid–water (60:20:20, v/v) (Rouser *et al.*, 1964). The plates were air-dried. For quantitative determination of phospholipids, the plates were developed in solvent A or B and sprayed with the Molybdenum Blue reagent (Skipski and Barclay, 1969), and the results were quantitated using a Joyce, Loebl & Co. Chromoscan densitometer. For qualitative determinations, spots were visualized with iodine vapor, ninhydrin, Dragendorff reagent, periodate–Schiff reagent, and sulfuric acid–acetic acid reagent (Skipski and Barclay, 1969).

TABLE I: Reagents for the Characterization of Lipids (Skipski and Barclay, 1969).[a]

Reagent	Test for	Results of Test on Mixed Phospholipids
Ninhydrin	Phosphatidylethanolamine (free NH_2 groups)	+
Periodate–Schiff	Phosphatidylinositol (α-diglycols)	−
Dragendorff	Phosphatidylcholine	+
CH_3CO_2H–H_2SO_4	Cholesterol and its esters	−
Molybdenum Blue	Phospholipids	+
H_2SO_4–Cr_2O_7	Carbon compds	+
I_2	Unsaturated compds	+

[a] The reagents were sprayed onto thin layer chromatograms.

For phosphorus analysis and identification of individual phospholipids, bands were eluted from developed chromatograms with chloroform–methanol (2:1, v/v) saturated with water. Analysis of the phosphorus content of samples was performed by the method of King (1932) in the absence of hydrogen peroxide.

Infrared spectra of KBr disks containing approximately 1.5% (w/w) phospholipid were determined with a Beckman IR-12 spectrophotometer calibrated against CO_2 and H_2O vapor. Spectra were compared with those of Rouser *et al.* (1963).

The pH and temperature dependence of the 695-nm absorbance band of oxidized cytochrome *c* and the oxidized complex were monitored with a Beckman DB-GT spectrophotometer, and a W and W 1100 recorder. Temperature was monitored with a Yellow Springs Instrument Co. telethermometer.

Ultracentrifugation experiments were performed at room temperature in a Beckman Model E ultracentrifuge with an AN-D two-cell rotor at 56,000 rpm. Samples of cytochrome *c*, phospholipid, or complex were solubilized in 0.025 M sodium deoxycholate, pH 8.3, at concentrations of 0.1–0.5% w/w (1–5 mg/ml). In one experiment the complex was dispersed in water by ultrasonication. The complex was freshly pre-

[1] Kaminsky, L. S., Henderson, J. J., and Ivanetich, K. M. (1973), manuscript in preparation.

TABLE II: Phospholipid Composition of Mixed Beef Heart Mitochondrial Phospholipid and of the Complex of Cytochrome *c* with These Phospholipids.[a]

Source	Solvent for Tlc	% of Total Phosphorus			% of Total Phospholipid		
		CL[b]	PE	PC	CL	PE	PC
Phospholipid[f]		20.0	36.6	40.4	11.5	42.0	46.5
Phospholipid[g,h]		17.6	41.6	40.7	9.7	45.7	44.8
Phospholipid	A	17.2 ± 2.4	35.9 ± 4.0	46.5 ± 3.5	9.5	39.4	51.1[c]
Phospholipid	B	15.5 ± 2.6	37.8 ± 2.6	46.9 ± 4.4	8.4	40.8	50.7[c]
Complex	A	15.6 ± 5.0	33.8 ± 4.3	48.6 ± 5.6	8.6	37.5	54.0[d]
Complex	B	15.5 ± 2.1	36.7 ± 5.1	46.0 ± 1.7	8.6	40.6	50.8[c]
Complex[i]		15	41	26[e]			

[a] Assays were performed using the Molybdenum Blue reagent on thin layer chromatograms of the phospholipids. The solvent systems are described in the text. [b] Abbreviations used in the table are: CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine. [c] Average of five determinations. [d] Average of seven determinations. [e] Also contained 13% phosphatidylinositol, 5% phosphatidic acid. [f] Fleischer *et al.*, 1967. [g] Fleischer and Rouser, 1965. [h] Rouser *et al.*, 1964. [i] Das and Crane, 1964.

pared immediately before each experiment. All solutions were filtered through 0.45-μ Millipore filters prior to ultracentrifugation. The schlieren optical system was used with Kodak high-speed infrared film type 135-20 or Kodak type 1-N spectroscopic plates and a Wratten No. 25 red filter.

Low voltage electrophoresis of samples of phospholipid or complex, dispersed by ultrasonication or solubilized in 0.025 M sodium deoxycholate, was performed on cellulose acetate strips in 0.02 M sodium phosphate buffer, pH 7.2, using a Pleuger Power Supply type CVC-D. The protein was located with 0.2% Amido Black in 2% acetic acid and the phospholipid with the Molybdenum Blue reagent.

Results

The mixed heart mitochondrial phospholipids from a number of beef hearts consistently showed three components on thin layer chromatography in the four solvent systems. Phosphatidylcholine, phosphatidylethanolamine, and cardiolipin were characterized with the color reagents shown in Table I and by their infrared spectra. No trace of phosphatidylinositol or cholesterol or its esters was noted. The quantities of each phospholipid present are given in Table II and are shown to be similar to previously published values. There is an apparent error in the literature concerning the assay for cardiolipin. Cardiolipin contains two phosphorus atoms, and consequently assays for this phospholipid relying on an analysis of phosphate or the molybdenum spray reagent give values twice as great as the actual amount of cardiolipin. Similarly, since cardiolipin contains twice as many carbon atoms as either phosphatidylcholine or phosphatidylethanolamine its analysis using charring methods again yields values twice as large as the amount of cardiolipin. This was confirmed by elution of the bands from the chromatograms and subsequent phosphorus analysis which yielded values of 32.2% for phosphatidylcholine (39.6% P), 59.2% for phosphatidylethanolamine (54.6% P), and 8.6% for cardiolipin (15.6% P).

Thin layer chromatography of the purified complex yielded the same three components as the phospholipid mixture with virtually identical R_F values and with very similar relative concentrations (Table II). The cytochrome *c* component of the complex remained at the origin of the thin layer plates.

Phosphorus analysis of the complex yielded a value of 0.112 mg of P/mg of cytochrome *c*, which represents the binding of 44 atoms of phosphorus per molecule of cytochrome *c* equivalent to 42 molecules of phospholipid per molecule of cytochrome *c*.

TABLE III: Solubility of the Cytochrome *c*–Phospholipid Complex and Its Components in Aqueous and Nonaqueous Solvents.[a]

Solvent	Solubility		
	Complex	Cytochrome	Phospholipids[b]
H_2O or 1 M HCl	−	+	−
Isooctane	∓	−	−
$CHCl_3$–CH_3OH (4:1)	+	−	+
$(CH_3)_2C{=}O$ or 95% C_2H_5OH	−	−	−
$(C_2H_5)_2O$	+	−	−
0.025 M sodium deoxycholate			
pH 5	−		−
pH 8.3	+	+	+
pH 11	+	+	+
0.05 M sodium dodecyl sulfate, pH 7.3	+	+	
1% Triton N-101, pH 4–5	−		
5% Triton N-101, pH 4–5	±		

[a] Solubility was determined visually and spectrophotometrically where: + indicates a concentration of cytochrome *c* or complex $>1 \times 10^{-5}$ M, − indicates $<5 \times 10^{-6}$ M, and ± indicates $\sim 8 \times 10^{-6}$ M. [b] Mixture isolated from beef heart mitochondria.

The solubility properties of the complex are summarized in Table III. The complex is insoluble in water but is solubilized by sodium dodecyl sulfate, sodium deoxycholate, and to a lesser extent by Triton N-101. The complex differs from both its constituents in that it is soluble in diethyl ether. Unlike many other cytochrome *c*–phospholipid complexes, this complex is only very slightly soluble in isooctane (Das and Crane, 1964; Das *et al.*, 1965).

The 695-nm absorbance band of ferricytochrome *c* was

TABLE IV: A Comparison of the Effect of Solvents in Extracting Cytochrome *c* from Mitochondria and in Solubilizing the Cytochrome *c*–Phospholipid Complex.[a]

Solvent Conditions	Solubility of Complex	Extraction of Cyt *c* from Mitochondria
0.15 M KCl	+	+[b]
0.3% $Al_2(SO_4)_3 \cdot 17H_2O$	+	+[c]
0.5 M CH_3CO_2H, 5°, 1 hr	−	−[d]
0.5 M CH_3CO_2H, 5°, 1 hr, adjust pH to 6.3 with 2 M NH_4OH, 5° overnight	±	+[d]
0.145 M Cl_3CCO_2H, 3–4 hr	−	±[e]
Adjust pH of filtrate to 7.3 with 10% NaOH	±	+[e]
20% sucrose–0.25% saponin	−	−[f]
20% sucrose–0.25% saponin + acetone	−	−[f]
20% sucrose–0.25% saponin + 0.1 M NaCl	−	+[f]
0.1 M NaCl	±	+[f]
0.05 M glycylglycine–H_2O (1:1)	−	−[g]
0.05 M glycylglycine–0.5% sodium deoxycholate (1:1)	+	+[g]
0.3 ml of 0.02 M Tris buffer, pH 8	−	−[h]
0.3 ml of 0.02 M Tris buffer, pH 8 0,18 ml of 20% cholate, 0.42 ml of 10% sodium deoxycholate, 0.1 ml of saturated $(NH_4)_2SO_4$, mix 1:1 with 95% C_2H_5OH	+	+[h]

[a] Solubilization of the complex may involve disruption of the complex and solubilization of the chytochrome *c* or solubilization of the complex itself. Solubility was determined visually and spectrophotometrically. The signs +, −, and ± are as for Table III. [b] Jacobs and Sanadi, 1960. [c] Margoliash and Walasek, 1967. [d] Hagihara *et al.*, 1958. [e] Keilin and Hartree, 1952. [f] Morrison *et al.*, 1960. [g] Ball and Cooper, 1959. [h] Blair *et al.*, 1963.

retained on complex formation. In Figure 1 the effect of temperature on the 695-nm absorbance of a deoxycholate solution of the complex is compared with the effect of temperature on cytochrome *c* in a solution of the same concentration of deoxycholate and in aqueous solution. The transition temperatures (temperature corresponding to the absorbance value midway between the maximal and minimal absorbance values) were 31° for the complex, 25° for cytochrome *c* in deoxycholate, and 40° for cytochrome *c* in aqueous solution. The data shown in Figure 1 were used to calculate the equilibrium constants for the liganding of methionine-80 to the heme iron of ferricytochrome *c*. Calculation of the equilibrium constants is based on the following assumptions. The methionine-80–heme iron bond is fully formed when the 695-nm band is fully developed and the bond is fully dissociated when this absorbance band is fully quenched. Plots of the logarithm of the equilibrium constants against the inverse of the absolute temperature were biphasic, and separate values were calculated for the enthalpy changes

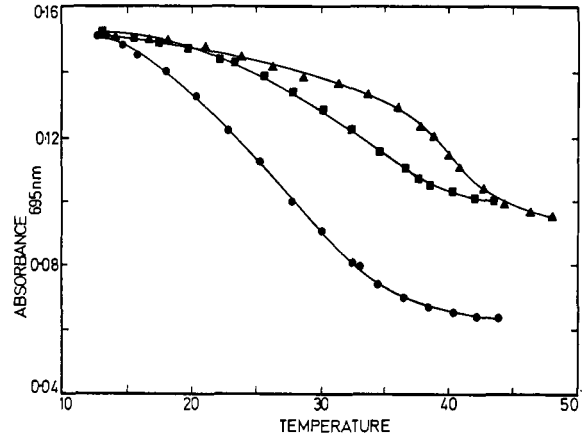

FIGURE 1: The effect of temperature on the 695-nm absorbance of: (▲) cytochrome *c* in phosphate buffer; (●) cytochrome *c* in 0.025 M deoxycholate; (■) cytochrome *c*–phospholipid complex in 0.025 M deoxycholate, pH 8.3.

at the high- and low-temperature ranges. For the deoxycholate-induced solution of the complex, ΔH° = 37.1 and 84.3 kcal/mol; for cytochrome *c* in deoxycholate solution, ΔH° = 39.2 and 99.1 kcal/mol; and for cytochrome *c* in aqueous solution at the same pH and ionic strength as the other solutions, ΔH° = 23.5 and 47.2 kcal/mol.

The influence of pH on the 695-nm band of the complex is compared with that of cytochrome *c* in Figure 2. The extinction coefficients at 695 nm of the complex are higher than those of cytochrome *c* at all pH values studied, and the two undergo a similar bleaching of absorbance at higher pH values. One value for the extinction coefficient at 695 nm of cytochrome *c* was determined in a 0.025 M deoxycholate solution; at pH 8.5 the extinction coefficient of this solution at 695 nm was 480.

Cytochrome *c* can be extracted from mitochondria with a number of different solutions. The effect of these solutions on the lipid–cytochrome *c* complex was investigated, and the results are shown in Table IV. A positive result indicates that either the complex or the cytochrome *c* from the complex has

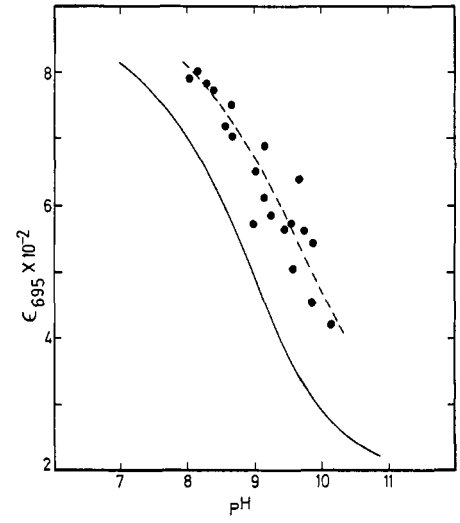

FIGURE 2: The effect of pH on the extinction coefficient at 695 nm of: (—) cytochrome *c* in water; (●----●) cytochrome *c*–phospholipid complex in 0.025 M deoxycholate, 25°.

TABLE V: Spectral Properties of Cytochrome *c* and the Cytochrome *c*–Phospholipid Complex.

Sample/Solvent	pH	Oxidized Spectrum			Reduced Spectrum			
		$\frac{A_{Soret}}{A_{530}}$	λ_{max}, Soret	$\epsilon \times 10^5$, Soret	$\frac{A_{550}}{A_{520}}$	$\frac{A_{Soret}}{A_{550}}$	$\frac{A_{550}}{A_{535}}$	λ_{max} Soret
Complex/0.025 M sodium deoxycholate[c]	8.3	9.8	410	1.11	1.6	3.1	5.0	417
Complex/0.025 M sodium deoxycholate	11.0	8.5	407					
Cytochrome *c*/0.025 M Na_2HPO_4	8.4	9.3	410	1.06	1.7	3.8	4.8	416
Cytochrome *c*/0.025 M sodium deoxycholate	8.4	10.2	410	1.13				
Cytochrome *c*/0.025 M sodium deoxycholate	10.5	11.5	407	1.06	1.8	3.8	4.7	416
Complex/$(C_2H_5)_2O$		~10	409					
Complex[a]/isooctane		8.4	410					
Complex/isooctane		9.1	410					
Complex/H_2O[b]	6.5–9.0		410					
Complex/H_2O[b]	11.0		408					

[a] Complex prepared by the method of Das and Crane (1964): mix 6 mg of cytochrome *c* per 2 ml of H_2O with 4.5 ml ol 65% ethanol and 15 mg of phospholipid sonicated per 2 ml of H_2O; dilute to 15 ml with water; add 15 ml of isooctane; shake 15 min; centrifuge at 2000 rpm for 5 min to collect the complex. [b] Sonicated. [c] Complex, cytochrome *c*–mitochondrial phospholipid complex.

been solubilized, as evidenced by the development of a pink coloration. A very close correlation was observed between the solubilization of the complex and the extraction of cytochrome *c* from mitochondria.

In Table V the spectral characteristics of the complex are compared with those of cytochrome *c*.

The results of the ultracentrifugation studies indicate that the complex of cytochrome *c* and phospholipids is intact when solubilized by 0.025 M sodium deoxycholate at pH 8.3. The complex has an uncorrected sedimentation coefficient of 0.1 S and sediments less rapidly than either cytochrome *c* (1.0 S) or phospholipid (0.5 S) under the same conditions. In some experiments ultracentrifugation of the solubilized complex yielded rapidly sedimenting peaks characterized by sedimentation coefficients varying between approximately 9 and 20 S. These peaks, which represent less than 3% of the total sample, could result from either aggregation of the complex or the presence of well-dispersed but incompletely solubilized material.

The integrity of the sonicated or solubilized complex was also demonstrated by the results of low voltage electrophoresis experiments. The solubilized complex was less mobile than the phospholipid fraction and more mobile than cytochrome *c* in sodium deoxycholate solution. The ultrasonically dispersed complex remained at the origin in contrast to the ultrasonicated phospholipid mixture or cytochrome *c*. Prolonged electrophoresis at low voltage does, however, result in the partial dissociation of the complex.

Discussion

It is evident from our electrophoresis and ultracentrifugation data that ferricytochrome *c* interacts with mixed beef heart mitochondrial phospholipids to produce an essentially homogeneous complex under the conditions of our experiments. The ratio of the phospholipids in the complex is identical with that isolated from the mitochondria indicating that there is no preferential binding of any component of the phospholipid mixture by cytochrome *c*. Our findings are not, however, necessarily significant with regard to the binding of cytochrome *c* in the mitochondrion since we do not know whether all three phospholipids are present, and in what ratio, at the cytochrome *c* binding site. These results are not in accord with the observation by Reich and Wainio (1961) that only the phosphatidylethanolamine fraction from a mixture of mitochondrial phospholipids was effective in binding to cytochrome *c*. A cytochrome *c*–total mitochondrial phospholipid complex prepared by Das and Crane (1964) was reported to contain phosphatidylinositol and phosphatidic acid in addition to the three phospholipids found in our complex. These compounds were identified only by R_F values and probably reflect decomposition products of phosphatidylethanolamine or cardiolipin known to occur with the silica gel chromatography technique used, rather than the presence of these lipids which are not normally found in beef heart mitochondrial phospholipids (Fleischer and Rouser, 1965; but *cf.* Awasthi *et al.*, 1971).

Reich and Wainio (1961) proposed that the bile salts sodium cholate and sodium deoxycholate displace phospholipid molecules from the complex. That this does not occur to any large extent is suggested by our low-voltage, short term electrophoresis experiments which reveal no displacement of lipid from the complex in the presence of deoxycholate. In our opinion the data of Reich and Wainio (1961) could be interpreted as a solubilization of the complex rather than of the phospholipids from the complex. Our ultracentrifugation experiments indicate that in contrast to many lipoproteins which float on centrifugation, the complex of cytochrome *c* with phospholipid sediments slowly. The sedimentation coefficient of the complex (0.1 S) compared with that of cytochrome *c* in sodium deoxycholate (1.0 S) or with the phospholipids in sodium deoxycholate (0.5 S) may indicate the presence of not only phospholipid but also deoxycholate in the complex, since deoxycholate also lowers the uncorrected sedimentation coefficient of cytochrome *c* from approximately 1.9 S in aqueous solution (Margoliash and Schejter, 1966)

to 0.5 S in 0.025 M sodium deoxycholate. The presence of a single peak and the absence of any peak corresponding to cytochrome*c* –sodium deoxycholate indicate the integrity of the complex under the conditions of the experiments.

The lack of solubility of the complex in water and the requirement for a detergent to achieve solubilization make valid comparisons of the complex with cytochrome *c* difficult. In most cases we have used cytochrome *c* in both buffer and in detergent solution to effect comparisons with the complex.

The 695-nm absorbance band of ferricytochrome *c* has been assigned to a charge transfer complex of methionine-80 and the heme iron (Eaton and Hochstrasser, 1967). The absorbance at 695 nm is a measure of the integrity of this bond and is readily quenched by denaturants which produce a dissociation or a weakening of the bond (Schejter and George, 1964; Sreenathan and Taylor, 1971). In the mitochondrion, where cytochrome *c* performs its function of biological electron transport, this absorbance band persists (Chance *et al.*, 1968). In view of the lability of the methionine–iron bond (Schejter and George, 1964; Wilson and Greenwood, 1971; Kaminsky *et al.*, 1972a) and its significance for biological activity of cytochrome *c* (Chance *et al.*, 1968), its retention in the complex supports the applicability of the complex as a model for endogenous cytochrome *c*. Our studies on the effects of thermal changes on the 695-nm absorbance band reveal that sodium deoxycholate considerably destabilizes cytochrome *c*, as evidenced by the lowering of the transition temperature, and that the lipid–protein interaction of the complex causes a partial reversal of this destabilization. This reversal probably results from the partial exclusion of the detergent from interactions at the protein surface by the lipids of the complex. The decreased stability of the complex in deoxycholate solution relative to cytochrome *c* in aqueous solution could possibly be a consequence of a slight weakening of the heme crevice by the lipids or more probably of the incomplete elimination of the detergent from the protein surface. The ΔH° increase, associated with the decrease in stability, in the order aqueous cytochrome *c* solution, deoxycholate complex solution, and deoxycholate cytochrome *c* solution is consistent with our earlier study of the thermodynamics of the heme crevice opening of cytochrome *c* (Kaminsky *et al.*, 1973[2]). The thermal denaturation of cytochrome *c* is accompanied by an unfavorable enthalpy change which becomes more unfavorable as the detergent enhances the thermal denaturation.

In contrast to the thermal effects the phospholipid–protein interactions completely protect the cytochrome *c* from deoxycholate induced susceptibility to denaturation by conditions of high pH. Throughout the pH range of our investigation the extinction coefficient of the complex was slightly higher than that of the native protein. This may result from the displacement of the equilibrium to a more fully liganded form of the heme iron.

The effect on the complex of a series of solvents, previously shown to be responsible for the extraction of cytochrome *c* from mitochondria, could be a consequence of disruption of the complex with subsequent solubilization of the cytochrome *c* or of the solubilization of the complex itself. We have not distinguished between these two possibilities, but in either case the close correlation between the extraction of cytochrome *c* from mitochondria and solubilization of the complex by the various solvents is consistent with the applicability of a cytochrome *c*–mitochondrial phospholipid complex as a model for cytochrome *c in vivo*.

[2] Kaminsky, L. S., Miller, V. J., and Davison, A. J. (1973), manuscript submitted for publication.

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References

Ambe, K. S., and Crane, F. L. (1959), *Science 129*, 98.
Awasthi, Y. C., Chuang, T. F., Keenan, T. W., and Crane, F. L. (1971), *Biochim. Biophys. Acta 226*, 42.
Ball, E. G., and Cooper, O. (1959), *J. Biol. Chem. 226*, 755.
Blair, P. V., Oda, T., Green, D. E., and Fernandez-Moran, H. (1963), *Biochemistry 2*, 756.
Chance, B., Lee, C. P., Mela, L., and Wilson, D. F. (1968), Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, University of Tokyo Press, pp 353–356.
Das, M. L., and Crane, F. L. (1964), *Biochemistry 3*, 696.
Das, M. L., Haak, E. D., and Crane, F. L. (1965), *Biochemistry 4*, 859.
Eaton, W. A., and Hochstrasser, R. M. (1967), *J. Chem. Phys. 46*, 2533.
Edwards, D. L., and Criddle, R. S. (1966), *Biochemistry 5*, 583.
Fleischer, S., and Rouser, G. (1965), *J. Amer. Oil Chem. Soc. 42*, 588.
Fleischer, S., Rouser, G., Fleischer, B., Casu, A., and Kritchevsky, G. (1967), *J. Lipid Res. 8*, 170.
Hagihara, B., Morikawa, I., Tagawa, K., and Okunuki, K. (1958), *Biochem. Prep. 6*, 1.
Hanahan, D. J., Dittmer, J. C., and Warashina, E. (1957), *J. Biol. Chem. 228*, 685.
Jacobs, E. E., and Sanadi, D. R. (1960), *J. Biol. Chem. 235*, 531.
Kaminsky, L. S., Byrne, M. J., and Davison, A. J. (1972a), *Arch. Biochem. Biophys. 150*, 355.
Kaminsky, L. S., Yong, F. C., and King, T. E. (1972b), *J. Biol. Chem. 247*, 1354.
Keilin, D., and Hartree, E. F. (1952), *Biochem. Prep. 2*, 1.
Kimelberg, H. K., Lee, C. P., Claude, A., and Mrena, E. (1970), *J. Membrane Biol. 2*, 235.
King, E. J. (1932), *Biochem. J. 26*, 293.
Machinist, J. M., Das, M. L., Crane, F. L., and Jacobs, E. E. (1962), *Biochem. Biophys. Res. Commun. 6*, 475.
Margoliash, E., and Frohwirt, N. (1959), *Biochem. J. 71*, 570.
Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 114.
Margoliash, E., and Walasek, O. F. (1967), *Methods Enzymol. 10*, 339.
Marinetti, G. V., Erbland, J., and Stotz, E. (1958), *J. Biol. Chem. 233*, 562.
Morrison, N., Hollocher, T., Murray, R., Marinetti, G., and Stotz, E. (1960), *Biochim. Biophys. Acta 41*, 334.
Nicholls, P., Mochan, E., and Kimelberg, H. K. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3*, 242.
Norton, R. M. (1958), *Rev. Pure Appl. Chem. 8*, 161.
Quinn, P. J., and Dawson, R. M. C. (1969), *Biochem. J. 115*, 65.

Reich, M., and Wainio, W. W. (1961), *J. Biol. Chem. 236*, 3058.
Rouser, G., Galli, C., Lieber, E., Blank, M. L., and Privett, O. S. (1964), *J. Amer. Oil Chem. Soc. 41*, 836.
Rouser, G., Kritchevsky, G., Heller, D., and Lieber, E. (1963), *J. Amer. Oil Chem. Soc. 40*, 425.
Schejter, A., and George, P. (1964), *Biochemistry 3*, 1045.
Skipski, V. P., and Barclay, M. (1969), *Methods Enzymol. 14*, 531.
Sorland, F. B. (1963), *Comp. Biochem. 3*, 1.
Sreenathan, B. R., and Taylor, C. P. S. (1971), *Biochem. Biophys. Res. Commun. 42*, 1122.
Sun, F. F., and Crane, F. L. (1969), *Biochim. Biophys. Acta 172*, 417.
Wilson, M. T., and Greenwood, C. (1971), *Eur. J. Biochem. 22*, 11.

Cofactor Activity of Protein Components of Human Very Low Density Lipoproteins in the Hydrolysis of Triglycerides by Lipoprotein Lipase from Different Sources†

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ABSTRACT: The protein component of very low density lipoproteins of human plasma with carboxyl-terminal glutamic acid was a potent activator of the hydrolysis of triglycerides in a lecithin-stabilized emulsion by highly purified lipoprotein lipase from human and rat post-heparin plasma and cows' milk, and by crude preparations of the enzyme from cows' milk and rat adipose tissue. The protein components with carboxyl-terminal serine and alanine also had slight but detectable activity with enzyme preparations from all sources, except that purified from milk. At high concentrations these two proteins inhibited enzyme activity. Heparin stimulated the activity of both impure and purified preparations of the lipase from cows' milk in the absence of cofactor protein and increased the sensitivity of the enzyme to stimulation by the protein component with carboxyl-terminal glutamic acid. These effects were not observed with purified lipase from rat post-heparin plasma. At high concentrations, the protein components with carboxyl-terminal serine and alanine inhibited the stimulatory activity of the component with carboxyl-terminal glutamic acid. Heparin abolished this effect in the case of the impure lipase from cows' milk, but not with the purified lipases from cows' milk and rat post-heparin plasma. Of three subfractions of the component with carboxyl-terminal alanine, one caused greater stimulation of lipoprotein lipase than the others. This difference could not be attributed to contamination of the former with the component with carboxyl-terminal glutamic acid.

Recent studies have shown that certain polypeptides common to triglyceride-rich lipoproteins (chylomicrons and VLDL)[1] and HDL of human plasma promote the hydrolysis of emulsified triglycerides by impure preparations of lipoprotein lipase in cows' milk protein and extracts of rat adipose tissue (Havel *et al.*, 1970; LaRosa *et al.*, 1970). One polypeptide, R-Glu, was particularly active in this process; a second polypeptide, R-Ala, also had some activity, but it varied among preparations. With enzyme in cows' milk, large amounts of R-Ala were inhibitory and, in addition, they inhibited activation by R-Glu (Havel *et al.*, 1970). Subsequently, evidence was presented to suggest that activity with the milk enzyme of preparations of R-Ala purified by ion-exchange chromatography results from contamination with R-Glu (Brown and Baginsky, 1972). In addition, purified preparations of lipase from post-heparin plasma of dog, rat and man were reported to be activated chiefly by a third polypeptide, R-Ser, and to a lesser extent by R-Glu, while R-Ala was inactive (Ganesan *et al.*, 1971).

We now report a comparative study of the effect of these three polypeptides upon the hydrolysis of triglycerides emulsified with lecithin by purified lipoprotein lipase from human and rat post-heparin plasma, the enzyme from rat adipose tissue and by both the crude and purified enzyme from cows' milk.

Experimental Procedure

Preparation of Lipoprotein Lipase. Lipoprotein lipase was purified from the post-heparin plasma of male human donors or male Sprague–Dawley rats by published techniques (Fielding, 1969, 1970; Nillson-Ehle *et al.*, 1971) to provide an electrophoretically homogeneous product that was free of detectable amounts of lipoprotein polypeptides (Fielding, 1969;

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[1] Abbreviations used are: VLDL, very low density lipoproteins; HDL, high density lipoprotein; R-Glu, R-Ala, R-Ser, apolipoprotein species with carboxyl-terminal glutamic acid, alanine, and serine, respectively; R_1-Ala, R_2-Ala, R_3-Ala, species of R-Ala with zero, one, and two residues of sialic acid, respectively.