Effect of Temperature on Urocanase from a Psychrophile, *Pseudomonas putida*†

Daniel H. Hug* and John K. Hunter

ABSTRACT: Urocanase from *Pseudomonas putida* was assayed for catalytic activity spectrophotometrically at different temperatures. The enzyme exhibited a marked ability to sustain its catalytic activity as the temperature was lowered from 45 to 0°. The reaction rate at 0° was 30% of the rate at 30°, the temperature optimum. This effect was observed with intact cells, crude cell extract, and purified enzyme. The Arrhenius plot was curvilinear. As the temperature of urocanase was raised, the Michaelis constant and the absorbance near 238 nm increased. These results suggest that the enzyme opposes the expected effect of temperature changes on the reaction by alterations in the shape of the protein. The faculty of urocanase from *P. putida* to function well at low temperatures is a factor in the ability of this microorganism to grow on a histidine medium at 0°.

The photoactivation of urocanase from *Pseudomonas putida* was described (Hug *et al.*, 1971). The "inactive" form of urocanase was found to be more stable to heat than the active enzyme (Hug and Roth, 1971). To compare the two forms further, we recently studied the effect of assay temperature on their catalytic activities. Although the temperature activity curves of the two enzyme forms were similar (Figure 1), the effect of temperature on enzyme activity was unusual. A surprisingly high enzyme activity persisted as 0° was approached. This type of enzyme behavior might be expected for a psychrophilic bacterium (Stokes, 1967). Psychrophiles are defined (Ingraham, 1958) as microorganisms which grow reasonably well at 0°. *P. putida* A.3.12 grew readily at 0° on histidine. The purpose of this investigation was to study the effect of temperature on urocanase, an enzyme which is needed to provide energy, carbon, and nitrogen when the organism grows on histidine. Urocanase is the second enzyme on the catabolic pathway of histidine.

Materials and Methods

General. P. putida A.3.12 was grown aerobically in a medium which contains 0.2% L-histidine. Growth conditions, irradiation by black light lamps for photoactivation, chemicals, and cell disruption have been described (Hug and Roth, 1971). The effect of temperature on growth was determined on 2% agar slants of the histidine medium by incubation in constant-temperature baths from 0 to 42°. Bovine serum albumin was the standard for protein determination (Lowry *et al.*, 1951).

Enzymes and Assays. Enzyme preparation (crude), purification, and the assay of urocanase by measurement of the absorbance of urocanate have been described (Hug and Roth, 1971). The total volume for assay was 1.0 ml in a 1.0-ml water-jacketed microcuvet instead of 3.0 ml in a standard cuvet. However, the concentrations of the components were the same. For intact cells, the assay was performed in the presence of 10 μl of cell suspension (100 mg, wet weight/ml). Spectrophotometric assay of urocanase activity in intact cells has been employed before (Schlesinger *et al.*, 1965). Crude and purified bacterial urocanase contained about 2 and 0.1 mg of protein/ml.

Crude rat liver urocanase was prepared by homogenization of 65 g of fresh rat liver for 5 min in a cold Waring Blendor with 260 ml of 0.05 M potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 1° at 1500*g* for 10 min, the supernatant fluid was centrifuged at 1° for 30 min at 22,000*g*, and the supernatant fluid (50 mg protein/ml) was stored at −20°.

Temperature. A constant-temperature bath (±0.01° control) and circulator were used to hold the water-jacketed cuvets and sample chamber at selected temperatures. In some experiments, two cuvets were maintained simultaneously at different temperatures or the temperature in one cuvet was rapidly changed (10° change in 30 sec) by appropriate connections to two water baths. The temperature was monitored with two thermistor probes in the cuvet contents when spectrophotometric measurements were made. At temperatures of 40° or higher, the reaction was initiated by addition of enzyme to avoid heat inactivation during preliminary manipulation.

The pH of phosphate buffer (7.4) changed by only 0.11 pH unit in the range from 10 to 50°. The complete reaction mixture (crude enzyme) varied by 0.08 pH unit over the same range. Thus it was unnecessary to adjust the buffer for each temperature especially because there is a broad optimum activity region from pH 7.2 to 8.0 (George and Phillips, 1970).

The extinction coefficient of the substrate, which is the basis of the assay, decreased linearly by 3.9% with increasing temperature in the range from 10 to 50° at pH 7.4. Correction, therefore, was not made for this effect.

Michaelis Constant. The Michaelis constant was determined for different temperatures. The mean of two to four assays was determined for each of 5–18 substrate concentrations. The equation of the best line of double reciprocal plots was obtained by the method of least squares. The K_m value was calculated from this equation.

Difference Spectrum. Difference spectra of purified bacterial urocanase (fraction 5, 1 ml, 0.11 mg, in each of two cuvets) were determined manually. One cuvet (the blank) and the cuvet chamber were maintained at 10°. The other cuvet was adjusted in turn to 10, 25, 30, 35, 40, and 45° and a difference spectrum was obtained at each temperature. The 10°/10°

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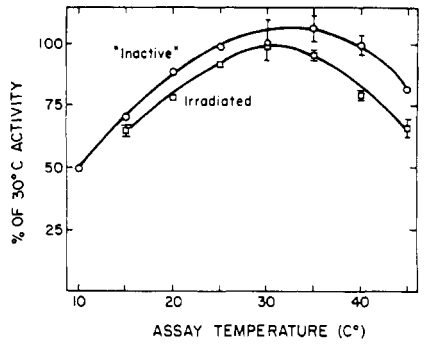

FIGURE 1: Effect of assay temperature on the catalytic activity of "inactive" and photoactivated *P. putida* urocanase: (□) photoactivated crude enzyme, 7 μl; each point is the mean of three assays; (○) "inactive" crude enzyme, 40 μl; each point is the mean of two assays. The error bars indicate the average deviation from the mean.

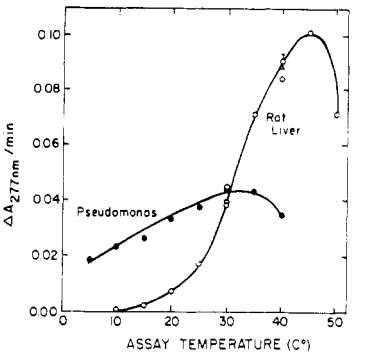

FIGURE 3: Effect of assay temperature on the catalytic activity of rat liver urocanase. Each point is the mean of three assays. The error bar (average deviation) exceeded the symbol size only once. (○) Crude rat liver urocanase, 33 μl; (●) purified *P. putida* urocanase, 33 μl, is shown for direct comparison.

spectrum (base line) was used to make minor corrections in the other spectra.

Results

Effect of Temperature on Growth. A series of slants of histidine medium inoculated with *P. putida* were incubated in an ice–water bath and in various constant-temperature baths. After 11 days, the organism had grown luxuriantly at 0, 4, 8, and 20°, slightly at 37°, but not at all at 42°. When histidine was omitted, the yeast extract in the medium supported only slight growth.

Comparison of Photoactivated and Inactive Urocanase. When the assay temperature was varied, the temperature–activity curves for the "inactive" enzyme and the photoactivated enzyme were similar (Figure 1). Because the active form was 20 times more active than the "inactive" form, the data were normalized to 100 at 30°. The Q_{10} (20–30°) was 1.3 and 1.2 for photoactivated and "inactive" forms, respectively.

Effect of Assay Temperature on Bacterial Urocanase. We assayed urocanase at different temperatures (Figure 2) in active extract from freshly grown cells, photoactivated extract, photoactivated intact cells, and photoactivated purified enzymes. These reaction rates did not double or triple for a rise of 10° as is typical of chemical reactions. Instead, these rates changed only moderately. The Q_{10} for 15–25° was 1.22 ± 0.07, for 20–30° it was 1.33 ± 0.11, and for 25–35° it was 1.08 ± 0.05 based on 10–13 experiments for each value (standard deviations given).

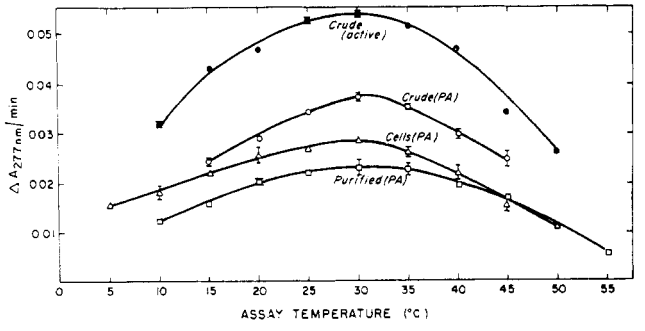

FIGURE 2: Effect of assay temperature on the catalytic activity of *P. putida* urocanase. (●) Crude active urocanase, 13 μl. The crude enzyme was prepared from freshly harvested cells containing active urocanase; ultraviolet irradiation increased its activity by only 13%; each point is the mean of three assays. One other experiment not given was essentially the same. (○) Crude photoactivated urocanase, 7 μl; each point is the mean of three assays. Four other experiments were essentially the same. (△) Intact photoactivated cells, 10 μl; each point is the mean of two assays. One other experiment was essentially the same. (□) Purified *P. putida* photoactivated urocanase, 50 μl; each point is the mean of two assays. Three other experiments were essentially the same. The error bars show the average deviation of the mean.

Effect of Assay Temperature on Rat Liver Urocanase. We wanted to assure ourselves that these low Q_{10} values for bacterial urocanase were not due to an effect of temperature on the pH of the reaction mixture, absorbance of the substrate, instrumental performance, or some other extraneous factor. Therefore, we studied the effect of temperature on another urocanase, one from rat liver (Figure 3). This enzyme was compared to the purified bacterial enzyme at concentrations which catalyzed equal reaction rates at 30°. Temperatures above or below 30° caused marked changes in the rat enzyme activity but only moderate changes in the bacterial enzyme activity. The Q_{10} for rat urocanase was 5.62 at 15–25°, 4.92 at 20–30°, and 3.30 at 25–35° compared to values of 1.22, 1.33, and 1.08, respectively, for the bacterial enzyme.

Activity at 0°. An assay of urocanase at 0° was performed with special precautions to avoid condensation of moisture on the cuvet. This problem was monitored and successfully avoided. The rat enzyme had almost no activity at 0° but the bacterial enzyme exhibited 30% of its 30° activity. The urocanase activities (ΔA/min) at 30 and 0° were 0.0694 and 0.211 for photoactivated crude *P. putida* enzyme (20 μl), 0.0539 and 0.0156 for active crude enzyme (13 μl, freshly grown cells, not irradiated), and 0.0482 and 0.0006 for rat enzyme (33 μl), respectively.

Preincubation of Urocanase. If heat inactivation is responsible for the relative temperature independence (Figure 2), then preincubation of the enzyme at different temperatures would affect enzyme activity. After preincubation, all assays were done at 25°. Substantial irreversible heat inactivation of bacterial urocanase did not occur until the temperature reached 40–45° for 10 min (Figure 4). When crude photoactivated urocanase was preincubated for a longer time at 35° and assayed at 30°, the remaining activity was 100% after 30 min, 94% after 60 min, and 84% after 100 min. We conclude that urocanase is relatively stable below 40°. Inactivation of 50% occurred at 50.8° (10 min). Thus, heat inactivation does not explain the moderate effect of temperature on the enzyme reaction velocity below 40°.

Crude rat liver urocanase was less stable to heat (Figure 4). This enzyme had a temperature optimum at 45° (Figure 3), a temperature which inactivates the enzyme by 80% in 10 min. These findings are probably explained by substrate protection.

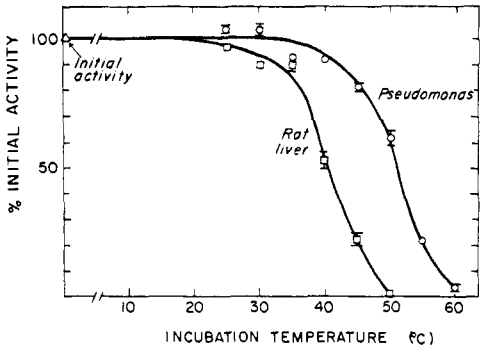

FIGURE 4: Thermal stability of urocanases. (○) Purified *P. putida* urocanase, 33 μl, was diluted to assay conditions, photoactivated and stored in an ice–water bath, incubated 10 min at the indicated temperature, rapidly cooled in an ice–water bath, and warmed to 25° in a water-jacketed cuvet, and the assay was initiated by addition of substrate. Each point is the mean of two assays except the initial activity which is the mean of four. (□) Crude rat liver urocanase, 33 μl; preincubation was 10 min at the indicated temperature; each point is the mean of three asays. The error bar is the average deviation of the mean.

Effect of Temperature Decrease during the Assay. When the temperature of the assay mixture was rapidly lowered to 30° during the course of the reaction, the velocity shifted to a higher rate without a lag. This was observed even though the enzyme had been partially irreversibly inactivated. During a 1.5-min shift from 45 to 30°, the velocity increased by 17 ± 5.7% (mean of seven assays); from 50 to 30° (2 min), the increase was 34 ± 8% (three assays); from 52.5 to 30° (2.5 min), the increase was 72 ± 3% (two assays).

Arrhenius Plot. Temperature–activity data for three rat liver enzyme experiments are presented in Figure 5 as Arrhenius plots. Below the temperature region where thermal inactivation becomes important, the plot is linear which is typical of enzyme or chemical reactions. The activation energy was 27.3 ± 3.9 kcal/mol (mean of five determinations).

For the bacterial enzyme (Figure 6) the plots were not linear, which indicated the activation energy was dependent on temperature. The slope of the tangent drawn at 17.5° indicated the activation energy was 8.9 ± 0.6 kcal/mol (mean of six determinations) which was a third of the rat urocanase value.

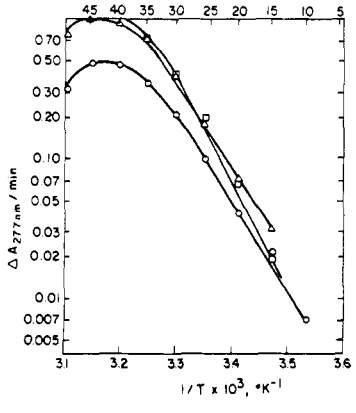

FIGURE 5: Arrhenius plot of the catalytic activity of rat liver urocanase. The reciprocal of the assay temperature was plotted against the enzyme activity on a logarithmic scale. Each point of two experiments was the mean of three assays and for another (○) the mean of two assays. For each experiment, 33 μl of crude rat liver urocanase was assayed.

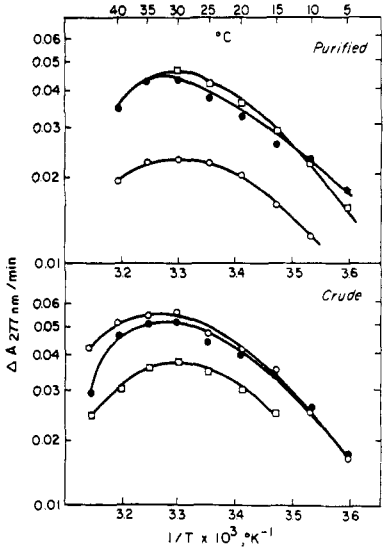

FIGURE 6: Arrhenius plot of the catalytic activity of *P. putida* urocanase. The reciprocal of the assay temperature was plotted against the enzyme activity on a logarithmic scale. Each point of two experiments for the purified enzyme was the mean of two assays and for another (□) the mean of three assays. Each point for the experiments with the crude enzyme was the mean of three assays.

Effect of Temperature on the Michaelis Constant. The apparent K_m for bacterial urocanase was determined at different assay temperatures (Figure 7). The K_m increased with temperature, which, at low substrate concentration, would tend to keep the overall reaction velocity relatively temperature independent. Even though a lower temperature should be expected to result in a slower reaction velocity, this effect is resisted by an increase in enzyme–substrate affinity (Baldwin and Hochachka, 1970). The K_m values of some enzymes from poikilothermic animals and bacteria have been shown to increase with temperature (Hochachka and Lewis, 1970, 1971; Baldwin and Hochachka, 1970; Paule, 1971; Wernick and Künnemann, 1973). The K_m of enolase from a thermophile decreased as temperature was increased (Stellwagen *et al.*, 1973). A temperature-dependent change in enzyme–effector affinity was found for fructose 1,6-diphosphatase (Gonzalez, *et al.*, 1972).

Effect of Temperature on Difference Spectrum. As the temperature was increased, the difference spectrum (read against the enzyme at 10°) developed positive peaks near 231 and 238 nm (Figure 8). The difference spectrum was observed even

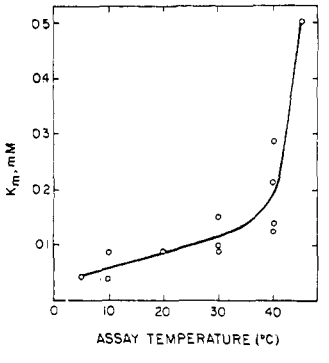

FIGURE 7: Effect of temperature on the Michaelis constant of *P. putida* urocanase. The mean correlation coefficient (r) of the 12 kinetic experiments was 0.991 (range, 0.925–0.999). These data represent 301 enzyme assays.

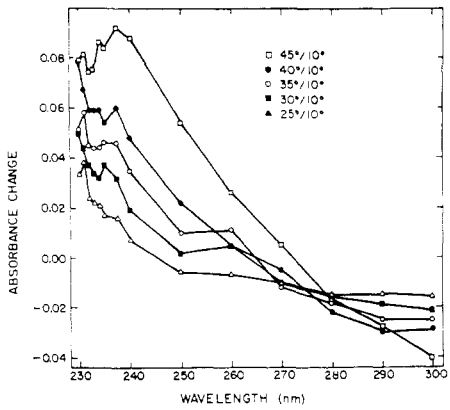

FIGURE 8: Difference spectra of purified *P. putida* urocanase at different temperatures. The absorbances of urocanase for each temperature were measured against urocanase at 10° as a blank.

at 25 and 30°, temperatures which are well below the irreversible thermal inactivation range (45–60°). Glazer and Smith (1961) reported that several proteins have a peak in the difference spectrum near 235 nm when denatured protein was compared to native protein; they attributed this peak to changes in the peptide backbone.

Discussion

The temperature optimum for *P. putida* urocanase was 30°; the activity did not decrease markedly as temperature was decreased from 30 to 0° (Figure 2). The fact that the latter property was exhibited by intact cells indicated that the transfer of substrate into the cell does not become limiting in this system at low temperature. Because purified enzyme exhibited this property, other enzymes, inhibitors, or activators need not be invoked to explain the moderate effect of temperature changes on velocity.

Irreversible heat inactivation does not account for the moderate response of the bacterial enzyme to temperature change (Figure 4). However, the nonlinear Arrhenius plot (Figure 6) suggests that more than one form of the enzyme may occur. The Arrhenius plots of typical enzymes are linear, but for some enzymes the plots have a sharp break at a transition temperature (Kim and Graves, 1973; Suelter, 1967). A listing of such enzymes has been given (Massey *et al.*, 1966; Paule, 1971). A curved Arrhenius plot was reported for acetylcholinesterase (Baldwin and Hochachka, 1970; Wilson and Cabib, 1956). The bacterial urocanase plots also appear to be smooth curves (Figure 6). Nonlinear plots have been related to a transiton between two or more stable conformational forms dependent on temperature and these forms exhibited different kinetic behavior (Massey *et al.*, 1966; Suelter, 1967; Koster and Veeger, 1968; Baldwin and Hochachka, 1970; Paule, 1971).

As temperature was raised (0–35° range) these changes were noted in bacterial urocanase: (1) an unexpectedly small change in activity (Figure 2); (2) an increase in K_m (Figure 7); (3) an increase in absorbance at 230–240 nm (Figure 8); (4) a decrease in activation energy (Figure 6). At 40–45°, activity decreased (Figure 2), heat inactivation appeared (Figure 4), K_m increased markedly (Figure 7), and the difference spectrum revealed a peak at 238 nm (Figure 8). These results support a suggestion that regulation of urocanase activity in response to temperature changes takes place by means of conformational changes in the enzyme molecule. Sedimentation studies yielded evidence that an unfolded form of thyroglobulin was produced from the native form by reduction of temperature from 23 to 2° (Schneider *et al.*, 1971).

A change from higher temperatures to 30° during the reaction resulted in an unexpected increase in the velocity. A change in conformation that caused substrate affinity to increase (Figure 7) might oppose the expected effect of the lower temperature on the velocity. For these shifts, we suggest that the enzyme overcompensates for a reduction in temperature. At lower temperatures (0–30°) some similar compensation might also occur.

Hochachka and Lewis (1971) have discussed the proposal that low substrate concentrations are responsible for low Q_{10} values. The Q_{10} values for *P. putida* urocanase were 1–1.4. When we increased substrate concentrations by 50%, the Q_{10} values did not change. Higher concentrations were not used because we wanted to avoid substrate inhibition, which begins at double substrate concentration.

Inasmuch as *P. putida* grew on histidine at 0°, we conclude that a histidine catabolic pathway, which includes urocanase, operates at this temperature. Urocanase from *P. putida* functions well at 0°. We conclude that this behavior contributes to the ability of this organism to grow on histidine at 0°. The protein molecule is flexible and can be altered by various external stimuli. Perhaps one way this cell can cope with an adverse environmental temperature is by adjustment of catalytic properties through temperature-dependent changes in the molecular shape of the enzyme.

Acknowledgment

We thank John M. Heysinger for his contributions to this investigation.

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Pancreatic Nonspecific Lipase, an Enzyme Highly Specific for Micelles†

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ABSTRACT: Nonspecific lipase activity in extracts of rat pancreas has been studied using phthalate esters as selective substrates. Evidence indicates that the enzyme binds anionic micelles at a site different from the hydrolytic site. Inhibition studies implicate sulfhydryl and amino groups as active components of the binding site, hydroxyl and imidazole groups as components of the hydrolytic site. Kinetic studies suggest that apparent K_m for nonpolar substrates is determined by the micelle-forming agent used, bile salts or anionic detergents. Both K_m(app), which was the same for a variety of phthalates, and V_{max}(app), which was not, were linearly proportional to the concentration of bile salt used to solubilize the substrates. Difficulties in making "obvious" interpretations of kinetic studies with substrates as mixed micelles are discussed.

In 1966 Mattson and Volpenhein reported the presence in rat pancreatic juice of an enzyme capable of hydrolyzing esters of both primary and secondary alcohols. Later (Mattson and Volpenhein, 1968) they suggested the trivial name "nonspecific lipase" for his enzyme activity. Among the esters reportedly hydrolyzed by pancreatic "nonspecific lipase" are 2,3-dioleylbutane and isopropyl oleate (Mattson and Volpenhein, 1966), 2-monoglycerides (Mattson and Volpenhein, 1968), erythritol tetraoleate (Mattson and Volpenhein, 1972a,b), methyl oleate, ethylene glycol dioleate, triolein, pentaerythritol tetraoleate, and adonitol pentaoleate (Mattson and Volpenhein, 1972b), bis(2-ethylhexyl) phthalate, and isopropyl palmitate (Albro and Thomas, 1973).

It now seems certain that nonspecific lipase is a different enzyme (or enzymes) from pancreatic lipase (EC 3.1.1.3) (Mattson and Volpenhein, 1966, 1968; Desnuelle and Savary, 1963) and pancreatic cholesterol esterase (EC 3.1.1.13) (Hyun *et al.*, 1969; Albro and Thomas, 1973). Likewise, evidence has been presented that nonspecific lipase activity is probably not associated with trypsin, α-chymotrypsin, carboxypeptidase, or phospholipase A (Mattson and Volpenhein, 1966). Nonspecific lipase has not been obtained in any high degree of purity up to the present time, although it probably was separated from pancreatic lipase by Morgan *et al.* (1968), and a 36-fold purification that did not separate it from cholesterol esterase has been reported (Albro and Thomas, 1973).

The nonspecific lipase from rat pancreas is strongly activated by taurocholate (Mattson and Volpenhein, 1966), and, unlike cholesterol esterase (Vahouny *et al.*, 1965), by deoxycholate as well as cholate (Albro and Thomas, 1973). The acceptable substrates are apparently those that can be presented in micelles (Albro and Thomas, 1973; Morgan *et al.*, 1968). As has been pointed out by Hyun *et al.* (1969), there are great difficulties encountered in attempting to apply enzyme kinetics derived for aqueous systems to systems involving dispersed substrates. It is the purpose of the present report to discuss studies of the role of micelles in the properties of nonspecific lipase activity in extracts of rat pancreas.

Materials and Methods

Buffer Salts. Hepes[1] was either Sigma H-3375 or the equivalent product from Nutritional Biochemicals Corp. Mes and Taps were Sigma No. M-8250 and T-5130, respectively. Tris was enzyme grade from Schwarz/Mann. Phosphate buffer was prepared from mono- and dibasic sodium phosphates, Baker and Adamson reagent grade.

Surfactants. Sodium taurocholate was Sigma T-0750; sodium desoxycholate was Fisher J-285; sodium glycocholate was from Nutritional Biochemicals; sodium cholate was prepared by titrating Eastman 4952 cholic acid with sodium hydroxide to give an aqueous solution 0.232 M in cholate at pH 8. Tween 20 and Tween 80 were from Nutritional Biochemicals; Brij 35 and Cutscum were from Fisher; sodium dodecyl sulfate was Sequanal grade from Pierce Chemical Co.; cetylpyridinium chloride and cetyltrimethylammonium bromide were Eastman P-5361 and P-5650, respectively; Triton X-100 was purified grade from Packard Instrument Co. Other dispersing agents included gum acacia (gum Arabic), Fisher G-85, and bovine serum albumin (fraction V), Sigma No. A4503.

Substrates. Bis(2-ethylhexyl) phthalate, dimethyl phthalate, and isopropyl palmitate labeled with ^{14}C in the carbonyl

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid; BBOT, 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene; Egmee, ethylene glycol monoethyl ether; BEHP, bis(ethylhexyl) phthalate; cmc, critical micelle concentration; E-600, diethyl *p*-nitrophenyl phosphate.