Effect of Modification of Individual Cytochrome c Lysines on the Reaction with Cytochrome b_5†

Siong Ng, Michael B. Smith, Harry T. Smith, and Francis Millett*

ABSTRACT: The rate of reduction of cytochrome c by liver microsomal cytochrome b_5 was found to be strongly inhibited by high ionic strength, indicating that the reaction probably involves complementary charge interactions between positively charged lysine groups on cytochrome c and negatively charged carboxyl groups on cytochrome b_5, as proposed by F. R. Salemme [(1976) *J. Mol. Biol. 102,* 563]. The reaction rates between cytochrome b_5 and cytochrome c derivatives modified at single lysine residues to form trifluoroacetylated or trifluoromethylphenylcarbamoylated lysines were studied to determine the role of individual lysines in the reaction. The only modifications that decreased the reaction rate were those of lysines immediately surrounding the heme crevice, lysines 8, 13, 25, 27, 72, and 79. Modification of lysines 22, 55, 99, and 100 had no effect on the rate. We propose that lysines 13, 25, 27, 72, and 79 of cytochrome c form complementary charge interactions with the cytochrome b_5 carboxyl groups Asp-48, Glu-43, Glu-44, Asp-60, and the most exposed heme propionate, respectively. The same modifications that affect the cytochrome b_5 activity also affect the cytochrome oxidase activity of cytochrome c, indicating that the pattern of complementary charge interactions is rather similar for the two complexes.

Cytochrome c is probably the most completely characterized electron transport protein known, but the mechanism by which it transports electrons from cytochrome c_1 to cytochrome oxidase in the mitochrondrial membrane is not well understood. One of the major reasons for this situation is the lack of detailed structural information about its physiological electron donor and acceptor. The recent x-ray determination of the crystal structure of cytochrome b_5 in both redox states (Argos and Mathews, 1975) gives us a unique opportunity to study electron transport between two well-characterized proteins, since cytochrome b_5 is known to rapidly reduce cytochrome c (Rogers and Strittmatter, 1974). The rate constant for the reduction of cytochrome c by cytochrome b_5 is nearly as large as that for the reduction of cytochrome c by cytochrome c_1 (Strittmatter, 1964; Yu et al., 1973).

Although cytochrome b_5 was first discovered in the membrane of the endoplasmic reticulum, a very similar form of cytochrome b_5 has recently been found in other organelles, including the outer membrane of the mitochondrion (Borgese and Meldolesi, 1976). Furthermore, Matlib and O'Brien (1976) have suggested that the reaction between cytochrome b_5 and cytochrome c might have some physiological significance because, under conditions of high intermembrane ionic strength, cytochrome c is released from the inner membrane and can transport electrons from cytochrome b_5 located on the inner surface of the outer membrane to cytochrome oxidase on the inner membrane. The cytochrome b_5 is reduced by NADH-cytochrome b_5 reductase, also located on the inner surface of the outer membrane. Ferguson-Miller et al. (1976) have recently shown that low concentrations of intermembrane ATP are especially effective at dissociating cytochrome c from the inner membrane.

Liver microsomal cytochrome b_5 isolated by detergent extraction is a single polypeptide chain consisting of two distinct regions, an amino terminus of about 95 amino acids which includes the heme prosthetic group, and a carboxyl terminus of about 45 hydrophobic amino acids which apparently serves to "anchor" the protein to the membrane (Spatz and Strittmatter, 1971). Mild treatment with proteolytic enzymes cleaves the intact enzyme into two fragments, corresponding to the two regions of the intact enzyme. Cytochrome c reacts as well with the water-soluble proteolytically cleaved protein as it does with the intact detergent isolated protein, illustrating that the active site of cytochrome b_5 is probably not affected by the hydrophobic anchor, or by binding to a membrane (Strittmatter et al., 1972). The x-ray crystallographic study was carried out on the proteolytically cleaved protein (Argos and Mathews, 1975). Salemme (1976) has recently compared the x-ray crystallographic structures of cytochrome c (Swanson et al., 1977) and cytochrome b_5 (Argos and Mathews, 1975) and proposed that they can form a complex dominated by complementary charge interactions between four negatively charged groups surrounding the heme crevice of cytochrome b_5 and four positively charged groups surrounding the heme crevice of cytochrome c. In the proposed complex, the heme groups of the two proteins nearly lie in the same plane, with their edges separated by 8.4 Å.

Since the interaction between cytochrome b_5 and cytochrome c is proposed to involve four positively charged lysine groups on cytochrome c, one way to verify the involvement of these groups is to specifically modify one of them with a reagent which would remove the positive charge. We have recently used ethylthioltrifluoroacetate to prepare five different singly trifluoroacetylated cytochrome c derivatives modified at lysines 13, 22, 25, 55, and 99, respectively (Staudenmayer et al., 1976, 1977). We have also used trifluoromethylphenyl isocyanate to prepare six different trifluoromethylphenylcarbamoyllysine (TFC-lysine)[1] derivatives modified at residues 8, 13, 27, 72, 79, and 100, respectively (Smith et al., 1977). We report here on the effect of these modifications on the reaction of cytochrome b_5 with cytochrome c.

† From the Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701. *Received April 7, 1977.* Supported by U.S. Public Health Service Grant GM-20488.

[1] Abbreviations used are: TFA, trifluoroacetyl; TFC, trifluoromethylphenylcarbamoyl; Tris, tris(hydroxymethyl)aminomethane; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

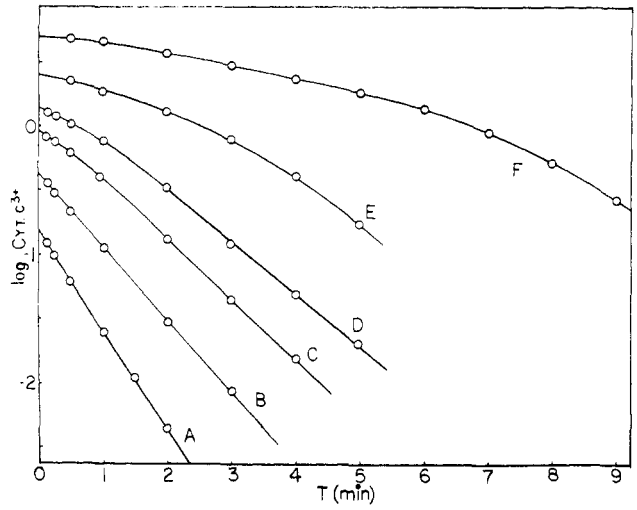

FIGURE 1: Logarithmic plot of the concentration of ferricytochrome *c* as a function of time after the addition of microsomes. The assay medium contained 0.02 M phosphate, pH 7.0, 0.200 M sucrose, 50 μM NADH, and 3.8×10^{-10} M cytochrome b_5. The total cytochrome *c* concentrations were 0.2 μM (A), 0.5 μM (B), 1.0 μM (C), 1.5 μM (D), 2.7 μM (E), and 5.5 μM (F).

Experimental Procedure

Materials. Horse heart cytochrome *c* (type VI) and NADH were obtained from Sigma Chemical Co. EDTA and Tris were obtained from Fisher Scientific Co. The specifically trifluoroacetylated (TFA) derivatives were prepared according to the procedures of Staudenmayer et al. (1976, 1977), while the TFC derivatives were prepared by the procedure of Smith et al. (1977). All derivatives were chromatographed a final time on a 1.5 × 10 cm column of Whatman CM32 carboxymethyl-cellulose eluted with 0.08 M phosphate buffer (pH 6.0). Fractions were analyzed by ^{19}F NMR techniques (Staudenmayer et al., 1977) to determine their purity before they were pooled. The derivatives were passed through a Bio-Gel P-60 column immediately prior to the enzyme kinetics studies to remove any possible polymeric material. The derivatives were never lyophilized at any point in the purification.

Cytochrome b_5 Assays. The source of the cytochrome b_5 activity was a beef liver microsomal fraction prepared according to the procedure of Strittmatter et al. (1972). The preparation could be stored in 50% glycerol at −20 °C for months with no change in NADH-cytochrome *c* reductase activity. The microsomal preparation was treated with 5% deoxycholate (1 mg/mg of protein) and diluted with buffer immediately prior to use. The assay medium contained 0.1 to 10 μM ferricytochrome *c*, 20 mM phosphate, pH 7.2, 200 mM sucrose, and 50 μM NADH. The rate of reduction of cytochrome *c* following addition of microsomes was followed on a Cary 14 spectrophotometer at 420 nm using 4-cm cells for the lower cytochrome *c* concentrations and 1-cm cells for the higher concentrations. The final cytochrome b_5 concentration was 3.8×10^{-10} M, as determined according to the procedures of Rogers and Strittmatter (1974).

Results

It has been well established that the NADH-cytochrome *c* reductase activity of liver microsomes results from the NADH-cytochrome b_5 reductase–cytochrome b_5 system (Strittmatter et al., 1972). At high concentrations of cytochrome *c*, however, the rate of cytochrome *c* reduction is limited by the rate of reduction of cytochrome b_5 by cytochrome b_5 reductase, rather than by the rate of reduction of cytochrome *c* by cytochrome b_5 (Strittmatter et al., 1972). We therefore studied the reaction at low concentrations of cytochrome *c* where the cytochrome b_5 to cytochrome *c* step would be expected to be rate limiting. The logarithm of the ferricytochrome *c* concentration is plotted in Figure 1 as a function of time after addition of enzyme for several concentrations of total cytochrome *c*. At concentrations below about 0.5 μM the reaction is nearly pure first order in ferricytochrome *c*, but at higher concentrations it is mixed zero and first order. The rate constant of the first-order component of the reaction decreased somewhat at higher cytochrome *c* concentrations, indicating that the product, ferrocytochrome *c*, might be inhibiting the reaction as discussed by Smith et al. (1974) for the mitochondrial cytochrome *c* reductase reaction. However, the extent of the first-order component of the reaction becomes progressively smaller at higher cytochrome *c* concentrations, and becomes more difficult to measure accurately. Because of the complexity of the effect of product on the reaction, we have limited further discussion to the initial velocity of the reaction. It is understood, however, that the apparent maximum velocity is not determined by the cytochrome b_5–cytochrome *c* reaction. The ratio V_{max}/K_m obtained by extrapolating V/S to $V = 0$ on an Eadie–Hofstee plot is probably the most unambiguous kinetic parameter that can be measured under these conditions. It was found to be numerically equal to the pseudo-first-order rate constant measured at the lowest cytochrome *c* concentrations. Furthermore, the constant V_{max}/K_mE_0 obtained by dividing V_{max}/K_m by the cytochrome b_5 concentration E_0 was found to be 3×10^7 M^{-1} s^{-1} (0.1 M phosphate, pH 7.0), very similar to the second-order rate constant for the reaction between the two purified proteins measured by stopped-flow techniques, 4.7×10^7 M^{-1} s^{-1} (Strittmatter, 1964). Although "V_{max}/K_mE_0" is almost certainly related directly to the cytochrome b_5–cytochrome *c* reaction, it is used here as an empirical parameter for comparison purposes only, and cannot be interpreted in the usual manner appropriate for classical Michaelis–Menten kinetics in the absence of a more complete kinetic analysis of the reaction.

The effect of ionic strength on the reaction is shown in Figure 2. Increasing NaCl concentrations have very little effect on the apparent maximum velocity, but cause a large decrease in V_{max}/K_m indicating that the reaction between cytochrome b_5 and cytochrome *c* probably involves an electrostatic interaction between the two proteins. Eadie–Hofstee plots of the reaction between cytochrome b_5 and derivatives of cytochrome *c* modified at individual lysine residues are shown in Figure 3. As expected, none of the modifications affected the apparent maximum velocity, but the rate constant V_{max}/K_m was decreased by modification of those lysines immediately surrounding the heme crevice of cytochrome *c*, lysines 13, 25, 27, 72, and 79 (Figure 16, Swanson et al., 1977). The TFC-Lys-8 derivative also had decreased activity toward cytochrome b_5, indicating that lysine-8 at the top of the heme crevice might play a role in the reaction. The reaction was not affected by modification of lysine-22 on the right side of cytochrome *c*, lysine-55 at the bottom of the "left channel", lysine-99 at the rear of the molecule, or lysine-100, also at the rear, indicating that none of these lysines plays a role in the reaction with cytochrome b_5.

Discussion

The strong ionic strength dependence of the rate constant for reduction of cytochrome *c* by cytochrome b_5 provides good evidence for the importance of complementary charge inter-

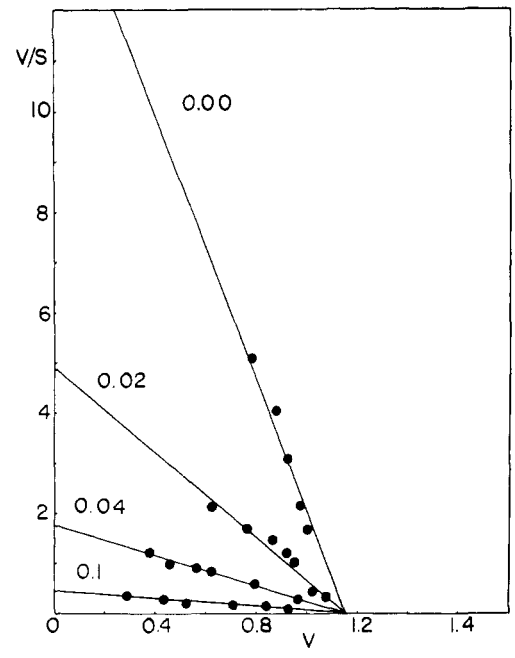

FIGURE 2: The dependence of the rate of reduction of cytochrome c by cytochrome b_5 on NaCl. All solutions contained 0.02 Mops, pH 7.2, 50 μM NADH, 0.200 M sucrose, and the indicated concentrations of NaCl. The initial velocities were measured in μM cytochrome c reduced per minute. S is in μM cytochrome c.

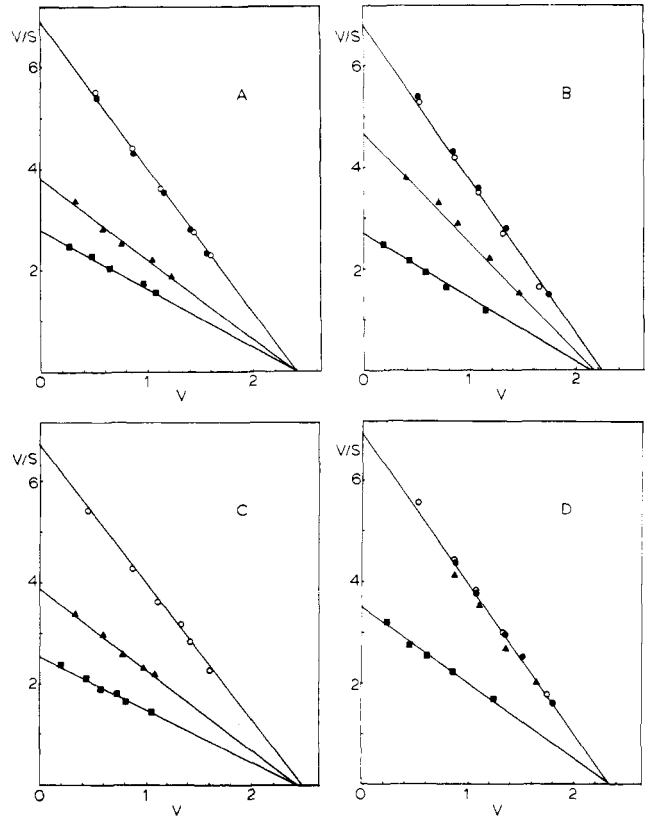

FIGURE 3: Rate of reduction of cytochrome c derivatives by cytochrome b_5. All assay mixtures contained 0.02 M phosphate, pH 7.0, 0.200 M sucrose, 50 μM NADH, and 3.8×10^{-10} M cytochrome b_5. Initial velocities were measured in μM cytochrome c reduced per minute. (A) Native cytochrome c (○); TFA-Lys-22 (●); TFC-Lys-27 (▲); TFC-Lys-72 (■). (B) TFA-Lys-99 (●); TFC-Lys-8 (▲); TFC-Lys-13 (■). (C) TFA-Lys-25 (▲); TFA-Lys-13 (■). (D) TFC-Lys-100 (●); TFA-Lys-55 (▲); TFC-Lys-79 (■).

actions between the positively charged lysine residues surrounding the heme crevice of cytochrome c and the negatively charged carboxyls surrounding the heme crevice of cytochrome b_5. Salemme (1976) proposed that there are four such interactions between the cytochrome c lysines 13, 27, 72, and 79 and the cytochrome b_5 carboxyl groups of Asp-48, Glu-44, Asp-60, and the most exposed heme propionate, respectively. It is significant that both of these groups of charged residues are highly conserved in different species of cytochromes c and b_5, respectively. Furthermore, specific modification of each of the four cytochrome c lysines with a reagent which removed the positive charge led to a decrease in the reaction rate constant. The decrease in the reaction rate could in principle be due either to the absence of the complementary charge interaction in the modified protein, or to steric interference of the modified group with the electron transport reaction. The latter possibility seems unlikely at least for the lysine-13 derivatives, since modification with the relatively bulky TFC group caused the same decrease in reaction rate as modification with the TFA group. Modification of lysine-25 also led to a decrease in the reaction rate, which is not surprising, since this lysine lies close to lysine-27, and would appear to be correctly positioned to form a complementary charge interaction with Glu-43 of cytochrome b_5. The decrease in rate upon modification of lysine-8 at the top of cytochrome c is somewhat more difficult to justify since it is somewhat further from the heme crevice than lysine-13. However, a distant charge interaction with the carboxyl group of cytochrome b_5 Asp-53 seems possible. The absence of any decrease in the rate due to modification of lysine-22 at the far right side of cytochrome c, lysine-55 at the bottom left side, or 99 and 100 at the back of the molecule indicates that these regions are probably not involved in the reaction, and provides a good check on our methodology.

Since we were not able to separately measure a V_{max} or K_m for the cytochrome b_5–cytochrome c reaction, we cannot tell whether the lysine modifications affected V_{max} or K_m, or even whether a true Michaelis complex was formed between the two proteins. However, in our recent studies of the effect of specific lysine modifications on the reaction with cytochrome oxidase (Staudenmayer et al., 1976, 1977; Smith et al., 1977), only the Michaelis constant K_m was affected, and Ferguson-Miller et al. (1976) have shown that the kinetically determined K_m value is identical with the dissociation constant of a stable ferricytochrome c–cytochrome oxidase complex. It is of interest that each of the derivatives that decreases the rate of the reaction with cytochrome b_5 also increases the K_m value of the reaction with cytochrome oxidase, and furthermore, that the magnitude of the change is nearly the same for the two reactions (Table

TABLE I: Enzymatic Activity of Cytochrome c Derivatives.

Derivative	Cytochrome b_5 act. V_{max}/K_mE_0 (M^{-1} s^{-1})	Cytochrome oxidase act. K_m (μM)
Native	3.0×10^8	0.051[a]
TFA-Lys-13	1.1×10^8	0.25[a]
TFA-Lys-22	2.9×10^8	0.057[b]
TFA-Lys-25	1.7×10^8	0.14[a]
TFA-Lys-55	2.9×10^8	0.051[b]
TFA-Lys-99	3.1×10^8	0.046[a]
TFC-Lys-8	2.1×10^8	0.12[c]
TFC-Lys-13	1.1×10^8	0.35[c]
TFC-Lys-27	1.7×10^8	0.080[c]
TFC-Lys-72	1.2×10^8	0.14[c]
TFC-Lys-79	1.6×10^8	0.22[c]
TFC-Lys-100	3.0×10^8	0.048[c]

[a] Staudenmayer et al. (1977). [b] Staudenmayer et al. (1976). [c] Smith et al. (1977).

I). Apparently, the pattern of complementary charge interactions is rather similar for the two complexes.

One of the most significant features of the hypothetical complex between cytochrome b_5 and cytochrome c proposed by Salemme is that the hemes of the two proteins are nearly coplanar, and the closest approach between the two is 8.4 Å. Since there are no amino acid side chains between the two hemes which could function as intermediate electron carriers, electron transfer from cytochrome b_5 to cytochrome c would appear to involve a direct transfer of electrons from heme edge to heme edge, possibly by a thermally activated tunnelling mechanism (Hopfield, 1974; Jortner, 1976).

References

Argos, P., and Mathews, F. S. (1975), *J. Biol. Chem. 250,* 747.

Borgese, N., and Meldolesi, J. (1976), *FEBS Lett. 63,* 231.

Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1976), *J. Biol. Chem. 251,* 1104.

Hopfield, J. J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3640.

Jortner, J. (1976), *J. Chem. Phys. 64,* 4860.

Matlib, M. A., and O'Brien, P. J. (1976), *Arch. Biochem. Biophys. 173,* 27.

Rogers, M. J., and Strittmatter, P. (1974), *J. Biol. Chem. 249,* 895.

Salemme, F. R. (1976), *J. Mol. Biol. 102,* 563.

Smith, H., Staudenmayer, N., and Millett, F. (1977), *Biochemistry 16* (preceding paper in this issue).

Smith, L., Davies, H. C., and Nava, M. (1974), *J. Biol. Chem. 249,* 2904.

Spatz, L., and Strittmatter, P. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1042.

Staudenmayer, N., Ng, S., Smith, M. B., and Millett, F. (1977), *Biochemistry 16,* 600.

Staudenmayer, N., Smith, M. B., Smith, H. T., Spies, F. K., and Millett, F. (1976), *Biochemistry 15,* 3198.

Strittmatter, P. (1964), On Rapid Mixing and Sampling Techniques in Biochemistry, Chance, B., Eisenhardt, R. H., Gibson, Q. H., and Lunberg-Holm, K. K., Ed., New York, N.Y., Academic Press, pp 71–84.

Strittmatter, P., Rogers, M. J., and Spatz, L. (1972), *J. Biol. Chem. 247,* 7188.

Swanson, R., Trus, B. L., Mandel, N., Mandel, G., Kallai, O., and Dickerson, R. E. (1977), *J. Biol. Chem. 252,* 759.

Yu, C. A., Yu, L., and King, T. E. (1973), *J. Biol. Chem. 248,* 529.

Complete Primary Structure of the Major Component Myoglobin of Pacific Common Dolphin (*Delphinus delphis*)†

Chi-Chin Wang, Romualdo Avila, Barry N. Jones, and Frank R. N. Gurd*

ABSTRACT: The complete amino acid sequence of the major component myoglobin from Pacific common dolphin, *Delphinus delphis,* was determined by the automatic Edman degradation of several large peptides obtained by specific cleavages of the protein. More than 80% of the covalent structure was established by the degradation of the apomyoglobin and five peptides from: (1) cyanogen bromide cleavage at the two methionine residues, (2) trypsin cleavage of the acetimidated apomyoglobin at the three arginine residues, and (3) 2-*p*-nitrophenylsulfenyl-3-methyl-3′-bromoindolenine cleavage at the two tryptophan residues. The rest of the sequence was determined by use of the peptides prepared from further digestion of the central cyanogen bromide peptide with staphylococcal protease and trypsin. The primary structure of this myoglobin proved identical with that from the Atlantic bottlenosed dolphin, *Tursiops truncatus,* but showed four substitutions with respect to the sequence reported for the Black Sea dolphin which has also been given the designation *Delphinus delphis.*

This report presents the application of the automatic Edman degradation procedure to the determination of the primary structure of myoglobin from the Pacific common dolphin, *Delphinus delphis.* The general strategy and analytical procedures had been established and utilized for the determination of several myoglobin sequences recently reported (Dwulet et al., 1975), 1977; Bogardt et al., 1976; Jones et al., 1976; Lehman et al., 1977). In addition, the protein was cleaved at the tryptophan residues (Fontana, 1972) to produce a long peptide to assist in confirming some amino acid substitutions found between this protein and that from the same named species from the Black Sea (Kluh and Bakardjieva, 1971). Other Cetacean myoglobin sequences reported include common porpoise (Bradshaw and Gurd, 1969) and sperm whale (Edmundson, 1965).

Experimental Section

Materials

The major component of Pacific common dolphin myoglobin was isolated from frozen muscle tissue by the procedure of Hapner et al. (1968). Phosphate buffer, pH 6.5, ionic strength 0.1, was used at room temperature to fractionate the crude

† From the Department of Chemistry, Indiana University, Bloomington Indiana 47401. *Received May 19, 1977.* This is the 83rd paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper, see Rothgeb and Gurd (1978). This work was supported by Public Health Service Research Grant HL-05556. R. Avila is supported by Latin American Scholarship Program of American Universities.