THE BIOCHEMISTRY OF CYTOCHROME C IN RELATION TO MATERNALLY INHERITED PHENOTYPES OF NEUROSPORA

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1961

ACKNOWLEDGMENTS

I appreciate greatly the opportunity of studying at The California Institute of Technology; particularly it has been most rewarding to be under the guidance of Dr. Herschel K. Mitchell. His scientific excellence, patience, and understanding have made the years both profitable and pleasurable. The counsel of Dr. Jerome R. Vinograd has also been especially helpful. I would like to thank Mary B. Mitchell for the strains of Neurospora used in this work and all members of the Division of Biology whose assistance and friendliness have meant so much to me.

For financial support I thank the Arthur McCallum Fund, The California Institute of Technology and the Public Health Services.

My heartfelt thanks go to my wife, Willa Mae, for her direct and indirect help and encouragement with this work.

This work I respectfully and
affectionately dedicate to my mother
and to the memory of my father.

ABSTRACT

Cytochrome *c* from Neurospora was isolated and its properties studied with respect to its role as a protein accumulated in the extrachromosomal mutants, *poky* and *mi 3*. All samples of cytochrome isolated using several different procedures were electrophoretically heterogeneous. Further, electrophoretic differences were observed in the cytochrome *c* isolated from the different strains of Neurospora although cytochrome *c* from *poky* and wild type appeared homogeneous during sedimentation and had very similar sedimentation coefficients. In contrast to wild type Neurospora, cytochrome *c* in homogenates of 2.5 day old *poky* was found to remain largely in the supernatant fraction after high speed centrifugation suggesting that the enzyme in *poky* is not bound to mitochondria or any other particulate, subcellular structure.

Lipid extracts from *poky* were found to contain up to 36 times the amount of total free fatty acids present in similar extracts from wild type although the chemically bound fatty acids of the two strains were similar. The interaction of free fatty acids with mammalian cytochrome *c* was studied in detail and it was suggested that they may cause an unfolding of the protein portion of the cytochrome *c* molecule thereby exposing the heme to direct interaction with such compounds as hydrogen peroxide or molecular oxygen. Thus the large amounts of free fatty acid present in *poky* might allow its cytochrome *c* to react directly with molecular oxygen thereby allowing cytochrome *c* to function as the terminal oxidase in place of cytochromes $a + a_3$ present in wild type Neurospora but not found in *poky*.

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INTRODUCTION

One of the problems of modern biology concerns the biochemical mechanisms involved in the transmission of heritable specificity from generation to generation. Numerous papers have outlined the apparent relation between genes and the structure of specific proteins (1,2,3,4,5,6) however, the phenomenon of extrachromosomal inheritance suggests the existence of heritable specificity not directly controlled or transmitted by genes.

Some of the most studied and genetically stable examples of extrachromosomal inheritance are found in Neurospora crassa. Mitchell and Mitchell (7) have isolated mutants with two distinct phenotypes, poky and mi 3, that are inherited through a non-genic mechanism and which were found to be stable through a large number of vegetative transfers. Crosses between either poky or mi 3 and wild type Neurospora using the mutants as the perithecial or female parent yield progeny of only the mutant type with rare exceptions. About one wild type per 1,000 poky were found in the cross described above for poky x wild type. The infrequent wild progeny are considered to be due to reciprocal crossing and not reversion of poky since crosses of poky x poky did not yield any wild types among more than 20,000 progeny.

Both poky and mi 3 are characterized by relatively slow growth and aberrations in many biochemical systems when compared with wild type Neurospora (8). For instance alkaline phosphatase appears to be consistently about 50 per cent higher in the different poky re-isolates and it has been found that young poky contains about 30 per cent more DNA per unit weight of mold than wild type. However, it should be noted that this difference is probably primarly due to variations in structural elements such as hyphal walls, thus in total dry weight, rather than in the amount of DNA present in poky and wild type. Ribonucleic acid and polysaccharide are found in about the same total concentration in poky and wild type but the distribution in the cells is different. The large granules or mitochondrial fraction are relatively deficient (two fold or more) in these components. On the other hand poky contains approximately twice as much riboflavin and niacin as wild type, the excess niacin being found in the soluble fraction whereas the riboflavin (as FAD) is relatively concentrated in the mitochondria. Perhaps the most striking difference in the biochemical composition of poky or mi 3 and wild type is in the cytochrome system. Young poky accumulates up to 16 times the amount of cytochrome c found in wild type but lacks cytochromes b and a + a_3. Mi 3 also accumulates cytochrome c but to a smaller extent than poky. It contains cytochrome b and has detectable amounts of cytochrome a_1, which is not present in wild type, however, like poky, it lacks

cytochromes a + a_3. Also, Herzenberg (9) purified and partially characterized an ether soluble, acidic material from poky which he called pokonic acid. This material was found to prevent the reduction of Neurospora or horse heart cytochrome c by ascorbic acid and was apparently important in the destruction of cytochrome c in poky. Subsequent unpublished work by Dr. H. K. Mitchell and Dr. Carl Stevens demonstrated that pokonic acid was primarily free fatty acid and that it caused cytochrome c to become very sensitive to destructive oxidation by hydrogen peroxide.

Little is known of the chemical nature or mechanisms involved in extrachromosomal inheritance and how they differ from or are integrated with genic systems. Observations on the extrachromosomal mutants of Neurospora and the rather similar "petites" in yeast (10) suggest that the gross organization of subcellular systems, particularly mitochondria, may be involved. However, these observations do not exclude the possibility that extrachromosomal mutations might also cause changes in the structure of specific proteins. Cytochrome c accumulated in both poky and mi 3 seemed to have many desirable physical and chemical properties for a comparison with cytochrome c from wild type and to offer an approach to further investigate apparent biochemical aberrations of these mutants. Accordingly studies were undertaken on cytochrome c and factors thought to effect its synthesis, breakdown or physiological function in Neurospora.

PART I

THE INTERACTION OF FATTY ACIDS WITH CYTOCHROME *C*

A. MATERIALS AND METHODS

Cytochrome *c*

Sigma Chemical Company type II horse heart cytochrome *c* was used throughout the work reported on the interaction of fatty acids with cytochrome *c*. The material was determined to have a molecular weight of about 16,800 on the basis of one molecule of iron per molecule of protein. Iron was determined by the *o*-phenanthroline method of Drabkin (11) with slight modification (see p. 77). This molecular weight is in fair agreement with the 16,500 originally reported by Keilin and Hartree (12). However, this preparation of cytochrome *c* was observed to contain a colorless protein component which moved towards the positive pole in free boundary electrophoresis at pH 8.60 in barbital buffer. Such a material was also observed and removed by chromatography on IRC 50 resin by Margoliash (13). In the present work removal of the colorless component by continuous flow paper electrophoresis at pH 8.0 yielded a product found to have a molecular weight of 13,000 on the basis of iron content. This value is in agreement with the molecular weight of highly purified cytochrome *c* obtained with ion exchange resins reported in the paper by Margoliash mentioned above.

Cytochrome solutions were diluted to the desired molar concentration on the basis of the extinction coefficient for the Soret band of oxidized cytochrome *c* reported by Margoliash

and Frohwirt (14). The final cytochrome concentration for all reactions, unless otherwise specifically mentioned (as for the effect of palmitic acid on the reducibility of cytochrome *c*; see table 3) was about 0.02 mM, a concentration which for fully oxidized cytochrome *c* gave an optical density of 2.12 at a wave length of 407 mμ .

The cytochrome used always contained small amounts of ferrocytochrome *c* and exact concentrations were determined for fully oxidized cytochrome *c* by making the solutions 0.1 mM in potassium ferricyanide by a 100:1 dilution from a stock solution. When checking the spectrum of fully oxidized cytochrome the same concentration of potassium ferricyanide was used in the reference cell of the spectrophotometer.

Margoliash and Frohwirt reported the Soret band peak of oxidized cytochrome *c* to be at 410 mμ in 0.1 M phosphate buffer at pH 6.8. The spectrophotometer used in this study indicated the peak to be at about 407 mμ in pH 8.6, 0.05 M tris-(hydroxymethyl)-aminomethane buffer, thus this wave length was used throughout this work.

Fatty Acids

California Biochemical Corporation CfP grade fatty acids were used without further purification. The purity of each sample of fatty acid was checked by forming the methyl ester with diazomethane, then quantitatively analyzing the sample with an Aerograph gas chromatograph. The details of this

procedure are given under the section on "the free fatty acid content of poky and wild type Neurospora" (see p. 112) in part II of this thesis. In all cases the purity was found to be excellent with no more than trace amounts of closely related fatty acids detected.

Solutions of fatty acids were made up in absolute ethanol, generally so that 0.1 ml of the ethanolic solution added to a final volume of 4.0 ml of cytochrome c would give the desired final concentration of the fatty acid. It was determined that 0.3 ml or three times the amount of ethanol generally added, had no apparent effect on the rate or extent of the hydrogen peroxide reaction with untreated or fatty acid treated cytochrome c.

Buffers

The buffer used in all experiments described in part I of this thesis, unless otherwise specified, was 0.05 M tris-(hydroxymethyl)-aminomethane, (tris), neutralized to pH 8.60 with HCl. Tris was obtained as Sigma "7-9" Biochemical Buffer.

Buffers used in the determination of the effects of pH on the palmitic acid-hydrogen peroxide reaction with cytochrome c (see figure 4) were as follows:

pH 6.8	through 7.6	NaH_2PO_4 plus NaOH
7.8	" 8.8	tris " HCl
8.8	" 9.5	NH_4Cl " NaOH
9.6	" 10.0	glycine " NaOH
10.5	" 11.0	$NaHCO_3$ " NaOH

All buffers were made up to 0.05 molar, however, no attempt was made to maintain constant ionic strength.

Hydrogen Peroxide

Hydrogen peroxide was diluted from a refrigerated stock solution of Superoxol (Merck and Co.) determined to be 33.0 per cent hydrogen peroxide by the sodium thiosulfate method outlined by Vogel (15). Hydrogen peroxide solutions were always made up immediately before use by dilution of the stock solution into cold, distilled water. Unless otherwise specifically stated, as for the "effects of hydrogen peroxide concentrations", the stock solution was diluted to 0.1 per cent concentration. Thus, a final concentration of 0.736 mM hydrogen peroxide resulted from the addition of 0.1 ml of the 0.1 per cent solution to a final volume of 4.0 ml of cytochrome solution.

General Procedure

Unless otherwise specifically stated 0.1 ml of ethanolic solution of fatty acid was added to a final volume of 4.0 ml of cytochrome c in cuvettes or small test tubes and mixed by covering with "Saran Wrap" then inverting 10 times. The

solution of cytochrome c plus fatty acid was allowed to stand for 15 to 30 minutes before use. Then, 0.1 ml of hydrogen peroxide solution was quickly added and the solutions were mixed by again inverting the tubes or cuvettes, and the time of reaction measured with a stop watch.

All spectrophotometric determinations except those for the effect of temperature were done on the Cary model 11 MS recording spectrophotometer. Spectrophotometric measurements requiring temperature control were performed with a Beckman model DK2 spectrophotometer.

A slight decrease in the rate of reaction was noted when hydrogen peroxide was added within 5 minutes of the addition of fatty acid. No differences were detected with the addition of hydrogen peroxide from 5 to 60 minutes after the addition of fatty acid.

B. RESULTS

Figure 1 was taken directly from tracings made with a Cary recording spectrophotometer and illustrates the hydrogen peroxide induced decline in absorption at 407 mμ for cytochrome c alone and for cytochrome c in the presence of 0.20 mM and 0.60 mM palmitic acid. Samples containing palmitic acid show a sharp decline in Soret band absorption although samples containing no palmitic acid show little change under similar conditions.

The fall in optical density does not reach its maximum rate immediately but rather is characterized by an initial lag as seen for both palmitic acid treated samples. Also, the absorption for palmitic acid treated samples is somewhat greater than 2.12, the optical density at 407 mμ of a 0.02 mM sample of oxidized cytochrome c, because of the hyperchromic effect discussed in connection with table 3.

Samples having large changes in Soret band absorption always undergo corresponding visible changes in color from red to green. Figure 2 illustrates the changes in the spectrum of cytochrome c treated with 0.6 mM palmitic acid and 0.6 mM palmitic acid followed by the addition of hydrogen peroxide to give a 0.736 mM final concentration. The latter spectrum was taken 20 minutes after the addition of hydrogen peroxide so that the sample would undergo very little further change. Hydrogen peroxide also causes the formation of a

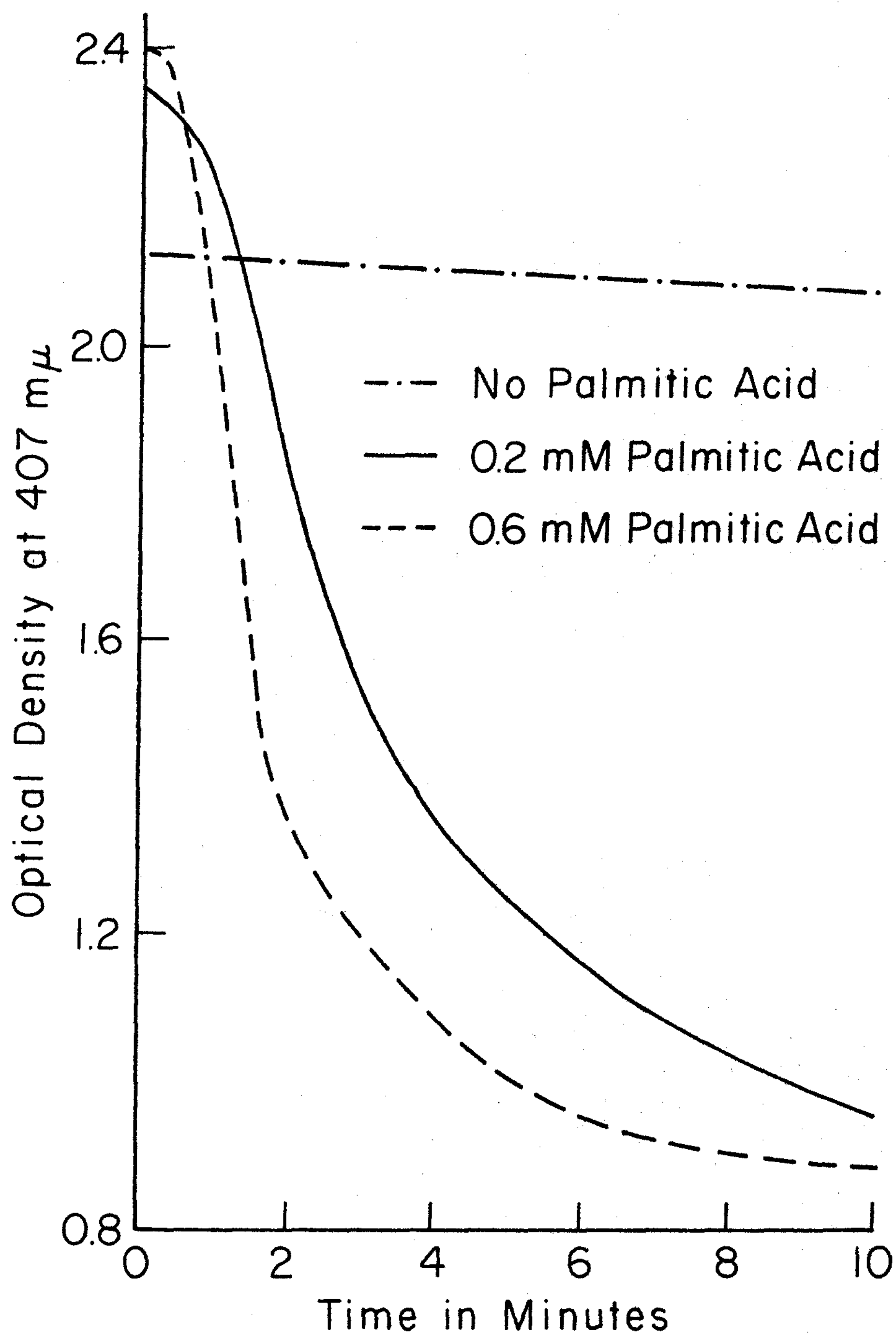

Figure 1. The decline of Soret band absorption of 0.02 mM untreated and palmitic acid treated cytochome c caused by 0.736 mM hydrogen peroxide.

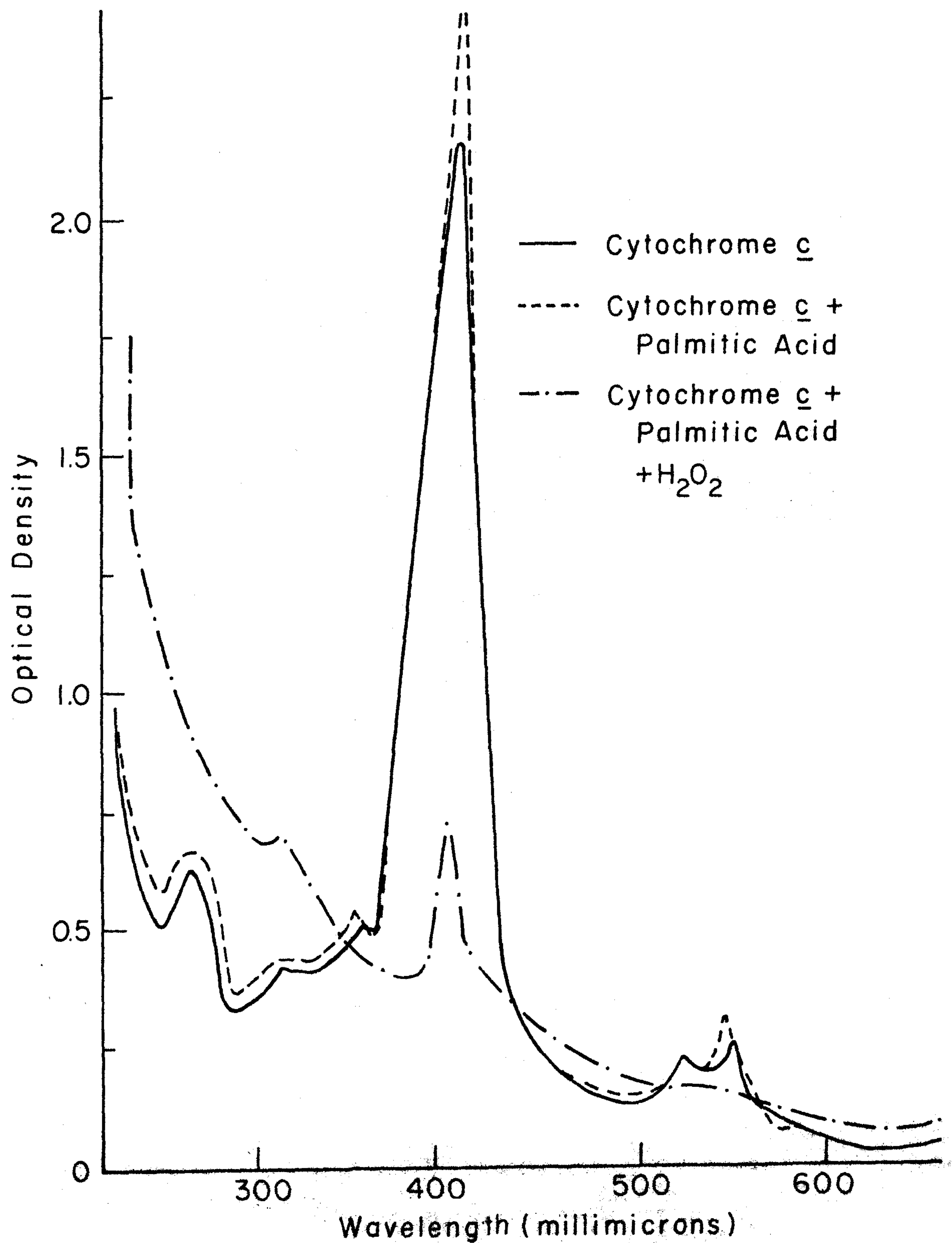

Figure 2. The spectrum of 0.02 mM cytochrome c in pH 8.6 tris buffer, cytochrome c plus a final concentration of 0.6 mM palmitic acid, and cytochrome c plus palmitic acid followed by treatment with hydrogen peroxide to give a final concentration of 0.736 mM.

small but variable amount of a troublesome, finely divided white substance that was precipitated by centrifugation before this spectrum was taken.

Samples not exposed to hydrogen peroxide or other effective oxidants contain some cytochrome c in the reduced form as evidenced by the peaks in the 550 mμ region of the above spectra. Hydrogen peroxide easily oxidizes the iron of cytochrome c to which no fatty acid has been added, however, the determination of its effect on treated samples is complicated by the loss of the characteristic cytochrome spectrum. Attempts to measure the oxidation state of the iron in samples of reduced cytochrome c to which fatty acid and then hydrogen peroxide are added indicate that the iron probably undergoes very rapid oxidation.

Effects of Hydrogen Peroxide Concentration

Figure 3 illustrates the effect of hydrogen peroxide concentration on 0.02 mM cytochrome c with and without palmitic acid added to give a final concentration of 0.20 mM. The values recorded as percentages were obtained from the observed optical densities after 15 minutes with hydrogen peroxide and were compared with values for potassium ferricyanide oxidized blanks containing neither palmitic acid nor hydrogen peroxide. It may be seen that at low concentrations of hydrogen peroxide, values of more than 100 per cent are recorded. This phenomenon is due to a hyperchromic effect of palmitic acid on cytochrome c mentioned above.

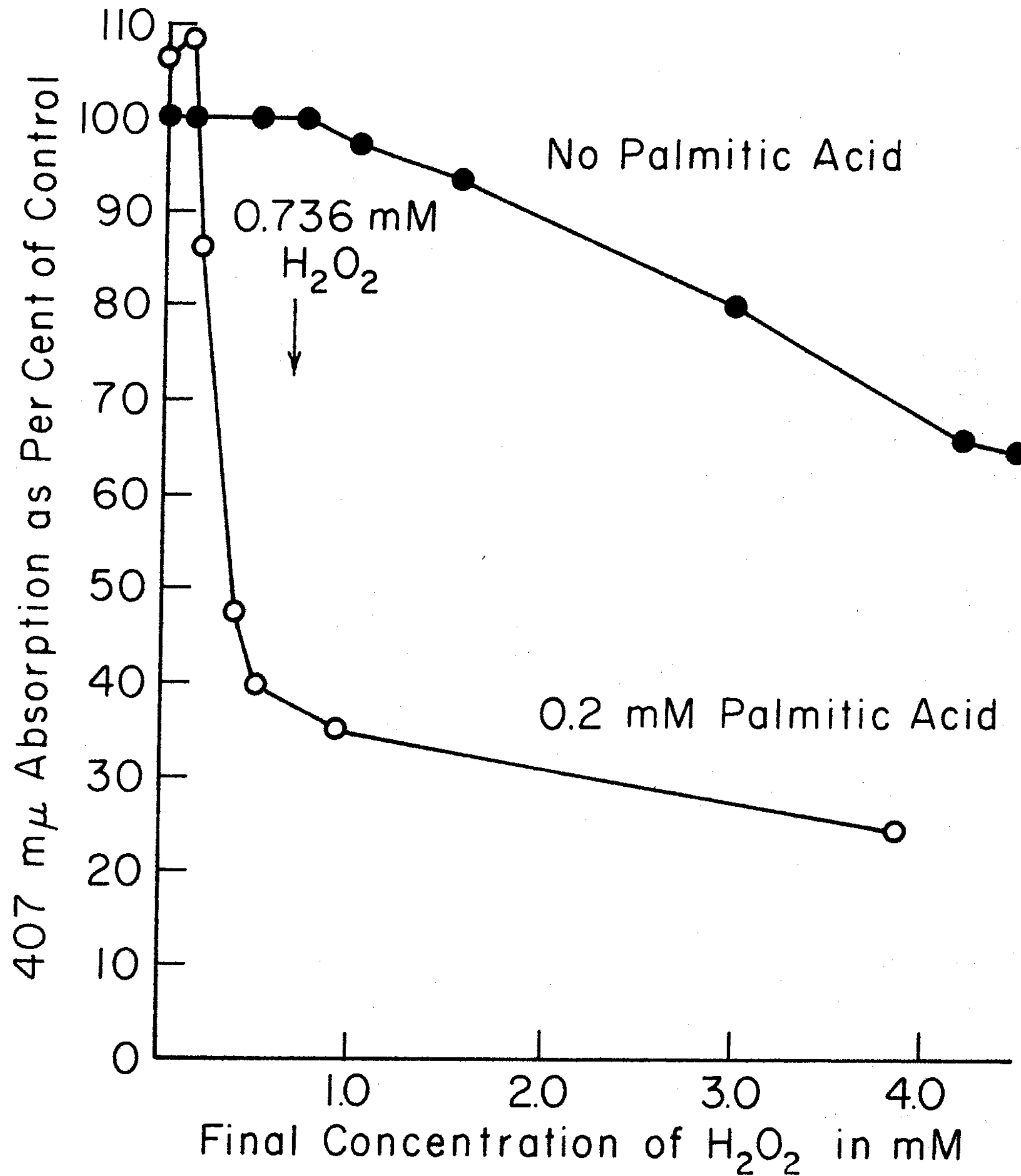

Figure 3. The effect of hydrogen peroxide on 0.02 mM cytochrome c and 0.02 mM cytochrome c with 0.02 mM palmitic acid. See text for a description and explanation of the procedure.

The concentration of hydrogen peroxide generally used was 0.736 mM and is indicated in the figure. This concentration arose from the addition of 0.1 ml of a 0.1 per cent solution of hydrogen peroxide to a solution of cytochrome c to give a final volume of 4.0 ml. At this concentration there is only a very slow decline in the Soret band absorption of samples containing no palmitic acid but a rapid decrease in absorption of treated samples as can also be seen in figure 1.

Effects of Hydrogen Ion Concentration

The dependence of the cytochrome destroying reaction on hydrogen ion concentration is shown in figure 4. Hydrogen peroxide was added to palmitic acid treated and untreated solutions of cytochrome c at different hydrogen ion concentrations. The final concentration of palmitic acid used was 0.20 mM. The Soret band absorption of samples was measured 15 minutes after the addition of hydrogen peroxide and recorded as the per cent of a potassium ferricyanide oxidized blank at the same pH in a manner similar to that used for figure 3.

A sharp break in reactivity is seen at about pH 7.8. Reactions carried out below this pH were consistently turbid, while those at hydrogen ion concentrations between pH 8.0 and 10.0 were not. The difference between palmitic acid treated and untreated cytochrome below this pH is likely due to turbidity of the treated samples rather than differences in reactivity of the cytochrome produced by palmitic acid. At pH

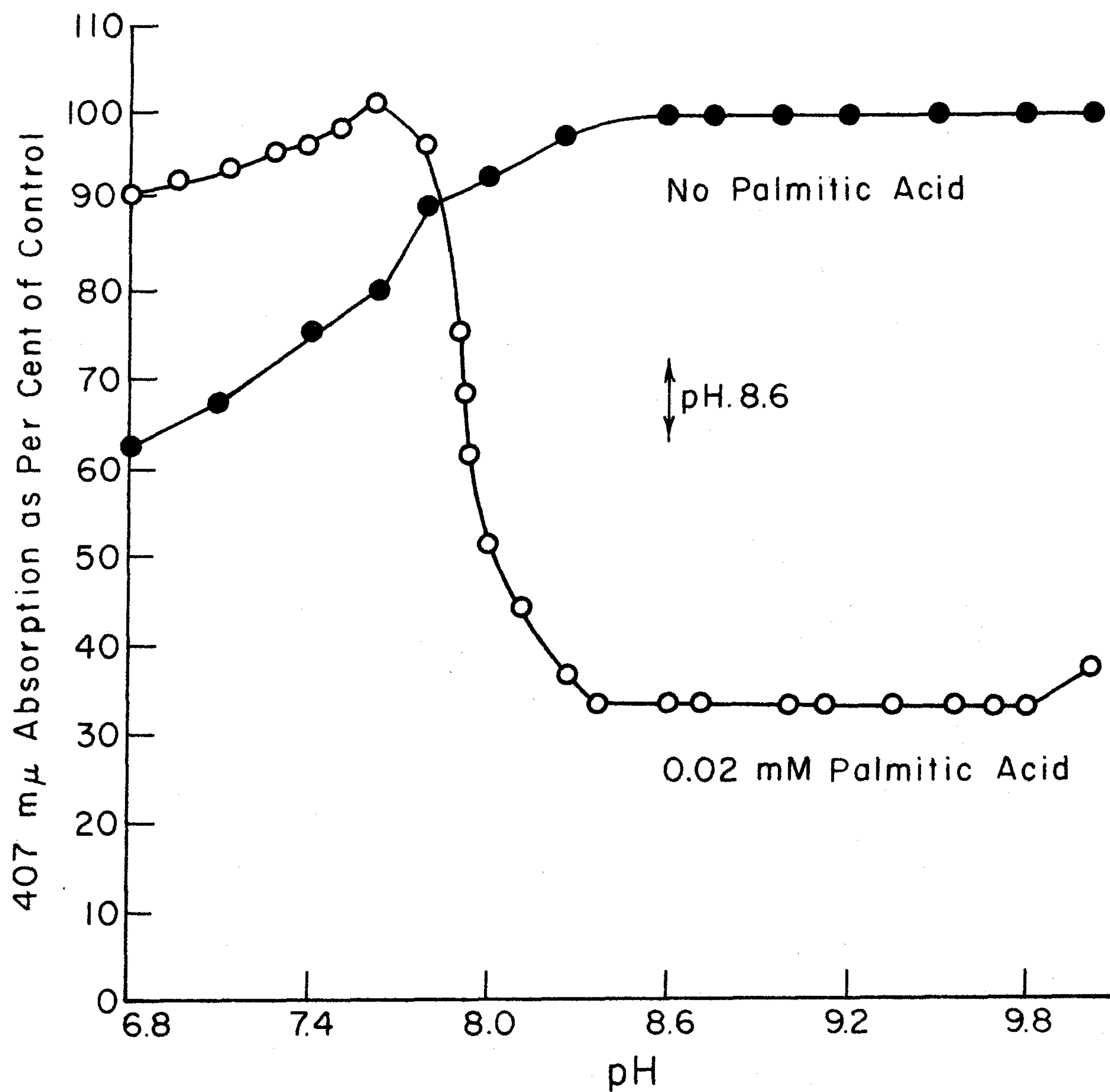

Figure 4. The effect of hydrogen ion concentration on the hydrogen peroxide oxidation of 0.02 mM cytochrome _c_ with and without added palmitic acid.

values of 10.0 and above an increasing amount of flocculent material precipitable by centrifugation occurred thus preventing accurate spectrophotometric analysis of these samples.

Effects of Temperature

The reaction of hydrogen peroxide with cytochrome c treated with 0.2 mM palmitic acid was carried out at controlled temperatures in a Beckman DK-2 Spectrophotometer and the data plotted giving curves similar to those of figure 1 for each temperature. The maximum rate of reaction was determined graphically and recorded in figure 5 according to the Arrhenius equation:

$$\ln k = - \frac{E_a}{R} \frac{1}{T} + c$$

where k is the activation rate constant (per min.). R is the gas constant (1.987 cal. per degree per mole). T is the absolute temperature, E_a is the activation energy (cal. per mole), and c is a constant of integration.

It may be seen that the maximum rate of the reaction increases rapidly with relatively small increases in temperature. The maximum rate is increased by approximately a factor of 2 with an increase in temperature from 24° to 29° C.

The heat of activation was calculated as 25,200 calories per mole by application of the Arrhenius equation to the data of figure 5. However, this value is of questionable significance because of the complex nature of the reaction and the

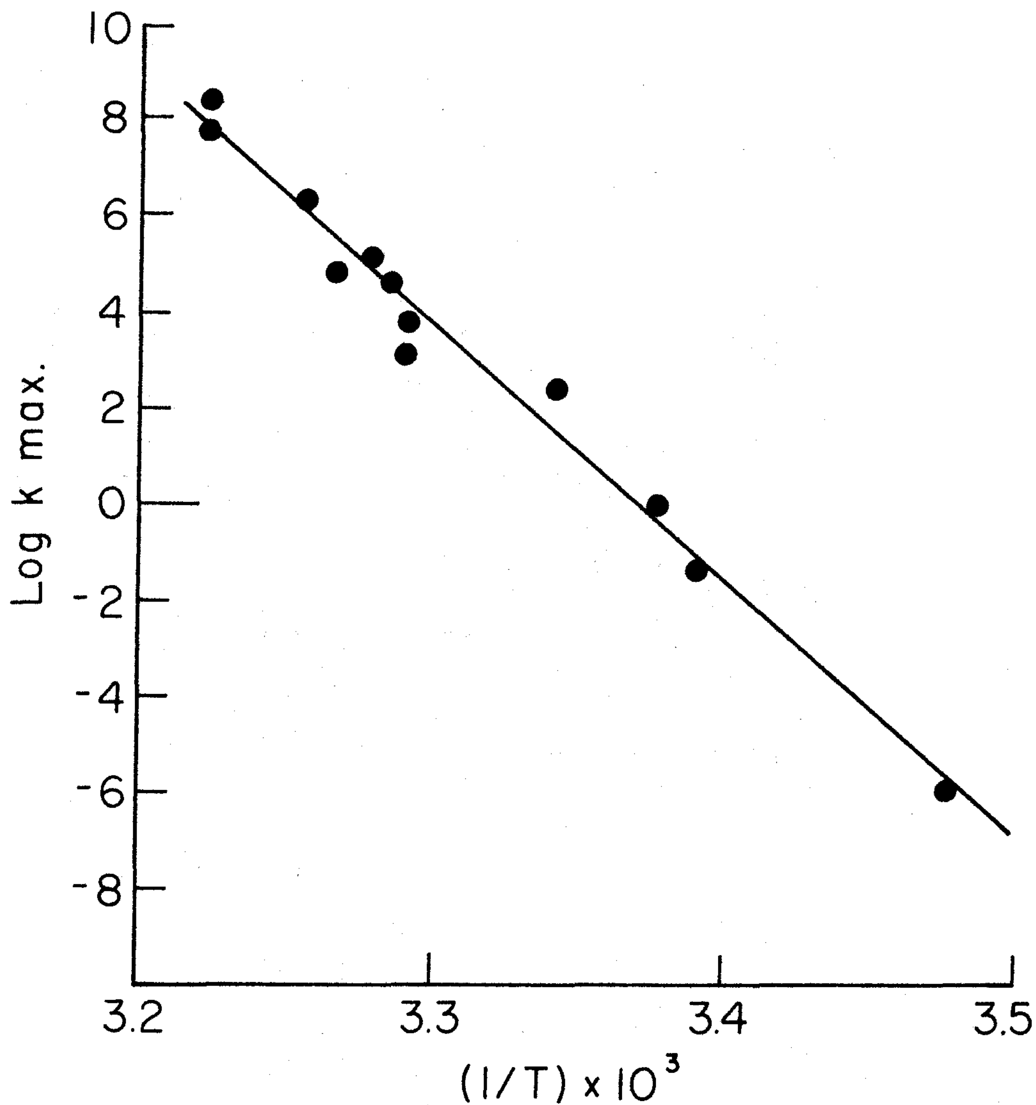

Figure 5. Effect of temperature on the hydrogen peroxide oxidation of palmitic acid treated cytochrome c. The final concentrations used were 0.02 mM cytochrome c, 0.20 mM palmitic acid and 0.736 mM hydrogen peroxide.

possible necessity for a correction of the data for the presence of colored or insoluble products.

Effectiveness of Common Fatty Acids

The maximum rates of reactions for various concentrations of fatty acids were determined from recordings of the decline in Soret band absorption similar to those shown in figure 1 and are presented in figures 6a and 6b. All reactions were run at room temperature (about 26° C.) with 0.02 mM cytochrome c and 0.736 mM hydrogen peroxide.

Some variations in rates were observed, undoubtedly due in part to variations of temperature and hydrogen peroxide concentrations. With the exception of lauryl sulfate all curves were extended into the region of limiting solubility for the compound being tested.

The highest maximum rates were obtained with lauric acid and its non-fatty acid analogue, lauryl sulfate, but at relatively high concentrations compared with palmitic acid. Myristic acid was found to be about intermediate in activity between palmitic and lauric acid. Stearic acid was quite insoluble and had a relatively small affect in the test system. The C_{18} unsaturated acids, oleic, linoleic, and linolenic acids, were found to be roughly similar in solubility being more soluble than either palmitic or myristic acid. Their maximum rates were also relatively high.

It is difficult to obtain a satisfactory basis for

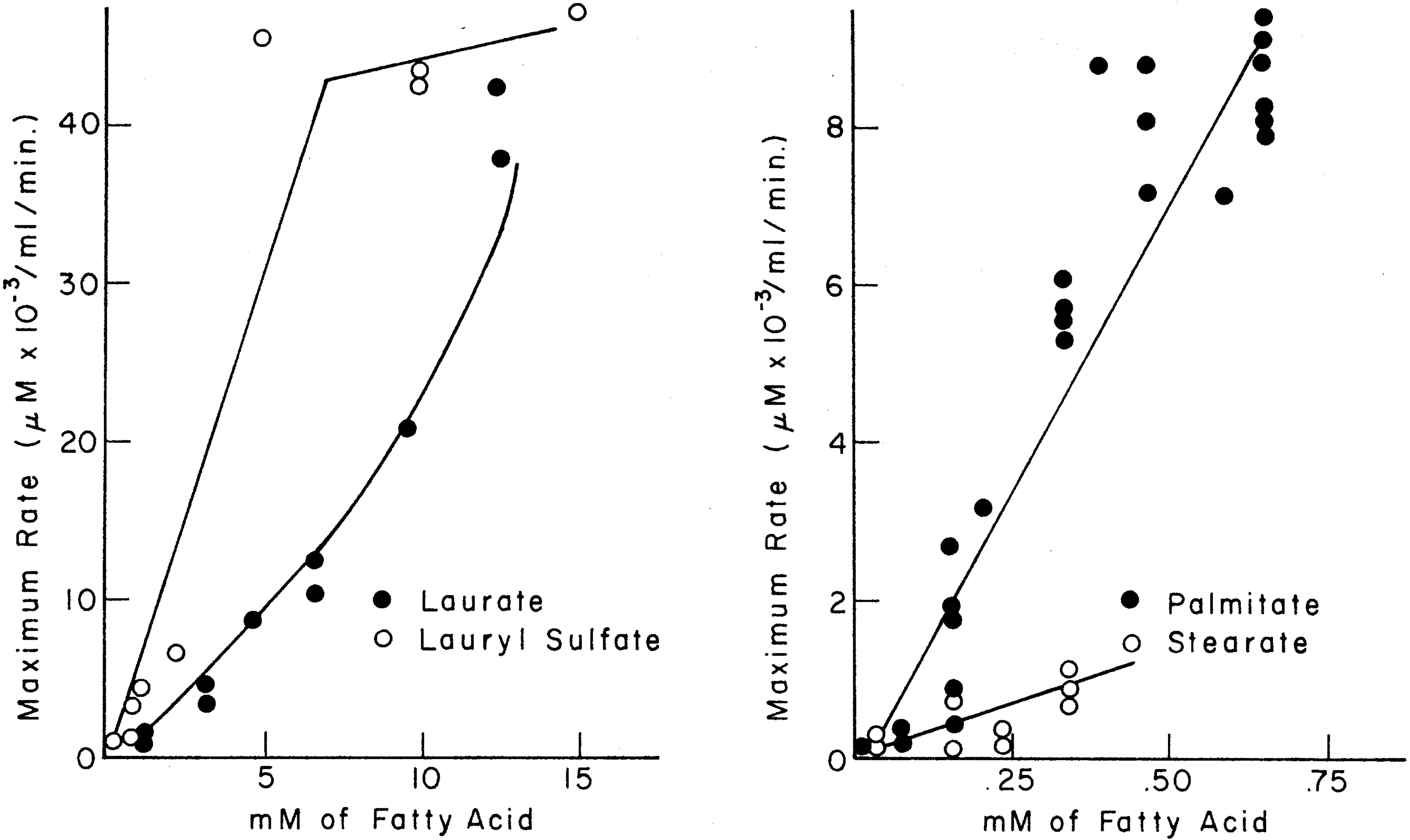

Figure 6a. Maximum rate of destruction of cytochrome c obtained with hydrogen peroxide and various final concentrations of common fatty acids and lauryl sulfate.

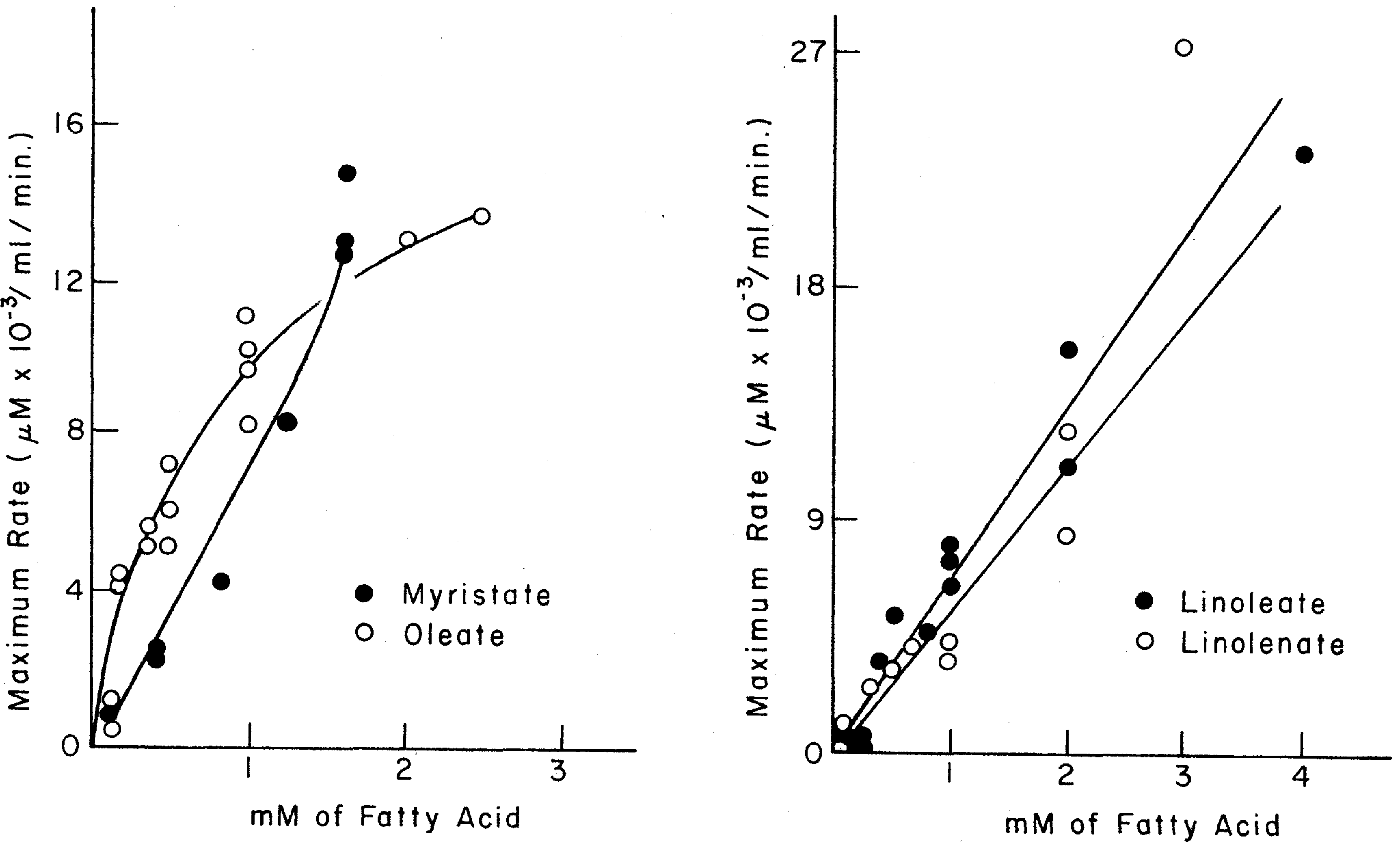

Figure 6b (concluded)

comparison of the relative activities of the fatty acids used, however, these data suggest a relation between activity of the acid and its molecular size. Figure 7 presents the relative rates obtained directly or by extrapolation to final concentrations of 1.0 mM for the saturated fatty acids. Palmitic and stearic acids were insoluble at concentrations of 1.0 mM, however, values were obtained for these compounds by extrapolation of the data of figures 6a and 6b. On this basis lauric, myristic and stearic acids are seen to have 8.7, 45.0 and 21.7 per cent, respectively of the activity of palmitic acid. The corresponding values for the 18 carbon unsaturated compounds, oleic, linoleic and linolenic acids are 51.4, 47.2, and 37.7 per cent respectively.

Other compounds tried but found to be inactive included cholic acid, desoxycholic acid, xylene sulfonic acid, traumatic acid, camphoric acid, pimelic acid, suberic acid, cinnamic acid, chaulmoogric acid, methyl palmitate and tripalmitin. Saturated straight chain acids shorter than lauric acid were also tested and found to be inactive. However, complete concentration curves were not done for these compounds.

Effect of Isobutyl and n-Butyl Alcohol on Palmitic Acid Activity

It was suggested that the fatty acids might be effective by the formation of a micellar structure which might involve, or could react with, cytochrome c. The addition of alcohols of borderline solubility to such a system might be expected to

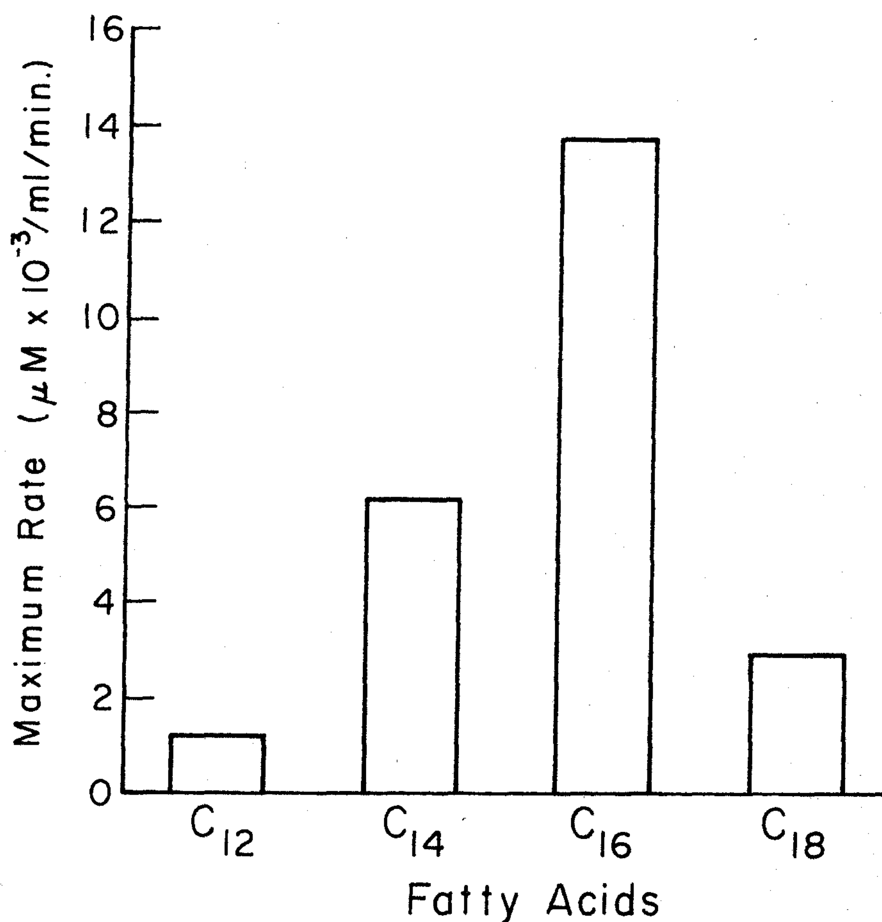

Figure 7. The maximum rates obtained or expected with 1.0 mM final concentrations of the saturated fatty acids:

C_{12} lauric acid
C_{14} myristic acid
C_{16} palmitic acid
C_{18} stearic acid

The corresponding rates at 1.0 mM of the 18 carbon unsaturated fatty acids used are:

oleic acid: 7.1 μM x 10^{-3}/ml/min.
linoleic acid: 6.5 μM x 10^{-3}/ml/min.
linolenic acid: 5.2 μM x 10^{-3}/ml/min.

have a considerable effect, although it is not clear whether this might stabilize or disrupt the structure.

Accordingly 0.2 ml of isobutyl alcohol and n-butyl alcohol were added to test solutions of a final volume of 4.0 ml containing 0.16 mM and 0.64 mM palmitic acid. Reactions were run as previously described and the maximum rates determined. The results are recorded in table 1.

TABLE 1. THE EFFECT OF ISOBUTANOL AND n-BUTANOL WITH PALMITIC ACID

0.2 ml alcohol	mM conc. pal. acid	Maximum Rate in $\mu M \times 10^{-3}$ with alcohol	cyto. c/ml/min* expected without alcohol
iso-butanol	0.00	0.05	0.03
	0.16	6.04	1.76
	0.64	25.5	8.47
n-butanol	0.00	0.05	0.03
	0.16	-	1.76**
	0.64	19.4	8.47

*Taken from the data for figure 5.
**Turned soapy after 5-10 minutes.

The activity of palmitic acid seems to be considerably increased by the presence of normal and isobutyl alcohol although similar quantities of ethyl alcohol had no such effect. Other alcohols were not tried with the exception of isoamyl alcohol which was found to have a prohibitively low solubility.

Stability of a Possible Cytochrome *c*-
Palmitic Acid Complex

Preliminary work indicated that a stable complex was formed between palmitic acid and cytochrome *c*. Only part of the palmitic acid added to unpurified cytochrome *c* at pH 8.6 then taken to low pH could be recovered by repeated ether extractions. Accordingly experiments were begun to test the stability of the complex to electrophoresis.

A 0.02 mM sample of cytochrome *c* at pH 8.6 was treated with palmitic-1-C^{14} acid in ethanol. The final concentration of palmitic acid was 0.30 mM with a specific activity of 3.56 x 10^4 cpm per μM. The solution was then lyophilized to remove about half of the water present. Centrifugation of this concentrated solution resulted in a loss of 17.7 per cent of the cytochrome and 61.6 per cent of the palmitic acid as an insoluble red precipitate. The solution was then dialyzed for 24 hours against three changes of 0.05 M, pH 8.0 tris buffer and run in a Beckman model CP continuous flow paper electrophoresis cell. After completion of the electrophoresis of the palmitic-1-C^{14} acid treated cytochrome, the curtain of the electrophoresis cell was washed by running 15 liters of fresh buffer for 24 hours and the electrophoresis repeated with untreated cytochrome *c*. The distribution of the cytochrome *c* from the two runs was determined spectrophotometrically and labeled samples counted to determine the amount of palmitic-1-C^{14} present.

Table 2 indicates the distribution and recovery of palmitic acid and cytochrome _c_ during the entire experiment. Of the 3.44 μM of cytochrome _c_ remaining after lyophilization and dialysis about 56 per cent was recovered in the collection tubes of the cell, the remainder presumably being lost during manipulation of the samples and by adsorption to the paper of the cell. In contrast less than 1 per cent of the 0.93 x 10^6 CPM in the palmitic acid of the sample was recovered after electrophoresis. Most of the palmitic acid was apparently lost by adsorption and by migration into the recirculated buffer of the electrodes. This migration of labeled palmitic acid into the electrode vessels, eventually to be recirculated throughout the cell, presumably accounts for the low levels of radioactivity detected in all fractions from the electrophoresis of this sample.

It is seen in figure 8 that about 80 per cent of the palmitic acid present in the collection tubes after electrophoresis was found in fractions 23, 24 and 25 although these fractions contained less than 2 per cent of the total recovered cytochrome _c_ as determined by absorption at 407 mμ.

Another point of interest in the data of table 2 is the absence of radioactive material in the dialyzate of samples labeled with palmitic-1-C^{14} acid. Although these observations were originally presumed to reflect the stability of a palmitic acid-cytochrome _c_ complex such an interpretation seems doubtful. Floch, Lees, and Stanley (16) pointed out that lipids in

TABLE 2. DISTRIBUTION AND RECOVERY OF CYTOCHROME C AND PALMITIC ACID DURING ELECTROPHORESIS

	Palmitic Acid		Cytochrome *c*	
	CPM	% of original sample	$mM \times 10^{-5}$	% of original sample
original sample	2.24×10^6	--	420	--
sample after lyophilization, centrifugation, and dialysis	0.93×10^6	41.0	344	82.3
precipitate from centrifugation	1.38×10^6	61.6	---	17.7*
dialyzate	20	0	0	0
total of all tubes	8.9×10^3	0.3	192	46.0

*Calculated by difference.

See text for a description and explanation of the experiment and data.

general are not dialyzable. Further, when a solution containing 0.30 mM labeled palmitic acid but no cytochrome c was made up and dialyzed as above, less than 0.5 per cent of the label was found in the buffer outside the 8/32 Visking dialysis bag after 24 hours.

Figure 8 indicates that palmitic acid and cytochrome c were separated during electrophoresis, however, the fatty acid had a profound effect on the mobility of the protein. As is seen in the figure, untreated cytochrome c migrates rapidly towards the negative electrode of the cell, however, the palmitic acid treated cytochrome exhibited a net migration towards the positive electrode. Further, the treated cytochrome c underwent a much greater spreading during electrophoresis and was distributed in a greater number of fractions than the untreated cytochrome c.

Hyperchromic Effect and Decrease in Reducibility

Palmitic acid was found to have two effects on cytochrome c independent of the increase in susceptibility to hydrogen peroxide. One, the hyperchromic effect on the Soret band absorption of cytochrome c, has previously been mentioned. The other, a decrease in the susceptibility of cytochrome c to reduction by ascorbate, was first noted by Herzenberg (9).

A sample of cytochrome c in pH 8.6, 0.05 M tris buffer was oxidized with potassium ferricyanide (0.125 mM final concentration), then dialyzed against two changes of the same

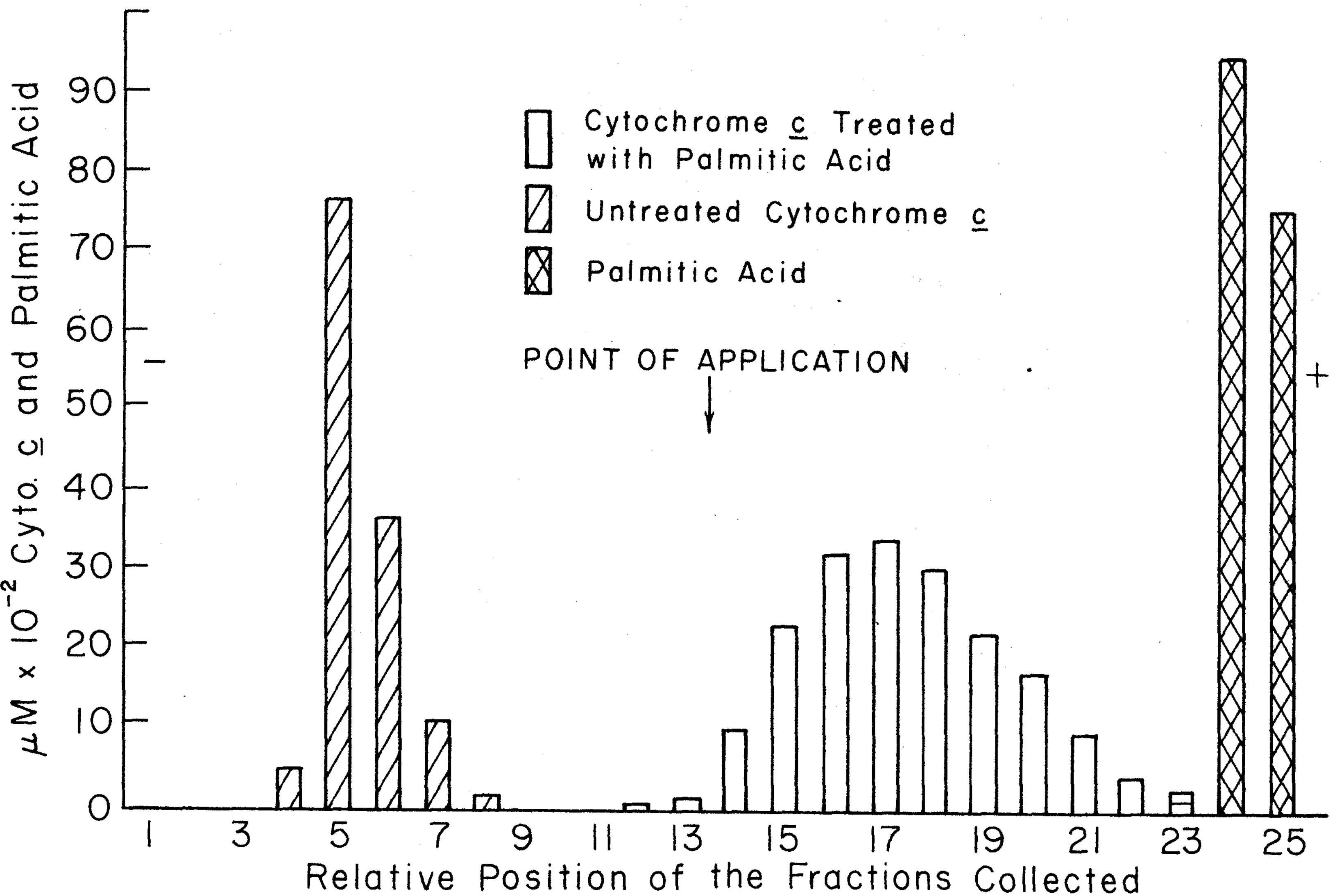

Figure 8. The distribution of palmitic-1-C^{14} acid treated cytochrome c, palmitic acid, and untreated cytochrome c after continuous flow electrophoresis.

buffer in the cold for 36 hours. The spectrum of the cytochrome c was examined for complete oxidation and the sample diluted to give a final concentration of 0.02 mM.* The amount of hyperchromic effect at 407 mμ produced by different concentrations of palmitic acid was determined by comparing the absorption of treated and untreated samples and is reported as the percentages in table 3.

The influence of palmitic acid on the reduction of cytochrome c by ascorbate was determined by comparisons of the absorption at 550 mμ ** of palmitic acid treated samples and untreated controls. Freshly prepared, cold ascorbate at pH 6.0 was added to give a final concentration of 0.05 per cent in samples of the potassium ferricyanide oxidized cytochrome c described above. The difference in optical density at 550 mμ between an oxidized sample and the resulting reduced cytochrome c was taken as 100 per cent reduction. The 550 mμ absorption of samples treated first with palmitic acid then ascorbate as outlined above were determined and the increase in optical density over fully oxidized controls expressed as the percentages of complete reduction in table 3.

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*Fully oxidized samples of cytochrome c allowed to stand in solution occasionally develop some reduced form, presumably by auto-reduction. (Boeri, E., 17).

** λ maximum for the α band of reduced cytochrome c is at 550 mμ.

TABLE 3. THE HYPERCHROMIC EFFECT AND DECREASED ASCORBATE REDUCIBILITY OF PALMITIC ACID TREATED CYTOCHROME C

Palmitic acid mM concentration	Hyperchromic effect at 407 mμ, % of controls	% of reduction by ascorbate
0.00	100.0	100
0.05	100.5	100
0.10	101.3	100
0.15	103.4	-
0.20	105.3	94
0.25	107.1	-
0.30	108.0	67
0.40	109.4	56
0.60	111.0	17

The first detectable effect on the ascorbate reduction of cytochrome c became apparent at about 0.02 mM palmitic acid or a molar ratio of 10:1 palmitic acid to cytochrome c. In contrast some increase in Soret band absorption occurred at half this concentration of palmitic acid.

The maximum concentration of palmitic acid tested is close to the region of limiting solubility at which samples had a visibly soapy appearance. However, the relatively small increase in optical density in the spectral regions adjacent to the Soret band, as evidenced in figure 2, makes it seem unlikely that the increase in absorption is due to particulate material formed in the sample.

Nature of the Reaction

Three methods were attempted to investigate the nature of the destructive oxidation of cytochrome c by hydrogen peroxide in the presence of palmitic acid and the nature of the reaction products formed. The first, an attempt to evaluate the number of moles of hydrogen peroxide consumed per mole of cytochrome c destroyed, did not give satisfactory results. Technical difficulties in measuring the small amount of hydrogen peroxide and cytochrome c remaining after the reaction had run to completion prevented dependable determination of these parameters. However, the data indicated that at least two and probably considerably more than two moles of hydrogen peroxide were involved in the destruction of each mole of cytochrome c.

The fate of palmitic acid during the reaction and some properties of the reaction products were also investigated. Hydrogen peroxide was added to 500 ml of 0.02 mM cytochrome c to which palmitic acid had been added to give a 0.30 mM final concentration. After 4 hours the sample was dried by lyophilization, then hydrolyzed with 6 N HCl in a sealed tube at 100° C. for 18 hours. The ether extract of the hydrolyzate was analyzed by gas chromatography* and shown

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*The procedures used for gas chromatography are described in Part II, the section on "The Free Fatty Acids of Poky."

to contain much (72 per cent) of the palmitic acid expected for quantitative recovery. Although a 28 per cent loss may seem high, it is likely that it resulted during analysis of the small amount of palmitic acid involved rather than by destruction during the reaction.

The third type of experiment performed was to measure the effect of hydrogen peroxide on cytochrome c that had been subjected to hydrolysis by proteolytic enzymes. Cytochrome c was dissolved in 0.05 M tris buffer at pH 8.2 so that a concentration of 0.02 mM was given by the addition of 0.4 ml to 3.6 ml of the starting cytochrome c solutions. To 3.6 ml portions of the solution of cytochrome c were added 0.1 ml of Worthington Biochemical Corporation trypsin or chymotrypsin dissolved in cold distilled water in concentrations that would give 1/5 by weight the amount of cytochrome c present in the sample. The absorption at 407 mμ was followed for 30 minutes at room temperature, then the pH adjusted to about 8.5 by the addition of 0.2 ml of 2.10 M un-neutralized tris. Hydrogen peroxide was then added as usual, and the decline in absorption at 407 mμ followed. The average results for two trials with trypsin and with chymotrypsin are shown in figure 9. The experiment was repeated using boiled solutions of trypsin and chymotrypsin which were found to have no effect.

Although the total initial loss was different in the two cases, both trypsin and chymotrypsin hydrolyzed samples demon-

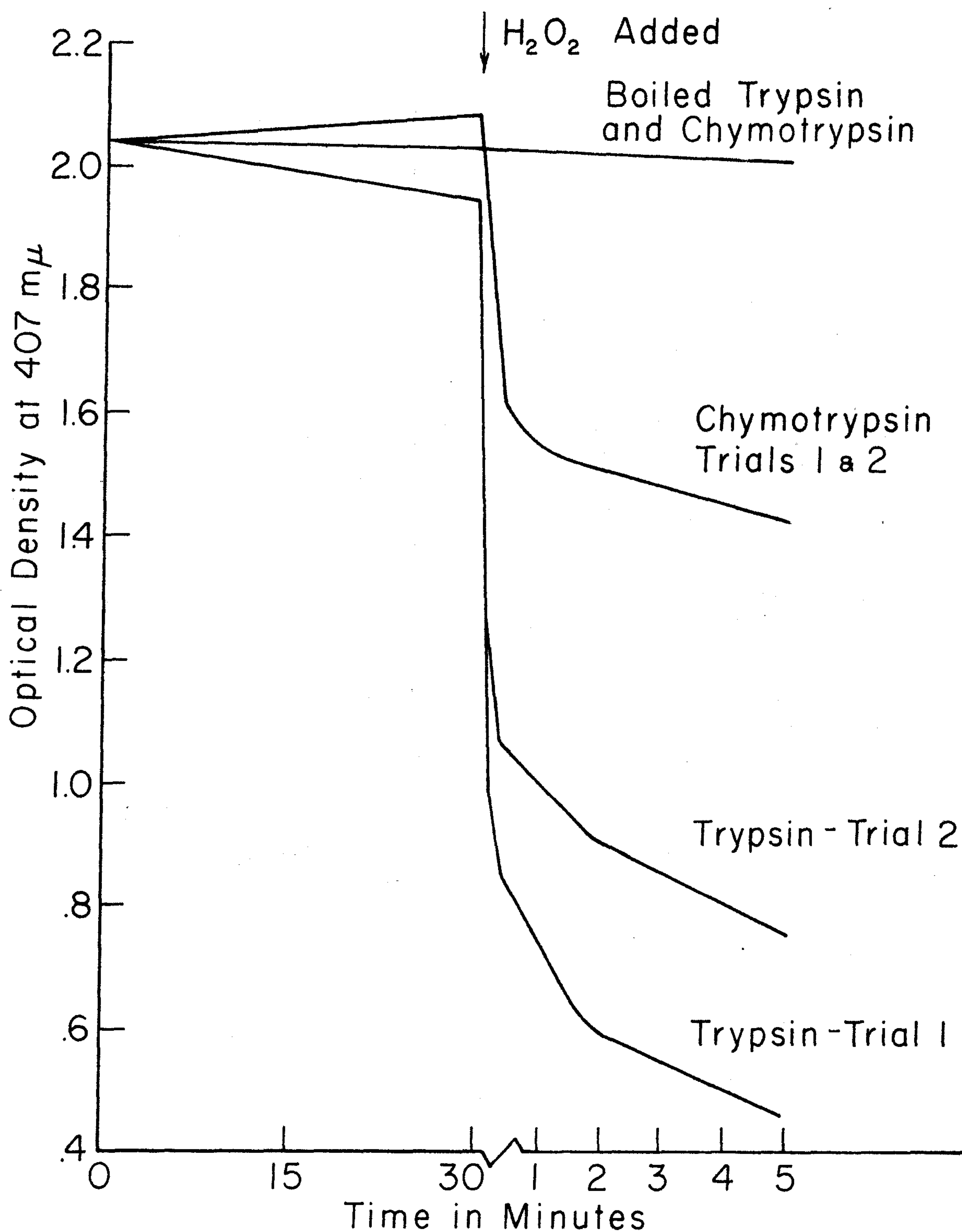

Figure 9. The effect of hydrogen peroxide on cytochrome c that had been subjected to hydrolysis by the proteolytic enzymes, trypsin and chymotrypsin.

strated marked declines in Soret band absorption following the addition of hydrogen peroxide. Further, after the initial sharp drop in optical density both types of samples seemed to level off at about the rate of decline given by controls containing boiled trypsin or chymotrypsin. Also of interest is the slight but apparently real difference in the optical density of trypsin and chymotrypsin treated samples before the addition of hydrogen peroxide.

C. DISCUSSION

Purified horse heart cytochrome c is a relatively small, basic protein having a molecular weight of about 13,000 and an isoelectric point of 10.65 at 0° C. (Theorell and Åkesson, 18). It contains one heme of the protoporhyrin IX type per molecule and about 93 amino acids including 18 molecules of lysine and 3 of histidine. (Leaf, Gillies and Pirrie, 19). Heart muscle-cytochrome c is stable to dilute acids, even mineral acids, 0.1 N alkali and boiling. At physiological pH it is not auto-oxidizable. Ferricyanide and cupric ions oxidize ferrocytochrome c to ferricytochrome, while hydrogen activated by platinum or palladium, dithionite, cysteine, p-phenylenediamine, ascorbic acid, catechol and pyrogallol reduce ferricytochrome to ferrocytochrome.

The molecular shape of horse cytochrome c has been described as a prolate elipsoid with a frictional ratio of 1.34 (f/fo) (Ehrenberg and Paléus, 20), and with molecular dimensions of a = 18 Å and b = 98 Å (Neurath, 21). By working with scale models Ehrenberg and Theorell (22) have concluded the most probable configuration for the heme peptide from proteolytic digestion is as an α-helix with a left hand spiral. The configuration provides a near perfect "fit" for thioether bonds between the vinyl groups of the planar protoporphyrin ring and two cysteine residues of the peptide. Four of the six coordinative valences of iron are occupied with the nitrogen atoms of the tetrapyrrole, however, the other two are apparently

coordinated with oxygen or nitrogen containing groups of the protein. Amino acid analysis, titration curves, and spectrophotometry strongly suggest that the two valence bonds of iron that are not directed in the plane of tetrapyrrole ring are coordinated with two imidazole rings of histidine (Theorell and Åkesson, 23). A hypothetical cross section through the molecule has been visualized as in figure 10 which is reproduced from the English translation of Hugo Theorell's Nobel Prize Lecture (1956).

Thus Theorell (24) considered it extremely probable that the heme plate in cytochrome c is surrounded by peptide spirals on all sides in such a way that the heme iron is entirely screened off from contact with oxygen gas. This is offered as an explanation as to why reduced iron in cytochrome c cannot be oxidized directly by oxygen gas for it cannot come in contact with the iron atom. Electrons are probably passed in and out to the iron atom via the imidazole groups.

With this proposed structure of cytochrome c in mind and the known interaction of fatty acids with other proteins (25,26,27,28,29) a probable explanation of their effect on cytochrome c is that they somehow interact with the protein moiety of the cytochrome molecule in such a way that the heme is more directly exposed to attack by hydrogen peroxide. This hypothesis seems to be supported by many of the previously described results in that they suggest a mechanism by which the helical peptide chain is at least partially unfolded from around the cytochrome heme.

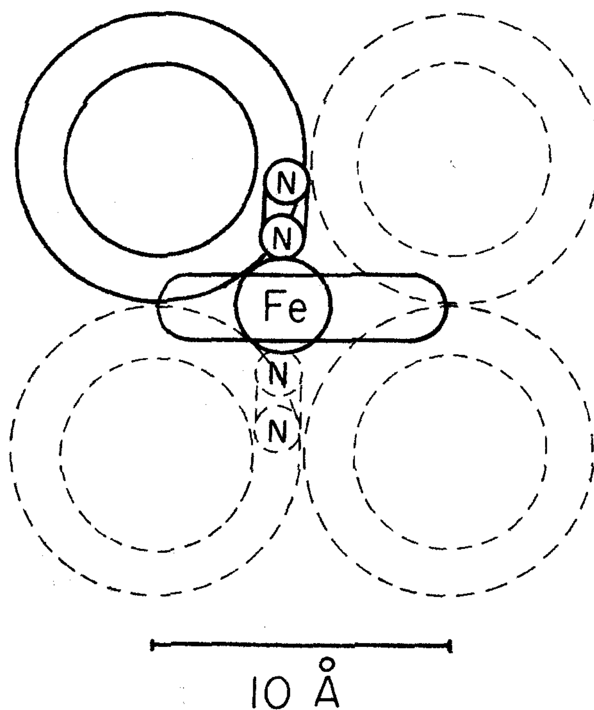

Figure 10. Hypothetical section through a cytochrome c-molecule. The solid lines show the hemopeptide. The dashed lines show the parts of the natural molecule split off with pepsin. The region between the outer and inner circles is taken up by the side chains of the amino acids. The four peptide chains surround the iron atom, making it inaccessible to oxygen gas (24).

If fatty acids do cause exposure of the heme then it might be reasoned that digestion by proteolytic enzymes such as trypsin and chymotrypsin might produce many of the same effects. Such treatment would hydrolyze much of the protein and presumably expose the tetrapyrrole ring. Indeed, the data in figure 9 shows that proteolytic digestion does cause cytochrome to become highly susceptible to destructive oxidation by hydrogen peroxide similar to treatment with fatty acids.

Further, if the hypothetical mechanism is correct then it seems likely that molecular oxygen would be able to oxidize reduced cytochrome c directly in the presence of fatty acids. Accordingly, 24 ml of 0.1 mM solution of cytochrome c was made up in degassed 0.05 M tris buffer at pH 8.6, reduced with ascorbic acid and dialyzed against two 1000 ml changes of degassed buffer in the cold under N_2. The spectrum was checked to assure complete reduction,then the sample was quickly divided into three 8 ml portions. Two were made 0.6 mM by the addition of palmitic acid in 0.2 ml of ethanol. Then one of these treated samples and the untreated portion were placed in 100 ml beakers and put into a desiccator. The desiccator was quickly evacuated and refilled with oxygen gas and allowed to stand at room temperature for 1 hour after which the spectra of all three samples were recorded. Meanwhile the other palmitic acid treated sample was placed in a sealed cuvette and its spectrum recorded immediately as well as at the end of the 1 hour period.

The first spectrum of the treated sample kept free of oxygen insofar as was possible was that of completely reduced cytochrome c. However, after 1 hour it indicated the presence of some ferri form, possibly due to oxidation by small amount of molecular oxygen not completely excluded from the system. In contrast, the spectrum of the palmitic acid treated sample exposed to oxygen indicated about 50 per cent of the oxidized form although no oxidized cytochrome was apparent in the untreated portion which had also been exposed to oxygen.

These results probably represent the same phenomenon observed by Rabinovitz and Boyer (30) who noted a decrease in the characteristic absorption peaks of ferrocytochrome c with the addition of α-tocopheryl phosphate or sodium dodecyl sulfate. They also reported an inhibition of the rat heart succinoxidase system probably due principally to the prevention of the interaction of the succinic dehydrogenase with cytochrome c.

The conclusion is that fatty acids do allow molecular oxygen to oxidize reduced cytochrome c directly, thus supporting the proposed mechanism. It follows that tryptic digestion might have essentially the same effect on cytochrome c by hydrolysis of the protein thus exposing the heme. However, some differences were noted between fatty acid and treated and trypsin hydrolyzed samples of cytochrome c.

The foregoing experiment was repeated using a 1 hour

digestion at room temperature with 0.01 per cent trypsin instead of the fatty acid treatment of ferrocytochrome c. In this case digestion seemed to be paralleled by an increase in the amount of ferricytochrome c present in the sample as judged by its spectrum. After 1 hour no reduced cytochrome could be detected in the sample. Its spectrum was that expected for completely oxidized cytochrome c. Although this result (repeated several times) might be due to traces of molecular oxygen present in the system it seems possible that the digestion itself might destroy interaction between the protein and heme of cytochrome c necessary for the characteristic absorption spectrum of reduced cytochrome c. In this case change in the absorption spectrum from reduced to oxidized cytochrome c might not reflect a change in the oxidation state of the iron. With this in mind it seems necessary to question the effect of fatty acid in decreasing the apparent amount of reduction of cytochrome c obtained with ascorbic acid (table 3).

The rupture of the coordinated bonds of iron directed out of the plane of the tetrapyrrole ring to some portion of the protein, presumably imidazole rings, might well be expected to disrupt a two step transmission of electrons to iron and thus lend support to the mechanism proposed by Theorell as well as the proposed explanation of the effect of fatty acids on cytochrome c. However, it also seems likely that a gross change in the tertiary structure of the protein

might cause changes in the characteristic spectrum of reduced cytochrome _c_ so that it did not reflect the oxidation state of the heme iron. In this case, the data of table 3, based on spectral observations might not represent the amount of reduction actually obtained. This problem might be resolved by use of some means other than characteristic absorption to measure the oxidation state of the iron, such as coupling with indicators or electron spin resonance, or possibly by measurement of the physiological effectiveness of fatty acid treated cytochrome _c_.

The interaction of fatty acids with cytochrome _c_ does cause some changes in absorption as seen in that portion of table 3, presenting data on the hyperchromic effect of the Soret band peak of oxidized cytochrome. Treatment with different amounts of palmitic acid led to an increase in absorption at 407 mμ but did not change the wave length of maximum absorption. Such an effect might be produced by a change in the electronic environment of the heme as would result from an unfolding of the protein from around the heme of cytochrome _c_. An analogy might be made between this observation and the situation with deoxynucleic acid in which "melting" of its tertiary structure by increased thermal motion or chemical agents leads to a hyperchromic effect in the region of 260 mμ.

The data present very little evidence for the nature of the binding between fatty acids and cytochrome _c_. However,

Rowley and Wainio (31) concluded that bonding between cytochrome c and desoxycholate in solution was primarily ionic in nature. During electrophoresis of cytochrome c at pH 8.55 they found that the mobility changed from cationic with increasing amounts of desoxycholate and calculated the maximum number of bound anions to be 12. This value was in close agreement with the number of excess positive charges (+13) calculated to be present on the protein at this pH. Also, cytochrome and desoxycholate were observed to separate during electrophoresis. Such observations agree with the findings presented in figure 8 where palmitic acid was reported to affect the electrophoretic mobility of cytochrome c but to be eventually completely separated from it.

The sharp breaks in activity at about pH 8.0 and 10.0 seen in the pH curve of figure 4 might be expected to reflect some specific form of the protein. However, comparison of these data with the spectrophotometric titration curves of ferricytochrome c reported by Theorell and Åkesson (32) indicate that they do not correspond to any one specific form of the cytochrome. In fact, it may be of significance that this is quite precisely the region of inner conversion of two of the pH dependent spectral forms of cytochrome c observed. Direct titration curves of cytochrome (Theorell and Åkesson, 23) show a break in the pH 9 to 10 region as expected for titration of basic amino acids.

Figures 6a and 6b present the maximum rates of destruction

of cytochrome c by hydrogen peroxide obtained at various concentrations of the common fatty acids and sodium lauryl sulfate. These data suggest an interesting apparent specificity more clearly shown for the saturated fatty acids in figure 7 where palmitic acid is seen to have the greatest activity on a molar basis. The shorter fatty acids, lauric and myristic as well as the longer stearic acid, have considerably less activity, perhaps indicating a certain degree of specificity of the protein for fatty acid. Such a relation suggests a requirement for a certain size and/or shape of fatty acid molecule. The hypothesis that the length of the hydrocarbon chain may somehow determine the extent of interaction is supported by the results with the unsaturated 18 carbon compounds, oleic, linoleic and linolenic acids. All are considerably more active than the 18 carbon saturated compound, stearic acid. This might be expected since the cis double bonds present in these compounds would tend to turn the hydrocarbon chain back upon itself thereby possibly shortening the effective chain length.

A close correlation seems to exist between the rates obtained for the saturated fatty acids at a concentration of 0.1 mM (figure 7) and the second class of apparent association constants (k_2) for the interaction of human serum albumin with fatty acid anions at pH 7.45 reported by Goodman (25). Goodman equilibrated varying quantities of each fatty acid in a two phase system of n-heptane and an aqueous solution of serum albumin in phosphate buffer at pH 7.45. The

concentration of unbound fatty acid in each aqueous phase containing serum albumin was determined from the concentration in heptane, then compared with results from identical distribution experiments performed in the absence of albumin. Apparent association constants for the interaction of human serum albumin with fatty acid anions are reported in table 4 which is reproduced from Goodman's paper (25). It was determined that human serum albumin has about 2, 5, and 20 binding sites related to k_1, k_2, and k_3 respectively. The relative values of k_2 based on palmitic acid were calculated from Goodman's data and compared with similar values for the data of figure 7. The results are presented in table 5. The correlation for the saturated fatty acids seems more than coincidental even though similar values for the unsaturated compounds, oleic and linoleic acids do not exhibit the same close agreement.

The possibility that fatty acid micelles and not the free acids are the active forms responsible for the effects noted has been considered. It could be imagined that the molecules of cytochrome c are adsorbed to the surface of fatty acid micelles in such a manner as to distort their tertiary structure and cause the partial unfolding of the polypeptide chain from around the heme. Unfortunately, no information is available concerning critical micelle concentrations under the conditions employed. Granath (33) has indicated that micellar size, shape, and concentration of formation

TABLE 4. The apparent association constants for the interaction of human serum albumin with fatty acid anions at pH 7.45, ionic strength 0.160, 23°, with three classes of binding sites, $n_1 = 2$, $n_2 = 5$, and $n_3 = 20$ (25).

Fatty acid anion	k_1'	k_2'	k_3'
laurate	1.6×10^6	2.4×10^5	6×10^2
myristate	4.0×10^6	1.4×10^6	2×10^2
palmitate	6.0×10^7	3.0×10^6	1×10^3
stearate	8.0×10^7	8.0×10^5	1×10^3
oleate	1.1×10^8	4.0×10^6	1×10^3
linoleate	1.3×10^7	2.5×10^6	2.5×10^3

TABLE 5. The relative activities of the saturated fatty acids based on palmitic acid and calculated from values of k_2, the second class of apparent rate constants (table 4), Goodman (25) and the maximum rate constants at 1.0 mM concentration (figure 7)

Fatty acid	k_2	k max. at 1.0 mM
lauric	8.0%	8.7%
myristic	46.7%	45.0%
palmitic	100.0%	100.0%
stearic	26.7%	21.7%

depend chiefly on length of the hydrocarbon "tail" of the detergent molecule, concentration of the detergent, temperature, concentration of salts present and pH of the solution. It was reasoned that the addition of slightly soluble alcohols such as isobutyl or n-butyl alcohol might interfere with possible micelle formation. Thus, if micelle formation was necessary for activity then such alcohols might be expected to reduce the effect of the fatty acid. The data of table (1) indicates that both isobutyl and n-butyl alcohol markedly increase rather than decrease the activity of palmitic acid. This would be the expected result if free acid and not micelles were active and the alcohol increased the effective concentration of free acid by inhibiting micelle formation. However, there is some reason to question the foregoing assumptions in that it is not clear that the alcohols would interfere with, rather than stabilize, fatty acid micelles (Schick and Fowkes, 34) or especially micelle-like aggregations of protein and fatty acid.

Large increases in the amount of either fatty acids or cytochrome c seem to lead to the formation of an insoluble complex. Table (2) presenting the data for the stability of the cytochrome c-palmitic acid complex indicates that 17.7 per cent of the cytochrome c and 61.6 per cent of the palmitic acid was lost as an insoluble red precipitate after lyophilization during this experiment. These results are in agreement with those of Michelazzi (35) who observed precipitation of

cytochrome c when it was added to lipid extracts of yeast or liver. He also reported that such treatment produced spectral changes of both oxidized and reduced cytochrome c similar to those outlined above.

The products formed during the oxidation of fatty acid treated cytochrome c have not been chemically identified. However, hydrogen peroxide might be expected to cause oxidation of the porphyrin ring. The visible change in color of cytochrome solutions from red to green during the reaction (see figure 2) strongly suggests an oxidative cleavage of the porphyrin ring yielding at least in part a compound or group of compounds related to the bile pigments, particularly the green or blue-green verdins (chapter IV, Lemberg and Legge, 36). Although such compounds would be expected to undergo further oxidation in the presence of excess hydrogen peroxide it seems likely that they might occur in sufficient amounts to account for the color observed. Further oxidation would be expected to shift their absorption maxima to shorter wave lengths, thus, the solution would transmit light of longer wave length and have a color shift towards the red.

PART II

STUDIES ON NEUROSPORA

A. MATERIALS AND METHODS--GENERAL

The Strains

Strains of _Neurospora crassa_ number WP-3178 _a_, _po_-3627-1_a_, _po f_-3627-4A, _mi 3_-3543-2a and C115-R-3 have been used. WP-3178 a is a wild type strain. _Po_-3627-1A is the "maternally inherited" strain called _poky_ and isolated from an old wild type culture by Mitchell and Mitchell (7). It is characterized by relatively slow growth compared with wild type and an accumulation of cytochrome _c_ plus a deficiency of cytochromes _a_ and _b_. _Po f_-3627-4A carries genetic suppressor of _poky_ described by Mitchell and Mitchell (37) which restores the growth rate of _poky_ to nearly that of wild type but has little if any effect on the amounts of the cytochromes present. _Mi 3_-2543-2A is also a "maternally inherited" strain characterized by relatively slow growth and an aberrant cytochrome system in which cytochrome _c_ is accumulated (38).

Two gene mutants of _Neurospora crassa_ have been described (Mitchell, Mitchell and Tissieres, 38) which have aberrant cytochrome systems. One of these (strain C115) contains an excess cytochrome _c_, an abnormally low level of cytochrome _b_ and little or no cytochrome _a_. The second strain (C117) contains an excess of a heme component with the absorption spectrum of cytochrome _c_. Unlike wild type, a substance with spectral properties similar to cytochrome _e_, is also present. However, C117 is deficient in cytochrome _c_ and _a_ and was not used in this work.

Culture of Neurospora

Stock cultures of the different strains were maintained on agar slants of minimal medium by frequent transfer and storage of mature cultures at 10° C. The nutrient used for slants and liquid culture was made up from a 50 x stock solution as described by Vogel (39).

Larger amounts of mold were grown in liquid culture in five gallon pyrex carboys fitted with aeration and exhaust tubes. Sterile air was supplied by filtration through cotton or Kerby air filters at rates sufficiently high to keep the 15 liter contents of each carboy in constant motion and to cause the formation of many small bubbles. Growing cultures were maintained in a 25° C. constant temperature room. However, the temperature of the liquid nutrient in the carboys was somewhat below 25° C. due to cooling by the air being forced through them.

The conidia spores and some hyphae from one six inch test tube slant of a mature stock culture were used to inoculate each carboy. Spores were suspended in distilled water by agitation, then drawn into a sterile 20 ml hypodermic syringe and injected directly into the carboys through their exhaust tubes. The exhaust tubes were capped by inverted six inch test tubes.

Some variation in growth rate between carboys frequently occurred, probably in part due to variations in the amount of innoculum, aeration, and the temperature of the nutrient.

Cultures grown in carboys were harvested within a few minutes of the termination of aeration by straining the nutrient through a strong cotton bag which was then quickly placed in about 50 per cent cracked ice in water. The washing removed traces of the nutrient and quickly cooled the tissue to nearly 0° C. As much water as possible was then removed by squeezing and twisting the bag. The resulting press-dry mat of mycelia was then either used immediately or wrapped in heavy gauge aluminum foil and stored at a -27° C.

Grinding Mold for Extraction

The resistance of fresh Neurospora mycelia to fragmentation by Waring blenders and similar devices made them nearly useless in the preparation of large quantities of tissue. Satisfactory fragmentation was obtained with a Nossel shaker but the quantities that could be handled were prohibitively small. The most satisfactory method found for up to 75 gram quantities of fresh mold was that used by Herzenberg (9). The press-dry mycelial mats, 1/2 part by weight of washed sand, and 2 parts of 0.05 M, pH 7 phosphate buffer containing 75 grams per liter of mannitol were mixed in a mortar and ground in the cold for about 10 minutes. The resulting slurry was centrifuged at about 1,800 x g for 10 minutes and the precipitate re-ground and centrifuged twice more, each with the addition of 1 part of phosphate-mannitol buffer. This method of grinding was used for preparation of mitochondrial, microsomal and supernatant fractions.

By far the most satisfactory method for total extraction was to grind the frozen, press-dry mycelial mats with about 2 parts of cracked, dry ice in a power driven corn mill (Quaker City Mill Model F-3E, Fisher Scientific Company). This device has two circular friction plates between which the frozen tissue and dry ice was forced. The result of the grinding was a fine frozen powder of dry ice and mycelia. The dry ice was allowed to sublimate away as CO_2 gas in a deep freezer so that only a fine frozen flour remained. Microscopic examination and results from extraction procedures indicate excellent rupture of structural elements present in the tissue. Shearing forces exerted on ice crystals during grinding probably destroy such fine cellular structures as nuclei and mitochondria as well as the hyphal walls. In addition this procedure has the advantage of keeping the tissue in a frozen state until the extraction is actually started. These factors plus the relative ease with which it could be applied to even kilogram quantities of tissue made it the most satisfactory method of grinding used.

B. NEUROSPORA CYTOCHROME C

Materials and Methods

Isolation of Cytochrome c by Extraction with Ammonium Hydroxide

Two procedures were used for the isolation of cytochrome c from Neurospora during these investigations. The ammonium hydroxide extraction described in this section was used in all cases for the preparation of large amounts of cytochrome c used for free boundary electrophoresis, sedimentation, and molecular weight determinations.

The flow chart of figure 11 indicates the procedure used to obtain semi-purified cytochrome c from ammonium hydroxide extracts of Neurospora. The methods were evolved through many modifications in the early stages of the work and contain techniques and conditions found by experience to be satisfactory. For instance, a primary objective was the extraction and subsequent isolation of all or nearly all of the cytochrome c present in the intact mold, thereby decreasing the possibility of a differential loss of components. It was found that use of the resin, IRC-50, used by Margoliash (40,41) led to relatively high losses (30 per cent or more) of cytochrome c as determined by the spectrum of the recovered material. In contrast, 90 to 95 per cent of the cytochrome c applied could be recovered from acid washed Hyflo Super-cel.

Care was taken to maintain temperatures near 0° C. throughout the procedure. Insofar as was possible all steps

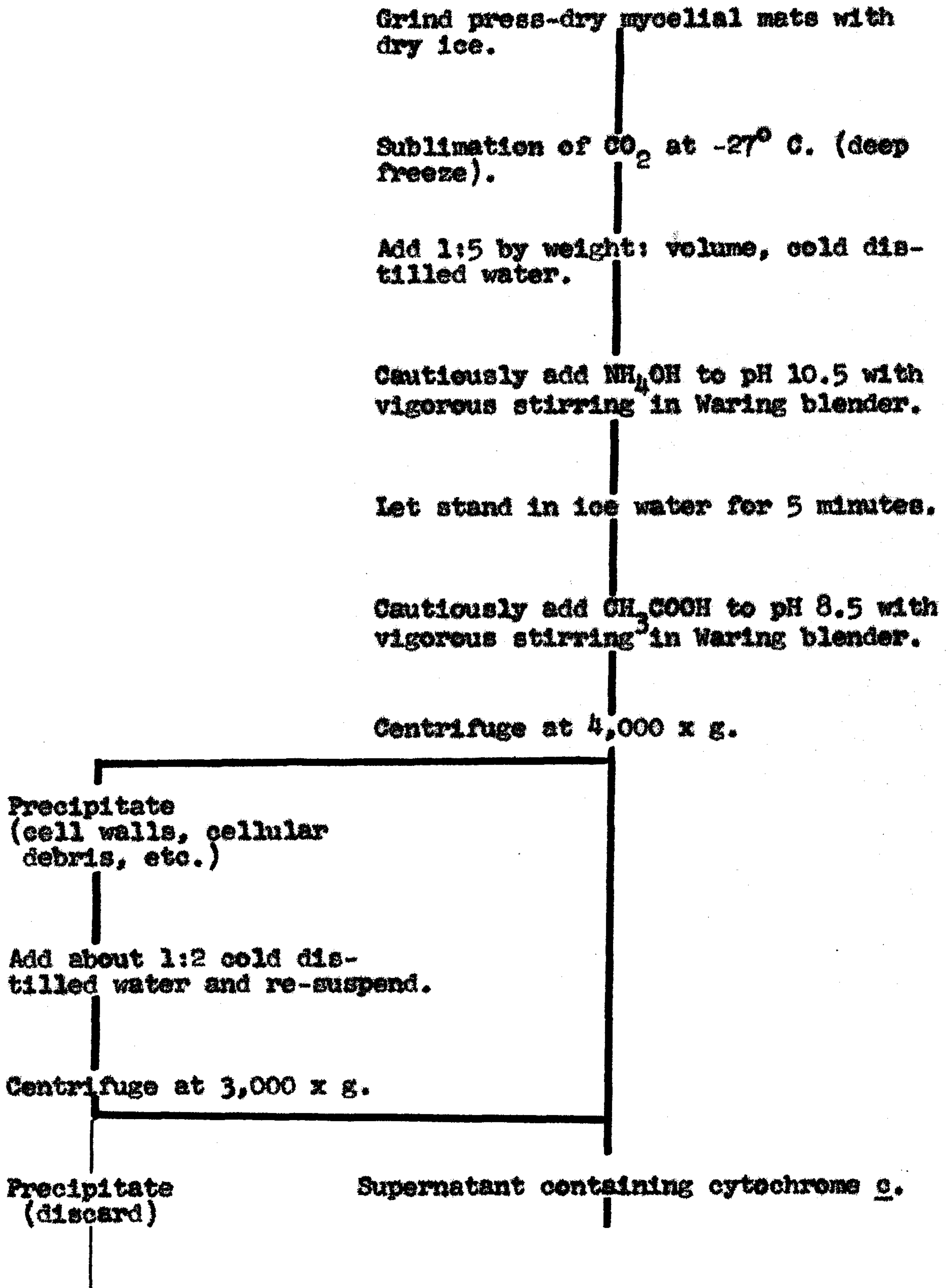

Figure 11

Flow diagram of purification procedure.

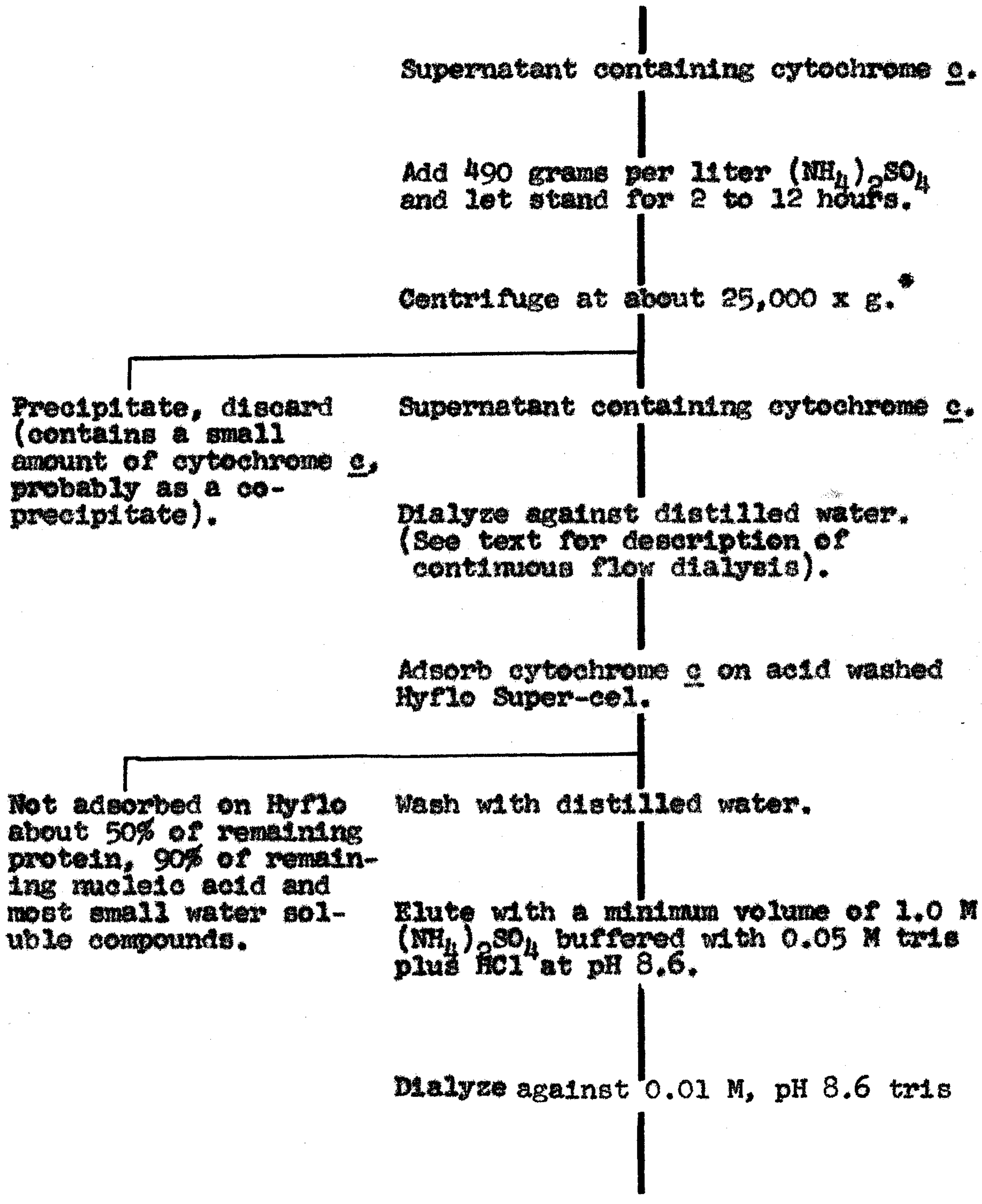

Figure 11 (continued)

*Early isolation employed a Spinco model L preparative centrifuge however, the large volumes involved were most easily handled with a Lourd's continuous flow centrifuge at 25,000 x g.

Dialyze against 0.01 M, pH 8.6 tris.

Lyophilize to dryness.

Store at -27° C. as a dry powder until used.

Figure 11 (concluded)

were carried out either in a cold room held at about 4° C. or in vessels surrounded by ice. Ammonium hydroxide and acetic acid were added in a fume hood in a warm room; however, solutions were kept on ice except during the actual addition of these compounds.

A hand held spectroscope (Dr. C. E. Bleeker, Nederlandse Optiek-en Instrumenfabriek; Zeist, Nederland) was an indispensable aid in following the distribution of small amounts of cytochrome c during the course of fractionation. A powerful but variable light source in the form of a microscope lamp equipped with an iris diaphragm and a variable power supply was set up to direct light through containers placed above it. In this manner solutions, precipitates or intact tissues in which the cytochrome c had previously been reduced, usually by the addition of a small amount of crystalline sodium hydrosulfite, could be quickly and easily examined. The width of the characteristic absorption band of reduced cytochrome c at 550 mμ indicated the amount of carrier present.

Treatment with ammonium hydroxide.--The addition of 5 parts of cold distilled water to 1 part of ground Neurospora at -27° C. yielded a lumpy half-frozen suspension. Homogenation of the suspension in a blender (Waring Model CB-3, Los Angeles Chemical Company) provided a viscous material of uniform consistency and a means of assuring vigorous stirring during the addition of ammonium hydroxide and acetic acid. Such stirring is important in avoiding local concentration

gradients, particularly for acetic acid. Neurospora cytochrome c is irreversibly denatured under conditions of acid pH (pH 4 to 5 and below) as determined by its spectrum. Further it is apparently important to maintain relatively high pH to inhibit proteolytic enzymes known to be present in Neurospora and effective in the hydrolytic destruction of cytochrome c (9). Homogenates of poky taken to near neutrality at this point tended to turn green and to lose the characteristic cytochrome c spectrum. This is in agreement with Herzenberg's observation of rapid proteolysis at pH 7.0 but none at pH 8.2.

Low speed centrifugations (4,000 x g) were generally performed in a model PR-2 refrigerated International centrifuge (International Equipment Company; Boston, Mass.), although several continuous flow centrifuges were tried. The large amount of precipitate involved limited the usefulness of the continuous flow devices for this centrifugation. Some loss of cytochrome c with the precipitated cellular debris inevitably occurred in this step. Further, there is a significant difference between poky and wild type Neurospora in this regard. A much higher percentage of the total cytochrome c present in intact wild type is lost, possibly reflecting the differences in the total amount of cytochrome c in poky and wild type Neurospora.

Ammonium sulfate fractionation.--The amount of ammonium sulfate added (about 70 per cent saturated at 0° C.) represents a compromise between two undesirable situations. Although

purified cytochrome c is soluble in low concentrations in 80 to 100 per cent saturated ammonium sulfate, increasing amounts are lost with increasing concentrations of ammonium sulfate, probably due primarily to co-precipitation with other proteins. On the other hand lower concentrations of ammonium sulfate fail to precipitate a great deal of the protein. Indeed, when concentrations of ammonium sulfate of 40 per cent saturation or less were used and when the hydrogen ion concentration of subsequent solutions was allowed to drop to near pH 7.0 the red color of cytochrome c was replaced by green suggesting the proteolytic activity previously mentioned. In contrast, when 70 per cent ammonium sulfate was used at this point, subsequent solutions seemed quite stable despite the pH. This observation indicates that the proteolytic enzyme or enzymes involved may have been largely removed with 70 per cent saturated ammonium sulfate.

The ammonium sulfate precipitate was most conveniently removed with a Lourdes model LRA refrigerated centrifuge equipped with a CFR-1 continuous flow head. (Lourds Instrument Corp.; Brooklyn, N.Y.). A centrifugal force of 25,000 x g could easily be obtained with this centrifuge and supernatant fractions were nearly free of turbidity.

Continuous flow dialysis.--Dialysis of up to 10 liter quantities of 70 per cent saturated ammonium sulfate solutions from the preceding step represented a difficult technical problem. Direct dialysis of this material against distilled

water or dilute buffer was complicated by the high osmotic pressures involved as well as the large amount of dialysis tubing required to contain the large volumes.

A satisfactory solution to this problem was the construction of the continuous flow pressure dialysis system described below and depicted in figure 12. Basically this involved an 18 foot length of 8/32 inch (inflated diameter) seamless cellulose tubing (Visking Co., Chicago, Ill.) drawn through a similar length of 3/4 inch polyethylene tubing in such a way that the ammonium sulfate solutions were very slowly passed through the dialysis tubing at the same time that cold, distilled water was passed in the opposite direction through the polyethylene tubing. About 5 pounds per square inch pressure was applied to the ammonium sulfate solution through a dynamic pressure regulator that would prevent the increase in pressure above this value. It was found that new 8/32 inch Visking tubing would usually withstand at least 15 pounds per square inch of pressure. The use of positive pressure within the system acted against the osmotic pressure gradient in the uptake of water into the solution and even led to slight reduction in volume after lower salt concentrations had been obtained.

Some difficulty was experienced in attaching the dialysis tubing to the glass intake tube in such a manner as to prevent rupture of the dialysis tube at this point. The most satisfactory technique was to insert the glass tube into the dialysis tube, then wrap both with electrical tape (Scotch Brand, No. 33)

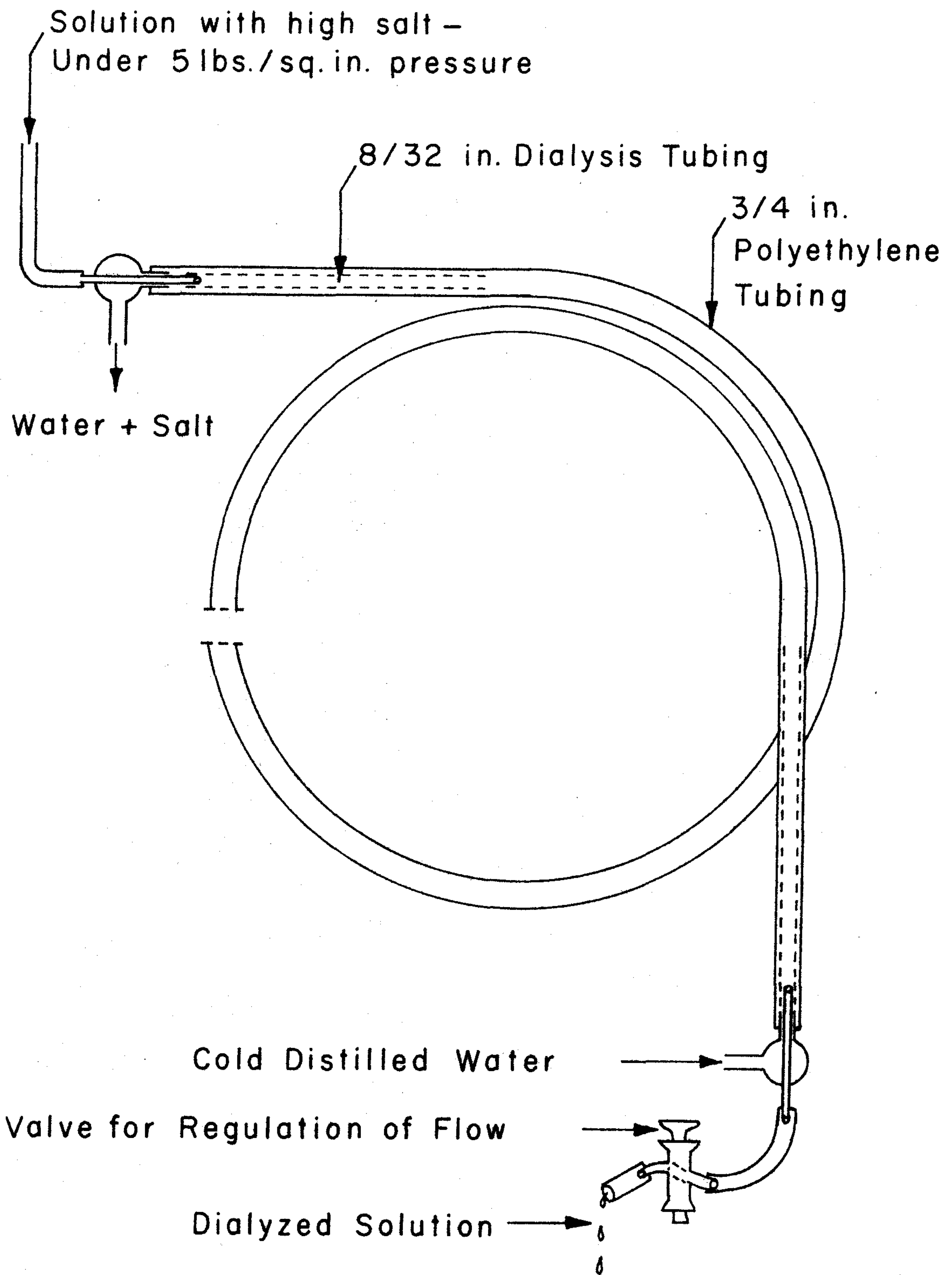

Figure 12. Diagram of "continuous flow dialysis" set up.

so that a single layer of tape covered the section adjacent to the region of strain for the dialysis tube. Strong thread was then wound over the electrical tape to within about 1/4 inch of its edge over the dialysis tubing. The electrical tape used was quite plastic as well as resistant to water and salt. It would stretch with increasing pressure within the dialysis tube so that no localized point of stress was created on the dialysis tube thus preventing its rupture.

It was always necessary to test the apparatus thoroughly for pin holes or weak spots in the dialysis tubing. However, when a satisfactory set up was attained the dialysis generally progressed with surprising ease. The flow rate of ammonium sulfate solution through the apparatus was adjusted as low as possible, preferably to a few drops per minute. By passing the same solutions through the apparatus several times quite low concentrations of salt were obtained.

Set ups employing larger diameter Visking tubing such as the 24/32 inch tubing, coiled in open containers of distilled water or proportionally larger polyethylene or rubber tubes were not successful. Even when no external pressure was applied, kinks, pin holes, and weak spots in the dialysis tubing almost invariably led to its rupture and, of course, the loss or extreme dilution of the ammonium sulfate solution.

<u>Chromatography on Hyflo Super-cel</u>.--As previously mentioned acid washed diatomaceous earth sold under the trade name of Hyflo Super-cel (Johns Manville Corp., Los Angeles,

Calif.) was found to be most useful in concentration and purification of cytochrome c solutions containing low quantities of salt and having hydrogen ion concentration near neutrality. This material was found to have the extremely useful property of tenaciously binding either mammaliam or Neurospora cytochrome c so that it could be used to effect the nearly quantitative recovery of cytochrome c from extremely dilute solutions. Hyflo Super-cel on which cytochrome c had been adsorbed could be washed with very large volumes of distilled water with no detectable loss of cytochrome. Yet this material had the unique property of allowing nearly quantitative recovery by elution with solutions of high pH or high concentrations of salt.

Specifically, a volume of about 500 cm^3 of Hyflo was suspended in 0.1 M HCl in distilled water. This acidic suspension was then poured into a 7.5 x 55 cm chromatographic column fitted with a porous sintered glass plate, then washed with distilled water until the effluent was near pH 6. The large chromatographic column was convenient in that a vacuum could be easily applied to increase the rate of flow and that it would contain a relatively large quantity of the dilute solution of cytochrome c. The acid wash of the Hyflo was found to be important for the removal of contaminating materials and was necessary to assure a satisfactory rate of flow. Although relatively high rates of flow were easily attained with acid washed Hyflo, exposure to solutions of high pH caused a drastic decrease in rate to prohibitively

low levels.

It was found that cytochrome c was adsorbed from neutral solutions to Hyflo at concentrations of ammonium sulfate below about 0.05 M. Thus, samples from the continuous flow dialysis described above were either diluted to sufficiently low concentrations of salt (usually by a factor of 2-5) or dialyzed further against 0.01 M, pH 8.6 tris buffer. This dialysis was accomplished by placing the solutions into 1 5/8 inch heavy wall Visking tubing and then into 10 x 18 inch jars filled with .01 M, pH 8.6 tris buffer.

Hyflo was found to have a maximum capacity for retaining cytochrome c that was dependent upon the purity of the material applied so that the lower the purity the less the total amount of cytochrome c that could be adsorbed from solution. Thus, experience and care were necessary to prevent over loading the Hyflo used so that cytochrome c was not lost at this point. The Hyflo with the adsorbed cytochrome c was then washed by running a large volume of distilled water through the column. It was found that almost all remaining traces of small water soluble organic compounds such as sugars and amino acids, most of the nucleic acid, and much of the protein present in solutions after dialysis were not retained on the Hyflo.

Elution of the cytochrome c was accomplished with 1 M $(NH_4)_2SO_4$ in 0.05 M tris buffer adjusted to pH 8.6. Elution could also be accomplished with 0.1 M ammonium hydroxide or

similar basic solutions but this had the decided disadvantage of causing the marked decrease in flow rate through the Hyflo described above. The cytochrome c usually is eluted from the Hyflo in a sharp red band so that it may be recovered in relatively concentrated solution.

Lyophilization and storage.--The solution from the preceding step was then easily dialyzed against 0.01 M tris-hydrochloric acid at pH 8.6 to low salt concentrations, lyophilized to dryness on a VirTis Freeze Dryer (The VirTis Co., Yonkers, N.Y.) and stored as a red, dry powder at -27° C. until used. The cytochrome c of the resulting semi-purified preparations was found to account for 20 to 30 per cent of the total protein present as based on the millimolar extinction coefficient for the Soret band peak of mammalian cytochrome c (14), an assumed molecular weight of 16,500 and a Biuret determination of total protein.

Isolation of "Soluble" Cytochrome c of Poky

Much of the cytochrome c present in poky is apparently not bound to any particulate, subcellular structure for it remains in the supernatant after fractionation of homogenates by centrifugation (see fig. 13). The procedure outlined below was designed to isolate this "soluble cytochrome c" from poky and fast poky. It proved completely unsatisfactory for wild type Neurospora which contains much smaller amounts of

cytochrome c per unit weight of tissue and in which the cytochrome c present is almost completely bound to the mitochondrial fraction. Also, only trace amounts of cytochrome were recovered in attempted isolations from mi 3 using this method, although, like poky, mi 3 accumulates cytochrome c. This may reflect a primary difference in the cellular organization of the two mutants.

The method outlined in figure 13 requires minimum manipulation of the extracted cytochrome c and attempts to avoid such questionable procedures as high pH and relatively long periods of dialysis. Further, it has the advantage of being relatively easy and quick to perform. On the other hand, it has the decided disadvantage of failing to extract much of the total cytochrome c present in the poky and fast poky and was found to be unsatisfactory for wild type and mi 3. It may be of particular significance that primarily only one type or class of cytochrome c is extracted, namely the so-called "soluble cytochrome c."

Electrophoresis

Since some electrophoretic heterogeneity was observed early in this work several types of electrophoresis apparatus were employed in an attempt to find the most satisfactory method of analysis. Each of the three methods finally chosen as an analytical and/or preparative procedure had certain advantages and disadvantages not shared by the other two methods.

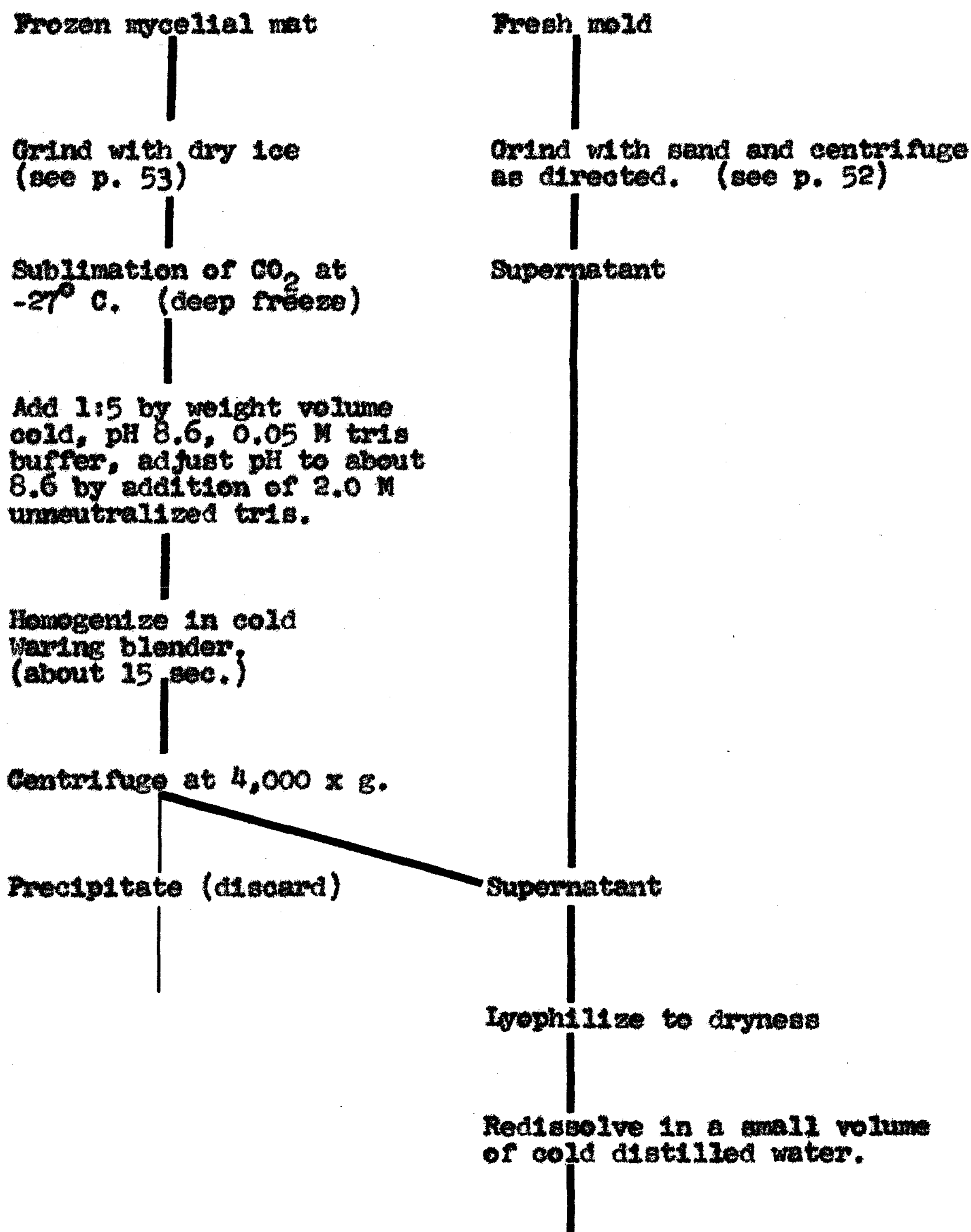

Figure 13. Isolation of Soluble Cytochrome c from Poky

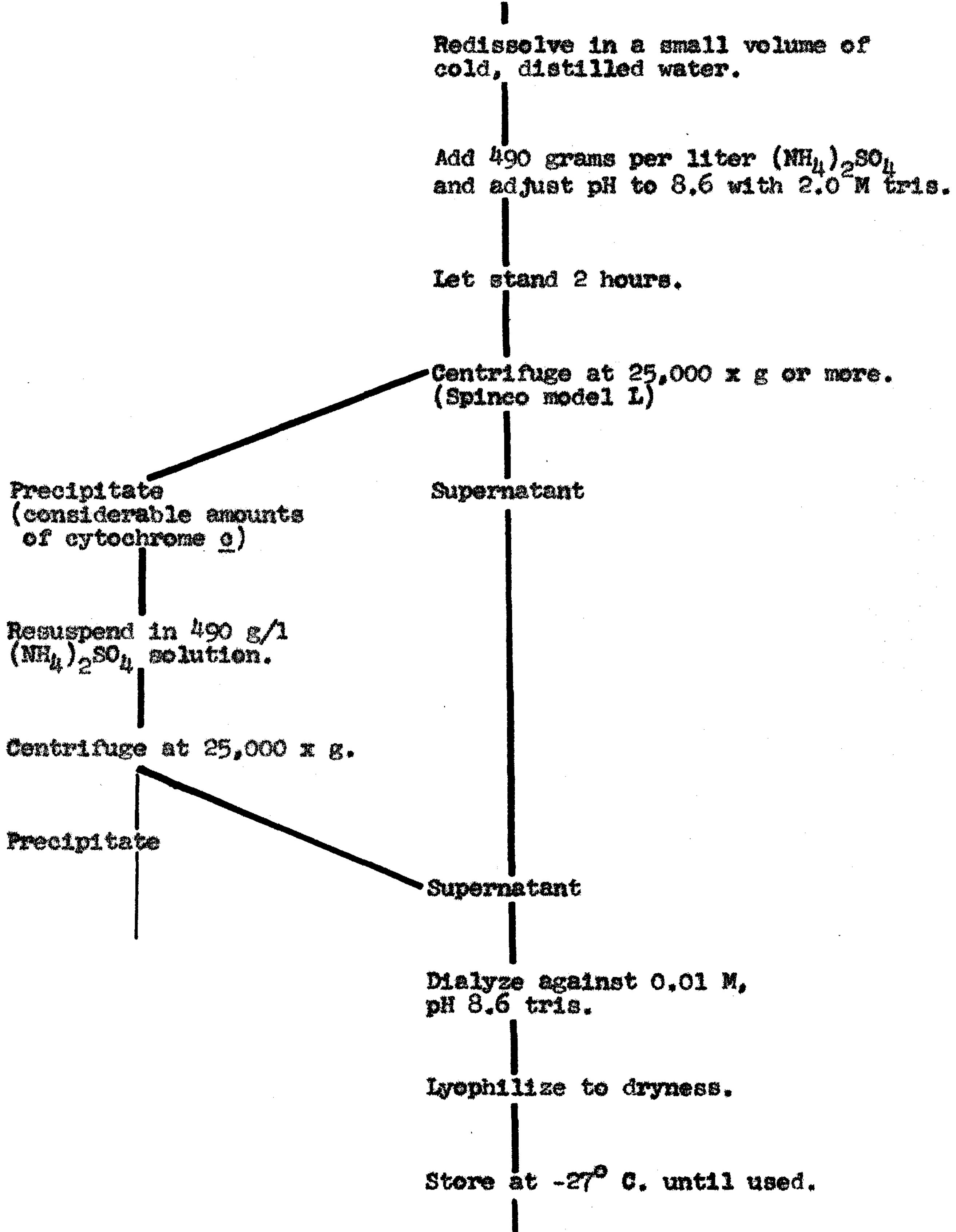

Figure 13 (concluded)

For instance, both Neurospora and mammalian cytochrome c are adsorbed rather badly to filter paper generally used for paper electrophoresis. Attempts to use one dimensional paper electrophoresis even with albumin treated paper were unsuccessful due to "tailing" of the cytochrome c applied.

Continuous flow paper electrophoresis.--The problem of "tailing" on paper was largely overcome by use of a continuous flow paper electrophoresis apparatus shown in figure 14 (model CP - Continuous Flow Paper Electrophoresis Cell, Spinco Division, Beckman Instrument, Inc., Belmont, California) in which that portion of the paper constantly exposed to the sample soon became saturated with protein. Further, the current and the flow of buffer down across the paper curtain could be maintained for some time after the application of the sample had stopped so that much of the material remaining on the paper was washed off into the collection tubes.

Relatively large amounts of the semi-purified cytochrome c obtained by ammonium hydroxide extraction could be conveniently further purified and analyzed in the same operation. Material collected in the primary band of cytochrome c (figure 18) is thought to be pure or nearly pure cytochrome c as defined by its spectrum.

In general results from repeated experiments using continuous flow electrophoresis as an analytical tool were quite

reproducible. However, some difficulty was experienced in preventing lateral displacement of the entire elution curve as a result of improper adjustment of solvent flow down the curtain. Care must be taken to assure a vertical flow of solvent straight down the primary curtain so that material applied near the top of the sheet without current will be collected directly below the point of application.

A further hazard in the use of continuous flow electrophoresis as an analytical tool is differential loss of minor components due to adsorption on the paper, the percentage of loss being much higher for components present in small amounts as compared with major components. This difficulty is particularly important for the analysis of very small samples.

The results of runs on the continuous flow electrophoresis apparatus were conveniently determined spectrophotometrically, using the Soret band peak at 407 mμ to determine cytochrome c as well as absorption at 280 mμ and 260 mμ as a measure of the amount of total protein and nucleic acid present. The amount of cytochrome c recovered in each tube was calculated from the total volume in that tube, the molar extinction coefficient of horse heart cytochrome c and an assumed molecular weight of 16,500. In general, however, data are expressed as the total number of milliliters recovered in each tube multiplied by the optical density of that tube giving a value which is directly proportional to the amount of material present. A distribution curve is obtained by plotting this value versus

the number of the collection tube. The apparatus has 32 collection tubes numbered consecutively from left to right.

The most satisfactory buffer used for electrophoresis was 0.5 M tris (hydroxymethylaminomethane sold as Sigma 7-9, Biochemical Buffer, Sigma Chemical Co., St. Louis, Missouri) neutralized to pH 8.0 with hydrochloric acid. Tris has the advantage of near maximum buffering capacity at this pH and relatively low ionic strength and thus low conductivity. Phosphate buffer proved unsatisfactory for continuous flow electrophoresis because of its relatively high conductivity in concentrations having satisfactory buffering capacity. Adequate potential and temperature control became difficult with increasing conductivity of the buffer. Veronal buffers seemed to lack sufficient buffering capacity in the strengths tried.

Continuous flow electrophoresis was performed with a reservoir containing 15 liters of the tris buffer described above. Buffer was returned from the electrodes into the reservoir to be remixed and recycled. The buffer in the reservoir was replaced by fresh buffer in runs lasting over 36 hours.

Settings for the model CP are listed below:

Right side balances for lateral flow on the primary curtain	6.3 units
left side balances for lateral flow on the primary curtain	6.1 units
top curtain flow rate setting	8.2 units

sample feed rate setting	6.0 units
voltage held constant	600 volts
amperage	89 mAmps
flow rate over the primary curtain	12 cm/hour
time of equilibration with current before the sample was applied	12 hours

All runs were done in a cold room held at about + 4° C. and with cold water at the same temperature circulated through the cooling plate of the electrophoresis cell.

Free boundary electrophoresis.--All free boundary electrophoresis was performed with a portable Tiselius electrophoresis apparatus (model 38-Perkin-Elmer Corp., Norwalk, Connecticut) equipped with a schlieren optical system. Satisfactory photographs of dark red solutions were obtained by using a red filter and a very sensitive panchromatic film, Royal X-Pan (Eastman Kodak Co., Rochester, N.Y.) developed in DK 60a for 6 minutes.

Buffers used in free boundary electrophoresis were as follows:

Buffer	pH	salt form	concentration	NaCl added
succinate	5.8	Na^+	0.05 M	0.025 M
phosphate	6.8	Na^+	0.05 M	0.025 M
phosphate	7.1	Na^+	0.05 M	0.025 M
phosphate	7.4	Na^+	0.05 M	0.025 M
barbital	8.0	Na^+	*μ = 0.1	yes

Buffer	pH	salt form	concentration	NaCl added
tris	8.0	Cl^-	0.05 M	0.05 M
barbital	8.6	Na^+	$^*\mu = 0.1$	yes
glycine	9.5	Na^+	0.05 M	0.05 M

$^*\mu$ indicates ionic strength

All buffers were made up with deionized water. Samples were dialyzed against 1 liter of the same buffer used in electrophoresis for 18 to 24 hours.

All the reported data are from runs using 0.4 to 0.6 per cent cytochrome c as determined by its spectrum (37).

The distance of migration of cytochrome c was determined by projection of the schlieren images from photographic negatives onto suitable squared graph paper. A calibrated transparent scale was mounted with the negative in the photographic enlarger used for projection so that both the schlieren picture and the scale were projected simultaneously and at the same magnification. In this manner the distance of migration from some fixed point of reference within the cell was given directly.

Starch gel electrophoresis.--Both continuous flow and free boundary electrophoresis require relatively large amounts of material and lack convenience of application. A method of seemingly high resolution and advantage as an analytical technique was developed using the method of starch gel electrophoresis outlined by Smithies (42). The tris buffer used in

continuous flow (0.05 M tris, pH 8.0) proved satisfactory for starch gel. Hydrolyzed starch was obtained from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada, and was mixed with buffer using the recommended procedure and proportions.

Electrophoresis was performed in a 4° C. constant temperature room with 300 volts held constant, applied to a 0.7 x 7.3 x 25 cm block of starch gel. Typically the current was about 45 milliamps. Some small rise in temperature of the blocks was noted after several hours of electrophoresis.

Two procedures were used in photographing the starch gel blocks after electrophoresis. One, the staining procedure recommended by Smithies (42) involved the use of aniline blue black (National Aniline Division, Allied Chemical Corp., New York, N.Y.) to detect cytochrome c as well as the other proteins in the block.

A system was worked out whereby cytochrome c could be semi-specifically detected by virtue of the intense Soret band absorption of its heme. An RFL 2 General Electric photoflood lamp was mounted below a vessel containing a mixture of the dyes, tetrabromphenolsulfonphthalein and methylene blue in concentrations sufficiently high to make the lamp appear as a blue circle of light of moderate intensity when viewed through the solution. The starch blocks were then mounted above the light source and photographed with transmitted light.

The solution of dye removes nearly all the light between the wave lengths of 450 to 650 mμ. Further, tetrabromphenol has an absorption maximum near 370 mμ so that primarily only light in the region of 410 mμ is transmitted. Since this is the spectral region of Soret band absorption, portions of the blocks containing cytochrome c transmitted relatively little light and appear as dark or deep red spots on a light background. A 35 mm single lens reflex camera with Plus X Kodak film was used to photograph the blocks. Since this film has very low sensitivity to red light above 640 mμ, positive prints appear as black spots on a white background and sensitivity seems comparable to that obtained with the protein stain mentioned above.

Sedimentation

The sedimentation constants reported were determined using highly purified samples of poky and wild type cytochrome c obtained from the primary elution bands from continuous flow electrophoresis. The concentrations of cytochrome c used were between 0.4 and 0.7 per cent as determined from Soret band absorption. The buffer was 0.05 M pH 8.0 tris, containing 0.05 M NaCl and was the same as used at that pH for free boundary electrophoresis.

Sedimentation data were obtained with an analytical ultracentrifuge (model E, Spinco Division, Beckman Instrument Co., Palo Alto, Calif.) using a schlieren optical system and an

AH6 light source. All reported runs were at 59,780 rpm in an An-D head with a standard 3 mm cell. Temperature was maintained within ± 0.1° C. between 4° C. and 7° C. for various runs. Red sensitive 103 F spectroscopic plates (Eastman Kodak Co. Rochester, N.Y.) were developed in D-11 developer for 6 minutes and were used in all photographs. Unless otherwise specifically stated all photographs were made with a red, #29 Wratten Filter. A blue filter was used to detect the presence of absorbing material sedimenting at a slower rate than the primary component. All plates were analyzed on a micro comparator sold as a Toolmaker microscope with a 15 power projection attachment (model M2001, The Gaertner Scientific Corp., Chicago 14, Ill.).

Determination of Iron

The method was adapted from that of Drabkin (11) in which a red complex is made by the ferrous ion and orthophenanthroline. From 12 to 15 mg of cytochrome c in 3 ml of water, 2.0 ml of 0.169 M sodium hydroxide and 0.5 ml of 30 per cent hydrogen peroxide were mixed, allowed to stand over night at room temperature, and then heated at 90° C. for 10 minutes. The hot tubes were cooled by immersion in water, 1.0 ml of 1 N hydrochloric acid cautiously added with stirring, and the solutions again heated at 90° C. for 10 minutes. After cooling, 0.2 ml of 50 per cent saturated ammonium acetate, 0.4 ml of fresh, cold, 1 per cent ascorbic acid and 2.0 ml of 0.1 per

cent orthophenanthroline were added. The solution was adjusted to a volume of 10.0 ml and the optical density at 500 mμ determined after 90 minutes at room temperature. All solutions were made up with deionized water. The unknown values were determined by comparison with a standard curve made at the same time.

Results

Continuous Flow Electrophoresis

The frozen, semipurified cytochrome c isolated by the ammonium hydroxide extraction (see fig. 11) from poky, fast poky, mi 3, and wild type Neurospora was dissolved in cold 0.05 M, pH 8.0 tris buffer to give a final concentration of about 0.3 per cent cytochrome c as determined by absorption at 407 mμ. These solutions were then made 0.5 mM in potassium ferricyanide by a 100:1 dilution from a stock solution and dialyzed against two changes of the tris buffer for at least 24 hours. Spectral examination of the solution before and after dialysis indicated that cytochrome c was present only in the ferri form and that no more than trace amounts of potassium ferricyanide remained after dialysis. The solutions of cytochrome c were then run on the continuous flow electrophoresis as shown for poky in figure 14 and with the results indicated in figure 15. Continuous flow electrophoresis of cytochrome c from wild type, mi 3, and fast poky gave distribution patterns very similar to that shown for poky in

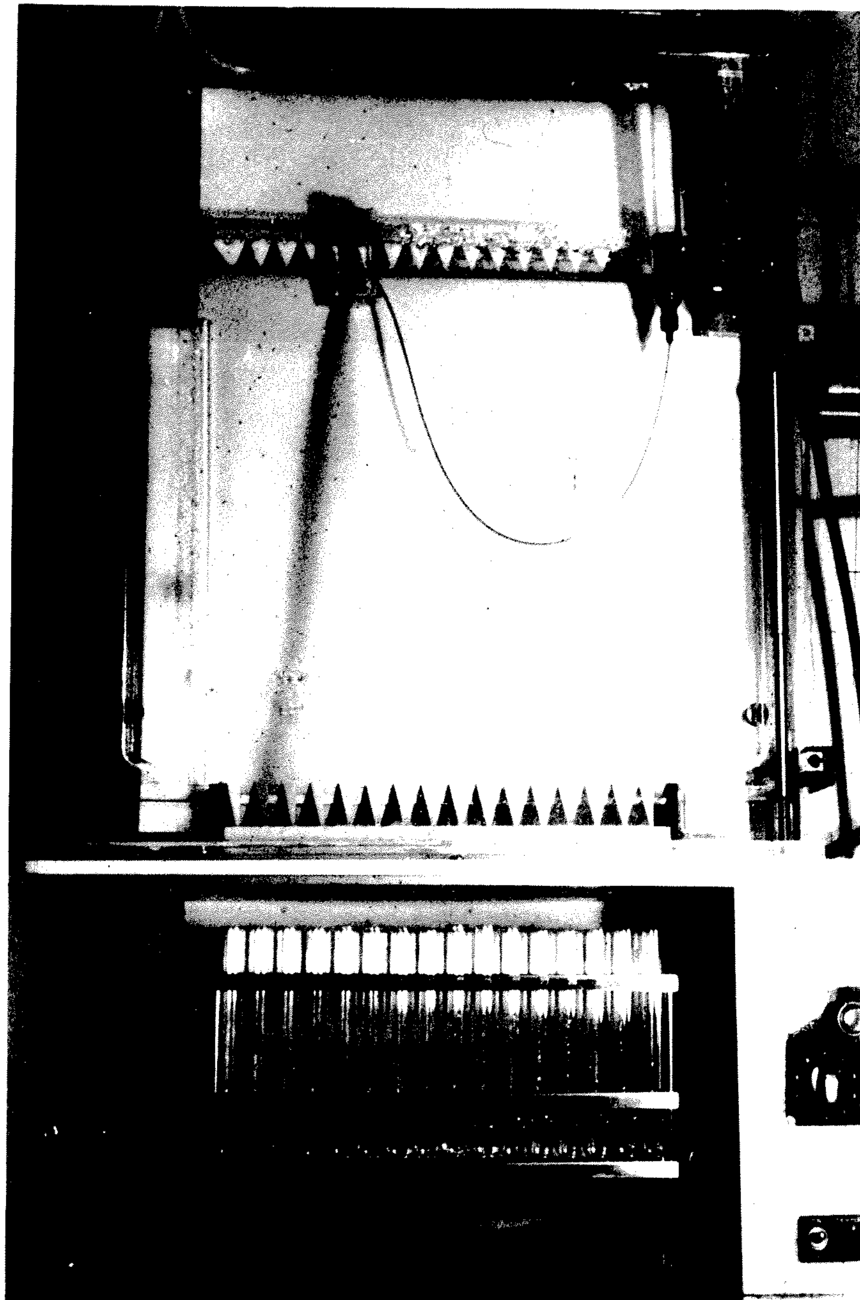

Figure 14. Continuous flow electrophoresis cell showing the electrophoresis of semipurified _poky_ cytochrome _c_ prepared by ammonium hydroxide extraction.

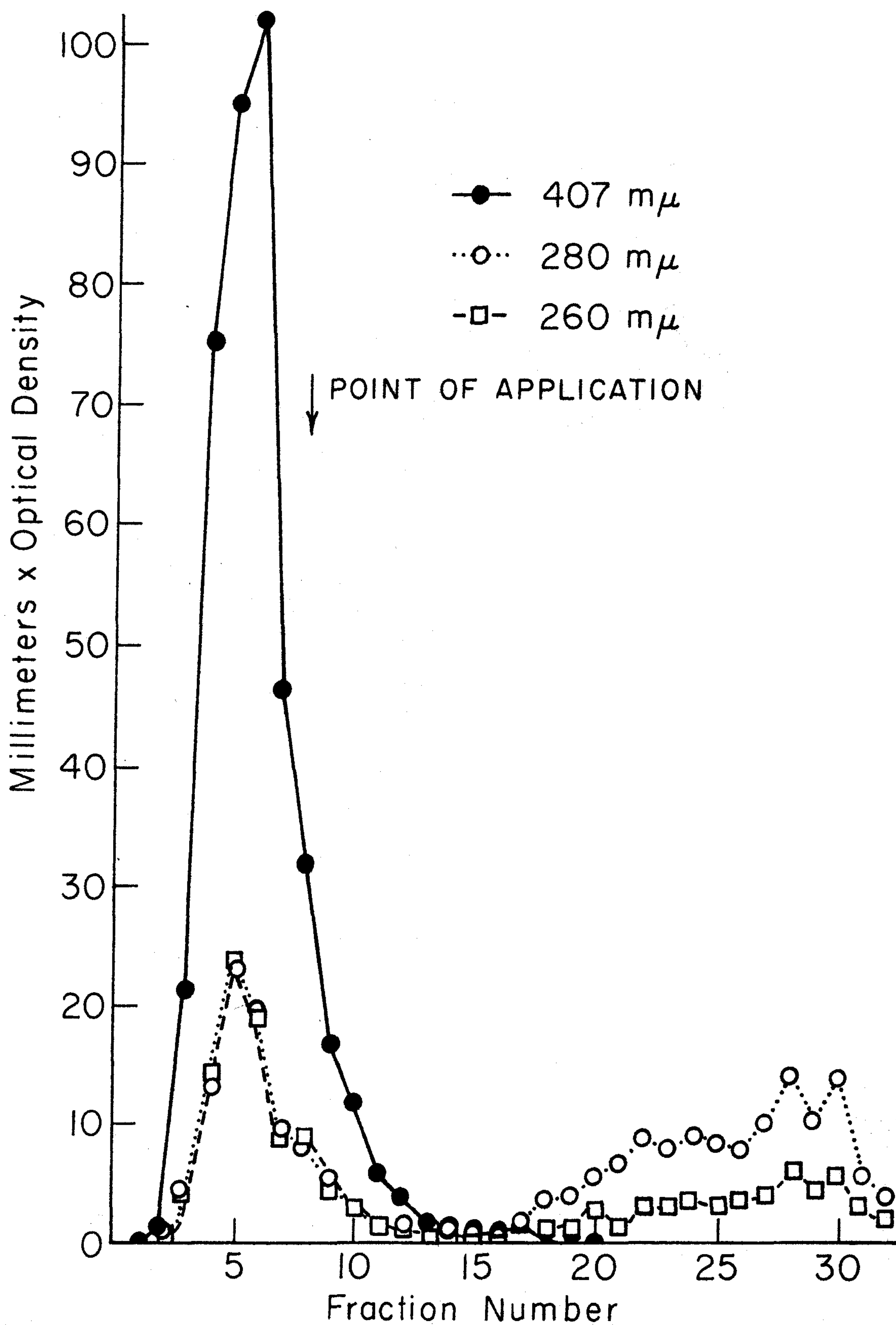

Figure 15. The distribution of materials absorbing at 407, 280, and 260 mμ from continuous flow electrophoresis of semipurified poky cytochrome c.

figure 15. Most of the cytochrome c from all samples run under the standard conditions was collected in tubes numbered 4, 5, and 6 of the continuous flow electrophoresis cell, however, all samples also contained apparently more acidic cytochrome that was distributed in a "tail" towards the positive pole of the cell. The amounts of cytochrome c found in tubes 8 to 20 of the cell were found to vary somewhat for different samples of cytochrome c, however, the variability between preparations for the different strains seemed no greater than the variability for the same strain.

The reproducibility of the results from continuous flow electrophoresis was checked by rerunning cytochrome c collected in different tubes of the cell. Material from tubes 3, 7, and 9, 10, and 11 were concentrated by lyophilization, redialyzed and consecutively rerun with the results shown in figure 16. These three fractions did not contain equal amounts of cytochrome c so that it was necessary to express the results in relative amounts found in each tube rather than in milliliters x optical density as used for figure 15. The relative amount of cytochrome for each tube of each fraction was calculated by totaling the amount of cytochrome recovered in all tubes for that fraction and then expressing the amount in each tube as a percentage of the total amount recovered. Thus the percentage values for all the tubes of each of the three factions equals 100 per cent. The results indicate repeated electrophoresis of the cytochrome from dif-

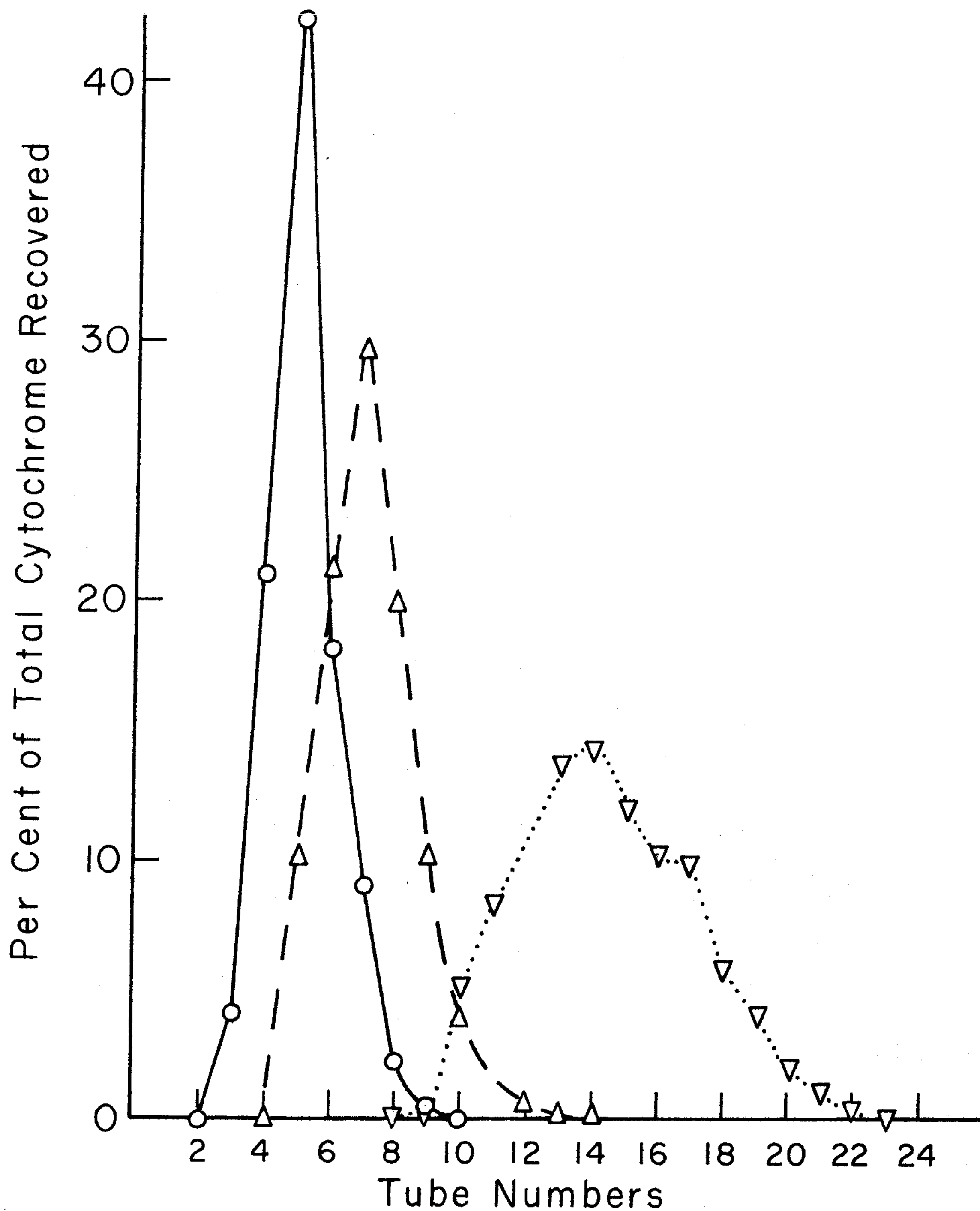

Figure 16. The relative distribution of *poky* cytochrome *c* during the reelectrophoresis of material collected in different factions of the continuous flow electrophoresis represented by figure 15.

——————— reelectrophoresis of material collected in tube 3 of the run represented by figure 15.

——— ——— material from tube 7.

········ material from tubes 9, 10 and 11.

ferent portions of the distribution curve shown in figure 15 tended to migrate about as they did in the original run. Tube number 5 contained the most cytochrome for the rerun of tube number 3 of the original run and the material from tube 7 of the original run was collected primarily in tube number 7 of the rerun. Cytochrome collected in tubes 9, 10, and 11 of the original run were distributed over a rather broad region from about tubes 9 through 23 of the rerun, however, no material from this fraction was found to migrate into tubes 4, 5, and 6 thus indicating that no cytochrome c with the mobility of the primary type of cytochrome in the original sample was present in this fraction.

Cytochrome c from tubes, 4, 5, and 6, of the electrophoresis of the original sample and the same tubes from the electrophoresis of two other preparations of poky cytochrome c were combined, concentrated by lyophilization and stored at -27° C. to be used as highly purified poky cytochrome c for free boundary and starch-gel electrophoresis, molecular weight determinations and ultracentrifugations. Similar fractions were collected from the continuous flow electrophoresis of cytochrome c from wild type, mi 3 and fast poky.

Free Boundary Electrophoresis

Free boundary electrophoresis of highly purified cytochrome c from tubes 4, 5, and 6 of the continuous flow electrophoresis of poky cytochrome c was carried out in buffers of

various hydrogen ion concentrations and the mobilities were calculated from the equation:

$$\mu = \left(\frac{\Delta x}{\Delta t}\right) A \left(\frac{K}{IR}\right)$$

μ = mobility with the dimensions of cm^2 $volt^{-1}$ $sec.^{-1}$

Δx = distance of boundary travel in cm.

Δt = time

A = cross sectional area of the electrophoresis cell in cm^2.

K = conductivity cell constant

I = current in amperes

R = resistance of the buffer in ohms

Figure 17 presents a plot of mobility versus pH from which it may be seen that the isoelectric point of the major component (A peak of figure 18) in this preparation is at about pH 8.0. Although this mobility seems to be dependent to some extent on the buffer used.

Preparations of highly purified <u>poky</u> cytochrome <u>c</u> invariably contained three components, as shown in figure 18, when electrophoresis was carried out in 0.05 tris + 0.05 M NaCl at pH 8.0. In this system the A component has a mobility of 0.97×10^{-5} cm^2 $volt^{-1}$ $sec.^{-1}$ towards the negative pole of the cell while the mobility of the B component is 1.1×10^{-5} cm^2 $volt^{-1}$ $sec.^{-1}$ towards the positive pole. The C component was calculated to have a cathodic mobility of about 6.1×10^{-5} cm^2 $volt^{-1}$ $sec.^{-1}$ Recognition of the three components was much

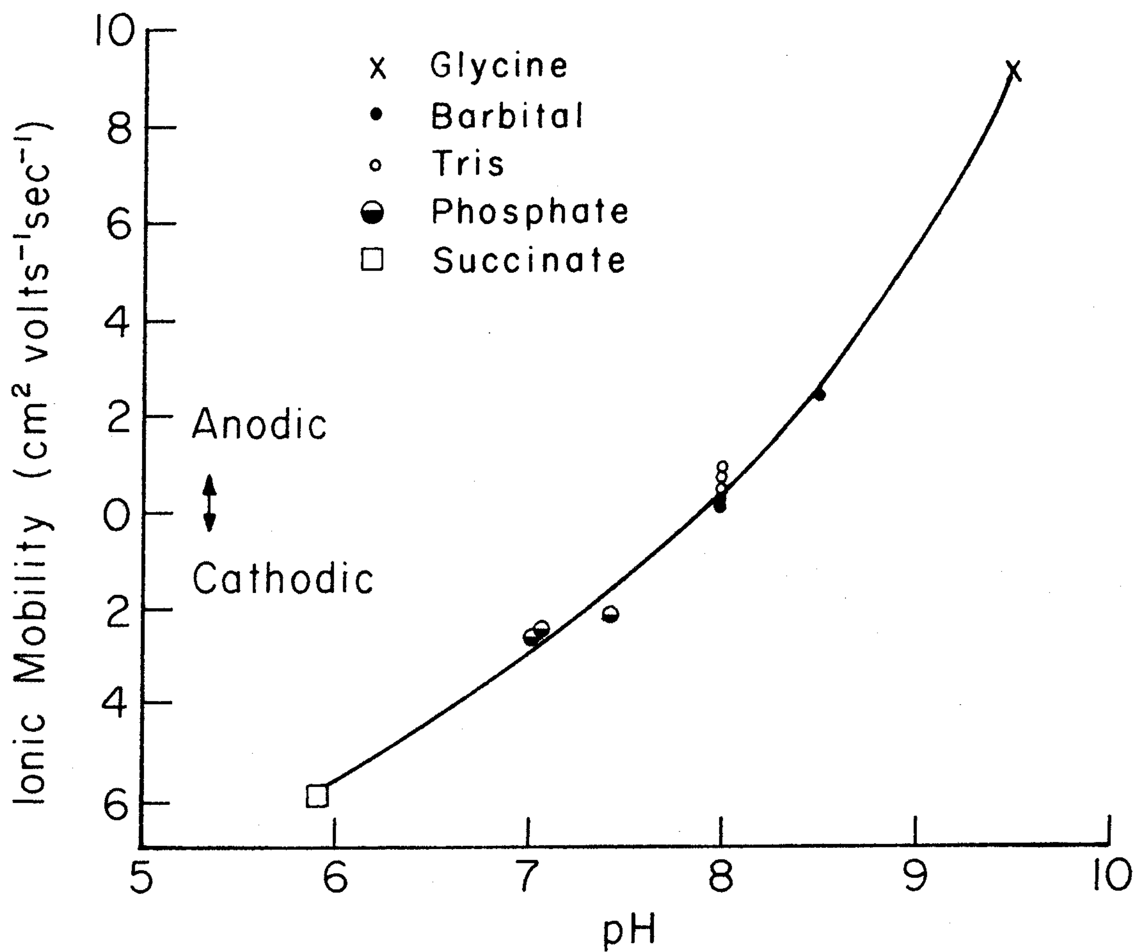

Figure 17. Ionic mobility of highly purified cytochrome _c_, from the descending limb of the electrophoresis cell.

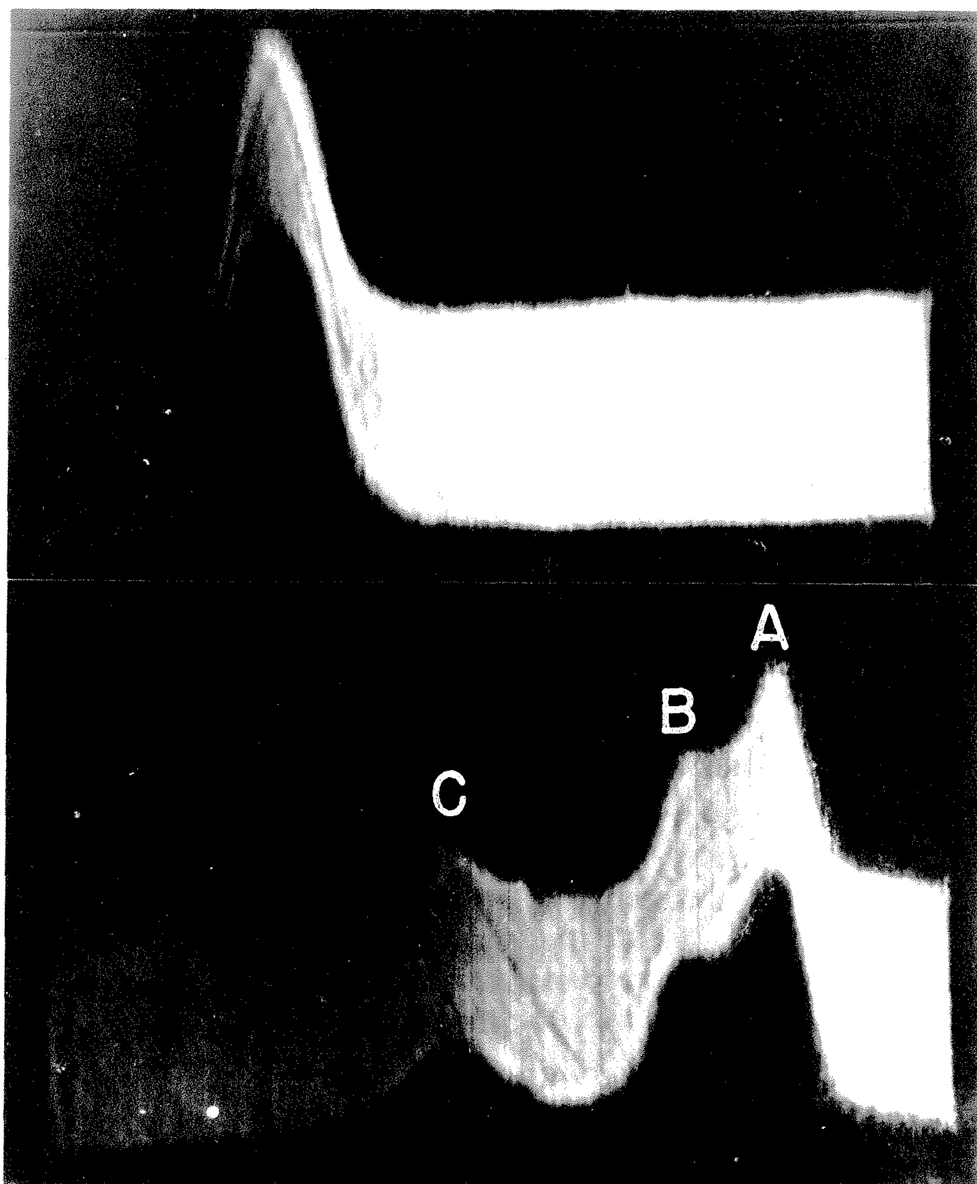

Figure 18. Schlieren image from the ascending (upper) and descending (lower) limbs during the free boundary electrophoresis of highly purified poky cytochrome c.

more difficult at other hydrogen ion concentrations where the B component was usually seen only as an irregularity in the curve or not seen at all and the C component was detected only as red color migrating into the ascending limb of the electrophoresis cell but not associated with any specific peak.

Free boundary electrophoresis of semipurified cytochrome c with pH 8.0 tris buffer was complicated by the presence of contaminating materials, however, red color was observed to migrate rapidly towards the positive pole of the cell similar to the electrophoresis of highly purified cytochrome c described above. A red color gradient indicating cytochrome c was associated with a low peak in the semipurified material and was calculated to have a cathodic mobility of about 6×10^{-5} cm^2 $volt^{-1}$ $sec.^{-1}$ compared with an anodic mobility of about 1×10^{-5} cm^2 $volt^{-1}$ $sec.^{-1}$ for the primary cytochrome c peak. Thus, free boundary electrophoresis suggests the presence of at least three electrophoretically distinguishable species of cytochrome c prepared with ammonium hydroxide extraction. The mobility of these peaks have the ratio of -1:+1:+6 in the pH 8.0, 0.05 M tris +0.05 M NaCl buffer used.

Starch Gel Electrophoresis

Starch gel electrophoresis was carried out with cytochrome from poky, fast poky, mi 3, and wild type purified by continuous flow electrophoresis and stored as a dry powder at -27° C. until used. Samples were diluted to appropriate con-

centrations, then dialyzed against 0.05 M tris buffer at pH 8.0 and run as previously described. Figure 19 depicts the results of these runs where apparent differences in the preparations are seen. The preparations from mi 3 and wild type apparently contain primarily only one detectable electrophoretic species of cytochrome c although both exhibit "tailing" that probably cannot be explained solely on the basis of smearing due to lack of electrophoretic resolution in the starch. Similar materials from poky seem to contain three types of cytochrome c, probably identical with those seen in free boundary studies (figure 18). Fast poky cytochrome c produces a streak with significant amounts of red material moving towards the positive pole of the starch block but with little or no cytochrome moving as rapidly as the fastest component of poky cytochrome.

The unusual electrophoretic heterogeneity of all samples as indicated by distribution curves from the continuous flow electrophoresis and the "tailing" on starch gel warranted considerable doubt concerning the stability of Neurospora cytochrome c during the isolation using extraction with ammonium hydroxide. Accordingly "soluble" cytochrome c from poky and fast poky was prepared by the method outlined in figure 13 and analyzed by starch gel electrophoresis with the results shown in figures 20a and 20b. Since these preparations had not been purified by continuous flow electrophoresis it was necessary to photograph the unstained blocks with transmitted

Figure 19. Starch gel electrophoresis of cytochrome c from the four strains of Neurospora: l. to r. wild type, fast poky, poky and mi 3.

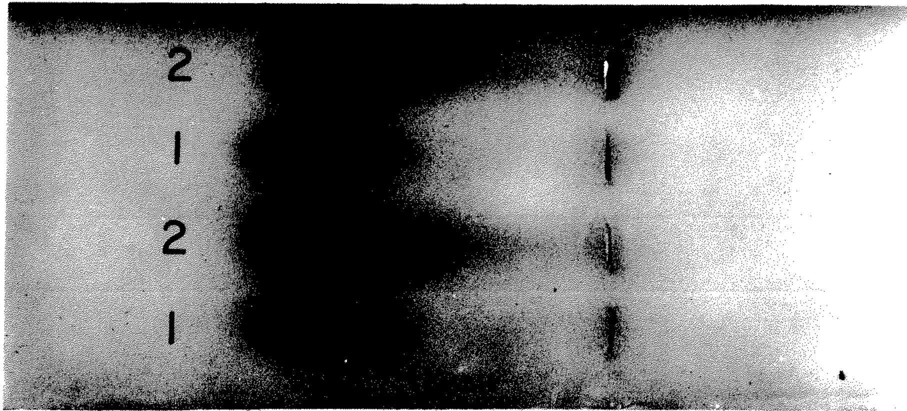

Figure 20a. Unstained starch gel block after electrophoresis of "soluble" cytochrome _c_ from _poky_ (1) and _fast poky_ (2).

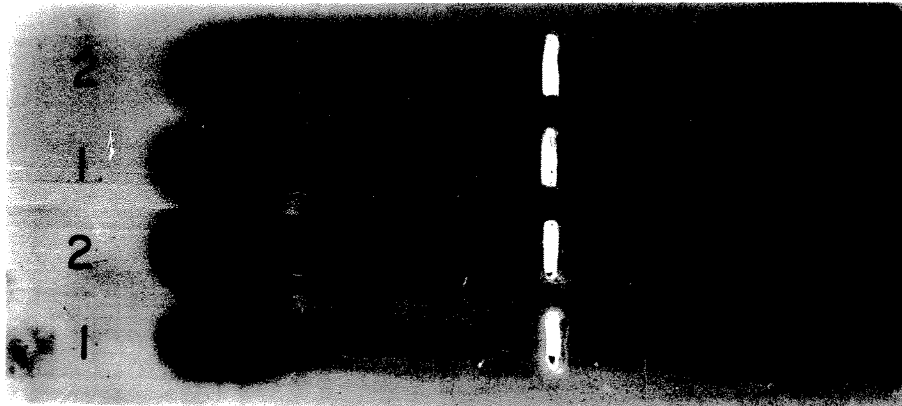

Figure 20b. The same block as shown in figure 20b after staining. "Soluble" _poky_ cytochrome _c_ (1) and "soluble" _fast poky_ cytochrome _c_ (2).

blue light to determine the regions containing cytochrome _c_ as shown in figure 20a. The blocks were then cut, stained, and photographed again with transmitted light as shown in figure 20b. These figures also suggest electrophoretic heterogeneity of the samples although there is somewhat less tailing of the cytochrome _c_ than observed for samples prepared with ammonium hydroxide. Both _poky_ and _fast poky_ cytochrome _c_ seem to contain three electrophoretic species similar to those seen in figure 19 for cytochrome _c_ extracted from _poky_ with ammonium hydroxide. "Soluble" _fast poky_ cytochrome _c_ apparently contains relatively more of the second cytochrome component than _poky_, although the cytochrome concentrations in the two samples apparently differed slightly.

Since cytochrome _c_ contains heme iron that might exist in either the ferri or ferro form the possibility was considered that two of the electrophoretic components of _poky_ cytochrome might represent the oxidized and reduced forms of the enzyme. The spectrum of _poky_ cytochrome _c_ prepared by ammonium hydroxide extraction was taken directly and with buffers containing cysteine and potassium ferricyanide as described later in this paper (see p. 96). These spectra are recorded on figure 21 and indicate that the preparation of _poky_ cytochrome _c_ does contain a small but significant amount of reduced cytochrome _c_ although it was treated with potassium ferricyanide before purification by continuous flow electrophoresis. This observation suggests an oxidation-

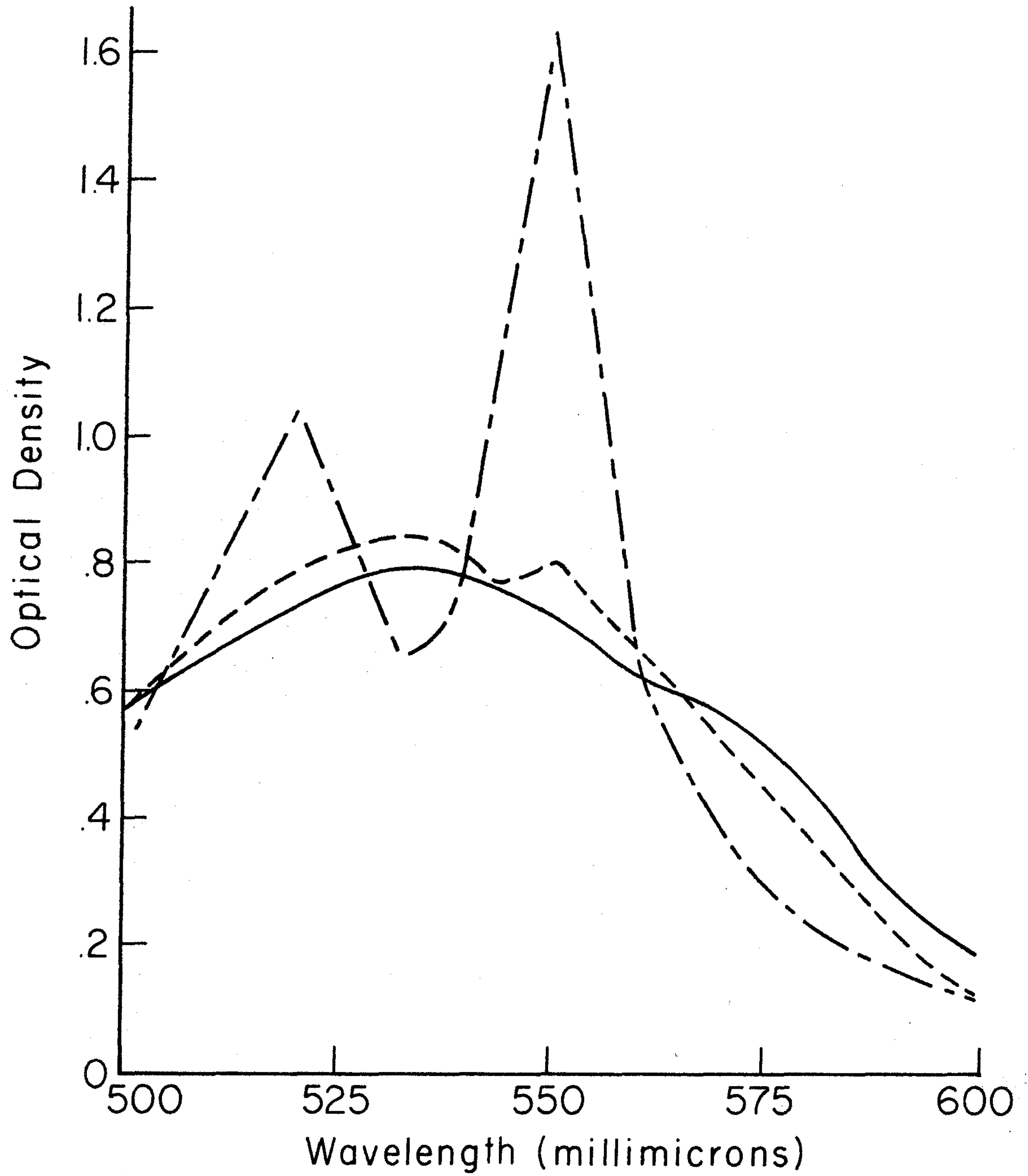

Figure 21. The spectra of purified poky cytochrome c used for starch gel electrophoresis.

- - - - diluted directly into buffer
——— oxidized with potassium ferricyanide
— - — reduced with cysteine

reduction reaction within the protein possibly related to auto-reduction described by Boeri (17).

Sedimentation

The sedimentation coefficients for highly purified samples of _poky_ and wild type cytochromes _c_ from tubes, 4, 5, and 6 of the continuous flow electrophoresis were calculated using the equation:

$$S_{obs} = \frac{2.303}{\omega^2} \frac{\log r}{t}$$

where $\frac{\log r}{t}$ = slope for the plot of log r versus t

and S_{obs} = observed sedimentation coefficient

ω^2 = angular velocity in radians/sec.

r = the radius of rotation

t = time in seconds

The observed values for S were related to water at 20° C. and are listed below:

S_{w20} wild type cytochrome _c_ = 2.12×10^{-13} sec.

S_{w20} _poky_ cytochrome _c_ = 2.20×10^{-13} sec.

These values seem in reasonable agreement with sedimentation coefficients found for mammalian cytochrome _c_. Edsall (43) lists values for the sedimentation coefficient of cow, horse and pig heart cytochrome _c_ as 2.5, 2.1, and 2.3 $\times 10^{-13}$ sec. respectively.

The samples of cytochrome c seemed homogeneous to centrifugation in that only one peak was detected. Boundary spreading occurred at a fairly rapid rate so that attempts to analyze the shape of the curves for polydispersion were not successful due to difficulty in establishing a base line. Figure 22 presents the schlieren image for poky cytochrome c at time intervals of 96 minutes.

Cytochrome c has an intense Soret absorption band in the blue region of the spectrum. Thus the presence of absorbing materials that were sedimented at a slower rate than cytochrome c would probably have been detected by inserting a blue filter in the optical system near the light source. This system gave a sharp change in the amount of transmitted light over the cytochrome containing boundary. However, no absorbing material was detected in solution above the cytochrome boundary of highly purified poky or wild type cytochrome c or in preparation of semipurified poky cytochrome as shown in figure 23. This finding indicates no very light heme-containing proteins or peptides are present in these preparations.

Determination of Molecular Weight by Iron

Highly purified poky cytochrome c from continuous flow electrophoresis was lyophilized and diluted to give a 2.0 per cent solution of cytochrome and then extensively dialyzed against pH 8.0, 0.01 M tris buffer made up with deionized water.

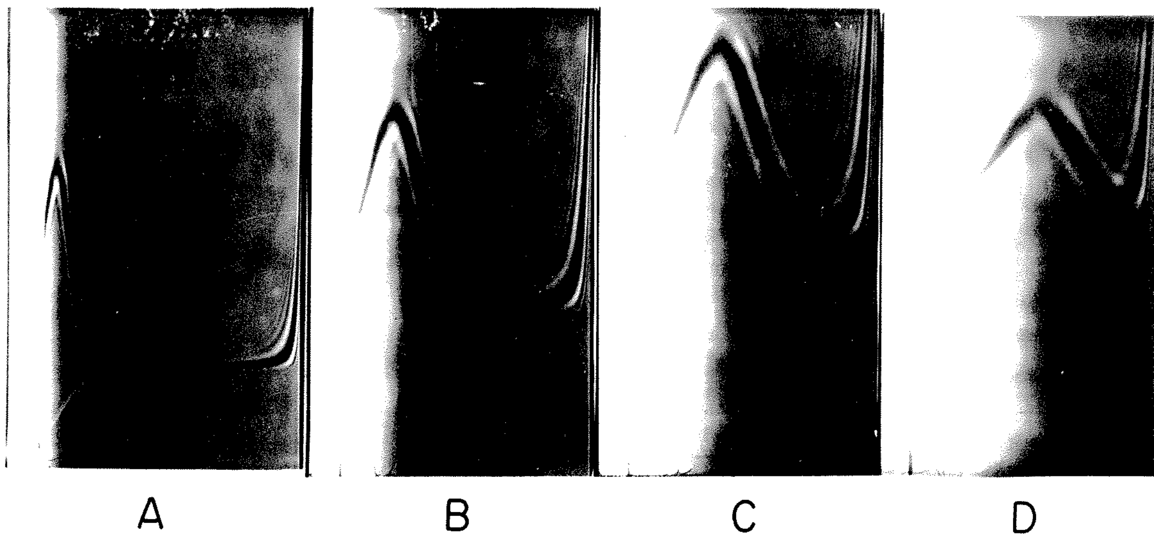

Figure 22. Ultracentrifugation of highly purified poky cytochrome c.

A 32 minutes, phase plate angle at 50°
B 128 minutes, phase plate angle at 30°
C 224 minutes, phase plate angle at 20°
D 320 minutes, phase plate angle at 20°

Figure 23. Poky cytochrome c photographed with blue light indicating very little or no absorbing material above the cytochrome c boundary.

Three determinations of iron were made as previously described (p. 77) using similar portions of the buffer used in the dialysis as blanks. The minimum molecular weight of poky cytochrome c was calculated to be 13,700 for 0.408, 0.394, and 0.407 per cent iron found in the three samples.

Spectrum

Portions of the dialyzed solution used for the determination of molecular weight above were diluted to suitable concentrations with 0.05 M tris buffer at pH 8.0 and the visible spectra from 400 mμ to 600 mμ taken with a Model 11 MS Cary Recording Spectrophotometer (Applied Physics Corp., Pasadena, Calif.). The oxidized spectrum was taken by dilution of the cytochrome with tris buffer which had been made 0.1 mM in potassium ferricyanide. Portions of this buffer were used also in the reference cell of the spectrophotometer. The only detectable difference from the oxidized spectrum of horse heart cytochrome c in 0.1 M phosphate buffer at pH 6.8 reported by Margoliash and Frohwirt (14) was in the position of the Soret band maxima. They reported the peak to be at 410 mμ , however the maximum for poky cytochrome c was found to be at 407 mμ. As previously mentioned (p. 6) under similar conditions horse heart cytochrome c was also found to have a maximum at 407 mμ.

The spectrum for reduced poky cytochrome c was taken by diluting the stock solution of cytochrome with 0.05 M, pH 8.0

tris buffer which was 0.05 M in cysteine. Positions of the maximum for reduced *poky* cytochrome *c* were at 416 mμ, 520-521 mμ and 550 mμ and are essentially identical with those reported for horse heart cytochrome *c* (14). However, the millimolar extinction coefficients were found to be somewhat lower as indicated in table 6. The significance of the variations in the extinction coefficients of reduced *poky* and horse heart cytochrome *c* is not known but may represent unreduced cytochrome *c* present in the preparation from *poky*.

TABLE 6. Position of the maximum in the visible spectrum and the related millimolar extinction coefficients for horse heart cytochrome *c* at pH 6.8 (14) and *poky* cytochrome *c* at pH 8.0.

Form of Cytochrome	Horse Heart Cytochrome *c*		Poky Cytochrome *c*	
	wave length (mμ)	millimolar extinction coefficient	wave length (mμ)	millimolar extinction coefficient
oxidized	410	106.1	407	104
	528	11.2	528	11
reduced	416	129.1	416	120
	520.5	15.9	520-521	15
	550.25	27.7	550	24

Discussion

Many of the properties of cytochrome c isolated from sources other than Neurospora were discussed in Part I (p. 36) and will not be repeated here. In general cytochrome c from Neurospora seems similar to that isolated from mammalian sources. Horse heart cytochrome c purified by column chromatography, on IRC 50 resin was found to have a molecular weight of about 12,400 (40) although Neilands (44) reported an approximate molecular weight by sedimentation and diffusion of 18,000 for Ustilago sphaerogena cytochrome c. The molecular weight determined for poky cytochrome c, assuming 1 mole of iron per mole of protein, was 13,700. The sedimentation coefficient also seems similar to that of mammalian cytochrome c. Atlas and Farber (45) found average sedimentation coefficients for horse, pig and beef heart cytochrome c to be 2.1, 2.3 and 2.4×10^{-13} seconds respectively while the value found for poky cytochrome c was 2.2×10^{-13} seconds. The spectra of oxidized and reduced cytochrome c was similar to that found for horse heart cytochrome by Margoliash and Frohwirt (14). The isoelectric point of horse heart cytochrome c found by Theorell and Åkesson (18) was at pH 10.65 with a cathodic mobility of about 2.3×10^{-5} cm^2 $volt^{-1}$ $sec.^{-1}$ at pH 8.0 and Neilands (44) reported the isoelectric point of Ustilago cytochrome c to be near pH 7.0. The isoelectric point of poky cytochrome c was found to be at about pH 8.0, suggesting

a greater similarity of structure between cytochrome _c_ from _poky_ and the rust fungus, Ustilago, than for cytochrome _c_ from mammalian sources.

No significant difference was found between preparations from _poky_, _fast poky_, _mi 3_ or wild type cytochrome _c_ during continuous flow electrophoresis, however, all exhibited an interesting electrophoretic heterogeneity in that they contained a rather small proportion of apparently acidic cytochrome _c_ detected as a "tail" of material absorbing at 407 mμ migrating more towards the positive pole of the cell. Material from three tubes containing the highest concentrations of cytochrome _c_ were taken for further analysis. Free boundary electrophoresis indicated that this sample probably contained at least three distinct species of cytochrome _c_, although the schlieren images of the ascending and descending limbs of the cell were strangely dissimilar in that resolution of the peaks was visible only in the descending limb. Generally self sharpening effects in the ascending limb give the sharpest resolution of boundaries. The observation of these differences between the two limbs of the cell strongly suggests a complex system in which interaction between proteins or between protein and some other material dissolved in the system might be involved. Although the nature of this difference between the two limbs of the electrophoresis cell remains unexplained it should be noted that three apparently red spots also occur during starch gel electrophoresis and

that they seem to be similar in amount and distribution to the components seen in the descending limb of the free boundary electrophoresis cell.

Starch gel electrophoresis of samples of poky, fast poky, mi 3 and wild type show serious "tailing" probably due to electrophoretic heterogeneity in the samples. Rather marked differences were seen in cytochrome isolated from the different types of Neurospora, however, these differences should probably be considered with caution since the stability of the preparations before and after isolation seems questionable. Semipurified samples of "soluble" cytochrome isolated from poky and fast poky show less streaking and three qualitatively although not quantitatively similar spots. However, these preparations of cytochrome from poky and fast poky are objectionable in that they contain only the cytochrome *c* that is apparently not bound into subcellular structures in the mold. Further, a much larger proportion of the total cytochrome *c* was lost in the 70 per cent ammonium sulfate precipitate removed from this sample than was observed for cytochrome *c* extracted with ammonium hydroxide. Although this may represent only co-precipitation of cytochrome *c* with other material it is possible that a selection of a certain type of cytochrome *c* may have occurred during this step of the procedure. This possibility seems to be strengthened by Neilands' (44) observation that cytochrome *c* from Ustilago was precipitated by saturated ammonium sulfate even

though purified Neurospora cytochrome c is not precipitated from dilute solution saturated with ammonium sulfate. The conclusion seems justified that poky cytochrome c contains at least three electrophoretically distinct components. Although there is some question concerning the stability of Neurospora cytochrome c, the heterogeneity is present in preparations made under apparently mild condition involving essentially only freezing, grinding, lyophilization, ammonium sulfate fractionation, and dialysis.

The question of the electrophoretic heterogeneity of cytochrome c is not limited to preparations from Neurospora. Numerous reports exist stating the observation of two or more components in preparations of cytochrome c isolated from various sources. Nunnikhoven (46) observed two electrophoretic components in yeast cytochrome c that had been purified using IRC 50 ion exchange resin and then analyzed by paper electrophoresis. Horio (47) observed two fractions of cytochrome c-like proteins in extracts from Pseudomonas aeruginosa. Nozaki, et al. (48) observed two elution peaks for cytochrome c isolated from baker's yeast when samples were chromatographed on XE-64 resin. Yamanaka, et al. (49) observed two chromatographic fractions in preparations of cytochrome c from cow, horse, and pig heart, but concluded that only one component represented the "native" enzyme. Westcott and Dickman (50) observed three components in horse heart cytochrome c purified on XE-64 resin and then analyzed by electrophoresis at pH 8.4

but only one component during electrophoresis at pH 7.3 or 10.7. Paléus and Theorell (51) observed three components in recrystallized reduced beef heart cytochrome c at pH 7.2.

The nature of the heterogeneity of cytochrome c has often been related to a denaturization-like process that might take place in isolated cytochrome c. The observation of auto-reduction in purified preparations of horse heart cytochrome c by Boeri (17) and the detection of significant amounts of reduced cytochrome c in samples thought to contain only the oxidized form of the Neurospora enzyme are particularly interesting. Coupled with the electrophoretic data they suggest the occurrence of oxidizable sites other than reduced iron in the cytochrome molecule. Indeed, as previously discussed Theorell (24) has suggested that histidine may be involved in transporting electrons to the heme iron. It seems possible that other oxidizable groups such as the sulfhydryl moeity of cysteine residues in other portions of the molecule might somehow be capable of causing the reduction of the heme iron so that there would be at least several or possibly many oxidation-reduction states of the protein as a whole.

Another previously unmentioned difference between preparations of cytochrome c from various strains of Neurospora was found for the cytochrome c of C115. In preparation of ammonium hydroxide extracts from this strain, no detectable cytochrome c remained in solution during fractionation with 70 per cent saturated ammonium sulfate but, in contrast to

cytochrome _c_ extracted from the other strains of Neurospora, was present in the precipitate. This observation suggests a possible difference in the protein of C115.

An interesting oxidation-reduction of both horse heart and Neurospora cytochrome _c_ was observed by heating dilute solutions of reduced cytochrome _c_ containing small amounts of sodium hydrosulfite. Before heating, solutions invariably contained broad absorption bands at 550 $m\mu$ as seen with a hand spectroscope. However, after heating and then cooling the absorption band could not be detected although it could be reinduced by the addition of fresh sodium hydrosulfite into the system.

The results described above contribute to the characterization of Neurospora cytochrome _c_ but offer little basis for predicting the relation between the heterogeneity of the protein and mechanisms involved in its synthesis, or its role in the inheritance of the _poky_ phenotype.

C. THE DISTRIBUTION OF CYTOCHROME c WITHIN POKY

Haskins et al. (52) reported that poky accumulated up to 3 per cent of its total dry weight as cytochrome c in 2 day cultures. During the preparation of mitochondria from poky it was observed that much of this cytochrome c was not bound to particulate fractions, but rather remained in the supernatant and could not be precipitated even at a relatively high centrifugal force.

Materials and Methods

Preparation of Subcellular Fractions

A sample of 112.1 grams of fresh 3 day poky mycelia was ground in two portions with phosphate-mannitol buffer and sand as previously described (p. 52). A small sample of the tissue was found to lose 84.9 per cent of its weight when dried in a vacuum over P_2O_5. The precipitate from the centrifugations following grinding contained primarily sand, unground mycelia, mycelial walls, and undoubtedly some nuclei. It was found to weigh 52.6 g. not including the sand present and was designated as cellular debris, etc.

The combined supernatants were then centrifuged at an average centrifugal force of 45,000 x g for 10 minutes. The resulting precipitate contained primarily mitochondria but was undoubtedly contaminated by some nuclei which did not come down in the preceding precipitate. Attempts to frac-

tionate nuclei and mitochondria by sedimentation were not successful probably because of the relatively small size of nuclei from Neurospora mycelia.

The opalescent, red supernatant from the preceding centrifugation was in turn centrifuged at an average of 110,000 x g for 2 hours. The precipitate was a red translucent jelly-like material corresponding to the microsomal fraction. The supernatant from the microsomal fraction was a clear red solution whose spectrum approximated that of primarily oxidized cytochrome c. Its spectrum was not significantly changed by the addition of ascorbate.

Determination of RNA and Protein

Known portions of each of the four fractions (cellular debris etc., mitochondria, microsomes and soluble fractions) were put through part of the extraction procedure recommended by Ts'o and Sato (53). Aliquots to be analyzed for protein and RNA were left overnight in 5 ml of 0.5 N TCA at 0° C., washed twice with 70 per cent ethanol (5 ml) at 3.5° C., twice with 0.1 per cent perchloric acid in ethanol (5 ml) at 3.5° C., twice with 1:2 ether:ethanol (5 ml) at 50° C., and twice with 0.2 N $HClO_4$ (5 ml) at 3.5° C. The precipitate from the above procedure was left for 72 hrs. in 4 ml of 1.0 N perchloric acid at 3.5° C. with occasional stirring, extracted and washed twice more, each time with 1 ml of 1.0 N perchloric acid. The phosphorous content of the combined supernatants

from the 1.0 N perchloric acid hydrolysis of ribonucleic acid were analyzed for phosphoreus by the method of Fiske and Subbarow (54) as outlined on page 115. The amount of RNA was calculated by assuming that it contained 9 per cent phosphorous (55).

The protein remaining after the 1.0 N perchloric acid extraction of RNA was determined by the Biuret method* (56) and by the Nessler method for total nitrogen (57). The values obtained by the two methods agreed within about 10 per cent and were averaged to calculate total protein present in the fractions, assuming the total protein contains 16 per cent nitrogen. The mitochondrial fraction undoubtedly contained some RNA, protein and DNA from contaminating nuclei that would be included, as a source of error in the values calculated for this fraction. Although the amount of error from this source has not been estimated, it is probably relatively small but would supposedly tend to lower the RNA:protein ratio for this fraction.

Determination of Cytochrome _c_

The cytochrome _c_ present in the first three precipitated fractions was extracted by suspending the precipitates in cold distilled water and bringing the pH cautiously to about 10.5

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*The Biuret method for total protein was not applied to the fraction containing cellular debris etc. because of insoluble interferring substances.

with concentrated ammonium hydroxide while the suspensions were being vigorously agitated in a Waring blender. After standing 10 minutes in ice water the pH of the suspensions was reduced to about 8.6 by the addition of acetic acid in the Waring blender. The suspensions were then centrifuged and the precipitates washed once with 0.05 M tris buffer at pH 8.6. Ammonium sulfate was added to the combined supernatants of each of the three ammonium hydroxide treated fractions and also to the soluble fraction to give a concentration of 152 mg. per ml. The samples were placed in the cold for 18 hrs. then centrifuged at 45,000 x g for 10 min. Supernatants were clear and the amount of cytochrome c present was determined from their spectra in a manner patterned after that used by Rosenthal and Drabkin (58). The precipitates from the centrifugation following the extraction and from the ammonium sulfate treatment contained only traces of cytochrome c as indicated by examination with a hand spectroscope.

Results and Discussion

The results are summarized in table 7 and expressed in mg. of RNA, protein and cytochrome c per gram dry weight of the intact tissue. The values obtained for the RNA:protein ratio in mitochondria, microsomes, and supernatant were 0.102, 0.383, and 0.018 respectively and seem to fall into the range found from other sources given below. For tobacco leaves the weight ratio of RNA:protein for mitochondria calculated from

TABLE 7. THE DISTRIBUTION OF RNA, TOTAL PROTEIN AND CYTOCHROME c IN THE SUBCELLULAR FRACTIONS OF *POKY*

in mg. per gram dry weight of the original tissue

	RNA	Protein	RNA:protein*	Cyt. c
Cellular debris	---	6.4 mg N**	---	2.93
Mito-chondria	13.0	127.4	0.102	1.58
Microsomes	38.3	100.0	0.383	0.67
Supernatant	0.75	41.6	0.018	10.62
Total				15.8

*Ratio by weight of RNA to protein

**Based on nitrogen determination only and expressed as mg N per gram dry weight of tissue

the data of McClendon (59) is 0.114 and 0.412 for the combination of microsomes and the soluble fraction. Ts'o and Sato (53) found the RNA:protein ratio for mitochondria from pea seedlings stems and tips to be 0.17 and 0.40 respectively. The corresponding values for the soluble fractions from stems and tips were 0.026 and 0.05 respectively.

In contrast to the distribution of RNA and protein the distribution as well as the amount of cytochrome c in *poky* seems quite unusual. The total amount of cytochrome c accounted for was 15.8 mg. per gram dry weight of intact

tissue. This is in close agreement with the amount obtained from similar but frozen samples of poky and is essentially identical to the value of 1.6 mg. per gram reported for 3 day cultures by Haskins *et al.* (52). Of the total amount of cytochrome *c* 10 per cent was recovered in the mitochondrial fraction and 67 per cent in the soluble fraction, the total protein of which was estimated to consist of 25.5 per cent cytochrome *c*. In contrast, Schneider and Hogeboom (60) found over 50 per cent of the total cytochrome *c* of liver homogenates to be associated with the mitochondrial fraction. Calculations from the data of Beinert (61) for liver tissue fractionated in isotonic sucrose indicate that 70 per cent of the endogenous cytochrome *c* was recovered with the mitochondria while only 16.5 per cent was recovered in the soluble fraction.

Also of possible importance is the small but significant amount of cytochrome *c* found in the microsomal fraction. The spectrum of the microsomal cytochrome was essentially identical to that of cytochrome *c* and indicated that it amounted to about 4.2 per cent of the total cytochrome *c* isolated. Although it would not seem unlikely that this amount of cytochrome *c* could have been due to contamination of the microsomal fraction by mitochondria, fragments of broken mitochondria or by the adsorption of cytochrome *c* to the microsomes, the data of Beinert (61) seem to make this a questionable point. In his experiments, in which labeled cytochrome *c* was added to isotonic sucrose solutions in which homogenation of rat livers

was carried out, much but not all of the microsomal cytochrome c could be accounted for by adsorption.

Data similar to that of table 7 does not exist for wild type Neurospora. However, estimation of the distribution of cytochrome c in the cellular fractions of 2.5 day old cultures ground only twice with sand in phosphate mannitol buffer indicate that about half or more of the total cytochrome c present remained in the cellular debris with much of the rest in the mitochondrial fraction. Not more than 25 per cent of the total was found in the soluble fraction. The total amount of cytochrome c present was about 0.2 mg per gram dry weight. However, these figures should be taken as approximate values, for the cytochrome c was not extracted at high pH nor was an ammonium sulfate fractionation performed. Estimations were made with a Zeist hand spectroscope by comparing the width of the 550 mμ absorption band of reduced cytochrome with samples of known concentration. The difference in the amount of cytochrome c remaining in the cellular debris of poky and wild type may reflect differences in the extent of fragmentation accomplished by grind with sand.

In contrast to wild type Neurospora and higher organisms in which cytochrome c is largely bound to mitochondria, these results suggest that much if not all of the cytochrome c of poky is not structurally incorporated into any particulate, subcellular fraction. A comparative study of the biochemical and morphological properties of the mitochondrial fractions

of _poky_ and wild type might disclose apparent disorders in _poky_ related to the organization rather than the specific composition of _poky_ mitochondria. Of particular interest is the lipid composition of this fraction.

D. THE FREE FATTY ACID CONTENT OF *POKY* AND WILD TYPE NEUROSPORA

While working with *poky*, Herzenberg (9) observed and isolated an ether soluble acidic substance which produced an immediate change in Neurospora or horse heart cytochrome *c* so that it was no longer reducible by ascorbate. The acidic material also caused horse heart cytochrome *c* to become much more susceptible to destruction by low concentrations of hydrogen peroxide. Dr. Carl Stevens identified the active substance as primarily free fatty acid. Accordingly 3 day and 6 day cultures of *poky* and 2.5 day old cultures of wild type Neurospora were examined for their lipid and free fatty acid content.

Materials and Methods

Extraction and Determination of Total Lipids

The water content of frozen pressed dry samples was determined by drying a small portion *in vacuo* over P_2O_5 and found to be 84.2, 84.5, and 86.4 per cent for wild type, 3 day *poky* and 6 day *poky* respectively. Frozen tissues ground as previously described was extracted for total lipid by the procedure recommended by Floch, Lees and Stanley (16) used with slight modification. The frozen powder was suspended in 2:1 $CHCl_3:CH_3OH$ at -27° C. then centrifuged. The precipitate was resuspended in cold $CHCl_3:CH_3OH$ and centrifuged twice more, then the process was repeated with 2:1 $CHCl_3:CH_3OH$ at room temperature an additional 3 times. The combined washings

were at least 20 times greater by volume:weight than the tissue originally used and formed one phase. Sufficient salt solution containing 0.02 per cent $CaCl_2$, 0.07 per cent $MgCl_2$, 0.29 per cent NaCl, and 0.37 per cent KCl was added to give a two phase system of the composition 8:4:3 $CHCl_3$:CH_3OH: salt solution including the weight of the frozen tissue as salt solution.

The lower chloroform phase was removed and washed once with pure upper phase made by allowing the equilibration at room temperature of 8:4:3 $CHCl_3$:CH_3OH: salt solution. Finally the lower chloroform phase was evaporated to dryness under vacuum in a rotary flash evaporator made by Lab Glass and Instrument Corporation of New York. Ice water was maintained in the receiver trap and temperature in the bath of the evaporation chamber was kept at 30° C. Vacuum was maintained by a cold water aspirator.

The evaporation chamber of the flash evaporator was thoroughly washed with several portions of redistilled 30-60° C. ligroin, the combined washings filtered, and the volume adjusted to exactly 50 ml. The extract was stored under N_2 gas at 0° C. until used. All steps were carried out quantitatively so that portions of the lipid sample could be related to specific amounts of the original tissue.

Considerable amounts of material came out of solution when the lipids in ligroin were cooled to 0° C., a small portion of which could not be redissolved upon warming the solutions. The amount of insoluble material did not seem to be

the same for all the samples although no specific determination of this was made. Also extracts of poky were green in color while those of wild type were light yellow.

The total lipid content of the extracts was determined by evaporating a known volume of the extract in ligroin under vacuum in tarred pans.

Determination of Ergosterol

The ergosterol content of the total lipid extracts was measured by the procedure of Sobel, Mayer, and Kramer (62). Samples were taken to dryness under vacuum and redissolved in chloroform. Sufficient reagent grade glycerol α, γ dichlorohydrin containing 4 per cent acetyl chloride was added to give a ratio of 2:3 chloroform:dichlorohydrin reagent and the absorption at 500 $m\mu$ was recorded after 1 hour at room temperature. A standard curve was established using C grade ergosterol obtained from the California Corporation for Biochemical Research. The sample of ergosterol was of questionable purity, thus the absolute amounts of ergosterol recorded may be in error although the relative values found for poky and wild type Neurospora should not be effected. Also it should be recognized that this colorimetric determination is not specific for ergosterol and that other related steriods present in the total lipid extract might influence the determination. Indeed, the spectra for the reaction carried out on the lipid extracts were not identical with those obtained

with known ergosterol, although the resemblances were much closer than with known calciferol.

Determination of Lipid Phosphorous

The total phosphorous content of the lipid extracts was determined by the method of Fiske and Subbarow (54) as modified by Stewart and Hendry (63).

Organic material was digested by heating with a small amount of concentrated sulfuric acid followed by the addition of a drop of 30 per cent hydrogen peroxide to the slightly cooled samples. The process was repeated until the samples remained colorless after heating. Caution was exercised to avoid excess fuming of the sulfuric acid during heating and to prevent spattering upon addition of the hydrogen peroxide. After complete clearing had been accomplished samples were cooled and diluted nearly to volume. Then aqueous molybdate followed by the reducing agent, 1-amino-2-napthol-4-sulfonic acid was added and the optical density at 670 mμ determined after heating in a boiling water bath for 10 minutes. The unknown values were determined by comparison with a standard curve made at the same time.

Fatty Acid Analysis

Methyl esters of free fatty acids in hydrolyzed and unhydrolyzed portions of the lipid extracts were formed by reaction with diazomethane generated by decomposition of nitroso-

methyl urea with 70 per cent KOH in water as outlined by Hickinbottom (64). Nitrosomethyl urea was synthesized and recrystallized as outlined by Weygand (65) and stored at -27° C.

Convenient 5 to 10 ml portions of the lipid samples to be treated with diazomethane without preliminary hydrolysis were evaporated to dryness under vacuum in 40 ml graduated centrifuge tubes. About 10 ml of diethyl ether was added to each tube and an ethereal solution of diazomethane distilled into the tubes until the solution took on the characteristic yellow-green color of diazomethane. The ether and excess diazomethane were then removed by placing the test tubes in beakers of warm water and directing a gentle stream of N_2 gas into the top of the tube. Diazomethane should be generated only in ether in small amounts and should be handled only in a fume hood equipped with a powerful exhaust fan. When free of diazomethane and ether, samples were diluted in a known, convenient amount of methyl butyrate and stored under N_2 gas in the cold until used.

The procedure used for hydrolyzed samples differed only in that portions of the lipid extract in ligroin were evaporated to dryness in vacuum then treated with 5 ml of 1.0 M KOH and allowed to stand with occasional stirring at 100° C. for 5 hours. The samples were then acidified with 10 N H_2SO_4 and extracted 3 times with diethyl ether. The volumes of the combined ethereal extracts were adjusted to about 10 ml by evaporation under N_2 gas and the samples were treated with diazomethane as described above.

Quantitative analysis of the methyl esters of base hydrolyzed and unhydrolyzed portions of the total lipid extract were performed with an Aerograph model A-100-C gas chromatograph (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) fitted with a 10 millivolt full span Varian G-10 recorder to which a 1 millivolt adapter had been attached. Standard curves for the amounts of various fatty acid methyl esters and their retention times were worked out using fatty acid methyl esters or fatty acid obtained in CfP grade from the California Corporation for Biochemical Research. For fatty acids not available as methyl esters, the esters were formed via the diazomethane procedure.

Insofar as was possible identical conditions for chromatography were maintained for all samples. A 10 foot x 1/4 inch stainless steel LAC 446* on Chromosorb Column at 200° C. and 83 ml/min. of helium were used with the recorder set at 5 millivolts. Retention times and the relation between curve areas and amounts of fatty acid esters were routinely rechecked throughout the work and found not to undergo significant change.

Fatty acid esters from the lipid extracts of poky and wild type Neurospora were identified by retention times and checked by co-chromatography with known compounds. A small peak comprising less than 1 per cent of the total fatty acid

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*LAC 446 is a glycol-adipate polymer supplied by the Wilkens Instrument and Research, Inc.

and with the approximate retention time expected for arachidic acid was detected but is not reported as such since no sample of known arachidic acid was available for co-chromatography. No arachidonic acid was detected in any sample.

Results and Discussion

The results of the analyses of the lipid extracts are recorded in table 8 where all data have been reported on a dry weight basis. It should be noted, however, that dry weights for young and old poky and wild type do not necessarily provide an equivalent basis for comparison. For example, somewhat more of the total dry weight of 2.5 day wild type probably consists of hyphal walls and similar structural elements than for poky.

The data indicate that 3 day and 6 day poky had 68 and 58 per cent more total lipid than 2.5 day wild, however, by the same comparison for lipid phosphate, 3 day poky was 36 per cent greater than 6 day poky and wild type. In contrast to the data for lipid phosphate the results of the glycerol α, γ dichlorohydrin reaction expressed as ergosterol indicated essentially identical amounts for 3 day poky and wild type but a 37 per cent increase for 6 day poky.

A most striking difference between poky and wild type is the amount of free fatty acid present in poky as seen in that portion of table 8 which lists the data for unhydrolyzed lipid samples. For example, linoleic acid, the most prevalent fatty

	Sample	Total Lipid	Pi	Ergo-sterol	Total Fatty Acid	Myris-tic	Pal-mitic	Palmit-oleic	Stea-ric	Oleic	Lin-oleic	Lin-olenic
Total Content (hydrolyzed)	2.5 day wild	51.4	1.17	9.6	35.50	0.176	6.42	1.72	1.17	3.35	19.45	3.21
	3 day poky	86.2	1.59	9.5	66.75	0.264	11.3	3.62	0.72	3.93	33.3	13.62
	6 day poky	81.4	1.17	15.0	53.36	0.114	8.13	3.11	1.22	3.17	30.5	7.12
Free Fatty Acid (unhydrolyzed)	2.5 day wild				1.36	0.00	0.34	0.26	0.00	0.00	0.76	0.00
	3 day poky				30.74	0.00	3.68	2.01	trace	1.13	16.35	7.57
	6 day poky				24.14	0.00	3.08	1.28	0.12	1.24	16.15	2.27
Bound Fatty Acid	2.5 day wild				34.14	0.176	6.08	1.46	1.17	3.35	18.69	3.21
	3 day poky				36.01	0.264	7.62	1.61	0.72	2.80	16.95	6.05
	6 day poky				29.25	0.114	5.05	1.83	1.10	1.93	14.35	4.85

TABLE 8. The Lipid Composition of Poky and Wild Type in mg/g Dry Weight

acid in all samples, is present in young and old *poky* as a free acid in concentrations about 21 times greater than found in wild. The free linoleic acid of wild type represents about 3.9 per cent of the total linoleic acid but 49 and 53 per cent of the total linoleic acid of 3 and 6 day *poky* respectively.

The excess of total lipid found in *poky* as compared with wild type is apparently not due solely to an accumulation of free fatty acids. The amount of non-fatty acid lipid may be calculated by subtracting the total fatty acid content (hydrolyzed samples) from the total lipid and is 15.9, 19.5, and 28.0 mg per gram dry weight for 2.5 day wild, 3 day *poky* and 6 day *poky*. The per cent of the total lipid other than free fatty acids represented by the non-fatty acid portion of the total lipid can be calculated by dividing the above figures by the total lipid content less the total free fatty acid (unhydrolyzed samples) and is 32, 35, and 49 per cent for wild type, 3 day *poky* and 6 day *poky* respectively. Although young *poky* and wild type seem similar in this respect there is apparently a substantial increase in the non-fatty acid lipid present in old *poky*. This relation seems to reflect the value expressed as the ergosterol content and can probably justifiably be considered as a check on the type of calculations involved.

The percentage of non-fatty acid lipid other than ergosterol and phosphate may be calculated from the values given

by subtracting the ergosterol plus phosphorous from the total amount of non-fatty acid lipid. The resulting value is 32 per cent for wild type but 43 and 42 per cent for 3 and 6 day poky respectively. The difference presumably represents the accumulation in poky of some lipid component not specifically determined. Unfortunately these calculations would be strongly affected by errors in the ergosterol determination as discussed above.

Differences can also be seen in the absolute amounts and percentages of individual free and bound fatty acids in wild type and young and old poky. Table 9 lists the amounts of individual free and bound fatty acids expressed as percentages of the total free or bound fatty acid for that sample. For example, it may be seen from table 8 that wild type contained 0.76 mg of free linoleic acid per gram dry weight of tissue and had a total free fatty acid content of 1.36 mg per gram dry weight. Thus table 9 indicates that linoleic acid comprises 56.0 per cent of the total free fatty acid in wild type. Similarly, wild type contained a total of 34.14 mg per gram dry weight of bound fatty acid of which 54.7 per cent was linoleic acid.

The accuracy of percentages calculated from small absolute values such as these for the free fatty acids of wild type are probably subject to considerable error. Despite this, there seems to be a correlation between the bound fatty acid composition of the three samples, especially between the 3 and 6 day

TABLE 9. AMOUNT OF INDIVIDUAL FREE AND BOUND FATTY ACIDS EXPRESSED AS PERCENTAGE OF THE TOTAL FREE OR BOUND FATTY ACID

(See the text for an explanation of calculations)

Type of Sample	2.5 day wild	3 day poky	6 day poky
Myristic			
free	0.0	0.0	0.0
bound	5.2	7.3	3.9
Palmitic			
free	25.0	12.0	12.3
bound	17.8	21.8	17.3
Palmitoleic			
free	19.0	6.5	5.2
bound	4.3	4.6	6.2
Stearic			
free	0.0	0.0	0.0
bound	3.4	2.1	3.7
Oleic			
free	0.0	2.7	5.1
bound	9.8	7.8	6.6
Linoleic			
free	56.0	53.2	65.8
bound	54.7	47.0	49.0
Linolenic			
free	0.0	24.6	9.2
bound	9.4	16.8	16.6

poky. Further, there seems to be considerably more variation in the composition of the free and bound fatty acid fractions than was expected from the accuracy of the techniques used. This might suggest that the free fatty acids of poky have not arisen directly from the bound fatty acids by degradative processes in the living mold.

A very interesting aspect of the presence of free fatty acids in poky is the effect they might have on the large amounts of cytochrome c accumulated in this mutant. The observations concerning the effect of palmitic acid on horse heart cytochrome c presented in Part I indicate that the enzyme can react directly with oxygen in the presence of free fatty acid. It seems possible that cytochrome c plus fatty acid might actually function as the terminal oxidase in place of cytochromes a and a_3 which are apparently not present in poky. No specific data are available, however, the rather large amounts of both cytochrome c and free fatty acid present in poky seem to warrant further consideration of this point. The interaction of free fatty acids with protein surely involves enzymes other than cytochrome c. It seems likely that an aberration in lipid metabolism of the type described for poky would be reflected by irregularities in the formation or functions of many lipid containing structures. Although the mechanism by which cytochrome c is normally bound to the mitochondrial fraction is not known, it seems likely that

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