STUDIES ON A CYTOCHROME DESTROYING SYSTEM IN NEUROSPORA

Thesis by

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ABSTRACT

The results of investigations on a cytochrome destroying system in Neurospora are presented. It is shown that the destruction is complex and dependent on the action of several enzymes as well as non-catalyzed reactions.

Poky particles exhibit a rapid and extensive hydrolysis of their own and added proteins whereas fast poky and wild-type particles do so to a much smaller extent. When cytochrome c is added to poky particles or particle-derived preparations, heme-peptides are produced. Several heme-peptides are partially characterized. Evidence is presented which suggests that poky particles, succinate and cytochrome c, when incubated together in air, produce a verdohemochrome.

An ether soluble substance is found in pokyand fast poky particles which combines with cytochrome c so that the iron is not reducible by ascorbate and the heme group is destroyed on reduction by hydrosulfite and reoxidation by oxygen. Purification of this substance and some of its chemical properties are described. It has been named "pokonic acid."

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INTRODUCTION

A number of mutants of Neurospora crassa, described by Mitchell, et al., grow slowly on complete or minimal media and have cytochrome systems which differ from that of wild type[1] (see fig. 1). In the course of measuring succinoxidase activity of a particle preparation prepared from one of these strains, poky, it was noted that the flask containing the particle preparation, succinate, and cytochrome c turned from red to green during the incubation. When wild-type particles were incubated with succinate and particles prepared from poky, the characteristic absorption bands of all the cytochromes disappeared. Hemin and heme-proteins other than cytochromes were not affected by incubation with poky particles and succinate. For this reason, the system in poky particles was named "Cytochromase." This activity was not demonstrated in wild type and was found to decrease with the age of the mold in poky until, in a 6 day-old culture, none was found. The supernatant from the high speed centrifugation by which the particles were prepared exhibited a strong inhibitory effect on the cytochromase activity.[2]

The enzymatic properties usually associated with the cytochromes are compared for poky and wild type in fig. 2.

The work in this thesis was undertaken to attempt to characterize the cytochromase reaction. A brief review of

the literature on the properties and structure of cytochrome c is included here.

A. Discovery and Isolation of Cytochrome c

Cytochromes were first described by MacMunn, under the names histohaematin and myohaematin.[3] Using a spectroscope he observed, in a large variety of tissues, absorption bands which are characteristic of what Keilin later called cytochromes.[4]

Cytochrome c was first extracted as a distinct pigment in 1925 by Keilin and shown to be responsible for the absorption bands at 520 and 550 mμ of the in situ cytochrome spectrum.[4] In that and subsequent work (Keilin,[5] Keilin and Hartree[6]) cytochrome c was shown to be a heme-protein which is quite stable to heat, acid and base but not to organic solvents, is not oxidizable by air but can be reversibly oxidized and reduced with certain oxidizing and reducing agents.

Since it is relatively stable and abundant, cytochrome c has been isolated and purified in many different ways. The most generally used method is that of Keilin and Hartree in which cytochrome c is extracted from minced heart muscle with 25% trichloracetic acid (TCA) at pH 4 and fractionally precipitated with ammonium sulfate and more TCA. This yields a product containing 0.34% iron.[6] In 1940, Theorell and Åkesson obtained cytochrome c with an iron content of 0.43% by prolonged electrophoresis in alkaline solution.[7] Keilin and Hartree later prepared cytochrome c with a 0.43% iron content

by a further fractional precipitation with ammonium sulfate, at pH 10, of their 0.34% preparation and showed this to be as active, on an iron basis, as the less pure preparation.[8]

With the advent of ion-exchange resins, the preparation of purified cytochrome c has become a much easier task, and preparations containing a higher percentage composition of iron have been obtained which still retain the same degree of biological activity per iron atom. Paléus and Neilands, in 1950, used a column of finely ground IRC-50, a carboxylic type of cation exchanger, to purify a 0.34% iron preparation of cytochrome c. They adsorbed the cytochrome c on the resin from 0.1 M ammonium acetate at pH 9 and then continued washing with this solution. First, a brown fraction with low iron content moved rapidly down the column, then, much later, two red fractions followed. A third red fraction was eluted with 0.5% ammonium hydroxide. These 3 fractions assayed at 0.401, 0.466 and 0.353% iron respectively with the bulk of the material being in the 0.466% fraction. Unfortunately, no tests of enzymatic activity were made on these preparations.[9] Neilands, later, using a modification of this method, obtained a purified preparation which did show enzymatic activity in a succinoxidase system but the data to compare this activity with iron content were not given.[10] Margoliash, also using IRC-50, investigated a number of elution systems. With a somewhat complicated procedure involving neutral and basic elutions, he obtained a preparation containing 0.465% iron which had the same activity

per atom of iron in a succinoxidase system as the original 0.34% starting material. He showed also that TCA at pH 4.5 changes cytochrome c slowly into a spectroscopically identical but chromatographically and enzymatically different form. This changed cytochrome catalyzed the oxidation of ascorbate and was less effective in a cytochrome oxidase system than the original cytochrome c.[11]

B. Properties and Structure of Cytochrome c

1. Mode of Linkage of Porphyrin to Protein.

Cytochrome c was shown to be a heme-protein complex by Keilin's first work on cytochromes.[4] Theorell first proposed that the porphyrin was covalently linked to the protein by thioether bonds between the side chains at positions 2 and 4 of the porphyrin and 2 cysteine residues of the protein.[12] (See fig. 3.) He later withdrew this proposal and looked on the cysteine adduct of porphyrin as an artifact of hydrolysis in which an addition of the cysteine occurred across the double bonds of the vinyl groups.[13] Zeile and Meyer, by hydrolyzing under conditions which should prevent this addition, showed that Theorell's original proposal was probably correct. In addition, they synthesized a dicysteine adduct of protoporphyrin which was identical to porphyrin c in all its properties except for the optical rotation.[14,15]

Paul has recently reinvestigated the nature of the protein-porphyrin linkage through the use of metal salts to cleave the

thioether linkage. By heating cytochrome c at 60° C. for 80 minutes in ca. 10^{-2} M silver sulfate in ca. 10% acetic acid he has obtained heme-free protein and hematohemin c. This he showed to be an isomer of hematin in which the side chains at positions 2 and 4 are α-hydroxyethyl groups. He thus concluded that the 2 cysteine residues are connected through thioether links to the secondary carbon atoms of side chains 2 and 4 of the porphyrin.[16,17,18]

2. Bonds Between Iron and Protein.

In this section, the mode of attachment of the iron to the protein of cytochrome c will be discussed. Iron has 6 coordinative valences directed towards the corners of a regular octahedron. The 4 in 1 plane are occupied with the 4 nitrogen atoms of the tetrapyrrole, and the other 2 can coordinate with oxygen or nitrogen-containing groups of the protein. Attempts to decide which groups are involved in this coordination in native cytochrome c have been made by several different methods.

a. Optical properties of cytochrome c. Theorell and Åkesson found that the absorption spectrum of ferricytochrome c varied with the hydrogen ion concentration.[19,20] They described types I - V which appeared in order from acid to basic solutions of the heme-protein. By plotting the change from type to type as a function of pH, they obtained a "spectrophotometric titration curve" with transition pK values of 0.42, 2.50, 9.35 and 12.76. Type I had the same absorption spectrum

as that of hemin c in acid solution and thus was interpreted as showing that in strongly acidic solutions, the iron of cytochrome c is attached only to the 4 porphyrin nitrogen atoms. Types II and V complex with fluoride and cyanide, respectively, and are interpreted as having 1 of the 2 remaining valences of the iron complexed with a group on the protein and the other one available for combination with negative ions. The fact, that on reduction types I, II and V form carbon monoxide compounds, lends support to this interpretation. Type III, the physiologically important one, existing around neutrality, would have both of the 2 iron valences not occupied with the porphyrin bound to the hemochromogen-forming groups in the protein. It does not form an azide or cyanide complex, according to Theorell and Åkesson, testifying to the firmness of the complexing with the protein. Type IV is similar to type III in not complexing with cyanide or azide in the oxidized form nor with carbon monoxide in the reduced form. The pK of transition between III and IV is 9.35 thus leading to the interpretation that the dissociation of the imino nitrogen of an imidazole has been effected which would not be expected to release 1 of the coordinative valences of the iron.[19,20]

Later work showed that ferricytochrome does form both the cyanide and the azide complex through a wide range of hydrogen ion concentrations, including that of neutrality.[21,22,23] Although the equilibrium of the reaction between cyanide and ferricytochrome c is only reached over several hours, it is

still difficult to see why Theorell could not find the cyanide complex. Kinetic data on the formation of the complex led George and Tsou to the conclusion the iron is bound firmly on both sides to protein. They were not able to ascribe this binding to any specific groups of the protein.[23]

b. Titration curves. Theorell and Åkesson found on titrating beef cytochrome containing 0.43% iron that only 1 imidazole group is titrated in the normal titration range of imidazole (pH 5.5 to 8.5). Since chemical analysis showed 3 histidines per mole, they concluded that the remaining 2 imidazoles are probably complexed with the iron and are thus unavailable for titration in this range. They also stated that the titration curve in this region is the same for a 0.41%-iron-containing preparation of horse heart cytochrome c.[20] Margoliash reported, in 1955, that horse heart cytochrome, with an iron content of 0.465%, has 4 histidines per mole, determined chemically.[21] This leaves the validity of such titration data open to question.

c. Magnetic studies. By measuring the paramagnetic susceptibility of a molecule, the number of unpaired electrons can be determined. Studies of this sort on cytochrome c by Theorell and Åkesson[24] are consistent with the conclusion that the iron is bound firmly to 2 groups of the protein in neutral solution, but do not give any help in deciding which are the hemochromogen-forming groups of the protein.

d. Oxidation-reduction potentials. A number of investigators have determined the redox potential of cytochrome c at various hydrogen ion concentrations. There has been general agreement, in the relatively recent literature, on a value of 0.262 ± .005 volts at pH 7. The results of potentiometric titrations by different investigators do not agree so well. Stotz et al. found the E'_o to be constant at about 0.262 volts from pH 5 to 8.[25] Essentially the same result was obtained by Wurmser and Fillitti-Wurmser.[26] Rodkey and Ball reported a constant redox potential for cytochrome c, 0.260, from pH 1.75 to 7.8.[27] These workers used cytochrome c which had an iron content of 0.34%, or less. Paul, using an electrophoretically homogeneous preparation containing 0.43% iron, found that the dE'_o/dpH is 0 from pH 4 to approximately pH 7. At pH 6.86 the dE'_o/dpH started increasing until it reached the value of 0.06 volts at pH 7.5.[28] This indicates a heme-linked group with a pK of 6.86. Theorell states that this ". . . fits in very well with the imidazole linkage theory because a histidine imidazole linked to ferroheme by covalent bonds is expected to have its pK near 7."[29]

The reasons for discrepancy between the results reported in the 2 recent papers in this field, Rodkey and Ball[27] and Paul,[25] are not clear. It is worth noting that the measurements of the former were made with a preparation of cytochrome containing only 0.27% iron while the latter used one containing 0.43% iron. The material removed, when going from the prepara-

tion with the lower percentage of iron to the higher, comes off under alkaline conditions[7,8] or with addition of cyanide.[22] Electrophoretic investigations of Tint and Reiss on preparations of cytochrome c from beef, horse, chicken and pig hearts indicated that there was probably a single impurity in each of these with very similar electrophoretic mobility properties.[30] It is interesting to speculate that this impurity plays some biological role in the function of cytochrome c, is responsible for the conflicting results mentioned above and is responsible for the non-reactivity of "endogenous"[31] cytochrome c with cyanide.

e. Chemical determination of positions and reactivity of histidines. Paul reacted 0.43%-iron-containing cytochrome c from beef heart with 1-fluoro-2,4-dinitrobenzene (FDNB) in 66% ethanol and excess sodium bicarbonate. On hydrolysis, he found 0.54 moles (corrected) of free histidine per mole of cytochrome. He interpreted this result as proving that at least 1 imidazole is complexed with the iron.[18] Margoliash, using a preparation of horse heart cytochrome c with an iron content of 0.465%, attempted to determine the positions and reactivities of the histidines in the molecule.[21] There were 4 moles of histidine per mole of cytochrome in his preparation. He reacted this cytochrome with FDNB in 66% ethanol and excess sodium bicarbonate for 24 hours. In a separate experiment he drastically oxidized with hydrogen peroxide so as to destroy

the heme and reacted this (presumably iron free) product with FDNB under the same conditions. He then hydrolyzed these dinitrophenyl-proteins (DNP-proteins) and estimated free histidine and the 3 possible DNP-derivatives of histidine. One mole of free histidine and 1 mole of imidazole-DNP-histidine was found in each, per mole of cytochrome c. In the unoxidized DNP-protein hydrolyzate he found 1 mole of α-DNP-histidine and nearly 2 moles of the bis-DNP-derivative. He thus concluded that one of the unreactive imidazoles was available to the FDNB only after peroxide oxidation and was therefore presumably bound to the iron. Why there was still 0.26 mole of α-DNP-histidine in the hydrolyzate of the oxidized DNP-protein was not discussed. That the peroxide treatment might affect more than the heme was not considered either, but, from the data given on the yields of ε-DNP-lysine, this seems to be the case. Before peroxide, about 20 moles of this lysine derivative were found, while after the oxidation only 16.7 moles were reported. It should be noted that the imidazole purported to be coordinated with the iron was part of a histidine whose α-amino group was free to react with FDNB.[21]

Another approach to the problem of the positions of the histidines with relation to the heme group was the one used by Tsou,[32] Paléus,[33] and Tuppy and Bodo.[34,35,36] Tsou found that if cytochrome c was digested with various proteolytic enzymes, products could be obtained which were autoxidizable

and unable to substitute for cytochrome c in a succinoxidase or cytochrome oxidase preparation using p-phenylenediamine as substrate. He found the molecular weight of the pepsin-modified cytochrome obtained from horse heart cytochrome c was 2500, based on the iron content (2.21%) and that it contained histidine.[32] No quantitative data on histidine content were given. Paléus prepared pepsin-modified cytochromes from beef, chicken and salmon heart cytochromes and found 1 mole of histidine, in each, per atom of iron.[33] These modified products all had absorption maxima in the reduced form at 520 and 550 mμ (as in cytochrome c), and thus, would be expected to have 2 groups coordinated with the iron. Therefore, at least 1 group other than histidine should be involved.[33]

Spectrometric and titrimetric studies on these heme-peptides indicated that there were 2 groups coordinated with the iron but, again, no conclusions could be drawn with respect to the nature of these groups.[37]

Tuppy and Bodo prepared heme-peptides from horse cytochrome c by partial hydrolysis with sulfuric acid or with trypsin.[34,35,36] After removal of the heme group by the silver salt method of Paul,[17] they determined the sequence of the amino acids in the peptides. They found there was 1 histidine adjacent to 1 of the cysteines which were attached to the porphyrin by their sulfur atoms. This was the only histidine present in the molecule but, again, on reduction, the 520 and 550 mμ absorption bands of the hemochromogen-

type spectrum of cytochrome c appeared.[34,35] They repeated this work with beef and pig heart cytochrome c and obtained the same results with these as with the horse heart cytochrome. The only histidine they found in the peptide, in each case, had its α-amino group joined in a peptide bond with threonine.[36]

Tuppy and Paléus,[38] and Paléus et al.,[37] reinvestigated the pepsin-modified cytochromes obtained from beef, chicken and salmon heart cytochromes c. The results of their amino acid sequence studies, coupled with the earlier ones of Tuppy and Bodo,[34,35,36] showed that 13 amino acid residues are arranged in the following sequence in beef heart cytochrome c.

$$^{a}\text{Val-Glu}(NH_2)-^{b}\text{Lys-CySH-Ala-Glu}(NH_2)\text{-CySH-His-Thr-Val-Glu-}^{c}\text{Lys}^{d}$$

The abbreviations are those of Brand and Edsall[39] and the arrangement is described as suggested by Sanger.[40] The amino terminal group is represented on the left. From "a" to "c," the sequence has been shown to be the same in salmon heart cytochrome[37] and from "b" to "d" in horse and pig heart cytochrome.[34,35,36] In the pepsin-modified cytochrome from chicken heart, a serine residue was found in place of the alanine residue.[37]

If the histidine imidazole that Margoliash concluded is complexed with the iron[21] is compared with the histidine which Tuppy and Bodo found,[36] a discrepancy arises. The imidazole

that Margoliash found was part of a histidine with a free α-amino group while Tuppy and Bodo showed that their histidine did not have a free α-amino group. From Margoliash's work, this α-amino group did not react with FDNB until the cytochrome was altered in some way (alcohol or TCA at pH 4.5) and made enzymatically inactive. It is possible that some type of rearrangement occurred in the molecule under these conditions which could explain the discrepancy in the results and conclusions.

MATERIALS AND METHODS

The strains used in this work, and their characteristics, are described in table 1.

Table 1. Neurospora Strains Used in This Work

Strain	Origin	Growth	Mode of Inheritance
poky[41]			
po 3627-2a	spontaneous	slow	cytoplasmic
po 1803-4A	spontaneous	slow	cytoplasmic
fast poky[42]			
pof 3627-4A	spontaneous (in a *poky* strain)	medium	*po*: cytoplasmic *f*: chromosomal
C115[1]			
2173-3A	spontaneous	very slow	chromosomal
C117[1]			
2159-2a	spontaneous	slow	chromosomal
mi 3[1]			
2110-2	spontaneous	medium	cytoplasmic
Wild type			
W 2293-2a	-	fast	-
WP 1400-4A	-	fast	-

The characteristic cytochrome spectra of these strains are given in fig. 1.

The materials used in this work, other than standard reagents, are listed here.

Cytochrome c from horse and cow heart, Sigma Chemical Co., St. Louis, Mo.

Tri-n-octylamine, British Hydrological Corp., High Path, London S.W. 19, England.

Amberlite XE-97 (IRC-50), Rohm and Haas Co., Philadelphia, Penn.

Dowex 1 - X8, Cl^-, 200-400 mesh, AR, Bio-Rad, Berkeley, Calif.

Lysozyme, crystallized from egg white, Armour Laboratories, Chicago, Ill.

Micrococcus lysodeikticus, courtesy of Dr. R. L. Lester.

Growth and Harvesting of Mold

All strains were maintained on agar solidified minimal medium, 20 ml. in 125 ml. Erlenmeyer flasks.[43] Cultures for experimental use were grown in liquid minimal medium in 1, 7 or 15 liter volumes, in bottles equipped with facilities for forced sterile aeration, in a room maintained at 25° C. Inoculations were made with conidia suspended in sterile distilled water. The mold was harvested by filtration through cheese cloth with manual pressure to remove the excess medium. The mold was then washed 3 times with at least 20 volumes of distilled water and finally pressed between paper towels to remove excess water, and weighed.

Particle Preparations

The washed mold was ground in an ice cold mortar with 2 parts, by weight, of 0.05 M, pH 7 phosphate-mannitol buffer (57 ml. of 0.5 M Na_2HPO_4, 43 ml. of 0.5 M KH_2PO_4, 75 g. of mannitol and water to make 1 liter) and 1/2 part, by weight, of washed sea sand. The well ground slurry was centrifuged for 5 minutes at an acceleration of 2,000 x g. The precipitate was then re-ground with 1 volume of the phosphate-mannitol (P-M) and centrifuged again. The combined supernatants were sedimented in a Spinco model L preparative ultracentrifuge under an average field of 40,000 x g for 10 minutes in the cold. The packed particles were then washed twice with at least 10 volumes of P-M, by resuspension and recentrifugation. Finally, the particles were resuspended in P-M. The concentrations of particles, throughout this thesis, are expressed as g./ml. to mean g. of moist mold per ml. of particle suspension.

Breakdown of Particles and Added Proteins

The particles and any additions were incubated at 35° C. in 0.05 M phosphate buffer (pH 7) with shaking in air. Immediately after mixing, and at intervals thereafter, samples of 0.2 ml. were pipetted into 0.2 ml. of 15% trichloracetic acid (TCA). The precipitated samples were kept at 5° C. overnight and then centrifuged at about 2,000 x g for 10 minutes. Aliquots of the supernatant were analyzed for amino nitrogen by

means of a quantitative ninhydrin procedure. During the course of this work, 3 different methods were used for this determination. They were those of Moore and Stein,[44] Troll and Cannan[45] and Cocking and Yemm.[46] The principles in all of these methods are the same but each later one, in turn, was technically more desirable. In some experiments, a total nitrogen determination was made on the TCA precipitates. This was done by a direct Nesslerization in which the sample was refluxed with 0.2 ml. of sulfuric acid for 3 hours, cleared with Perhydrol, diluted to 30 ml. with water and the color developed with 5 ml. of Nessler's reagent. After standing 20 minutes at room temperature, the amount of color was determined in a Klett colorimeter with a blue filter (5433) and the values compared with a standard curve obtained in the same experiment with known amounts of ammonium sulfate.

Desoxycholate Preparation

A particle suspension was prepared as described on p.16 except that the washings were made with 0.05 M phosphate (pH 7). The final suspension was made 1 g./ml. One volume of this was well mixed at 0° C. with 1 volume of neutralized 0.5% w/v desoxycholic acid. (This was made by dissolving the desoxycholic acid in the minimum volume of 6 M sodium hydroxide, diluting with 0.05 M phosphate buffer to almost the proper volume and then adding 1 M hydrochloric acid to pH 7. The solution was then diluted to volume with phosphate

buffer. This solution soon gels but it is usable in this form.) The dispersed particle preparation was centrifuged at 25,000 rpm for 20 minutes and the precipitate discarded. The opalescent supernatant was kept at 5° C. overnight and the copious precipitate which formed was packed by centrifugation, washed once with phosphate buffer and once with distilled water. The precipitate keeps at least 6 months in a deep freeze.

For use, the preparation was dissolved in a small volume of buffer at pH 8 and any remaining precipitate removed by centrifugation. It was then reprecipitated at pH 7, washed once with buffer at pH 7, and then redissolved in 0.1 M TRIS at pH 8.2. In experiments on amino nitrogen release, and decrease in TCA insoluble nitrogen, the desoxycholate preparations were made by dispersing the packed washed particles, as above, in a volume of the neutralized desoxycholate such that the final concentration of particles is 1 g./ml. This was centrifuged in the cold at 25,000 rpm and the supernatant used.

Silver Salt Splitting of the Porphyrin-Peptide Thioether Bonds

The porphyrins of the heme-peptides were removed from the peptides by a modified method of Paul[17] and Tuppy and Bodo.[34] Up to 10 mg. of heme-peptides were dissolved in 1 ml. of water, then 2 ml. of glacial acetic acid and 6.3 ml. of water containing 50 mg. of silver sulfate were added and this mixture

was incubated at 60° C. for 90 minutes. It was then dried *in vacuo* and taken up in 5 ml. of 87% acetic acid. Insoluble material (silver salt and porphyrin) was removed by centrifugation. To the 5 ml. of 87% acetic acid containing the silver-peptides was added 0.5 ml. of 30% hydrogen peroxide. After 20 minutes at room temperature, water (5 ml.) was added and the solution dried *in vacuo* at 50° C. The residue was taken up in 20 ml. of water, sulfuric acid added to bring the pH to 3, and hydrogen sulfide gas bubbled through the solution at 60° C. for 3 minutes. The silver sulfide was removed by centrifugation and the remaining sulfuric acid was removed by extracting the solution twice with 6 ml. of a 5% solution of tri-n-octylamine in chloroform and then once with chloroform, leaving free peptide(s) in neutral solution.

Histidine Determination

The method used was adapted from that of Jorpes, in which a red color is obtained with histidine and diazotized sulfanilic acid.[47] A stock sulfanilic acid solution was made by dissolving 0.9 g. of sulfanilic acid in 9 ml. of concentrated hydrochloric acid and 91 ml. of water. At the time of the determination, 1.5 ml. of this solution plus 7.5 ml. of freshly prepared 5% sodium nitrite were mixed at 0° C., diluted, after 5 minutes, to 50 ml. with water, and kept cold. One ml. of this diazo reagent was mixed with 1 ml. of water containing from 1 to 10 μg. of histidine and allowed to stand at room

temperature for 30 minutes. Then 1 ml. of 5% sodium carbonate was added and, in 4 to 6 minutes, the optical density at 480 mμ determined with a Beckman DU spectrophotometer. The unknown values were determined by comparison with a standard curve.

Iron Determination

The method used was adapted from that of Drabkin, in which a red complex is made by ferrous ion and orthophenanthroline.[48] From 1 to 10 μg. of heme iron in 1 ml. of water, 0.4 ml. of 0.167 M sodium hydroxide and 0.1 ml. of Perhydrol were mixed, allowed to stand at room temperature for 3 hours and then heated at 90° C. for 10 minutes. Then 0.2 ml. of M hydrochloric acid was added and the solution again heated at 90° C. for 10 minutes. After cooling, 0.8 ml. of 5% ammonium acetate, 0.08 ml. of fresh 1% ascorbic acid and 0.4 ml. of 0.1% orthophenanthroline were added and the optical density at 500 mμ was read in a Beckman DU spectrophotometer. The unknown values were determined by comparison with a standard curve made at the same time.

Chromatography

Ninhydrin (0.2%) in pyridine was used as a general reagent for the detection of amino acids and peptides. Most of these compounds gave a purple color with this reagent on paper, but aspartic acid gave a blue color; glycine, phenylalanine and histidine gave a brown color; and proline gave a yellow color.

Isatin (0.2%) in 4% acetic acid in acetone gave a blue color with proline.[49] The diazo reagent of Ames and Mitchell was used to obtain a red color with histidine and tyrosine[50] and the periodate procedure of Metzenberg and Mitchell was used to reveal the presence of threonine, serine and cystine (cysteine) by showing them up as white spots on a purple background.[51] The azide-iodine spray was used to detect the sulfur containing amino acids, methionine and cystine (cysteine), as white spots on a brown background.[52] Through the use of 2-dimensional chromatography, with propanol-ammonia (3 volumes of n-propanol to 1 volume of 1 M ammonium hydroxide) in the first direction and propanol-acetic (3 volumes of n-propanol to 1 volume of 1 M acetic acid) in the second direction, and the above sprays, the amino acids in protein or peptide hydrolysates were identified.

RESULTS

Breakdown of Preparations and Added Proteins

Poky particles, when incubated in buffer at pH 7, exhibit a rapid and extensive release of TCA soluble, ninhydrin reacting material, whereas wild-type particles exhibit little of this release. Paper chromatography showed that this ninhydrin reacting material is a complex mixture of amino acids and peptides. The time course of this release with fresh and aged poky particles, with and without added cytochrome c is shown in fig. 4. The rate and extent of this release decreases with the age of the mold (fig. 5). To compensate for possible variations in yield of particles from the mold of different ages, the basis for comparison is the total nitrogen content of the particle preparation used. The actual variation in yield of particles is indicated by an average deviation of 13% in yield in a number of separate experiments over a period of $2\frac{1}{2}$ years.

Various proteins, other than cytochrome c, can act as substrates for this proteolytic process. An indication of their relative activities in the system is given by the data summarized in table 2. By examination of table 3, it can be seen that wild type and fast poky particles demonstrated this breakdown to a very much smaller extent than poky particles and that dispersing the particles with desoxycholate increased the activity somewhat. The breakdown of particle preparations and added cytochrome c was also followed, in some experiments, by

Table 2. Comparison of Action of poky Particles on Various Proteins

Particle Preparation	Added Protein	µg. Amino Nitrogen Released per g. of Mold
fresh	-	64
aged	-	20
"	beef heart cytochrome c	47
"	egg albumen	10
"	hemoglobin	34
"	catalase	74
"	chymotrypsin	34
"	casein	98
"	protamine	112

Particles prepared from po 1803-4A (age 3 days). Aged particles were obtained by incubating fresh particles in 0.05 M P-M buffer (pH 7) for 2.5 hours at 35° C., centrifuged for 5 minutes at an acceleration of 40,000 x g and resuspended in original volume of P-M. Incubations were in P-M at 35° C. for 1 hour. All additions were 4.2 mg. of protein per g. of mold.

Table 3. Changes in Nitrogen Distribution

Strain	Age of Mold (in days)	Preparation	% Decrease of TCA Insoluble Nitrogen	% of TCA Insoluble Nitrogen Appearing as TCA Soluble Amino Nitrogen
WP 2293-2a	3	Particles	12	2.5
		DOC[1]	9.7	11
pof (average of 6 strains)	2.5	Particles	18	3.6
		DOC	16	3.3
po 1803-4A	2.5	Particles	52	25
		DOC	76	44
po 1803-4A	4.5	Particles	44	14
		DOC	61	20

Preparations from 0.2 grams of mold incubated in 1.2 ml. of 0.05 M phosphate buffer (pH 7) at 35° C. for 1 hour.

[1]Desoxycholate dispersed.

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the less sensitive method of determining the decrease, during the incubation, of TCA insoluble nitrogen. The extent of this decrease is given.

Cytochrome Destruction by Particle Preparations

Fresh poky particle preparations, from 0.2 g. of moist 3 day-old mold, when shaken with 0.2 mg. of cytochrome c and 1 mg. of sodium succinate in 0.5 ml. of 0.05 M phosphate buffer (pH 7) at 35° C., cause a destruction of the cytochrome c. In 1½ hours, the original pink color of the cytochrome starts to change to a brown color and after 2½ hours the color

has changed to a bright green. With a hand spectroscope it can be seen, after this time, that the absorption bands of reduced cytochrome c at 550 and 520 mμ have disappeared and a new band at 650 mμ is present. Both succinate and air are necessary for the appearance of the green color. If succinate is omitted, the incubation mixture is orange (absorption band at 530 mμ) at the end of 2½ hours. Ascorbate will not serve as a substitute for succinate. Incubation under nitrogen results in a final pink color with absorption bands at 520 and 550 mμ. The color changes noted above served as a semi-quantitative index of cytochrome destruction by particle preparations. A spectrophotometric method could not be used due to the turbidity and the variable changes in turbidity during the incubation.

The destruction of cytochrome by poky particle preparations involves at least 2 separate reactions. The first of these, not requiring succinate or air, makes the cytochrome no longer reducible by ascorbate and causes it to be destroyed on reduction by hydrosulfite and reoxidation with air. The second reaction, in which the green color appears, requires succinate and air. Cytochrome preincubated with particles, as above, for 2½ hours without succinate, turns green in 5 to 10 minutes after the addition of succinate while particles preincubated in buffer alone, as above, for 2½ hours require 15 to 20 minutes after the addition of cytochrome and succinate for the green color to appear. Some change in the par-

ticles occurs during the preincubation period, in addition to the change in the cytochrome, as indicated by the decrease in time required for the color change with added cytochrome and succinate.

The time required for the color change to occur when fresh poky particles are incubated with cytochrome and succinate as described above, increases to about $3\frac{1}{2}$ hours with particles prepared from 4 to 5 day-old mold. The reaction does not occur with particles prepared from 6 day-old poky. The time required for the color change to occur is not changed by varying the relative amounts of cytochrome and particle preparation used but if too much cytochrome is used for the amount of particle preparation, some unchanged cytochrome is left.

The particles can be stored in the frozen state for periods of at least 6 months with no loss in activity. Boiling for 2 minutes was sufficient to destroy all the activity.

The cytochrome destroying activity described above was found also in particles prepared from 3 day-old C117. Particle preparations from 2, 3, 4 and 5 day-old wild type, 2, 3, 4, and 5 day-old fast poky, 2 and 4 day-old mi 3 and 3 day-old C115 did not have this activity when assayed in the same manner as the poky particle preparations. Neither freezing nor preincubation of the particles from wild type or fast poky evokes the cytochrome destroying activity. It has been noted, however, by Dr. Francis Haskins, that the contents of Warburg

flasks containing fast poky particles, cytochrome c and succinate in P-M retained a pink color for at least 4 or 5 hours after mixing but were brownish green after 18 to 24 hours. This was probably an example of the same type of cytochrome destruction as is exhibited by poky particles as it did not occur in flasks containing, in addition to the particles, cytochrome, and succinate, the soluble fraction of the mold homogenate, which contains an inhibitor of the cytochrome destroying system in poky.

Cytochrome Destruction by Dispersed Particle Preparations

Poky particle preparations, after they were dispersed with desoxycholate (preparation of desoxycholate solution described on p. 17), were no longer able to carry out the destruction of cytochrome ending in the production of the green compounds. They were, however, still able to carry out the non-succinate requiring step, or something very similar to that step. Furthermore, a long incubation was no longer necessary. The freshly prepared particles from 0.2 g. of moist 3 day-old poky were dispersed in 0.2 ml. of 0.5% sodium desoxycholate in 0.05 M phosphate buffer (pH 7). This was added to 0.2 mg. of cytochrome c in 0.3 ml. of 0.05 M phosphate buffer (pH 7). After incubation for 20 minutes at 35° C., a small quantity (1-5 mg.) of sodium hydrosulfite was added and the mixture well shaken with air. The absorption bands of reduced cytochrome c could not be seen, although

in a control mixture, not containing the dispersed particle preparation, the bands could be seen clearly. The orange product present after the 20 minute incubation could not be reduced by ascorbate. Experiments carried out as above, in which 3 day-old wild type and 2 and 4 day-old fast poky dispersed particle preparations were used, did not show any evidence of a similar cytochrome destruction.

Desoxycholate dispersed particle preparations from poky, fast poky and wild type developed a copious precipitate when kept overnight in a refrigerator. These precipitates were packed by centrifugation, washed once with 0.05 M phosphate buffer (pH 7), and resuspended in this buffer so that the preparation from 1 g. of moist mold was suspended in 1 ml. Cytochrome (0.2 mg. in 0.3 ml. of the phosphate buffer) incubated at 35° C. with 0.2 ml. of each of these suspensions for 20 minutes showed that cytochrome destruction (detected by non-reducibility by ascorbate and absence of reduced absorption bands of cytochrome after hydrosulfite reduction and shaking with air) had occurred in the mixtures containing the poky and fast poky preparations, but had not occurred in the mixture containing the wild-type preparation.

In all the experiments on cytochrome destruction reported above with particulate, dispersed and dispersed-precipitated preparations, the reaction was in buffer at pH 7 and required at least 15 to 20 minutes for completion. Boiling for 2 minutes of any of these preparations destroyed the activity.

The particulate, dispersed or dispersed precipitated preparations from 0.2 g. of moist poky or fast poky in 0.5 ml. of 0.1 M TRIS (pH 8.2) containing 0.2 mg. of cytochrome c, caused an immediate change in the cytochrome so that it was no longer reducible by ascorbate and was destroyed by reduction with hydrosulfite and reoxidation by air. This reaction was not affected by prior boiling of the preparations for 1 hour in buffer at pH 7 or 8.2 or in 6 M hydrochloric acid. The material responsible for the immediate change in the cytochrome at pH 8.2 was then shown to be extractable into ether from aqueous solution at pH 8.2. This material is henceforth referred to as "pokonic acid." It was named pokonic acid because it was first isolated from poky, and chemical studies, to be described later, indicate it is a keto acid. The isolation of, and assay procedures developed for pokonic acid are described in the following sections.

Isolation of Pokonic Acid

Pokonic acid has been purified from poky and fast poky mycelium. A similar and possibly identical substance has been purified from wild-type Neurospora. Washed mold was frozen and then extracted with 5 parts, by weight, of boiling water. After removing all excess liquid, the moist residue was extracted 4 times with 2 parts, by weight, of acetone. The acetone was removed by distillation, the remaining brown liquid was acidified and extracted with peroxide-free ether

which was then evaporated to yield a brown oil. Pokonic acid has been isolated from this material by a column fractionation and also by a counter-current distribution.

In the column procedure,[53] Hyflo Supercel, washed with concentrated hydrochloric acid and then with water and dried, was impregnated with 98% methanol, 2 ml. per 3 g. of Supercel. The seemingly dry powder was slurried in ligroin (60° - 70° C.) and packed into a column 2 by 10 cm. A few mm. of dry Supercel were placed on top of the column. The ether extracted brown oil from 20 g. of moist mold was dissolved in 1 ml. of the ligroin, added slowly to the top of the column and the column developed with this solvent. A yellow, blue fluorescent oil travelled at the front and was collected in the first 3 ml. fraction. The second 3 ml. fraction contained the pokonic acid, a colorless oil which crystallized on standing. The third 3 ml. fraction had a small amount of this same material.

Pokonic acid was also isolated from the ether extract by a counter-current distribution between ligroin (60° - 70° C.) and 98% methanol. Absolute methanol (250 ml.), ligroin (250 ml.) and water (5 ml.) were equilibrated. Into each of 12 separatory funnels (60 ml. capacity) were placed 10 ml. of the upper phase. The ether extract from 250 g. of mold was dissolved in 15 ml. of the lower phase and placed in the first funnel. After equilibration, the lower phase was transferred to the second funnel and fresh lower phase was added to the first funnel. This process was repeated through 12 funnels

until 14 fractions of the lower phase had been collected in tubes and then the upper phase fractions were transferred to tubes. A large amount of brown oil was found in the first few fractions of the ligroin phase, after the solvent had been evaporated, and in the last fraction of the methanol phase. The pokonic acid was distributed in fractions 7 to 13 of the methanol phase.

Assay of Pokonic Acid

A completely reproducible assay for pokonic acid has not been developed but a procedure which gives semi-quantitative results has been used. The assay, based on the combination of cytochrome withpokonic acid, reduction by hydrosulfite and reoxidation by air, is performed as follows: The sample, containing approximately 50 to 250 µg. of pokonic acid is added in up to 0.1 ml. of ethanol to 450 µg. of cytochrome c in 3 ml. of 0.1 M TRIS (pH 8.2). Then, 0.2 ml. of a freshly prepared solution of sodium hydrosulfite, 10 mg./ml. in 0.1 M TRIS (pH 8.2) is added and the solution well shaken with air. After 20 minutes at room temperature, the optical density at 414 mµ (the maximum of the Soret absorption peak of reduced cytochrome c) is determined in a Beckman DU spectrophotometer in a cell with a 1.0 cm. light path. The results of such an assay, with a once recrystallized (from ligroin) pokonic acid fraction from a counter current distribution purification, are shown in fig. 6.

Another assay procedure has been tried which may prove to be more satisfactory. This is based on the enhanced destruction of cytochrome c by hydrogen peroxide, in the presence of pokonic acid. Pokonic acid, 25 to 250 µg., was added to 450 µg. of cytochrome c in 1.5 ml. of 0.1 M TRIS (pH 8.2). Then 1.5 ml. of 0.1 M TRIS (pH 8.2), containing 1.5 µM of hydrogen peroxide was added and mixed. After 15 minutes, the optical density was determined at 410 mµ (the maximum of the Soret absorption peak of oxidized cytochrome c) in a Beckman DU spectrophotometer in a cell with a 1.0 cm. light path. An optical density change of about 0.4 occurs under these conditions with 100 µg. of pokonic acid, as compared with a change of 0.065 without pokonic acid.

Pokonic Acid in Particles

The experiments on destruction of cytochrome c by poky particle preparations, at pH 7, indicated that there was a lag before cytochrome destruction commenced (see p. 24). Fast poky particle preparations, at pH 7, did not destroy cytochrome except after a long lag, greater than 5 hours (see p. 27), or after dispersal with desoxycholate and storage overnight in the refrigerator (see p. 28). Wild-type particle, dispersed, or dispersed precipitated preparations did not destroy cytochrome c (see p. 28). The isolation from poky and fast poky of pokonic acid, an ether soluble substance which causes a destruction of cytochrome c that

is similar to the destruction by the particle or particle derived preparations, suggested that pokonic acid might be responsible, in part, for the destruction of cytochrome by the particles. Poky, fast poky and wild-type particles were prepared (as described on p. 16), brought to pH 1 or 2 with hydrochloric acid and extracted with 3 parts, by volume, of ether. The acidification was necessary to prevent serious emulsion formation. Another portion of particles was incubated for 3 hours in P-M at 35° C., acidified and extracted with ether in the same way. A third portion of particles was dispersed with desoxycholate (as described on p. 17), acidified, and extracted with ether in the same manner as above. Each of the dried ether extracts was taken up in ethanol and the ethanol solutions were assayed for pokonic acid by the hydrosulfite procedure (see p. 31). The results of this experiment are given in table 4.

Chemical Properties of Pokonic Acid

Pokonic acid is soluble in ether, acetone, ethanol, ligroin, toluene and dilute sodium hydroxide. It is very sparingly soluble in water. Titration of a recrystallized sample of the acid by Professor Mitchell showed that it has a pK between 4 and 6 and a neutralization equivalent of 290 to 300. This pokonic acid preparation reacted rapidly with 2,4-dinitrophenylhydrazine in 2 M hydrochloric acid to give a yellow precipitate which turned brownish red when the mixture was made

Table 4. Pokonic Acid Liberation

Preparation	po 3627-2a (age: 3½ days)	pof 3627-4A (age: 4 days)	W 2293-2a (age: 4 days)
Fresh particles	550	125	40
Aged particles (incubated 3 hours at 35° C. at pH 7)	1750	900	210
Fresh particles dispersed with sodium desoxycholate	1250	1200	475

Numerical values in the table are µg./g. of ether extractable pokonic acid, as assayed by the hydrosulfite method.

alkaline. A control preparation (not containing pokonic acid) did not show these changes. Less than 1% of a solution of pokonic acid in ethanol formed a bisulfite addition complex with saturated aqueous sodium bisulfite. The complex was formed by shaking 0.5 ml. of pokonic acid in ethanol with 0.5 ml. of saturated aqueous sodium bisulfite for 10 hours in a closed tube at 35° C. The precipitate was washed with 1 ml. of saturated aqueous sodium bisulfite, 1 ml. of ethanol and 1 ml. of ether. The precipitate was then dissolved in 1 ml. of M hydrochloric acid and the solution extracted with ether. The ether extract was assayed for pokonic acid.

Chromatography of pokonic acid on paper with propanol-ammonia (4 parts of n-propanol to 1 part of M ammonium hydroxide) showed that it has an Rf, in this solvent, of 0.8 (detected by activity of the ethanol eluate, in cytochrome destruction, by the hydrosulfite method). Material with this same Rf reacts with periodate to give a white spot,[51] with 15% phosphoric acid to give a dark brown to black spot[54] and with phosphomolybdic acid to give a blue spot.

The purest preparations of pokonic acid which have been obtained give a sharp melting point at 58° to 59° C. without decomposition.

It has been shown, as described in previous sections, that pokonic acid combines with cytochrome, in a reversible manner, so that the heme of the cytochrome is destroyed on

reduction with hydrosulfite and reoxidation with air. The heme destruction also occurs, after combination of the pokonic acid and cytochrome, with a concentration of hydrogen peroxide low enough to cause only a negligible heme destruction in the absence of pokonic acid (see p. 32). Further evidence that pokonic acid combines with cytochrome c and changes its properties is given by the fact that after addition of pokonic acid to cytochrome, ascorbate no longer reduces the cytochrome.

Cytochrome c is very strongly adsorbed on the cation exchange resin, IRC-50, in the hydrogen or ammonium form, and is not eluted by 0.1 M TRIS at pH 8.2. A mixture of 10 mg. of cytochrome c in 2 ml. of 0.1 M TRIS (pH 8.2) plus 3 mg. of pokonic acid in 0.1 ml. of ethanol was allowed to stand for 5 minutes at room temperature. It was then placed on the top of a 1 by 10 cm. column of IRC-50, in the ammonium form, (equilibrated with 4 M ammonium formate at pH 8.2 and washed free of the salt with water). The red material was washed right through this column with water. This material was then placed on the top of a 1 by 10 cm. column of Dowex 1, a strong anion exchange resin, in the chloride form. Approximately $\frac{1}{2}$ of the red material was adsorbed on the top $\frac{1}{2}$ cm. of the column and the rest was washed right through with water. This latter material was then adsorbed strongly onto the first column (IRC-50). The red material which was adsorbed on the Dowex 1 column could not be eluted with 4 M ammonium chloride

at pH 7 or 9, 0.1 or 4 M sodium hydroxide or pyridine. It was eluted with 2 M sodium salicylate at pH 7. Thus, pokonic acid combines with cytochrome c and changes its properties on ion exchange resins.

A hot water extract of 0.2 g. of moist poky, concentrated to 0.1 ml., completely inhibited the destruction of 450 µg. of cytochrome c by 250 µg. of pokonic acid, as measured by the hydrosulfite method (see p. 31). By using the same method, it was found that 700 µg. of cholesterol inhibited by 60 to 80% the destruction of 450 µg. of cytochrome c by 250 µg. of pokonic acid.

Pokonic Acid Inhibition of Lysozyme

Lysozyme activity was determined by measuring the decrease in turbidity of a suspension of cells of Micrococcus lysodeikticus in 0.1 M acetate buffer at pH 5.6. For each assay, 150 µg. of lysozyme was added to a series of tubes containing 0.2 to 2.5 mg. of pokonic acid in 0.5 ml. of 0.1 M TRIS (pH 8.2). These mixtures, after standing 5 minutes at room temperature, were added to 0.8 mg. of dried M. lysodekticus cells in 4.5 ml. of 0.1 M acetate buffer (pH 5.6) in Klett colorimeter tubes. The turbidity of the suspension in each tube was measured every 5 minutes for 20 minutes with a Klett colorimeter equipped with a blue filter (5433). The lysozyme activity was inhibited less than 5% by 0.2 mg. of pokonic acid, 30% by 0.5 mg. of pokonic acid, 68% by 0.8 mg. of pokonic

acid, and 77-79% by 1.5 - 2.5 mg. of pokonic acid in the above experiment. No inhibition was observed if the pokonic acid and lysozyme were not mixed at pH 8.2 before being brought to pH 5.6. The inhibition is reversed, as with cytochrome destruction, by removal of the pokonic acid by extraction with ether. The ether extract contained active pokonic acid (as measured in a cytochrome destruction assay) and active lysozyme was left in the aqueous solution.

Soluble Inhibitor

An inhibitor of the destruction of cytochrome c by the preparations from poky and fast poky is present in a hot water extract of all the strains of mold used in this work and of rat liver and kidney. No other tissues were tried. Only the inhibitor from Neurospora has been studied.

A hot water extract of 0.05 g. of mold (wild type, poky or fast poky) prevents the destruction of 0.2 mg. of cytochrome c in 0.5 ml. of 0.05 M phosphate buffer (pH 7) by a particulate, dispersed or dispersed precipitated preparation of poky. The test for cytochrome destruction was carried out in the same manner as on p. 25 for the particulate preparation and as on p. 27 , for the dispersed and dispersed precipitated preparations.

The inhibitor is stable to boiling for at least 1 hour at pH 5, 7 and 9, is dialyzable and is not stable to ashing in a porcelain crucible. The following procedure can be used

as the basis for further work on the purification and isolation of the inhibitor.

Frozen wild-type (WP 1400-4A) mycelium (250 g.) was homogenized in a blendor with 300 ml. of boiling water and then filtered. The precipitate was washed with an additional 200 ml. of boiling water and the filtrate and washings combined. These were evaporated to 100 ml. with a stream of filtered air directed at the solution maintained at 70° C. This concentrate was kept at 5° C. for 24 hours and then centrifuged to remove a precipitate which had formed. To the supernatant, 2 parts, by volume, of acetone were added. The resultant dark brown precipitate which formed was packed by centrifugation after standing 1 hour at room temperature. This precipitate was dissolved in 25 ml. of water and treated with three 200 mg. portions of Norite until colorless. This colorless material was stored in the deep freeze for over 2 years with little or no loss in activity.

Heme-Peptide Formation and Purification

Heme-peptides were prepared and purified in several different ways during the course of this work. No entirely satisfactory method for preparation and purification was found but the following can be used as the basis for further work.

Throughout these studies, red color and absorption in the region of 405 to 415 mμ was used as an index of the loca-

tion of heme-containing compounds.

Horse heart cytochrome c (145 mg.), containing 0.32% iron, and the desoxycholate preparation from 50 g. of fast poky were incubated in 20 ml. of 0.1 M TRIS (pH 8.2) for 2 hours at 35° C. Then the desoxycholate preparation from another 25 g. of fast poky in 10 ml. of TRIS was added and the mixture incubated 1½ hours longer. Finally, the desoxycholate preparation from another 25 g. of fast poky in 5 ml. of water was added, the pH adjusted to 8.2 with sodium hydroxide and the incubation continued for 1 hour. The extent of cytochrome destruction, during the incubation, was determined, on small aliquots, by the hydrosulfite method (see p. 27). At the end of the incubation, all of the cytochrome had been destroyed (measured by this method). That this is not an adequate criterion for measuring the extent of proteolysis of cytochrome c was not known at the time of this experiment and undoubtedly contributed to the low yield of heme-peptides which was obtained. The digestion mixture was frozen and stored overnight. It was then fractionated as shown in fig. 7.

Fraction A_1 was eluted with 2 M sodium salicylate (pH 7) after attempts at elution with 0.1 and 4 M sodium hydroxide, 0.1 M hydrochloric acid, 0.1 and 4 M ammonium chloride, and pyridine had failed.

Two of the heme-peptide fractions were studied (A_2, B_3). The material in the others was not investigated further for

one of the following reasons. It was not recovered from the column eluate (A_1), it precipitated and would not dissolve in water after storage in the freezer (A_3), or it was present in amounts of 1 mg. or less (B_1, B_2, B_4).

The red material in fraction A_2 was found to move as a cation by paper electrophoresis in 0.03 M TRIS at pH 8.2. No red material with a cationic mobility under these conditions was found in fraction A_s, from which A_2 was derived. Therefore, the material with cationic mobility was not present before being passed through the Dowex 1 chloride column but the effluent of that column contained what appeared to be a single cationic material. Apparently, the passage through the Dowex 1 column changed the charge characteristics of the heme-containing substances.

Fraction A_2 was subjected to quantitative iron and histidine analyses and qualitative amino acid analysis (procedures given in the methods section). The maximum molecular weight, based on the iron content (1.3%), was 4250. The figure is the maximum molecular weight because any non-iron containing impurities would tend to make the apparent molecular weight higher. The histidine analyses gave 1.9 moles of histidine per atom of iron in fraction A_2. The other amino acids found were cystine, aspartic acid, glutamic acid, lysine, glycine, alanine, threonine, valine, leucine, isoleucine, proline, and a trace of phenylalanine.

Fraction B_3 was subjected to paper electrophoresis at

pH 8.2 in 0.03 M TRIS. It moved as a weaker anion than glycine and probably was carried along by electroendosmosis. After the heme group was removed and the remaining peptide oxidized (cysteine residues to cysteic acid residues) as described on p. 18 , only 1 ninhydrin reacting spot was found by paper electrophoresis in 0.03 M TRIS (pH 8.2). This represented a compound with a greater cationic mobility than lysine. Paper chromatography of the oxidized peptide in propanol-acetic (2 parts of n-propanol to 1 part of M acetic acid) revealed only 1 ninhydrin reacting material (Rf 0.9).

After storage of the oxidized peptide in the deep freeze for 3 days, the paper electrophoresis and chromatography was repeated under the same conditions. This time, only a trace of the cationic and high-Rf material could be found but there were several ninhydrin reacting spots representing anionic materials on the paper electrophoresis strip and several ninhydrin reacting spots on the chromatogram with Rf values ranging from less than 0.1 to 0.6. The remaining portion of the oxidized-peptide fraction was then hydrolyzed in 6 M hydrochloric acid in a sealed tube at 105° C. for 24 hours. After removal of the acid, the hydrolysate was chromatographed in propanol-ammonia and propanol-acetic (3 parts of n-propanol to 1 part of M ammonium hydroxide or acetic acid). A number of amino acids could be detected on these chromatograms but, in addition, there was a large amount of ninhydrin reacting material which moved as a streak with an Rf of 0 to 0.2 in

each solvent. A portion of the hydrolysate was rehydrolyzed in 6 M hydrochloric acid in a sealed tube at 105° C. for an additional 24 hours. After removal of the acid and chromatography as above, the streaked ninhydrin reacting material was found in undiminished amount. It was shown that the chromatograms were not overloaded by spotting just enough material to give a barely perceptible color with ninhydrin. The streaked material was still present. No diazo reacting spot, to indicate the presence of histidine (see p. 18) was found on any chromatograms of these hydrolyzates. The sensitivity of the diazo spray reagent (0.1 μg. of histidine) indicated that less than 0.5% of the hydrolyzate was histidine.

DISCUSSION

The Mechanism of Cytochrome Destruction

The following is offered as an interpretation of the mechanism of cytochrome destruction by poky particle and particle derived preparations.

The destruction of cytochrome c by poky particles involves at least 3 reactions. Proteolytic activity results in extensive breakdown of the particles and in the hydrolysis of added cytochrome c. Pokonic acid changes the properties of the heme group. A succinate-requiring system produces a verdohemochrome.

Studies of the proteolytic reaction have been presented which show that poky particles hydrolyze their own, and added, proteins to yield peptides and amino acids. When cytochrome is used as substrate, heme-peptides are produced.

The green compound formed from cytochrome by the particulate system and succinate is thought to be a verdohemochrome (see fig. 8) because of its similarity to verdohemochrome as described by Kench[55] and Theorell and Swedin.[56] Kench added ascorbic acid continuously over a period of 2 hours to a solution of hemin (buffered at pH 7 to 8) which was being aerated with a stream of oxygen during this time. By this procedure (a coupled oxidation), he obtained material whose pyridine-

chromogen had a maximum absorption at 650 mμ. This material was identified as verdohematin. A similar verdohemochrome was formed with globin.[55] The formation of a verdohemochrome from cytochrome c by a manganese-catalyzed coupled oxidation of dihydroxymaleic acid has been reported by Theorell and Swedin.[56] The requirement for both succinate and oxygen by the poky particle system for the production of the green compound (maximum absorption at 650 mμ) makes it seem likely that here, too, a coupled oxidation occurs.

Haskins et al. reported finding a compound with a spectrum similar to biliverdin (see fig. 8) in an incubation mixture of poky particles, succinate and cytochrome c.[2] Biliverdin is produced by a loss of iron from verdohemochrome as shown in fig. 8.[57]

The changed properties of the heme group after reaction with pokonic acid may be due to the pokonic acid (1) replacing one or both of the groups normally coordinating with the iron or (2) preventing one or both of these groups from coordinating with the iron by combining with the group(s) and allowing another group in the protein or peptide to coordinate with the iron. These explanations lead to a possible explanation of why ether extraction of the product of the reaction of purified pokonic acid with cytochrome results in the recovery of cytochrome while extraction of the particle produced heme-peptides does not result in heme-peptides which are stable to hydrosulfite and can be reduced by ascorbate. In the former

case, when the pokonic acid is removed, the groups normally coordinated with the iron resume their normal function. This is likely because cytochrome c is a relatively rigid structure. In the latter case, due to the non-rigidity of the heme-peptide, the groups normally coordinated with the iron do not resume their normal function after the pokonic acid is removed.

The Role of Pokonic Acid in the Destruction of Cytochrome by poky Preparations

Several questions, regarding the relationship of pokonic acid to the destruction of cytochrome c by poky particles, are raised.

Is pokonic acid involved?

There is a positive correlation between the amount of ether extractable pokonic acid in the particle preparation and the speed with which the particle preparation (plus succinate) will produce verdohemochrome.

It seems unlikely that merely removing a piece of the protein of the cytochrome from the heme portion of the molecule is sufficient to labilize the heme group because heme-peptides produced by digestion of cytochrome c with trypsin, pepsin and papain are not subject to the interesting heme destruction exhibited by poky-produced heme-peptides. It is interesting, too, that poky-produced heme-peptides are not reducible by ascorbate while trypsin, pepsin and papain-

modified cytochromes are. These differences appear to be due to pokonic acid.

Are the products of the action of poky particles on cytochrome different from the products of the reaction of purified pokonic acid with cytochrome?

Purified pokonic acid has been shown to react with intact cytochrome c changing the properties of the heme in the same way that poky particles do. However, the reaction with purified pokonic acid can be reversed (by extraction with ether) allowing the recovery of cytochrome which is stable to hydrosulfite and which can be reduced by ascorbate. Ether extraction of the products of the action of poky preparations on cytochrome neither makes those products stable to hydrosulfite nor reducible by ascorbate. Thus, there is a difference between the products in question.

To what can the difference be attributed?

In the case of the particle preparation, it seems necessary to postulate a different mode of combination than that which occurs in the reaction of purified pokonic acid with cytochrome c. The postulation is reasonable because, while the particulate reaction (1) occurs slowly, (2) is stopped by boiling and (3) takes place at pH 7 (not at pH 8.2), the reaction with purified pokonic acid goes very rapidly at pH 8.2 (only slightly at pH 7) and is not affected by boiling. This suggests that in a particulate or dispersed system, the

reaction of pokonic acid with cytochrome c, or derivatives thereof, is enzymatically mediated. The product(s) of this enzymatic reaction could have the pokonic acid bound in such a way as to make it non-extractable by ether.

Another possible explanation for the observed difference is that when pokonic acid reacts with cytochrome or a heme-peptide, it displaces the normal iron-coordinating groups. In intact cytochrome, the rigid structure of the molecule makes it probable that, on removal of pokonic acid (by ether extraction), the original iron-coordinating groups resume their usual function. In a heme-peptide, where the usual iron-coordinating groups may be held less rigidly, or may be absent, removal of the pokonic acid would not be likely to restore the normal iron coordination.

With the available evidence, it is not possible to choose between these explanations.

The Reaction of Purified Pokonic Acid with Cytochrome c

Pokonic acid is an acidic compound and cytochrome is a very basic protein (isoelectric point about pH 10.5). It is conceivable that pokonic acid combines with some basic group or groups of the cytochrome molecule making the cytochrome less basic. This would explain why pokonic acid-reacted cytochrome passes through a column of IRC-50 while cytochrome is adsorbed very strongly under the same conditions. The fact that pokonic acid reacts with lysozyme (a

basic protein), lends weight to this supposition.

If the histidine which is generally believed to be coordinated with the iron of cytochrome reacts with pokonic acid, the iron would become autoxidizable. Then, if the iron is reduced, reoxidation by oxygen would produce a peroxide which would then destroy the labilized heme group. The greatly enhanced destruction of cytochrome by small amounts of hydrogen peroxide in the presence of pokonic acid supports this hypothesis.

The Role of Inhibitors

The presence in poky of about 10 times as much cytochrome c as in wild type, in spite of the presence of a cytochrome destroying system in poky particles, is made possible by a soluble inhibitor found in the mold. This inhibitor probably prevents the proteolysis of the particles from occurring, thus, preventing the liberation of pokonic acid and the hydrolysis of the cytochrome (and the rest of the proteins of the mold). A single experiment did, in fact, indicate that a function of the inhibitor is to inhibit the proteolysis.

In fast poky, although the particles do not have proteolysis activity, desoxycholate dispersion does give a preparation which produces heme-peptides from cytochrome while, in wild type no treatment of the particles has resulted in the appearance of proteolytic activity. These facts may be explained by assuming that an inhibitor which is soluble in

poky is released from fast poky particles by desoxycholate while the inhibitor, in wild type, may be so bound to the particles that desoxycholate will not release it and allow the particles to exhibit proteolysis.

There is also a soluble inhibitor of the purified pokonic acid reaction with cytochrome c. This may be the same inhibitor as above. Its role, if any, is not known.

The Inheritance of poky Characteristics

Along with many of the other characteristics of poky, the cytochrome destroying system is cytoplasmically inherited. It is possible that the particles are the carriers of the genetic determinant(s) for, at least, this system. The genetic determinant may be merely a deficiency in the production of some complex which binds the inhibitor of proteolysis. The term, genetic determinant, does not imply that there is a specific factor, analogous to a chromosomal gene, but the genetic determinant may be a particular metabolic state which does not allow for the production of the inhibitor binder.

Terminal Oxidase

The terminal oxidase of poky is not known. It is possible that a small amount of pokonic acid is released, perhaps bound specifically to a protein, which reacts with cytochrome c so as to make a complex whose oxidation-reduction properties are such that this complex can act as a terminal oxidase.

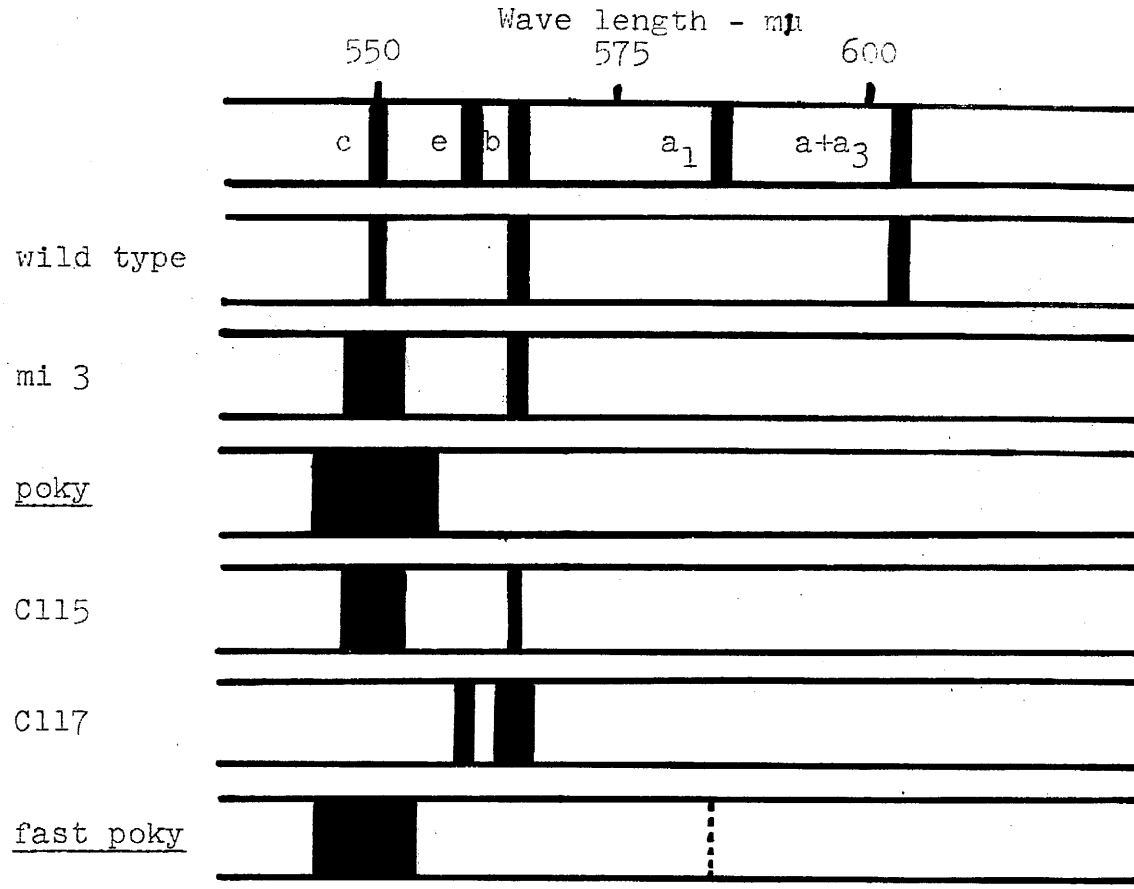

Figure 1. Diagrammatic Representation of Cytochrome Absorption Bands. Relative concentrations of cytochromes are indicated by the width of the bands, e.g., the ratio of cytochrome c concentration in wild type, mi 3 and poky is approximately 1:5:15.

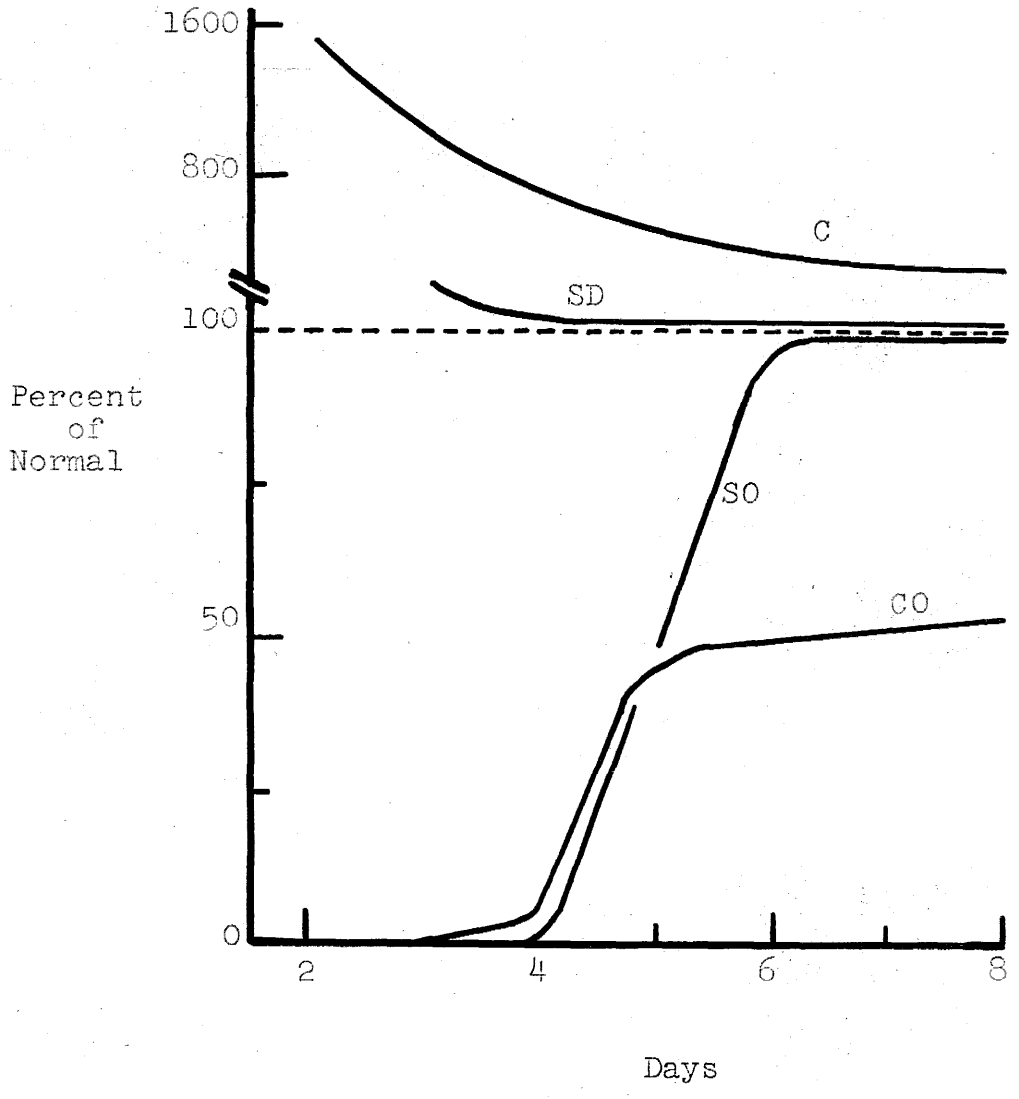

Figure 2. Comparison of Enzymes Related to Cytochromes in poky and Wild Type.[2] Diagram showing cytochrome c content (C), succinic dehydrogenase activity (SD), succinic acid oxidase activity (SO), and cytochrome oxidase activity (CO) of poky as compared to wild type. The latter is represented by the horizontal dotted line at the level of 100 percent.

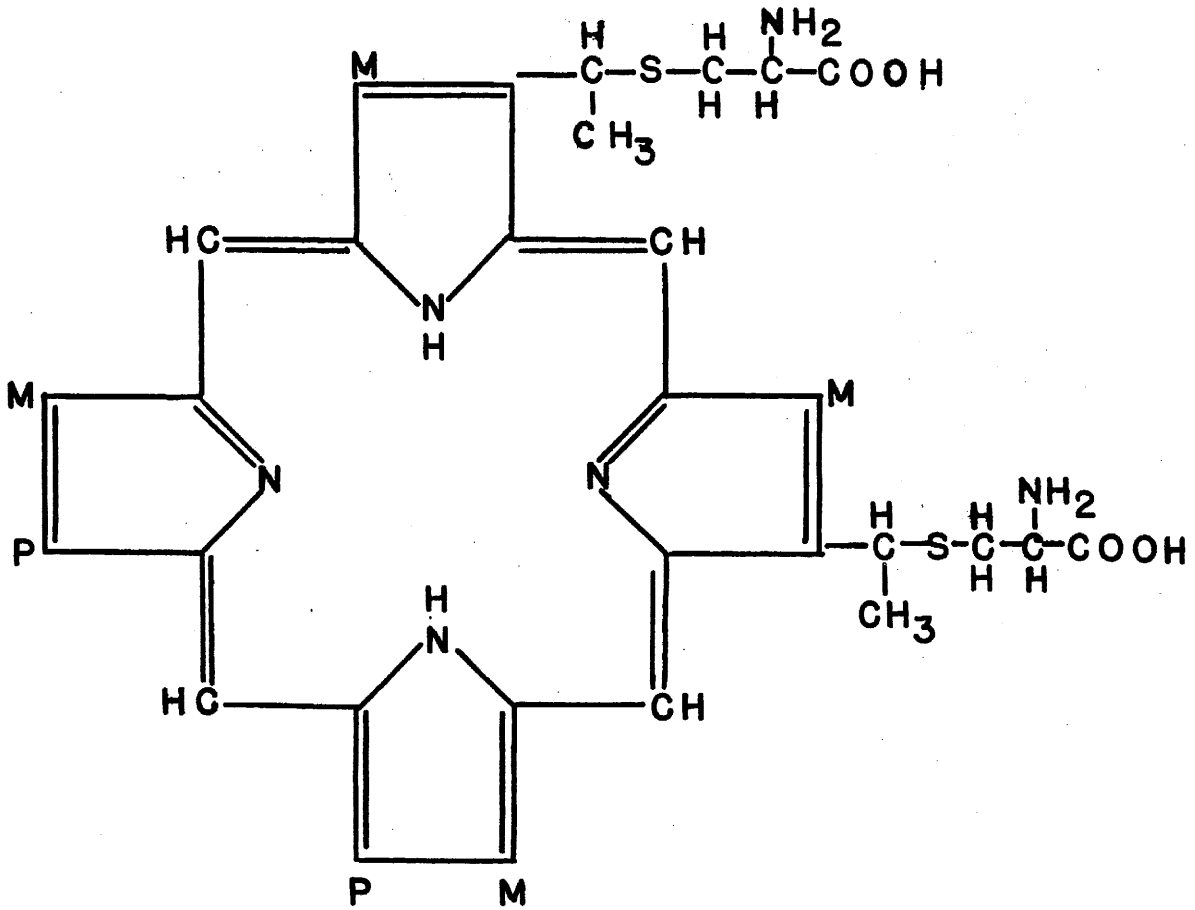

Figure 3. Porphyrin c.[12]

M = Methyl

P = Propionic Acid

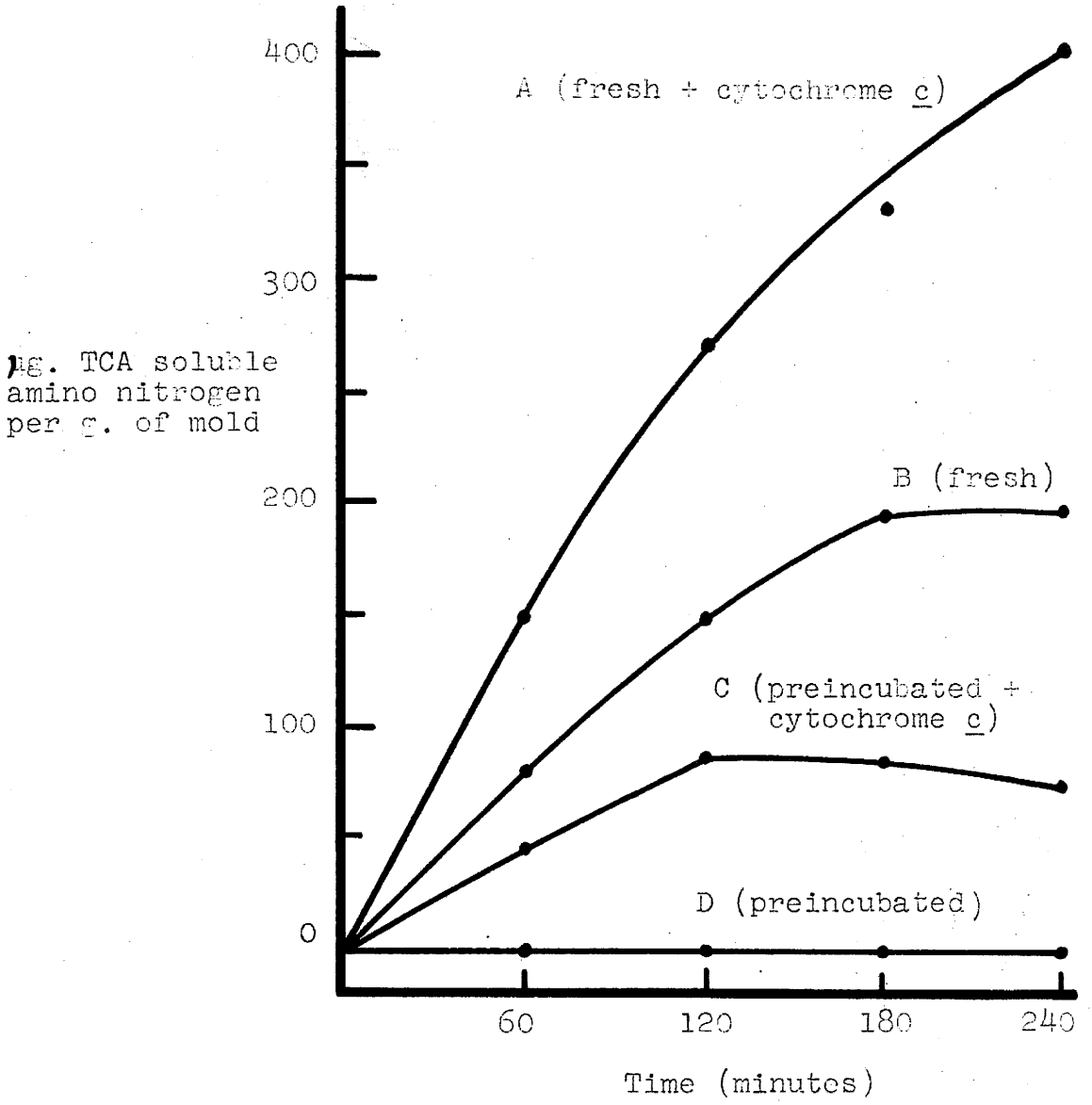

Figure 4. Time Course of Proteolysis. A and B contained fresh particles from po 1803-4A (3 day old) and C and D contained particles from the same lot, preincubated in P-M at 35° C. for 150 minutes, packed by centrifugation and resuspended in P-M. In addition, B and D contained 2.5 mg. cytochrome c. All tubes contained particles from 0.25 g. of mold and all tubes were made up to 2 ml. with P-M.

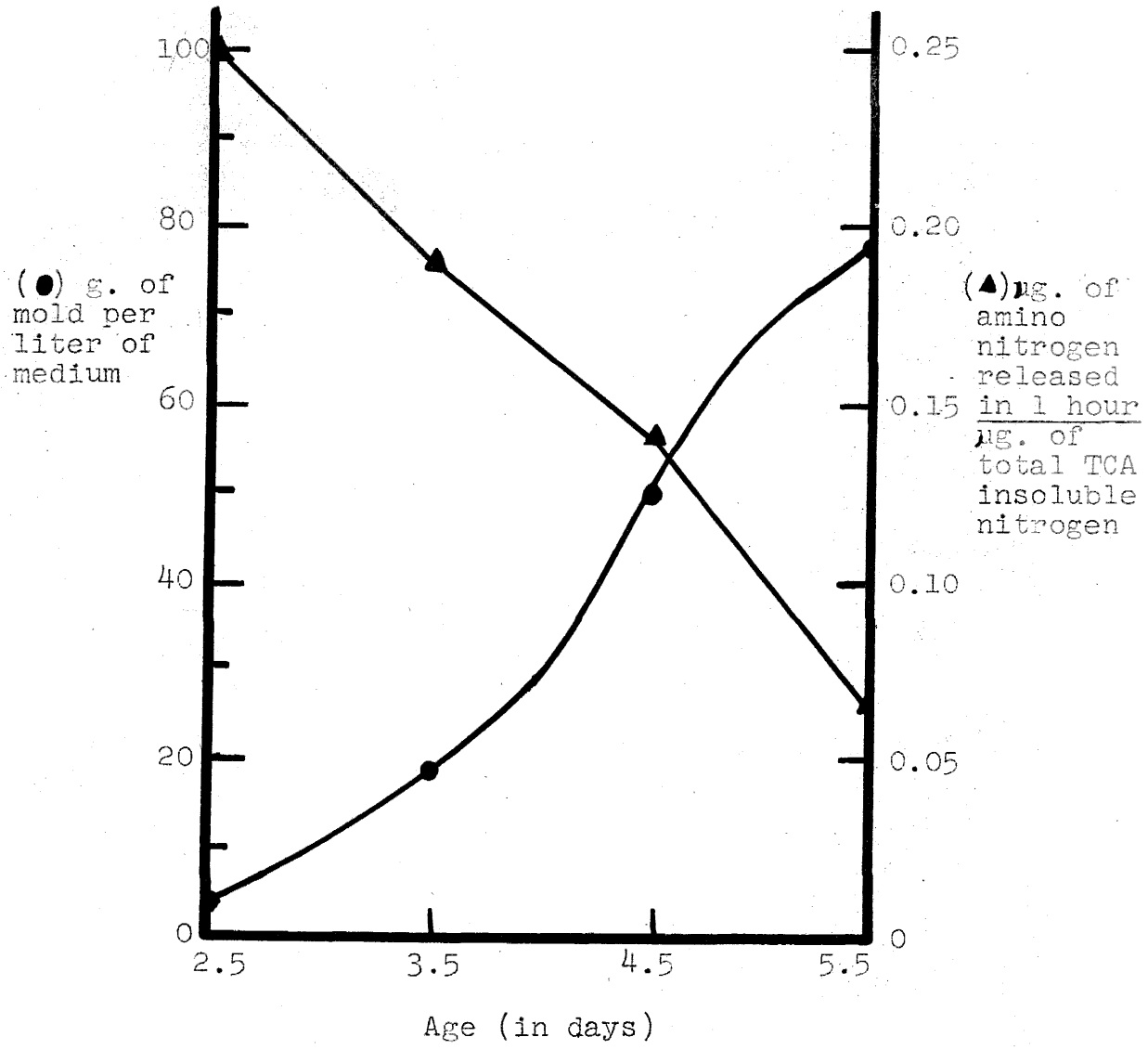

Figure 5. Relation of Growth to Particle Amino Nitrogen Release. Particles incubated in P-M at 35° C.

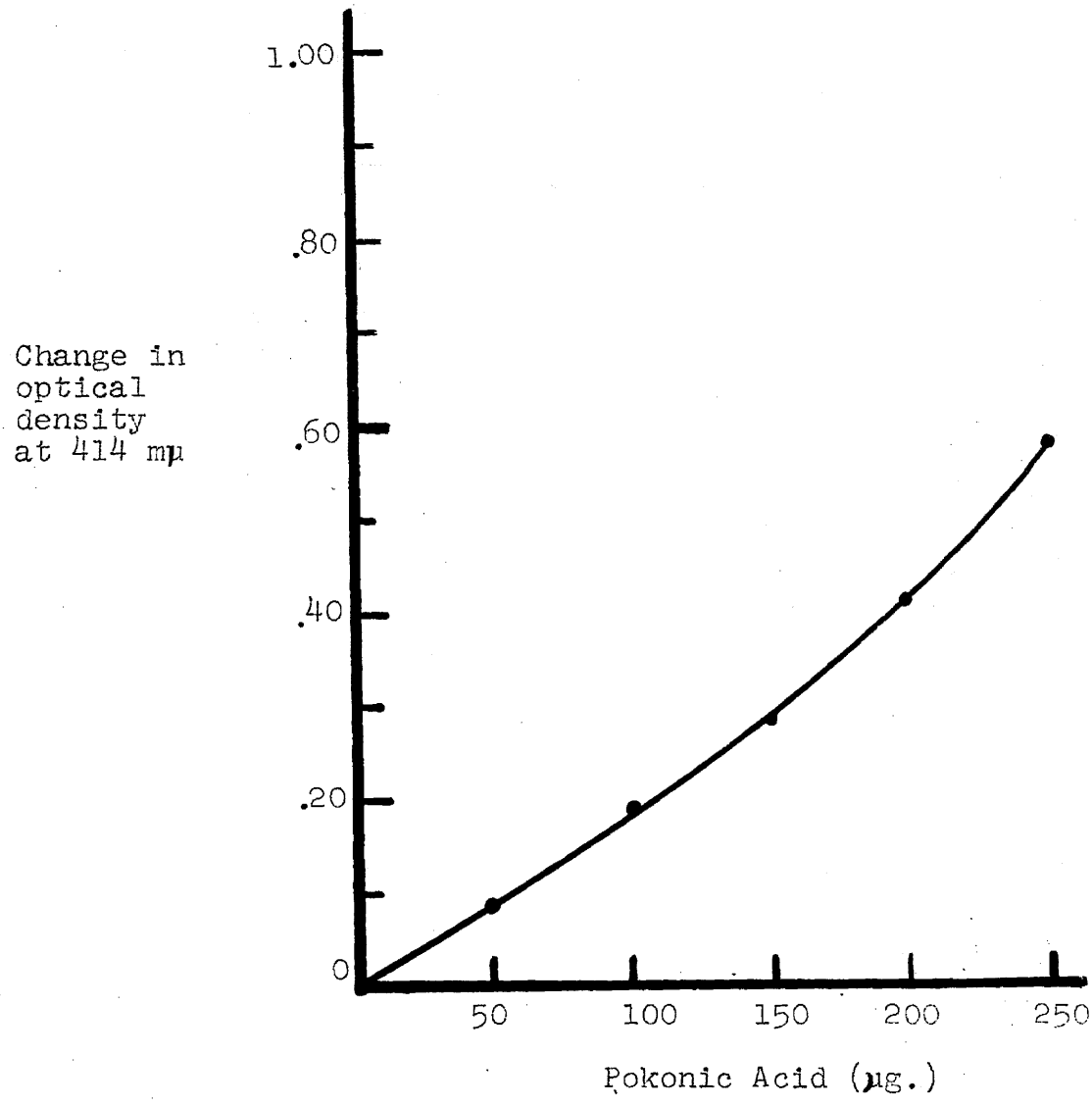

Figure 6. Pokonic Acid Assay - Hydrosulfite Method. Pokonic acid, in less than 0.1 ml. of ethanol, was added to 450 μg. of cytochrome c in 3 ml. of 0.1 M TRIS (pH 8.2). Sodium hydrosulfite, 0.2 ml. of a freshly prepared solution containing 10 mg. per ml. in 0.1 M TRIS (pH 8.2) was then added and the mixture shaken well. After 20 minutes the optical density was determined at 414 mμ in a 1 cm. cell in a Beckman DU spectrophotometer.

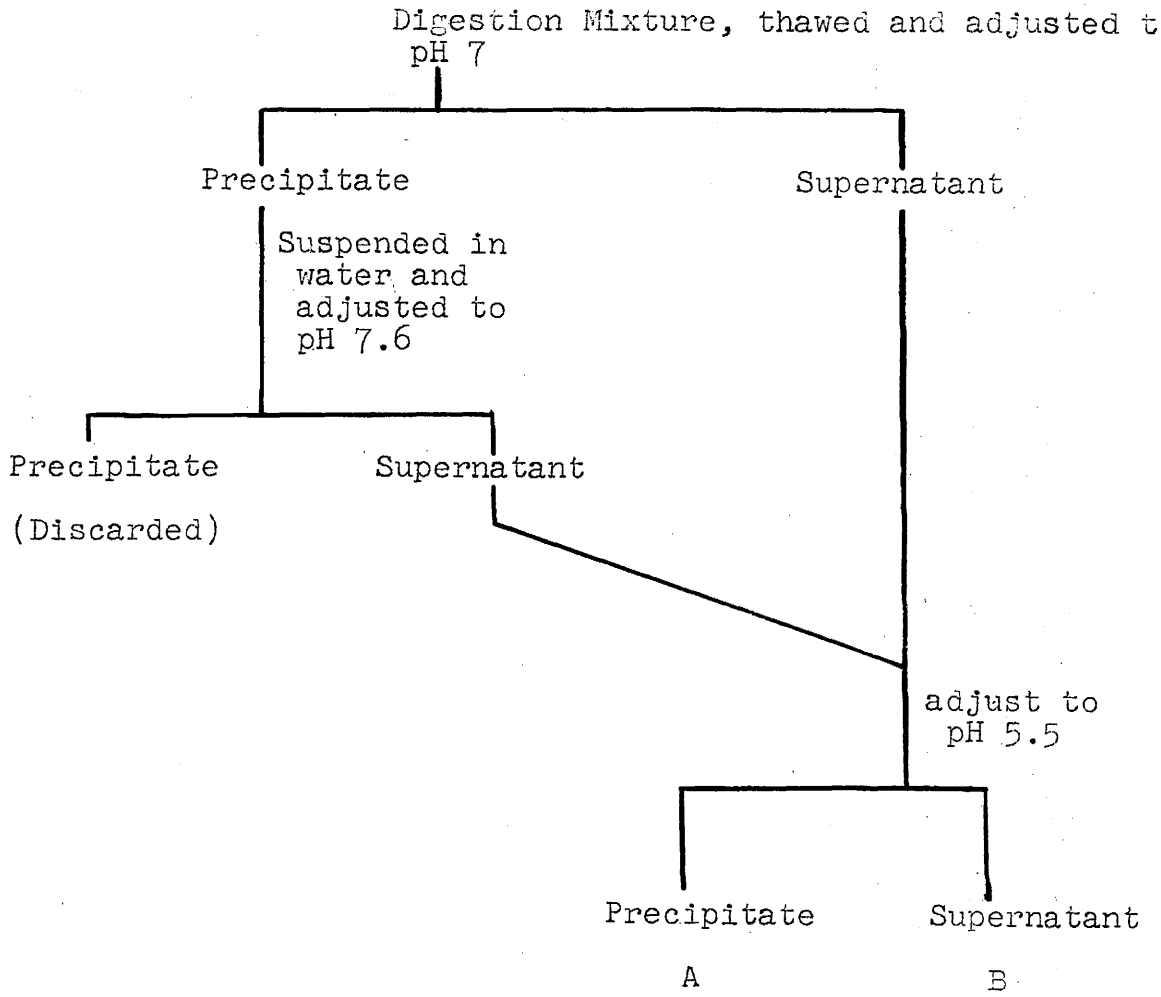

Fig. 7. Heme-Peptide Purification (continued).

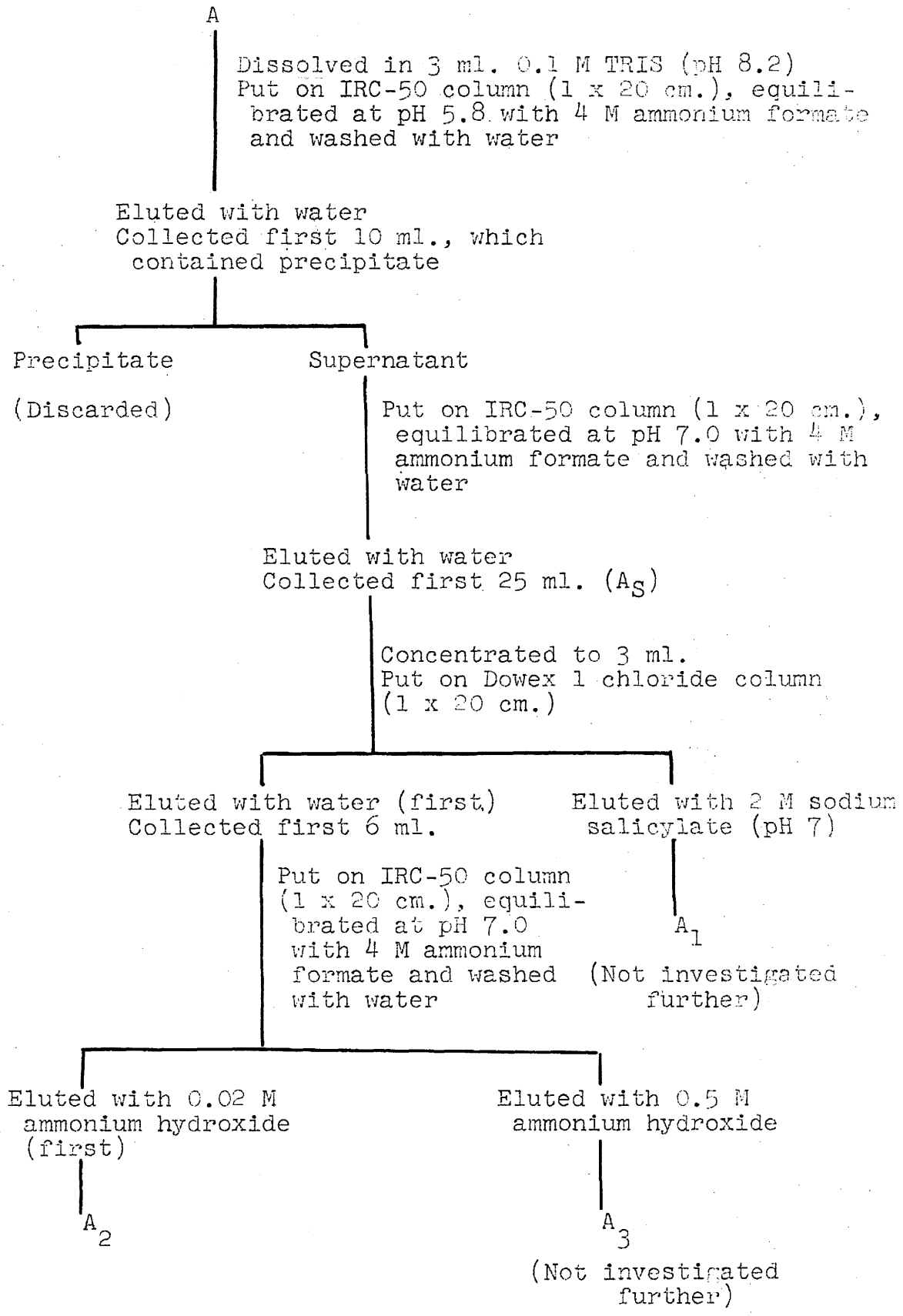

Fig. 7. Heme-Peptide Purification (continued).

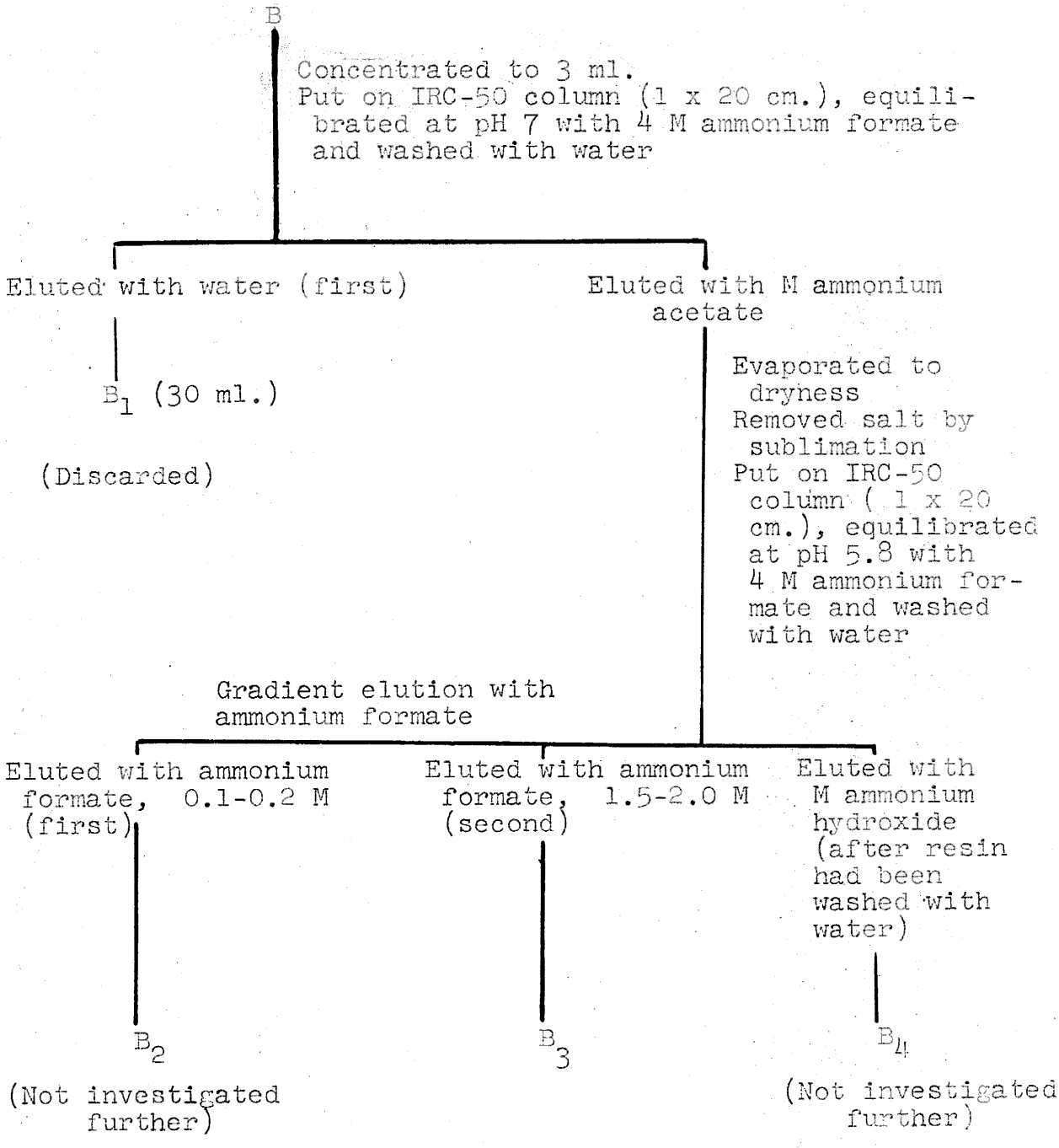

Fig. 7. Heme-Peptide Purification (concluded).

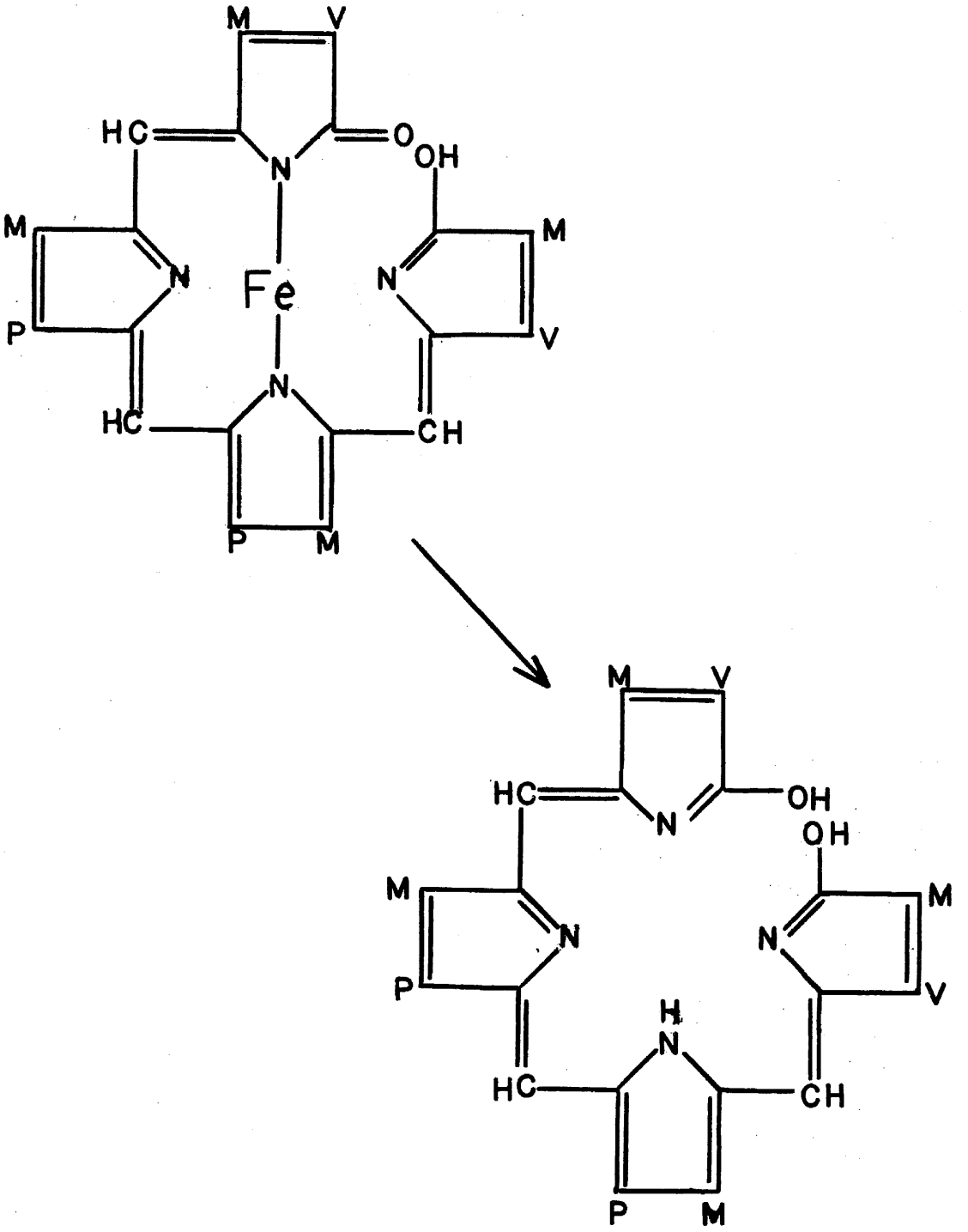

Figure 8. Formation of Biliverdin from Verdohemochrome.[57]

M = Methyl

P = Propionic Acid

V = Vinyl

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