injury to the myocardium; whether these cardiac effects are due to a direct action of the venom upon the myocardium or coronary vessels or are secondary to the hypotension remains to be determined (*8*).

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Purification of Folic Acid

Abstract. Various purification procedures for folic acid were investigated. The criteria of purity were a negative Bratton-Marshall test and the absence of fluorescent spots on paper chromatograms. Since no method provided a pure product, a procedure consisting of cellulose column chromatography followed by filtration through charcoal was developed.

Commercial folic acid contains a number of impurities, principally photochemical decomposition products. At least one of these substances, 2-amino-4-hydroxypteridine-6-aldehyde, has an intense effect on certain enzymes. It strongly inhibits the enzyme (or enzymes) of milk that oxidizes xanthine (*1–3*), xanthopterin (*1–3*), and 2-amino-4-hydroxypteridine, (*3*), as well as liver xanthopterin (*1*) and quinine oxidase (*1*). This report (*4*) describes the purification of folic acid by chromatography on cellulose powder followed by filtration through charcoal.

Various other purification procedures have been investigated—washing with dilute HCl solution (*5*), charcoal treatment and recrystallization (*6*), and crystallization of calcium foliate followed by charcoal treatment (*7*). The purity of the products was studied by paper chromatography and by determination of *p*-aminobenzoylglutamic acid by the Bratton-Marshall procedure (*8*). Since folic acid undergoes photochemical decomposition (*7*), all operations were carried out under dim illumination.

Descending paper chromatograms, 30 cm long, or more, were prepared; 2-cm² spots of neutral sodium foliate solution on Whatman No. 1 paper and two solvent systems—0.2*M* sodium phosphate buffer (*p*H 7.0) saturated with isoamyl alcohol (*9*) and *n*-butanol:acetic-acid:water (4:1:5) (*5*)—were used. When the phosphate buffer was used, 0.1-mg samples of folic acid were chromatographed. Three ultraviolet-fluorescing spots (R_f 0.48, 0.33, and 0.13, respectively) and a single ultraviolet-absorbing spot [R_f 0.40 (sodium foliate)] were present on chromatograms of the commercial product.

When the *n*-butanol:acetic-acid:water system was employed, denser (0.25 mg) spots were used, since sodium foliate does not move in this solvent. Three fluorescent spots (R_f 0.38, 0.26, and 0.11, respectively), and a single ultraviolet-absorbing spot (R_f 0) were visually detectable on chromatograms of commercial folic acid. The first two spots were decomposition products of the pteridine portion of the folic acid molecule (*5*).

The absence of fluorescent spots on the chromatograms and a negative test for diazotizable amine (*p*-aminobenzoic acid and *p*-aminobenzoylglutamic acid) were employed as the criteria of folic acid purity.

Since none of the purification procedures that were studied yielded a pure product, the problem was investigated, and the method described below was developed. The procedure involves a combination of cellulose chromatography and filtration through charcoal.

The cellulose column is prepared in the following manner. Two hundred grams of Whatman standard-grade cellulose powder are mixed with 0.1*M* phosphate buffer (*p*H 7) saturated with isoamyl alcohol. After it has stood for ½ hour, the mixture is poured into a tube (7.5 by 55 cm) plugged with cotton. The cellulose is packed to a height of 40 cm by suction, covered with a circle of heavy filter paper (Eaton-Dikeman No. 627-030), and compressed with a plunger to a final height of 37 cm. The column is then washed with 500 ml of buffer and placed in a dark room. The remaining operations should be carried out under dim illumination.

Commercial folic acid (550 mg) is suspended in 30 ml of water, and sufficient 1*N* NaOH is added to dissolve the folic acid. The solution [*p*H 7 (universal indicator paper)] is introduced into the column, and sodium foliate is moved down from the top with three 2-ml portions of the isoamyl-alcohol–saturated buffer. The column is then eluted with buffer at the rate of about 75 ml/hr. The yellow foliate band, which is clearly visible, is collected between approximately 550 and 730 ml of effluent. Since *p*-aminobenzoylglutamic acid is eluted just before folic acid, collection of the folic acid fraction should not be started until the effluent is distinctly yellow. If the folic acid band is so uneven that the folic acid fraction exceeds 200 ml, it probably will be contaminated with *p*-aminobenzoylglutamic acid.

Cellulose chromatography reduces the content of *p*-aminobenzoylglutamic acid (from 1 to 2 percent in commercial folic acid) to less than 0.02 percent. It also removes most, but not all, of the fluorescent material present in the folic acid. To remove the remaining trace, the folic acid is filtered through charcoal, as described below.

The eluate from the cellulose column is placed in a 250-ml polyethylene bottle, acidified to *p*H 2 (universal indicator paper) with 3*N* HCl, and centrifuged at 0°C in an International PR-1 refrigerated centrifuge. The sediment is then suspended in water and dissolved with sodium hydroxide, as previously described. It is important that the solution be completely clear, otherwise filtration will be extremely slow.

A charcoal column is prepared, an 8- by 100-mm chromatography tube being fused at the top to a 25- by 200-mm section of tubing. The tube is plugged with cotton and filled to a depth of 1 cm with cellulose powder, which serves to trap charcoal particles. A mixture of 0.5 g of Darco G-60 decolorizing charcoal and 1 g of cellulose powder is then slurried in water, poured into the tube, and held in place with a cotton plug. After the column has been washed with 25 ml of 6*N* HCl and 100 ml of water, the folic acid solution is filtered through the charcoal by suction. The column is washed until the effluent is colorless. Filtration through charcoal removes all of the fluorescent material remaining after cellulose chromatography.

Folic acid is recovered from the charcoal filtrate as follows. The solution is acidified to *p*H 2 with 3*N* HCl and centrifuged at 0°. The sediment is washed four times by stirring with 50-ml portions of 1-percent acetic acid and by centrifugation at 0°; it is then suspended in water and lyophilized. A pale yellow powder is obtained in a 70- to 75-percent over-all yield.

The product, which is completely pure according to the criteria that have been described, should be stored in the dark.

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Significance of Mitochondria for Porphyrin and Heme Biosynthesis

Abstract. It is concluded that mitochondria are involved in three steps of porphyrin and heme biosynthesis—first, in the formation of δ-aminolevulinic acid from glycine and active succinate; second, in the synthesis of protoporphyrin; third, in the incorporation of iron into the porphyrin ring—that is, in heme formation.

It has long been believed that nonnucleated erythrocytes have no mitochondria. By phase-contrast microscopy, the senior author (*1*), in 1955, identified mitochondria in the basophilic stippled cells formed in lead poisoning. Recently Brunner *et al.* (*2*), Braunstein *et al.* (*3*), and Seno *et al.* (*4*) have reported that mitochondria are also present in reticulocytes. Measurements which we made of the high oxygen consumption and cytochrome-*c* content of these two blood cells support this conclusion (*5*). Chicken erythrocytes which have distinct mitochondria near the nuclear membrane showed higher oxygen consumption and cytochrome-*c* content than even the basophilic stippled cells of lead poisoning (*5*). Basophilic stippled cells have a high concentration of δ-aminolevulinic acid and protoporphyrin (*5*) and are as effective in synthesizing protoporphyrin from glycine (*5*) as are avian red cells and reticulocytes (*6*). Since in all these red-cell types the presence of mitochondria can be correlated with protoporphyrin synthesis, the exact role played by the mitochondria in this synthesis was investigated (*7*).

Hemolysates of washed chicken erythrocytes were made according to the method of Dresel and Falk (*8*). Mitochondria were prepared from rabbit bone marrow or rat liver by Schneider's method (*9*). Addition of bone-marrow mitochondria to a chicken red-cell hemolysate incubated with glycine doubled the amount of protoporphyrin synthesized, but this ability to stimulate synthesis of protoporphyrin was destroyed by prior homogenation of bone-marrow mitochondria (Fig. 1*A*). Addition of liver mitochondria, however, had the effect of decreasing markedly protoporphyrin formation, due to the high content of an oxidative deaminase for glycine in liver mitochondria.

When δ-aminolevulinic acid was used as substrate, basophilic stippled cells, reticulocytes, and chicken erythrocytes synthesized much protoporphyrin but little coproporphyrin or uroporphyrin (Fig. 1*B*). Mature rabbit erythrocytes, however, produced much uroporphyrin and coproporphyrin, with little protoporphyrin. This can be explained by the presence of mitochondria in the former cells, since, when liver mitochondria were added to preparations devoid of mitochondria, such as mature rabbit erythrocytes or the supernatant obtained by centrifuging chicken red-cell hemolysates, the mixture converted δ-aminolevulinic acid to protoporphyrin. In a typical experiment a chicken red-cell hemolysate supernatant (20 ml) was incubated for 4 hours with δ-aminolevulinic acid (2 mg), then rat-liver mitochondria were added and the incubation was continued for an additional 4 hours. Porphyrins were then determined and compared with values found in the controls—that is, in preparations with no added mitochondria. It was found that the yield of isolated coproporphyrin decreased promptly, whereas the yield of protoporphyrin was markedly elevated as compared to that of the controls. No protoporphyrin was produced by incubation of coproporphyrin with these mitochondria. The activity residing in the mitochondria could be extracted by water or phosphate buffer from a mitochondria acetone powder. Mitochondria from rabbit liver, chicken erythrocytes, bone marrow, and mesenteric lymph nodes possessed this activity but mitochondria from kidney, heart muscle, and intestinal mucosa did not. Similar effects were found when porphobilinogen was used as substrate.

It is believed that iron is only inserted

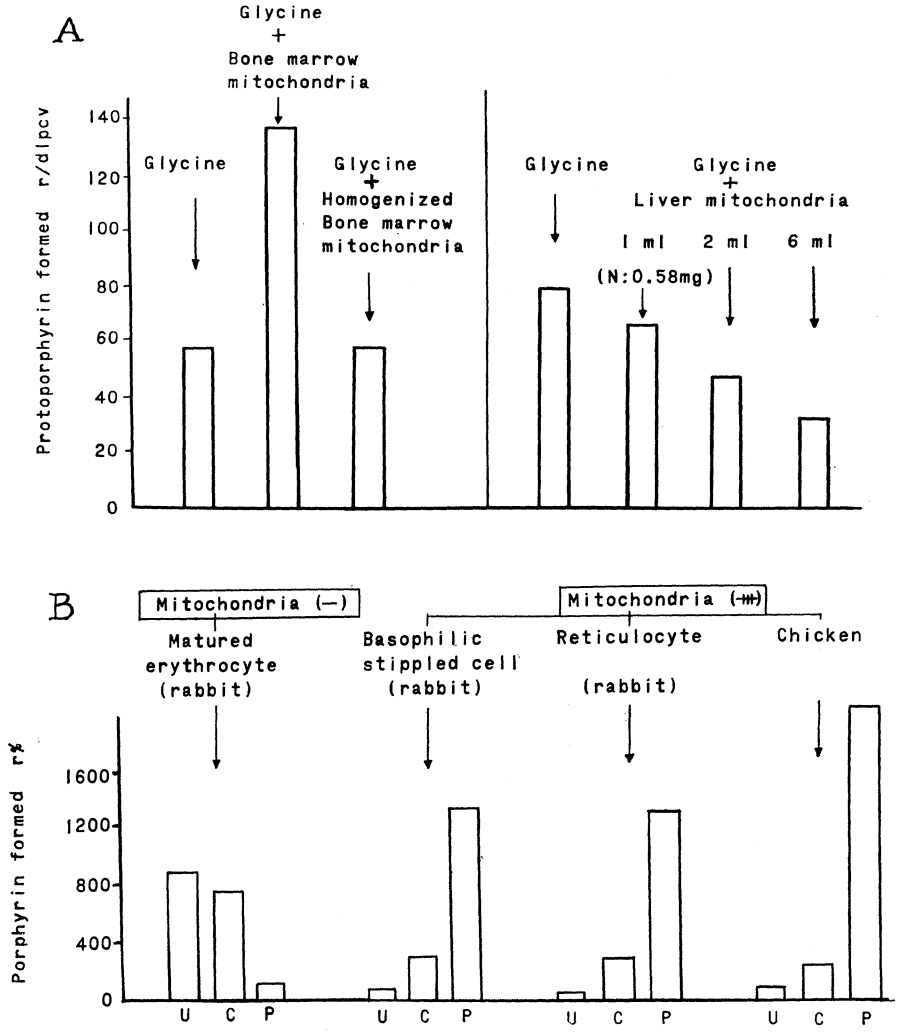

Fig. 1. (*A*) Effect of bone-marrow mitochondria and liver mitochondria on protoporphyrin biosynthesis from glycine in chicken red-cell hemolysate. Glycine, 0.028*M*; incubation period, 2 hours. (*B*) Differences in level of various porphyrins biosynthesized from δ-aminolevulinic acid (0.645 mmole) in erythrocytes containing mitochondria and in cells containing no mitochondria.