THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 61: 226–232, 1973

Vol. 61, No. 4
Printed in U.S.A.

THE MOLECULAR BASIS OF THE ACTION OF CHLOROQUINE IN PORPHYRIA CUTANEA TARDA*

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ABSTRACT

A single therapeutic dose of chloroquine, a 4-aminoquinoline, administered to patients with porphyria cutanea tarda (PCT) results in a massive porphyrinuria with subsequent remission of the disease. In the present investigations, an experimental animal model of porphyria was utilized to elucidate the initial biochemical events involved in the therapeutic response of patients with PCT to chloroquine. Rats were made chemically porphyric by treatment with 3,5 dicarbethoxy 1,4 dihydro 2,4,6, trimethyl pyridine (DDC) and subsequently treated with chloroquine. As in patients with PCT, chloroquine caused a massive porphyrinuria in the experimental animals. The increased excretion of porphyrin was associated with a marked decrease in hepatic porphyrin content without changing the activity of δ-aminolevulinic acid synthetase, the rate controlling enzyme of porphyrin biosynthesis. In addition, there was no biochemical evidence of hepatotoxicity in the experimentally porphyric animals during chloroquine treatment. The validity of the present model system for the study of the effects of chloroquine in PCT is supported by the similarity of the response of the chemically porphyric animals and PCT patients to primaquine, an 8-aminoquinoline. In both instances, primaquine has no effect on porphyrin excretion. In vitro data based on spectrophotometric and gel filtration studies demonstrated that porphyrins form a unique molecular complex with chloroquine. Further, equilibrium dialysis studies demonstrate that chloroquine causes the release of tissue-bound porphyrins. The in vitro studies, in association with the results from the animal model, suggest that the initial event following chloroquine administration to patients with PCT is a release of bound hepatic porphyrin and its rapid elimination.

Chloroquine (7-chloro-4-[4-dimethylamino-1-methylamino] quinoline) is one of several drugs that were developed during World War II for the treatment of malaria; it has subsequently been shown to be of therapeutic value in a number of unrelated diseases [1]. In 1957 London [2], and subsequently Sweeney, Saunders, Dowdle, and Eales [3], and Felsher and Redeker [4], reported on the unique effect of chloroquine administration to patients with porphyria cutanea tarda, a photocutaneous syndrome that results from a marked elevation of hepatic porphyrin production. The disease is characterized by marked increases in the concentrations of hepatic and urinary porphyrins, primarily of the eight and seven carboxyl configuration. The administration of a single therapeutic dose of chloroquine to these patients may result in an acute increase in urinary porphyrin excretion and a fall in hepatic porphyrin content. These initial events have been associated with a variable degree of hepatotoxicity. The porphyrinuria is followed in a few days by a clinical remission. The biochemical and clinical improvement persists for months to years. It is of interest that Felsher and Redeker have shown that the structurally related antimalarial primaquine (8-[4-amino-1-methylbutylamino-6-methoxy] quinoline) does not elicit this clinical response [4].

The mechanism of the clinical response is unclear. Cohen, Phifer, and Yielding [5] demonstrated that chloroquine forms a complex with hydroxyferriporphyrin and other nonmetalloporphyrins. Our report provides further evidence based on spectrophotometric and gel filtration studies, for the existence of a molecular complex between porphyrins and chloroquine. In addition, data obtained by equilibrium dialysis studies and from chemically porphyric animals treated with chloroquine, suggest that the resulting complex may be responsible for the clinical and biochemical response of patients with porphyria cutanea tarda receiving chloroquine.

Manuscript received March 15, 1973; in revised form May 11, 1973; accepted for publication May 18, 1973.

This investigation was supported in part by USPHS Grant AM 11296 from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland.

A part of this work has been presented in abstract form, Clin Res 16:258, 1968.

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MATERIALS AND METHODS

Porphyrins were obtained as methyl esters from the Sigma Chemical Company and hydrolyzed prior to use [6]. The corresponding porphyrinogens were prepared by the method of Mauzerall and Granick [7]. DDC (3,5

dicarbethoxy 1,4 dihydro 2,4,6, trimethyl pyridine) was purchased from K and K Laboratories and recrystallized from methanol. Chloroquine, primaquine, and 4-amino-7-chloroquinoline were kindly provided by Dr. Ascher of the Sterline-Winthrop Research Institute. Sephadex resins were purchased from Pharmacia Fine Chemicals.

For studies of spectral absorbance, a Shimadzu MPS/50L recording spectrophotometer was used. Fluorescence measurements were made with an Amin-co-Bowman Spectrophotofluorometer. The pH was monitored with a Radiometer pH meter (Copenhagen).

Porphyrins were extracted from urine, feces, blood, and tissues by phase partition [8] and were determined spectrophotometrically using appropriate correction factors [9] or by flurometry. Chloroquine was extracted by the method of Brodie, Udenfriend, Dill, and Chenkin [10] and determined flurometrically as suggested by Udenfriend [11].

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall [12] with crystalline human albumin as standard.

Sprague-Dawley female rats weighting 100-160 gm were used in those studies involving animals. Food was removed 24 hr prior to, and withheld for the duration of, the study. Experimental chemical porphyria was induced by a single intraperitoneal injection of DDC (300mg/kg) solubilized in polyethylene glycol 300. Quinoline compounds (equivalent to 100 mg/kg chloroquine) were administered subcutaneously in 0.9% NaCl 24 hr after the DDC was given. Twelve hr later, the animals were killed by decapitation.

Preparation of subcellular fractions of the liver was carried out at 0-4° C in the following manner. Following killing of the animals, the livers were immediately removed and placed in 0.9% NaCl. Subcellular fractions utilized to study the distribution of porphyrin and chloroquine were prepared from 25% liver homogenates essentially by the method of Novikoff and Heus [13] in TKM-sucrose (0.1 M Tris-HCl, pH 7.5; 0.05 M KCl; 0.01 M $MgCl_2$; and 0.25 M sucrose). The preparation of the nuclear fraction was modified to further purify this fraction. The initial 750 $g \times 10$ min pellet was resuspended in 2% Trition X-100-TKM-sucrose, centrifuged at 600 $g \times 10$ min and the resulting pellet was washed and resuspended in TKM-sucrose. All subcellular fractions were resuspended in TKM-sucrose so that 1 ml of final suspension was equivalent to 1 gm of liver, wet weight. Mitochondria used for studies of oxidative phosphorylation were prepared by the method of Schneider and Hogebloom [14].

δ-Aminolevulinic acid synthetase activity was determined in liver homogenates by the method of Marver, Tschudy, Perlroth, and Collins [15]. Acid phosphatase activity was determined by the method of Berthet and DeDuve [16], the phosphate liberated in this fashion was determined by the method of Fiske and Subbarow [17]. Oxidative phosphorylation was evaluated polarographically [18] using a Clark oxygen electrode (Yellow Springs Instrument Co.). The respiratory control index (RCI) was utilized as a measure of mitochondrial integrity [19]. Serum glutamic-oxalacetic transaminase (SGOT) was measured by the method of Karmen [20]. Serum glutamic-pyruvic transaminase (SGPT) was determined by the method of Wroblewski and LaDue [21].

Sections for electron microscopy were prepared in the following manner: Immediately after death of the animals, the livers were removed, specimens taken and placed in 3% glutaraldehyde 0.1 M Na cacodylate, pH 7.4, and held at 4° C overnight [22]. This was followed by 2 hr postfixation in 1% osmium tetroxide. All tissues for electron microscopy were dehydrated in ethanol and embedded in epoxy resin [23]. Thin sections were double stained with uranyl acetate and lead citrate and examined in a Siemens Elimshop 1A electron microscope.

RESULTS

Effect of Chloroquine on Chemically Porphyric Rats

The results of chloroquine administration to chemically porphyric rats are summarized in Figure 1. As indicated, there is an increase in serum and urinary prophyrins with a concomitant decrease in hepatic porphyrin content. In order to ascertain whether there was intracellular selectivity in the distribution of chloroquine action, subcellular fractions of liver were prepared, and the porphyrin content of each determined (Fig. 2). Although the nuclei showed the greatest concentration of porphyrin, the principal sites of porphyrin release from the hepatocytes following chloroquine were the mitochondrial and lysosomal subcellular fractions. It is of interest that these subcellular fractions have been previously shown

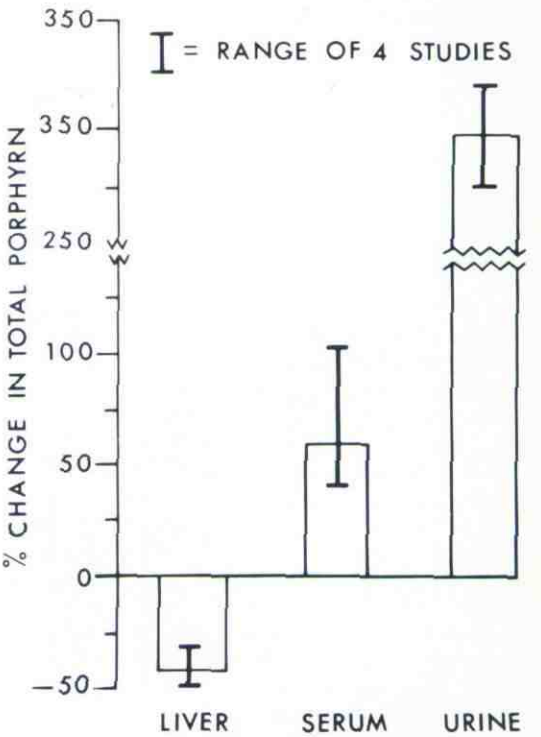

FIG. 1: The effect of chloroquine treatment of chemically porphyric rats on serum, hepatic, and urinary content of porphyrin. Each series of studies represents three to six animals per study for both the chloroquine-treated and saline-treated chemically porphyric animals. The "0" level porphyrin values for the chemically porphyric animals were, 39 μmoles/24 hr urine; 1.83 μmoles/100 ml serum; 56 μmoles/liver. For details see *Materials and Methods*.

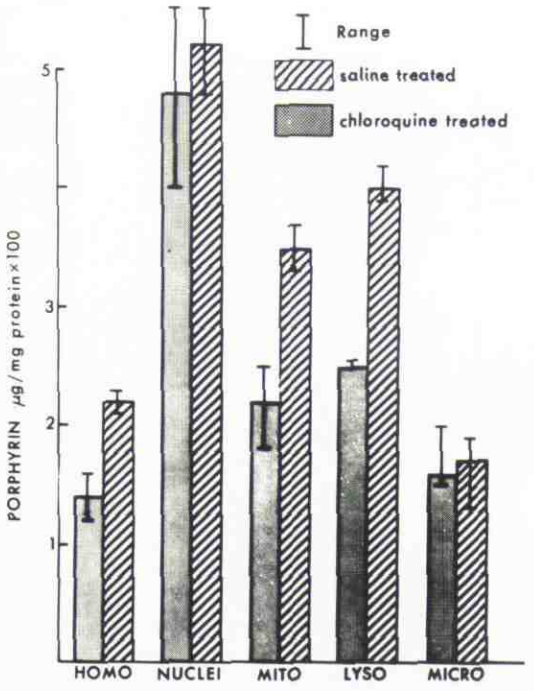

FIG. 2: The effect of chloroquine on the subcellular distribution of porphyrins in the livers of chemically porphyric rats. For details see *Materials and Methods*.

to selectively concentrate chloroquine in rat liver [24].

In order to ascertain whether the organellar specificity of chloroquine on the release of hepatic porphyrin was associated with specific biochemical changes, the following studies were done. Liver mitochondria were isolated from chloroquine- and saline-treated chemically porphyric animals and from control animals similarly treated. The functional integrity of these mitochondria was evaluated by measuring oxidative phosphorylation, and determining the respiratory control index, with the substrates succinate, β-hydroxybutyrate, and α-ketoglutarate. In each instance, there was no difference between the mitochondria isolated from the experimental animals and those from the controls. The specific ADP/O ratios were: 1.3 $\pm$ 0.10 for succinate, 2.6 $\pm$ 0.14 for β-hydroxybutyrate, and 2.5 $\pm$ 0.20 for α-ketoglutarate; the respiratory control index was 4.4 $\pm$ 0.5 ($\pm$ represents the range of three separate experiments). Similarly, when chloroquine and/or porphyrins were added to normal rat liver mitochondria no effect was observed on either oxidative phosphorylation or the respiratory control index. Although these data strongly suggest that the mitochondria, isolated from chloroquine-treated chemically porphyric animals, are entirely normal, we can not exclude the possibility that mitochondria which have been damaged may not separate in the usual manner. Likewise, the experiments in which normal mitochondria were exposed to chloroquine and/or porphyrins without effecting biochemical function do not exclude the possibility of a significant interaction under physiologic conditions, as the ability of these molecules to reach the inner mitochondrial membrane under these conditions in unknown. This latter point may be of particular significance as Ruzicka and Crane [25] have recently shown that chloroquine interrupts the electron transport chain in submitochondrial particles by blocking the chain between NADH dehydrogenase and coenzyme Q. In addition to mitochondrial function, lysosomal function and serum transaminase levels were examined in experimental and control rats; in each instance there was no significant difference between the various groups of animals.

Identical studies to those reported above were carried out with the antimalarial primaquine. The results of these studies were analogous to those reported for patients with PCT in that primaquine did not effect the excretion of porphyrin in the chemically porphyric animals.

In order to determine whether the increased excretion of porphyrins, by the chemically porphyric animals following chloroquine treatment, was due solely to the release of hepatic porphyrin or was in addition associated with an increase in porphyrin production, the activity of δ-aminolevulinic acid synthetase, the rate-limiting enzyme of heme biosynthesis, was measured. The level of activity of this enzyme was determined in six groups of normal, DDC-treated, and DDC-chloroquine-treated animals. The enzyme activities were 18 (range 9–27), 300 (230–370), and 280 (210–355) mμmoles δ-aminolevulinic acid/hr/gm liver, respectively. These studies indicate that in experimental porphyria the increment in the excretion of porphyrins following chloroquine administration does not reflect an accelerated production of porphyrins.

Morphologic examination of liver specimens taken from chloroquine-treated porphyric animals, as well as appropriate control animals, was performed in order to evaluate whether any histologic changes had occurred subsequent to chloroquine therapy that had not been detected in the biochemical studies. Routine gross examination, as well as light microscopic examination of hematoxylin and eosin-stained liver sections failed to demonstrate any significant difference between the livers of the various animals. Electron microscopic examination of liver tissue taken from chloroquine-treated porphyric animals, however, showed an increased number of lamellar structures within the hepatic lysosomes as compared to control liver tissue. Although the origin of these "onion skin" structures has not been definitely determined, it is likely that they represent the residual matrix of degraded membranes. It is of interest that similar lamellar structures have been identified in leukocytes [26] and cultured fibroblasts and macro-

phages [27] following exposure to chloroquine. In the current study, lamellar structures were not seen in the absence of chemical porphyria.

In Vitro Interaction of Chloroquine with Porphyrins

The results of our studies, in which chemically porphyric rats were treated with chloroquine, indicated that the subsequent porphyrinuria was due neither to an increased production of porphyrin nor to a general hepatotoxicity. We therefore explored the possibility that the porphyrinuria was the direct result of a chemical reaction between the porphyrins with chloroquine. For these studies, we utilized spectrophotometric, gel filtration, and dialysis techniques.

The results of the determination of the difference absorption spectrum between coproporphyrin I and coproporphyrin I with chloroquine are shown in Figure 3. This study demonstrates that chloroquine induces a shift in the absorbance maximum of coproporphyrin I and indicates that a physical

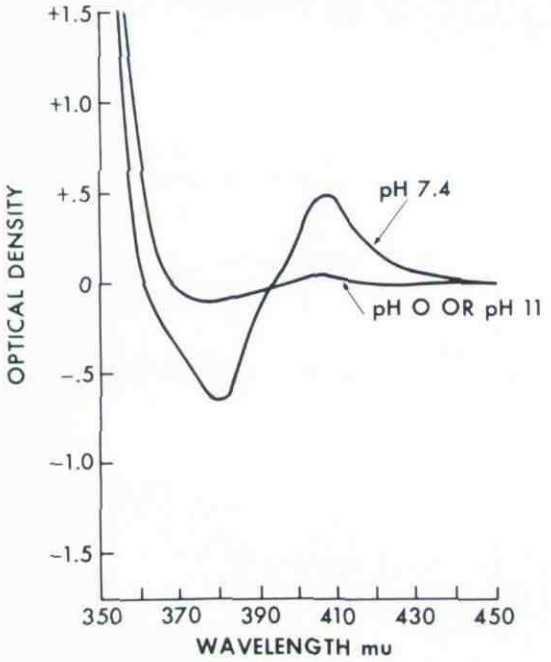

FIG. 3: The effect of pH on the difference absorption spectrum between coproporphyrin and coproporphyrin with chloroquine. For these studies a dual-beam spectrophotometer was used. Initially both the sample and reference cuvettes were made up with 0.5 ml of 20 μM coproporphyrin I in 0.01 M Na phosphate at various pHs. The difference in absorbance between each pair of cuvettes was zero. Subsequently, to each sample cuvette of coproporphyrin I, chloroquine diphosphate was added to give a final concentration of 80 μM; the volume and ionic strength of the reference cuvette was maintained by the addition of a KCl solution. The difference in absorbance between the sample and reference cuvettes was again determined at each pH. A 1-cm light path was used in these studies; all recordings were made at 25° C.

interaction has occurred between this porphyrin and chloroquine. Similar results were obtained when uroporphyrin III or protoporphyrin IX were used in place of coproporphyrin I; specifically in the presence of chloroquine the porphyrin spectra exhibited a relative decrease in absorbancy at ~408 mμ. It should be noted that chloroquine has no absorbance over this energy range. The above findings are in agreement with those of Cohen, Phifer, and Yielding [5] who determined the effect of chloroquine on the spectra of various porphyrins using direct spectrophotometric evaluation. In the present study, the spectrum of chloroquine was examined (not shown). In the presence of porphyrin chloroquine exists in a monoprotonated form; additional spectrophotometric studies of the porphyrin–chloroquine complex showed it to be stable from a pH of 4.5 to 9.0 in aqueous solution and to dissociate in a reversible manner outside this pH range. Similar studies utilizing the water-insoluble chloroquine base and coproporphyrin I were carried out in diethyl ether. A comparable spectral shift was observed in this aprotic solvent but the change in absorbancy was of lesser magnitude than that seen in aqueous solution. When primaquine or 4-amino-7-chloroquinoline was substituted for chloroquine there was no spectrophotometric evidence for the interaction of either with the porphyrins studied. Likewise, there was no evidence of a molecular interaction between porphyrinogens and chloroquine in the near ultraviolet spectrum; concentrations of coproporphyrinogen from 1.2×10^{-7} M to 7×10^{-6} M and chloroquine from 5×10^{-6} M to 2×10^{-5} M were used for these studies. It is of interest that chloropromazine (2-chloro-10[3-dimethyl-aminopropyl]-phenothiazine), a molecule known to participate in charge transfer interactions [28], induces a shift in the porphyrin spectrum similar to that observed with chloroquine. However, the magnitude of the shift in the absorbance spectrum of the porphyrins is only 1–5 percent of that induced by chloroquine, when studied at comparable molar concentrations.

The chloroquine–porphyrin complex was separated by Sephadex gel filtration. A Sephadex column was prepared and equilibrated with a buffer containing chloroquine diphosphate at pH 7.4. Following equilibration, uroporphyrin III in the same buffer was applied to the column and eluted with the equilibration solution (Fig. 4). The peak concentration of the chloroquine corresponds to that fraction that contains porphyrin. Immediately following the "peak" there is a "trough" and a subsequent return to the equilibrated chloroquine concentration. This biphasic pattern is indicative of the formation of a porphyrin–chloroquine complex. The peak represents that amount of chloroquine removed from the elution fluid which participates in the formation of the complex; the trough represents that amount of chloroquine removed from the elution fluid that is

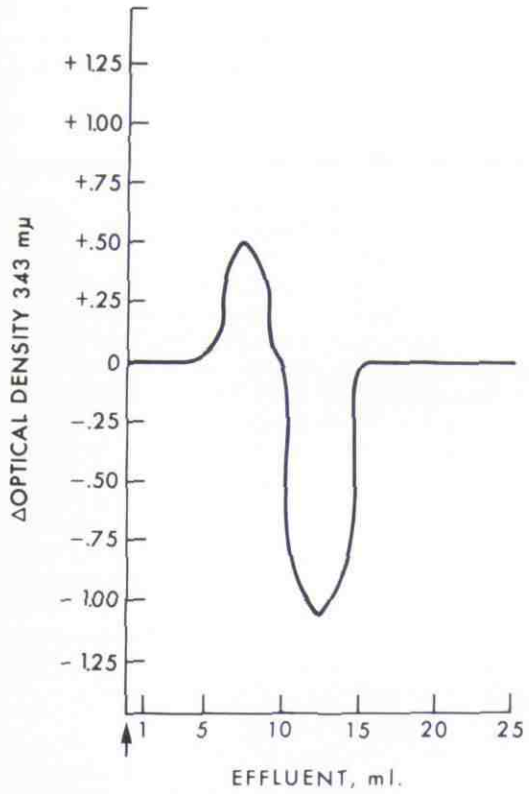

FIG. 4: Demonstration of a uroporphyrin III–chloroquine diphosphate complex by Sephadex gel filtration. A Sephadex G-25F column (20 × 1 cm) was equilibrated with 3.3×10^{-5} M chloroquine diphosphate in 0.01 M Na phosphate, pH 7.4. Following equilibration, 1 ml of 6.6×10^{-6} M uroporphyrin III (↑) in the equilibration solution was layered on the column and eluted with the same solution. Chloroquine content of the effluent was monitored spectrophotometrically at 343 mμ.

required to reestablish the equilibrated concentration. Porphyrins placed on a phosphate-equilibrated Sephadex column interact with the gel and are not eluted with eluant volumes equal to 10 times the bed volume. Chloroquine placed on a similarly prepared Sephadex column forms an aggregate or polymer, as evidenced by 70 percent of the chloroquine being recovered in the void volume.

The molecular size of the porphyrin–chloroquine complex was investigated with the use of different exclusion sizes of Sephadex gels, G-10 to G-75. The columns were prepared and treated in a manner identical to that described for the isolation of the complex. In each instance, the complex was identified spectrophotometrically in the void volume, suggesting the formation of a large polymer or aggregation of the complex. At a constant chloroquine concentration of 3.3×10^{-5} M and a porphyrin concentration of 6.6×10^{-6} M, the chloroquine to porphyrin molar ratio varied from 1.5 to 2 within the complex.

In order to determine whether the interaction of chloroquine with porphyrin would result in the release of tissue-bound porphyrins in vitro, a series of equilibrium dialysis studies were done. Liver homogenate was initially saturated with coproporphyrin and subsequently dialyzed against an isotonic salt solution, pH 7.4, with and without chloroquine. As shown in Figure 5, chloroquine acted in a concentration-dependent manner in effecting the release of porphyrin from the liver homogenate. These results may be explained by the formation of a complex between chloroquine and coproporphyrin which renders the porphyrin more water-soluble or alternatively, by a direct competition between chloroquine and porphyrin *for specific binding sites.*

DISCUSSION

The present study was carried out in an attempt to clarify the mechanism of action of chloroquine in patients with PCT. In particular, is the massive porphyrinuria observed in PCT patients following chloroquine administration solely the result of a hepatotoxic reaction, as has been suggested [3], or is the hepatotoxicity a coincidental and controllable aspect of chloroquine therapy? In order to investigate specific biochemical aspects of the chloroquine effect, it was necessary to utilize an experimental animal model of porphyria for these studies. It should be noted, however, that although the animal disease differs from PCT, in the activities of heme biosynthetic enzyme activity and in the specific hepatic porphyrins present, they both exhibit marked increases in hepatic porphyrin content. The validity of the use of the animal model in these studies is supported by our observation that chemically porphyric animals responded

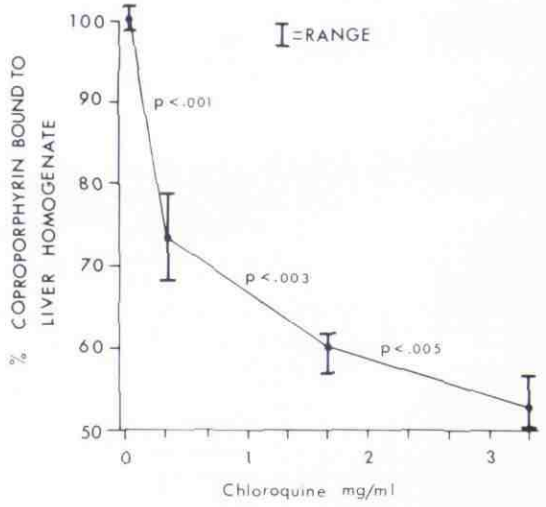

FIG. 5: Equilibrium dialysis studies demonstrating the effect of chloroquine on the release of tissue-bound coproporphyrin I. Five ml of a 20% liver homogenate saturated with coproporphyrin I (17 μmoles/ml) were dialysed against four volumes of a solution containing 0.14 M KCl, 0.005 M Na phosphate, pH 7.4, and chloroquine in the concentration indicated. Dialysis was carried out at 4° C.

to both chloroquine and primaquine in a manner identical to that observed in patients with PCT [4]; that is, chloroquine caused an increased excretion of porphyrin and primaquine was without effect.

The immediate effects of chloroquine administration to chemically prophyric rats are, as in PCT patients, an increased urinary and fecal excretion of porphyrin and a concomitant decrease in the hepatic porphyrin content. That this was not the result of a change in the rate of hepatic porphyrin production was suggested by the finding that the level of activity of the rate-controlling enzyme of porphyrin biosynthesis, δ-aminolevulinic acid synthetase, was unchanged following chloroquine treatment. In addition, it did not appear that the marked increase in porphyrin excretion by the porphyric animals could be attributed to hepatotoxicity following chloroquine. This conclusion is based on our findings that the SGOT, SGPT, and LDH levels were not increased following chloroquine, nor was there any abnormality in intracellular organelle function. In particular, mitochondrial and lysosomal functions were identical to control animals. In addition, light and electron microscopic examination of the animals' livers showed no evidence of toxic change.

From the results of the in vivo studies, it is apparent that the porphyrinuria, which followed chloroquine administration to chemically porphyric rats, required neither hepatotoxicity nor biochemical changes in porphyrin biosynthesis. It therefore seemed reasonable to evaluate alternative means by which porphyrin could be removed from liver by chloroquine. In particular, the possibility of a direct interaction between chloroquine and porphyrin, which could account for the porphyrinuria, was examined. Initial spectrophotometric studies confirmed the findings of Cohen, Phifer, and Yielding [5] as to the existence of a molecular interaction between chloroquine and the various porphyrins. In addition, the molecular complex was further isolated and characterized with the use of Sephadex gel filtration. Of particular interest in these studies was the finding that none of the chemical analogs of chloroquine reacted with any of the porphyrins tested. This result parallels the in vivo results seen in patients with PCT given chloroquine or primaquine, in that only chloroquine, and not primaquine, has been shown to release porphyrins from the livers of patients with the disease. The striking similarity between the spectrophotometric data and the clinical situation was likewise observed in dialysis studies in which chloroquine was demonstrated to cause the release of prophyrin from porphyrin-saturated liver homogenate. It is apparent from the above discussion that there is a specific chemical interaction between porphyrins and chloroquine and that it is sufficient to effect the acute release of porphyrins from hepatic tissue.

It is evident from the above considerations of the results of this study that the release of hepatic porphyrins following chloroquine therapy in chemically porphyric rats, and by analogy in patients with PCT, is not dependent on the production of severe hepatotoxicity as has been suggested [3]. Both the in vivo and in vitro experiments described in this report support this conclusion. In addition, recent clinical reports [29, 30], describing the successful treatment of PCT patients with chloroquine without apparent liver injury, are also consistent with the above conclusion. The intriguing question as to the mechanism by which chloroquine effects a long-term remission in patients with PCT remains unanswered and is beyond the capacity of the experimental model used for these studies. Whether it is due to the removal of the porphyrin per se, or iron as has been recently proposed [30], or to some other mechanism, will only be answered with additional studies. Lastly, it should be pointed out that until further information is available as to safe dosage, chloroquine should be utilized with caution for the treatment of PCT.

We wish to acknowledge the support and encouragement of Professor Rudi Schmid in the preparation of this manuscript. In addition, we wish to thank Drs. Neal Castagnoli, Jr., and Donald L. Schneider for their advice and helpful suggestions.

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