In vitro inhibition of protein synthesis in the retinal pigment epithelium by chloroquine*

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There is substantial evidence that the primary structure involved in chloroquine retinopathy is the retinal pigment epithelium (RPE) and that this drug, like the phenothiazines, stores in RPE in high concentration. Experiments reported here show that in concentrations of approximately $5 \times 10^{-6}$ M chloroquine inhibits protein synthesis in RPE without inhibiting amino acid uptake as measured with $^{14}$C-labeled phenylalanine, lysine, and leucine. Similar effects are observed with hydroxychloroquine, desethylchloroquine, and quinine. These findings strongly suggest that after storage in RPE the direct toxic effect of the drug is inhibition of protein synthesis.

Key words: Chloroquine, desethylchloroquine, hydroxychloroquine, inhibition, melanin, photoreceptor, protein synthesis, retinal pigment epithelium, retinopathy.

Ocular abnormalities are known to result from the long-term, high-dosage administration of chloroquine employed in the treatment of rheumatoid arthritis, discoid and systemic lupus erythematosus, and other collagen-related diseases. The first case histories of chloroquine retinopathy were reported by Hobbs, Sorsby, and Friedman1 in 1959, and since then numerous reports have appeared confirming both the clinical observations and the etiology as resulting from chloroquine therapy (see review by Nylander2).

The clinically observed pigmentary changes in the retina, the electrophysiologic changes, as well as the sparse human histopathologic data all suggest that the primary site of chloroquine retinopathy is in the retinal pigment epithelium (RPE) with subsequent degeneration of retinal receptors. An unusual feature is the retention of nearly normal dark adaptation until the very late stages of the disease.

Experimentally induced chloroquine retinopathy in cats3 and rabbits4 suggested an initial toxic effect on the RPE. Histologic analysis in experimental animals revealed a thickening of the pigment epithelial cell layer with migration of pigment as far as the outer nuclear layer, and concomitant atrophy of the photoreceptors.
Materials and methods

Chloroquine diphosphate, hydroxychloroquine sulfate, and desethylchloroquine were generously supplied by the Sterling-Winthrop Research Institute, Rensselaer, N. Y. Quinine sulfate and sodium penicillin G, United States Pharmacopoeia grade, were obtained from the Pharmacy Department, University of Chicago Hospitals. Puromycin dihydrochloride and cycloheximide were purchased from the Sigma Chemical Company, St. Louis, Mo. L-phenylalanine, L-leucine, and L-lysine dihydrochloride were obtained from Nutritional Biochemicals Company, Cleveland, Ohio, and 1-3-phenylalanine-C\(_{14}\) (U), 1-lysine-C\(_{14}\) (U) monohydrochloride, and L-leucine-C\(_{14}\) (U) were purchased from Amersham/Searle Corporation, Chicago, Ill., in the highest specific activity available. 1,4-bis [2-(4-methyl-5-phenyloxazoly) ]-benzene (Dimethyl POPOP) and Beckman Bio-Solv BBS-3 aqueous solubilizer were obtained from Beckman Instruments, Inc., Lincolnwood, Ill., and 2,3-diphenyloxazole (POPOP) was obtained from Packard Instrument Company, Inc., Downers Grove, Ill. Crystallized bovine plasma albumin was purchased from Armour Pharmaceutical Company, Kankakee, III.

Fresh beef eyes were obtained from a local slaughter house and RPE was prepared in an isotonic-isosmotic sodium-potassium phosphate buffer, pH 7.4, containing 5.0 mM glucose, by a method standard in our laboratory. After centrifuging at 600 x g for 10 minutes in a Sorvall refrigerated centrifuge, the cells were very gently resuspended in ice cold buffer, now containing 500 units of sodium penicillin G per milliliter.

RPE from one to two eyes was incubated at 37° C for two and one-half hours in 10 ml pyrex beakers containing 2 mM \(^{14}\)C-amin acid in the presence or absence of drug. Final volume was adjusted to 1.2 ml. Reactions were terminated with trichloroacetic acid (TCA) final concentration five per cent), containing 1 mg per milliliter of carrier amino acid and the mixture was transferred to conical centrifuge tubes. Beakers were rinsed once with five per cent TCA and proteins were allowed to precipitate overnight at 4° to 6° C. After washing the proteins with five per cent TCA containing 0.5 mg per milliliter of carrier amino acid, once at room temperature, once while heating to 90° C for 15 minutes, and once again at room temperature, proteins were solubilized with 1.5 to 2.0 ml of three per cent sodium hydroxide while heating to 50° C. to 60° C. for two to three hours. Hydrogen peroxide was added dropwise to oxidize and decolorize melanin. The yellowish hydrosolate was transferred to 5 ml volumetric flasks along with two distilled water washings of the centrifuge tube and final volume was adjusted to 5 ml. Duplicate 1.0 ml aliquots were counted in 15 ml of a toluene-based scintillant containing 150 ml per liter of Beckman Bio-Solv BBS-3 solubilizer, 5.0 Gm per liter of POPOP, and 300 mg per liter dimethyl POPOP. A Packard model No. 3375 Luid Scintillation Counter was employed and the efficiency was determined by the automatic external standardization system (AES). In the past, we have checked the Packard AES by comparing its results with those obtained by adding internal radioactive standards (containing accurately known numbers of disintegrations per minute) to various volumes of the yellowish hydrosolate. The corrected values obtained by each method did not differ by more than two per cent for any determination.

The rate of l-phenylalanine-\(^{14}\)C incorporation into RPE protein was determined by incubating the cells in 50 ml Erlenmeyer flasks in a final volume of 30 ml at the same concentrations as described above. Duplicate 1.2 ml aliquots were removed at the appropriate times and protein precipitated with TCA (final concentration equals five per cent). Assay procedures for the determination of incorporated radioactivity were the same as outlined above.

The in vitro uptake of l-phenylalanine-\(^{14}\)C into RPE cells was determined from the 30 ml incubations. Duplicate 1.2 ml aliquots were removed at the appropriate times and placed into 15 ml round-bottom polystyrene centrifuge tubes, kept under ice, diluted with 3 ml of ice-cold buffer, and centrifuged at 5,000 x g for 10 minutes in a Sorvall refrigerated centrifuge (0° C.). Supernatant solutions were decanted and the tubes were inverted and allowed to drain for several min-
Fig. 1. Effect of chloroquine, puromycin, and cycloheximide on the in vitro incorporation of 1-phenylalanine-14C into retinal pigment epithelial protein.

utes. After the walls of the centrifuge tubes had been wiped dry with cheesecloth, the RPE cells were vigorously resuspended in 2.5 ml of ice-cold buffer and protein was precipitated with TCA (final concentration equals five per cent). The supernatant obtained on centrifugation of the protein precipitate was combined with the three TCA washings of the precipitate and added to 25 ml volumetric flasks. Protein was solubilized in three per cent sodium hydroxide and the radioactivity was assayed as described above. The volumetric flasks were adjusted to 25 ml. with distilled water and the 1-phenylalanine-14C content released from RPE cells was assayed by counting duplicate 1.0 ml aliquots in 15 ml of the same toluene-BBS-3 scintillant described previously.

Counts obtained from samples incubated for 15 minutes, one hour, or two and one-half hours at 4° C. were used in calculating normal and drug-inhibited amino acid incorporation. Protein was assayed by a modification of the Lowry procedure \(^9\) with bovine plasma albumin used as a standard. Statistical significance was computed by Student's \(t\) test.

Results

Fig. 1 demonstrates our initial results obtained by investigating phenylalanine incorporation into RPE protein. Some inhibition of incorporating activity was consistently observed at 10^{-4}M chloroquine concentration, and 10^{-3}M chloroquine inhibited phenylalanine incorporation approximately 75 per cent. Puromycin and cycloheximide, known inhibitors of protein synthesis, were studied in parallel to chloroquine to demonstrate the amino-acid incorporating properties of bovine pigment epithelium. Both agents significantly inhibited phenylalanine incorporation into protein at 10^{-5}M, while practically all of the activity was eliminated at 10^{-4}M. Chloroquine depressed both the initial rate and the total amount of phenylalanine incorporated (Fig. 2). Five minutes after introduction of labeled amino acid significant inhibition was observed. There was a similar depression of total incorporating ability at the end of three to four hours of incubation. Chloroquine inhibited phenylalanine incorporation into protein without affecting the entry of that amino acid into the RPE cell (Fig. 3). This suggests that an equivalent amount of amino acid was available for incorporation into protein in both control and chloroquine-treated preparations. Hydroxycchloroquine and desethylchloroquine, the latter a metabolite of chloroquine, also inhibited phenylalanine incorporation into protein (Fig. 4) to the same degree as chloroquine. Likewise, quinine caused significant inhibition at 5.3 \times 10^{-4}M.
Fig. 2. Effect of chloroquine on the in vitro rate of L-phenylalanine-14C incorporation into retinal pigment epithelial protein. Data are plotted as the Mean obtained from five experiments ± the standard deviation.

Fig. 3. L-phenylalanine-14C recovery from retinal pigment epithelium during the in vitro inhibition of protein synthesis by chloroquine. Data are plotted as the Mean obtained from five experiments.

In order to eliminate the possibility that chloroquine inhibits only phenylalanine incorporation into protein, these studies were extended to include the incorporation of L-leucine-14C and L-lysine-14C. The results in Figs. 5 and 6 indicate that these two amino acids are not only incorporated into RPE protein but also that their incorporation is significantly inhibited by chloroquine, hydroxychloroquine, and desethylchloroquine at 5.0 x 10^-4M. Cycloheximide at 10^-3M, which has become our standard of protein synthesis inhibition, also decreased leucine and lysine incorporation into protein. Moreover, equimolar combinations of chloroquine with either hydroxychloroquine or desethylchloroquine resulted in an additive inhibition of incorporation for both amino acids. This was particularly significant for leucine (Fig. 5).

Discussion

When considering the mechanism of the toxic side-effects of a drug whose time scale is that of chloroquine, one must look for subtle effects, most of which eventually turn out to be inhibition of a single enzymic reaction. When one has isolated such a system and one can work with pure enzyme, substrate, and inhibitor one can describe relationships among these components with precision. One can write Michaelis-Menten kinetics and draw Lineweaver and Burke plots. In the early stages of such an investigation, however, one must be content to study multi-enzyme systems and to use cell suspensions or tissue homogenates as their source.

In this intermediate type of study which lies between the use of the intact animal and that of a single isolated enzyme, one expects the amount of inhibitor required for a given effect to be less than that required in the whole animal and greater...
than that required in the isolated enzyme system. It has been customary in the past to use a dilute cell suspension or homogenate with optimal substrate, and to specify the per cent inhibition of the reaction under study by given molarity of inhibitor.

Because the plot of per cent inhibition versus concentration of inhibitor in such systems is often sigmoidal, the concentration which produces 50 per cent inhibition is frequently specified as a parameter of the system ([I]_{50}), for this is the part of the sigmoid curve most affected by small changes in concentration.

From system to system the [I]_{50} varies depending on characteristics of the system and on precise experimental conditions. The inhibition of glycolysis by iodoacetate...
has been studied intensively, and has been found to result from alkylation of the SH groups in phosphoglyceraldehyde dehydrogenase. Mackworth found 60 per cent inhibition of this enzyme from rabbit muscle acetone powder extract by $2 \times 10^{-4}$M iodoacetate. Holzer found 50 per cent inhibition by $5 \times 10^{-4}$M iodoacetate in a similar preparation. This is exactly the same order of magnitude as the chloroquine concentration found in our experiments to give 50 per cent inhibition of phenylalanine and leucine incorporation into RPE protein.

Similarly, in an homogenate of rat liver used as a source of succinic dehydrogenase, Ackermann and Potter found that the $[I]_{50}$ for malonate was $1 \times 10^{-3}$M. Under the experimental conditions of Brown, who measured the effectiveness of sulfonamides in inhibiting folate synthesis by effect on the growth of E. coli, the $[I]_{50}$ for sulfanilamide was $2.1 \times 10^{-3}$M. For sulfathiazole the figure was $2.5 \times 10^{-5}$M. When a purified enzyme preparation was used, the inhibitory concentrations were an order of magnitude less.

It is true that where an inhibitor has very high affinity for a binding site, as cyanide ion does for the iron of cytochrome, the $[I]_{50}$ is very much lower than the figures quoted above. In rat brain homogenate as a source of cytochrome oxidase, the $[I]_{50}$ for cytochrome c disappearance is $2 \times 10^{-4}$M cyanide. On the other hand, 250 mg. of KCN is the acutely fatal dose for man. No one suggests that chloroquine possesses such toxic properties. We contend that it is a moderately active inhibitor whose $[I]_{50}$ is directly comparable to that of other moderately active inhibitors when used in cell suspensions.

The dose of chloroquine which causes retinotoxic effects in humans is 250 mg. of the diphasphate given from one to three times per day. This dose of 0.48 mM, if evenly distributed through the 41 kilograms of body water of a 68 kilogram patient, would result in an immediate drug concentration of $1.17 \times 10^{-4}$M which would occur as much as three times a day. Furthermore, as was demonstrated in our laboratory, including that of the retinal pigment epithelium, has a high affinity for chloroquine and other polycyclic aromatic compounds. We demonstrated that 10 mg. (dry weight) of RPE pigment adsorbed 38 per cent of 2.5 μmoles of chloroquine to which it was exposed in vitro. Animal experiments by Bernstein and co-workers confirmed by many later reports, described accumulation of chloroquine in ocular tissues after in vivo administration. As was shown in our laboratory for other compounds, this high-level accumulation is a function of the presence of melanin and occurs only in melanin-containing tissues. McChesney, Bank, and Sullivan showed that in pigmented rats, after 40 mg. per kilogram of chloroquine six days a week for thirteen weeks, the whole eyes contained a weight of chloroquine equivalent to $2.5 \times 10^{-3}$M. A very conservative estimate would place the concentration in pigmented tissues at ten times this figure.

A major problem lies in determining how such a high concentration of drug on a pigment reservoir affects the tissue immediately adjacent to it—in the case of RPE, the adjacent 8 μ of tissue. We know that multiple doses allow accumulation of polycyclic compounds on melanin. In the rats of McChesney, Bank, and Sullivan which received a dose some ten times the usual high-level human dose, most of the accumulation occurred during the first five to six weeks. The first week after cessation of drug, 14 per cent of the store was lost from the eye and during the second week an additional 5.3 per cent was lost. This agrees in essence with the early findings in our laboratory on loss of chlorpromazine from the uvea of pigmented rabbits after a single dose. We were able to demonstrate a bimodal depletion curve whose flex came at about the end of the first week. The slope of this first portion of the curve was 10 per cent per day.

In the clinical situation where human toxic effects take place drug is given one
to three times a day. During the time immediately after a dose, when blood levels are high, the drug accumulates on tissue melanin and gives cumulative tissue levels higher than the maximum momentary blood level. When blood levels drop the drug comes off the pigment, creating a concentration gradient which drops sharply as the distance from the pigment granule increases. The unanswered question is: What is the actual concentration gradient inside the pigment-granule-rich RPE cell? Experiments are under way in our laboratory to answer this question. Meanwhile, one observation is worth recalling. In experiments done by us in the past which examined the competition between chlorpromazine and acridine orange for uveal melanin, we concluded that all adsorption sites were not equivalent. Strongly adsorbed acridine orange markedly reduced the pigment capacity for holding chlorpromazine. This suggests that some sites absorb more strongly than others, and when all sites are occupied, the subsequent elution occurs more readily from the weaker adsorption sites. This means that neither the rate of loss described above by McChesney, Bank, and Sullivan, which calculates to 0.08 per cent per hour nor our figure for chlorpromazine which calculates to 0.4 per cent per hour is likely to be correct. By making the first measurements one week or even (in our case) one day after cessation of administration, one loses an hypothetical early part of the curve which is operative immediately as the blood level begins to fall. We have recounted above how it is probable from the figures of McChesney, Bank, and Sullivan that the pigmented tissues of the eye in their high dosage, experimental animals must reach $2.5 \times 10^{-2}$M. We have shown that initial tissue levels in normal humans on potentially toxic doses of chloroquine must be in the vicinity of $1.2 \times 10^{-2}$M. These values straddle the experimental value obtained by us in the paper where we show that $1 \times 10^{-4}$M chloroquine measurably inhibits protein synthesis in retinal pigment epithelium. It is very probable that, in the immediate vicinity of a high concentration store of low overall capacity as in the retinal pigment epithelium, inhibitory concentrations are reached.

One problem, indeed, is generated by this very probability. Our experiments and most others like it run for only a few hours. The effects observable in this period are relatively gross effects. In chloroquine retinopathy we are dealing with effects so gradual and so marginal that only a fraction of the population at risk experiences retinopathy (this could be genetic), and a year of steady dosage is commonly required for the effects to be manifest. This last suggests that the concentration of drug in the RPE is somewhat under $1 \times 10^{-4}$M, where in vitro inhibitory effects are observable in several hours but greater than $1 \times 10^{-4}$M to which all tissues—pigmented and nonpigmented alike—are subjected daily.

The data presented in this paper clearly demonstrate that chloroquine inhibits amino acid incorporation into RPE protein at concentrations which are pharmacologically reasonable without affecting the in vitro transport of amino acids into the cell. A possible mechanism of inhibition may involve interactions with nucleoproteins, nucleic acids, or polyribosomes, which are the templates for cellular protein synthesis. Recently, chloroquine has been reported to inhibit the in vitro incorporation of amino acids into protein by a cell-free preparation of liver microsomes and a polynucleotide-directed liver ribosomal system. Chloroquine inhibition of phenylalanine incorporation into RPE protein at very short periods of incubation (Figs. 2 and 3), suggests an immediate interaction with those macromolecules essential in directing protein synthesis and, therefore, is in agreement with those results. However, our data cannot exclude a possible mode of action by chloroquine on DNA or RNA synthesis when the drug is present in the RPE cell in vivo, as has been reported for some bacterial cultures.
Although hydroxychloroquine has been reported to be less toxic than chloroquine during long-term therapy, reports of macular degeneration and a "bulls-eye" retina after hydroxychloroquine have appeared.\textsuperscript{2,29} Furthermore, hydroxychloroquine and desethylchloroquine, the major metabolite of chloroquine, concentrate in the eye of the pigmented rat.\textsuperscript{12,13} Both of these compounds in our experiments significantly inhibited amino acid incorporation into RPE protein in vitro, suggesting interaction with these molecules essential for protein synthesis. In addition, combinations of chloroquine with hydroxychloroquine or desethylchloroquine additively inhibited amino acid incorporation into proteins suggesting that combined therapy may not result in lower retinal toxicity. More important, metabolism of the parent drug to its mono-deethylated derivative may not inactivate the drug but only prolong the toxicity of the parent compound.

On the basis of all the foregoing we suggest that two factors are involved in chloroquine toxicity. The first is high local concentration of the drug over long time periods due to adsorption on melanin and gradual elution. The second is exertion of toxicity by inhibition of protein synthesis.

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