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Mechanism of Chloroquine Transport in the Isolated Retina

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SUMMARY

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The feasibility of using the isolated rat retina to study transport processes was ascertained. The incubated tissue was characterized by a constant extracellular space, high steady-state levels of energy reserves, and high ratio of cellular K^+ to Na^+. The mediated transport system for chloroquine was experimentally resolved from total drug uptake and was distinguished from the nonsaturable uptake component as well as from tissue binding. The saturable uptake process had an apparent K_m and V_{max} of 2.4 mM and 8 nmoles/mg, dry weight, per minute, and resulted in retinal accumulation of chloroquine relative to its concentration in the medium. The uptake was independent of monovalent cations and displayed a Q_{10} of 1.62. Metabolic inhibitors and ouabain strongly enhanced the rate of chloroquine uptake. Exposure of the retina to these substances abolished the characteristics of the saturable transport system and resulted in drug uptake by a markedly stimulated, nonmediated process, apparently because of increased permeability of the plasma membrane. The saturable uptake increased at high pH, the data indicating an uncharged form of the drug during transport. Both uptake and exodus of chloroquine displayed the phenomenon of countertransport. Cellular disruption altered the sensitivity of uptake to temperature and pH, and abolished drug accumulation as well as countertransport. Quinacrine, amphetamine, and cocaine, but not biogenic amines, competitively inhibited the uptake of chloroquine and transaccelerated its transport. The results provide evidence for the existence in the retina of a mediated transport system for drugs structurally related to basic amines.

INTRODUCTION

We recently characterized an active transport system in leukocytes for the uptake of benzomorphans (1, 2) and other central nervous system-active drugs (3, 4). Although the transport-related binding affinities of the drugs in leukocytes correlated well with the ease of their transfer across the blood-brain barrier (5), the nature of the investigated compounds suggested a study of their transport in cellular preparations from the nervous system. The experimental design and kinetic interpretation of data on transport favor the use of unicellular suspensions over cellular preparations containing extracellular space. Although synaptosomes have recently been used to investigate the uptake of CNS drugs (6), the availability of isolated neural cells with acceptable trans-

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port-related viability, i.e., with a nonleaking plasma membrane, is severely limited (7, 8).[2] Swelling, caused by increasing uptake of water during incubation, presents a particular disadvantage in evaluating uptake processes in brain slices (9). Furthermore, in view of the structural and functional heterogeneity of brain tissue, comparable populations of cells in the individual slices must be assured.

For these reasons we investigated the feasibility of utilizing the isolated rat retina, a conveniently available, structurally well-defined neural tissue, as a model to study the uptake of CNS drugs. Initial experiments revealed that the water content of both whole and quartered rat retinae was constant during incubation for up to 60 min at 30° (10). Although retinal uptake of endogenous compounds, including biogenic amines (11), γ-aminoisobutyric acid (12, 13), and amino acids (13–16), has been studied, no information is available on the transport of drugs in this tissue. Extensive stereospecific binding of narcotic drugs in the rat retina has recently been demonstrated (17).

The selection of chloroquine [7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline] as a model drug in the present study was based in part on the retinal effects of this compound. Chloroquine retinopathy has been described in humans (18, 19) and induced in animals, including the rat (20, 21). Moreover, chloroquine represents a basic amine, a common structural feature of all the drugs recognized by the mediated transport systems previously described in leukocytes (2, 4) and synaptosomes (6).

MATERIALS AND METHODS

Materials. The drugs used in this study either were purchased from the Hospital Pharmacy, University of Michigan, or were kindly provided by Drs. H. H. Swain and J. H. Woods, Department of Pharmacology, University of Michigan Medical Center. [*ring*-3-^{14}C]Chloroquine (4.48 mCi/mmole), [*carboxyl*-^{14}C]inulin, Protosol tissue solubilizer, and Omnifluor scintillant were obtained from New England Nuclear Corporation. [*G*-^{14}C]Sucrose was a gift from Dr. G. V. Jourdian, Department of Biological Chemistry, University of Michigan. Enzymes and biochemicals were purchased from Boehringer/Mannheim and Sigma Chemical Company.

Isolation of retina. Male Sprague-Dawley rats weighing 300 g were anesthetized with hexobarbital (250 mg/kg intraperitoneally), and the eyeball was extirpated with curved scissors and hemisected along the ora serrata with a razor blade. The lens and vitreous humor were removed, and the remaining tissue was immersed in buffer kept in a Petri dish on ice. The buffer medium contained Tris-HCl, 35 mM; NaCl, 110 mM; KCl, 4 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 0.75 mM; KH_2PO_4, 1.2 mM; glucose, 10 mM; and sucrose, 78 mM. The final pH and the measured osmolarity of the buffer were 7.4 and 374 mOsmoles/kg, respectively. The retina was freed from the eye cup by severing the optic nerve with a wire loop and gently lifted into the medium. To increase the number of achievable determinations, in most experiments the dissected retinae were further divided into four equal segments by means of a scalpel. The total dissection of one retina lasted 1–1.5 min. The dissected tissue was usually suspended in buffer and incubated at 30° with oxygen bubbling through the medium. Further experimental details are given below or in the figure and table legends.

Retinal contents of water, potassium, sodium, phosphocreatine, and ATP. To measure the water content at various times of incubation, the retina was carefully lifted from the medium using a wire loop, blotted with filter paper, and weighed on a Cahn electrobalance. Samples were freeze-dried overnight, and the measured dry weight was used to calculate water content. In experiments designed to determine tissue swelling, some of the retinae were dissected on a block of paraffin rather than in buffer medium.

For the determination of retinal K^+ and Na^+, the incubated tissue was filtered as described in the experiments on drug uptake, washed twice with 10 ml of ice-cold

[2] J. E. Dyer and F. Medzihradsky, unpublished observations.

0.32 M sucrose, then freeze-dried and weighed. The dried tissue was mixed with 10 μl of distilled water and digested with 50 μl of concentrated HNO_3 for 1 hr at 60°. After dilution with 5 ml of 15 mEq/liter of LiCl, used as internal standard, the samples were measured in a flame photometer against appropriate standards of potassium and sodium.

For the determination of PC[3] and ATP, retinae were quickly collected on a nylon screen as described below and frozen in liquid N_2. At −20° the retinae were lifted off the screen and ground in a 1-ml, all-glass Potter-Elvehjem homogenizer with 100 μl of 0.1 N HCl in absolute methanol. The samples were warmed to 0°, 500 μl of 0.02 N HCl were added, and the homogenization was completed. Aliquots of the homogenate were analyzed for protein. To 400 μl of the homogenate were added 60 μl of 3 N $HClO_4$–1 mM EDTA, and the samples were mixed, centrifuged at 1700 × g for 5 min, decanted, and then stored at −70° until analysis. The concentrations of PC and ATP were determined by enzymatic analysis, measuring the fluorescence of reduced nicotinamide adenine dinucleotides (22). The reaction mixture (pH 7.5) contained Tris-HCl, 100 mM; bovine serum albumin, 0.01%; $MgCl_2$, 5 mM; glucose, 1 mM; NADP, 0.05 mM; ADP, 0.025 mM; glucose 6-phosphate dehydrogenase, 3 μg/ml; hexokinase, 4 μg/ml; and creatine phosphokinase, 8.0 μg/ml.

Protein. Protein was determined according to Lowry *et al.* (23), using an adapted microprocedure (24). A conversion factor of 500 μg of protein per milligram of retinal dry weight was established.

Determination of extracellular space and intracellular pH. After initial incubation of whole or quartered retinae in buffer medium for 30 min, [^{14}C]inulin or [^{14}C]-sucrose was added at a final concentration of 0.4% or 0.1 mM, respectively. In experiments with radiolabeled sucrose, the sucrose in the standard buffer medium was replaced with mannitol. Following a 3-min incubation, radioactivity in the retina was determined as described below.

Intracellular pH was determined using ^{14}C-labeled 5,5-dimethyloxazolidine-2,4-dione (kindly provided by Dr. H. N. Christensen, Department of Biological Chemistry). After a 30-min incubation with retina, the distribution of the radiolabeled compound between intra- and extracellular water was determined and evaluated as described (25).

Uptake of radiolabeled drug. The dissected tissue was suspended in 1 ml of buffer medium and incubated for 60 min at 30° with oxygen bubbling through the medium. An appropriate amount of drug, dissolved in 250 μl of buffer, was then added, and the suspension was incubated further at 30°. At given times the incubations were terminated by collecting the retina under gentle suction on a 333-mesh nylon screen cut out over a standard (2.4-cm diameter) stainless steel support screen (Millipore). The retina was rapidly washed with 10 ml of ice-cold buffer, removed from the screen with a small bent spatula, placed in a 30 × 10 mm test tube, and freeze-dried overnight at a temperature gradient of 20° to −70° at 0.1 mm Hg. In some experiments the initially incubated retina was filtered and quickly washed, and the screen was immersed in a fresh incubation vial containing the appropriate solution. The empty screen was carefully lifted out of the vials with pointed forceps. The dried samples were weighed, placed in a counting vial, and mixed with 10 μl of distilled water. After the addition of 0.3 ml of Protosol, the vials were heated for 1 hr at 60° and cooled to room temperature, and 10 ml of toluene-based Omnifluor scintillation mixture were added. The radioactivity was determined in a Packard liquid scintillation spectrometer, model 3320, and was corrected for quenching by appropriately established curves. Mean counting efficiencies were 69% for ^{3}H and 90% for ^{14}C.

In most experiments uptake was terminated while linear; however, in some cases the uptake at equilibrium was determined. Inhibition constants (K_i) of drug uptake were estimated from Dixon plots of the data. The concentration of chloroquine during uptake was 1 mM, well below the

[3] The abbreviations used are: PC, phosphocreatine; DNP, 2,4-dinitrophenol.

determined K_m of the drug. For studies of countertransport, the concentration of the radiolabeled drug was less than 5% of that of the unlabeled compounds.

RESULTS

Characteristics of isolated retina. The average weight of the retina was 6.6 mg, and the water content was 78% ± 2% of the wet weight. The latter value was determined immediately after isolation, with care to avoid contact of the tissue with the aqueous phase, and remained constant during incubation of the tissue for up to 60 min. Extracellular space, determined with 0.4% [^{14}C]inulin and 0.1 mM [^{14}C]sucrose, was 23% ± 3% and 22.4% ± 4% of the wet weight, respectively. Incubation with 78 mM sucrose, the concentration present in the standard buffer medium, gave similar values for extracellular space. The extent of the latter was considered in calculating all results on retinal content or uptake. The previously reported water content of the isolated retina was 82–85% of its wet weight in the rat (13) and 80% in the rabbit (26). Swelling of the rat retina was "slight" in bicarbonate buffer and ranged up to 165% in "different test media" (26). The extracellular space of isolated retina amounted to 39% of its wet weight in the rat (13) and 29% in the rabbit (26).

Retinal contents of the energy reserves PC and ATP were depleted immediately following isolation but recovered 4- and 2-fold after incubation in the buffer medium for 60 min (Table 1 and Fig. 1). The levels reached were similar to those found in the retinae of quickly frozen rat eyes[4] and in the mouse retina *in vivo* (27). Dissecting the retina into four equal segments did not affect the concentration of either PC or ATP (Table 1). Omitting glucose from the incubation medium caused a significant decrease in the tissue contents of both PC and ATP. Furthermore, exposure of the isolated retina to various metabolic inhibitors resulted in severe depletion of the energy reserves (Table 1).

During incubation for up to 120 min, the ratio of K^+ to Na^+ in the retina remained constant at about 4:1 (Table 1). This value is higher than the range (1.2–3.0) measured in rabbit retinae *in vitro* (26). Although metabolic inhibitors caused pronounced depletion of cellular K^+ and an increase in cellular Na^+, high concentrations of chloroquine had no effect on the distribution of these ions (Table 1).

Saturability of chloroquine uptake. Total retinal uptake of chloroquine increased with time and concentration (Figs. 2 and 3). From the linear portion of uptake, obtained at high concentrations of drug in the medium, a kinetic constant for the nonsaturable component of the process (K_{ns}) was calculated. Correcting the total uptake by this factor (0.42 nmole/3 min/mg, dry weight) resulted in plots representing diffusion and the saturable component of the transport system (Figs. 2 and 3). The latter displayed an apparent K_m and V_{max} of 2.4 mM and 8 nmoles/min/mg, dry weight (Fig. 4). At 30 min, retinal accumulation of chloroquine by the saturable process was 4-fold relative to the 1 mM concentration in the medium. The possibility that such distribution reflected a gradient in hydrogen ion concentration across the plasma membrane was excluded by the determined intracellular pH of 7.1.

Temperature dependence of chloroquine uptake. At decreased temperatures the rate of chloroquine uptake was inhibited noncompetitively (Fig. 5). An Arrhenius plot of uptake at various temperatures was linear, and between 20° and 30° its slope corresponded to a Q_{10} of 1.62.

Effects of metabolic inhibitors on uptake of chloroquine. Incubation of the retina with any of several metabolic inhibitors or ouabain resulted in a pronounced increase in the uptake of chloroquine (Fig. 6), whereby both K_m and V_{max} of the uptake process became elevated.[4] Anaerobic conditions caused little change in the extent of the effect. Enhancement of chloroquine uptake by DNP[3] or ouabain was most pronounced after exposure of the retina to the action of these compounds for 5–10 min. At that time the retina was characterized by considerably depleted contents of PC and ATP and an impaired ratio of K^+ to Na^+ (Figs. 7 and 8). The effect weakened as

[4] D. J. Bednarczyk and F. Medzihradsky, unpublished observations.

TABLE 1

Retinal content of energy reserves, potassium, and sodium

Isolated whole or quartered retinae were incubated for 60 min at 30° in the buffer medium described in the text, or for 30 min in the buffer followed by a 30-min incubation in the presence of the compounds listed below. Experimental details are described under MATERIALS AND METHODS. In separate experiments, retinae were incubated for 120 min in glucose-free incubation medium, or frozen in liquid nitrogen immediately after isolation. Following each of these treatments, the retinae were quickly filtered and placed in liquid nitrogen, and the frozen tissue was subjected to analysis as described under MATERIALS AND METHODS. Values are the means and standard deviations of four to eight determinations using different retinae.

Conditions	Tissue concentration of			
	PC	ATP	K^+	Na^+
	nmoles/mg, dry wt		*μEq/mg, dry wt*	
No incubation	3.3 ± 1.6	3.4 ± 0.2		
Buffer medium, whole retinae	12.3 ± 2.4	6.8 ± 0.8		
Buffer medium, quartered retinae	13.3 ± 1.5	6.4 ± 2.6	2.25 ± 0.18	0.53 ± 0.21
Buffer medium, no glucose	2.4 ± 0.2	2.9 ± 0.2		
NaF (30 mM)	1.1 ± 0.4	1.0 ± 0.2		
KCN (3 mM)	0.6 ± 0.2	1.3 ± 0.3		
NaF (30 mM) + KCN (3 mM)	0.5 ± 0.3	0.4 ± 0.1	0.69 ± 0.08	3.62 ± 0.30
DNP (3 mM)	0.2 ± 0.2	0.8 ± 0.2	0.38 ± 0.05	3.12 ± 0.26
Ouabain (1 mM)	7.8 ± 2.8	4.9 ± 2.3	0.34 ± 0.11	2.84 ± 0.24
Chloroquine (8 mM)			2.12 ± 0.16	0.49 ± 0.10

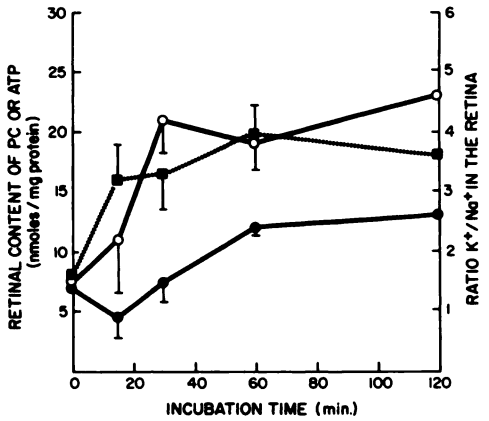

FIG. 1. *Viability of isolated retinae*

Retinal contents of phosphocreatine (○——○) and ATP (●——●) and the K^+:Na^+ ratio (■-----■) were determined immediately following dissection and after various times of incubation in the standard medium. Further treatment of tissue and analytical methods are described under MATERIALS AND METHODS. Values are the means and standard deviations of four experiments, in which duplicates were run for each point of observation.

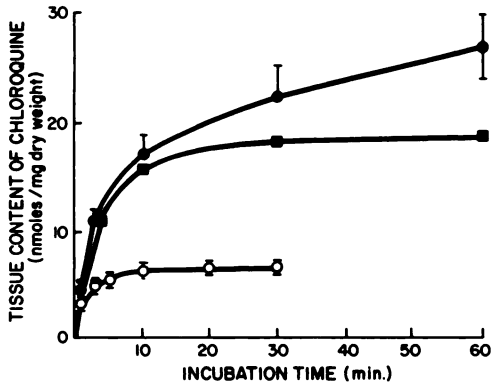

FIG. 2. *Time course of chloroquine uptake in retina*

Quartered retinae were initially incubated for 60 min in the standard incubation medium as described under MATERIALS AND METHODS. After the addition of 1 mM radiolabeled chloroquine, the tissue content of radioactivity was determined at various times. Plotted are the total retinal uptake of [^{3}H]chloroquine (●——●) and uptake corrected for the nonsaturable component (■——■). In addition, uptake by previously homogenized retinae was determined (○——○). Experimental details for incubation and quantitation of drug uptake are described under MATERIALS AND METHODS. Values were corrected as described in the text. Values are the means and standard deviations of at least eight determinations using different preparations of retinae.

cellular K^+:Na^+ ratios approached 0.5. Although in control retinae, at high concentrations of chloroquine, uptake was proportional to the amount of drug in the medium (Fig. 3), after exposure to DNP

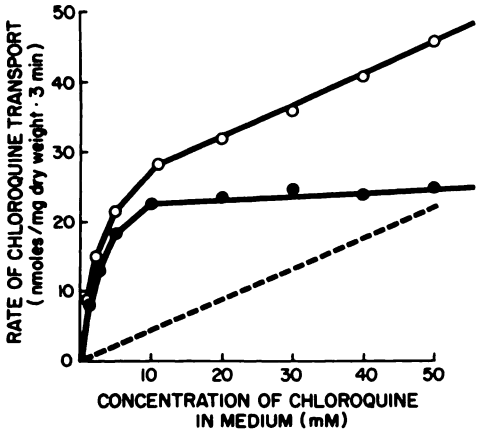

FIG. 3. *Saturability of chloroquine uptake*

After initial incubation of the tissue as described in the legend to Fig. 2, retinal uptake of chloroquine at 3 min was determined at various concentrations of the drug in the medium. Plotted are total uptake (○——○) and uptake by the saturable (●——●) and nonsaturable (- - -) transport components. The method of resolving the individual components is described under RESULTS. Values are the means of four experiments.

the tissue became saturated at these concentrations. Sucrose space in retinae previously incubated with 3 mM DNP was 26.5% ± 3.9% of wet weight, a value not statistically different from results obtained with control tissue. Furthermore, no change in intracellular pH was measured in treated retinae.

Effect of pH on chloroquine uptake. First the K_{ns} values for uptake at various pH were determined. At pH 6.4, 6.8, 7.0, 7.4, 7.8, and 8.3, the corresponding constants were 0.50, 0.48, 0.40, 0.42, 1.62, and 1.98 nmoles/3 min/mg, dry weight. These factors were used to correct the total uptake of chloroquine for the nonsaturable transport component at each pH. With rising pH, experimentally limited to 8.3 because of insolubility of the drug, the rate of saturable uptake of chloroquine increased. Between pH 7.5 and 8.3 the V_{max} increased proportionally; however, the K_m remained constant (Fig. 9). Similar pH dependence was characteristic of the transport system in leukocytes translocating drugs with

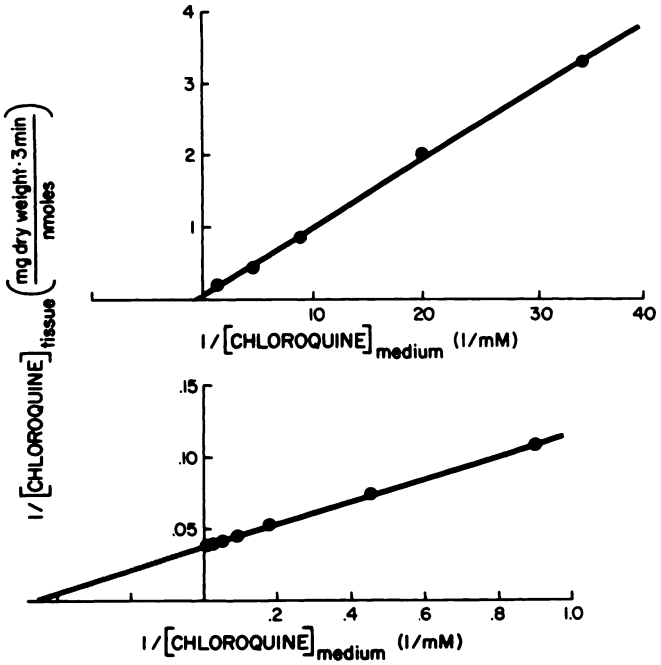

FIG. 4. *Lineweaver-Burk plot of chloroquine uptake by the saturable transport component*

The lower and upper graphs show uptake at limited and extended concentration ranges of the drug in the medium, respectively. The apparent K_m and V_{max} were 2.4 mM and 8 nmoles/min/mg dry weight. Values are the mean results obtained in four experiments.

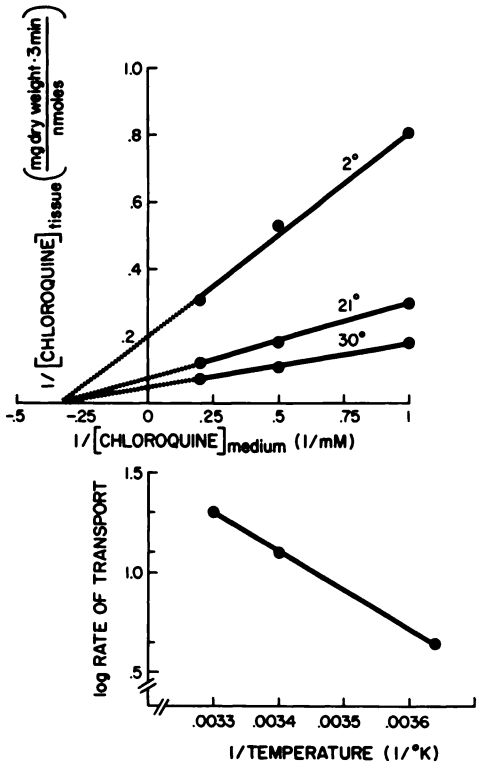

FIG. 5. *Effect of temperature on chloroquine uptake*

Quartered retinae were initially incubated for 60 min at 30° in the standard buffer medium, and then for 30 min at the indicated temperatures. Radiolabeled chloroquine was added, and uptake at 3 min was determined. The upper graph shows Lineweaver-Burk plots of chloroquine uptake by the saturable transport process. The lower figure represents an Arrhenius plot of the data. Between 20° and 30° the slope of the line corresponds to a Q_{10} of 1.62. Values are the means of four experiments.

pK_a values between 8 and 10 (4). By titration, the lower pK_a of chloroquine was determined as 8.3. The unchanged cellular permeability in the retina during short-term exposure to altered pH was ascertained by the steady ratio of K^+ to Na^+. Exposure of the retina to metabolic inhibitors altered the pH dependence of drug transport. Whereas in control tissue the ratio of the rate of chloroquine uptake at pH 8.2 and 7.4 was 2.3, in retina treated with DNP or ouabain it was 1.3 and 1.0, respectively.

Ion dependence of chloroquine uptake. After initial incubation in the standard buffer, the retinae were filtered and washed with 0.32 M sucrose. The uptake of radiolabeled chloroquine was determined using a buffer in which NaCl replaced KCl or an isosmotic amount of mannitol replaced NaCl. The results showed no statistically significant difference in the uptake of chloroquine relative to the control.

Efflux of chloroquine. Exodus of chloroquine was rapid, and at equilibrium 57% of the drug was released into the medium (Fig. 10). Analysis of the data showed that for the first 3 min the rate of exodus was linear.

Countertransport during retinal uptake and efflux of chloroquine. Exposing the tissue to 8 mM chloroquine for 30 min prior to addition of the radiolabeled drug resulted in uptake rates which were signifi-

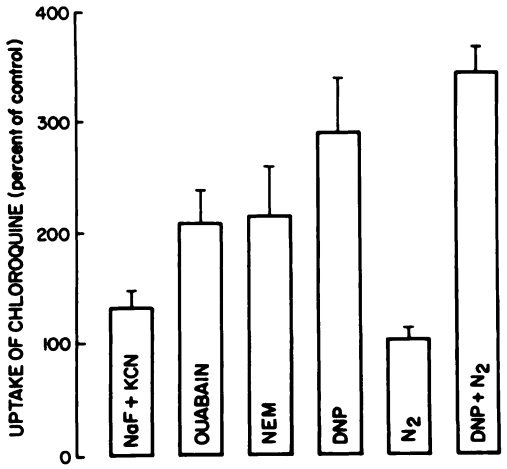

FIG. 6. *Effects of metabolic inhibitors and ouabain on chloroquine uptake*

After initial incubation of retinae in the standard buffer medium, the tissue was incubated for 30 min under anaerobic conditions (N_2) or in the presence of either 1 mM ouabain, 3 mM *N*-ethylmaleimide (NEM), 30 mM NaF plus 3 mM KCN, or 3 mM DNP. To maintain isotonicity, the content of sucrose in the buffer medium was decreased proportionally to the added amount of inhibitor. In control experiments the retinae were incubated for the same period of time in the standard buffer medium. After these treatments the uptake of radiolabeled chloroquine (1 mM) was determined at 3 min. Values are the means and standard deviations of eight determinations.

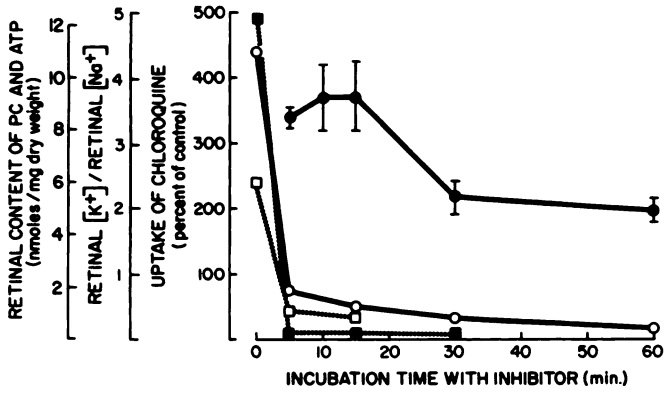

FIG. 7. *Onset and metabolic correlates of effect of DNP on chloroquine uptake*

Isolated retinae were exposed to DNP as described in the legend to Fig. 6. Prior to the addition of the inhibitor, and after incubation of the retina in its presence for the indicated lengths of time, tissue concentrations of PC (■-----■) and ATP (□-----□), as well as K^+ and Na^+, were determined. From the latter two values the cellular K^+:Na^+ ratio was calculated and plotted (○——○). Also determined was the retinal uptake of radiolabeled chloroquine (●——●), and expressed as a percentage of uptake in control tissue, which had been incubated for the same period of time in the absence of inhibitor. Experimental details are described under MATERIALS AND METHODS. Values are the means and standard deviations of four determinations.

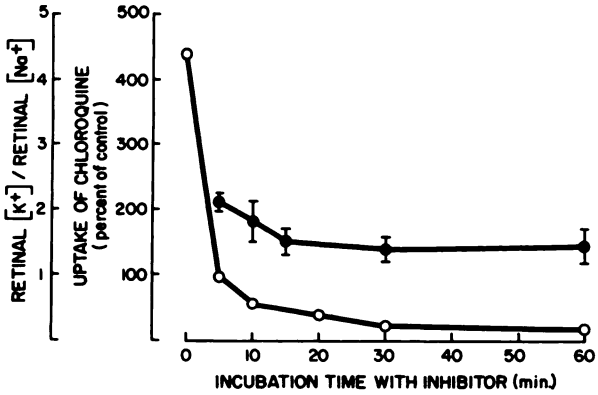

FIG. 8. *Onset and metabolic correlates of effect of ouabain on chloroquine uptake*

The experimental conditions were the same as described in the legend to Fig. 7. Prior to addition of the inhibitor and after incubation of the retina in its presence for various periods of time, tissue contents of K^+ and Na^+ were determined and expressed as the K^+:Na^+ ratio (○——○). Also determined was the uptake of radiolabeled chloroquine (●——●) compared to control tissue, which had been incubated for the same period of time in the buffer medium without inhibitor. Values are the means and standard deviations of four determinations.

cantly higher relative to controls, which had initially been incubated in the absence of drug (Fig. 11). Exposure of the retina to high concentrations of chloroquine did not affect tissue permeability (Table 1). Countertransport was also demonstrated during efflux of chloroquine: the rate of exodus of radiolabeled chloroquine was proportional to the external concentration of the unlabeled drug (Fig. 11). Initial incubation of the retina with DNP or ouabain abolished countertransport.

Uptake of chloroquine in disrupted tissue. Uptake of the drug in these experiments was expressed on the basis of protein and correlated with results obtained with intact tissue by using the conversion factor described under MATERIALS AND

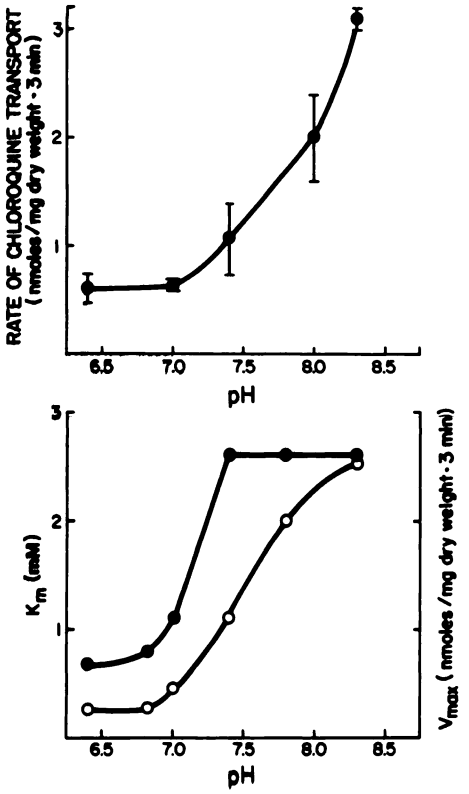

FIG. 9. *Effect of pH on uptake of chloroquine*
After the usual incubation of retinae in the standard buffer, pH 7.4, the hydrogen ion concentration of the medium was changed to the indicated values by the addition of aliquots of the same buffer medium, which had been adjusted to the appropriate pH. The rate of radiolabeled chloroquine uptake was determined at 3 min (upper graph). From these data, K_m (●——●) and V_{max} (○——○) were estimated graphically and plotted against pH (lower graph). Shown are results of representative experiments, in which the standard deviation was less than ±10%.

METHODS. After 30 min of incubation the uptake of chloroquine by homogenized retinae amounted to 28% of control values (Fig. 12). Chloroquine uptake in disrupted tissue was insensitive to changes in temperature, and the uptake at pH 8.2 was not significantly different from that at pH 7.4. Furthermore, disruption of the retina abolished countertransport during the uptake of chloroquine.

Interaction of drugs and biogenic amines with retinal uptake of chloroquine. Quinacrine (6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridine), a compound structurally similar to chloroquine, competitively inhibited the uptake of the latter drug (Fig. 13). Competitive interaction was also obtained with amphetamine and cocaine. The corresponding K_i values, obtained from Dixon plots of the uptake data (Fig. 14), were 4.0 mM for quinacrine, 2.5 mM for amphetamine, and 1.5 mM for cocaine. In addition, several compounds exhibited noncompetitive inhibition of chloroquine uptake, e.g., methadone, codeine, imipramine, norepinephrine, and 5-hydroxytryptamine. However, dopamine, at concentrations from 8 μM to 12.8 mM, had no effect on retinal chloroquine uptake.

DISCUSSION

The isolated retina was able to recover and then maintain high steady-state concentrations of both PC and ATP. During prolonged incubation at 30° no swelling of the the tissue was observed. Important evidence of the transport-related viability of the preparation during incubation was the high, constant tissue K^+:Na^+ ratio. This

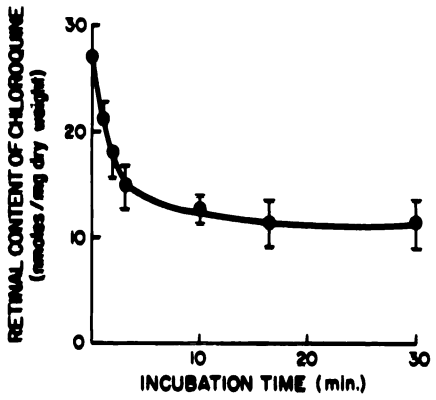

FIG. 10. *Exodus of chloroquine from retina*
After initial incubation of isolated retinae for 60 min in the buffer medium described in the text, the tissue was incubated for 30 min with 1 mM radiolabeled chloroquine. The samples were rapidly diluted 15-fold with the buffer medium, and the retinal content of radiolabeled drug was determined at the times indicated by quick filtration of the tissue as described under MATERIALS AND METHODS. Presented are results of a representative experiment, each point being the mean and standard deviation of four determinations.

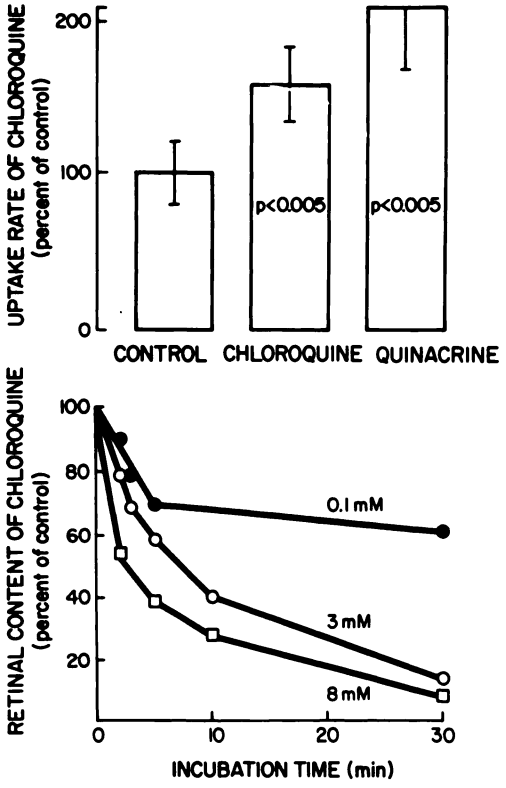

FIG. 11. *Transacceleration of chloroquine transport*

After incubation of isolated retinae in the buffer medium as described under MATERIALS AND METHODS, the tissue was incubated for 30 min in the presence of 8 mM chloroquine or 8 mM quinacrine. The uptake of radiolabeled chloroquine (upper graph) was determined as described in the text. The concentration of added radiolabeled chloroquine was less than 5% of that present during the initial incubation of tissue. In efflux experiments the conditions were the same as in Fig. 10, except that the initial uptake medium was diluted 9-fold, using solutions of unlabeled chloroquine in the buffer medium to give the final concentrations of the drug indicated in the lower graph. Values are the means and standard deviations of eight determinations.

ratio, in addition to the cellular content of K^+, was recently described as a markedly sensitive measure of the presence of a nonleaking plasma membrane (28). The cellular content of these cations also indicated optimal conditions for the synaptosomal transport of biogenic amines (29). Thus the tissue concentrations of energy reserves, the distribution of monovalent cations, and the constant extracellular space during incubation indicated that the isolated retina fulfills the criteria for a tissue preparation suitable for studying cellular transport. An electroretinogram of isolated rat retina has been published (30).

Retinal transport of chloroquine occurred both by a saturable process and by an uptake component related to diffusion. The basic amine, with pK_a values of 8.3 and 10.4, was expected to exhibit nonspecific binding to tissue (31, 32). Accordingly, the concentration-dependent total drug uptake in the isolated retina did not reach equilibrium within 1 hr. The saturable component of chloroquine transport

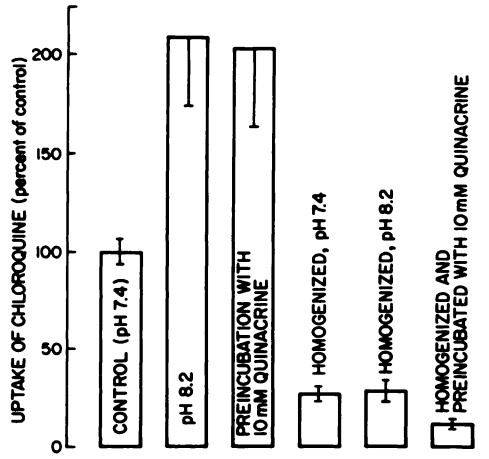

FIG. 12. *Uptake of chloroquine in disrupted retinae*

Retinae were disrupted in an all-glass Potter-Elvehjem homogenizer and then incubated with radiolabeled chloroquine (1 mM) for 30 min at either pH 7.4 or 8.2. The latter pH was achieved by addition to the incubation medium of aliquots of the buffer medium that had been adjusted to appropriate pH values. In separate experiments, homogenized retinae were incubated for 30 min with 10 mM quinacrine before drug uptake was determined. The incubation of disrupted retinae was terminated by filtration through glass fiber filters (Reeve Angel, No 984-H) soaked in 0.9% NaCl saturated with amyl alcohol. Control experiments, with intact tissue, were carried out as described in the legends to Figs. 9 and 11. The data obtained with intact and disrupted tissue were correlated by the use of a conversion factor equaling 500 μg of protein per milligram of retinal dry weight. Values are the means and standard deviations of eight determinations.

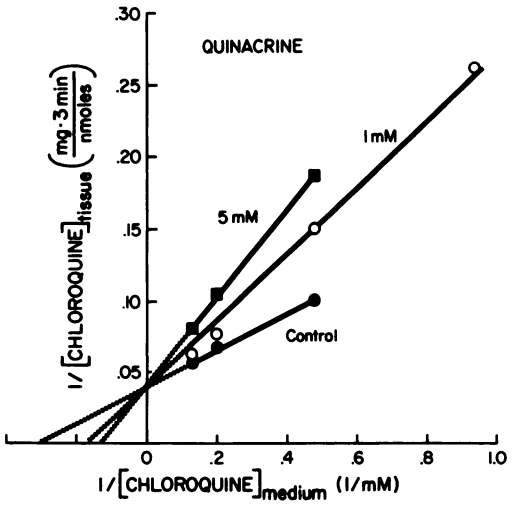

FIG. 13. *Competitive inhibition by quinacrine of retinal uptake of chloroquine: Lineweaver-Burk plot*

Uptake was determined as described under MATERIALS AND METHODS. Each point represents the mean of four determinations carried out with different preparations of retinae.

was resolved by applying a correction factor (33) by analogy with Fick's law for diffusion. In addition to diffusion, this factor is likely to have reflected processes of nonsaturable drug-tissue interaction. Additional resolution of transmembrane transport from nonspecific binding was obtained by using disrupted tissue. The results indicated that the saturable uptake of chloroquine was dependent on an intact plasma membrane of retinal cells. In addition to abolishing retinal drug accumulation, membrane perturbation altered the pH and temperature dependence of drug uptake.

The apparent K_m for saturable chloroquine uptake was considerably higher than the affinity constants which characterized the transport of drugs in leukocytes (4) and synaptosomes (6). However, the uptake *in vitro* of various amino acids in various tissues, including brain slices, also occurred at concentrations in the millimolar range (33–35). At the lowest achievable concentration of chloroquine, limited by the specific radioactivity of the compound, no saturable drug uptake was detectable. A significant effect of metabolism during short-term incubation of the isolated retina, as carried out in this study, was discounted. After chronic administration to rats, more than 88% of the chloroquine in the eye as well as in other tissues was present unchanged, the rest being desethylchloroquine (32, 36).

Despite its relatively high value, the Q_{10} for chloroquine uptake cannot be interpreted as strong evidence of mediated transport. The translocation of a permeant by a process of diffusion can conceivably result in the dissociation of numerous hydrogen bonds and thus exhibit a temperature coefficient similar to that of a chemical reaction (37). Metabolic inhibitors and ouabain unexpectedly increased the rate of chloroquine uptake. As a result of inhibitor action, the characteristics of mediated

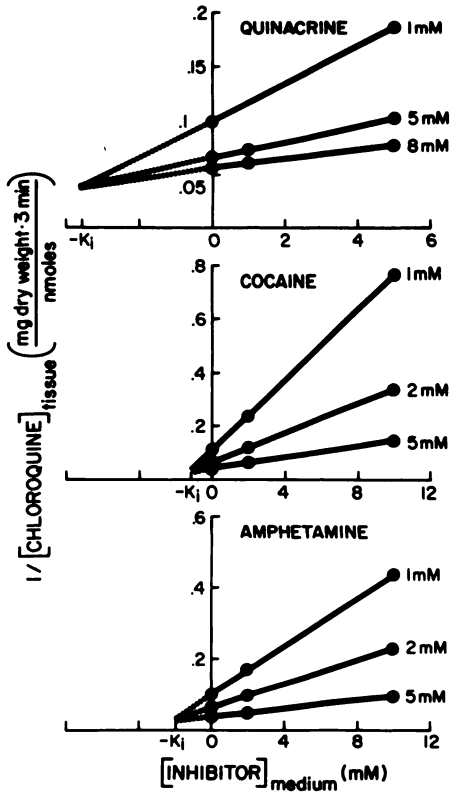

FIG. 14. *Dixon plots for estimation of inhibition constants*

The uptake of radiolabeled chloroquine was determined at different concentrations of the drug and inhibitors. The values of the inhibition constants are listed under RESULTS. Values are the means of four determinations.

transport were abolished (or became masked), and chloroquine uptake occurred primarily by a markedly enhanced, nonmediated process displaying saturation even at very high concentrations of the drug. Neither an enlarged extracellular space nor a shift in intracellular pH accounted for the observed effect. In the isolated rat diaphragm DNP and sodium arsenate exerted a similar effect on glucose transport (38, 39), and DNP likewise affected the uptake of α-aminoisobutyric acid (40). Neither phenomenon was further studied by the authors. The previously reported uptakes of amino acids and of γ-aminoisobutyric acid in the retina were inhibited by metabolic poisons.

At cellular concentrations of K^+ and Na^+, reflecting increased perturbation of the plasma membrane (28), the effect of the inhibitors weakened, and in frozen-thawed retinae it was abolished.[4] By depleting ATP concentrations and by direct inhibition of $(Na^+ + K^+)$-ATPase, the investigated metabolic poisons and ouabain can alter the cellular distribution of K^+ and Na^+, thus decreasing the transmembrane potential and possibly causing perturbations in the plasma membrane (41, 42). The latter effect can, in turn, profoundly affect membrane function related to transport. Incubation of brain slices with ouabain or cyanide for more than 40 min caused irreversible inhibition of amino acid uptake (43, 44). The latter findings were interpreted as permanent effects of the inhibitors on the uptake system, such as changes in membrane structure. In sum, these observations indicate that the effects of inhibitors in the retina are linked to the vesicular nature of the cell and are due to induced structural changes in the plasma membrane, resulting in increased permeability toward chloroquine. The marked enhancement of nonmediated uptake prevented assessment of the effects of metabolic inhibitors on the saturable transport system. Inhibition of the latter process would have fulfilled a criterion of active transport, being responsible for the retinal accumulation of chloroquine against its concentration gradient.

Evaluation of the pH dependence of transport was made difficult by the complexity of the tissue and the existence of two pK_a values of chloroquine. Nevertheless, the horizontal slope of the curve representing the change in K_m with pH (Fig. 9) suggests (45) that, as a result of drug-carrier interaction, and like the uptake of drugs in leukocytes (2), chloroquine is transported with the involved amine group present in its uncharged form. The uptake of chloroquine in frozen-thawed or homogenized retinae, in contrast to normal tissue, was unaffected by rising pH, suggesting that the increase in uptake at higher pH was not due to enhanced protein binding of the drug. The observed pH dependence is seemingly in agreement with diffusion as the underlying process of drug transport (e.g., refs. 46 and 47). However, a positive correlation with pH of a mediated uptake process, as described in the retina, leukocytes, and synaptosomes, is quite plausible. Binding of the investigated drugs, structurally all related to basic amines, with pK_a values in the range of 8–10, to carriers in the plasma membrane is likely to occur in a lipophilic environment, thus depending on the relative lipid solubility of the compounds at a given hydrogen ion concentration.

Efflux of chloroquine from retinae initially followed first-order kinetics. In view of the substantial degree of dilution required to observe true efflux (37), the relatively large amount of drug retained by the tissue in equilibrium could reflect secondary recapture of the solute from the medium. During both efflux and uptake of chloroquine countertransport was demonstrated, whereby a compound on a given side of a membrane accelerates the translocation of the solute present on the opposite side of the barrier (48, 49). Countertransport is predicted for systems in which a substrate is bound to a mobile membrane component, but not to fixed binding sites. In contrast to the process of diffusion, governed by Fick's law, during countertransport the solute actually moves against its concentration gradient. Therefore the demonstration of this phenomenon pre-

sents strong evidence for the involvement of a carrier mechanism in the transport process under observation. Initial tissue disruption abolished countertransport during both uptake and efflux of chloroquine.

The saturable uptake of chloroquine was competitively inhibited by quinacrine, which exhibited a K_i similar to the K_m of chloroquine uptake. The latter relationship suggests that the two drugs bind to a common carrier (37). Competitive inhibition was also exhibited by amphetamine and cocaine. All three drugs transaccelerated both the uptake and efflux of chloroquine, further suggesting their recognition by a common transport carrier. On the other hand, no competitive interaction was obtained with 5-hydroxytryptamine, norepinephrine, or dopamine. Dopamine was previously identified as the predominant biogenic amine in the retina (50). In contrast to retinal uptakes of amino acids, the transport process described here was independent of Na^+ in the medium. All the drugs transported in the retina represent basic amines and, except for amphetamine, bear little resemblance to known endogenous constituents of this tissue. Similar structural specificity and ion dependence characterized the transport system for CNS drugs in leukocytes (2, 4). However, in contrast to the findings in leukocytes, and despite the observed accumulation of drug in retina, dependence of uptake on metabolic energy could not be ascertained in this tissue. Nevertheless, both transport systems share certain characteristics and differ from those responsible for the cellular uptake of biogenic amines and amino acids. Their presence indicates that the entry of drugs into cells frequently occurs by mediated transport, leading to cellular accumulation of these compounds. Despite the relatively wide specificity of these transport systems, the high cellular concentration attained could conceivably result in specific effects of the drugs.

The previously investigated processes of drug transport in retina, leukocytes, and synaptosomes displayed many characteristics indicative of mediated and/or active transport: saturability, cellular accumulation of the solute, high temperature coefficient, dependence on glucose, inhibition by metabolic poisons, correlation of uptake with cellular ATP concentration, inhibition by structural analogues, countertransport, and adverse effects of cell perturbation. Despite such strong evidence for carrier-mediated transport as the underlying mechanism, additional evidence would be desirable to confirm this conclusion. Although rendered improbable by the data presented here and in the previous studies, it should be ascertained that the observed kinetic relationships do not reflect binding of the drugs to intracellular macromolecular reactive sites, whose existence for various compounds is postulated by advocates of the sorption theory (51, 52). Such forthcoming studies should also establish that the rate-limiting phenomenon under observation is transport, defined as a process by which a solute is transferred from one phase to another, in the same initial and final states in the two phases (37).

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