Transport and Binding of Chloroguanide-Triazine by Rat Liver Slices[1] (34375)

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A recent investigation on the biliary excretion of weak organic bases has shown that certain tertiary amine compounds are excreted in bile by a process of active transport (1). For example, after intravenous administration in rats, the antimalarial drug chloroguanide-triazine (CGT) is readily transported from blood to bile against a large concentration gradient; transport occurs by a saturable process, and other tertiary amines that are known to be secreted into bile depress the excretion of CGT through competition for the secretory process. Tertiary amines appear to be transported by the same process that transports a number of quaternary amines (2, 3), since the latter compounds inhibit the biliary excretion of CGT in a competitive manner (1).

Very little is known about the uptake of organic bases into liver tissue. Although one quaternary amine, procaine amide ethobromide, has been shown to be accumulated by rat liver slices mainly by a process of active transport (4), tertiary amines have not been investigated in this regard.

In the present study, the hepatic transport of CGT is investigated in the liver slice preparation. Slices of rat liver are shown to take up the amine by both active transport and passive diffusion; and accumulation of the amine in the tissue appears to be the result of both active transport and tissue binding.

Materials and Methods. Male Sprague-Dawley rats (300–340 g) were decapitated and the livers immediately removed, rinsed quickly in cold Krebs-Ringer phosphate solution, and placed in chilled beakers. Slices of liver, 0.5 mm thick, were prepared with a Stadie-Riggs microtome. Three slices, each weighing 140–200 mg, were suspended in 10 ml of Krebs-Ringer phosphate solution (pH 7.4) containing 1 g glucose/liter and various concentrations of chloroguanide-triazine-^{3}H hydrochloride (CGT). In some experiments, the following compounds were added to the incubation mixture to investigate their effect on the tissue uptake of CGT: procaine amide ethobromide hydrobromide (PAEB), oxyphenonium bromide, quinine hydrochloride, procaine amide hydrochloride, probenecid, *p*-acetylaminohippuric acid (PAAH), iodoacetic acid, and 2,4-dinitrophenol. The mixtures, contained in 50-ml beakers, were shaken in a Dubnoff metabolic shaker (90 oscillations/min) at 37° in an atmosphere of oxygen. When incubations were carried out in a nitrogen atmosphere, the Krebs-Ringer phosphate solution was previously saturated with nitrogen by bubbling the gas through the solution for 20 min.

After incubation, liver slices were removed from the beakers, blotted on slightly moistened filter paper, and weighed. For measurement of radioactivity, slices (450–600 mg) were homogenized in 8 ml of distilled water using a Thomas tissue grinder fitted with a

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Teflon pestle. Two milliliters of 10% trichloroacetic acid was added to the homogenate to precipitate proteins and the resulting mixture centrifuged for 10 min at 900*g*. A 0.5-ml aliquot of the resulting clear supernatant fluid was added to 15 ml of a scintillation medium (5) and the radioacticity measured with a Packard Tri-Carb liquid scintillation spectrometer, model 3375. Samples of the incubation medium were similarly treated with trichloroacetic acid, centrifuged, added to the scintillation medium, and counted. In all measurements of radioactivity, the total number of counts was at least 40 times the background, and corrections for quenching were made by the external standardization method. Results were expressed as a slice/medium (S/M) concentration ratio of CGT, the concentration in slices being calculated in terms of wet tissue weight. When known amounts of ^{3}H-labeled CGT were added to rat liver slices and the resulting mixtures subjected to the procedure described above, a recovery of 88% (SE $\pm$ 1.4 in 36 determinations) was obtained. Recoveries from the incubation medium were complete, 99–101%. The data presented in this paper have been corrected for the incomplete recovery from liver.

To determine whether CGT is taken up by liver slices without undergoing metabolic alteration, slices were incubated aerobically for 2 hr in Krebs-Ringer phosphate solution containing ^{3}H-labeled CGT. The slices were then removed from the medium and homogenized in 10 ml of absolute ethanol. The resulting homogenate was heated at 80° for 5 min to complete the precipitation of proteins. After cooling, the mixture was centrifuged and the clear supernatant fluid transferred to a clean test tube. The fluid was evaporated to a small volume under a current of air and then applied to strips of Whatman 3MM filter paper. Chromatograms were developed ascendingly with one of the following solvent systems: (A) methylethylketone:water:diethylamine (90:10:0.5); or (B) *n*-butanol:glacial acetic acid (9:1) saturated with water. For controls, pure ^{3}H-labeled CGT was added to the alcoholic extract of liver slices that had been incubated in the absence of CGT. Radioactive spots were detected with a Vanguard model 880 automatic chromatogram scanner. Both the experimental and control preparations showed single spots with nearly identical R_F values in each of the solvent systems, thus indicating that CGT was not appreciably metabolized by rat liver slices (R_F values: system A, 0.34; system B, 0.64).

The binding of CGT to homogenates of rat liver was estimated by either ultrafiltration or ultracentrifugation. Details of these procedures have been described previously (4, 6). Homogenates (5–40% w/v) in Krebs-Ringer phosphate solution containing CGT were prepared with a Sorvall Omnimixer. Ultrafiltration of homogenates through sacs of Visking cellulose casing was accomplished by centrifugation of the sacs at 3000 *g* in a Sorvall model RC-2 centrifuge at 0°. Ultracentrifugation of homogenates at 0° was carried out for 24 hr at 30,000 rpm in a Spinco model L ultracentrifuge (rotor No. 40; average rotational centrifugal force 59, 360*g*).

Tritium-labeled CGT and unlabeled CGT were kindly provided by Parke, Davis & Co.; PAEB and procaine amide by Squibb Institute for Medical Research; oxyphenonium by Ciba Pharmaceutical Products, Inc.; and probenecid by Merck Institute for Therapeutic Research.

Unless otherwise indicated, results are expressed as the mean $\pm$ SE.

Results. When rat liver slices were incubated with 3.5×10^{-7} *M* CGT, the drug readily entered the tissue. After 1 hr, the compound attained a slice/medium (S/M) concentration ratio of 7.6, and after 4 hr a ratio of 12.4 (Fig. 1). Lower S/M ratios were obtained on raising the concentration of CGT in the incubation medium. For example, on increasing the concentration 10-fold, to 3.5×10^{-6} *M*, the 4-hr S/M ratio declined from 12.4 to 9.4; and on increasing the concentration 1000-fold, to 3.5×10^{-4} *M*, the S/M ratio declined to a value of 6.4 (Fig. 1). These results indicated that liver slices can accumulate CGT against an apparent concentration gradient and that uptake occurs at least in part by a process that can be saturated.

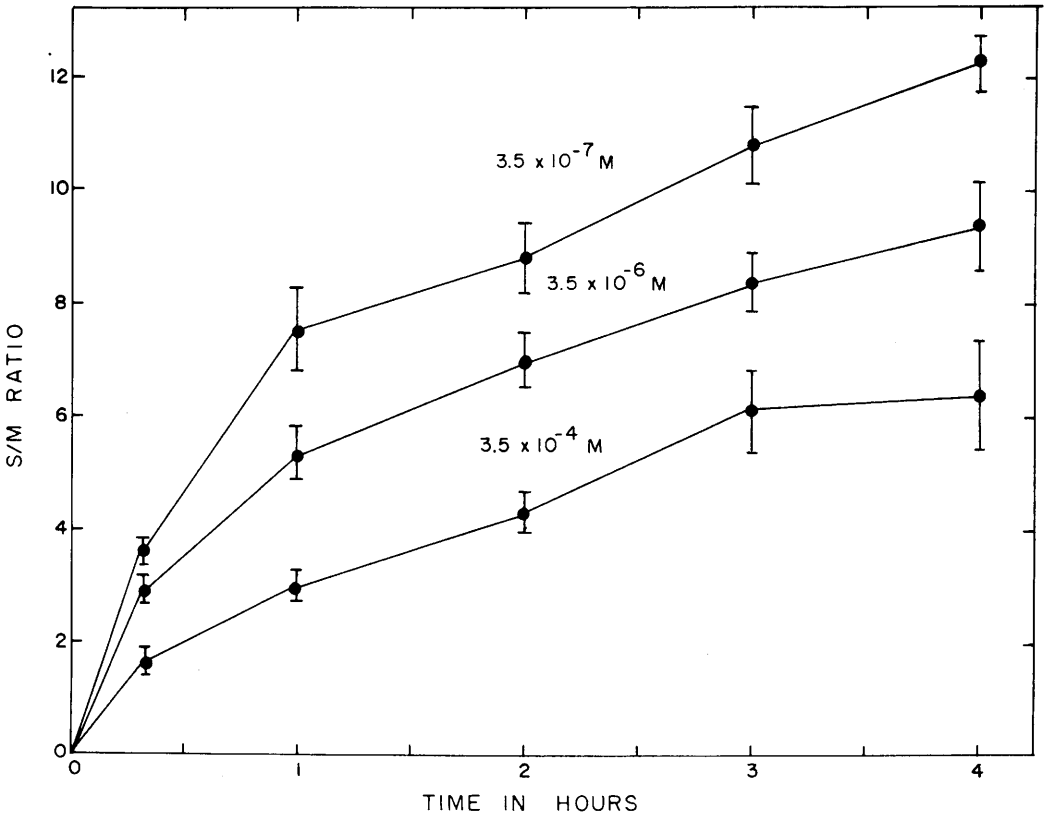

FIG. 1. Influence of concentration on time course of uptake of CGT by rat liver slices. Each point is the mean of 3–7 experiments. Brackets indicate SE.

Evidence that the hepatic uptake of CGT is to some extent dependent on cell metabolism was seen in experiments in which liver slices were exposed to metabolic inhibitors or anaerobic conditions. In the presence of 3.5×10^{-4} *M* iodoacetate or 2,4-dinitrophenol, or in an atmosphere of 100% nitrogen, the 2-hr S/M ratio of the drug was depressed from 7.0 to values of 4.3–5.0 (Fig. 2). With either dinitrophenol or a nitrogen atmosphere, significant inhibition of CGT uptake was seen after 1 hr; whereas with iodoacetate, inhibition was not apparent until after 2 hr of incubation.

The uptake of CGT by liver slices was also depressed by a number of tertiary and quaternary amine compounds (Fig. 3). For instance, the tertiary amines, procaine amide and quinine, and the quaternary amines, oxyphenonium and procaine amide ethobromide (PAEB), when present in a concentration of $3.5 \times 10^{-4} M$, depressed the 2-hr S/M ratio of CGT ($3.5 \times 10^{-6} M$) from a value of 7.0 to values of 3.2–4.7. With all four amines, inhibition was marked throughout the entire incubation period.

In contrast to the inhibitory effect of cationic compounds, two organic anions, *p*-acetylaminohippuric acid (PAAH) and probenecid, had no effect on the hepatic uptake of CGT when present in a concentration 100 times that of the latter drug (Fig. 3).

To investigate the possible binding of CGT to components of hepatic tissue, homogenates of rat liver, prepared in Krebs-Ringer phosphate solution and containing $3.5 \times 10^{-6} M$ CGT, were subjected to ultrafiltration through a Visking cellulose membrane (6). The degree of binding to 5, 10, 20, and 40% homogenates of the tissue was 23, 35, 53, and 68% respectively. To estimate the binding to undiluted tissue, these data were

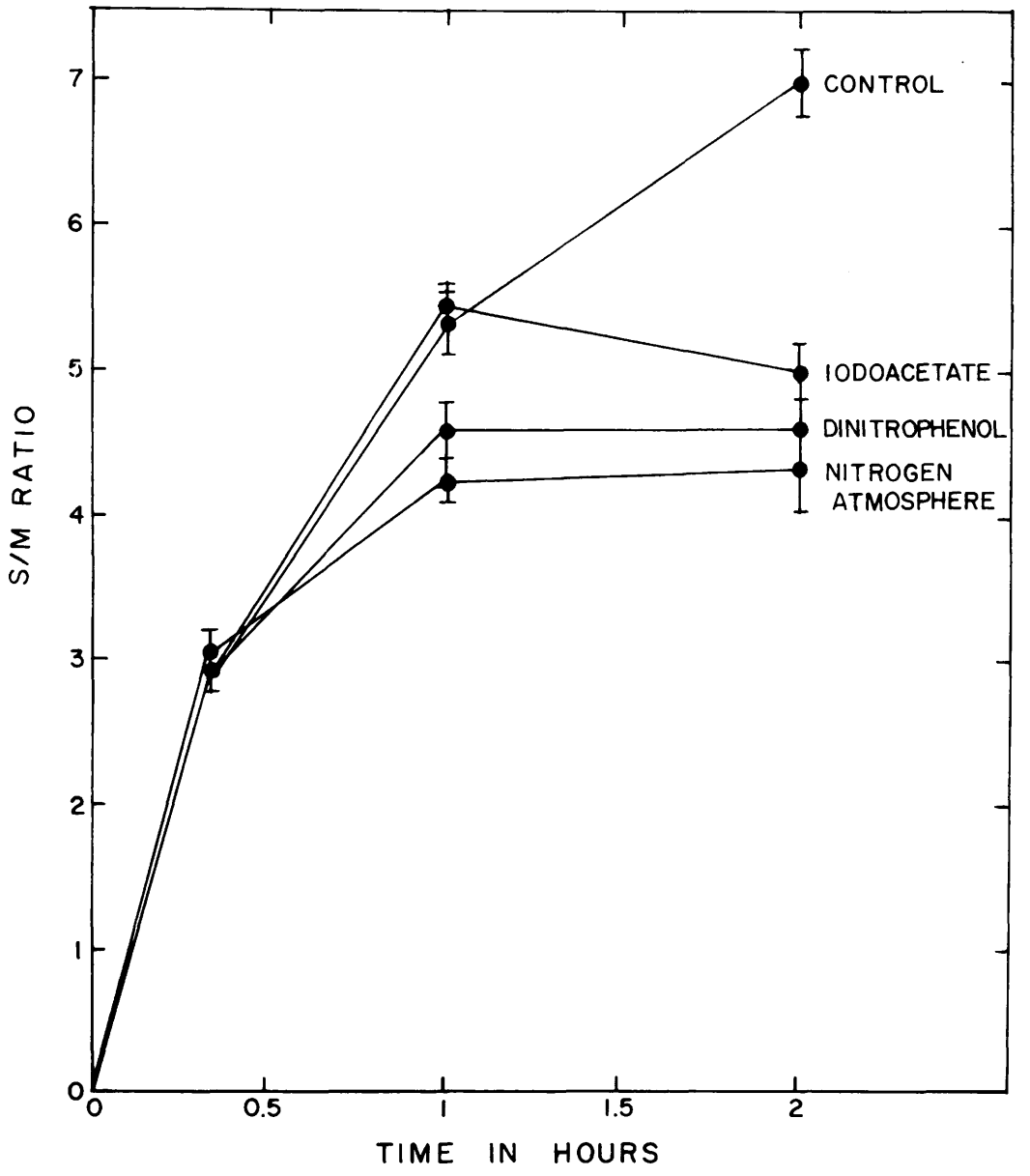

FIG. 2. Effect of metabolic inhibitors or a nitrogen atmosphere on uptake of CGT by liver slices. Initial concentrations were: CGT, $3.5\times10^{-6}M$; other substances, $3.5\times10^{-4}M$. Each point is the mean of three experiments. Brackets indicate SE.

extrapolated graphically (6) to find the extent of binding for a 100% homogenate (Fig. 4). A value of 85% binding was obtained.

Binding was also assessed by ultracentrifugation (4). After centrifugation of 40% homogenates of liver containing $3.5\times10^{-6}M$ CGT, the concentration of CGT in the water of the particulate material was 7.3 ± 0.02 times that in the supernatant fluid. From this ratio it could be calculated that the extent of binding of CGT to the particulate material was 86%, a value similar to that obtained by extrapolation of the ultrafiltration data.

To determine whether substances which

depress the uptake of CGT by liver slices can also depress binding of the drug to tissue components, the ultracentrifugation procedure was repeated in the presence of $3.5 \times 10^{-4}M$ iodoacetic acid, 2,4-dinitrophenol, quinine, or PAEB. Neither of the metabolic inhibitors influenced the binding of CGT; the particulate material/supernatant fluid ratio was 7.2 ± 0.2 in the presence of iodoacetic acid and 7.8 ± 0.02 in the presence of dinitrophenol. In contrast, quinine depressed the ratio to a value of 5.7 ± 0.07, and PAEB depressed the ratio

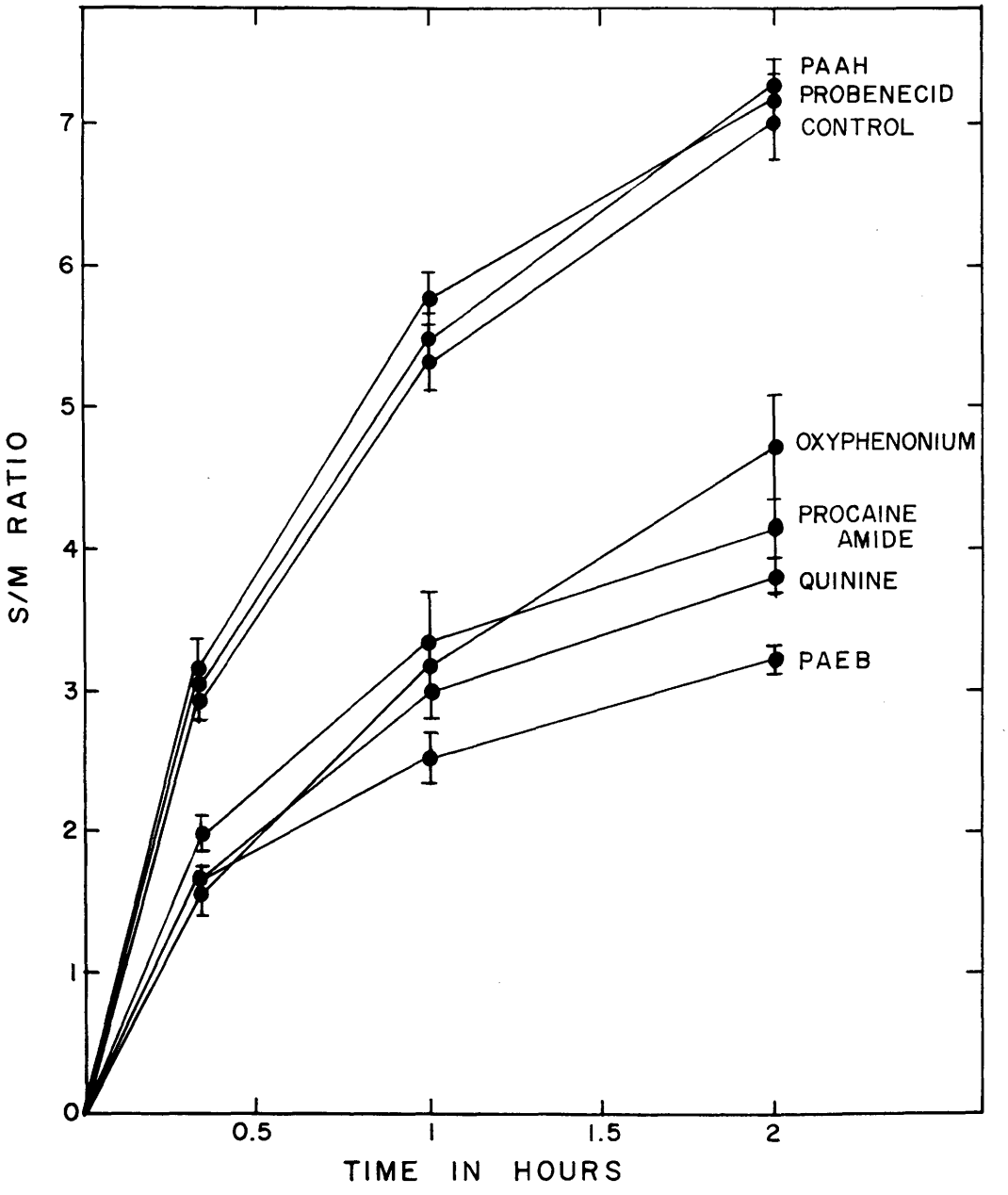

FIG. 3. Effect of organic cations and anions on uptake of CGT by liver slices. Initial concentrations were: CGT, $3.5 \times 10^{-6}M$; other substances, $3.5 \times 10^{-4}M$. Each point is the mean of three experiments. Brackets indicate SE.

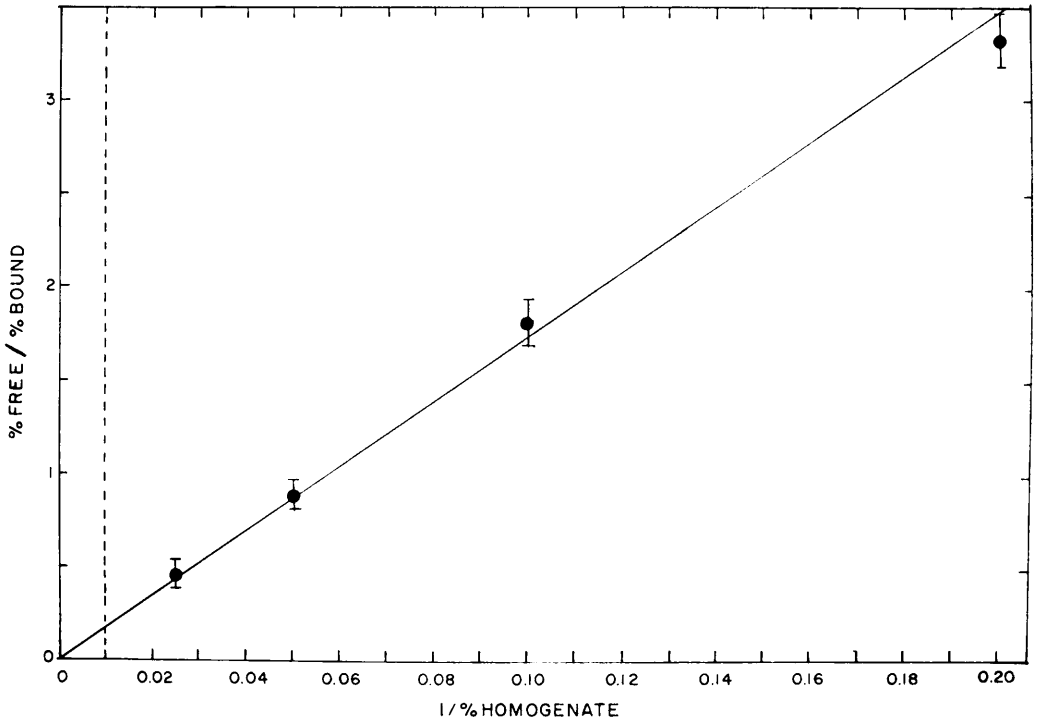

FIG. 4. Binding of CGT to homogenates of rat liver. Percentage binding for $3.5\times10^{-6}M$ CGT was measured by ultrafiltration of 5, 10, 20, and 40% homogenates. The intercept of the curve on the vertical broken line indicates the free/bound ratio for a theoretical 100% homogenate. Each point is the mean of three experiments. Brackets indicate SE.

to a value of 5.4±0.02.

Discussion. The uptake and accumulation of CGT by rat liver slices appears to involve three processes: active transport, simple diffusion, and tissue binding. Direct evidence of binding is provided by results obtained on subjecting liver homogenates to ultrafiltration or ultracentrifugation; with either technique, about 85% of the CGT is bound when the concentration of total drug is 3.5×10^{-6} *M*. An indication of binding in the intact liver slice is seen in data obtained in the presence of metabolic poisons or a nitrogen atmosphere (Fig. 2); under these conditions, CGT ($3.5\times10^{-6}M$) attains a steady-state S/M ratio of 4.3–5.0. When these data are expressed in terms of tissue water (77% of the wet slice weight) (4), thereby converting them to the same basis as the homogenate binding data, the slice water/medium ratios at the steady state are 5.6–6.5. From these ratios it may be calculated that binding in the tissue slice is 82–85%. Thus two widely different lines of evidence suggest that accumulation of CGT in liver is in large part accounted for by tissue binding.

But binding does not account for all the drug accumulated by liver slices. There appears to be, in addition, accumulation by active transport. The best evidence of this is seen in the data of Figs. 1 and 2, in which CGT ($3.5\times10^{-6}M$) attains a S/M ratio of 7.0 after 2 hr and 9.4 after 4 hr, values considerably greater than the steady-state ratios of 4.3–5.0 obtained in the presence of metabolic inhibitors or a nitrogen atmosphere. The latter agents presumably block energy sources needed for active transport and allow CGT to attain a steady-state distribution between slice and medium that is determined simply by diffusion and tissue binding.

An additional line of evidence suggesting the presence of an active transport process for CGT is the finding that iodoacetic acid

and dinitrophenol do not interfere with the binding of the drug to liver homogenates. Thus these agents depress the S/M ratio of CGT solely by interfering with an energy-dependent accumulating mechanism.

Analysis of other portions of this study is complicated by the fact that the processes of active transport and binding are difficult to distinguish on a kinetic basis. For example, both processes have the characteristic of being saturable. Moreover substances may compete with one another for transport as well as for binding sites. Thus the data of Fig. 1, showing that the S/M ratio of CGT declines as the initial concentration is raised, indicates saturation of either the transport process or the binding process, or both. Similarly, the inhibition of CGT uptake by such organic cations as oxyphenonium, procaine amide, quinine, and PAEB (Fig. 3), may be interpreted as competition for either active transport or binding, or both. That the saturation and competition seen in this study probably involve both active transport and binding is suggested by the following considerations: 1. In the intact rat, it has been shown that CGT, PAEB, procaine amide, and quinine are actively transported from blood to bile, and that the four cations compete with one another for the transport process (1); and 2. The binding of CGT to liver homogenates is depressed by PAEB, and it is known that PAEB binds to liver homogenates (4).

The uptake of CGT by liver slices resembles in many respects the secretion of this drug from blood into bile. In both of the systems: transport occurs against a concentration gradient; the transport process is saturable; transport is inhibited by certain tertiary and quaternary amines which appear to share the same transport mechanism; and transport is not inhibited by such organic anions as probenecid or PAAH—substances secreted into bile by a separate active transport process (3, 7).

Summary. When rat liver slices were incubated aerobically at 37° with 3.5×10^{-7} *M* chloroguanide-triazine (CGT), the compound readily entered the tissue against an apparent concentration gradient. After 2 hr, the slice/medium concentration ratio was 9, and after 4 hr about 12. Uptake of CGT into slices occurred by a process that became saturated at high concentrations of the drug. Uptake was diminished by anaerobic conditions and by metabolic inhibitors such as iodoacetate and 2, 4-dinitrophenol. Uptake was also inhibited by certain tertiary and quaternary amine compounds, which pre- iodoacetate and 2,4-dinitrophenol. Uptake process. These results suggested that CGT is taken up by liver slices at least in part by an active transport process that is closely related to the process which secretes CGT and certain other tertiary and quaternary amines into bile *in vivo*. CGT also entered liver slices by a process of diffusion. The compound was highly bound to homogenates of rat liver, suggesting that part of the accumulation seen in liver slices results from binding to tissue components.

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