Quantification of the Binding Tendencies of Cholestyramine I: Effect of Structure and Added Electrolytes on the Binding of Unconjugated and Conjugated Bile-Salt Anions

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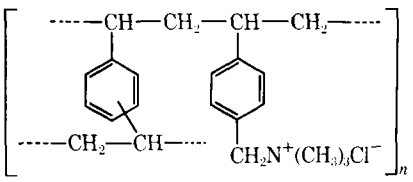
Abstract □ The binding of conjugated and unconjugated bile salts to cholestyramine from aqueous media was investigated and the data plotted according to the Langmuir adsorption equation. Higher affinity constants were obtained with the dihydroxy rather than the trihydroxy derivatives. Glycine conjugation was found to alter the affinity constants only slightly, whereas conjugation with taurine markedly increased the affinity of cholestyramine for the bile salt. The addition of bicarbonate or chloride anions to the system produced a significant decrease in the extent of binding of the trihydroxy bile-salt anions; the effect being more pronounced in the case of the chloride ion.

Keyphrases □ Cholestyramine—bile-salt anion binding □ Temperature effect—cholestyramine binding □ Structure, bile salts—cholestyramine binding □ Electrolyte effect—cholestyramine binding □ UV spectrophotometry—analysis

Cholestyramine is a pharmacologically important anionic-exchange resin, which is chemically equivalent to a commercial ion-exchange resin.[1] The basic quaternary ammonium-exchange functionalities (I) in the resin are attached to a styrene divinylbenzene copolymer skeleton.



(I)

It has been demonstrated in animals (1–6) and clinically in man (7–11) that cholestyramine is capable of increasing the fecal excretion of endogenous bile salts, thereby significantly decreasing the extent of absorption of fats and fatty materials. This resin also possesses the ability to lower plasma cholesterol levels by binding bile-salt anions in the small intestine (11–13). The reduction in bile-salt concentration results in a decrease in the extent of intestinal absorption of exogenous cholesterol as well as an increase in the hepatic metabolism of endogenous cholesterol into additional bile salts (14, 15).

The *in vitro* binding tendencies of cholestyramine for bile-salt anions has received limited attention (16–18). These reports are informative, but the experimental designs do not reflect the conditions known to exist in the fluids of the upper regions of the small intestine, where only conjugated bile salts are present. In addition, no studies have described the complete characteristics of the adsorption or binding process nor the influence of the structure of the bile salt thereon.

The purpose of this study was to quantitatively determine the influence of structure, temperature, and electrolyte concentration on the binding tendencies of cholestyramine for conjugated and unconjugated bile-salt anions.

EXPERIMENTAL

Materials—The sodium salts of cholic acid,[2] deoxycholic acid,[2] glycocholic acid,[3] glycodeoxycholic acid,[3] and taurocholic acid[3] were dried *in vacuo* for at least 48 hr. prior to use. The cholestyramine[4] employed in this study was pharmaceutical grade, the particle size of which was 100% > 100 mesh, 80% > 200 mesh. Reagent grade sodium chloride, sodium bicarbonate, and concentrated sulfuric acid were used as received.

Procedure for Adsorption Studies—One of the experimental objectives in the adsorption studies was to maintain the concentration of the bile salt within the range generally accepted as being physiologic (19, 20), yet below that which would induce micelle formation. When these two criteria are satisfied, the isotherms obtained were expected to indicate only monomolecular adsorption while still retaining physiologic significance.

A series of aqueous solutions of each bile salt was prepared over the concentration range of 0.75–4.5 m*M*. Twenty-five-milligram samples of cholestyramine were accurately weighed and placed into 50-ml. glass-stoppered conical flasks together with a 25.0-ml. portion of bile-salt solution of varying concentrations. A control flask containing a similar quantity of bile-salt solution at each concentration was also prepared. The latter solutions served as blanks which upon concomitant assay yielded the necessary Beer's law relationship. All flasks were closed securely and mechanically shaken[5] at 25 or 37° until equilibrium was established. Equilibration was determined by means of repetitive sampling and was found to occur within a 24–48-hr. period. The equilibrated samples were subjected to Millipore filtration (0.45-μ pore size), the filtrates suitably diluted, and the equilibrium bile-salt concentration determined spectrophotometrically (see *Assay Procedure*).

The protocol for those studies designed to determine the binding tendencies of cholestyramine for bile-salt anions in the presence of added sodium chloride or sodium bicarbonate was essentially the same with the exception that the concentration of the bile salt was held constant at 3.0 m*M*. The adsorption phenomenon was studied at 50-, 100-, and 150-m*M* concentrations of sodium chloride or bicarbonate.

In the desorption studies, 25.0-ml. quantities of each bile-salt solution were prepared and equilibrated with 25.0 mg. of cholestyramine in 125-ml. glass-stoppered conical flasks. After equilibration the samples were diluted with 50.0-ml. portions of distilled water and shaken until equilibrium was again established. The solutions were then filtered, appropriately diluted, and assayed for bile-salt concentration.

[1] Dowex 1-X2, Dow Chemical Co., Midland, Mich.

[2] Obtained from Mann Research Laboratories, New York, NY 10006. Enzymatic grade (reported to be >99% pure by TLC).

[3] Obtained from Calbiochem, Los Angeles, CA 90063. Grade A.

[4] Generously supplied by Merck and Co., Inc., Rahway, NJ 07065

[5] Precision Shaker, Precision Scientific Co., Chicago, IL 60647

Table I—Effect of Temperature on the Binding of Unconjugated and Conjugated Bile-Salt Anions to Cholestyramine

Bile Salt[a]	x/m (Moles of Bile Salt Bound per gram of Cholestyramine × 10^4) 25°	37°
Trihydroxy anions		
Cholate	37.7	37.7
Taurocholate	36.1	36.4
Glycocholate	34.9	36.0
Dihydroxy anions		
Deoxycholate	36.3	37.4
Glycodeoxycholate	37.4	38.3

[a] The initial concentration of bile salt was 4.0 m*M*.

Assay Procedure—The equilibrium concentration of unbound bile salt present in solution was determined spectrophotometrically by essentially the same procedure described by Eriksson and Sjovall (21, 22). The method involves heating an appropriately diluted bile-salt sample in a 65% sulfuric acid medium. All absorbance readings were determined utilizing a recording spectrophotometer.[6] A 65% sulfuric acid solution, treated in an identical manner, served as the blank. All of the bile salts under investigation followed a Beer's law relationship at their respective wavelengths of maximum absorbance (dihydroxy derivatives, 385 mμ; trihydroxy derivatives, 320 mμ).

The amount of bile salt bound to cholestyramine was calculated from the difference between the initial concentration of bile salt introduced into the system and the concentration present in solution at equilibrium.

RESULTS AND DISCUSSION

The monomolecular adsorption of adsorbate molecules from solution, at constant temperature, onto an adsorbent may be described by the following Langmuir-type equation (23):

$$\frac{x}{m} = \frac{k_1 k_2 C_{eq.}}{1 + k_1 C_{eq.}} \qquad \text{(Eq. 1)}$$

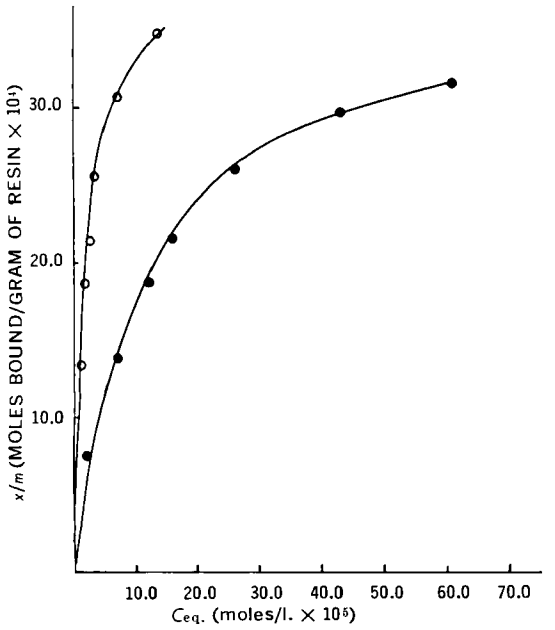


Figure 1—*Adsorption isotherms for the binding of unconjugated bile-salt anions to cholestyramine at 25°. Key:* ○, *deoxycholate;* ●, *cholate.*

[6] Beckman model DB-G, Beckman Instruments, Inc., Fullerton, Calif.

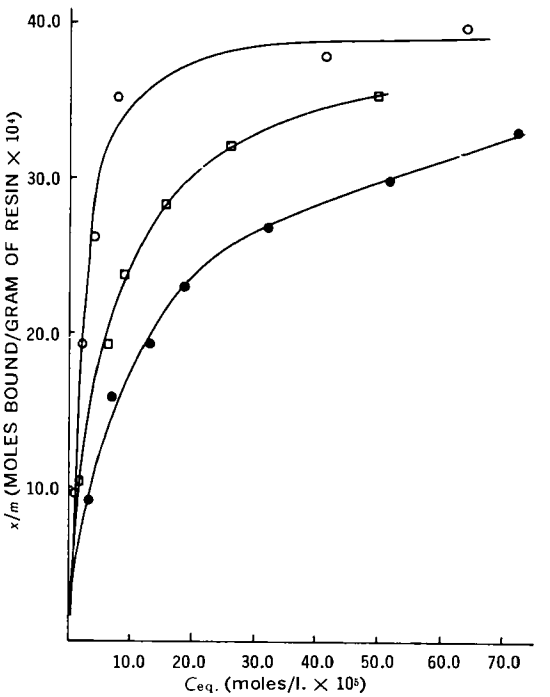


Figure 2—*Adsorption isotherms for the binding of conjugated bile-salt anions to cholestyramine at 25°. Key:* ○, *glycodeoxycholate;* □, *taurocholate;* ●, *glycocholate.*

or upon rearranging Eq. 1,

$$\frac{C_{eq.}}{x/m} = \frac{1}{k_1 k_2} + \frac{C_{eq.}}{k_2} \qquad \text{(Eq. 2)}$$

where $C_{eq.}$ is the concentration of adsorbate remaining in solution at equilibrium; x the amount of adsorbate bound to the adsorbent, and m the amount of adsorbent employed. The constant k_1 may be defined as the adsorption coefficient or affinity constant and is related to the magnitude of the forces involved in the binding process. The Langmuir-capacity constant, k_2, indicates the apparent maximum amount of adsorbate that can be adsorbed per unit weight of adsorbent. Equation 2 suggests that a plot of $C_{eq.}/(x/m)$ *versus* $C_{eq.}$, on rectilinear coordinates, should yield a straight line from which one can obtain the constants k_1 and k_2.

All adsorption experiments were determined in aqueous solution. Under these conditions, the pH of the system before and after equilibration with cholestyramine remained constant for each bile salt and was essentially independent of the initial bile-salt concentration. The pH of these solutions varied from 5.40 to 6.80 depending on the nature of the bile salt. Based on reported pKa values (24) for the bile acids employed in this study, at concentrations below their respective critical micelle concentrations (CMC), it was determined that the bile acids were present almost entirely in the dissociated form (*i.e.*, from 96.4 to 99.2%).[7]

Effect of Temperature on the Adsorption Process—The binding to cholestyramine of unconjugated and conjugated dihydroxy and trihydroxy bile-salt anions, at an initial concentration of 4.0 m*M*, was conducted at 25 and 37°. The results of these studies, expressed as the moles of bile-salt anion bound per gram of chole-

[7] It should be noted that according to the investigation of Ekwall *et al.* (24) the pKa values for bile salts are subject to variation depending upon the concentration of salt in solution. This variation results from the ability of these physiologic surfactants to undergo association (micellization) in solution. The pKa's for these surfactants appear to remain constant below their respective CMC values, where only monomers are present in solution, and tend to increase in magnitude above these critical concentrations. The reported range of pKa values for the salts used in this investigation are: glycocholate, 2.78–4.35; taurocholate, 1.56–3.33; glycodeoxycholate, 2.46–3.98; cholate, 4.95–4.98; and deoxycholate, 4.97–5.17. The latter two values were determined at concentrations below their respective CMC's. At the pH's of the equilibrated bile-salt cholestyramine samples the percent bile acid existing in the ionized form, based on the highest reported pKa values, were calculated to be 98.8, 99.2, 99.6, 97.7, and 96.4, respectively.

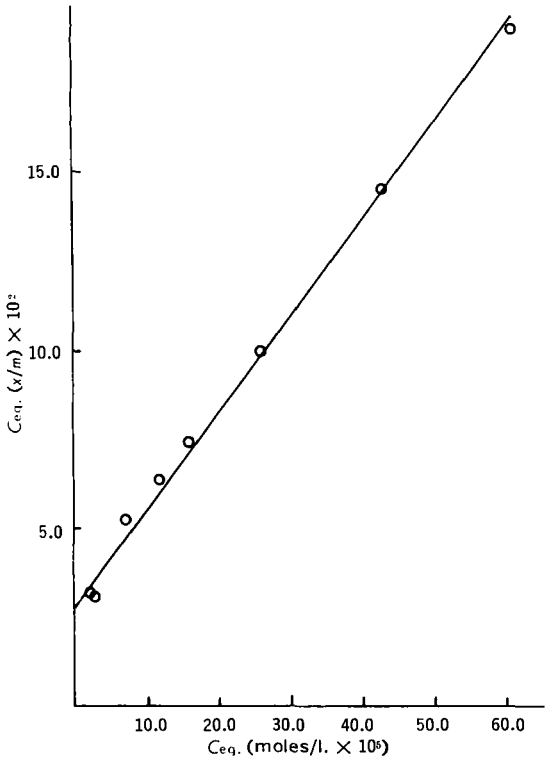


Figure 3—*Langmuir adsorption isotherm for the binding of cholate anion to cholestyramine at 25°.*

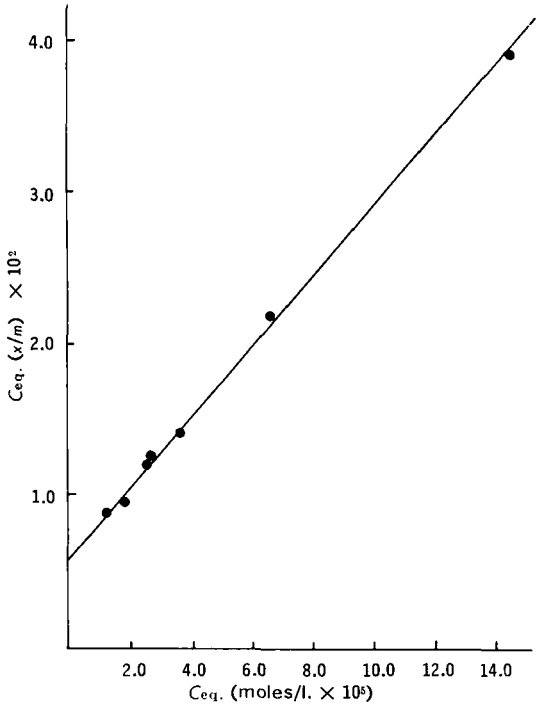


Figure 4—*Langmuir adsorption isotherm for the binding of deoxycholate anion to cholestyramine at 25°.*

styramine, are listed in Table I. It can be seen from this table that the binding process is not significantly influenced by temperature. Similar results were obtained when the experiments were repeated at an initial bile-salt concentration of 2.0 m*M*. In view of the apparent temperature independence of the adsorption process all subsequent experiments were performed at 25°.

Effect of Bile-Salt Structure on the Adsorption Process—The adsorption isotherms describing the binding tendencies of cholestyramine for unconjugated (cholate and deoxycholate) bile-salt anions and their corresponding amino acid conjugates (taurocholate, glycocholate, and glycodeoxycholate) at 25° were plotted according to Eq. 1 and are shown in Figs. 1 and 2, respectively. The curves show a tendency to reach a plateau at high $C_{eq.}$ values indicating that the system is approaching the limiting monomolecular exchange capacity of cholestyramine for the particular bile-salt anion.

Figures 3–5 show the excellent linearity observed with all of the adsorbate anions under investigation and demonstrate the adherence of the binding process to the Langmuir-type adsorption isotherm (Eq. 2). The adsorption constants k_1 and k_2, obtained from the intercept and slope values of these figures, are reported in Table II. The capacity constant, k_2, expressed as the number of moles of bile-salt anion adsorbed per mole equivalent of cholestyramine, would be unity if all of the available binding sites on cholestyramine were occupied by the adsorbate molecules. The fact that the k_2 values listed in this table are somewhat less than unity suggests that some of the sites are unavailable to the bile-salt anions. This is in agreement with the porous nature of the cholestyramine particle, which allows the possibility of binding positions on the interior surfaces of the resin bead (25).

An examination of the affinity constants, k_1, obtained for the three trihydroxy bile-salt derivatives indicates that they tend to decrease in the following order: taurocholate > cholate ~ glycocholate. The enhanced affinity shown by the taurocholate anion may reflect the greater acidity of its sulfonic acid group over that of the carboxyl group present in the other two bile-salt derivatives. As a result of conjugating the bile salt with either taurine or glycine, the anionic charge on the side chain of the molecule becomes more distantly located with respect to the cyclopentanophenanthrene or steroid nucleus, thus facilitating the movement of the chain (19). The amino acid taurine, in addition to possessing a sulfonic acid group, also contains one more carbon in its chain than does the carboxyl-containing glycine molecule. The greater elongation of the side-chain of the taurocholate anion probably also contributes to the enhanced interaction of this molecule with cholestyramine, possibly by providing the optimal orientation of the anionic site with respect to the binding sites on the resin.

In agreement with the data for the cholate derivatives, a similar affinity constant for the deoxycholate anion and its glycine conjugate was observed.

The affinity constants for the two dihydroxy bile salts (deoxy-

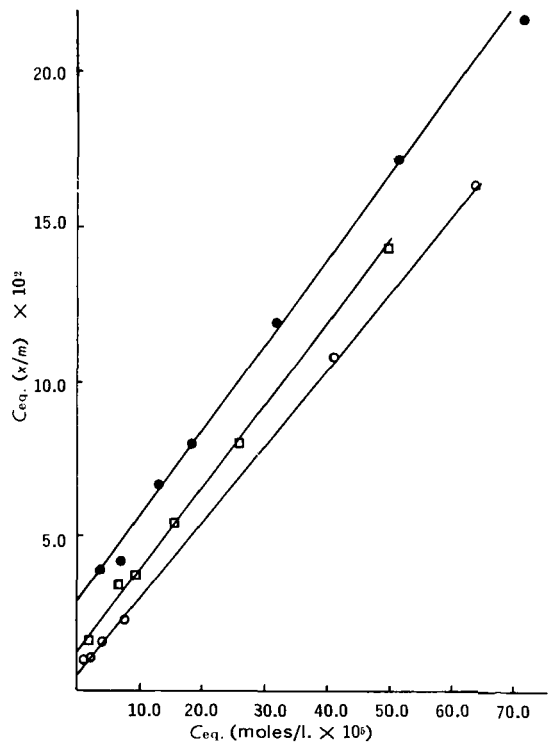


Figure 5—*Langmuir adsorption isotherms for the binding of conjugated bile-salt anions to cholestyramine at 25°. Key:* ○, *glycodeoxycholate;* □, *taurocholate;* ●, *glycocholate.*

Table II—Langmuir Adsorption Constants for the Binding of Conjugated and Unconjugated Bile-Salt Anions to Cholestyramine at 25°

Bile Salt	k_1 (l./mole of Bile Salt × 10^{-4})	$(k_2)^a$ (Moles of Adsorbate Bound per Mole Equiv. of Resin)
Cholate	1.01	0.843
Taurocholate	1.99	0.874
Glycocholate	0.891	0.863
Deoxycholate	4.53	0.875
Glycodeoxycholate	4.25	0.941

[a] Based on a monomer equivalent weight for cholestyramine of 230.

cholate and glycodeoxycholate) were found to be approximately four times those of the corresponding trihydroxy derivatives (cholate and glycocholate), demonstrating that the former anions interact significantly more strongly with the binding sites on cholestyramine (see Table II). The observed differences cannot be accounted for solely on the basis of geometric differences. Structurally, both types of anions contain α-hydroxyl groups in the 3 and 12 positions of the steroid nucleus, with the trihydroxy salts possessing an additional 7-α-hydroxyl group. The presence of this additional hydroxyl group causes an increased degree of polarization of the cyclopentanophenanthrene ring system (26).

Although the primary mode of interaction of bile-salt anions to cholestyramine is most probably electrostatic in nature (*i.e.*, between the negatively charged carboxyl groups of the bile-salt anions and the positively charged quaternary ammonium groups on cholestyramine), the data suggest the existence of secondary binding forces. These forces, being nonelectrostatic in nature, would involve interactions between the hydrophobic regions of the adsorbate and adsorbent molecules. The results obtained in this investigation are consistent with this hypothesis in that the more hydrophobic dihydroxy anions are more strongly held by the polystyrene matrix of the anion-exchange resin than are their more polar trihydroxy counterparts. A similar occurrence has been reported by Rudman and Kendall (26) in their studies on the binding of a series of bile-salt derivatives to plasma proteins. These investigators found that the greater the number of hydroxyl groups on the steroid nucleus, the lower the affinity of the bile salt to the protein molecule.

Experiments designed to study the desorption characteristics of the bile salt-resin complex showed that the binding process was essentially nonreversible under the conditions employed in this investigation.

Effect of Added Electrolytes on the Adsorption Process—When cholestyramine is orally administered it must traverse a significant portion of the gastrointestinal tract before it reaches the region of the small intestine where the physiologic bile-salt anions are present. In view of the possible influence of other anions on the binding capacity of cholestyramine for bile salts, two physiologic anions were introduced separately into the binding system. The influence of varying concentrations of sodium chloride and bicarbonate on the binding capacities of cholestyramine are shown in Tables III and IV, respectively. The data in these tables indicate that the trihydroxy bile-salt anions are significantly reduced in their extent of binding to cholestyramine and that the degree of

Table III—Effect of Varying Concentrations of Sodium Chloride on the Binding of Bile-Salt Anions to Cholestyramine at 25°

Bile Salt[a]	x/m (Moles of Bile Salt Bound per gram of Cholestyramine × 10^4) mM Concn. Sodium Chloride 0.0	50.0	100.0	150.0
Cholate	25.9	12.4	9.80	8.65
Taurocholate	28.6	18.7	15.3	12.2
Glycocholate	26.8	14.1	10.7	9.18
Deoxycholate	25.6	25.5	23.5	21.5
Glycodeoxycholate	26.5	24.9	22.8	22.3

[a] The initial concentration of bile salt was held constant at 3.0 m*M*.

Table IV—Effect of Varying Concentrations of Sodium Bicarbonate on the Binding of Bile-Salt Anions to Cholestyramine at 25°

Bile Salt[a]	x/m (Moles of Bile Salt Bound per gram of Cholestyramine × 10^4) mM Concn. Sodium Bicarbonate 0.0	50.0	100.0	150.0
Cholate	25.9	14.9	13.4	13.3
Taurocholate	28.6	22.2	18.4	14.6
Glycocholate	26.8	16.8	12.1	10.5
Deoxycholate	25.6	26.9	25.3	24.1
Glycodeoxycholate	26.5	27.9	26.5	25.7

[a] The initial concentration of bile salt was held constant at 3.0 m*M*.

binding decreases with increasing concentrations of electrolyte. The observed reduction can best be attributed to the existence of a competition between the chloride or bicarbonate anion and the bile-salt anion for the available binding sites on the resin particle as well as to the possible screening effect of these electrolytes on the binding process. In agreement with the proposed greater contribution of nonelectrostatic forces to the binding of dihydroxy bile-salt anions to cholestyramine, these anions were found to be little influenced by the addition of either electrolyte.

The consistently higher x/m values obtained in the presence of the bicarbonate anion as compared to the chloride ion, at equimolar concentrations, cannot be accounted for based on the slightly greater degree of dissociation of the bile salts produced by the more alkaline bicarbonate environment. A more plausible explanation is based on the inherent differences in selectivity of cholestyramine for various small ions (25). This resin displays more of a preference for chloride than for bicarbonate anions, which parallels the observed differences in the degree of competition of these electrolytes on the bile-salt cholestyramine binding process.

The possible influence of other physiologic substances on the binding characteristics of cholestyramine for bile-salt anions will be explored in subsequent communications in an attempt to explain the relatively low *in vivo* efficiency exhibited by cholestyramine. A more complete understanding of the factors effecting these interactions may lead to the development of a resin possessing greater selectivity and affinity.

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Barbiturate Mortality in Hypothyroid and Hyperthyroid Rats

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Abstract □ Thyroid state was found to affect the mortality in rats of five barbiturates. Male rats were thyroidectomized or injected on 5 days with L-triiodothyronine 0.2 mg./kg. Hypo- and hyperthyroidism were characterized by appropriate changes in body weights, temperatures, and basal metabolism rates. Effects of the treatments on drug metabolism systems were indicated by alterations in hexobarbital sleeping time and zoxazolamine paralysis time. The 24-hr. mortality rates of hexobarbital, thiopental, amobarbital, pentobarbital, and phenobarbital were significantly ($p < 0.05$) increased in hyperthyroid rats. In hypothyroid rats, mortality rates were unchanged except for a significant decrease seen with thiopental.

Keyphrases □ Barbiturate mortality rates—thyroid effect □ Triiodothyronine thyrotoxicosis—barbiturate lethality □ Thyroidectomy—barbiturate lethality

The acute lethality of certain drugs was significantly increased in hypoexcretory animals, which were either anuric or cholestatic (2). The intensity and duration of action of many drugs might also be altered by impaired excretion or homeostatic mechanisms. The thyroid gland, which exerts significant control over metabolic processes and other body functions, could have significant influence on drug actions which depend on the functional state of the thyroid. Hyperthyroid animals have been found to be susceptible to the toxicity of some pharmacological agents; Carrier and Buday (3) have compiled a list of substances whose toxicity was increased by hyperthyroidism. Seyle (4) has reported a diminished sensitivity of hyperthyroid animals to the toxicity of a number of nitriles. Administration of thyroxine to rats has been shown to accelerate or inhibit various drug-metabolizing enzymes (5, 6). Hypothyroidism and hyperthyroidism delayed the removal of pentobarbital from rat tissues after intravenous administration (7).

The purpose of this investigation was to evaluate the effect of altered thyroid state on the acute mortality of selected barbiturates in the rat.

MATERIALS AND METHODS

Drug solutions were prepared so that the desired dosage was injected intraperitoneally in a volume of 0.01 ml./g. of body weight. Sodium hexobarbital,[1] sodium thiopental,[2] sodium amobarbital,[3] and sodium phenobarbital[4] were dissolved in distilled water just prior to use. Zoxazolamine[5] was suspended in 1% sodium carboxymethylcellulose. L-Triiodothyronine[6] was dissolved in a small volume of 0.75 *N* sodium hydroxide; the resulting pH of the solution when made to volume with distilled water was 9.0. The dosages of all drugs are expressed as the respective salts.

Male Sprague-Dawley (Simonsen) rats were housed five per cage and fed Wayne Lab-Blox[7] and tap water *ad libitum*. Groups of 20–30 rats weighing 80 to 100 g. were thyroidectomized under pentobarbital anesthesia. Another group of rats was sham-operated at the same time and served as euthyroid controls. All operated animals were allowed 30 days for recovery and development of hypothyroidism.

Hyperthyroidism was induced in groups of 20–30 rats, weighing 180 to 220 g., by intraperitoneal injection of L-triiodothyronine, 0.2 mg./kg., daily for 5 days. Control animals were injected for 5 days with an equal volume of dilute sodium hydroxide solution (pH 9.0).

At various times during and after induction of the altered thyroid state, the body weights and temperatures of randomly selected animals were recorded; basal metabolic rates were determined utilizing a modified Phipps and Bird metabolism apparatus. All basal metabolic rates were measured between 9 a.m. and 3 p.m. to reduce time-of-day variation. Duration of the loss of the righting reflex due to intraperitoneal administration of either

[1] Evipal, Winthrop Chemical Co., New York, N. Y.
[2] Sodium Pentothal, Abbott Laboratories, N. Chicago, Ill.
[3] Sodium amobarbital, USP, Ruger Chemical Co.
[4] Sodium phenobarbital, Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Flexin, McNeil Laboratories.
[6] California Corporation for Biochemical Research.
[7] Allied Mills, Inc.