band was scraped separately, extracted with methanol, and concentrated. The extract from the second band yielded 160 mg of Solid 5 (P).

**Solid 5 (P) and Hyrcanoside (I)**—Recrystallization from methanol yielded hyrcanoside, mp 205–208°; IR $\nu_{max}$ (KBr): 3430 (OH), 2940 (aliphatic CH), 2870 (aldehyde CH), 1740 (aldehyde carbonyl), 1720 (unsaturated lactone), and 1620 (olefin) $cm^{-1}$. The NMR (dimethyl sulfoxide) spectrum was complicated due to the presence of numerous methylene and hydroxy protons. However, it clearly showed the presence of the methyl and vinyl protons and also the aldehyde proton; UV $\lambda_{max}$ ($CH_3OH$) (log $\epsilon$): 230 (4.10), 290 (1.65), and 322 (1.54) nm. Comparison (IR, UV, NMR, mixed melting point, and cochromatography in three different solvent systems[6]) of the isolated Solid 5 (P) with an authentic sample of hyrcanoside showed the two to be indistinguishable.

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[6] The solvent systems used were: 1, chloroform–ethanol (2:1); 2, chloroform–tetrahydrofuran–*N*-methylformamide–methanol (50:50:7:18); and 3, chloroform–acetone–methanol (6:2:2).

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# Pharmacology of Malnutrition III: Binding of Digoxin to Normal and Kwashiorkor Serum


**N. BUCHANAN *x, L. A. VAN DER WALT ‡, and B. STRICKWOLD ***



**Abstract** □ Digoxin binding to normal and kwashiorkor serum was studied and found to be inferior in the latter. Digoxin should be used with care in hypoalbuminemic patients.



**Keyphrases** □ Digoxin—serum protein binding, normal and kwashiorkor serum □ Protein binding—digoxin, normal and kwashiorkor serum □ Malnutritic serum—digoxin binding, compared to normal serum □ Cardiotonic agents—digoxin, serum protein binding, normal and kwashiorkor serum


Circulating digoxin is known to be approximately 25% bound and 75% free in serum, the sole binding protein being albumin (1). Cardiac disease, associated with rheumatic fever, hypertension, and cardiomyopathy, is commonly seen in developing countries with concomitant poor nutrition. An *in vitro* study was thus performed to assess the effect of hypoalbuminemia on digoxin binding.

## EXPERIMENTAL

Normal serum (albumin concentration of 3.75 g/100 ml) was obtained, with consent, from a patient hospitalized for an orthopedic disorder. Pooled kwashiorkor serum (albumin concentration of 2.4 g/100 ml) was obtained from several children admitted to a metabolic unit for various studies. Such serum was collected prior to the child's receiving therapy to avoid the presence of any competitive binders.

Tritiated digoxin[1] had a specific activity of 10,000 mCi/mmole and a mass concentration of 88,000 μg/ml. This solution was diluted with deionized water so that ultimately aliquots of the diluted solution could be added to 5-ml batches of normal and kwashiorkor serum, producing final digoxin concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 5 ng/ml.

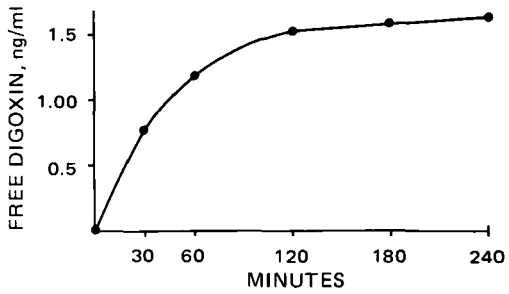


**Figure 1**—*Curve showing the equilibration time for $^3H$-digoxin to be 180 min, there being no statistically significant increase in the free digoxin concentration between the 3- and 4-hr samples* ($p > 0.2$).

The serum was then dialyzed by equilibrium dialysis (2) at 37°, using 0.4 ml of serum for each dialysis. The equilibration time for $^3H$-digoxin was 180 min (Fig. 1), but in practice each run was extended to 240 min. Ten dialyses were performed at each concentration; the digoxin concentrations in the original serum, dialyzed serum, and dialyzate were assessed by liquid scintillation counting[2]. The efficiency for tritium was 38.6%, and each specimen was assayed to give a counting error of 0.7% or less. The amount of digoxin in each sample was calculated directly from these data.

[1] Radiochemical Centre, Amersham, United Kingdom.

[2] Packard Tri-Carb liquid scintillation counter.

Table I—Results of Equilibrium Dialysis Studies on the Binding of $^3$H-Digoxin to Normal and Kwashiorkor Serum

| Total Serum Digoxin Concentration, ng/ml | Normal Serum | | | Kwashiorkor Serum | | |
|---|---|---|---|---|---|---|
| | Free Digoxin, ng/ml | Bound Digoxin, ng/ml | Digoxin Bound, % | Free Digoxin, ng/ml | Bound Digoxin, ng/ml | Digoxin Bound, % |
| 0.1 | 0.084 (0.00)[a] | 0.015 (0.00) | 15 | 0.098 (0.00) | 0.006 (0.00) | 5.8 |
| 0.2 | 0.162 (0.001) | 0.038 (0.00) | 19 | 0.18 (0.00) | 0.013 (0.001) | 8.8 |
| 0.3 | 0.236 (0.008) | 0.064 (0.00) | 21.3 | 0.28 (0.015) | 0.017 (0.001) | 5.8 |
| 0.4 | 0.292 (0.009) | 0.108 (0.005) | 27 | 0.035 (0.002) | 0.029 (0.005) | 7.7 |
| 0.5 | 0.374 (0.006) | 0.126 (0.007) | 25.2 | 0.48 (0.025) | 0.042 (0.002) | 8.0 |
| 1.0 | 0.876 (0.08) | 0.108 (0.006) | 11 | 0.94 (0.02) | 0.059 (0.002) | 5.9 |
| 5.0 | 4.48 (0.29) | 0.24 (0.2) | 5.0 | 4.64 (0.21) | 0.06 (0.001) | 1.3 |

[a] Values in parentheses represent standard deviation.

## RESULTS

The bound and free digoxin concentrations at various total serum digoxin concentrations are shown in Table I. The free concentrations differed significantly between the two groups ($p < 0.01$), as did the bound concentrations ($p = 0.005$).

The differences in binding up to 1 ng/ml are depicted in Figs. 2 and 3, where lines of best fit are employed. In normal serum, only a small amount of digoxin was bound.

From Table I, it can be seen that there was a progressive rise in the amount bound up to 0.5 mg/ml, where there was about 25% binding, after which there was a relative fall off. In kwashiorkor serum, the degree of binding was appreciably less.

## DISCUSSION

A considerable difference of opinion exists concerning the digoxin concentrations associated with toxicity. Doherty and Kane (3) found toxicity to be associated almost invariably with a serum concentration of greater than 3 ng/ml. Cree *et al.* (4) felt that concentrations of approximately 1.5 ng/ml were therapeutically adequate. Zeegers *et al.* (5) observed a mean concentration of 1.6 ± 0.7 ng/ml in nonintoxicated patients as opposed to 4.4 ± 0.9 ng/ml in intoxicated patients. Butler (6) observed an overlap in serum concentrations between nonintoxicated and intoxicated groups; 85% of the latter had serum concentrations above 2 ng/ml.

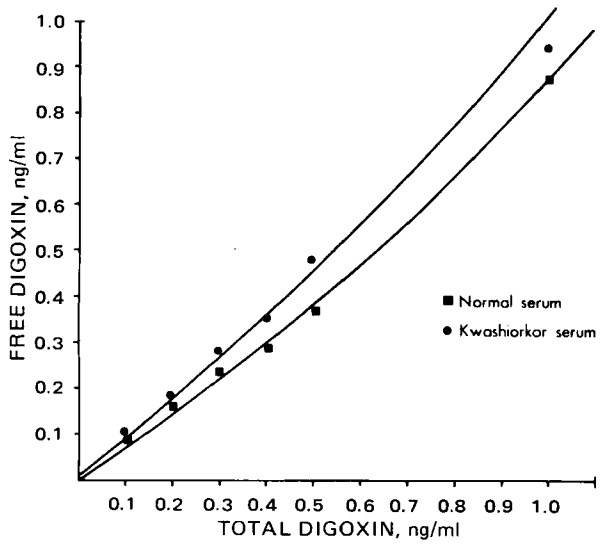


**Figure 2**—*Relationship of free digoxin in normal serum compared to that in kwashiorkor serum. The difference is significant* (t = *3.39*, n = *5*, p < *0.01*).

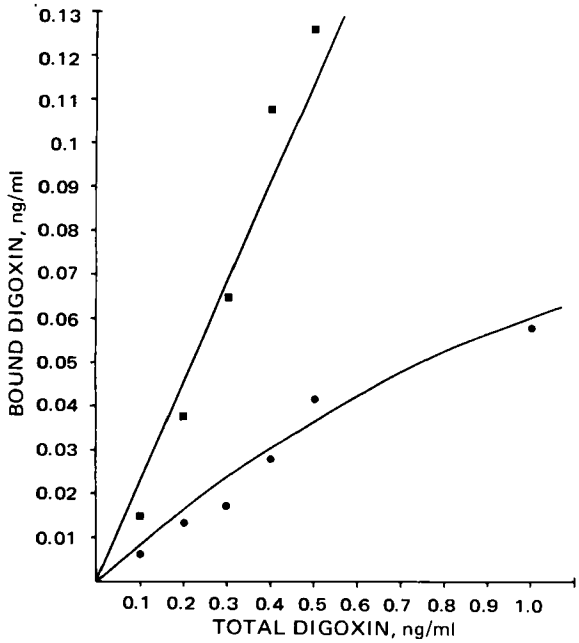


**Figure 3**—*Difference between the amounts of bound digoxin in normal and kwashiorkor serum. The difference is significant* (t = *4.08*, n = *5*, p = *0.005*).

The problem of digitalis toxicity is rendered even more complex because the tissue (cardiac muscle) digoxin concentrations probably are of most importance. The heart to serum concentration is known to vary from 17:1 to 35:1 (mean of 29:1) (6). The variability is as yet unexplained, although such factors as renal function play a part (7).

It is of interest to speculate that this variability may be related to the amount of free digoxin present in the serum and, hence, to the serum albumin concentration. The present study demonstrates that about 25% of serum digoxin is protein bound in normal serum and that binding is saturated at about 0.5 ng/ml total concentration. Thereafter, a progressive increase in the free concentration is observed. This situation is even more marked in the presence of hypoalbuminemia.

In the context of malnutrition, it is known that the serum albumin, serum potassium, and the total body potassium concentrations are depressed (8). These factors, especially when present concomitantly, are liable to produce digitalis intoxication and should probably be considered when treating malnourished persons with cardiac disease.

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# Influence of pH and Route of Injection on Acute Toxicity of Tetracycline in Mice

**ROBERT T. WIVAGG, JAMES M. JAFFE, and JOHN L. COLAIZZI ˣ**

**Abstract** □ Some $LD_{50}$ determinations for tetracycline hydrochloride in mice were carried out over a range of pH values, using both the intraperitoneal and the subcutaneous routes of injection. Depending on the pH of the formulation, either water or a solvent system of water and 60% (v/v) propylene glycol was employed thus ensuring complete solution of the drug at all pH values tested. Higher $LD_{50}$ values were obtained with the subcutaneous route than with the intraperitoneal route. With either route of administration, there was a trend toward lowest $LD_{50}$ value occurrence at the isoelectric pH of tetracycline. Actual statistical significance was achieved for the intraperitoneal route only when $LD_{50}$ values obtained at the isoelectric pH were compared with either of the more acidic pH values.

**Keyphrases** □ Tetracycline—effect of pH and route of injection on acute toxicity, mice □ pH—effect on acute toxicity of tetracycline, mice □ Injection route—subcutaneous *versus* intraperitoneal, effect on acute toxicity of tetracycline, mice □ Toxicity—tetracycline, effect of pH and route of injection, mice □ Antibiotics—tetracycline, effect of pH and route of injection on acute toxicity, mice

The acute toxicity of a drug is usually defined in terms of an $LD_{50}$ value, which is the milligrams per kilogram dose of a drug that, on the average, will kill one-half of the group of test animals of a certain species under specified and controlled conditions following administration of a single dose (1). In the required acute toxicity testing performed on new drug entities, two factors that can influence the experimental results with a parenterally administered drug are its formulation and route of injection (2). For example, it has been demonstrated that intraperitoneal injections of a nitrogen mustard in mice at two different pH values resulted in different $LD_{50}$ values, which could be accounted for on the basis of absorption rate differences resulting from pH–partition effects (3, 4). Other studies revealed that the route of injection may have either substantial effects on acute toxicity, as with procaine, or relatively small effects, as with isoniazid (5).

For the tetracycline class of antibiotics, maximum lipid solubility occurs at the isoelectric pH ($pH_{iso}$); for tetracycline itself, it occurs around pH 5.6 (6). The relative lipid solubility of tetracycline then decreases as the pH is raised or lowered on either side of the $pH_{iso}$. Thus, the objective of this study was to determine if there is a significant difference in acute toxicity values, as determined in mice receiving tetracycline injections, when the formulation pH is altered or when the route of parenteral administration is changed.

## EXPERIMENTAL

**Preparation of Tetracycline Solutions**—Aqueous solutions of tetracycline hydrochloride[1] or solutions containing 60% (v/v) propylene glycol[2] were prepared immediately before injection. Both solutions contained 20 mg of tetracycline hydrochloride/ml. The propylene glycol–aqueous vehicle was necessary due to the minimal water solubility of tetracycline hydrochloride around the $pH_{iso}$, which resulted in marked precipitation when water alone was employed as the solvent. The pH values of the solution were adjusted using 1 *N* NaOH to 2.45, 8.20, and 11.00 in water and to 3.00, 5.50, and 9.00 in the propylene glycol–water solvent mixture.

**$LD_{50}$ Determinations**—The $LD_{50}$ was determined by the method of Litchfield and Wilcoxon (7) as suggested by the Food and Drug Administration (8). This method employs logarithmic probability graph paper and nomographs as the basis of the calculation.

For each test (*i.e.*, for each different pH of a given solvent system by a particular route of administration), 25 male albino mice[3], ~20 g, were randomly divided into five groups of five animals. The mice were injected with the appropriate dose of tetracycline hydrochloride on a milligrams per kilogram basis, employing the appropriate pH–solvent system. Doses for every one of the six pH–solvent systems tested were administered to each of the five groups in the following manner: one dose at the approximate $LD_{50}$ value, previously estimated experimentally, and two doses above and two doses below this median point.

The exact doses were determined by converting the experimentally estimated doses to log scale and separating each dose by ap-

[1] Tetracycline hydrochloride, lot 174-010, donated by Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N.Y.

[2] Propylene glycol, Ruger Chemical Co., Irvington, N.Y.

[3] Swiss-Webster male albino mice, Hilltop Farms, Scottdale, Pa.