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METABOLISM OF CARDIAC GLYCOSIDES—I METABOLISM OF DIGITOXIN-7αT BY NORMAL RABBITS AND RABBITS WITH HEART FAILURE*

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Abstract—A method of obtaining specifically labeled digitoxin-7αT from *Digitalis purpurea* plants previously fed pregnenolone-7αT is described.

The metabolism of digitoxin-7αT by normal rabbits and by rabbits with surgically induced heart failure was investigated. Digitoxin-7αT was injected into both groups of rabbits, and the urinary excretion of radioactivity was evaluated. By means of countercurrent distribution and thin-layer chromatography, a difference in metabolism of digitoxin-7αT between the two groups was observed.

The data show that rabbits with heart failure required much longer periods of time to excrete an equal amount of radioactivity as compared with the normal rabbits. Further, although the radiochromatographic patterns of the two groups were qualitatively identical, the profiles showed that in the group with heart failure the conversion of digitoxin-7αT to its relatively more polar metabolites was substantially hindered.

IN RECENT years, many reports have appeared concerning the metabolism of digitalis glycosides[1–3] which have been randomly labeled by hydrogen exchange, as described by Wilzbach.[4] The problems encountered in obtaining cardiac glycosides that have radiochemical homogeneity when they have been randomly labeled by the Wilzbach technique have been described by Wartzburg *et al.*,[5] who also have recently succeeded in synthesizing specifically labeled digoxin-12α-T. We have found randomly labeled digitoxin lacking for our work in studying the metabolism and excretion of the glycoside. Therefore, a method was developed to biosynthesize specifically labelled digitoxin from pregnenolone-7αT, that had a sufficiently high specific activity.

The excretion and biotransformation of cardiac glycosides have been previously reported by several investigators, among them Okita *et al.*,[6] Cox and Wright,[7] Okita,[8] Wright,[9] and Brown *et al.*[10] However, it has yet to be shown that normal animals and animals with heart failure metabolize or excrete cardiac glycosides differently. As an example of surgically altered glycoside metabolism, Marcus *et al.*[11] have reported that bilaterally nephrectomized dogs, injected with randomly labeled digoxin, maintained higher blood levels of radioactivity than did the control dogs.

It has previously been suggested that a slowed excretion of cardiac glycosides may account for the sensitivity to the glycosides that is frequently seen in patients with severe cardiac decompensation.[12] Considering the aforementioned possibility, we

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undertook to produce severe heart failure in rabbits and then to determine if a change in the metabolism of digitoxin-7αT occurs.

METHODS

Biosynthesis of digitoxin-7αT. Specifically labeled digitoxin was biosynthesized by modifying the experiment described by Tschesche and Lilienweiss[13] in which they used labeled pregnenolone glucoside as the substrate; 4·75 μg of pregnenolone-7αT, with a specific activity of 8·95 mc/mg, was added to 25 ml water. By means of blank runs we showed that this weight of pregnenolone would remain suspended and/or dissolved in 25 ml water. Cut leaves of *Digitalis purpurea* were placed in the water and maintained for 22 days. The leaves were then removed, chopped roughly, and subjected to a continuous hot methanol extraction for 8 hr. The methanol extract was evaporated to dryness and redissolved in a solution of 80% methanol in water and partitioned five times against equal volumes of hexane. The methanol–water layer was evaporated to dryness and chromatographed on a 1·8 cm × 38·0 cm silica gel column. The column was first eluted with a solution of ethyl acetate:benzene (80:20), saturated with water, until 20 fractions of 5 ml each were collected. An aliquot of each fraction was counted in a Tri-Carb liquid scintillation spectrometer, and peak activities were determined. The peaks occurring after the start of the second eluting solution of 2% methanol in ethyl acetate were combined, evaporated to dryness, and placed on a thick-layer silica gel H (Merck) plate, and unlabeled digitoxin was spotted on both edges of the plate. After development in acetone:cyclohexane:acetic acid (49:49:2) the zone of digitoxin-7αT was located by spraying the carrier digitoxin, after masking the radioactive zone, with Zimmerman's reagent. The radioactivity in this zone was eluted with methanol, dried, and subjected to paper chromatography as described by Wells *et al.*[14] The zone of activity that corresponded to digitoxin was cut out and the paper eluted three times with 10 ml methanol. These methanol fractions were combined, dried, and reapplied to a second paper chromatography system as described by Gisvold and Wright.[15] Again the radioactive zone that corresponded to digitoxin was cut out and eluted in the manner described above. At this point 16 mg of carrier digitoxin was added to the radioactive material, and the mixture was crystallized four times from aqueous ethanol until a constant specific activity of 0·376 μc/mg was attained.

Metabolism of digitoxin-7αT. Three virgin New Zealand albino rabbits were anesthetized with halothane, and heart failure was successfully induced by aortic constriction, as described by Gertler *et al.*[16] The presence of heart failure in rabbits used in this study was substantiated by criteria described by Zühlke *et al.*[17] Heart failure was evident within 8 days after the surgery. Three similar rabbits served as controls, with two undergoing sham operations. The average weight for all six animals was 3·2 ± 0·22 kg. Eight days after operation each animal received, in the identical muscle site, an intramuscular injection per kg of 0·22 mg digitoxin-7αT. This dose level permitted the animals to receive approximately 545,000–622,000 disintegrations/min (dpm). The intramuscular route of administration did not evoke the overt signs of drug toxicity that were observed with the intravenous route. Standard rabbit metabolic cages were utilized to prevent mixing of urine and feces. Urine was collected and stored at −6°. The collections continued for 3–5 weeks. An aliquot of each collection before freezing was taken and counted for radioactivity to determine the rate of excretion of radioactivity in each rabbit.

The thawed urine samples were combined and filtered through glass wool and reduced to dryness by lyophilization; they were then distributed through 99 countercurrent transfers in ethyl acetate:cyclohexane:ethanol:water (7:3:3:7). K values were determined for peaks of activity which were obtained from the countercurrent distribution. The less polar peak obtained in the distribution was tested for homogeneity by silica gel H thin-layer chromatography in an ethyl acetate:butanol system (90:10) and by eluting the plate twice for a total running time of 90 min.

RESULTS

The biosynthesis of digitoxin-7αT. Figure 1 shows the radiochromatographic profile of the methanol extract of *D. purpurea* on a silica gel column. It is evident that three major peaks (A, B, and C) were detectable. Peak A was subsequently shown to be unconverted pregnenolone-7αT by crystallization with pregnenolone. Peak B, which accounted for approximately 8 per cent of the originally applied radioactivity, was not identified but was shown to be a conversion product of pregnenolone-7αT.* Peak C was subjected to chromatography and crystallization with carrier digitoxin, as

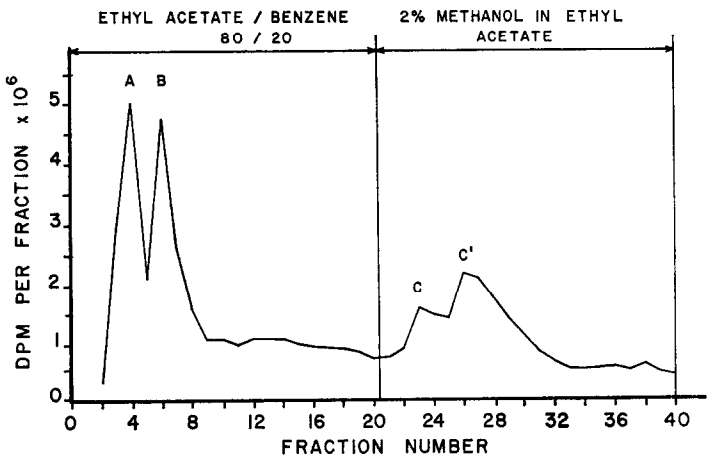

FIG. 1. Radiochromatogram of methanol extract of *Digitalis purpurea*, previously fed pregnenolone-7αT on silica gel column, with ethyl acetate:benzene (80:20) saturated with water and followed by 2 % methanol in ethyl acetate to elute the column.

described under Methods, until a constant specific activity of 0·376 μc/mg was obtained. While the conversion to digitoxin is low from a preparative point of view, it seems to be the easiest method[18] for making the labeled glycoside. In theory, material of higher specific activity could be obtained by using correspondingly more active pregnenolone and/or a more efficient isolation procedure.

Metabolism of digitoxin-7αT. Figure 2 shows the per cent accumulation of the recovered radioactivity in urine for the normal and the heart failure groups; in the normal group 50 per cent of the injected radioactivity was recovered in approximately 16 days and 64 per cent by day 22. However, in the animal with heart failure, a

* Careful examination of the labeled pregnenolone used as substrate excluded the possibility that peak B was a contaminant. Experiments are now being carried out to elucidate the structure of this major conversion product.

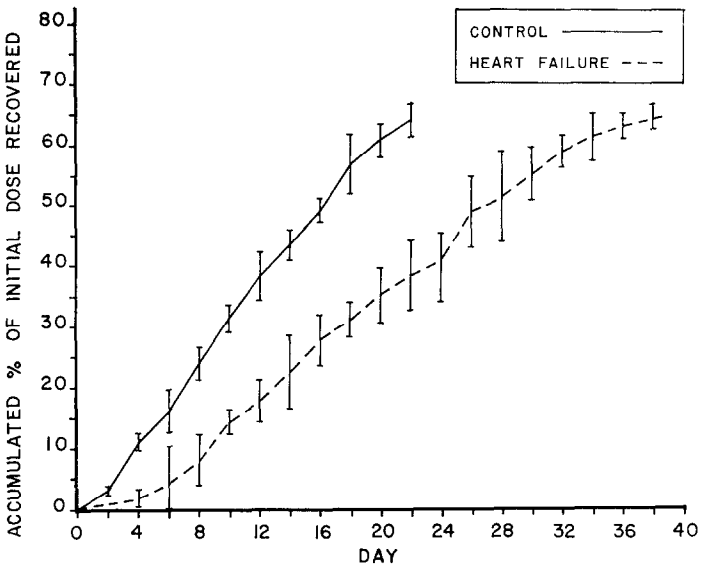

FIG. 2. Accumulative per cent of radioactivity recovered from the urine of normal rabbits as compared to rabbits with heart failure. Vertical lines designate standard error.

greatly diminished excretion was observed. This group required approximately 27 days to excrete 50 per cent of the initial amount of radioactivity and 38 days to excrete 64 per cent.

The lyophilized urines were subjected to countercurrent distribution. In all six cases the radioactivity was resolved into three major peaks. Figure 3 shows typical chromatographic profiles obtained from the urines of a control animal and the animal

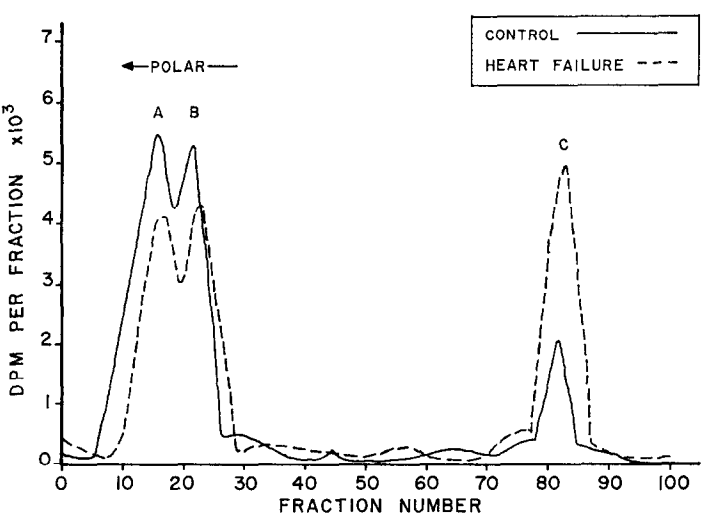

FIG. 3. Typical radiochromatogram obtained by a countercurrent distribution of urine from a normal rabbit and a rabbit with heart failure. The system was ethyl acetate:cyclohexane:ethanol:water (7:3:3:7).

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN URINE

Rabbit	Total activity in urine (dpm)	Peak A and B		Peak C		Recovery from CCD (%)
		Total dpm	% of activity	Total dpm	% of activity	
Normal (sham operated)	383,980	273,520	71·3	110,460	28·7	100·0
Normal	332,400	255,320	76·8	70,140	21·1	97·9
Normal (sham operated)	370,770	257,310	69·4	97,150	26·2	96·6
Mean values*	362,360 ± 5,100	262,050 ± 5,730	72·5 ± 2·21	99,570 ± 11,830	25·3 ± 2·25	98·2 ± 0·98
Heart failure	323,700	181,260	55·9	158,220	48·8	104·7
Heart failure	408,230	195,140	47·8	187,780	46·0	93·8
Heart failure	309,290	175,680	56·8	167,220	54·1	100·9
Mean values*	347,050 ± 30,880	183,950 ± 6,360	53·5† ± 2·86	171,110 ± 8,730	49·6‡ ± 2·37	99·8 ± 3·19

* Mean ± S.E.
† Significantly different from % activity under peaks A and B of normal at 5 per cent level.
‡ Significantly different from % activity under peak C of normal at 5 per cent level.

with heart failure. The K values of peaks A, B, and C were 0·17, 0·27, and 4·9, respectively, in the control animal and were very similar to the values (0·18, 0·29, and 5·2) of the animal with heart failure.

The combined amounts of radioactivity under peaks A and B from the control animals and from the animals with heart failure were compared. A similar comparison with peak C was made between the two groups of animals. Table 1 shows that in the control animals 72·5 ± 2·21 per cent of the recovered radioactivity was located under peaks A and B, with 25·3 ± 2·25 per cent of the activity under peak C. These three peaks constitute approximately 98 per cent of the recovered urinary radioactivity from the controls. In rabbits with heart failure, peaks A and B represent 53·5 ± 2·86 per cent of the recovered radioactivity, and peak C represents 49·6 ± 2·37 per cent. The three peaks constitute approximately 99 per cent of the recovered urinary radioactivity from the animals with heart failure. Table 1 also shows that the amounts of radioactivity under peaks A and B (combined) and peak C of the controls are significantly different at the 5 per cent level from the corresponding peaks from the animals with heart failure.

The peak tubes of peaks A and B, from both groups of animals, were applied to two thin-layer chromatography systems, described under Methods. However, each peak produced only one area of radioactivity. Further, the presence of a glucuronide conjugate has been excluded because incubation of peaks A and B with Ketodase (Warner-Chilcott), a system for cleaving glucuronides, did not result in an alteration in mobility of either peak with thin-layer chromatography. It has been shown that substances normally present in rabbit urine did not inhibit this enzyme system (Layne *et al.*[19]). To date, it has not been possible to identify the exact chemical nature of polar peaks A and B.

Approximately 132,000 dpm of peak C from both groups of rabbits were applied along with digitoxin, digitoxigenin-bis-digitoxoside, and digitoxigenin-mono-digitoxoside standards on silica gel H plates and developed for 90 min in the system and manner described under Methods. Table 2 shows that peak C radioactivity could be

TABLE 2. THIN-LAYER RADIOCHROMATOGRAPHIC PROFILE OF PEAK C

Compound	R_f	Per cent of recovered activity*	
		Normal	Heart failure
Digitoxigenin-mono-digitoxoside	0·81	2 ± 0·58 (2)†	4 ± 1·11 (2)
Digitoxigenin-bis-digitoxoside	0·72	18 ± 1·00 (2)	18 ± 0·82 (2)
Digitoxin	0·63	80 ± 2·05 (3)	78 ± 3·14 (3)

* Mean ± S.E.
† Values in parentheses designate number of tests.

further resolved into three separate peaks that corresponded to the three co-spotted standards. Table 2 shows that in the normal and heart failure groups, the mono-glycoside appeared, respectively, as 2 and 4 per cent, the bis-glycoside as 18 and 18 per cent, and digitoxin as 80 and 78 per cent of the total recovered radioactivity from the plate. It is apparent that peak C of both groups contained essentially the same percentage of all three compounds.

Although the lack of sufficient quantities of the mono- and bis-glycosides precluded crystallization of the mono- and bis-glycosides from peak C, the successful crystallization of digitoxin-7αT to constant specific activity from peak C was accomplished by adding carrier digitoxin.

DISCUSSION

In two previous studies it was reported that the total urinary excretion of radioactivity from labeled digitoxin by patients with congestive heart failure (Doherty and Perkins[20]) and the total urinary excretion of radioactivity by normal patients (Marcus *et al.*[12]) were essentially the same. The excretion rate of radioactivity for normal rabbits agrees closely with data presented by Brown *et al.*[21] for normal rats, although in that study urine collection was continued for only 24 hr. The rates of excretion of radioactivity in the present study (Fig. 2) show that 50 per cent of the initial dose was recovered in approximately 16 days from the control group of rabbits, as compared with 50 per cent excretion of the initial dose in approximately 27 days from the group of rabbits with heart failure. As shown in Fig. 2, approximately 64 per cent of the initial dose of radioctivity was recovered in the urine from both groups of rabbits, although it required 16 days longer for the rabbits with heart failure to excrete this amount.

The rabbits used in this study were in extreme heart failure and, as was suggested by Marcus *et al.*,[12] a severely decompensated heart could possibly alter renal excretion rates. Our data would support this view. However, unlike the suggestion of Marcus *et al.*[12] that metabolism of digoxin is not impaired in patients with heart failure, our results shown in Table 1 and Fig. 3 suggest that impairment of the biochemical conversion of digitoxin-7αT to more polar metabolites does occur in rabbits with heart failure. It is possible that this impairment has been overlooked until now because in previous studies the urine is first extracted by chloroform. This extraction removes most of the less polar compounds but leaves the polar ones. The polar compounds left behind may very well be our peaks A and B (Fig. 3).

Table 1 shows that in the control group, digitoxin and its polar metabolites (peaks A and B) are excreted into the urine at a ratio of approximately 1:3 by the time 64 per cent of the initial dose of radioactivity is recovered. Conversely, the group with heart failure excreted digitoxin and its polar metabolites (peaks A and B) in a ratio of 1:1 by the time 64 per cent of the initial amount of radioactivity was recovered. The calculated K values from Fig. 3 for peak A in the normal and heart failure cases were 0·17 and 0·18. Peak B values in the normal and heart failure cases were 0·27 and 0·29. Consequently, because of the similarities of the K values of Peak A from both groups and the K values of peak B from both groups, and further, because of our inability to resolve either peak from each group by thin-layer chromatography into additional peaks, we must conclude that the impairment of the conversion of digitoxin-7αT to its subsequent polar metabolites by rabbits with heart failure is of a quantitative, not qualitative, nature.

Peak C (Fig. 3) was carefully studied, as described in Methods and Results. An analysis of the compounds under peak C was made to ascertain if the ratios of the compounds were different for either group of animals. Repke[22] has described the stepwise degradation of digitoxin to its bis-and mono-digitoxosides. In our study it was shown that this degradation occurred to the same degree in both groups (Table 2).

The relative amount of each compound is the same in both cases. Thus it appears that the impairment to the metabolism of digitoxin is not in the stepwise cleavage of the polysaccharide moeity, since no accumulation of any one compound occurs.

Recently Marcus *et al.*[11] have reported that in two patients with renal failure there appeared to be an increase in the water-soluble metabolites of digoxin in the stool. It was shown in the same report that impaired renal function produced a significantly higher and prolonged blood level of radioactivity after injections of labeled digoxin. With these data and the data reported here, it could be suggested that in the rabbits with heart failure the observed slowed excretion rate may be accounted for by the hindered rate of conversion of digitoxin to its more polar, water-soluble metabolites. Thus the rate of appearance of radioactivity in the urine would be greatly hindered because the renal clearance rate of digitoxin could be much less than that of its subsequent metabolites.

Present studies are underway with large quantities of low specific activity digitoxin-7αT to facilitate isolation, crystallization, and total chemical analysis of peaks A and B. These studies are designed to elucidate the possible metabolic and/or physiologic alterations that may have occurred to produce the reported impairment of conversion and excretion of digitoxin-7αT in rabbits with heart failure.

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