The Fixation of Radioactive Digitoxin by Isolated Hearts

By A. Sjoerdsma, Ph.D., M.D., and C. S. Fischer, M.D.

The nature of digitoxin fixation in isolated hearts was studied with C\textsuperscript{14} labelled digitoxin. Fixation was greatest in the early stages of perfusion. The amount of drug fixed was measured directly. A considerable percentage of the digitoxin in cardiac muscle was changed to other substances. These substances are more firmly bound to the heart than digitoxin.

The preparation of pure, randomly labelled, radioactive digitoxin in this laboratory\textsuperscript{1} has made available a sensitive tracer technic particularly suited to studying the fate of digitoxin in isolated organs and intact animals. In contrast to other methods of assay, this new technic enables one to follow the unchanged drug as well as metabolic products formed after interaction with body tissues. The extreme sensitivity permits experiments with digitoxin in amounts which are not lethal to the intact animal. Furthermore, in perfusion experiments on isolated hearts, the concentration of labeled digitoxin is low.

The work of previous investigators has not settled the problem of digitoxin fixation in cardiac muscle. Conclusions drawn were only by inference since the results depended on biologic assay experiments. With the radioactive technic, direct measurement of digitoxin uptake can be carried out on homogenates of hearts previously perfused with radioactive solutions. The percentage of conversion to other substances can also be determined by this direct method. Furthermore, studying the radioactivity of the perfusion fluids of isolated heart preparations enables one to judge the rapidity of drug fixation by heart muscle. With these possibilities in mind we studied the fixation of radioactive digitoxin by isolated hearts of rats, guinea pigs, rabbits and cats.

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Methods

A small amount of radioactive digitoxin with a specific activity of 350 counts per minute per \( \mu g \) was supplied in a stock solution containing 50 \( \mu g \) of drug per cc. of 95 per cent ethanol. For perfusion, digitoxin was diluted with Ringer-Locke solution to a concentration of 0.2 \( \mu g \) per cc. The hearts were perfused by the Langendorff\textsuperscript{2} technic through a cannula inserted into the aorta. A two-way stopcock above the cannula permitted rapid change from control to radioactive solutions.

After stabilization of the rate and rhythm, the hearts were perfused with radioactive digitoxin and six successive 25 cc. samples of perfusate were collected. Control specimens were drawn from the side arm of the cannula. Immediately following perfusion, a 10 per cent water homogenate of the hearts was made and 1 cc. volumes of homogenate and perfusion fluids were dried on flat copper discs in an area of 10 sq. cm. Measurements of the total radioactivity were carried out on these preparations.

To determine the percentage of total radioactivity due to unchanged digitoxin, extractions were done on 5 cc. aliquots of control and perfusate solutions, and on 2 to 10 cc. aliquots of heart homogenate. Each sample was shaken for 15 minutes in a glass-stoppered centrifuge tube containing 1 mg. of nonradioactive digitoxin in 20 cc. of reagent grade chloroform. After centrifuging for 10 minutes, the water phase was discarded and the chloroform fraction filtered. The residue was re-extracted twice with chloroform, filtered, and the filtrate added to the original. The total was then evaporated to dryness to remove excess water. The dried material was redissolved in 30 cc. of chloroform.
and adsorbed on a 5 by 50 mm. adsorption column using alumina, which had previously been washed successively with distilled water, 95 per cent ethanol and chloroform. Thirty cc. of 1 per cent ethanol in chloroform was added to the column, followed by 50 cc. of 10 per cent ethanol in chloroform. This latter fraction was collected, dried, redissolved in 10 per cent ethanol in chloroform, transferred to a 5 cc. beaker with a Pasteur pipet, and again evaporated to dryness. Finally, the extract was suspended in 1.2 cc. of 20 per cent ethanol in water and plated on a copper cup in a circular depression 10 sq. cm. in area.

By this method of extraction, recovery of radioactive digitoxin from Ringer-Locke solution, perfusate and water was 85 to 100 per cent but only 50 to 70 per cent from 10 per cent heart homogenate.

Triplicate samples were counted on copper cups and discs in the internal Geiger counter as described by Kelsey, with a difference between samples of less than 10 counts per minute. This represents a maximum error of 10 per cent, the more active samples affording measurements of greater accuracy. A self-absorption correction factor was used with heart homogenate whereas self-absorption by residues from Ringer-Locke and perfusion solutions was negligible.

**Results**

Digitoxin in the low concentration used produced little physiologic effect. A slight depression of contractile force was noted in most cases, probably due to the alcohol in the solution. Notwithstanding the small amounts of the drug, the radioactivity was sufficient to enable accurate analysis.

By comparing the radioactivity of the solutions before and after perfusion, it was possible to obtain a measure of the rapidity of digitoxin fixation. Such data, representing an average of...
three to six experiments on each species, is given in figure 1. Fifteen to 20 minutes was required for rat and guinea pig perfusions, and 5 to 10 minutes for rabbits and cats. The bars depict the total radioactivity (expressed as µg. equivalents of digitoxin) of control digitoxin and perfusate fluids, while the cross-hatched portion refers to recoverable digitoxin. Blank experiments demonstrated small but variable losses of radioactivity in the apparatus. Hence, the values obtained do not lend themselves to an accurate calculation of total drug fixation but are given rather to show the general curves of uptake. Graphs of the four species are similar in configuration; there was a rapid initial uptake of carbon¹⁴ followed by a slow but persistent fixation through the course of the perfusion. The amount of digitoxin in the perfusates tended to parallel the total radioactivity, although toward the end of the perfusions the percentage of digitoxin in the perfusates was less for rabbits and cats than for rats and guinea pigs.

In table 1 are shown the uptakes of digitoxin as derived from direct measurement of heart homogenate activity. The total carbon¹⁴ fixed by rat and guinea pig hearts was remarkably constant and averaged an equivalent of 1.49 and 1.76 µg. of digitoxin per Gm. of heart, respectively. Fifty-nine and 68 per cent of the radioactivity was present as unchanged digitoxin. The fixation by rabbit and cat hearts was variable but the averages were .65 and .83 µg. per Gm. of heart, with corresponding percentages of 70 and 62 as digitoxin. The total fixation of digitoxin varied directly with the heart weight, so that the large cat hearts exhibited the greatest fixation.

The mode of digitoxin fixation to heart muscle is unknown. To learn whether or not digitoxin is reversibly bound, hearts of the same four species were perfused as before with 150 cc. of radioactive digitoxin solution, after which the perfusion was continued with 50, 200, 450 or 1000 cc. of nonradioactive Ringer-Locke. The hearts were then homogenized and total and digitoxin-recoverable carbon¹⁴ measured. The results as given in table 2 again indicate that rat and guinea pig uptakes are less variable than those of rabbits and cats. The rabbit hearts washed with 50 and 450 cc. and the cat

<table>
<thead>
<tr>
<th>Animal</th>
<th>Volume of Wash</th>
<th>Total C¹⁴</th>
<th>Extractable Digitoxin*</th>
<th>Digitoxin as % of Total C¹⁴</th>
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<td>0.48</td>
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<tr>
<td></td>
<td>450</td>
<td>1.49</td>
<td>†</td>
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<tr>
<td></td>
<td>1000</td>
<td>1.49</td>
<td>†</td>
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<td>200</td>
<td>1.76</td>
<td>0.51</td>
<td>26</td>
</tr>
<tr>
<td></td>
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<td>1.76</td>
<td>0.39</td>
<td>23</td>
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<td></td>
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<td></td>
<td>450</td>
<td>0.25</td>
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<td>48</td>
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</table>

* Values expressed as digitoxin equivalent (µg./Gm. of heart).
† Value statistically invalid (less than .015 µg.)
‡ No explanation can be offered for these high values except individual variation from the mean as described in the text.
heart washed with 50 cc. contained more activity than the average for the species without washing. Nevertheless, the carbon$^{14}$ present as digitoxin exhibited a progressive decline in the animals studied. It was particularly easy to wash digitoxin from rat hearts while the other hearts retained significant activity even after 450 to 1000 cc. of wash. Another phenomenon observed was the tendency toward a progressive diminution of the digitoxin to total carbon$^{14}$ ratio as the washing proceeded from 0 to 1000 cc.

**Discussion**

In the isolated hearts of the four species studied, the uptake of radioactive digitoxin was relatively greater in the early stages of the perfusion. This is in agreement with the experiments of Straub$^1$ and others on the Starling cat preparation and the excised frog heart. He states that the frog heart may absorb a fatal dose of digitoxin from the solution in one minute while the systolic arrest appears only after 10 minutes. Straub believed that the receptive capacity of heart muscle might be exhausted after one passage of digitoxin through it, providing the concentration is sufficiently high. Our results with a low concentration of radioactive digitoxin can be interpreted in terms of these two hypotheses. Following the initial rapid fixation, absorption of the drug from the solution continued because the receptive capacity of the heart had not yet been exhausted. The absence of typical physiologic effect noted in our experiments is undoubtedly due in part to the small amount of digitoxin in solution; however, the delay in digitoxin action observed by Straub may also be a factor. Recent work by Friedman and Bine$^5$, $^6$ with the embryonic duck heart tends to contradict earlier experiments. Using lanatoside C and digitoxin they demonstrated that with increasing concentrations of the drugs there was a progressive decrease in the time taken for occurrence of digitalis effect. The same authors suggest that previous data may be attendant on penetration of the glycosides into cells of the adult heart.

Measuring the fixation of radioactive digitoxin by isolated hearts gave no clue as to the reason for differences in species sensitivity. The only correlation made was that hearts tended to remove digitoxin from solution in direct relation to their mass. We have no explanation for variable uptakes of rabbit and cat heart.

The reversibility of digitoxin fixation has been a subject of great controversy for years. The studies of Issekutz,$^7$ Straub$^1$ and others on the frog heart indicated that the digitalis effect persists despite prolonged washing, and that small doses lower the threshold requirement even after a long intervening wash period. The implication was that digitoxin is irreversibly bound to heart muscle. Hatcher's work$^4$ with intact cats led to the conclusion that digitoxin is bound to heart muscle for several weeks. The greater susceptibility of Starling cat preparations from animals pretreated with digitoxin gave further continuity to these ideas.$^4$ Other authors have emphatically declared that the combination of digitoxin with heart muscle is not irreversible.$^5$ Kingsepp$^5$ performed washout experiments with digitoxin using isolated frog ventricles and showed that the action of digitoxin can be completely reversed by thorough washing. With several other glycosides the effects were more easily reversible. Paff and Johnson$^8$ have demonstrated the same phenomenon on chick hearts. In all these studies the important criticism is that the results depended entirely on the observation of biologic effects and in no case could it be definitely proved that digitoxin was actually washed out. Hence, the dichotomy of storage in the heart versus persistence of effect was insoluble. The radioactive technic surmounts these difficulties. Our studies on the reversibility of fixation demonstrates that the amount of both total radioactivity and digitoxin can be markedly lowered by washing, but that within the limits of the experiments some digitoxin always remains, except with the rat heart. With this animal, the total radioactivity and digitoxin are readily and completely washed out. It is of interest that the rat heart is also most resistant to digitoxin. In addition it was found that digitoxin is less strongly bound than its metabolites, the latter term referring to the radioactivity not accounted for by extractable digitoxin. It is possible that this fraction consists in part of digitoxin bound in the tissues, to the tissue protein.
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for example. The nature of these metabolic products awaits further investigation. Experiments now in progress show that the radioactivity not ascribable to unchanged digitoxin appears in the initial water phase of the extraction and in the 1 per cent eluate, which paper chromatographic analyses indicate consists primarily of digitoxigenin.

SUMMARY

Isolated hearts of four mammalian species were perfused by the Langendorff technic with 150 cc. of Ringer-Locke solution containing 0.2 \mu g. of radioactive digitoxin per cc. The most rapid uptakes of digitoxin from the perfusing fluid occurred during the early stages of perfusion. There was a relatively constant fixation of radioactive digitoxin by rat and guinea pig hearts, whereas the uptake by rabbit and cat hearts varied considerably. About 50 to 70 per cent of the total radioactivity could be extracted from the hearts as digitoxin. Experiments on the reversibility of digitoxin fixation clearly demonstrated that a considerable percentage of the digitoxin fixed in heart muscle is changed to other substances, the nature of which is unknown. The concentration of these metabolites and unchanged digitoxin in the heart can be lowered appreciably by washing, but with the exception of the rat heart, a small quantity always remains. Digitoxin is bound less firmly to heart muscle than its metabolites.

REFERENCES

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