was isolated and was shown to be related to vaccinia virus by cross hemagglutination tests and cross neutralization tests. The infected colony as well as harvests of other viral agents prepared from mice in the colony have been destroyed.

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## Intracellular Distribution of Digitoxin.* (20345)

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Recently we have demonstrated in various animals(1) that digitoxin readily leaves the blood stream to enter the various organs in quite significant amounts. However, the possible intracellular fate of digitoxin administered to the intact animal has never been determined because of the technical difficulties heretofore involved in such a study. Yet, in view of the uncertainty concerning both the site and mode of action of this glycoside, such information might be of considerable value. This is particularly so since the intracellular site of various enzymes now has been determined with a high degree of certainty.

On the introduction of the ultracentrifugal method for routinely isolating and obtaining intracellular components(2,3) and a microsensitive assay for the quantitative detection of digitoxin(4), it appeared possible that some information could be obtained concerning the intracellular fate of parenterally administered digitoxin. The results of a study using these technics are reported in this communication.

*Methods.* 1. *Separation and Collection of Intracellular Components.* The methods used, except for slight modifications, are those which have been described by Schneider and Hogeboom(3). The rats used were Long-Evans strain males, weighing 200 to 250 g. Rat liver was obtained five minutes after the intravenous injection of digitoxin (1 mcg. per gram of body weight) and rinsed in isotonic sucrose until the washings appeared free of gross blood. The liver was then forced through a tissue press into a beaker containing isotonic sucrose, the pulp weighed and sufficient sucrose solution added to prepare a 10% homogenate. Hearts were grossly dissected free of vessels and valves, weighed and minced with a scissor into the homogenizer containing sucrose. The homogenate was spun at 600 g for 10 minutes, the supernatant removed and the sediment washed, then respun again at the same speed and for the same period. The sediment obtained was identified by staining and microscopic study as the nuclear and debris fraction (Nw fraction)† The supernatant and washings were combined and spun at 5000 g for 10 minutes. The sediment was washed again and respun at 5000 g for 10 minutes. The sediment obtained was identified as the mitochondrial fraction (Mw2 fraction), the supernatant fluid identified as the fraction $S_1$ (no mitochondria were seen in this fluid) described by Schneider and Hogeboom(3). Similar fractions were obtained from the hearts of rats given the same quantity of digitoxin, except that an additional sedi-

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† The nomenclature used in labelling these fractions is that of Schneider and Hogeboom(3).

TABLE I. The Digitoxin Content of Liver and Heart Cell Fractions Immediately after Its Administration.

| Total organ dig., μg/g | Fraction Nw: Dig., μg/g | Fraction Nw: % of total in all fractions Dig. | Fraction Nw: % of total in all fractions Nitrogen | Fraction Mw2: Dig., μg/g | Fraction Mw2: % of total in all fractions Dig. | Fraction Mw2: % of total in all fractions N. | Fraction $S_1$: Dig., μg/g | Fraction $S_1$: % of total in all fractions Dig. | Fraction $S_1$: % of total in all fractions N. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | A. Liver homogenate | | | | | |
| 5.0 | .004 | .2 | — | .042 | 2.0 | — | 2.0 | 98 | — |
| 2.5 | N.D. | N.D. | 6.3 | N.D. | 0 | 8.3 | 1.8 | 100 | 85 |
| 2.5 | .150 | 7.4 | 2.0 | .03 | 1.5 | 5.8 | 1.8 | 91 | 92 |
| 1.3 * | .018 | 1.9 | 6.2 | .01 | .9 | 4.5 | .9 | 97 | 89 |
| 2.5 | .024 | 1.7 | 2.1 | N.D. | 0 | 8.1 | 1.4 | 98 | 90 |
| 2.1 | .138 | 10.2 | 9.8 | .01 | .7 | 15.9 | 1.2 | 89 | 75 |
| 2.65 | .056 | 3.6 | 5.3 | .015 | .09 | 8.5 | 1.5 | 96.5 | 86 |
| | | | | B. Heart homogenate | | | | | |
| | | | | | | | Fraction $S_2$ | | |
| 2.2 | .11 | 8.5 | — | .06 | 3.2 | — | 1.1 | 85 | — |
| 2.2 | .06 | 4.8 | 27 | .02 | 1.6 | 17 | 1.1 | 89 | 50.0 |
| 2.0 | .12 | 5.5 | 29 | .05 | 2.5 | 19 | 2.0 | 92 | 44.2 |
| 2.13 | .096 | 6.3 | 28 | .04 | 2.4 | 18 | 1.4 | 88.7 | 47.1 |

* Liver perfused with isotonic sucrose *in situ* before homogenization.

mentation of the submicroscopic particles of fraction $S_1$ at 22000 g resulted in the sediment labelled fraction Pw and the supernatant $S_2$. The heart homogenates were centrifuged in a Spinco horizontal head which gives particularly well defined layers. All of the fractionations were done at a temperature between 0 and 5°C. The nitrogen of the fractions was determined by direct nesslerization(5).

2. *Extraction of Digitoxin for Bio-Assay.* The Nw, Mw2, Pw, and aliquots of the S fractions were diluted to 10 ml. with Tyrode's solution, then shaken with 25 ml of $CH_2Cl_2$ for 1 hour at room temperature, allowed to chill overnight at 4°C—or shaken in the cold and centrifuged immediately. The aqueous layer was removed and the protein and $CH_2Cl_2$ layers separated by filtration. The $CH_2Cl_2$ filtrate was evaporated to dryness; $C_2H_5OH$ (95%) was added and evaporated twice to remove traces of $CH_2Cl_2$ from the slight residue. The residue was taken up in Tyrode's media, and assayed for its digitoxin content by the embryonic duck heart method(4). In each experiment, a portion of the intact tissue (or homogenate) also was analyzed for its digitoxin content in order to determine what fraction of the total amount of digitoxin in the tissue was present in each fraction isolated. For control purposes, identical analyses both of intracellular components and whole organ fragments of animals not receiving digitoxin were carried out in each experiment. None of these control samples was found to affect the contractions of the embryonic duck heart preparation.

*Results.* 1. *The Deposition of Digitoxin in the Hepatic Cell.* As Table I shows, digitoxin when administered to the intact rat appears to enter the interior of the cell. Thus the nuclear and mitochondrial fractions contained 0.056 and 0.015 μg of digitoxin per gram of tissue respectively, 5 minutes after injection of the drug. The deposition of digitoxin, however, in these particular fractions of the homogenate was notably low compared to its accumulation (1.5 μg/g) in the aqueous soluble fraction $S_1$. This amounted to approximately 96% of the total digitoxin found in all of the fractions extracted. The nitrogen assay of these same fractions suggested that the major portion of cellular protein also was present in this same fraction.

2. *The deposition of digitoxin in the cardiac cell.* As in the above findings, only the supernatant fraction $S_2$ was found to contain a significant amount of digitoxin (Table I, B). Thus this fraction contained approximately 89% of all digitoxin recovered from the four fractions analyzed. The nitrogen analyses of these same fractions are interesting in that they demonstrate that the deposition of digitoxin is not a simple protein adsorption

phenomenon. For, while the digitoxin content of soluble protein fraction $S_2$ is 35 times as great as that of mitochondrial fraction Mw2, the nitrogen content of $S_2$ is less than 3 times that of Mw2.

*Discussion.* The present results indicate that although digitoxin when administered to the intact animal was capable of entering various cellular components, it appeared to concentrate only in that fraction ($S_1$ or $S_2$) of liver or heart containing the soluble constituents of the cell and extracellular fluid. The trace amounts of digitoxin found in the nuclear and mitochondrial fractions suggest that digitoxin probably does not exert its characteristic action either at these intracellular stations, or upon the energy producing enzyme-substrate complexes found therein. Preliminary experiments on the influence of digitoxin on the oxygen uptake of the various fractions appear to confirm this view. Although it is possible that the drug may have a catalytic effect at the levels found, there has been no work reported to substantiate this hypothesis. In fact, all evidence appears to dissociate the glycoside effect from energy producing systems(10). Another possibility, that low recovery is due to very rapid inactivation or destruction of the drug by mitochondrial enzymes during fractionation may be eliminated since digitoxin is not destroyed during 4 hours of incubation under similar conditions. It is possible, however, that the great amount of digitoxin present in fraction $S_1$ may be stored for later use by the other components of the cell. Studies concerning this possibility are now in progress.

Our interest naturally is centered in the elements of the soluble fraction. According to Hogeboom(6) this fraction is a highly complex mixture containing all the enzymes involved in anaerobic glycolysis, isocitric dehydrogenase, acid and alkaline phosphatase, catalase, cytochrome C and others. Besides these constituents, adenosine deaminase also has been found in this fraction(7). It is of interest that Snellman and Gellotte(8) recently have found that digitoxin is an inhibitor of this enzyme. Finally, in the heart it is this fraction too, which contains the proteins actin and myosin,—two substances which contribute fundamentally to the contractile dynamics of the cardiac cell.

Quite recently, after completion of this report, Harvey and Pieper(9) published a preliminary report about the intracellular distribution of radioactive digitoxin in the guinea pig heart. They report, contrary to our own findings, that the cellular fractions consisting of cellular debris, and of mitochondria, contained the greatest radioactivity and the aqueous soluble fraction, the least. The reason for this discrepancy between their results and ours is not apparent from their precis, but it should be noted that we have analyzed the cellular fractions after injection of the *intact* animal and their fractions were obtained from the isolated heart perfused apparently with a fluid other than blood. Nevertheless, it may be interesting if confirmation of this work could show that accumulation of digitoxin in guinea pig mitochondria, as opposed to the slight deposition in rat mitochondria, provides a basis for the extreme sensitivity of the guinea pig to glycosides, as opposed to the notorious insensitivity of the rat.

*Summary.* The hearts and livers of animals given intravenous digitoxin were homogenized and separated by centrifugation into nuclear, mitochondrial and homogeneous supernatant fractions. More than 85% of the digitoxin was found in the supernatant fractions of both heart and liver, while the mitochondria of the organs contained 3 and 0.1%.

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