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CONVERSION OF DIGOXIN TO DIGOXIGENIN BY LIVER TISSUE *IN VITRO**

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Abstract—An essentially quantitative extraction method for the recovery of microgram quantities of ^{3}H-digoxin from tissue has been used to study the conversion of digoxin to its genin by liver tissue *in vitro*. Contrary to a previous report of rapid genin conversion of digitoxin *in vitro*, only trace amount of genin was formed with digoxin as the parent glycoside. Similar results were obtained with both slices and homogenates under aerobic and anaerobic conditions.

THE EXTREME variation in both the effective and the toxic dosage of cardiac glycosides from one patient to another is still a perplexing problem in the daily practice of clinical medicine. Similarly, the large variation in species response to these drugs is well known but not adequately understood. The fairly recent availability of microchemical techniques and radioactive glycosides has now made it possible to investigate the metabolism of the compounds in an effort to explain these phenomena.[1] The earlier work of Hilton[2] indicated that cardiac glycosides could be rapidly converted to their genin form *in vitro*. However, considering the persistance of unchanged glycosides in the blood,[3] it is difficult to believe that such a rate of hydrolysis occurs *in vivo*. The present report is concerned with initial investigations on the biotransformation of cardiac glycosides by means of tritium-labeled digoxin.

METHODS

Male Holtzman albino rats weighing 200 to 250 g were starved overnight prior to use. The animals were sacrificed by cervical fracture and the liver quickly excised and placed in an ice-cold solution of the appropriate incubation system. Liver tissue was then prepared as either tissue slices of less than 1 mm thickness, sectioned in the Stadie-Riggs tissue slicer, or homogenized in Teflon and glass with 9 volumes of Krebs-Ringer phosphate solution.[4] In some cases certain cofactors were incorporated into the Krebs-Ringer phosphate solution as indicated in the tables. Likewise, in many incubations Tris buffer (Sigma Chemical Co., St. Louis, Mo.) was substituted for phosphate buffer to yield a final concentration at 37° of 0·0247 M (pH of 7·4).

Each incubation experiment consisted of 0·5 g of liver slices or its weight equivalent in homogenate in a total volume of 5 ml in a 20-ml beaker. Two μg of tritiated digoxin having a specific activity of 10·5 μc/mg (generously supplied through the courtesy of

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Burroughs-Wellcome Ltd., Tuckahoe, N.Y.) was added to each incubation beaker in a total volume of 0·1 ml of 95% ethyl alcohol. Incubation was carried out at 37° in a Dubnoff shaking metabolic incubator for the appropriate time intervals stated in the tables. At the termination of the incubation period, the incubates were extracted through the multiple solvent extraction method yielding aqueous alcohol-, CCl_4-, and $CHCl_3$-soluble fractions as previously described.[5] The $CHCl_3$-soluble extract was then separated on alumina chromatography columns.[5]

Radioassay was performed with a liquid scintillation spectrometer (model 720, Nuclear Chicago Corp., Chicago, Ill.). Through the course of these investigations, intermittent determination of the mean background counting rate to an error of less than 1% was made; minimal variation was found from one counting period to another. The background was then considered as 'known' for the counting of all samples to a 95% confidence limit, as outlined by Loevinger and Berman.[6] All samples were counted for 30 min, and those significant at this 5% level (approximately 1·6 times background) were accepted as meaningful values. Samples with lesser counting rates were considered to be without activity, since the counting time necessary to obtain meaningful data would be prohibitive. All samples with significant activity were corrected for quenching by the addition of a known amount of tritiated toluene as internal standard. In some cases, samples with a counting rate of greater than 200 net cpm were corrected by the channels–ratio method.[7] In each independent group of experiments, at least duplicate assays of the standard tritiated digoxin added to the system were performed.

Some of the incubations had additional substrates present in solution, according to methods of previous investigators.[8] These were present in a final concentration of 11·5 mmoles/l glucose, 4·9 mmoles/l monosodium glutamate, 4·9 mmoles/l sodium pyruvate, and 5·4 mmoles/l sodium fumarate. Some of the incubations had either NADPH (reduced triphosphopyridinenucleotide) or NADH (reduced diphosphopyridine-nucleotide) present in solution (both purchased from California Corp. for Biochemical Research, Los Angeles, Calif.).

RESULTS

As shown in Table 1, recovery from various control systems was essentially complete with only an occasional trace amount of radioactivity found in the nondigoxin fractions. This was found to be reasonably consistent both with and without tissue present in the incubate.

Table 2 shows minimal conversion of digoxin to its genin in the 1-hr incubations described. When phosphate buffer was supplanted by Tris buffer, it appears that a somewhat greater amount of genin and aqueous alcohol-soluble material (probably digitoxose) is formed. There were no apparent differences noted when any one of the four organic substrates (glucose, glutamate, fumarate, pyruvate) was absent from the system, but more genin was found when all four were present in Tris buffer. Results with homogenates and slices were essentially identical.

A slightly greater amount of genin production was noted in the presence of oxygen as compared to incubation under nitrogen (Table 3). However, only slight metabolism was noted, and there appeared to be no further change after 10 to 20 min.

When NADPH was added to incubates not containing organic substrates, a considerable drop in total recovery was noted with no striking change in genin

TABLE 1. AEROBIC INCUBATION OF ^{3}H-DIGOXIN IN CONTROL SYSTEMS*

Gas phase	Incubation time (min)	Tissue	Buffer	Substrates†	Precolumn fractions		Column fractions			Total recovery (%)
					Aqueous alcohol soluble	CCl_4-soluble	$CHCl_3$-soluble	Genin fraction	Digoxin + digitoxosides fraction	
	0	None	None	—	0	0	0	0	1,919 ± 27	96·0 ± 1·4
	0	Homog.	Tris	—	0	0	0	1 ± 3	1,917 ± 29	95·9 ± 1·4
O_2	60	Boiled‡ Homog.	Phosphate	—	0	0	2 ± 3	3 ± 4	1,905 ± 33	95·5 ± 1·8
O_2	60	Boiled Homog.	Tris	—	1 ± 2	0	1 ± 3	0	1,912 ± 48	95·7 ± 2·4
O_2	60	None	Phosphate	+	0	0	0	0	1,902 ± 31	95·1 ± 1·5
O_2	60	None	Tris	+	2 ± 4	0	1 ± 3	2 ± 4	1,907 ± 34	95·6 ± 1·8

* Data expressed as mean ± standard deviation in nanograms or nanogram-equivalents of ^{3}H-digoxin; 2 μg of ^{3}H-digoxin used in each experiment; 8 experiments per group with 2 experiments per animal.

† Added substrates (+) in final concentration are: 11·5 mmoles/l glucose, 4·9 mmoles/l Na glutamate, 4·9 mmoles/l Na pyruvate, and 5·4 mmoles/l Na_2 fumarate;[8] not added (—).

‡ Homogenate placed in boiling water for 1 min prior to incubation.

TABLE 2. AEROBIC INCUBATION OF ^{3}H-DIGOXIN WITH LIVER HOMOGENATES AND SLICES*

No of experiments	Tissue	Buffer	Substrates	Precolumn fractions		Column fractions			Total recovery (%)
				Aqueous alcohol soluble	CCl_4-soluble	$CHCl_3$-soluble	Genin fraction	Digoxin + digitoxosides fraction	
8	Homog.	Tris	−	5 ± 6	0	1 ± 3	6 ± 4	1,866 ± 37	93·9 ± 1·9
8	Homog.	Phosphate	−	2 ± 3	0	0	5 ± 5	1,863 ± 27	93·5 ± 1·6
8	Homog.	Tris	+	21 ± 8	0	2 ± 3	23 ± 9	1,799 ± 35	92·3 ± 2·0
8	Homog.	Phosphate	+	7 ± 6	0	0	9 ± 6	1,825 ± 31	92·0 ± 1·8
8	Slices	Tris	+	20 ± 7	0	3 ± 5	23 ± 9	1,803 ± 50	92·4 ± 2·3
8	Slices	Phosphate	+	6 ± 5	0	1 ± 3	8 ± 5	1,805 ± 39	91·0 ± 1·9
4	Homog.	Tris	+ (no glucose)	2 ± 4	0	0	7 ± 0	1,835 ± 31	92·2 ± 1·3
4	Homog.	Tris	+ (no glutamate)	5 ± 3	0	0	9 ± 2	1,854 ± 20	93·4 ± 0·8
4	Homog.	Tris	+ (no fumarate)	7 ± 1	0	0	10 ± 2	1,808 ± 42	91·2 ± 2·0
4	Homog.	Tris	+ (no pyruvate)	6 ± 4	0	0	10 ± 0	1,799 ± 28	90·8 ± 1·3

* Data expressed as mean ± standard deviation in nanograms or nonogram-equivalents of ^{3}H-digoxin; 2 μg of ^{3}H-digoxin used in each incubation. All incubations under 100% O_2 for 60 min. Added substrates are same as those noted in text and Table 1. Groups of 4 are single experiments from same 4 rats, whereas groups of 8 are duplicate experiments from different rats.

TABLE 3. AEROBIC AND ANAEROBIC INCUBATION OF ^{3}H-DIGOXIN WITH LIVER HOMOGENATES*

Gas phase	Incubation time (min)	Precolumn fractions		Column fractions			Total recovery (%)
		Aqueous alcohol soluble	CCl_4-soluble	$CHCl_3$-soluble	Digoxigenin fraction	Digoxin plus digitoxosides	
100% N_2	10	2 ± 3	0	0	2 ± 4	1,891 ± 25	94·7 ± 1·3
100% N_2	20	4 ± 4	0	2 ± 3	6 ± 4	1,837 ± 48	92·4 ± 2·3
100% N_2	40	7 ± 5	0	0	8 ± 6	1,835 ± 77	92·6 ± 3·8
100% N_2	180	4 ± 5	0	2 ± 3	5 ± 5	1,828 ± 34	91·9 ± 1·8
100% O_2	10	2 ± 5	0	0	12 ± 2	1,877 ± 37	94·6 ± 2·0
100% O_2	20	7 ± 5	0	0	12 ± 11	1,890 ± 27	95·4 ± 1·2
100% O_2	40	7 ± 5	0	0	16 ± 4	1,869 ± 47	94·6 ± 2·5
100% O_2	180	7 ± 5	0	0	18 ± 3	1,857 ± 42	94·1 ± 2·5

* Data expressed as mean ± standard deviation in nanograms or nanogram-equivalents of ^{3}H-digoxin; 2 μg of ^{3}H-digoxin used in each experiment. All incubations with 10% liver homogenates. Tris buffer supplanting the phosphate buffer in the Kreb's-Ringer solution and substrates added to each incubation in same concentrations as in Tables 1 and 2, 4 rats used for aerobic incubations and 4 rats used for anaerobic incubations.

TABLE 4. AEROBIC INCUBATION OF ^{3}H-DIGOXIN IN THE PRESENCE OF NADPH*

No. of experiments	NADPH added	Precolumn fractions		Column fractions			Total recovery (%)
		Aqueous alcohol soluble	CCl_4-soluble	$CHCl_3$-soluble	Digoxigenin fraction	Digoxin plus digitoxosides fraction	
6	1×10^{-3} M	0	0	4 ± 2	12 ± 7	1,607 ± 19	81·2 ± 1·4
5	5×10^{-4}	0	0	5 ± 5	13 ± 3	1,658 ± 14	83·8 ± 0·9
6	None	0	0	4 ± 4	6 ± 5	1,853 ± 56	93·2 ± 2·8

* Data expressed as mean ± standard deviation in nanograms or nanogram-equivalents of ^{3}H-digoxin; 2 μg of ^{3}H-digoxin used in each experiment. All incubations with 10% liver homogenates under 100% O_2 for 60 min with Tris buffer supplanting the phosphate buffer in the Krebs-Ringer solution. No organic substrates added. Control and experimental tissue obtained from each of same 6 rats.

production (Table 4). This was not noted in a limited number of similar experiments with NADH.

DISCUSSION

In 1950 Hilton[2] published experiments on the breakdown of digitoxin by preparations from organs which had been dried and 'defatted' with ethylene chloride. Digitoxin remaining in the incubates at various time intervals was determined by polarographic techniques. Using the reaction rate constant found for liver and the formula used for its calculation, one concludes that the extract from 1 g of dried liver hydrolyzed 97% of the original 0·1 mg of digitoxin to its genin in 60 min. Considering the persistence of unchanged digitoxin in the body,[3] it is questionable whether such a reaction rate occurs *in vivo*. Wright[1] later found metabolic conversion of both digoxin and digitoxin by isolated perfused rat livers, but no quantitative figures were given. Lauterbach and Repke have reported more recently that liver slices, but not homogenates, can metabolize both digoxin and digitoxin.[8]

The latter investigators reported strong or complete inhibition of hydrolysis when nitrogen was substituted for oxygen in their incubation system.[9] The studies reported herein appear to confirm this finding, although the amount converted to genin is extremely small (Table 3). One of the many experiments performed by Lauterbach and Repke included ATP, NAD, and NADP added to a rat liver homogenate system containing digitoxin.[8] After 6 hr, only unchanged digitoxin was found, but digitoxin recovery from the incubates was not quantitative. It is unlikely that the nonrecoverable material of Lauterbach and Repke or the nonrecovered fraction of our own NADPH experiments (Table 4) represents conversion to volatile products. Since the alumina chromatography columns used in the present experiments were not extensively washed with more polar solvents after elution of the digoxin and digitoxosides fraction, it may be possible that a more polar material (perhaps a conjugate) was formed in our NADPH experiments. Although the tissue residues remaining after extraction in these NADPH experiments were not combusted for radioactivity determinations, we have combusted many drug-extracted liver residues and have found no significant radioactivity.[10]

Now that the biotransformation reactions of digitalis glycosides have been brought to light by vigorous investigations within the past few years,[1, 3, 8, 9] perhaps a more meaningful appreciation of the link of digitalis metabolism and its cardiotonic mechanism can be anticipated. However, since the reaction rates are extremely low and it is difficult to isolate the products quantitatively, some investigators have had to use 'recovery conversion factors' in estimating metabolites.[8] It is hoped that application of the separation techniques established with colorimetric assay[1, 8, 9] coupled with radiotracer techniques will now allow quantitative data at dosages more consistent with therapeutic levels.

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