of the substantia nigra. However, unless there is a marked difference, the present study would indicate that the phenothiazines do not act upon tyrosinase. Since only phenelzine of the MAO inhibitors had any effect on the enzyme, it would appear that there is little significance to the inhibition observed with it.

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Biochemical Pharmacology, 1965, Vol. 14, pp. 633–636. Pergamon Press Ltd., Printed in Great Britain.

Cholesterol as a presumed metabolite of digoxin and digitoxin*

(*Received* 21 *October* 1964; *accepted* 20 *November* 1964)

GVOZDJAK *et al.* have reported that daily administration of digitoxin to rats for a week results in a definite gradual increase of adrenal cholesterol.[1] This was attributed to the possible conversion of digitoxin to cholesterol. However, the experimental data did not allow a direct demonstration of this conclusion, nor could the possibility of this conversion occurring elsewhere with transport to the adrenal be excluded. The present study was therefore undertaken to test this hypothesis with more direct evidence. Both *in vivo* and *in vitro* systems were used to study the possible conversion of both tritium-labeled glycosides, digitoxin and digoxin, to cholesterol.

METHODS

In vivo

Adult male Sprague-Dawley rats, 150–200 g, were starved overnight prior to use. Random-labeled ^{3}H-digoxin (55·8 μc/mg) or ^{3}H-digitoxin (23·3 μc/mg) were injected i.p. in 47·5% ethanol. The ^{3}H-digoxin had a radiochemical purity of 98·49 ± 0·05%.† Unfortunately, the ^{3}H-digitoxin was subject to marked degradation after its preparation. Most of this conversion was to the *bis*-digitoxoside. However, paper chromatography revealed that at least 3/4 of the material on the strips was digitoxin and its digitoxosides, 2/3 of this being digitoxin. Although impure, the material therefore still sufficed for the purposes of the present study. These purities were determined by radiochemical analysis following quantitative extraction from paper chromatograms.[2, 3]

Six hr after injection, each animal was killed by decapitation and the blood collected in a heparinized beaker. Both adrenal glands and the liver were rapidly removed and placed in ice-cold 0·9% NaCl. After weighing, the adrenal glands were homogenized in 2 ml of ice-cold 0·9% NaCl and the liver in 5 ml of the saline solution. Plasma was obtained from 1,000 *g* blood centrifugation for 10 min.

* Supported by U.S. Public Health Service Research Grant GM-09784.

† Expressed as mean ± standard error of 3 chromatographic assays.

The total cholesterol content of each of these tissues was then extracted by the method of Sperry and Webb[4] with only minor modifications. The precipitated digitonides were washed with 0·5 ml of 1 : 1 acetone–ethanol, centrifuged at 1,000 *g* for 10 min, and the supernatant decanted. This cholesterol precipitate was termed the first precipitate. A second sample of each extract was precipitated and washed twice (second precipitate). A third extract sample was precipitated and then washed three times (third precipitate). Each of these precipitates was dehydrated with 1 : 2 acetone–ether and then dried at room temperature for 30 min. Subsequent solution of the dried precipitates was accomplished by gentle heating in 3 ml methanol.

After cooling, the solution was reconstituted to 3 ml with methanol and thoroughly mixed. Radioassay was performed by adding 1 ml of the methanolic solution to 19 ml toluene and counting to 5% error[5] in a liquid scintillation spectrometer with later quench corrections. The total counting volume contained 4 g 2,5-diphenyloxazole (PPO) per liter and 50 mg 1,4-*bis*-2(5-phenyloxazolyl)-benzene (POPOP) per liter. Colorimetric determination of cholesterol content was performed on another 1-ml aliquot taken to dryness and assayed according to Courchaine *et al.*[6] Supernatants to be assayed for radioactivity were evaporated at 50°–60° until thoroughly dry. The residue was then solubilized in 2 ml methanol, diluted to a total volume of 20 ml with the appropriate toluene–phosphor solution, and counted as above.

In vitro

For each experiment, four male Sprague-Dawley rats weighing 150–200 g were fed *ad libitum* prior to use. After cervical fracture, one adrenal from each of the four animals was rapidly removed, weighed, and maintained in ice-cold 0·9% saline. Likewise, 3- to 5 g liver samples were removed from each of two animals and the combined liver remained in ice-cold 0·9% NaCl for the brief period prior to homogenization.

Homogenization and incubation of the combined adrenals was performed with the media, cofactors, and conditions used by Goodman *et al.*[7] the only modification being the different substrate utilized. Homogenization and incubation of the 500-*g* supernate of pooled liver was performed according to Bucher and McGarrahan.[8] When a glycoside served as substrate, approximately 40,000 dpm of either digoxin ($4{\cdot}13 \times 10^{-4}$ μmole) or digitoxin ($1{\cdot}01 \times 10^{-3}$ μmole) was used per beaker. Control experiments on cholesterol synthesis were also performed with approximately 1·5 μc of sodium acetate-2-^{14}C (0·25 μmole) used per beaker. All incubations were carried out at 37° in a Dubnoff shaking metabolic incubator for 2 hr under oxygen.

After incubation, enzymatic activity was terminated by addition of 5 ml of 1 : 1 acetone–ethanol. Nonradioactive carrier cholesterol was then added to each sample and cholesterol isolation performed according to the techniques stated in the above *in vivo* section.

RESULTS

The data obtained from the *in vivo* studies are shown in Table 1. The first liver cholesterol precipitates after ^{3}H-digoxin administration and one of the first adrenal cholesterol precipitates from ^{3}H-digitoxin were the only precipitates with any radioactivity. Even in these cases the precipitates washed further showed that no isotope incorporation had occurred. The radioactivity found in the acetone–ethanol extraction supernates verify that substantial amounts of glycoside or metabolites were still present in the tissues even after the 6-hr interval.

Table 2 shows that cholesterol synthesis from acetate occurred in all cases and that constant specific activities were readily obtained by repeated precipitation. No conversion of glycosides to cholesterol occurred, although these incubations were performed concurrently with the acetate incubations. In these incubations, and in the *in vivo* studies, the presence of significant radioactivity in the extraction supernates argues well for the lack of glycoside-to-cholesterol conversion.

DISCUSSION

The data of Tables 1 and 2 quite obviously refute the original postulation by Gvozdjak *et al.* that digitoxin may be converted to cholesterol in the rat.[1] This is likewise true for digoxin. Since the original postulation was made, Gvozdjak and Niederland have ascribed the increase in adrenal cholesterol following digitoxin administration to "non-specific" causes.[9] This latter conclusion was based on hourly adrenal cholesterol assays after i.p. administration of digitoxin to rats. Since their

highest cholesterol values occurred at 5 to 7 hr after digitoxin administration, it is a fortuitous circumstance that the 6-hr interval was arbitrarily chosen for the *in vivo* studies reported herein.

Although it might be argued that glycoside conversion to cholesterol could have proceeded along further metabolic and/or excretory pathways, the *in vitro* studies (Table 2) do not substantiate such a

TABLE 1. CHOLESTEROL RADIOASSAY AFTER INTRAPERITONEAL ADMINISTRATION OF ^{3}H-DIGOXIN AND ^{3}H-DIGITOXIN*

Glycoside administered†	Tissue	Rat no.	Specific activity of isolated cholesterol (dpm/mg) 1st precipitate	2nd precipitate	3rd precipitate	First extraction supernate (dpm)
^{3}H-Digoxin	Adrenals	1	0	0	0	3,179
		2	0	0	0	3,321
	Liver	1	417	0	0	1,021
		2	38	0	0	5,934
	Plasma	1	0	0	0	4,941
		2	0	0	0	2,855
^{3}H-Digitoxin	Adrenals	3	0	0	0	414
		4	467	0	0	802
	Liver	3	0	0	0	491
		4	0	0	0	859
	Plasma	3	0	0	0	186
		4	0	0	0	741

* All data at 6 hr after injection.

† Digoxin dose was 0·5 mg/kg or 27·9 μc/kg. Digitoxin dose was 0·4 mg/kg or 9·32 μc/kg.

TABLE 2. DIGOXIN AND DIGITOXIN AS SUBSTRATES FOR *IN-VITRO* CHOLESTEROL SYNTHESIS BY RAT LIVER AND ADRENALS*

Substrate	Tissue	Experiment no.	Specific activity of isolated cholesterol (dpm/mg) 1st precipitate	2nd precipitate	3rd precipitate	First extraction supernate (dpm)	Initial radioactivity in 1st supernate (%)
^{3}H-Digoxin	Adrenals	1	0	0	0	5,736	100·8
		2	0	0	0	5,224	97·9
		3	0	0	0	3,750	70·3
	Liver	1	0	0	0	1,659	62·2
		2	0	0	0	1,731	64·9
		3	0	0	0	2,048	76·8
^{3}H-Digitoxin	Adrenals	4	0	0	0	3,715	69·5
		5	0	0	0	3,812	71·5
		6	0	0	0	4,956	51·1
	Liver	4	0	0	0	1,781	66·8
		5	0	0	0	1,801	67·5
		6	0	0	0	2,764	92·9
Sodium acetate-2-^{14}C	Adrenals	3	5,525	1,650	1,600	390×10^3	87·6
		4	5,600	3,428	3,500	298×10^3	67·0
		6	4,848	1,333	1,357	369×10^3	82·9
	Liver	3	9,250	8,434	8,428	189×10^3	42·4
		4	9,350	6,800	6,789	270×10^3	60·7
		6	7,070	8,650	8,700	353×10^3	79·4

*All incubations in O_2 for 2 hr at 37°.

position. There is no question that cholesterol synthesis occurred, since acetate was readily incorporated into the product. On the other hand, digoxin and digitoxin were not incorporated into cholesterol.

Acknowledgement—The authors would like to express their gratitude to Drs. Stanley T. Bloomfield and James Murphy of Burroughs-Wellcome Ltd. for the ^{3}H-digoxin used in these investigations. Similarly, thanks are due to Dr. George T. Okita, Northwestern University, for the ^{3}H-digitoxin used.

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Biochemical Pharmacology, 1965, Vol. 14, pp. 636–638. Pergamon Press Ltd., Printed in Great Britain.

Effects of *d*-Amphetamine and Chlorpromazine on Oxidised (NAD) and Reduced ($NADH_2$) Nicotinamide Adenine Dinucleotide Levels in Rat Brain

(*Received* 16 *September* 1964; *accepted* 7 *November* 1964)

LARGE doses of nicotinamide increase liver, brain and spleen NAD.[1, 2] Nicotinic acid has a similar effect on blood NAD.[3, 4] Reserpine or chlorpromazine, given prior to nicotinamide, maintain elevated liver NAD levels.[5] The ability of brain tissue to control NAD metabolism is reflected by the absence of this reserpine effect in brain and because nicotinamide itself causes only a 50–75% increase in brain NAD[2] but an 800–900% increase in the liver.[1] Furthermore, when peripheral NAD stores are severely depleted by dietary deficiences,[6] no change in brain NAD occurs, even in terminally deficient animals.[2]

We have investigated the effects of *d*-amphetamine sulphate and chlorpromazine hydrochloride on rat brain NAD and $NADH_2$ levels to determine if the influence of these drugs on behaviour could be correlated with changes in NAD metabolism.

Drug solutions were prepared in 0·9% NaCl so that the required dose was contained in 0·2 ml/100 g body weight. Groups of two or three male Wistar rats of approximately equal weight (90–120 g) were used. The order in which the animals were injected intraperitoneally, killed and the brains dissected, extracted and assayed, was randomised, using either a 3 × 3 Latin Square design or a table of random numbers. 0·9% NaCl was used as control. The method for extracting and estimating the nicotinamide