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Biochemical Lesion of Diphtheria Toxin in the Heart *

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Clinical and experimental investigations have shown that the myocardium is highly susceptible to the action of diphtheria toxin (1, 2). Both in man and the guinea pig, one of the earliest alterations in the heart muscle exposed to this toxin is fatty degeneration (2, 3). This morphological observation along with recently acquired evidence that fatty acids are a major metabolic substrate of the heart (4) suggested that the toxin may exert its effect on the myocardium by interfering with fatty acid oxidation.

Experiments were designed to test this hypothesis by ascertaining the effect of diphtheria toxin on *1*) the capacity of the myocardium to oxidize long-chain fatty acids, *2*) the integrity of component parts of the fatty acid oxidation pathway in the heart, and *3*) the concentration of myocardial carnitine (DL-γ-trimethylamino-β-hydroxybutyrate), a compound known to effect a stimulation of long-chain fatty acid oxidation in the heart (5).

Methods

White, male guinea pigs weighing 250 to 300 g were used. A standard diet consisting of 50% Purina rabbit chow and 50% oats supplemented with fresh cabbage was used except as indicated. The experimental animals were divided into 4 groups as follows: *a*) Diphtheria-toxin [1] animals were injected with toxin subcutaneously, the dose being adjusted so that fatty degeneration of the myocardium could be produced regularly in surviving animals within 5 days after treatment; *b*) diphtheria-antitoxin [1] animals were injected intraperitoneally with 500 U of antitoxin; *c*) diphtheria-toxin and -antitoxin animals were injected with 500 U of antitoxin 24 hours before administration of the toxin; and *d*) fasting animals were deprived of food and given water ad libitum. Each experimental animal used for an enzymatic assay was tested simultaneously with a paired untreated mate as a control. For all other determinations, groups of experimental animals were compared with groups of controls.

Five to six days after injection of the toxin or the beginning of the fast period, the guinea pigs were sacrificed by a blow on the head. The entire heart was removed immediately, the great vessels were trimmed off at their origin, and the cardiac cavities were freed of blood. A transventricular block of myocardium was placed in 10% neutral, buffered formalin solution and kept at room temperature for at least 24 hours. Frozen sections, 14-μ thick, were prepared and stained as follows: *a*) hematoxylin and eosin, *b*) oil-red O dissolved in acetone alcohol, and *c*) oil-red O followed by hematoxylin. Histological examination was performed on all hearts except for those used for quantitative analysis of lipids and carnitine.

For quantification of lipid fractions in the myocardium, hearts were homogenized in water and lipids extracted by the method of Folch and associates (6). Triglycerides were determined according to the procedure of van Handel and Zilversmit (7), and phospholipids by a modification of the method of Youngburg and Youngburg (8).

Carnitine assays on trichloroacetic acid extracts of myocardium were done by the method of Mehlman and Wolf (9).[2]

To measure the capacity of the myocardium to oxidize selected substrates, a modification of the method of Snyder and Godfrey was used (10). Hearts were homogenized in calcium-free Krebs-Ringer phosphate solution, pH 7.4, at 2 to 4° C with a conical ground-glass homogenizer. By using 7 to 9 ml of the Krebs-Ringer solution per heart, a protein concentration ranging from 8 to 15 mg per ml of homogenate was obtained. The homogenate was filtered through a double layer of cheesecloth into an ice-cold container and used immediately. Reactions were carried out in 25-ml Erlenmeyer flasks equipped with removable glass vessels and sealed with rubber serum stoppers. Appropriate amounts of C^{14}-labeled substrate and of nonradioactive cofactor were added to each flask, the flask was sealed, and the reac-

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[1] Diphtheria toxin, lot #706396, and diphtheria antitoxin, lot #G-138-B, were supplied by Eli Lilly and Co., Indianapolis, Ind.

[2] Performed by Dr. George Wolf, Nutrition Laboratory, Massachusetts Institute of Technology, Cambridge, Mass.

tion was started by injection of 1 ml of the homogenate into the flask through the rubber stopper. The flasks were agitated at a rate of 30 strokes per minute in a Dubnoff shaking incubator at 30° C. After the appropriate time interval, which was 30 minutes unless indicated otherwise, the reaction was terminated by injecting 0.15 ml of 4 N H_2SO_4 into the reaction mixture. For collecting CO_2 evolved during the incubation, Hyamine, (*p*-[diisobutylcresoxyethoxyethyl] dimethyl benzylammonium hydroxide), 0.2 ml, was injected into the removable glass vessel after the reaction was terminated, and the flasks were agitated in the incubator for 1 hour. The glass vessel was then removed and placed in 10 ml of phosphor solution consisting of *p*-dioxane, anisole, 1,2-dimethoxyethane, PPO (2,5-diphenyloxazole), and POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene]. Radioactivity of the CO_2 collected in the Hyamine was determined with a Packard Tri-Carb liquid scintillation spectrometer.

The concentration of free fatty acids in the myocardial homogenates used for assessing rates of fatty acid oxidation was determined by the method of Dole as modified by Trout, Estes, and Friedberg (11). Before the addition of radioactive fatty acid, the myocardium from both treated and control animals contained 4 to 5 μmoles of free fatty acid per g wet weight of tissue. Since identical amounts of radioactive fatty acid of the same specific activity were added to the reaction mixtures of control and experimental animals, the specific activities of the reaction mixtures at the beginning of the incubation period were considered equal. To determine whether or not the specific activity changed during the incubation period, time course experiments were done over an interval of 5 to 45 minutes. During the incubation, the reaction rates were linear and a fixed ratio of $C^{14}O_2$ evolved was maintained between control and experimental animals. This was interpreted as indicating that significant net lipolysis with dilution of the free fatty acid pool did not occur during the incubation period.

Activity of the long-chain fatty acid activating enzyme was assayed by the method of Kornberg and Pricer (12), in microsomal [3] and mitochondrial fractions. These were obtained by differential centrifugation, and appropriate samples of each fraction were used (13).

Protein concentration was measured by the biuret method (14). After completion of the reaction, the solution was filtered through a layer of Celite in a Buchner-type funnel. This was necessary to remove lipids which produced turbidity of the solution, especially in the treated animals. The results obtained by this procedure agreed closely with those obtained by Kjeldahl nitrogen determination (15).

Radioactive substrates—carboxyl-labeled palmitic, oleic, and linoleic acids,[4] succinate-2,3-C^{14},[5] and glucose-U-C^{14} [6]—were obtained commercially. Palmityl CoA-1-C^{14} was prepared by the procedure of Kornberg and Pricer (12). Nonradioactive palmitic, oleic, and linoleic acids,[7] DL carnitine,[5] ATP,[8] coenzyme A,[9] and crystalline bovine serum albumin [10] were also obtained commercially. The radioactive fatty acids were assayed by gas-liquid chromatography. They were 95% chemically pure and 98% radio pure. The unlabeled fatty acids were over 98% pure.

The statistical significance of the data was evaluated by applying the *t* test to the mean differences between experimental and control groups or between experimental and control pairs (16).

Results

A. Histology

In all animals given diphtheria toxin, sections of ventricular myocardium stained for neutral fat with oil-red O showed groups of myofibers stippled with fine red droplets distributed uniformly throughout the cytoplasm. The amount of muscle involved varied from 25 to 50% of the transventricular section. Septal and free walls of the right and the left ventricles were equally involved as were all mural layers. No stainable fat was demonstrated in control animals or in animals given antitoxin alone or antitoxin followed by toxin. The myocardium of 3 of 10 fasted animals contained stainable fat. Necrosis, myocytolysis, fibrosis, or cellular exudation was not encountered in the myocardium of any of the animals regardless of treatment.

B. Biochemistry

Triglyceride and phospholipid concentrations. The triglyceride concentration in the hearts of guinea pigs given diphtheria toxin was greater than that in the hearts of control animals. In a group of six treated animals the triglyceride con-

[3] The term microsomal fraction does not imply the presence of microsomes as in the case of the liver. As used in this work, the term designates that fraction of the myocardial homogenate obtained after removal of the nuclear and mitochondrial fractions by centrifugation procedures.

[4] Nichem Corp., Bethesda, Md.

[5] California Corp. for Biochemical Research, Los Angeles, Calif.

[6] New England Nuclear Corp., Boston, Mass.

[7] Applied Science Laboratories, Pennsylvania State University, University Park, Pa.

[8] Sigma Chemical Co., St. Louis, Mo.

[9] Pabst Laboratories, Milwaukee, Wis.

[10] Armour Pharmaceutical Co., Kankakee, Ill.

TABLE I

*Myocardial palmitate-1-C^{14} oxidation by animals given diphtheria toxin**

Exp. no.	$C^{14}O_2$		
	Control	Toxin	Difference
	μmoles/g protein/30 minutes		
1	3.76	2.40	1.36
2	3.67	0.97	2.70
3	3.86	2.68	1.18
4	1.38	0.44	0.94
5	3.96	1.12	2.84
6	2.22	0.98	1.24
7	2.19	0.98	1.21
8	1.06	0.47	0.59
9	4.21	1.12	3.09
10	1.69	0.60	1.09
11	4.91	2.60	2.31
12	1.06	0.66	0.40
13	3.45	1.36	2.09
14	2.31	0.65	1.66
15	4.84	1.32	3.52
16	2.33	1.52	0.81
Mean	2.93	1.24	1.69
SE	0.37	0.18	0.24
p			<0.01

* Each reaction mixture contained palmitate-1-C^{14}, 100 mμmoles (100,000 cpm), and 8 to 15 mg of guinea pig heart homogenate in 1 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4. Final reaction volume was 1.02 ml. Incubations were at 30° C for 30 minutes.

centration ranged from 4.41 to 11.73 mg per g wet weight of myocardium (mean = 7.41; SE = 1.37), whereas in the control group of five animals the concentration ranged from 1.03 to 2.25 mg per g wet weight of myocardium (mean = 1.38; SE = 0.22). The difference in myocardial triglyceride concentration between the treated and control groups is statistically significant ($p < 0.01$).

A significant difference in phospholipid concentration of the myocardium between the control and treated animals was not demonstrated. In the group of control animals the concentration ranged from 17 to 18 mg per g wet weight of myocardium (mean = 17.6; SE = 0.1) and in the group of treated animals from 15 to 20 mg per g (mean = 18.2; SE = 1.9).

Long-chain fatty acid oxidation. In all animals treated with diphtheria toxin, the rate of oxidation of palmitate-1-C^{14} by the myocardium was decreased below that of the paired controls (Table I). As a group, the animals given toxin had a mean rate of palmitate oxidation, which was less than half that of the control animals. The mean difference between the control and experimental pairs is statistically significant. Experiments using oleic or linoleic acids gave similar results (Table II).

Addition of ATP in final concentrations of 2×10^{-4}-9×10^{-3} moles per L or of CoA in final concentrations of 10^{-4}-4.5×10^{-3} moles per L to the oxidative assay of palmitic acid failed to increase its depressed rate of oxidation by the myocardium of the animals given toxin or to augment its oxidation rate in the paired controls. At the highest concentrations used, the oxidation rates were markedly depressed in both the experimental and control animals.

Assay of the long-chain fatty acid activating enzymes in the microsomal and in the mitochondrial fractions of the myocardium from toxin-treated animals revealed no decrease in activity in either fraction when compared with the control animals.

Guinea pigs given antitoxin only or antitoxin before administration of toxin failed to show any significant differences from their paired controls in the oxidation rate of palmitic acid. In three experiments in each of which an antitoxin-treated and a toxin-antitoxin-treated animal were paired with the same control, the mean rates of palmitate-1-C^{14} oxidation in micromoles per gram protein per 30 minutes were 2.98, 2.76, and 2.86, respectively. These differences are not significant.

Loss of body weight by the animals given diphtheria toxin raised the possibility that failure to eat was responsible for the defective oxidation of palmitic acid. To investigate this, guinea pigs were fasted for periods of time equal to those

TABLE II

*Myocardial fatty acid oxidation by toxin-treated animals**

Substrate	$C^{14}O_2$	
	Control	Toxin
	μmoles/g protein/ 30 minutes	
Palmitate-1-C^{14}	1.52	0.57
Oleate-1-C^{14}	1.59	0.48
Linoleate-1-C^{14}	2.99	0.95

* Each reaction mixture contained 12.5 mg of guinea pig heart homogenate in 1 ml of calcium-free Krebs-Ringer phosphate solution, pH 7.4, and palmitate-1-C^{14}, 100 mμmoles (100,000 cpm), oleate-1-C^{14}, 80 mμmoles (120,000 cpm), or linoleate-1-C^{14}, 70 mμmoles (110,000 cpm). Final reaction volume was 1.02 ml. Incubations were as given in Table I.

required for fatty degeneration of the myocardium to appear after the administration of diphtheria toxin. Body weight loss was approximately 20% of the initial weight in both toxin-treated and fasted animals. In striking contrast to the toxin-treated guinea pigs, the fasted animals showed an increased rate of palmitic acid oxidation by the myocardium as compared with the paired controls (Table III). The mean rate of oxidation in the fasted group was almost 40% higher than in its control group. The mean difference between the fasted and control pairs is statistically significant. Stimulation of fatty acid oxidation by fasting has also been demonstrated in other tissues (17).

TABLE III

*Effect of fasting on $C^{14}O_2$ production from palmitate-1-C^{14}**

Exp. no.	$C^{14}O_2$		
	Control	Fasted	Difference
	μmoles/g protein/30 minutes		
1	2.90	3.19	0.29
2	1.85	2.47	0.62
3	1.69	2.39	0.70
4	0.98	2.33	1.35
5	0.80	1.39	0.59
6	2.76	3.85	1.09
7	4.00	4.66	0.66
8	1.68	2.28	0.60
9	1.45	2.23	0.78
10	0.80	1.42	0.62
Mean	1.89	2.62	0.73
SE	0.33	0.32	0.30
p			<0.025 >0.01

* Conditions as in Table I.

Palmityl CoA oxidation. Table IV shows that the rate of palmityl CoA-1-C^{14} oxidation by myocardial homogenates of toxin-treated animals did not differ from that of the paired controls.

It has been suggested that palmityl CoA has marked surface active properties that might compromise the integrity of the mitochondrial membrane (18). Therefore, bovine serum albumin, which protects mitochondria against surface active agents, was used in conjunction with the palmityl CoA (Table V). The oxidation rate of unbound palmityl CoA in the toxin-treated guinea pig was 16% less than that of the control, whereas when albumin-bound palmityl CoA was used as substrate, the rate of oxidation by the toxin-

TABLE IV

*Effect of toxin on metabolism of various substrates**

Substrate	No. of experiments	$C^{14}O_2$	
		Control	Toxin
		μmoles/g protein/30 minutes	
Palmityl CoA-1-C^{14}	5	1.6 ± 0.2†	1.7 ± 0.2
Succinate-2, 3-C^{14}	5	35.8 ± 6.6	35.9 ± 4.5
Glucose-U-C^{14}	5	2.5 ± 0.8	2.6 ± 0.8

* Each reaction mixture contained 8 to 15 mg of guinea pig heart homogenate in 1 ml of calcium-free Krebs-Ringer phosphate solution, pH 7.4, and palmityl CoA-1-C^{14}, 90 mμmoles (80,000 cpm), succinate-2, 3-C^{14}, 1,500 mμmoles (440,000 cpm), or glucose-U-C^{14}, 1,000 mμmoles (200,000 cpm). Final reaction volume was 1.02 ml. Conditions of incubation were as given in Table I.

† Mean ± SE.

treated animal was depressed 45%. In contrast, the oxidation rate of free palmitic acid by the toxin-treated animal was less than one-third that of the paired control whether albumin was present or not.

From these data, it may be inferred that the myocardial oxidation rate of palmityl CoA is depressed in the toxin-treated animal when "albumin-protected" mitochondria are assayed. The depression, however, is not due to a defect in the β-oxidation mechanism, tricarboxylic acid cycle, or electron transport system, because the rate of palmityl CoA oxidation by the same toxin-treated animal does not differ significantly from control levels when unprotected mitochondria are assayed.

Succinate and glucose oxidation. In Table IV are listed the rates of oxidation of succinate-2,3-C^{14} and glucose-U-C^{14} by the myocardium of the

TABLE V

*Effect of bovine serum albumin on the rate of oxidation of palmitate-1-C^{14} and palmityl CoA-1-C^{14}**

	$C^{14}O_2$			
	Palmitate-1-C^{14}	Percentage of decrease	Palmityl CoA-1-C^{14}	Percentage of decrease
	μmoles/g protein/30 minutes			
Control	41.0		57.4	
Toxin	12.0	70.6	48.3	15.9
Control plus albumin	26.8		81.2	
Toxin plus albumin	8.4	68.5	45.0	44.6

* Each reaction mixture contained palmitate-1-C^{14}, 1,000 mμmoles (1,000,000 cpm), or palmityl CoA-1-C^{14}, 900 mμmoles 800,000 cpm), and 11.0 mg of guinea pig heart homogenate in 1 ml of calcium-free Krebs-Ringer phosphate solution. 2% bovine serum albumin, 0.3 ml, was added as shown. Final reaction volume was 1.5 ml. The standard incubation conditions were used.

TABLE VI

*Effect of DL-carnitine on rate of oxidation of palmitate-1-C^{14} by control and toxin-treated guinea pigs**

	$C^{14}O_2$								
	Control		Diphtheria toxin		Differences				
Exp. no.	1	+Carn.† 2	3	+Carn.† 4	1–3	2–1	4–3	4–1	2–4
	μmoles/g protein/30 minutes								
1	3.76	4.56	2.40	5.09	1.36	0.80	2.69	1.33	−0.53
2	3.67	4.85	0.97	3.21	2.70	1.18	2.24	−0.46	1.64
3	1.38	2.92	0.44	0.84	0.94	1.54	0.40	−0.54	2.08
4	2.22	3.28	0.98	2.43	1.24	1.06	1.45	0.21	0.85
5	2.19	3.04	0.98	2.94	1.21	0.85	1.96	0.75	0.10
6	1.06	1.31	0.47	1.17	0.59	0.25	0.70	0.11	0.14
7	1.69	2.48	0.60	1.93	1.09	0.79	1.33	0.24	0.55
8	1.06	1.31	0.66	0.79	0.40	0.25	0.13	−0.27	0.52
9	3.45	5.43	1.36	3.98	2.09	1.98	2.62	0.53	1.45
10	4.84	4.89	1.32	4.78	3.52	0.05	3.46	−0.06	0.11
Mean	2.53	3.41	1.02	2.72	1.51	0.88	1.70	0.18	0.69
SE	0.42	0.47	0.18	0.49	0.31	0.19	0.34	0.18	0.26
p					<0.01	<0.01	<0.01	<20.0	<0.025
								>10.0	>0.01

* Each reaction mixture contained palmitate-1-C^{14}, 100 mμmoles (100,000 cpm), and 8 to 15 mg of guinea pig heart homogenate in 1 ml of calcium-free Krebs-Ringer phosphate solution, pH 7.4. DL-Carnitine, 10^{-3} mmoles, was added where indicated. Final reaction volume was 1.02 ml. Standard conditions of incubation were used.

† DL-Carnitine.

animals given diphtheria toxin and by their paired controls. Significant differences between the experimental and control animals in the rates of oxidation are not evident for either substrate. These data are in agreement with those obtained with palmityl CoA which indicate that defects in the tricarboxylic acid cycle or in the electron transport system are not responsible for the decreased rate of palmitic acid oxidation by the myocardium of animals treated with diphtheria toxin. Also evident from the data is the integrity of the glycolytic pathway in the toxin-treated animals.

Carnitine concentration and effect. Carnitine, a compound that is normally present in relatively large amounts in muscular tissue (19), exerts a stimulatory action on the rate of oxidation of long-chain fatty acids in several tissues including the myocardium (20). In the guinea pigs given diphtheria toxin, there was a striking decrease in the concentration of carnitine in the heart. The mean concentration in a group of seven toxin-treated animals (mean = 237 μg per g wet weight; SE = 15) was only one-third of that present in the group of control animals (mean = 691 μg per g wet weight; SE = 160). This difference is statistically significant ($p < 0.01$).

To ascertain whether or not exogenous carnitine was capable of increasing the depressed oxidation rate of palmitic acid in the toxin-treated animals, carnitine was added to the oxidative assays. The addition of carnitine effected an elevation of the depressed oxidation rate in the toxin-treated animals as well as a stimulation of the rate in the paired controls (Table VI). The mean rate of oxidation by the hearts of the toxin-treated animals was increased from a depressed level of 1.02 to one of 2.72 μmoles per g protein per 30 minutes, whereas the rate of the controls was elevated from a mean of 2.53 to one of 3.41 μmoles per g protein per 30 minutes. Although the rate achieved by the stimulated controls was higher than that of the stimulated toxin-treated animals, the increment was almost two times greater in the latter (1.70 versus 0.88 μmoles per g protein per 30 minutes). This difference is statistically significant ($p < 0.025$).

Discussion

Fatty degeneration of the myocardium in guinea pigs inoculated with diphtheria toxin was manifested biochemically in the heart muscle by *1*) a depressed rate of oxidation of long-chain fatty acids, *2*) a decrease in concentration of car-

nitine, and *3*) an excessive accumulation of triglycerides.

Long-chain fatty acid oxidation. Previous investigations have suggested that diphtheria toxin interferes with the synthesis of cytochrome b (21), or with the formation of ATP (22–24). A defect in either of these could have explained the depressed rate of long-chain fatty acid oxidation. The demonstration, however, that succinate and glucose were oxidized at unimpaired rates by the heart of the guinea pig treated with diphtheria toxin is inconsistent with the hypothesis that the toxin impairs the synthesis of cytochrome b. An adverse effect upon ATP formation by diphtheria toxin appears equally improbable, because the rate of glycolysis was not impeded, and added ATP did not elevate the depressed oxidation rate of the long-chain fatty acids in the toxin-treated animal. Furthermore, ATP and CoA are obligatory for a carnitine-effected stimulation of long-chain fatty acid oxidation (18). Since neither ATP nor CoA was added to the oxidative assays in which carnitine was used, the restorative effect of carnitine on the depressed oxidation rate of free palmitic acid in the toxin-treated animals indicated the presence of sufficient endogenous ATP and CoA.

Exclusion of abnormalities in the β-oxidation mechanism of long-chain fatty acids, in the tricarboxylic acid cycle, and in the electron transport system indicated that the defect responsible for the impaired metabolism of long-chain fatty acids in myocardium affected by diphtheria toxin was at a step before the oxidative pathways in the mitochondria. Examination of the activating mechanism of the long-chain fatty acids, however, disclosed no abnormality: the activity of long-chain acylthiokinase was not depressed, and addition of either ATP or CoA to the oxidative assay of free palmitic acid had no restorative effect. Thus it appeared that the defect in the oxidation of palmitic acid by the heart of the toxin-treated animal was located at a point beyond the formation of palmityl CoA and before the β-oxidation of its acyl group.

Role of carnitine. Recent investigations have suggested that carnitine stimulates oxidation of palmitic acid or palmityl CoA by facilitating the transport of the palmityl CoA from the extramitochondrial cytoplasm to the site of fatty acid oxidation within the mitochondrion (18, 25). In the case of palmityl CoA, the carnitine-effected stimulation can be shown only with the albumin bound CoA ester and not with the unbound ester (18). This difference has been attributed to the disruptive effect of unbound palmityl CoA on the mitochondrial membrane. Thus, although disrupted mitochondria can oxidize long-chain fatty acids, a carnitine stimulatory effect on the rate of oxidation can only be demonstrated when the integrity of the mitochondrial membrane is maintained.

In the animals given diphtheria toxin, there was a marked decrease in the concentration of carnitine in the myocardium. When carnitine was added to the reaction mixture of palmitic acid and cardiac homogenate from toxin-treated animals, the depressed rate of oxidation of palmitic acid was invariably increased. These observations, together with the demonstration of a defect in the oxidation of albumin-bound palmityl CoA, suggest that diphtheria toxin may depress the rate of oxidation of long-chain fatty acids in the heart by interfering with the metabolism of carnitine. Whether or not carnitine administered *in vivo* has a remedial effect on the depressed oxidation rate of long-chain fatty acids in the myocardium of toxin-treated animals is under study.

Fatty degeneration of myocardium. Biochemically, fatty degeneration of the myocardium may be equated to the presence of an increased triglyceride concentration in the muscle fibers (26). In the accumulation of myocardial triglycerides related to diphtheria toxin, one or more of the following mechanisms was considered: *1*) increased rate of extraction from the blood; *2*) accelerated synthesis; *3*) depressed rate of utilization, i.e., oxidation; *4*) decreased rate of release into the blood; and *5*) a shift in concentration between the various classes of lipid without an increase in total lipid content, i.e., phanerosis.

Myocardial extraction of plasma free fatty acids has been demonstrated by arteriovenous difference measurements (4, 27), but uptake of circulating triglycerides by the heart remains to be proved (28). Since the fatty acid activation mechanism was intact and triglyceride synthesis apparently possible in the heart affected by diphtheria toxin, plasma fatty acids could serve as a source of the triglycerides (29). Regardless

of the rate at which the triglycerides were synthesized, they would eventually accumulate to abnormally high levels if their rate of utlization were disproportionately decreased. Triglyceride utilization in muscle proceeds mainly by hydrolysis of the glyceride bond followed by oxidation of the fatty acid moieties (28). A marked depression of the rate of long-chain fatty acid oxidation would then favor accumulation of triglycerides provided hydrolysis of the triglycerides with the accumulation of the freed fatty acids or with the formation of phospholipids did not occur. Evidence was obtained that the concentrations of free fatty acids or of phospholipids were not increased over the controls in the toxin-treated animals.

A decreased rate of release of triglyceride into the blood appears to play an important role in fatty degeneration of the liver (30). It is unlikely, however, that this factor is pertinent with respect to the heart, not only because release of lipids has not been shown by arteriovenous measurement across the heart (4, 27), but also because the myocardium does not function as a storage organ like the liver (26). Finally, phanerosis has been implicated (31) and disclaimed (26) as a mechanism in fatty degeneration for many years. Recent experiments on the isolated, perfused, anaerobic heart suggest that this mechanism can produce fatty degeneration under certain circumstances (32). The increase in myocardial triglyceride concentration without an associated change in phospholipid concentration, however, excludes phanerosis as a major mechanism of fatty degeneration associated with diphtheria toxin. Thus, available evidence is consistent with the hypothesis that fatty degeneration of the myocardium effected by diphtheria toxin is due to an accumulation of triglycerides synthesized in the myocardium from fatty acids that cannot be otherwise utilized because of a block in the oxidation of long-chain fatty acids.

Summary

Fatty degeneration of the myocardium was induced in the guinea pig with diphtheria toxin. The morphologic lesion was expressed biochemically in cardiac homogenates by *1*) a marked depression in the rate of oxidation of long-chain fatty acids, *2*) a markedly decreased concentration of carnitine, and *3*) an excessive accumulation of triglycerides.

Exogenous carnitine had a restorative effect *in vitro* on the depressed rate of palmitic acid oxidation by myocardial homogenates of the toxin-treated animal.

A biochemical basis for myocardial fatty degeneration in the guinea pig given diphtheria toxin is postulated.

References

1. Friedberg, C. K. Diseases of the Heart, 2nd ed. Philadelphia, W. B. Saunders, 1956, p. 906.
2. Gukelberger, J. Neue experimentelle Arbeiten uber Beginn und Ausbreitung der diphtheritischen Schadigung des Herzmuskels. Z. ges. exp. Med. 1936, **97**, 749.
3. Warthin, A. S. The myocardial lesions of diphtheria. J. infect. Dis. 1924, **35**, 32.
4. Ballard, F. B., W. H. Danford, S. Naegle, and R. J. Bing. Myocardial metabolism of fatty acids. J. clin. Invest. 1960, **39**, 717.
5. Fritz, I. B., E. Kaplan, and K. T. N. Yue. Specificity of carnitine action on fatty acid oxidation by heart muscle. Amer. J. Physiol. 1962, **202**, 117.
6. Folch, J., I. Ascole, M. Lees, V. A. Meath, and F. N. LeBaron. Preparation of lipid extracts from brain tissue. J. biol. Chem. 1951, **191**, 833.
7. Van Handel, E., and D. B. Zilversmit. Micromethod for the direct determination of serum triglycerides. J. Lab. clin. Med. 1957, **50**, 152.
8. Hawk, P. B., B. L. Oser, and W. H. Summerson. Practical Physiological Chemistry. New York, McGraw-Hill, 1954, p. 589.
9. Mehlman, M. A., and G. Wolf. Studies on the distribution of free carnitine and the occurrence and nature of bound carnitine. Arch. Biochem. 1962, **98**, 146.
10. Bressler, R., and S. J. Wakil. Studies on the mechanism of fatty acid synthesis. XI. The product of the reaction and the role of sulfhydryl groups in the synthesis of fatty acids. J. biol. Chem. 1962, **237**, 1441.
11. Trout, D. L., E. H. Estes, Jr., and S. J. Friedberg. Titration of free fatty acids of plasma: a study of current methods and a new modification. J. Lipid Res. 1960, **1**, 199.
12. Kornberg, A., and W. E. Pricer, Jr. Enzymatic synthesis of the coenzyme A derivatives of long chain fatty acids. J. biol. Chem. 1953, **204**, 329.
13. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. Manometric Techniques, 3rd ed. Minneapolis, Burgess, 1959, p. 188.

14. Gornall, A. G., C. J. Bardawill, and M. M. David. Determination of serum proteins by means of the biuret reaction. J. biol. Chem. 1949, **177**, 751.
15. Hawk, P. B., B. L. Oser, and W. H. Summerson. Practical Physiological Chemistry. New York, McGraw-Hill, 1954, p. 880.
16. Dixon, W. J., and F. J. Massey, Jr. Introduction to Statistical Analysis, 2nd ed. New York, McGraw-Hill, 1957, p. 119.
17. Geyer, R. P., E. J. Bowie, and J. C. Bates. Effects of fasting and pyruvate on palmitic acid metabolism. J. biol. Chem. 1953, **200**, 271.
18. Fritz, I. B., and K. T. N. Yue. Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. J. Lipid Res. 1963, **4**, 279.
19. Fraenkel, G. S., and S. Friedman. Carnitine *in* Vitamins and Hormones, R. S. Harris, G. F. Marrian, and K. V. Thimann, Eds. New York, Academic Press, 1957, p. 73.
20. Fritz, I. B., and E. Kaplan. A survey of the effects of carnitine on fatty acid oxidation by tissue preparations. Fed. Proc. 1960, **19**, 223.
21. Pappenheimer, A. M., Jr. The pathogenesis of diphtheria *in* Mechanisms of Microbial Pathogenicity, J. W. Howie and A. J. O'Hea, Eds. Cambridge, University Press, 1955, p. 40.
22. Kato, I., and A. M. Pappenheimer, Jr. An early effect of diphtheria toxin on the metabolism of mammalian cells growing in culture. J. exp. Med. 1960, **112**, 329.
23. Fonnesu, A., and C. Severi. Oxidative phosphorylation in mitochondria from livers showing cloudy swelling. J. biophys. biochem. Cytol. 1956, **2**, 293.
24. Cessi, C., L. Montanaro, and F. Serafini-Cessi. The effect of diphtheria toxin on the incorporation of (^{32}P) phosphate into acid-soluble adenosine nucleotide of liver of guinea pig. Biochem. J. 1963, **87**, 35 p.
25. Bremer, J. Carnitine in intermediate metabolism. The metabolism of fatty acid esters of carnitine by mitochondria. J. biol. Chem. 1962, **237**, 3628.
26. Dible, J. H., and W. W. Gerrard. The source of the fat in experimentally produced fatty degeneration of the heart. J. Path. Bact. 1938, **46**, 77.
27. Gordon, R. S., Jr. Unesterified fatty acid in human blood plasma. II. The transport function of unesterified fatty acid. J. clin. Invest. 1957, **36**, 810.
28. Dole, V. P., and J. T. Hamlin III. Particulate fat in lymph and blood. Physiol. Rev. 1962, **42**, 674.
29. Kennedy, E. P. Metabolism of lipids. Ann. Rev. Biochem. 1957, 119.
30. Recknagel, R. O., B. Lombardi, and M. C. Schotz. A new insight into pathogenesis of carbon tetrachloride fat infiltration. Proc. Soc. exp. Biol. (N. Y.) 1960, **104**, 608.
31. Dixon, K. C. Fatty deposition: disorder of the cell. Quart. J. exp. Physiol. 1958, **43**, 139.
32. Orth, D. H., and H. E. Morgan. The effect of insulin, alloxan diabetes, and anoxia on the ultrastructure of the rat heart. J. Cell Biol. 1962, **15**, 509.