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Liver lipid response to intravenous rare earths in rats

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SUMMARY

Intravenous injection of rare earths with low atomic numbers produces fatty infiltration in the liver of rats. This infiltration is maximal 48 hours later and is characterized by an increase in neutral fat esters. Total cholesterol and phospholipids of the liver in cerium-treated rats were similar to those of control livers. Cerium was found to be effective in producing extremely high levels of liver fat in female rats of several strains tested. Male rats treated in the same way did not exhibit the consistent response or the high liver-lipid levels that were seen in the females. After the castration of male rats, their response resembled that of females. Testosterone caused a significant reduction in the fatty infiltration induced by cerium in both intact and ovariectomized females. Hypophysectomy prevented fatty livers in both females and males, whereas adrenalectomy did so only in males. Choline and methionine had no protective effect against cerium. Tracer experiments involving cerium^{144} demonstrated that most of the radioactivity appeared in the acid-soluble fraction of the liver and that the lipid fraction contained essentially no tracer.

The group of elements known as the rare earths has received relatively little attention in biological systems. The ability of these metals to behave like colloids at physiological pH has suggested some therapeutic possibilities (1), their increased industrial use (2) stresses the importance of acquiring a better biochemical understanding of this group of elements.

In a series of experiments designed to evaluate the clinical usefulness of different rare earths, a conspicuous blanching was observed in the livers of rats injected with cerium. Additional work showed that this blanching was the result of fatty infiltration (3). The initial studies showed levels of total liver lipid to be as high as 15 per cent within 48 hours after administration of cerium. We believe that no chemical or dietary means so far shown to cause fatty livers can do so as rapidly and extensively as a single injection of cerium. It appears that some of the rare earths would be useful experimental tools in the study of lipid metabolism.

This report represents the first detailed study of the various factors that influence development of the cerium-induced fatty liver. The data show the effect of dosage, time, endocrines, and lipotropic agents on the development of this condition. The data also demonstrate that the fatty infiltration caused by rare-earth elements of low atomic number is characterized by an increase in the content of esterified neutral fat.

METHODS

Animals. Male and female rats of the Sprague-Dawley (S-D), Charles River (CR), Carworth Farms Nelson (CFN), and Wistar (W) strains weighing 180 to 250 g. were maintained on Rockland rat pellets. Water was available at all times. Hypophysectomized animals were obtained from the Charles River Breeding Laboratories, but gonadectomies and adrenalectomies were performed by the usual surgical procedure in our laboratory. The success of each adrenalectomy was determined by the water tolerance test of Beatty *et al.* (4).

Preparation of Rare-Earth Solutions. All stock solutions were prepared quantitatively from the corresponding oxides (5). The procedure consisted of dissolving the oxides, with the exception of that of cerium, in either hydrochloric or nitric acid. The difficulty encountered with cerium was circumvented by using the following modified method (6). Equal weights of cerium oxide and sodium iodide were boiled vigorously in concentrated hydrochloric acid until the

TABLE 1. EFFECT OF CERIUM DOSE ON SURVIVAL TIME AND TOTAL LIVER LIPIDS IN CFN MALE AND FEMALE RATS

Cerium Administered	Sex	Total Liver Lipids in Wet Tissue		
mg./kg. body wt.		*per cent ± S.D. 24 hours*	*per cent ± S.D. 48 hours*	*per cent ± S.D. 72 hours*
0.0	F		6.11 ± 0.94 (7/7)	
0.5	F		6.60 ± 0.47 (3/3)	
1.0	F	8.10 ± 1.47 (3/3) *	11.69 ± 2.98 (3/3)	8.19 ± 2.27 (3/3)
2.0	F	10.23 ± 1.13 (3/3)	15.82 ± 1.30 (3/3)	17.17 (1/3)
3.5	F	9.77 ± 0.87 (3/3)	11.67 ± 0.43 (2/3)	(0/3)
4.5	F	9.93 ± 2.24 (3/3)	14.24 ± 1.90 (2/3)	(0/3)
0.0	M		5.34 ± 0.33 (3/3)	
3.5	M		7.93 ± 2.43 (3/3)	
7.0	M		9.54 ± 1.04 (3/3)	
14.0	M		9.16 ± 1.04 (3/3)	

* The ratio in parenthesis indicates (number of rats surviving/number of rats receiving dose).

cerium was dissolved and most of the iodine was evolved. Sodium nitrite was added and the mixture was heated again to ensure the complete removal of the iodine from the solution. The acid was removed by gentle evaporation and the residue was dissolved in 6 N HCl. Various dilutions of the stock rare-earth solutions, adjusted to pH 4, were used for all animal injections.

Injection of Rare Earths. Unless otherwise stated, 3.5 mg. of element per kilogram of body weight were injected into the tail vein. The volume of rare-earth solution administered was between 0.1 and 0.5 ml. per rat depending on the body weight of the rat and on the rare-earth solution used. Control animals were injected in the same way with an equivalent volume of saline. The following rare earths were used: cerium, lanthanum, praseodymium, neodymium, samarium, gadolinium, dysprosium, holmium, lutetium, and yttrium. To determine the distribution of cerium in the liver at the peak of fatty infiltration, two rats received 12 microcuries of cerium^{144} intravenously.[1]

Treatment of Animals. One group of animals received intramuscular injections of testosterone propionate or estrone (0.2 mg. in 0.1 ml. saline per rat per day for 16 days). To evaluate the protective effect of lipotropic agents, one group of rats received choline (100 mg. per kg. body weight, intraperitoneally) 1 hour before and 24 hours after the cerium injection; another group received methionine (7 mg. per kg. body weight, intravenously) 5 minutes before the cerium injection.

Analytical Procedures. The rats were killed by decapitation at various periods (usually 48 hours) after injection of the rare-earth solution. A 3-g. portion of the liver was removed, weighed, covered with 95 per cent ethanol, minced, and allowed to dehydrate overnight. The sample was transferred to a glass extraction thimble and the lipids were extracted for 4 hours with a 2:1 (v/v) ethanol:ether mixture in Soxhlet continuous extractors. The combined ethanol and ethanol:ether extracts were evaporated under infrared lamps and extracted with chloroform. Total lipids were determined gravimetrically on aliquots of the chloroform solution. The quantity of lipid present was expressed as percentage of the wet tissue weight, because the moisture content of the liver was not significantly altered by the rare-earth treatment. The per cent water in three control and five cerium-treated livers was 72.4 ± 0.8 and 72.9 ± 1.8, respectively. Ester equivalents (7) were measured in the neutral and phospholipid fractions, which had been separated from each other on a silicic acid column; chloroform and methanol were used as eluting solvents, essentially according to the method of Borgström (8). Mallinckrodt AR silicic acid (100 mesh) was activated by drying at 110°C for 24 hours before use. Phospholipid phosphorus was determined on all fractions by the method of King (9), using Elon as the reducing agent (a substitute for aminonaphtholsulfonic acid), prepared according to Harris and Popat (10). The method of Nieft and Deuel was adapted with slight modification for the measurement of total cholesterol (11) in the neutral fat fractions.

The livers of the rats that received cerium^{144} were separated into trichloracetic acid (TCA) soluble, lipid, and protein fractions. Weighed portions of the

[1] Cerium^{144} obtained from the Oak Ridge National Laboratory.

liver were homogenized in cold 10 per cent TCA containing 0.4 M $MgCl_2$ (12), centrifuged at 4°C, and the supernatant (acid-soluble fraction) was decanted. The residue was washed twice with the TCA solution and the supernatants were combined with the acid-soluble fraction. The lipids in the residue were extracted as described previously. The lipid-free residue, designated as the protein fraction, was dissolved in a boiling HNO_3:HCl (3:1 v/v) acid mixture. Aliquots of the various fractions were radioassayed with a scintillation detector (Nuclear-Chicago). The results were calculated as percentage of total radioactivity in the liver.

RESULTS

Table 1 shows the effects of dose and time after intravenous injection of cerium on the development of fatty livers and on survival of CFN rats. Only the total lipids of livers from survivors is given in the table. For female rats the maximum fat values usually appear at 48 hours after injection, and a dose as low as 1.0 mg. causes a significant increase (13) ($p < 0.01$) in liver lipids within 24 hours. Mortality increases with dose and with time. Because of less response and greater tolerance to cerium by male rats than by female rats, doses up to 14 mg. per kg. were used in four groups of males and all the animals survived. Liver lipids were significantly elevated by 7 and by 14 mg. of cerium per kilogram when compared with the saline group ($p < 0.01$) but no significant difference occurred among the three treated groups.

Variability in the fatty-liver response of male S-D rats to cerium in some early experiments prompted a series of tests in which the fatty-liver response in several strains of females and males was studied. Table 2 demonstrates that the degree of cerium-induced fatty liver is definitely sex dependent. Males are less responsive than females to fatty infiltration in all the strains that were compared. S-D and CFN males showed little or none of this response to cerium, whereas males of the W and CR strains showed an intermediate but significant response.

When castration of S-D rats preceded the cerium treatment, a lipid response similar to that of females was observed (Table 3). On the other hand, testosterone caused a significant reduction in liver fat in both intact and in ovariectomized females. Estrogen, when administered to castrated males, had no additional effect on the liver-lipid response to cerium.

In an attempt to see whether a parallelism exists to other types of chemically induced fatty livers, the role of the adrenals and of the pituitary on the fatty infiltration was examined. By comparison with Table 2, Table 3 indicates that adrenalectomy prevents fatty liver infiltration due to cerium in male rats of both the S-D and CR strains, whereas adrenalectomy only slightly reduces the fatty infiltration due to cerium in female rats. A similar comparison with intact animals shows that hypophysectomy prevents the occurrence of fat accumulation due to cerium in both male and female rats (Table 3).

Table 4 demonstrates that neither choline nor methionine has any protective effect against the fatty liver caused by cerium under the conditions of our experiments.

A survey of the ability of other rare earths to cause fatty livers is reported in Table 5. The data show that the fatty infiltration caused by rare earths is a biological property of the first third (At. No. 57 to 62) of the elements in this series, commonly called the cerium group of rare earths.

Silicic acid fractionation of the total lipids obtained from rats injected with rare earths showed significant increases in neutral lipid ester equivalents when compared with those of the controls (Table 6), whereas the levels of cholesterol and phospholipid in livers of cerium-treated animals were similar to controls. A more detailed study of the specific lipid components in these fractions is in progress.

TABLE 2. TOTAL LIVER LIPIDS OF MALE AND FEMALE RATS OF DIFFERENT STRAINS 48 HOURS AFTER INTRAVENOUS INJECTION OF CERIUM

Cerium Administered	Strain	Sex	Number of Rats	Total Liver Lipids in Wet Tissue	Probability *
mg./kg. body wt.				*per cent ± S.D.*	
0	S-D	M	13	4.72 ± 0.75	
3.5	S-D	M	14	6.23 ± 2.21	
0	S-D	F	2	6.25 ± 0.16	
3.5	S-D	F	15	12.39 ± 1.26	<0.01
0	CR	M	8	5.60 ± 0.71	
3.5	CR	M	7	9.71 ± 2.22	<0.01
0	CR	F	5	6.32 ± 0.80	
3.5	CR	F	6	12.83 ± 2.52	<0.01
0	CFN	M	6	5.30 ± 1.08	
2	CFN	M	6	5.90 ± 1.76	
0	CFN	F	7	6.11 ± 0.94	
3.5	CFN	F	12	11.35 ± 1.84	<0.01
0	W	M	3	4.64 ± 0.25	
3.5	W	M	4	10.49 ± 2.62	<0.02

* The probability that the difference between the observed mean and the saline control for each group is due to chance.

TABLE 3. EFFECT OF GONADECTOMY, ADRENALECTOMY, AND HYPOPHYSECTOMY ON TOTAL LIVER LIPIDS 48 HOURS AFTER INTRAVENOUS INJECTION OF CERIUM

Cerium Administered	Strain	Sex	Number of Rats	Total Liver Lipids in Wet Tissue	Probability [a]
mg./kg. body wt.				*per cent ± S.D.*	
			Intact		
0	S-D	M	13	4.72 ± 0.75	
3.5	S-D	M	14	6.23 ± 2.21	
			Castrated		
0	S-D	M	4	5.73 ± 0.43	
3.5	S-D	M	7	10.01 ± 1.22	<0.01*
3.5	S-D #	M	7	10.12 ± 1.35	<0.01****
			Intact		
0	S-D	F	2	6.25 ± 0.16	
3.5	S-D	F	15	12.39 ± 1.26	<0.01****
3.5	S-D ##	F	10	10.99 ± 1.19	<0.02**
			Ovariectomized		
0	CFN	F	8	7.20 ± 0.67	
3.5	CFN	F	8	14.93 ± 1.84	<0.01****
3.5	CFN ##	F	8	11.61 ± 1.28	<0.001***
			Adrenalectomized		
0	CFN	F	4	5.68 ± 0.19	
3.5	CFN	F	5	11.95 ± 1.16	<0.01*
0	S-D	M	4	4.11 ± 0.32	
3.5	S-D	M	5	4.37 ± 0.48	
0	CR	M	5	4.50 ± 0.30	
3.5	CR	M	2	4.33 ± 0.58	
3.5	CR	F	4	9.09 ± 2.88	
			Hypophysectomized		
0	CR	M	5	4.93 ± 0.50	
3.5	CR	M	3	5.54 ± 0.29	
0	CR	F	7	5.03 ± 0.32	
3.5	CR	F	6	5.34 ± 0.32	

[a] The probability that the difference is due to chance between: the observed mean and the saline (intact or castrated) or cerium control*; the observed mean and the cerium control**; the cerium ovariectomized and the saline control***; and the observed mean and the saline control****.

0.2 mg. estrone or ## testosterone injected intramuscularly per rat per day.

TABLE 4. EFFECT OF CHOLINE OR METHIONINE ON LIVER FAT DEPOSITION DUE TO CERIUM IN FEMALE CFN RATS

Cerium Administered	Treatment	Number of Rats	Total Liver Lipids in Wet Tissue
mg./kg. body wt.			*per cent ± S.D.*
0	None	7	6.11 ± 0.94
3.5	None	5	11.70 ± 0.90
3.5	Methionine *	5	13.32 ± 2.23
3.5	Choline †	4	10.41 ± 1.01

* 7.0 mg./kg. injected intravenously 5 minutes before cerium injection.

† 100 mg./kg. injected intraperitoneally 1 hour before and 24 hours after cerium injection.

The distribution of cerium^{144} in the TCA-soluble, lipid, and protein fractions of the liver at the peak of fatty infiltration was 94.0, 0.1, and 5.9 per cent, respectively.

DISCUSSION

The intravenous administration of rare earths with low atomic numbers (57 to 62) shows a pronounced effect on fat metabolism in the rat. Investigations of respiration by liver slices [2] together with studies of toxicity (5) and distribution (14) have indicated that the heavier rare earths behave more like yttrium and the lighter rare earths more like lanthanum. This is borne out by the liver-fat values for yttrium and lanthanum with respect to those shown for the other rare earths used (Table 5).

The difference we found between male and female rats in the liver-lipid response is also illustrated in reports by other investigators who have used ethionine (15, 16), ethanol (17), and low protein diets (18), rather than cerium, to produce fatty livers. Our data demonstrate that testosterone is definitely a causative factor in this sexual difference. Hormonal influence on lipid metabolism has not been clearly defined (19, 20) but the recently reported *in vitro* studies of Perry and Bowen (21) indicate that the gonads definitely have a role in the biosynthesis of lipids. These workers have demonstrated that castration in the male, but not in the female, is followed by an increased incorporation of 2-C^{14}-acetate into the fatty acids of liver and adipose tissue. Testosterone treatment in the male and estrogen treatment in the female resulted in the decrease and in the increase, respectively, of the incorporation of acetate into liver lipids. Clear-cut interpretation of other data of this type is often made difficult by the complicated pituitary-adrenal-gonad relationships (22).

In the light of known lipid metabolism, the inability of the lipotropic agents to protect against cerium-induced fatty livers indicates that the rare-earth type of fatty liver is nonspecific. The molar ratios of choline:cerium (33:1) and of methionine:cerium (2:1) were such that the quantity of lipotropic agents was well in excess of that of cerium. Methionine has been shown to protect against the ethionine-induced fatty liver even when the molar ratio of methionine to ethionine was 1:4 (16).

The fatty liver induced by rare earths is similar to the fatty infiltration caused by ethanol (23, 24) in that hypophysectomy and adrenalectomy prevent the deposition of fat. Other types of "nonspecific" fatty livers, e.g., phosphorus (25), are also similar in this respect. Some workers have implied that hormones of the pituitary exhibit lipid mobilizing properties (26). Mallov and Bloch (24) have reported that pituitary tissue homogenized in saline, when injected intravenously, can cause a significant increase in total liver fat, but we could not demonstrate any such influence on lipid metabolism under supposedly identical experimental conditions.

Experiments ruled out the possibility that a reduced food intake after the rare-earth injection and fasting for 24 hours before killing might cause some of the observed fatty infiltration. Total liver lipids in two rats fasted as long as 72 hours showed values similar to those of control animals (6.34 and 7.11 per cent).

A considerable effort has been expended in these laboratories in studying the distribution of cerium when administered intravenously to dogs, although very little work has been done in the rat. Our studies indicate that most of the cerium in the rat liver is associated with the TCA-soluble fraction, but we believe that the high cerium^{144} activity in the acid-soluble fraction is a result of solubilization of cerium by the low pH of the TCA solution. Other related studies in our laboratories have suggested the ability of rare earths to complex with proteins at physiological pH. The fact that the lipids contain no radioactivity seemingly rules out the possibility that any strong cerium chelate complex is formed between fatty acid carboxyl and phosphate hydroxyl groups in lecithin or other conjugated lipids. If such a complex were formed, fatty acid transfer from the liver via phospholipids might be seriously impaired. That such complexes are not formed *in vivo* and then dis-

[2] A separate report by E. A. Cress and G. C. Kyker, in preparation.

TABLE 5. TOTAL LIVER LIPIDS OF CFN FEMALE RATS 48 HOURS AFTER INTRAVENOUS INJECTION OF DIFFERENT RARE EARTHS

Rare Earth	Dose Administered	Number of Rats	Total Liver Lipids in Wet Tissue	Probability *
	mg./kg. body wt.		*per cent ± S.D.*	
None	0	7	6.11 ± 0.94	
Lanthanum	2	2	12.43 ± 0.92	<0.001
Cerium	2	3	15.82 ± 1.30	<0.001
Praseodymium	2	4	14.70 ± 1.40	<0.001
Neodymium	3.5	3	14.10 ± 1.45	<0.001
Samarium	3.5	3	12.53 ± 1.57	<0.001
Gadolinium	3.5	3	5.47 ± 0.65	
Dysprosium	3.5	3	6.57 ± 0.71	
Holmium	3.5	3	6.44 ± 0.94	
Lutetium	3.5	3	5.74 ± 0.51	
Yttrium †	3.3	2	5.01 ± 0.40	
Yttrium †	12.5	2	4.33 ± 0.31	

* The probability that the difference between the observed mean and the saline control for each group is due to chance.

† Animals were sacrificed 24 hours after injection of yttrium.

sociated by the strong acid during fractionation gains support by the absence of radioactivity in the total lipid fraction of dog liver, which had not been subjected to TCA extraction after an intravenous dose of tagged cerium.[3]

It is of interest to note that the elevated lipid level in rat livers is a result of glyceride infiltration. At the time when there was a maximal fatty-liver response, the phospholipid and cholesterol content of the livers remained essentially unaltered. The high ester-bond to phosphorus ratio is difficult to interpret. The methods were validated by theoretical results on several purified compounds and a commercial sample of lecithin purified by the same silicic acid method gave an ester to phosphorus ratio of 2.05; also, a commercial preparation without further purification showed a ratio of 1.88. Marinetti *et al.* (27) report an unidentified phospholipid fraction, with phosphatidic acid properties, that gave an unexplained high ester to phosphorus ratio of 3.58; they also used a silicic acid column to separate this fraction from rat liver lipids. The phospholipid fraction separated under our conditions represents a complex mixture of naturally occurring polar lipids. Proteolipids or sulfatides might possibly explain the high ratio, since Gaitonde and Richter (28, 29) have detected small but significant amounts of sulfur-containing lipoproteins in lipid extracts of rat liver. Any contaminating neutral lipid

TABLE 6. SEPARATION OF LIVER LIPIDS INTO NEUTRAL AND PHOSPHOLIPID FRACTIONS ON SILICIC ACID

Treatment	Number of Rats	Total Liver Lipids in Wet Tissue	Neutral Lipid per Gram Wet Tissue		Phospholipid per Gram Wet Tissue	
			Ester	Cholesterol	Ester	Phosphorus
		per cent ± S.D.	*μ eq. ± S.D.*	*mg. ± S.D.*	*μ eq. ± S.D.*	*μ eq. ± S.D.*
Saline control	3	5.63 ±0.20	67.7 ±19.7	3.26 ±0.30	103.1 ±14.2	34.6 ±8.5
Cerium-treated (2 mg./kg. body wt.)	3	15.33 ±2.50	432.5 ±82.2	3.14 ±0.92	94.1 ±14.0	36.0 ±2.3

[3] Unpublished data.

was ruled out by control experiments and the absence of any ester-positive material in the last 28 ml. of chloroform (four-column fractions) prior to the methanol elution of the phospholipids. There is also the possibility of some change during extraction or chromatography that would result in a loss of phosphorus but this seems highly improbable to us.

Experiments are currently under way to determine to what extent mobilization or biosynthesis or both account for the enormous influx of glycerides in the liver of rats treated with cerium. Also the early results of other studies of fatty infiltration suggest quite a different response to cerium in various species.

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