Fatty Liver in the Rat After Intravenous Infusion of Orotic Acid. (32328)

LEO H. VON EULER AND H. G. WINDMUELLER (Introduced by John G. Bieri)
Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

Since the original observation by Standerfer and Handler(1) that rats fed a purified diet containing 1% orotic acid show fatty change of the liver after 28 days, numerous studies of the mechanism of action involved in this phenomenon have been reported(2-9). In all these studies orotic acid was given by mouth. Even after large oral doses, only minute amounts of orotic acid could be detected in the liver. This has suggested the possibility that fatty liver is induced by a metabolite of orotic acid produced by the intestinal mucosa or by the intestinal microflora. Earlier work from this laboratory(10) showed that bacteria are not necessary for fatty liver induction by orotic acid, but a mucosal role has not been ruled out. To minimize this possible role we have given orotic acid to rats intravenously, and have succeeded in producing fatty livers by this route. The major difficulties were related to the low solubility of orotic acid.

Method. Male Osborne-Mendel rats, weighing 130-150 g were used. Water and a balanced purified, purine-free diet, designated W-7* were offered *ad libitum* to animals housed in modified metabolic cages (Fig. 1).

We modified a continuous infusion technique, described by Little *et al*(12) and Eve *et al*(13) in which a swivel joint is used to allow the rats relatively unrestrained movement within the cage (Figs. 1 and 2). The modification consisted of cannulating the saphenous vein at the mid-thigh level, under direct vision, instead of catheterizing one of the superficial tail veins. Under ether anesthesia, a polyethylene catheter,† drawn out to an outside diameter of about 0.012 inches, was inserted about 3 inches into a saphenous vein. This placed the tip in the inferior vena cava at about the level of the renal veins, as verified at the end of each experiment. The other end of the catheter was tunneled subcutaneously to a point on the tail about 3 cm from the base. The catheter was secured to the tail in the same manner as described by Little *et al*, and their technique of connecting the catheter with the infusion pump was used, except that the plastic spiral section in the cage (Fig. 1, ref. 13) was omitted. This reduced the volume between the pump and the rat and diminished the likelihood of back-up of blood in the narrow catheter tip. Although such back-up was always temporary, it led to clotting and obstruction of the catheter on several occasions. We further reduced the incidence of this complication by pumping a heparinized 5% glucose solution through the catheter during the insertion procedure. A Harvard infusion pump model #975, modified to accept four 50 ml glass syringes, was used in most of the experiments (Fig. 2). When the syringes were nearly empty, a second pump with filled syringes was started and connected without cessation of flow. In the earlier experiments, 10 ml were infused every 24 hours. This volume was later increased to 15 ml without apparent effect on the well-being of the animals. Food consumption of 10-15 g per day permitted the animal to gain weight, although not as fast as non-catheterized controls.

The ammonium salt of orotic acid was used, because it is more soluble than the sodium salt and is well tolerated by the rat. To the calculated amount of free orotic acid, enough NH_4OH was added to bring the pH to 7.4. In this way, as much as 4.4 mg orotic acid per ml (28 mM) could be kept in solution at room temperature for several months.

* The W7 diet differs from R1, described in ref. 11, only in that the fat-soluble vitamins are mixed in with the diet, in the following amounts (mg/kg diet): Micro A "30", 400 (30,000 USP units/g, Nopco Chem. Co.); vitamin D3, 0.08; dl-α-tocopherol acetate, 100; menadione 1.

† *Intramedic* polyethylene tubing, No. PE60, Clay-Adams, Inc., New York.

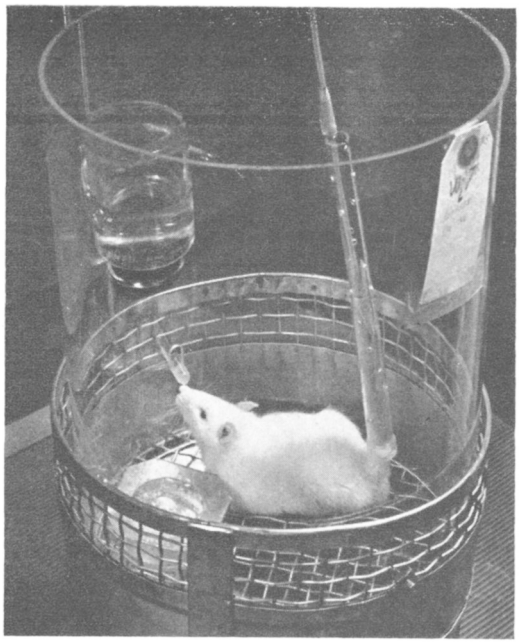

FIG. 1. Photograph demonstrating freedom of movement of rat within the cage.

The pH of the NH_4Cl control infusions was not adjusted and was 6.0. The NH_4Cl infusions were 29 mM, except in one experiment (90 mM). In 5 experiments adenine, as the free base, was added to orotic acid before bringing the solution to pH 7.4 with NH_4OH. To most infusions enough dextrose was added to make the solution isotonic with plasma.

After 6-15 days, the rats were decapitated and the liver removed. Total lipid extracts were prepared(14) and total fatty acids determined by titration after saponification, acidification and extraction into n-hexane.

Results and discussion. The major changes observed when rats are fed 1% orotic acid in the diet are 1) fat accumulation in the liver, not detectable before 3 days and reaching levels of 20-30% of wet weight in 10 days (1,6); 2) markedly reduced plasma lipid levels(2,6,15) and disappearance of detectable circulating β-lipoprotein due to complete inhibition of β-lipoprotein release into plasma from the liver(7); 3) 4-fold increase in total uridine nucleotides and a 50% reduction of total adenine nucleotides in the acid-soluble pools of the liver after 1-2 days(8,16) and 4) reversibility of both the lipid and nucleotide changes in 1-2 days after adenine is added to the diet in a molar ratio of adenine:orotic acid of 0.19:1.

All of these findings have now been duplicated in rats receiving orotic acid by the intravenous route. The orotic acid infusions which did not contain adenine caused grossly fatty livers which in every instance were confirmed by chemical analyses (Table I). These values are similar to the ones found in orotic acid-fed rats(2,15). There was no overlap between the control and orotic acid groups, and the lowest total fatty acid value for the latter group, 212 μmoles/g wet liver, is well outside the normal range for this laboratory. NH_4Cl caused no deviation from normal total fatty acid values. As with orotic acid-fed rats, fat does not begin to accumulate in the liver of animals infused with orotic acid until after the third day. When adenine was present in the infusion in a molar ratio of adenine:orotic acid of 0.22:1, it prevented the fatty change, but when the ratio was 0.11:1 or less, fatty livers were observed. No circulating plasma β-lipoprotein was detected by a sensitive immunoelectrophoretic method(7) in 4 rats after continuous infusion of 44-65 mg orotic acid per 24 hours for 7 days. The acid soluble nucleotide pools in the liver were examined in several infused animals and showed changes identical to those seen in orotic acid-fed rats.

When rats are fed a 1% orotic acid diet (about 100 mg orotic acid per day), as little as 40% may be absorbed from the gut; the rest is excreted in the feces unchanged, or

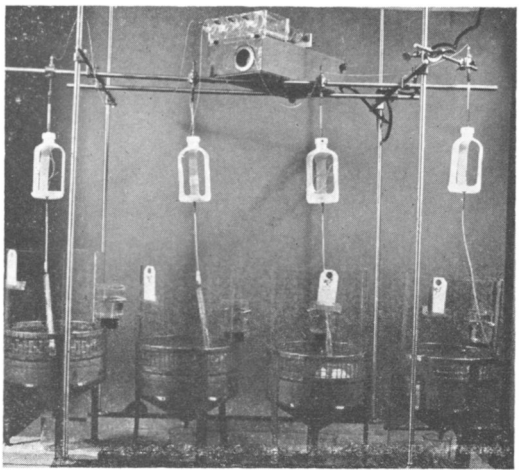

FIG. 2. Set-up for 4 simultaneous infusions.

TABLE I. Liver Total Fatty Acid Content Following Intravenous Orotic Acid Infusion.

Group	Days of treatment	Infused per 24 hr: Amount	Infused per 24 hr: Vol (ml)	Glucose present in infusion	Liver total fatty acids (μmoles/g fresh tissue)
Control	7	500 mg dextrose	10		73
	8	23 mg NH_4Cl	15	+	78
	8	" "	15	+	85
	8	" "	15	+	113
	8	48 mg "	10	—	87
Orotic acid	6	44 mg orotic acid	10	—	212
	6	" " "	10	—	288
	8	" " "	10	+	283
	8	" " "	10	+	436
	9	" " "	10	+	417
	11	53 mg " "	10	+	310
	10	65 mg " "	15	+	718
	10	" " "	15	+	770
	15	" " "	15	+	567
	15	" " "	15	+	847
	15	" " "	15	+	903
Orotic acid + adenine	8	44 mg orotic acid + 8.3 mg adenine[a]	10	—	85
	8	*Idem*	10	—	112
	8	44 mg orotic acid + 4.2 mg adenine[b]	10	—	145
	8	*Idem*	10	—	386
	8	44 mg orotic acid + 2.1 mg adenine[c]	10	—	430

Molar ratio adenine : orotic acid is
[a] 0.22:1 [b] 0.11:1 [c] 0.06:1

as bacterial metabolites(10). Up to 20% of the administered dose is excreted unaltered in the urine. Thus, on a 1% orotic acid diet, the rat may metabolize less than 50 mg/24 hr. Data from a long-term infusion with 65 mg orotic acid/24 hr indicated about 35% excretion in the urine, while fecal excretion was negligible. In these experiments, the rats metabolized no more than 33-43 mg orotic acid, an amount sufficient to cause a fatty liver if delivery is spread over 24 hours. The data in Table I suggest that there is a crude proportionality between the dose of orotic acid and the total fatty acid content of the liver, and that lower doses would be insufficient to cause a fatty liver. Attempts to produce fatty livers by intraperitoneal administration of orotic acid were unsuccessful. No effect was seen when 21 mg lithium orotate in 1 ml was injected twice daily to 100 g rats. A higher dose of 60 mg per injection, on the other hand, was not tolerated and most animals died within a few days with accretions in the peritoneal cavity and urinary crystals.

Sidransky *et al*(9) found that by varying the quantity, composition and timing of the diet relative to the orotic acid, they could critically affect the development of fatty livers in female Wistar rats. Thus, if diet and orotic acid were fed at different times of the day, fatty livers did not develop. The interpretation given was that the basal purified diet must be given together with the orotic acid, in order to observe the phenomenon. Their results might also suggest that 1% orotic acid is near the minimal effective dose and that anything that interferes with the steady supply of orotic acid to the liver will prevent fatty liver induction. The data presented in this paper support this view and also make it seem unlikely that the mucosal cells play an essential role in fatty liver induction since, when infused, only a small portion of the orotic acid presumably reaches the intestinal mucosa.

Summary. Continuous intravenous infusion of 44-65 mg orotic acid per 24 hours in 120-180 g rats caused fatty liver, liver nucleotide changes and disappearance of plasma β-lipoprotein, effects which were indistinguishable from those caused by feeding a 1% orotic acid diet. Thus a method for continuous, controlled administration of

orotic acid, independent of the diet, is now available for the study of fatty liver induction. The results suggest that the intestine and its contents do not play a necessary role in causing orotic acid induced fatty liver.

The technical assitance of Mr. Albert E. Spaeth is gratefully acknowledged.

1. Standerfer, S. B., Handler, P., Proc. Soc. Exp. Biol. & Med., 1955, v90, 270.

2. Windmueller, H. G., J. Biol. Chem., 1964, v239, 530.

3. Handschumacher, R. E., Creasy, W. A., Jaffe, J. J., Pasternak, C. A., Hankin, L., Proc. Nat. Acad. Sci., U. S., 1960, v46, 178.

4. Marchetti, M., Puddu, P., Caldarera, C. M., Biochem. J., 1964, v92, 46.

5. Rajalakshmi, S., Sarma, D. S. R., Sarma, P. S., ibid., 1961, v80, 375.

6. Creasey, W. A., Hankin, L., Handschumacher, R. E., J. Biol. Chem., 1961, v236, 2064.

7. Windmueller, H. G., Levy, R. I., ibid., 1967, v242, 2246.

8. von Euler, L. H., Rubin, R. J., Handschumacher, R. E., J. Biol. Chem., 1963, v238, 2464.

9. Sidransky, H. Verney, E., Lombardi, B., J. Nutr., 1963 v81, 348.

10. Windmueller, H. G., McDaniel, E. G., Spaeth, A., Arch Biochem. Biophys., 1965, v109, 13.

11. Williams, J. N., Jr., J. Nutr., 1961, v73, 199.

12. Little, J. R., Brecher, G., Bradley, T. R., Rose, S., Blood, 1962, v19, 236.

13. Eve, C., Robinson, S. H., J. Lab. & Clin. Med., 1963, v62, 169.

14. Folch, J. Lees, M., Sloane Stanley, G. H., J. Biol. Chem., 1957, v226, 497.

15. Windmuller, H. G., Biochem. Biophys. Res. Commun., 1963, v11, 496.

16. Marchetti, M., Puddu, P., Caldarera, C. M., Biochim. Biophys. Acta, 1962, v61, 826.

Received May 8, 1967. P.S.E.B.M., 1967, v125.

Effect of Adrenalectomy and of Dexamethasone upon Circadian Distribution of Mitosis in the Cornea of Rats—I.* (32329)

S. S. CARDOSO AND A. L. FERREIRA (Introduced by J. W. Fisher)

Departments of Pharmacology and Morphology, Faculty of Medicine of Ribeirão Prêto, University of São Paulo, Brazil

Corticosteroid participation in the general control mechanisms responsible for the distinct circadian mitotic and metabolic fluctuations, which occur in mammalians, has been established. Thus, potassium excretion by the kidneys and neutrophil and eosinophil levels in the blood have distinct diurnal changes which are related either to mineral or glucocorticoid activity(1-7).

Mitotic activity and its distinct circadian variations, with highest levels in the late morning and lowest levels at night, have been established in rats and mice, and are related to adrenal function(7-10).

RNA and enzyme synthesis can be stimulated by administration of glucosteroids(11-14), while DNA synthesis and mitosis, in general, have been reported to be inhibited by these hormones(14-17). Whether this inhibitory effect could in any way be related to the low levels of mitosis which are found in tissues of mammals, coinciding in time with periods of rising concentrations of circulating glucocosteroids(18,19), has not been established.

The well documented mitotic stimulation found in the corneal epithelium of rats, following partial hepatectomy, is apparently related to adrenal gland function, as judged by both its absence in adrenalectomized animals and by its reestablishment in adrenalectomized animals treated with dexamethasone (20,21).

Evidence has recently been found that glucosteroids have a stimulatory effect upon mitosis in the corneal epithelium of rats. Thus, the administration of dexamethasone, a potent glucosteroid, to adrenalectomized animals resulted in the appearance of a significantly greater number of cells undergoing

* This work was supported in part by a Grant (CY-6473) from Nat. Inst. Health, USPHS and from Fun dação de Amparo à Pesquisa do Estado de São Paulo, Brazil.