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Further Studies on the Time Course of Rerepression[1] of Two Rat Liver Enzymes (37884)

B. SZEPESI, C. A. SLAYTON, AND O. E. MICHAELIS, IV

Nutrition Institute, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705

It has been shown that second episode of starvation–refeeding results in higher levels of NADP-linked hepatic dehydrogenases than is obtained after one starvation–refeeding episode (1). Such "increased inducibility" can be prevented by feeding a high-fat diet during the first refeeding (1). It has also been shown that in order to obtain this effect of the high-fat diet, it must be fed for the first 2 days following starvation (2). Refeeding the high-fat diet during only the first day of refeeding was ineffective in preventing the "increased inducibility" produced by a subsequent starvation–refeeding episode (2). In the experiments reported here, the relative effectiveness of the high-fat diet in preventing "increased inducibility" was tested as a function of the time the diet was given to the animals during the first refeeding. The dependence of the effect of the high-fat diet on *de novo* RNA synthesis was tested by treatment of half of the fat-fed rats with 8-azaguanine.

Methods and Materials. Male, specific pathogen-free Wistar rats were purchased from Carworth Laboratory Animals, Inc., of Vincentown, NJ. Rats were shipped under such conditions as to prevent stress due to heat, food, or water restriction and infection. The animals were housed individually in screen-bottom cages and were kept in an air-conditioned (21–23°) humidity-controlled environment with light-dark cycles of 12 hr (lights off from 6 PM until 6 AM).

Rats were subjected to one (group 1) or two (groups 2–8) starvation–refeeding cycles. Group 1 was starved for 2 days and was refed the inducer (65% glucose) diet for 3 days. The inducer diet contained 65% glucose, 25% casein, 5% corn oil, 4% Jones–Foster salt mix, and 1% Vitamin Fortification Mixture.[2] Groups subjected to two starved–refeed cycles were starved for 2 days and refed for 2 days the same diet (groups 2–4) or two different diets (each diet was fed for 1 day—groups 5–8), then starved for 2 days and refed for 3 days the inducer diet. Groups 2–8 differed from each other in the treatment during the first refeeding; these were: 2 days, inducer diet for group 2; 2 days 35% fat diet for groups 3 and 4; 1-day 35%-fat diet followed by a day of inducer diet for groups 5 and 6; and 1 day of inducer diet

[1] The term derepression is used in this paper to denote the process(es) operating during starvation which render glucose 6-phosphate dehydrogenase and malic enzyme inducible during refeeding an inducer diet. Hence, the term rerepression is used here to denote the effect of a treatment in counteracting those processes which render glucose 6-phosphate dehydrogenase and malic enzyme inducible. These are operational definitions and do not imply a particular mechanism of action.

[2] All dietary ingredients were purchased from Nutritional Biochemicals Corp., Cleveland, OH, with the exception of Mazola corn oil which was purchased in local stores and beef tallow which was rendered in our laboratories. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture, nor does it imply its approval to the exclusion of other products that may also be suitable.

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followed by a day of 35% fat diet for groups 7 and 8. Groups 4, 6, and 8 were treated with 8-azaguanine during the feeding of the 35%-fat diet. 8-Azaguanine was dissolved in dilute alkali (15 mg/ml, pH = 10, in NaOH) and administered in 0.5-ml doses intraperitoneally at 8 AM and 8 PM. The first injection of 8-azaguanine was administered at the time the rats were given the 35%-fat diet, and the last injection was given at the time the 35%-fat diet was taken away. The 35%-fat diet contained 35% fat (beef tallow, corn oil, lard, 1:1:1 by weight), 25% casein, 35% glucose, and salt mix and vitamins as described above.

Food intakes and body weights were recorded at each dietary change or the termination of the experiment. Food intakes were calculated as grams of food eaten per 100 grams body weight per day. Body weight changes were calculated as percent change per day per rat (or grams change per 100 grams of body weight per day).

Rats were killed in the early morning by decapitation. The preparation of liver homogenates and enzyme assay were done as described by Freedland (3). Student's t test was used to ascertain the statistical significance of differences. Any difference between two means having a t value greater than what is calculated for $P < 0.05$ was designated as "significant."

Results and Discussion. Ever since the induction of rat-liver glucose 6-phosphate dehydrogenase (G6PD) and malic enzyme (ME) by starvation–refeeding was first described (4), there has been a considerable amount of work devoted to elucidate the mechanism of these inductions and their dietary consequences. Recently, a shorter (5) and a more expanded (2) model was put forth in order to characterize, describe, predict, and test the behavior of these enzymes under various conditions. Briefly, it was suggested that G6PD and ME are derepressed by stress, both at the transcriptional and translational levels. The data can best be explained by assuming that the genes which code for G6PD and ME have repressor systems containing lipid corepressors and that the corepressors are destroyed by stress such as starvation. Upon refeeding for 3 days, a high-carbohydrate adequate-protein and low-fat diet, enzyme levels of 3–5 times those found in *ad lib.*-fed rats can be measured. If feeding of the high-carbohydrate diet is continued, G6PD and ME levels will return to normal in a little over a week. A second starve–refeed episode, however, will lead to a greater enzyme induction with G6PD than is produced by one starve–refeed episode even 3 weeks after the first starvation (6). An explanation consistent with the model presented (1, 2) is that endogenous liver lipids (which reach three times normal levels during refeeding) can affect translational rerepression, but that transcriptional rerepression requires exogenous (dietary) lipids. Indeed, the inclusion of sufficient amounts of dietary fat during the first refeeding can prevent the "increased inducibility" during the second refeeding (1). It is within this framework that the time course of transcriptional rerepression is examined here.

The data are summarized in Table I. Comparison of groups 1 and 2 shows that all three enzymes were induced to a greater extent by a second starve–refeed episode. Feeding of the 35%-fat diet during the first refeeding (group 3) completely prevented the "increased inducibility" during the second refeeding. Treatment with 8-azaguanine during the feeding of the 35%-fat diet had a different degree of effectiveness of reversing the effect of the high-fat diet on the inducibility of the three enzymes. Thus, treatment with 8-azaguanine reversed the effect of the high-fat diet totally in the case of G6PD, partially in the case of ME, but was ineffective with 6-phosphogluconate dehydrogenase (6PGD). Since 8-azaguanine is incorporated into RNA made *de novo* resulting in the formation of nonfunctional RNA (7, 8), the data indicate that rerepression requires *de novo* RNA synthesis for G6PD and ME, but not for 6PGD. It is reasonable to assume that the *de novo* RNA synthesis is required for *de novo* protein synthesis, perhaps for the synthesis of the aporepres-

TABLE I. Effect of a High-Fat Diet Fed During the First Refeeding on the Inducibility of Three Rat-Liver Enzymes During the Second Refeeding.

Treatment[a]	Weight at start (g)	Weight at killing (g)	Relative liver size (liver wt × 100)/ (body wt)	Enzyme activity[b] U/100 g body wt		
				G6PD[c]	6PGD	ME
Group						
One cycle						
(1) 2*,3(65G)	144 ± 2[d]	145 ± 2	6.23 ± 0.18	107 ± 5.32	21.5 ± 2.51	51.8 ± 2.40
Two cycles						
(2) 2*,2(65G),2*,3(65G)	140 ± 3	146 ± 4	6.51 ± 0.19	128 ± 3.65	30.4 ± 1.48	64.7 ± 3.67
(3) 2*,2(35F),2*3(65G)	147 ± 3	163 ± 3	6.29 ± 0.21	103 ± 8.14	17.9 ± 2.18	47.1 ± 4.98
(4) 2*,2(35F)[e],2*,3(65G)	148 ± 4	144 ± 5	6.12 ± 0.07	128 ± 5.15	21.3 ± 1.54	58.5 ± 1.41
(5) 2*,1(35F),1(65G),2*,3(65G)	144 ± 3	156 ± 3	6.35 ± 0.17	121 ± 4.64	25.2 ± 1.07	63.6 ± 4.39
(6) 2*,1(35F)[e],1(65G),2*,3(65G)	147 ± 3	148 ± 2	6.32 ± 0.16	138 ± 7.16	23.6 ± 1.07	74.8 ± 5.41
(7) 2*,1(65G),1(35F),2*,3(65G)	146 ± 4	156 ± 4	6.33 ± 0.07	104 ± 5.28	22.9 ± 1.69	53.3 ± 4.21
(8) 2*,1(65G),1(35F)[e],2*,3(65G)	150 ± 5	152 ± 6	6.50 ± 0.10	131 ± 3.78	23.4 ± 1.05	58.5 ± 1.41

[a] Numbers outside of parentheses refer to the duration (in days) of a treatment. Starvation is starred. Numbers in parentheses refer to diet fed: (65G) = 65%-glucose (inducer) diet; (35F) = 35%-fat diet.

[b] One unit of enzyme activity is defined as that amount of enzyme which can produce 1 μmole of measured product (NADPH) per minute under the conditions of the assay.

[c] Abbreviations: G6PD = glucose 6-phosphate dehydrogenase; 6GPD = 6-phosphogluconate dehydrogenase; ME = malic enzyme.

[d] Standard error of the mean. Each group contained six rats.

[e] 8-Azaguanine was administered during this period.

sor (if the aporepressor is lost during starvation) or an enzyme to synthesize the corepressor, or both.

In a series of experiments (groups 5–8), the suppression of "increased inducibility" was studied as a function of when the high-fat diet was fed. If the *de novo* protein necessary for rerepression were made during the first day of refeeding, then the "increased inducibility" should be abolished by feeding the high-fat diet during either day of the first refeeding. If, however, this protein was made during the second day of refeeding, then the high-fat diet should be effective if fed during the second day, but ineffective if fed during the first day of refeeding. Examination of the data shows that rerepression of G6PD is accomplished during Day 2 of the first refeeding and that the effect of the high-fat diet can be reversed by 8-azaguanine treatment. The effect of 8-azaguanine indicates that the *de novo* RNA necessary for rerepression of G6PD is made during the second day of refeeding, as is the RNA necessary for the enzyme overshoot (9). The results indicate that the rerepression of malic enzyme follows a similar time course, although the effect of the high-fat diet is not completely abolished by 8-azaguanine (group 8). Apparently, 6PGD can be rerepressed by the high-fat diet on either day and the effect of the high-fat diet is not reversed by 8-azaguanine. This would suggest that the 6PGD aporepressor is not destroyed during starvation or, alternately, that the corepressor of 6PGD can be made without the necessity for *de novo* RNA synthesis.

The indication from previous (9) as well as from present data that the *de novo* RNA synthesis required for the enzyme overshoot and rerepression occur about the same time is compatible with the lack of endogenous transcriptional rerepression. Indeed, if endogenous fat were available for transcriptional rerepression, the enzyme overshoot could not occur because (presumably) a few molecules of completed repressors could shut off specific *de novo* RNA synthesis before a substantial induction of G6PD or ME could occur.

Recently, it has been reported that enzyme induction upon refeeding (i.e., within hours after refeeding) is inhibited by actinomycin D when given immediately after refeeding (10). It was suggested, therefore, that the *de novo* RNA synthesis required for the enzyme induction (and, by implication, for the enzyme overshoot) is accomplished within a short time after refeeding. This interpretation of the data would conflict with the interpretation that when 8-azaguanine is used the *de novo* RNA synthesis needed for the enzyme overshoot is accomplished between 24 and 48 hr after refeeding (9). It should be remembered that the enzyme changes reported in the work in which actinomycin D is used are not the same as the enzyme overshoot, because the latter is not noticeable until 24 hr after refeeding. Since actinomycin D is known to have a number of side effects (11–15) including the disaggregation of polysomes (14), it is entirely possible that actinomycin D prevents the early change in G6PD because it prevents polysomal aggregation.

Summary. Rerepression of G6PD, 6PGD, and ME can be accomplished by feeding a high-fat diet to starving rats. When such regimen is followed, a second episode of starvation–refeeding will no longer cause "increased inducibility" of these enzymes. Treatment of the rats with 8-azaguanine during the feeding of the high-fat diet prevented the effect of the high-fat diet on G6PD inducibility, decreased the effect on ME inducibility, but had very little effect on 6PGD inducibility. The results indicate that transcriptional rerepression requires exogenous fat and *de novo* RNA synthesis, but that 6PGD can be rerepressed without *de novo* RNA synthesis. The results further indicate that the *de novo* RNA synthesis necessary for the rerepression of G6PD and ME occurs during the second day of refeeding and coincides with the time course of *de novo* RNA synthesis requirement for the enzyme overshoot.

1. Szepesi, B., Vegors, R., and DeMouy, J. M., Nutr. Rep. Int. 5, 281 (1972).

2. Szepesi, B., Nutr. Rep. Int. **9**, 415 (1973).
3. Freedland, R. A., J. Nutr. **91**, 489 (1967).
4. Tepperman, H. M., and Tepperman, J., Diabetes **7**, 478 (1958).
5. Szepesi, B., Nutr. Rep. Int. **7**, 133 (1973).
6. Szepesi, B., and Michaelis, O. E., IV, Fed. Proc. **31**, 709 (1972).
7. Zimmerman, E. F., and Greenberg, S. A., Mol. Pharmacol. **1**, 113 (1965).
8. Kwan, S. W., and Webb, T. E., J. Biol. Chem. **242**, 5542 (1967).
9. Szepesi, B., and Freedland, R. A., Proc. Soc. Exp. Biol. Med. **135**, 709 (1970).
10. Sassoon, H. F., Dror, Y., Watson, J. J., and Johnson, B. C., J. Nutr. **103**, 321 (1973).
11. DeMatteis, F., Biochem. J. **100**, 15P (1966).
12. Earl, D. C. N., and Korner, A., Arch. Biochem. Biophys. **115**, 437 (1966).
13. Grey, R. D., and Moog, F., Nature **211**, 418 (1966).
14. Sociro, R., and Amos, H., Biochim. Biophys. Acta **129**, 406 (1966).
15. Yamada, C., Clark, A. J., and Swendseid, M. E., Science **158**, 129 (1967).

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