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# The *in Vitro* Stability of Rat Liver Glucose-6-Phosphate Dehydrogenase as a Function of Diet (41743)

BELA SZEPESI AND JULIAN SAFIR

*Carbohydrate Nutrition Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705*

*Abstract.* Rat liver glucose-6-phosphate dehydrogenase (G6PD) activity was studied in starved–refed rats given diets containing three levels of fat (35%, high-fat; 5%, low-fat; 0%, fat-free). Elution characteristics from DEAE-cellulose, $K_m$ for glucose-6-phosphate, pH optimum, and molecular weight appeared to be similar. During storage or heat denaturation, stability apparently was lowest of G6PD of livers from rats refed the high-fat diet. Heat stability was enhanced by the addition of NADP, but some differences due to diet persisted. Titration of a constant amount of enzyme with heating gave inconsistent results: in two of four experiments rats refed the high-fat diet had an equivalence point twice that of rats refed the fat-free diet. This difference disappeared if the antibody titration was carried out in the cold. The diet-induced instability of the G6PD, as measured *in vitro*, was reversible by changing the diet of the rats.

Glucose-6-phosphate dehydrogenase of rat liver (EC 1.1.1.49; G6PD) can be induced by starvation–refeeding (1), increasing the dietary carbohydrate level within moderate ranges (2, 3), and substituting fructose (4–7) or digestible disaccharides (8) for glucose or starch in the diet. Induction in the starve–refeed experiment can be blocked by increasing the dietary intake of fat (9–16), particularly fats containing polyunsaturated fatty acids (12, 13, 15–17). The first set of studies using antibodies raised against G6PD indicated that enzyme activity was increased by an increase in the rate of synthesis of titratable enzyme protein (18–20); there was no indication of a conversion from inactive to active species to account for the increased activity observed. This was later questioned by Kelley *et al.* (21) who used rat liver G6PD in a radial immunodiffusion experiment and by Hizi and Yagil who used monospecific antibody with mouse G6PD (22). Both studies demonstrated a lack of correlation between enzyme activity and the rate of enzyme synthesis. The lack of correlation is demonstrable chiefly with rats that had been fed a high-fat diet. While a lack of correlation between enzyme activity and titratable enzyme protein does not prove an inactive/active conversion, it implies the presence of enzymatically inactive, but immunologically active enzyme. When Wolfe and Holten (23) repeated the experiments of Hizi and Yagil (22) and Kelley *et al.* (21), they found no evidence of a conversion of inactive to active enzyme and, in fact, they reported confirmation of their earlier findings that increased enzyme activity is always proportional to titratable enzyme protein.

In some of our studies we found that anti G6PD rabbit serum would remove almost twice the activity from DEAE eluants prepared from livers of rats fed a high-fat diet as compared to the amount of enzyme activity removed from corresponding DEAE eluants prepared from livers of rats fed a fat-free diet. The difference appears to be due to nonspecific precipitation of G6PD (i.e., not antibody-directed precipitation) and is due to the greater loss of enzyme activity in samples from rats fed a high-fat diet. The authors cannot say whether this explains the difference between the reports of Kelley *et al.* and Hizi and Yagil (21, 22) on the one hand and the reports of others (18–20, 23–25).

**Materials and Methods.** *Animals and diet.* Male, Wistar rats weighing 150–175 g were purchased from Hilltop Laboratory Animals[1] of Scottdale, Pennsylvania. Following receipt, the animals were equilibrated to their new

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.



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environment for 2–3 days during which time their ration consisted of a standard laboratory chow diet. The rats were then starved 2 days, refed for 2 days one of three diets, and were then killed between 8:00 and 10:00 AM. The animals had free access to distilled water all the time. Temperature, lights (dark between 6:00 PM and 6:00 AM), and humidity were controlled by automated devices. Rats were assigned to three equal-sized groups which differed from each other in the diet refed. The composition of the three diets refed was as follows (g/kg diet): HF (high-fat); casein, 250; glucose, 350; salt mix (AIN salt mix, no sucrose), 40; vitamins (Teklad Vitamin Mix No. 1), 10; fat, 350 (corn oil:beef tallow:lard, 1:1:1): FF (fat-free); casein, 250; glucose, 700; salt mix, 40; vitamins, 10: 65G (low-fat); casein, 250; glucose, 650; salt mix, 40; vitamins, 10; corn oil, 50. Rats were stunned by a blow to the head and killed by decapitation. The carcasses were exsanguinated. Livers were quickly removed, rinsed, blotted, and chilled over ice, then frozen and kept at −20°C until used.

*DEAE-cellulose chromatography.* A 10% liver homogenate in 10 m*M* Tris buffer (5 m*M* mercaptoethanol, 1 m*M* EDTA, 10 μ*M* NADP, pH 7.5) was prepared with a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 30 min at 30,000*g*, the resultant clear supernatant was drawn off, diluted 1:10 with the homogenizing buffer and was applied to a column containing coarse-mesh DEAE (washed with NaOH, HCl, water, fined, and equilibrated in the 10 m*M* Tris buffer). The column was serially eluted with the same buffer containing 0.025 *M* $MgCl_2$ and 0.1 *M* $MgCl_2$. Liver G6PD was eluted in the 0.1 *M* $MgCl_2$ fraction. This eluant was used for all subsequent experiments. The purpose of this procedure was to separate G6PD from 6-phosphogluconate dehydrogenase in order to improve the accuracy of the G6PD assay. Conditions of the elution were optimized to ensure complete recovery of G6PD activity, but the extent of purification (i.e., the change in specific activity) was of secondary importance.

*Purification of G6PD, preparation of antibody.* Rat liver G6PD was purified to homogeneity as previously described (25). Antibody against this protein was raised in rabbit (25). The antibody was shown to be monovalent by crossed rocket immunoelectrophoresis (26).

*Immunotitration of G6PD.* Immunotitration of G6PD was performed in the 10 m*M* Tris buffer (0.1 *M* $MgCl_2$) in one of three ways: (i) by adding a constant amount of enzyme and increasing antibody concentration and (ii) by adding a constant amount of antibody and increasing the amount of enzyme added. The enzyme-antibody mix was heated at 37°C for 30 min, cooled, and allowed to stand 2 days before enzyme activity was determined. The third method of immunotitration (iii) consisted of assaying the enzyme–antibody mix for enzyme activity 2 hr after the addition of the antibody, without heating the enzyme–antibody mixture upon addition of the antibody.

**Results.** *DEAE-cellulose chromatography of liver G6PD.* The results of the DEAE-cellulose chromatography are summarized in Table I. Enzyme preparations from all dietary treatments had essentially the same eluting characteristics and were purified to about the same extent.

*Immunotitration of the partially purified G6PD.* Immunotitration of enzyme from rats refed the fat-free (FF) or low-fat diet (65G)

TABLE I. PARTIAL PURIFICATION OF RAT LIVER G6PD[a] BY DEAE-CELLULOSE CHROMATOGRAPHY

| Diet refed[b] | Grams liver used | Specific activity[c] (units/mg protein) after DEAE | Number units used | Percentage recovery | Purification |
|---|---|---|---|---|---|
| 0% fat (FF) | 12 | 2.83 | 30.6 | 109 | 2.9× |
| 5% fat (65G) | 12 | 2.10 | 63.2 | 105 | 3.1× |
| 35% fat (HF) | 24 | 0.51 | 46.6 | 110 | 2.5× |

[a] G6PD, Glucose-6-phosphate dehydrogenase.
[b] Rats were starved 2 days and refed 3 days. For the complete description of the diets, see Materials and Methods.
[c] One unit of enzyme activity = amount of enzyme capable of producing 1 μmole of NADP/min under the conditions of the assay.

proceeded as expected. When increasing amounts of enzyme were added to a constant amount of antibody (as reconstituted serum in the 10 m*M* Tris buffer), an equivalence point of 90–110 mU G6PD activity neutralized/100 μl immunized serum was obtained. Excess antibody neutralized all enzyme activity; in the presence of enzyme excess, the curve of activity added vs activity recovered had a slope of nearly 1.0. DEAE eluants from rats refed the 65G diet had similar equivalence points to those refed the FF diet. This was not the case, however, with the DEAE eluants from rats refed the high-fat (HF) diet (Fig. 1). In a series of experiments, equivalence point varied from 90–220 mU G6PD activity neutralized/100 μl serum and the slope of the activity added vs activity recovered line (enzyme excess conditions) varied from 0.2–0.6. In four subsequent experiments (Table II), we decided to compare DEAE eluants only from the HF- and FF-refed groups. In two experiments, the equivalence points were twice as high for the HF group as for the FF group (Table II). Various mixing experiments gave equally inconsistent results. It appeared that G6PD from rats refed the high-fat diet was either different in some way from the enzyme found in the FF-refed rats or that enzyme from rats refed the HF diet disappeared nonspecifically under some conditions. These observations led us to compare some of the physical/chemical properties of G6PD from HF-fed or FF-fed rats.

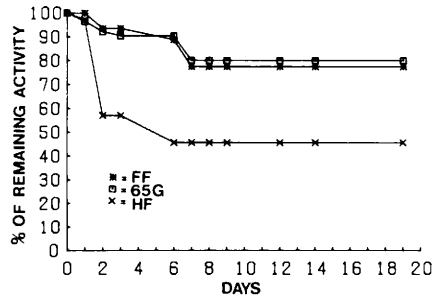


FIG. 1. Stability of rat liver glucose-6-phosphate dehydrogenase activity as a function of diet. The enzyme was serially eluted from DEAE-cellulose (0.025 and 0.10 *M* $MgCl_2$) and stored at 4°C in a cold room. Diets: fat-free diet (FF); 65% glucose diet (65G); high-fat diet (HF). For a complete description of diets, see Materials and Methods.

*Physical/chemical properties of G6PD from rat liver.* Glucose-6-phosphate dehydrogenase from rats refed the HF or the FF diet had the following characteristics: elution from DEAE, same pattern; $K_m$ for glucose-6-phosphate, $1.1 \times 10^{-5}$ *M* vs $2.4 \times 10^{-5}$ *M;* pH optimum, 7.8 vs 8.0. Chromatography with Sephadex gels suggested a molecular weight greater than 100,000 and all the enzyme activity was recovered as one peak. This would indicate that all the active enzyme that can be assayed is present in the dimer fraction or in aggregates of higher molecular weight than the dimer (monomer molecular weight $\simeq$ 60,000).

Although the properties of enzyme obtained from rats refed the HF or FF diets were similar with respect to elution, $K_m$, pH optimum, and minimum molecular weight, the stability of the DEAE eluants differed markedly (Fig. 2). Enzyme isolated from the HF-fed rats was unstable and lost nearly half of its activity during the first 2 days of storage. Repetition of the experiment yielded a similar conclusion although from somewhat different data (Table III). In that experiment enzyme activity disappeared continuously, but activity loss was three times greater in the HF homogenate than in the FF homogenate (34 vs 11.5%/100 hr). An equal volume mixture of the two homogenates lost activity at a somewhat lower rate than the predicted average rate of loss (20.1 vs 23.7). Heat stability appeared to be a function of the amount of dietary fat, with the group fed the highest amount of fat having the least stable enzyme (Table IV). Addition of NADP increased heat stability. The stabilizing effect of NADP appeared greater with the enzyme isolated from the FF-fed rat inasmuch as there was no loss of enzyme activity in that group when heated 20 min at 45°C. Enzyme from the HF group still lost nearly 20% of its activity during the same treatment.

*Reversibility of in vitro stability of liver G6PD by diet.* Experiments were conducted to determine whether the effect of the HF diet depended on prior starvation and whether the diet effect was reversible (Table V). Enzyme obtained from rats fed the HF diet was less stable whether fed *ad libitum* (group 1) or starved–refed (group 2). In this series of experiments, loss of G6PD activity during *in vitro* storage was twice as fast in preparations from rats fed the HF diet as in preparations

TABLE II. ANTIBODY TITRATION OF DEAE-ELUANT WITH RABBIT ANTI-G6PD SERUM[a]

| Diet refed | Experiment | Equivalence point (mU activity neutralized by 100 μl reconstituted rabbit anti-G6PD serum) | Ratio of HF/FF equivalence points |
|---|---|---|---|
| 0% Fat (FF) | 1 | 104 | |
| | 2 | 148 | |
| | 3 | 90 | |
| | 4 | 98 | |
| 35% Fat (HF) | 1 | 220 | 2 |
| | 2 | 128 | 1 |
| | 3 | 170 | 2 |
| | 4 | 110 | 1 |

[a] Enzyme, antibody, and buffer were mixed in siliconized tubes, heated at 37°C for 30 min, and then cooled in ice. Enzyme activity was determined 2 days later.

from rats fed the FF diet. The prior effect of feeding the HF diet was reversible by feeding the FF diet both in *ad libitum*-fed rats (group 6) as well as in starved–refed rats (groups 4, 5).

**Discussion.** Our data suggest that either part or all of the G6PD isolated from rats refed an HF diet is less stable than the enzyme isolated from rats refed a FF diet. The interpretation of Fig. 2 is that either part of the enzyme is unstable (and disappears in 2–4 days of storage) or that during this time a destabilizing factor disappears from the solution. In experiments subsequent to that depicted in Fig. 2, the loss of activity never stopped, which would imply that rats fed the HF diet have a less stable G6PD.

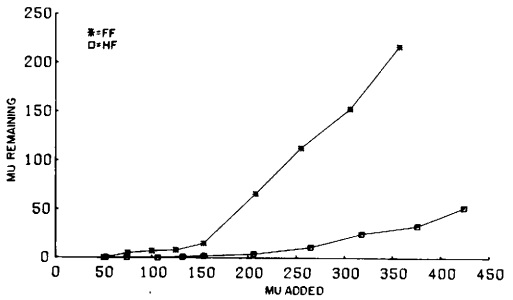


FIG. 2. Stability of DEAE effluents. Immunotitration of rat liver glucose-6-phosphate dehydrogenase following serial elution from DEAE-cellulose. All vials contained 100 μl reconstituted rabbit serum in buffer and 0.1 *M* $MgCl_2$, DEAE eluant (enzyme), and buffer with 0.1 *M* $MgCl_2$. This mixture was heated for 1/2 hr at 37°C, cooled in ice, kept at 4°C for 2 days, and assayed for enzyme activity. Diets: fat-free diet (FF); high-fat diet (HF).

Other workers inferred the existence of an NADP removing enzyme which can destabilize G6PD activity (27–29). In our work we could not confirm or deny this hypothesis. In some (but not all) of our DEAE eluants added NADP was found to stabilize the enzyme from the animal refed the HF diet, but only when the enzyme was briefly heated with NADP. It is difficult to assess the meaning of this in the absence of further work. Mixing experiments with HF and FF eluants containing G6PD indicated that enzyme activity disappeared during cold storage as if each eluant lost activity independently of the other. This observation would argue against a destabilizing factor in the eluant. Diet reversal studies indicate that whatever mechanism may be responsible

TABLE III. RATE OF DISAPPEARANCE OF G6PD ACTIVITY IN STARVED–REFED RATS[a]

| Diet refed | Disappearance of activity (% per 100 hr) |
|---|---|
| FF | 11.5 |
| HF | 34.0 |
| HF + FF (equal volumes) | 20.1 |

[a] Rats were starved and refed as described under Materials and Methods. "Enzyme" refers to the 0.1 *M* $MgCl_2$ eluant from a DEAE column.

for causing the enzyme instability *in vitro* it is reversible by diet *in vivo*. Thus, in rats refed the HF diet for 2 days and then the FF diet for 4 days liver G6PD in the DEAE eluant was more stable than the enzyme isolated from rats fed the HF diet. Likewise, in rats fed *ad libitum* (4 days) the FF diet G6PD was stable in the DEAE eluant, whereas the enzyme prepared from rats fed the HF diet was unstable.

The difference in stability of G6PD activity from rats fed the HF or FF diet might explain in part the results obtained by Hizi and Yagil (22) and Kelly *et al.* (21). It is very likely that enzyme prepared from rats fed the HF diet would lose more activity than predicted from the amount of antibody added. This could be interpreted in various ways depending on the experimental protocol used. When this nonspecific precipitation is eliminated by storage and/or heating, the remaining enzyme is the more stable variety and activity removed by the antibody is proportional to protein removed by the antibody. However, if a relatively fresh homogenate is used (21, 22) a greater amount of enzyme protein is precipitated than is expected on the basis of the enzyme activity removed. It should be emphasized that this discrepancy occurs only with rats fed an HF diet; and, in fact, antibody precipitation of rats fed chow or other high-carbohydrate diets gives the expected results (18–20, 25).

Whether this explanation of different findings regarding the nature of induction of G6PD is correct must await further experiments from the laboratories in question. In recent work (C. S. Nace and B. Szepesi, unpublished data), antibody precipitation of G6PD, obtained from HF-fed animals and treated to remove nonspecifically precipitable protein, showed a pattern of many fast peaks upon SDS–disk gel electrophoresis, indicating the possible presence of breakdown fragments. Such fast-moving material was almost absent in rats fed the FF diet. These observations would suggest that antibody precipitable material (i.e., enzyme) may be overestimated in rats fed the HF diet if the antibody is added to fresh homogenate (even if partially purified) and in particular, when the homogenate–antibody mix is heated. In experiments where antibody was added without heating, the equivalence point was 90–100 mU activity removed/100 μl immunized serum for both HF- and FF-fed rats.

TABLE IV. HEAT STABILITY OF DEAE ELUANTS[a]

| Diet refed | Percentage activity lost during heating for 15 min | | |
|---|---|---|---|
| | 40°C[b] | 45°C | 50°C |
| 0% Fat (FF) | 6.5 | 13.1 | 19.9 |
| 5% Fat (65G) | 15.7 | 27.9 | 31.3 |
| 35% Fat (HF) | 29.3 | 35.1 | 52.8 |

| | Heat stability of DEAE eluants in 1 m*M* NADP | |
|---|---|---|
| | 1 m*M* NADP added | Percentage activity lost during heating for 20 min at 45°C |
| 0% Fat (FF) | No | 30 |
| 0% Fat (FF) | Yes | 0 |
| 35% Fat (HF) | No | 58 |
| 35% Fat (HF) | Yes | 19 |

*Note.* For the complete diet composition and experimental protocol, see Materials and Methods.

[a] Recovery from DEAE was between 100 and 110%.

[b] Incubation temperature.

TABLE V. REVERSIBILITY OF IN VITRO INSTABILITY OF LIVER G6PD BY DIET[a]

| Group | Treatment[b] | Percentage loss after 350 hr storage at 0–5°C[c] |
|---|---|---|
| 1 | 4 (HF) | 79 |
| 2 | 2*, 2 (HF) | 75 |
| 3 | 2*, 2 (FF) | 43 |
| 4 | 2*, 2 (HF), 2 (FF) | 40 |
| 5 | 2*, 2 (HF), 4 (FF) | 26 |
| 6 | 4 (HF), 4 (FF) | 35 |
| 7 | 4 (FF) | 36 |

[a] Diet formulations are described fully under Materials and Methods. HF, high-fat diet; FF, fat-free diet.

[b] Numbers depict day of treatment, letters in parentheses the diet fed. Starvation is denoted by an asterisk.

[c] Livers were homogenized, the homogenate centrifuged, the clear supernatant was applied to a DEAE column and eluted serially with 0.025 *M* $MgCl_2$ and 0.1 *M* $MgCl_2$. G6PD was eluted in the 0.1 *M* $MgCl_2$ fraction. In all treatments, the same amount of liver was homogenized and the same amount of 0.1 *M* $MgCl_2$ was used for the final elution.

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