Effect of Food Restriction on Metabolic Alterations in "Control Animals" Used in Studies on Biotin-deficient Rats [1,2]

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ABSTRACT Different types of "control animals" used in investigations on biotin-deficient rats have been studied. The effect of restricted food intake (10 g), offered as one, two, or four equal meals a day, on metabolic patterns of control animals was investigated. Although the rate of glycogen synthesis in livers of control animals was not altered, it was considerably greater in diaphragms of animals given a restricted amount of food than in animals fed ad libitum. This increase was even more significant in animals fed multiple meals. The synthesis in vitro of fatty acids from labeled glucose or acetate by adipose tissues from animals given a restricted amount of food as a single meal was greatly increased compared with animals fed ad libitum. This increase was less marked in animals given multiple meals. The present study shows that metabolic alterations are induced in control animals as a consequence of food restriction. In view of these findings the noncritical use of "pair-fed," "pair-weighed" and "trained-fed" animals as "controls" in nutritional studies could lead to erroneous conclusions.

It is known that biotin deficiency results in a marked reduction in food intake of animals. To restrict food intake of control animals, pair-feeding (1, 2), pair-weighing (3, 4) and trained-feeding (4) techniques have been employed. Recently, we (5) observed systematic oscillations in body weight, liver weight, the level of hepatic glycogen and spontaneous activity of control animals given a restricted amount of food (10 g) as a single meal or as multiple meals a day. In view of these systematic oscillations the effect of food restriction on the synthesis in vivo of glycogen, and the synthesis in vitro of fatty acids in various tissues of control rats, has been studied.

METHODS AND MATERIALS

Animals. Nineteen-day-old weanling male rats of the Sprague-Dawley strain weighing 32 to 38 g were used. The animals were housed individually in metal cages with raised wire screens in a temperature-regulated room. Biotin deficiency was produced by feeding ad libitum a basal diet (6) containing 71% glucose, 20% spray-dried egg white, 4% corn oil and all the other nutrients; biotin was not added to the diet. The control animals were cured of biotin deficiency by injecting intramuscularly 100 μg biotin in physiological saline three times a week during the 2-week curative period. During this treatment the visible symptoms of biotin deficiency progressively disappeared. Water was available to animals at all times.

Experimental design. Sixty deficient animals, after being fed the basal diet for 5 weeks, were divided into five groups of 12 animals/group, as shown in table 1. The curative treatment and various feeding schedules were carried out for 2 weeks. Animals fed 10 g once (group 3), 5 g twice (group 4) and 2.5 g four times (group 5) a day, completely ate their individual offering in about 6 hours, 30 and 15 minutes, respectively, as reported in our earlier study (5). These animals (groups 3 to 5) consumed their total food allotment of 10 g every day during these 2 weeks. The average food intake of deficient animals fed ad libitum (group 1) and control (group 2) animals in week 2 of the treatment was about 10 and 17 g, respectively.

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TABLE 1
Experimental design [1]

Group	Biotin status	Feeding pattern
1	Deficient	Ad libitum
2	Cured-normal	Ad libitum
3	Cured-normal	Restricted food intake, 10 g once a day (10 g × 1)
4	Cured-normal	Restricted food intake, 5 g twice a day at 12-hour intervals (5 g × 2)
5	Cured-normal	Restricted food intake, 2.5 g four times a day at 6-hour intervals (2.5 g × 4)

[1] Sixty animals were fed ad libitum an egg-white diet for the first 5 weeks of the experiment. The animals were then grouped (12 in each group) and fed the same diet for another 2 weeks as indicated above. Animals in control groups were cured of biotin deficiency.

On the last day of the treatment the control animals in groups 3, 4 and 5 were given 2 g of food at the zero hour (12 noon), which was consumed in about 15 minutes. This procedure brought the animals (groups 3 to 5) to the same nutritional status. At this time food cups were removed from animals in groups 1 and 2. All animals were then fasted for 24 hours, but water was available at all times. At the end of the fast, six animals from each group were decapitated and exsanguinated; the glycogen in livers and diaphragms was determined as described below. Adipose tissues from control animals (groups 2 to 5) were used to study in vitro synthesis of fatty acids as described below. The remaining six animals from each group (groups 1 to 5) received, intragastrically, 5 mmoles glucose containing 1.5 μCi labeled glucose [4] in a 3-ml solution per animal. After 3 hours the animals were killed, and the radioactivity and amount of glycogen in livers and diaphragms were determined.

Determination of glycogen. Immediately after decapitation about 300 mg liver and 100 mg diaphragm were removed; they were quickly weighed on a torsion balance,[5] transferred to tubes containing 2 ml of hot 30% KOH and digested in a boiling-water bath for 30 minutes. Glycogen was coprecipitated with Na_2SO_4 from the KOH digest using 95% ethanol (7), and the precipitate was washed once with 65% ethanol, as suggested by Fong et al. (8). The precipitate containing the radioactivity was dissolved in water and reprecipitated with 95% ethanol. After repeating this treatment, the precipitate was dissolved in water and glycogen was estimated by the anthrone method (9). Radioactivity was determined by counting an aliquot with 15 ml of Bray's scintillation solution (10) in a scintillation counter [6] and a counting efficiency of about 65%.

In vitro synthesis of fatty acids. As mentioned above, after the 24-hour fast six animals from groups 2 to 5 (table 1) were decapitated, and the epididymal adipose tissues were rapidly removed; one fat pad was used for studying fatty acid synthesis from glucose; the other pad was used for the incorporation of acetate into fatty acids. About 100 mg of the tissue were weighed on a torsion balance and immediately transferred to a 25-ml Erlenmeyer flask containing 3 ml of calcium-free Krebs-Ringer biocarbonate buffer, pH 7.4 (11). The buffer contained per milliliter: a) 5 μmoles glucose-U-^{14}C (0.180 μCi) and 0.1 unit of insulin [7]; or b) 10 μmoles labeled acetate [8] (0.179 μCi), 5 μmoles of glucose and 0.1 unit of insulin. Glucose and insulin are required for optimal synthesis of fatty acids from labeled acetate, or pyruvate-2-^{14}C, or pyruvate-3-^{14}C by adipose tissue (12). The levels employed were the same as used in earlier studies (12, 13).

Each flask was gassed with 95% O_2–5% CO_2 and sealed with a rubber serum stopper having a hanging polyethylene well which contained a 2 cm by 2 cm piece of Whatman no. 1 filter paper. The flasks were then shaken in a reciprocating water bath (90 strokes/minute) at 38° for 3 hours. At the end of the incubation period, through the rubber stopper, 0.1 ml of 25% KOH was introduced with a syringe and needle onto the filter paper; similarly, 0.5 ml of 0.2 N H_2SO_4 was added to the incubation medium to stop the reaction and to release CO_2. To ensure complete liberation and trapping of CO_2, shaking was continued for another 30 minutes. The filter

[4] Glucose-U-^{14}C (specific activity 3.83 mCi/mmole), New England Nuclear Corporation, Boston, Mass.
[5] Roller-Smith Precision Balances, Newark, N. J.
[6] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.
[7] Crystalline pork insulin, Eli Lilly and Company, Indianapolis, Ind.
[8] Acetate-U-^{14}C (specific activity 28.2 mCi/mmole), Nuclear-Chicago Corporation, Des Plaines, Ill.

paper containing trapped $^{14}CO_2$ was transferred to a counting vial and allowed to dry; the paper was flattened, and 10 ml of toluene scintillation solution (13) was added and counted in a scintillation counter [9] as described by Buhler (14). The overall efficiency of the method was calculated to be 24% using the buffer containing $NaH^{14}CO_3$ (13).

After incubation the adipose tissue was treated essentially as described by Cahill et al. (15). The tissue was rinsed three times in physiological saline and transferred to a tube containing 15 ml chloroform–methanol solution (2:1, v/v), and the lipids were extracted with constant shaking for 6 hours. To remove radioactive contamination of nonlipid material, the lipid extract was washed with 4 ml of "salty wash" and then with 7.5 ml of "salty wash upper phase" (16). After adding two or three boiling chips, the solvent was evaporated to dryness in a water bath at 60°.

The lipids were saponified by refluxing in 10 ml of 3% methanolic KOH at 80 to 85° for 45 minutes. The tubes were allowed to cool and 10 ml water was added to each tube. The nonsaponifiable lipids were removed by three 5-ml extractions with petroleum ether (BP 30 to 60°). The saponified lipid fraction was acidified with concentrated HCl (tested with Congo red test paper). The fatty acids were extracted with three 5-ml portions of petroleum ether, and each portion was transferred to a scintillation vial. The combined extract was evaporated under a stream of O_2-free N_2. The fatty acids were dissolved in 10 ml of toluene scintillation solution (13), and radioactivity was determined in a scintillation counter [10] and a counting efficiency of 70%.

RESULTS

The effect of various feeding patterns on the synthesis in vivo of glycogen in livers of control animals is shown in table 2. Restricted feeding, as a single meal or multiple meals (groups 3 to 5), had no effect on the repletion of hepatic glycogen compared with ad libitum feeding (group 2). The incorporation of labeled glucose into glycogen supported this finding. A very marked reduction in the repletion of glycogen in livers of biotin-deficient rats was observed.

In contrast to the finding in the liver, a marked increase in glycogen repletion was observed in the diaphragm of animals given a restricted amount of food (table 3, groups 3 to 5) compared with animals fed ad libitum (group 2). Furthermore, this increase was most marked in animals given two or four meals a day compared with animals receiving a single meal (compare groups 4 and 5 with group 3). The radioactivity incorporated into glycogen correlated with these observations. A marked reduction in the repletion of glycogen in diaphragms of biotin-deficient animals was observed.

The effect of feeding patterns on the synthesis in vitro of fatty acids from labeled glucose and its oxidation to $^{14}CO_2$ is shown in table 4. Biotin-deficient animals were not used in these studies because under our experimental conditions they had hardly any subcutaneous or epididymal fat. Glu-

[9] See footnote 6.
[10] See footnote 6.

TABLE 2

Effect of feeding patterns on glycogen synthesis in livers of biotin-deficient and control animals [1]

Group	Biotin status	Feeding pattern	Glycogen per gram liver after hours				P
			0	3	Difference	3	
			mg	*mg*	*mg*	*dpm × 10⁻³*	
1	Deficient	Ad libitum	0.1 ± 0.02 [2]	1.9 ± 1.1	1.8	7 ± 6	< 0.001
2	Cured-normal	Ad libitum	8.4 ± 2.0	31.1 ± 3.0	22.7	87 ± 6.6	
3	Cured-normal	10 g × 1	0.4 ± 0.1	28.3 ± 4.8	27.9	89 ± 16.3	ns [3]
4	Cured-normal	5 g × 2	1.5 ± 0.3	23.3 ± 1.3	21.8	82 ± 5.2	ns
5	Cured-normal	2.5 g × 4	1.2 ± 0.2	29.8 ± 1.2	28.6	105 ± 5.2	ns

[1] The animals were fasted for 24 hours and given 5 mmoles (1.5 μCi) of labeled glucose in 3 ml by stomach tube.
[2] Each result is the mean ± SE of the mean of six animals.
[3] ns = not significant.

cose oxidation and incorporation into fatty acids was markedly greater in adipose tissues of control animals given restricted amount of food (table 4, groups 3 to 5) than in animals fed ad libitum (group 2). This increase appeared to be more in animals fed a single meal (group 3) than in animals fed multiple meals (groups 4 and 5). As seen in table 5, when labeled acetate instead of glucose was used, similar results were obtained except that this increase was even more marked in animals fed a single

TABLE 3

Effect of feeding patterns on glycogen synthesis in diaphragms of biotin-deficient and control animals [1]

Group	Biotin status	Feeding pattern	Glycogen per gram diaphragm after hours				P
			0	3	Difference	3	
			mg	*mg*	*mg*	*dpm × 10⁻³*	
1	Deficient	Ad libitum	0.3 ± 0.1 [2]	0.8 ± 0.3	0.5	3 ± 1.7	< 0.01
2	Cured-normal	Ad libitum	1.2 ± 0.1	5.1 ± 0.5	3.9	13 ± 1.2	
3	Cured-normal	10 g × 1	0.4 ± 0.1	5.9 ± 0.3	5.5	20 ± 1.2	< 0.01
4	Cured-normal	5 g × 2	0.6 ± 0.1	7.2 ± 1.0	6.6	29 ± 4.1	< 0.01
5	Cured-normal	2.5 g × 4	0.7 ± 0.1	7.9 ± 0.4	7.2	30 ± 2.2	< 0.001
3 and 4							< 0.05
3 and 5							< 0.01
4 and 5							ns [3]

[1] The animals were fasted for 24 hours and given 5 mmoles (1.5 μCi) of labeled glucose in 3 ml by stomach tube.
[2] Each result is the mean ± SE of the mean of six animals.
[3] ns = not significant.

TABLE 4

Effect of feeding patterns on the synthesis in vitro of fatty acids from glucose-U-^{14}C and its oxidation to $^{14}CO_2$ by adipose tissues of control animals

Group	Biotin status	Feeding pattern	Glucose-U-^{14}C [1] converted to			
			$^{14}CO_2$	P	Fatty acids	P
			mμmoles of glucose converted/100 mg tissue in 3 hr			
2	Cured-normal	Ad libitum	708 ± 60 [2]		1019 ± 163	
3	Cured-normal	10 g × 1	1897 ± 309	< 0.01	3181 ± 579	< 0.01
4	Cured-normal	5 g × 2	1201 ± 151	< 0.02	2028 ± 364	< 0.05
5	Cured-normal	2.5 g × 4	1122 ± 84	< 0.01	1831 ± 260	< 0.05
3 and 4				ns [3]		ns
3 and 5				< 0.05		ns
4 and 5				ns		ns

[1] The buffer contained per milliliter: 5 μmoles (0.180 μCi) labeled glucose and 0.1 unit insulin.
[2] Each result is the mean ± SE of the mean of six animals.
[3] ns = not significant.

TABLE 5

Effect of feeding patterns on the synthesis in vitro of fatty acids from acetate-1,2-^{14}C and its oxidation to $^{14}CO_2$ by adipose tissues of controls animals

Group	Biotin status	Feeding pattern	Acetate-1,2-^{14}C [1] converted to			
			$^{14}CO_2$	P	Fatty acids	P
			mμmoles of acetate converted/100 mg tissue in 3 hr			
2	Cured-normal	Ad libitum	751 ± 78 [2]		879 ± 79	
3	Cured-normal	10 g × 1	2238 ± 297	< 0.001	4440 ± 317	< 0.001
4	Cured-normal	5 g × 2	1303 ± 115	< 0.01	3124 ± 295	< 0.001
5	Cured-normal	2.5 g × 4	1354 ± 227	< 0.05	2454 ± 227	< 0.001
3 and 4				< 0.02		< 0.02
3 and 5				< 0.05		< 0.001
4 and 5				ns [3]		ns

[1] The buffer contained per milliliter: 10 μmoles (0.179 μCi) sodium acetate, 5 μmoles glucose and 0.1 unit insulin.
[2] Each result is the mean ± SE of the mean of six animals.
[3] ns = not significant.

meal (group 3) than in animals fed multiple meals (groups 4 and 5).

DISCUSSION

To overcome differences in food intake between biotin-deficient and control animals, pair-feeding (1, 2), pair-weighing (3, 4) and trained-feeding (4) techniques have been used. Patel and Mistry (5) observed systematic oscillations in the steady-state level of hepatic glycogen of control animals given a restricted amount of food (10 g) as a single meal a day. Control animals, however, given four equal meals 6 hours apart were able to maintain the level of hepatic glycogen in the range observed in animals fed ad libitum (5). This could be a result of increased synthesis or decreased breakdown of hepatic glycogen. Since the rate of glycogen synthesis in the liver was not altered in animals fed multiple meals (table 2, groups 4 and 5), the effect observed in the earlier study (5) could have been a result of decreased breakdown of glycogen. Also, no change in glycogen synthesis in the liver was observed in animals given a single meal (table 2, group 3).

In contrast to the findings in the liver, glycogen synthesis was markedly increased in diaphragms of control animals given a restricted amount of food as a single meal compared with animals fed ad libitum (table 3, group 2 compared with group 3). A similar increase in the diaphragms of meal-fed rats compared to nibbling animals has been observed by Leveille and Chakrabarty (17). The significance of this finding in relation to energy storage has been discussed by these authors. Furthermore, this increase was even more marked in animals given multiple meals than in animals given a single meal (table 3, groups 4 and 5 compared with group 3). It is possible that the avoidance of a prolonged postabsorptive state between two successive meals, and the rapid influx of a substantial amount of dietary glucose at regular intervals result in the maintenance of a high level of glycogen synthetase activity, the rate-limiting enzyme, on the pathway of glycogen synthesis.

A similar increase in lipogenesis also was observed in animals given a restricted amount of food when compared with animals fed ad libitum (tables 4 and 5, groups 3, 4 and 5 compared with group 2). As mentioned earlier, these results are maximal responses obtained under optimal conditions of fatty acid synthesis by the adipose tissue. Whether the criterion was fatty acid synthesis or CO_2 production from labeled substrates such as glucose or acetate, the results were similar. The enhanced lipogenic capacity of adipose tissues from meal-fed rats compared with nibbling animals has been observed by many investigators (18–21). The significance of this finding in meal-fed rats in terms of energy storage has been discussed by Leveille and Hanson (20). This would also explain the increase in lipogenesis induced in the adipose tissue of control animals fed a single meal. It is of interest to note, however, that in the present study lipogenesis was greater in control animals even after feeding four meals a day than in animals fed ad libitum. The explanation offered above for the increased synthesis of glycogen in diaphragms of animals fed multiple meals would also explain this observation.

The usefulness and limitations of the pair-feeding technique have been discussed by Mitchell (22). Very little is known about metabolic alternations induced, by pair-feeding or restricting food intake, in rats used as controls in studies on biotin deficiency; however, in the field of nutrition some scattered observations have been reported. In a study on the effect of pantothenic acid, protein, and calorie intake on the respiration of duodenal mucosa of the rat, Vitale et al. (23) pointed out that the pair-feeding technique might not be an adequate or satisfactory method of separating the metabolic effects of nutritional deficiency from the associated inanition which sometimes accompanies it. Castor and Armstrong (24), in studying the effect of X-irradiation on loss of potassium from rats, cautioned that the noncritical use of the pair-feeding technique might constitute a distinct hazard in the interpretation of the data. They suggested an alternate approach, namely, to use the animal fed ad libitum as a standard for comparison, and to express the effects of the treatment as deviations from the control state. In studying the response of lipogenesis to repletion of pyridoxine in deficient rats, Radhakrishnamurty et al. (25) reported

marked differences in the incorporation of labeled acetate into fatty acids by adipose tissues from animals fed ad libitum and pair-fed animals.

In the light of marked metabolic alterations observed in animals fed a restricted amount of food given as a single meal, the noncritical use of "pair-fed," "pair-weighed" and "trained-fed" rats as "controls" in nutritional studies could lead to erroneous conclusions. In the present study control animals fed a restricted amount of food in four equal meals 6 hours apart were still not comparable to animals fed ad libitum with respect to alterations in metabolism. Whether increasing the number of meals offered to control animals would overcome the limitations of pair-feeding needs further investigation.

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