v15, 457.
7. Dixon, M., Webb, E. C., *Enzymes,* Academic Press, Inc., 1958.
8. Gillis, M. B., Norris, L. C., Heuser, G. F., *J. Nutr.,* 1948, v85, 195.
9. Fiske, G. H., Subbarow, Y., *J. Biol. Chem.,* 1925, v66, 375.
10. Michaelis, M., *Biochem. Z.,* 1931, 139, 234.
11. Gillis, M. B., Norris, L. C., Heuser, G. F., *Poultry Sci.,* 1953, v32, 997.
12. ———, *ibid.,* 1949, v28, 283.
13. Adams, D. H., Whitaker, V. P., *Biochem. Biophys. Acta,* 1950, v4, 543.

Received December 13, 1963. P.S.E.B.M., 1964, v115.

Feeding Frequency: A Factor in Dietary Protein Utilization.* (29115)

CLARENCE COHN, DOROTHY JOSEPH, LOUISEA BELL AND NORMAN A. FRIGERIO
Department of Biochemistry,† Michael Reese Hospital and Medical Center, Chicago, Ill. and Biology Division, Argonne National Laboratories, Lemont, Ill.

With a constant food intake, the rate of ingestion of the diet (periodicity of food consumption or load of nutrients to be handled per unit of time) alters the intermediary metabolism of fat and carbohydrate(1,2,3). With fewer meals, an increased percentage of dietary calories is retained as body fat. The manner in which the body handles dietary protein is likewise subject to the influence of feeding frequency; a decrease in number of daily feedings results in the organism depositing less protein and water and excreting more urinary nitrogen(4). Presumably these changes occur as a result of enzymatic adaptations. The results of the present experiments have yielded data consistent with the concept that there is a limit to the quantity of ingested amino acids that can be handled per unit of time by an organism for anabolic purposes.

Methods. In the 2 experiments to be described, male Holtzman rats, received when 100-120 g in weight, were given a moderate carbohydrate-20% protein-diet(4), either *ad libitum* in solid form or in a distilled water homogenate by stomach tube for one week. Force feeding was carried out before 8:45 A.M. and after 4:15 P.M.; half the day's allotment of nutrients was given on each occasion. A stomach tube, but no food, was passed into the animals with free access to the diet at the same times. This protocol served to accustom to the diet and "tubing" the rats with free access to food and to adapt the force-fed ones to the procedure. Distilled water was freely available at all times to all animals.

In the first experiment, the influence of feeding frequency on urinary excretion of N^{15} after the ingestion of N^{15} labelled protein was evaluated. After the week of adaptation, 3 animals eating *ad libitum* and 3 force-fed ones were individually housed in metabolism cages for 5 days. On the sixth day, each animal with free access to food was given the usual diet into which was incorporated a trace amount of N^{15} enriched yeast protein. On the next 2 days, the basic diet without tracer was fed; the amount of diet consumed by each animal was weighed for each of the 3 days. The twice per day "tubing" procedure was continued during this period. The tube-fed rats were individually pair-force-fed against a control animal a homogenized liquid diet that contained 0.7 g of diet per ml. Urine was collected from all animals daily, from 9 A.M. to 9 A.M. from the force-fed ones and from 2 P.M. until 2 P.M. from those eating *ad libitum.* These periods were chosen because of the different eating times (night *vs* day) of the 2 groups. The urines were analyzed for urea N on the Autoanalyzer(5) and for urea N^{15} by mass spectrometry to an accuracy of 0.1%. The

* Supported by grants from U.S.P.H.S. and N.S.F.
† This department is supported in part by Michael Reese Research Foundation.

TABLE I. Food Intake of Rats Eating *Ad libitum* and Those Pair-Force-Fed Against Them.*†

Day	Rats 1A‡	1F‡	2A‡	2F‡	3A‡	3F‡
1	10.5	10.5	10.9	11.2	12.3	12.6
2	12.7	12.6	13.2	13.3	14.2	14.0
3	12.4	12.6	13.7	13.3	14.6	14.7

* Exp 1.

† Amounts given are in g/day. On day 1, the diet contained 1 mg/g of N^{15} enriched yeast protein.

‡ Rats 1F, 2F and 3F were pair-force-fed against rats 1A, 2A and 3A respectively.

TABLE II. Urea N and Urea N^{15} Excretion by Rats Eating *Ad libitum* and by Those Pair-Force-Fed Against Them.*†

Day		Rats 1A‡	1F‡	2A‡	2F‡	3A‡	3F‡
1	Urea N	27.0	39.0	29.5	37.7	32.0	41.6
	" N^{15}	12.3	33.9	16.3	29.8	12.6	33.3
2	" N	19.7	42.6	25.4	39.4	33.1	37.4
	" N^{15}	6.4	16.7	9.5	7.1	13.3	6.7
3	" N	18.6	35.5	21.7	40.4	31.6	41.8
	" N^{15}	3.2	4.9	7.6	7.2	8.7	6.7
Total for 3 days							
	Urea N (mg)	239	436	299	464	414	520
	Urea N^{15}	21.9	55.5	33.4	44.1	34.6	46.7

* Trace amounts of N^{15} labelled protein were included in the diet on day 1 only.

† Results are expressed as percent of nitrogen or N^{15} ingested and excreted on the particular day.

‡ Rats 1F, 2F and 3F were pair-force-fed against rats 1A, 2A and 3A respectively.

urinary N^{15} was in turn related to the N^{15} consumed.

The second experiment was designed to study the effect of feeding frequency on the activities of 2 hepatic urea cycle enzymes. Rats were force fed or allowed to eat *ad libitum* for 9 days after the period of adaptation. On the day enzymatic activities were evaluated, since the force-fed animals had had no food for 18 hours, both groups of animals were given 5 ml of diet by stomach tube early in the morning and killed 4 hours later. Liver homogenates were made and the activities of the enzymes—arginine synthetase system and of arginase—determined as described by Brown and Cohen(6).

Results and discussion. The food intake of the animals during the 3 day experimental period and the results of the first experiment are given in Tables I and II. The data show that with a similar caloric, dietary protein and N^{15} intake, a decrease in feeding frequency was accompanied by an increase in both urinary urea N and urea N^{15}. From the figures for the dietary intakes and urinary excretions of N^{15} and from the lack of differences in the fecal nitrogen excretions of rats eating *ad libitum* and those force fed(4), it is apparent that a smaller proportion of the ingested isotope is retained by the body as the number of meals/day are reduced. Less of the yeast nitrogen than the regular dietary nitrogen was excreted in the urine on the day the isotope was fed; the reasons for this are unknown. The increased excretion of the isotope by the force-fed animals occurred primarily on the day of its ingestion; on the next 2 days, was more or less similar for the two groups although the urea N output was greater in the tube-fed animals. Thus, the data indicate that on any given day less dietary nitrogen is retained in the body (as protein ?) under forced feeding conditions. Whether this results from an increased turnover of body protein or "shunting" of the ingested amino acids into the Krebs urea cycle is not evident from the data.

The hepatic arginine snythetase system is the rate limiting "enzyme" in the urea cycle (7); enhanced urea formation should be reflected by an increase in activity of the "enzyme." Forced feeding was associated with an increase in activity of the synthetase system but was without appreciable effect on the activity of arginase (Table III). These

TABLE III. Hepatic Urea Cycle Enzyme Activities as Influenced by Feeding Frequency.*†

Manner of feeding	Arginase	Arginine synthetase
Ad libitum (14)‡ (group 1)	19,950 ± 1,480	25.2 ± 4.7
Force fed (16)‡ (group 2)	23,500 ± 1,440	41.9 ± 3.2
Enzyme activities of group 2/group 1	1.18	1.66
t values (group 1 vs group 2)	1.23	2.97
p values (group 1 vs group 2)	>.4	<.01

* Results are means ± S.E. Unit of activity = micromoles ($\times 10^3$) of product formed/mg of protein N in homogenate supernatant/minute.

† Homogenates and assays ere made as described by Brown and Cohen(6).

‡ No. of rats studied.

changes are comparable to those described by Schimke as accompanying an increased protein content of the diet(8). In the N^{15} experiment, results were similar to those showing that an increase in the percentage of dietary protein results in a greater excretion of dietary amino acid N^{15}(9). Thus, it may be seen that using 2 measures of metabolic changes, forced feeding results in metabolic adjustments not unlike those observed with a greater protein intake. Furthermore, the results are in accord with the hypothesis that there is a limit to the magnitude of the load of ingested protein that an animal can handle, per unit of time, for protein synthetic purposes.

The rat was chosen for our experiments because under the usual laboratory conditions it eats its food in small amounts on many occasions, albeit at night. Force feeding permits limitation of the number of periods during which food is ingested as well as allowing for quantitative control of food intake. Previous studies have yielded evidence that the handling the animals receive during this procedure, the resultant gastric distension and the procedure *per se* have no significant influence on body metabolism(4,10). Furthermore, studies in the adrenalectomized animal and in normal rats eating *ad libitum* or force-fed suggest no participation of the adrenal in the metabolic alterations accompanying forced feeding. Moreover, regardless of whether forced feeding is accomplished at night or during the day, the rat responds metabolically in an identical fashion. We can only conclude that the changes in metabolism that accompany this method of food ingestion are a result of the frequency with which the day's allotment of calories is ingested, not the forced feeding *per se*. Aside from the rat, the cow, pig and sheep(4) appear to respond in a similar fashion to the rate of food ingestion.

Man likewise has been studied under conditions correlating protein utilization, protein load and number of feedings. Wu and Wu (11) found that 0.85 g more of nitrogen was retained when 12.33 g of nitrogen were fed 4 times rather than twice daily. When nitrogen intake was reduced to 7.92 g daily, the number of feedings did not influence nitrogen balance. Similarly, young college girls given approximately 63 g of protein per day retained one gram more of the ingested nitrogen when consuming it 3 times per day as compared to twice a day(12). However, if the protein load was reduced to 33 g per day, feeding frequency (*3 vs* 6 times) did not influence nitrogen balance(13).

Summary. Urinary excretion of urea N^{15} and the activities of hepatic urea cycle enzymes were measured in rats eating *ad libitum* and in those pair-force-fed against them. The force-fed animals excreted more of the dietary N^{15} as urea and exhibited a greater hepatic arginine synthetase activity than their controls with free access to food. The results suggest that there is a limit to the magnitude of the load of dietary protein that an organism can utilize per unit of time period for protein synthetic purposes. When the limit is exceeded, "excess" nitrogen is excreted in the urine as enzymatic reactions adapt themselves to disposing of the nonutilized nitrogen.

1. Tepperman, J., Brobeck, J. R., Long, C. N. H., *Yale J. Biol. and Med.,* 1942, v15, 855.

2. Cohn, C., Joseph, D., *Metabolism,* 1960, v9, 492.

3. Fabry, P., Petrasek, R., Kujalova, V., Holeckova, E., *Adaptation to Changed Pattern of Food Intake,* Statni Zdravotnicke Nakladatelstvi, Prague, 1962.

4. Cohn, C., Joseph, D., Bell, L., Oler, A., *Am. J. Physiol.,* 1963, v205, 71.

5. Skeggs, L. T., *Am. J. Clin. Path.,* 1957, v28, 311.

6. Brown, G. W., Jr., Cohen, P. P., *J. Biol. Chem.,* 1959, v234, 1769.

7. Friedland, R. A., Sodikoff, C. H., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 394.

8. Schimke, R. T., *J. Biol. Chem.,* 1962, v237, 459.

9. Sprinson, D. B., in *Use of Isotopes in Biology and Medicine,* Univ. of Wisconsin Press, Madison, 1948, p182.

10. Cohn, C., Joseph, D., Shrago, E., *Metabolism,* 1957, v6, 381.

11. Wu, H., Wu, D. Y., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 78.

12. Leverton, R. M., Gram, M. R., *J. Nutr.,* 1949, v39, 57.

13. Shortridge, L. J., Linkswiler, H., *Fed. Proc.,* 1963, v22, 320.

Received December 16, 1963. P.S.E.B.M., 1964, v115.