Gross Composition and Variation of the Components of Baboon Milk during Natural Lactation [1]

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ABSTRACT The characterization of natural baboon milk was undertaken to provide information on the dietary requirements of infant and lactating female baboons (*Papio anubis, P. cynocephalus and P. papio*). The gross composition of the mature milk (days 36 to 279) was as follows: (in grams per 100 ml) total solids, 14.4; lipids, 5.0; crude protein, 1.6; lactose, 7.3; and ash, 0.26. The specific gravity and pH were 1.027 and 7.18, respectively. The mammary secretion obtained during the first 5 days of lactation was richer in crude protein and poorer in carbohydrates than the mature milk, but the lipid and ash concentrations were not appreciably different. Variations in those components with each other, with length of lactation and with milk yield were also determined; as lactation progressed, the only significant change in composition was the increase in lipid concentration.

Adequate lactation is of critical importance to the maintenance of any mammalian species. The quantity and composition of the milk not only determine all the nutriments which the infant receives, but also reflect the nutritional status of the mother at a time when her requirements are especially stringent. Human lactation has been studied in detail (1), but, despite the extent to which subhuman primates are now being used as models for man in health research (2), very little is known about lactation in any other primate. A few reports of the gross composition of primate milk have been published (3–8); of these, two were concerned with baboon milk. Van Zyl (5) determined the cholesterol, lipid phosphorus, lipid and iodine contents, and Vice et al. (7) determined only the lipid content.

Baboons are among the most used of laboratory primates, because of their many physiological similarities to man (9, 10). It is, therefore, important that their nutritional requirements be known. To better elucidate the requirements of both infant and lactating baboons, we have analyzed baboon milk more completely. This paper reports the gross chemical composition of the milk obtained when lactation was maintained by the suckling of the infant. The variations of the components with the length of lactation, with the size of the sample and with each other are also presented, together with an indication of the changes occurring in the gross composition of the milk in the final stages of lactation.

MATERIALS AND METHODS

Animals. The 26 lactating baboons (*Papio anubis, P. cynocephalus* and *P. papio*) used in this study were fed a diet of a standard biscuit (11) and water, ad libitum. Their infants were all born naturally.

Milk samples. At approximately weekly intervals the baboon to be milked was sedated with 0.75 mg/kg 1-(1-phenylcyclohexyl)piperidine hydrochloride,[2] and the infant was removed. The nipples were prepared as described elsewhere (12) and, approximately 20 minutes later, 20 IU of a purified oxytocic preparation [3] were injected intravenously. The milk flow began almost immediately, and the samples were obtained by gentle manual massage of the mammary tissue and stripping of the teat in one motion with the thumb and forefinger. More than 85% of the samples contained between 3 and 12 ml of milk. To prevent its rejection, the infant was re-

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[2] Sernylan, Parke, Davis and Company, Dallas, Texas.

[3] P.O.P., Armour-Baldwin Labs., Omaha, Nebraska.

TABLE 1
Composition of natural baboon milk

Property or constituent	Days 0–5 (4) [1]	Days 6–11 (4)	Days 12–35 (7)	Days 36–279 (21)	Human milk [2]
Specific gravity	1.027 ± 0.006 [3] (4) [4]	1.024 ± 0.003 (3)	1.027 ± 0.002 (12)	1.027 ± 0.003 (44)	1.031 ± 0.002
pH	6.98 ± 0.15 (3)	7.17 ± 0.16 (4)	7.17 ± 0.19 (11)	7.18 ± 0.21 (42)	7.1 [5]
Total solids, g/100 ml	14.6 (2)	15.3 (2)	14.0 ± 1.5 (12)	14.4 ± 1.6 (44)	12.9 ± 1.1
Lipids, g/100 ml	5.1 (2)	5.8 (2)	4.6 ± 1.2 (12)	5.0 ± 1.8 (43)	4.5 ± 1.0
Crude protein (N × 6.38), g/100 ml	2.3 (2)	1.7 (2)	1.5 ± 0.2 (12)	1.6 ± 0.3 (41)	1.1 ± 0.5
Anhydrous lactose (direct), g/100 ml	6.8 (2)	7.4 (2)	7.7 ± 0.7 (11)	7.3 ± 0.8 (43)	7.1 ± 0.4
Ash, g/100 ml	0.27 (2)	0.25 (2)	0.28 ± 0.06 (11)	0.26 ± 0.09 (43)	0.20 ± 0.02

[1] Numbers of baboons lactating during each period are shown in parentheses.
[2] From Macy, I. G. 1949 Amer. J. Dis. Children, *78:* 589.
[3] Mean ± SD.
[4] Numbers of determinations are shown in parentheses.
[5] From Modde, H., E. Wedler and O. Zacharias 1961 Arch. Gynaekol., *196:* 343.

turned to the mother before she recovered from the sedation.

Some suckling infants were killed for other purposes; milking of their mothers was continued at approximately weekly intervals until the milk supply dried up.

Analytical methods. The specific gravity of the milk was determined at 24° with a micropycnometer (13) and the pH [4] was measured, prior to storage at − 20°. For determinations of the gross chemical composition, the samples were allowed to reach room temperature and were well mixed before portions were removed for analysis.

The total solids and ash were determined by AOAC methods (14), and lipids were determined by the Röse-Gottlieb method (15). Crude protein was determined by the micro-Kjeldahl procedure (16) and calculated as N × 6.38. The carbohydrate content was measured by the Nelson-Somogyi method (17) modified for use with milk by deproteinizing 0.1 ml with 0.9 ml 10% tungstic acid, diluting to 10 ml with water, centrifuging and performing the analysis on 1 ml of the supernatant solution.

Correlation coefficients were calculated between the values obtained for day of lactation, weight of sample, specific gravity, pH, total solids, lipids, crude protein, carbohydrate and ash, using an IBM 1130 computer.

The carbohydrate composition of two samples of mature milk (after 22 and 211 days of lactation) was investigated by a modification of one of the methods of Jenness et al. (18). After precipitation of the protein by addition of 5 volumes of 95% v/v ethanol, the milk was centrifuged. The supernatant solution was diluted with an equal volume of water and deionized by passage through a column of mixed ion-exchange resins [5] and elution with water. The eluate was separated by paper chromatography using ethyl acetate–pyridine–water, 10:4:3, v/v/v (18) and the upper layer of ethyl acetate–pyridine–water, 5:2:7, v/v/v (19) as developing solvents. Carbohydrates were detected

[4] Miniature combination electrode and model LS pH meter, E. H. Sargent and Company, Chicago, Illinois.
[5] Amberlite IR 120 (H^+) and IR 4B (OH^-), Mallinckrodt Chemical Works, St. Louis, Missouri.

by spraying the developed chromatograms with silver nitrate and ammonia (20). The samples were then combined and lyophilized; the resultant solid was twice recrystallized from water to give colorless prisms; and the melting point and infrared spectrum were determined. The melting point and infrared spectrum of authentic lactose monohydrate [6] were also determined, as was the melting point of a mixture of the milk sugar and lactose monohydrate.

RESULTS

The mean values for the major properties and constituents of baboon milk from various lactation periods, their standard deviations and the number of determinations made are shown in table 1. Mean values for mature human milk (21, 22) have been included for comparison.

The sign and significance level [7] of the correlation coefficients calculated between the day of lactation, weight of sample, specific gravity, pH, total solids, lipids, crude protein, lactose and ash are shown in table 2.

Table 3 shows the individual analyses of the milk samples obtained after final removal of the infants; the total length of lactation and the number of days after the suckling stimulus was removed are both shown.

[6] Mann Research Laboratories, Inc., New York, New York.
[7] Diem, K. (editor) 1962 Scientific Tables, ed. 6. Geigy Pharmaceuticals, Ardsley, New York, p. 61.

TABLE 2

Sign and significance [1] of the correlation coefficients between the properties and constituents of natural baboon milk [2]

	Specific gravity	Yield	pH	Total solids	Lipids	Crude protein	Lactose	Ash
Day of lactation	− ns	+ ***	− ns	+ ns	+ **	+ ns	− ns	− ns
Specific gravity		− ns	+ *	− ***	− ***	+ ns	+ ns	+ ns
Yield			− ***	+ ns	+ *	− ns	− ns	− ns
pH				− ns	− **	− ns	+ **	− ns
Total solids					+ ***	+ *	− ns	− ns
Lipids						+ ns	− **	+ ns
Crude protein							− ns	+ **
Lactose								− *

[1] ns = not significant; *** = $P < 0.001$; ** = $P < 0.01$; and * = $P < 0.05$.
[2] From 56 to 63 pairs of values.

TABLE 3

Composition of baboon milk as the supply was allowed to dry up by removal of infant

Property or constituent	Sample designation								
	A	B	C	D	E	F	G	H	I
Days after removal of infant	1	1	9	9	9	14	14	14	14
Total days of lactation	4	64	33	73	200	118	138	149	156
Specific gravity	1.026	1.022	1.032	1.020	1.020	1.022	1.010	1.010	1.010
Yield, g	11.2	5.2	5.2	8.0	12.0	11.8	4.5	5.3	5.3
pH	6.71	7.49	6.98	7.37	7.12	7.32	7.44	7.28	7.40
Total solids, g/100 ml	13.6	16.8	13.1	18.2	25.5	19.5	25.3	29.3	29.0
Lipids, g/100 ml	3.9	7.4	2.8	9.5	13.9	10.4	17.2	20.2	20.3
Crude protein (N × 6.38), g/100 ml	2.3	1.3	3.8	4.0	5.8	1.8	3.2	3.5	2.7
Anhydrous lactose (direct), g/100 ml	6.9	7.9	5.6	4.7	4.2	7.0	4.8	5.3	5.1
Ash, g/100 ml	0.34	0.24	0.58	0.53	0.60	0.20	0.34	0.38	0.29

The only carbohydrate detected in mature baboon milk had a chromatographic mobility identical with that of lactose, with both solvent systems used. That it was lactose was confirmed by its melting point of 210 to 213° (uncorrected). Authentic lactose monohydrate [8] melted at 210 to 214°, and a mixture of the two melted at 210 to 214°. The infrared spectra of the milk sugar and lactose monohydrate were also identical.

DISCUSSION

Natural lactation produced mature baboon milk which was more concentrated than mature human milk. It contained more lipids and lactose, and considerably more crude protein and ash than human milk; the pH, however, was similar in the two types of milk. No differences were found between the values from milk obtained from baboons classified as *Papio anubis*, *P. cynocephalus* and *P. papio*. Of the 64 samples analyzed, nine were obtained from one baboon, and eight from each of two other animals. Six samples from each of these animals were analyzed in the 36- to 279-day lactation period. No other baboon contributed more than three samples.

In the human, the mammary secretion during the first 5 days after parturition is considered as colostrum, and during the next 5 days as transitional milk (23). The composition of the milk changes most rapidly through this period, and some color change also occurs. Samples of baboon milk taken 1 day after parturition were the same color, white, with a very slight yellow cast, as those taken several months later and, because of the probability of rejection of the infant after too early a sedation of the mother, an insufficient number of early milk samples was obtained for analysis to indicate whether colostrum was produced for similar periods in the two species. The protein concentration was considerably higher, and the lactose concentration was lower, in this early milk; this is characteristic of human colostrum, but neither the fat nor ash concentrations changed appreciably. No clarification was achieved by considering the relative amounts of each component in the total solids.

As lactation progressed, both the sample size and the lipid concentration in these samples increased considerably. There resulted a small positive correlation ($P < 0.05$) between the sample size and the lipid concentration, in contrast to the negative correlation reported between the sample size and lipid content of human milk [9] (24). The pH decreased markedly with a larger sample, but did not significantly change as lactation progressed. The pH was also correlated with the lipid and carbohydrate contents.

The total solids were extremely dependent on the lipid content, and the specific gravity was in turn lowered by increases in these. The lack of correlation between the lipids and protein indicated that only a small portion of either could be in the form of lipoprotein, but there was a significant positive correlation between the protein and the ash. Significant negative correlations were found between the carbohydrates and the lipid, protein and ash. Somewhat different correlations were found in a detailed study of the milk of Japanese women.[10]

The milk used in the present study was assumed to be representative of normal baboon milk, despite the necessity of sedating the animals to remove the infant from the breast and obtain the milk. Unless oxytocin was also used, only 1 to 2 ml of milk could be expressed. In the cat and rabbit, anesthesia lowered the milk yield, but the yield could be returned to normal by the use of oxytocin (25, 26); the effect on the milk composition was not, however, reported. The low yield of baboon milk was to be expected from a primate whose infants suckle almost continuously; van Zyl [11] has milked unsedated baboons without the use of oxytocin and was able to obtain only about 6 ml of milk; and van Wagenen et al. (4) could obtain only similar quantities from rhesus monkeys. The trauma caused by such a procedure, or by allowing the milk to accumulate in the breast for several hours, would be unphys-

[8] See footnote 6.
[9] Saito, K., E. Furuichi, S. Kondo, G. Kawanishi, I. Nishikawa, H. Nakazato, Y. Noguchi, T. Doi, A. Noguchi and S. Shingo 1965 Studies on human milk. Reports of Research Laboratory, no. 69, Snow Brand Milk Products Company Ltd., Tokyo, Japan.
[10] See footnote 9.
[11] Van Zyl, A., personal communication.

iological and might also cause changes in the milk composition.

The concentration of lipids in the milk was found to be approximately 5 g/100 ml, and only increased gradually as lactation progressed (the lipid concentration of the seven samples taken after more than 200 days of lactation was 6.1 ± 2.1 g/100 ml). The baboon milk obtained by van Zyl (5), however, had a lipid concentration of 10.6 ± 2.0%. The mean of the values he obtained during the first 3 months of lactation was 3.8%, but the concentration increased very rapidly thereafter. Several samples contained more than 15% lipids, a concentration not normally found with species which nurse frequently (6). It is probable that the infants of those baboons were partially weaned, since the results in table 3 show that as the milk supply was allowed to dry up, the lipid content could rise as high as 20 g/100 ml. The milk lipid concentration found in the present study was also much closer to the values reported for the human (21) and other primates, namely orangutan (3.5%) (3), rhesus macaque (3.9%) (4), chimpanzee (3.7%) (6) and gorilla (2.17%) (6).

As the milk supply dried up after the complete removal of the infant, the protein and ash concentrations also increased and the lactose decreased. These changes in composition appeared to occur more quickly the longer the previous lactation. With a total solids content of nearly 30 g/100 ml, the milk became extremely viscous.

The concentrations of these other components of the baboon milk used in this study were also close to those of human milk (21), and to those reported for other primates (3, 4, 6), except for the prosimian *Galago crassicaudatus* (8).

The values obtained not only serve as a base line for studies of the nutrition of infant and female baboons, but are also prerequisite for the preparation of substitute milks for the more successful raising of infant baboons away from their mothers.

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Gross Composition and Variation of the Components of Baboon Milk during Artificially Stimulated Lactation [1]

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ABSTRACT For a study of the early nutritional requirements of baboons (*Papio anubis, P. cynocephalus* and *P. papio*), lactation was induced and maintained by treatment with oxytocin and repeated milking after the removal of their infants by cesarean section. The gross composition of this milk (from days 11 to 35) was as follows: (in grams per 100 ml) total solids, 14.3; lipids, 4.8; crude protein, 2.0; lactose, 7.0; and ash, 0.30. The specific gravity and pH were 1.027 and 7.44, respectively. The secretion obtained during the first 36 hours was richer than the later milk in crude protein and ash, and poorer in lipids and lactose. Variations of these constituents and properties with each other, with the sample weight and with the length of lactation were determined. As lactation progressed, the pH increased significantly and the concentrations of lipids and crude protein decreased.

As part of a program to maintain and study baboons in captivity, it was necessary to raise infant baboons away from their mothers. Some of these were born naturally, but most were delivered by cesarean section under germfree conditions approximately 2 weeks before full term. These infants have been raised on a commercial human milk formula, modified on the basis of a partial analysis of milk from baboons whose lactation was induced and maintained in the absence of their infants by administration of exogenous oxytocin (1).

Because of the marked disparity between the lipid content of this milk and that reported for other simian primates (2–4) and for humans (5), it was decided to study baboon lactation in more detail. The composition of milk obtained from baboons whose infants were removed by cesarean section and who were caused to lactate by frequent milking after oxytocin administration, and some of the factors affecting this lactation are reported in this paper.

MATERIALS AND METHODS

Animals. The 30 baboons (*Papio anubis, P. cynocephalus* and *P. papio*) used during this study were fed a diet of a standard biscuit (6) and water, ad libitum. Their infants were removed by cesarean section at a conception age of 164 ± 2 days. Full term is 175 ± 11 days (7).

Milk samples. Immediately after the operation, the animals were milked by gentle manual massage of the mammary tissue and stripping of the teat in one motion with the thumb and forefinger, but a sample could rarely be obtained at this stage. Six hours later the animals were sedated with 0.75 mg/kg 1-(1-phenylcyclohexyl) piperidine hydrochloride [2] and injected intravenously with 20 IU of a purified oxytocic preparation [3] immediately before milking. Milking was continued in the same way every 12 hours thereafter, usually by hand, but some samples were taken with a milking machine developed to reduce the trauma inherent in this procedure (8).

Fifteen of the baboons (12 *Papio anubis* and *P. cynocephalus*, and 3 *P. papio*) gave milk in sufficient quantities for chemical analyses to be performed without pooling the samples and for a sufficient time for lactational trends to be established. Since samples containing more than 1 ml could

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[2] Sernylan, Parke, Davis and Company, Dallas, Texas.

[3] P.O.P., Armour-Baldwin Labs., Omaha, Nebraska.

TABLE 1

Composition of artificially stimulated baboon milk

Property or constituent	16-hour pool [1]	16 hours [2]	28 hours	Day 2	Day 3
Specific gravity	—	1.038(3) [3]	1.029 ± 0.006 [4](8)	1.025 ± 0.002(7)	1.025 ± 0.002(6)
Yield, g	—	—	4.6 ± 2.4 (9)	6.8 ± 2.5 (11)	6.4 ± 2.4 (12)
pH	—	—	7.04 ± 0.15 (6)	6.98 ± 0.13 (7)	7.21 ± 0.24 (6)
Total solids, g/100 ml	11.7	15.1 (3)	15.4 ± 1.6 (8)	15.8 ± 2.0 (11)	16.8 ± 2.2 (11)
Lipids, g/100 ml	0.9	5.3 (3)	5.7 ± 1.8 (8)	6.1 ± 2.1 (11)	7.6 ± 2.0 (11)
Crude protein (N × 6.38), g/100 ml	4.85	2.6 (2)	2.6 ± 0.6 (5)	2.4 ± 0.6 (8)	2.4 ± 0.6 (10)
Anhydrous lactose (direct), g/100 ml	4.95	6.5 (4)	7.1 ± 0.3 (7)	7.0 ± 0.6 (10)	6.5 ± 0.7 (12)
Ash, g/100 ml	0.63	0.30 (3)	0.28 ± 0.05 (6)	0.27 ± 0.11 (9)	0.28 ± 0.05 (10)

	Day 4	Day 5	Days 6–10	Days 11–35	Days 36–63
Specific gravity	1.025 ± 0.003(6)	1.026 ± 0.004(7)	1.027 ± 0.003(41)	1.027 ± 0.003(70)	1.029 ± 0.004(14)
Yield, g	6.3 ± 2.2 (9)	8.6 ± 3.3 (11)	7.1 ± 2.9 (48)	5.6 ± 2.4 (82)	4.8 ± 1.7 (19)
pH	7.15 ± 0.23 (6)	7.01 ± 0.22 (7)	7.31 ± 0.20 (41)	7.44 ± 0.19 (69)	7.46 ± 0.17 (14)
Total solids, g/100 ml	16.0 ± 1.9 (9)	15.5 ± 1.8 (10)	15.2 ± 2.3 (45)	14.3 ± 1.8 (82)	13.2 ± 0.6 (19)
Lipids, g/100 ml	6.9 ± 1.8 (8)	6.0 ± 2.2 (10)	5.6 ± 2.1 (44)	4.8 ± 1.5 (82)	4.2 ± 0.6 (19)
Crude protein (N × 6.38), g/100 ml	2.0 ± 0.4 (7)	2.2 ± 0.4 (8)	2.0 ± 0.4 (38)	2.0 ± 0.4 (61)	1.6 ± 0.1 (8)
Anhydrous lactose (direct), g/100 ml	6.8 ± 0.8 (8)	6.6 ± 0.6 (7)	7.1 ± 0.5 (40)	7.0 ± 0.6 (74)	7.6 ± 0.2 (10)
Ash, g/100 ml	0.27 ± 0.05 (9)	0.28 ± 0.04 (10)	0.28 ± 0.05 (36)	0.30 ± 0.70 (69)	0.31 ± 0.04 (14)

[1] Mixture of eight small samples of approximately equal volume; one was taken 6 hours and the others 16 hours after cesarean section.
[2] Mean of individual determinations.
[3] Numbers of determinations are shown in parentheses.
[4] Mean ± SD.

rarely be expressed within 30 hours of the cesarean section, a pool of the early translucent secretion was made from one sample taken 6 hours, and seven samples taken 16 hours after the operation; three larger samples of white milk were also obtained 16 hours after the operation and were analyzed individually. The largest samples of milk were obtained about 5 days after the operation, and contained 5 to 10 ml. Milking was usually suspended when the yield had dropped back to about 1 ml.

Analytical methods. The analyses were performed as reported in a previous paper (9) except that the pH was measured on a different pH-meter.[4]

The gross chemical composition of 195 of the samples obtained was determined completely or almost completely, that is, with seven or more of the nine constituents or properties (day of lactation, weight of sample, specific gravity, pH total solids, lipids, crude protein, carbohydrate and ash) known. Correlation coefficients were calculated for these values, using an IBM 1620 computer.

The carbohydrate composition of the pool of early milk and two samples of more mature (22-day) milk was investigated as described previously (9).

RESULTS

In table 1 are shown the mean values for the major properties and constituents of this baboon milk, their standard deviations and the number of determinations made. The mean values for the same properties and constituents of the secretion obtained during the first days of lactation are also shown.

The signs and significance levels [5] of the correlation coefficients between the day of lactation, weight of sample, specific gravity, pH, total solids, lipids, crude protein, carbohydrate and ash are shown in table 2. In table 3 are summarized the analyses of the milk obtained from day 11 through day 35 of lactation, for each baboon lactating during this period.

The only carbohydrate detected in the earliest and more mature milk samples was lactose, determined as described previously (9).

DISCUSSION

Although the milk was taken under distinctly abnormal conditions, and was highly selected in that only a small proportion of the samples from 15 of the baboons were of sufficient size for analysis, there was a considerable similarity between its gross chemical composition and that of milk obtained under more normal conditions (9). The milk obtained in the present study was slightly richer in crude protein and ash, and slightly poorer in lactose than the natural milk; the only major difference was that the pH of the milk was higher during artificially maintained lactation. The standard deviations of the components were also similar in the two kinds of milk, and no differences were

[4] Model G pH meter, Beckman Instruments, Inc., Fullerton, California.

[5] Diem, K. (editor) 1962 Scientific Tables, ed. 6. Geigy Pharmaceuticals, Ardsley, New York, p. 61.

TABLE 2

Sign and significance [1] of the correlation coefficients between the properties and constituents of artificially stimulated baboon milk [2]

	Specific gravity	Yield	pH	Total solids	Lipids	Crude protein	Lactose	Ash
Day of lactation	+ ns	− **	+ ***	− ***	− ***	− ***	+ *	+ ns
Specific gravity		− *	+ ***	− ***	− ***	+ ***	+ ns	+ ***
Yield			− ***	+ ns	+ *	− ***	+ ns	− ***
pH				− ***	− ***	+ ns	+ ns	+ ***
Total solids					+ ***	+ ns	− ns	− ***
Lipids						− ns	− *	− ***
Crude protein							− ***	+ ***
Lactose								− *

[1] ns = not significant; *** = $P < 0.001$; ** = $P < 0.01$; and * = $P < 0.05$.

[2] From 125 to 195 pairs of values.

TABLE 3

Composition of 11- to 35-day milk from each baboon whose lactation could be maintained into this period

	Baboon no.				
	A 486	A 516	2996	A 448	D 36
Age of baboon,[1] years	9	7	> 10	7	> 10
Parity	⩾ 2	⩾ 2	⩾ 1	⩾ 2	5
Day lactation terminated	11	15	17	22	22
Specific gravity	1.030(1)[2]	1.032 ± 0.003[3](4)	1.027 ± 0.001(8)	1.029 ± 0.002(9)	1.024 (2)
Yield, g	10.8 (1)	6.2 ± 0.9 (4)	8.1 ± 3.0 (8)	4.2 ± 1.1 (12)	4.8 ± 1.3 (3)
pH	7.33 (1)	7.56 ± 0.07 (4)	7.53 ± 0.04 (8)	7.53 ± 0.23 (9)	7.40 (2)
Total solids, g/100 ml	14.8 (1)	13.7 ± 0.8 (4)	15.1 ± 0.7 (8)	13.6 ± 0.6 (12)	15.7 ± 2.3 (3)
Lipids, g/100 ml	4.9 (1)	3.5 ± 0.8 (4)	5.5 ± 0.8 (8)	4.0 ± 0.5 (12)	6.9 ± 2.9 (3)
Crude protein (N × 6.38), g/100 ml	2.5 (1)	2.7 ± 0.2 (4)	1.8 ± 0.1 (8)	2.4 ± 0.2 (6)	1.6 ± 0.1 (3)
Anhydrous lactose (direct), g/100 ml	7.0 (1)	7.0 ± 0.2 (4)	7.3 ± 0.2 (8)	6.7 ± 0.2 (10)	7.4 (2)
Ash, g/100 ml	0.35 (1)	0.40 ± 0.05 (4)	0.27 ± 0.02 (8)	0.37 ± 0.05 (12)	0.24 ± 0.04(3)

	Baboon no.				
	A 581	1805	D 26	A 515	A 481
Age of baboon,[1] years	7	10	8	7	9
Parity	⩾ 2	⩾ 1	3	⩾ 2	⩾ 2
Day lactation terminated	26	31	34	47	63
Specific gravity	1.028 ± 0.002(7)	1.028 ± 0.002(7)	1.028 ± 0.004(12)	1.029 ± 0.002(11)	1.026 ± 0.001(9)
Yield, g	5.0 ± 1.3 (7)	4.1 ± 1.1 (7)	7.1 ± 2.9 (15)	5.2 ± 1.1 (15)	7.4 ± 2.1 (10)
pH	7.34 ± 0.21 (7)	7.40 ± 0.22 (7)	7.45 ± 0.22 (11)	7.45 ± 0.19 (11)	7.28 ± 0.14 (9)
Total solids, g/100 ml	12.6 ± 1.0 (7)	14.9 ± 0.8 (7)	14.3 ± 1.8 (15)	13.5 ± 1.0 (15)	14.7 ± 0.7 (10)
Lipids, g/100 ml	3.8 ± 0.7 (7)	5.1 ± 0.5 (7)	5.2 ± 2.0 (15)	4.0 ± 0.7 (15)	5.8 ± 0.8 (10)
Crude protein (N × 6.38), g/100 ml	2.0 ± 0.2 (4)	2.1 ± 0.1 (3)	1.6 ± 0.1 (12)	2.4 ± 0.3 (10)	1.6 ± 0.1 (10)
Anhydrous lactose (direct), g/100 ml	6.8 ± 0.7 (6)	7.1 ± 0.4 (6)	7.6 ± 0.5 (14)	6.3 ± 0.6 (15)	7.2 ± 0.5 (8)
Ash, g/100 ml	0.32 ± 0.04 (7)	0.30 (2)	0.23 ± 0.02 (13)	0.34 ± 0.03 (9)	0.26 ± 0.02 (10)

[1] Estimated from dentition.
[2] Numbers in parentheses indicate number of determinations.
[3] Mean ± SD.

found between the values obtained from baboons classified as *Papio anubis*, *P. cynocephalus* and *P. papio*.

The earliest secretion was translucent and almost colorless and was replaced by the white milk after about 24 hours of lactation. This precolostrum had a higher specific gravity, was richer in crude protein and ash, and poorer in lipids and lactose than the corresponding mature milk. Similar tendencies are shown during human lactation as colostrum is replaced by mature milk (5), but the time taken is much longer. The lactational trends observed under these conditions may therefore indicate the changes which occur as baboon precolostrum is replaced by colostrum and by milk but, as the timing would be different (10), insufficient evidence has been obtained to determine when this change occurs in baboons under normal conditions.

As lactation progressed further, the samples became smaller. They showed, however, significant[6] decreases in the total solids, lipid and crude protein concentrations, and increases in the lactose concentration and pH with time. With smaller volumes were shown increases in the pH, crude protein and ash, but a decrease in the lipid concentration. This last result, contrasting with the increase found in the lipid concentration of smaller samples of human milk[7] (11) was probably because there was a closer correlation of the lipid concentration with the length of lactation than with the sample size.

The total solids were extremely dependent on the lipid content, and the specific gravity was in turn lowered by increases in these. Most of the other correlations found could be attributed to the mutual changes of the properties and constituents as lactation progressed. The lack of correlation between lipids and protein indicated that only a small portion of either could be in the form of lipoprotein. The negative correlation found between pH and ash was remarkable in that a positive correlation was found between the pH and ash of the milk of Japanese women in a detailed study by Saito et al.[8]

Most of these correlations were similar to those found for natural baboon milk; the most striking differences were those with the length of lactation, which might have been because somewhat different lactation periods were being considered, and with the ash. The mineral content is being further investigated to clarify this difference.

The samples obtained between days 11 and 35 of artificially maintained lactation were considered as representative of the mature milk. There was little change in the composition through this period, but there were considerable differences from one baboon to another. These were not related to the estimated ages of the animals or to the time lactation could be maintained. The two baboons characterized as *Papio papio*, D 26 and D 36, were born and raised in captivity, and their previous pregnancies had been terminated naturally; while the other baboons were captured in Africa as adults, and six of them had had a previous pregnancy terminated by cesarean section. The milk from D 26 and D 36 during the 11 to 35-day lactation period was slightly richer in lactose and poorer in ash than the milk from the other baboons, but during the whole lactation period was not significantly different (t test) in composition.

The repeated administration of the sedative and exogenous oxytocin to obtain milk in the absence of the infant appeared to effect little change on the gross composition of the milk from the normal (9). When human lactation has been induced in the absence of parturition (12), or occurred spontaneously from hyperthyroidism (13), the composition of the milk was not the same as in normal lactation (5). The milk-like fluid obtained from a rat mammary carcinoma (14) after estradiol treatment was also different from normal rat's milk (15). It is, therefore, probable that the induced lactation of the present study gave milk by a mechanism similar to that of normal lactation and that the baboon milk obtained by Vice et al. (1) had an abnormally high lipid concentration because milking was insufficiently

[6] See footnote 5.

[7] Saito, K., E. Furuichi, S. Kondo, G. Kawanishi, I. Nishikawa, H. Nakazato, Y. Noguchi, T. Doi, A. Noguchi and S. Shingo 1965 Studies on human milk. Reports of Research Laboratory, no. 69, Snow Brand Milk Products Company, Ltd., Tokyo, Japan.

[8] See footnote 7.

frequent to maintain adequate lactation (9).

The advantages of milking baboons under these conditions are not only that information may be gained on very early lactation in this primate, but also that many lactation studies requiring frequent milking (see 16) may be performed without harm to the infant from frequent sedation and handling.

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Metabolism of Thiamine and Thiamine Tetrahydrofurfuryl Disulfide to 4-Methylthiazole-5-Acetic Acid in Conventional and Germfree Rats under Various Dosing Conditions [1]

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ABSTRACT The urinary excretion of thiamine and 4-methylthiazole-5-acetic acid was studied in both conventional and germfree rats under various dosing conditions of thiazole-2-^{14}C-labeled thiamine or thiamine tetrahydrofurfuryl disulfide. The urine was fractionated by Amberlite CG-50 chromatography and the thiazole acid was determined by the isotope dilution method. Thiamine, in an oral dose larger than 1.5 mg/rat, was predominantly excreted in the urine after conversion to the thiazole acid, whereas most of the injected thiamine was eliminated unchanged. The thiamine moiety of thiamine tetrahydrofurfuryl disulfide was also converted to the thiazole acid upon oral ingestion but less was excreted as the thiazole acid and more as the unchanged thiamine, comparing with the administration of thiamine. Time course study on the urinary metabolite pattern showed that most of the thiazole acid was excreted during the first 3 hours after oral ingestion of both vitamins. The thiazole acid was also identified as a metabolite of thiamine in germfree rats and there was no essential difference in the urinary metabolite pattern between conventional rats and germfree ones. This finding indicates that intestinal microflora are not primarily responsible for the conversion of thiamine to the thiazole acid in the rat.

Since the pioneering work of McCarthy et al. (1) using labeled thiamine, numerous papers have appeared concerning the metabolic fate of thiamine (2–11). Most of the injected thiamine, particularly in a dose of unphysiological excess, was excreted in the urine as unchanged thiamine (1–6). Recently, Neal and Pearson (7) reported the occurrence of 2-methyl-4-amino-5-pyrimidinecarboxylic acid as a urinary metabolite of pyrimidine-labeled thiamine. Little is known, however, of the chemical nature of the catabolic products derived from the thiazole moiety of the vitamin, although the presence of more than a dozen metabolites has been detected chromatographically (2, 10). In 1963 Takenouchi and Aso (4) indicated that after massive dosing of thiazole-labeled thiamine or thiamine propyl disulfide (TPD),[3] a considerable amount of the administered radioactivity was eliminated into the urine as unknown substances. Among these nonthiamine metabolites the major unidentified metabolite was designated "substance X," and subjected to identification studies but the chemical structure remained unsolved. Meshi and Sato (9) also reported similar results on the urinary metabolites and demonstrated that the metabolic pattern was remarkably affected by route of administration. During the course of our studies on the metabolic fate of thiamine and its alkyl disulfides, we confirmed their findings and identified 4-methylthiazole-5-acetic acid (MTA) as a major constituent of these thiochrome reaction negative products in the urine from the rats fed TPD.[4] Recently, we have estab-

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[3] Thiamine propyl disulfide (TPD) is 2-(2-methyl-4-aminopyrimidin-5-yl)methylformamido-5-hydroxy-2-penten-3-yl propyl disulfide. Thiamine tetrahydrofurfuryl disulfide (TTFD) is the tetrahydrofurfuryl homologue of thiamine propyl disulfide (fig. 3). The detailed descriptions concerning the chemical and biological properties of these compounds may be found in the following literatures. Johnson, B. C. 1955 Ann. Rev. Biochem., *24*: 419; Nutr. Rev., *16*: 336 (1958); Nutr. Rev., *18*: 181 (1960); Kawasaki, C. 1963 Vitamins Hormones, *21*: 69.

[4] Suzuoki, Z., K. Nishikawa, T. Matsuo and H. Tanaka 1965 Urinary metabolites of thiamine propyl disulfide-^{35}S in rats. Vitamins, 32: 128 (abstract).

lished the identity of MTA as thiamine metabolite (12) and further clarified that the urinary excretion of MTA depends on not only the intake level of the vitamin but also on the route of administration. The present report describes the results obtained with both conventional and germfree rats receiving thiamine or thiamine tetrahydrofurfuryl disulfide (TTFD) [5] under various dosing conditions.

MATERIALS AND METHODS

Young adult male rats, including conventional Sprague-Dawley rats, conventional Wistar rats [6] and specific pathogen-free Sprague-Dawley (JCL) rats,[7] were used. The latter were kept in specific pathogen-free quarantine throughout the experiments. The animal rooms were maintained at 24° with controlled humidity. All rats were fed the usual laboratory ration [8] ad libitum. The rats were administered thiazole-2-^{14}C-labeled thiamine hydrochloride or TTFD hydrochloride [9] (usually about 2 μCi dissolved in 0.2 ml of water) in a single dose, orally by stomach tube or parenterally by injection. The rats were then kept individually in stainless steel metabolism cages with screen-bottoms and bottles (containing 2 ml of 1 N hydrochloric acid) for separate collection of feces and urine. The rats, though fasted, were allowed water freely after the administration. The urine was collected at appropriate intervals, adjusted to a pH of 2.5 to 3.5 and stored in the frozen state until the subsequent chemical analyses.

Two germfree rats were used for the comparative studies. The animals were male F_1 adults of the germfree MS-Wistar rats which were established in the Laboratory of Germfree Life Research, Nagoya University School of Medicine. The rats were fed thiazole-^{14}C-labeled thiamine hydrochloride (8.4 mg in 2.1 ml) or TTFD hydrochloride (6.6 mg equivalent calculated as thiamine hydrochloride in 1.6 ml) by stomach tube. The rats were then kept in individual metabolism cages with the collection device of glass sphere type (13) for separating urine and feces.[10] All procedures were carried out inside the germfree plastic isolator.

The materials and methods used for chemical procedures were the same as those reported previously (12, 14, 15), but several points are described below. TPD and TTFD [11] are alkyl disulfide derivatives of the thiol-type thiamine (fig. 3). These compounds are not only better absorbed from the intestinal tract but readily converted to thiamine, causing much higher blood thiamine levels as compared with the administration of thiamine (16–18). Thiamine was determined by a modified procedure of the thiochrome method (19) in which cyanogen bromide was used as the oxidizing agent in place of potassium ferricyanide. The fluorometry was carried out by using a spectrophotofluorometer [12] (excitation wavelength 375 mμ: emission wavelength 430 mμ). These modifications greatly improved the specificity of the fluorometry. The urine samples from the individual rats were subjected to liquid scintillation counting of radioactivity (12) as well as thiamine determination. The samples were then pooled for each group and an aliquot was taken for the subsequent chromatographic examination.

The qualitative and quantitative analyses of MTA in urine were performed by the isotope dilution method. To aliquots (usually 5 to 10 ml) of the urine samples which contained 20,000 to 100,000 cpm of the radioactivity were added 10 to 30 μmoles of the nonradioactive standard of MTA [13] dissolved in methanol. The mixture was then concentrated to about 0.5 ml by a rotary evaporator and subjected to the

[5] See footnote 3.

[6] Bred in Animal Supply Center of Kyoto University and offered for the present studies through the courtesy of Dr. M. Fujiwara of Kyoto University, School of Medicine.

[7] Purchased from CLEA Japan, Inc., Tokyo. The rat is bred as closed colony of the COBS rats of CRCD ® strain, introduced in 1964 from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts.

[8] Mouse laboratory chow, No. CE-2, CLEA Japan, Inc., Tokyo. The thiamine content is about 7 mg/kg.

[9] These radioactive samples generously supplied by Dr. M. Nishikawa and Mr. T. Toga, of the Isotope Laboratory, Takeda Chemical Industries, Ltd., are gratefully acknowledged.

[10] The urine samples were slightly contaminated by small amounts of feces because feces, more viscous and more fluid in germfree rats, adhered to the glass sphere of the separating device.

[11] See footnote 3.

[12] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Inc., Silver Spring, Maryland.

[13] The authentic MTA was synthesized by Drs. H. Hirano and T. Tsujikawa, Chemical Research Laboratories, Takeda Chemical Industries, Ltd., according to the method of Cerecedo, L. R., and J. G. Tolpin 1937 Studies on thiazoles. I. 4-Methylthiazole-5-acetic acid and some of its derivatives. J. Amer. Chem. Soc., 59: 1660. The kind supply of this sample is gratefully acknowledged.

ion-exchange chromatography using Amberlite CG-50 column (12, 14, 15). MTA was readily eluted from the column as fraction II by washing with distilled water, whereas thiamine was not eluted unless the column was treated with 0.1 N hydrochloric acid or 0.1 N perchloric acid (fraction III). A typical elution pattern is illustrated in figure 1. The main portions of fraction II gave an ultraviolet absorption spectrum identical to that of MTA (absorption maximum, 254 mμ; molecular extinction in 0.1 N HCl, 4570) (14) and had a constant specific radioactivity (cpm/E_{254} mμ). These portions were then pooled and evaporated to dryness in vacuo. The residue was dissolved in 0.2 to 0.5 ml of hot water or hot 0.1 N hydrochloric acid solution and allowed to stand. MTA soon crystallized out as needles. The specific radioactivity, measured by weight (cpm/mg) or optical density (cpm/E_{254} mμ), became constant during repeated recrystallization procedures. The amount of MTA in the urine was calculated from the dilution under either one of the following two assumptions. In the experiments with dosages smaller than 1.5 mg/rat, the amount of the original MTA in the urine sample could be neglected as compared with the amount of the added carrier. In the experiments with larger dosages of 5 mg or more, however, the specific radioactivity of the urinary MTA was assumed to be the same as that of the administered label, because the amount of the endogenous MTA can be neglected in comparison with the radioactive MTA formed from the ingested thiamine. These assumptions might introduce, at maximum, 2% of underestimation. The results obtained by the dilution method indicated that about 85 to 90% of the radioactivity found in fraction II was accounted for by MTA at a dose higher than 0.5 mg/rat. In fraction III there was recognized little or no discrepancy between the amounts of thiamine estimated from radioactivity and those determined by the

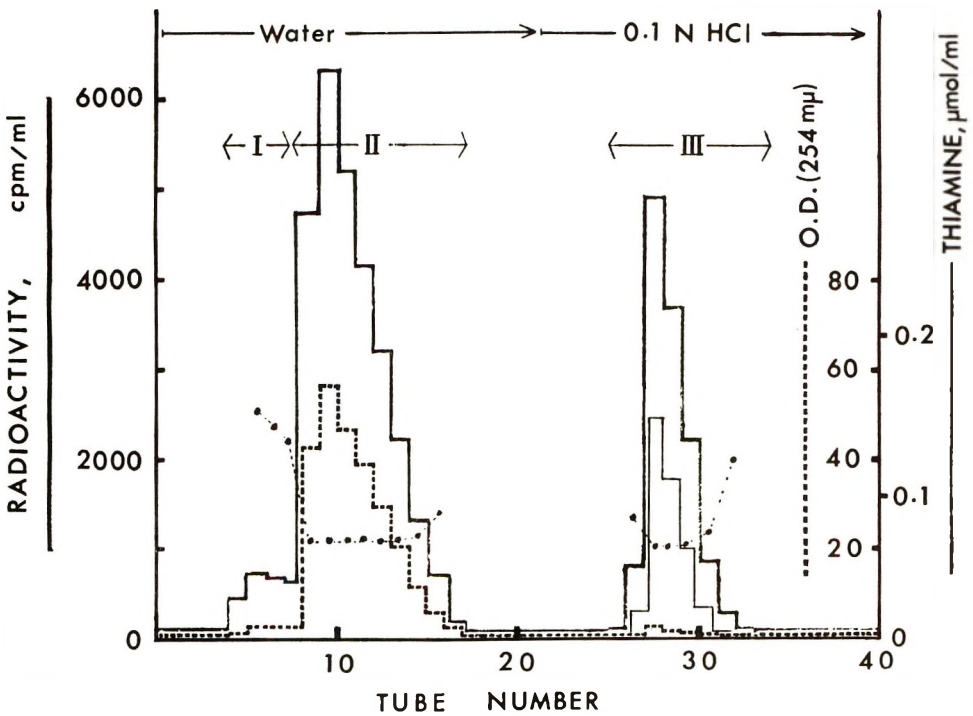

Fig. 1 Chromatographic elution pattern of the 24-hour urine from a germfree rat receiving labeled thiamine (8.4 mg). Column, Amberlite CG-50, H form, 1 × 22 cm. One tube 3.16 ml. The applied sample and recovery are indicated in table 6. The dots show the specific radioactivities of MTA (cpm/E_{254}) and thiamine (cpm/μmole) in arbitrary scale.

thiochrome method on higher intakes. Under such experimental conditions the percentage distribution of the urinary radioactivity in fractions II and III can be taken as an approximate index for conversion of thiamine to MTA. In the experiments presented in tables 2 and 3, the exact amount of urinary thiamine derived from the administered thiamine was calculated from the specific radioactivity (cpm/μmole from the thiochrome method) of thiamine eluted in the peak portion of fraction III. Constancy of the specific radioactivity was checked after further purification by paper chromatography (*n*-butanol/acetic acid/water (4:1:5) by volume) or paper electrophoresis (0.1 M acetate buffer, pH 4.6) and, if necessary, the fraction was purified by these procedures until the specific radioactivity reached a constant value. When the thiamine content was low, 1 μmole of the authentic thiamine was added as carrier to the urine sample before application on Amberlite CG-50 column to facilitate the thiochrome assay. A typical example is presented in figure 1 and table 6.

RESULTS

Preliminary experiments. Table 1 summarizes the results on the urinary excretion of thiamine and radioactivity as influenced by administration route. When thiamine was given by a single injection of 5 mg, about 90% of the administered radioactivity was excreted in the urine during the first 24 hours. Determination by the thiochrome method also showed that the urinary thiamine eliminated in the same duration amounted to about 80% of the injected thiamine. Chromatographic examination further indicated that about 90% of the urinary radioactivity was recovered in the thiamine fraction (fraction III in fig. 1) with less than 10% in the MTA fraction (fraction II). These results showed that most of the injected thiamine was excreted as unchanged thiamine. When thiamine was given orally, about 40% of the administered radioactivity was excreted in the 24-hour urine; whereas, the amounts measured by the thiochrome method corresponded to only about 10% of the dose. Upon chromatographic separation, about 85% of the urinary radioactivity was eluted in fraction II (table 1)

TABLE 1

Influences of the administration route of thiamine on urinary excretion [1] of radioactivity and thiamine

Rat			Administration		Radioactivity			Thiamine [4]	
Strain	Body wt	No.	Dose	Route [2]	II	III			
	g		*mg*		*% of dose*	*% in fractions [3]*		*mg*	*% of dose*
Wistar (conventional)	187	2	5	PO	38.3	83	13	0.372	7.5
	175	2	5	IP	89.1	3	91	3.735	74.6
Sprague-Dawley, JCL (specific pathogen free)	170	6	5	PO	49.3 ± 8.6	85	14	0.559 ± 0.092	11.2 ± 1.8
	280	2	5	IP	84.3	5	95	4.036	80.7
	285	1	4	IV	91.0	9	90	3.356	83.9

[1] Data are obtained on the 24-hour urine and expressed as mean (± SD).
[2] PO, oral; IP, intraperitoneal injection; IV, intravenous injection.
[3] Percentage of the recovered ^{14}C in fractions II and III obtained by the chromatography (see fig. 1).
[4] Determined by the thiochrome method.

whose main component has been already identified as MTA (12). Therefore, the observed discrepancy, demonstrating the occurrence of nonthiamine metabolites, can be explained mainly by the presence of the thiazole acid. From these results it is concluded that thiamine was excreted predominantly as MTA after oral administration, but it was eliminated largely unchanged following parenteral administration.

Effect of dosage levels. The urinary excretion pattern of the orally administered thiamine was investigated at various dosage levels ranging from nutritionally adequate amounts (50 μg) to unphysiological excess (41 mg) (table 2). The urinary excretion of radioactivity increased from 10% at the 50 μg dose to about 40% at the 0.5 mg dose and maintained the same magnitude at larger doses. Thiamine content of the 24-hour urine corresponded to 7 to 14% of each dose. The amounts of both MTA and thiamine were then determined by isotope dilution. Only 2.9 and 2.7% of the dose were excreted as MTA and thiamine at the physiological dose of 50 μg, respectively. The excretion as MTA was increased by increasing the dose to 1.5 mg and attained a plateau of about 30%, but the excretion of the radioactive thiamine was less than 10% in all the tested levels except the 0.5 mg dose. Therefore, the excretion of MTA exceeded that of thiamine 4 to 8 times at the higher dose levels. This feature of the metabolic pattern to depend on the dose is more clearly visualized when the results are expressed by percentage distribution of the urinary radioactivity. Namely, thiamine and MTA accounted for 26 and 27% of the radioactivity at the 50 μg dose and the remaining half was unidentified. At the higher doses, MTA made up 74% of the radioactivity whereas thiamine accounted for only 9 to 18% and the remaining radioactivity of 7 to 17% was due to unidentified metabolites.

Similar experiments were performed with thiazole-labeled TTFD (table 3). The urinary excretions of radioactivity, thiamine and MTA were enhanced with increasing dose to 1.5 mg and were thereafter maintained at a plateau or somewhat declined in the higher doses. The excretion of the radioactivity was about 20% higher at all the intake levels when compared with the results on thiamine. The most notable difference observed between thiamine and TTFD was the finding that, unlike thiamine, the thiamine moiety of TTFD was excreted more as thiamine rather than as MTA. Namely, the excretion of thiamine exceeded that of MTA at the lower dose levels of TTFD, being still comparable to the latter even at the higher dosage levels which induced the increased excretion of MTA. These results indicate that TTFD is more resistant to biological conversion to MTA than is thiamine.

Time course of the urinary metabolite pattern. Figure 2 shows the excretion curves of the radioactivity, ^{14}C-MTA and total thiamine after oral administration of labeled thiamine or TTFD in the rats. Unexpectedly, about two-thirds of the thiazole acid excreted within 48 hours was recovered in the first 3-hour urine samples in both cases. Following the ingestion of

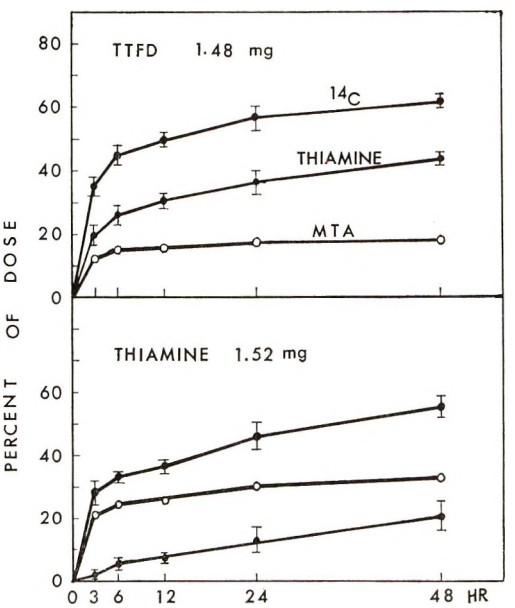

Fig. 2 Cumulative excretion of the urinary radioactivity, MTA and thiamine after oral administration of labeled thiamine and TTFD. Each group consists of 3 male rats of the Sprague-Dawley strain, weighing about 200 g. Bars indicate standard deviation. Thiamine was determined by the thiochrome method and MTA by the isotope dilution method.

TABLE 2

Relation of dosing amounts of thiamine and urinary excretion of radioactivity, MTA and thiamine

Dose[1]: Thiamine·HCl, mg/rat [2]	41	6	1.5	0.5	0.15	0.05
Excreted in the 24-hour urine						
Radioactivity, % of dose	41.3 ± 6.1 [3]	41.6 ± 3.6	38.8 ± 4.0	43.8 ± 2.3	19.5 ± 2.3	10.6 ± 2.1
Thiamine,[4] mg	2.74 ± 1.00	0.480 ± 0.207	0.113 ± 0.066	0.0695 ± 0.0133	0.0121 ± 0.0081	0.00504 ± 0.00065
% of dose	6.7	8.0	7.5	14.0	8.0	10.0
Percent of recovered ^{14}C in chromatography [5]						
Fraction I	2.8	2.0	2.2	1.4	4.9	7.5
Fraction II	82.1	79.7	86.8	57.0	61.7	42.3
Fraction III	15.1	18.2	11.0	41.6	33.3	48.7
Determination by isotope dilution						
MTA,						
% in fraction II	89.9	93.9	85.0	86.8	89.9	64.2
% of urinary ^{14}C	73.8	74.8	73.7	49.5	55.5	27.1
% of dose	30.5	31.1	28.6	20.4	10.2	2.9
Thiamine,						
% in fraction III	100	100	84.9	84.0	72.1	53.0
% of urinary ^{14}C	15.1	18.2	9.3	34.9	24.0	25.8
% of dose	6.2	7.6	3.6	14.4	4.4	2.7

[1] In a single oral dose by stomach tube.
[2] Each group consists of 3 male rats of the Sprague-Dawley strain weighing about 185 g.
[3] Mean ± SD.
[4] Determined by the thiochrome method.
[5] Conditions for the chromatography are the same as those shown in figure 1.

TABLE 3

Relation of dosage amounts of TTFD and urinary excretion of radioactivity, MTA and thiamine

Dose[1]: TTFD, mg/rat [2]	45.5	10.5	6.0	1.5	0.5	0.15	0.05
Excreted in the 24-hour urine							
Radioactivity, % of dose	49.7 ± 9.2 [3]	59.5 ± 1.8	51.5 ± 6.6	68.9 ± 10.9	45.7 ± 3.6	24.4 ± 4.5	13.5 ± 0.9
Thiamine,[4] mg	9.02 ± 0.19	3.17 ± 0.15	1.51 ± 0.31	0.521 ± 0.091	0.206 ± 0.019	0.028 ± 0.0143	0.00537 ± 0.00113
% of dose	19.8	30.2	25.3	34.7	41.2	18.7	10.8
Percentage of recovered ^{14}C in chromatography [5]							
Fraction I	2.1	1.7	1.7	8.8	3.2	4.7	6.4
Fraction II	55.0	41.2	48.7	44.9	29.2	30.0	26.4
Fraction III	42.2	53.8	48.3	46.2	65.1	60.0	55.7
Determination by isotope dilution							
MTA,							
% in fraction II	83.1	79.6	80.3	83.1	83.6	71.0	45.8
% of urinary ^{14}C	45.7	32.8	39.1	37.8	24.4	21.3	12.2
% of dose	22.7	19.5	20.1	25.7	11.2	5.2	1.6
Thiamine,							
% in fraction III	96.0	92.5	93.5	86.6	84.9	68.6	39.0
% of urinary ^{14}C	40.5	49.8	45.1	40.0	55.2	41.2	21.7
% of dose	20.1	29.6	23.3	27.6	25.2	10.1	3.0

[1] In a single oral dose by stomach tube. The doses are expressed as milligram equivalent to thiamine·HCl.
[2] Each group consists of 3 male rats of the Sprague-Dawley strain weighing about 185 g.
[3] Mean ± SD.
[4] Determined by the thiochrome method.
[5] Conditions for the chromatography are the same as those shown in figure 1.

thiamine, 21.4% of the dose was eliminated as MTA and only 2.0% as thiamine within the first 3 hours but the next 3-hour urine contained 3.0% as MTA and 3.3% as thiamine. After the administration of TTFD, 11.8% of the dose was excreted as MTA and 19.7% as thiamine for the first 3 hours and 3.0% as MTA and 6.6% as thiamine for the next 3 hours, respectively. The excretion as thiamine exceeded the excretion as MTA in all the tested periods. Table 4 shows the percentage distribution of radioactivity among the 3 fractions separated by the column chromatography as well as the percentage of MTA determined by isotope dilution. In both cases the percentage of the thiamine fraction (fraction III) increased with time whereas that of MTA (fraction II) declined.

Identification of MTA as a thiamine metabolite in germfree rats. The finding that thiamine ingested orally, but not injected, was largely catabolized to MTA (table 1), prompted us to carry out the experiments using germfree rats to ascertain whether intestinal microflora were involved in the thiamine breakdown. As shown in table 5, considerable amount of

TABLE 4

Time course changes in metabolite pattern after oral administration [1] of thiamine and TTFD

Time, hour		0–3	3–6	6–12	12–24	24–48
Thiamine·HCl						
Radioactivity, % of the recovered ^{14}C in the chromatography [2]						
Fraction I		2.3	4.2	4.7	2.1	2.3
Fraction II		93.2	68.5	56.1	52.5	38.0
Fraction III		3.9	25.5	37.4	44.3	58.0
MTA,[3]	% of urinary ^{14}C	75.6	57.6	42.1	46.9	29.7
	% of dose	21.4	3.0	1.2	4.4	5.2
TTFD						
Radioactivity, % of the recovered ^{14}C in the chromatography [2]						
Fraction I		1.7	3.7	2.8	1.9	5.7
Fraction II		43.2	36.1	27.0	25.8	25.3
Fraction III		51.4	58.2	68.5	60.3	67.7
MTA,[3]	% of urinary ^{14}C	33.1	31.0	21.2	21.7	19.3
	% of dose	11.8	3.0	0.9	1.6	0.9

[1] The rats and doses are shown in the legend of figure 2.
[2] Conditions for the chromatography are the same as those shown in figure 1.
[3] Measured by the isotope dilution method.

TABLE 5

Urinary excretion of radioactivity and thiamine after oral administration of labeled thiamine or TTFD to the germfree rat

Dose	Thiamine·HCl, 8.4 mg [2]		TTFD, 6.6 mg [3]	
Body wt	417 g		323 g	
Time, hour	0–24	24–48	0–24	24–48
Urinary ^{14}C				
% of dose	18.8	7.5	39.6	6.1
% of recovery [1] in				
Fraction II	64.5	21.4	29.3	16.4
Fraction III	29.8	78.6	70.6	82.6
Urinary thiamine [4]				
mg	0.606	0.536	2.204	0.400
% of dose	7.2	6.4	33.4	6.1

[1] Conditions for the chromatography are the same as those shown in figure 1.
[2] A small amount of thiamine solution (less than one-fourth of the total dose) was spilled during feeding by stomach tube because of the technical difficulty that arose through use of the plastic isolator. Thus, the data expressed as percentage of dose are somewhat underestimated.
[3] Milligram equivalent to thiamine·HCl.
[4] Determined by the thiochrome method.

TABLE 6

Chromatographic data [1] on the urine [2] from a germfree rat that received labeled thiamine

Sample applied	Radioactivity		Thiamine·HCl	MTA
	cpm 144,000	% 100	μmole 0.18 + 1.0 [3]	μmole 3.2 + 180 [3]
Fraction I	7,700	5.3	—	—
Fraction II	88,000	61.3	—	180
Fraction III	40,600	28.3	1.11	—
Recovery		95%	94%	98%

[1] Figure 1 shows the conditions for chromatography.
[2] The 24-hour urine sample from the rat that received thiamine (table 5).
[3] The underlined values were added as carriers.

radioactivity was recovered in fraction II where MTA was eluted. Especially in the first 24-hour urine of the rat receiving thiamine, about two-thirds of the radioactivity was found in fraction II. To an aliquot (46,300 cpm) of the fraction were added 53 mg of an authentic sample of MTA and subjected to crystallization. The MTA was crystallized out from hot water. The specific activities were found to be 869 ± 11, 863 ± 14, 838 ± 9 and 848 ± 38 cpm/mg after the successive recrystallization procedures, respectively. Calculation from the expected value (873 cpm/mg) showed that 96% of the radioactivity in fraction II was identified as MTA. The identity was further confirmed by another experiment. To an aliquot of the same urine sample were added 1 μmole of thiamine and 180 μmoles of MTA as carriers and then the mixture was fractionated by the chromatography (table 6 and fig. 1). The major portion of fraction II gave a specific radioactivity of 102 to 106 cpm/E_{254} mμ for each tube. From the pooled solution the crystals of MTA were obtained whose specific activity was 99 cpm/E_{254} mμ or 2850 cpm/mg. Calculation based on the dilution revealed that MTA accounted for 91% of the radioactivity in fraction II and therefore, for 58.7% of the radioactivity in the 24-hour urine. The peak portions of fraction III also showed a constant specific radioactivity, that is, 100, 103 and 111 cpm/μg of thiamine determined by the thiochrome method, respectively (fig. 1). The specific activity did not vary significantly after further purification by paper chromatography (*n*-butanol/acetic acid/water (4:1:5) by volume). The result indicated that 97% of the radioactivity in fraction III was ascribed to thiamine which in turn accounted for 28.9% of the radioactivity in the 24-hour urine. The excretion pattern after administration of TTFD was also very similar in both conventional and germfree rats, although the excretion as thiamine was somewhat greater in the germfree rat (table 5). Therefore, it was concluded that thiamine in a large oral dose was excreted principally as MTA in the urine in germfree rats, and thus the intestinal microflora were not primarily responsible for the thiamine breakdown in the rat.

DISCUSSION

The previous study has established the identity of MTA as a thiamine metabolite (12). In the present work we have reported that MTA is predominantly excreted only after a large oral dose of thiamine with most of the injected vitamin being eliminated unchanged into the urine. Hitherto many investigators (1–11) have studied the metabolism of thiamine, but none have reported the occurrence of the thiazole acid. Most of these investigations involved either parenteral administration or physiological doses. This would be one of the reasons why MTA has not been found previously. Takenouchi and his coworkers (4, 8) first pointed out the urinary excretion of considerable amounts of nonthiamine metabolites after massive administration of thiazole-labeled thiamine, TPD or TTFD. Although the metabolites were not identified, the chromatographic behaviors which they described on the major component (substance X) resembled those of MTA. Recently, Meshi and Sato (9) reported that the orally ingested thia-

mine was largely excreted as nonthiamine metabolites, of which 4-methyl-5-(2-hydroxyethyl) thiazole (MHT) was identified by isotope dilution. It still remained unsolved how much radioactivity is accounted for by MHT, however, since quantitative data were not presented.

The fact that thiamine was readily converted to MTA after oral administration but only poorly after parenteral administration raised the question whether intestinal microflora were responsible. This possibility was decisively excluded by the finding that MTA was produced in germfree rats to about the same degree as in the conventional rats. This fact is consistent with the result of Neal and Pearson (7) reporting identification of 2-methyl-4-amino-5-pyrimidinecarboxylic acid (PCA) as a thiamine metabolite in the urine of germfree rats as well as conventional rats. The urinary excretion of both counterparts of the vitamin in germfree rats is an unequivocal evidence supporting the view that thiamine cleavage occurs in the mammal. However, any thiamine-splitting enzyme has not yet been recognized in mammalian tissues. Another possibility also should be considered that nonenzymic cleavage of the thiamine molecule might take place in the intestinal tract, for example, by the sulfite decomposition (20, 21). In any event, elucidation of the mechanism is a problem left for future study.

Previously, we clarified that MHT, when injected into the rat, was excreted quantitatively into the urine after conversion to MTA (14). Neal and Pearson (7) reported the formation of PCA from 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) in the rat. These facts suggest that a possible metabolic sequence of thiamine catabolism would be breakdown to HMP and MHT followed by oxidation of both primary alcohols to the corresponding carboxylic acids (fig. 3). Both counterpart alcohols have been reported to be thiamine metabolites in the rat (2, 9), rabbit (3) and man (22). We could not confirm the occurrence of MHT as a thiamine metabolite, however, and Pearson and his coworkers (7, 11) also reported their failure to identify these compounds. Hence, it is suggested that both MHT and HMP, even if formed in the rat, are oxidized so rapidly that most of them are excreted as the corresponding carboxylic acids.

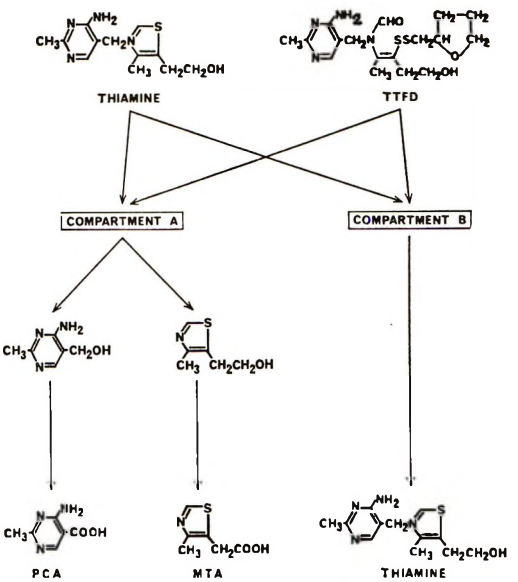

Fig. 3 A possible metabolic sequence of thiamine and TTFD in the rat.

The present findings obtained on the metabolite pattern of thiamine can be explained reasonably by an assumption that there are two distinct metabolic compartments which influence the fate of thiamine. As illustrated in figure 3, injected thiamine would enter preferentially into compartment B which favours the renal excretion without chemical conversion whereas most of the orally fed thiamine would enter into compartment A which is responsible for splitting the thiamine molecule. Although the nature of the compartments is obscure, compartment A might have some relation to the intestinal tract because the formation of MTA is most active immediately after the oral ingestion of the vitamin. This view is supported by the findings of Meshi and Sato (9) that the percentage of MHT is the highest in portal venous blood among the tested tissues after oral administration of ^{35}S-labeled thiamine. Compartment A may also be related to detoxication, since the excretion of MTA is enhanced to about 75% of the urinary metabolites by increasing the dose to 1.5 mg or more. In this connection, TTFD behaved rather differently from thi-

amine; namely, upon oral administration, the formation of MTA was far less with TTFD than with thiamine. In other words, the fate of orally fed TTFD resembled to a considerable degree that of injected thiamine. This feature of TTFD would be also explicable by assuming that the thiamine moiety of TTFD enters into compartment B more readily than thiamine even under the oral administration. At present much evidence has been accumulated for the higher affinity to various cells as well as better intestinal absorption of thiamine alkyl disulfides than that of thiamine (16–18). These characteristics are ascribed to passive transport due to their lipophilic property (17). On the contrary, thiamine is proposed to be absorbed by an active transport process (18, 23–25). Therefore, the mechanism underlying intestinal absorption would be quite different for thiamine and TTFD, and this would be one of factors responsible for the difference in the metabolic behavior. It has been well-established that thiamine alkyl disulfides, unlike thiamine, are quite stable to attack by thiaminase I and II (EC. 2.5.1.2 and EC. 3.5.99.2). It is, therefore, anticipated that TTFD might be more resistant to the mammalian breakdown (enzymic or non-enzymic), and such resistance would be an additional cause for the observed difference in the metabolic patterns.

According to the report of Meshi and Sato (9), the hydrolytic cleavage of thiamine to MHT occurs in the rabbit, mouse and guinea pig as well as in the rat, although the rat is the most active in this metabolic conversion. Since our present findings have been obtained solely with the rat, further studies should be made on the formation of MTA in the other mammals.

ACKNOWLEDGMENT

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4-Methylthiazole-5-Acetic Acid — A Urinary Metabolite of Thiamine [1]

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ABSTRACT A compound identified as 4-methylthiazole-5-acetic acid (TAA) has been isolated from the urine of rats receiving injections of either ^{14}2C-thiazole-labeled thiamine or ^{14}2C-thiazole-labeled oxythiamine. Rats injected daily for 8 to 20 weeks with 40 μg of ^{14}C-thiamine excreted 9.5% of their daily dose as TAA. Rats injected daily with 80 μg of ^{14}C-oxythiamine excreted 12.1% of their daily dose as TAA. Rats injected with 80 μg oxythiamine + 40 μg ^{14}C-thiamine excreted only 3.9% of their daily intake as TAA suggesting that oxythiamine inhibits this pathway of thiamine degradation.

In previous studies from this laboratory, 22 metabolites of thiamine have been reported (1) to occur in rat urine. Of these, only thiamine and pyrimidine carboxylic acid have been unequivocally identified (2). We now wish to report the isolation and identification of 4-methylthiazole-5 acetic acid ("thiazole acetic acid") as a urinary metabolite of both thiamine and oxythiamine. This compound has been previously reported by Imai et al. (3) to be the main metabolic product of thiazole (4-methyl-5-β-hydroxyethyl thiazole) present in the urine of the rat.

MATERIALS AND METHODS

The animals, diets, techniques used in the fractionation of urine by column chromatography and subsequent thin-layer chromatography of each radioactive peak have been described previously (4). Three groups of 6 female weanling rats of the Sprague-Dawley strain were fed a thiamine-deficient diet and injected intraperitoneally daily with 40 μg thiamine and/or oxythiamine as follows: group 1, 40 μg 2-^{14}C-thiazole thiamine; group 2, 40 μg ^{14}C-thiamine + 80 μg unlabeled oxythiamine; and group 3, 40 μg unlabeled thiamine + 80 μg ^{14}C-oxythiamine. For the development of thin-layer chromatograms the following solvent systems were used: *n*-propanol–water–1 M acetate buffer, pH 5.0 (70:20:10, by volume), secondary butanol saturated with water; methanol–chloroform–10% ammonium hydroxide (16.25:8.75:1, v/v); 2-propanol–ammonium hydroxide–water (20:1:2, v/v); ethanol–ammonium hydroxide–water (18:1:1, v/v). The Dowex-1-Cl column was prepared as described by Neal and Pearson (1). Potassium bromide micro and macro pellets were used in infrared spectroscopic studies of thiamine and oxythiamine metabolites. A Perkin-Elmer Model 337 Grating Infrared Spectrophotometer was used for recording the infrared spectra.

RESULTS AND DISCUSSION

In our previous study (4) it was found that one of the main components of the first radioactive peak to emerge from the Amberlite GC-50 resin had an R_F value of 0.80 (*n*-propanol–acetate buffer–water system) on thin-layer plates of microcrystalline cellulose. Because Amberlite CG-50 resin was in the H^+ form and the unknown compound was eluted by distilled H_2O shortly after the hold-up volume, the compound was considered to be acidic or neutral. In addition, the injection of 2-^{14}C-thiazole into the rat (prepared by bisulfite cleavage of ^{14}C-thiamine according to the

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[3] A portion of these data was taken from a thesis submitted by M. R. Ariaey-Nejad to the graduate faculty of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

method of Williams et al. (5)) yielded a major metabolite with chromatographic properties that were identical with those of the unknown metabolite. Since Imai et al. (3) found thiazole acetic acid to be the major urinary metabolite of this compound, we assumed that this was the case and set about collecting firm evidence for this assumption.

The unknown radioactive band was scraped from cellulose plates and eluted with a small volume of water. Separate preparations were made from the urines of rats which received ^{14}C-thiamine or ^{14}C-oxythiamine. An authentic sample[4] of 4-methylthiazole-5-acetic acid was spotted with the unknown metabolite obtained from ^{14}C-thiamine and ^{14}C-oxythiamine on cellulose thin-layer plates and their R_F values were compared in five solvent systems. In addition, mixtures of the unknown with authentic 4-methylthiazole-5-acetic acid were chromatographed. After development, the plates were viewed under ultraviolet light (peak intensity 253 mμ) to visualize the authentic sample. The thin-layer plates were then placed in contact with X-ray film to locate the position of the unknown radioactive compounds. The unknown material isolated from the urines of all three groups of rats had an R_F value identical to that of the authentic sample in five different solvent systems. In addition, a mixture of the unidentified compound and the authentic sample gave a single ultraviolet absorbing radioactive spot. The results of these autoradiograms are shown in table 1.

Infrared spectroscopy. Although the chromatographic evidence strongly favored the conclusion that the unknown was 4-methylthiazole-5-acetic acid, an infrared spectrum was obtained for further confirmation. This required the isolation and rigorous purification of microgram amounts of this compound from urine. The procedure devised for this purification is summarized below. As it developed, removal of contaminating urinary pigments proved to be a difficult task.

Preparative cellulose thin-layer chromatography of the Amberlite CG-50 peak I was carried out in the *n*-propanol–acetate buffer–water solvent system. The unknown band ($R_F \pm 0.80$) was localized by autoradiography, removed by scraping, and eluted from the cellulose with distilled water. Although this material always gave a single sharp peak when repeatedly chromatographed on Amberlite CG-50 and Dowex AG1-X8, the infrared spectrum continued to show the presence of considerable amounts of a yellow impurity. In an

[4] A generous amount of authentic 4-methylthiazole-5-acetic acid was supplied by Dr. R. A. Neal who synthesized it according to the procedure of Cerecedo, L. R., and J. G. Tolpin 1937 Studies on thiazole. I. 4-Methylthiazole-5-acetic acid and some of its derivatives. J. Amer. Chem. Soc., 59: 1660.

TABLE 1

R_F values of thiazole acetic acid (TAA)[1] and unknown compound obtained from the urine of three groups of rats

Solvent systems	TAA	Group 1	Group 2	Group 3	Mixture[2]
		^{14}C-thiamine	^{14}C-thiamine + oxy-thiamine	^{14}C-oxy-thiamine + thiamine	
n-Propanol – acetate buffer – H_2O (7 : 1 : 2)	0.86	0.86	0.86	0.86	0.86
Methanol – chloroform – 10% NH_4OH (16.25 : 8.75 : 1.0)	0.44	0.44	0.44	0.43	0.43
Sec. butanol saturated with H_2O	0.88	0.88	0.88	0.88	0.88
2-Propanol – NH_4OH – water (20 : 1 : 2)	0.26	0.26	0.26	0.26	0.26
Ethanol – NH_4OH – water (18 : 1 : 1)	0.52	0.50	0.51	0.50	0.49

[1] TAA = synthetic 4-methylthiazole-5-acetic acid.
[2] Mixture indicates a combination of unknown compound from 3 groups and synthetic TAA.

attempt to remove this impurity, the sample was lyophilized, the residue was taken up in 0.2 ml of distilled water and spotted on Whatman no. 40 acid-washed filter paper. An authentic sample of thiazole acetic acid was also spotted on the same sheet and ascending chromatography was carried out in a solvent system consisting of methanol–chloroform–10% ammonium hydroxide (189:101:10, v/v) for 8 hours. The sample was visualized on the paper chromatogram by its ultraviolet quenching characteristics and radioautography. The R_F value was identical to that of the authentic sample. A nonradioactive yellow fluorescent material was cleanly separated from the radioactive metabolite and was left behind when the radioactive band was eluted from the paper with 2 ml of distilled water. Other nonradioactive impurities were removed by extracting the water solution with two, 2-ml portions of dry ethyl ether. Only about 5% of the radioactivity appeared in the ether layer. To remove ammonium salts from the solution of the unknown metabolite, it was chromatographed on a column of Amberlite CG-50 that had been extensively washed with double glass distilled water. The sample was eluted with distilled water. The single radioactive peak was collected, and its volume reduced, under vacuum, to 2 ml. The concentrate was again applied to another column of Amberlite CG-50 fol-

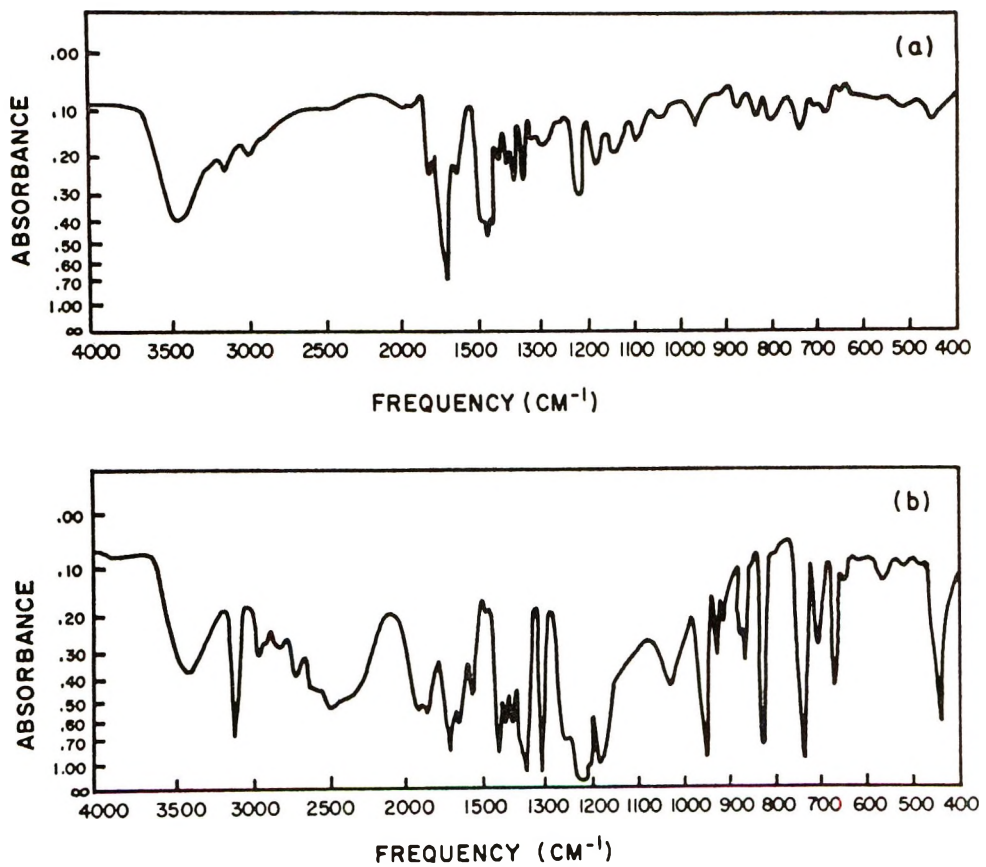

Fig. 1 Infrared absorption spectrum of the unknown metabolite obtained from the urine of rats injected with ^{14}C-thiamine and ^{14}C-oxythiamine in comparison with that of an authentic sample. Both spectra were obtained with a Perkin-Elmer IR 337 spectrophotometer, Grating system. a) Approximately 200 μg (on the basis of radioactivity) of the unknown compound in a 300 mg KBr pellet (slow scanning); and b) 500 μg synthetic 4-methylthiazone-5-acetic acid (TAA) in a 300 mg potassium bromide pellet (slow scanning).

lowing the same procedure as above, until the compound had been cycled four times in this fashion. On the basis of its radioactivity the final product was estimated to contain approximately 200 μg of the unknown metabolite.

The infrared spectrum of this purified product is compared with that of an authentic sample of thiazole acetic acid in figure 1. Although a trace of impurity is still present, the fingerprint region of the spectrum of the unknown is similar to that of the authentic compound. The absorption peak characteristic of functional groups of 4-methylthiazole-5-acetic acid is clearly detectable in the spectrum. For example, the broad peak at 3400 cm^{-1} represents the acid hydrogen bonding ($-$ OH . . . . . $O^{=}$) and a relatively sharp peak at 1700 cm^{-1} is specific for the carbonyl group of the molecule.

Crystallization. Further evidence as to the identity of the unknown metabolite was obtained by crystallization of a mixture of the unknown and authentic 4-methylthiazole-5-acetic acid to constant specific activity. Ten milligrams of the authentic compound were mixed with about 100 μg (on the basis of radioactivity) of relatively pure unknown compound obtained directly from cellulose thin-layer chromatograms. The mixture was first chromatographed on an Amberlite CG-50 column that had been extensively washed prior to application of the sample. The compound was eluted with distilled water as a sharp radioactive peak. This was collected, its volume reduced under vacuum to 2 ml and lyophilized. The residue was dissolved in 0.3 ml of boiling water, which upon cooling yielded crystals of thiazole acetic acid. The crystals were harvested by centrifuging and the yellow supernatant solution was removed with a Pasteur pipet. A sample of the crystals was dissolved in 1 ml of 0.01 N HCl by warming and a 0.1 ml aliquot of this solution was diluted to 2 ml with another portion of 0.01 N HCl. The optical density of this solution was measured in a Beckman DB-G Spectrophotometer at 256 mμ and the concentration of thiazole acetic acid determined by comparison with a standard curve obtained using the authentic compound. After measurement of the optical density, 1 ml of the solution was counted in a Nuclear-Chicago liquid scintillation counter for 100 minutes. The remaining crystals were redissolved in 0.2 ml of boiling water, and the above procedure was repeated 5 times. The results are shown in table 2. The specific activity increased slightly after the second crystallization which coincided with removal of some yellow pigment but remained constant thereafter.

On the basis of chromatographic data, the infrared data, and the recrystallization data, we consider that the unknown compound has been demonstrated beyond reasonable doubt to be 4-methylthiazole-5-acetic acid.

From the quantitative point of view, the radioactivity of this metabolite represents 9.5% of the daily dosage for the rats which were injected with 40 μg thiazole-2-^{14}C-thiamine alone, 3.9% of the daily intake for the rats which were injected with a mixture of thiazole-2-^{14}C-thiamine + oxythiamine (40 μg + 80 μg), and 12.1% of the daily dose for the rats which were injected with thiazole 2-^{14}C-oxythiamine + thiamine (80 μg + 40 μg). Thus, oxy-

TABLE 2

Specific activities of 5 recrystallizations of a mixture of authentic 4-methylthiazole-5-acetic acid and the unknown urinary metabolite

Crystallization no.	Thiazole acetic acid [2] in sample			
	cpm [1]	*μg*	*cpm/μg*	*cpm/μmole*
1	157	18.5	8.4	1312
2	232	26.0	8.9	1390
3	265	27.5	9.6	1453
4	170	18.0	9.4	1468
5	372	38.0	9.8	1406

[1] Radioactivity was determined in a Nuclear-Chicago liquid scintillation counter.
[2] Determined spectrophotometrically in a Beckman DB-G spectrophotometer.

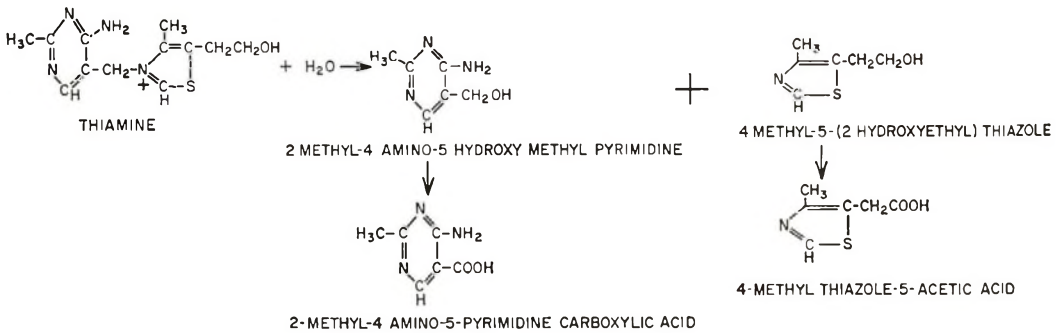

Fig. 2 A proposed cleavage pathway for thiamine catabolism in the rat.

thiamine yields somewhat more of this metabolite than does thiamine and also appears to inhibit this pathway of thiamine degradation.

Although there is considerable literature concerning the thiaminase enzymes in the lower vertebrates, invertebrates and plants, little is known of these enzymes in the mammalian organism. It is now obvious that such enzymes do exist. It seems logical to expect that 4-methyl-5-β-hydroxyethyl thiazole ("thiazole") is an obligatory intermediate in the generation of thiazole acetic acid from thiamine. In previous studies we failed to detect this compound, but in these studies using thin-layer chromatography it would appear that a small amount of thiazole is present in urine. Thus, the sequence for the cleavage degradation of thiamine seen in figure 2 would seem to be a reasonable one. An alternative pathway would be the formation of thiamine acetic acid with subsequent cleavage to the pyrimidine moiety and thiazole acetic acid. At the present time, however, we have no evidence that thiamine acetic acid is formed in mammalian systems.

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Glutamic Acid Metabolism in the Lactating Dairy Cow [1]

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ABSTRACT A single dose of L-glutamic acid uniformly labeled with carbon-14 was injected into a lactating dairy cow via the portal vein or the jugular vein in four experiments, and the time course of the isotope was followed in respired CO_2, plasma glutamate, plasma glucose and milk constituents. In each experiment the specific activity of plasma glutamate decreased with a half-time of approximately 5 minutes during the first 30 minutes after injection. Expired CO_2 and plasma glucose became labeled rapidly with maximal specific activities occurring at about 12 minutes in CO_2 and 20 minutes after the injection in plasma glucose. Between 34 and 41% of the injected dose appeared in respired CO_2 in the first 3 hours. Milk constituents contained 3 to 8% of the injected dose after 3 hours and 12 to 15% after 48 hours. Lactose accounted for 46 to 54% of all carbon-14 recovered in milk during 48 hours; casein, 27 to 37%; and albumin, 4 to 5%. The specific activity of citrate was very high, whereas that of milk fat was negligible. Aspartate was the most highly labeled amino acid in casein except for glutamate, indicating extensive interconversion of these two amino acids. Proline and arginine had very low specific activities, which means that their synthesis from glutamate was nominal. It is concluded that the amount of carbon from the plasma glutamate pool utilized for gluconeogenesis exceeds that incorporated into milk protein.

Amino acids are specifically required for synthesis of protein, but many of these compounds also play a critically important role as glucogenic precursors when glucose is limiting. These are competing processes for several amino acids which may be utilized either in gluconeogenesis or in protein synthesis.

The present study was undertaken to assess the metabolic fate of a glucogenic amino acid under conditions in which there is a large daily precursor requirement for both protein and carbohydrate. The lactating cow provides a unique model for this type of study since the formation of lactose and milk protein is virtually continuous, and approximately equal amounts of organic precursor [3] are required for each process. Furthermore, the glucose required for lactose synthesis (1, 2) must be derived largely by gluconeogenesis since the amount of alimentary glucose in the ruminant is limited (3, 4).

Glutamic acid was selected for the present study since it is the major component of milk protein,[4] comprising 19.8% of all milk protein (5), including 22.4% of the casein (6), and it is also a glucogenic amino acid (7, 8). Its metabolic fate was assessed by following the time course of appearance of isotope from glutamate-^{14}C in respired CO_2, blood glucose and the major constituents of milk. It was of interest also to determine how conditions which influence gluconeogenesis, such as withholding food or providing an exogenous supply of a glucogenic precursor, affect the metabolic fate of glutamic acid.

The studies reported here show that more of the carbon from glutamate appeared in lactose than in milk protein

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[3] Based on an average composition of milk containing 4.8% lactose (42% carbon in lactose), 3.3% casein and 0.7% albumin (54% carbon in protein), each liter of milk would contain 1.68 g atoms carbon in lactose and 1.78 g atoms carbon in protein.

[4] An undetermined part of the "glutamate" in casein is actually present as glutamine. Barry (34) has estimated that there are approximately equal amounts of each amino acid in casein and has shown that the specific activities of the two amino acids in casein are nearly the same 3 hours after intravenous injection of ^{14}C-glutamate. In view of this rapid interconversion, the two amino acids are considered here as a single component.

when the cow was not fed and that this relationship was not appreciably changed by feeding the cow, or by providing substrate amounts of propionic acid intravenously. It is concluded that the processes involved in gluconeogenesis sequester a greater part of the carbon flux through the glutamate pool than do processes of protein synthesis during milk formation in the cow.

MATERIALS AND METHODS

Animal. All experiments were carried out with a mature lactating Jersey cow (356 kg body weight) which had a plastic catheter implanted in the portal vein (9). On the day preceding each experiment, a second catheter was inserted into the jugular vein. These catheters permitted the intravenous injection of ^{14}C-glutamate and withdrawal of blood samples over precisely timed intervals with minimum disturbance of the cow. This approach was critically important in the lactating animal, especially for studies of gluconeogenesis, since emotional disturbance can interfere with glucose metabolism and obscure normal relationships. For this reason also, the cow had been accustomed to the experimental area and to all procedures used during the experiment.

Diet. The cow received a high quality alfalfa hay ad libitum (about 12 kg/day ingested) and a grain concentrate[5] (3.3 kg, twice daily, given at each milking). This ration supplied protein and energy in excess of the NRC recommended allowance (10). When "unfed," the animal had received a full ration on the day preceding the experiment, but no food was available from 14 hours before the experiment. Though this treatment does not represent a fasted state or even a postabsorptive state, as it would in a monogastric animal, at the time of the experiment rumen fermentation would have subsided. This procedure provided a more standard condition than the fed animal in which, depending on the level and rate of intake on the morning of the experiment, there would be variable amounts of CO_2 produced in rumen fermentation. A longer period of fasting was rejected to avoid a situation that would appreciably interfere with milk formation.

Radioactive glutamic acid (L-glutamic acid-U-^{14}C). Protein was extracted from a *Chlorella pyrenoidosa* culture grown in an atmosphere of $^{14}CO_2$ of high specific activity (25.5 mCi/mmole) and was hydrolyzed in 6 N HCl for 16 hours. Amino acids were separated by chromatography on a column of Dowex-50 (8% cross linked) ion exchange resin, eluting with hydrochloric acid (11). Successive fractions were examined by paper chromatography and autoradiographic techniques, and the pure fractions containing a single amino acid were combined and concentrated under reduced pressure. Purity of the sample was checked by standard procedures of chromatography (see below for solvent system) and autoradiography.

Experimental procedures. Each experiment began at 8 AM to avoid diurnal effects. The cow was connected by face mask to the respiration apparatus for one-half hour before injection of ^{14}C-glutamate. When propionate was administered, the infusion was started 30 minutes before injection of labeled glutamate. The cow was completely milked, after which the labeled glutamate was intravenously administered over a period of 2 minutes. The syringe and catheter were then flushed twice with 15 ml of sterile saline, during an additional minute. Following the first 3-hour period of sample collection, the cow was milked, and then intermittent samples of respired air, blood and milk were collected for 2 days.

Experiment 1: The cow was unfed overnight (14 hours) and L-glutamate-U-^{14}C was injected via the portal vein.

Experiment 2: The cow was unfed overnight (14 hours), and propionate (neutralized to pH 7.0 with NaOH) was infused via the portal vein at the rate of 0.44 mole/hour for 4 hours, commencing 30 minutes before portal injection of L-glutamate-U-^{14}C.

Experiment 3: The cow was fed just before the experiment and was infused with propionate as in experiment 2; the L-glutamate-U-^{14}C was injected via the portal vein.

[5] Feed contained 16% crude protein and consisted of wheat mill run, rolled barley, cane molasses, cottonseed meal, ground grain sorghums, and copra meal in a commercial mix for which the exact composition is not available.

Experiment 4: The cow was unfed (14 hours) and L-glutamate-U-^{14}C was injected via the jugular vein. In experiments 1, 3 and 4 the cow received feed 3 hours after ^{14}C-glutamate injection and was then returned to the normal feeding regimen.

An interval of at least 1 week elapsed between each experiment. Since more than 50% of the ^{14}C had left the cow as CO_2 and milk products in 2 days (52 to 63% of that injected), and the traces of ^{14}C that may have remained in CO_2, glucose and milk products after 7 days were too small to be detected by our methods, it is concluded that residual ^{14}C from one experiment did not affect the results of succeeding experiments.

Analytical methods. Expired CO_2 was collected by drawing air from a face mask through flow meters to an infrared CO_2 analyzer and to a spray tower for absorption of respiratory CO_2 in 4% NaOH solution. Absorbed CO_2 was precipitated as $BaCO_3$ and counted at "infinite-thickness" for calculation of the specific activity. Total radioactivity recovered in respiratory CO_2 was calculated from total air flow, CO_2 concentration and specific activity data.

Blood samples (15 ml) were collected and stored on ice in tubes containing 1 mg each of heparin and sodium fluoride. Samples were obtained at frequent intervals during the first 3 hours after glutamate injection. The blood was centrifuged within a few minutes of collection, and the plasma was stored at −15° until analyzed.

Plasma glucose concentration (table 1) was measured by the glucose oxidase method, and the specific activity of plasma glucose was determined after isolation of the glucosazone (12).

The specific activity of plasma glutamate was assayed by a procedure adapted from that used for algal amino acids (13). The plasma (3 ml) was deproteinized with 5% $ZnSO_4$—0.3 N $Ba(OH)_2$. The filtrate was concentrated to 1 ml under reduced pressure and transferred to a column of Dowex-50 resin (H^+ form; 0.7 cm diameter by 5 cm long). Ten milliliters of distilled water removed free sugars, and the amino acids were eluted with 4 N NH_4OH. The eluate was concentrated in vacuo over concentrated sulfuric acid to remove excess ammonia. The concentrated solution was then spotted onto paper and chromatographed in a solvent containing isobutyric acid, 2000 ml; 1-propanol, 200 ml; 2-propanol, 60 ml; 1-butanol, 60 ml; H_2O, 750 ml; 17 N NH_4OH, 100 ml; and ethylenediaminetetraacetic acid (EDTA), 1.0 g. This system gave good separation of the glutamic acid, free from other amino acids. A pure segment of the glutamate, located by a chromatogram scanner, was cut from the chromatograph, eluted, and diluted to 2 ml. One milliliter was used to measure glutamate concentration (14), and the other portion was used for assay of radioactivity at "infinite-thinness." Counting efficiency at "infinite-thinness" was established by plating increasing aliquots (5 to 50 μliter) of a ^{14}C-glucose solution that had been standardized against the National Bureau of Standards sodium bicarbonate-^{14}C standard (Standard No. 4924). This standardization was accomplished by mixing carrier glucose with an aliquot of the ^{14}C-glucose, com-

TABLE 1

Conditions of experiments

	Exp. no.			
	1	2	3	4
Injected dose, mCi	0.96	0.72	0.72	0.72
Injection route	portal	portal	portal	jugular
Conditions	fasted	fasted and propionate	fed and propionate	fasted
CO_2 production, g atoms carbon/hr	2.91	3.14	3.75	3.45
Mean blood glucose, mg/100 ml	49	53	52	53

busting with Van Slyke reagent, precipitating the CO_2 as $BaCO_3$ and counting at "infinite-thickness."

The milk collected at 3 hours and the pooled samples collected between 3 and 48 hours were separated into major components by methods described elsewhere (15, 16). Casein amino acids were separated, purified and crystallized as described earlier (17). All samples were oxidized by the Van Slyke wet-combustion technique; the evolved CO_2 was trapped, precipitated as barium carbonate and counted at "infinite-thickness" to determine specific activity of milk constituents. The internal labeling pattern of milk citrate was determined by the stepwise method of decarboxylation and oxidation (18). The ^{14}C assay was made on a low background Geiger counter, using duplicate planchettes counted for 20 minutes or 10,000 counts to reduce the counting error to 1% or less.

All data have been presented as standardized specific activities derived by dividing the specific activity of the samples (μCi per gram atom carbon) by the tracer dose (mCi), and have the dimensions μCi per gram atom carbon per mCi injected.

RESULTS

Plasma glutamate. The turnover of plasma glutamate was very rapid as shown by the time course of specific activity plotted in figure 1. During the first 30 minutes after injection, the specific activity decreased with a half-time of 4 to 6 minutes, in all experiments. After 30 minutes, when less than 10% of the ^{14}C-glutamate remained in the plasma, the rate of disappearance decreased, presumably reflect-

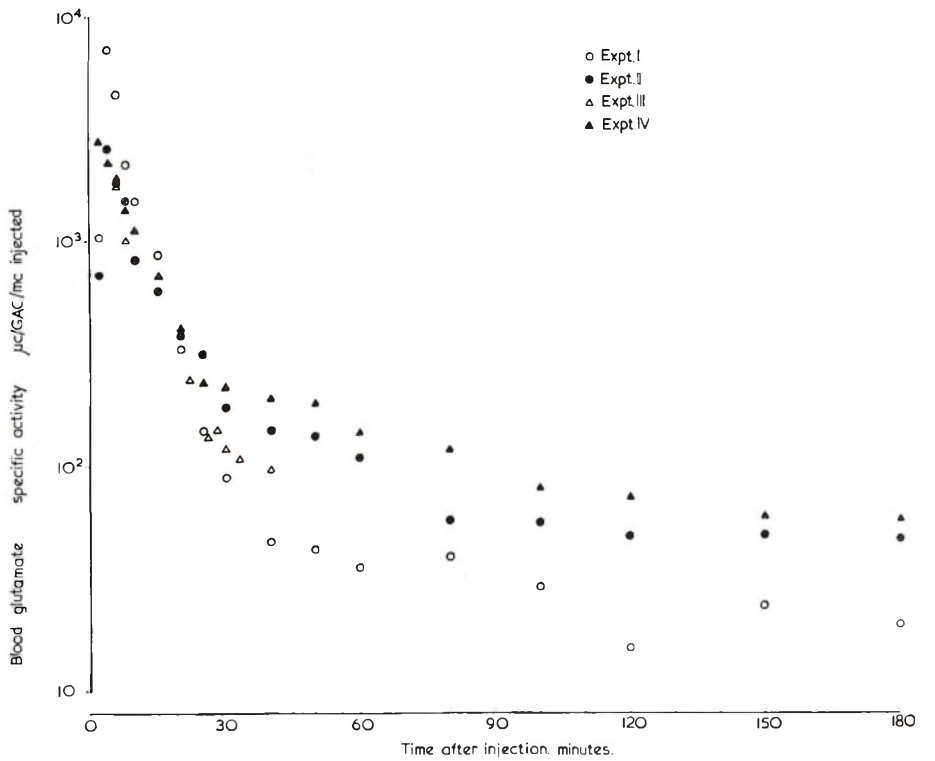

Fig. 1 Specific activity of plasma glutamate (μCi per gram atom carbon per mCi injected) during the first 3 hours after intravenous injection of U-^{14}C-glutamate into the cow. The ^{14}C-glutamate was injected into portal blood in experiments 1, 2 and 3 and into jugular blood in experiment 4. Other experimental variables were: cow unfed in experiment 1; unfed but received propionate intraportally in experiment 2; fed and received propionate intraportally in experiment 3; and unfed in experiment 4.

ing substantial reentry of labeled glutamate into the plasma pool. The plasma glutamate specific activity declined at a slightly slower rate when injection was made via jugular (exp. 4) rather than via the portal vein. The average standardized specific activity for plasma glutamate, calculated for 24 hours after injection was 24 and 22 units in experiments 1 and 2, respectively.

Respired CO_2. Carbon-14 appeared in the respired CO_2 within the first minute after injecting labeled glutamate and reached a maximum specific activity in 10 to 14 minutes (fig. 2). The curve for change of specific activity with time was similar for all treatments, but it had the lowest value when the cow was fed and intravenous propionate administered (exp. 3). During this latter experiment the CO_2 output of the cow was greater than it was at any other time. The cumulative percentage of the injected dose appearing in CO_2 during the first 3 hours ranged from 34 to 41% (table 3). During the 24 hours after glutamate injection, the average standardized specific activity of CO_2, estimated from continuous measurements during 3 hours and intermittent measurements later, was 2.30 and 1.85 units for experiments 1 and 2, respectively. The transfer quotient (specific activity ratio for CO_2/plasma glutamate) indicates that 8 to 10% of the respiratory CO_2 comes from oxidation of carbon that equilibrates with the plasma glutamate pool.

Plasma glucose. As with respired CO_2, carbon-14 from glutamate appeared rapidly in plasma glucose (fig. 3). The first sample, collected within 3 minutes after injection of the ^{14}C-glutamate, contained isotope, and the maximal specific activity occurred between 15 and 25 minutes. This maximum occurred somewhat later, and the

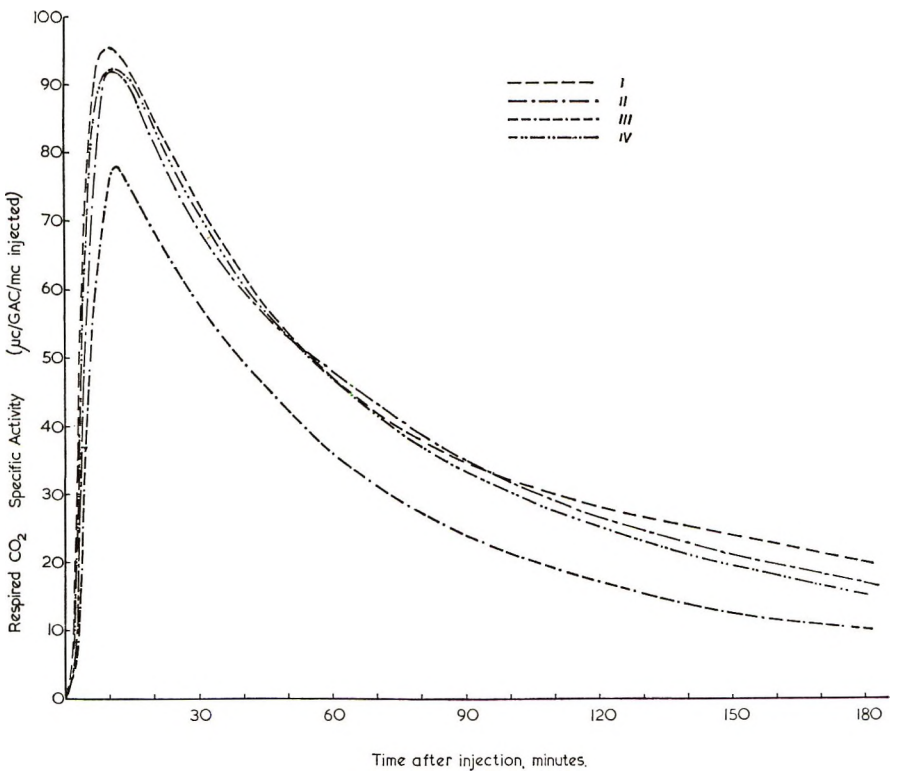

Fig. 2 Specific activity of CO_2 in respired air (μCi per gram atom carbon per mCi injected) after intravenous injection of U-^{14}C-glutamate into the lactating cow. See figure 1 for experimental conditions.

value was of lower magnitude than in respired CO_2, in all experiments.

The specific activity in glucose was greatest when ^{14}C-glutamate was injected into portal blood, producing a value that was nearly equal to that in respired CO_2 (exp. 1, figs. 2 and 3). Lower values resulted when ^{14}C-glutamate was injected into jugular blood (exp. 4) and when propionate was infused into the fed animal (exp. 3). The average standardized specific activity of plasma glucose, estimated from periodic measurements over 24 hours after injection, was 2.0 and 1.7 units for experiments 1 and 2, respectively. The nearly equal specific activity values for CO_2 and plasma glucose indicate that about the same percentage of carbon in CO_2 and glucose arose from the glutamate pool.

Milk constituents. The specific activity of the major milk constituents, shown in table 2, reflects the relative utilization of carbon from glutamate for biosynthesis of the various components. The specific activity of citrate always exceeded that of all other milk constituents and, in the milk collected at 3 hours, was two to three times as great as that of protein or lactose. These results indicated extensive synthesis of citrate from glutamate carbon, presumably reflecting the close metabolic relationship between citrate and α-ketoglutarate, derived from glutamate, in the tricarboxylic acid cycle. Lactose and casein had intermediate specific activities, but in more than half of the samples lactose had a greater specific activity than casein. Albumin had a lower specific activity than casein which would be anticipated in view of its lower glutamic acid content. In all experiments fat had only very low specific activities even after 48 hours which demonstrated that very little of the carbon from glutamate was utilized for fat synthesis in the lactating cow.

The cumulative recovery of carbon-14 in respired CO_2 and in milk constituents is listed in table 3. Forty to forty-five percent of the carbon-14 injected appeared in respired CO_2 and milk products during the

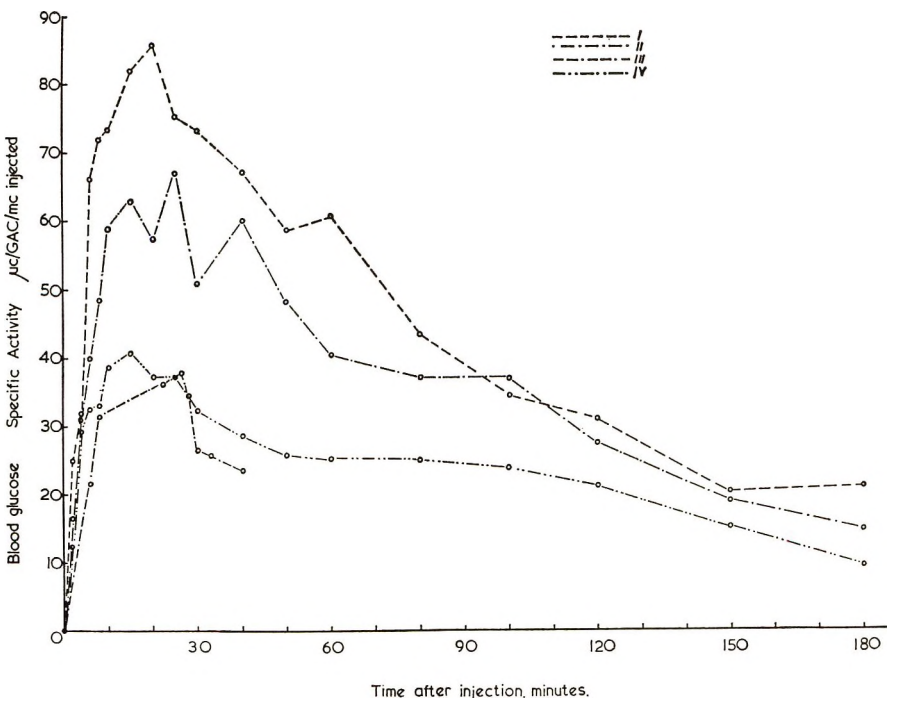

Fig. 3 The time course of plasma glucose specific activity (μCi per gram atom carbon per mCi injected) after intravenous injection of U-^{14}C-glutamate into the lactating cow. See figure 1 for experimental conditions.

first 3 hours, the larger amount appearing after injection of glutamate into jugular blood. From figure 2 it is apparent that considerable amounts of isotope were still appearing in the respired CO_2 3 hours after injection of the labeled glutamate so that the cumulative percentage oxidized would continue to increase significantly at later times. Continuous measurements of CO_2 production were not made, but on the assumption that the rate was constant over 24 hours it may be estimated that an additional 12 to 18% of the injected dose appeared in expired CO_2 during the subsequent 21 hours.

The total carbon-14 appearing in milk constituents during 48 hours was 11 to 15% of that injected. Among the milk constituents, lactose accounted for 46 to 54% and casein, 25 to 37% of the total carbon-14 recovered in milk. In all experiments the total activity in lactose relative to casein increased with time, indicating that at later times after injection the carbon from glutamate was used to a greater extent for glucose than for milk protein synthesis.

Table 4 lists the specific activities for several amino acids recovered from milk casein collected during the first 3 hours, and from 3 to 48 hours after ^{14}C-glutamate injection. In all experiments the greatest specific activity occurred in glutamic acid, although in several cases the specific activity of aspartic acid was nearly as great. This relationship would be anticipated since both amino acids are closely linked metabolically to intermediates of the tricarboxylic acid cycle. In addition, the high specific activity in aspartate may reflect the intervening role of aspartate in the transfer of carbon out of the mitochondria during gluconeogenesis (19, 20). Serine, alanine and glycine had lower specific activities than aspartic acid but greater than proline and arginine. Although glutamate is known to be an active precursor of proline and arginine in the rat (21), the specific activities of these three amino

TABLE 2

Specific activities of major milk constituents following intravenous injection of U-^{14}C-glutamate in each of four experiments

	Exp. no.			
	1	2	3	4
	μCi per gram atom carbon per mCi injected			
Milk 1 [1]				
Lactose	4.92	6.67	7.89	3.80
Casein	3.35	4.52	8.71	7.23
Albumin	1.78	3.03	5.74	3.20
Citrate	11.78	18.00	19.47	22.61
Fat	0.12	0.11	0.17	0.18
Milk 2 [2]				
Lactose	2.06	2.26	1.16	1.10
Casein	1.11	1.21	0.83	1.21
Albumin	0.83	0.88	0.77	0.99
Citrate	3.88	4.30	1.87	3.92
Fat	0.24	0.33	0.17	0.22

[1] Milk 1 was collected 3 hours after intravenous injection of ^{14}C-glutamate.
[2] Milk 2 contained pooled samples of milk collected between 3 and 48 hours after labeled glutamate was injected.

TABLE 3

Cumulative recovery of carbon-14 in respired air and milk constituents following intravenous injection of U-^{14}C-glutamate in each of four experiments

	Percentage of injected dose appearing in product							
	Exp. no.							
	1		2		3		4	
Respired CO_2 (3 hr)	37.6		38.2		34.2		41.4	
Time of sample	3 hr	48 hr	3 hr	48 hr	3 hr	48 hr	3 hr	48 hr
Milk constituents								
Lactose	1.50	6.66	1.52	6.09	3.48	6.73	1.30	7.29
Casein	0.90	3.35	0.91	3.07	3.39	5.44	2.18	5.07
Albumin	0.10	0.47	0.12	0.44	0.45	0.84	0.26	0.71
Citrate	0.13	0.48	0.15	0.46	0.31	0.50	0.28	0.66
Fat	0.07	1.21	0.05	1.33	0.14	1.03	0.12	1.28
	2.70	12.17	2.75	11.39	7.77	14.54	4.14	15.01
Milk and CO_2	40.3		41.0		42.0		45.5	

TABLE 4

Specific activities of casein amino acids recovered from milk 3 hours and 48 hours following intravenous injection of U-^{14}C-glutamate in the cow [1]

	Exp. no.						
	1		2		3		4
Time of sample	3 hr	48 hr	3 hr	48 hr	3 hr	48 hr	3 hr
	μCi ^{14}C per gram atom carbon per mCi injected						
Amino acids							
Glutamate	9.68	3.97	12.6	4.36	37.8	2.65	27.0
Aspartic	6.66	2.56	11.3	—	17.9	1.73	15.1
Serine	2.26	2.36	6.22	1.60	—	1.27	5.46
Alanine	2.68	1.65	5.12	1.99	6.56	0.88	4.08
Glycine	—	1.49	3.43	1.75	5.40	1.27	—
Proline	1.24	0.87	1.54	0.77	3.80	0.61	2.87
Arginine	0.46	0.66	0.08	0.72	0.88	—	—

[1] See Methods for details of experiments.

acids show that only 10 to 20% of the proline and arginine had been derived from glutamate in the cow. Very low levels of isotope were observed in valine, threonine, leucine, lysine and histidine, which are known to be essential amino acids for the cow (22); therefore, their significant synthesis from glutamate would not be expected.

Citrate. Because of the very high specific activity in citrate, which exceeded that in casein glutamate in the 3-hour samples, some citrate samples were partially degraded; and, from the intramolecular distribution of isotope, an attempt was made to establish the metabolic relationship between this compound and glutamate. The results shown in table 5 provide only partial clues but these indicate that much of the citrate was derived directly from α-ketoglutarate by CO_2 fixation, which agrees with evidence from perfused mammary gland experiments (23). This route incorporates CO_2 carbon into the C-6 position of citrate which had the lowest specific activity in the molecule. The remaining carbons of citrate would be derived directly from glutamate, via α-ketoglutarate.

Although the specific activity of citrate was greater than that of casein-glutamate at 3 hours it was less in the later milk samples (see tables 2 and 4). These relationships presumably reflect the greater time interval between synthesis and secretion in milk for casein compared with citrate.

TABLE 5

Distribution of carbon-14 in citrate isolated from milk after ^{14}C-glutamate injection

	Exp. no.		
	1	2	3
	μCi per gram atom carbon per mCi injected		
C_1+C_5 [1]	10.45	12.89	13.27
$C_2+C_3+C_4$	16.32	20.12	19.20
C_6 [1]	0.24	0.52	1.02
Total specific activity	11.68	14.44	14.19
Carbon-14 recovery in degradation	97.1%	80.2%	72.9%

[1] C_1 and C_5 designate terminal carboxyls; C_6 designates the middle carboxyl.

DISCUSSION

Glutamic acid is the most abundant component of milk protein comprising 19.8% of the amino acid residues (5). On this basis, one might anticipate extensive incorporation of ^{14}C-glutamate into milk protein. In fact, only 3.5 to 6.3% of the carbon-14 from glutamate was recovered in milk proteins during 48 hours after adding the labeled glutamate to the plasma pool, which indicates that there was extensive utilization of glutamate carbon for many processes other than protein synthesis. On the assumption that the ^{14}C-glutamate had all been removed from the body pool at 48 hours and from the percentage recovery of carbon-14 in milk protein, one may estimate that the flux through the glutamate pool must have been some 16 (100/6.3) to 29 (100/3.5) times greater than the amount of glutamate actually in-

corporated into the protein fractions of milk.

More intriguing, in view of the large glutamate content of milk protein, was the extensive recovery of carbon-14 in the lactose of milk. In all experiments, whether the cow was fed or not, the isotope recovered in lactose over 48 hours was greater than in milk protein; in the nonfed cow receiving ^{14}C-glutamate portally, the differential was nearly twofold in favor of lactose. Thus, it is evident that gluconeogenesis acquires a larger fraction of the glutamate carbon than does protein synthesis during milk formation. One could argue that the greater recovery of isotope in carbohydrate relative to protein simply reflects the transient effects of milking and was, therefore, an experimental artifact. The cow was completely milked just before ^{14}C-glutamate injection; thus, if this condition resulted in a preferential stimulation of gluconeogenesis, then more of the label would be transferred to carbohydrate soon after the injection, when the pool was still highly labeled. This explanation must be rejected, however, as the ratio of carbon-14 recovered in lactose/protein was always greater after 48 hours than it was after 3 hours (table 3).

The most reasonable explanation for the results observed would be that the rate and extent of glutamate catabolism in the cow is much greater than the rate and extent of its utilization for protein synthesis. Several factors support the conclusion that glutamate has a very active and diverse metabolic role. It has been shown that glutamate disappeared from the plasma with a half-life of about 5 minutes during the initial period after injection of the labeled glutamate (fig. 1). This change was accompanied by the very rapid appearance of carbon-14 in respired CO_2 and plasma glucose (figs. 2 and 3), demonstrating that tissue uptake and metabolism of glutamate paralleled its rapid disappearance from the plasma. Consequently, carbon of maximal specific activity appeared in respired CO_2 in about 12 minutes and in plasma glucose in about 20 minutes after the labeled glutamate was added to the plasma glutamate. The high specific activity in citrate and in aspartate, which is derived from oxaloacetate, demonstrated that the carbon from glutamate entered extensively into reactions of the tricarboxylic acid cycle. Since the cycle is intimately involved in gluconeogenesis (20, 24, 25) through the intervening role of oxaloacetate, it is apparent that these relationships favor extensive transfer of carbon from glutamate into carbohydrate synthesis in the intact animal.

A perennial question that has plagued investigators working with intact animals concerns the interpretation of isotope data when labeled carbon from a compound equilibrates extensively with active metabolic pools in the body. Under these conditions, product-precursor relationships become diffuse, and the physiological significance of isotope transfer among metabolites is difficult to assess accurately. This problem has been considered by various investigators (26–29), most recently by Krebs et al. (30) in relation to gluconeogenesis from ^{14}C-lactate and ^{14}C-acetoacetate in kidney slices. Krebs et al. concluded that when there was a "crossing over" of carbon between substrates of respiration and gluconeogenesis (in the tricarboxylic acid cycle), the fate of a label does not reflect the *net* fate of a labeled metabolite. It is this phenomenon which accounts for the extensive transfer of isotope from some even-chain fatty acids to carbohydrate even though animals lack a pathway for the net synthesis of glucose from these substrates. This matter has been reviewed by Weinman et al. (29) and has been discussed for the ruminant in relation to the paradoxical behavior of butyrate-^{14}C (31, 32). Unlike the case with butyrate, there is a pathway for net conversion of carbon from glutamate to glucose, via the tricarboxylic acid cycle. This fact, together with the rapid appearance of substantial levels of carbon-14 in plasma glucose and the recovery of 6 to 7% of the isotope in lactose during the same interval (48 hours) that milk protein acquired 3 to 6% of the glutamate-^{14}C, clearly demonstrates a glucogenic role for plasma glutamate in the lactating cow and one which, quantitatively, is more important in gluconeogenesis than in protein synthesis.

The transfer quotient, which is the specific activity ratio of plasma glucose/plasma glutamate, provides one means for

estimating the precursor role of glutamate in glucose synthesis. In experiments 1 and 2, respectively, the standardized specific activities over 24 hours were 2.0 and 1.7 for plasma glucose, and 24 and 22 for plasma glutamate. The ratios for these figures indicate that approximately 8% of the glucose carbon had been derived from plasma glutamate. This value does not necessarily reflect the overall role of glutamate as a glucogenic precursor in the cow since it is valid only for glutamate which equilibrates (in specific activity) with the plasma pool. Within certain cells, the labeled glutamate entering from the plasma pool may be diluted by glutamate derived from protein turnover or glutamate synthesized from unlabeled precursors in the cell. These processes would reduce the specific activity of the glutamate at the site of gluconeogenesis, and the estimate of glucose synthesis based on plasma glutamate would then be too low.

The specific activity of glutamate recovered from casein (see table 4) in the 3-hour and 48-hour milk samples was considerably lower than the average value measured in plasma glutamate. This difference reflects a dilution by the mammary gland which has been shown to incorporate carbon from fatty acids and carbohydrate into glutamate (33). Thus, an estimate of the quantitative precursor role of glutamate in the mammary gland based on plasma glutamate specific activity would be erroneously low, and it seems likely that a similar situation would occur in all tissues, including the liver where most gluconeogenesis occurs. It would be necessary to have the specific activity of glutamate at the site of gluconeogenesis to get a more accurate estimate of its precursor role in the cow. This information was not obtained; thus, the estimate based on plasma glutamate, while almost certainly too low, is the best value available.

The transfer quotient for casein based on the specific activity of mammary gland (casein) glutamate (table 4) ranged from 0.23 to 0.36 (specific activity ratio for casein/glutamate) for the various milk samples. The lower figure corresponds closely to the glutamate composition of casein (22.4%), and the higher values reflect the presence of carbon-14 in other amino acids that the cow synthesizes.

Any quantitative values for product-precursor relationships are necessarily approximations in an organism as complex as the intact mammal. In any case, such evaluations would be applicable only to those conditions under which the measurements were carried out, since many variables could affect these relationships and lead to different results. For example, data from experiments 3 and 4 indicate that availability of additional gluconeogenic material and injection of labeled glutamate into peripheral circulation to avoid an initial passage of the amino acid through the liver are both factors which divert somewhat more glutamate toward protein synthesis. Recoveries of carbon-14 in milk casein and albumin were higher in both experiments, but there was no simultaneous decrease in lactose. Although the quantitative aspects of glutamate utilization in the lactating cow are uncertain, it seems clear that when there are both protein synthesis and gluconeogenesis, the latter process will acquire a greater part of the glutamate carbon, even in those conditions which presumably provide ample levels of alternate glucogenic substrate.

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Influence of Environmental Temperature and Dietary Fat on Backfat Composition of Swine [1]

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ABSTRACT Three groups of growing barrows were exposed to environmental temperatures of 0 to 5° and 25 to 30° and fed diets containing either no supplemental fat, 10% corn oil, or 10% beef tallow. Food intake was restricted on all treatments to 2 kg/pig per day. In animals fed the unsupplemented diet and to a lesser extent in those fed the tallow diet at either environmental temperature, there was a temperature gradient from outer to inner backfat layer inversely related to total fat unsaturation (iodine value). With the corn oil-supplemented diets, no clear relationship between temperature gradient of backfat layer and degree of fat unsaturation was established. Although the pigs exposed to the cold temperature exhibited greater backfat unsaturation than those exposed to the warm environment with all dietary treatments, the backfat from pigs fed the unsupplemented diet and exposed to the cold environment nevertheless decreased in total unsaturation. It was concluded that the relationship between depot fat unsaturation and environmental temperature is influenced by the intake of polyunsaturated fatty acids which are not synthesized by the pig.

Henriques and Hansen (1) and Dean and Hilditch (2) reported an inverse relationship between environmental temperature and extent of depot fat unsaturation in swine. These workers also showed that a temperature gradient existed from outer to innermost backfat layer and that the degree of unsaturation of these fat stores was also inversely related to the temperature of the tissue in which the fat was embedded. Consequent to these findings, Hilditch and Williams (3) postulated that depot fat saturation is conditioned by the temperature of the tissue in which fat is deposited. In support of this hypothesis, Hilditch pointed to the relative lack of insulation on the skin of the pig, which was probably responsible for the body temperature gradient of this species, and contrasted this with the absence of differences in composition of depot fat from various sites in the hen, a species that derives considerable insulative protection from feathers. Some doubt concerning a direct relationship between body temperature and degree of depot fat saturation was expressed by Fisher et al. (4), who found an increase in depot fat unsaturation in hens maintained in a cold environment despite the absence of any change in body temperature measured at the side of the depot fat sampling. More recent studies from our laboratory (5) have suggested that level of food intake, particularly as it reflects environmental temperature, may be an important influencing factor relative to fatty acid composition of depot fat. In light of these observations, the present study was undertaken to reinvestigate the relationship between environmental temperature and backfat composition in swine under conditions of different dietary fat and restricted food intake.

EXPERIMENTAL

General. Three trials were carried out using Yorkshire barrows maintained at environmental temperatures of 0 to 5° and 25 to 30°, respectively, for 6 weeks.

Four barrows per treatment were fed 2 kg/day of diets containing either a) no added fat, b) 10% corn oil or c) 10% beef tallow. The composition of the experimental diets is given in table 1. The environmental chambers were constructed to house four pigs, which was the number used in each of the treatment groups. Each

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TABLE 1

Composition of experimental diets

	Trial		
	1	2	3
	%	%	%
Ground yellow corn	72.1	62.1	62.1
Soybean meal (50% protein)	25.5	25.5	25.5
Corn oil	—	10.0	—
Beef tallow	—	—	10.0
Limestone	0.9	0.9	0.9
Defluorinated rock phosphate	0.5	0.5	0.5
Mineral mix [1]	0.5	0.5	0.5
Vitamin premix [2]	0.5	0.5	0.5
Total fat, %	3.15	14.01	13.65
Fatty acids:			
$C_{14:0}$	tr	tr	2.64
$C_{16:0}$	13.33	12.61	21.02
$C_{16:1}$	tr	tr	4.17
$C_{18:0}$	2.14	3.00	20.35
$C_{18:1}$	29.86	26.88	34.37
$C_{18:2}$	52.78	52.97	16.27
$C_{18:3}$	1.89	4.53 [3]	1.18

[1] Contained not less than: (in percent) NaCl, 94.5; Co, 0.015; Cu, 0.050; I_2, 0.015; Fe, 0.200; Mg, 0.600; (SO_4^{2-}), 0.050; and Zn, 0.800.

[2] Contributed the following: (per kilogram of diet) vitamin A, 1508 IU; vitamin D_2, 660 IU; riboflavin, 5.28 mg; calcium pantothenate, 9.68 mg; niacin, 23.76 mg; choline chloride, 26.4 mg, and vitamin B_{12}, 22 μg.

[3] The peak for $C_{18:3}$ was not sharply defined, and the value obtained appears unduly high in view of the low linolenic acid content of corn oil (ca. 1%).

chamber was 3 by 1.8 by 2.5 m and contained a feed trough and a conventional automatic waterer. The cold chamber was maintained at 0 to 5° with an air-cooled refrigeration unit, and the warm chamber was maintained at 25 to 30° temperature with a thermostatically controlled heater. Each chamber was provided with an electric fan to allow three air changes per hour, with manually controlled electric lights and with a 7-day temperature recorder.

Sampling and temperature measurements. Subcutaneous fat temperature readings were taken at the end of each environmental temperature exposure; fat biopsies were carried out before and after each exposure to a different environmental temperature. The animals were placed in a squeeze chute, and the area between the second and third rib, 10 cm from the midline, was clipped of all hair and then thoroughly cleaned and disinfected. After a 1-cm incision had been made through the skin, a thermistor was inserted, and temperatures were recorded at every centimeter depth. For the biopsy, a no. 3 cork borer was inserted directly through the skin to the muscle layer to obtain a fat sample which was severed from the muscle layer and removed under mild suction (applied by mouth to the open end of the cork borer). After removal of the sample, the antiseptic [(*o*-carboxyphenyl)thio] ethylmercury sodium salt [2] was applied to the area. The adipose cores were stored in glass vials under nitrogen at − 5°.

Lipid extraction. The frozen adipose cores were cut in half longitudinally to facilitate analysis of fatty acids from the inner and outer layers, and by centimeter intervals corresponding to the sites at which temperatures were recorded. The material for analysis was extracted in a Potter-Elvehjem homogenizer with a small volume of chloroform–methanol (2:1, v/v) as described by Folch et al. (6). The homogenate was filtered through Whatman no. 40 filter paper and washed with sufficient solvent to ensure complete extraction. The crude extract was mixed thoroughly with 0.2 times its volume of water and centrifuged into two phases without interfacial fluff. The upper phase was removed by siphoning and the interface washed three times with small amounts of chloroform–methanol–water (3:47:48, v/v/v). The final extract was dried with a vacuum evaporator at 35°, dissolved in hexane, washed into a small test tube, sealed under nitrogen and stored at − 5°.

Saponification and esterification. The hexane extract was dried under nitrogen at a temperature of 35° and saponified with 3 ml of freshly prepared 10% potassium hydroxide in methanol for 4 hours in a water bath at 60°. The resultant mixture, after cooling, was diluted with water, shaken with hexane and centrifuged. The fatty acids were then liberated by addition of 0.5 ml concentrated hydrochloric acid. The free fatty acids were extracted, by thoroughly mixing and centrifuging the acidified solution three times with 2 ml of hexane, and dried under nitrogen. Two millimeters of boron trichloride-methanol

[2] Merthiolate.

reagent was added to the dried fatty acids. The esterification was carried out in a boiling water bath for 3 minutes. The methyl esters were extracted with hexane, washed with distilled water and stored under nitrogen at − 5°.

Gas chromatography. Separation and analysis of the methyl esters of the fatty acids were carried out on a hydrogen flame ionization instrument.[3] A 1.82-m long column, 6.25 mm in diameter and containing 80 to 100 mesh Gas Chrome A coated with 17% ethylene glycol succinate polyester, was used at a temperature of 185°, a detector temperature of 230° and an injector temperature of 230°. The nitrogen, hydrogen and air flow rates were 25, 25 and 385 ml/minute, respectively. The identification of the methyl esters of the straight-chain, unsaturated and saturated fatty acids was made on the same column by comparing the retention times of the peaks of the sample with those of known reference esters. The weight percent of the various methyl esters was calculated from the relative areas under the peaks, determined with a Disk integrator. Total unsaturation was expressed in terms of iodine values calculated from the fatty acid composition.

RESULTS AND DISCUSSION

Body weight changes. Animals on the same diet (table 2) exposed to a temperature of 25 to 30° gained weight significantly faster ($P < 0.01$) than their counterparts exposed to a temperature of 0 to 5°. The barrows receiving 10% corn oil and maintained at 25 to 30° were significantly heavier in weight (table 2) than those fed the 10% beef tallow ($P < 0.05$) or the unsupplemented diet ($P < 0.01$). At a temperature of 0 to 5° animals on the fat-supplemented diets gained significantly more weight ($P < 0.01$) than those on the unsupplemented diet. This is undoubtedly a normal reflection of the lower calorie intake with the unsupplemented diet. Although a significant difference ($P < 0.05$) was observed in the weight gains between tallow- and corn oil-supplemented groups at the higher environmental temperature, no such difference was noted at the lower temperature. This suggests that the relatively saturated tallow fat may have been better utilized at the low environmental temperature than at the higher temperature. The possibility cannot be ruled out, however, that the greater initial weight of the corn oil-fed pigs might have unduly influenced the weight gain of this group of animals.

Backfat unsaturation and body temperature. Table 3 shows temperatures at 1, 2 and 3 cm of backfat depth and the calculated iodine values of the fat at the same site. A definite temperature gradient was evident: The first centimeter of backfat had the lowest temperature; and the third centimeter (next to the muscle layer), the highest. There were considerable differences in body temperature not only between the two environmental groups on the same diet, but also among the three dietary treatments at the same environmental temperature. From the point of view of the major objectives of this study, attention is called to the clear inverse relationship between the body temperature and degree of backfat unsaturation for the barrows receiving the diet without added fat. On this treatment, an inverse relation-

[3] Model 5300, Barber-Colman Company, Rockford, Illinois.

TABLE 2

Weight gain and feed intake of barrows fed no added fat, corn oil or beef tallow for 6 weeks at environmental temperatures of 0 to 5° and 25 to 30°

	Trial 1 (no added fat)		Trial 2 (corn oil)		Trial 3 (beef tallow)	
	0–5°	25–30°	0–5°	25–30°	0–5°	25–30°
Feed per day per animal, kg	2	2	2	2	2	2
Initial weight, kg	75.8	74.5	80.1	81.6	74.3	73.4
Gain, kg	6.6	18.8 [3]	15.1 [3]	28.5 [1,4]	16.8 [3]	23.9 [1,2]
Backfat core thickness, cm	3.0	3.1	3.0	3.2	3.0	3.0

[1,2,3] Values with the same superscript are not significantly different from each other at $P < 0.01$.
[4] Values with the same superscript are not significantly different from each other at $P < 0.05$.
Four animals per group.

TABLE 3

Body temperature and total unsaturation (iodine values) at each centimeter depth of backfat from barrows exposed to temperatures of 0 to 5° and 25 to 30° for 6 weeks and fed diets containing 10% corn oil, 10% beef tallow and no added fat

Environmental temperature	Measurement	Backfat depth		
		1 cm	2 cm	3 cm
			No added fat	
25–30°	Body temperature [1]	32.9 ± 1.0	37.2 ± 0.7 [a]	38.4 ± 0.4 [a]
	Iodine value [2]	76.3	72.8	69.1
0–5°	Body temperature	25.8 ± 0.8	28.9 ± 0.8 [b]	30.4 ± 0.8 [b]
	Iodine value	77.6	73.5	71.9
			10% corn oil	
25–30°	Body temperature	27.9 ± 0.7 [c]	31.9 ± 0.6 [d]	37.1 ± 0.9
	Iodine value	82.1	85.6	76.1
0–5°	Body temperature	23.1 ± 0.8	26.6 ± 1.0 [c]	33.1 ± 0.1 [d]
	Iodine value	78.2	82.7	81.3
			10% beef tallow	
25–30°	Body temperature	36.2 ± 0.8 [f]	39.1 ± 0.7 [e]	39.6 ± 0.4 [e]
	Iodine value	72.6	71.4	70.9
0–5°	Body temperature	30.0 ± 0.2	34.4 ± 0.3 [f]	38.8 ± 0.4 [e]
	Iodine value	76.3	75.1	75.4

[1] Temperature values with the same superscript are not significantly different within the same dietary treatment; all other temperatures within each dietary treatment are significantly different ($P < 0.05$).

[2] Calculated from the fatty acid composition.

ship was noted for both the low and high environmental temperatures in conformity with the earlier observations of Henriques and Hansen (1) and of Dean and Hilditch (2). Also, the degree of unsaturation was slightly greater for fat from cold-exposed pigs as compared with that from warm temperature-exposed animals fed the diet with no added fat. No relationship between total fat unsaturation and body temperature, however, was noted for the groups of swine receiving corn oil-supplemented diets, nor was there any relationship for these groups between fat unsaturation and environmental temperature.

The results for the beef tallow-supplemented pigs showed a slight trend toward an inverse relationship between body temperature and backfat unsaturation. There was a pronounced difference in fat unsaturation between the cold and warm temperature-exposed groups in line with the observations for the groups fed the unsupplemented diets.

The degree of fat unsaturation for almost all backfat samples from the animals fed the unsupplemented or tallow diets was less than that for the backfat from the animals fed the corn oil-supplemented diets.

Changes in fatty acid composition of inner and outer fat layers. The fatty acid composition (table 4) of the inner and outer fat layers of swine before exposure to dietary or temperature treatment was similar to that reported by others (2, 7, 8). The major fatty acids were palmitic, stearic, oleic and linoleic acids. Myristic, palmitoleic and linolenic acids were also present in small concentrations as were trace amounts of lauric and arachidonic acid. There were no major differences between inner and outer fat layers before the start of the experimental treatments, although the outer layer was slightly more

TABLE 4

Average concentration with standard error of major fatty acids (weight percent of total methyl esters) in inner and outer fat layers of all barrows prior to treatment (total of 24 animals)

Fatty acid	Fat layer	
	Inner	Outer
	%	%
Palmitic	21.6 ± 0.5	20.4 ± 0.5
Stearic	11.9 ± 0.4	10.2 ± 0.5
Oleic	45.8 ± 0.8	47.6 ± 0.7
Linoleic	13.1 ± 0.3	13.1 ± 0.4
Iodine value [1]	74.6	77.6

[1] Calculated from the fatty acid composition.

TABLE 5

Percentage changes in fatty acid composition as a result of different dietary fats and environmental temperature exposure [1]

Fatty acid	Backfat layer	No added fat		10% corn oil		10% beef tallow	
		0–5°	25–30°	0–5°	25–30°	0–5°	25–30°
Palmitic	inner	6.9	11.6	−6.9	2.3	−8.3	1.8
	outer	7.4	13.7	−5.4	−3.9	−6.8	4.4
Stearic	inner	22.7	25.2	6.7	15.1	−8.4	7.6
	outer	0.9	11.8	3.9	0.0	−8.8	18.6
Oleic	inner	−8.3	−10.5	−5.7	−15.2	8.3	2.0
	outer	−6.1	−9.9	−5.2	−12.2	8.2	−0.4
Linoleic	inner	−3.8	1.5	27.4	38.1	−12.2	−18.3
	outer	7.6	11.4	29.0	54.9	−10.7	−17.6
Iodine value [2]	inner	−6.2	−8.2	5.9	2.3	0.7	−6.0
	outer	−1.4	−3.9	11.4	9.8	0.3	−7.5

[1] All changes are calculated on the basis of the preexperimental values given in table 4.
[2] Calculated from the fatty acid composition.

unsaturated as a result of lower palmitic and stearic acid concentrations and a slightly higher oleic acid level.

Table 5 shows the percentage change in fatty acid composition following exposure to the two environmental temperatures and the restricted consumption of the three differently supplemented diets. There were considerable increases in the palmitic and stearic acid content with the unsupplemented diet, particularly in the inner fat layer, irrespective of environmental temperature. These changes were accompanied by a decreased oleic acid concentration to the extent that the total degree of unsaturation decreased in the inner and, to a somewhat smaller extent, in the outer fat layer. The results from swine fed the corn oil-supplemented diet showed a highly increased linoleic acid content, probably reflecting the fatty acid composition of corn oil, a concomitant decrease in oleic acid content and a somewhat smaller decrease in palmitic acid. Despite the relatively large increase in linoleic acid, the total degree of unsaturation increased only slightly in the inner fat layer, but in the outer fat layer the total degree of unsaturation increased substantially. This reflects the absolute concentration of the fatty acids, with linoleic being present at only about one-fourth the concentration of oleic acid.

The fat from the tallow-fed barrows showed an appreciable decrease in linoleic acid content at both environmental temperatures; and, at the low environmental temperature, a decrease in palmitic and stearic acids and an increase in oleic acid. The overall change at 0 to 5° was a negligible increase in total unsaturation. The animals exposed to the higher environmental temperature showed, in addition to the marked decrease in linoleic acid, variable increases in palmitic and stearic acid and negligible changes in oleic acid, leading to a small decrease in total unsaturation.

The cold-exposed pigs within each of the three dietary treatments had a greater degree of backfat unsaturation than the warm-exposed animals. This finding is particularly noteworthy in view of the absolute reduction in total unsaturation which occurred in the backfat of all the pigs fed the unsupplemented diet and some of those fed the tallow-supplemented diet.

GENERAL COMMENTS

The results of this study show that the effects of cold and warm temperatures on fatty acid composition of depot fat are more complex than suggested by earlier works (1, 2). In the present investigation, different dietary fats were used as additional experimental variables, and all animals received the same feed daily, but in restricted amounts. This contrasts with earlier studies (1, 2) in which feed was consumed ad libitum and the diet contained no supplemental fat. Under these

conditions it is perhaps not too surprising that the degree of total fatty unsaturation should have varied inversely with environmental temperature. Melting point and physical consistency of fats are dependent on fatty acid unsaturation and, in the absence of dietary polyunsaturated fatty acids which are not synthesized by swine, the extent of depot fat unsaturation would depend essentially on the oleic acid synthesized by the animal. When the diet contains polyunsaturated fatty acids, however, depot fat unsaturation is no longer a function of oleic acid concentration alone, but depends also on intake and catabolism of the polyunsaturated fatty acids. Under these conditions, the physical characteristics of the fat will depend to a greater extent on structural features of the triglycerides and less on total unsaturation.

In the context of a more complex fatty acid profile, as it existed for the corn oil-fed pigs in this study, the concept of "total unsaturation" no longer has the same meaning as originally ascribed to it by early workers in this field. This is suggested by the results in table 5 which show relatively small changes in total fat unsaturation among certain dietary groups; whereas changes of rather large magnitude occurred, often in opposite directions, for individual fatty acids.

In conclusion, the present results confirm that cold temperature exposure results in greater depot fat unsaturation than exposure to warm temperature, irrespective of dietary fat (table 5). However, the iodine value of the backfat of the pigs kept on the unsupplemented diet showed a distinct decrease when the animals were changed from a moderate to a cold environment. Thus, the effect of environmental temperature on depot fat unsaturation is not simple.

ACKNOWLEDGMENT

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Postnatal Nutritional Deprivations as Determinants of Adult Rat Behavior toward Food, Its Consumption and Utilization [1]

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ABSTRACT During the first 7 weeks of life, rats were subjected either to restriction of the quantity of diet of optimal composition or to protein-deficiency malnutrition to learn more about the animals' behavior toward food several months following complete nutritional rehabilitation. The day following parturition female rats were fed a low protein diet (12% casein) for the duration of the nursing period (3 weeks). This resulted in a decreased milk supply and a consequent retardation in the growth of the pups. At weaning, some of the restricted pups were fed for 4 weeks a severely protein-deficient diet (5% casein) which prevented any weight gain during this period (designated low protein). Others were given a diet of optimal composition but in restricted amount so they did not grow during the 4-week experimental period (designated restricted). All animals were then given the normal control diet ad libitum. When adult weight had been reached (6 months of age), the previously low protein and restricted rats were smaller than the controls. Expressed on the basis of body weight$^{0.75}$, food intake was significantly greater in the previously low protein rats. It is believed this may be accounted for by altered body composition, with a larger percentage of metabolically active tissue in the previously low protein rats. When food spillage was measured in rats fed ad libitum, there was no difference between controls and previously restricted or low protein rats. However, when food availability was limited to 1 hour each day, there was a threefold increase in food spillage in previously low protein, male rats and a significant but much smaller increase in previously restricted, male rats as compared with controls. No difference in food consumption or food spillage was observed in female rats. The food spillage of previously malnourished rats is believed to be one of several manifestations of elevated excitement under stress that have been noted in other studies with adult animals that were severely malnourished in early life. The present studies also confirm and extend previous observations from our laboratories which conclusively show that postnatal nutritional stunting of adult body size is not necessarily correlated with behavioral development. Behavioral abnormalities in the adult that are due to postnatal nutritional deprivations, however, always appear to be accompanied by a stunting of body size. Nitrogen balance studies with both casein and wheat gluten showed no effect of early postnatal malnutrition on nitrogen retention in the adult.

A restriction in the quantity of food consumed by a rat in the first 3 weeks of life determines not only growth rate during that time, but also subsequent growth characteristics when unlimited food is available. For example, Jackson and Stewart (1) intermittently removed rat pups from mothers so that total nursing time was reduced. Following weaning and subsequent unlimited availability of food, ultimate body size was reduced. Similarly, Kennedy (2) observed that after varying the number of pups suckling one lactating female, size at weaning was greatly affected, and subsequent growth and ultimate body size were less for the rats nursed in large litters than for those nursed in small litters. Schultze (3) employed still another technique for restricting food intake of nursing rats, which was to feed the mothers an amino acid diet that had been shown to be relatively poor for growth and lactation. However, this nutritionally marginal diet was fed to the mothers during the gestation period as well as during lactation, so that relative contribution of poor maternal nutrition during these two periods cannot be assessed. Nevertheless, Schultze pre-

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sumed that the permanent stunting he observed was due to the nutritional restriction that had been imposed during the first few weeks of life. Extension of studies dealing with nutritional restrictions in early life has been carried out by numerous investigators. Notable among these, by reason of their intensive investigation of the subject, has been the work of Widdowson and McCance (4, 5). Chow and Lee (6) and Lee and Chow (7) have greatly extended the investigation of the combined maternal nutritional deprivation during gestation and lactation.

There is general agreement that permanent stunting of body size can be caused by a relatively short period of nutritional restriction during the early life of the rat. Apparently this period does not have to encompass the first 3 weeks of life, for permanent growth effects have been noted when severe restriction was imposed for varying periods of time immediately following weaning of rats that had been normally nursed (1, 8). There is evidence in both of these studies which indicates that preweaning nutritional restriction was more effective than postweaning restriction in causing subsequent body-weight stunting.

Kennedy (2) and Widdowson and McCance (5) found a decreased efficiency of food conversion in rehabilitating slower growing rats that had been nursed in large litters. On the other hand, Heggeness and his associates (9), who have used a modification of the large litter vs. small litter technique for obtaining wide differences in preweaning food intake, found no difference in the efficiency of food utilization for growth in the postweaning stage of growth.

Body composition of rats is affected by their early postnatal nutrition. Heggeness et al. (9) found equal concentrations of protein in the carcasses of both fast-growing and slow-growing rats at weaning age, but considerably higher fat in the fast-growing animals. Widdowson and McCance (5), however, found that fast-growing animals at 3 weeks of age accumulated more fat than slower-growing animals, but this difference in body composition disappeared some weeks after weaning. Another characteristic noted in adult rats following nutritional rehabilitation from severe protein restriction in early life is an altered behavior toward food (10). Careful studies of food consumption and body composition in adult rats that have reached a plateau in body weight, but differ in ultimate size because of nutritional restriction in early life, have not been made; and it is this area of study, as well as a more detailed examination of behavior toward food, that forms the basis for the present report.

MATERIALS AND METHODS

Previous publications from this laboratory dealing with the behavioral effects of postnatal nutritional restrictions in rats have involved the use of the large litter–small litter technique of obtaining wide differences in milk consumption during the nursing period (8, 10). In the present study, mothers of newborn rats of the Holtzman strain were switched from a purified diet containing 25% casein to one with 12% casein on the day following parturition (table 1). Litter size was uniformly adjusted to eight pups. At 3 weeks of age, the young rats were weaned. Those from mothers which had received the 25% casein diet continued to be fed this diet. These have been labeled C–C indicating control diet during both the nursing and postweaning periods. Those from mothers which had received the 12% casein diet were divided into three groups. One group received a 25% casein diet at weaning (R-C for restricted preweaning and control diet postweaning). A second group received the 25% casein diet at weaning but in restricted quantity for 4 weeks so as to maintain constant body weight (R-R for restricted preweaning and restricted 4 weeks postweaning). The third group received a 5% casein diet for 4 weeks postweaning (R–LP for restricted preweaning and low protein postweaning). All rats at 3 weeks of age were individually caged, and at 7 weeks all were receiving the 25%-casein purified diet. Both male and female offspring were used. There were approximately 20 rats in each dietary group at weaning; the exact numbers are given in the legends for the growth curves (figures 1 and 2). The females were discarded after 26 weeks; and the R-C group was discontinued at an early age and is not included

TABLE 1

Diet composition

	25% casein diet	12% casein diet	5% casein diet
	g	*g*	*g*
Major components			
Casein [1]	25	12	5
Glucose monohydrate [2]	53	66	73
Hydrogenated vegetable oil [3]	15	15	15
Salt [4]	4	4	4
B-vitamins in sucrose	2	2	2
Fat-soluble vitamins in corn oil	1	1	1
Total	100	100	100

B-vitamins in 2.0 g sucrose	*mg*
Thiamine·HCl	0.40
Riboflavin	0.80
Pyridoxine·HCl	0.40
Ca pantothenate	4.00
Niacin	4.00
Inositol	20.00
Biotin	0.02
Folic acid	0.20
Vitamin B_{12}	0.003
Menadione	1.00

Fat-soluble vitamins in 1.0 g corn oil	*mg*
Vitamin A acetate	0.31
Vitamin D (calciferol)	0.0045
α-Tocopherol	5.00

[1] Vitamin-Test Casein, General Biochemicals, Chagrin Falls, Ohio.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Primex, Procter and Gamble Company, Cincinnati.
[4] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutr., *14*: 273.

in experimental studies reported in this paper, but was used in behavioral studies published separately (11, 12). At 23 weeks of age food intake was measured daily for 1 week; and approximately 1 week following this, food was provided for only 1 hour each day for 5 days. Food consumption and food spillage were measured, and the rats' behavior during the 1-hour/day feeding was observed.

After the 1-hour/day feeding period, 12 male rats from the control group and 11 from the R-LP group were placed in individual metabolism cages; each group was divided into two subgroups. Diets containing 10% protein from either casein or wheat gluten were fed to each of the subgroups for 14 days. During the final 6 days, urine and feces were collected daily and analyzed individually for nitrogen. Food intake was measured so that nitrogen balance could be calculated. The male rats were then fed the 25% casein control diet for 3 weeks, and the animals were killed by etherization. They were bled as completely as possible, and the entire intestinal tract was stripped out, leaving all omental and mesenteric tissue. The carcasses were blotted, weighed, then dried in an oven at 100° for 48 hours and weighed; 100 ml of 6 N HCl/100 g body weight were added to each dried carcass in a beaker, and this was autoclaved at 121° for 16 hours. The resulting hydrolysate was adjusted to a uniform volume, and aliquots were taken for nitrogen analysis (micro-Kjeldahl) and lipid (petroleum ether extraction) analysis.

RESULTS

Growth curves for male and female rats are shown in figures 1 and 2. Male rats were subjected to several experimental procedures that did not involve females, and the effects of these interim studies are readily seen in the male growth curves. Females were discontinued after 26 weeks and males at 32 weeks of age. Rats of the R–C treatment were discontinued following behavioral studies, but prior to the initiation of the food studies reported in this communication.

Ad libitum food consumption covering a 5-day period for three groups of male and female rats at 23 weeks of age is given in table 2. Food intake is expressed both in terms of daily intake/100 g body weight (BW) and per $BW^{0.75}$. In males there was a tendency for food consumption to be elevated in the R–R and R–LP groups, with the latter showing the greater effect. Percentage increase over controls was cut approximately in half when food consumption was expressed on the basis of $BW^{0.75}$. No apparent differences were seen in the groups of females. There was a general tendency for the experimental groups to spill more food than the controls (table 3).

One week following the ad libitum food consumption measurements, the controls and two experimental groups (R–R and R–LP) were permitted access to food for only 1 hour each day. Average food intake for 5 consecutive days is given in table 2. Again there was a tendency for the two

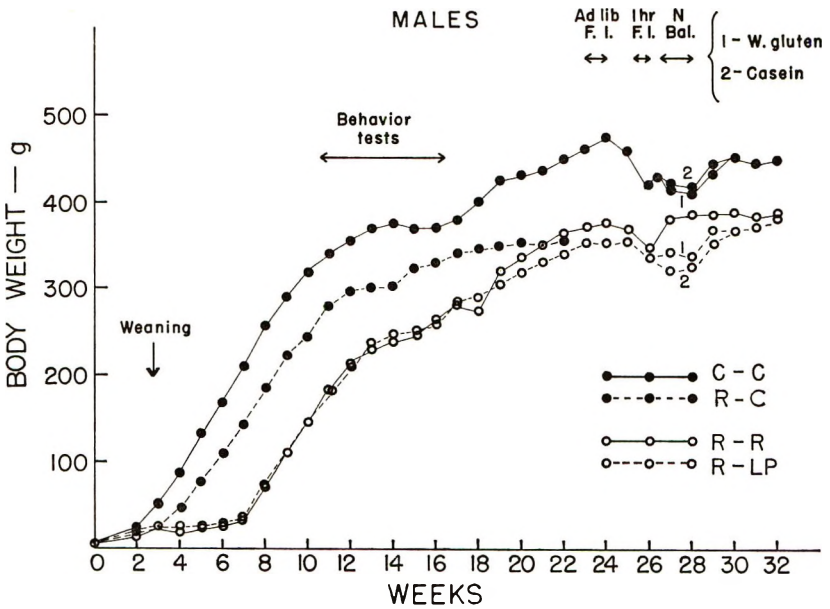

Fig. 1 Growth curves of male rats that had been malnourished during the early weeks of life. Nutritional treatment designation for the experimental rats are: C, control; R, restricted amount of normal diet; and LP, low protein (5% casein). The first letter denotes preweaning treatment and the second letter, 4-week postweaning treatment. Other labels on the graph designate times when various experimental procedures were conducted. In the interval 26–28 weeks wheat (W) gluten or casein was fed at a level of 10% protein in the diet. F.I. = food intake.

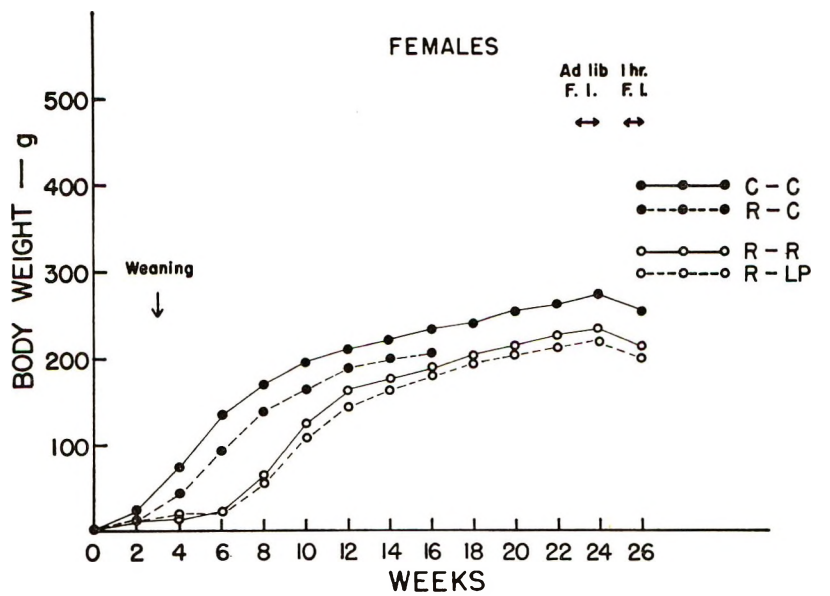

Fig. 2 Growth curves of female rats that had been malnourished during the early weeks of life. Nutritional treatment designations and abbreviations are explained in the legend for figure 1.

TABLE 2

Food intake of adult rats measured over 5-day periods under conditions of ad libitum feeding and also during a feeding schedule permitting access to food for only 1 hour daily

Group	No. of rats	5-day body wt change [1]	Food intake/ 100 g body wt	Food intake/ body $wt^{0.75}$
		g	*g/day*	*g/day*
		Males — ad libitum		
C–C (control)	12	455–471	4.3	0.203
R–R (restricted)	11	365–373	4.9 *	0.216
R–LP (low protein)	12	347–355	5.2 ***	0.224**
		Females — ad libitum		
C–C	12	262–265	5.5	0.220
R–R	12	224–228	5.4	0.208
R–LP	12	211–213	5.8	0.218
		Males — 1-hr feeding		
C–C	12	442–421	1.8	0.082
R–R	11	362–344	2.2 **	0.094
R–LP	12	342–337	2.5 ***	0.107 **
		Females — 1-hr feeding		
C–C	12	265–254	2.3	0.091
R–R	12	227–217	2.6	0.101
R–LP	12	211–201	2.8 **	0.107

[1] Average body weight at the beginning and end of the 5-day period.
* $P < 0.05$ as compared with C–C.
** $P < 0.01$ as compared with C–C.
*** $P < 0.001$ as compared with C–C.

TABLE 3

Food spillage of adult rats measured over 5-day periods under conditions of ad libitum feeding and also during a feeding schedule permitting access to food for only 1 hour daily.

Group	No. of rats	Food spillage as % food intake
		%
Males — ad libitum		
C–C (control)	12	2.9
R–R (restricted)	11	2.2
R–LP (low protein)	12	3.5
Females — ad libitum		
C–C	12	3.2
R–R	12	4.7
R–LP	12	5.1
Males — 1-hr feeding		
C–C	12	4.5
R–R	11	6.1 *
R–LP	12	12.6 ***
Females — 1-hr feeding		
C–C	12	5.6
R–R	12	7.0
R–LP	12	5.2

* $P < 0.05$.
*** $P < 0.001$.

experimental groups to consume more food, with the males showing greater differences than the females. Statistically the only group differing significantly from the controls was the male R–LP group ($P < 0.01$). Even more striking was the increased food spillage for this group as compared with controls. Experimental groups of male rats had elevated amounts of food spilled, but the male R–LP spillage was much greater than others. The amount of food spilled was not the only indication of an altered eating behavior for the experimental animals. All rats consumed food voraciously when first placed in the cage. After a short time, however, the control animals appeared satiated and left the feeder cup to lie down; but the experimental animals, for the most part, remained in constant contact with the feeder for the full hour that it remained in the cage. Rapid eating and a tendency to hoard food under conditions of restricted feeding have been described many times as a consequence of early food deprivation in rats (literature reviewed by Bronfenbrenner, 13). Another behavioral characteristic that has been

mentioned by others is an elevated level of activity of early deprived rats during the period of limited access to food. This has been observed consistently and readily differentiates the control from either R–R or R–LP rats, but is not present when continued access to a nutritionally adequate diet is provided. During the 5 days of 1-hour/day feeding, the controls lost 7.7% of their initial body weight; and the R–R and R–LP groups lost 6.8 and 5.6%, respectively. The smaller weight loss of the experimental groups correlates well with an apparently higher level of food intake, but does not reflect their elevated daily activity.

The next study was the measurement of nitrogen retention by the nitrogen balance method using a good protein (casein) and a poor protein (wheat gluten) at a level of 10% protein in the diet. These two proteins were fed to groups of six rats each from the control and the R–LP treatments. The rats were simultaneously housed in individual metabolic cages and fed one of the 10% protein diets. After 7 days, urine and feces were collected, and food consumption was measured daily for 7 days. During the last 5 days, collections were used for determining nitrogen balances, and the results are given in table 4. Small positive balances were obtained, with casein providing the greater nitrogen retention. No significant differences in nitrogen retention were obtained between control and R–LP groups for either test protein.

Subjecting the animals to restricted food intake followed by a nitrogen balance study caused them to lose weight. Therefore, a 4-week rehabilitation period was provided, and this appeared (see fig. 1) to be sufficient to stabilize body weights for the 3 groups (C, R–R and R–LP). All rats in the 3 groups were killed, and body composition was determined (table 5). Percentage protein was slightly higher for the two experimental groups than the controls, and fat was somewhat higher for the controls. The various dietary manipulations during the last 2 months of this study resulted in large changes in body weight for the controls and lesser changes for the two experimental groups. This may have influenced final body composition, particularly fat; therefore, too much emphasis should not be given to the interpretation of these results.

TABLE 4

Nitrogen balance studies in male rats with casein and wheat gluten at dietary levels of 10% protein from each source

Group [1]	No. of rats	Body wt	Protein source	Food intake	Nitrogen			
					Intake	Urine	Feces	Retention
		g		*g/day*	*mg N/100 g body wt/day*			
C–C	6	415	Casein	10.9	44	27	3	14 ± 3.1 [2]
R–LP	6	324	Casein	10.8	54	32	4	18 ± 3.0
C–C	6	412	Gluten	10.0	39	28	3	7 ± 2.6
R–LP	5	337	Gluten	10.5	50	35	4	11 ± 3.2

[1] C–C indicates control diet pre- and postweaning; R–LP, dam's diet restricted preweaning, low protein diet for 4 weeks postweaning, and control diet from 50 days of age. At approximately 27 weeks of age, rats were fed either casein or wheat gluten for 14 days; urine and feces were collected and analyzed individually for nitrogen during the final 6 days.
[2] Mean ± SE.

TABLE 5

Body composition, male rats, 32 weeks of age

	Control (15) [1] (C–C)		Restricted (16) (R–R)		Low protein (18) (R–LP)	
	g	*% carcass*	*g*	*% carcass*	*g*	*% carcass*
Body wt	445 ± 12.1 [2]		387 ± 26.4		378 ± 9.0	
Carcass [3]	413 ± 11.8		359 ± 7.5		349 ± 8.2	
Protein	86 ± 2.1	20.8 ± 0.36	79 ± 1.3	22.0 ± 0.27	76 ± 1.7	21.8 ± 0.25
Fat	63 ± 2.4	15.3 ± 0.52	43 ± 2.4	11.4 ± 1.6	44 ± 3.4	12.9 ± 2.6

[1] Number of rats in parentheses.
[2] Mean ± SE.
[3] Wet weight of body minus blood and intestinal tract.

DISCUSSION

Growth. The general shape of the growth curves and the stunting of adult body weight for all groups that had a restricted food intake during the first 3 weeks of life follows a thoroughly established pattern. The stunting in ultimate body size is reflected in decreases in total carcass protein and fat in the early restricted rats, as would be predicted from the studies of cellular response to malnutrition by Winick and Noble (14) and of adipose tissue cellularity by Knittle and Hirsch.[3] It is probable that the difference in fat content between control and experimental groups was greatly minimized by the larger weight loss of the controls, due to experimental manipulations prior to terminating the study.

The extent of restriction during the first 3 weeks of life that was caused by feeding the lactating dam a 12% casein (10% protein) diet was large. The controls weighed an average of 50 g, whereas the restricted weighed 24 g at weaning (3 weeks). It is evident that this preweaning restriction was not comparable in all respects to the more common practice of having the lactating dam nurse large litters of 18 or more pups. In the latter case, milk intake is reduced from the first day of nursing; however, with the dam fed a low protein diet, there is a decreased production of milk, but this does not show up in a decreased body size of the pups until about day 5 of life.[4] Furthermore, with large litters an optimal diet is fed to the dam; thus, during the third week of life when the pups are receiving an increasing amount of their food from the diet provided the dam, it is a food of good quality. Thus, large-litter restriction is truly a restriction of a diet of optimal composition from day 1 to day 21 of life. With the dam fed a low protein diet, however, the pups consume increasing amounts of the dam's protein-deficient diet during the third week. Thus they consume a restricted amount of a good diet after the first few days of life, but are gradually weaned to a deficient diet during the third week. Presumably this situation more accurately simulates the case of the human infant in the beginning stages of marasmus. This is even more accurately represented by those rat pups which were completely weaned to the very low protein (5% casein) diet and consumed this for an additional 4 weeks.

Food consumption. As pointed out in the introduction, there is conflicting evidence regarding the efficiency of food utilization for growth of rats that had been underfed in early life. Apparently no measurements of food consumption have been made in adult rats that had been restricted in the early postnatal period. However, Lee and Chow (7) have reported a decreased feed efficiency (body weight gain per day per food intake) up to 20 months of age for rats which were progeny of dams fed restricted amounts of food during both gestation and lactation. The progeny of restricted dams consumed more food per kilogram body weight in rats weighing in the range of 400 to 500 g (7, 13).

In the present study, early postnatal dietary deficiencies resulted in an increased food intake for male, but not for female, rats. Changing the practice of biweekly weighing and filling feed cups to daily filling of feeders resulted in a temporary increase in food intake and growth of the controls as reflected in the growth curves (fig. 1). Changes in food intake in male rats appeared to be related to the type of nutritional deficiency, but not to the degree of body stunting. Expressed on the basis of intake per 100 g body weight, food consumed by R–R and R–LP males was significantly higher than controls. When expressed on the basis of $BW^{0.75}$, however, this effect tended to disappear in the R–R but remained in the R–LP males. This, of course, is the more correct manner of calculating food intake since $BW^{0.75}$ more accurately correlates with total metabolic rate. Unfortunately, food intake was not measured for R–C rats; but, judging from the minimal effect observed with R–R rats, it seems justified to assume that dietary restriction during only the first 3 weeks of life would have no effect on food intake of the rehabilitated adult male rat.

Food intake over a long period of time, as represented in this type of ad libitum measurement, must reflect the satisfaction

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[3] Knittle, J., and J. Hirsch 1967 Infantile nutrition as a determinant of adult adipose tissue metabolism and cellularity. Clin. Res., 15: 323.

[4] Barnes, R. H., C. S. Neely, E. Kwong, B. A. Labadan and Slávka Franková. Unpublished results.

of overall metabolic requirements if the diet is nutritionally optimal in its composition. This is the case in the present study; therefore, food intake should be directly correlated with total metabolic rate, which in turn is correlated with surface area or $BW^{0.75}$. The deviation from this correlation, which was noted in the case of the male rats restricted preweaning and then fed a low protein diet for 4 weeks postweaning (R–LP), might be accounted for by the slight increase in protein content of the R–R and R–LP carcasses which would be indicative of a greater proportion of the body weight composed of metabolically active tissues. More carefully controlled studies of body composition will have to be conducted in order to verify this hypothesis. Some details of food consumption in both ad libitum and 1-hour feeding are brought out in figure 3 which gives the daily consumption pattern for 5 consecutive days.

The 1-hour feeding schedule resulted in a smaller daily food intake which obviously was not sufficient to provide maintenance requirements of both male and female rats, as shown by their declining weight. Percentage increase in the intake of previously malnourished animals was greater than was the case for ad libitum feeding, but individual variation was great, and the only group differing significantly from the controls was the R–LP males. Increased food intake under conditions of 1-hour daily feeding has been noted in earlier studies reported from this laboratory (10). In this previous report it was noted that ad libitum food intake was not altered and, furthermore, if 1-hour daily feeding was continued for several weeks, differences in food intake disappeared. In these previous studies, nutritional deprivation was induced only during the first 3 weeks of life. From the present results one would anticipate that this form of malnutrition might not be sufficient to induce measurable changes in the ad libitum intake of the adult rat.

By far the most striking change related to food intake is the pattern of food spillage. Spillage has been expressed as a percentage of the total food consumed. This seems to be a logical basis, but the same results were obtained if spillage was expressed as a function of $BW^{0.75}$. The fact that no differences were observed in the rats fed ad libitum, despite the significant difference

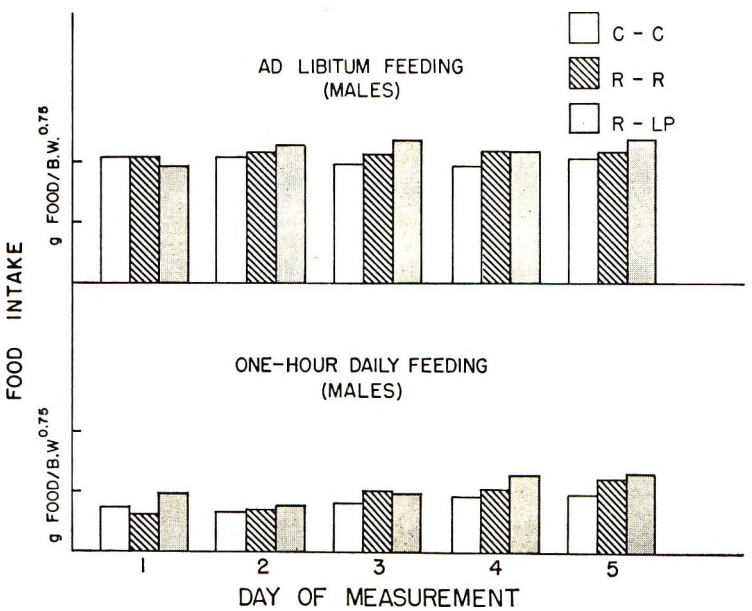

Fig. 3 Ad libitum and 1-hour/day food consumption for 5 consecutive days. Abbreviations are explained in the legend for figure 1.

in food intake, argues against spillage being an obligatory correlate of intake. On the other hand, the extremely large spillage of the R–LP rats and the lesser but still highly significant elevation in the R–R rats under conditions of 1-hour daily feeding, makes this a very interesting phenomenon. Figure 4 shows food spillage for 5 consecutive days, and this type of presentation brings out certain characteristics not evident in the tabular presentation of data. In the 1-hour feeding, both controls and R–R animals appear to be adapting with progressively less spillage on succeeding days while the R–LP rats continue to have markedly elevated spillage. More subjective, but nevertheless highly consistent, has been the observation that R–LP rats remained in contact with the feeder jar during most of the hour it was in the cage, while the C–C rats ate during the first part of the hour and then moved to another part of the cage away from the feeder. A remarkable feature of the food spillage pattern is its similarity to other behavioral testing with the same rats which has shown wide differences between C–C and R–LP rats with R–R rats being intermediate (11, 12). Furthermore, in one of these studies (12) R–LP rats exhibited an elevated state of excitability (or emotionality) when exposed to a stressful condition. The 1-hour feeding schedule is obviously another example of a stressful condition, and the increased food spillage of the R–LP rats can be interpreted as an exhibition of an elevated state of excitement. In other words, increased food spillage in a previously malnourished rat need not be specifically related to a food drive, but may be a manifestation of a more general behavioral abnormality that can be elicited in a variety of ways. It is our current belief that elevated excitement under stress is one of the most consistent and most dominating behavioral abnormalities caused by severe malnutrition in early life. Food spillage as an example of this abnormal behavior could be an excellent test that could be quantitatively measured without special equipment or specially trained personnel. This simple test might be very helpful in studying details of biochemical and nutritional components of behavioral development.

It is of particular interest that with this behavioral test which is quantitatively affected by the extent of previous malnutrition, it was in male but not female rats that significant differences were obtained, just as we have found in other forms of behavioral testing (8).

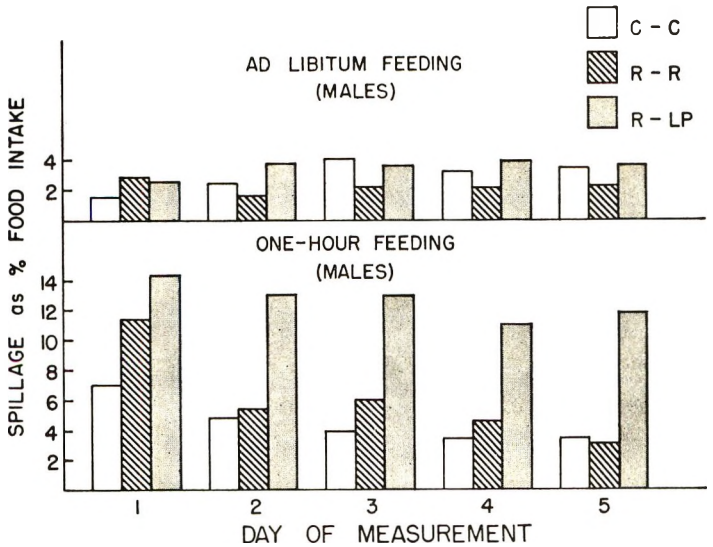

Fig. 4 Daily food spillage for 5 consecutive days for male rats being fed either ad libitum or during 1 hour/day. Abbreviations are explained in the legend for figure 1.

Finally, the nitrogen balance studies with good and poor quality protein diets gave no indication of inferior nitrogen retention in previously malnourished rats. The primary reason for looking at this aspect of metabolism was the report of Lee and Chow (7) that progeny of rats that had been partially deprived of food during both gestation and lactation showed poor retention of nitrogen long after nutritional rehabilitation, particularly when ingesting a poor quality protein. The present results indicate that if such a metabolic abnormality exists, its cause is not due to nutritional deprivation during the early postnatal period.

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Influence of Malnutrition in Early Life on Exploratory Behavior of Rats [1]

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ABSTRACT Development of exploratory behavior during the preweaning period and the intensity of exploratory activity in adults were studied in rats subjected to nutritional deprivation in early life. In the preweaning period, the dams of experimental animals were fed a low protein diet (12% casein) to suppress milk production. Control rats were provided a well-balanced basal (25% casein) diet. At 3 weeks of age the pups were weaned and divided into 3 treatment groups. One group was fed an extremely low protein diet (5% casein), a second group was fed the basal diet in restricted amount to prevent any growth, and the third group was provided basal diet ad libitum. After 4 weeks, protein- or calorie-restricted animals were rehabilitated by feeding the basal diet. Control rats were fed the basal diet throughout the entire experimental period. Protein restriction of lactating rats soon affected growth and behavior in their offspring. Lower body weight and slower development of exploratory behavior were found in experimental animals from day 10 of life. After weaning, severe protein or calorie restriction caused an elevation of spontaneous activity in male rats and calorie-restricted females. Animals fed the low protein diet during only the preweaning period did not show increased intensity of exploratory activity in the postweaning period. After nutritional rehabilitation, exploratory activity declined rapidly in all treatment groups. At day 75 and day 85 of life, all previously malnourished rats showed a very low exploratory drive. A consistent tendency to reduce spontaneous activity was found both in the group that was malnourished only in the preweaning period and in rats that also had been restricted for 4 weeks after weaning. At day 85 of life, all experimental groups differed significantly from normally fed rats. Similar trends in behavioral changes were observed in males and in females.

Numerous observations give presumptive evidence of the long-term effect of nutritional deprivation in early life on mental development of children. Cravioto and Robles (1), and Cravioto (2) found that the lower scores in psychomotor tests of protein-calorie-malnourished children remained lower than in normally fed children even after realimentation, when clinical symptoms of undernutrition had disappeared. Various studies have shown alterations in brain activity of undernourished preschool children (reviewed by Coursin, 3, 4). Stress often has been given to early developmental periods in which brain is developing very rapidly (5).

Many experimental studies with animals have provided detailed information of the effect of calorie or protein restrictions on behavior. Cowley and Griesel (6, 7) studied the effect of a low protein diet on maternal behavior, exploratory behavior and performance in the Hebb-Williams maze in two filial generations of white rats. The low protein diet they used contained approximately 13% protein, and the control diet, 20% protein. They observed no significant differences in exploratory behavior either in the parent generation or in the first filial generation. In the Hebb-Williams test, the parent generation which had been retained on a low protein diet from weaning did not differ significantly from animals fed the control diet. They reported, however, that the low protein diet affected growth and "intelligence" of the first filial generation rats, reared from mothers fed the low protein diet from the age of 21 days.

The effect of different forms of nutritional deprivation on learning behavior of rats in a water maze was studied by Barnes et al. (8). Rats nutritionally deprived from birth to 11 weeks of age were nutritionally rehabilitated, and from 6 to 9 months of

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age visual discrimination in a Y water maze was measured. Male rats that were subjected to this extreme degree of malnutrition made significantly more errors than did normal controls. Male animals which were deprived only before or after weaning (3 weeks of age) gave intermediate results. No significant differences were found among groups of female rats. Further work from the laboratory (9, 10) has also shown that baby pigs, malnourished from the third to the eleventh week of life by feeding an extremely low protein diet, exhibited behavioral abnormalities in classical conditional-response and conditional-avoidance studies when tested several months after rehabilitation. Underfeeding a diet of optimal nutritional content so as to cause stunting of body growth to the same extent and for the same duration as with the pigs fed a low protein diet, however, resulted in behavioral responses similar to those of control animals.

Despite increasing numbers of experimental studies with laboratory animals, inconsistencies in our knowledge of behavioral deficits remain; and generalization of results to predict behavior-nutrition relationships in man is practically impossible. The cause is not in the inability of research workers to reveal all discrete changes in behavior, but rather in the great behavioral complexity of any given test. There are many variables, such as age at which deprivation starts, duration of exposure to the nutritional deprivation, degree of nutrient restriction, as well as further variables such as genetic properties of the deprived animals, lactating ability of mothers and psychological effects of environment, that must be considered and controlled when possible. For example, it has been shown [3] that increased stimulation (handling) in early life modifies to a great extent the effect of early nutrition on behavior of rats.

In the present study, we concentrated our attention on the type of restriction and the time during which restriction was imposed. We compared the effects of feeding a diet with a subnormal protein level 1) in the preweaning period, followed by a period of severe protein restriction; 2) in the preweaning period, followed by a period of severe calorie restriction; or 3) in the preweaning period alone.

EXPERIMENTAL

Pregnant female rats of the Holtzman strain were used in these experiments. All offspring were born within 24 hours. Litter sizes were adjusted so that every mother nursed eight rats. During the nursing period, some of the lactating females were fed a low protein diet (11) containing 12% casein to suppress milk production. The control group received 25% casein in the diet. After weaning at 21 days of age, the pups nursed by mothers receiving the 12% casein diet were divided into three groups.[4] In the first group, the dietary protein level was lowered to 5%. The second group was fed the basal control diet (25% casein) in limited amounts, about 1 to 3 g daily, to maintain body weight as it was at weaning. A third group was restricted during the preweaning period only; after weaning the rats were fed the control diet ad libitum. From day 50 of life, all underfed rats were changed to the 25% casein diet and fed ad libitum. The nutritional plan is given in table 1, and growth curves are published elsewhere (11).

In analyzing behavioral characteristics, two procedures were used. The method of exploratory activity is described in the present study. Intensity of exploratory activity was previously demonstrated to be highly sensitive to changes in nutrition of growing or adult rats (12–14). In observing the development of exploratory behavior we used an empty cage 27 by 34.5 by 45.5 cm, the bottom of which was divided into six equal squares. This was placed behind a one-way mirror so that the observer could not be seen. At 10, 14 and 21 days of age, the intensity and quality of exploratory activity were evaluated during 6-minute test periods. The number and duration of activities were recorded for a

[3] Franková, S. 1966 Influence of nutrition and stimulation in early infancy on the exploratory behaviour of adult rats. VII International Congress of Nutrition, Hamburg, p. 310 (abstracts of papers).

[4] In the abbreviation system described previously (11), the first letter denotes preweaning dietary treatment; the second, dietary treatment during 4 weeks postweaning. Thus, C–C indicates control diet pre- and postweaning; R–C, dam's diet restricted preweaning and control diet postweaning; R–LP, dam's diet restricted preweaning and low protein diet postweaning; and R–R, dam's diet restricted preweaning and calorie-restricted diet postweaning. All groups received control diet ad libitum from day 50.

TABLE 1

Experimental plan for nutritional deprivations during the preweaning and postweaning periods

Group	Preweaning Day 1–21 (dam's diet)	Experimental treatment postweaning	
		Day 22–49	From day 50
R–LP	Protein-deficient (12% casein)	Protein-deficient (5% casein)	Normal (25% casein)
R–R	Protein-deficient (12% casein)	Normal, restricted quantity (25% casein) [1]	Normal (25% casein)
R–C	Protein-deficient (12% casein)	Normal (25% casein)	Normal (25% casein)
C–C	Normal (25% casein)	Normal (25% casein)	Normal (25% casein)

[1] Only group with restricted intake; all others fed ad libitum.

given period: number of squares traversed, number of head-up reactions, number and duration of standing up reactions (supported or perfect without support), and duration of total inactivity. After weaning, the length of the cage was doubled and the bottom divided into eight squares. Observations were prolonged to 9-minute periods. Intensity of exploratory activity was measured at age 50 days, which was the end of the severe protein or calorie restriction period; and also after rehabilitation, at 75 and 85 days of age. All rats were housed individually and maintained at constant room temperature, 23°. For statistical evaluation of differences among experimental groups, the nonparametric Wilcoxon test was used (15).

RESULTS

The low protein diet given to lactating females caused a decrease in growth rate of suckled animals. At 10 days of age, growth differences between control and low protein groups were significant. Therefore, we started behavioral observations at this time. During the period of severe protein or calorie restriction after weaning, growth curves of both malnourished groups were identical (11). Rehabilitation had similar effects on growth of both calorie-restricted and protein-restricted animals.

Development of spontaneous exploratory behavior under normal conditions has a very regular course (12, 16). At 10 days of age it is typical for normally fed rats to be very mobile. They start to explore their new surroundings and only seldom are inactive in the test situation. Exploratory behavior has two components: horizontal and vertical. Characteristic horizontal components are walking, running and sniffing. Vertical components of exploratory activity at day 10 are manifested by attempts to turn the head up, before the animal is able to stand on its hind legs. In the control group 100% of the young rats displayed this type of behavior, but in the low protein group only 53% of rats tried to turn their heads up. Differences between groups were highly significant. In the low protein group duration of inactivity was negatively correlated with body weight at 10 days of age ($r = -0.60$), which indicated that heavier rats were more active. By day 14 the repertoire of spontaneous activities included walking, running, turning heads up, grooming, and usually unsuccessful attempts to stand up unsupported. Significant differences in all criteria were found. Protein-restricted animals showed very low levels of exploratory activity. Only 14% of undernourished rats attempted to stand up, whereas in 87.5% of control rats standing-up reactions were recorded. The last observation before weaning was done on the twenty-first day. Statistically significant differences in duration of inactivity, number of head-up reactions and number of unsupported standing-up reactions were observed. Results of all preweaning behavioral observations are given in figure 1. Results obtained in males and females during the preweaning period were combined because behavioral differences between sexes were not evident at this age.

After weaning, when severe protein or calorie restriction was still being imposed, striking changes in spontaneous behavior were observed. In male rats the

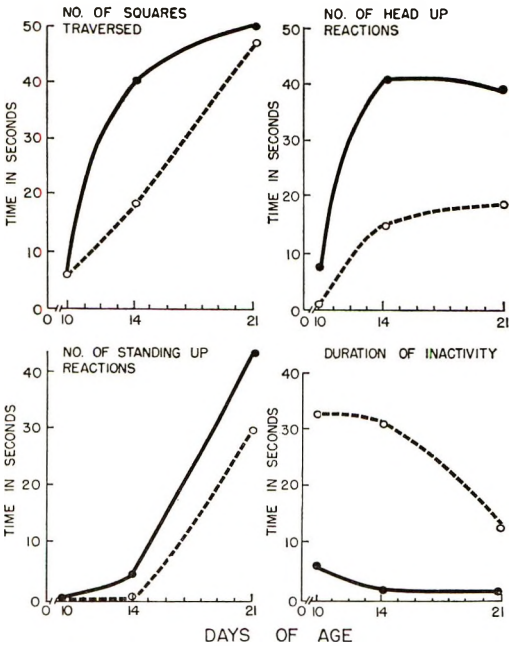

Fig. 1 The development of different spontaneous exploratory activities during the first 3 weeks of life and the effect of inadequate milk for suckling the young. Solid line, rat pups suckling dams with adequate milk supply. Broken line, rat pups suckling dams fed a low protein diet to reduce milk supply.

intensity of exploratory activity increased dramatically (table 2). Significant differences were found between control group (C–C) and the postweaning low protein group (R–LP) or calorie-restricted (R–R) animals. Highest indices of exploratory activity were recorded in R–R males, which differed significantly not only from controls, but also from the R–LP group (P = significant at 5% level). Even in their home cages, R–R animals were restless; they climbed on the walls of cages throughout the day. Different results were obtained when behavioral patterns of male R–C rats, restricted only in preweaning period, were evaluated. Horizontal activity, expressed as number of squares traversed, was elevated as compared with control group. In number and duration of standing-up reactions, R–C rats did not differ from control animals significantly, but they reached lower values as compared with both R–LP or R–R groups. The R–C animals spent significantly less time grooming than control rats. Most striking was the duration of inactivity in the R–C group. All animals of this group were inactive for long periods during the test, and duration of inactivity was significantly higher as compared with all experimental groups. Total body immobility of more than 5 seconds was classified as inactivity.

In females, exploratory activity level in all tested groups exceeded values recorded for males. Behavioral differences between malnourished and normal females were less evident than in males. Female R–LP rats did not differ from controls either in the number of squares traversed or in the number and duration of stand-

TABLE 2

Exploratory activity at 50 days of age in male and female rats

Treatment [1]	No. of rats	No. of squares traversed	No. of standing-up reactions	Duration of standing-up reactions	Duration of grooming	Duration of inactivity	
				sec	*sec*	*sec*	%
			Males				
C–C	10	48.1 ± 6.2 [2]	33.0 ± 4.8	31.8 ± 5.1	126.3 ± 26.6	5.3	33 [3]
R–C	8	91.7 ± 9.8	36.1 ± 7.8	29.7 ± 6.1	40.8 ± 10.9	48.7	100
R–LP	10	69.7 ± 3.7	59.6 ± 4.0	58.2 ± 5.8	61.7 ± 16.1	0.0	0
R–R	10	110.1 ± 3.1	85.3 ± 8.5	89.0 ± 12.4	25.7 ± 7.9	1.1	10
			Females				
C–C	8	99.4 ± 8.4	80.5 ± 11.3	67.5 ± 9.8	84.6 ± 10.7	0	0
R–LP	8	81.5 ± 11.5	80.0 ± 11.3	78.5 ± 5.5	44.6 ± 10.6	0	0
R–R	9	132.8 ± 12.5	117.0 ± 10.6	115.9 ± 19.7	10.6 ± 1.9	0	0

[1] C–C indicates control diet pre- and postweaning; R–C, dam's diet restricted preweaning and control diet postweaning; R–LP, dam's diet restricted preweaning and low protein diet postweaning; and R–R, dam's diet restricted preweaning and calorie-restricted diet postweaning. All groups received control diet ad libitum from day 50.

[2] Mean ± SE.

[3] Percentage of animals exhibiting inactivity.

up reactions. The only difference was found in duration of grooming, which was lower in underfed rats. Exploratory activity of R–R rats was markedly higher as compared with C–C and R–LP females. Statistically significant differences were found between C–C and R–R females in all criteria except inactivity. There were no periods of inactivity for female rats in any group. Females restricted only in preweaning period (R–C) were not tested for intensity of exploratory activity at age 50 days.

After rehabilitation, exploratory activity of all previously malnourished groups tends to decrease very rapidly (table 3). At 75 days of age there were significantly fewer standing-up reactions of shorter duration in R–LP and R–R male groups as compared with values obtained in the control group. The number of squares traversed was also lower in all rehabilitated groups, but the differences were not statistically significant. Duration of inactivity increased markedly in R–LP and R–R groups. At 85 days results were much more distinct. Differences between previously malnourished and control rats were even greater, indicating that the drop in spontaneous activity below values for the control group was not transient. In all criteria rehabilitated male rats differed significantly from values of control rats. In the preweaning-restricted group (R–C), exploratory activity level showed a consistent tendency to be lower than that of controls. At 85 days of age, all recorded activities for previously malnourished groups were significantly below the level of control animals but did not differ significantly among themselves.

There was also a tendency toward a reduced intensity of exploratory activity in rehabilitated females. At 75 days of age the lowest values for number and duration of standing-up reactions were recorded in R–R females. Differences in number of squares traversed were not significant. Lower values for exploratory activity were recorded at 85 days in all rehabilitated groups. Female R–C rats reduced their exploratory activity less than did R–LP and R–R groups. The tendency toward a

TABLE 3

Exploratory activity after rehabilitation in male and female rats

Treatment groups [1]	No. of rats	No. of squares traversed	No. of standing-up reactions	Duration of standing-up reactions	Duration of grooming	Duration of inactivity	
				sec	*sec*	*sec*	%
			Males, day 75				
C–C	11	79.2± 8.7 [2]	51.0±6.9	68.2±10.5	44.4±10.8	8.0	18 [3]
R–C	8	63.7± 9.2	31.1±6.0	39.5± 9.2	59.1±18.7	51.2	20
R–LP	11	66.1±11.2	25.9±4.1	25.5± 5.5	57.3±16.1	67.3	80
R–R	10	42.5± 8.4	24.3±4.5	27.4± 5.4	45.1± 9.1	59.0	70
			Males, day 85				
C–C	11	74.3± 5.1	50.3±5.0	77.2± 8.9	39.0±10.5	12.9	9
R–C	10	44.2± 7.7	25.8±5.8	36.5± 9.0	26.4± 9.5	84.0	70
R–LP	11	52.0± 6.7	46.7±3.4	27.2± 3.8	45.6±12.9	83.5	83.3
R–R	10	38.5± 7.8	26.1±4.1	32.1± 6.1	59.6±22.7	84.0	81.8
			Females, day 75				
C–C	8	116.0± 7.5	91.6±8.7	148.5±13.2	24.2± 8.2	0	0
R–C	8	115.7±11.2	79.3±9.6	92.3±12.3	22.4± 8.1	0	0
R–LP	9	102.6±14.3	91.6±8.7	67.3±11.2	46.6±16.3	27.0	22.2
R–R	7	103.2±16.2	51.4±8.4	49.0± 7.9	44.7± 8.5	12.8	42.8
			Females, day 85				
C–C	8	122.1± 8.2	77.6±9.8	132.9±21.8	10.7± 4.6	0	0
R–C	8	95.8± 4.2	55.9±9.5	91.7±16.5	23.5± 4.8	11.2	8.0
R–LP	7	86.6±11.4	47.6±6.9	78.5±12.3	27.8± 7.6	14.1	28.6
R–R	7	86.1± 9.6	49.8±5.3	71.7± 8.7	53.0± 8.4	17.3	28.6

[1] See footnote 1 in table 2 for dietary treatment abbreviations.
[2] Mean ± SE.
[3] Percentage of animals exhibiting inactivity.

lower intensity of exploratory activity was marked in all rehabilitated females, although not as much as in male rats.

DISCUSSION

Rat pups, dams of which were fed a low protein diet from the beginning of lactation, showed early deficits in the development of exploratory behavior. Low exploratory activity and long duration of inactivity were recorded on the tenth day. Statistically significant lower values for active exploration were found in all tests in the preweaning period.

Slower development of spontaneous behavior had been observed previously in different experimental models of malnutrition, as in using extremely large-sized litters [5] or in feeding mothers 50% of the food intake of control rats during both gestation and lactation (17). Similar results have been reported by Simonson et al.[6] Low exploratory activity and prolonged inactivity periods could be the consequence of slower brain development. During the second and third weeks of postnatal life, brain of rats becomes myelinated. Dobbing (18) found significantly lower accumulation of brain cholesterol in 12- to 21-day-old rats nursed in large-sized litters. Accumulation of cholesterol in the brain during the first weeks of life could serve as a good index of the structural development of brain. It is possible that structural and functional retardation of brain maturation resulted in low exploration because of poor locomotor coordination.

Deleterious effects of nutritional restriction in the period of most rapid growth and development appear to be clear, but many problems concerning the influence of dietary deficiencies on the adult rat's behavior remain unsolved. Effects of protein deficiency on spontaneous activity and learning behavior of adult animals have been studied under a number of experimental conditions, but results have not always been consistent. In an early study, Bernhardt (19) described an effect of a low protein diet on escape from a water maze. He reported that rats fed a low protein diet were poorer in maze learning than control rats, but differences were not significant. According to Pilgrim et al. (20), there were no marked differences between groups of rats about 34 days old fed low protein, high protein or basal diets for 40 to 50 days. Cowley and Griesel (6, 7) did not find significant differences in exploratory activity of animals on high or low protein diets. However, some methodological comments could be made: their so-called low protein diet contained about 13% protein, and their control or "high protein" diet contained about 20% protein. It is possible that in both experimental groups, the protein levels were less than optimal. In addition, the difference between 13 and 20% protein was probably too small to influence behavior significantly. As a measure of exploratory activity, they used the number of units entered by the rat during a 3-minute training phase. Not only was 3 minutes too short an observation period with a complex new environment, but also the number of units entered may not be enough to detect changes in the intensity of exploratory drive.

When exploratory activity of rats under different nutritional or stimulation conditions was analyzed, it was necessary to evaluate horizontal as well as vertical components of exploratory behavior (17). In previously malnourished rats the horizontal component, namely, the number of squares traversed or units entered, was not influenced as much as vertical components of exploration expressed as number (or duration) of standing-up reactions. Intensity of standing-up reactions appears to be a better measure of exploratory drive.

When considering the effect of protein deficiency on rat behavior, it is important to pay attention to the age at which protein restriction is initiated. In the preweaning period, rats maintained on a decreased food intake showed very low exploratory activity; however, after weaning those with severe protein restriction showed increased exploratory activity. Increased exploratory activity of rats fed a low protein diet after weaning has been described by Lát and Faltová (21), Franková (14) and Collier and Squibb (22). When ad libitum feeding is allowed, decrease of dietary protein

[5] See footnote 3.

[6] Simonson, M., W. Yu, J. K. Anilane, R. Sherwin and B. F. Chow 1967 Studies of development in progeny of underfed mother rats. Federation Proc., 26: 519 (abstract).

means that fat or carbohydrate concentration increases. Lát and Faltová (21), comparing the effects of proteins and carbohydrates on exploratory activity and learning, established that with increased protein level of the diet and consequent decrease in carbohydrate, exploratory activity was lowered and vice versa. Similar results have been reported by Franková (14), who studied the influence of a change in ratio between fat, protein and carbohydrate on excitability of the central nervous system. Higher concentrations of dietary protein lowered exploratory activity in adult rats, whereas decreased amount of protein with an increased fat content stimulated exploratory behavior. According to Collier and Squibb (22), a low protein diet increased total body activity of rats in a revolving drum. The effect was greater with a low protein-high fat diet than with a low protein-high carbohydrate diet. All of the above experiments were carried out with adult rats but nevertheless support the present findings.

Enhancement of spontaneous activity was most striking during calorie restriction. Numerous experimental studies have evaluated the effect of food deprivation on rat behavior. Most of them have concluded that food deprivation increases exploratory activity (reviewed by Brožek and Vaes, 23).

After rehabilitation when a good diet was provided, exploratory activity of previously malnourished rats dropped far below the values for control, normally fed rats. The low protein or calorie-restricted diets increased exploratory activity temporarily, only during the time that restriction was imposed. After 25 to 35 days of rehabilitation spontaneous activity reached only about 50% of the activity level of normally fed rats. Furthermore, the two experimental groups did not differ from rats that were underfed only during the preweaning period. In these experiments the intensity of exploratory activity was tested at 75 and 85 days of age. However, in some previous experiments,[7] low exploratory activity was observed even at 300 days of age for rats which had been maintained on a restriction schedule during the preweaning period alone. It is concluded that undernutrition in the first weeks of life may cause uniform long-term effects manifested by a decrease of exploratory drive. This may occur despite either a period of hyperactivity due to severe protein or calorie restriction after weaning or rehabilitation with a well-balanced diet fed ad libitum from 21 days of age.

When the effects of early malnutrition on male and female rats were compared, more striking differences were consistently found in males than in females. There is not general agreement whether changes in nutrition affect females to the same extent as males. In the present studies spontaneous activity of females was affected in the same direction as in male rats, but to a lesser extent.

Regarding the results of numerous studies of early undernutrition on animal behavior, one conclusion is evident. In order to get more detailed information about the influence of early restriction, analysis of different characteristics of behavior is necessary. Also it would be of great value to be able to compare results from different animal species.

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Effect of Malnutrition in Early Life on Avoidance Conditioning and Behavior of Adult Rats [1]

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ABSTRACT The influence of malnutrition in infancy upon the development of certain behavioral characteristics was investigated by studying conditioned avoidance learning and extinction in adult rats subjected to nutritional deprivation in early life. In the preweaning period, young rats suckled dams fed a low protein diet (12% casein) which suppressed milk production. At 3 weeks of age the pups were weaned and divided into 3 treatment groups. One group was fed a low protein diet (5% casein). The second group was provided a basal diet (25% casein) but in restricted amount to maintain the same body weight as at weaning. The third group was changed to the basal diet ad libitum. After 4 weeks, protein- and calorie-restricted animals were rehabilitated by feeding them the basal diet. Control rats were fed the basal diet throughout the entire experimental period. From 95 days of age, all rats were subjected to avoidance conditioning. They learned to jump on a vertical screen when a conditioned stimulus (sound) was presented. The unconditioned stimuli, shocks from electrified floor grids, occurred after presenting the conditioned stimulus for 10 seconds. Duration of the latent periods and spontaneous activity throughout the periods of observation were recorded. After 6 tests of conditioning, 1 extinction experiment was carried out. There were no significant differences between control and previously malnourished rats in the learning rate expressed in terms of the duration of latent periods, but striking behavioral disturbances developed in groups of rats which had been restricted both before and after weaning. During 12 days of the experiment, increased excitability was recorded in these rats. Behavioral abnormalities were manifested as inadequate stereotyped movements and inability to delay or to extinguish the fixed conditioned reactions. Animals restricted in the preweaning period only did not show signs of disturbances which characterized the behavior of rats restricted both before and after weaning.

Various studies have shown the deleterious effect of nutritional deficiencies in early life on behavior and intellectual development. Lower than normal performance in psychomotor and intelligence tests has been reported in the acute phase of malnutrition and after nutritional therapy (1–4).

Despite an increasing number of studies on the characteristics of learning efficiency of malnourished children or experimental animals, little is known about the real causes of learning deficits. Damage to the central nervous system during the period of rapid brain growth could directly influence learning ability, but other possible interpretations cannot be excluded. For example, motivational or emotional disturbances could be responsible for poor learning efficiency. Lack of motivation, impairment of concentration, low exploratory drive, apathy and other such factors can contribute to low scores on intelligence tests.

It has been shown that nutritional deprivation in the first weeks of life may cause a decrease of exploratory activity which continues long after rehabilitation with a normal, well-balanced diet (5–7). In the present experiment the rate of learning and extinguishing of avoidance responses to painful stimuli and changes in spontaneous behavior were studied in adult nutritionally rehabilitated rats which had been nutritionally deprived during the first 3 or 7 weeks of life.

EXPERIMENTAL

Lactating female rats of the Holtzman strain were divided into two groups: One group was fed a low protein diet containing 12% casein to suppress milk production

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(diet composition published elsewhere, 8). To the other group, a basal diet with 25% casein was provided. The control group was formed by weaning these rats to the 25% casein diet fed ad libitum (C–C, six rats). After weaning at 21 days of age, three experimental groups were formed with the male progeny of the protein-restricted dams. For one group, dietary protein was lowered to 5% (R–LP, six rats). The second group was fed the basal diet in restricted amount to maintain body weight as it was at weaning (R–R, six rats). A third group was restricted during the preweaning period only, and after weaning these animals were fed the control diet ad libitum (R–C, seven rats). From age 50 days, the first and second groups were fed the basal diet containing 25% casein. All rats were housed individually. They were weighed once a week and, with the exception of the observations of the intensity of exploratory activity at 50, 75 and 85 days, they were not used for any other experiment. At day 95 of life, avoidance conditioning was imposed. Growth curves and other experimental details have been described separately (8).

For these studies, a cage 35 by 35 by 35 cm with a Plexiglas front wall and a floor of electric grids was used. The top of the cage was covered with a Plexiglas plate. Adjacent and parallel to one wall, a wire mesh screen was suspended. The weight of the rat hanging on this screen would break an electric connection. The cage was placed behind a one-way mirror so that the observer could not be seen by the animals.

At the beginning of the first test the rat was placed in the cage, and it was left there for 20 minutes to adapt to the new environment. From the second test onward the adaptation periods were restricted to 5 minutes. During the adaptation period, the number of spontaneous jumps and the duration on the screen were recorded. The rats learned to jump onto the screen to avoid electric shocks from the grids. As a conditioned stimulus (CS) a tone from an electric oscillator having a frequency of 800 cycles/second was used. After 10 seconds the unconditional stimulus (UCS), electric shocks from the grids, joined the conditioned stimulus, and both CS and UCS acted until the rat jumped on the screen, or for a maximum time interval of 150 seconds. After the rat had jumped on the screen, or after 150 seconds if the animal failed to jump, CS and UCS were automatically switched off. The intensity of shocks varied from 0.3 to 0.5 ma, enough to provoke escape movements. The time from the onset of the CS until the rat jumped on the screen was recorded as the "latent period." After the rat jumped on the screen, the time during which it remained there was also recorded. Since the screen was not fixed to the wall, it was uncomfortable to remain there for long, and control rats frequently returned to the grid floor with little delay. Nevertheless, duration of time spent on the screen was one of the important behavioral characteristics that was influenced by early malnutrition. After the rat jumped off the screen, the intertrial interval, 60 seconds long, was imposed. During this time, the animals jumped on the screen spontaneously. Latency to the first spontaneous jump in every intertrial interval, number of jumps and total time spent on the screen were recorded. The next CS was not presented until the animal had spent 15 seconds quietly on the floor of the cage. If some of the rats continued jumping at the end of the intertrial interval that period was prolonged and all further jumps were recorded separately as "after intertrial interval." A total of six tests was carried out with every animal every second day. In every test, seven sessions involving the CS and UCS were presented.

Two days after the sixth test, all rats were subjected to one extinction experiment. The animal was kept in the cage for 3 minutes without stimuli. Two CS were then presented without UCS, followed by 15 minutes without any further stimuli. The number of spontaneous jumps on the screen, duration on the screen, number of standing-up reactions and number of squares crossed (the floor was divided into four equal squares) in every consecutive minute were recorded.

This method of avoidance conditioning emphasizes the significance of spontaneous behavior throughout the process of learning. The free-moving animal was not restricted in performing various activities which were typical for a given individual.

Often they reflected different motivations or changes in emotionality.

RESULTS

Mean duration of latent periods in six consecutive tests are shown in figure 1. Except for the first test, only slight differences in latent periods were recorded between rats nutritionally deprived during the first 7 postnatal weeks of life. Most rats learned promptly to escape from the electric grid within the 10-second time interval of CS so that most latencies were less than 10 seconds from the second test onwards. Thus, the actual number of shocks was very small. A consistent tendency to shorter latent periods was exhibited by control rats as well as rehabilitated groups which were restricted both before and after weaning. The R–C group, underfed during the preweaning period only, differed slightly from the remaining groups. These animals shortened their latent periods more slowly than the other groups. Statistically significant differences were found between R–C and the other groups from the fourth to the sixth tests.

In the course of conditioning, striking changes in spontaneous behavior developed in rats which had been restricted both before and after weaning (R–R and R–LP groups). These differences between rehabilitated and control groups (C–C) were evident from the third test. In the adaptation periods, control animals explored their environment or waited quietly until the onset of the first CS, but animals of R–R and R–LP groups started to jump spontaneously on the screen despite the absence of CS or UCS, and they also spent prolonged time on the screen. Figure 2 shows the mean time spent on the screen. The number of ineffectual jumps during the adaptation periods is given in table 1 (section a). Statistically significant differences between rehabilitated and control groups were found in the second half of the conditioning tests. In rats restricted only in preweaning period (R–C groups), no significant changes in spontaneous activity during the adaptation period were recorded. Actually, they were somewhat less active than rats of the control group. A similar trend in spontaneous activity was found when behavior during the inter-

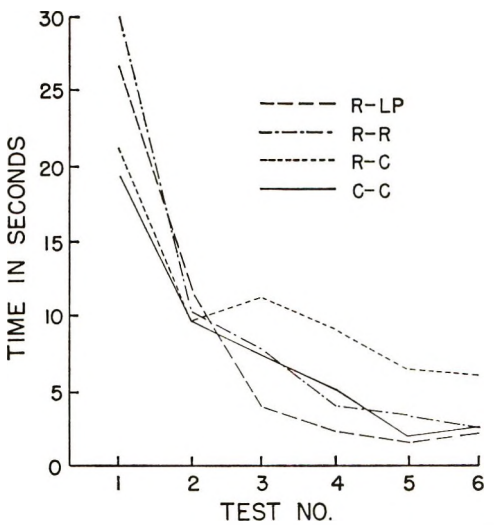

Fig. 1 Mean duration of latent periods (time from the onset of CS until the rat jumped on the screen) in six consecutive tests. Group designations are R–LP, dam's diet restricted preweaning and young rats fed a low protein diet for 4 weeks postweaning; R–R, dam's diet restricted preweaning and for 4 weeks postweaning fed a control diet in restricted quantity to prevent increase in weight; R–C, dam's diet restricted preweaning and control diet ad libitum postweaning; and C–C, control dietary treatment both pre- and postweaning. All rats had been rehabilitated and were 95 days of age at the start of these tests.

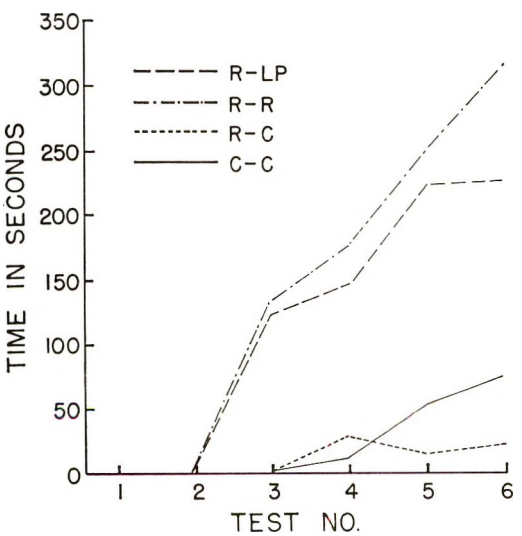

Fig. 2 Average time per rat spent on the screen during the adaptation periods for each of the six test sessions. All rats had been rehabilitated and were 95 days of age at the start of these tests. Abbreviations are explained in legend for figure 1.

TABLE 1

Number of spontaneous (ineffectual) jumps

Group [1]	No. of test						Mean values	
	1	2	3	4	5	6	Tests 1 to 3	Tests 4 to 6
a) In the adaptation periods (20 min for first test, 5 min for subsequent tests, before initial CS)								
C–C	0.00	0.16	0.00	0.50	2.50	2.00	0.05	1.66
R–C	0.14	0.14	0.00	0.50	0.66	0.83	0.09	0.66
R–R	0.00	0.16	1.83	2.66	5.16	10.16	0.66	5.99
R–LP	0.16	0.00	1.16	4.83	7.00	6.33	0.44	6.05
b) In the intertrial intervals (60-sec period between each trial)								
C–C	2.17	3.83	6.00	5.66	8.50	8.80	4.00	7.65
R–C	2.28	4.14	5.71	4.00	4.42	5.70	4.04	4.71
R–R	1.83	4.50	4.00	7.00	8.50	12.00	3.44	9.17
R–LP	3.00	5.50	6.16	9.16	12.83	18.00	4.88	13.33
c) After the intertrial intervals (following 45 sec of intertrial period and until rat remained quiet for 15 sec)								
C–C	0.16	0.50	1.50	0.66	3.00	4.00	0.72	2.55
R–C	0.57	0.71	1.57	0.25	0.85	1.57	0.95	0.89
R–R	0.00	1.50	2.00	5.16	6.50	12.16	1.17	7.94
R–LP	0.00	2.00	4.00	7.00	22.16	24.00	2.00	17.72
d) Total number of all ineffectual jumps								
C–C	2.33	4.49	7.15	6.82	14.00	14.80	4.65	11.87
R–C	2.99	4.99	7.28	4.75	5.93	8.11	5.08	6.26
R–R	1.83	6.16	7.83	14.82	20.16	34.32	5.16	23.10
R–LP	3.16	7.50	11.32	20.99	41.99	48.33	7.32	37.00

[1] C–C rats received control diet pre- and postweaning; R–C, dam's diet restricted preweaning and control diet ad libitum for 4 weeks postweaning; R–R, dam's diet restricted preweaning and control diet restricted to prevent growth postweaning; and R–LP, dam's diet restricted preweaning and low protein diet postweaning. All groups received control diet ad libitum from day 50.

Pair tested	Statistical significance in tests 4 to 6			
	a	b	c	d
C–C and R–C	not significant	not significant	$P = 0.05$	$P = 0.05$
C–C and R–R	$P < 0.01$	not significant	$P < 0.01$	$P < 0.02$
C–C and R–LP	$P = 0.50$	$P < 0.05$	$P < 0.01$	$P < 0.01$
R–C and R–R	$P < 0.01$	$P < 0.02$	$P < 0.01$	$P < 0.01$
R–C and R–LP	$P < 0.02$	$P < 0.01$	$P < 0.01$	$P < 0.01$

trial intervals was observed. Rats of the R–R and R–LP groups made more spontaneous jumps on the screen than the C–C and R–C groups (table 1, section b). During the intertrial intervals the number of jumps performed within 60 seconds gradually increased in all animals. The highest values were recorded for animals of R–R and R–LP groups which differed significantly from R–C and C–C groups in the second half of the experiment (table 1, section b).

After 60 seconds of the intertrial interval the next CS was applied, but only if the rat had remained on the cage floor for at least 15 seconds. If not, all further jumps were recorded and designated as "after intertrial intervals." From table 1, section c, it is apparent that for C–C and R–C rats it was seldom necessary to prolong the intertrial interval and few jumps occurred in the fifth and sixth tests. However, marked increases in jumping activity were observed in R–R and R–LP groups. In R–LP group an average of 24 jumps was recorded in the sixth test.

Table 1, section d, gives the mean number of all jumps called "ineffectual" because they did not represent reactions which would be effective in stopping the sound and preventing shocks. Putting all these jumps together, highly significant differences between the combined R–R and R–LP groups and the C–C group were ob-

served as well as between the R–C group, and the R–R and R–LP groups.

Marked differences among groups were also noted when the time interval (latency) to the first spontaneous intertrial jump was evaluated. All experimental groups showed a tendency to shorten the time interval during the course of conditioned learning (table 2). The values for the R–R and R–LP groups decreased considerably faster than those for the C–C and R–C groups. In animals of the R–C group, decreased latency to the first spontaneous jump occurred more slowly than in control animals.

Defecation scores provided additional information about the behavioral changes which developed in rehabilitated groups in the course of conditioning. Number of fecal pellets was recorded separately for the adaptation periods and for periods of actual conditioning. Although the differences among groups were relatively small, they were consistent, and differences between the R–R and R–LP rats and C–C animals were statistically significant (table 3).

Stereotyped jumping on and off the screen persisted in animals of R–R and R–LP groups throughout the extinction experiment. These animals did not perform any form of activity other than jumping during the 15-minute extinction period. After jumping down from the screen they remained motionless near the wall on which the screen was hung. Animals of the C–C and R–C groups showed fewer spontaneous reactions from the beginning of the test, and they spent significantly less time on the screen as compared with the R–R and R–LP groups. Figure 3 gives the mean duration of climbing on the screen in each consecutive minute of the extinction period. When the time spent on the screen decreased, spontaneous exploratory activity, characterized by walking and standing-up reactions increased again, as illustrated in table 4. Animals of the R–C group spent less time on the screen as compared with all other experimental groups; however, they showed only slight spontaneous exploratory activity.

DISCUSSION

The results of the avoidance conditioning experiment reported here confirm previous findings of both authors on the long-term effect of nutritional deprivations imposed in early life on various types of animal behavior after nutritional rehabilitation (6–13). The conditions of the present experiment were simple enough to enable practically all animals to learn the given task which was to jump on the screen during the presentation of a conditioned "warning" acoustical stimulus to avoid painful shocks. Latent periods, measured from the onset of the CS until the rat jumped on the screen, gave one quantitative index of the learning ability of the rats. Since the mean latent periods in all groups dropped below 10 seconds of the CS interval very quickly, the actual noxious stimulation was imposed mostly during the first or second tests. Only slight, insignificant differences in the rate of conditioned learning were observed between the control group and all

TABLE 2

Mean latency to the first spontaneous jump during intertrial periods

Group [1]	1		2		3		4		5		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	sec		*sec*		*sec*		*sec*		*sec*		*sec*	
C–C	49.56	3.56	49.06	3.06	42.30	3.07	40.27	4.96	30.15	5.61	35.63	6.63
R–C	47.20	2.42	42.68	2.91	38.53	2.91	47.23	2.84	42.99	5.34	39.86	4.35
R–R	49.81	4.09	43.62	3.85	45.20	2.82	32.55	2.70	27.18	3.52	16.70	2.41
R–LP	53.00	2.99	41.35	3.36	41.11	4.83	31.38	5.40	15.50	4.34	12.40	1.43

[1] See footnote 1, table 1.

Statistical significance in tests 4 to 6

C–C and R–C	$P < 0.02$
C–C and R–R	P = not significant
C–C and R–LP	$P < 0.05$
R–C and R–R	$P = 0.01$
R–C and R–LP	$P < 0.05$

TABLE 3

Defecation scores as expressed by the number of fecal pellets found during two periods of the avoidance conditioning

Group [1]	Adaptation period	Conditioning period	Total
C–C	1.08	2.33	3.41
R–C	2.23	4.01	6.24
R–R	2.88	2.66	5.54
R–LP	2.61	2.66	5.27

[1] See footnote 1, table 1.

Statistically significant differences		
Adaptation period	C–C and R–R	$P = 0.01$
	C–C and R–LP	$P = 0.05$
Total	C–C and R–C	$P < 0.01$
	C–C and R–R	$P < 0.05$

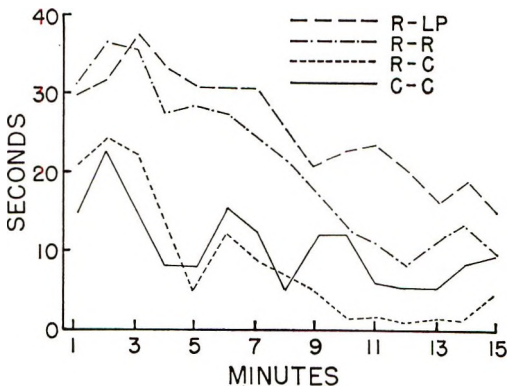

Fig. 3 Extinction of conditioned avoidance responses during 15 minutes following two CS without accompanying UCS. Responses are indicated by the average time in seconds that each rat remained on the screen during each consecutive minute. All rats had been rehabilitated and were 95 days of age at the start of these tests. Abbreviations are explained in legend for figure 1.

rehabilitated groups. Nevertheless, striking behavioral disturbances appeared in R–R and R–LP rats, which had been deprived both before and after weaning. The disturbances were manifested by changed spontaneous behavior from the third or the fourth tests onward.

The following is a description of the typical behavioral activity of normally fed control (C–C) rats. Immediately after the initiation of the CS, rats quickly performed the avoidance reaction, climbed on the screen and spent a short time there. Since it was uncomfortable to remain on the screen, which was not rigidly fixed, rats usually jumped down before long; thus, they spent a major portion of the intertrial interval on the cage floor either relatively inactive or exploring their surroundings. At the end of the intertrial interval rats would approach the end of the cage near the screen, and at the onset of the next CS they would jump on the screen again. The rats apparently had learned that the only jump which could protect them from shocks was associated with the CS signal. All other jumps had been ineffectual; therefore, the animals learned gradually to inhibit all other spontaneous jumps and to delay the learned protective reaction throughout the intertrial interval. Most of the animals of the C–C group behaved this way at least from the first to the fourth tests.

During the adaptation period, C–C rats waited near the screen, or they explored their environment and only seldom jumped on the screen. In contrast, rats of the R–R and R–LP groups jumped with increasing frequency on the screen during the adaptation phase and spent more and more time there in subsequent tests. They continued jumping not only in the presence of the conditioned stimuli but also during the intertrial intervals. Latency to the first intertrial spontaneous jump decreased rapidly with each test session. Since they were not able to stop jumping at the end of the intertrial interval, it was often

TABLE 4

Extinction of conditioned response (15 minutes)

Spontaneous activity	Groups [1]			
	C–C	R–C	R–R	R–LP
Time spent on the screen, sec	160.3	131.9	317.1	385.9
Duration of standing-up reactions, sec	48.9	5.4	0.1	1.2
Number of squares traversed	16.0	5.1	0.0	0.0

[1] See footnote 1, table 1.

necessary to prolong the observation and to record all further reactions until the animal spent at least 15 seconds on the floor.

From the fourth through the sixth test, marked signs of anxiety were observed in R–R and R–LP groups. These included rapid respiration, pilo-erection, trembling and increased defecation. Their behavior became chaotic, and these rats did not perform any activity other than inadequate, stereotype, persistent jumping regardless of the conditions of the external environment. This type of behavior could be described as an example of an experimental neurosis. According to Wolpe (14), "An animal is said to have an experimental neurosis if it displays unadaptive responses that are characterized by anxiety, that are persistent and that have been produced experimentally by behavioral means (as opposed to direct assault on the nervous system by chemical or physical agencies such as poisonings or extirpations)." Warren (15) defined adaptive response as any response appropriate to the situation. In our experimental set-up, the reaction appropriate to the situation was the jump onto the screen only during the presence of the conditioned stimulus.

The question could arise about the reason for the development of such pathologic behavior. Shocks by themselves did not necessarily provide a conflict situation; jumps during the action of the CS effectively prevented the shocks; thus, the number of actual shocks was relatively small. The fixed intervals between stimuli, however, could contribute to the neurotic behavior. Several authors have produced experimental neurosis by rhythmic stimulation using positive stimuli alone. According to Anderson and Parmenter (16), the waiting-time interval itself becomes an additional conditioned stimulus to anxiety response. Furthermore, animals had to delay their learned conditioned responses. Years ago it was observed in Pavlov's laboratory that forced delay of conditioned reactions could provoke general excitation and symptoms of experimental neurosis (17). This finding has been confirmed by several other authors working with the method of delayed conditioned responses (Petrova, 18, Majorov, 19, and as reviewed by Ivanov-Smolenski, 20). Pavlov and his associates assumed that a disturbance of cortical inhibitory processes was responsible for the development of neurotic symptoms in animals.

Behavioral characteristics similar to those described in the present experiment with rats have been observed by Barnes and associates (10–13) in pigs that were malnourished from the third to the eleventh week of life by being fed extremely low protein diets. Conditioned responses and their extinction have been studied several months after nutritional rehabilitation. There were no substantial differences in learning between early restricted and control animals, as expressed by the rate of establishing a classical Pavlovian conditioned response. Previously malnourished pigs, however, were not able to extinguish a once-learned response with facility and during the extinction process exhibited exaggerated anxiety and excitatory behavior. Highly elevated emotionality was characterized by increased vocalization, defecation and urination in the experimental conditions that were used.

In a previously reported experiment (7) we had measured the intensity of spontaneous exploratory activity at 75 and 85 days of age, using the same rats that have been described in this report on avoidance conditioning. All rats that had been nutritionally deprived in the first weeks of life (R–R, R–LP and R–C groups) showed low excitability levels after rehabilitation with a basal, well-balanced diet. In the relatively simple experimental situation in which behavior was tested, the previously malnourished rats exhibited poor spontaneous exploratory activity and were inactive for long periods of time. Thus, the excitability level was raised only when the conditions became more complicated and when stressful stimuli were imposed.

Marked differences were observed in behavioral responses to avoidance conditioning when the effect of food restriction both during the suckling period and after weaning (R–R and R–LP) was compared with that of restriction in the suckling period alone (R–C). The excitability of the R–C group decreased gradually throughout the conditioning process, as characterized by longer latent periods during tests 4 to 6,

prolonged latencies to the first spontaneous intertrial jump, inactivity before conditioning, low spontaneous intertrial activity and rapid extinction of conditioned response. On the other hand, excitability of R–R and R–LP groups increased progressively in successive conditioning tests. It was evident that behavioral disturbances were much more severe in rats malnourished not only before weaning, but also during the 4 weeks after weaning, whether the postweaning restriction was in the form of calorie or protein deficiency. Even though there was a fairly consistent tendency for R–R rats to perform more like normal controls (C–C), the differences between the R–R and R–LP groups were not statistically significant.

Many authors have stressed the importance of the first days of postnatal life in which growth and development of rat's brain proceeds most rapidly, and they evaluated highly the significance of malnutrition in this period (21, 22). The present as well as previous experiments demonstrate that the behavior of rats can be affected not only by changing the nutritional state during the preweaning period, but also by changes in quantity or quality of the diet after weaning (23–25). Results of previous studies by Barnes and his associates (9) have shown that in rats a greater effect on behavior is exerted when both pre- and postweaning nutritional restriction is imposed rather than preweaning deficiency alone. Our present findings support this conclusion.

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Adaptation in Monosaccharide Absorption in Infant and Adult Rats [1]

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ABSTRACT The influence of carbohydrate intake on the ontogeny of intestinal monosaccharide transport in the rat was examined. Nutritionally adequate, carbohydrate-free or 60% glucose diets were provided to litters of rats starting 10 days following parturition. Absorptive rates for glucose were determined in the young animals and adults (mothers) following stomach loading after 8 to 18 weeks. Intestinal transport rates for monosaccharides were sensitive to the presence of dietary carbohydrate. Rate of glucose and galactose absorption in all animals fed the carbohydrate-free diets was 75% of that in animals fed high glucose intakes. Similar differences in absorptive rates in young animals were also present in isolated intestinal loops in situ. In young animals glucose absorptive rates increased to greater than control levels promptly, following addition of glucose to the carbohydrate-free diet.

The purpose of these experiments was to examine the influence of carbohydrate intake on intestinal sugar transport. Studies of others (1–4) suggest that the capacity of the intestine to absorb sugar is related to calorie intake, feeding pattern and diet composition. The observation that sugars absorbed largely by diffusion, such as xylose (5) and sorbose (6), are not altered by procedures which change glucose absorption suggests that only actively transported sugars are influenced. The influence of diet on the absorption of sugars which compete with glucose for absorption has not been studied.

Calorie deficiency appears to be an important stimulus for a nonspecific adaptive increase in intestinal absorptive capacity. Absorptive rates for glucose (7), amino acids (7) and fat (6) are elevated in rats fed calorically restricted diets. Kujalova and Fabry (6) reported that the elevation in glucose absorption observed in intermittently starved rats was reversed by ad libitum realimentation. The feeding of diets diluted with nonabsorbable bulk also resulted in an increased glucose absorption rate (1). Dowling (1) suggested that these procedures have in common a net reduction of calories.

That intestinal adaptations may be specific is indicated by observations that the glucose absorptive capacity responds to the carbohydrate intake independently of calorie deficiency. In rats adapted to intermittent starvation on a carbohydrate-free diet, intestinal glucose absorption did not differ from controls fed the same diet ad libitum (6). Hahn and Koldovsky (2) reported that rats weaned to a high fat, low carbohydrate diet have lower absorptive capacities for glucose than animals weaned to standard diets containing 60% carbohydrate. It has not been demonstrated whether this depression can be induced in adults or whether it is reversible when induced in young animals.

The pattern of food intake also influences intestinal absorptive capacity. Intermittent starvation induced an increase in glucose absorption rate, whereas continued feeding of the same amount of food, divided into equal daily rations, was followed by a gradual decrease to levels below control (6). These findings led Kujalova and Fabry to suggest that the stimulus for increased absorption in intermittent starvation was due to adaptations induced by the increased intake during the periods of food abundance.

The experiments described here were undertaken to characterize further the in-

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fluence of dietary carbohydrate on intestinal sugar absorptive functions in infant and adult rats fed nutritionally adequate diets. Do infant rats respond to dietary modifications as do adults? Are the responses reversible? What are the effects on the transport of sugars other than glucose?

METHODS

Pregnant female rats of the Holtzman strain were obtained.[4] At 10 days following parturition, either a carbohydrate-free or a 60% carbohydrate diet was provided to mothers and young. Pups were separated from the dams at 20 days of age. No other foodstuff, except water (and, until weaning, milk), was available to the animals. The control diet contained 60% glucose, 15% fat (Crisco[5]), 21% casein, 4% Wesson's salt mixture (5) and a complete vitamin supplement.[6] The carbohydrate-free diet contained 35% casein, 59% fat (Crisco), 6% salt mixture plus the vitamins in amounts to maintain the vitamin-to-calorie concentration ratio equal to that of the control diet. Analysis of the acid hydrolysate of the casein for glucose and galactose by enzymatic methods failed to detect galactose. The glucose content of the casein was 0.004% by weight. Some young rats weaned to the carbohydrate-free diet were shifted to a diet containing 30% carbohydrate at 8 weeks of age. All diets contained 7 to 8 mg nitrogen/kcal.

Glucose and galactose absorptions were measured in vivo by the method described by Cori (8). Eighty minutes after receiving a stomach load of 3 ml of 30% glucose or galactose, animals were decapitated, and the contents of the stomach and small intestine were quantitatively recovered for analysis. Absorption coefficients for glucose were determined in adults and in young fed the respective diets for 8 weeks, and again in young animals at 18 weeks. One group of young reared on the carbohydrate-free diet was fed the 30% glucose diet for either 2 or 4 weeks prior to measurement of absorption rates. Galactose-absorptive capacities were measured after 10 weeks.

To determine absorption rates independently of such variables as stomach emptying and intestinal propulsive activity, sugar-absorption rates were also determined in isolated intestinal loops in situ (9). These studies were carried out in rats anesthetized with pentobarbital at 13 weeks. The loops were isolated between ligatures located 2 cm below the common bile duct and 3 cm proximal to the ileocecal valve. Four to five milliliters of 4% glucose or 10% galactose in 0.9% saline were placed in the loop, and after 20 minutes the residual content was collected and analyzed by enzyme-specific methods.

After recovery of luminal contents, small intestines were blotted and weighed before and after drying to constant weight at 105 to 110°. Glucose and galactose were measured enzymatically (10) in protein-free filtrates (11) of intestinal and stomach contents using enzyme systems commercially available.[7] Difference between administered and recovered monosaccharide was considered absorbed and absorptive rates were recorded as milligrams per 100 g body weight per hour and milligrams per 100 g dry weight of intestine per hour. Statistical evaluation employed the t test for small samples of unequal size (12).

RESULTS

Intestinal weights. Intestinal wet and dry weights were not different in animals fed the diets employed. Intestinal dry weight per 100 g body weight in various groups of animals fed carbohydrate ranged between 0.52 ± 0.08 and 0.59 ± 0.10 and in the experimental animals from 0.45 ± 0.062 to 0.62 ± 0.16. Only in one subgroup (galactose test meal animals, 14 weeks of age) was there a significant difference ($P = 0.02$) between control and experimental animals. In this group the dry gut weight-to-body weight ratio of experimental animals was 15% lower than control. The difference appeared to be related to a proportionally greater gain in

[4] The Holtzman Company, Madison, Wisconsin.

[5] Procter and Gamble Company, Cincinnati, Ohio.

[6] Vitamin content as milligrams per kilogram of diet: menadione, 50; thiamine, 10; pyridoxine, 40; calcium pantothenate, 25; nicotinamide·HCl, 50; p-aminobenzoic acid, 50; folic acid, 0.6; biotin, 0.6; riboflavin, 20; inositol, 100; vitamin B_{12}, 0.01; choline chloride, 1000; and 19,000 units of vitamin A and 1900 units of vitamin D added as USP cod liver oil. Crisco used to provide the fat content contained 104 mg/g of tocopherol (Victor Emil, personal communication).

[7] Obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

TABLE 1

Effect of diet on glucose absorption following stomach loading

Time fed diet [1]	Diet	Body wt	Absorption rates [3]	
			100 g body wt	100 g dry intestine
weeks		*g*	*mg/hr*	*mg/hr*
8	60% CHO (9) [2]	152 ± 10	247 ± 11	434 ± 27
	CHO free (7)	156 ± 6	191 ± 14 **	323 ± 16 **
10	CHO free 8 weeks, then 30% CHO for 2 weeks (8)	148 ± 8	293 ± 16 *	580 ± 33 **
	CHO free 6 weeks then 30% CHO for 4 weeks (6)	142 ± 8	293 ± 19 *	476 ± 36
18 [4]	60% CHO (14)	260 ± 11	192 ± 7	370 ± 18
	CHO free (13)	275 ± 9	136 ± 6 **	293 ± 18 **

[1] Diet indicated was available from 10 days of age.
[2] Number of animals per group.
[3] Mean value ± SE.
[4] Mean values for absorption per unit dry intestine weight are for seven animals in each group.
* Mean value significantly different from control fed carbohydrate, $P < 0.05$.
** Mean value significantly different from control fed carbohydrate, $P < 0.01$.

body weight than in gut weight. In view of the findings with six other subgroups of younger and older animals, this was considered to be spurious. In the other subgroups the body weight (table 1), dry gut weight and ratio of dry intestinal weight to body weight showed no significant difference between control and experimental animals. In the galactose test meal group the change in the ratio of dry intestine to body weight was only half the magnitude of the difference in absorptive coefficients observed and did not appear to be related to the changes in absorption rate.

Monosaccharide absorption following stomach loading. Adults fed the carbohydrate-free diet for 8 weeks had glucose absorption rates approximately 75% of that of animals fed the 60% carbohydrate diet. Adult animals on the 60% carbohydrate diet absorbed 181 ± 5 mg glucose/100 g body weight per hour as compared with 150 ± 7 mg for animals on a carbohydrate-free diet. Absorption in milligrams per 100 g intestinal dry weight was 336 ± 28 and 250 ± 23 for control and experimental groups, respectively. Values for animals fed carbohydrate-free diets were significantly lower ($P < 0.05$) than those for control animals by either method of comparison.

In young animals reared on the carbohydrate-free diet for 8 weeks, absorptive rates were similarly decreased below that of controls fed the high glucose diets. Differences were of the same order of magnitude whether computed in terms of body weight or gut weight (table 1).

Young rats weaned on the carbohydrate-free diets and shifted at 8 weeks to a 30% carbohydrate diet had absorption rates 2 weeks later significantly greater per unit body weight or per unit dry intestine than animals fed the 60% carbohydrate diet exclusively. After 4 weeks, absorption was significantly different when compared in absorptive rates per unit body weight but not per unit of intestinal dry weight (table 1).

Impaired glucose absorption persisted in animals fed the carbohydrate-free diet for 18 weeks. At this time absorptive rates were depressed by about the same magnitude as at 8 weeks (table 1), although absolute values were lower at the later age.

The absorption of galactose, a sugar which competes with glucose for transport in the intestine (4), was also significantly lower per unit body weight or per unit intestinal dry weight in animals reared without, as compared with animals fed, dietary glucose. Animals fed the carbohydrate-free diet for 13 weeks absorbed 130 ± 6 mg as compared with 197 ± 6 mg/100 g body weight per hour for the control group. When calculated on a basis of dry weight of intestine, values were 282 ± 13 and 379 ± 25 mg/g for animals fed the carbohydrate-free diet and 60% carbohydrate

TABLE 2

Sugar absorption in intestinal loops in situ [1]

Test sugar	Diet	100 g wet intestine	100 g dry intestine
		mg/hr	*mg/hr*
Glucose	60% CHO	42.2 ± 1.2 [2]	187 ± 4.9 [2]
	CHO free	30.3 ± 2.2 [3]	141 ± 7.5 [3]
Galactose	60% CHO	84.7 ± 3.4	nd [4]
	CHO free	63.5 ± 2.1 [3]	nd

[1] There were eight animals in each group, but only seven in the group fed the high carbohydrate diet and given galactose as the test substance. Animals were 13 weeks old.
[2] Mean value ± SE.
[3] Difference from control animals significantly different $P < 0.001$.
[4] nd = intestinal dry weight not determined.

diet, respectively. By both methods of comparison values are significantly different ($P < 0.005$). There were 9 animals in each galactose test meal group.

Absorption from isolated intestinal loops in situ. The absorption of both galactose and glucose from intestinal loops in situ was also significantly reduced in rats on the carbohydrate-free diet (table 2) when compared with animals on high glucose intakes. Differences were of the same order of magnitude as that observed following stomach loading (table 2).

DISCUSSION

The rats in these studies were fed a nutritionally adequate diet as evidenced by its composition, the appearance of the animals and the normal growth of all animals. The diet-related differences in glucose and galactose absorption were of about the same magnitude whether calculated per unit body weight or per unit dry intestine weight, and whether measured by stomach loading in waking rats or in isolated loops in situ.

Adaptations in absorptive capacity developed with no change in intestinal weight. There were no significant differences in dry intestine-to-body weight ratios except in the galactose test meal subgroup, and here the difference was not great and could not account for the differences in absorption rates observed. The animals on the carbohydrate-free diet showed depression of galactose absorption per unit of intestinal weight in stomach loading and isolated loop experiments.

The parallel decrease of absorptive rate for both galactose and glucose induced by feeding the carbohydrate-free diet was of about the same magnitude. This implies a modification in the total capacity of the monosaccharide transport system, rather than a specific effect on glucose, and suggests that the adaptations that develop may be quantitative rather than qualitative in nature. The minimal amount of dietary carbohydrate necessary to induce this response is not known and cannot be determined from these data. These studies support the idea that the quantitative capacity of the intestine for active transport of sugar is specifically sensitive to the presence of carbohydrate in the diet.

Of particular interest in this context is the prompt elevation of absorptive capacity to greater than control levels when carbohydrate is added to the diet of rats weaned on carbohydrate-free diets. After 2 weeks on a diet containing 30% glucose, absorption rates were 134% of the controls and 180% of that of rats continued on the carbohydrate-free diet throughout the experimental period. It would be of interest to know whether such selective adaptive changes in absorption, as found here for glucose, occur for other substrates. That this may be so is suggested by the findings of others of maintenance of body weight on diets of widely varying composition, and by the reports of enhanced absorption of amino acid (7) and fat (6) in response to changes in nutritional status.

The ontogeny of sugar absorption in the infant rat is not influenced permanently by the presence or absence of dietary glucose in the postweaning period, but the magnitude of absorptive capacity can vary in response to the content of dietary carbohydrate fed to immature and adult rats.

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Inhibitory Effects of Alcohol on Intestinal Amino Acid Transport in vivo and in vitro [1]

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ABSTRACT The effect of ethanol on the active transport of amino acids in the rat intestine was studied in vitro and in vivo. It was shown that 0.5 and 2.0% ethanol significantly inhibited the active transport of L-phenylalanine in everted sacs in vitro by 60 and 84%, respectively. The latter concentration completely abolished the active transport of L-methionine. At this concentration ethanol reduced tissue respiration by only 19%. In studies in vivo, 250 mg alcohol/100 g body weight given by stomach tube significantly inhibited the intestinal absorption of L-phenylalanine by about 50% but did not modify absorption of D-phenylalanine, an amino acid that is not actively transported. The possible significance of these findings is discussed in relation to the nutritional deficiencies and fatty liver that occur in chronic alcoholism.

It has been reported that ethanol,[2] in concentrations comparable to those used in studies in vivo, inhibits the active transport of Na^+ and K^+ in several tissues from different species (1–4). This appears to be due to the inhibitory effect of ethanol on the $(Na^+ + K^+)$-activated ATPase (5, 6), an enzymatic activity related to the active transport of Na^+ and K^+ across the cellular membrane (7, 8). Several authors have shown that the active transport of amino acids in the intestine depends on the presence of a fully operative active transport of Na^+ or $(Na^+ + K^+)$-activated ATPase, or both (9–12). It was, therefore, conceivable that ethanol would also inhibit the active transport of amino acids. This might explain the protein deficiency and liver steatosis that appear in humans and animals that consume ethanol with apparently balanced diets (13).

The effect of ethanol on the active transport of amino acids was studied in vitro in everted sacs of intestine and in vivo on amino acid intestinal absorption into the blood. Ethanol was found to inhibit the active transport of amino acids both in vitro and in vivo.

METHODS

The method used to measure active transport of amino acids in vitro was that described by Wilson and Wiseman (14) in everted sacs of intestine. Male albino rats of the University of Chile strain, weighing 250 to 300 g (4 to 6 months), were fed a balanced diet ad libitum; they were decapitated; the small intestine was stripped by teasing it from the mesentery; and the everted jejunum was used. The incubation medium was saturated with oxygen and contained 118 mM NaCl; 4.7 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 2.5 mM $CaCl_2$; 25 mM Tris-HCl, pH 7.4; 5 mM of ^{3}H-L-amino acid [3] (2×10^4 cpm/μmole); and 0.2% glucose. Sacs of 4 to 5 cm were filled with 0.8 to 1.0 ml of the incubation medium and closed, weighed and promptly incubated in flasks containing 10 ml of the same medium as in the sacs. The flasks were shaken in a water bath at 37° (70 strokes/minute). After 1 hour the sacs were removed and their contents drained. The radioactivity of an aliquot of the inner and outer incubation fluids was then determined in a liquid scintillation spectrometer using a phosphor solution in toluene [4] and was corrected for quenching by internal standardization. The ratio of the

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[2] The words alcohol and ethanol are used interchangeably.

[3] The Radiochemical Centre, Amersham, England.

[4] Liquifluor, Nuclear-Chicago Corporation, Des Plaines, Illinois.

final radioactivity of amino acid inside the sac to final radioactivity in the incubation medium was used as a measure of the active transport. At the beginning of the incubation this value equals 1.0; any value of more than 1.0 after the incubation period indicates an active transport (9). The recovery of total radioactivity after the incubation (inside plus outside the sac) was 95 to 97%.

To study the effect of ethanol on active amino acid transport in vivo, ^{3}H-L-phenylalanine was given by stomach tube, as a 5 mM solution (1 ml/100 g body weight with 2×10^6 cpm/μmole). Alcohol, when given, was administered with the amino acid solution at a concentration of 25% (w/v) in a final dose of 250 mg of ethanol per 100 g of body weight. At 5, 10, 20 and 60 minutes after the administration of the labeled amino acid, 0.10 ml of blood was extracted from the tail vein by pipet into 4.9 ml of 96% ethanol. The precipitate was centrifuged, and an aliquot of the clear supernatant was mixed in a 3:7 proportion with the phosphor solution in toluene and then counted in a liquid scintillation counter. Quenching was corrected by internal standardization. Previous studies had shown that 99 to 100% of the phenylalanine present in whole blood was extracted by this method.

The in vivo effect of alcohol on passive transport across the intestine was determined using D-phenylalanine, an amino acid that is not actively transported in the intestine (15). D-Phenylalanine, at a concentration of 20 mg/ml, was given by stomach tube in a dose of 20 mg/100 g of body weight. When alcohol was given, it was administered as a 25% solution (w/v) with the amino acid, as in the L-amino acid study, in a final dose of 250 mg ethanol/100 g of body weight. At different times after the administration, 0.10 ml of blood was extracted from the tail vein, mixed with 0.10 ml of 0.15 M NaCl and centrifuged at 3000 rpm for 10 minutes. Phenylalanine was determined fluorometrically in 50 μl of the supernatant by the micromethod of Wong et al. (16). Blanks of blood from the same animal extracted before the administration of the D-phenylalanine also were analyzed.

Alcohol in the lumen of the rat jejunum was collected by washing the contents of the jejunum with saline, and its concentration was measured enzymatically by the method of Kaplan and Ciotti (17). The rats were fasted for 18 hours before all experiments but allowed to drink water. Oxygen consumption of small segments of jejunum was determined in an air-saturated solution of the same composition as in the everted sac experiments. For this an oxygen electrode [5] was used attached to a recorder.

RESULTS

The results in table 1 indicate that under our experimental conditions the amino acid accumulation in the serosal side *is* an active process. Inhibitors of cell metabolism such as cyanide, dinitrophenol and fluoride abolished the amino acid accumulation. The effect of alcohol on the active transport of L-phenylalanine in vitro is shown in figure 1. In concentrations as low as 0.5%, ethanol significantly inhibited the active amino acid accumulation by 60% ($P < 0.05$). At 2% the inhibition was of 84% ($P < 0.005$). At 0.3% the inhibition (25%) was not significant ($0.1 > P > 0.05$). The effect of ethanol on the active transport of L-methionine was also studied. As shown in table 2 the inhibition at 2% alcohol was nearly 100%. The control ratios of the concentrations (inside/outside) obtained with L-methionine were significantly lower than those found using L-phenylalanine. This observation is in agreement with the data of Wiseman (18)

TABLE 1

Effect of different metabolic inhibitors on the active accumulation of ^{3}H-L-phenylalanine in everted intestinal sacs, in vitro

Exp.	Inhibitor	Amino acid ratio [1] (inside/outside)
A	—	2.3
	10^{-4} M dinitrophenol	1.05
	10^{-3} M KCN	1.09
B	—	2.05
	9×10^{-3}M NaF	0.95

[1] A ratio higher than 1.0 is, by definition, due to an active transport (9). The values are the average of two determinations for the same animal.

[5] Clark oxygen electrode, Yellow Springs Instrument Company, Yellow Springs, Ohio.

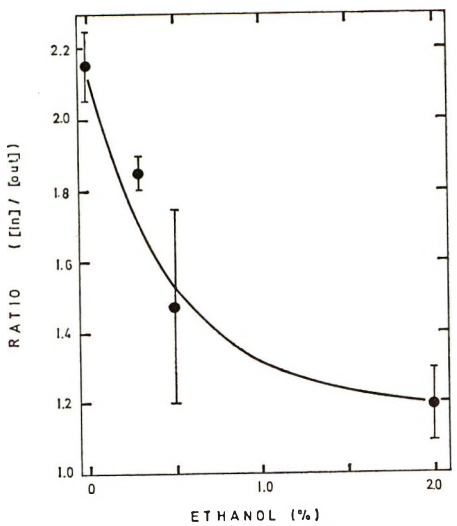

Fig. 1 Effect of ethanol on the active accumulation of ^{3}H-L-phenylalanine in everted sacs of rat small intestine. The vertical bars represent the SE of the mean of at least five experiments.

TABLE 2

Effect of 2% ethanol on the active accumulation of ^{3}H-L-methionine in everted intestinal sacs, in vitro

Exp. no.	Amino acid ratio (inside/outside) [1]	
	Control	2% ethanol
1	1.53	0.97
2	1.62	1.09
3	1.40	0.94
Avg	1.52	1.00

[1] The values given are the average of two determinations for the same animal.

and of Chang et al. (19) who also observed smaller ratios for L-methionine.

Since ethanol may conceivably inhibit the active transport of amino acids by interacting with the oxidative metabolism of the intestinal tissue, its effect on tissue respiration was studied. The amino acid present in the incubation medium was L-phenylalanine. As shown in figure 2, ethanol at 2% produced only a slight inhibition of tissue respiration (19%). Cyanide (10^{-3} M), at a concentration that inhibits the active transport of amino acids as much as 2% ethanol, inhibited the tissue respiration by nearly 80%.

Alcohol also inhibited the intestinal absorption of L-phenylalanine in rats in vivo (fig. 3). A dose of 250 mg alcohol/100 g rat weight was given with the amino acid. In the presence of alcohol absorption was lower than that of the controls at all times studied and was significantly different from the controls at 5 and 60 minutes ($P < 0.025$ and $P < 0.01$, respectively). Under the same conditions alcohol did not prevent the passive absorption of D-phenylalanine; rather a small, but nonsignificant, increase was noted (fig. 4).

The effect of alcohol on the active transport of L-phenylalanine in vivo is in agreement with its effect on the everted sacs in vitro, taking into consideration the concentrations of alcohol that are likely to reach the intestine after the dose of alcohol given. In preliminary experiments the alco-

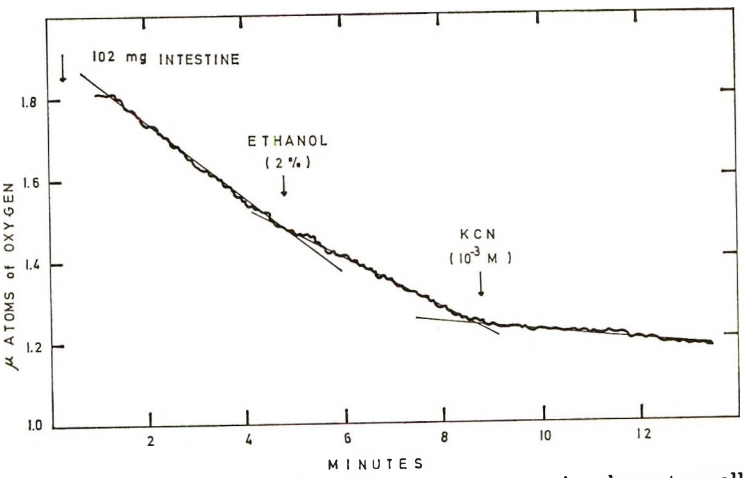

Fig. 2 Effect of ethanol and cyanide on oxygen consumption by rat small intestine. Tracing of the oxygen electrode of one representative experiment.

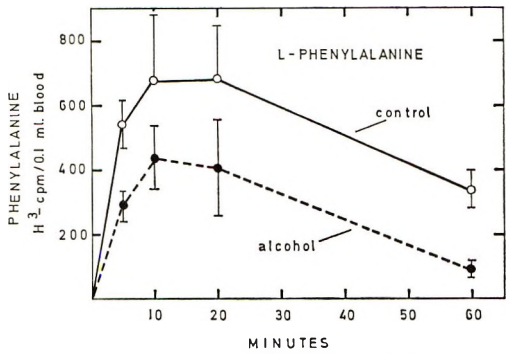

Fig. 3 Effect of ethanol on intestinal L-amino acid absorption in vivo. ^{3}H-L-phenylalanine was given orally with or without ethanol (250 mg ethanol/100 g animal). Absorption was followed by the appearance of radioactivity in the blood. Values represent the average ± SE of at least four animals.

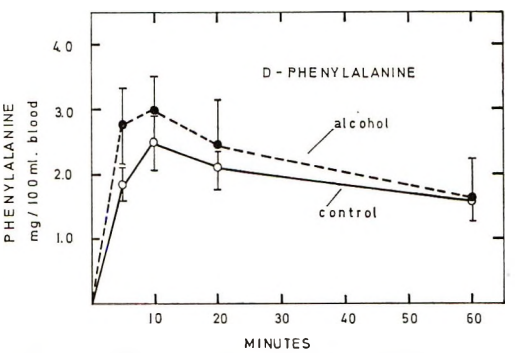

Fig. 4 Effect of ethanol on intestinal D-amino acid absorption in vivo. D-Phenylalanine was given orally with and without ethanol (250 mg/100 g animal). Phenylalanine was determined in the blood by a fluorometric method (16). Values represent the average ± SE of at least six animals.

TABLE 3

Concentration of ethanol in the jejunal lumen after oral administration of 200 mg ethanol/100 g rat weight

Time after administration [1]	Mean value	Range 3 exp.
minutes	%	%
5	1.30	1.60–0.90
10	1.75	2.70–0.98
20	1.40	1.65–0.78
60	0.65	0.68–0.60

[1] Alcohol was given as a 20% (w/v) solution.

hol concentration in the jejunal lumen was determined after a dose of 200 mg ethanol/100 g body weight given as a 20% (w/v) solution. It was found that even 1 hour after administration, the concentration of alcohol in the intestine was 0.65%. Higher values were observed at 5, 10 and 20 minutes, with a maximum of 1.75% at 10 minutes (table 3).

DISCUSSION

The inhibitory effect of alcohol on the active transport of amino acids in the intestine is in keeping with the observations by several authors (9–12) that a fully operative active transport of Na^+ or ($Na^+ + K^+$)-activated ATPase is necessary for maximal active amino acid transport. Israel, Kalant and co-workers have observed that ethanol inhibits both ion transport and ATPase in several tissues of different species (1–3, 5). The inhibitory effect on amino acid transport was seen both in vitro and in vivo at concentrations of alcohol that are within the range of those that may exist in the intestine after the consumption of moderate to large doses by humans.

As shown, not all the alcohol given orally is absorbed in the stomach; some passes to the small intestine where the active absorption of amino acids takes place. The actual concentration of alcohol in the intestinal fluid will depend on the speed of absorption from the stomach and of emptying of it. Data by Harichaux and Moline (20) show that alcohol, in concentrations higher than 5%, hastens the emptying of the stomach. It is also conceivable that, under some conditions, alcohol might attain a higher concentration in the intestine when the same final dose is given in a large volume rather than in concentrated form. This might occur since the absorption of alcohol in the stomach may be slower if given in larger volume and because emptying of the stomach is promoted by a certain degree of gastric distension.

While this work was in progress, Chang et al. (19) reported that ethanol and other alcohols inhibited the active transport of amino acids in vitro in the rat intestine. These workers found no significant effect at 1% alcohol, but a significant inhibition was found at 3% for a series of amino acids, including phenylalanine and methionine. Under our experimental conditions in vitro, 0.5% ethanol inhibited the active transport of L-phenylalanine by 60% ($P <$

0.05). For methionine, a concentration of 2% ethanol completely abolished active accumulation. Using the small intestine of the hamster, Spencer et al. (21) found that 4.6% ethanol inhibited the active transport of glycine and L-proline. Chambers et al.[6] have reported that 45 mM alcohol (0.2%) inhibits by 40% the uptake of α-aminoisobutyric acid in perfused rat liver.

The small inhibitory effect of 2% alcohol on oxygen consumption of the intestine compared with the marked inhibitory effect of 10^{-3} M cyanide would at least indicate that ethanol does not inhibit the active transport of amino acids by a direct interference in the oxidative metabolism of the tissue. This is in keeping with data in other tissues that alcohol at the concentrations used is not an inhibitor of mitochondrial respiration or oxidative phosphorylation (22, 23).

Alcohol also inhibited active amino acid absorption in vivo (L-phenylalanine) without modifying passive absorption (D-phenylalanine). Chang and co-workers[7] reported that 50% alcohol given orally to rats inhibited the absorption of the L- as well as the D- forms of phenylalanine. It is known, however, that alcohol given at these concentrations produces gross histological alterations in the intestinal mucosa (24). This damage might result in changes in the passive as well as active absorption of solutes.

Studies by Lieber et al. (13) have shown that a long-term administration of ethanol given with balanced liquid diets as isocaloric replacement for sucrose produces fatty liver. These authors have therefore proposed that under their conditions alcohol produces liver steatosis per se and that nutritional factors are not important. Nevertheless, if alcohol would have inhibited the active absorption of amino acids in the intestine, as shown in our study, an effective nutritional deficiency could arise. This idea was supported by the fact that the animals receiving alcohol in their liquid diets did not grow at the same rate as the sucrose controls (13). The same group of investigators has also reported that supplementation with methionine or choline in relatively large amounts reduced by 70 to 80% the accumulation of liver triglycerides induced by alcohol (25).

Our findings might also explain the recent observations by Wallgren et al. (26) that rats given alcohol solutions by stomach tube once a day (in the daytime) showed markedly less fatty liver than rats given the same amount of ethanol as the only source of fluid. As shown by these authors, the rats drink most of their liquid in a 2- to 3-hour period, around midnight. Thus, the rats given alcohol as the only fluid source consume it at approximately the same time they normally eat. It is therefore not unlikely that under these conditions amino acid absorption might have been impaired.

Further studies are in progress to determine the relative importance of the present findings in human liver steatosis and also to explore the mechanism(s) by which alcohol produces its effect.

ACKNOWLEDGMENTS

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Utilization of Carotene, Vitamin A and Triglyceride Following Oral Intake of Triton

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ABSTRACT Triton has been suggested as a nutritional adjunct to modify the absorption of fat. The postulated mode of action implied that metabolism of the fat-soluble vitamins might also be impaired. Experiments were conducted with rats to study the effects of dietary Triton WR-1339 on the utilization of β-carotene, vitamin A, trioleate and oleic acid. The absorption of vitamin A and utilization of the provitamin were not impaired by the prefeeding of Triton. Triton did not alter the radioactivity in blood, liver or depot fat following oral administration of ^{14}C-triolein or ^{14}C-oleic acid. The data suggest that Triton WR-1339 at levels below 5% has little practical significance for inducing a controllable steatorrhea.

The use of compounds to alter fat absorption has practical significance in the management of a number of disease conditions. Surface active agents have been studied in this regard. Some such as polysorbate 80 (Tween 80) have been reported to increase lipid absorption (1), whereas others (Triton WR-1339) have been reported to decrease lipid absorption (2,3). Since changes in fat absorption may have significant effects on the utilization of fat soluble vitamins and on the absorption of fat soluble drugs it was considered desirable to study the metabolism of specific lipids in relation to altered fat absorption.

Ideally, a detergent should limit fat absorption and not be absorbable. It has been reported (3,4) that Triton WR-1339 meets both requirements and, therefore, was chosen for further study. The utilization of dietary carotene, vitamin A and triglyceride following the feeding of Triton is reported.

MATERIALS AND METHODS

Male rats of the Wistar strain, weaned at 21 days of age, fed either ground stock cubes or a semipurified vitamin A-free diet (5) having the following composition: (in percent) vitamin-free casein, 18; dried brewer's yeast, 8; sugar, 65; salt mix USP XIV, 4; and cottonseed oil, 5. The cottonseed oil contained per 100 g: vitamin E, as *dl*-α-tocopheryl acetate, 60 mg; vitamin K as 2-methyl-1,4-naphthoquinone, 1.5 mg; and vitamin D_2, 8000 units. The experimental designs are shown below. Rats were killed at specified times, and liver vitamin A was determined by the Carr-Price reaction following saponification of the tissue (6). The data were statistically analyzed by analysis of variance (7).

Experiment 1. This experiment was designed to study the utilization of a single oral dose of β-carotene or vitamin A in the rat during the feeding of Triton. Sixty rats were fed the semipurified vitamin A-free diet to which stabilized vitamin A was added, equivalent to 180 μg vitamin A alcohol per kilogram of diet (a level of vitamin previously shown to give adequate growth with a minimum of liver stores). The animals were divided into 6 groups of 10 each. Triton 2% was added to the basal diet of groups 2, 4 and 6. On day 17 test animals in groups 3 and 4 were given orally by stomach tube 0.5 ml cottonseed oil containing 4.268 mg of all *trans* β-carotene; groups 5 and 6 received 0.5 ml cottonseed oil containing 455 μg of vitamin A alcohol; and groups 1 and 2 received only 0.5 ml cottonseed oil. Forty-eight hours later the animals were killed and the liver removed.

Experiment 2. This experiment was designed to study the effect of Triton on the utilization of vitamin A and the provitamins in a practical type diet, containing 2046 μg of vitamin A and 3960 μg β-carotene/kg. The basal diet was ground stock

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TABLE 1

Effect of dietary Triton on the utilization of a single oral dose of carotene or vitamin A

	Group					
	1	2	3	4	5	6
Dietary Triton	nil	2%	nil	2%	nil	2%
Oral supplement	nil	nil	carotene	carotene	vitamin A	vitamin A
Initial body wt, g	59 ± 2 [1]	59 ± 2	59 ± 2	59 ± 2	59 ± 2	58 ± 2
Final body wt, g	128 ± 4	131 ± 4	116 ± 4	124 ± 5	132 ± 3	123 ± 5
Liver wt, g	7.45 ± 0.36	7.34 ± 0.35	6.46 ± 0.40	7.21 ± 0.33	7.39 ± 0.32	7.17 ± 0.36
Vitamin A, μg/g liver	1.4 ± 0.2	1.5 ± 0.2	21.1 ± 2.1	18.7 ± 2.3	21.7 ± 1.5	23.2 ± 2.2
μg/liver	9 ± 1	11 ± 1	131 ± 13	136 ± 18	146 ± 8	160 ± 7

[1] SE of the mean.

cubes containing 3% corn oil. Sixty rats were divided into 3 groups. Group 1 received the basal diet and served as untreated controls. Group 2 received the basal diet to which 2% Triton was added. Group 3 was fed the basal diet containing 5% Triton. Animals were fed the above regimens for 28 days and liver vitamin A stores determined.

Experiment 3. To study the effects of dietary Triton on absorption and tissue deposition of radioactive triglycerides and fatty acids, two separate experiments of 36 animals each were conducted. The results from both experiments were combined. Half the animals were fed a ground cube diet containing 3% corn oil; the remainder were fed this diet containing 2% Triton. A radioactive triglyceride dosing solution was prepared by mixing 0.1 mCi glyceryl trioleate-1-^{14}C [1] with 12.5 ml of inactive glyceryl trioleate. A similar fatty acid dosing solution was prepared by adding 0.1 mCi oleic acid-^{14}C to 12.5 ml of inactive oleic acid. After 26 days all animals were dosed by stomach tube with 0.25 ml of either solution containing 2.0 μCi of ^{14}C. In both experiments, three animals on each treatment were killed at time intervals of 3, 6 and 12 hours after dosing. Blood, liver and a sample of abdominal fat were removed from each animal.

Tissues were assayed for radioactivity by the following procedures. Fat samples (approximately 2 to 5 g) were homogenized in 50 ml of a mixture of 2:1, chloroform–methanol. Appropriate aliquots were transferred to scintillation counting vials and evaporated to dryness. Individual livers were homogenized with 100 ml of the chloroform – methanol mixture, filtered through sintered glass and made to a known volume. Blood serum was shaken with chloroform and methanol, and aliquots were transferred to counting vials. All samples were counted in a liquid scintillation counter using 10 ml toluene containing 0.5% 2,5-diphenyloxazole (POP) and 0.03% 1,4-bis [2-(5-phenyloxazolyl)]-benzene (POPOP).

RESULTS

The effect of dietary Triton on the utilization of a single oral dose of carotene or vitamin A is shown in table 1. Neither Triton, nor the source of vitamin A, had a significant effect on the final body weight or liver weight. Oral administration of vitamin A increased liver stores of the vitamin and Triton exerted no significant effect on storage. Similar results were observed when β-carotene was administered as a source of vitamin A.

Feeding a practical type diet containing up to 5% Triton for 28 days produced no significant effects on the final body weight, liver weight or liver vitamin A stores (table 2).

In experiment 3 adult rats were fed a ground cube diet with or without added Triton for 26 days before oral administration of the radioactive triglyceride or fatty acid. Utilization of the radioactive materials was established for the control animals and is shown in table 3. Three hours after dosing, the blood and liver contained the highest observed level of radioactivity which decreased with time. In contrast, the radioactivity in the depot fat increased

[1] Obtained from Radiochemical Centre, Amersham, England.

TABLE 2

Effect of Triton on the utilization of vitamin A from a practical diet

	Group		
	1	2	3
Triton as percentage of diet	nil	2	5
Initial body wt, g	54.5 ± 1.3 [1]	54.6 ± 1.2	54.5 ± 1.5
Final body wt, g	139.1 ± 3.8	141.1 ± 3.9	132.8 ± 3.8
Liver wt, g	6.9 ± 0.3	7.3 ± 0.3	6.8 ± 0.3
Vitamin A, μg/g liver	45.6 ± 1.0	45.4 ± 1.2	45.8 ± 1.3
μg/liver	312.1 ± 10.9	328.4 ± 10.9	305.0 ± 10.7

[1] SE of the mean.

TABLE 3

Effect of Triton on the tissue distribution of ^{14}C following oral administration of triolein or oleic acid

		Group			
		1	2	3	4
Diet supplement		nil	Triton	nil	Triton
Oral dose		Triolein [1]	Triolein [1]	Oleic acid [2]	Oleic acid [2]
Body wt, g		455 ± 11 [3]	469 ± 9	469 ± 14	464 ± 10
^{14}C activity					
Blood, dpm/ml serum	3 hr	2391 ± 355	2226 ± 282	1753 ± 393	1667 ± 436
	6 hr	864 ± 137	1331 ± 286	662 ± 190	539 ± 128
	12 hr	398 ± 636	541 ± 103	439 ± 75	439 ± 70
Fat, dpm/g	3 hr	1432 ± 211	1124 ± 122	619 ± 154	642 ± 119
	6 hr	1450 ± 153	1578 ± 162	1051 ± 145	892 ± 194
	12 hr	2277 ± 119	2558 ± 274	2204 ± 205	2764 ± 99
Liver, dpm/g	3 hr	20833 ± 2340	15924 ± 1219	8619 ± 1397	8796 ± 2730
	6 hr	13063 ± 1258	12690 ± 1958	8038 ± 1281	8731 ± 1695
	12 hr	6960 ± 694	7116 ± 1105	6051 ± 1088	6240 ± 466

[1] Triolein dosing solution (0.25 ml) contained 4.34×10^6 dpm.
[2] Oleic acid dosing solution (0.25 ml) contained 4.54×10^6 dpm.
[3] SE of the mean.

throughout the experimental period. The distribution patterns of ^{14}C observed in the animals receiving dietary Triton were similar to those observed for control animals. Triton did not limit the absorption of radioactive oleic acid or triolein.

DISCUSSION

Triton (WR-1339) when administered parenterally induces a hyperlipemia (8). Janicki et al. (9) demonstrated that this effect does not occur as a result of enhanced absorption of dietary fat. More recently (3) it was suggested that fat absorption was delayed when Triton was administered orally with fat, the effect being mediated by the inhibition of lipolysis (4). These results have suggested (10) another approach to the practical management of fat absorption in certain disease conditions. It was further stated that if the material has a potent effect on lipolysis, then production of a predictable and controlled steatorrhea could become feasible. The mode of action of oral Triton inhibiting lipolysis, as demonstrated by its effect on pancreatic lipase, suggested by Tidwell et al. (4), theoretically could seriously impair the metabolism of fat-soluble vitamins. Triton would thus simulate conditions observed in cystic fibrosis. Certo (11) stated that pancreatic enzyme deficiency occurs in more than 80% of patients with cystic fibrosis and that all foods, fats in particular, are poorly absorbed. Similarly, low levels of vitamin A and vitamin E have been observed in these patients (12, 13). If Triton had a

similar effect on the absorption of fat-soluble vitamins its use would be contraindicated, or supplementation during therapy would be required. The present studies clearly indicate, however, that the feeding of up to 5% Triton in the diet of the rat does not impair carotene or vitamin A metabolism. These findings would not be expected from considerations of the proposed mechanism of action. Studies were therefore undertaken to examine directly the effects of Triton on the utilization of fat using radioactive substrates. The appearance rate of ^{14}C in the serum and liver and deposition in the body fat following Triton ingestion does not lend support to the concept that this agent is effecive in inducing a controllable steatorrhea. The present findings regarding fat absorption following oral administration of Triton are similar to those observed following parenteral administration of Triton (9) and would support the concept that Triton does not modify fat absorption.

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Virus Involvement in the Avian Heart: Effect on Protein Synthesis [1]

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ABSTRACT The effects of a Newcastle disease virus on myocardial synthesis of protein were observed in young, rapidly growing chicks. Biochemical parameters, selected to reflect this vital process, were tissue protein, DNA, RNA and free amino acids. The results showed that protein synthesis is rhythmic in the avian heart, with significant yet-to-be explained diurnal changes in nucleic and free amino acids. The virus infection resulted in a highly significant depression of heart size, protein, DNA, RNA and free amino acids. These occurred in the very early stages of the infection and were correlated with the intensity of disease involvement. The changes suggest possible patterns for cardiac irregularities observed in humans exposed to viral diseases.

To our knowledge, there are no reports on the effect of a virus infection on protein synthesis in the heart of a young, growing animal. There are, however, several reports concerning nucleic and free amino acid changes in the cardiac cell under various types of stress. For example, high environmental temperatures reduced heart weight and increased DNA in chick embryo tissue (1). Forced muscular effort in the rat increased amino acid levels (2) and the size of the mitochondria (3). Nutritional deficiencies have been used to produce a cardiomegaly in the rat for the study of hyperplasia and hypertrophy of cells (4, 5). In these cases myocardial DNA was decreased as a result of the nutritional stress whereas heart weight, RNA and protein increased. Takahashi (6) studied protein synthesis in dogs with experimental coronary occlusions. Water, fat and changes in structural total protein were observed to increase whereas contractile and mitochondrial protein decreased due to destruction of cardiac muscle fibers. In the infarcted area DNA increased whereas RNA first decreased then increased. The increase in protein synthesis in the infarcted area was considered to be one of the reparative mechanisms.

In the study reported here, the effect of a Newcastle disease virus (NDV) infection in cardiac tissue was observed in relation to changes in diurnal patterns and levels of DNA, RNA and free amino acids in control and NDV-infected chicks during the latter part of the incubation stage of the disease.

METHODS

A total of 300 White Leghorn male chicks was used in this replicated study. They were maintained from day of hatch in all-wire community cages in air-conditioned rooms with constant light; water and a normal chick diet (7) were provided ad libitum throughout the experiments. All necessary tasks were performed between 0800 and 0900 hours each day by a single caretaker; thereafter no one entered the rooms.

When the chicks were 28 days old they were divided into two comparable groups — one to remain as the control and the other to be infected with Newcastle disease virus. The control chicks remained in the same laboratory; those to be inoculated were transferred to similar but isolated quarters; all diet and management conditions were identical for both groups. The NDV was a 10^{-3} concentration of the Rutgers-Grun strain, injected intramuscularly (0.1 ml/chick). The inoculations took

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place from 0745 to 0815 hours in both experiments.

Forty-eight hours after inoculation, control and NDV chicks were killed in lots of 5 (trial 1) or 10/group (trial 2); the hearts were removed, bisected, thoroughly blotted, weighed and frozen. This procedure was repeated at 1200, 1600, 2000 and 2400 hours, and at 0800 hours the following day, for two reasons: to observe possible periodicity changes in the controls, and to observe any changes occurring during the latter stage of the incubation period of the NDV which we have previously defined as zero to 72 hours postinoculation. The remaining NDV chicks were held for an additional 5 days to observe the rate of mortality or degree of NDV involvement (7). The entire heart was used for the biochemical analyses, and the samples were on an individual basis. Total protein, DNA and RNA determinations were according to Wannemacher et al. (8), and the free amino acids by the method of Squibb (9).

RESULTS

Mortality, determined by holding representative chicks for the entire disease cycle, was 23% in trial 1 and 42% in trial 2, indicating a higher degree of involvement during the latter trial. Statistical analyses of the data showed that in the controls there were no significant differences between trials with respect to diurnal patterns of the various parameters. During the period under study food and water intake were the same for control and NDV chicks, and clinical symptoms could not be observed in the infected birds.

Heart weight and total quantities of DNA, RNA, protein and free amino acids (heart weight × milligrams per gram wet tissue) for the controls are shown in figure 1. Heart weight had a definite diurnal rhythm with the peak ($P < 0.01$) at 2000 hours, but body weight did not show a rhythm, with the result that there was a significant ($P < 0.05$) increase in the heart-to-body weight ratio at 2000 hours. There was a 36% increase in total DNA, the peak occurring at 2000 hours ($P < 0.01$). RNA increased 16% ($P < 0.01$) in phase with DNA, and the free amino acids increased 32% ($P < 0.01$) with a distinct

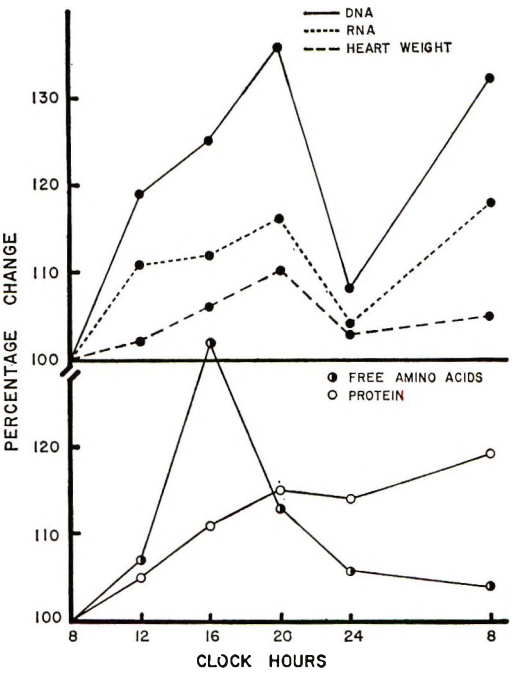

Fig. 1 Diurnal patterns of heart weight and total quantities of DNA, RNA, protein and free amino acids in noninfected chicks. Each point represents the average of 10 chicks of trial 2.

peak at 1600 hours. The significant increase in tissue protein ($P < 0.01$) was linear over the 24-hour period.

Biochemical alterations related to myocardial NDV involvement are evident from the curves shown in figure 2. Here the data of the infected birds were calculated in terms of the controls at each sampling period. The NDV infection significantly depressed heart size, DNA, RNA, total protein and the free amino acids ($P < 0.01$ to < 0.001) in terms of cellular concentrations as well as total quantities. The average slope of the oscillations showed that the greatest effects occurred 72 hours postinoculation, or just prior to the time of first mortality.

DISCUSSION

The results shown here leave no doubt that there are diurnal rhythms of DNA, RNA and free amino acids in the heart of the young growing chick. Squibb (10) has made similar observations in avian liver and breast muscle. Moreover, Lyons et al.

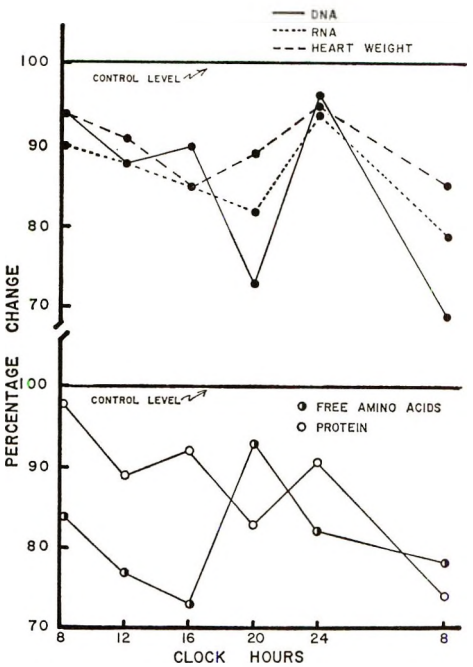

Fig. 2 Diurnal levels of heart weight and total quantities of DNA, RNA, protein and free amino acids in chicks infected with Newcastle disease virus. Values are in terms of controls at each sampling period. Each point represents the average of 10 chicks of trial 2.

(11) have noted rhythms in the mature rat heart although the magnitude of change has not been as great. Further, it is obvious that unless periodic effects are considered, the interpretation of data can be confounded through changes in linearity of the oscillations or shifts or desynchronization of patterns in relation to clock hours (10).

The patterns of the curves for DNA, RNA and the free amino acids in figure 1 merit comment since they apparently do not conform to the usual 24-hour cycle observed in other tissues (10). Subsequent trials [2] have revealed these same patterns, suggesting that in the avian heart the diurnal rhythms for these parameters may be less than 24 hours, given the same lighting and management conditions that prevailed in the trials reported here.

Since the parameters are considered a reasonable reflection of protein synthesis in a tissue, the oscillations of the parameters are an indication of the magnitude and pattern of the synthesis of protein taking place in the heart. The significant changes in total quantities of DNA in the noninfected chicks are of interest, as these changes were observed in an organ whose cells apparently do not divide but rather increase in size in the postfetal state (4, 5, 12). The 36% rise in DNA in the growing chick heart at 2000 hours is in striking contrast to the 11% increase observed in the mature rat heart (11). Korecky and French (13) found that DNA increased in enlarged hearts of anemic rats and attributed the phenomenon to hyperplasia. Grimm et al. (14) point out the difficulty of interpreting changes in DNA and RNA levels in the rat heart; their results suggest that in early postnatal life myocardial growth is due to both hyperplasia and hypertrophy. None of these reports, however, deals with the phenomenon of periodicity.

Our observations on the normal avian heart indicate that the nucleic acid levels in the myocardium undergo significant diurnal changes which would be difficult to attribute to hyperplasia. But if cardiac cells do not proliferate in the manner of other tissues, the diurnal increases in total quantities of DNA could be a reflection, at least in part, of a metabolic function for this nucleic acid, as suggested by Stroun et al. (15).

In the chicks infected with Newcastle disease virus the biochemical changes which occurred in the apparent absence of histopathologic alterations document the profound myocardial involvement resulting from this infection. Our Rutgers-Grun strain of Newcastle disease virus — an RNA virus in the same group as parainfluenza and mumps — produces an infection with three definite phases. The incubation stage extends from zero to 72 hours when there are no clinical symptoms evident; the active involvement period is from 72 to 144 hours, followed by initiation of recovery if the chick survives. Mortality can range from zero to 100% depending on the concentration of the inoculum. The clinical syndrome includes loss of weight and slight-to-severe respiratory symptoms, with or without paralysis.

[2] Squibb, R. L., M. M. Lyons and W. R. Beisel 1968 unpublished data.

The extent of the NDV involvement in the heart during the incubation stage is striking, as shown in figure 2 where the curves are all significantly below control levels. Moreover, it can be seen from the figure that these changes were taking place prior to the first sampling period. Statistical analysis revealed that there was a greater depression of protein synthesis in trial 2, which correlates with the higher level of mortality.

Although the data document the events, pathways are not elucidated. It has been shown, however, that NDV is able to penetrate cultured monkey cardiac cells and synthesize viral RNA (16). Despite the fact that total quantities of protein, nucleic acids and free amino acids were reduced in our trials, the ratios of these to the DNA molecule were not significantly altered by the virus, which also reduced the size of the heart.

Finally, the early profound alterations in the biochemical parameters associated with protein synthesis noted here suggest a possible pattern for cardiac irregularities observed in humans exposed to certain viral diseases.

ACKNOWLEDGMENT

Marie Utzinger was responsible for the care and management of the chicks and the biochemical analyses.

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Utilization of Calcium and Phosphorus from Hydrous and Anhydrous Dicalcium Phosphates [1]

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ABSTRACT Studies were conducted with chickens and turkeys to determine factors which affect the nutritional availability of phosphorus from dicalcium phosphates. Incorporation of orally administered ^{45}Ca and ^{32}P from either $^{45}CaH^{32}PO_4$ or $^{45}CaH^{32}PO_4 \cdot 2H_2O$ into bone was 25 to 50% greater from the hydrous form than from the anhydrous form in 1-week-old birds. For chickens the differences in incorporation of ^{45}Ca and ^{32}P from anhydrous and hydrous dicalcium phosphate decreased gradually, so that at 5 weeks of age, equal amounts of ^{45}Ca and ^{32}P were incorporated into the femur when chicks were fed either form of the phosphate. In turkeys, the incorporation of ^{45}Ca and ^{32}P was greater when supplied in the hydrous form than in the anhydrous form throughout the 5-week experimental period. Further studies were conducted with 1-week-old chicks and turkeys fed controlled amounts of feed and killed at 1, 2 and 4 hours after oral dosing. Incorporation of ^{45}Ca and ^{32}P into the femur from anhydrous dicalcium phosphate was again less at each time interval than incorporation from hydrous dicalcium phosphate. Measurement of the rate of solution for $CaHPO_4 \cdot 2H_2O$ at pH 3 to 7 indicated that the differences in incorporation may be due in part to differences in solution rates. The solution rate and the amounts of ^{45}Ca and ^{32}P incorporated into the bone were both higher with hydrous dicalcium phosphate.

Scott et al. (1), Griffith et al. (2) and Gillis et al. (3) have observed early death, inferior growth and poor bone development with turkey poults when anhydrous dicalcium phosphate was used as the phosphorus source in experimental diets. Similar symptoms, although less severe, were noted with young chicks. These effects did not occur when hydrous dicalcium phosphate was fed in experimental diets. Gillis et al. (3, 4) also reported that the solubility of dicalcium phosphate in weak acids and neutral ammonium citrate was not a reliable index for the estimation of nutritionally available phosphorus. These authors did suggest, however, that the differences in phosphorus availability may be attributed in part to the differences in the degree of hydration or crystalline modifications existing for the two dicalcium phosphates.

The primary objectives of the present study were 1) to assess physiological factors which caused the utilization of calcium and phosphorus from the two dicalcium phosphate sources to differ in turkeys, compared with chickens, and 2) to examine physical characteristics of dicalcium phosphates which appeared to relate to phosphorus availability.

MATERIALS AND METHODS

Birds. Hubbard White Mountain cockerels and Keithley-McPherrin White toms were housed in electrically heated brooders.

Diets. The basal diet used in the chick experiments contained the following ingredients: (expressed as grams/100 per g) glucose,[2] 64.01; isolated soybean protein,[3] 26.0; glycine, 0.30; methionine, 0.50; cellulose,[4] 3.0; corn oil, 4.0; choline chloride, 0.25; NaCl, 0.60; KCl, 0.60; $MgSO_4$, 0.30, plus the following: (expressed as milligrams per 100 g) niacin, 7.50; Ca pantothenate, 2.12; inositol, 17.2; *p*-aminobenzoic acid, 11.00; thiamine·HCl, 1.32; riboflavin, 1.32; pyridoxine·HCl, 0.8; folic acid, 0.40; menadione, 0.20; biotin, 0.04; vitamin B_{12}, 0.02; vitamin A concentrate (5000 USP units/g), 200.0; vitamin D_3 concentrate (15,000 ICU/g), 13.30; *d*-α-tocopheryl acetate concentrate, NF (250 mg/g), 70.0; $MnSO_4 \cdot H_2O$, 50.0; $FeSO_4 \cdot 7H_2O$, 57.4; $CuSO_4 \cdot 5H_2O$, 1.8; $CoCl_2 \cdot 6H_2O$,

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[1] Supported by Public Health Service Research Grant no. AM-04740 from the National Institute of Arthritis and Metabolic Diseases.
[2] Corn Products Company, New York.
[3] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis, Minnesota.
[4] Solka Floc, Brown Company, Berlin, New Hampshire.

1.1; $ZnCl_2$, 1.1; KI, 1.1; and $Na_2MoO_4 \cdot 2H_2O$, 0.11. The composition of the basal diet used in the turkey experiments was as follows: (expressed as grams per 100 g diet) glucose, 58.97; isolated soybean protein, 32.0; glycine, 0.38; methionine, 0.63; corn oil, 4.0; cellulose, 3.0; choline chloride, 0.25; NaCl, 0.60; KCl, 0.40; $MgSO_4$, 0.30, plus the following: (expressed as milligrams per 100 g) niacin, 6.6; Ca pantothenate, 2.65; *p*-aminobenzoic acid, 4.4; riboflavin, 1.1; pyridoxine·HCl, 1.1; thiamine·HCl, 1.1; folic acid, 0.22; inositol, 22.0; biotin, 0.04; *d*-α-tocopheryl acetate concentrate, NF (250 mg/g), 25.0; menadione, 1.1; vitamin A concentrate (5000 USP units/g), 260.0; vitamin D_3 concentrate (5000 USP units/g), 30.0; $MnSO_4 \cdot H_2O$, 50.0; $FeSO_4 \cdot 5H_2O$, 57.4; $ZnCl_2$, 1.0; $CoCl_2 \cdot 6H_2O$, 0.4; $CuSO_4 \cdot 5H_2O$, 1.8; KI, 6.2; and $Na_2MoO_4 \cdot 2H_2O$, 0.11. Phosphorus was added at the expense of glucose as either $CaHPO_4$ or $CaHPO_4 \cdot 2H_2O$ [5] at levels ranging from 0.10 to 0.50% for the chick and 0.30 to 1.0% for the turkey. The calcium level was maintained constant (chicks, 0.8%; turkey poults, 1.4%) in all diets by varying $CaCO_3$.

Bone analysis. After 21 days, 10 birds from each group were killed, and the right femur was removed, extracted with ethanol and diethyl ether and dried.

The bones were ashed and calcium was determined in an atomic absorption spectrophotometer.[6] Bone phosphorus was analyzed by the method of Fiske and Subbarow (5).

Determination of solubility, purity and rate of solution. An apparatus (fig. 1) was constructed to determine the relative rates of solution for $CaHPO_4 \cdot 2H_2O$ and $CaHPO_4$. The inner chamber (A) could be filled with 50 ml of buffered solution (D), and the outer chamber (B) could be filled with 100 to 150 ml of buffered solution (C). The dicalcium phosphates were added to the inner chamber, and transfer of calcium and phosphorus through a cellophane membrane (G) was measured by taking samples from the solution in the outer chamber. Solutions in the two chambers were agitated by magnetic stirrers (E, F). It was assumed that the rate of transfer into the outer chamber could be used as an index of the rate of solution of the compound in the inner chamber. Experiments were performed at three pH's (3, 5 and 7) in several 0.1 M buffer systems (acetate, citrate and maleate). Anhydrous or hydrous dicalcium phosphate was added in amounts of 40 and 50.4 mg, respectively. Before addition, both compounds were passed through a 200-wire mesh screen to assure uniform particle size. Purity of the dicalcium phosphates was determined chromatographically (6) and by combustion analysis. The AOAC method (7) was used to determine phosphate solubility.

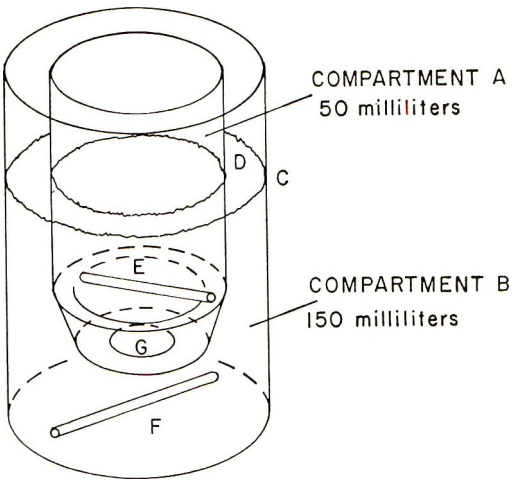

Fig. 1 Diagram of diffusion apparatus. The transfer of calcium through cellophane membrane (G) was measured as calcium moved from compartment A into compartment B with time.

Preparation of $^{45}CaH^{32}PO_4$ *and* $^{45}CaH^{32}PO_4 \cdot 2H_2O$. Hydrous and anhydrous dicalcium phosphate labeled with ^{45}Ca and ^{32}P were prepared in the manner described by Jensen and Rathleu (8). The hydrous dicalcium phosphate was prepared by mixing a solution prepared from $NaHPO_4 \cdot 2H_2O$ and $H_3{}^{32}PO_4$ with a solution of $CaCl_2$ containing ^{45}Ca at room temperature (25°). Anhydrous dicalcium phosphate was prepared by mixing the labeled solutions at 100°. Purity of the samples was estimated by the weight loss upon ignition of an air-dried sample.

[5] J. T. Baker Chemical Company, Phillipsburg, New Jersey.

[6] Model 214, Perkin-Elmer Corporation, Norwalk, Connecticut.

Influence of age and growth on incorporation of ^{45}Ca and ^{32}P into bone. One group of sixty 1-day-old chicks and sixty 1-day-old turkeys was fed diets ad libitum containing phosphorus (0.4%, chicken; 0.8%, turkey) in the form of $Ca(H_2PO_4)_2 \cdot H_2O$. Extra calcium (0.8%, chicken; 1.4%, turkey) was added as $CaCO_3$. The monocalcium phosphate was chosen to assure that all birds received an available source of phosphate. At 7, 21 and 35 days, 20 chickens and 20 turkeys were chosen at random and dosed orally with gelatin capsules containing either 50.4 mg $^{45}CaH^{32}PO_4 \cdot 2H_2O$ or 40 mg $^{45}CaH^{32}PO_4$. The compounds were prepared so that 0.5 to 1.0 μCi of ^{45}Ca and ^{32}P were present in each dose. Twenty-four hours after dosing, the birds were killed and the right femur was removed for analysis. After ashing in porcelain crucibles at 600° for 12 hours, the bones were dissolved in 1 N HCl. One-milliliter aliquots were then mixed with 20 ml scintillation fluid and counted in a dual channel liquid scintillation counter.[7] The composition of the scintillation fluid was *p*-xylene, 200 ml; 1,4-dioxane, 600 ml; 2-ethoxyethanol, 600 ml; naphthalene, 112 g; 2,5-diphenyloxazole (PPO), 14 g; and 1,4-*bis*-2-(4-methyl-5 - phenyloxazolyl)-benzene (methyl-substituted POPOP), 0.7 g.

Incorporation of ^{45}Ca and ^{32}P into bone at different times after administration. One-week-old chickens and turkeys were studied as described in the preceding experiment. In these studies, however, the birds were killed at 1, 2 and 4 hours after oral administrations of either $^{45}CaH^{32}PO_4 \cdot 2H_2O$ or $^{45}CaH^{32}PO_4$. The birds had been previously starved for six hours and were then allowed to consume a small quantity of food (5 to 10 g) before dosing. Determinations of pH of the contents of the gastrointestinal tract were also obtained from the birds in this experiment.

RESULTS AND DISCUSSION

Bone ash values (table 1) demonstrated that greater amounts of anhydrous than hydrous dicalcium phosphate were required to give equivalent bone ash values. About 50% more phosphorus was required from anhydrous dicalcium phosphate than from the hydrous form to obtain bone ash

[7] Model 314 EX, Packard Instrument Company, La Grange, Illinois.

TABLE 1

Analysis of femur ash from 3-week-old chicks and turkeys

Phosphorus source	P in diet [1]	Ash	% in ash		Ca/P ratio
			Ca	P	
	%	%			
		Chicks			
$CaHPO_4$(AR)	0.5	38.3 ± 5.1 [2]	36.1 ± 1.1	15.4 ± 0.8	2.34 ± 0.04
	0.4	34.5 ± 0.4	36.9 ± 0.9	16.4 ± 0.7	2.25 ± 0.06
	0.3	25.8 ± 1.7	29.7 ± 1.0	15.0 ± 0.5	1.97 ± 0.11
	0.2	17.7 ± 1.2	30.1 ± 0.8	15.2 ± 0.6	1.97 ± 0.08
$CaHPO_4 \cdot 2H_2O$(NF)	0.4	35.8 ± 1.2	34.0 ± 0.6	15.9 ± 0.6	2.14 ± 0.11
	0.3	31.2 ± 1.4	34.6 ± 1.1	15.6 ± 0.3	2.21 ± 0.09
	0.2	25.0 ± 0.7	28.3 ± 0.7	15.7 ± 0.4	1.81 ± 0.03
	0.1	18.2 ± 1.2	25.2 ± 1.5	16.0 ± 0.6	1.58 ± 0.13
		Turkeys			
$CaHPO_4$(AR)	1.0	36.7 ± 0.9	35.7 ± 1.0	15.6 ± 0.6	2.29 ± 0.11
	0.8	34.6 ± 0.8	35.7 ± 0.9	15.8 ± 0.5	2.36 ± 0.08
	0.6	31.3 ± 2.3	33.3 ± 1.1	15.9 ± 0.6	2.09 ± 0.08
	0.4	26.6 ± 2.7	32.1 ± 1.2	15.7 ± 0.7	2.04 ± 0.10
$CaHPO_4 \cdot 2H_2O$(NF)	0.6	38.0 ± 0.6	35.2 ± 1.1	16.4 ± 0.4	2.15 ± 0.07
	0.5	32.8 ± 0.8	35.3 ± 1.1	15.8 ± 0.5	2.24 ± 0.08
	0.4	29.8 ± 0.5	32.4 ± 0.9	15.9 ± 0.7	2.04 ± 0.06
	0.3	25.9 ± 2.0	32.1 ± 0.8	16.0 ± 0.3	2.00 ± 0.07

[1] Percentage of diet as phosphorus supplied from inorganic phosphate source.
[2] Mean ± SE.

of 35% for the turkey. For the chicken, 35% bone ash values were obtained with approximately the same amounts of the two phosphate sources; however, differences between the two phosphate sources for the chick were considerably greater at lower phosphate levels.

Analysis of bone calcium and phosphorus (table 1) demonstrated that the percentage of calcium in femur ash tended to decrease in response to low dietary phosphorus levels. The percentage of phosphorus in ash remained relatively constant with all treatments. These results yielded low calcium-to-phosphorus ratios in bones of most of the birds fed diets low in phosphorus. Since only calcium and phosphate were analyzed, evidence of isomorphic substitution could not be determined. If forms of calcium phosphate other than hydroxyapatites were deposited upon the initiation of calcification, perhaps low plasma phosphorus levels due to low phosphorus intake could have affected the form of calcium phosphate in bone (9).

Even though both calcium phosphates have similar solubility in some solutions (3, 4, 10, 11), the rate at which the two compounds went into solution appeared to be different (fig. 2). From the physical observations made to determine indirectly the rates of solution in acetate and other buffer systems (citrate and maleate), the differences in the rates of transfer for calcium from $CaHPO_4 \cdot 2H_2O$ compared with $CaHPO_4$ closely approximated the relative rates of incorporation found in bone when the two compounds were compared (fig. 3). The calcium transfer rate from anhydrous dicalcium phosphate was much slower than the transfer rate from hydrous dicalcium phosphate. The difference in the rates of calcium transfer from the two compounds was not altered greatly by increasing hydrogen ion concentration, although the amounts transferred did increase as the pH decreased.

At 24 hours, the percentage dose of ^{45}Ca incorporated per gram of femur in 1-week-old birds was 5.45% (hydrous) to 3.90% (anhydrous), and 2.8% (hydrous) to 1.81% (anhydrous) for the chicken and turkey, respectively. The values for ^{32}P incorporated per gram of femur were 5.45% (hydrous) to 4.30% (anhydrous), and 3.30% (hydrous) to 2.25% (anhydrous) for the chicken and turkey, respectively. These values give perhaps a better indication of the final incorporation rates, since no determinations of food transit times were made.

The amounts of ^{45}Ca and ^{32}P incorporated into bone were greater when the labeled dicalcium phosphate was supplied in the hydrous form than the anhydrous form to both chickens and turkeys at 1 to 3 weeks of age. In figure 4, the incorporation ratios

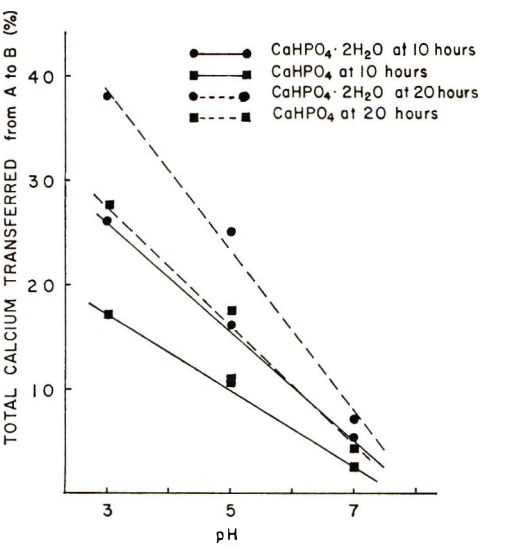

Fig. 2 Rate of solution of $CaHPO \cdot 2H_2O$ and $CaHPO_4$ in 0.1 M acetate buffer as measured by the transfer of calcium through a cellophane membrane. See figure 1 for diagram of apparatus.

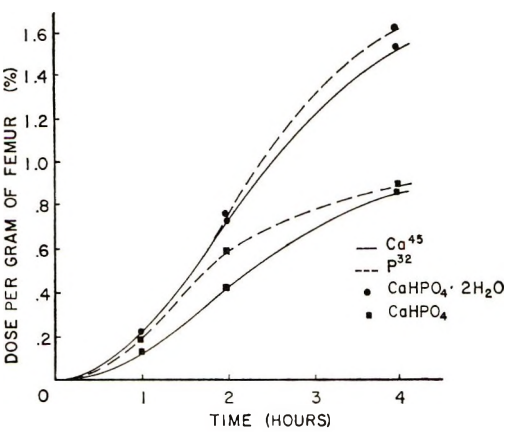

Fig. 3 Incorporation of ^{45}Ca and ^{32}P into the femur of the 1-week-old chick.

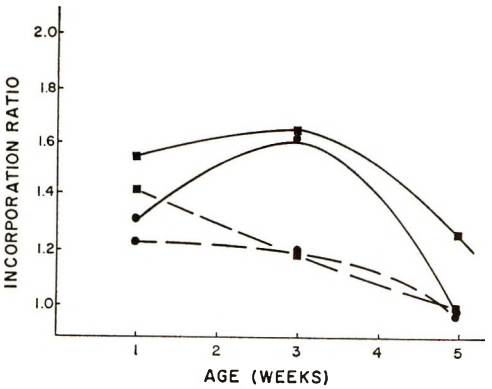

Fig. 4 Incorporation ratio for calcium and phosphorus. The ratio of the percentage dose of ^{45}Ca per gram femur obtained for $^{45}CaH^{32}PO_4 \cdot 2H_2O$ to that obtained for $^{45}CaH^{32}PO_4$, and the ratio of the percentage dose of ^{32}P per gram femur obtained for $^{45}CaH^{32}PO_4 \cdot 2H_2O$ to that obtained for $^{45}CaH^{32}PO_4$ are plotted versus the age of birds. A ratio of 1.0 indicates that ^{45}Ca or ^{32}P was incorporated in equal amounts from both sources of phosphorus. Each point represents an average of 10 birds. The value of the calcium for the turkey is indicated as ■——————■, and for the chicken as ■— — — — — —■. The value of the phosphorus ratio for the turkey is indicated as ●——————●, and for the chicken as ●— — — — — —●.

of ^{45}Ca and ^{32}P for $^{45}CaH^{32}PO_4 \cdot 2H_2O$ to $^{45}CaH^{32}PO_4$ were plotted versus age of the birds. The ratios were obtained by determining the percentage dose of the isotopes incorporated per gram of femur. Correction was made for differences in body weights, but these differences were insignificant, since the birds were fed the same chick or poult diets.

At 7 days, 55% more ^{45}Ca from the hydrous source was incorporated in the turkey, and 40% more in the chick, than from the anhydrous source. At 21 days, ratios of 1.60 and 1.20 were observed for the turkey and chick, respectively. After 35 days, the two dicalcium phosphates provided calcium, incorporated in equivalent amounts into the femur of the chick. For the turkey, however, calcium from the anhydrous source continued to be less available than from the hydrous source. The ratio of ^{32}P incorporation from the hydrous–anhydrous source also yielded similar values at 7 and 21 days, except differences were not as great as the values for ^{45}Ca incorporation in the first week of growth. After 35 days in both species, the two dicalcium phosphates provided phosphorus, incorporated in equivalent amounts into the femur.

These observations reflected the differences in the developmental growth of chickens and turkeys. The increased availability of anhydrous dicalcium phosphate with age was perhaps due to the extension of the intestines with growth and alterations in food transit time (12). The high mortality observed with turkeys, but not with chickens, fed anhydrous dicalcium phosphate in previous studies (1–3) may be related to the observation that the availability of the phosphorus from anhydrous dicalcium phosphate increased gradually with increasing age of the growing chick, but was limited throughout the early growth of the turkey.

The pH and length for various sections of the intestine from 1-week-old birds were also determined (table 2). The values obtained indicated that the content of the gastrointestinal tract of the turkey was slightly more acidic than that of the chicken; thus, the more severe deficiency symptoms observed with turkeys fed anhydrous dicalcium phosphate could not be attributed to differences in intestinal pH. The distance of food transit in the intestine was assumed to be equal for both chicks and turkeys, since the length of intestine was the same in both species.

Food transit in the digestive tract of young birds has been shown to be very rapid (12). The slower solution rate of anhydrous dicalcium phosphate could ac-

TABLE 2

Values of pH from intestinal segments

Segment	Young chick, avg length		Young turkey, avg length	
	pH [1,2]	*cm* [1]	*pH* [1,2]	*cm* [1]
Proventriculus	4.28	—	3.58	—
Gizzard	4.10	—	3.37	—
Duodenum	6.21	14.1	5.97	13.0
Proximal intestine [3]	6.44	28.9	6.27	29.3
Distal intestine [4]	7.14	28.8	6.69	27.9
Ceca (right)	6.46	6.25	6.69	8.9

[1] Average of 10 values.
[2] Values were obtained by placing microelectrode directly to exposed intestinal contents.
[3] Section from the duodenum to the site of the residual yolk sac.
[4] Section from the yolk sac to the opening of the ceca.

count for the lack of availability of calcium and phosphorus in young birds if the compound was only partially solvated in the region of calcium and phosphorus absorption.

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Relation of Zinc and Calcium Concentrations in Hair to Zinc Nutrition in Rats [1,2]

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ABSTRACT Zinc concentration decreased substantially in newly grown hair of nearly all rats depleted of zinc by being fed diets containing approximately 2 ppm zinc. Lowered zinc concentrations in hair occurred with significantly greater frequency than decreased growth rates. Some rats with markedly decreased zinc concentrations in hair, however, maintained growth rates that did not differ from those of pair-fed controls consuming the same diet but supplemented with 20 ppm of zinc. It is concluded that zinc concentrations in hair are dependent upon zinc intake, but that they do not necessarily reflect the severity of the metabolic effects of zinc deficiency as manifested by impaired growth rates. Lowered concentrations of zinc in the hair of zinc-depleted rats were associated with elevated concentrations of calcium.

The concentration of zinc in hair is about 10 times that in visceral organs and about 200 times that in plasma. A previous study (1) showed that zinc concentrations decreased significantly in the hair of rats when zinc intake in the diet was low. Miller et al. (2) observed a decline in the zinc concentrations in the hair of calves fed diets containing minimal concentrations of zinc. Studies of human hair also have shown that changes in zinc concentration may occur. Lowered concentrations of zinc were found by Strain et al. (3) in the hair of Egyptian youths exhibiting the syndrome of arrested growth, enlargement of liver and spleen and anemia, attributed by Prasad et al. (4) to zinc deficiency. A study of the hair of Iranian villagers (5) demonstrated a prevalence of decreased concentrations of zinc compared with the hair of well-nourished urban residents. The difference was attributed to the lower intake of available zinc by the villagers. Evidence provided by these studies suggests that the concentration of zinc in hair depends upon zinc intake, although the nature and degree of the dependence have not been established.

The signs of zinc deficiency are nonspecific and often equivocal. Proof that zinc is a limiting factor in nutrition may be difficult to obtain, and examination of hair for its zinc concentration has potentialities as an aid to the detection of zinc deficiency. The purpose of this study was to learn more about the relationship between zinc nutrition and zinc in hair. In particular, it seemed necessary to establish whether the lowered concentrations of zinc in hair are closely related to the metabolic disturbances associated with zinc deficiency, or whether they are a largely passive response to the lowered intake of zinc in the diet.

EXPERIMENTAL

Rats of a local Sprague-Dawley strain weighing 40 to 60 g were fed a diet similar to that described by Forbes and Yohe (6), modified in that 2 parts casein and 1 part gelatin supplied protein at a level of 14.8%. The casein was extracted with 0.1% disodium ethylenediaminetetraacetate (EDTA) solution several times to remove most of the zinc, and then with water to remove the EDTA. The diet contained all other essential constituents and supported a satisfactory rate of growth when zinc was added. The diet included 1% by weight of $CaHPO_4$. Zinc concentrations in the diets were measured, following digestion with nitric and perchloric acids, either by extraction with dithizone in carbon tetrachloride according to Wolff

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[2] Some of the studies here presented were described in a preliminary report published in the Proceedings of The Third Symposium on Human Nutrition and Health in the Near East held in Beirut May 15, 1967.

(7) or by atomic absorption spectrophotometry. They varied between 1.5 and 2.6 ppm (table 1). Control rats from the same litter paired according to sex and weight were fed the low zinc diet supplemented with zinc carbonate to provide in four of five experiments a zinc concentration approximating 20 ppm. This amount is nearly twice the estimated minimal requirements of the rat (6) but well below toxic concentrations. It approximates the dietary zinc level at which zinc concentrations in hair remain stable. In series 9, the control diet contained 30 ppm. The food consumption of the controls was adjusted each day to that of the experimental rats. Rats were housed in stainless steel cages. Deionized water was provided ad libitum.

Hair was sheared with electric clippers from the ventral surface within 1 to 11 days after the rats were started on the diets. A second shearing confined to the same area provided hair grown during the experimental period. Under "periods of study" (table 1), the numbers designate the times of the first and second shearings (as number of days after start of experiment). In series 9, a second harvest of hair was obtained from 12 of the original 24 rats. The initial shearings were delayed in four experiments for 5 to 11 days to enable hair "in process" to be extruded and removed.

The hair was washed with a sulfonated fatty acid detergent (Lux Liquid) in water (approximately 0.1% solution, v/v), and rinsed with deionized water until no evidence of foaming was detected. Two washings with 95% ethanol and one with ethyl ether followed. The hair was then allowed to equilibrate with air at room temperature for several days. Duplicate samples of 50 to 100 mg were digested with nitric acid followed by perchloric acid until the digests were colorless. The acids were fumed off, and the residue was dissolved in 2 ml deionized water. Care was taken to avoid loss of zinc through overheating or prolonged heating of the residue during the fuming-off.

[3] Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer, Perkin-Elmer Corporation, Norwalk, Connecticut.

TABLE 1

Effects of low zinc intake upon growth and zinc concentrations in hair

Series	No. of rats [1]	Zinc content of diet		Period of study [2]	Weight gain [3]		Zinc in hair [4]			
							Initial		Final	
		Control	Deficient		Control	Deficient	Control	Deficient	Control	Deficient
		ppm	*ppm*	*days*	*g/day*	*g/day*	*μg/g*	*μg/g*	*μg/g*	*μg/g*
9	24	30.0	1.77	5–25	1.62 ± 0.55	1.25 ± 0.39	155 ± 12.4	165 ± 40.8	188 ± 22.3	131 ± 30.7
	12			26–63	1.52 ± 0.35	0.86 ± 0.25	187 ± 19.5	134 ± 22.1	197 ± 21.6	115 ± 28.7
10	22	21.0	1.66	10–36	1.56 ± 0.34	0.93 ± 0.54	183 ± 14.5	185 ± 11.4	163 ± 14.4	107 ± 14.1
11	20	22.5	1.98	1–17	1.42 ± 0.29	0.28 ± 0.32	179 ± 9.7	195 ± 31.3	169 ± 7.5	140 ± 17.8
16	28	18.8	1.54	11–60	0.83 ± 0.13	0.58 ± 0.30	174 ± 11.6	159 ± 14.5	164 ± 17.7	101 ± 13.2
17	16	20.5	2.62	1–33	1.17 ± 0.12	0.56 ± 0.14	169 ± 11.7	174 ± 8.4	156 ± 28.5	100 ± 5.1

[1] Control and deficient rats were pair-fed. One deficient rat died in series 10 and two in series 16. These and their controls were omitted from the statistical evaluation.
[2] The first figure is the interval between the start of the diet and the initial shearing (see text); the second is the day of the next shearing. In series 9, hair was harvested during a second experimental period between days 26 and 63.
[3] Calculated for the intervals shown in period of study.
[4] Means and SD.

The zinc concentration in the digest was measured with an atomic absorption spectrophotometer [3] equipped with a zinc-calcium hollow cathode emission lamp. Measurements were made at 213.8 mμ using an acetylene-compressed air mixture in proportions that formed a blue flame which became white when fuel flow was slightly increased or auxiliary air flow slightly decreased. The readings were recorded on a Moseley chart recorder and corrected for a blank which had been carried through the digestion procedure. Comparison was made with zinc standards prepared from zinc sulfate heptahydrate crystals dissolved in water.

Zinc concentrations in hair and growth rates were considered to be significantly decreased when they differed from the means of the controls by more than two standard deviations.

RESULTS

Table 1 summarizes the studies made of five groups of rats. The expected decrease in growth rate occurred when diets low in zinc were fed. Growth rates of deficient and control rats were lower than those previously reported (1) in which the deficient diets had contained more zinc. No close correlation, however, between zinc content of the diet within the range used in these experiments and rate of growth or other signs of zinc deficiency was evident. The occurrence of lesions of the skin on the paws and around the eyes, bleeding into stomach and intestine, and susceptibility to other effects of low zinc intake varied considerably. Litter size was a noticeably important determinant; pups from litters of 11 or more were more vulnerable than those from litters of 6 to 10. Growth rates of control rats also were subnormal because of the curtailed intake of food resulting from paired feeding.

In agreement with previous observations (1), the concentration of zinc in hair decreased substantially in the rats consuming the low zinc diets. A new and interesting observation was the promptness with which the changes in zinc concentrations of hair occurred. In series 9 and 11, these declined by 20 and 25% in 20- and 17-day periods, in series 10 and 17, by more than 40% in 33 to 35 days. Longer periods of zinc deprivation, however, were accompanied by only moderate additional changes, as a third shearing in series 9 demonstrates.

Zinc concentrations in the hair of control rats receiving 20 ppm of zinc tended to decrease slightly during the experimental period. This contrasts with the increase that occurred in series 9 in the hair of control rats that were fed diets containing 30 ppm of zinc. A similar result with the higher intake was observed previously (1).

Significantly decreased concentrations of zinc in hair occurred in 43 of the 52 rats fed low zinc diets. The frequency of decreased concentrations, however, varied between groups. All rats in series 17 showed a decrease (table 1). In series 9, however, only 8 of 12 had significantly lowered zinc concentrations after 20 days on the diet, although eventually all fell below the 2 SD limit.

Significantly decreased zinc concentrations in hair occurred more consistently than decreased growth rates in the zinc-depleted rats (table 2). Only half of the depleted rats gained weight at rates significantly lower than those of the controls. Generally, the rats undergoing the greatest losses of zinc from hair were those that made the poorest weight gains. Coefficients of correlation between rate of gain and decrease in zinc concentration in hair per day were significant in two experiments. In series 16, $r = -0.69$ was significant at $P < 0.02$, and in series 17 a significant correlation ($r = -0.61$, $P < 0.02$) was demonstrated when controls and deficient rats were included in calculations. Correlations between zinc concentration in hair and growth were not significant in the remaining groups. Similar tests applied to the longer term experiments with higher zinc intakes described in our preceding report (1) also failed to demonstrate a significant correlation. The variability of the effect of low zinc intake upon growth was mainly responsible. Again, zinc concentrations in hair were more consistently changed in the longer experiments than growth rates.

The relation between concentrations of zinc and calcium in hair. Like zinc, little is known about the calcium content in

TABLE 2

Statistical summary

Series	Frequencies				P [2]
	Weight gain		Decrease in zinc		
	No. of rats				
	Total	< 2 SD [1]	Total	< 2 SD [1]	
9	12	0	12	8	< 0.05
	6	3	6	6	ns
10	10	7	10	9	ns
11	10	10	10	9	ns
16	12	4	12	9	< 0.05
17	8	4	8	8	0.05
All series	52	26	52	43	< 0.001

[1] The number of rats among those fed low zinc diets differing from the means of the controls by more than 2 SD. (See footnote 1 to table 1.)

[2] Probability that the frequency of significant change in rate of weight gain or decrease of zinc in hair differed. Tested by the chi-square method.

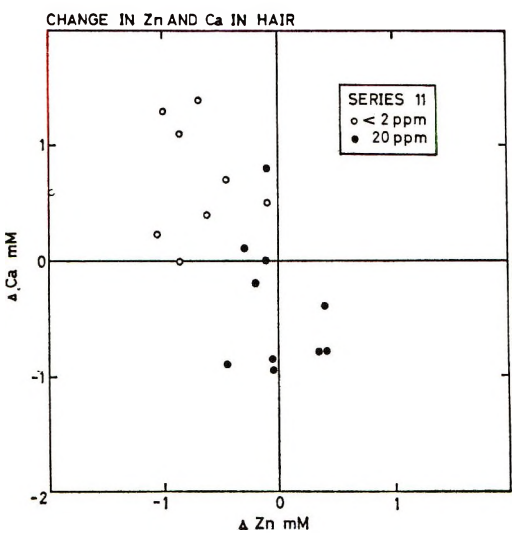

Fig. 1 Changes in calcium concentration in newly grown hair compared with changes in zinc concentration in rats fed diets containing low or normal concentrations of zinc. Δ Ca and Δ Zn are expressed as mmoles per day.

hair, the extent of its dependence upon calcium metabolism, or correlation with concentrations of zinc. Figures 1 and 2 show typical results in two experiments in which calcium and zinc concentrations were measured. Concentrations of calcium increased, at times substantially, as those of zinc decreased in hair of depleted rats. Coefficients of correlation calculated in three of five experiments (series 11, 9, and eight deficient rats in series 16) were − 0.53, − 0.65 and − 0.94. These are significant at $P < 0.05$, < 0.001 and < 0.01 levels, respectively. No correlation was demonstrated in series 10, however, which differed from the others in that calcium concentrations in hair decreased during the experiment. This trend was, however, less marked in the zinc-depleted rats than in the controls.

DISCUSSION

The concentration of zinc in most of the visceral organs and tissues studied undergoes little change in young rats fed diets

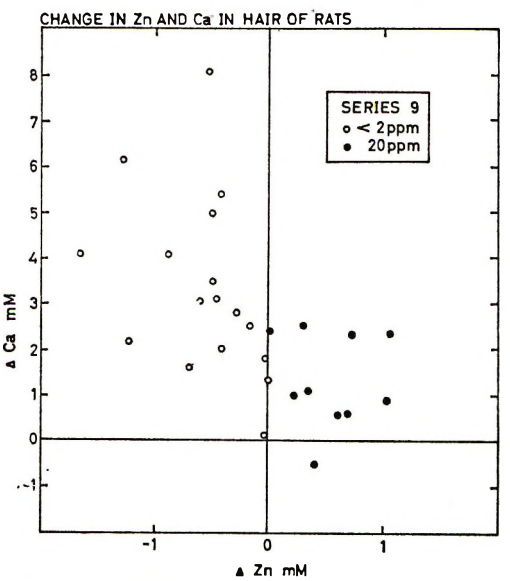

Fig. 2 Results of an experiment similar to that shown in figure 1. Note the large increment of calcium in some zinc-deficient rats.

low in zinc for 8 to 10 weeks (1). By contrast, the present study shows that the concentration of zinc in hair decreases rapidly and extensively. The zinc concentration decreased by 40 to 50% within 17 days in one experiment and within 20, 26 and 32 days in other experiments. Subsequently, zinc concentrations became stable at approximately half of the concentrations found in control samples of hair sheared from the same rat before the deficient diets took effect. Occasionally, further declines occurred as the experiments continued during a second month.

The lower concentrations of zinc in the hair of depleted rats observed in these experiments are explained by the lowered zinc concentrations in the diets. These were less than 2 ppm in four of five experiments, whereas in the earlier experiments (1) the diets contain about double this amount. The importance of zinc intake as a determinant of zinc concentration in hair also is supported by analyses of the hair of the control rats. When controls were fed diets containing 30 ppm of zinc, the concentration of zinc in hair rose substantially, whereas at 20 ppm zinc concentrations in the hair remained stable. It may be inferred that the intake of 20 ppm in the diet was equivalent to that during the first weeks of life, since the hair removed at the first shearing had grown during the neonatal period.

The relationship of the changes occurring in the zinc concentrations in hair in the zinc-depleted rats to the effects of zinc depletion upon growth rates is of some interest, since growth failure is one of the earlier and more prominent manifestations of zinc deficiency (8). Decreased concentrations of zinc in hair occurred with a much higher frequency (83% of the rats) than did depressed growth rates (50%). The difference is highly significant when tested by the chi-square method ($P < 0.001$).

Some rats in each experimental group were able to maintain growth approximating that of the controls when fed diets containing less than 2 ppm of zinc. Zinc concentrations in the hair of such rats decreased to the same extent as in the rats that developed signs of deficiency, an indication that they were not obtaining zinc from extradietary sources. It must be concluded, therefore, that the concentration of zinc in hair depends mainly upon the quantity of zinc consumed in the diet, but that it does not necessarily define the state of zinc nutrition. A similar conclusion concerning the significance of the concentration of zinc in serum was reached by Mills et al. (9).

No correlation between zinc concentration in hair and that in liver could be demonstrated in the rats of series 16 and 17. Coefficients of correlation were less than 0.25.

The mechanism by which zinc or other metals enters hair is unknown. Bates (10) suggests that this may be by adsorption, a proposal that is supported by observations of Kennington (11) on the uptake of sodium from dilute solutions by hair. The dependence of zinc concentration in hair upon dietary intake of zinc would then be governed by the concentration of zinc available to the hair follicle. However, hair contains as much calcium as zinc. The reciprocal relationship between zinc and calcium in the hair of zinc-depleted rats demonstrated in these studies indicates that the concentration of zinc in hair may depend upon not only the concentration of zinc available to the follicle but also the concentration of calcium. Calcium is known to aggravate the severity of zinc deficiency; Forbes and Yohe (6) believed this effect to occur at the cellular level, although Forbes later attributed it to a depressant effect on intestinal absorption (12). It appears probable that a competition between zinc and calcium for certain chemical groupings in hair may exist. It is conceivable that a similar competition may exist elsewhere and that this could affect movement of zinc within the tissues.

Follis (13) observed atrophy of hair follicles and extensive skin changes as manifestations of zinc deficiency. It is possible that the onset of deterioration was responsible for the lowered zinc concentrations. This explanation, however, seems less satisfactory than one based on a decreased concentration of zinc in skin.

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Excretion of 4-Pyridoxic Acid during Deoxypyridoxine and Pyridoxine Administration to Mongoloid and Non-Mongoloid Subjects [1]

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ABSTRACT The excretion of 4-pyridoxic acid (4PA) was studied in mongoloid subjects and controls of similar age and weight living in the same cottages and eating the same diet. The base-line excretion of 4PA was similar in the two groups. On the first day of deoxypyridoxine (DP) administration, a fourfold increase in 4PA excretion occurred in both groups; but on continued DP administration, 4PA excretion increased significantly less in mongoloid subjects compared with controls. On pyridoxine and DP administration loading, a significantly greater excretion of 4PA occurred in mongoloids compared with controls. The above data suggest that mongoloid subjects have a smaller dissociable pool of vitamin B_6 and that the enzymes of vitamin B_6 catabolism have greater activity in mongoloid subjects than in controls.

A previous study of tryptophan metabolism in mongoloid subjects showed that when tryptophan loads were given during administration of the vitamin B_6 antagonist, deoxypyridoxine, significantly greater amounts of the metabolites xanthurenic acid and 3-OH-kynurenine were excreted by mongoloid subjects compared with controls (1). When deoxypyridoxine was administered, an increased excretion of oxalic acid was also noted in mongoloid subjects compared with controls (2). These results were interpreted as indicating a greater susceptibility to vitamin B_6 deficiency induced by deoxypyridoxine in mongoloid subjects than controls. One explanation for the previous results could be greater excretion of the principal end product of vitamin B_6 metabolism, 4-pyridoxic acid, by mongoloid subjects compared with controls, and as a consequence, a greater vitamin B_6 deficiency. The present report is a study of serial 4-pyridoxic acid excretion in mongoloid and control subjects during deoxypyridoxine administration and during vitamin B_6 loading. The results indicate that, compared with controls, mongoloid subjects have significantly different 4-pyridoxic acid excretion patterns during deoxypyridoxine administration and a significantly greater excretion of 4-pyridoxic acid following pyridoxine loading.

METHODS

The subjects studied were all 10- to 14-year-old residents at the Higginsville State School, Higginsville, Missouri. Patients with Down's syndrome were matched for age and weight with non-mongoloid, mentally retarded controls living in the same cottage. All subjects received the same diet from the staff dietitian which was judged to be adequate for all essential nutrients. Patients with Down's syndrome were diagnosed by their physical characteristics and chromosome karyotype. During urine-collection periods, patients were under continuous supervision and voided every 2 hours. The urine was collected under toluene, kept cool, then frozen until analysis. Deoxypyridoxine [3] was administered orally, 200 mg/day for 11 days, after the initial 24-hour collection was obtained. Twenty-four-hour urine collections were obtained on days 1, 2, 4 and 10 of deoxypyridoxine administration. On day 11, 100 mg pyridoxine·HCl was administered intramuscularly; a 24-hour urine collection was obtained; and 4-pyri-

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doxic acid excretion was measured by the fluorometric method of Reddy et al. (3) following separation by column chromatography. The 4-pyridoxic acid standard [4] was purified by repeated crystallization until only one fluorescent spot was present on paper chromatography. It was determined that deoxypyridoxine did not interfere with the fluorometric determination of 4-pyridoxic acid. It is essential for good results that the pH be correct in the lactonizing and delactonizing steps, or variable values will be obtained. The figures in table 1 for days 1 through 11 are net increases in excretion above base-line levels. They were obtained by subtracting the base-line excretion of each individual from the total excretion of each day.

RESULTS

Excretion of 4-pyridoxic acid during deoxypyridoxine administration. As shown in table 1, the base-line excretion of 4-pyridoxic acid (4PA) was similar in the two groups. Mongoloid subjects excreted an average of 83 mμmoles/kg per 24 hours and control subjects 90 mμmoles/kg per 24 hours.

During the first day of deoxypyridoxine administration, an average fourfold increase in excretion of 4PA occurred in both groups. Mongoloids excreted an average increase of 343 mμmoles/kg per 24 hours and controls 367 mμmoles/kg per 24 hours of 4PA. The increase, compared with the base line, was highly significant in both groups, but there was no significant difference between groups.

During the second day of deoxypyridoxine administration, a 2.3-fold increase in the excretion of 4PA occurred for mongoloids and a threefold increase for con-

[4] Sigma Chemical Company, St. Louis.

TABLE 1

Excretion of 4-pyridoxic acid (4Pa) during deoxypyridoxine administration [1]

Body wt	Base line	Day 1	Day 2	Day 4	Day 7	Day 10	Day 11
kg				*mμmoles/kg/42 hr*			
				Controls			
34.6	77	536	237	155	145	137	7810
25.0	104	540	430	137	107	156	11,460
23.0	92	583	243	192	288	162	7910
43.2	49	315	181	104	81	103	5910
35.0	94	150	96	71		53	5860
24.6	136	358	198	415		164	6160
23.0	125	379	171	327		165	6060
39.0	44	79	651	651		521	7010
Mean ± SEM	90 ± 11	367 ± 65	278 ± 63	256 ± 69	155 ± 46	182 ± 50	7270 ± 728
				Mongoloids			
29.6	101	619	353	52	53	2	12,620
24.2	96	694	444	96	90	66	12,720
23.6	87	347	347	60	111	41	12,220
41.8	61	409	173	50	47	70	9770
35.0	33	183	93	45		61	6820
28.0	143	207	47	36		82	10,320
25.0	103	145	61	133		97	9920
39.0	42	86	54	2		2	8920
Mean ± SEM	83 ± 13	343 ± 77	196 ± 56	59 ± 14	75 ± 15	52 ± 13	10,410 ± 721
	$P < 0.35$	$P < 0.45$	$P < 0.2$	$P < 0.01$		$P < 0.01$	$P < 0.005$

[1] Base line 24-hour urine collections were obtained; then oral deoxypyridoxine, 200 mg/day, was given for 11 days. Twenty-four-hour urine collections were obtained on days 1, 2, 4, 7 and 10. On day 11, 100 mg pyridoxine was given intramuscularly, and a 24-hour urine collection was obtained. The 4PA was determined as described in the text and the excretion expressed as mμmoles/kg per 24 hours. The results for days 1 through 11 were excretions above base-line values and were obtained by subtracting the base-line value of each individual from the total 4PA excretion for each day.

trols. Excretion by mongoloid subjects increased an average of 196 mμmoles/kg per 24 hours compared with 278 mμmoles/kg per 24 hours for controls. The difference was not significant.

During day 4 of deoxypyridoxine administration the increase in excretion for mongoloid subjects averaged 59 mμmoles/kg per 24 hours, whereas for controls the average increase was 256 mμmoles/kg per 24 hours. A comparison of the net increase for the two groups showed that mongoloid subjects excreted a significantly lower amount of 4PA than controls ($P < 0.01$).

On day 10 of deoxypyridoxine administration, 4PA excretion was still increased above the base-line values in both groups but was greater in controls than mongoloid subjects. The increased excretion in mongoloid subjects averaged 52 mμmoles/kg per 24 hours, compared with controls who excreted an average of 182 mμmoles/kg per 24 hours. As shown in table 1, the greater excretion by control subjects was significant ($P < 0.01$).

On day 7, urine collections from only four subjects in each group were obtained. The average increase in excretion for mongoloid subjects was 75 mμmoles/kg per 24 hours and for controls 155 mμmoles/kg per 24 hours. No statistical analysis was carried out in this small group.

Excretion of 4-pyridoxic acid during deoxypyridoxine administration and following pyridoxine administration. The average increase in excretion of 4PA for 24 hours following the intramuscular administration of 100 mg pyridoxine was 10,410 mμmoles/kg per 24 hours in mongoloid subjects and 7,270 mμmoles/kg per 24 hours in controls. The larger excretion of 4PA by mongoloid patients compared with control subjects following this large load of pyridoxine was significant ($P < 0.005$).

DISCUSSION

The base-line excretion of 4PA was similar in both mongoloid and control groups. This suggested that the intake and average degradation of pyridoxine were similar in the two groups. The administration of the vitamin B_6 antagonist, deoxypyridoxine, resulted in a marked increase in excretion of the principal end product of vitamin B_6 metabolism, 4PA, in both groups. After administration of 4-deoxypyridoxine, the vitamin B_6 antagonist is phosphorylated (4) and competes with the phosphorylated form of vitamin B_6 for binding sites on proteins. This results in a displacement of pyridoxal phosphate. The free pyridoxal phosphate is then acted on successively by a phosphatase (5) and an aldehyde oxidase (6) to form the end product, 4-pyridoxic acid. The equal increase of 4PA excretion in both groups following the initial administration of deoxypyridoxine suggests that similar pools of easily dissociable B_6 vitamers were available for conversion to 4PA. As much larger amounts of 4PA were formed on pyridoxine loading in both groups, the activity of vitamin B_6 degradative enzymes was not maximal when deoxypyridoxine alone was given. Equal pool size of easily dissociable vitamin B_6 in the two groups is a reasonable explanation for the similar increases in 4PA excretion on initial deoxypyridoxine administration. On continued administration of deoxypyridoxine, the excretion of 4PA became significantly less in mongoloid subjects compared with controls. Reasons for the decreased excretion could be tighter binding of pyridoxal phosphate to protein, or decreased tissue stores and earlier depletion of vitamin B_6 in the mongoloid subjects compared with controls.

Johansson et al. (7) have presented evidence for a small, easily dissociable and a larger, more slowly dissociable pool of tissue vitamin B_6. As previous work (1) showed a greater degree of abnormality in the tryptophan load test in mongoloids during deoxypyridoxine feeding, a smaller pool of slowly dissociable tissue vitamin B_6 in mongolism could explain the present and previous work. The recent report of Coburn and Seidenberg,[5] showing decreased amounts of pyridoxal phosphate in leukocytes in patients with Down's syndrome, is in keeping with the above interpretation.

The principal end product of vitamin B_6 metabolism is 4-pyridoxic acid. In the present study between 45 and 50% of the

[5] Coburn, S. P., and M. Seidenberg 1968 Leukocyte pyridoxal phosphate levels in Down's syndrome and other retardates. Federation Proc., 27: 554 (abstract).

100 mg dose of pyridoxine was excreted as 4PA. In the studies of Reddy et al. (3) there was an excretion of approximately 50% of the administered dose as 4PA. Rabinowitz and Snell (8) found that 90% of administered pyridoxine was excreted as 4PA. Johansson et al. used tritiated pyridoxine in rat (9) and human (7) studies. They observed that with small doses of pyridoxine, 10 to 12% of the administered pyridoxine was excreted as 4PA the first day, and pyridoxal in approximately one-third the amount of 4PA was also excreted. Though other end products of vitamin B_6 metabolism are present in the urine and may be excreted in different amounts by the mongoloid subject compared with controls, the present work was concerned with the measurement of the 4PA parameter of vitamin B_6 metabolism. It is quite possible that differences in vitamin B_6 metabolism other than those described here will be found.

The administration of 100 mg pyridoxine to both groups was followed by a significantly greater excretion of 4PA by mongoloid subjects. This large quantity of pyridoxine should have utilized the full capacity of the involved enzymes to metabolize this load. The reason for this greater excretion could be a greater rate of conversion of pyridoxine to pyridoxal, or a more active aldehyde oxidase, or both factors in mongoloid subjects. During administration of smaller amounts of pyridoxine than used in this study, Gershoff et al. (10) noted that mongoloid subjects excreted a larger amount of 4PA and less pyridoxine than controls. The difference in 4PA excretion in the present study was demonstrable only after the administration of a pyridoxine load larger than that available for degradation from the competitive action of deoxypyridoxine. This suggests that mongoloid subjects have greater potential activity of vitamin B_6 degradation enzymes that is demonstrable only when substrate loads are presented that would utilize this capacity. It is not possible to relate this finding to the altered physical features and mental function present in mongolism, but it adds to the growing number of chemical differences that will allow correlations of accumulated data pertinent to the factors involved in the mental retardation to be made in the future.

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Effect of Dietary Fluoride on the Pattern of Food Intake in the Rat and the Development of a Programmed Pellet Dispenser [1]

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ABSTRACT The effect of excessive amounts (200 to 600 ppm) of dietary fluoride on the amount and pattern of food consumption in the rat was investigated. Rats fed diets containing fluoride ate the same number of meals each day as did control rats, although rats fed 400 or 600 ppm fluoride ate less diet than controls. The rats fed 400 or 600 ppm fluoride spent a longer time actually consuming each meal. An apparatus is described which made it possible to subject control rats to the abnormal dietary intake pattern which was observed in rats receiving the high fluoride diets. Using this programmed pellet dispenser, it was observed that the drop in liver glucose 6-phosphate dehydrogenase noted in rats fed fluoride could be duplicated in control animals, whereas a conventional type of "pair-fed" control group showed no significant change in the activity of this enzyme. These studies indicated that the effect of fluoride in depressing the activity of this liver enzyme was therefore secondary to its effect on food intake. This apparatus would have similar utility in studying any dietary condition where food intake is altered.

The effects of excessive amounts of dietary fluoride ingested by laboratory animals have been extensively studied and reviewed (1, 2). One consistent response has been a depressed food intake (3) at least partially related to plasma fluoride concentrations (4). In rats fed sufficient fluoride to depress food intake, there are a number of alterations in lipid [2] and carbohydrate metabolism (5), including a decline in the activity of liver glucose 6-phosphate dehydrogenase (6). As the level of this enzyme is known to respond to alterations in either amount or pattern of food intake (7–9), it was postulated that the effect of fluoride on the activity of this enzyme, and by inference on other metabolic changes, was secondary to a primary effect of fluoride on food intake. We have also observed that there is a change in the food intake pattern in the rat fed fluoride which cannot be duplicated by a conventional pair-fed control group.

This paper elaborates on the effects of fluoride on food intake in the rat and describes a feeding apparatus which provides control animals with their diet in a time sequence identical to the ad libitum pattern of the rats fed fluoride. This device could, of course, be used in any nutritional study where a particular diet depresses or alters the pattern of food intake.

METHODS

Animals and diets. Female rats of the Holtzman strain weighing 140 g at the start of the experiments were housed in individual cages in an air-conditioned room with a 12-hour light-dark cycle. All animals were given distilled water ad libitum and were fed the semipurified diet shown in table 1 to which the indicated amount of fluoride was added as NaF. In those experiments in which pelleted diets were fed, this diet was shipped to a commercial laboratory [3] to be formed into 4.8-mm, 97-mg pellets. It was not necessary to add a binding agent to pellet the diet; but because of concern about oxidation during the pelleting process, the normal

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[1] Supported in part by a research grant from the Aluminum Company of America, the Aluminum Company of Canada Ltd., the Anaconda Aluminum Company, the Electric Reduction Company of Canada, the Kennecott Copper Corporation, the Monsanto Chemical Company, the Ormet Corporation, the Tennessee Valley Authority, the Victor Chemical Works, Reynolds Metals Company, the Kaiser Aluminum and Chemical Corporation, the Harvey Aluminum Company, the U.S. Steel Corporation of Delaware, the Tennessee Corporation, and in part by a venture grant from the Nutrition Foundation Inc. of New York.

[2] Zebrowski, E. J. 1965 Ph.D. Thesis, University of Wisconsin, Madison.

[3] P. J. Noyes Company, Lancaster, New Hampshire.

TABLE 1
Composition of experimental diet

Ingredient	% of diet
Casein	23.5
Sucrose	66.0
Salts B [1]	5.0
Corn oil	5.0
Vitamin premix [2]	0.5
	100.0

Supplementary additions
1.0 g choline·HCl/kg diet in 25% ethanol solution
0.125 g α-tocopherol/kg diet in corn oil
NaF added to the fluoride diets at the level indicated

[1] Harper, A. E. 1959 J. Nutr., 68: 405.
[2] The vitamin premix contained per kilogram: inositol, 20 g; Ca pantothenate, 4 g; niacin, 2 g; menadione, 0.8 g; riboflavin, 0.6 g; thiamine·HCl, 1.2 g; pyridoxine·HCl, 0.5 g; biotin, 20 mg; folic acid, 40 mg; vitamin B_{12}, 2 mg; vitamin A, 800,000 IU; and vitamin D, 200,000 IU.

vitamin content was doubled, and 50 mg/kg vitamin C were added.

Record of food consumption. The amount and pattern of food intake were determined with a commercially available electronic balance [4] based on that described by Spengler (10) and a multipoint recorder.[5] The chart speed was 1 inch/hour, and a recorder deflection of 1 cm was equivalent to 3 g of diet. In most cases the signal from four balances was recorded simultaneously. Periods of eating were estimated to the nearest 5 minutes, and amounts of diet consumed per meal, to the nearest 0.5 g. A "meal" was defined as a period of continuous consumption which exceeded 0.5 g. Consumption after an interruption in food intake for more than 5 minutes was recorded as a new meal.

Enzyme assays. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities of rat liver were estimated by a modification (6) of the two substrate method of Glock and McLean (11). The protein content of the liver homogenates was determined with biuret reagent (12), and an enzyme unit was defined as 1 μmole NADP reduced per minute in the reaction mixture.

Programmed pellet dispenser. The apparatus shown in figure 1 was developed to present the animals with any predetermined pattern of diet consumption. It was constructed from the following parts: A: pellet dispenser,[6] B: feeder,[7] C: dual signal

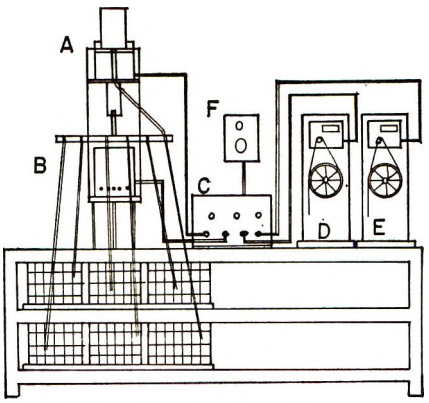

Fig. 1 Schematic drawing of the programmed pellet dispenser. When in use, the apparatus is also dispensing pellets to six cages on the other side of the rack. See text for details of construction.

controller,[8] D and E: variable interval timers (1 mm/second) [9] connected to a roll of 16-mm movie film, and F: time switch.[10] In operation, a hole punched in the movie film activates timer D or E which starts dispenser A and feeder B, causing them to dispense pellets through plastic tubes leading to six individual rat cages. The other timer (D or E) controls the dispensing of pellets in a different pattern to six more cages. If only one timer is used, the feeder can be set to feed up to 12 animals. The number of pellets given each animal and the pattern of their distribution during the feeding period are controlled by punching holes at the appropriate spacings in the roll of movie film. As the length of film is not an endless roll, timer F shuts off the entire apparatus at the end of the evening feeding period, and the tape is rewound daily.

RESULTS

The electronic balance was used to determine the diet consumption pattern of rats fed varying amounts of fluoride. A

[4] Viterra Electronic Instruments, Wallisellen, Zurich, Switzerland.
[5] Speedomax H, Leeds and Northrup Company, Philadelphia, Pennsylvania.
[6] Model PD-109A, Davis Scientific Instruments, North Hollywood, California.
[7] MTA Selecto feeder, Modern Teaching Associates, Milwaukee, Wisconsin.
[8] Needed only when two timers are activating the same Selecto feeder, details of construction are available from the author upon request.
[9] Model PT-1A, Ralph Gerbrands Company, Arlington, Massachusetts.
[10] Model T101, Intermatic time switch, International Register Company, Chicago, Illinois.

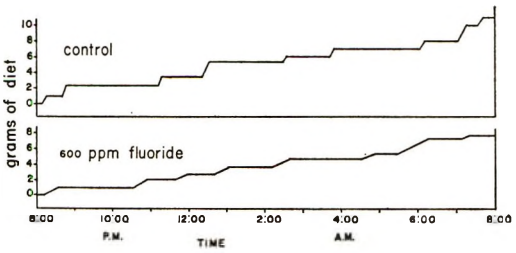

Fig. 2 Pattern of food intake of a single rat fed the control diet and one fed the 600-ppm F diet for 4 days. The decreased slope of the line during the consumption of each meal is characteristic of the animals fed fluoride. The lights in the animal room were off from 8:00 PM until 8:00 AM, and in the case of these two rats no meals had been consumed during the preceding 12 hours.

140-g female rat was placed in each cage and allowed to adjust to the new environment for 3 to 4 days while consuming the control diet. Following this, it was given either the control diet or the 200-, 400- or 600-ppm F diet for a 4-day experimental period. In most cases, the signal from four cages was recorded simultaneously so that a rat from each group could be observed at the same time. The data obtained indicated not only that the rats fed the 400- or 600-ppm F diets ate less, but also that their food consumption pattern was changed. This change, which is illustrated in figure 2, was characterized by an increase in the time that the rats spent in consuming each meal. When the records of food consumption were examined in detail, it was possible to derive considerable information about the characteristic manner in which the rats fed different amounts of fluoride consumed their diet. The data obtained on day 4 from a number of experiments are summarized in table 2. It can be seen that the measurable parameters which can be influenced by fluoride ingestion are the amount of diet consumed and the time taken to consume the diet, whether this is expressed as total time, minutes per gram of diet or minutes per meal. As the statistically significant increase in the number of meals eaten by the 200-ppm F group on day 4 was not seen on the other days, it was not considered to be related to fluoride ingestion. The data in table 3 compare some of the responses seen in the control and the rats fed 600-ppm F as the period of exposure to fluoride was continued. The observation that would appear most significant is that, although there was a significant increase in the minutes needed to consume a gram of diet by the 600-ppm F group on day 1, this measure, as well as the total time spent eating the diet containing fluoride, continued to increase over the 4-day experimental period. Preliminary studies have indicated that the total time spent consuming the diet does not increase appreciably beyond day 4, and it has been shown (3) that food intake itself begins to increase in rats of this size fed fluoride after about 1 week.

The distribution of the meals into various time periods was also considered, and the data for the control and the 600-ppm F group are shown in figure 3. It can be seen that the general pattern of distribution was the same for both groups. The

TABLE 2

Effect of fluoride ingestion on pattern of food intake [1]

Measurement	Fluoride added to diet			
	0	200	400	600
	ppm	*ppm*	*ppm*	*ppm*
No. of meals/day	9.0 ± 0.5 [a] [2]	11.0 ± 0.4 [b] [3]	7.3 ± 0.3 [a]	8.3 ± 0.7 [a]
Diet, g/day	10.6 ± 1.0 [a]	11.2 ± 0.5 [a]	6.2 ± 0.2 [b]	6.5 ± 0.4 [b]
Minutes eating/day	73.3 ± 6.5 [a]	85.0 ± 8.6 [a]	105.0 ± 15.3 [b]	143.3 ± 20.2 [b]
Meal, g	1.0 ± 0.11	1.0 ± 0.05	0.9 ± 0.03	0.8 ± 0.08
Minutes/meal	8.0 ± 0.6 [a]	7.7 ± 0.6 [a]	14.2 ± 1.4 [ab]	17.6 ± 0.30 [b]
Minutes/g	7.0 ± 0.5 [a]	7.8 ± 1.0 [a]	16.9 ± 2.0 [b]	22.0 ± 2.7 [b]

[1] Female rats, 140 g, were placed in the electronic balances described in the text. There were 9 rats in each group; however, the data from each rat were not usable each day because of mechanical problems with the balances.

[2] There was an average of seven usable records from each group per day, and the data presented are from day 4 of the experiment and represent the mean ± SE for from 5 to 9 rats/group.

[3] Means within each horizontal column having different superscripts are significantly different at $P \leq 0.05$.

TABLE 3

Effect of dietary fluoride and number of days diet was offered on pattern of food intake [1]

	Diet	Days on experiment			
		1	2	3	4
Diet, g/day	Control	13.0 ± 0.7 * [2]	11.6 ± 1.7 *	11.6 ± 1.0 *	10.6 ± 1.0 *
	600 ppm F	6.3 ± 0.9	6.7 ± 0.5	6.5 ± 0.6	6.5 ± 0.4
Minutes eating/day	Control	92.1 ± 8.6	75.0 ± 10.7	85.0 ± 7.1	73.3 ± 6.5 *
	600 ppm F	74.3 ± 10.5 [a 3]	95.6 ± 16.5 [ab]	107.5 ± 17.5 [ab]	143.3 ± 20.2 [b]
Minutes/g	Control	7.0 ± 0.5 *	6.5 ± 0.5 *	7.7 ± 0.8 *	7.0 ± 0.8 *
	600 ppm F	12.1 ± 0.7 [a]	14.2 ± 2.3 [a]	17.4 ± 2.0 [ab]	22.0 ± 2.7 [b]

[1] Same experimental conditions as table 2.
[2] Significant differences between control and 600-ppm F groups at any day are indicated by an asterisk.
[3] Values are means ± SE; means within each horizontal column having different superscripts are significantly different at $P \leqq 0.05$.

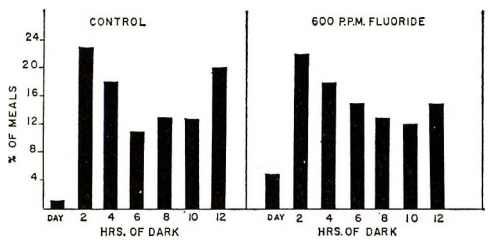

Fig. 3 Distribution of meal consumption into consecutive 2-hour periods. The number of meals consumed during the entire 12-hour period when the lights were on have been grouped together as the day consumption. The data include all of the control and 600-ppm F rats from each of the 4 days.

rats ate very few meals during the daylight hours and exhibited the greatest eating activity in the first 2 hours after the lights were turned off. There is also an indication, at least in the control animals, that there was a second peak of eating activity toward the end of the 12-hour dark period.

The data obtained from monitoring food consumption in the electronic balances made it evident that any attempt to differentiate the primary effects of fluoride on metabolic processes from secondary effects resulting from the changes in amount or pattern of food intake would be complicated by the nature of these changes, and such differentiation could not be accomplished by a normal pair-feeding type of experiment. With a normal pair-fed control group in an experiment, the animals with food intake restricted to that of some experimental group will consume all of their diet in a relatively short time. This type of "stuff and starve" intake pattern is known to influence the levels of many enzymes, including liver glucose 6-phosphate dehydrogenase (7) which we have found decreased in rats fed fluoride (6). In an attempt to circumvent this disadvantage, the programmed pellet dispenser described in the Methods section was developed. Using the data on food consumption obtained from the electronic balances, control animals could be subjected to a pattern of food intake which was nearly identical to that of the group fed fluoride.

To test this method of feeding control animals in experiments in which a dietary component influences food intake, the effect of fluoride on liver glucose 6-phosphate dehydrogenase was reinvestigated. For a 1-week period, 140-g rats were fed 1) the control diet ad libitum, 2) the 600-ppm F diet ad libitum, 3) an amount of control diet equal to that eaten by the fluoride group the previous day (the pair-fed group), or 4) the control diet in a pelleted form from the programmed dispenser. The animals fed from the dispenser were given an intake pattern which was based on the consumption of the 600-ppm F group on day 4 of the experiment shown in tables 2 and 3. They were fed 6.6 g/day, at a rate of 22 minutes/g. This amount was divided into eight meals which were given 0.5, 1.5, 3.0, 4.5, 6.0, 8.0, 9.5 and 11.0 hours after the lights in the room were turned off. This distribution of meals was based on the meal distribution observed in figure 3. At the end of the 7-day experimental period, the rats were killed and liver enzyme activity determined. The

TABLE 4

Effect of dietary fluoride and method of feeding on pentose cycle dehydrogenases [1]

Group	Wt change	Food intake	Enzyme activity	
			Glucose 6-phosphate dehydrogenase	6-phospho-gluconate dehydrogenase
	g/week	*g/day*	*unit/mg protein*	
Control	+35	13.0	3.53 ± 0.24 [2]	4.73 ± 0.37
600 ppm F	−12	7.0	1.95 ± 0.10	4.24 ± 0.28
Pair fed	−8	7.2	4.00 ± 2.10	5.21 ± 0.40
Control, fed by programmed dispenser	−11	6.6	1.39 ± 0.17	3.94 ± 0.28

[1] Six rats per group, see text for details.
[2] Values are means with SE indicated for enzyme activities.

results of this experiment are shown in table 4. The data indicate that both the pair-fed group and the group fed by the programmed dispenser had food intakes and changes in body weight which were similar to those of the 600-ppm F group. Although the data indicate that the group fed fluoride and the pair-fed group consumed slightly more diet on an average than the rats fed from the programmed dispenser, the unavoidable spillage of the loose diet in these groups compared with the pelleted diet would have made the weekly intake essentially the same. It was observed that the rats ate the pellets as they were delivered to the cages and did not allow a number to accumulate before eating them. The diet consumption of the control group in this experiment was a few grams higher than that of the controls on day 4 of the electronic-balance experiment, but was not statistically different than the 4-day average consumption in that experiment. In contrast to the effects on body weight and food intake, only the group on the programmed dispenser with an intake patterned after that of the 600-ppm F group exhibited the same changes in enzyme activity. That is, these rats had a dramatic drop in the level of glucose 6-P dehydrogenase, and only minor changes in 6-phosphogluconate dehydrogenase. In contrast to this, the pair-fed animals actually showed a slight increase in the activities of both enzymes.

DISCUSSION

The data obtained in this experiment, indicating that normal rats consume their diet at a rapid rate in about 10 distinct meals/day mainly at night, agree well with data published by others (10, 13, 14) using a similar experimental design. Using a slightly different apparatus, LeMagnen and Tallon (15) found almost as many meals consumed in the daylight as in the night hours. The reason for this discrepancy is not clear, as experiments dealing with amino acid imbalances [11] have also shown that at least 80%, and usually more, of the meals are consumed during the night; and earlier experiments in which voluntary pellet consumption of mice was recorded (16) also indicated a clear 24-hour cycle of heavy and light eating activity.

The total eating time of the rats fed high dietary levels of fluoride was increased even though total consumption was decreased; this observation is in sharp contrast to the observations of Köneg et al. (14). They restricted food intake by dietary calcium gluconate, quinine·HCl, or by SeO_2 in the drinking water; both the total time spent eating and the minutes needed to consume a gram of diet were decreased. This difference in response would seem to point to some basic physiological difference in the way in which dietary fluoride and these other dietary additions depress food intake and suggests that comparative studies of this type might contribute much to the understanding of a number of toxicities and deficiencies which cause depression of food intake. When these differences in the manner in which

[11] Perez, L. C. 1968 Ph.D. Thesis, University of Wisconsin, Madison.

different food intake depressants affect the pattern of consumption are considered, the ability to duplicate the actual pattern of consumption of the experimental animals may be rather important in the appropriate control of an experiment.

The apparatus which is described for dispensing pellets in any preset pattern can be simply constructed at moderate cost. Although a device for feeding a single rat a preset pattern of pellets has been described (9), and a simple recycling timer could be used to activate the Selecto feeder used in the programmed pellet dispenser, the simple technique of punching holes at varying intervals in the moving tape offers a great deal of flexibility. The value of such an apparatus is illustrated by the data in table 3: a standard pair-fed control group did not duplicate the decrease in liver glucose 6-P dehydrogenase seen in the rats fed fluoride, but actually showed a slight increase above ad libitum controls. As both amount and pattern of diet intake will influence this particular enzyme, the large standard error observed in the pair-fed group probably indicates that the amount of diet consumed was just sufficient to cause an increase even though this pattern of intake ("stuff and starve") is one which promotes an elevation of the enzyme. In contrast to this group, the control group fed the same amount of diet in the same pattern as the fluoride-fed rats from the programmed dispenser showed an enzyme activity effect which was similar to that of the rats fed fluoride.

These data clearly indicate that the effect of dietary fluoride on liver glucose 6-phosphate dehydrogenase previously observed (6) was not a direct effect of tissue fluoride on the enzyme-forming system, but rather the response observed was an indirect one, mediated through the change in the amount and pattern of food intake. It has also been shown [12] that many of the alterations in concentrations of metabolic intermediates seen in animals fed fluoride can be duplicated by imposing the appropriate pattern of food intake on the control animals.

These data, therefore, suggest that many of the metabolic studies which have been done on animals subjected to dietary deficiencies or toxicities in which pair-fed animals have been used as a presumably adequate control might well be reinvestigated.

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