Dental Abnormalities in Rats Attributable to Protein Deficiency during Reproduction[1]

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ABSTRACT Experiments were conducted with 2 strains of caries-susceptible rats and a strain of caries-resistant rats to determine the influence of a low-protein diet during the reproductive cycle on the development and maintenance of the dental structures. In the offspring of all 3 strains, the low-protein diet caused high mortality during lactation, very low body weights at weaning, reduction in the size of the molars, delay in third molar eruption, high frequency of missing cusps on third molars and increased susceptibility to carious lesions in the occlusal sulci and on the smooth surfaces of the molars. A supplement of 1.0% DL-methionine to the low-protein diet throughout the reproductive cycle led to striking reductions in the frequency of the various abnormalities. Post-weaning administration of this supplement or of an adequate protein diet to offspring of protein-deficient females was too late to correct the abnormalities. Supplements of 0.5 or 2.0% sodium dihydrogen orthophosphate or 1.0% sodium sulfate under comparable conditions were ineffective. The ability of nutritional abnormalities to cause deviations from the genetically established blueprint for development of the dental structures was clearly demonstrated in these studies. However, the deviations from this blueprint were not sufficiently great to mask the genetic identity of any strain.

Vitamin A deficiency, fluoride supplementation and a low calcium-phosphorus ratio during pregnancy and lactation in the rat have been shown by Paynter and Grainger (1) to result in significantly smaller molars in the offspring; the low calcium-phosphorus ratio produced significant alterations in the shape and size of the occlusal fissures. In later studies, vitamin A deficiency had no influence on caries-susceptibility (2).

Holloway et al. (3) observed that protein deficiency during pregnancy and lactation in Harvard caries-susceptible rats resulted in delayed eruption of the third molars, reduction in the size of the molars, failure of minor cusps on the third molars to develop and increased caries-susceptibility. The question arises in the latter study whether the protein deficiency produced in the pregnant and lactating mothers was the primary or the most important nutritional problem actually imposed upon the offspring.

The present series of experiments was undertaken to determine whether such supplements as DL-methionine, phosphate or sulfate or the substitution of egg albumen for casein would alter the occurrence of the above abnormalities in the offspring of protein-deficient caries-susceptible rats. In addition, a caries-resistant strain was tested to determine whether a sufficiently increased caries-susceptibility could be induced among the offspring by this nutritional procedure to offset their inherited caries-resistance.

EXPERIMENTAL

Five experiments were conducted.[1] The control diet P_3 contained adequate protein as casein and was capable of supporting an excellent rate of reproduction in our strains of rats. The standard low-protein diet P_5 contained 8% casein; the rest of the casein had been isocalorically replaced with sucrose. The components of diets P_3 and P_5 and their various supplemented modifications are presented in table 1. The specific details of each experiment are listed in the first 3 vertical columns of tables 2 and 3 and repeated in tables 5 and 6. For accuracy in referring from text to tables, the groups have been labeled consecutively through the 5 experiments.

The first experiment was composed of 3 groups of breeding females from the Harvard caries-resistant strain. The 6 con-

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TABLE 1
Composition of diets in grams

Ingredients	P_3	P_5	P_6	P_7	P_{12}	P_{13}	P_{14}	P_{15}	P_{17}	P_{18}	P_{19}	P_{20}	P_{21}
Sucrose	67	83	83	83	83	83	67	67	83	83	83	83	83
Crude casein	16	—	—	—	—	—	16	16	—	—	—	—	—
Vitaminized casein[1]	8	8	8	—	10	—	8	8	8	8	8	8	8
Vitaminized egg albumen[2]	—	—	—	8	—	10	—	—	—	—	—	—	—
Vitaminized corn oil[1]	5	5	5	5	5	5	5	5	5	5	5	5	5
Salt mixture[1]	4	4	4	4	4	4	4	4	4	4	4	4	4
Dessicated liver	2	2	2	2	2	2	2	2	2	2	2	2	2
Cellulose[3]	15	15	15	15	15	15	15	15	15	15	15	15	15
DL-Methionine	—	—	0.3	—	—	—	—	—	—	—	1.0	—	1.0
$NaH_2PO_4 \cdot H_2O$	—	—	—	—	—	—	0.5	2.0	0.5	2.0	—	—	—
Na_2SO_4	—	—	—	—	—	—	—	—	—	—	—	1.0	1.0

[1] Prepared as described in J. Dent. Res., 26: 47, 1947.
[2] Same levels of vitamins added as for casein.
[3] Cellu Flour, The Chicago Dietetic Supply House, Inc., Chicago, Illinois.

trol females in group 1 were fed diet P_3. The 10 females in group 2 were maintained with low-protein diet P_5. The 10 females in group 3 were fed diet P_7 with 8% egg albumen instead of casein. The second experiment was similar except that the females in groups 5 and 6 were fed diets P_{12} and P_{13}, with 10% casein and 10% egg albumen, respectively. There were 6, 9 and 9 females in groups 4 through 6, respectively.

Experiment 3 was similar to experiment 1 with 2 exceptions. Harvard caries-susceptible rats were used instead of Harvard caries-resistant rats. The females in group 9 were included to test the influence of a 0.3% supplement of DL-methionine to the low-protein diet. There were 6, 10, 10 and 9 female rats in groups 7 through 10, respectively.

Experiment 4 was designed to test 0.5 and 2.0% supplements of sodium dihydrogen orthophosphate to diets P_3 and P_5 during the reproductive cycle and after weaning. Six groups of females were used: 3 with the adequate casein level in diet P_3 and 3 with 8% casein in diet P_5. There were 5, 7, 7, 8, 8 and 8 female rats of the Harvard caries-susceptible strain in groups 11 through 16.

The subjects in experiment 5 were representatives of the mutant albino strain which is at least as caries-susceptible as the Harvard caries-susceptible strain. This experiment was designed to test the protein-deficiency relationship in another strain and to evaluate the influence of supplements of 1.0% DL-methionine and 1.0% sodium sulfate alone and together. Groups 17 through 21 were fed as follows: group 17, the control diet P_3; group 18, the low-protein diet P_5; and groups 19 through 21, the low-protein diet supplemented with 1.0% DL-methionine, 1.0% sodium sulfate, and a combination of 1.0% DL-methionine and 1.0% sodium sulfate, respectively (diets P_{19}, P_{20} and P_{21}). Each group contained 8 female rats.

The general details of the 5 experiments closely resembled each other and involved 2 generations of rats as in the earlier study by Holloway et al. (3). Young adult female rats were selected from the appropriate colony and subdivided into the required groups with littermate representation. More females were placed in protein-deficient groups to offset the higher mortality rate among the offspring. These females were fed the appropriate control or experimental diet for a 28-day stabilization period before mating with a closely related male. While males and females were caged together, both received the female's diet. Males were removed after receiving any experimental diet for 6 days and were returned to the stock colony for a repletion period with the adequate protein diet P_3.

When females were visually demonstrable to be pregnant, they were separated into individual cages with shavings as bedding. Within 24 hours after parturition, the offspring were reduced to 6. When possible, surplus pups were used to increase small litters of the same group to 6 to make the stress upon lactating mothers

TABLE 2

Influence of protein restriction during reproductive cycle on weaning weight, eruption time of third molars and morphology of third molars in Harvard caries-resistant rats[1]

Group no.	Diet		No. of rats weaned		Weight at weaning				Eruption time of lower third molars		Weight when third molars erupted				% rats with malformed third molars
					Males		Females				Males		Females		
	Developmental	Post-developmental	Males	Females	Avg	P[2]	Avg	P	Avg	P	Avg	P	Avg	P	
					g		*g*		*day*		*g*		*g*		
							Experiment 1								
1A	P_3	P_3	18	32	49.1 (2.2)	—	49.0 (1.5)	—	38.0 (0.2)	—	98.3 (7.0)	—	89.9 (2.9)	—	0
1B	P_3	P_5							38.4 (0.3)	> 0.05	49.8 (2.0)	<< 0.01	56.2 (2.7)	<< 0.01	0
2A	P_5	P_5	23	23	20.0 (1.1)	<< 0.01	20.7 (1.0)	<< 0.01	40.2 (0.3)	<< 0.01	37.5 (1.9)	<< 0.01	34.8 (1.9)	<< 0.01	43
2B	P_5	P_3							40.4 (0.6)	< 0.01	80.8 (5.2)	< 0.05	66.5 (5.8)	< 0.01	64
3A	P_7	P_7	28	31	19.3 (1.1)	<< 0.01	18.0 (1.1)	<< 0.01	40.7 (0.4)	<< 0.01	35.5 (2.0)	<< 0.01	33.3 (2.1)	<< 0.01	44
3B	P_7	P_3							41.8 (0.6)	<< 0.01	66.7 (7.0)	< 0.01	68.8 (4.7)	< 0.01	67
							Experiment 2								
4A	P_3	P_3	20	17	55.0 (1.2)	—	50.1 (1.4)	—	37.8 (0.3)	—	98.7 (6.6)	—	82.2 (4.7)	—	14
4B	P_3	P_{12}							37.4 (0.3)	> 0.05	64.5 (4.4)	< 0.01	66.3 (3.7)	< 0.05	8
5A	P_{12}	P_{12}	40	32	26.3 (0.8)	<< 0.01	28.1 (1.2)	<< 0.01	39.2 (0.3)	< 0.01	48.2 (1.7)	<< 0.01	47.7 (2.4)	<< 0.01	38
5B	P_{12}	P_3							38.8 (0.4)	> 0.05	73.0 (2.9)	< 0.01	70.8 (4.0)	> 0.05	20
6A	P_{13}	P_{13}	29	20	24.7 (1.6)	<< 0.01	23.2 (1.4)	<< 0.01	40.3 (0.4)	<< 0.01	38.2 (2.1)	<< 0.01	38.6 (4.2)	< 0.01	44
6B	P_{13}	P_3							39.9 (0.5)	< 0.01	69.2 (5.0)	< 0.01	66.0 (3.5)	< 0.05	0

[1] Figures in parentheses are standard errors.

[2] Probability based on *t* values. Each comparison was made with the control group in the same experiment.

TABLE 3

Influence of protein restriction with various supplements during the reproductive cycle on weaning weight, eruption time of third molars and morphology of third molars in caries-susceptible rats[1]

Group no.	Diet		No. of rats weaned		Weight at weaning				Eruption time of lower third molars		Weight when third molars erupted				% rats with malformed third molars
					Males		Females				Males		Females		
	Developmental	Post-developmental	Males	Females	Avg	P[2]	Avg	P	Avg	P	Avg	P	Avg	P	
					g		*g*		*day*		*g*		*g*		
							Experiment 3								
7A	P_3	P_3	17	19	48.8 (0.9)	—	46.8 (0.8)	—	34.3 (0.3)	—	77.8 (5.4)	—	81.3 (4.0)	—	0
7B	P_3	P_5							35.1 (0.4)	> 0.05	54.2 (2.7)	< 0.01	51.0 (2.1)	$<< 0.01$	0
8A	P_5	P_5	48	57	20.5 (0.8)	$<< 0.01$	21.6 (0.9)	$<< 0.01$	39.5 (0.4)	$<< 0.01$	38.9 (2.2)	$<< 0.01$	38.9 (2.1)	$<< 0.01$	53
8B	P_5	P_3							38.2 (0.3)	$<< 0.01$	71.1 (5.0)	> 0.05	63.9 (3.4)	< 0.01	48
9A	P_6	P_6	46	51	25.4 (0.7)	$<< 0.01$	25.0 (1.0)	$<< 0.01$	37.9 (0.2)	$<< 0.01$	49.9 (2.3)	< 0.01	48.5 (2.3)	$<< 0.01$	37
9B	P_6	P_3							37.7 (0.3)	$<< 0.01$	76.7 (3.3)	> 0.05	73.6 (4.6)	> 0.05	22
10A	P_7	P_7	44	42	19.2 (0.6)	$<< 0.01$	17.9 (0.6)	$<< 0.01$	40.0 (0.2)	$<< 0.01$	34.8 (1.7)	$<< 0.01$	35.6 (2.0)	$<< 0.01$	48
10B	P_7	P_3							39.0 (0.3)	$<< 0.01$	74.7 (4.1)	> 0.05	69.1 (3.1)	< 0.05	47
							Experiment 4								
11A	P_3	P_3	28	25	51.4 (1.2)	—	47.5 (1.2)	—	34.6 (0.3)	—	98.3 (4.3)	—	85.3 (5.6)	—	11
11B	P_3	P_{14}							34.5 (0.5)	> 0.05	102.5 (4.0)	> 0.05	90.2 (3.5)	> 0.05	6
11C	P_3	P_{15}							34.7 (0.2)	> 0.05	87.9 (5.3)	> 0.05	83.0 (5.2)	> 0.05	17
12A	P_{14}	P_{14}	22	22	53.9 (1.8)	> 0.05	50.9 (1.6)	> 0.05	34.6 (0.3)	> 0.05	108.6 (3.8)	> 0.05	93.6 (2.9)	> 0.05	5
12B	P_{14}	P_3							34.3 (0.3)	> 0.05	107.3 (7.7)	> 0.05	93.3 (4.5)	> 0.05	5

13A	P_{15}	P_{15}	27	38	49.5 (0.9)	> 0.05	45.8 (1.1)	> 0.05	35.5 (0.1)	< 0.01	97.9 (3.9)	> 0.05	87.1 (1.3)	> 0.05	3
13B	P_{15}	P_3							35.5 (0.2)	< 0.05	100.4 (3.2)	> 0.05	88.9 (3.3)	> 0.05	7
14A	P_5	P_5	31	24	20.7 (1.0)	<< 0.01	20.0 (1.2)	<< 0.01	39.4 (0.4)	<< 0.01	38.6 (2.7)	<< 0.01	38.0 (3.4)	<< 0.01	76
14B	P_5	P_{17}							38.9 (0.5)	<< 0.01	36.8 (1.6)	<< 0.01	32.3 (2.3)	<< 0.01	76
14C	P_5	P_{18}							39.1 (0.4)	<< 0.01	34.5 (2.9)	<< 0.01	37.8 (3.4)	<< 0.01	76
15A	P_{17}	P_{17}	29	27	21.3 (0.9)	<< 0.01	21.5 (1.0)	<< 0.01	38.0 (0.4)	<< 0.01	39.1 (1.7)	<< 0.01	37.9 (1.6)	<< 0.01	71
15B	P_{17}	P_5							38.8 (0.8)	<< 0.01	40.1 (1.5)	<< 0.01	39.4 (2.4)	<< 0.01	74
16A	P_{18}	P_{18}	17	15	18.8 (1.2)	<< 0.01	20.2 (1.3)	<< 0.01	40.1 (0.5)	<< 0.01	32.0 (2.3)	<< 0.01	38.4 (1.6)	<< 0.01	71
16B	P_{18}	P_5							39.5 (0.4)	<< 0.01	39.7 (1.9)	<< 0.01	34.0 (1.9)	<< 0.01	71
							Experiment 5								
17A	P_3	P_3	35	27	43.2 (1.6)	—	39.5 (1.5)	—	39.5 (0.4)	—	89.3 (1.2)	—	68.8 (2.9)	—	6
17B	P_3	P_{21}							40.0 (0.4)	> 0.05	57.5 (2.1)	<< 0.01	53.3 (3.3)	< 0.01	21
18A	P_5	P_5	26	15	16.8 (1.0)	<< 0.01	15.1 (1.0)	<< 0.01	43.4 (0.6)	<< 0.01	30.5 (3.0)	<< 0.01	29.4 (1.7)	<< 0.01	80
18B	P_5	P_3							42.4 (0.4)	<< 0.01	63.6 (6.7)	< 0.05	56.7 (3.0)	< 0.05	94
19A	P_{19}	P_{19}	25	21	21.7 (0.8)	<< 0.01	19.7 (0.7)	<< 0.01	43.1 (0.3)	<< 0.01	36.9 (2.6)	<< 0.01	43.4 (2.3)	<< 0.01	35
19B	P_{19}	P_3							40.3 (0.4)	> 0.05	73.0 (5.8)	< 0.01	68.6 (2.0)	> 0.05	35
20A	P_{20}	P_{20}	18	20	17.7 (1.1)	<< 0.01	17.5 (0.9)	<< 0.01	42.8 (0.5)	<< 0.01	40.0 (2.4)	<< 0.01	32.7 (3.0)	<< 0.01	71
20B	P_{20}	P_3							42.7 (0.6)	<< 0.01	69.9 (5.4)	< 0.01	47.8 (1.9)	< 0.01	71
21A	P_{21}	P_{21}	31	22	25.5 (1.0)	<< 0.01	24.7 (1.3)	<< 0.01	40.4 (0.6)	> 0.05	34.9 (1.4)	<< 0.01	44.8 (3.9)	<< 0.01	50
21B	P_{21}	P_3							40.4 (0.4)	> 0.05	74.5 (3.1)	< 0.01	64.9 (3.7)	> 0.05	50

[1] Figures in parentheses are standard errors.
[2] Probability based on t values.

more uniform and to provide a more nearly equal opportunity to individual offspring.

Since the growth rate of pups born to protein-deficient females was very slow, they could not adapt to independent life before 25 days of age. Hence all offspring in control and experimental litters were weaned at 25 days.

Ordinarily only one litter was taken from each female fed diet P_3. Occasionally second litters were taken to increase the number of control subjects. However, routinely 2 and occasionally 3 litters had to be taken from each female in protein-deficient groups in order to have adequate subjects for post-weaning comparisons. This procedure proved to be justifiable since no statistically significant differences were observed between first and second or third litters for the various parameters tested.

After weaning, each litter in experiments 1, 2, 3 and 5 was subdivided into 2 approximately equal subgroups: one-half always continued to be fed the same diet as the mother. Among offspring whose mothers were fed control diet P_3, the second subgroup was transferred to low-protein diet P_5 or P_{12} to test the post-weaning influences of the diets. The second subgroup of offspring whose mothers were fed any experimental diet was transferred to diet P_3. The latter subdivision made possible post-developmental comparisons of the dental effects of the 2 diets. These subgroups are labeled A and B in the tables.

A different post-weaning subdivision of the offspring was used in experiment 4 where dietary supplements of 0.5 and 2.0% sodium dihydrogen orthophosphate were tested. These supplements were of special interest during and after tooth development because of current evidence that soluble phosphates exert a cariostatic influence in rodents (4). In control group 11 and experimental group 14 fed low-protein diet P_5, each litter was divided into 3 subgroups, A, B and C; the first (A) as usual continued to be fed the maternal diet. The second and third subgroups (B and C) were transferred to the diets supplemented with 0.5 and 2.0% sodium dihydrogen orthophosphate, respectively, for post-developmental evaluations. The usual subdivision into subgroups A and B was practiced with respect to the offspring from experimental groups 12, 13, 15 and 16.

After weaning, the rats were housed singly or in pairs of the same sex in screen-bottom cages. The appropriate diet and tap water were provided ad libitum. Each rat was weighed at weaning, and every 2 weeks until killed. *In vivo* examinations were conducted periodically to determine when the third molars were fully erupted and to inspect the rate of progression of carious lesions. In subgroups fed diet P_3 in experiments 1, 2 and 3, representative rats were killed when the third molars were fully erupted and were used for precise measurements of the mesio-distal and bucco-lingual dimensions of the molars.

The remaining offspring in experiments 1 and 2 were killed at 250 days of age, those in experiment 3 at 100 days of age, and those in experiments 4 and 5 at 80 days of age. The skulls were fixed in 95% ethyl alcohol and scored for carious lesions by the method of Shaw et al. (5).

RESULTS

Well-being and fertility of breeding females. During the 28-day pre-mating period in the 5 experiments, the females receiving all diets were able to maintain their body weights. Increases in body weight during pregnancy were appreciably lower with the low-protein diets than with the control diet, P_3. Only 9 of the 168 breeding females failed to deliver living young. One was a control in experiment 5; 3 were in phosphate-supplemented, adequate protein groups of experiment 4, and the remaining 5 were fed low-protein diets in experiments 4 and 5.

Birth weight and survival to weaning. In experiment 1 with Harvard caries-resistant rats, the average birth weight for the 6 offspring per litter of control group 1 was 37.3 g; 15.5% of these offspring died prior to weaning. The average birth weight per litter in group 2 was 31.6 g; 62.2% died prior to weaning. The average birth weight in group 3 was 34.4 g; 37.2% of these pups died before weaning. In experiment 2, the increase in casein from 8 to 10% in group 5 had no significant influence on birth weight but de-

creased pre-weaning losses to 18.3%. Comparable increase in egg albumen in group 6 also had no significant influence on birth weight but increased pre-weaning deaths to 59.2%.

In experiment 3 with Harvard caries-susceptible rats, the average body weight for the litters at birth in control group 7 was 39.7 g. Among protein-deficient groups 8 through 10, the average birth weights of the litters were 32.4, 31.1 and 30.5 g, respectively. The pre-weaning mortality among these offspring was 0, 21.3, 25.4, and 29.8%.

In experiment 4, the average litter weights at birth were typical of early experiments with both normal and low-protein diets. The phosphate supplements did not appear to have any influence on reproductive ability or on birth weights. The mortality rate prior to weaning for groups 11 through 16 was 4.1, 4.7, 4.9, 19.2, 23.8 and 42.1%. The 2.0% phosphate supplement to the low-protein diet apparently increased the death rate among the sucklings.

In experiment 5 with mutant albino rats, the average litter weight at birth for control group 17 was 39.5 g, whereas the averages for low-protein groups 18 through 21 varied from 32.6 to 33.4 g. The frequency of deaths among the offspring prior to weaning was 11.6, 35.6, 2.5, 17.3 and 14.7% for the 5 groups, respectively. The benefit of the 1.0% DL-methionine supplement to the low-protein diet in group 19 in reducing mortality was remarkable, although no influence on birth weight was observed. The sulfate supplement in group 20 caused a smaller reduction in mortality; the combination of sulfate and DL-methionine in group 21 was not as effective as DL-methionine alone.

Weaning weights. Data for the number of rats weaned and their weaning weights are presented in the fourth through ninth vertical columns of tables 2 and 3. In all 3 strains, a low-protein diet during the reproductive cycle resulted in significant reductions in body weight at weaning at 25 days of age. Offspring of low-protein females routinely weighed less than one-half the body weights of their first cousins whose mothers were fed diet P_3 through the reproductive cycle. Increase in protein from 8 to 10% in experiment 2 did not have any significant influence on weaning weights. The 0.5% phosphate supplement in groups 12 and 15 of experiment 4 tended to cause slight increases in weaning weights, but there was a trend toward a reduction when a 2.0% phosphate supplement was used in groups 13 and 16.

The offspring of the females in group 9 of experiment 3 with the 0.3% DL-methionine supplement were slightly heavier at weaning than the rats in the other protein-deficient groups 8 and 10. This difference was significant for the females ($P < 0.05$) and highly significant for the males ($P < 0.01$). Supplementation with 1.0% DL-methionine with or without sulfate in groups 19 and 21 of experiment 5 resulted in highly significant increases in weaning weights for both males and females. The sulfate supplement may have augmented the methionine effect to produce larger offspring in group 21 at weaning. However, this increase may have been due to the small number of rats weaned per litter as a result of higher mortality rate in this sulfate-methionine supplemented group. Even with these increases in body weight due to the methionine and sulfate supplements, the offspring were still extremely penalized. Supplementation with 1.0% sodium sulfate in group 20 produced no significant increase in weaning weights.

Eruption time of third molars. The data for the eruption times of lower third molars are presented in the tenth and eleventh vertical columns of tables 2 and 3. Strong inter-strain differences in the eruption times of third molars were observed among control rats. The mandibular third molars of the Harvard caries-resistant rats in control groups 1 and 4 in experiments 1 and 2 erupted at 37.4 to 38.4 days of age. Among the Harvard caries-susceptible rats in control groups 7 and 11 of experiments 3 and 4, the mandibular third molars erupted at 34.3 to 35.1 days of age. This difference between strains was highly significant ($P < 0.01$). In the mutant albino strain used in experiment 5, the mandibular third molars erupted still later, with averages of 39.5 to 40.0 days for the control rats in group 17. These values again were different by

highly significant amounts from either of the 2 strains. Eruption time for the maxillary third molars was invariably 0.5 to 1.0 days later than for the mandibular opponents in each strain. Comparable significant differences were observed for eruption times of the maxillary third molars between strains.

Wherever a borderline protein deficiency was imposed throughout the reproductive cycle in the 5 experiments, a highly significant retardation in the time of third molar eruption was observed among the offspring. Irrespective of the normal eruption time in the 3 strains, protein deficiency caused delays of from 2 to 5 days. Invariably when the offspring of deficient mothers were transferred to diet P_3 at weaning, the same delay in third molar eruption was observed as in the brothers and sisters continued with a protein-deficient diet. Conversely when offspring of mothers fed a normal diet P_3 were transferred at weaning to inadequate diet P_5, their third molars erupted at the usual date expected for that strain among control littermates continued with diet P_3. These data indicated that the cause and sequence of events leading to delayed eruption had been established before weaning and that eruption time for third molars under these experimental circumstances was independent of the post-weaning adequacy of dietary protein.

This lack of post-weaning influence led to enormous variations in body weight when third molar eruption occurred. These data are presented in the twelfth through the fifteenth vertical columns of tables 2 and 3. A good example of this contrast in body weights may be noted in group 2 of experiment 1 where the males continued with diet P_5 weighed 37.5 g at the time of third molar eruption in contrast with 80.8 g for the littermates transferred to diet P_3.

The slight increase in protein levels in experiment 2 reduced the delay in third molar eruption from 2 to 3 days to 1.5 to 2 days. The phosphate supplements in experiment 4 had no beneficial influence on tooth eruption. An interesting observation was made with respect to the offspring of females in group 13 who were fed the control diet with a 2% phosphate supplement. In subgroups 13A and 13B a delay in eruption of approximately one day was observed which was significant ($P < 0.05$) for one subgroup and highly significant ($P < 0.01$) for the other subgroup. This observation, coupled with slightly lower weaning weights, suggested that this level of phosphate may have been slightly disadvantagous during this period of the rat's life.

The third molars of the offspring of group 9 in experiment 3 where the diet was supplemented with 0.3% DL-methionine erupted approximately a day earlier than for their counterparts in group 8 fed diet P_5 without the supplement. This earlier eruption time was significantly different for the males ($P < 0.05$) but not for the females. Even with this methionine supplement, third molar eruption was still significantly later than for controls in group 7 ($P < 0.01$).

Among offspring of females in group 19 of experiment 5 fed the low-protein diet supplemented with 1.0% DL-methionine (diet P_{19}), third molar eruption was significantly delayed for subgroup A continued with diet P_{19} but was not delayed for the subgroup B transferred to diet P_3. In addition in group 2 where the females were fed the low-protein diet supplemented with both 1.0% DL-methionine and 1.0% sodium sulfate (diet P_{21}) no significant delay was observed in third molar eruption in either subgroup A continued with diet P_{21} or in subgroup B transferred to diet P_3. Sulfate alone did not hasten third molar eruption in group 20.

Missing cusps on third molars. The small mesiolingual cusp of the maxillary third molars and the small mesiobuccal cusp of the mandibular third molars occasionally are not present in normal rats. In vertical column 16 of tables 2 and 3, the relatively infrequent absence of these cusps in control groups of experiments 2, 4 and 5 is recorded, whereas their absence was not observed in the control groups of experiments 1 and 3. The frequency with which these minor cusps failed to develop was greatly increased among the offspring of females fed all unsupplemented and phosphate-supplemented protein-deficient diets.

In experiment 3, group 9, a slight tendency toward a lower incidence of missing

cusps appeared to have occurred among the rats on the 0.3% DL-methionine supplemented regimen than was present among the offspring of protein-deficient females in group 8. In experiment 5, groups 19 and 21, the 1.0% DL-methionine supplement with or without the 1.0% sodium sulfate supplement caused appreciably lower incidences of missing cusps than in protein-deficient group 18. The 1.0% sodium sulfate supplement in group 20 was ineffective.

Mesio-distal and bucco-lingual dimensions of molars. The data for the average sizes of maxillary molars in experiments 1, 2 and 3 are presented in table 4 in terms of their mesio-distal and bucco-lingual dimensions. The frequent failure of minor cusps to develop among the offspring of protein-deficient females was not an isolated phenomenon, but was probably just a readily visible gross indication of more profound influences on the size of the molars. Throughout these 3 experiments, the molars of the offspring of protein-deficient females were smaller than the molars from control rats. This reduction in size was least for the first molars where the differences varied from insignificance to highly significant and greatest for the third molars where the differences were almost always highly significant. The reductions for the third molars were sufficiently great that they could be unerringly detected by the naked eye. Although only data for the maxillary molars were presented, data of comparable levels of significance were obtained for the mandibular molars.

Precise measurements were not made for the sizes of the molars in the fourth and fifth experiments. As in experiments 1 through 3, the third molars of offspring from protein-deficient females were visibly smaller than those of controls. Phosphate and sulfate supplements had no detectable influence on molar size. However, the offspring in groups 19 and 21 with the 1.0% DL-methionine supplement were clearly larger than those of offspring in groups 18 and 20 although not as large as those in control group 17.

Presumably the end of the spectrum with respect to missing cusps and reduced molar size would be the failure of third molars to develop. One subject was observed in group 11 of experiment 4 which had no gross evidence of mandibular third molars when killed at 80 days of age. Furthermore, no evidence of these molars could be observed radiographically (fig. 1) or by careful dissection. A comparable control is shown in figure 2. In the previous study, one rat that was born to a protein-deficient mother was found to have no maxillary third molar (3). In the course of examination of approximately 40,000 representatives of these strains over the past decade, no other rat with one or more missing third molars has been observed. Although those 2 isolated rats are insufficient to prove a causative relationship, they suggest that in the extreme situation environmental circumstances may be able to prevent tooth development.

Post-weaning gain in body weight. The results for the average body weight increases during the post-weaning caries assay periods are presented in vertical columns 5 and 6 of tables 5 and 6. The offspring of protein-deficient females that were transferred to diet P_3 at weaning grew well but rarely had overcome their initial handicap by the end of the experimental periods. The offspring of protein-deficient mothers that continued to be fed the maternal diet invariably grew very poorly after weaning. During this post-weaning period, diets P_7 and P_{13} containing 8 and 10% egg albumen, respectively, supported slightly better growth among the Harvard caries-resistant rats in experiments 1 and 2 than did diets P_5 and P_{12} with the same casein levels. The increases in protein level from 8 to 10% by the use of diets P_{12} and P_{13} in experiment 2 resulted in better post-weaning growth, especially among the offspring of the females fed diet P_{13} containing egg albumen. However, diet P_7 did not appear to have any advantage over diet P_5 with respect to post-weaning growth for the Harvard caries-susceptible rats in experiment 3.

The offspring in group 9 of experiment 3 fed diet P_6 with the 0.3% DL-methionine supplement grew better than those without this supplement, although still distinctly less rapidly than those fed control diet P_3. In group 19 in experiment 5, the supplement of 1.0% DL-methionine to the

TABLE 4

Influence of protein restriction on the mesio-distal and bucco-lingual dimensions of the maxillary molars of rats expressed in millimeters[1]

Group no.	Diet	No. of rats	First molar				Second molar				Third molar			
			m-d[2]		b-l[3]		m-d		b-l		m-d		b-l	
			Avg	P[4]	Avg	P	Avg	P	Avg	P	Avg	P	Avg	P
							Experiment 1							
1	P_3	11	3.27 (0.036)	—	2.15 (0.013)	—	2.37 (0.026)	—	2.13 (0.015)	—	1.61 (0.029)	—	1.61 (0.026)	—
2	P_5	4	3.28 (0.110)	> 0.05	2.23 (0.022)	> 0.05	2.31 (0.054)	> 0.05	2.05 (0.018)	< 0.01	1.31 (0.047)	$<< 0.01$	1.45 (0.020)	$<< 0.01$
3	P_7	11	3.17 (0.049)	> 0.05	2.09 (0.030)	> 0.05	2.19 (0.030)	< 0.01	1.99 (0.030)	< 0.01	1.34 (0.044)	$<< 0.01$	1.41 (0.029)	$<< 0.01$
							Experiment 2							
4	P_3	10	3.39 (0.049)	—	2.22 (0.043)	—	2.41 (0.025)	—	2.18 (0.015)	—	1.59 (0.030)	—	1.64 (0.017)	—
5	P_{12}	17	3.24 (0.028)	< 0.05	2.12 (0.013)	< 0.05	2.32 (0.016)	< 0.01	2.06 (0.043)	< 0.05	1.42 (0.020)	< 0.01	1.47 (0.019)	$<< 0.01$
6	P_{13}	11	3.19 (0.045)	< 0.05	2.09 (0.015)	< 0.05	2.30 (0.023)	< 0.01	2.05 (0.012)	$<< 0.01$	1.47 (0.037)	< 0.05	1.49 (0.031)	< 0.01
							Experiment 3							
7	P_3	12	3.24 (0.038)	—	2.06 (0.016)	—	2.35 (0.025)	—	2.06 (0.015)	—	1.59 (0.039)	—	1.54 (0.016)	—
8	P_5	26	3.14 (0.022)	< 0.05	2.04 (0.014)	> 0.05	2.24 (0.014)	< 0.01	1.97 (0.013)	< 0.01	1.35 (0.026)	$<< 0.01$	1.39 (0.017)	$<< 0.01$
9	P_6	27	3.10 (0.027)	< 0.01	2.02 (0.015)	> 0.05	2.24 (0.020)	< 0.01	1.99 (0.016)	< 0.01	1.37 (0.025)	< 0.01	1.45 (0.014)	< 0.01
10	P_7	23	3.07 (0.024)	< 0.01	1.99 (0.012)	< 0.01	2.16 (0.013)	$<< 0.01$	1.93 (0.012)	$<< 0.01$	1.30 (0.015)	$<< 0.01$	1.39 (0.012)	$<< 0.01$

[1] Figures in parentheses are standard errors.
[2] m-d = mesio-distal dimension.
[3] b-l = bucco-lingual dimension.
[4] Probability based on *t* values.

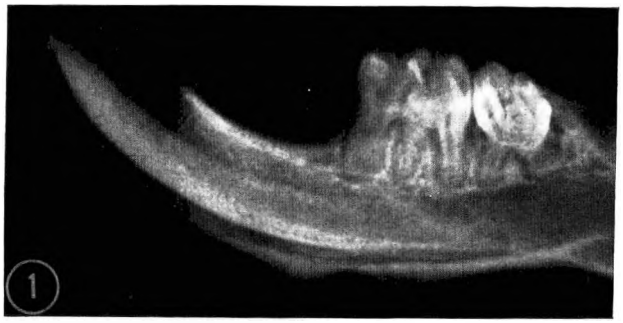

Fig. 1 Radiograph of a mandible of rat in experiment 4 in the subgroup where the mother was fed protein-deficient diet P_5 throughout pregnancy and lactation and continued to be fed the same diet after weaning. Note absence of third molar although first and second molars are present and well formed. × 5.

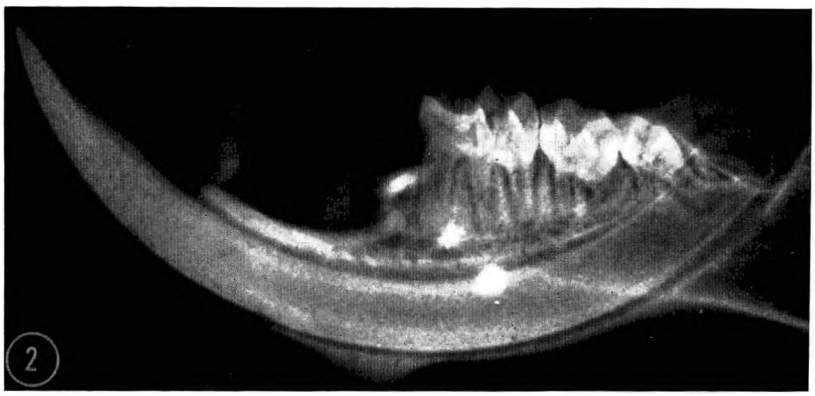

Fig. 2 Radiograph of a mandible of a rat whose mother was fed control diet P_9 throughout pregnancy and lactation and continued to be fed the same diet after weaning. Note normal position of third molars. × 5.

low-protein diet (P_{19}) resulted in a modest improvement in the growth rate. The combined supplement of DL-methionine and sodium sulfate in group 21 promoted even better growth among males but had no added benefit for females. Likewise, the sulfate supplement in group 20 resulted in a slightly improved growth rate for males but not for females when comparison was made with the offspring fed the low-protein diet (P_5) in group 17. In experiment 4, all rats in the 3 groups 14 through 16 fed low-protein diets grew poorly. The phosphate supplements did not appear to help or hinder their growth.

Carious lesions in the occlusal sulci of the molars. The data for percentage of caries-free rats, number of carious molars, number of carious lesions and extent of carious lesions are presented in the seventh through thirteenth vertical columns of table 5 and in the seventh through twelfth columns of table 6. In experiments 1 and 2, dental caries activity, as measured by all 4 of the above parameters, was very low, as would be expected for the Harvard caries-resistant strain. The general caries-resistance of the population in experiment 2 tended to be appreciably higher than that of the population in experiment 1. This intra-strain variation was evidenced by a higher frequency of caries-free rats throughout experiment 2 and also by an extremely low incidence of dental caries in the control group in experiment 2. However, in both experiments the offspring of females fed the protein-deficient diet during the reproductive cycle had a significantly higher dental caries incidence than the offspring of control rats. The

TABLE 5

Influence of protein restriction during the reproductive cycle on the dental caries incidence in Harvard caries-resistant rats[1]

Group no.	Diet		No. of rats	Avg wt increase		% caries-free rats	No. of carious molars		No. of carious lesions		Extent of carious lesions[3]	
	Developmental	Postdevelopmental		Males	Females		Avg	P[2]	Avg	P	Avg	P
				g	*g*							
						Experiment 1						
1A	P_3	P_3	18	350	214	44	1.2 (0.3)	—	1.4 (0.4)	—	2.7+ (1.0)	—
1B	P_3	P_5	11	167	158	36	1.4 (0.4)	> 0.05	1.4 (0.4)	> 0.05	2.2+ (0.7)	> 0.05
2A	P_5	P_5	14	181	138	29	3.1 (0.8)	< 0.05	5.6 (1.8)	< 0.05	20.6+ (6.7)	< 0.05
2B	P_5	P_3	15	299	198	40	1.5 (0.4)	> 0.05	1.9 (0.5)	> 0.05	5.6+ (1.7)	> 0.05
3A	P_7	P_7	16	238	172	6	4.1 (0.6)	< 0.01	5.7 (1.0)	< 0.01	19.6+ (4.2)	< 0.01
3B	P_7	P_3	15	325	202	27	3.0 (0.5)	< 0.01	4.7 (1.0)	< 0.01	16.9+ (4.3)	< 0.01
						Experiment 2						
4A	P_3	P_3	16	321	186	81	0.1 (0.1)	—	0.1 (0.1)	—	0.2+ (0.2)	—
4B	P_3	P_{12}	13	231	163	92	0.3 (0.3)	> 0.05	0.3 (0.3)	> 0.05	0.5+ (0.4)	> 0.05
5A	P_{12}	P_{12}	24	240	181	54	1.5 (0.4)	< 0.01	2.2 (0.8)	< 0.05	6.8+ (2.7)	< 0.05
5B	P_{12}	P_3	25	316	202	40	1.0 (0.3)	< 0.05	1.4 (0.5)	< 0.05	4.2+ (2.0)	< 0.05
6A	P_{13}	P_{13}	9	283	188	44	2.4 (0.8)	< 0.05	3.2 (1.1)	< 0.05	10.4+ (3.6)	< 0.05
6B	P_{13}	P_3	11	373	217	36	2.0 (0.6)	< 0.01	3.5 (1.3)	< 0.05	12.7+ (4.5)	< 0.05

[1] Figures in parentheses are standard errors.
[2] Probability based on *t* values.
[3] According to the "plus scoring" method of Shaw et al. (5).

increase in protein levels from 8% in experiment 1 to 10% in experiment 2 was insufficient to counteract the adverse influence of protein deprivation upon caries resistance. The question arises whether the additional offspring kept alive during lactation by the slightly higher protein levels may have been the ones most penalized during pregnancy and early lactation with respect to dental development.

The dental caries incidence among rats in experiments 3, 4 and 5 was a great deal higher than for the Harvard caries-resistant rats in the previous 2 experiments. No subjects in these 3 experiments were caries-free in contrast with the high percentage in Harvard caries-resistant populations. In addition to the differences between the absolute values for carious molars, carious lesions and extent of carious lesions, it must be remembered that these rats in experiment 3 were maintained for only 75 days and those in experiments 4 and 5 for only 55 days after weaning in comparison with 225 days for the caries-resistant rats.

In every comparison in experiments 3 through 5 with caries-susceptible rats, the average number of carious molars, average number of carious lesions and average extent of carious lesions were higher for the offspring of protein-deficient females than for those of control rats.

In experiment 4, supplementation of the adequate protein diet in groups 12 and 13 with 0.5 or 2.0% sodium dihydrogen orthophosphate during the reproductive cycle but not after weaning resulted in modest reductions in dental caries incidence. This reduction was small and not statistically significant for the number of carious molars, but the reductions were somewhat larger and statistically significant ($P < 0.05$) for the number of carious lesions and the extent of carious lesions. When the same supplement as the mother received was continued for the offspring after weaning in subgroups 12A and 13A, greater reductions were observed in all categories with the level of significance varying from $P < 0.05$ to $P < 0.01$ in various comparisons. Among the offspring of the subgroups 11B and 11C where the phosphate supplements were only provided post-weaning, minor reductions in dental caries incidence were observed in all comparisons which were not statistically significant.

In groups 14 through 16 of experiment 4 composed of offspring from mothers provided with low-protein diets during pregnancy, with or without phosphate supplementation, an increased dental caries incidence above the controls in group 11 was observed in every single comparison. However, the general increase in caries activity was less than in the previous 3 experiments. These increases attributable to protein deprivation varied from insignificant to highly significant. Unlike the results in adequate protein groups 11 through 13, there was no evidence that the phosphate supplements in protein-deficient groups 14 through 16 had any important influence upon caries activity as a result of being fed only during the reproductive cycle, only during the post-weaning period or throughout the entire experiment.

In experiment 3, there was no evidence that the 0.3% DL-methionine supplement had been of value in reducing the tendency toward a higher caries-susceptibility among the protein-deficient rats.

The expected high caries activity for the mutant albino strain was realized in experiment 5. The control rats in subgroup 17A with a history of diet P_3 during the reproductive cycle as well as post-weaning had a typical caries incidence. Their littermates in subgroup 17B which were fed diet P_{21} (low-protein supplemented with DL-methionine and sulfate) had a significantly higher number of carious molars, carious lesions and extent of carious lesions (P for all 3 comparisons < 0.01). This striking difference between littermates in these 2 subgroups was unlike any other comparison for controls in the preceding 4 experiments. Routinely, the dental caries incidence of controls fed a low-protein diet P_5 did not differ from their littermates fed adequate diet P_3 despite the different sucrose concentrations. One clear difference existed in this experiment: instead of diet P_5, the methionine and sulfate supplemented diet (P_{21}) had been substituted. These data suggest that the methionine and sulfate supplements had augmented the dental caries activity.

TABLE 6

Influence of protein restriction with various supplements during the reproductive cycle on the dental caries incidence in caries-susceptible rats[1]

Group no.	Diet		No. of rats	Avg wt increase		No. of carious molars		No. of carious lesions		Extent of carious lesions[3]	
	Developmental	Post-developmental		Males	Females	Avg	P[2]	Avg	P	Avg	P
				g	*g*						
						Experiment 3					
7A	P_3	P_3	12	213	133	6.7 (0.6)	—	11.2 (1.5)	—	31.5+ (4.9)	—
7B	P_3	P_5	12	92	69	5.8 (0.6)	> 0.05	8.6 (1.3)	> 0.05	25.5+ (4.6)	> 0.05
8A	P_5	P_5	30	87	81	7.3 (0.3)	> 0.05	14.7 (0.9)	> 0.05	54.8+ (4.7)	< 0.01
8B	P_5	P_3	33	178	137	7.3 (0.2)	> 0.05	14.3 (0.6)	> 0.05	47.7+ (2.9)	< 0.01
9A	P_6	P_6	35	125	99	8.1 (0.2)	> 0.05	16.8 (0.6)	< 0.01	59.5+ (2.8)	< 0.01
9B	P_6	P_3	32	199	135	7.1 (0.2)	> 0.05	13.8 (0.7)	> 0.05	46.9+ (2.8)	< 0.01
10A	P_7	P_7	27	90	88	7.1 (0.3)	> 0.05	12.2 (0.8)	> 0.05	41.5+ (3.8)	< 0.05
10B	P_7	P_3	30	190	137	7.3 (0.2)	> 0.05	13.7 (0.7)	> 0.05	46.2+ (2.9)	< 0.05
						Experiment 4					
11A	P_3	P_3	18	194	119	6.5 (0.5)	—	11.7 (1.1)	—	36.6+ (3.8)	—
11B	P_3	P_{14}	17	186	111	5.9 (0.4)	> 0.05	10.2 (1.3)	> 0.05	30.9+ (3.5)	> 0.05
11C	P_3	P_{15}	18	159	103	5.4 (0.3)	> 0.05	8.7 (0.8)	> 0.05	28.0+ (2.8)	> 0.05
12A	P_{14}	P_{14}	22	184	113	4.9 (0.4)	< 0.05	7.0 (0.9)	< 0.01	21.0+ (2.7)	< 0.01
12B	P_{14}	P_3	21	197	126	5.5 (0.3)	> 0.05	8.4 (0.7)	< 0.05	25.7+ (2.4)	< 0.05
13A	P_{15}	P_{15}	31	211	115	4.6 (0.3)	< 0.01	6.2 (0.7)	< 0.01	18.1+ (2.3)	< 0.01
13B	P_{15}	P_3	30	221	119	5.7 (0.2)	> 0.05	8.7 (0.6)	< 0.05	26.7+ (1.8)	< 0.05

14A	P_5	P_5	17	64	61	7.1 (0.2)	> 0.05	14.7 (0.8)	> 0.05	45.2 + (2.6)	> 0.05
14B	P_5	P_{17}	17	69	63	7.1 (0.2)	> 0.05	14.4 (0.7)	> 0.05	47.9 + (2.8)	> 0.05
14C	P_5	P_{18}	17	63	64	7.1 (0.2)	> 0.05	13.4 (1.1)	> 0.05	43.8 + (3.7)	> 0.05
15A	P_{17}	P_{17}	28	69	68	7.5 (0.2)	> 0.05	13.9 (0.5)	> 0.05	45.6 + (1.8)	> 0.05
15B	P_{17}	P_5	27	77	72	7.7 (0.3)	> 0.05	14.9 (0.7)	< 0.05	50.7 + (2.7)	< 0.01
16A	P_{18}	P_{18}	17	63	70	7.2 (0.4)	> 0.05	13.2 (1.1)	> 0.05	42.8 + (3.9)	> 0.05
16B	P_{18}	P_5	14	62	71	7.6 (0.2)	> 0.05	15.9 (1.0)	< 0.05	51.0 + (3.5)	< 0.05
						Experiment 5					
17A	P_3	P_3	31	164	98	6.4 (0.3)	—	11.5 (1.0)	—	38.3 + (3.1)	—
17B	P_3	P_{21}	28	91	68	9.0 (0.3)	<< 0.01	18.1 (1.0)	< 0.01	64.1 + (1.4)	<< 0.01
18A	P_5	P_5	15	50	55	8.4 (0.4)	< 0.01	18.0 (0.7)	<< 0.01	60.8 + (3.2)	<< 0.01
18B	P_5	P_3	18	145	109	8.1 (0.2)	< 0.01	15.5 (0.8)	< 0.01	52.9 + (3.1)	< 0.01
19A	P_{19}	P_{19}	20	76	71	8.7 (0.4)	< 0.01	18.0 (0.9)	<< 0.01	60.6 + (4.4)	< 0.01
19B	P_{19}	P_3	23	145	108	6.7 (0.3)	> 0.05	12.3 (0.7)	> 0.05	40.9 + (3.0)	> 0.05
20A	P_{20}	P_{20}	17	70	58	8.4 (0.3)	<< 0.01	18.2 (0.9)	<< 0.01	66.6 + (4.0)	<< 0.01
20B	P_{20}	P_3	17	152	104	7.8 (0.3)	< 0.01	16.9 (1.0)	<< 0.01	59.1 + (5.0)	< 0.01
21A	P_{21}	P_{21}	24	87	77	8.6 (0.3)	<< 0.01	17.7 (0.9)	< 0.01	58.5 + (3.6)	< 0.01
21B	P_{21}	P_3	26	170	100	6.0 (0.4)	> 0.05	10.6 (0.9)	> 0.05	33.0 + (3.3)	> 0.05

[1] Figures in parentheses are standard errors.
[2] Probability based on *t* values.
[3] According to the "plus scoring" method of Shaw et al. (5).

The offspring of the females in groups 18 and 20 fed the low-protein diet without or with a sulfate supplement had a significantly higher dental caries incidence ($P < 0.01$) than the controls in subgroup 17A with their complete history on diet P_3. The dental caries experiences in these 4 subgroups, 18A and B, 20A and B, were similar to each other and to the control subgroup 17B fed diet P_{21}.

The incidence of dental caries among the offspring of the females in groups 19 and 21 which were fed the low-protein diet supplemented with 1.0% DL-methionine (diet P_{19}) or the low-protein diet supplemented with 1.0% DL-methionine and 1.0% sodium sulfate (diet P_{21}) differed depending upon the post-weaning diet. The 2 subgroups fed the control diet P_3, 19B and 21B, had a dental caries incidence that was very similar to those of the control subgroup 17A fed P_3 post-weaning. This observation indicated that the DL-methionine supplement alone or with sulfate during tooth development had been able to counteract the caries-potentiating influence of the low-protein diet. However, in contrast, 2 subgroups, 19A and 21A, which continued to receive the maternal diet after weaning, developed an incidence of dental caries far in excess of their littermates and equal to that of the rats in groups 18 and 20 and to subgroup 17B fed diet P_{21}. These data indicated that the supplements have exerted a post-developmental caries-potentiating influence just as was observed in group 17. Since the sulfate supplement in groups 17 and 21 did not cause any added caries-potentiating influence beyond that demonstrated by the DL-methionine supplement alone in group 19, DL-methionine was apparently responsible for the post-developmental caries-potentiation.

These observations were especially interesting since DL-methionine appeared to operate in an adverse fashion post-developmentally although the same supplement appeared to act beneficially to reduce caries-susceptibility when fed during tooth development. Opposite effects of a supplement before and after tooth eruption need not be improbable due to the striking change in environment of the tooth with eruption into the oral cavity. Before the tooth erupted, the beneficial influence of methionine could have been mediated through partial correction of the deficiency; after tooth eruption, methionine may have acted in the oral environment to increase its cariogenic potential.

Carious lesions on smooth surfaces of molars. In tables 5 and 6 and in the preceding section, dental caries incidence referred only to carious lesions in the occlusal sulci of the molars. Ordinarily over the past decade, Harvard caries-resistant and Harvard caries-susceptible rats have not developed carious lesions on the buccal, lingual and proximal surfaces of the molars except on a very infrequent basis. This type is usually referred to as a smooth surface lesion or a lesion on the smooth surface in contrast with an occlusal lesion or a lesion in an occlusal sulcus.

With respect to the rats' resistance to smooth surface lesions, those of our strains have been quite unlike those of the Holtzman strain used by McClure (6). Even when we used McClure's diets in extensive, unpublished experiments with the Harvard strains, no evidence of susceptibility to smooth surface lesions was found. However, in the current experiments the frequency of smooth surface lesions was sufficiently great in certain groups to justify tabulation and comment.

In experiment 1 with Harvard caries-resistant rats, 2 of 89 offspring had lesions on the smooth surfaces. Both affected rats were offspring of females fed low-protein diets. None of the 98 offspring in experiment 2 developed smooth surface lesions. Possibly this difference from experiment 1 is not unexpected because of the lesser incidence of carious lesions in the occlusal sulci in experiment 2 which has been commented upon previously.

In experiment 3 with Harvard caries-susceptible rats, 1 of 24 offspring in control group 7 whose mothers were fed diet P_3 had smooth surface lesions. However, among the 187 offspring in groups 8 through 10 where the females were fed low-protein diets, 36 (19.3%) developed smooth surface lesions. In each subgroup 8B, 9B and 10B with offspring fed diet P_3 after weaning, the frequency of rats with smooth surface lesions was appreciably lower than for subgroups 8A, 9A

and 10A which had continued to be fed the same low-protein diet as the mother. There was no evidence that the 0.3% DL-methionine supplement had been of value with respect to smooth surface lesions.

In experiment 4, also with Harvard caries-susceptible rats, only 2 (1.3%) of 147 offspring from females in groups 11 through 13 fed diets P_3, P_{14} and P_{15} with normal protein levels developed any smooth surface lesions. Again there was an increase in frequency of rats with this type of lesion among protein-deficient groups 14 through 16, with 15 (10.9%) of 137 offspring affected. In view of the comparable caries-susceptibility with respect to occlusal lesions in experiments 3 and 4, we have no explanation for the apparently lower susceptibility to smooth surface lesions among the rats in experiment 4 than in experiment 3. Possibly this result may be an expression of hereditary differences within the strain between the 2 populations. On the other hand, the severity of protein deficiency may have been somewhat less in experiment 4 than in experiment 3.

The mutant albino rats in experiment 5 proved to be far more prone to develop smooth surface lesions under similar dietary circumstances than either Harvard caries-resistant or Harvard caries-susceptible rats. Among 59 offspring of control group 17 whose mothers had been fed diet P_3, 29 (49.2%) developed smooth surface lesions. Again a potentiating influence of low-protein diets during the reproductive cycle was observed. In offspring from mothers fed diet P_5 alone or with a 1.0% sulfate supplement (groups 18 and 20), the frequency of rats with smooth surface lesions increased to 69.7 and 64.7%, respectively. Among 43 offspring in group 19 with a 1.0% DL-methionine supplement, 21 (48.8%) had smooth surface lesions. Thus 1.0% DL-methionine alone appeared to be capable of reducing the frequency of rats with smooth surface lesions to the same level as in control group 17. However, a reduction below the frequency for controls was observed among 50 offspring of females in group 21 (1.0% DL-methionine and 1.0% sulfate supplements) with 16 (32.6%) of the rats affected. This reduction is small and may not be significant or may not be related to the sulfate supplement since unavoidable hereditary differences do occur even between 2 groups of littermate females. However, the possibility remains of a supportive effect of sulfate which needs to be examined further. As in experiment 3, subgroups fed diet P_3 post-weaning routinely had a lower frequency of rats with smooth surface lesions than the subgroups which were continued with the maternal low-protein diets.

These data considered together clearly indicate that low-protein diets during the reproductive cycle were responsible for an increased susceptibility to smooth surface lesions. This potentiating influence was true in the 2 strains (Harvard caries-susceptible and Harvard caries-resistant) that have been invariably highly resistant to smooth surface lesions as well as in the mutant albino strain which has been moderately susceptible to the development of smooth surface lesions. The rate of development of smooth surface lesions among the affected animals appears to be about the same for the Harvard caries-susceptible rats and the mutant albino rats. In the Harvard caries-resistant rats the final number and size of smooth surface lesions in affected rats was about the same as for affected rats in the other 2 strains. However, 3 to 4 times as long was necessary in the Harvard resistant strain to develop this level of caries activity on the smooth surfaces. Among the offspring of low-protein females in all 3 strains, the relative rate of destruction of tooth substance by smooth surface lesions was from 1/10 to 1/5 as rapid as by occlusal lesions.

DISCUSSION

These strains of rats have been well characterized in our laboratories with respect to their individual susceptibilities to the initiation and progression of carious lesions in the occlusal sulci and on the smooth surfaces of the molar teeth. Various attempts to increase the caries activity of representatives of the Harvard caries-resistant strain by massive oral inoculation with supposedly caries-producing microorganisms had relatively little influence (7). As a corollary, various efforts were

made to restrict the availability of caries-producing microorganisms to rats of the Harvard caries-susceptible strain but we found that a highly cariogenic flora could become established exceedingly easily (7).

These studies indicated that the genetic constitutions of these strains provided blueprints for the development and maintenance of the teeth which included determination of whether the rats would be susceptible or resistant to the carious process in the presence of the various oral factors necessary for high caries activity. These characteristics appeared to be extremely rigorously maintained in the midst of vigorous attempts to overcome them. Yet the hereditary tendency toward high caries susceptibility or resistance may be masked partially or prevented completely from revealing itself phenotypically by such procedures as penicillin administration, on the one hand, or sialoadenectomy, on the other.

In the present experiments, the inherited blueprints with respect to tooth development, including caries susceptibility, could not be achieved completely due to the superimposition of a borderline protein deficiency during the reproductive cycle. In all 3 strains of rats, the responses were similar. Birth weights of the offspring were not strikingly different between those from protein-deficient and control female rats. However, growth during the lactation period was severely restricted among the offspring of protein-deficient mothers. Protein restriction during the reproductive cycle resulted in small teeth, delayed third molar eruption, missing cusps on the third molars and increased susceptibility to carious lesions both in the occlusal sulci and on the smooth surfaces.

The responses were actually very similar among the 3 strains despite various existing strain differences. For example, although the normal eruption time for third molars was significantly different from strain to strain, the experimental treatment employed caused significant delays in third molar eruption in each strain. In all 3 strains, a normal diet post-weaning had no ability to cause a more nearly normal eruption time than the previous low-protein diet had predetermined during pregnancy and lactation. Again with respect to susceptibility to dental caries in the occlusal sulci and on the smooth surfaces, all 3 strains responded to the protein-deficient diet with an increased caries activity but did not lose their identities. Although the Harvard and mutant albino caries-susceptible rats developed high caries activities in the occlusal sulci beyond their normal expectations, the Harvard strain of caries-resistant rats, even with an increase in caries activity, never attained a comparable caries activity to that of the controls in the 2 caries-susceptible strains. Among the Harvard caries-resistant rats in experiments 1 and 2, only 2 offspring with smooth surface lesions were observed, both from females fed low-protein diets. This low incidence among the experimental rats may appear unimportant until considered in the light of the lack of any previously affected rats among several thousand progeny of this strain. In the Harvard caries-susceptible strain, a low frequency of offspring with smooth surface lesions was observed among control groups which was increased appreciably among the offspring of protein-deficient females. The rats of the mutant albino strain were one stage further along the same trend. Although the frequency of rats prone to development of smooth surface lesions was high in the control groups, further increases were observed for the offspring from females fed low-protein diets.

The limiting factor during the reproductive cycle in the causation of the various abnormalities did not appear to be phosphate or sulfate, both of which would be significantly reduced in the low-protein diet and both of which could be importantly related to development and maintenance of the teeth. However, a good dose response was observed with DL-methionine, with the 1.0% level providing definite evidence of effectiveness with respect to all abnormalities. Thus the overall metabolic problem produced during reproduction was associated with protein restriction and one of the limiting amino acids was methionine.

This type of experiment contains inherent difficulties. Chief among these is the inability to guarantee the same degree of

protein deficiency from strain to strain or from experiment to experiment within a strain. To achieve the desired objectives a sufficiently rigorous deficiency must be imposed to cause demonstrable abnormalities without the development of such a gross deficiency that conception will not occur, or that the offspring will be sufficiently deformed to be nonviable, as in the major teratogenic studies. The range of protein level in the diet that will fulfill these requirements appears to be narrow.

Furthermore, the imposition of a borderline protein deficiency upon pregnant and lactating rats does not guarantee that the abnormalities developing in their offspring would be primarily the influence of the protein deficiency. By reason of the nature of the mother-child interrelationship in the rat during tooth development, it is almost, if not, impossible to design experiments with rats to support the statement that a protein deficiency in the offspring caused the observed abnormalities. Possibly in these experiments, protein restriction during the reproductive cycle led to inadequate milk production and caloric insufficiency among the offspring. We cannot state whether the abnormalities were the primary result of protein deficiency in the offspring or the secondary result of either a caloric insufficiency or other metabolic sequelae. In any case, these experiments demonstrated profound and lasting disturbances in tooth development that were precipitated by a nutritional abnormality. This environmental factor prevented the organism from fulfillment of its genetic blueprint.

Further studies are in progress to determine the crucial time for causing these abnormalities and whether other borderline deficiency states, including caloric restriction, during the reproductive cycle will have comparable or different influences upon the development and maintenance of the dental structures.

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Pantothenic Acid Deficiency in Cholesterol-fed Hamsters[1]

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ABSTRACT A 5-week study of hamsters fed a pantothenic acid-deficient diet, with and without cholesterol, is reported. The adverse effect of pantothenate deficiency on growth was greater in the cholesterol-fed female hamster than in the male. Pantothenic acid deficiency did not prevent the accumulation of lipid in the liver of the hamster as it did in the rat. However, cholesterol feeding resulted in even greater increases in liver and plasma cholesterol of the hamster than those of the rat. The lack of cholesterol in the hamster adrenal points to an unusual path of steroid metabolism, which needs further study.

The golden hamster has certain characteristics that make it potentially valuable as a laboratory animal for study of lipid metabolism. It is somewhat smaller than the rat, has a normal plasma cholesterol level of about 100 mg/100 ml, and responds to the feeding of small amounts of cholesterol by a marked increase in plasma as well as liver cholesterol. Although both atherosclerotic lesions and gallstones have been reported for the cholesterol-fed hamster (1), its food requirements are not fully known.

Preliminary studies showed that the hamster requires pantothenic acid for normal growth. Studies of the effects of pantothenic acid deficiency on lipid metabolism in the rat and the dog revealed species differences. In rats, the deposition of fat in the liver which occurred when cholesterol constituted 1% of the diet was largely prevented by concomitant pantothenic acid deficiency (2). The reverse was found to be true in dogs; lipid accumulation in the liver occurred when cholesterol was added to the diet, with and without a pantothenic acid supplement (3).

A parallel study with hamsters was considered likely to yield further information concerning species differences in the effects of pantothenic acid deficiency on lipid metabolism. The present paper reports data for a 5-week study of hamsters fed a pantothenic acid-deficient diet, with and without cholesterol.

METHODS

Four groups of weanling hamsters weighing, initially, between 44 and 45 g each were fed the following diets: 1, basal control, which contained (in per cent) "vitamin-free" casein,[3] 17; salts,[4] 4; fat,[5] 13.5; sucrose, 62.5; vitamin A mix,[6] 1; vitamin B mix,[6] 1; diet 2, same as diet 1, but with 1% cholesterol substituted for 1% sucrose. Diet 3, same as diet 1, with pantothenate omitted from the vitamin B mix. Diet 4, same as diet 2 without pantothenate. Five grams of lettuce, assaying 1.07 μg of pantothenic acid/g, were given to each hamster twice weekly.[7]

Animals were caged individually on wire screening, and had access to food and

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[1] Data are taken from a Master's thesis in Nutrition filed by Nina L. Cohen at the University of California, Berkeley.

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[3] "Vitamin-Free" Casein, Nutritional Biochemicals Corporation, Cleveland.

[4] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mix for use in experimental diets. J. Nutrition, *14*: 273.

[5] Primex, partially hydrogenated vegetable oil. Procter and Gamble, Cincinnati.

[6] The vitamin mixes were as follows: vitamin B mix: thiamine, 25 mg; riboflavin, 40 mg; folic acid, 20 mg; pyridoxine·HCl, 20 mg; Ca pantothenate, 100 mg; nicotinamide, 100 mg; p-aminobenzoic acid, 100 mg; biotin, 2 mg; choline chloride, 5 g; inositol, 5 g; ascorbic acid, 1 g. Sucrose was added to make 100 g. Vitamin A mix: vitamin A concentrate, 6 g at 600,000 IU/g; vitamin D, 275 mg (Delsterol, E. I. Du Pont de Nemours, Delaware, Maryland), at 700,000 IU/g; vitamin E, 180 g mixed tocopherols; vitamin K, 1 g menadione. Cottonseed oil was added to make 2,000 g.

[7] Pantothenic acid was assayed microbiologically by means of the method of W. K. Moss and B. D. Davis 1950 J. Bacteriol., 60: 733.

water at all times. The data reported are for animals killed after being supplied with the diet for 5 weeks. Several additional groups fed for different periods of time showed similar differences in liver and plasma lipid. Data for the latter are not reported because only a small percentage of the pantothenate-deficient animals survived when fed cholesterol for more than 5 weeks.

The procedure at autopsy was essentially that described previously for the rat (4) except that the animals were anaesthetized with amytal, and heparinized plasma was collected by heart puncture. Tissues were stored at 0°C until they could be analyzed.

Total liver lipids were determined by a modification of the method of Bloor (5), and cholesterol by the method of Sperry and Webb (6).

RESULTS

Growth rates of the hamsters were not materially depressed by cholesterol feeding (group 2) nor by pantothenate deficiency alone (group 3). However, when cholesterol was added to the pantothenate-deficient diet (group 4), females ceased to grow after the second week of receiving the diet and had a mean weight of only 67 g at the end of the fifth week of the dietary regimen. This was in contrast to mean weights of 93 and 91 g for controls of groups 1 and 2, respectively, and of 86 g for the pantothenate-deficient females of group 3. Males fed cholesterol with the pantothenate-deficient diet continued to grow slowly and had a mean weight of 78 g at 5 weeks, in contrast to 85 g for males of group 1 and 81 g for males of groups 2 and 3.

Livers were significantly heavier for the cholesterol-fed animals of group 2 than for controls of group 1 ($P < 0.01$). Percentages of liver lipids were significantly higher in the cholesterol-fed animals of both sexes (groups 2 and 4) (table 1) than in those fed no cholesterol (groups 1 and 3) ($P < 0.01$). An even greater difference was observed between the percentages of total cholesterol in the livers of the cholesterol-fed and the control animals (table 1). Free cholesterol concentrations were also increased by cholesterol feeding. Differences between the pantothenate-deficient and pantothenate-fed groups were small. The higher percentages of liver lipid and cholesterol in the female than in the male hamsters reflected the larger size of the females, and, in those of group 2, larger livers. Plasma cholesterol values were increased consistently by cholesterol feeding, even when the animals were pantothenate-deficient. Standard errors were so great as to make the differences of doubtful significance. This was also true of sex differences in plasma cholesterol values.

Adrenals of the female hamsters were significantly smaller ($P < 0.01$) than those of males. Mean values for males and females, respectively, were: group 1, 21 and 15 mg; group 2, 20 and 13 mg; group 3,

TABLE 1

Liver lipids and plasma cholesterol

Group	Sex	No. of animals	Liver lipids			Plasma cholesterol	
			Total lipids	Total cholesterol	Free cholesterol	Total	Free
			% wet wt	*% wet wt*	*% wet wt*	*mg/100 ml*	
1	M	16	2.7 ± 0.21[1]	0.18 ± 0.02	0.09 ± 0.01[2]	94 ± 9	41 ± 4
	F	16	2.6 ± 0.10	0.16 ± 0.02	0.08 ± 0.01[3]	130 ± 17	53 ± 9
2	M	16	11.6 ± 0.29	3.8 ± 0.29	0.18 ± 0.01[3]	238 ± 25	89 ± 9
	F	16	14.5 ± 0.80	5.5 ± 0.27	0.20 ± 0.02[3]	294 ± 29	113 ± 8
3	M	16	3.0 ± 0.18	0.13 ± 0.01	0.09 ± 0.00[2]	99 ± 13	39 ± 4
	F	17	2.7 ± 0.14	0.15 ± 0.02	0.09 ± 0.00[2]	114 ± 19	36 ± 4
4	M	16	11.8 ± 0.27	3.8 ± 0.09	0.15 ± 0.01[3]	205 ± 42	75 ± 8
	F	17	13.6 ± 0.68	5.0 ± 0.19	0.18 ± 0.01[2]	211 ± 28	69 ± 5

[1] Mean ± SE.
[2] Seven animals.
[3] Six animals.

19 and 13 mg; group 4, 19 and 11 mg. Insofar as could be determined, the hamster adrenals contained no digitonin-precipitable sterol that gave a positive Liebermann-Burchard test, that is, no cholesterol.

DISCUSSION

The data presented confirm previous reports of differences between the lipid and cholesterol metabolism of the hamster and those of other, frequently used species of small laboratory animals (1, 2).

The basal lipid and cholesterol levels in liver and plasma were not greatly different from those of the rat, but the increases in liver and plasma cholesterol in response to cholesterol feeding were much greater in the hamster. If the diets can be considered to have been sufficiently deficient in pantothenic acid, the lack of a dietary source of Co A would apparently have less effect on fat and cholesterol accumulation in the hamster than in the rat (2).

The fact that no cholesterol could be demonstrated in the adrenals of the hamsters agrees with the observations of Shindler and Knigge (7), Schaefer et al. (8), and Marks et al. (9), who suggest that, in the hamster, short-chain compounds go directly to C-21 rather than to C-27 compounds. That pantothenic acid-deficient hamsters did not generally survive cholesterol feeding for more than 5 weeks might also indicate a difference in the intermediary metabolism of cholesterol of the hamster.

In view of these differences, this species offers a possible tool for study of intermediary steps in lipid metabolism.

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Effect of Dietary Lipids upon Mitochondrial Composition and Swelling[1]

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ABSTRACT Liver mitochondria from rats fed 15% corn oil or 7% cod liver oil were more sensitive to silver and were less sensitive to thyroxine as swelling agents than similar preparations from animals fed 15% beef fat. No significant differences in swelling response to phosphate or copper were observed. Mitochondria from chicks fed 4 or 8% corn oil were more sensitive to silver, copper, and mercuric ions as swelling agents than similar preparations from chicks fed 4 or 8% beef fat. Preparations of mitochondria in sucrose with 0.01% α-tocopherol were unchanged in their responses to swelling agents. Chick liver mitochondria prepared in sucrose with 0.01 M propyl gallate were more sensitive to copper, whereas rat mitochondria prepared with propyl gallate were less sensitive to thyroxine. Mitochondria from both chicks and rats prepared with added propyl gallate were unchanged in their sensitivity to silver.

Since first noting that the composition of fatty acids of the brain and erythrocytes were partially dependent upon the type of fat consumed (1), this laboratory has been pursuing the physiological implications of such changes (2–5), proceeding on the assumption that all components of the cell, including the lipid-containing enzymes and the subcellular structural lipids, could have their fatty acid compositions altered by diet (6). The influence of dietary lipids upon compositions of brain mitochondria and other tissues has been documented (7, 8).[2,3] Studies by Tapley (9) and by Lehninger et al. (10) as well as other investigators suggested that the use of *in vitro* mitochondrial swelling in response to very low concentrations of certain toxic ions or substances as a test system might be a worthwhile way of evaluating functional changes related to alterations in the fatty acids of mitochondrial membranes. Hayashida and Portman (7) reported that mitochondria from rats fed a diet deficient in essential fatty acids swelled more readily than those given an adequate diet. The degree of swelling appeared to be inversely related to the level of essential fatty acids in the mitochondria, suggestive of increased fragility associated with the essential fatty acid deficiency. The work presented here describes the differences obtained in the swelling response of mitochondria from rats fed diets considered to be nutritionally adequate, but containing different types of fat.

EXPERIMENTAL

Weanling male Sprague-Dawley rats were fed purified diets containing 15% beef fat, 15% corn oil or 7% cod liver oil as previously described (11), except that salt mixture 446 (12), which in addition furnished 0.356 ppm of Na_2SeO_3 and 1.07 ppm of $K_2Cr_2O_7$ to the total diet, was used instead of salts USP 14. Fat-soluble vitamins were supplemented orally. The animals were killed after 11 to 14 weeks. Mitochondria were prepared by a procedure taking into consideration the methods described by Witter et al. (13) and by Lehninger et al. (10). Liver homogenates were made in 9 parts of ice-cold 0.33 M sucrose containing 0.001 M EDTA at pH 7.0. Nuclei and debris were removed by centrifugation at $650 \times g$ for 7 minutes. Mitochondria were sedimented by centrifugation at $11{,}000 \times g$ for 15 minutes, washed and recentrifuged in 0.33 M sucrose, and resuspended in 0.33 M sucrose.

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Mitochondrial aliquots of about 50 μliter were added to 1-cm square cuvettes containing test substances in 3.0 ml of 0.3 M sucrose with 0.02 M Tris buffer at pH 7.4, and optical density readings were made in a Beckman Model B spectrophotometer at 520 mμ, at a temperature of 22 to 23°C (9, 10). Initial optical densities were maintained close to 0.450 and within a 7% range for any set of 6 cuvettes that were being read at the same time.

Crossbred Arbor-Acres × Vantress cockerals were fed essentially the diet of Bosshardt et al. (14, 15) for 28 to 30 days containing either corn oil or beef fat at 4 and 8% levels. The diets were isocalorically similar. Chick liver mitochondria were isolated and tested with swelling agents in the same manner as rat preparations.

Stock solutions of 3×10^{-4} M $AgNO_3$, 1×10^{-3} M $CuCl_2$, 3×10^{-4} M $HgCl_2$, and 0.045 M K^+ phosphate (pH 7.4) were prepared for addition to cuvettes to the final concentrations indicated. Thyroxine was freshly prepared at 1.2×10^{-3} M by dissolving in a slight excess of KOH, diluting to 9.5 ml and adding 0.5 ml of Tris-buffered sucrose.

Nitrogen content of mitochondria was determined by a modified Nessler procedure (16), and fatty acid compositions of lipids by gas-liquid chromatography (4, 17–19). Preliminary studies showed that samples of mitochondria from livers of rats from all 3 diet groups had the same ratios of initial optical density to total nitrogen.

RESULTS

Rat liver mitochondria prepared by the method described here were quite stable for 30 minutes in the absence of a swelling agent (fig. 1). The change in optical density was 6.3 ± 1.7% with mitochondria from the beef fat group, and 3.9 ± 1.2 with samples from the corn oil and cod liver oil groups. These differences were not enough to be significant.

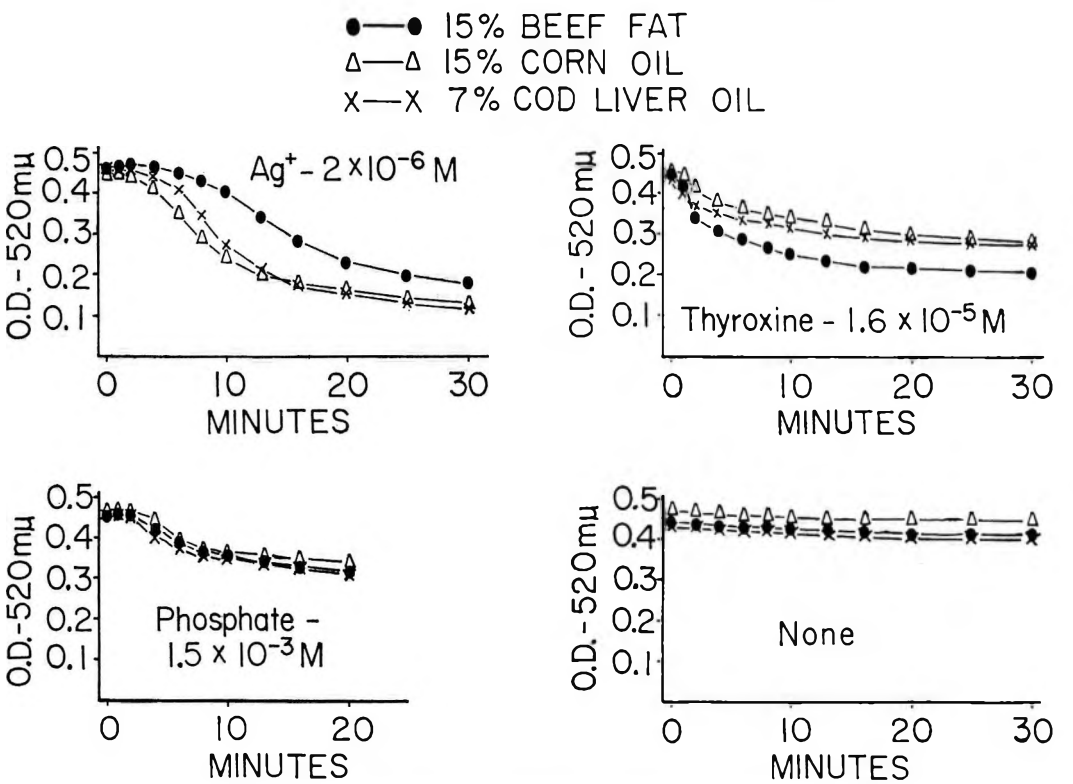

Fig. 1 Swelling of liver mitochondria from rats fed different lipid diets.

Using 2×10^{-6} M silver ion as a test substance, there was a slight initial increase in optical density followed by a rapid drop to about one-third of the original value (fig. 1). With mitochondria from rats fed 15% beef fat, 20.1 ± 1.9 minutes elapsed for a 50% decrease in optical density, in comparison with 13.0 ± 0.7 and 14.2 ± 1.3 minutes observed using preparations from rats fed 15% corn oil or 7% cod liver oil, respectively. On the other hand, the response obtained with 1.6×10^{-5} M thyroxine was greatest with mitochondria from rats fed 15% beef fat (fig. 1). After 30 minutes, the optical density of these mitochondria decreased 46.8 ± 2.4%, as compared with 32.7 ± 2.8 and 35.1 ± 2.6% obtained with particles from rats fed 15% corn oil and 7% cod liver oil, respectively. Swelling in response to 1.5×10^{-3} M phosphate was prompt, but no significant differences were observed among the 3 dietary groups. Preliminary tests with 1×10^{-5} M cupric ion also showed no differences in swelling among the mitochondria from various diet groups.

Chick liver mitochondria responded to the same degree with 3×10^{-6} M silver as rat preparations with 2×10^{-6} M silver (fig. 2). With 1×10^{-5} M cupric ion, the change in optical density was slow until after 15 to 20 minutes, whereas the rate of response to 4×10^{-6} M mercuric ion was rather uniform. Using 3×10^{-6} M silver, the optical density of mitochondria from chicks fed 8% beef fat decreased 46.0 ± 3.0%, as compared with 58.0 ± 0.9% observed with preparations from an 8% corn oil group. With 1×10^{-5} M cupric ion, the respective decreases in optical density were 33.0 ± 1.6% versus 43.8 ± 1.5%, and with 4×10^{-6} M mercuric ion, they were 37.9 ± 1.9% versus 45.7 ± 1.0%. In the absence of a swelling agent, the optical density of mitochondria from the group fed beef fat decreased 14.6 ± 1.3%, as compared with 12.4 ± 1.4% with preparations from the group fed corn oil. When 4% levels of beef fat

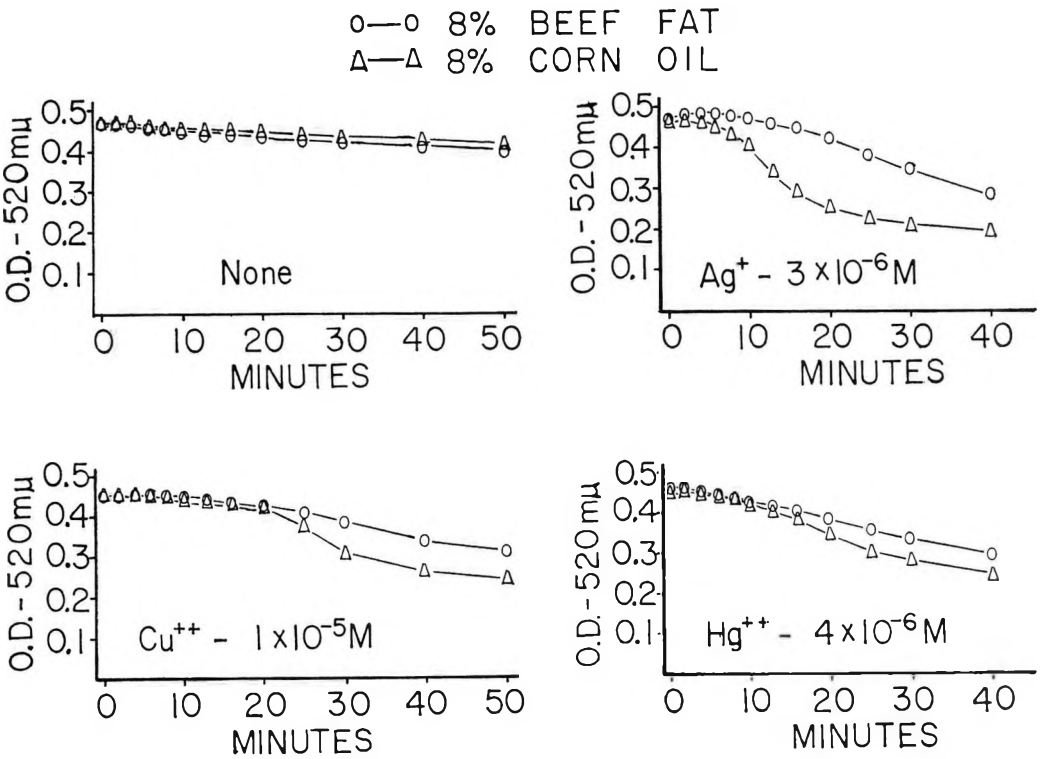

Fig. 2 Swelling of liver mitochondria from chicks fed different lipid diets.

and corn oil were fed to the chicks instead of 8%, the observed differences in mitochondrial swelling were even greater. Preliminary tests indicated that chick liver mitochondria did not swell in response to thyroxine, similar to mitochondria from newborn rats (20).

Several duplicate preparations of mitochondria were made in sucrose containing either 1×10^{-4} M propyl gallate, or 0.01% α-tocopherol suspension, to evaluate the effects of antioxidants upon the observed swelling. Chick liver mitochondria prepared in sucrose with 0.01% α-tocopherol were unchanged in their responses to silver and copper regardless of diet. The responses of rat liver mitochondria to silver and thyroxine were also unaffected by preparation in tocopherol. Both rat and chick mitochondria prepared with 0.01 M propyl gallate were unchanged in their sensitivity to silver, but 4 samples of chick mitochondria from each of the 8% beef fat and 8% corn oil groups prepared in propyl gallate resulted in 10.7 ± 3.0% and 8.2 ± 2.6% greater changes in total optical density, respectively, with copper as the test substance. On the other hand, rat liver mitochondria prepared with propyl gallate were significantly less sensitive to thyroxine, the decreases in total optical density being 11.1 ± 3.2%, 15.2 ± 2.1%, and 16.5 ± 1.6% lower than with corresponding control mitochondria prepared from animals on 15% beef fat, 15% corn oil and 7% cod liver oil, respectively. Seven comparisons were made for each diet.

Fatty acid compositions of the liver mitochondria are shown in table 1. Samples from rats fed beef fat were characterized by higher percentages of palmitoleic ($P < 0.01$), oleic ($P < 0.001$) and 5, 8, 11-eicosatrienoic acids ($P < 0.001$), with less linoleic ($P < 0.001$), and arachidonic ($P < 0.01$) acids, as compared with the corn oil group (21), whereas mitochondria from animals fed cod liver oil had higher levels of palmitoleic ($P < 0.001$), oleic ($P < 0.001$), 5, 8, 11, 14, 17-eicosapentaenoic ($P < 0.001$), 7, 10, 13, 16, 19-docosapentaenoic ($P < 0.005$), and 4, 7, 10, 13, 16, 19-docosahexaenoic ($P < 0.001$) acids, with much less linoleic ($P < 0.001$), and arachidonic ($P < 0.001$) acids, and slightly less 4, 7, 10, 13, 16-docosapentaenoic acid in comparison with the corn oil group, the last 3 having essential fatty acid configuration (22). Similarly, mitochondria from chicks fed beef fat had lower levels of the essential fatty acid members, linoleic ($P < 0.001$), arachidonic

TABLE 1

Fatty acid compositions of liver mitochondrial lipids from rats and chicks

Fatty acid	Rat diets			Chick diets	
	15% Beef fat (8)[1]	15% Corn oil (9)	7% Cod liver oil (10)	8% Beef fat (8)	8% Corn oil (8)
	%	%	%	%	%
16:0[2]	19.9 ± 2.0[3]	17.0 ± 1.4	23.3 ± 1.5	13.1 ± 0.5	12.5 ± 0.7
18:0	24.2 ± 1.4	22.3 ± 2.0	15.9 ± 1.2	24.2 ± 1.5	24.9 ± 1.0
16:1	2.5 ± 0.3	1.3 ± 0.2	6.3 ± 0.6	5.6 ± 0.6	1.0 ± 0.3
18:1	19.2 ± 0.9	7.4 ± 0.7	17.2 ± 0.7	30.8 ± 0.9	8.8 ± 0.9
18:2 (Δ 9, 12)	9.1 ± 0.7	19.6 ± 0.5	3.6 ± 0.2	8.0 ± 0.7	22.9 ± 1.4
20:3 (Δ 5, 8, 11)	2.8 ± 0.6	0.0	0.2 ± 0.05	6.0 ± 0.9	0.3 ± 0.1
20:3 (Δ 8, 11, 14)	1.3 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	1.3 ± 0.2	1.9 ± 0.4
20:4 (Δ 5, 8,11, 14)	11.8 ± 0.9	21.0 ± 1.9	5.3 ± 0.5	6.5 ± 0.9	20.4 ± 1.4
22:4 (Δ 7, 10, 13, 16)	0.2 ± 0.1	0.5 ± 0.1	0.1 ± 0.05	0.2 ± 0.05	0.8 ± 0.1
20:5 (Δ 5, 8, 11, 14, 17)	0.3 ± 0.1	0.3 ± 0.1	9.7 ± 0.9	0.5 ± 0.05	0.3 ± 0.1
22:5 (Δ 4, 7, 10, 13, 16)	0.5 ± 0.1	1.0 ± 0.2	0.2 ± 0.05	0.2 ± 0.03	1.4 ± 0.3
22:5 (Δ 7, 10, 13, 16, 19)	0.5 ± 0.1	0.4 ± 0.1	1.9 ± 0.2	0.2 ± 0.1	0.1 ± 0.05
22:6 (Δ 4, 7, 10, 13, 16, 19)	4.8 ± 0.9	3.4 ± 0.7	13.4 ± 1.4	0.6 ± 0.1	0.9 ± 0.1

[1] Number of samples in parentheses.
[2] The first figure represents the number of carbon atoms, the second the number of double bonds.
[3] ± SE.

($P < 0.001$), 7, 10, 13, 16-docosatetraenoic ($P < 0.001$), and 4, 7, 10, 13, 16-docosapentaenoic ($P < 0.002$) acids, with compensatory increases in monoenes ($P < 0.001$) and 5, 8, 11-eicosatrienoic ($P < 0.001$) acid, in comparison with samples from chicks fed corn oil.

DISCUSSION

In the present experiments, varying the fatty acid compositions of mitochondria resulted in altered responses to swelling agents. Changes in the fatty acids of mitochondrial membranes might have altered the rate of penetration of these active ions to their site of action. Collins reported that the fastest rate of exchange of radioactive phosphate in liver lecithin was in the fraction where both fatty acids of the glyceride molecule were unsaturated (23), supporting the possibility that membrane structures with more polyunsaturated fatty acids may transport certain substances more readily. Presumably, the actions of silver and copper ions may be related to their ability to penetrate and combine with sulfhydryl groups in mitochondria (9), but some difference in their modes of action is still suggested by the fact that the various diets used resulted in different responses to silver using both chick and rat preparations, whereas an altered response to copper due to diet was observed only with liver mitochondria from chicks but not from rats.

Since mitochondrial swelling could possibly be related to the peroxidation of structural polyunsaturated fatty acids (24, 25), several preparations were made with sucrose containing tocopherol or propyl gallate, to determine whether by protecting the structural lipids from peroxidation with antioxidants, swelling might be prevented or reversed under the present conditions. However, tocopherol had no effect upon these results, and propyl gallate actually increased the sensitivity of chick liver mitochondria to copper. On the other hand, propyl gallate partially reversed the effect of thyroxine upon rat mitochondria, especially with preparations from corn oil and cod liver oil groups, but had no effect when silver was the test substance. Possibly the difference in response to thyroxine may be related to the level of bound DPN on the mitochondrion (10), and that mitochondria having lower levels of polyunsaturated fatty acids, such as those from rats fed 15% beef fat, may be more able to retain bound DPN. Another possibility is that thyroxine may cause the release of a greater amount of the lipid-swelling factor described by Lehninger and Remmert (26) when mitochondria from rats fed beef fat were tested. This swelling factor was described as having a composition lower in polyunsaturated fatty acids than the mitochondria themselves (27). The particles obtained from animals fed beef fat could possibly elaborate a greater amount of this factor by having more of the fatty acids which resemble its composition. The effects of antioxidants upon swelling of mitochondria are not conclusive. It is not possible to state at this time whether the differences in swelling observed are related to membrane phenomena or to differences in the rate of peroxidation of the lipid membranes *in vitro*.

It nevertheless appears that variation in dietary fat, which can alter the fatty acid composition of mitochondria, can also affect certain aspects of mitochondrial function, suggesting that other subcellular functions might also be affected in a similar manner by diet.

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Biological Responses of Young Rats Fed Diets Containing Genistin and Genistein[1,2]

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ABSTRACT Young male rats were fed diets containing 0.1 and 0.5% of genistin or genistein for 4 weeks to determine the effects of these plant estrogens on weight gain, on the development of the adrenals, kidneys, spleens and testes and on certain aspects of the metabolism of copper, iron, zinc, calcium, phosphorus and magnesium. A dietary level of 0.5% of genistin or genistein resulted in significant decreases in weight gain and in the weights of the kidneys and spleens. Rats fed diets containing either plant estrogen had liver and spleen iron levels that were significantly higher than those of the controls, but hemoglobin and tissue copper levels were not affected by the feeding of either genistin or genistein. Levels of zinc in the bones and livers of the estrogen-fed rats were higher than the corresponding zinc levels of the controls. Genistin and genistein enhanced the deposition of calcium, phosphorus and magnesium in the bones of young rats. Bone Ca/P ratios were lower in the animals receiving diets containing the plant estrogens.

Within recent years numerous investigations have been directed towards the detection, isolation and characterization of naturally occurring plant estrogens. One of these compounds which has been studied is genistin, an isoflavone glucoside found in soybean meal. Genistin and its aglucone, genistein, have been shown to have adverse effects on reproduction, weight gain and the development of several internal organs of young mice (1–3). Previous results indicate that the effects of genistin and genistein on weight gain and on the development of the kidneys, ovaries and testes of the mouse are not the same as the effects of estradiol and stilbestrol (2, 3). These studies, however, suggest a further exploration of the biological responses of animals fed the plant estrogens utilizing the results of previously conducted studies with naturally occurring and synthetic estrogens as a basis for possible areas of investigation.

The administration of naturally occurring or synthetic estrogens has been shown to cause decreases in blood hemoglobin levels in several species of animals (4–6). Ramsay and Campbell (4) reported slight increases in liver non-heme iron levels of pullets injected with estradiol benzoate. Results of several studies have indicated that changes in copper and zinc levels occur in human subjects during pregnancy and following the administration of estrogens (7–11). Macola (12) reported that estrone caused small decreases in the inorganic and the total phosphorus content of the femurs of rats and an increase in the Ca/P ratio of the bone. Bone calcium values, however, were unchanged. Wentworth et al. (13) observed that levels of bone calcium and bone Ca/P ratios were higher in rats receiving estradiol benzoate than in control animals. Bone phosphorus remained essentially the same as the controls in their studies.

In view of these results, the present study was conducted to determine whether the feeding of genistin and genistein to immature rats had any effect on hemoglobin level, on the deposition of copper, iron and zinc in certain body tissues, and on bone mineralization. The effects of genistin and genistein on weight gain and on the weight of the adrenals, kidneys, spleen and testes were also observed to see whether the rat responded the same as the mouse does to these compounds.

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EXPERIMENTAL PROCEDURES

Young male Sprague-Dawley rats were used in this study. The animals were housed in individual wire-bottom cages, allowed free access to feed and water and kept on the dietary regimens for 4 weeks. A randomized block design consisting of 5 treatments (2 levels of genistin, 2 levels of genistein and one control) and 6 replications was used in this study. The rats were randomized into replications according to initial body weights. Test treatments and animals within a replication were randomly assigned to individual cages.

The basal diet used in the study consisted of the following (in per cent): casein, 19;[3] corn starch, 63;[4] vegetable oil, 10;[5] mineral mix, 4;[6] vitamin mix, 2;[7] cellulose, 2;[8] and oleum percomorphum.[9] Levels of genistin and genistein (0.1 and 0.5%) used in the study were substituted for equal amounts of starch in the basal diet.

A method similar to that outlined by Walter (14) was used to extract and prepare genistin and genistein from commercial soybean meal.

At the end of the experimental period, a blood sample was taken from the tail of each animal. Oxyhemoglobin determinations were made on the blood samples by the method of Shenk et al. (15).

At the end of the experimental period, all animals were killed, and the adrenals, kidneys, livers, spleens and testes were removed and weighed. The kidneys, livers and spleens were prepared for trace mineral analyses by wet-ashing with nitric and perchloric acids on a hot plate. The ash of each individual tissue was dissolved in 0.6 N HCl and brought to a volume of either 10 or 25 ml with redistilled water. Copper and iron determinations of the samples were made by the methods of Parks et al. (16) and Kitzes et al. (17), respectively, as modified by Matrone et al. (18). The method outlined by McCall et al. (19) was used for the determination of zinc.

Both femurs of each animal were also removed at the time the animals were killed. The bone samples were cleaned of muscle and connective tissue, dried, weighed and subjected to the wet-ash treatment previously mentioned. The ash of each pair of bones was dissolved in 0.6 N HCl, brought to a volume of 100 ml with distilled water and analyzed for calcium, phosphorus, magnesium and zinc by the methods of Weybrew et al. (20), Simonsen et al. (21, 22) and McCall et al. (19), respectively.

[3] Vitamin Test Casein, Nutritional Biochemicals Corporation, Cleveland.

[4] Globe Easy-flow Corn Starch 3366, Corn Products Sales Company, Greensboro, North Carolina.

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

[6] Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland.

[7] Each 100 g of the vitamin mix contained (in milligrams): biotin, 1; folic acid, 5; 0.1% vitamin B_{12} (with mannitol), 0.1; thiamine·HCl, 25; pyridoxine·HCl, 25; 2-methyl-naphthoquinone, 50; riboflavin, 50; nicotinic acid, 50; Ca pantothenate, 150; p-aminobenzoic acid, 500; (in grams): inositol, 5; choline chloride, 7.5; DL-methionine, 30; corn starch, 56.6. All vitamins and methionine were purchased from Nutritional Biochemicals Corporation, Cleveland.

[8] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[9] Each 1000 g of diet contained 24 drops of oleum percomorphum, Mead Johnson and Company, Evansville, Indiana.

TABLE 1

Weight gain and organ weights of young rats[1] fed genistin and genistein for 4 weeks[2]

Treatment	Weight gain	Organ weight (fresh-weight basis)			
		Adrenals[3]	Spleen	Kidneys[3]	Testes[3]
	g	*mg*	*mg*	*g*	*g*
Control	186 ± 4	53.8 ± 2.6	858.5 ± 72.7	2.21 ± 0.07	2.94 ± 0.08
0.1% Genistin	182 ± 2	51.5 ± 2.9	781.2 ± 29.7	2.18 ± 0.06	2.79 ± 0.09
0.5% Genistin	147 ± 6	49.6 ± 1.8	624.6 ± 47.2	1.82 ± 0.11	2.74 ± 0.08
0.1% Genistein	184 ± 6	49.9 ± 1.8	785.1 ± 22.6	2.16 ± 0.05	3.12 ± 0.18
0.5% Genistein	171 ± 4	54.4 ± 2.3	719.2 ± 19.9	2.02 ± 0.09	2.88 ± 0.09
LSD,[4] 0.05	13	6.5	123.6	0.18	0.32
LSD, 0.01	17	8.8	168.6	0.25	0.44

[1] Sprague-Dawley rats averaging 48 g in weight initially.
[2] Each figure represents mean of 6 animals with SE.
[3] Both organs together.
[4] Least significant difference at specified probability levels.

All data were subjected to an analysis of variance. Statements of significance are based on odds of at least 19 to 1 ($P \leq 0.05$). Least significant difference (LSD) values for each criterion studied were calculated and give an indication of the difference between 2 treatment means required to show significance.

RESULTS

Weight gain and tissue weights. The effects of dietary genistin and genistein on the weight gain and the weight of the adrenals, kidneys, spleens and testes of young rats are given in table 1. When a level of 0.5% of genistin was fed to young rats, a highly significant decrease ($P \leq 0.01$) in weight gain resulted, whereas a level of 0.5% of genistein was associated with a decrease in weight gain which was significant at the 5% level of probability. The weights of the kidneys and the spleens of rats fed 0.5% of genistin were markedly lower ($P \leq 0.01$) than those of the control animals. A level of 0.5% of dietary genistein resulted in decreases in the weights of the kidneys and the spleens which were significant at the 5% level of probability. The effects of genistin and genistein on the kidneys and the spleens were not significant after an adjustment was made for final body weight by covariance, indicating that the differences in kidney and spleen weights were probably associated with differences in body weight. In this study the weights of the adrenals or the testes of the controls were not significantly different from the weights of the adrenals or the testes of the animals receiving either genistin or genistein. The weights of the testes of the animals receiving genistin, however, were significantly lower ($P \leq 0.05$) than the weights of the testes of the rats receiving genistein.

Hemoglobin, copper and iron. Mean hemoglobin, copper and iron values for rats fed varying levels of genistin and genistein are shown in table 2. The feeding of either plant estrogen at levels that affected weight gain had no apparent effect on hemoglobin since the mean hemoglobin values of the rats on different treatments were essentially the same. The copper content of the kidneys, livers and spleens of animals receiving either plant

TABLE 2

Effects of genistin and genistein on hemoglobin, copper and iron values of young rats[1]

Treatment	Hemoglobin	Tissue and constituent					
		Kidneys[2]		Liver		Spleen	
		Cu	Fe	Cu	Fe	Cu	Fe
	g/100 ml blood	*μg/g dry weight*		*μg/g dry weight*		*μg/g dry weight*	
Control	13.1 ± 0.3	29.9 ± 3.3	152 ± 7	12.0 ± 0.5	299 ± 35	11.6 ± 1.4	689 ± 40
0.1% Genistin	13.2 ± 0.5	26.5 ± 3.3	176 ± 17	11.8 ± 1.1	357 ± 41	13.6 ± 1.2	755 ± 52
0.5% Genistin	13.0 ± 0.3	27.8 ± 3.4	156 ± 10	10.4 ± 1.3	406 ± 32	15.3 ± 1.6	826 ± 53
0.1% Genistein	13.2 ± 0.3	29.6 ± 1.8	161 ± 9	13.7 ± 0.6	422 ± 25	11.9 ± 0.9	864 ± 32
0.5% Genistein	13.2 ± 0.3	26.2 ± 3.5	165 ± 7	13.5 ± 1.6	433 ± 47	13.4 ± 1.2	851 ± 69
LSD,[3] 0.05	1.0	9.5	30	3.3	107	3.9	122
LSD, 0.01	1.4	13.0	41	4.5	146	5.3	166

[1] Each figure represents mean of 6 animals with SE.
[2] Both organs together.
[3] Least significant difference at specified probability levels.

estrogen were not significantly different from the copper content of the corresponding tissues of the controls. The feeding of genistin was associated with a decrease in liver copper, whereas dietary genistein resulted in an increase in liver copper. The effects of genistin and genistein on liver copper deposition were significantly different ($P \leq 0.05$). Neither estrogen caused any significant change in kidney iron, but the feeding of either genistin or genistein was associated with increases in the iron content of the livers and spleens which were significantly different ($P \leq 0.05$) from the levels of iron observed in these tissues in the control animals. Genistein had a greater influence on iron deposition than did genistin.

Zinc. The effects of genistin and genistein on bone and liver zinc deposition are given in table 3. Rats receiving diets containing either estrogen had higher concentrations of zinc in their bones than the controls, but an analysis of the data revealed that these increases in zinc levels were not statistically significant. The addition of genistin to the diet resulted in small decreases in liver zinc levels, but the feeding of genistein was associated with increases in liver zinc levels. The mean liver zinc content of rats receiving 0.5% of genistin was significantly different ($P \leq 0.05$) from the mean liver zinc content of rats fed 0.5% of genistein.

TABLE 3

Effects of genistin and genistein on zinc accumulation in bone and liver of young rats[1]

Treatment	Bone[2]	Liver[3]
	μg Zn/g dry weight	
Control	82.4 ± 35.7	65.1 ± 4.8
0.1% Genistin	72.0 ± 17.4	66.1 ± 4.2
0.5% Genistin	142.6 ± 33.4	58.6 ± 6.3
0.1% Genistein	136.4 ± 41.6	83.2 ± 10.7
0.5% Genistein	151.2 ± 48.7	90.3 ± 14.7
LSD,[4] 0.05	122.0	26.0
LSD, 0.01	171.0	35.5

[1] Each figure represents mean ± SE.
[2] Mean of 4 animals.
[3] Mean of 6 animals.
[4] Least significant difference at specified probability levels.

Calcium, phosphorus and magnesium. Mean values for bone calcium, phosphorus, magnesium and Ca/P ratios are given in table 4. Rats receiving 0.5% levels of genistin had mean bone calcium and phosphorus values which were significantly higher ($P \leq 0.05$) than the mean bone calcium and phosphorus values of the controls. The addition of 0.5% of genistein to the diet resulted in increased calcium and phosphorus levels in the bones which approached significance at the 5% level of probability. The effect of either plant estrogen on bone calcium and phosphorus was approximately the same. Further analyses of the data revealed a linear relationship between calcium or phosphorus deposition and the level of genistin or genistein added to the diet. Although bone calcium and phosphorus levels increased in the estrogen-fed rats, the Ca/P ratios of these animals were lower than those of the controls. Increasing levels of genistin and genistein were associated with significant linear increases ($P \leq 0.05$) in bone magnesium. Under the conditions of this study, both estrogens were associated with essentially the same amount of increase in bone magnesium.

TABLE 4

Effects of genistin and genistein on bone calcium, phosphorus, magnesium and Ca/P ratios of young rats[1]

Treatment	Bone constituent			
	Ca	P	Mg	Ca/P ratio
	mg/g dry weight			
Control	186.8 ± 7.8	40.2 ± 5.1	2.79 ± 0.08	4.97 ± 0.58
0.1% Genistin	194.4 ± 7.2	48.5 ± 2.2	3.15 ± 0.17	4.06 ± 0.28
0.5% Genistin	203.6 ± 4.0	56.2 ± 5.5	3.40 ± 0.06	3.78 ± 0.32
0.1% Genistein	199.5 ± 5.3	44.4 ± 3.2	3.11 ± 0.17	4.60 ± 0.32
0.5% Genistein	201.7 ± 6.9	51.2 ± 4.4	3.22 ± 0.13	4.04 ± 0.26
LSD,[2] 0.05	15.4	12.9	0.38	1.20
LSD, 0.01	21.0	17.6	0.52	1.63

[1] Each figure represents mean of 6 animals with SE.
[2] Least significant difference at specified probability levels.

DISCUSSION

Although both genistin and genistein caused significant decreases in weight gain when fed at 0.5% levels, the results indicate that the glucoside is more toxic than the aglucone. The reason for this difference in toxicity between the 2 compounds is not apparent. Potter and Kummerow (23) reported that purified alfalfa and soybean saponins inhibited the growth of chicks, but the sapogenins derived from these saponins had no effect on the growth of chicks. Thus, a similarity in action may exist between the conjugated and the unconjugated forms of these compounds with respect to toxicity properties.

The data also suggest that genistin exerts a greater effect on certain body tissues than genistein does. Since the weights of these tissues appeared to be related to body weight, the differences in the effects of the estrogens on a certain organ may be entirely accounted for by differences in the toxicity of the two estrogens on growth. In contrast with the results obtained with mice (2, 3), the testes weights of rats receiving levels of genistin and genistein which adversely affected weight gain were not significantly different from the testes weights of the controls. The depressing effects of these estrogens on the weights of the kidneys and the spleens were similar to those previously reported in mice (2, 3).

That hemoglobin levels of rats fed genistin and genistein were essentially the same as those of control animals could be interpreted to mean that iron absorption and utilization are apparently not adversely affected by these plant estrogens. The marked increases in the iron concentrations of the livers and spleens of rats receiving genistin and genistein suggest that these plant estrogens may have an influence on iron deposition.

Dukes and Goldwasser (6) reported that the administration of estradiol to rats resulted in decreased hemoglobin levels, but the results of this study indicate that genistin and genistein have no apparent effect on hemoglobin formation. These results, however, are in agreement with reports of other researchers (24, 25) that no changes in hemoglobin levels occurred in animals receiving either estradiol benzoate or stilbestrol.

The observed increase in bone and liver zinc associated with the feeding of genistin and genistein suggests the possibility that these plant estrogens also influence the movement of zinc in the animal body.

The results of this study indicate that genistin and genistein enhance the deposition of calcium, phorphorus and magnesium in the bones of young rats. Presumably, these plant estrogens could be influencing the absorption and utilization of calcium, phosphorus and magnesium or they could be affecting the mechanism (s) which controls the deposition of these minerals in the bones.

Some of the biological responses of young rats which are reported to be influenced by naturally occurring and synthetic estrogens are affected by dietary levels of genistin or genistein. The results of this study, however, tend to indicate that the overall actions of genistin and genistein on the criteria studied are not the same as would be expected to occur with either naturally occurring or synthetic estrogens.

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Relative Effects of Rapeseed Oil and Corn Oil on Rats Subjected to Adrenalectomy, Cold, or Pyridoxine Deprivation

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ABSTRACT Male rats that were adrenalectomized, sham-operated, maintained in the cold, or deprived of pyridoxine received 20% rapeseed oil or corn oil in a purified basal diet. Adrenalectomy did not alter the lower food intake and weight gain of rats fed rapeseed oil compared with those fed corn oil. Cold or pyridoxine deprivation had a more adverse effect with rapeseed oil than corn oil, although the total vitamin B_6 in the liver was independent of the type of dietary oil. Rats deprived of pyridoxine and fed rapeseed oil deposited a smaller proportion of fat than those fed corn oil. Analyses of carcass fatty acids showed that in rats deprived of pyridoxine, corn oil, but not rapeseed oil, increased the proportion of palmitic acid and reduced that of linoleic acid. Erucic acid was somewhat more concentrated in the carcass fat of rats not supplied with pyridoxine than in those that were.

In studies on the nutritional properties of rapeseed oil (1–3), rats fed the oil with an adequate basal diet in a satisfactory environment consumed less food and gained less weight than those fed corn oil. Information was lacking about the nutritional properties of rapeseed oil under more adverse conditions.

Adrenalectomy, low temperature and pyridoxine deprivation are conditions that may be implicated in some way with fat metabolism. Cohn et al. (4) showed a decrease in the proportion of fat in the tissues of adrenalectomized rats fed ad libitum. That such animals utilize fat differently was suggested by Engel and Engel (5), but was not confirmed by Perry and Bowen (6). Sellers et al. (7) demonstrated that varying the fat in the diet from 5.5 to 44.5% did not affect growth or survival in the cold, and that food intake was regulated according to its caloric value. The effect on rats maintained in the cold of an oil that usually reduces food intake is not known. Since pyridoxine may not be concerned specifically with essential fatty acids, but have a general effect on all fatty acids (8), the influence of rapeseed oil was studied in rats deprived of pyridoxine.

It is the purpose of this paper to compare rapeseed oil and corn oil in rats under conditions that may alter fat metabolism.

METHODS

Male, Wistar-strain rats of the Food and Drug colony were divided into groups of 10 in a randomized block design based on body weight. They were housed individually in screen-bottom cages, and supplied ad libitum with the following diet: (in per cent) rapeseed oil[1] or corn oil, 20; cornstarch, 30; sucrose, 20; casein, 19; vitamin mixture in casein, 1; salt mixture, USP XIV, 4; cellulose,[2] 6. The vitamin mixture contained: (in mg/kg of diet) thiamine·HCl, 10; riboflavin, 10; pyridoxine·HCl, 10; Ca pantothenate, 30; inositol, 500; niacin, 50; *p*-aminobenzoic acid, 100; biotin, 0.2; vitamin B_{12}, 0.02; choline tartrate, 2000; vitamin A, 1.5; vitamin D, 0.025; DL-α-tocopherol, 91.

For the first experiment, 6-week old rats were chosen. Two groups were adrenalectomized and thereafter supplied with physiological saline; 2 groups were sham-operated. All rats were fed a commercial ration until 8 weeks of age when the 4 operated groups and 2 groups of intact rats were fed the test diets containing rapeseed oil or corn oil, and maintained at room temperature, 21 to 23°C, while a

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[1] Golden rapeseed oil containing 37% erucic acid, obtained from the Saskatchewan Wheat Pool.

[2] Alphacel, Nutritional Biochemicals, Inc., Cleveland.

group fed each oil was housed at 4°C. Forty-six days later, the rats were anesthetized with ether, blood drained from a neck incision, and organs removed and weighed.

In the second experiment, rats of 23 to 24 days of age were fed rapeseed oil or corn oil in the same basal diet with or without pyridoxine in the vitamin mixture. Six weeks later the animals were killed as in the first experiment, and liver, adrenals, and gastrointestinal tract removed. The remaining tissue was ground, and the fat extracted by the procedure of Bligh and Dyer (9). Liver vitamin B_6 was determined by the method of Atkin et al. (10). Gas-liquid chromatography, as previously described (11), was used to analyze the fatty acids of the carcass fat.

RESULTS AND DISCUSSION

Figure 1 shows the effect of rapeseed oil and corn oil on the weight gains of adrenalectomized or sham-operated rats and of rats maintained at 4°C or at room temperature. Table 1 provides the data on mortality, food intake, weight gain and organ weights. Five of the 10 adrenalectomized rats fed rapeseed oil died in 1 to 31 days of the experimental feeding period and had a mean survival time of 10 days, whereas 3 of the adrenalectomized rats fed corn oil died in 16 to 36 days, and had a mean survival time of 26 days. All of the animals receiving rapeseed oil in the cold lost weight and died in 4 to 38 days with a mean survival time of 15 days, whereas one-half of those receiving corn oil in the cold died in 11 to 34 days with a mean survival time of 20 days. It appeared that the effects of cold were more acute when rapeseed oil rather than corn oil was fed, whereas the effects of adrenalectomy were inconclusive.

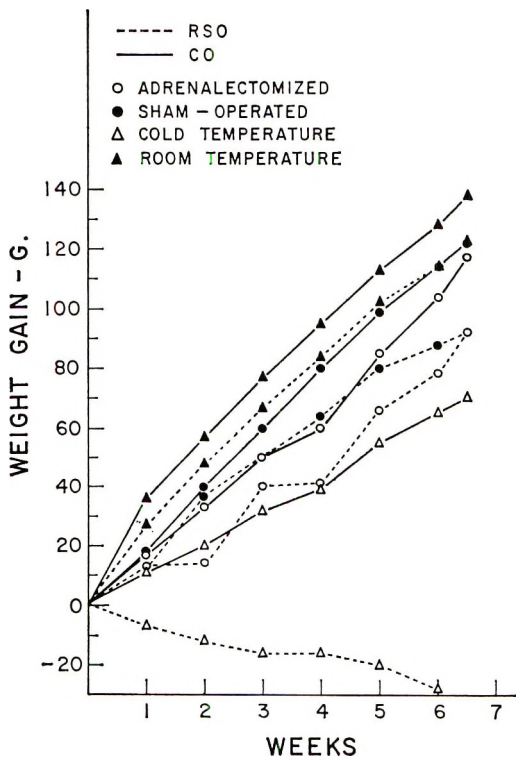

Fig. 1 Weight changes of rats that were adrenalectomized, sham-operated, kept in the cold or at room temperature, and supplied with rapeseed oil or corn oil in the diet.

As has been previously reported (1–3), rats fed rapeseed oil ate less food and gained less weight than those fed corn oil. When the weight gains of rats maintained at room temperature were adjusted for food intakes by a covariance analysis, the difference between oils was no longer significant. On the basis of food intakes and weight gains, it appeared that the response to dietary fats was not significantly altered by adrenalectomy. Perry and Brown (6) disagreed with the conclusion of Engel and Engel (5), by reporting that the utilization of fat was similar in adrenalectomized and normal rats. The body weights of sham-operated rats fed rapeseed oil were significantly lower ($P = 0.01$) than those of sham-operated rats fed corn oil and of both groups of unoperated rats at room temperature. In agreement with others (12, 13), the food or calorie intake increased and the weight gain decreased when rats were maintained in the cold. Sellers et al. (7) showed that variations in the fat content of the diet did not affect growth or survival in the cold, but the results of the present experiment demonstrated that the type of fat influenced the response of such animals.

The weights of livers and spleens of rats maintained in the cold followed the same trend as the body weights, and were lower ($P = 0.05$) than those of unoperated or sham-operated animals at room

TABLE 1

Effect of rapeseed oil and corn oil fed for 46 days to rats subjected to different treatments

Treatment	Dietary oil	Mortality	Food intake	Wt gain	Liver wt	Spleen wt	Adrenal wt	Testes wt
			g	*g*	*g*	*mg*	*mg/pair*	*g/pair*
Adrenalectomy	RSO[1]	5/10	439 ± 22[3]	82 ± 8	8.83 ± 0.94	0.63 ± 0.14	—	2.58 ± 0.16
	CO[2]	3/10	476 ± 17	115 ± 14	8.73 ± 0.85	0.65 ± 0.13	—	2.78 ± 0.06
Sham operation	RSO	0/10	470 ± 15	92 ± 8	9.40 ± 0.16	0.51 ± 0.02	42.1 ± 1.3	2.41 ± 0.07
	CO	0/10	518 ± 20	122 ± 11	10.06 ± 0.48	0.56 ± 0.07	42.5 ± 1.5	2.61 ± 0.04
Cold temperature	RSO	10/10	—	—	—	—	—	—
	CO	5/10	673 ± 20	70 ± 5	7.88 ± 0.20	0.40 ± 0.04	34.9 ± 2.5	2.24 ± 0.20
Room temperature	RSO	0/10	454 ± 10	123 ± 4	9.41 ± 0.19	0.57 ± 0.02	37.2 ± 1.3	2.47 ± 0.06
	CO	0/10	484 ± 13	138 ± 9	9.82 ± 0.26	0.54 ± 0.01	37.7 ± 1.6	2.57 ± 0.05

[1] Rapeseed oil.
[2] Corn oil.
[3] Mean ± SE.

temperature. Vahouny et al. (14) also showed a reduced absolute liver weight in rats housed in the cold. The adrenal weights of the sham-operated rats were larger than those of the unoperated rats, but there were no significant differences in testes weights.

As shown in table 2, the low food intake and weight gain of rats deprived of pyridoxine were still lower when rapeseed oil instead of corn oil was fed. The adrenal and liver weights of rats lacking pyridoxine were not further affected by the type of dietary oil. Since the levels of total vitamin B_6 in the liver appeared to be solely dependent upon the dietary supply of that vitamin, the resultant condition of rats fed rapeseed oil and deprived of pyridoxine was not related to the liver vitamin B_6 stores. The values for the proportion of fat in the carcass agreed with those reported earlier (15), in that rats deprived of pyridoxine had significantly less fat than the control animals. The reduction of carcass fat in the absence of dietary pyridoxine was most marked in the rats fed rapeseed oil. Brown (16) showed that rats better withstood the effects of pyridoxine deficiency when corn oil instead of hydrogenated shortening was fed.

The fatty acid composition of the carcasses is shown in table 3. The proportion of palmitic acid in the fat of animals supplied with pyridoxine was not significantly affected by the type of oil, but in those deprived of pyridoxine the acid was significantly higher in rats fed corn oil. A similar but less marked pattern was observed for stearic acid. Linoleic acid supplied by the dietary corn oil was retained in greater proportion in those animals that received pyridoxine than in those that did not, but the linoleic acid of rats fed rapeseed oil was not similarly affected. Johnston et al. (17) also observed that in rats fed corn oil but deprived of pyridoxine, stearic acid was higher and linoleic acid lower than in the control rats supplemented with pyridoxine. Swell et al. (18) on the other hand, noted the level of linoleic acid in the lipids of serum and adrenals to be higher in pyridoxine-deficient than in normal rats. The carcass linolenic acid was higher in rats fed rapeseed oil than in those fed corn oil, as was previously shown

TABLE 2

Effect of rapeseed oil and corn oil in rats deprived of or supplemented with pyridoxine for 6 weeks

Dietary oil	Dietary pyridoxine	Food intake	Wt gain	Adrenal wt	Liver wt	Total liver vitamin B_6	Carcass fat
		g	*g*	*mg/pair*	*g*	*µg*	*%*
RSO[1]	—	149 ± 8[2]	24 ± 5	27.6 ± 1.1	3.11 ± 0.23	15.3 ± 2.5	4.4 ± 0.2
RSO	+	351 ± 15	129 ± 7	34.9 ± 1.3	7.70 ± 0.38	93.7 ± 5.6	13.8 ± 0.6
CO[3]	—	195 ± 10	48 ± 6	24.8 ± 0.9	3.60 ± 0.28	16.2 ± 1.9	7.3 ± 0.2
CO	+	509 ± 16	209 ± 8	36.4 ± 1.2	9.51 ± 0.47	102.0 ± 7.0	13.0 ± 0.2

[1] Rapeseed oil.
[2] Mean ± SE.
[3] Corn oil.

TABLE 3

Carcass fatty acids of rats fed rapeseed oil or corn oil with or without pyridoxine for 6 weeks (% of total fatty acids)

Fatty acid[1]	Diet			
	RSO[2] minus vitamin B_6	RSO + vitamin B_6	CO[3] minus vitamin B_6	CO + vitamin B_6
12:0	0.2 ± 0.1[4]	0.6 ± 0.1	0.2 ± 0.1	0.5 ± 0.1
14:0	1.1 ± 0.2	1.7 ± 0.1	1.2 ± 0.3	1.2 ± 0.1
14:1	0.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
15:0	0.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
16:0	12.7 ± 0.6	18.0 ± 1.0	21.4 ± 0.9	15.9 ± 0.6
16:1	3.6 ± 0.3	6.2 ± 0.5	4.3 ± 0.3	4.2 ± 0.3
17:0	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
17:1	0.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.5 ± 0.2
18:0	3.4 ± 0.3	1.3 ± 0.1	5.6 ± 0.2	2.0 ± 1.2
18:1	35.6 ± 0.6	31.7 ± 0.6	30.5 ± 0.6	29.5 ± 0.2
18:2	13.7 ± 0.3	13.8 ± 0.4	33.7 ± 0.7	42.2 ± 0.6
18:3	3.3 ± 0.2	5.4 ± 0.2	0.4 ± 0.2	1.1 ± 0.1
20:0	0.5 ± 0.1	0.5 ± 0.1	—	—
20:1	10.2 ± 0.2	9.4 ± 0.3	0.7 ± 0.1	0.7 ± 0.1
20:2	0.6 ± 0.2	0.7 ± 0.1	0.1 ± 0.1	0.4 ± 0.2
20:4	1.8 ± 0.2	0.6 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
22:1	11.8 ± 0.3	8.8 ± 0.4	—	—

[1] The first figure represents the number of carbon atoms, the second the number of double bonds.
[2] Rapeseed oil.
[3] Corn oil
[4] Mean ± SE.

(19), but was somewhat reduced in the absence of pyridoxine. Eicosenoic acid, contributed by rapeseed oil, was deposited in similar proportions in rats deprived of and in rats supplied with pyridoxine. Erucic acid, also contributed by rapeseed oil but deposited in lower proportions than present in the dietary oil, was found in somewhat higher concentration in the fatty acids of the pyridoxine-deprived rat. This observation might be indicative of a slower rate of metabolism of erucic acid in the absence of dietary pyridoxine, or of a greater accumulation of this fatty acid when the fat contains a small proportion of C_{16} acids.

These experiments showed that when rapeseed oil constituted the entire source of dietary fat, with the exception of a small amount of oil in cornstarch, the responses of the rat to cold or pyridoxine deprivation were more pronounced than when corn oil was fed. The nutritional properties of rapeseed oil, which contains a smaller proportion of palmitic acid than most other vegetable oils, may be attributed to its low content of saturated acids and its high content of erucic acid. By the addition of a more saturated oil to rapeseed oil, food intake and weight gain of rats increased (20). It is likely that the effects of dietary rapeseed oli would be similarly

modified by the mixture of fats consumed by humans.

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Meat Diets

II. EFFECT OF THE AGE OF RATS ON THEIR ABILITY TO WITHSTAND THE LOW CALCIUM INTAKE INDUCED BY A DIET OF MINCED BEEF

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ABSTRACT Middle-aged piebald female rats, reared with a stock diet, were transferred to a diet of raw minced beef, deficient in calcium. They survived for long periods, sometimes over a year, without developing gross skeletal abnormalities, and with little or no demineralization. In contrast, weanling rats, fed the same meat, stopped growing after a few weeks, developed severe skeletal lesions and demineralization and usually died. The provision of calcium supplements to weanlings for graded periods, before restriction to meat without calcium, increased their ability to grow and thrive on the meat. With only 3 weeks of supplementation, for example, the rats all grew normally during an experiment lasting 4 months. Such rats appeared to be in perfect health, and had normally shaped bones. To a marked degree, however, the urge for growth had taken precedence over the demand for normal mineralization of the bones. Thus the ash content of their femurs was only one-half that of rats that had received calcium continuously. Severe stunting of growth, and fractures, occurred only when the ash content of the bones was one-third, or less, of the normal level. These observations emphasize the crucial influence of age, in rats previously adequately supplied with calcium, on their resistance to subsequent dietary deficiency of calcium. On a body weight basis the calcium demands of weanlings must be at least 20 times those of adults. Hypochromotrichia, due to an absolute or conditioned deficiency of copper, was observed in the middle-aged rats fed raw steak. It was cured by impure iron carbonate, containing copper. Calcium was also effective against hypochromotrichia, in these middle-aged rats, in a way not yet understood. In all the weanlings hypochromotrichia was prevented by supplements of copper.

An earlier paper (1) has reported experiments on the calcium deficiency, and on the apparent deficiency in iron, which is induced in young piebald rats by a diet of raw,[1] boneless beef. Thus the animals, if not given supplements, developed severe softening of the bones, as a sign of calcium deficiency. The defect in iron metabolism was indicated by anemia and dental depigmentation. The hair of the head and shoulders became grey. Adequate supplements of calcium carbonate prevented the bone softening, and large supplements of a medicinal grade of ferrous carbonate prevented the other abnormalities. The paper confirmed, and amplified, evidence as to interrelationships between the metabolisms of calcium and iron.

It was suggested, however, that copper might also be involved. Thus Hundley (3) has reported that hypochromotrichia occurs in copper deficiency, whereas iron deficiency has not been mentioned as a cause of this abnormality. Moore (4) reported that in meat-fed rats greyness was prevented completely by small doses of copper sulphate, but not by larger doses of pure ferrous ammonium sulphate. The ferrous carbonate used in the earlier work was found to contain enough copper, as impurity, to account for the prevention of anemia and hypochromotrichia. Ilan and Guggenheim (5) reported independently that copper prevents anemia in meat-fed mice. Moore (6) noted in rats that dental depigmentation is prevented by copper. The presence of copper as an impurity in the ferrous carbonate used in the earlier work, however, obviously could not account for the adverse effects of large intakes of iron on calcium metabolism,

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[1] Preliminary experiments (2) have indicated that some forms of cooked meat, but not all forms, are much less potent than raw meat in producing copper deficiency in rats. We have no evidence, however, that cooking alleviates the calcium deficiency induced by a meat diet.

which had been one of the main points under investigation.

A complex network of interrelationships must therefore be assumed between calcium, iron and copper, with probably the intervention of other nutrients. Another complicating factor is age, since the demands for the various minerals, and the relative pathological importances of low intakes, will vary according to the rate of growth.

This paper is mainly concerned with the influence of age on the severity of the lesions, due to calcium deficiency, in meat-fed rats. The liability of elderly human subjects to fractures induced by falls is a serious clinical problem. It is generally accepted that increased fragility of the bones is involved. Since skeletal lesions are readily induced in young rats by a meat diet, the feeding of meat to middle-aged rats appeared to offer a promising experimental approach to the study of the influence of dietary factors on senile bones. In experiments which will be described, however, it was found that mature rats could subsist, for very long periods and without the development of gross skeletal lesions, on meat diets which would rapidly cause growth cessation, decline and death in younger rats. Further experiments were planned, therefore, to examine more closely the effect of the age of rats on their resistance to calcium deficiency. A preliminary communication (7) has already described parts of these experiments.

A side issue arose, in our experiments on mature rats, in the consistent appearance of hypochromotrichia. This experiment was started before the importance of copper had been realized, and supplements of iron and calcium were tried for treatment of the greyness. This side issue was irrelevant to the interpretation of our observations on the effect of calcium deficiency, since the bones of the mature rats remained almost normal, irrespective of the presence or absence of hypochromotrichia. In the further experiments on the influence of age on resistance to calcium deficiency hypochromotrichia was prevented by supplements of copper.

MATERIALS AND METHODS

Rats. Purebred, piebald rats were used. In experiment 1 they were all females, and, with one exception, were mature. In experiment 2 the rats were weanlings, of both sexes.

Diet. In both experiments the diet was raw beef steak, minced up with such portions of attached adipose tissues as are usually supplied by the butcher. It contained 16.9 g of fat, 12.2 mg of calcium and 160 μg of copper/100 g wet weight. The steak, taken from the chine (shoulders) of British-reared steers or cows, was minced first by the butcher.[2] On arrival at the laboratory it was reminced to a fine state. The portions of meat to be supplemented with minerals were then kneaded by hand until the minerals were uniformly distributed. Unsupplemented meat was subjected to equally thorough kneading. The final state of the meat was a thick paste.

Supplies of pasted meat were made up fresh twice weekly, and were stored in a refrigerator until required. Portions of meat, in freshly washed food pots, were given daily to the rats. Daily inspection of the food left over by the rats indicated that the various components were eaten uniformly. The excess of food from the previous day was discarded.

In this regimen fat supplied nearly 70% of the caloric intake. There was no evidence, however, that the high intake of fat was injurious. In adolescent rats, given supplements of calcium and copper, weight increases up to 9 g daily were observed.[3] Since the body of the growing rat usually contains about 0.7% of calcium this rapid rate of growth corresponds to an incorporation of about 63 mg of calcium daily. A typical daily intake of 30 g of unsupplemented steak supplies less than 4.0 mg of calcium. In the absence of supplements, therefore, the deficiency of calcium induced in the growing rat was acute.

Mineral supplements. Calcium was provided, when experimentally required, as carbonate. It contained only a trace, 12.2 μg/g of copper. Iron was given to certain

[2] The exact nature of the steak does not seem to be highly relevant. The same abnormalities as are caused by steak have been observed in rats fed raw meat in the form of ox-heart, mutton or pork (6).

[3] In several experiments we have observed more rapid growth or heavier final body weights, in rats fed raw steak, with supplements of calcium and copper, than in controls given a liberal stock diet. Reproduction and lactation are satisfactory in rats fed steak, with calcium and copper (6).

of the mature rats as ferrous carbonate. It contained 324 μg/g of copper, and was supplied mixed with an equal quantity of glucose. Copper was given, to all the weanling rats, as sulphate.

Examination of bones, teeth, blood and hair. At the conclusion of each experiment, or on the natural death of the animals, the bones were inspected for obvious defects, such as fractures or deformity. Radiographs were taken of the whole rat, or of a dissected femur, or both. Femurs were weighed in the wet state, and were measured for length. They were then dried, defatted, and ashed. Calcium was estimated in ash from pooled groups in experiment 2.

It was found that the most informative index of the degree of decalcification was the ash content of the undried femur. Since the content of solid matter in highly decalcified bones was low, the expression of ash on a dry-weight basis would have obscured the full severity of the abnormality. The results for calcium tended merely to reflect the results for ash. For simplicity, therefore, the data presented on bones, apart from the mention of fractures and deformities, will be confined to ash percentages on a wet-weight basis.

The incisor teeth were examined for depigmentation. In experiment 1 blood was collected from the tails of the rats during life, or at the time of killing, for red blood cell counts, and for measurements of hemoglobin and the haematocrit. In experiment 2, in which the rats were given copper, and in which there was obviously no anemia, blood measurements were considered to be superfluous. In experiment 1 the hair on the head and shoulders was judged to be normal, or grey, by inspection. In experiment 2 the hair was invariably normal in color, and no records were kept for individual rats.

Mature rats

Experiment 1. Maintenance of mature female rats on a meat diet. The fats used in this experiment, with one exception, had been members, up to the age of 15 months, of a breeding colony. They had received a stock diet, adequate in calcium, and had each produced several litters. Since the stock diet was known to promote the accumulation of high stores of vitamin A it was considered unnecessary to provide special doses of this vitamin. At the end of the experiment the persistence of high reserves of vitamin A in the liver was confirmed by chemical estimations. No special provision of other vitamins was made. Some of the rats were given supplements of calcium carbonate, as 0.5% of the diet, either from the commencement of the experiment, or later. At a late stage 2 of the rats were given ferrous carbonate, as 2.5% of the diet, for the treatment of hypochromotrichia.

In contrast with the experience with weanlings, the mature rats could subsist on meat, without calcium supplements, for long periods. In many of the animals death occurred, through old age or disease, sometimes including abscess formation, without the development of marked skeletal abnormalities. Other rats, both with and without calcium supplements, were killed, while still in a fair state of health, after receiving the meat for more than a year. "Cage sores," or "pressure pads" (8, 9), which are difficult to prevent in aging rats, occurred, in varying severity, on the hind feet of all the animals.

The ash content of the undried femurs, with other particulars, is given in table 1. The ash content for 8 of the 12 rats not given calcium fell within the range 37.7 to 40.7%, which was not significantly different from the range 38.1 to 43.9% observed for 4 rats given calcium. The largest difference, between rat no. 3 (+ Ca) and rat no. 8 (no Ca), amounted to only 28%. Somewhat low ash percentages, 34.3 and 32.6, were observed for both the animals that received iron.

Hypochromotrichia was observed in all the rats not given mineral supplements. It was prevented, or cured, in 4 out of 5 rats given calcium, and in 2 rats given iron. Rat no. 5 differed from all the others in receiving meat, supplemented with 0.5% of calcium carbonate and adequate doses of vitamins A and D, from the time of weaning. It was in good health when killed, at an age of nearly 2 years. Hypochromotrichia was noticed in this rat 4 weeks after the commencement of the meat diet, but later the hair regained its

TABLE 1

Ash content of undried femurs, and color of the hair on the head and shoulders, in senile female rats that had been fed meat, with or without mineral supplements, for long periods

Mineral supplement	Rat no.	Diet fed			Died (D) or killed (K)	Abscess formation	Body weight			Hair: normal (N) or grey (G)	Femur ash content
		Stock diet	Meat only	Meat with minerals			Maximal	Final	Loss		
		days	*days*	*days*							%
$CaCO_3$, 0.5%	1	450		260	K		381	281	100	N	—
	2	450		445	K	+	343	233	110	N	38.1
	3	450	150	241	K		413	241	172	G	43.9
	4	450	150	295	K	+	361	321	140	N	43.8
	5	28		672	K		392	365	27	N	39.7
None	6	450	298		D		285	211	74	G	37.8
	7	450	324		D		374	286	88	G	40.0
	8	450	332		D	+	269	174	95	G	31.8
	9	450	348		D	+	371	217	154	G	37.7
	10	450	355		D	+	385	216	169	G	38.0
	11	450	358		D		362	242	121	G	38.8
	12	450	373		D		404	326	78	G	39.0
	13	450	388		D		399	266	133	G	39.0
	14	450	391		K		402	321	81	G	34.4
	15	450	446		K		362	304	58	G	40.7
$FeCO_3$, 2.5%	16	450	235	156	K		386	269	117	N	34.3
	17	450	235	199	D		399	327	72	N	32.6

normal color. Although hypochromotrichia appeared consistently in all the rats given unsupplemented meat, there was no evidence of anemia, and never more than slight dental depigmentation.

Growing rats

Experiment 2. The influence of age, at the commencement of calcium deficiency on the growth, survival and calcification of young rats. Weanling rats, weighing 40 to 49 g, were used, and were all given the meat diet from the commencement of the experiment. All received supplements of 10 μg copper/g of meat. Adequate doses of vitamins A, D, E and K were given weekly, separately from the basal diet. Six groups were made up, each of 3 males and 3 females. This arrangement was inconvenient for the application of conventional statistical methods, but it was desired to explore the influence of sex.

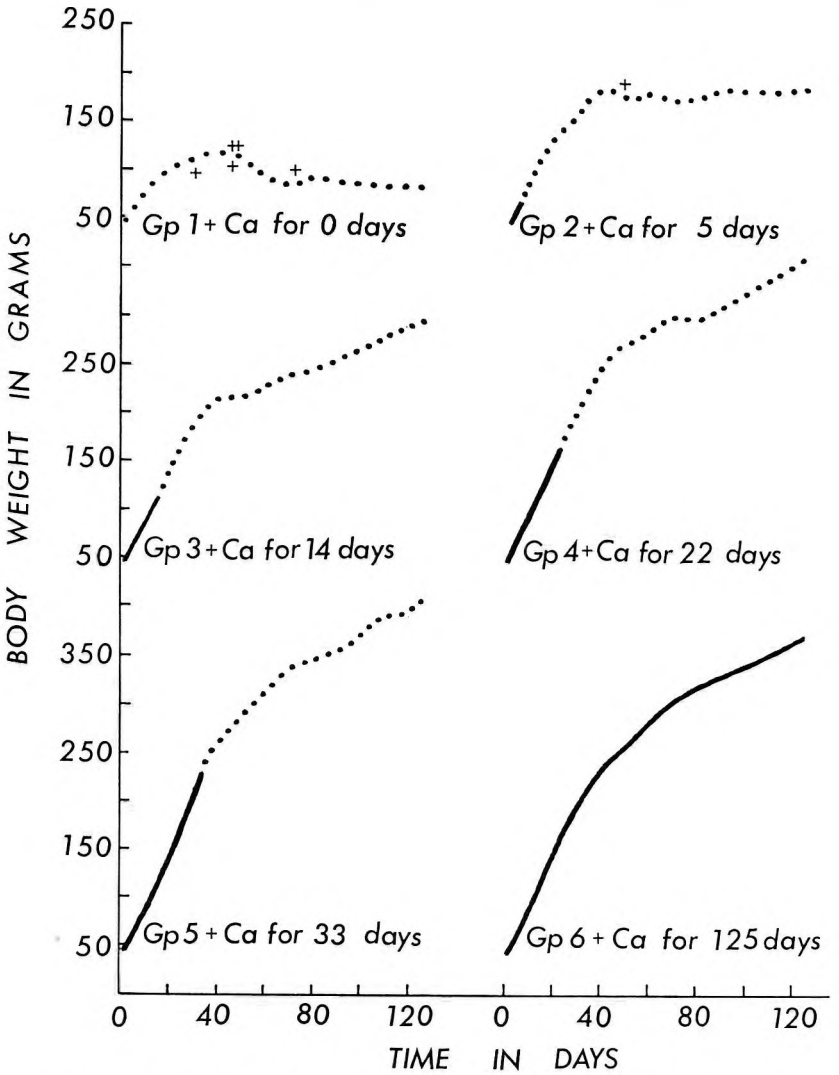

Fig. 1 (exp. 2) Average growth curves, for groups of 3 male and 3 female rats each, fed minced beef, low in calcium. The treatment of the different groups varied in the provision, to groups 2–6, of supplements of calcium carbonate, as 0.5% of the diet, for graded periods. Solid line indicates $CaCO_3$ given; dotted line indicates no $CaCO_3$ given.

TABLE 2

Mean ash percentages of the undried femurs of young rats fed meat, and given supplements of 0.5% calcium carbonate for different preliminary periods

Group no.	Days of calcium supplementation	Mean percentage of ash in femur			Skeletal fractures in group
		Males	Females	Males + females	
1	0	9.0	12.3	10.7	+
2	5	11.9	14.4	13.2	+
3	14	17.8	20.8	19.3	0
4	22	21.1	25.7	23.4	0
5	33	26.0	32.4	29.2	0
6	125	41.3	42.0	41.7	0

The treatment of the various groups differed in that calcium carbonate, as 0.5% of the diet, was given for graded periods from the commencement of the experiment. The times for which calcium was provided were as follows: (in days) group 1, 0; group 2, 5; group 3, 14; group 4, 22; group 5, 33; group 6 (controls), 125. The experiment was continued for 125 days. All the animals, even if suffering acute calcium deficiency, had normally pigmented hair and incisor teeth. During the experiment 5 rats died in group 1, and one rat in group 2. The remaining rats, with a few exceptions, kept in fair, or good health. Figure 1 shows the average growth curves for the combined sexes. After rats had died the curves were based on the mean of the averages for the survivors of each sex, rather than on the simple average for all the survivors. The latter part of the curve for group 1, however, relates to only a single surviving female.

In group 1 (no calcium supplement) a growth plateau was soon reached at a mean body weight of 120 g, and all but one of the rats died soon afterwards. At autopsy all the rats that died were found to be emaciated, and to have severe skeletal lesions, often with fractures of the ribs and hind leg bones. The one rat that survived was kept alive, beyond the conclusion of the rest of the experiment, for further investigation. In group 2 (Ca for 5 days) growth stopped at a mean body weight of about 180 g. One rat died, at this point, from lung hemorrhage. The remaining rats, before killing after 125 days, appeared in fair general health. At autopsy, however, skeletal lesions were found in every animal, usually in the form of fractured ribs, with collapse and deformity of the chest wall. Fat reserves were sparse. The rats of group 3 (Ca for 14 days) grew at a slightly subnormal rate, but at autopsy their bones, muscles, and fat deposits appeared to be normal. In groups 4, 5 and 6 (Ca for 22, 33 and 125 days respectively) growth was normal. All these rats were sleek and lively, and at autopsy were found to have normally shaped bones, normal muscles, and abundant fat deposits.

Observations on the ash percentages of the femurs are given in table 2. The degree of calcification in group 6, in which the animals received calcium throughout the experiment, was within the range, found in experiment 1, for adult animals receiving calcium. The difference between the sexes was insignificant. For the remaining groups the ash content of the femurs was invariably low, and was graded according to the number of days of calcium supplementation. The differences between groups were considerable, with about a fourfold difference between groups 1 and 6. In groups 1–5, moreover, a consistent difference was found between the sexes, with decidedly higher values in females than in males. The depth of the x-ray shadows (fig. 2) confirmed the chemical findings.

DISCUSSION

Calcification. It is well known that the demands for calcium, in any species, are largely determined by the rate of growth. In rats receiving adequate amounts of calcium Sherman and McCleod (10) studied the calcium content of the body in relation to age and growth. Calculations, based on

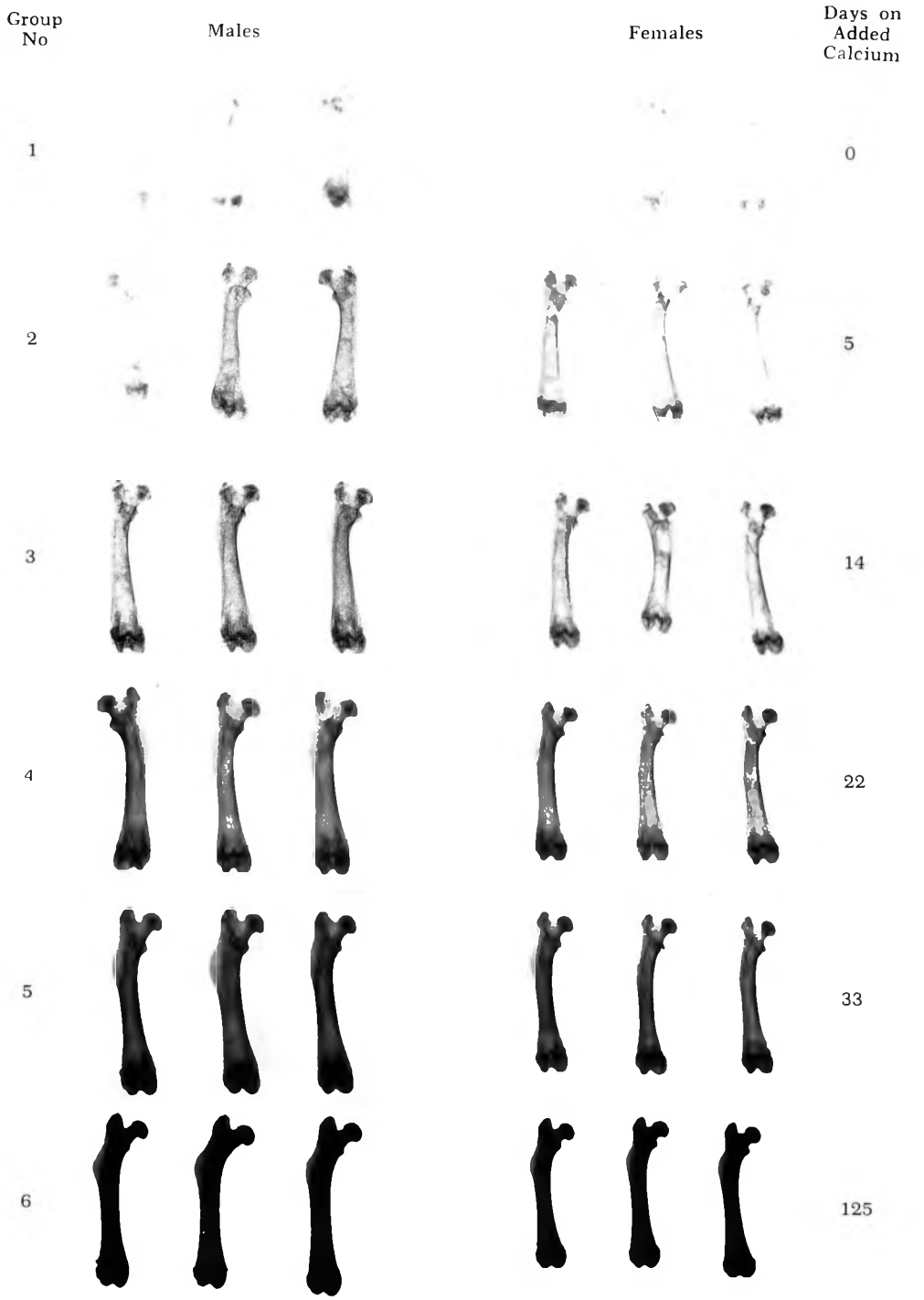

Fig. 2 (exp. 2) Radiographs of the femurs of rats fed meat with or without supplements of calcium carbonate for graded periods. In group 1 one female rat survived the experimental period, and was kept alive for further investigation.

their results, show that the rate of increment in the body calcium content in their rats aged 15 days was about 60 mg/100 g body weight daily. In rats aged 243 days the calcium increment decreased to only about 3.2 mg/100 g body weight daily. The calcium requirement of very young rats for body building must therefore be nearly 20 times greater, on a body weight basis, than that of the adult rats. We may suspect, moreover, that eventually the calcium increment in the adult rat will become zero, or even negative.

In the weanling meat-fed rat, of 50 g body weight, faced with a demand of about 30 mg of calcium daily for normal growth, and supplied with only about 2 mg in its unsupplemented diet, the development of acute calcium deficiency appears inevitable. No other explanation of the severe skeletal lesions appears to be required. The ability of mature rats to subsist on the same diet can be understood on the basis of their much smaller requirement of calcium for body building, or indeed the complete absence of such a requirement. Their prolonged survival and resistance to demineralization, however, indicates that the amount of calcium required by the adult for "metabolic turnover" can be reduced to a very low level. The amounts of calcium which need, like water, to enter and leave the body continuously must be minute.

The behavior of young rats given preliminary supplements of calcium was perhaps more unexpected than that of either the unsupplemented weanlings or the adults. These rats showed an ability for rapid growth, and for the maintenance of normal health, under conditions which greatly reduced the degree of mineralization of their bones. Thus the femurs in groups 4 and 5 had an ash content of only 56 and 70%, respectively, of the ash content for the "control" rats of group 6. In spite of such low ash content, these animals were indistinguishable from the controls, either during life or on gross postmortem inspection of their carcasses. In group 3 the ash content was only 46% of the control value, but the only obvious adverse effect was a slight retardation in growth. In group 2, however, a reduction of mineralization to 32% of the control value was associated with a cessation of growth at about one-half the control level, and with gross skeletal lesions.

These results suggest that the normally calcified rat, with a femur ash content of about 40%, has a safety margin of some 100% above the minimal ash content of 20% which appears consistent with a normal growth rate and an apparently good state of health. Further investigation seems desirable to find out whether rats, without this safety margin, can fully resist stresses such as reproduction, illness, old age, and mechanical pressure on the bones.

Sex difference. The consistent observation, in groups 1–5, that the femur ash content was higher in females than in males agrees with early observations by Sherman and McCleod (10). In rats of various ages these workers observed that the calcium content, expressed as a percentage of the body weight, was always higher in virgin females than in males, on an average by about 9%. Hammett (11) also noted a higher ash content in the bones of female rats than in males. In our experiments the higher femur ash content in the females appears readily explainable by their well-known lower growth rate. Thus in females growth would exert a lesser force than in males in the competition between growth and bone mineralization each to maintain its normal level.

Hypochromotrichia. The experiments that have been described, as already stated, were planned for the study of the effect of a low calcium intake, induced by a meat diet, on the bones of the rat. The hypochromotrichia observed in all the unsupplemented rats in experiment 1, however, also requires comment in relation to calcium metabolism. Thus the greyness was prevented in 4 out of 5 rats that were given calcium carbonate. In view of the dramatic action of copper, which was virtually absent from our calcium carbonate, in preventing hypochromotrichia, it appears improbable that calcium acts directly against this abnormality. More probably it acts indirectly, that is, by balancing the high phosphate content of meat, or by releasing copper from a porphyrin complex. This indirect action, however, is apparently unable to compensate

for the stress of growth. Thus Moore et al. (1) reported that calcium carbonate did not prevent hypochromotrichia in young rats, although impure iron carbonate, containing copper, was completely effective. The cure of hypochromotrichia in rats 16 and 17 (table 1), which were given iron carbonate, was presumably due to the presence of copper.

Our failure to observe anemia, or marked dental depigmentation, in any of the grey-haired rats in experiment 1 suggests that these lesions indicate different degrees of severity of copper deficiency. Thus hypochromotrichia is caused by a degree of copper deficiency which is insufficiently severe to cause marked anemia or dental depigmentation. We must conclude, therefore, that many of our rats were able to live long lives, and to maintain good general health, although suffering from chronic partial copper deficiency. It is interesting that this copper deficiency was manifest under conditions in which no evidence of calcium deficiency, as indicated by gross skeletal decalcification, could be detected. Thus a diet of meat, without supplements, produces in young rats signs of both calcium and copper deficiencies, but in mature rats only copper deficiency.

ACKNOWLEDGMENTS

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The Role of the Soft Pellets in the Production of Lactic Acid in the Rabbit Stomach

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ABSTRACT It is known from the literature that lactic acid is produced in the rabbit stomach and that the accumulation of the acid is accompanied, paradoxically, by an increase in pH. The bacteria in soft pellets are likely candidates for the role of lactic acid production; with this in mind the distribution of soft pellets and of lactic acid in the stomach was studied. It was found that pellets taken from the rectum contain lactic acid and that the pellets after ingestion are lodged in the fundus of the stomach. Consistent with this is the fact that the absolute amount and the concentration of lactic acid are highest in the fundus. The soft pellets possess a tough membrane which remains morphologically intact for at least 6 hours after ingestion. The membrane encloses a collection of bacteria including lactobacilli, and homogenates of the pellets possess marked amylase activity and a high level of phosphate buffer which maintains the pH of the homogenate at about 6.0 to 6.5. Consistent with these facts, the homogenates can form lactic acid from starch and from glucose. The high pH maintained by the buffer permits fermentation of carbohydrate to take place in intact pellets, even when the external medium of the stomach is strongly acid. The buffering action of the pellets can explain the apparent contradiction of an increase in pH in the stomach associated with lactic acid accumulation. It is concluded that the fundus of the rabbit stomach, loaded with soft pellets, is analogous to the rumens of sheep and cattle.

Carmichael et al. (1) showed that the stomach in normal rabbits and in rabbits in which coprophagy had been prevented, was more than one-half full of digesta at the end of a 24-hour fast. Some 13 years later Alexander and Chowdhury (2) became curious about the prolonged retention of food in the stomach and they thought that the circumstance would permit fermentation of carbohydrates. They showed that the rabbit stomach contained lactic acid, the amount of which was decreased by withholding food and increased by feeding, and that lactic acid was produced *in vitro* by inoculation of food with gastric contents. They thought it not unlikely that the lactic acid production was due to microbial fermentation, but did not consider coprophagy in this connection. Coprophagy could influence lactic acid levels in the stomach if the soft pellets themselves contained lactic acid; and also bacteria in the pellets are likely candidates for the role of production of lactic acid from carbohydrates of large molecular weight. With these considerations in mind lactic acid production in the rabbit stomach was studied again.

It has been suggested (3) that the soft pellets of rabbits contain bacteria but principally they have been regarded as chemical entities — so much protein, carbohydrate, fat, and vitamins (4, 5). In fact Thacker and Brandt (6) believe that the high protein content of soft pellets, relative to that of hard droppings, is due to the secretion, by the cecum of mucus-like protein onto the soft pellets. However, the results of the histological and bacteriological studies reported in the present study show that the soft pellet is a biological entity enclosed by a palpable membrane and consisting mainly of bacteria which account for the high protein content.

METHODS

Animals. Domestic rabbits of both sexes, weighing 1500 to 2000 g, were used for most experiments. Wild rabbits, however, were used for work on the effect of coprophagy on stomach pH since the onset and termination of coprophagy is well-defined in those animals (7). Both the domestic rabbits and the wild rabbits were

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fed a standard diet containing 21% protein; fresh greens were fed once a week.

In experiments during which oats were fed, the rabbits were maintained with that food for 4 to 5 days before the experiment. In some experiments coprophagy was prevented by fitting leather pants over the hind quarters.

Bacteriological. Standard techniques of culture in glucose, yeast extract, agar, thioglycollate media, and in acetic acid broth were used under aerobic and anaerobic conditions. Smears were stained with Gram stain.

Histological. Soft pellets from the rectum and from the stomach were either fixed intact in Bouin's fluid, or they were dissected in distilled water, the membranous envelopes washed and then transferred to Bouin's fixative. Portions of the alimentary canal from the colon to the posterior part of the rectum with soft pellets *in situ* were also fixed in Bouin's fluid. All tissues were embedded in paraffin, sectioned at 10 μ thickness, and stained with Heidenhain's iron hematoxylin and eosin, or by the Schiff's periodic acid procedure.

Chemical. Lactic acid was determined by the method of Barker and Summerson (8). Stomach contents and rectal pellets were prepared for lactic acid determination by trituration in a Potter-Elvehjem homogenizer.

Lactic acid production was studied *in vitro* in reaction mixtures containing substrate and homogenate in distilled water; the substrates were ground oats, potato starch, and glucose. Buffers were not used since it was found that the reaction mixtures could be maintained at the pH of choice by the buffering action of the homogenates themselves. When potato starch was used as a substrate it was first converted to the colloidal form by dissolving a paste in hot water.

Reactions in all instances were stopped by addition of trichloroacetic acid to a final concentration of 7%. Protein was partially removed by the combined actions of the trichloroacetic acid and centrifugation. The remaining protein was removed during the treatment with lime in the lactic acid method.

All pH measurements were made with the glass electrode. Since stomach contents were rarely liquid enough, the determinations were carried out on slurries made by the addition of a minimal quantity of distilled water.

Conventional protein was determined in rectal pellets by the Conway (9) microdiffusion method. The factor of 6.25 was used to convert nitrogen percentage to protein percentage.

Phosphate phosphorus was determined by the Fiske and Subba Row (10) method in cold water extracts of rectal pellets. The extracts were deproteinized and clarified by treatment with trichloroacetic acid and centrifugation at 25,000 × *g*. Sodium and potassium were determined in the supernatants by flame photometry.

Amylase was prepared by centrifugation, at 20,000 × *g* at 2°C, of a water extract of rectal pellet homogenate. Activity was measured iodometrically in a spectrophotometer at wave length 660 mμ, with colloidal starch as substrate.

RESULTS

Morphology of the soft pellet. When the soft pellets are passed they are glossy clusters in which the individual pellets are flattened where they contact one another. On being placed in distilled water or 0.1 N HCl, they imbibe water, separate, and become spheres similar to those found in the fundus of the stomach. The limiting membrane of the sphere is palpable, resists pressure and on being torn with a needle the contents of the pellet flow out into the liquid leaving a collapsed envelope. In the hind gut the membrane begins to form just posterior to the colon and by the time it reaches the lower rectum it has a well-defined morphology (fig. 1). The membrane is periodic acid Schiff-positive and the staining reaction is identical with that of the PAS-positive material in the epithelial cells of the hind gut (fig. 2).

The contents of the envelope as seen in the sections are largely microorganisms interspersed with undigested cell walls of plant material. The plant material can be separated out by gentle centrifugation for a minute or so. By repeated washing and centrifugation, adding the washings to the bacterial fraction each time, a reasonably clean separation of the pellet into bacterial and plant material fractions can be made.

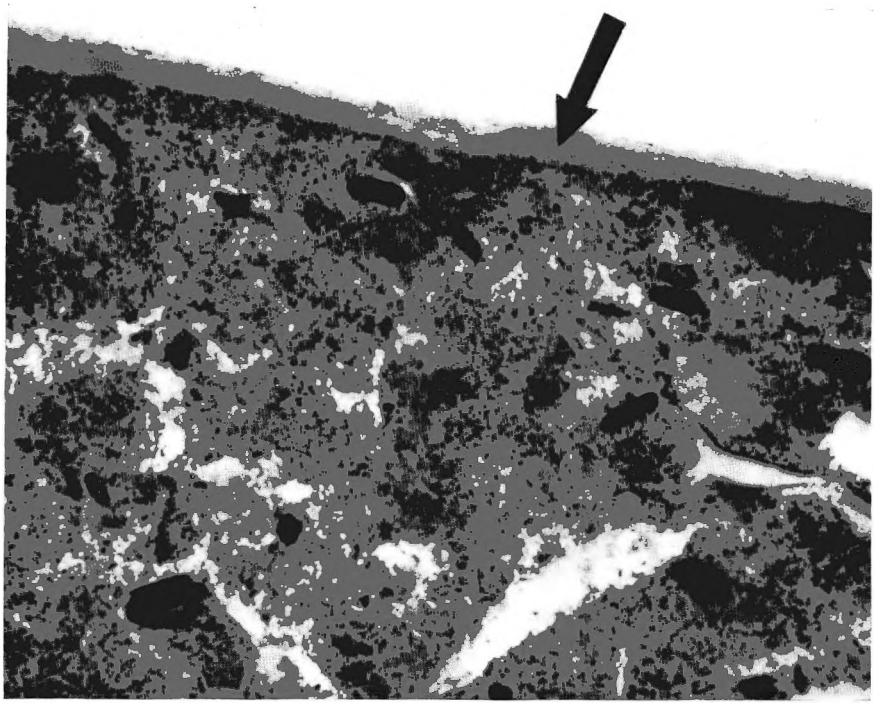

Fig. 1 Section of portion of a soft pellet showing fecal material bordered by a well-developed membrane. Hematoxylin and eosin. × 578.

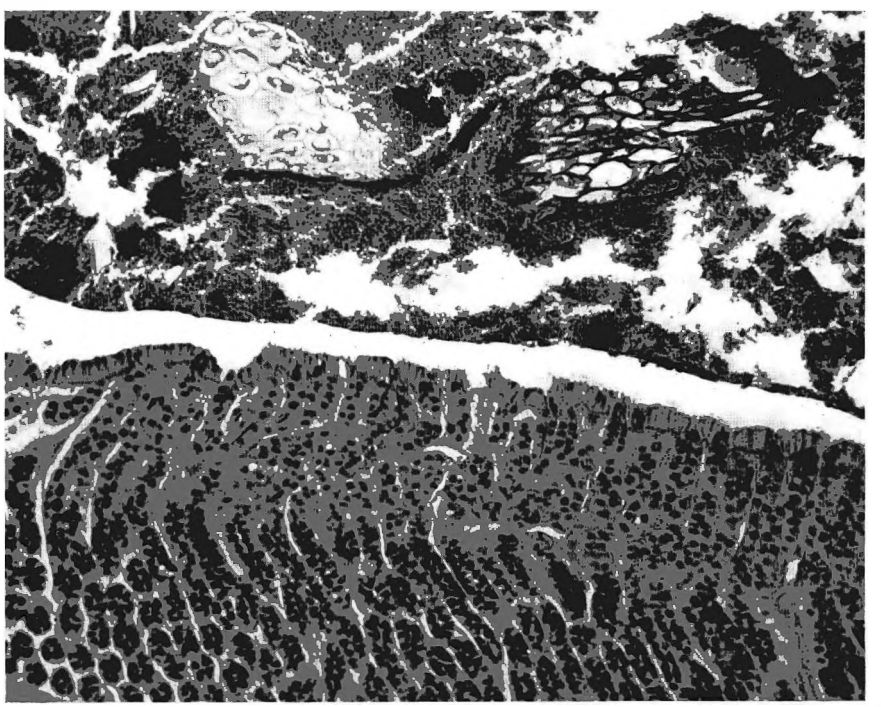

Fig. 2 Transverse section of upper rectum showing deposition of PAS-positive substance onto soft pellet material. Periodic acid-Schiff stain. × 128.

The bacteria constitute 56% of the dry weight of the pellet contents; conventional protein content of the bacterial fraction was found to be 36% and the plant fraction contained 11% protein. This gives an average figure of 24.4% protein for the pellet exclusive of envelope which agrees well with Eden's (3) figure of 28.5% for whole pellets.

Gram-stained smears of pellets taken from the rectum revealed a rich flora of Gram-positive cocci and coccobacilli — mainly diploids; and Gram-positive and Gram-negative filamentous and rod-like bacteria. Typical lactobacilli were isolated from pellet material cultured primarily on thioglycollate and sub-cultured in standard acetic acid broth (fig. 3). Anaerobic cultures consisted mainly of staphylococcus and enterococcus types.

The contents of pellets that had been in the fundus for 6 hours gave the same growth in nutrient media as the fresh rectal pellets did. This shows that the bacteria in the stomach, in the intact pellets at least, are viable up to 6 hours after ingestion.

The soft pellets in the stomach. The pellets are swallowed whole without mastication so that the enveloping membranes remain intact. The pellets pass to the fundic end of the stomach where they remain without mingling with the contents of the cardiac-pyloric part of the stomach until the stomach commences to empty. A few intact soft pellets can be found in the fundic contents 6 hours after ingestion but the majority are triturated by that time; even in that condition the pellet matter appears to be confined to the fundic end of the stomach.

Lactic acid content of the stomach and of the soft pellets from the rectum. The distribution of lactic acid in the stomachs of rabbits killed during coprophagy and the concentration of lactic acid in pellets taken from the rectum are shown in table 1.

The absolute amount of lactic acid in the fundus in all instances was higher than in the cardiac-pyloric part of the stomach, and in 8 out of 10 rabbits the concentration was higher in the fundus. The soft pellets taken from the rectum in

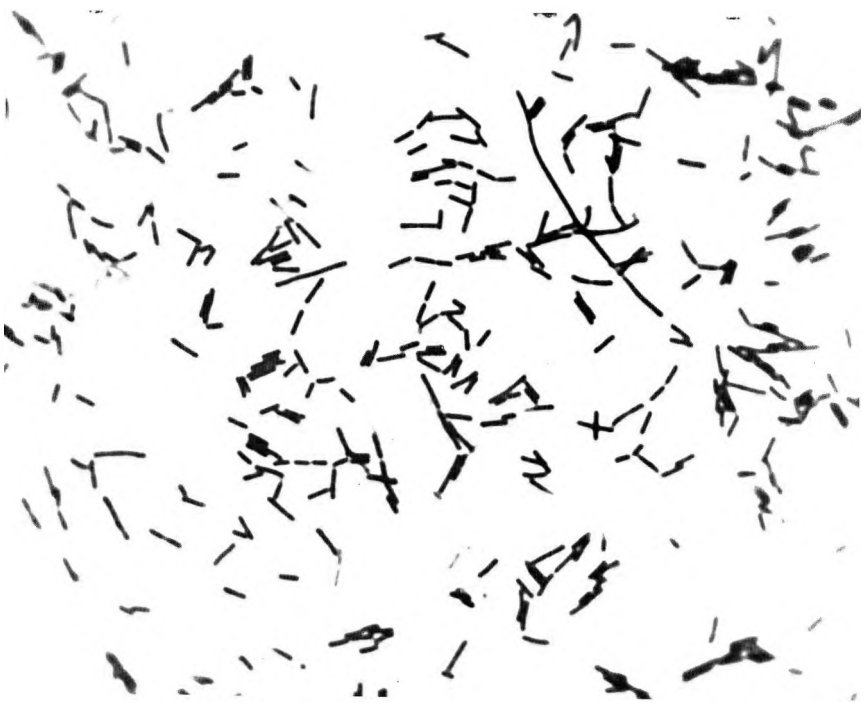

Fig. 3 Lactobacilli isolated from rectal pellet contents cultured primarily in Brewer's thioglycollate fluid medium, subcultured in standard acetic acid broth. Gram stain.

9 of the 10 rabbits contained lactic acid in as high or even higher concentration than in the fundus.

The level of lactic acid in stomach contents as a function of time. Accumulation of lactic acid in the stomach is a function of its rates of production and removal. In rabbits fed food containing 40 mg of lactic acid/25 g, there is a rapid disappearance of the lactic acid as the following data show (table 2).

By the time ingestion was complete (2 hours) the amount of lactic acid in the stomach had decreased to a low level. At 12.5 hours after commencement of ingestion a small increase in the lactic acid level was noted, associated with the presence of soft pellets in the fundus.

When oats were fed to rabbits allowed to practice coprophagy, despite the high rate of absorption, a relatively rapid accumulation of lactic acid took place soon after ingestion; thereafter the production declined sharply (table 3). When coprophagy was prevented the same marked increase occurred, but it took place some 6 hours later than in the controls. Doubtless the rapid accumulation of lactic acid in the stomachs of the controls was in part due to ingestion of lactic acid present in

TABLE 1

Amount and concentration of lactic acid in the stomach, and concentration of lactic acid in soft pellets taken directly from the rectum of 10 rabbits

Average total lactic acid		Average concentration of lactic acid		
In fundus[1]	In cardiac-pyloric part	In fundus	In cardiac-pyloric part	In rectal pellets from 9 rabbits
mg	*mg*	*mg/100 g dry wt*	*mg/100 g dry wt*	*mg/100 g dry wt*
2.5	1.2	47.3	34.6	41.3
(0.8–4.3)[2]	(0.2–2.9)	(23.0–76.4)	(9.7–62.9)	(21.1–72.3)

[1] Rabbits were fed ad libitum and were ingesting soft pellets when killed so that lactic acid in the fundus was determined in a mixture of food and soft pellets.
[2] Figures in parentheses indicate range.

TABLE 2

Amount of lactic acid in rabbit stomach as a function of time[1]

Rabbit	Time killed[2]	Soft pellets in fundus	Total lactic acid	
			In fundus	In cardiac-pyloric part
	hours		*mg*	*mg*
1 (Total intake of 37 mg lactic acid)	2	none	6.2	4.6
2 (Total intake of 40 mg lactic acid)	5	none	3.9	2.6
3	8	few	0.8	0.12
4	12.5	many	1.3	0.7
5	16.5	no formed pellets detectable	1.5	0.5
6	21.5	liquid pellet material	0.7	0.5
7	24.5	liquid pellet material	0.4	0.2

[1] Each rabbit consumed between 23 and 28 g of pelleted food, containing 40 mg lactic acid/25 g, over a period of 2 hours; lactic acid was incorporated in food in milk powder; amount of lactic acid determined by analysis.
[2] Rabbits were killed at the indicated times after starting to eat.

TABLE 3

Lactic acid content of stomachs from rabbits in which coprophagy was permitted or not permitted, and fed approximately 40 g of oats each

	Hours after commencement of ingestion													
	Coprophagy permitted							Coprophagy not permitted						
	2	5	8	11	13	14	22	2	5	8	11	13	14	22
Lactic acid, mg	2.0	11.5	23.0	3.5	4.7	8.0	nil	2.2	6.0	2.3	12.4	22.2	2.3	nil

the soft pellets but, as will be apparent from evidence given later, it is also likely that the soft pellets exerted a catalytic action on lactic acid accumulation.

Some of the lactic acid production in both instances was endogenous since ground-up oats suspended in distilled water and incubated at 38°C commenced to produce lactic acid at a steady rate after a lag period of 6 hours. The pH decreased from 6.00 to 3.95 during the test period of 23.5 hours (fig. 4).

In vitro formation of lactic acid by homogenates of rectal pellets. Suspensions of ground oats incubated at pH 5 to 6 with homogenates of rectal pellets formed lactic acid at a rapid rate which was linear with time (fig. 5). The rates were approximately the same under aerobic and anaerobic conditions (gas phases air or nitrogen). When colloidal starch, in excess, was used as a substrate the rate of lactic acid formation was slower than when oats were used as substrate (fig. 6). The glycolysis was strongly inhibited by a decrease in pH (fig. 7).

The slower rate of lactic acid production with starch as substrate may indicate that substrates other than starch, glucose for example, were available in oats. In support of this it was found with 3 different pellet homogenates that glucose was glycolyzed at a far more rapid rate than starch and that lactic acid can be formed from this substrate at pH 3 when the production from starch is nil. An example of these effects is given in table 4. It appears from this that the amylase present in the pellet homogenate is the rate-limiting enzyme at low pH and also because the amylase was strongly inhibited at low pH (fig. 7). The amylase, however, retained about 90% of its activity at pH 5.0.

In vitro formation of lactic acid by morphologically intact rectal pellets. It was noted that the homogenates of rectal pellets in the previous experiments had considerable buffering power. For example 7 g wet weight of rectal pellets made into a slurry with 5 ml of water had a pH of 6.4. Addition of 3 ml of HCl of pH 0.8 reduced the pH by less than one unit to 5.6. A suggestion as to the identity of the buffer was found in Eden's (3) analysis of rectal pellets. He noted considerable

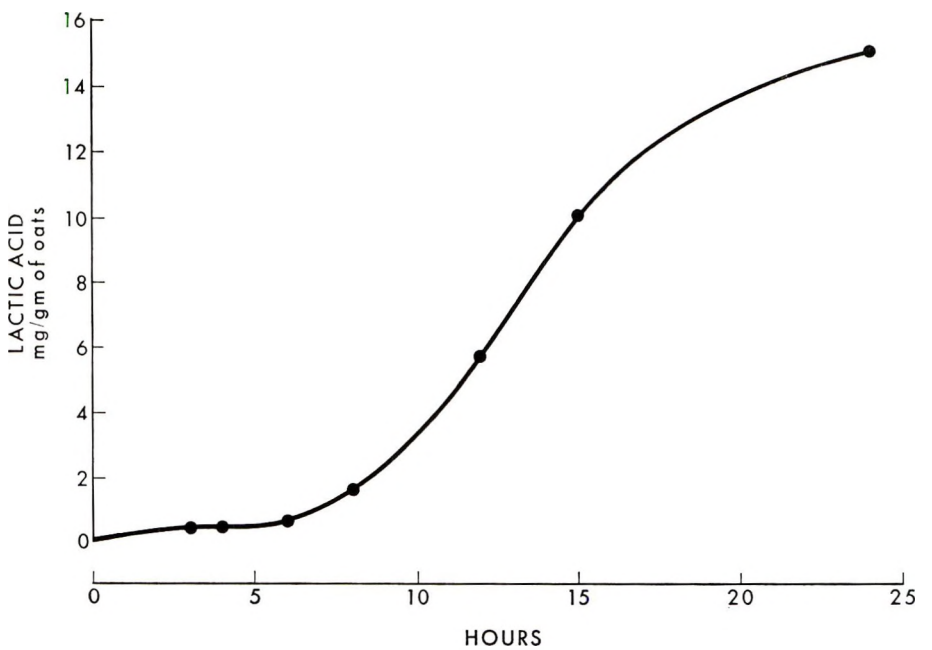

Fig. 4 Lactic acid production by ground oats suspended in distilled water. Reaction mixture: 4 g oats in 50 ml water; temperature 38°C; initial pH, 6.00; pH at 10 hours, 4.65; at 23.5 hours, 3.95.

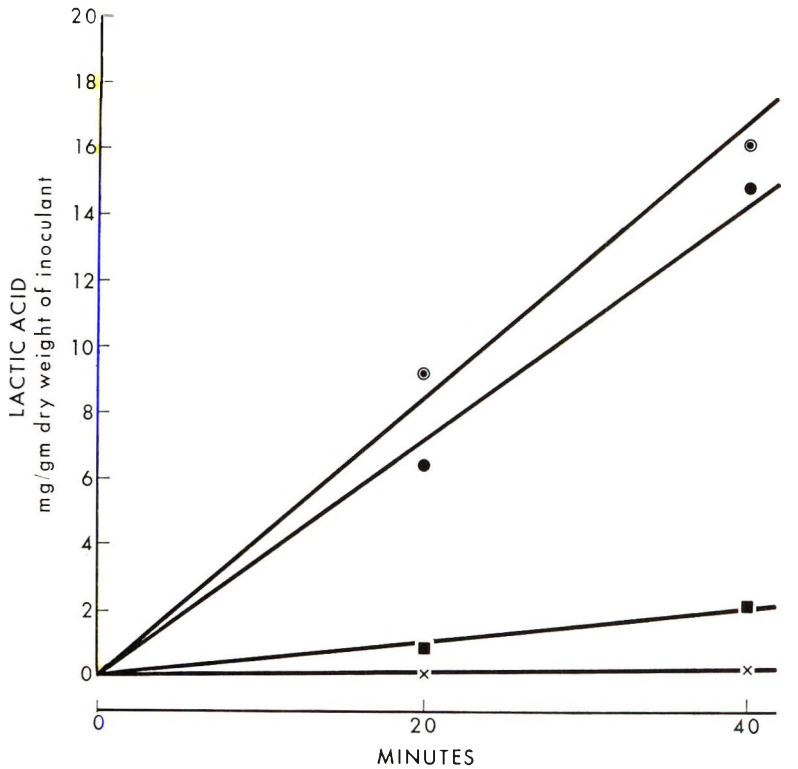

Fig. 5 Lactic acid production by a homogenate of rectal soft pellets with ground oats as substrate. Reaction mixture: 4 g ground oats suspended in 45 ml water; 5 ml homogenate of rectal soft pellets, final concentration 5.2 mg/ml; temperature 38°C; pH 5.15. Homogenate added at zero time. Key: · ——— ·, complete system, gas phase air; ⊙ ——— ⊙, complete system, gas phase N_2; ■ —— ■, homogenate alone, no substrate, gas phase N_2; × ———×, oats alone, no homogenate, gas phase air.

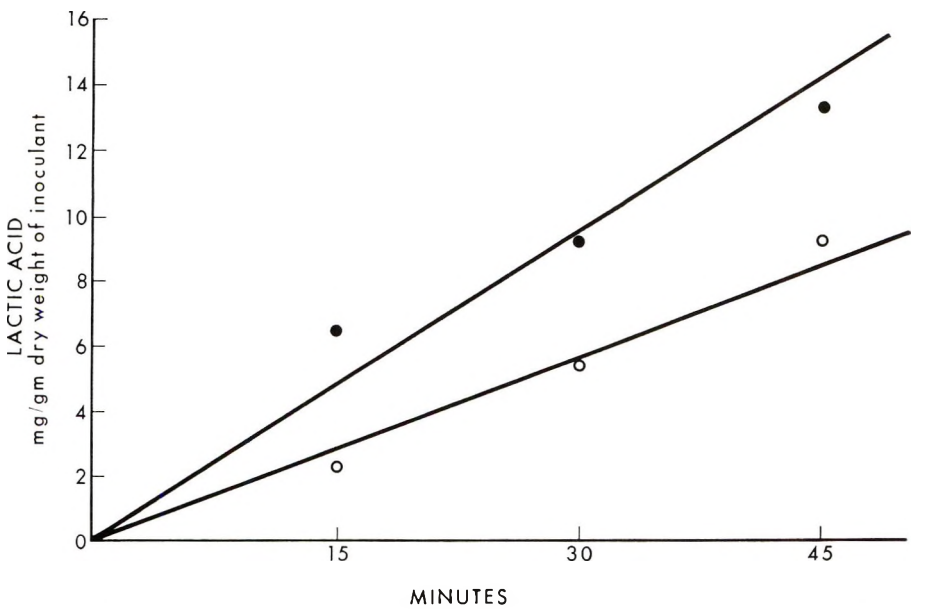

Fig. 6 Lactic acid production by a homogenate of rectal soft pellets with potato starch or oats as substrate. Reaction mixture: 500 mg starch or 4 g ground oats in 45 ml water:5 ml homogenate of rectal pellets, final concentration 9 mg/ml; temperature 38°C; pH 6.2; homogenate added at zero time. Key: · ——— ·, oats ○ ——— ○, starch.

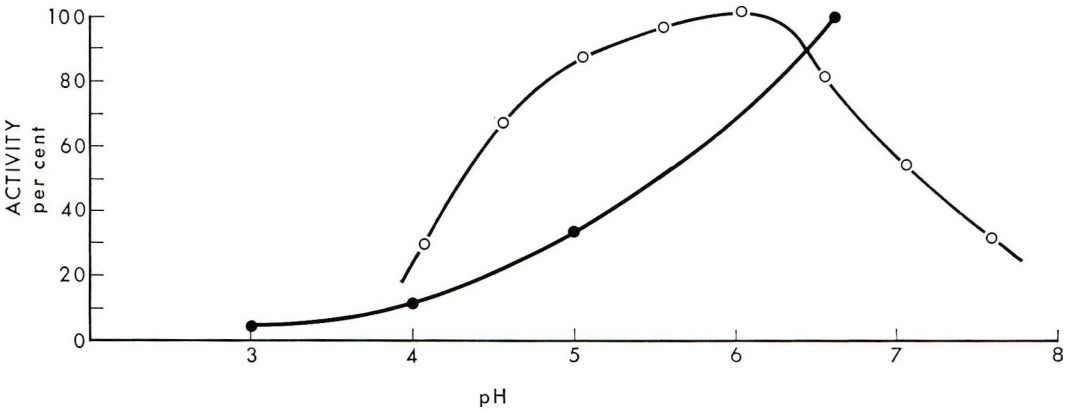

Fig. 7 Effect of pH on production of lactic acid from starch by a homogenate of rectal soft pellets, and on amylase activity of soft pellet preparations. Activities for lactic acid production calculated from 40-minute time curves; activities for amylase from 30-minute time curves. Activities calculated as a percentage of the maximal velocities observed. Glycolysis reaction mixture: 500 mg potato starch in 45 ml water; 5 ml homogenate of rectal pellets, final concentration 10.9 mg/ml; temperature 38°C. Amylase reaction mixture: 4 ml starch (2 mg/ml), 5 ml 0.1 M phosphate buffer, 1 ml 0.5 M NaCl, 6 ml enzyme preparation; temperature 30°C. Enzyme concentration limiting. Key: · ——— ·, lactic acid formation ○ ——— ○, amylase activity.

quantities of sodium, potassium, and phosphorus in the ash from soft pellets.

Some of the phosphorus is in the form of inorganic phosphate since a cold water extract of soft pellets from our rabbits gave a strong color with the Fiske and Subba Row method for inorganic P. The concentration of phosphate in the pellet would depend on the free water present and since the pellets imbibe water, that concentration must fall after ingestion. It was found that one gram of fresh pellets would take up 0.7 ml water; presumably something like this uptake occurs in the stomach; hence it would be reasonable to assume that for every gram of pellet in the stomach at least 0.7 g is free water. From the data in table 5 it can be concluded, if it is also assumed that most of the sodium and potassium is in the form of Na_2HPO_4 and KH_2PO_4, that pellet juice acts as a solution of phosphate buffer with a strength of about 0.13 M. Such a solution would have a powerful buffering action in the range pH 4 to 7, and no doubt calcium phosphate contributes to that buffering effect. This suggests that the internal pH of the pellet would be high enough to permit fermentation even when the external pH is acid, and actually it was found that intact pellets could ferment glucose and starch when placed in HCl of pH 2.4 (table 6).

When soft pellets were placed in the acid reaction mixture the pH increased steadily, and it was found that the inorganic P content of the reaction mixture likewise increased steadily so that the increase in pH could be ascribed to leakage of phosphate buffer from the pellets; if the

TABLE 4

Lactic acid production from starch or from glucose by a homogenate of rectal pellets[1]

Substrate	pH of reaction mixture	Rate of lactic acid production
		mg/g dry weight of inoculant/hour
Glucose	6.0	27.60
Starch	6.0	9.90
Glucose	3.0	0.53
Starch	3.0	nil

[1] Substrate concentration 5 mg/ml; reaction mixture 20 ml of substrate, 10 ml of homogenate (final concentration of 86 mg/ml dry weight of pellet material).

TABLE 5

Phosphate phosphorus, sodium, and potassium levels in rectal pellets

P	Na		K	
mg/g wet wt of pellet	*mg/g wet wt of pellet*	*Molarity as* Na_2HPO_4[1]	*mg/g wet wt of pellet*	*Molarity as* K_2HPO_4[1]
11.2	2.1	0.064	3.7	0.064

[1] Assuming 0.7 ml H_2O/g pellet.

pH was to be maintained between 2.4 and 4.0, for example, small amounts of 10 N HCl were added from time to time. When the pH was maintained within those limits glucose only was glycolyzed; if the pH was allowed to increase, starch was broken down to lactic acid.

The increase in pH *in vitro* brought about by soft pellets was paralleled by an increase in pH of the rabbit stomach during coprophagy. Before ingestion of soft pellets the whole of the stomach was uniformly acid (table 7), but as the pellets were ingested the pH increased, and the highest value was noted in the fundus.

TABLE 6

Concentration of lactic acid in intact soft pellets before and after incubation in starch or glucose at various pH levels[1]

Substrate	pH[2]	Lactic acid	
		Zero minutes	60 minutes
		mg/g dry wt of pellet	
Nil			0.42
Glucose	2.4–4.0	0.34	5.00
Starch			0.50
Glucose	2.2–4.3		7.80
Starch	2.2–4.3	0.55	0.67
Starch	3 –6.2		5.50
Starch	2.5–5.5	0.69	3.60

[1] Substrates 5 mg/ml, 25 to 30 intact pellets added to 20 ml substrate; temperature, 38°C.
[2] The figures indicate the range of pH observed in the substrate throughout the test run; 10 N HCl was added to bring the pH to the lower limit at the beginning of the test; additional HCl was added from time to time to keep pH within the upper limit.

TABLE 7

Effect of ingestion of soft pellets on pH of the stomach of wild rabbits in captivity[1]

Rabbit	Time A M	Rectal pellets	Fundic pellets	pH of fundus	pH of cardiac-pyloric portion of stomach
1	6.30	nil	nil	2	1.9
2	7.10	nil	nil	3.5	2.5
3	7.45	+	+	4.2	3.2
4	8.00	+	+	4.4	3.5
5	8.15	+	+	4.6	2.2
6	8.30	+	+	4.4	3.2
7	9.00	+	+	4.0	2.3
8	9.30	+	+	5.1	2.3

[1] Coprophagy commences at about 7.30 A M in wild rabbits.

DISCUSSION

Our results show that presence of lactic acid in the rabbit stomach is due to at least 3 processes: (1) ingestion of preformed lactic acid in soft pellets; (2) endogenous production by feeds such as oats; and (3) by fermentation carried out by the bacteria of the soft pellets. Alexander and Chowdhury (2) (see (11)) observed that as fermentation proceeded and lactic acid accumulated, the pH of the stomach increased despite the acid formation. It appears from our results that that increase was due to liberation of phosphate buffer from the soft pellets. This, no doubt, facilitates fermentation but the experiments with intact soft pellets, *in vitro*, show that fermentation proceeds even when the external environment is acid.

It is possible that the endogenous formation of lactic acid by oats is due to fermentation by microorganisms adhering to the husks.

Doubtless salivary amylase plays a part in glycolysis in the stomach, but a sustained production of amylase from bacteria is the more likely mechanism of starch breakdown since Coleman and Elliott (12) showed that the production of α-amylase by bacteria is a process of secretion rather than a passive release from lysed bacterial cells. It is perhaps significant that the amylase of pellet homogenate is adapted to acid conditions in that 88% of its activity is exhibited at pH 5.0, whereas salivary amylase is only 30% active at that pH. The 12 enzymes necessary to glycolyze the glucose units of the starch are undoubtedly supplied by the lactobacilli.

In conclusion, we consider that the fundus of the rabbit stomach, loaded with soft pellets, is analogous to the rumens of sheep and cattle in that it is a region of the stomach maintained at relatively high pH and capable of breaking down carbohydrate of high molecular weight. The analogy is strengthened because lactic acid is produced in large amounts in the rumens of sheep fed a diet rich in starch (13).

ACKNOWLEDGMENT

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Influence of Dietary Ca and P Levels on the Mg Requirement of the Chick[1,2]

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ABSTRACT The effect of the levels of calcium and phosphorus in the diet upon the magnesium requirement of young chicks was studied. When either the calcium or phosphorus content of the diet was increased, the quantity of magnesium required in the diet for maximal weight gain increased. When the levels of supplemental calcium and phosphorus in the diets were 0.6% Ca and 0.3% P, 0.6% Ca and 0.6% P, 0.6% Ca and 0.9% P, 1.2% Ca and 0.3% P, 1.2% Ca and 0.6% P, and 1.2% Ca and 0.9% P, the magnesium requirement for maximal growth was 461, 524, 562, 517, 577 and 594 ppm magnesium in the diet, respectively. A toxicity study was conducted and a level of 6400 ppm of magnesium in the diet caused a decrease in growth rate and an increase in mortality.

Tufts and Greenberg (1) showed that the magnesium requirement of the rat was increased by an increase of supplemental calcium in the diet. However, as calcium was increased in the diet, phosphorus also was increased. O'Dell et al. (2) indicated that in guinea pigs and rats the magnesium requirement was elevated with an increase of phosphorus in the diet. Finally, McAleese and Forbes (3) showed that the magnesium requirement of the rat was increased as the calcium content of the diet increased.

Nugara and Edwards (4) have shown that an increase of either calcium or phosphorus in the diet of the chick interfered with the metabolism of magnesium. This experiment was designed to estimate the magnesium requirement of the chick when the diet contained various levels of calcium and phosphorus.

EXPERIMENTAL

One-day-old White Plymouth Rock cockerels were used in this work. They were housed in electrically heated battery brooders with wire-mesh floors and were supplied feed and water ad libitum. The purified diets used were the same as described in an earlier publication (4). The basal diet contained (by analysis) 23 ppm magnesium and 0.15% phosphorus.

The experimental design of the magnesium requirement study was a 2 by 3 by 5 factorial involving 2 levels of calcium, 3 of phosphorus and 5 of magnesium.[3] A total of 720 chicks was used with 12 chicks/pen and 2 pens/treatment. When the chicks were 3 weeks old, they were weighed and 3 chicks were selected at random from each pen for obtaining samples for the analytical work. The chicks were killed and the left tibia removed for chemical analysis.

To determine the amount of supplemental magnesium that would have a toxic effect in the diet of the chick, the basal diet (0.3% supplemental phosphorus and 0.6% supplemental calcium) was fed to chicks supplemented with magnesium levels of zero, 1600, 3200, 6400 and 12,800 ppm. Tibia were obtained at the end of the 3-week experimental period and analyzed for ash and magnesium. Bone ash

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[3] The supplemental levels used were calcium (0.6 and 1.2%), phosphorus (0.3, 0.6 and 0.9%) and magnesium (50, 100, 200, 400, and 800 ppm). These levels were obtained by manipulation of sodium acid phosphate, potassium acid phosphate, sodium chloride, potassium chloride, tricalcium phosphate, and calcium carbonate. In all diets 0.6% Ca and 0.3% P were supplied as tricalcium phosphate. When the diets contained 0.6% P or 0.9% P the additional phosphorus was supplied one-half from sodium acid phosphate and one-half from potassium acid phosphate. All additional calcium above 0.6% was supplied from calcium carbonate. Sodium and potassium were kept constant at 0.30 and 0.315% of the diet by manipulating sodium chloride and potassium chloride when sodium acid phosphate or potassium acid phosphate were used. The various levels of magnesium were obtained by using magnesium carbonate.

was analyzed by the AOAC method (5) and magnesium by that of Simonsen et al. (6).

Edwards et al. (7) have indicated that the magnesium requirement was increased under normal contaminated laboratory conditions. Both the requirement and toxicity studies were conducted in a laboratory which was not fumigated.

RESULTS AND DISCUSSION

When the level of calcium and phosphorus in the diet was low (Ca, 0.6%; P, 0.3%), some of the chicks were able to survive to 3 weeks when only 50 ppm of supplemental magnesium was in the diet (table 1). The chicks that survived at this level of magnesium were actually able to grow at a fairly rapid rate, such that only moderate increases in weight gain were apparent from the addition of more magnesium. The requirement for maximal growth rate estimated by the method described by Almquist (8) was only 135 ppm magnesium in the diet.

When the supplemental phosphorus level was maintained constant at 0.3% and the calcium level increased to 1.2%, the number of survivors at low levels of supplemental magnesium was increased. The weight gain of survivors at the 50 ppm level of supplemental magnesium was lowered and sizeable increases in the rate of gain were apparent when 100 and 200 ppm of supplemental magnesium were added to the diet and the requirement was estimated at 225 ppm magnesium in the diet for maximal growth rate.

When the calcium level was maintained at 0.6 or 1.2% and the phosphorus level increased from 0.3 to 0.6 to 0.9%, the mortality was extremely high in the groups of chicks fed low levels of magnesium (table 1). The requirement for magnesium was also greatly increased. When the data were graphed, the slope of the response curve to magnesium supplementation increased tremendously as the level of phosphorus in the diet increased. This means that not only was the requirement for magnesium higher at these high phosphorus levels, but that the requirement is much more critical and a small deficiency may result in a marked decrease in growth rate.

When the phosphorus level was maintained constant and the level of supplemental calcium increased, the requirement for magnesium also increased. The effect of increasing the calcium level on the growth rate of chicks receiving low levels of magnesium was variable at the various phosphorus levels. This was probably due to the well known interference of calcium with phosphorus when only small amounts of phosphorus are present in the diet, such that the relationship between the 3 elements was extremely critical in some instances.

TABLE 1

Growth and mortality observed at three weeks when calcium, phosphorus and magnesium were varied in the diet

Supplemental Mg	Supplemental Ca and P levels, % of diet					
	Ca P	Ca P	Ca P	Ca P	Ca P	Ca P
	0.6 0.3	1.2 0.3	0.6 0.6	1.2 0.6	0.6 0.9	1.2 0.9
ppm	*g*	*g*	*g*	*g*	*g*	*g*
50	256(13)[1]	195(5)	105(22)	141(22)	—(24)	—(24)
100	278(14)	237(4)	177(20)	263(20)	88(23)	—(24)
200	281(3)	282(0)	279(5)	308(7)	201(17)	218(19)
400	290(0)	290(1)	268(3)	341(2)	240(5)	327(2)
800	281(2)	275(1)	264(0)	341(0)	228(2)	323(0)
Requirement estimate using method of Almquist (8)	135	225	207	295	261	420

[1] Number of chicks that died of the 24 chicks started on each treatment.

TABLE 2

Bone ash and magnesium in the tibiae when calcium, phosphorus and magnesium were varied in the diet

Supplemental Mg	Supplemental Ca and P levels, % of diet											
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
	0.6	0.3	1.2	0.3	0.6	0.6	1.2	0.6	0.6	0.9	1.2	0.9
	Bone ash[1]	Mg	Bone ash	Mg	Bone ash	Mg	Bone ash	Mg	Bone ash	Mg	Bone ash	Mg
ppm	%	%	%	%	%	%	%	%	%	%	%	%
50	37.8	0.61	29.5	0.61	36.2	0.49	42.7	0.30	—	—	—	—
100	34.3	0.72	28.1	0.70	35.6	0.57	40.3	0.51	34.1	0.53	—	—
200	34.3	0.81	29.0	0.74	35.5	0.73	41.4	0.67	37.5	0.70	41.1	0.50
400	35.0	0.90	28.0	0.86	33.2	0.82	41.8	0.84	35.9	0.88	39.2	0.85
800	34.6	1.05	27.2	0.71	35.3	0.97	39.8	0.83	34.9	0.85	38.9	0.87

[1] Bone ash expressed as percentage of fat-free dry tibia, magnesium expressed as a percentage of bone ash.

Plotting of the estimated magnesium requirement against the level of supplemental phosphorus in the diet indicates that the increased magnesium requirement is a linear response to the increase in phosphorus, but that the slope of the line is greater when the calcium level is high (1.2% vs. 0.6%), which then indicates an interaction between calcium and phosphorus on the magnesium requirement of the chick.

There was no statistically significant effect of the magnesium on the bone ash (table 2). The percentage of bone ash in the groups receiving 1.2% calcium and 0.3% phosphorus was very low since phosphorus was the limiting factor. Bone ash values of a reasonable magnitude were obtained in the groups receiving the diets supplemented with 1.2% calcium and 0.6% phosphorus. There was a tendency for the magnesium in the bone ash to be lowered when the calcium in the diet was increased from 0.6% to 1.2%, at any level of phosphorus in the diet. The same pattern was observed when the phosphorus content of the diet was increased. Therefore, it is evident that both high levels of calcium or phosphorus in the diet prevent the deposition of magnesium in the bone. Increasing the magnesium in the diet caused the bone magnesium to increase.

The results of the toxicity study are presented in table 3. It appears from these data that some quantity between 3200 and 6400 ppm of supplemental magnesium in the diet tested is toxic for chicks as measured by growth and mortality. A further increase in supplemental magnesium level to 12,800 ppm in the diet caused marked growth depression and increased mortality. Increasing dietary magnesium levels caused a decrease in bone ash. However, an increase in the magnesium content of the ash occurred. A possible explanation of these results might be that high dietary magnesium levels interfered directly with the process of calcification or the high dietary magnesium level may be preventing absorption of the calcium ion from the intestine since calcium and magnesium absorption from the intestine is by way of a common mechanism for divalent ions (9). Neuman and Neuman (10) have indicated that the magnesium ion is found on the crystal surface of the bone. They suggested that magnesium exchanges with surface calcium because it was adsorbed from solution by precipitates of basic calcium phosphate. Sobel et al. (11) demonstrated that in the young rat the diet

TABLE 3

Toxicity of magnesium to chicks[1]

Supplemental Mg	Avg wt 3 weeks	Mortality[2]	Bone ash[3]	Mg[4]
ppm	*g*		%	%
0	194	23	36.11	0.48
1600	306	2	31.55	1.07
3200	305	1	31.39	1.17
6400	262	6	27.39	1.48
12800	189	11	24.70	1.40

[1] Data for chicks fed 50, 100, 200, 400 and 800 ppm Mg are available in tables 1 and 2, under the column headed Ca, 0.6, P, 0.3.

[2] Twenty-four chicks were started on each dietary treatment.

[3] Bone ash expressed as percentage of fat-free dry tibia.

[4] Mg expressed as percentage of bone ash.

greatly influences bone formation. It appears that in the present experiment the high magnesium-low calcium diet may have caused magnesium to replace calcium by adsorption at the bone surface thereby resulting in the higher magnesium content of the bone (table 3).

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Erythrocyte Survival of Rats Deficient in Vitamin E or Vitamin B_6[1]

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ABSTRACT Rats were fed diets deficient in vitamin E or B_6, and the characteristic changes in growth rate, urinary creatine-to-creatinine ratios, and urinary xanthurenic acid output were observed. The maximal erythrocyte survival of rats fed a vitamin E-deficient diet for 138 days was less than values obtained from replete animals, as determined by the Cr^{51} method. After the diet was fed for 265 days, the difference was even greater. The bodies and organs of vitamin E-deficient rats contained more Cr^{51} than those of replete controls. Vitamin B_6 deficiency resulted in a small, but significant, increase in maximal survival time of erythrocytes. Whole-body Cr^{51} determinations did not reveal a difference between vitamin B_6-deficient and replete rats. The significance of these results is discussed in relation to blood destruction and sequestration in the reticuloendothelial system.

Although testicular damage, placental inadequacy and muscular atrophy are well known results of vitamin E deficiency in the rat (1), alterations in the life span of rat erythrocytes (RBC) have not become well known. In fact it has been stated that vitamin E deficiency is not characterized by increased destruction of RBC intravascularly (2, 3). The demonstration of a delayed anemia[2] and a decreased RBC survival time in monkeys (4) fed a vitamin E-deficient diet stimulated a similar study in rats. Since pyridoxine deficiency was shown to result in anemia in rats by Kornberg et al. (5) and later by Dinning and Day (6), a parallel study of this deficiency was included. The results of these studies are the substance of this report.

METHODS

Weanling male rats of the Holtzman strain were fed a basal diet deficient in both vitamin B_6 and vitamin E, consisting of the following: (in grams) vitamin-free casein, 18.6; sucrose, 67.4; lard,[3] 8.0; cod liver oil, 2.0; salt mix,[4] 4.0; (in mg) choline chloride, 100; inositol, 10; riboflavin, 0.8; thiamine·HCl, 0.5; Ca pantothenate, 2; nicotinic acid, 2; menadione, 0.44; folic acid, 0.8; and vitamin B_{12}, 0.01. Diet for control animals contained 10 mg pyridoxine·HCl/kg of basal mixture, and the rats received *dl*-α-tocopheryl acetate individually, 2 mg semi-weekly until body weight reached 100 g and 4 mg semi-weekly thereafter. Animals in groups fed deficient diets, received the basal diet lacking the appropriate supplement; all animals received their diet and water ad libitum.

After rats had been fed the vitamin B_6-deficient diet for 53 days or the vitamin E-deficient diet for 138 days and 265 days, blood from both deficient and replete rats was tagged with Cr^{51} as sodium chromate, and maximal survival times were determined as described previously (7). In addition whole-body counts were made using a 4 π liquid scintillation gamma detector, and the biological decay was represented as percentage of initial counts. Upon termination of the experiment, the animals were killed, and selected organs were removed and weighed. The gamma-emitting isotope content of each organ was determined with a scintillation crystal well-counter.

Xanthurenic acid in the urine of rats fed vitamin B_6-deficient and replete diets was determined (8) following a tryptophan load. Urinary creatine-to-creatinine ratios were obtained (9) for rats fed

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[2] Day, P. L., and J. S. Dinning 1956 Anemia and vitamin E-deficient monkeys. Federation Proc., 15: 548 (abstract).

[3] Stripped lard, Distillation Products, Inc.

[4] Salt mix, Hubbell, Mendel and Wakeman, J. Nutrition, 14: 273, 1937 puchased from Nutritional Biochemicals Corporation, Cleveland.

vitamin E-deficient and replete diets at two different times during the course of the study.

RESULTS AND DISCUSSION

The growth curves of the 3 groups of rats are shown in figure 1. The expected growth retardation resulting from vitamin B_6 deficiency was observed. Although the average body weights of vitamin E-deficient rats were significantly less than those of replete animals, muscular impairment was not grossly evident. Urinary creatine-to-creatinine ratios, however, supported the conclusion that muscle metabolism was disturbed. After vitamin E had been with-

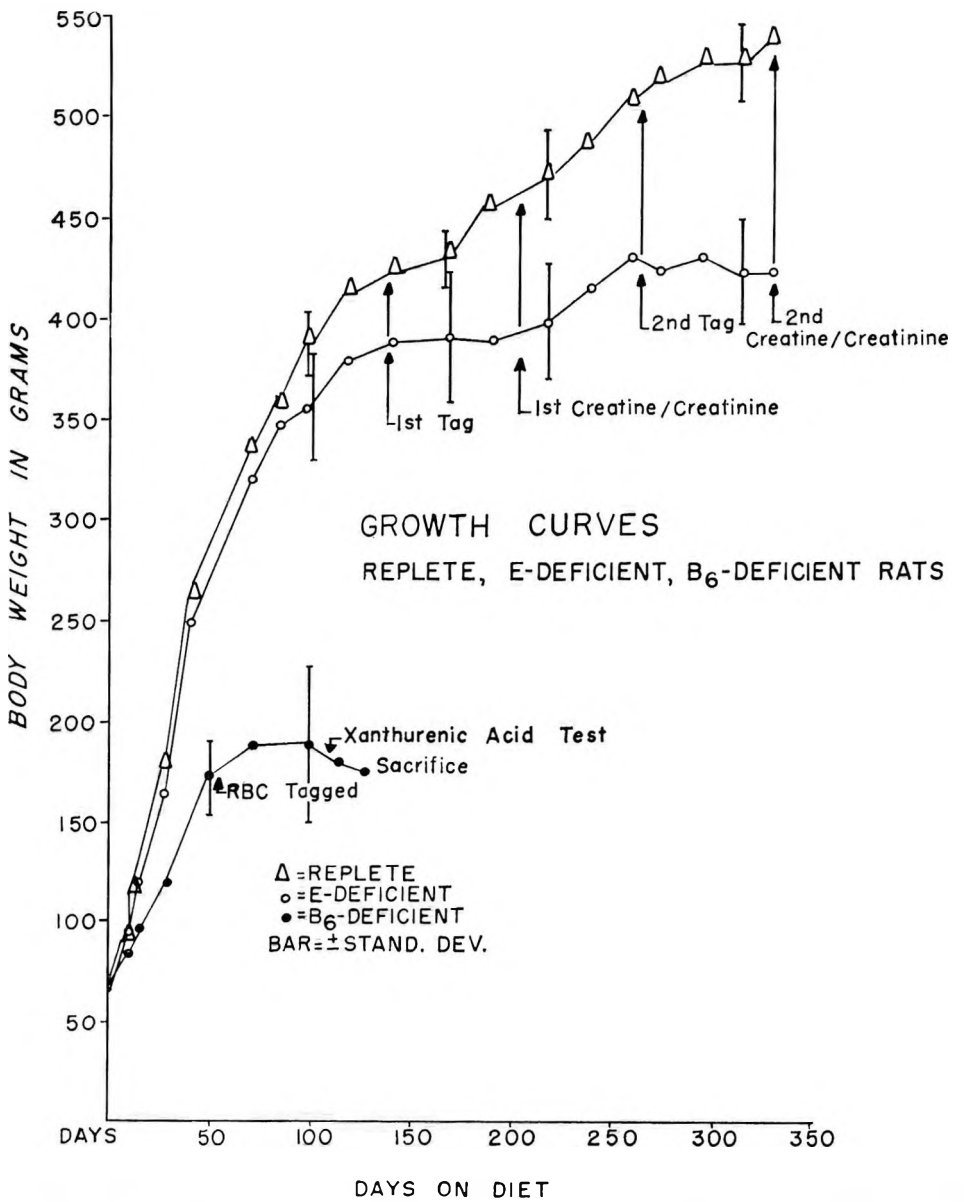

Fig. 1. Growth curves of replete, vitamin E-deficient and vitamin B_6-deficient rats. The vertical bars represent plus and minus one standard deviation.

TABLE 1

Mean maximal erythrocyte survival times of replete and deficient rats

Diet	First tag				Second tag			
	No. rats	Fed diet	Maximal survival	P[1] less than	No. rats	Fed diet	Maximal survival	P[1] less than
		days	*days*			*days*	*days*	
Replete	6	138	59	—	5	265	64	—
Vitamin E-deficient	10	138	53	0.01	10	265	53	0.02
Replete	6	53	59	—	—			
Vitamin B_6-deficient	13	53	62	0.02	—			

[1] *P* = probability from *t* test relating deficient to replete group.

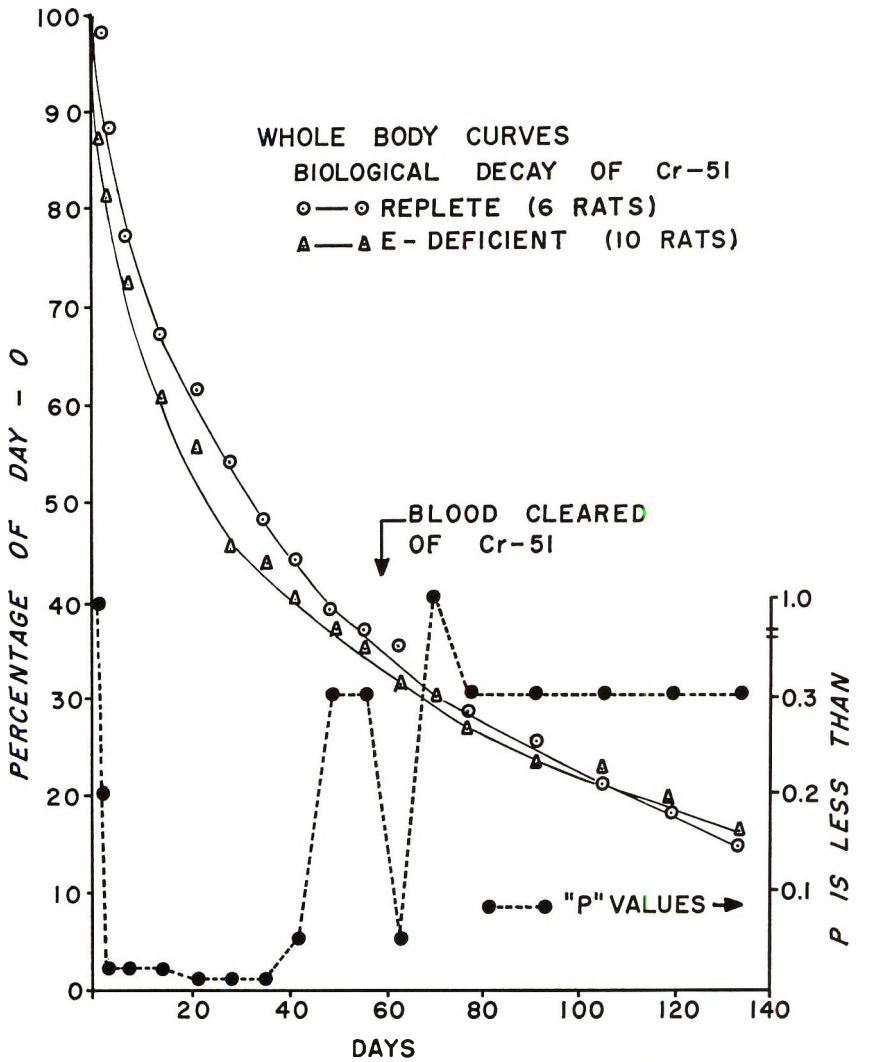

Fig. 2 Curves representing biological decay of whole-body Cr^{51} in vitamin E-deficient and replete rats. From zero to 60 days is period of erythrocytic destruction; beyond 60 days is period of reticuloendothelial sequestration. The curves are significantly different until about the fiftieth day (see curve of P values).

held 204 days, the mean ratio for replete rats was 0.19 (0.11 to 0.39), and for vitamin E-deficient rats it was 1.36 (0.85 to 2.10). After a total of 330 days, the values were 0.16 (0.03 to 0.31) and 3.48 (0.95 to 9.17), respectively. The increase from 1.36 to 3.48 for the vitamin E-deficient rats was statistically significant and validates the progressive nature of the deficiency. Urinary xanthurenic acid determinations were carried out after the rats had been fed the vitamin B_6-deficient diet for 110 days to verify the efficacy of the diet in producing this deficiency. Three animals from each group were selected and the following results obtained: vitamin B_6-deficient, 4.1 mg/24 hours (3.8 to 4.2); replete, 0.168 mg/24 hours (0.11 to 0.18).

The results obtained from an analysis of the RBC survival curves are summarized in table 1. The value and validity of the time of complete clearance of Cr^{51} from the blood for demonstrating fine differences in RBC survival have been discussed elsewhere (10), and for the reasons expressed there, only the averages of these

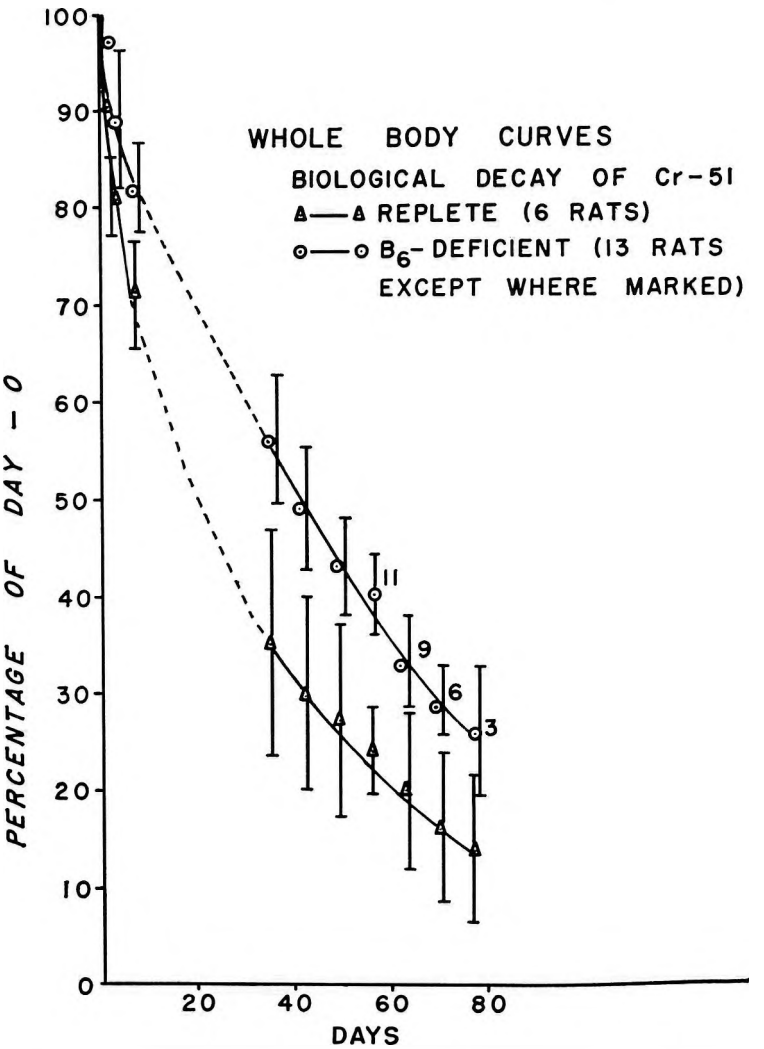

Fig. 3 Curves representing biological decay of whole-body Cr^{51} in vitamin B_6-deficient rats. Dotted portions of curves indicate period during which determinations could not be made.

times are reported here. It can be concluded that vitamin E deficiency shortens the survival time, statistically significant at the 0.01 level according to the *t* test, as early as 138 days with the deficient diet. After the rats had eaten the diets for 265 days, the absolute difference between RBC survival times of replete and vitamin E-deficient rats increase from 6 to 11 days, but due to increased variability, this difference is significant at the 0.02 level. Any comparisons of survival time should be made between groups of the same ages, as evidenced by the data in table 1. Vitamin B_6 deficiency prolonged the survival time of erythrocytes slightly, but significantly. Although hemoglobin synthesis is impaired by vitamin B_6 deficiency (6), the cells are capable of at least a normal life span. On the other hand, if life span is dependent upon envelop lipo-protein or upon intracellular enzyme content, vitamin B_6 deficiency does not interfere detrimentally with the synthesis of these constituents. Vitamin B_6 deficiency may actually reduce the catabolic phase of erythrocyte metabolism and protect the erythrocyte by prolonging the useful life of its constituents.

Whole-body count curves for vitamin E-deficient and B_6-deficient rats are reproduced in figures 2 and 3, respectively. These curves each represent the total gamma ray production by the nuclides present in each animal, only an insignificant part of which arises from nuclides other than Cr^{51}. Each curve is a composite of 3 primary processes, of which loss of Cr^{51} from the blood and loss from the reticuloendothelial (RE) organs are the most contributory. On this basis one may conclude from figure 2 that vitamin E deficiency is detrimental to RBC primarily if not entirely while they are in the circulation, since it is only during this period that the whole-body count curves are significantly different. Once the blood has been cleared of Cr^{51}, further loss by the vitamin E-deficient rats is slower than in replete animals, and although the difference is not statistically significant, data from organ counts given in table 2 strengthen this interpretation. In the case of vitamin B_6-deficient rats, however, the loss of Cr^{51} from the body occurs at a much slower rate than in replete animals. Due

TABLE 2

Weights and chromium content of organs of vitamin E-deficient and replete rats

	Liver			Spleen			Kidneys			Marrow		Body counts at death
	Weight	Activity		Weight	Activity		Weight	Activity		Weight	Activity	
	g	*count/mg*	*count/organ*	*g*	*count/mg*	*count/organ*	*g*	*count/mg*	*count/organ*	*mg*	*count/mg*	*count/min*
Replete	12.285	0.141	1711	0.680	10.35	6922	3.05	0.242	733	63	0.427	2238
Vitamin E-deficient	12.047	0.282	3317	0.953	13.91	13161	3.10	0.347	1075	50	0.750	8961
P less than	0.80	0.01	0.01	0.05	0.50	0.01	0.80	0.01	0.01	0.10	0.20	0.01

to death of deficient animals, counting could not be continued long enough beyond the sixtieth day to determine the nature of the whole-body curves during the period subsequent to blood clearance. One can conclude that Cr^{51}, either in the circulation or RE system is retained more effectively by the pyridoxine-deficient rats.

Organs obtained at death from replete and vitamin E-deficient animals were weighed, and Cr^{51} counts made on either the entire organ or an aliquot thereof. The results are summarized in table 2. The mean body weights are not included, but those of vitamin E-deficient animals were significantly less than those of replete rats. The data in table 2 demonstrate that more Cr^{51}, and therefore erythrocytic residua, is accumulated by the liver, spleen and kidneys of vitamin E-deficient rats than of replete rats. With the exception of the spleen, this is true both on a unit weight and total weight basis. The mean Cr^{51} content per milligram of spleen tissue was actually greater, but the difference was not statistically significant. The data support the conclusion, not well validated by whole body counts alone, that vitamin E-deficient rats held the Cr^{51} in the RE system more effectively than replete animals. Whether the RE system of vitamin E-deficient rats is more active in removing senile erythrocytes by phagocytosis, cannot be answered at the moment. It appears reasonably certain that, once the erythrocyte is sequestered in the liver, spleen and kidneys, the turnover is slower in vitamin E deficiency.

It is unfortunate that death of the animals receiving the vitamin B_6-deficient diet obviated a similar study of organ contents. The slope of the whole-body count curves suggest that the turnover rates of the RE organs are the same.

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Nutritive Value of Red Kidney Beans (*Phaseolus vulgaris*) for Chicks[1]

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ABSTRACT Inclusion of raw red kidney beans (*Phaseolus vulgaris*) at a level of 30% or above in the diet of chicks produced growth retardation and pancreatic hypertrophy. Autoclaving the kidney beans at 121°C for 30 minutes considerably improved weight gains and eliminated the pancreatic enlargement. A diet containing one per cent hemagglutinin (devoid of trypsin inhibitor) significantly lowered the growth rate of chicks without pancreatic enlargement. Supplementing the diet containing 50% red kidney beans with deficient essential amino acids did not result in growth performance equivalent to that with a control corn-soybean meal-type diet.

The poor utilization of protein due to the presence of certain toxic principles in a wide variety of seeds belonging to the family Leguminosae has been reported in nutritional studies of various experimental species. A recent review of the toxic factors present in edible legumes (1) has emphasized that most legumes contain hemagglutinins and trypsin inhibitors which are most likely responsible for the poor nutritive value of leguminous proteins in their raw state.

Liener (2) first indicated that purified hemagglutinin from raw soybeans exerted a growth-inhibiting effect on rats and suggested that these hemagglutinins may have some bearing on the nutritive value of legume proteins. It was later shown by Jaffé (3) and Honavar et al. (4) that the hemagglutinin purified from *Phaseolus vulgaris* had a deleterious effect on the growth of rats.

Chernick and his co-workers (5) reported that prolonged feeding of raw soybean meal induced in chicks an enlargement of pancreas and an increase in its proteolytic content. Tauber et al. (6) indicated that purified preparations of a trypsin inhibitor from lima beans directly inhibited the growth of mice. Lyman and Lepkovsky (7) have been able to produce pancreatic hypertrophy in chicks by feeding crystalline trypsin inhibitor.

Since little is known regarding the ability of the chick to utilize the protein of the kidney bean, the present study was undertaken to ascertain the toxicity of red kidney beans when included in the chick's diet as a source of protein.

EXPERIMENTAL

Male Columbian × Rhode Island Red chicks were reared in electrically heated battery brooders. Details of experimental period and replication are shown in table footnotes. Randomized complete block design was used in each experiment.

The composition of diets is given in table 1. Proximate analysis of red kidney beans indicated that the percentages of protein and fat were 21.2 and 0.9%, respectively. Feed and water were fed ad libitum. Conditions of soaking and autoclaving of red kidney beans are shown in appropriate table footnotes. Hemagglutinating and antitryptic activities of aqueous extracts of the kidney bean meal were determined by methods previously described (4).

The composition of essential amino acids from red kidney beans is shown in table 2. The essential amino acid values for the other dietary ingredients were obtained from table 3 of Waibel (8). Supplemental amino acids were added to the kidney bean rations in experiment 5 so that the total amino acid content of the kidney bean diet was equal to that of the control corn-soybean diet.

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TABLE 1

Composition of diets[1]

	A	B	C	D	E	F	G
	%	%	%	%	%	%	%
Ground yellow corn	54.2	48.7	43.2	32.2	17.4	51.4	16.4
Soybean meal, dehulled	30.0	28.0	26.0	22.0	16.8	31.8	16.8
Red kidney beans[2]	0.0	7.5	15.0	30.0	50.0	0.0	50.0
Constant ingredients[3]	15.8	15.8	15.8	15.8	15.8	16.8	16.8

[1] By calculation, diet A contains 22.9% of protein. Diets B, C, D, E, F and G are isonitrogenous with A.

[2] The red kidney beans used in these studies were purchased from Farmer Seed and Nursery Company, Faribault, Minnesota.

[3] Constant ingredients (in per cent) for diets A, B, C, D and E: fish solubles dried on soybean meal (100% equivalence), 3; dried whole whey, 3; alfalfa meal (17% protein, dehydrated), 3; stabilized bleachable fancy tallow, 3; dicalcium phosphate, 2; calcium carbonate, 1; iodized salt, 0.5; vitamin A (30,000 IU/g), 0.01; vitamin D_3 (15,000 ICU/g), 0.0075; vitamin E acetate (44 IU/g), 0.0125; menadione sodium bisulfite—63% USP (35.2 g/kg), 0.00625; vitamin supplement (4.4 g riboflavin, 8.8 g calcium pantothenate, 19.8 g niacin and 22 g choline chloride/kg), 0.075; choline chloride (25%), 0.075; vitamin B_{12} (44 mg/kg), 0.03; manganese sulfate (feed grade), 0.025; zinc sulfate (feed grade), 0.005; methionine hydroxy analogue calcium salt (90% purity), 0.05; and erythromycin thiocyanate (35.2 g/kg), 0.05. The constant ingredients (in per cent) for diets F and G are the same as above but excluding erythromycin thiocyanate and including one per cent of 30% chromic oxide bread.

TABLE 2

Essential amino acid composition of red kidney beans and the calculated analyses of essential amino acids in control and 50% red kidney bean diets used in experiment 5[1]

	Red kidney bean/g nitrogen	Control ration	50% Red kidney bean diet (exp. 5)
	mg	%	%
Arginine	359	1.501	1.418
Histidine	170	0.561	0.589
Isoleucine	383	1.188	1.295
Leucine	778	1.898	2.294
Lysine	410	1.338	1.443
Methionine	74	0.384	0.353
Cystine	(45)[2]	0.337	0.255
Phenylalanine	(360)[2]	1.129	1.207
Tyrosine	(181)[2]	0.801	0.713
Threonine	268	0.910	0.945
Tryptophan	(80)[2]	0.290	0.295
Valine	280	1.168	1.101
Glycine	248	0.990	0.971

[1] Amino acids in red kidney bean were determined on a Spinco Model 120 automatic amino acid analyzer following the procedure of Moore et al. (13). Due to acid hydrolysis, value for tryptophan could not be determined.

[2] Amino acid values for *Phaseolus vulgaris* are taken from Bressani et al. (14).

Statistical analyses of variance were made for each experiment and reported by means of the Duncan's multiple range test (9). Treatment values followed by the same letter are not significantly different at 0.05 level of probability.

RESULTS

The effect of different dietary levels of kidney beans on growth and pancreatic weights of the chicks is shown in table 3. Raw red kidney beans in experiment 1 at a level of 30% or more significantly lowered the growth rate and caused pancreatic hypertrophy as indicated by the pancreas weights. In experiment 2, the same parameters, however, were affected when the diet contained 15% raw red kidney beans. This variation in the experimental results between experiments 1 and 2 may be attributed to the age of birds; that is, younger birds were more susceptible to the toxicity.

The deleterious effect on growth rate and pancreas as influenced by the diet containing 50% raw red kidney beans is shown in tables 3 and 4. Autoclaving, but not soaking of kidney beans, resulted in improved growth and decreased pancreas weights.

A diet containing one per cent of purified hemagglutinin significantly lowered the growth rate but did not result in an enlarged pancreas (table 4).

Experiment 4 was conducted to evaluate the ideal heating time of red kidney beans while maintaining autoclaving temperature and pressure constant (table 5). Autoclaving from 5 to 240 minutes resulted in improved growth and decreased pancreas size; however, occurrence of lowest pancreas size at 30 minutes autoclaving allowed the tentative conclusion that this was a suitable heat treatment.

Data pertaining to the effect of supplementing the deficient essential amino acids in the diet containing 50% red kid-

TABLE 3

Influence of red kidney beans at different dietary levels on performance and pancreatic weights of chicks (exp. 1 and 2)[1]

Level and condition of red kidney beans	Weight gain		Efficiency[2]		Pancreas wt /kg body wt	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
%	g	g			g	g
0.0 Diet A	325a[3]	157a	0.53	0.63	3.71a	4.92a
7.5 Raw, diet B	317a	145ab	0.50	0.63	4.28a	5.87b
15.0 Raw, diet C	311a	136bc	0.50	0.59	4.25a	6.38b
30.0 Raw, diet D	287b	104d	0.45	0.52	5.83b	7.70c
50.0 Raw, diet E	181c	61e	0.37	0.37	8.24c	8.38c
50.0 Soaked[4]	—	71e	—	0.38	—	9.62d
50.0 Soaked and autoclaved[5]	—	120cd	—	0.51	—	6.04b

[1] Experiment 1: Treatment values are average of duplicate lots of 15 chicks each. Experimental period was from 9 to 28 days of age. Minimal significant differences between adjacent means for weight gains and pancreas weights were 15.7 and 1.16 g, respectively.
Experiment 2: Treatment values are average of triplicate lots of 8 chicks each. Experimental period was from 2 to 14 days of age. Minimal significant differences between adjacent means for weight gains and pancreas weights were 15.8 and 0.66 g, respectively.
[2] Ratio of gram body weight gain per gram feed eaten.
[3] Treatment values followed by the same letter are not significantly different at 0.05 level of probability.
[4] Soaked for 20 hours in cold tap water, dried at 65°C and ground.
[5] Treated as in footnote 4, plus autoclaving at 121°C, 15 pounds pressure for 30 minutes after soaking.

TABLE 4

Effect of raw and processed red kidney beans and of hemagglutinin on performance and pancreas weight of chicks[1] *(exp. 3)*

Treatment	Wt gain	Efficiency[2]	Pancreas wt /kg body wt
	g		g
Control (diet F)	109a[3]	0.62	4.87a
50% Red kidney beans			
Raw	18b	0.17	6.81bc
Soaked[4]	7b	0.07	7.11c
Autoclaved[5]	85c	0.51	4.78a
Soaked and autoclaved	71c	0.48	5.02ab
1.0% Hemagglutinin[6]	88c	0.56	5.65abc

[1] Treatment values are average of triplicate lots of 5 chicks each, except the last treatment which consisted of duplicate lots. Experimental period was from 2 to 14 days of age. Minimal significant differences between adjacent means for weight gains and pancreatic weights were 19.2 and 1.72 g, respectively.
[2] See table 3, footnote 2.
[3] Treatment values followed by the same letter are not significantly different at 0.05 level of probability.
[4] See table 3, footnote 4.
[5] Autoclaved at 121°C, 15 pound pressure for 30 minutes.
[6] The purified hemagglutinin was isolated from the red kidney beans by the technique outlined by Honavar et al. (4). One per cent of hemagglutinin was included in corn-soybean meal ration as in diet F at the expense of 1.1% ground yellow corn and 0.1% soybean meal increment such that this diet was isonitrogenous to diet F.

ney beans on the chick's performance are shown in table 6 (experiment 5). A comparison between the growth rate due to the control ration with that due to a diet containing autoclaved kidney beans fortified with deficient amino acids indicated that the amino acid supplementation failed to return the growth to normal. Thus, the combination of heating and (deficient) amino acids did not yield growth equivalent to that with the control ration.

DISCUSSION

The retardation of growth and the pancreatic hypertrophy observed among chicks fed the raw red kidney bean diet can be attributed to the toxic principles present in this legume. There is evidence that pure preparations of trypsin inhibitor have a direct growth-inhibiting effect on mice (6), and a host of investigators have reported pancreatic hypertrophy in chicks resulting from feeding of raw soybean

TABLE 5

Effect of red kidney beans with varying autoclaving time on performance and pancreatic weight of chicks[1] (exp. 4)

Treatment	Wt gain	Efficiency[2]	Pancreas wt/kg body wt
	g		*g*
Control (diet F)	116a[3]	0.59	4.42ab
50% Red kidney beans			
Raw	14d	0.10	6.05c
Autoclaved:[4]			
5 minutes	70bc	0.42	4.84b
15 minutes	62c	0.36	4.48ab
30 minutes	70bc	0.37	4.16a
1 hour	69bc	0.40	4.41ab
4 hours	74b	0.38	4.30a

[1] Treatment values are average of triplicate lots of 5 chicks each except the last treatment which consisted of duplicate lots. Experimental period was from 2 to 14 days of age. Minimal significant differences between adjacent means for weight gains and pancreas weights were 9.8 and 0.48 g, respectively.
[2] See table 3, footnote 2.
[3] Treatment values followed by the same letter are not significantly different at 0.05 level of probability.
[4] Autoclaved at 121°C, at 15 pounds pressure.

TABLE 6

Performance and pancreas weights of chicks as influenced by amino acid supplementation in kidney bean diet[1] (exp. 5)

Treatment	Wt gain	Efficiency[2]	Pancreas wt/kg body wt
	g		*g*
Control (diet F)	123a[3]	0.63	4.61a
50% Red kidney beans			
Raw	18b	0.13	6.83b
Raw + deficient amino acids[4]	22b	0.16	8.19b
Autoclaved[5]	83c	0.49	4.94a
Autoclaved + deficient amino acids[4]	85c	0.48	4.65a

[1] Treatment values are average of triplicate lots of 5 chicks/lot. Experimental period was from 2 to 14 days of age. Minimal significant differences between adjacent means for weight gains and pancreatic weights were 13.5 and 1.66 g, respectively.
[2] See table 3, footnote 2.
[3] Treatment values followed by the same letter are not significantly different at 0.05 level of probability.
[4] Deficient amino acids added were: methionine hydroxy analogue, calcium salt (90% purity), 0.113%; L-arginine·HCl, 0.083%; DL-valine, 0.134%; glycine, 0.019%; and L-phenylalanine, 0.010%.
[5] See table 4, footnote 5.

meal. Liener (10) has reviewed the thermolability of trypsin inhibitor from various legumes. The results of the present investigations indicate that the pancreas size returned to normal only after the red kidney beans were autoclaved before their inclusion in the diet. Our analyses indicated that the heat treatment involved in these experiments (30 minutes of autoclaving at 121°C) was sufficient to destroy all of the anti-tryptic and hemagglutining activity of the bean.

In contrast to studies with rats where it was found necessary to soak the beans prior to autoclaving in order to achieve satisfactory growth (4, 11), this study showed that preliminary soaking was unnecessary for chicks. The reasons for this difference in the response of the 2 species is unknown.

A diet containing 0.5% hemagglutinin (devoid of trypsin inhibitor) isolated from red kidney beans caused a high mortality among rats, according to Honavar et al. (4). In the present studies, however, there was no evidence of mortality among the chicks fed twice this level of hemagglutinin. From our analyses, the incorporation of one per cent of hemagglutinin in the diet is equivalent to about 30% raw

red kidney beans. Data in experiment 2 indicate a 33.8% growth depression among chicks fed a ration containing 30% raw red kidney beans, whereas in experiment 3, one per cent of the purified hemagglutinin equivalent to this level showed a 19.3% growth depression, as compared with the growth of chicks fed control diets in the respective experiments. It therefore appeared that the excess growth depression might have been due to toxic principles other than the hemagglutinin.

That the purified hemagglutinin significantly depressed growth in the absence of appreciable pancreatic hypertrophy is of interest since the growth depression caused by the soybean trypsin inhibitor is accompanied by marked pancreatic enlargement (12). This is further indicative of the multiplicity of toxic effects obtained with unheated legumes.

Since the growth performance of chicks fed the autoclaved kidney beans was subnormal compared to that with the control diet, an attempt was made to determine whether differences in dietary amino acid content might explain this effect. The results (table 6) show that equating the kidney bean diet with that of the control in terms of amino acid composition did not eliminate the growth differences. Thus, the unresolved growth depression noted with the autoclaved diet containing red kidney beans appears to be due to other factors such as a thermostable toxic principle, poor availability of amino acids other than those added or a dietary energy deficiency.

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The Utilization of Ethanol

II. THE ALCOHOL-ACETALDEHYDE DEHYDROGENASE SYSTEMS IN THE LIVERS OF ALCOHOL-TREATED RATS[1]

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ABSTRACT The levels of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ACDH) were determined at 2- to 4-week intervals in the livers of control rats and of treated rats given a 20% solution of ethanol as the sole drinking fluid during a 40-week period of observation. Both groups were fed an adequate purified diet. The levels of ADH in blood sera were also determined in some of the control and ethanol-treated rats, and some normal and alcoholic human subjects. The hepatic level of ADH in the ethanol-treated rats progressively increased above control values to a maximum after 26 weeks of ethanol consumption. A gradual decrease toward control values then occurred, perhaps associated with the development of fatty livers in the treated rats. The withdrawal of ethanol after 20 weeks of administration likewise was followed by a decrease in ADH levels to control values. Parallel, but less distinct changes were noted in the ADH levels of the sera of ethanol-treated rats. Detectable, but variable elevations in serum ADH were similarly observed in human alcoholics as compared with control subjects. The activity of ACDH in the livers of the ethanol-treated rats showed an increase followed by a gradual decrease to control levels similar to that observed for ADH but was a lesser magnitude.

Recent *in vitro* studies carried out in this laboratory (1) demonstrated that livers from rats maintained with a 20% ethanol solution as the sole drinking fluid used less acetate and more alcohol by way of the citric acid cycle than livers from untreated control animals. According to deductions from these experiments, it was suggested that an increased activity of some of the enzyme systems which lead to the formation of acetyl-CoA from ethanol could be a contributing factor to its better utilization. Whereas it is generally agreed that the first step in the chain of reactions of alcohol oxidation to CO_2 and H_2O, involving alcohol-dehydrogenase (ADH), is rate-limiting (2, 3), very little is known about the actual levels of this enzyme during the continuous administration of alcohol. Likewise, there is little information under such conditions with respect to the activity of acetaldehyde dehydrogenase (ACDH), the enzyme concerned with the oxidation of acetaldehyde to acetyl-CoA. It is known that this enzyme is extremely active since acetaldehyde appears to be metabolized as rapidly as it is formed from ethyl alcohol (2, 4).

Evidence presented in this paper which indicates that the prolonged treatment of rats with ethanol results in a steady increase in the levels of ADH, and to a lesser extent, of ACDH in the liver, a change which apparently is independent of food consumption. After the levels of both dehydrogenases attain a maximum, there is a gradual decline probably in proportion to the degree of hepatic damage induced by the continuous intake of ethanol.

MATERIALS AND METHODS

Diphosphopyridine nucleotide and hypoxanthine were purchased commercially,[3] as well as alcohol dehydrogenase,[4] which was crystallized twice. Ethanolamine,[5] was freshly redistilled before use. Other materials used were chemically pure grades.

Sixty male white Sprague-Dawley rats, weighing 100 to 120 g, were maintained for 4 weeks with a purified diet containing

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[3] Obtained from Nutritional Biochemicals Corporation, Cleveland.
[4] Obtained from Worthington Biochemicals Corporation, Freehold, New Jersey.
[5] Obtained from Paragon Testing Laboratories, Orange, New Jersey.

TABLE 1

Composition of purified diet

	%
Casein (purified)	24
Dextrin (white corn)	34
Sucrose	10
Sucrose, "vitaminized"[1]	10
Corn oil	15
Cod liver oil	1
Salt mixture (Wesson's)	4
Dried brewer's yeast	2

[1] "Vitaminized" sucrose contained per 1000 g: thiamine, 100 mg; riboflavin, 100 mg; pyridoxine·HCl, 100 mg; niacin, 400 mg; pantothenic acid, 400 mg; inositol, 10 g; folic acid, 20 mg; biotin, 10 mg; *p*-aminobenzoic acid, 3 g; choline chloride, 20 g; menadione, 300 mg; vitamin B_{12}, 10 μg; sucrose, 965 g.

adequate amounts of lipotropic factors and vitamins (table 1). The animals were divided into 2 groups of comparable weights. One group of 20 rats was given water and served as a control. The other group of 40 animals had access to 10% ethanol as the sole drinking fluid. After a 6-week period of adjustment, the concentration of alcohol was increased to 20% and kept at this level for the remaining 40 weeks of study. Both diet and fluids were given ad libitum. The animals were weighed weekly and their food consumption was recorded.

At intervals, 1 or 2 rats from each group, of as comparable weights as possible, were anesthetized with ether, the abdomens opened and the livers were perfused *in situ* with ice-cold 0.9% sodium chloride solution until blanched. Finally, the livers were removed, washed several times with cold saline and then used in making preparations of either ADH or ACDH or both.

Assay of alcohol dehydrogenase. The crude tissue preparations used in the assay of ADH were prepared by a modified procedure of Bonnichsen (5) and the ADH activity was measured spectrophotometrically at 340 mμ by the method of Theorell and Bonnichsen as applied to crude tissue preparations (5).

At the outset, semicarbazide was added to the reaction mixture to remove acetaldehyde as it was formed from alcohol by coupling with it, with the hope of driving the reaction completely to the right, thus offsetting the equilibrium constant for ADH which is known to be to the left in favor of ethanol. However, when it was found that the speed of the reaction was not faster in the presence of semicarbazide than in its absence, the addition of this coupling agent was discontinued in the subsequent experiments.

Assay of acetaldehyde dehydrogenase. The procedure followed for the extraction and purification of liver aldehyde dehydrogenase was essentially that of Racker as modified by Leder (6). This method, however, proved unsuitable for the assay of crude preparations or even for those with a fair state of purity because of contamination with varying quantities of ADH. To make possible the spectrophotometric determination of ACDH in the presence of ADH an inhibitor was sought that would inhibit the later but, at the same time, would not affect the activity of the former enzyme. Several substances were tested which would react with the zinc of enzyme or its sulfhydryl groups, or would compete with its substrate. Ethanolamine proved to be the most effective. The inhibitor was pipetted directly into the cuvettes to a final concentration of 0.5 M one minute before the addition of ethanol to the reaction mixture. Because of the marked nonenzymatic activity of ethanolamine a reagent blank determination was also carried out. The change in optical density was measured under the same conditions but without added enzyme or substrate. This value was subtracted from the increase in optical density obtained in the presence of both the substrate and enzyme. The spectrophotometric method as thus modified proved to be a rapid, simple and sensitive procedure for the assay of large numbers of samples of crude tissue preparations of ACDH. Also, it does not require the addition of the costly crystalline ADH. As a check on the spectrophotometric procedure, the assay of the tissue preparations for ACDH was conducted manometrically in the Warburg respirometer using the method described previously by Racker (7).

All prepartions used in the assay methods described above were tested for xanthine oxidase activity by a procedure outlined elsewhere (8), and were found to be completely devoid of this enzyme.

RESULTS

Typical activity curves for ADH in crude liver preparations of normal and alcohol-treated rats are presented in figure 1. The rates of reaction differed, being less in the normal as evident from the optical density readings at any given times (curves 2 and 3). Moreover, the overall characteristics of these curves are identical and follow a course similar to that of an authentic pure enzyme preparation (curve 4). The actual activities of ADH in units at different week intervals are given in figure 2. The curve for the alcohol-treated animals in-

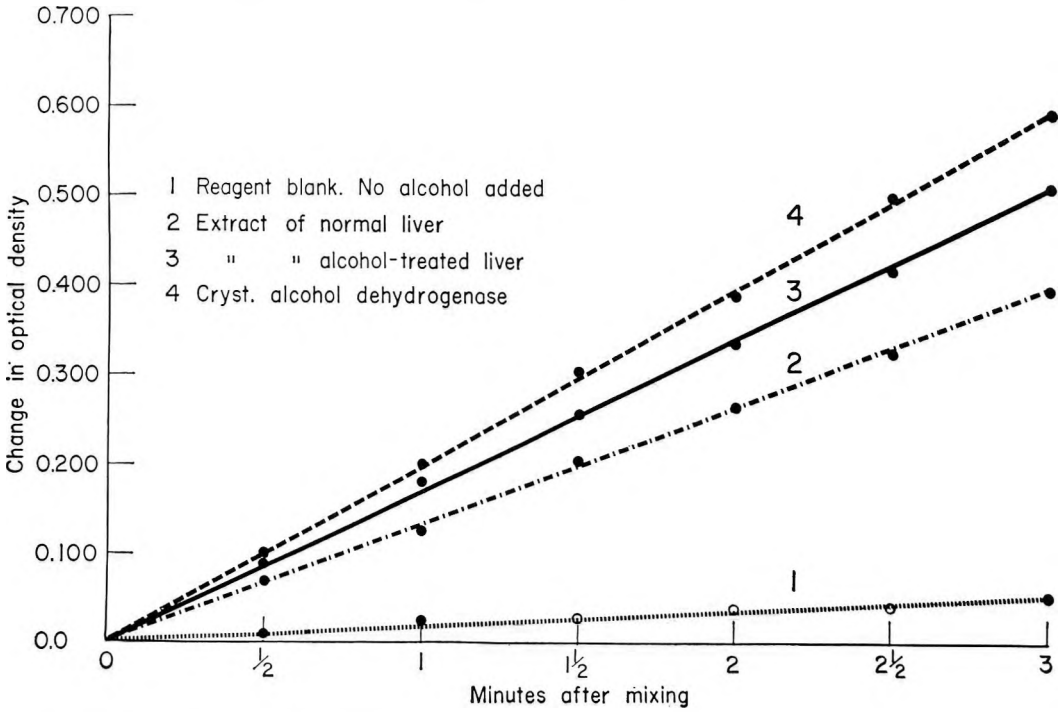

Fig. 1 Typical curves for alcohol dehydrogenase in crude extracts of livers of normal and alcohol-treated rats. (Bonnichsen method, 340 mμ). The blank contained buffer, DPN, and the enzyme preparation but no ethanol. The other tubes contained ethanol in addition and the enzyme preparations as indicated.

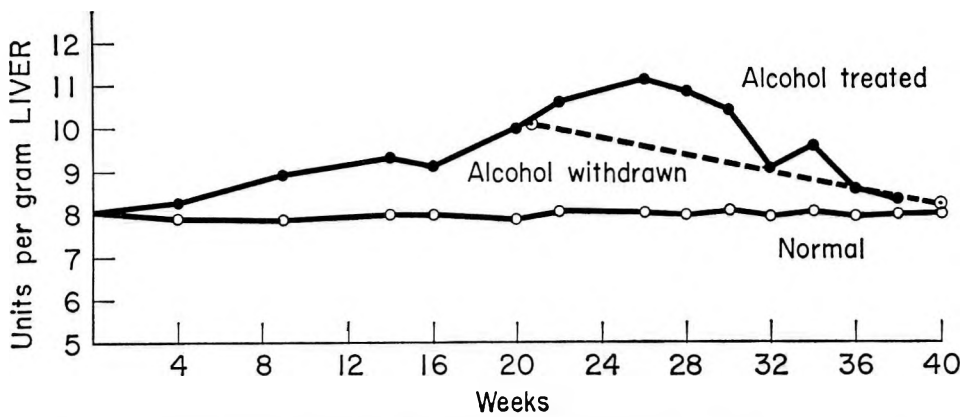

Fig. 2 Alcohol dehydrogenase levels in livers of normal and alcohol-treated rats.

dicates that the levels of the enzyme were consistently higher than those for the controls during most of the 40 weeks of the 20% alcohol treatment. Also, as the ingestion of ethanol is continued, the activity gradually increases in the livers of the treated rats and then diminishes until it approaches normal or even sub-normal values. Figure 2 further represents the effect of withdrawing alcohol from a group of 4 rats after 20 weeks of treatment when the enzyme activity of the rats killed had increased considerably above normal levels. In this case water was substituted for alcohol. After 18 weeks these animals exhibited relatively normal hepatic ADH activity and the gross appearance of their livers, with respect to size, color and fatty infiltration, was essentially normal. The significance of these observations will be discussed later.

To obtain further information, the ADH activity of the sera of some of the rats was measured at the time the livers were investigated. The results of these assays are presented in table 2. Whereas the absolute values for the enzyme activities are much less than those in the livers of the corresponding animals, the fact remains that the enzyme level in serum was higher in the alcohol-treated rats than in the controls. The values increased to a maximum at 22 weeks then decreased as observed with the liver preparations.

In addition to the determination of ADH in rats, the study was extended to human subjects at different stages of alcohol addiction. Sera of normal as well as of alcoholic subjects were assayed for ADH activity and the results are recorded in table 3. Differences occurred not only between normal and alcoholic subjects but there were also variations among the alcoholics themselves reflected by the extent of the disease. However, the actual levels of the enzymes are not as high as in the case of rat liver although the values are comparable to those for sera of rats. Plans have been made for investigating the activity of ADH in livers obtained from alcoholic human subjects at autopsy as soon as possible after death.

After establishing that long-term treatment with ethanol induces alterations in ADH levels in the rat, the study of the next step of alcohol oxidation, namely, the ACDH activity, appeared necessary. Since the crude liver preparations of ACDH in-

TABLE 2

Alcohol dehydrogenase (ADH) levels in sera of rats

Weeks on Experiment	Control		Alcohol-treated	
	No. of rats	ADH activity	No. of rats	ADH activity
		units/ml		*units/ml*
0	1	0.1	–	—
4	–	—	2	0.15–0.35
14	1	0.15	3	0.2 –0.8
22	1	0.3	2	0.85–1.2
32	1	0.15	3	0.25–0.9
36	–	—	1	0.28

TABLE 3

Alcohol dehydrogenase levels in human sera

Number of subjects	Normal	Alcoholic	Description of cases
	units/ml	*units/ml*	
8	0–0.4 Avg. 0.2	—	Non-drinkers or moderate drinkers (drinking on few occasions)
1	—	0.6	Delirium tremens
1	—	0.18	Very advanced Laennics cirrhosis; in prehepatic coma
2	—	0.8–1.5	Addict with very enlarged liver, no jaundice
2	—	0.4–1.0	Cirrhosis and jaundice: liver slightly enlarged
4	—	0.3–0.5	Under treatment 2–6 months after abstention, liver slightly palpable
6	—	0.1–0.25	Originally mild cases of addiction, one to 2 years abstention, treated and released from hospital

variably contain ADH activity, the inhibition of the latter is mandatory if a spectrophotometric assay procedure is adopted. This is because the method depends on the reduction of DPN to DPNH, a reversible reaction which is used by both dehydrogenases. As mentioned earlier, ethanolamine was found to be the best inhibitor tested. Figure 3 represents experiments conducted to ascertain which concentration would be necessary for optimal inhibition of ADH, assuming that levels up to 20 units/g would be encountered. Total inhibition of the crystalline ADH preparation was affected by a 0.5 M final concentration of ethanolamine, whereas the 0.1 M and 0.005 M concentrations produced approximately 40 and 25% inhibition, respectively.

The results of the spectrophotometric determination of hepatic ACDH in the presence of ethanolamine are represented in figure 4. The graphs show clearly that the activity of the enzyme in the livers of alcohol-treated rats was greater than that in the normal. To ascertain whether the apparent activity was actually enzymatic and not merely due to some "catalytic" action of ethanolamine, several tissue preparations were boiled prior to the assay. In figure 4, comparison between curve 3 and 4 or 5 demonstrates conclusively that a great part of the activity was due to the dehydrogenase. Indeed, boiling reduced the activity to the same level as that resulting from ethanolamine inhibition (curve 2).

The absolute activities of ACDH as measured by the spectrophotometric procedure are given in table 4. The data show that the changes in the levels of the dehydrogenase activity assume a pattern similar to that of ADH during long-term alcohol treatment, although to a lesser extent. Attention must be drawn to the fact that all determinations on ACDH were made during the latter part of the present study when the enzyme activity was thought to be high enough to permit accurate measurements.

To check the data obtained by the foregoing spectrophotometric method, the assay of ACDH for each tissue preparation was carried out manometrically in the Warburg apparatus. The results obtained by this technique are given in table 5. The data support those presented in table 4. Furthermore, the percentage increase in the activity of the dehydrogenase (4th columns, tables 4 and 5) in the alcohol-

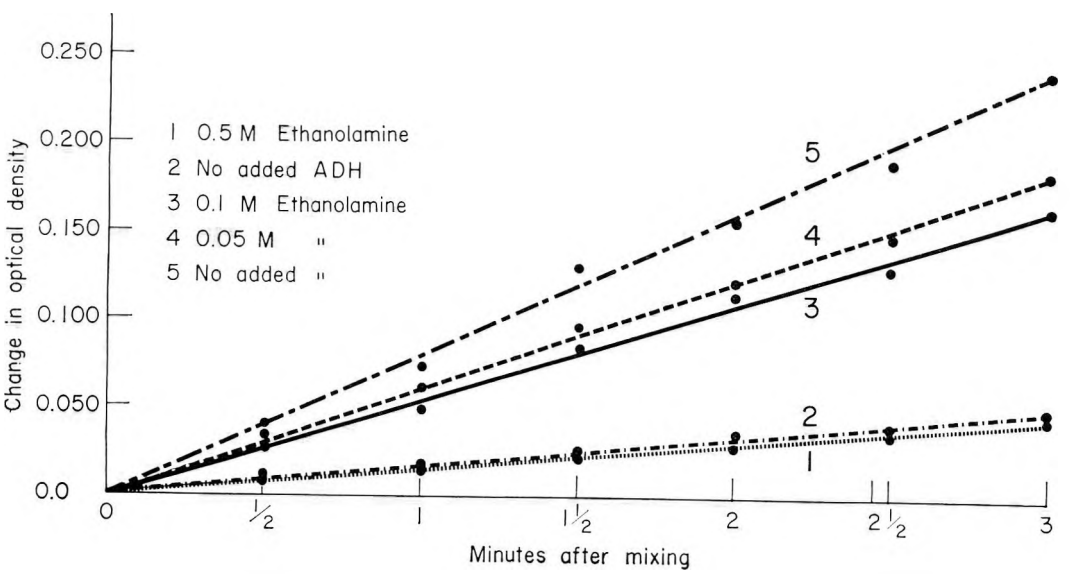

Fig. 3 Crystalline liver alcohol dehydrogenase activity. Inhibition by ethanolamine. The blank (line 2) contained buffer, DPN, and ethanol but no ADH. The other tubes contained ADH with varying amounts of ethanolamine as indicated.

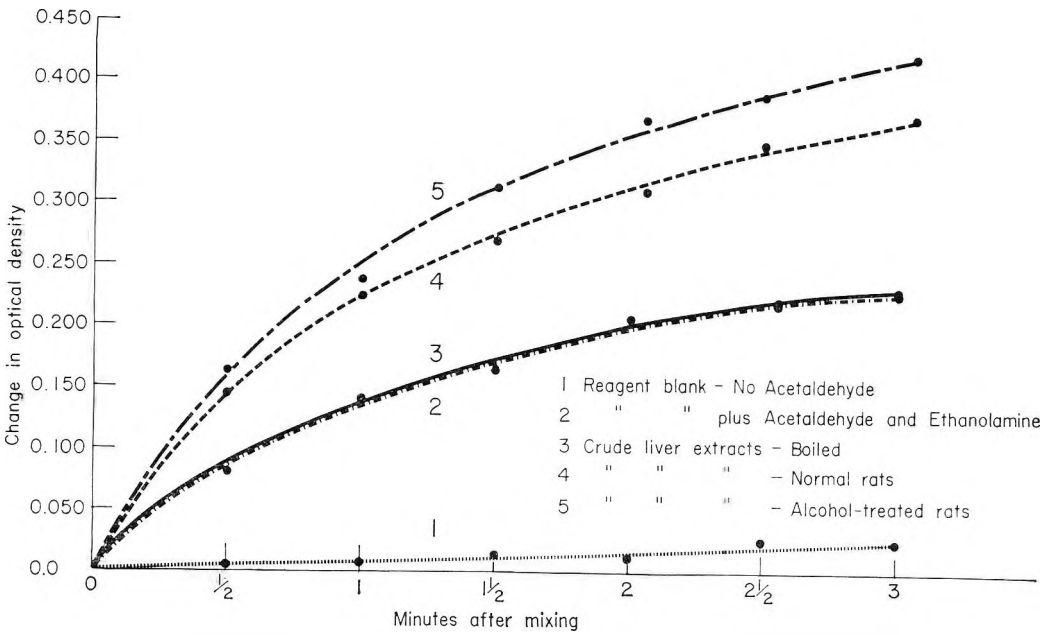

Fig. 4 Acetaldehyde dehydrogenase activity of crude liver extracts. The blank contained buffer, DPN, ethanolamine, and the liver enzyme preparations, but no acetaldehyde. The other tubes contained in addition acetaldehyde and the liver enzyme preparations as indicated. Ethanolamine was added in all cases to a 0.5 M final concentration.

treated over those of the normal agree closely, despite the data being given in different units.

Two other pertinent points may be mentioned. Whereas the average food intake of the alcohol-treated group was 20% less than the untreated, the effect of this difference becomes negligible if calculations were made on a gain in weight basis. In fact, the average weight of both groups was comparable and proportional to the amount of food ingested.

Also, the general appearance of the livers of the alcohol-treated rats was different from that of the normal; there was a gradual enlargement with indications of fatty infiltration, which was subsequently followed by a gradual diminution in the size of the organ as treatment with ethanol was prolonged. Near the end of the study there were signs of hepatic degeneration in the experimental animals. Further ex-

TABLE 4

Spectrophotometric assay (340 mμ) of acetaldehyde dehydrogenase activity of rat liver (ethanolamine added to 0.5 M final concentration)

Weeks on experiment	Acetaldehyde dehydrogenase activity		
	Normal	Alcohol-treated	Increase in alcohol-treated over normal average
	units/g liver		%
20	3450	3825	11.2
28	3500	4175	20.3
30	3650	4025	16.2
34	3480	3840	11.3
39	3200	3320	−3.9
Avg.	3456		

TABLE 5

Manometric determination of acetaldehyde dehydrogenase activity of rat liver

Weeks on experiment	Acetaldehyde dehydrogenase activity		
	Normal	Alcohol-treated	Increase in alcohol-treated over normal average
	μl CO_2/g liver in 20 min		%
28	131.0	156.6	20.2
30	132.6	150.8	15.6
34	130.4	143.0	9.7
39	128.8	133.4	2.4
Avg.	130.2		

periments to correlate the degree of liver damage with the changes in the levels of both ADH and ACDH in livers and sera of rats are being initiated.

DISCUSSION

It now appears well established that the major portion of alcohol metabolism takes place in the liver of most species, although the kidney may also play a minor role in some animals (9). The sequence of reactions that are probably involved in alcohol utilization, as reported in the literature, is summarized in figure 5. Two enzymes, namely ADH and catalase, are potentially capable of oxidizing ethanol to acetaldehyde. Likewise, several other enzymes could take part in acetaldehyde metabolism. However, there is ample evidence (4) that ADH and ACDH are the main enzymes primarily, if not exclusively, responsible for the first two steps of ethanol metabolism. Also, it is very likely that acetyl-CoA and not free acetic acid is the product of their immediate activities. Again, it is accepted as a fact that most of this acetyl-CoA is eventually metabolized by way of the citric acid cycle, whereas the rest is diverted to lipid biosynthesis and acetylation reactions. As stated previously, it is generally agreed that the first step (fig. 5) is the rate-limiting reaction in the utilization of alcohol. Recent studies (10) indicate that this limitation is predominantly dependent on the actual levels of ADH rather than on any other factor, especially in animals receiving an adequate diet. Smith and Newman (11) have, however, suggested that the DPN-to-DPNH ratio may be the actual limiting factor in the rate of alcohol metabolism in fasting and to some degree in fed animals. This observation has been confirmed by Raiko et al. (12).

Although the quality and amount of diet consumed by animals are frequently implicated in the production of metabolic deviations, the observed increased levels of ADH and ACDH in the ethanol-treated rats of the present study could hardly be associated with a diminished food or protein intake. This would appear logical if it

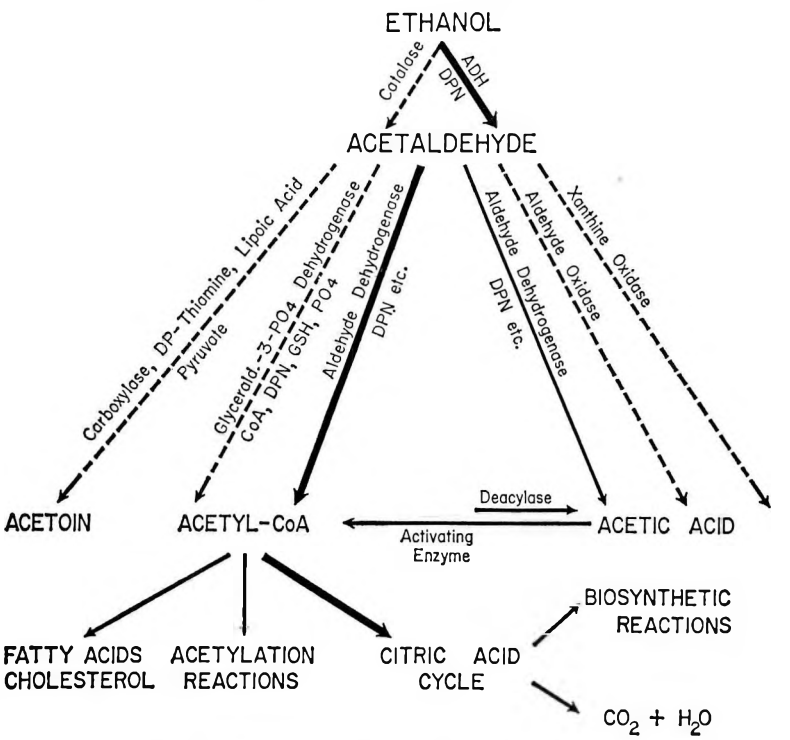

Fig. 5 Probable pathways of ethanol metabolism.

is assumed that reduction in diet consumption would cause a decrease in labile proteins of the body including enzymes. Actually, experiments mentioned earlier on xanthine oxidase activity, an enzyme which is considered by some workers as a sensitive index for protein deficiency, indicate that protein intake by the alcohol-treated rats was adequate in this respect. On this basis, it can be argued also that if there were a protein deficiency in the experimental group, the enzyme levels would be expected to increase still more if the alleged deficiency were corrected. Similar observations were made by Henley et al. (13) for the occurrence of alterations in the levels of certain enzymes of the liver under experimental conditions comparable to those in the present investigation. It appears, therefore, that there is a mechanism(s) other than a dietary effect which contributes to the observed increase in the activity of the two dehydrogenases. These observations on ADH in particular appear at variance with the recent results of Wartburg and Röthlisberger (14) which indicate that the activity of catalase rather than ADH was increased after treatment for 210 days with 10% alcohol. Even if catalase activity were to be higher as claimed, it has very little bearing on ethanol oxidation since, according to others (15), catalase participates to little, if any extent, in alcohol utilization. The apparent differences between the observations of Wartburg and co-workers (14) and the results of the present study, at least with respect to ADH, could be attributed to differences in the concentration of ethanol offered to rats.

It was thought desirable, in the light of the foregoing results, to evaluate the data on ADH in sera of rats in contrast with those in the livers of the same animals. If it could be assumed that the enzyme originates from the liver and that it is being continuously released into the blood stream, then it is very likely that the levels of serum would be proportional to those in the liver at any given time. Indeed, the pattern obtained in sera was similar to that in livers but with a lesser magnitude of activity. In view of this observation it was considered worthwhile to assess the status of the ADH in man as well in the different stages of alcoholism and to compare it with the data collected on the sera of rats. Here again the activity was also low but, in general, it was higher in some alcoholics than in the normal subjects. It appears that the levels of the enzyme in blood follow the course of the severity of the disease and the extent of hepatic cell damage, being higher in the mild or acute alcoholics and lower in the chronic, advanced or terminal cases. It is not clear, however, why the serum levels of ADH are not as high in the alcoholic with liver tissue destruction as, for instance, those of transaminase or lactic dehydrogenase in the damaging tissue diseases of heart, liver and muscle. It would not be unreasonable to assume that perhaps under these circumstances the enzyme molecule is not released intact like most of the DPN-linked enzymes but is disrupted, leaving only a small percentage unruptured to leak eventually into the blood stream. Consequently most of the enzyme reaching the blood would be inactive and, therefore, escape detection. If this is true, one would expect that zinc, which is considered an integral part of ADH and necessary for its enzymatic activity (16) would increase in the blood of certain alcoholics or be excreted in the urine in larger quantities than in normal individuals. This contention is in agreement with the observations (17, 18) that marked alteration(s) of zinc metabolism occur in patients with post-alcoholic cirrhosis, manifested in lowered concentrations of serum zinc and accompanied by a significant diminution in liver content of this element. In addition, marked zincuria in patients who have not as yet reached the terminal stages of the disease were also noticed.

Another explanation of the decrease in ADH level in the later stages of alcoholism is that the damaged liver cell has a decreased ability to form ADH. This was observed in the present experiments. After approximately 24 weeks of alcohol administration a progressive decrease in the ADH levels of the liver occurred (fig. 2). A similar decrease in the ADH levels in liver taken by biopsy from human alcoholics with cirrhosis was noted recently (19).

Another point pertinent to this discussion is the question of fatty livers, a

prominent feature of alcoholism. Several mechanisms can be offered to explain this phenomenon. It is possible that alcohol per se has a direct "toxic" effect on the liver which could produce as yet unknown alterations in its cells, thus rendering it more vulnerable to fatty infiltration (20). The increase in liver fat could also be due to an increase in fat formation as a result of excessive acetyl-CoA production from ethanol. Larger quantities of acetyl-CoA would be biosynthesized from alcohol if the levels of ADH and ACDH were elevated. This is particularly true if some step(s) in the reactions of alcohol oxidation beyond that of acetyl-CoA formation is retarded (1), thus diverting acetyl-CoA to fat production. The recent observation by Lansford et al. (21) that the administration of ethanol to rats is followed within 16 to 18 hours by a significant increase (3- to 4-fold) in liver triglyceride levels may be cited as further support for this concept. The lowered DPN-to-DPNH ratio observed during alcohol oxidation could also be involved in increasing fat formation in the liver.

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A Comparison of the Nutritional Value of Protein from Several Soybean Fractions[1,2]

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ABSTRACT Fractions studied were the following: full-fat soy flour (dehulled soybeans), soymilk (water-extract of soybeans), residue from water extraction, acid-precipitated curd from soymilk and whey protein left from the alcohol precipitation of the whey. All fractions were heated for one hour at 100°C and a series of protein concentrations in the diets (10, 20 and 30%) were tested to determine the quality of protein found in the various fractions. The residue contained the highest quality of protein as measured by growth and protein efficiency ratio. Diets containing residue were very acceptable to growing rats. Soymilk and curd protein were inferior to the residue and to the dehulled soybean protein. Poor growth was obtained with whey protein.

Soybeans and products derived from them have served as an important source of protein in the diet of millions of oriental people for nearly 5,000 years (1). The literature on the nutritive value of soybean protein has been reviewed by Markley (2) and Altschul (3). According to Wolf and Smith (4) food utilization of soybeans in the United States is still so new that many problems must be solved before their main pattern of use is established.

Much of the research on the nutritional value of soybean protein has been conducted on defatted soybean meal for animal feeding. The results are not applicable to several important soy products prepared for human use.

The work to be described was undertaken to evaluate the protein in soybean fractions obtained by processing the full-fat, dehulled soybean because equipment for solvent extraction is not readily available in many developing countries. Experiments were designed so that the results would indicate the relative nutritional value of various fractions of interest in human consumption and would suggest the direction in which emphasis should be placed in soybeans processed for food in developing countries.

EXPERIMENTAL METHODS

Male weanling rats of the Holtzman strain were used in these investigations. Throughout the studies, all test animals were kept in an air-conditioned room maintained at 21 to 22°C. Prior to being fed the test rations, the rats were fed a basal diet containing 15% casein for 2 days. During this 2-day adjustment period, the rats were housed in galvanized cages in pairs. Then the experimental animals were distributed, according to weight, into groups of 10 rats each and were housed in individual cages. The groups were then assigned, at random, to the experimental diets. During these studies, individual weights were recorded weekly. In addition, food intake was computed weekly for each group of rats. The test diets and water were given ad libitum with feed wastage being measured by collecting the total spillage 2 days each week. The wastage per week was then calculated from this 2-day spillage. Fresh water was given every other day, and the feed cups were checked each day and filled according to need during the 4-week test period. If there was any indication that food remaining in the cup had been soiled or contaminated, the cup was carefully cleaned before adding any new food.

In all studies, casein has been routinely used as a standard for comparison. The composition of the casein diets fed is shown in table 1. Since the fractions differ in protein and fat content from casein, adjustments were made in the amounts of dextrose and corn oil to maintain a con-

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[2] Approved for publication as Journal Paper no. 1322, by the Director of the New York State Agricultural Experiment Station, Geneva, New York.

TABLE 1

Composition of diets containing casein as a source of protein[1]

Ingredients	% crude protein as analyzed		
	10.15	20.20	29.95
Casein[2]	11.15	22.30	33.45
Dextrose[3]	57.20	40.05	24.40
Sucrose	12.00	12.00	12.00
Cellulose[4]	4.25	4.25	4.25
Salt mix[5]	4.00	4.00	4.00
Vitamin mix[6]	1.00	1.00	1.00
Corn oil[7]	9.00	15.00	19.50
A-D-E oil[8]	1.00	1.00	1.00
Choline chloride	0.40	0.40	0.40

[1] The casein diets are representative for the soybean fractions, as the fractions replaced casein and were then added at the expense of dextrose and corn oil to attain the desired crude protein and fat level.

[2] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] Cerelose (anhydrous dextrose no. 2401), Corn Products Company, Buffalo, New York.

[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[5] Hubbell et al. (6).

[6] Vitamin mix composition, mg/100 g of diet: thiamine·HCl, 1.0; riboflavin, 1.5; pyridoxine·HCl, 0.5; DL-Ca-pantothenate, 2.0; nicotinic acid, 3.0; biotin, 0.03; folic acid, 0.20; menadione, 0.40; inositol, 7.50; vitamin B_{12} (0.1% in mannitol), 4.0; *p*-aminobenzoic acid, 2.5; ascorbic acid, 0.4; and anhydrous dextrose to 100.

[7] Mazola, refined corn oil, Corn Products Company, Argo, Illinois.

[8] Composition, units/kg of diet: vitamin A, 20,000; vitamin D, 2,000; *dl*-α-tocopherol, 100; with corn oil as a carrier.

stant calorie-to-protein ratio. All other components of the diets remained constant. Each soybean fraction served as the only source of protein for a particular group of rats. Two separate shipments of Holtzman rats were used in the evaluation of the soybean fractions.

A series of protein concentrations (10, 20 and 30%) in the diets were tested to determine whether any of the fractions contained significant amounts of deleterious substances as measured by growth or appetite. The presence of such deleterious substances should be evidenced by reduction in food intakes or growth rates, or both, as the protein composition of the diets is increased. In addition to the criteria already discussed, protein efficiency ratios (gain in body weight per gram of protein consumed) were calculated and used in the evaluation of the various soybean fractions.

Figure 1 shows the distribution of solids and protein in the fractions. Certified Clark variety soybeans were used throughout this study in the preparation of the soybean fractions. Unextracted soybean flour was prepared by soaking dry soybeans overnight in 3 times their weight of water at room temperature. The hulls were removed by passing the soaked beans through a vegetable peeler[3] to loosen the hulls, which were then floated off in running water. The dehulled soybeans were drained, steamed one hour at 100°C in a heat exchanger, frozen at −40°C and freeze dried.[4]

Dehydrated soymilk powders were produced by grinding the soaked dehulled soybeans through an 023 screen of a disintegrator[5] with water, (8.3 liter/kg dry starting beans). The water-extractable solids were separated as a milk from the residue in a plate filter.[6] The soymilk was then cooked for one hour at 100°C, concentrated to 16% solids in a vacuum evaporator,[7] frozen at −40°C and freeze dried.

In the production of extracted soymilk, the residue was collected on the plate filter and washed with water until the filtrate became clear. The residue was then steamed at 100°C for one hour, frozen at −40°C and freeze dried.

The extracted soymilk was used in the preparation of soybean curd. The soymilk was cooked for one hour at 100°C in the heat exchanger and precipitation of the curd was then accomplished by adding glacial acetic acid (10 ml diluted to 400 ml with water/kg dry starting beans). The curd was separated from the whey by filtration on cheese cloth and then pressed to remove any residual whey. After pressing, the cake was frozen at −40°C and freeze dried.

The whey protein fraction was prepared by concentrating the filtrate from acid precipitation in a batch-type vacuum pan evaporator to 9.1% solids. Ethanol (95%) was added to yield a final alcohol concentration of 70%, based on weight. The solids precipitated by the alcohol were removed by filtration through filter paper on

[3] Model 14 Vegetable Peeler, Reynolds Electric Company, Chicago, Illinois.

[4] Model 200423 Freeze Drier, F. J. Stokes Machine Company, Philadelphia.

[5] Model RA-4-K53 Disintegrator, Rietz Manufacturing Company, Santa Rosa, California.

[6] Size 12 Filter Press (plate and frame), T. Shiver Company, Harrison, New Jersey.

[7] Four square foot laboratory agitated film evaporator, Blaw-Knox Company, Buflovak Equipment Division, Buffalo, New York.

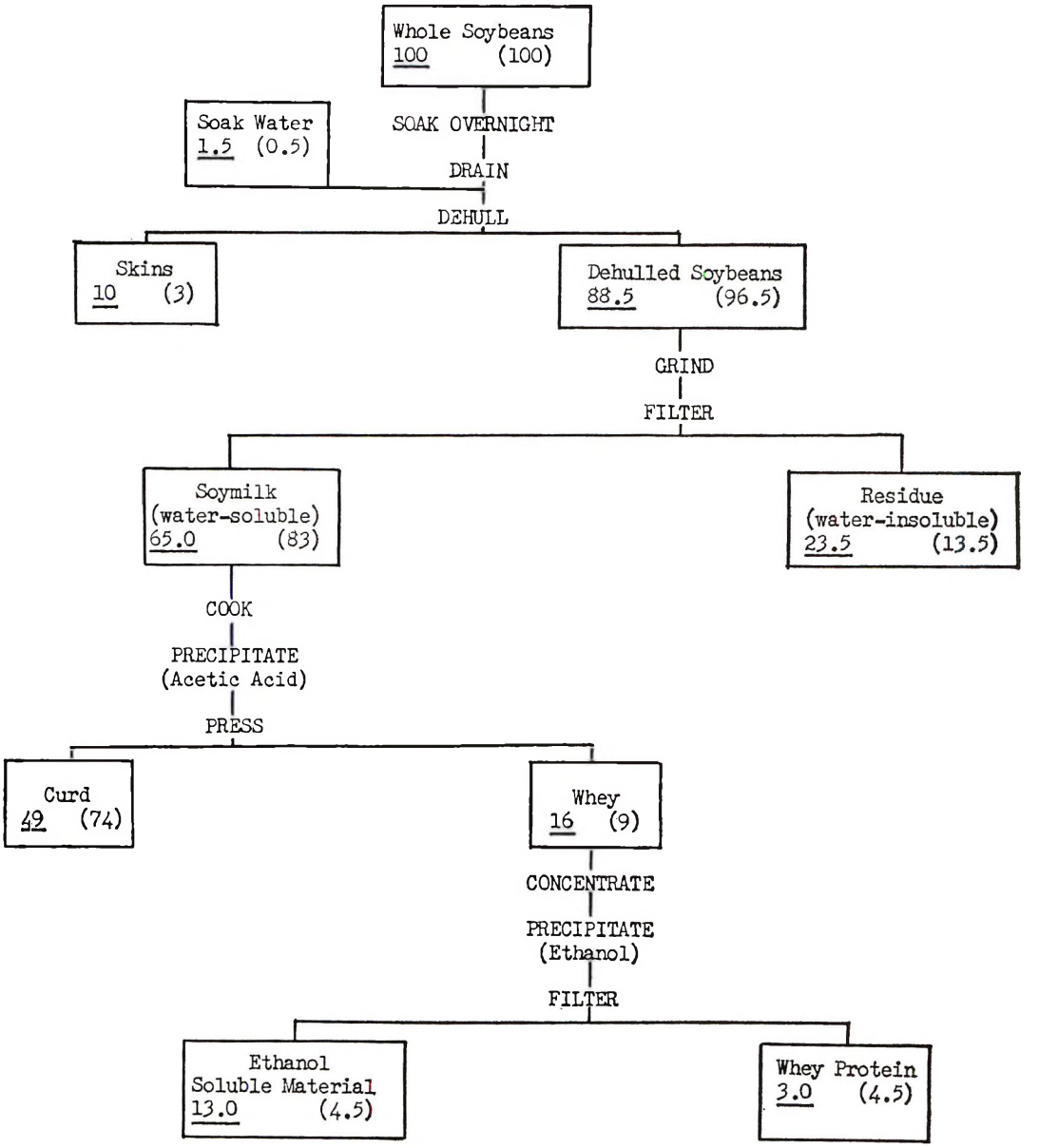

Fig. 1 Distribution of solids and protein in the fractions prepared from soybeans. The numbers underlined ——— represent the percentage of solids and the values in parentheses ( ) represent the percentage of the original soybean protein (nitrogen × 6.25) noted in each of the fractions.

a Buchner funnel and washed with 70% ethanol. The material was then frozen at − 40°C and freeze dried.

All samples were ground through a 40-mesh screen of a comminuting mill[8] before formulation for feeding tests. The fractions were analyzed for crude protein by the micro-Kjeldahl procedure and for moisture as outlined in AOAC (5). The fat content was determined by modification of an AOAC method in which the extraction is carried out with ethanol/chloro-

[8] Model D Comminuting Machine, W. J. Fitzpatrick Company, Chicago 7.

form. The diets were formulated and mixed, then analyzed for crude protein prior to starting the experiment. The analyzed protein value was used in determining the protein efficiency ratio (PER).

RESULTS AND DISCUSSION

The results obtained in the 2 studies when rats were fed diets containing 10% crude protein are summarized in table 2. In the first study, the rats consumed slightly more feed than they did in the second study and consequently there was a change in the average growth rate which cannot be ascertained from this table, as it contains only the average values. In the first study, faster growth rates were obtained which was a reflection of increased food consumption. The most striking results obtained in these studies were with the residue-fed rats. The rats receiving residue as a source of protein grew faster than those in any of the other groups receiving soybean fractions.

The feed consumption for the residue-fed rats was higher than that for any of the groups and they had the highest PER value (2.71), with the exception of the groups receiving the standard casein diet which had a PER of 2.86. The results indicate that the water-insoluble fraction contains an extremely high quality protein. The feed intake (18.22 g/day/rat) observed for rats receiving a diet containing residue as a source of protein no doubt partially explains the faster growth.

The growth and PER data indicate that the amino acid make-up of the residue or dehulled soybean fractions may be superior to the soymilk, curd and whey protein fractions fed in these studies. It is hoped to test the validity of this explanation in future studies.

The group of rats receiving the whey protein failed to grow at a rapid rate and during the 4-week study, 5 rats out of an initial 10 died. The diet was apparently very unpalatable to the rats, as they consumed only an average of 7.08 g/day. In an earlier study an attempt was made to feed whey solids; however, the whey solids were extremely hygroscopic and this resulted in the diet becoming very sticky.

Dehulled soybeans, soymilk and curd, as well as casein, were fed in diets designed to contain 20 and 30% crude protein, and the results are summarized in table 3. The casein standard was superior to the soybean fractions fed at these higher protein levels as it was at the 10% level.

The data obtained in providing diets to rats that contained 20 and 30% crude protein were very similar in the case of dehulled soybeans and curd. In addition, soymilk gave similar results at the 30% dietary crude protein level; however, at the 20% protein level, there was a slight depression in growth. This may be an indication that the water-soluble protein is slightly inferior to the acid-precipitated protein. Since the effect of increasing the

TABLE 2

A summary of the effect of soybean fractions upon growth of weanling rats when diets supplied 10% protein

Dietary protein, %	Diet designation by protein supplement					
	Casein[1] 10.15	Dehulled soybeans[1] 10.07	Residue[2] 9.93	Soymilk[1] 9.84	Curd[1] 10.00	Whey[3] 10.00
Average daily gain, g[4]	4.18 ± 0.14	3.95 ± 0.15	4.90 ± 0.18	2.63 ± 0.08	2.69 ± 0.12	1.37 ± 0.03
Average feed intake, g	14.34	15.59	18.22	12.70	12.20	7.08
Protein efficiency ratio	2.86	2.51	2.71	2.11	2.20	1.93

[1] Each datum represents an average of 20 rats from 2 studies of 10 each.
[2] Data represent an average of 40 rats from 2 studies of 20 each.
[3] Values represent an average for the 5 remaining rats from an initial group of 10.
[4] Average daily gain in grams ± SE of mean.

TABLE 3

A summary of the effect of soybean fractions upon growth of weanling rats when diets supplied 20 and 30% protein[1]

Dietary protein, %	Diet designation by protein supplement							
	Casein		Dehulled soybeans		Soymilk		Curd	
	20.20	29.95	19.90	29.87	19.66	29.61	19.89	29.75
Average daily gain, g[2]	7.19 ± 0.14	7.29 ± 0.14	6.39 ± 0.13	6.52 ± 0.13	5.69 ± 0.13	6.42 ± 0.15	6.39 ± 0.12	6.84 ± 0.16
Average feed intake, g	14.75	13.02	15.51	13.46	14.76	13.64	16.54	14.04
Protein efficiency ratio	2.41	1.87	2.07	1.62	1.96	1.59	1.94	1.64

[1] Each datum represents an average of 20 rats from 2 studies of 10 each.
[2] Average daily gain in grams ± SE of mean.

level of the fractions in the diets to supply 20 and 30% protein resulted in a similar response to that obtained with casein, there does not appear to be a significant quantity of deleterious substances in the dehulled soybean, soymilk and curd fractions after heating.

Although the results are not reported in table 3, the addition of enough residue to a diet to give a 20% protein level resulted in growth slightly lower than that obtained when residue was fed at the 10% protein level. The bulky physical characteristics of the residue no doubt played an important part in producing these results. Due to the bulkiness of the diet, the rats were unable to consume as much feed as when residue was added to the diet to supply 10% crude protein. In addition, residue was added at the expense of dextrose, which may have reduced the readily available digestible calories.

These results indicate that the residue (or water-insoluble fraction) contained the highest quality protein. In addition, an appetite or palatability factor appears to be present, since diets containing residue were more acceptable to the rats when fed ad libitum. Furthermore, the results show that as much residue as is possible should be left in the final product. The soymilk fraction, as well as the curd fraction, depressed appetite, but not to the extent that a whey protein-containing diet did. The consumption of the diet containing dehulled soybeans was intermediate. Since the dehulled soybean fraction is composed of residue and soymilk, this in effect supports the previous statement concerning the enhancement of appetite by the addition of residue to a diet to provide 10% crude protein.

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Utilization of Methionine by the Adult Rat

III. EARLY INCORPORATION OF METHIONINE-METHYL-C^{14} AND METHIONINE-2-C^{14} INTO RAT TISSUES[1]

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ABSTRACT The distribution of the *alpha-* and methyl carbons of methionine in water-soluble, fat-soluble, and protein fractions of 17 tissues of adult rats at early intervals after the administration of radiomethionine was investigated. The initial incorporation of the *alpha*-carbon of methionine was highest in the water-soluble fractions of pancreas and adrenal; of the methyl carbon, in liver. After 24 hours, however, bone marrow contained relatively greater concentrations of the *alpha-* and methyl carbons of this amino acid. Substantially greater concentrations of the methyl carbon of methionine were present in the fat-soluble fractions of liver and adrenals than in this fraction of other tissues. Although appreciable incorporation into tissue proteins had occurred 24 hours after the administration of *alpha*-carbon labeled methionine, only slight concentrations of radiocarbon arising from the methyl carbon appeared in these proteins.

In recent years, it has become increasingly clear that the need for certain amino acids cannot be explained purely on the basis of their roles in tissue protein synthesis. Particularly in the case of methionine, this former view must be extended to include other functions.

The roles of methionine as a lipotropic factor, as a source of sulfur, in detoxication, and as a methyl donor have been reviewed (1, 2). More recently, it has been reported that the methyl groups of carnitine (3), and ergosterol (4) are derived from methionine.

In a previous report from this laboratory (5), the high uptake of the *alpha*-carbon of methionine by bone marrow, pancreas, and adrenals suggested a priority of these tissues for the amino acid. In these studies the complexity of metabolic transformations of the methionine molecule occurring within the 24-hour period was evident. Whether these reactions reflected a need for the methionine molecule *in toto*, or the use only of compounds containing its *alpha*-carbon was not clear. It seemed, therefore, pertinent to us to study the early uptake of the *alpha-* and methyl carbons of methionine by tissues of the rat.

The objective of the present study was to investigate the presence of the *alpha-* and methyl carbons of methionine in tissues and tissue fractions immediately after the administration of the amino acid to provide additional data as to the major sites of its metabolic alterations and its primary use. To compare the early uptake of methionine derivatives with the situation occurring after equilibrium had been reached, we investigated the distribution of the *alpha-* and methyl carbons of the amino acid in tissues and tissue fractions at 24 hours. Observations are presented for adult rats killed 16, 20, 30 and 1440 minutes after the administration of methionine-2-C^{14} or methionine-methyl-C^{14}.

EXPERIMENTAL PROCEDURES

DL-Methionine-2-C^{14} (specific activity of 0.57 mc/mmole) and L-methionine-methyl-C^{14} (specific activity 1.84 mc/mmole) were used.[2] Proof of the position of labeling of these amino acids was supplied. L-Methionine-2-C^{14} could not be obtained commercially or prepared conveniently in

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[2] Tracerlab, Inc., Waltham, Massachusetts.

our laboratory at the time these experiments were conducted.

Adult, male rats of Wistar strain weighing approximately 300 g were fasted for 10 hours prior to the administration of the radioactive amino acids by stomach tube. Eighteen milligrams of DL-methionine-2-C^{14} were dissolved in distilled water and fed to 5 rats killed after 16 and 30 minutes. Since the specific activity of the methyl carbon-labeled compound was higher than that of the *alpha*-carbon labeled amino acid, and, to provide the same concentration of radiocarbon to all animals, 18 mg of a mixture of L-methionine-methyl-C^{14} and nonradioactive L-methionine dissolved in water were fed to rats killed at 20 and 30 minutes.

To 3 rats, killed at the end of 24 hours, were administered by stomach tube 18 mg of the radioactive amino acid contained in 4 g of a homogenized semipurified diet which provided in proper proportions all of the nutritive essentials required by adult rats (5).

Rats fed DL-methionine-2-C^{14} killed at 16 minutes and L-methionine-$C^{14}H_3$ killed at 20 minutes received 1,520,000 and 1,483,000 count/min, respectively. They absorbed 77.6 and 77.5%, respectively, of the administered dose. Rats killed at 30 minutes absorbed 88.7 and 95.7% of the aqueous solutions of DL-methionine-2-C^{14} and L-methionine-methyl-C^{14}, respectively. Rats killed after 24 hours received 2,599,000 count/min corresponding to DL-methionine-2-C^{14} or 5,931,000 count/min corresponding to L-methionine-methyl-C^{14}. The absorbed doses for these latter animals were 98.6 and 96.9%, respectively.

At the end of the experimental period, 17 organs and tissues were removed quickly, frozen immediately, and kept at −20°C until analysis was performed. After removal of water-soluble and fat-soluble components, tissue proteins were washed with trichloroacetic acid. The proteins were then hydrolyzed with 6 N HCl for 24 hours. Aliquots of the liquid tissue fractions were applied to filter paper and their activities measured with a Geiger-Mueller counter. Procedures for fractionation of tissues were described in a preceding paper (5).

The esophagus was tied with silk thread close to the stomach; the stomach was separated from the gastrointestinal tract. The small intestine was cut into 3 segments of approximately 35 cm each. The contents of the stomach, 3 segments of small intestine, the cecum, and large in-

TABLE 1

Specific activities of combined tissue fractions[1] of rats fed methionine-2-C^{14} and methionine-methyl-C^{14}

	Methionine-2-C^{14}		Methionine-methyl-C^{14}	
	16 min	24 hour	20 min	24 hour
	% absorbed dose/g tissue		*% absorbed dose/g tissue*	
Adrenals	0.42	0.24	0.14	1.07
Bone marrow	0.23	0.71	0.11	0.78
Brain	0.06	—	0.02	0.05
Heart	0.09	0.20	0.04	0.08
Kidney	0.33	0.57	0.05	0.37
Liver	0.26	0.49	0.71	0.97
Lungs	0.11	0.15	0.04	0.19
Muscle	0.01	0.05	0.02	0.04
Pancreas	0.64	0.32	0.05	0.34
Pituitary	—	—	0.13	—
Prostate	0.12	0.80	trace	0.14
Seminal vesicle	0.03	0.23	trace	0.04
Skin and hair	0.01	0.05	0	0.03
Spleen	0.05	0.42	0.04	0.37
Testes	trace	0.04	0.01	0.08
Thymus	0.33	—	0	0.37
Thyroid	0	—	0.01	0.39

[1] Includes radiocarbon in the following fractions: water-soluble, hexane-soluble, hydrolyzed protein, trichloroacetic acid filtrate after removal of protein, water residue after preparation of hexane fraction from ether and alcohol-soluble compounds, and alcohol washings of proteins.

testine were expressed individually into calibrated tubes. The inner walls of the tissues were rinsed with 3 successive aliquots of distilled water which were added to vials containing the tissue contents. These liquids were made to known volumes, and carefully shaken. Aliquots were taken immediately and applied to filter paper, dried, and counted with a gas-flow counter. The total activity remaining in the alimentary tract was subtracted from the administered dose to give the absorbed dose.

All data are presented as percentage of absorbed dose to eliminate differences in circulating activity due to differences in absorption of the DL- and L- isomers of the amino acid (6) and doses administered.

The comparative level of the isotope in various tissues may be calculated by multiplying the percentage of absorbed dose listed in table 1 by the absorbed dose (administered dose times per cent absorbed).

As data were obtained for only 2 time periods, it is not possible to determine when the peak of uptake of the radio-amino acids occurred.

RESULTS AND DISCUSSION

Table 2 presents the occurrence of radiocarbon in water-soluble, fat-soluble and hydrolyzed protein fractions of tissues.

Water-soluble tissue fraction. At 16 minutes, the concentration of radioactivity from the *alpha*-carbon of methionine in pancreas was approximately 2 times that of the next highest tissues: adrenals, thymus, kidney, and liver. Using S^{35}-labeled methionine, it has been shown by other workers (7, 8) that methionine localizes in the pancreas during active secretion. An acute deficiency of this amino acid does not appear to inhibit the secretory function of the pancreas, but reduces the amount of activity secreted into the small intestine (9).

The radiocarbon content of pancreas and adrenals of rats fed *alpha*-carbon labeled methionine decreased over the 24-hour interval, but remained high in kidney and liver. Activity in spleen, although low at 16 minutes, was high after 24 hours. The percentage of absorbed dose in bone marrow after 24 hours was higher than that of other tissues.

Twenty minutes after administration of the methyl carbon-labeled amino acid, the activities of the water-soluble fractions of liver and adrenals were higher than those of other tissues. After 24 hours, however, the specific activities of bone marrow, adrenals, liver, thyroid, thymus, spleen, pancreas, and kidney were high.

The high specific activities of water-soluble fractions of thyroid and thymus, 24 hours after feeding the methyl-labeled amino acid, are of special interest in view of the report by Charkey (10) that methionine decreases the oxygen consumption of chicks between 2 and 4 weeks of age. It has been suggested by this author that "methionine functions in metabolism as a coupling agent or by enhancing biosynthesis of such an agent, or of interfering otherwise with the functioning of the thyroid hormone as an uncoupler."

Fat-soluble tissue fractions. The lipid fractions of many of the tissues were radioactive. Axelrod and co-workers[3] reported the incorporation of amino acids into lipoid material by cell-free rat liver preparations. Hendler[4] has observed amino acid-lipid complexes in hen oviduct.

Sixteen minutes after administration of the *alpha*-carbon labeled amino acid, small concentrations of radiocarbon were observed in fat-soluble fractions of bone marrow and lungs (table 2). Early after administration of the methyl carbon-labeled amino acid, however, much greater concentrations of activity were observed in the liver than in other tissues. Substantially more of the methyl carbon of methionine was present in the fat-soluble fractions of adrenals and liver at 24 hours than in other tissues.

Hydrolyzed tissue proteins. In the hydrolyzed protein fraction, when *alpha*-carbon tagged methionine was fed, labeling occurred to a greater extent at 16 minutes in adrenals, bone marrow, and liver proteins than in other tissues (table

[3] Axelrod, B., J. L. Haining and T. Fukui 1959 Incorporation of amino acids into lipoidal material by cell-free rat liver preparations. Federation Proc., *18*: 184 (abstract).

[4] Hendler, R. W. 1960 Amino acid complexes formed in hen oviduct during protein synthesis. Federation Proc., *20*: 346 (abstract).

TABLE 2

Specific activities of water-soluble, fat-soluble and hydrolyzed protein fractions of rat tissues

Tissue	Water-soluble fraction				Fat-soluble fraction				Hydrolyzed protein fraction			
	Methionine-2-C^{14}		Methionine-methyl-C^{14}		Methionine-2-C^{14}		Methionine-methyl-C^{14}		Methionine-2-C^{14}		Methionine-methyl-C^{14}	
	16 min	24 hour	20 min	24 hour	16 min	24 hour	20 min	24 hour	16 min	24 hour	20 min	24 hour
	% absorbed dose/g tissue				*% absorbed dose/g tissue*				*% absorbed dose/g tissue*			
Adrenals	0.39	0.05	0.14	0.41	0	0	0	0.59	0.02	0.14	0	0.04
Bone marrow	0.17	0.54	0.08	0.76	0.03	0	0	0.01	0.02	0.04	0	0.01
Brain	0.04	—	0.01	0.02	0.01	—	trace	0.02	0	—	0	0.01
Heart	0.08	0.10	0.04	0.05	0.01	0	trace	0.03	0	0.10	0	trace
Kidney	0.30	0.34	0.04	0.28	0.01	0.02	trace	0.07	0.01	0.20	0	0.01
Liver	0.22	0.24	0.29	0.38	0.01	0.02	0.42	0.52	0.02	0.24	0	0.01
Lungs	0.09	0.05	0.02	0.09	0.02	0.01	0.01	0.08	trace	0.07	0	0.01
Muscle	0.01	0.01	0.02	0.02	trace	trace	trace	0.01	0	0.03	0	trace
Pancreas	0.62	0.25	0.04	0.30	0	0.02	0	0.02	0.01	—	0	0.02
Prostate	0.12	0.23	0	0.08	0	0.02	0	0.04	0	0.09	0	0.01
Seminal vesicle	0.03	0.12	0	0.02	trace	—	0	0.02	0	0.10	0	trace
Skin and hair	0.01	0.01	0	0.02	trace	trace	0	0.01	0	0.03	0	trace
Spleen	0.04	0.31	0.04	0.33	0	0.04	0	0.02	0.01	0.06	0	0.02
Testes	0	0.01	0.01	0.07	trace	0.01	0	0.01	0	0.02	0	0.01
Thymus	0.32	—	0	0.37	0	—	0	0	0	—	0	0
Thyroid	0	—	0	0.38	0	—	0	0	0	—	0	0

TABLE 3

Specific activities of total tissue fractions of rats fed methionine-2-C^{14} and methionine-methyl-C^{14}

Tissue fraction	Methionine-2-C^{14}		Methionine-methyl-C^{14}	
	16 min	24 hour	20 min	24 hour
	% absorbed dose		*% absorbed dose*	
Water-soluble	12.3	4.6	6.6	9.8
Fat-soluble	0.6	0.6	3.7	6.9
Hydrolyzed protein	0.2	5.8	0	0.6

2). By 24 hours, liver and kidney proteins contained higher percentages of the absorbed dose than other tissues. Only small concentrations of radiocarbon were observed in tissue proteins, even 24 hours after administration of the methyl-labeled methionine. Adrenal protein contained more radiocarbon than other proteins. It is apparent that labeling of tissue proteins occurred to a greater extent when the *alpha*-carbon tagged amino acid was fed than when methyl-C^{14}-methionine was administered.

Examination of this fraction by paper chromatography revealed that radioactive methionine was present in all tissue proteins. Whereas 52% of the radiocarbon in tissue proteins was present as methionine when the *alpha*-carbon labeled amino acid was fed, only 23% of the activity in tissue proteins was in this amino acid when the methyl-labeled compound was administered. This suggests that approximately one-half of the methionine which enters the protein molecule exchanges its methyl group with endogenous methionine.

Combined tissue fractions. Table 3 presents the percentage absorbed dose as total counts/minute in the combined water-soluble, fat-soluble, and hydrolyzed protein fractions of all tissues.

When *alpha*-carbon labeled methionine was administered, the concentration of radiocarbon in the water-soluble fraction decreased from 12.3% at 16 minutes to 4.6% at 24 hours. Simultaneously more carbon^{14} appeared in tissue proteins.

In contrast, when methyl-tagged methionine was fed, the concentrations of radiocarbon in the water-soluble fraction increased during the 24-hour interval. Incorporation of the methyl carbon into tissue proteins was slight, even after 24 hours.

Whereas, the concentration of the *alpha*-carbon of methionine in the fat-soluble fraction remained relatively constant, radiocarbon from the methyl carbon of methionine in this fraction increased during the 24-hour interval.

In addition to those tissue fractions already mentioned, others were obtained in the procedure by which tissue components were separated. These included: a trichloroacetic acid extract from which protein and glycogen had been removed (free amino acids and peptides not absorbed onto the protein might be expected to appear here), alcohol washings of the protein, and the water residue obtained by shaking the hexane solution of lipids with water and removing the hexane layer. The values for radiocarbon in these fractions were added to those of the fractions from individual tissues previously reported. The data are presented in table 1.

At the earliest time intervals highest concentrations of radiocarbon were contained in pancreas and adrenals after feeding the *alpha*-carbon labeled methionine; in liver, after administration of the methyl-labeled compound.

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Dental Caries of Rats Fed a Rice Diet and Modifications[1]

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ABSTRACT Because a diet with large amounts of white rice has been considered the possible cause of the high dental caries rate among children of Oriental ancestry in Hawaii, a study was made of the effect of a semi-purified diet high in rice, upon the teeth of 190 susceptible white rats. A preliminary experiment compared 2 degrees of fineness of diet ingredients. After washing and drying, white rice was made into a flour to pass a 48-mesh sieve, as did all other ingredients for the other 2 experiments. Hubbell-Mendel-Wakeman salt mixture was used at the low level of 1.2 g and cod liver oil at 0.2 g/100 g of basic diet. When the salt mixture was doubled, there was a marked improvement in dental conditions ($P < 0.01$) for number of carious teeth, number of carious areas and the caries score. Adding 15 times the amount of cod liver oil in the basic diet did not cause a statistically significant improvement. Results for increased salt mixture plus cod liver oil were similar to increased salt mixture alone. The good effect of doubling the salt mixture was verified in a second experiment, but the effect was nullified if 12% of powdered sucrose replaced an equal quantity of rice flour.

The high per capita consumption of white rice has been considered an important contributory factor in dental caries among rice eating groups in Hawaii (1). Larsen and co-workers (2) postulated that the potential acid ash of rice promoted dental decay. That the teeth of children of Oriental ancestry living in Hawaii show high DMF rates has been well established (3, 4). However, studies of various population groups in the Orient have indicated that high rice consumption is not necessarily concomitant with a high dental decay rate; in fact, quite the opposite has been reported (5, 6).

About one-half the people of Hawaii are of racial stocks that use rice as a staple and basic food. Prior to 1950, the per capita consumption of rice by these groups ranged from 91 kg (200 pounds) to more than 136 kg (300 pounds) per year, whereas the per capita consumption for all the people in the Islands was calculated to be about 55 kg (120 pounds) per year (7). Present consumption, according to the best estimates obtainable ('62),[2] is approximately 41 kg (91 pounds) per capita per year, which is much higher than in mainland U.S.A.

Rice has been used extensively as the principal ingredient in experimental diets to produce dental caries in rats. Hunt and Hoppert (8, 9) at first used coarsely ground, hulled, whole rice, but because they found the size of the food particles to be a factor in producing cavities in rats' teeth, they changed to what they term a "fine" rice diet (10). Their coarse rice was ground so that about 70% of the rice would be retained on a 20-mesh screen, whereas for the "fine" rice only 2 to 3% was retained. They found that even susceptible rats were more resistant to caries when fed the "fine" rice diet as compared with the coarse rice, and somewhat less susceptible when a rice flour made from polished rice passed through a silk bolting cloth was used (10). As we wished to use highly milled white rice of a known type, it was necessary to have it ground in Honolulu. The general aim of the experiments reported in this paper was to investigate the cariogenic effect of feeding rats a diet containing a large proportion of white rice ground to as fine consistency as possible, and simulating a natural diet low in its content of minerals and fat-soluble vitamins. Several modifications of the basic diet were tested.

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[2] Personal communication, John E. Porter, of Porter and McDonald, Rice Importers, Honolulu.

METHODS

Rats. The rats were bred in the Station colony from descendants of a strain susceptible to dental caries, which was originally obtained from the Naval Medical Research Institute, Bethesda, Maryland.

Diets. The basic diet (diets 4 and 4A) contained per 100 g the following foods: rice flour, 76.3; crude casein, 16.0; brewer's yeast, 3.0; cottonseed oil, 3.0; cod liver oil, 0.2; iodized salt, 0.3; salt mixture (11), 1.2.

Short grain white rice from California was thoroughly washed using 4 changes of tap water, as is commonly done by people of Oriental ancestry in Hawaii. It was then drained, air dried and ground. The coarser rice flour ground in our laboratory to pass a 28-mesh sieve was thus of a finer consistency than Hunt and Hoppert's "fine" rice (10). For the fine rice flour, the washed and dried rice was ground by a local commercial company which makes mochi flour from glutinous rice. It was requested that the resulting flour should pass a 60-mesh sieve, but because of the difficulties of grinding the rice, they could not assure us of this fineness. The aim was to have all ingredients in the diets (except diet 4) pass through a 48-mesh sieve. Samples of the rice flour first put through a 48-mesh sieve left only 0.7% of a 100-g sample on a 60-mesh sieve.

PROCEDURE

The aim of experiment 1 was to compare the effect of a somewhat coarse diet (all ingredients through a 28-mesh sieve, diet 4) with a finer diet containing the same proportion of ingredients, all of which would pass a 48-mesh sieve (diet 4A). For this experiment 13 littermate rats were fed each diet for 100 days. Since Hunt and Hoppert (9) had shown a marked difference in the cariogenic properties of their diets containing coarsely ground and "fine" rice, it was desirable to know whether our susceptible strain reacted differently to rice flour of 2 degrees of fineness.

For experiment 2, 11 litters of rats were divided into 4 groups of 21 each to achieve an approximately equal distribution of sexes from each litter. All ingredients in the diets were fine enough to pass a 48-mesh sieve. Diet 4B contained twice as much salt mixture as the basic diet 4A; diet 4C contained 15 times as much cod liver oil as diet 4A; and diet 4D had both increased salt mixture and cod liver oil as in diets 4B and in 4C. The cod liver oil contained 1700 USP units of vitamin A and 170 USP units of vitamin D. The purpose was to learn whether increasing a standard salt mixture and the fat-soluble vitamins would affect the caries rate in the rats.

Dietary studies (12, 13) have shown Hawaii families consuming large amounts of rice to have a relatively low intake of calcium, and because 2% of the Hubbell salt mixture, which furnishes a good supply of calcium and phosphorus, has been found adequate in purified diets, the quantity was purposely used at a low level of 1.2 g/100 g of rice diet 4A, and was increased to 2.5 g/100 g for diet 4B. Previous analyses in this laboratory of California short grain, thoroughly washed rice have shown it to be very low in calcium (7, 14), but relatively high in phosphorus.

For experiment 3, 10 litters of rats were divided among 4 groups of 20 each and fed the following diets: group 1 received the basic diet 4A; group 2, diet 4B with additional salt mixture as for experiment 2; group 3, diet 4E, same as 4B but with 12% of the rice flour replaced with an equal amount of powdered sucrose containing 3% of cornstarch. Group 4 received diet 4A as for group 1, plus daily supplements of fresh whole milk. For the first 2 weeks, each rat was fed a daily supplement of 2 ml pasteurized whole milk; then 5 ml daily for 3 weeks, and 10 ml daily for the remainder of the experiment.

The aim here was to recheck the effect of the addition of a complete salt mixture, and to learn what effect sucrose might have when it replaced part of the rice flour, since it may well be that in human diets it is the use of sweets in addition to white rice that results in a high incidence of dental caries. Also it was desired to learn whether the addition of a small quantity of milk daily influenced the dental caries picture in these rats.

All rats were fed the experimental diets at 21 or 22 days of age. They were housed in individual cages, fed the diets ad libitum and provided with local tap water which is low in total minerals and fluoride (F^- 0.05 ± 0.01). All jaws were prepared and examined using the method of caries evaluation previously described by Miller and Schlack (15).

Prior to evaluating the data by the analysis of variance, the original observations were, in all cases, subjected to transformation according to Snedecor (16, pp. 314–321). The specific transformations used were $(n + 3/8)^{1/2}$ for the numbers (n) of defective teeth and for the numbers of carious areas, and the $\log_{10}(s + 1)$ for the caries scores (s). In the tables the transformed values for square roots for numbers of teeth and number of carious areas are referred to as SR and the logs for caries scores as L.

RESULTS AND DISCUSSION

The results are summarized in tables 1 to 5. For experiment 1, although there were fewer carious teeth, carious areas, and a lower score for the finely ground diet as compared with the more coarsely ground diet (table 1), the differences did not prove to be statistically significant, perhaps because there were too few rats and the coefficient of variation after transformation was greater than usual. However, since there appeared to be some improvement in the incidence and severity of dental caries, and we wished to rule out any effect due to coarse particles, the finer diet (4A) was used for experiments 2 and 3.

TABLE 1

Summary of results of feeding a rice diet of two degrees of fineness

Category of interest	Group 1	Group 2
Diets	4 (Ingredients through 28-mesh sieve)	4A (Ingredients through 48-mesh sieve)
No. of rats	13	13
Rats with caries	12	12
Carious teeth/rat	5.23	3.77
Carious areas/rat	8.62	5.77
Caries scores/rat	15.15	9.46

For experiment 2, feeding diets 4B and also 4D, containing 2 times as much salt mixture as the basic diet 4A, resulted in improved dental conditions that were statistically significant ($P < 0.01$) for the usual 3 criteria of number of teeth, number of carious areas, and extent of carious areas (caries score) (tables 2 and 3). Although the amount of salt mixture in the basic diet 4A appeared adequate for normal growth, it was not sufficient to promote good dental conditions. The consistency of the results for the diets with in-

TABLE 2

Summary of results of feeding a rice diet with additional cod liver oil and mineral mixture

Category of interest	Group 1	Group 2	Group 3	Group 4
Diets	4A	4B (salt mixture)	4C (cod liver oil)	4D (salt mixture + cod liver oil)
No. of rats	21	21	21	21
Rats with caries	20	13	18	12
Carious teeth/rat	3.24	1.10	2.43	1.05
Carious areas/rat	4.62	1.19	3.28	1.19
Caries scores/rat	8.28	1.67	4.62	1.33
Mean wt, males, g	381	388	401	388
Mean wt, females, g	250	259	265	254

TABLE 3

Mean effects and interaction for the salt mixture and cod liver oil factors added to the basic diet

Effect or interaction	Mean effect or interaction		
	Teeth	Carious areas	Score
Salt mixture	−0.2969[1]	−0.3991[1]	−0.2146[1]
Cod liver oil	−0.0756	−0.0969	−0.0822
Salt mixture × cod liver oil	0.0613	0.0880	0.0484

[1] $P < 0.01$.

creased salt mixture (4B in both experiments 2 and 3, and 4D in experiment 2) is evident from the data in tables 2 and 4.

These results are in marked contrast to those obtained by Shaw (17) who found that increasing the salt mixture in his cariogenic diet from 4 to 6% by weight had no effect in reducing dental decay in cotton rats. It appears unlikely that the fluoride content of the mineral mixture used in the experiments here reported was the factor which caused such a marked improvement in dental conditions in our rats, as Shaw and Sognnaes (18) found no decrease in dental decay when their white rats had sufficient fluoride added to provide 25 ppm F^- in the purified diet which was fed postweaning. These authors found that even 25 ppm F^- in the diet during the developmental period had only a slight effect. When the mineral mixture was doubled in our experiments, only 5.4 ppm F^- was supplied.

Just which element or combination of elements in our salt mixture caused the

TABLE 4

Summary of the results of feeding a rice diet and modifications

Category of interest	Group 1	Group 2	Group 3	Group 4
Diets	4A	4B (salt mixture)	4E (4B with 12% sugar)	4A + milk
No. of rats	20	20	20	20
Rats with caries	17	12	16	19
Carious teeth/rat	2.65	1.00	3.05	3.45
Carious areas/rat	3.25	1.00	4.35	4.90
Caries scores/rat	5.55	1.40	7.00	7.95
Mean wt, males, g	371	368	377	376
Mean wt, females, g	243	246	243	252

TABLE 5

Transformed (SR and L) means, mean differences between basic diet with increased salt mixture and other diets, and differences required for significance

Group	Diet	Teeth		Carious areas		Scores	
		SR[1]	Mean difference	SR	Mean difference	L[2]	Mean difference
2	4B	1.0887		1.0887		0.2945	
1	4A	1.6462	0.5575[3]	1.7812	0.6925[3]	0.7023	0.4078[3]
3	4E	1.7109	0.6222[3]	1.9361	0.8474[3]	0.6994	0.4049[3]
4	4A + milk	1.8842	0.7955[3]	2.1709	1.0822[3]	0.8419	0.5474[3]
Highly significant difference between any two diets			0.4384		0.6318		0.3092

[1] The transformed values for square roots for numbers of teeth and number of carious areas are referred to as SR.
[2] L indicates logs for caries scores.
[3] $P < 0.01$.

improved dental conditions cannot be determined from these experiments.

McClure and co-workers (19), using an entirely different type of diet with high proportions of skim milk powder and cornstarch with glucose and liver powder, noted that adding 4% of the Osborne-Mendel salts with NaF omitted caused a statistically significant decrease in smooth surface caries. Many years ago Agnew and co-workers, using either wheat or corn as the basic cereal in their diets, observed that an adequate supply of phosphorus was an important factor in the prevention of dental caries, and that an adequate supply of vitamin D prevented or delayed dental caries in rats (20). McClure and Muller (21) tested several calcium and phosphorus salts and suggest that the relative solubility of the phosphates in the oral cavity may be responsible for the cariostatic action.

There was little evidence that increasing the cod liver oil by 15 times had any real effect on decreasing tooth decay, as only for the extent of the carious areas (caries score) did the results approach significance. No evidence was noted of interactive effects between amounts of salt mixture and of cod liver oil levels (table 3). It may be concluded that for these rats and under the conditions of these experiments, neither increased vitamin A nor vitamin D altered the dental conditions.

The results for experiment 3 are summarized in tables 4 and 5. Again, as in experiment 2, increasing the salt mixture improved the dental conditions as judged by the 3 criteria to the extent that the changes were highly significant ($P < 0.01$). But, when 12% of sugar replaced an equal weight of rice flour in diet 4E, increasing the salt mixture was not effective and the teeth conditions were so much worse than with diet 4B that there was a highly significant difference ($P < 0.01$) for all 3 criteria.

No satisfactory explanation can be given for the poor condition of the teeth when small amounts of milk were added to the rice diet. Table 4 shows the teeth were poorer than with the basic rice diet 4A, although the differences did not prove to be statistically significant (table 5). Either sufficient additional minerals were not provided by the milk, or it helped to promote oral conditions favorable to dental decay. Parents in Hawaii have often insisted to the author that their children have "plenty of milk," but they still have serious dental decay. If sugar had been added to the diet of group 4, as for group 3, it may safely be speculated that the teeth would have been in even poorer condition.

Mellenby (22) has demonstrated the good effect of vitamin D in reducing dental decay in temperate climates such as England. When she visited Hawaii in 1951, she expressed great surprise that despite the long hours of sunshine in Hawaii, there was a high incidence of dental decay among young school children. The habit of frequent consumption, or almost continuous eating during waking hours, of sweets and other sticky carbohydrate foods by children in Hawaii probably plays the most important role in promoting dental decay (23).

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