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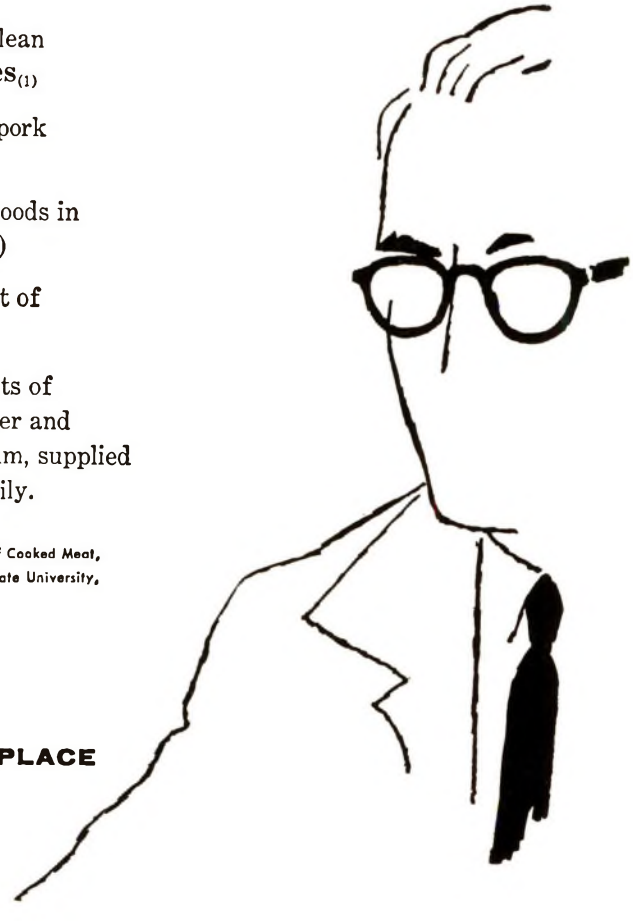

1. Leverton, R. M., and Odell, G. V.: The Nutritive Value of Cooked Meat, Oklahoma Agricultural Experiment Station, Oklahoma State University, Miscellaneous Publication MP-49, 1958.

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Relation of Diet to Rumen Volatile Fatty Acids, Digestibility, Efficiency of Gain and Degree of Unsaturation of Body Fat in Steers[1]

J. C. SHAW, W. L. ENSOR, H. F. TELLECHEA AND S. D. LEE
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In previous reports we have shown that the molar proportions of the rumen volatile fatty acids (VFA), and especially acetic acid and propionic acid, can be controlled by diet to a remarkable degree, and that the molar proportions of these two acids are closely related to the fat content of milk. It was concluded that their proportions in the rumen could be used to predict the relative effect of a ration upon the fat content of milk (Ensor et al., '59; Shaw, '58, '59; Shaw et al., '57, '59). The latter suggestion was based in part on the observations of McCarthy et al. ('58), working with perfused goat rumina, that the molar proportion of VFA in the rumen represents both relative production and absorption. In these studies it was also noted that the molar proportion of the rumen VFA did not change appreciably with time after feeding. These observations have since been confirmed and extended in *in vivo* studies by Ensor.[2] The greatest and most rapid decrease in the fat content of milk was achieved by means of a ration of finely ground (and pelleted) hay plus steam-heated corn (Shaw, '58; Ensor et al., '59). This was accompanied by decreases in the molar proportion of rumen acetate and increases in the molar proportion of rumen propionate of considerable magnitude. Balch and Rowland ('57) had noted that the feeding of equal parts of a finely ground hay and a mixed concentrate effected a marked decrease in the molar proportion of rumen acetate and an increase in the molar proportion of rumen propionate; our studies showed that steamed corn accentuates this effect. For reviews of earlier work see Balch et al. ('55) and Shaw et al. ('59).

It next became of interest to determine whether changes in the molar proportion (production) of these acids would be reflected in changes in the efficiency of gain in body weight. Armstrong and Blaxter ('57a, b) and Armstrong et al. ('57) had demonstrated with sheep that the heat increment of added acetic acid was greater than that of added propionic or butyric acid. This, combined with the fact that the rumen acid production could now be controlled to such a remarkable degree and could affect animal production (fat content of milk) led to the hope that control of efficiency of gain in body weight might also be achieved by controlling rumen acid production. This report deals with such a study. A brief report has been issued on some of the earlier phases of the study (Ensor et al., '59).

EXPERIMENTAL

Sixteen Holstein steer calves, each of which had been maintained for two months on a daily ration of 4 pounds of a commercial concentrate mixture plus alfalfa hay fed ad libitum were divided as evenly as possible according to weight into two groups of 8 animals each. A change-over to experimental rations was effected in three days. The animals in both groups received U. S. no. 1 alfalfa hay, corn meal and linseed oil meal in the proportions 1:1:0.04 and were allowed free access to salt blocks. For group 1 the hay was chopped (approximately 2″ length) and ground corn was

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[1] Scientific article no. A774, contribution no. 3035 of the Maryland Agricultural Experiment Station.

[2] Ensor, W. L. 1959 Effect of feeding ground hay and heated grains alone and in various combinations to cows upon rumen organic acids and fat content of milk. Doctorate thesis, University of Maryland.

used. For group 2 the same hay was used but was finely ground and then pelleted; the corn consisted of flaked corn which had been subjected to steam for several minutes in the process of flaking. Studies in progress at that time had already demonstrated that steam-heated corn when fed with ground hay had a greater effect on the rumen VFA than either steam-heated or unheated corn.[3]

The same hay was used for all animals, alternate bales of hay being ground and pelleted, leaving half of the hay to be chopped. Care was taken in both the grinding and the chopping to keep the leaf loss to a minimum. Pelleted linseed oil meal was used for both groups; this meal had been heated to over 200°C during processing. After 91 days of feeding it was necessary to obtain additional alfalfa hay, the hay selected being of a similar quality and processed in the same manner as the first lot.

All steers were fed individually according to body weight at 100% of Morrison's ('56) recommended total digestible nutrients for growing beef cattle. There was no "weigh-back"; any feed left uneaten was mixed with the regular allowance at the next feeding, this allowance being decreased when necessary to insure complete consumption. The feed intake of all animals (per 100 pounds of body weight) was regulated according to the amount eaten by the animal consuming the least. During feeding the animals were locked in closed mangers. They were maintained on clean concrete without access to bedding at all times. Weights were taken for three consecutive days at the beginning, at 56 and 91 days (the latter coinciding with the end of the first lot of hay) and at the end of the trial. In addition, body weights were obtained weekly for the calculations of the changes in feeding which were made at the end of each week.

Rumen fluid was obtained from three animals in each group after they had been on the experimental rations for 56 days. The samples were obtained 4 hours after feeding by means of a large-bore rumen tube. Immediately after centrifuging, the supernatant was analyzed for rumen VFA by a modification of the technique of Wiseman and Irvin ('57). Samples of feed were taken weekly from which composite samples were prepared for analysis. Uniform sampling was facilitated by the physical form of the feed (pelleted hay, chopped hay, ground or flaked corn, pelleted linseed oil meal).

Digestion trials were conducted with all the animals after they had been on the experimental rations for 87 days. Total collection of feces was made and weighed for three consecutive days. Five per cent aliquots of the daily collections from each steer were dried in a hot forced-air oven at 70°C for a minimum of 48 hours and equilibrated with atmospheric moisture for 7 days to bring them to air dryness, the weights being taken before placing them in the oven and after equilibration. All samples were ground and composite aliquot samples were taken from the daily aliquot sample from each steer. Throughout the trial, samples of the different feedstuffs were taken at random at the time of feeding. Composite samples were prepared for analysis similarly to the feces. Proximate analyses of the feed and feces samples were carried out according to A.O.A.C. methods ('50) with modifications for nitrogen according to Scales and Harrison ('20).

Final live weight and carcass weights and grades were obtained. Visceral and subcutaneous fat were obtained from two animals in each group and, following purification, were analyzed for iodine number by the method of Rosenmund and Kuhnhenn ('23).

RESULTS

The proximate analyses of the feeds used are shown in table 1 and the digestion coefficients in table 2. Note that the digestibility of total carbohydrate and fat was about the same for the two groups, whereas grinding the hay and heating the corn enhanced the digestibility of crude protein by approximately 12%. An analysis of variance resulted in a statistical significance at the 1% level. The increased digestibility of the protein was accompanied by an increase in the average digestibility of the total dry matter of the ration, although this increase was not

[3] See footnote 2, page 203.

TABLE 1

Percentage composition of feeds used in experiment (dry-matter basis)

Feed	Dry matter	Crude protein	Ether extract	Crude fiber and nitrogen-free extract	Ash
	%	%	%	%	%
Alfalfa hay no. 1					
2″ Chopped	87.2	16.5	1.2	73.7	8.6
Ground and pelleted	89.2	17.0	1.6	72.6	8.8
Alfalfa hay no. 2					
2″ Chopped	90.5	19.0	1.5	71.0	8.5
Ground and pelleted	87.3	18.1	1.5	70.7	9.8
Ground corn	87.1	8.4	4.5	86.3	.8
Flaked corn	88.3	8.1	4.3	86.8	.8
Linseed oil meal	88.6	34.7	1.5	58.4	5.4

TABLE 2

Average digestion coefficients obtained with the steers during the feeding trial (dry-matter basis)

Group[1]	Ration	Dry matter	Crude protein	Ether extract	Crude fiber and nitrogen-free extract
1	2″ Chopped alfalfa hay no. 1 + ground corn + linseed oil meal (1:1:0.04)	67.7 ± 3.3[2,3]	55.3 ± 4.6[4]	68.4 ± 4.0	72.3 ± 3.0
2	Ground and pelleted alfalfa hay no. 1 + flaked corn + linseed oil meal (1:1:0.04)	69.9 ± 3.3[3]	66.6 ± 4.4[4]	68.8 ± 5.3	71.8 ± 3.2

[1] Eight steers per group.
[2] Standard deviation.
[3] Difference between group means not statistically significant.
[4] Difference between group means significant at 1% level.

statistically significant. As shown in table 3, the rumen VFA were altered greatly by feed processing, the molar proportion of acetate decreasing and that of propionate increasing with the grinding of the hay and the steam-heating of the corn. This was accompanied by more than a two-fold increase in the concentration of total rumen fluid VFA.

Animal performance. Data on animal performance are shown in table 4. The steers receiving the ground and pelleted hay and flaked (steamed) corn (group 2) gained an average of 2.44 pounds per animal per day during the 116-day period, compared with 2.00 pounds for the control group. The difference was significant at the 1% level. These gains were made with 77.7 pounds less feed dry matter per 100 pounds of body weight gain than those of the control group, the increased efficiency being significant at the 1% level. The rate of gain was relatively uniform throughout the total period, groups 1 and 2 averaging 2.05 ± 0.14 and 2.55 ± 0.19 pounds per day respectively during the first 56 days. The average feed dry matter per 100 pounds of gain during this period was 399.0 ± 28.0 and 329.7 ± 28.5 pounds, respectively, for groups 1 and 2. The differences between the two groups in both daily gain and efficiency of gain were

TABLE 3

Rumen volatile fatty acids (VFA) of steers fed experiment rations

Group[1]	Volatile fatty acids					VFA in total rumen fluid
	C_2	C_3	C_4	C_5	C_{6+}	
	Molar % of total					*mg/100 ml*
1	68.2 ± 0.7[2]	16.3 ± 4.8	10.9 ± 1.0	2.5 ± 1.7	2.0 ± 2.4	580.4 ± 151.5
2	47.2 ± 3.0	41.1 ± 4.2	9.2 ± 0.9	1.9 ± 0.3	0.8 ± 0.7	1357.4 ± 184.0

[1] Eight steers per group.
[2] Standard deviation.

TABLE 4

Feed intake, body weight gains, and dressing percentage during 116-day feeding experiment (feed data on dry-matter basis)

Group	Average initial weight	Average daily gain in weight	Average dressing percentage	Total feed intake				Average pounds feed 100 pounds gain
				Hay	Corn	LOM[1]	Total	
	pounds	*pounds*		*pounds*	*pounds*	*pounds*	*pounds*	
1	274.8 ±47.0[2]	2.00[3] ±0.18	53.6[4] ±1.17	4558	4549	188	9295	501.9[3] ±40.9
2	276.4 ±41.6	2.44[3] ±0.14	55.1[4] ±1.20	4708	4714	186	9608	424.2[3] ±34.6

[1] Linseed oil meal.
[2] Standard deviation.
[3] Difference between group means significant at 1% level.
[4] Difference between group means significant at 5% level.

highly significant statistically. At the end of 91 days, just before the change to the second lot of hay, group 2 had gained 0.45 pounds more per animal per day than group 1.

No differences were observed in carcass grades between the two groups. It was apparent that the greater gains in group 2 were due to more rapid growth. Dressing percentage was somewhat higher for group 2 than group 1.

The iodine number of the fat of two animals in each group is shown in table 5. The animals in group 2 exhibited a higher degree of unsaturation of fat than the two animals in the control group, the differences being greater in the visceral than in the subcutaneous fat. Although values were obtained on only two animals in each group, good agreement was obtained within each group, leaving little doubt that the values represented a real difference in unsaturation of fat due to diet.

TABLE 5

Iodine number of body fat of steers

Group	Steer no.	Iodine number	
		Visceral fat	Subcutaneous fat
1	10	34.7	45.4
	12	35.9	42.9
2	11	41.9	47.1
	14	42.9	46.1

DISCUSSION

The 22% increase in body weight gain obtained by grinding and pelleting the hay and flaking (steam-heating) the corn was achieved with a 15.3% increase in efficiency of gain, presumably due in part to the greater energy provided by the increase in digestibility of the protein and, to a greater degree, to the decrease in the molar proportion of rumen acetate and the increase in the molar proportion of rumen propionate. The significance of the more than two fold increase in the concentration of total rumen VFA is difficult to appraise. We have observed, in unpublished work, that rations consisting solely of ground and pelleted hay induced as much as a two fold increase in total rumen VFA but did not effect marked changes in either the molar proportions of rumen VFA or in the fat content of

milk. Balch ('58) noted that incubating ground hay and concentrates with rumen contents *in vitro* gave a greater increase in total VFA than the addition of rumen contents to a mixture of long hay and concentrates, thus suggesting that fermentation *in vivo* would be more rapid in the former case. The ideal technique to appraise the effect of diet on total VFA production would appear to be the perfused rumen (McCarthy et al., '58). In earlier studies (Shaw, '58; Ensor et al., '59) we had noted that when as little as 4 pounds of steam-heated corn was fed with 30 pounds of ground and pelleted alfalfa hay, there was a marked increase in the molar proportion of rumen propionate and a similar marked decrease in the molar proportion of rumen acetate. It would appear that the change in the rumen microorganisms (Leffel et al., '56, Eusebio et al., '59), which occurs in cattle fed low milk-fat producing rations, results in the dissimilation of the hay to a greater proportion of propionate since the feeding of as little as 4 pounds of steamed corn per day with 28 to 32 pounds of ground and pelleted hay induces a remarkable increase in the molar proportion of rumen propionate (Shaw, '58; Ensor et al., '59). This increase was of too great a magnitude to have been achieved entirely from the dissimilation of the small amount of steamed corn which was fed. Thus it appears that the available energy of hay is increased when it is ground and fed with steam-heated corn. Unheated grains also induce this change when fed with ground hay but to a lesser extent (Shaw, '58).

There had been reason to hope that a diet which would enhance the proportion of propionate produced in the rumen would effect an increase in the efficiency of beef production. The 12% increase in the digestibility of the protein, on the other hand, was more unexpected. It seems logical to conclude that this was due to a change in rumen microorganisms since, as was noted earlier, we have observed that microbial metabolism is altered markedly by such diets.

When using such rations with milking cows we observed in unpublished studies that iodine values as high as 75 were obtained for milk fat. Since it was now known that such rations effect marked changes in rumen microbial metabolism it was conceived that the cause might be simply a lesser degree of hydrogenation of the long-chain unsaturated fatty acids of the hay and corn by the rumen microorganisms. It was reasoned that if this was the case such diets might also be expected to result in an increase in the long-chain unsaturated fatty acids in body fat. The data in table 5 shows that there was indeed such an increase, especially marked in the case of the visceral fat. Thus it is possible by dietary means to exert considerable influence on the degree of unsaturation of both milk fat and body fat, presumably by decreasing the hydrogenation of unsaturated fatty acids within the rumen.

The results of this study indicate that both rate and efficiency of body weight gain in beef cattle may be controlled to a remarkable degree by controlling rumen microbial metabolism. It is conceived that this may be accomplished not only by certain feed processing procedures such as the grinding of hay and cooking of concentrates, but also by providing certain feed additives to alter the proportional (and perhaps total) production of rumen VFA. For example, in a recent study (Shaw and Ensor, '59) it was noted that the addition of either cod liver oil or linoleic acid to the diet of cows markedly decreased the ratio of acetate to propionate in the rumen and increased total rumen VFA concentration. This was accompanied by large decreases in the fat content of the milk.

CONCLUSIONS

A ration differing from the basal ration only in that the hay was ground and pelleted and the corn steamed in the process of flaking, effected the following results when fed to steer calves:

1. A marked decrease in the molar proportion of rumen acetate and an equally marked increase in the molar proportion of rumen propionate.
2. An increase of 12% in the digestibility of protein.
3. An increase of 22% in body weight gain.
4. An increase of 15.3% in efficiency of utilization of feed.

5. An increase in the degree of unsaturation of body fat.

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The Protein Requirement of the Growing Chick Determined with Amino Acid Mixtures

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When diets are formulated from natural ingredients containing conventional amounts of fat, chick growth is maximized with a concentration of 20% protein and this value forms the recommendation of the National Research Council ('54). Recent work has shown, however, that this level of protein is distinctly suboptimal when low-fiber experimental diets of the glucose-casein type are used (Hogan et al., '53; Scott et al., '57; and Hinners and Scott, '60). While the original observation by Hogan et al., ('53) was made on a casein-arginine purified diet, the subsequent work noted above has demonstrated that other intact proteins (isolated soybean protein; a mixture of soybean meal, casein and gelatin; and fishmeal) are also unable to satisfy the protein need of the young chick at the 20% level and that the requirements under these conditions are of the order of 35%. With egg-white protein and a synthetic type ration a requirement of 25% protein for growth has recently been found (Hinners and Scott, '60). The high requirement for protein in purified diets is related to the high level of readily available energy in diets of this type (Scott et al., '57). Similarly, when the energy content of a natural diet is increased markedly by supplemental fat, a simultaneous increase in protein above the 20% level must be made if growth depression is to be avoided (Biely and March, '54).

Recently, Klain et al., ('58, '60) determined the amino acid requirements of the chick using an amino acid mixture containing approximately 3.2% of total nitrogen (20% protein). This amino acid mixture, containing the essential amino acids for the chick and glutamic acid to supply the remainder of the nitrogen requirement, resulted in a daily gain of 7 to 8 gm and a gain-to-feed ratio of 0.5 from 7 to 14 days of age. These results could not be improved by increasing this amino acid mixture to provide 30% protein. However, when the essential amino acids of this mixture were increased over twofold and the mixture was supplemented with alanine, serine, aspartic acid and proline, so as to bring the total protein to approximately 36%, the daily gain and feed efficiency were increased to 10 to 12 gm and 0.8, respectively.[1] Since the protein level of this improved diet was considerably higher than the earlier diet, and since all essential amino acids were present at approximately twice the minimum requirement levels previously reported, it seemed desirable to determine the minimum protein level required for this efficient mixture that would support maximum performance of the chick in order to obtain a true estimate of amino acid requirements. In addition, the nitrogen requirement was studied on another highly efficient amino acid mixture.

EXPERIMENTAL

The composition of the two amino acid mixtures used is given in table 1. These mixtures differed primarily in that alanine, serine and aspartic acid were eliminated in mixture 2, since it had been found that the absence of these three amino acids from the mixture did not affect the performance of the chicks.[2] Thus, the ratio of essential to non-essential amino acid

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[1] Klain, G. J., H. M. Scott and B. C. Johnson 1959 The effect of highly efficient amino acid diets on individual amino acid requirements. Federation Proc., *18*: 532 (abstract).

[2] Unpublished data.

TABLE 1

Composition of amino acid mixtures

	Mixture 1	Mixture 2
L-Arginine·HCl	1.54[1]	1.97[1]
L-Histidine·HCl	0.61	0.78
L-Lysine·HCl	1.46	1.87
L-Tyrosine	0.82	1.05
L-Tryptophan	0.20	0.25
L-Phenylalanine	0.70	0.89
L-Cystine	0.47	0.60
DL-Methionine	0.21	0.27
L-Threonine	0.68	0.87
L-Leucine	1.98	2.53
L-Isoleucine	0.86	1.10
L-Valine	0.98	1.25
Glycine	0.59	0.75
L-Glutamic acid	8.56	10.95
L-Proline	2.00	0.86
L-Aspartic acid	1.06	—
DL-Alanine	1.01	—
DL-Serine	2.05	—
Total	25.78	26.04

[1] Per cent in diet when amino acid mixture was fed at 3.2% nitrogen level.

nitrogen is higher in mixture 2 than in mixture 1, whereas the ratio of the essential amino acids to one another is the same in both mixtures.

To establish the nitrogen requirement, the chicks were fed graded levels of the amino acid mixture in question, equivalent to the nitrogen levels indicated in the corresponding tables. The criteria used to establish this requirement in experiment 1 were (1) average daily gain per chick, (2) protein content of the carcass, and (3) efficiency of feed utilization. In experiment 2 the additional criteria, (4) nitrogen retained and (5) fat content of the carcass, were also used. The nitrogen and fat content of the chick carcass were determined by the method of Rand.[3]

Experiment 1. The basal diet, except for level of fat and the amino acid mixture, was similar to that previously described by Klain et al. ('58). The level of dietary fat used in this experiment was 10%. Details concerning the type of chicks, their selection and care are to be found in the same report. Two replicates were made of three chicks in each treatment group, and the carcass protein content was determined on each replicate group. In varying the nitrogen from 3.2 to 7.2% it was assumed that at least the lowest level would be inadequate for chick growth.

Experiment 2. Chicks were housed individually in electrically heated wire cages equipped with pyrex dishes for the collection of excreta. Five chicks, carefully selected for uniformity of weight at 9 days of age, were fed each experimental diet for 5 days. Excreta were collected under 1 N HCl, blended, brought to volume and aliquots taken for nitrogen determination by the Kjeldahl method. On termination of the experiment, chicks were fasted for 24 hours, and the two nearest the mean weight of each treatment group were selected for carcass nitrogen and fat determination. The basal diet differed from that of the first experiment in that corn oil was increased to 15% at the expense of corn starch. Based on the results of the first test it was decided to vary the nitrogen content of the diets from 1.6 to 5.6%.

RESULTS

The experimental design and results of experiment 1 are presented in table 2. On examination of the data it becomes evident that the average gain on the lowest level of nitrogen (diet 1) equals that on the next two higher levels. Furthermore, the nitrogen levels in diets 4, 5 and 6 resulted in growth depression which was accentuated by each increment of nitrogen.

The carcass analysis revealed that the chicks fed diet 1 stored the least amount of protein, despite the fact that the gains with the first three diets were equal. Thus it appears that the chicks on the lowest level of dietary nitrogen stored more body fat than the chicks on the higher nitrogen levels. The results obtained in experiment 2 confirm this assumption.

It is worth noting that efficiency of feed utilization improved with increments of nitrogen in the diets, reaching a plateau at the 4.8% level (diet 3). Considering average gain as the sole criterion for the nitrogen requirement, it is apparent that the 3.2% level met the need for nitrogen. However, it is obvious that the diet was most efficiently utilized at the 4.8% nitrogen level. This finding was unexpected, since it has been generally assumed that the amino acid levels which support maxi-

[3] Rand, N. T. 1957 The utilization of fat by the growing chick. Ph.D thesis, University of Illinois.

TABLE 2

The effect of dietary nitrogen levels upon weight gain, feed utilization and carcass composition of the chick (exp. 1)

Diet no.	Dietary N level[1]	Gain/chick/day[2]	Gain/feed	Av. feed consumption[2]	Carcass protein[2]
	%	gm		gm	%
1	3.2	10.6 ± 0.31[3]	0.65	113	16.83
2	4.0	10.4 ± 0.30	0.70	104	17.61
3	4.8	10.9 ± 0.40	0.78	97	17.26
4	5.6	9.4 ± 0.27	0.77	88	17.86
5	6.4	9.0 ± 0.34	0.78	80	17.90
6	7.2	7.7 ± 0.36	0.75	72	17.59
7	Control[4]	12.0 ± 0.54	0.75	112	17.63

[1] Amino acid mixture no. 1.
[2] Average of two replicates of three chicks/treatment, from 7 to 14 days of age.
[3] Mean ± standard error.
[4] Isolated soybean protein (Drackett Assay Protein C-1) plus methionine and glycine substituted for the amino acid mixture to furnish 30% protein. N × 6.25).

mum growth will also result in maximum efficiency of feed utilization.

The results of the second experiment are given in table 3. Weight gains increased with increasing nitrogen levels up to about the 3.2% level, with perhaps a slight growth depression at the 4.8 and 5.6% levels. Feed utilization reached a maximum at the 4.0% level of nitrogen, which was also the level at which maximum nitrogen retention was obtained. This is in agreement with the requirement of 25% protein as egg white for growth reported by Hinners and Scott ('60). The nitrogen content of the carcass was not altered by varying the nitrogen level in the diet except for a slight increase at the two highests levels. Carcass fat was inversely related to the level of nitrogen in the diet. This observation was not surprising in view of the higher energy intake relative to gain on the lower nitrogen levels.

It is obvious that the voluntary consumption of food was influenced to a great extent by the nitrogen content of the diets. In both experiments, feed intake decreased as the level of dietary nitrogen increased except at the lowest level (1.6%) of nitrogen in experiment 2. It would seem, therefore, with these amino acid mixtures, at least, that the chick was able to compensate to a large degree for a low concentration of nitrogen (3.2%) by consuming more of the diet.

While maximum gain was attained at approximately 3.2% of nitrogen with both amino acid mixtures, maximum feed utilization was reached at a lower level of nitrogen with amino acid mixture no. 2 than with mixture no. 1. In comparing the two

TABLE 3

The effect of dietary nitrogen level on weight gain, feed utilization, nitrogen retention and carcass composition (exp. 2)

Treatment no.	N level[1]	Gain/chick/day	Gain/feed	Av. feed consumption	N retained	Carcass protein[2]	Carcass fat
	%	gm		gm	gm	%	%
1	1.6	7.5 ± 0.47[3]	0.374	100	0.714	16.91	13.16
2	2.4	10.8 ± 0.64	0.489	112	1.326	17.16	11.15
3	3.2	12.6 ± 0.99	0.588	107	1.659	16.91	9.71
4	4.0	13.3 ± 1.08	0.623	107	1.837	16.88	7.96
5	4.8	11.6 ± 0.87	0.592	99	1.818	18.18	3.84
6	5.6	11.7 ± 0.39	0.621	94	1.889	17.48	3.92
7	Control[4]	18.8 ± 0.47	0.852	111	—	—	—

[1] Amino acid mixture no. 2.
[2] N × 6.25.
[3] Mean ± standard error.
[4] Isolated soybean protein (Archer-Daniels-Midland Co., Cincinnati) plus methionine and glycine to supply 30% protein replaced amino acid mixture.

amino acid mixtures (table 1) it can be seen that mixture no. 2 contains a higher level of essential amino acids than mixture no. 1 at any given nitrogen level. Thus it becomes apparent that the requirement for essential amino acids, expressed as absolute intake, is a function of the weight and rate of gain of the chick and that under the conditions specified the chick consumed sufficient amounts of the diet to obtain these required amino acids. This probably is possible only when a well balanced amino acid mixture is used. Under these conditions, chicks fed a low nitrogen diet voluntarily ate more food (and stored more body fat as a result) in an effort to obtain the required amino acids. Thus amino acid requirements expressed as percentage of the diet are valid only when they insure maximum utilization of feed, even though lower concentrations may permit optimum gain and nitrogen retention.

SUMMARY

The nitrogen requirement of chicks was determined using crystalline amino acid diets. For optimum efficiency of feed utilization the requirement was found to be approximately 4.0% nitrogen. It was observed, however, that a lower nitrogen level (3.2%) resulted in equally good growth and nitrogen retention, but lower feed utilization. Chicks were able to gain satisfactorily on this low nitrogen diet by voluntarily eating more of the ration. This has the effect of increasing the absolute intake of dietary amino acids.

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Quantitative Aspects of Lysine Deficiency and Amino Acid Imbalance[1]

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The recent studies of Harper ('59) and Munaver and Harper ('59) have focused attention on the dearth of direct information on the subject of amino acid imbalance which may be vitally important in various aspects of protein nutrition, as well as in the estimation of amino acid requirements. Several thought-provoking hypotheses are suggested by Harper on the basis of his experimentation. It was the object of the present investigation to test these theories with an independently designed series of experiments. The results, which confirm the essential aspects of Harper's observations, permit a different interpretation of certain aspects of amino acid imbalance as defined by Harper ('59).

GENERAL PROCEDURES

Week-old Vantress cockerels were used in the first three trials and crossbred cockerels (N.H. ♂ × Columbian ♀) in the last. Duplicate groups of 7 chicks per lot were assigned to each dietary treatment. For the first week the chicks were fed a standard starting ration at the end of which time they were selected by weight for assignment to the treatment groups. The experimental diets and water were given ad libitum and fed for a two-week experimental period until the animals were three weeks old. In table 1 are shown the composition of the basal ration and that of the lysine-free amino acid mixture[2] used to produce the amino acid imbalance.[3] The dietary protein, sesame meal, when properly supplemented with lysine, is of very high biological value for the

TABLE 1

Composition of basal ration and of lysine-free amino acid mixture used to produce an amino acid imbalance

Basal ration	
Ingredients	Amount
	%
Sesame meal (46.3% protein)	23.76
Mineral mix[2]	4.94
Corn oil	3.00
B-vitamin mix[3]	0.15
Vitamin A, D and E mix[3]	0.10
Choline chloride	0.20
Glucose[4]	to 100

Amino acid mixture[1]	
Amino acid	Amount
	%
DL-Tryptophan	2.08
DL-Methionine	2.34
L-Cystine	2.66
DL-Isoleucine	8.56
DL-Threonine	8.05
DL-Valine	10.77
L-Arginine·HCl	8.05
DL-Phenylalanine	6.75
L-Tyrosine	3.37
L-Glutamic acid	23.56
L-Histidine HCl·H_2O	3.89
L-Leucine	6.49
Glycine	13.43
Total	100.00

[1] When 8.18% L-lysine is added, this mixture is adequate to support growth of chicks (Fisher and Johnson, '57).

[2] Based on the mineral requirements reported by Scott ('59) and consisting of: $Ca_3(PO_4)_2$, 0.85; KH_2PO_4, 1.05; NaCl, 0.80; $CaCO_3$, 1.90; Fe-gluconate, 0.052; $MgSO_4$, 0.25; $MnSO_4H_2O$, 0.02; KI, 0.001; $CuSO_4$ anhyd., 0.00128; $ZnCO_3$, 0.01 and $Na_2MoO_4 \cdot 2H_2O$, 0.001% (in % of total diet).

[3] For composition see Fisher and Johnson ('56).

[4] Cerelose.

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[2] This amino acid mixture when supplied with lysine will support adequate growth in chicks (Fisher and Johnson, '57).

[3] For materials supplied, the authors wish to thank Merck Sharp and Dohme, Rahway, New Jersey and E. I. duPont and Company, Wilmington, Delaware.

growing chick (adequate in all other amino acids). All experiments reported hereafter were carried out with the same supply of sesame meal; a lysine content of 2.7% of the protein was assumed in all calculations (Block and Weiss, '56).

EXPERIMENTAL AND RESULTS

Trial 1. The first experiment was designed to test whether an amino acid imbalance could be produced with the growing chick under conditions similar to those which will bring about an imbalance in the growing rat. At the same time the growth response to the variables under investigation, namely, protein and lysine level, was studied over the range of 11 to 26% for protein, and at 2.7, 4 and 7% of the protein for lysine. Previous work from this laboratory has suggested the soundness of expressing the lysine requirement on the basis of protein level rather than as a percentage of the total diet (Fisher et al., '59). Combinations of the three lysine levels with the two protein levels of 11 and 23% were also studied in the presence of the lysine-free amino acid mixture.

The complete design for trial 1, together with the results obtained, is shown in table 2. These data show that (1) the addition of the lysine-free amino acid mixture depressed growth when lysine was supplied at 4% of the dietary protein level, (2) the depression, in per cent, was greater with 11% dietary protein than on the 23% protein level and (3) no depression occurred when lysine was supplied either at the 2.7 or the 7% level as a percentage of the dietary protein.

The lysine consumption and efficiency data provide interesting information regarding the imbalance resulting from the addition of the lysine-free amino acid mixture. In general, regardless of the addition of the imbalancing amino acid mix-

TABLE 2

Growth, feed and lysine consumption, and lysine efficiency of chicks receiving various levels of protein, lysine and a supplement of a lysine-free amino acid mixture[1]

Protein level	Lysine level[2]	Amino acid supplement[3]	Final body weight	Av. feed consumption/ bird	Av. lysine consumption/ bird	Lysine efficiency gain/gm lysine consumed
%	%	% of diet	gm	gm	gm	
11	2.7	—	116 ± 2[4]	148	0.44	14.8
11	2.7	4.0	115 ± 3	135	0.40	14.2
11	4.0	—	148 ± 4	219	0.97	39.9
11	4.0	4.0	129 ± 4	164	0.72	25.5
11	7.0	—	258 ± 11	418	3.23	46.1
11	7.0	4.0	250 ± 9	346	2.67	51.2
14	2.7	—	129 ± 4	175	0.67	28.1
14	4.0	—	167 ± 4	238	1.34	42.7
14	7.0	—	284 ± 10	451	4.42	39.3
17	2.7	—	129 ± 11	161	0.74	25.3
17	7.0	—	340 ± 6	449	5.34	43.1
20	2.7	—	144 ± 4	191	1.04	32.6
20	7.0	—	363 ± 6	466	6.68	36.8
23	2.7	—	142 ± 4	169	1.05	31.3
23	2.7	4.0	146 ± 3	196	1.22	29.1
23	4.0	—	268 ± 8	393	3.62	43.8
23	4.0	4.0	245 ± 10	348	3.20	42.1
23	7.0	—	381 ± 7	483	7.77	35.0
23	7.0	4.0	378 ± 8	458	7.36	36.3
26	2.7	—	161 ± 4	219	1.52	33.3
26	4.0	—	295 ± 6	427	4.44	41.8
26	7.0	—	395 ± 5	496	9.02	31.6

[1] Results are from duplicate groups of 7 birds maintained on experimental rations from 7 to 21 days of age. Average starting weight for all groups, 110 gm.

[2] Expressed as free L-lysine as a percentage of the sesame protein content of the diet; L-lysine HCl (95%) was the actual supplement.

[3] Lysine-free, for composition see table 1.

[4] Mean ± standard error.

ture, there appears to be a close relationship between feed and therefore lysine *consumption* and growth rate. For example, the lysine consumption, and the efficiency and growth of the animals receiving 11% protein–4% lysine together with the amino acid mixture is essentially the same as for the birds on the 14 and 17% protein and 2.7% lysine diets (see italicized data, table 2). If one considers that the addition of the 4% amino acid mixture to the 11% protein diet has raised the total protein to 15%, then the lysine level can actually be expressed as only 2.9% of the total protein (or the same as for the 14% protein–2.7% lysine group). It appears then that, aside from the depressed food and lysine *intake*, there is no evidence that the imbalance impaired lysine *utilization*. Thus, the ratio of lysine to the total protein or the effective amino acids may be the determining factor in the production of an imbalance, which is really an exaggeration of the existing lysine deficiency.

The lack of a growth-depressing effect of the lysine-free amino acid mixture at the 7% level of lysine needs no explanation, since this concentration of lysine is in excess of the requirement for maximum growth (Fisher et al., '59). The lack of effect at the 2.7% lysine level may be rationalized on the basis of an inadequate amount to permit growth but an adequate amount to meet the maintenance requirement. Since the very low lysine maintenance requirement of the chicken (Leveille and Fisher, '59) is adequately met at the 2.7% level, even the addition of a lysine-free amino acid mixture is not likely to induce a deficiency.

In confirmation of Harper's observations, the data in table 2 show a greater depression due to the addition of the 4% amino acid mixture to the 11% protein–4% lysine diet (13%) than with the 23% protein–4% lysine diet (8%).

Trial 2. As a further check on the relationship between lysine level and growth depression due to the addition of a lysine-free amino acid mixture, the next experiment was set up. It was designed to study the growth-depressing activity of the lysine-free-amino-acid mixture at protein levels of 11 and 23%, each combination being tested with 8 increments of lysine. Tested also was the hypothesis that the same growth depression could be evoked at the 23% as at the 11% protein level if the amount of amino acid mixture were added proportionally to the protein content (4% in an 11% protein diet is equivalent to 8.4% amino acid mixture in the 23% protein ration). The complete design is shown together with the results in table 3.

It can be seen that at both protein levels the absolute growth depression due to the amino acid mixture increased as growth increased with higher increments of lysine up to the point where lysine was in excess. As expected, some growth improvement occurred at the highest levels of lysine in the presence of the amino supplement, since the latter could be utilized to the extent that lysine was provided in surplus of the amount needed to balance the dietary protein.

The hypothesis concerning growth depression on the high- versus the low-protein diet was substantiated by this experiment. Although the addition of the 4% amino acid mixture to the 11% protein diet (in the presence of 4.3% lysine) produced an 18% growth depression, the same supplementation (4%) to the 23% protein diet only caused a 6% depression. When the amino acid supplementation was adjusted proportionally to the protein level, the 8.4% amino acid mixture produced a 15% depression in line with the depression observed on the low-protein diet.

The data in table 3 indicate that the lysine requirement of the chick is approximately 6% of the protein at an 11% protein level and 5% at a protein level of 23%. Although the decreased requirement for lysine when expressed as a percentage of the protein is in line with the decrease observed by Grau ('48), the absolute values herein reported are higher and agree with those of Hill ('53) and the value calculated from the relationship between requirement and carcass amino acid composition (Fisher and Scott, '54).

Trial 3. The third experiment in this series was designed to determine the nature of the nitrogenous supplement necessary to produce a growth depression. To-

TABLE 3

Effect of protein and lysine level on the growth depression induced in chicks by a lysine-free amino acid mixture[1]

Protein level	Lysine level[2]	Amino acid supplement[3]	Final body weight	Change in growth[4]	Av. feed consumption/ bird	Change in feed intake[4]	Av. lysine consumption/ bird
% of diet	*% of sesame protein*	*% of diet*	*gm*	*gm*	*gm*	*gm*	*gm*
11	2.7	—	102 ± 2[5]		136		0.40
11	3.1	—	108 ± 3		142		0.49
11	3.5	—	122 ± 3		196		0.75
11	4.3	—	161 ± 6		245		1.16
11	5.1	—	212 ± 8		351		1.96
11	5.9	—	252 ± 8		400		2.59
11	6.7	—	274 ± 7		455		3.35
11	7.5	—	256 ± 7		398		3.28
11	2.7	4.0	99 ± 2	−3	112	−24	0.35
11	3.1	4.0	108 ± 2	0	143	+1	0.49
11	3.5	4.0	114 ± 3	−8	145	−51	0.56
11	4.3	4.0	132 ± 3	−29	174	−71	0.82
11	5.1	4.0	182 ± 7	−30	316	−35	1.77
11	5.9	4.0	220 ± 10	−32	338	−62	2.19
11	6.7	4.0	277 ± 7	+3	416	−39	3.05
11	7.5	4.0	298 ± 8	+42	424	+26	3.50
23	2.7	—	138 ± 6		179		1.11
23	3.1	—	172 ± 4		256		1.82
23	3.5	—	214 ± 8		329		2.65
23	4.3	—	314 ± 10		432		4.07
23	5.1	—	374 ± 7		482		5.65
23	5.9	—	392 ± 9		499		6.78
23	6.7	—	376 ± 15		465		7.16
23	7.5	—	390 ± 9		495		8.53
23	2.7	4.0	134 ± 4	−4	186	+7	1.15
23	3.1	4.0	162 ± 6	−10	218	−38	1.55
23	3.5	4.0	196 ± 7	−18	279	−50	2.24
23	4.3	4.0	294 ± 5	−20	406	−26	3.82
23	4.3	8.4[6]	268 ± 11	−46	370	−62	3.65
23	5.1	4.0	358 ± 10	−16	467	−15	5.47
23	5.9	4.0	376 ± 7	−16	491	−8	6.65
23	6.7	4.0	388 ± 7	+12	519	+54	7.99
23	7.5	4.0	378 ± 9	−12	451	−44	7.84

[1] Results are from duplicate groups of 7 birds maintained on experimental rations from 7 to 21 days of age. Average starting weight for all groups, 96 gm.

[2] Expressed as free L-lysine as a per cent of the sesame protein content of the diet; L-lysine HCl (95%) was the actual supplement.

[3] Lysine-free, for composition see table 1.

[4] On addition of amino acid supplement.

[5] Mean ± standard error.

[6] The 8.4% in a 23% protein ration is equivalent to 4% in an 11% protein ration.

ward this end, a single essential amino acid, a single non-essential amino acid, a mixture of non-essential amino acids, and zein (which is essentially lysine-free) were each compared in growth-depressing activity to the amino acid mixture used in trials 1 and 2. This experiment was carried out at the 11% protein level only, with lysine levels of 5.1 and 7%. The experimental design and the results are given in table 4.

It is evident that glutamic acid, leucine and the mixture of non-essential amino acids depressed growth, but in a manner unrelated to the lysine deficiency. That glutamic acid alone or the mixture of non-essential amino acids would also create an imbalance was unexpected, although Nasset ('57) and Johnson and Fisher ('59) have pointed out the possibility of creating an imbalance with non-essential amino acids when the essential ones were provided only in minimal or submarginal amounts. In connection with the major theme of the present report, it must be emphasized that this latter type of im-

TABLE 4

The comparative growth depressing effect of different nitrogen sources added to a lysine-deficient ration[1]

Nitrogen supplement[2]	Protein level	Lysine level[3]	Final body weight	Change in growth	Av. feed consumption /chick
	%	%	*gm*	*gm*	*gm*
None	11	5.1	177 ± 7[4]		288
L-Glutamic acid, 4.63%	11	5.1	154 ± 6	−23	237
L-Leucine, 4.11%	11	5.1	122 ± 6	−55	174
Zein, 4.63%[5]	11	5.1	*158* ± 5	*−19*	255
Non-essential amino acid mixture, 3.49%[6]	11	5.1	163 ± 5	−14	261
Essential amino acid mixture, 4.0%[7]	11	5.1	*158* ± 6	*−19*	237
None	11	7.0	226 ± 7		378
L-Glutamic acid, 4.63%	11	7.0	180 ± 10	−46	287
L-Leucine, 4.11%	11	7.0	132 ± 13	−94	185
Zein, 4.63%[5]	11	7.0	*274* ± 9	*+48*	390
Non-essential amino acid mixture, 3.49%[6]	11	7.0	190 ± 9	−36	281
Essential amino acid mixture, 4.0%[7]	11	7.0	282 ± 8	+56	408

[1] Results are from duplicate groups of 7 birds maintained on experimental rations from 7 to 21 days of age. Average starting weight for all groups, 98 gm.

[2] Except for zein, the nitrogen supplements were added on an isonitrogenous basis.

[3] Expressed as free L-lysine as a percentage of the sesame protein content of the diet; L-lysine HCl (95%) was the actual supplement.

[4] Mean ± standard error.

[5] The protein zein is lysine-free.

[6] The non-essential amino acid mixture had the following composition: 21.5% DL-serine, 14.3% L-proline, 21.5% L-aspartic acid, 14.3% DL-alanine, 7.2% glycine, 21.2% glutamic acid.

[7] This is the same lysine-free mixture used in all previous experiments (see footnote 3, table 2).

balance is different and distinct from the one which involves an exaggeration of the deficiency for the most limiting amino acid which in this case was lysine.

Zein supplementation produced the same depressing effect as the essential amino acid mixture on the low-lysine level (5.1%) and improved growth to the same extent at the high- and adequate-lysine level (7.0%). It may therefore be expected that the depressing action is dependent upon the extent to which the imbalancing supplement is either in itself, or in combination with the other dietary protein, well balanced except for the one limiting amino acid.

Trial 4. This experiment was carried out to test the hypothesis that the growth depression or imbalance produced in trial 3 by the non-essential amino acid mixture is a reflection of a generalized protein or total essential amino acid deficiency as suggested by the work of Nasset ('57). As shown in table 5, the design was to repeat the feeding of the non-essential amino acid mixture at the same ratio to the dietary protein as in trial 3 but with the protein level set at 23%. As controls, unsupplemented and essential amino acid (lysine-free) supplemented groups were included in the experiment.

The results (table 5) indicate that the growth depression due to the mixture of non-essential amino acids is related to the dietary protein level. At first glance it might appear that the intermediate growth depression observed with the non-essential amino acids at the inadequate 4% lysine level represents the same type of imbalance as observed with the essential amino acid supplement. However, reference to trial 3 (table 4) and the inability of lysine to overcome the growth depression at the 11% protein level suggest that the depression on the 23% pro-

TABLE 5

The effect of a high protein level on the growth-depressing activity of supplemental mixtures of essential or non-essential amino acids[1]

Amino acid supplement[2]	Protein level	Lysine level[3]	Final body weight	Av. feed consumption /bird
	%	%	*gm*	*gm*
None	23	4.0	258 ± 5[4]	403
Non-essential amino acid mixture, 7.35%[5]	23	4.0	228 ± 8	349
Essential amino acid mixture, 8.4%[6]	23	4.0	164 ± 7	262
None	23	7.0	333 ± 6	459
Non-essential amino acid mixture, 7.35%[5]	23	7.0	349 ± 8	471
Essential amino acid mixture, 8.4%[6]	23	7.0	331 ± 9	442

[1] Results are from duplicate groups of 7 birds maintained on experimental rations from 7 to 21 days of age. Average starting weight for all groups, 79 gm.

[2] The non-essential and the essential amino acid mixtures were supplied isonitrogenously; the amounts used are in the same ratio to dietary protein as was previously the case for the 11% protein diet (table 4).

[3] Lysine was added as a percentage of the dietary protein level, except that in the case of the essential amino acid mixture at the higher lysine level the latter was related to dietary protein (23%) plus the essential amino acid mixture (8.4) or 7.0% lysine of 31% total protein + free amino acids.

[4] Mean ± standard error.

[5] For composition of mixture see footnote 4, table 4.

[6] This is the same lysine-free mixture used in all previous experiments (see footnote 3, table 2).

tein-4% lysine diet is still the result of a general protein deficiency rather than of a specific lysine deficiency.[4]

GENERAL DISCUSSION

The data collected in these studies indicate that a growth depression fitting the definition for amino acid imbalance of Harper ('59) can be produced in the growing chick. The conditions under which such an imbalance may be expected to occur can be summarized as follows. Whenever the addition of a protein or amino acid supplement decreases the ratio of the most limiting amino acid to total potentially available protein (or total effective amino acids) such that the most limiting amino acid in terms of *total-protein* will be present in amounts below the minimum requirement for optimum performance. In specific terms, (a) there is an optimum ratio between protein level and the most limiting amino acid (namely the requirement at a specific protein level), (b) an imbalance is created by a nitrogen-containing supplement when it consists of a well-balanced mixture of amino acids (except for the limiting amino acid) or when it forms a well-balanced mixture by virtue of complementing the original dietary protein or nitrogen source, and finally (c) in order to produce the imbalance the limiting amino acid must be supplied in amounts *above* the maintenance and *below* the minimum requirement for optimum growth.

It has been suggested by Harper ('59), Kumta and Harper ('60), Griminger et al. ('56) and by Sauberlich and Salmon ('55) that the most limiting amino acid in cases of imbalance becomes less available either through excretion with the excess amino acids that cannot be utilized for growth due to the limitation of one essential acid, or through increased catabolism. This explanation is not completely satisfactory since it was shown in several comparisons (table 2, italics) that lysine utilization was not necessarily impaired by the creation of an imbalance through the amino acid supplementation. If comparisons are made between widely differing protein levels with reference only to equal lysine consumption, supporting evidence can be found for an impaired lysine efficiency using diets supplemented with the incomplete amino acid mixture. This interpretation of the latter comparison tends to disregard, how-

[4] Twenty-three per cent protein with 4% lysine is, in terms of adequately balanced protein, approximately equivalent to only 13% protein with 7% lysine.

ever, the effects which protein level and caloric density will exert on food intake over and above any limitations imposed by the lysine deficiency.

A new interpretation for the imbalance should therefore center on finding an explanation for the reduced food consumption which is such an integral part of the imbalance phenomenon as observed in the chick and in the rat (Kumta and Harper, '60). Anderson and Combs ('52) have reported an impaired glucose tolerance in chicks fed single amino acid excesses which influenced the dietary amino acid balance. It might be suggested that the homeostatic defence mechanism against the build-up of free amino acids and nitrogenous and other degradation products resulting from the catabolism of the excess amino acids (from a supplement producing the imbalance) involves the regulation of food intake. Since the *imbalance* discussed herein may of course also be considered as an *exaggeration of a specific amino acid deficiency,* the relationship of food consumption in the present case to the food refusals with a variety of other nutrient deficiencies needs serious exploration and elucidation.

The final experiment, considered in conjunction with trial 3 (tables 4 and 5), indicates the complexity of the problem of imbalance and the extent of our ignorance of its various facets. Having demonstrated a type of imbalance involving a certain relationship between protein level and a single limiting essential amino acid, the final data illustrate another type of imbalance concerned with the relationship between total effective protein (or essential amino acids) and a mixture of non-essential amino acids. In the former case the ratio of lysine to total protein was equally important in terms of the imbalance at high protein levels as well as at low levels, whereas for the latter condition the relationship between non-essential to essential amino acids was important primarily at low and suboptimal essential amino acid levels. If the results of table 5 were considered by themselves without reference to the previous data (table 4) with the 11% protein level, the erroneous conclusion might have been drawn that the non-essential amino acids at the 23% protein level also created an imbalance of the same type as produced by the essential amino acid mixture.

It seems clear from these last experiments that Harper's definition of amino acid imbalance will have to be revised in order to include the type of imbalance that can exist between essential and non-essential amino acids and undoubtedly other types as well which are as yet ill defined. Since under practical feeding conditions animals and man may easily consume excess amounts of individual amino acids, it may prove of considerable importance to study the extent to which such excessive intakes might also create an "imbalance" in terms of the total effective protein (or amino acids) consumed, and to ascertain the extent to which the term imbalance applies as contrasted by a pharmacological depression or toxicity which will not respond to nutrient change.

Finally, an extension of the concept of an optimum ratio for essential limiting amino acids to total effective protein might offer an explanation for the unusually high arginine requirement of the chick on a casein diet as compared to the requirement determined with other protein sources (Synder et al., '56; O'Dell et al., '58). Casein contains a greater excess of essential (and also non-essential) amino acids above the requirement than, for example, soybean meal protein when both provide equivalent protein levels expresses as nitrogen $\times$ 6.25. When casein is fed to provide 20% protein (N $\times$ 6.25), only arginine is not in excess of the requirement; it is, in fact, deficient. The total excess of essential amino acids other than arginine over the requirement of the chick is 4.73% (compared with only 2.67% for 20% soybean protein). Thus, optimum growth might not be achieved until arginine is supplemented not only to meet the requirement for a standard 20% protein diet, but to balance the excess essential amino acids. This might be even more critical if the casein diet is also supplemented, as is frequently done, with the next limiting amino acid in casein, namely cystine (or methionine).

SUMMARY

A series of studies on amino acid imbalance have been carried out in growing chicks. The following observations were recorded:

1. The chick is as susceptible to an amino acid imbalance as has been reported for the growing rat.

2. An imbalance could be created through the addition of an essential amino acid mixture deficient in one amino acid (lysine) which was also limiting in the dietary protein.

3. It could be shown that the imbalance is due to an altered ratio of the limiting amino acid to the total available protein or essential amino acids.

4. The imbalance, through the addition of an amino acid mixture deficient in the one limiting amino acid, was most pronounced when the level of the limiting amino acid was such that it permitted growth above maintenance but below the optimum growth rate.

5. The imbalance manifested itself in a reduced feed consumption and therefore a reduced consumption of the most limiting amino acid, but there was *no* evidence that the limiting amino acid was utilized less well than in the case where no imbalance was created.

6. The amino acid composition of nitrogenous mixtures which will produce an imbalance appears to be specific for essential amino acids that are either in themselves or in combination with the dietary protein sufficiently balanced as to permit growth when the single amino acid limitation is overcome. Single amino acid supplements or a mixture of non-essential amino acids produced an imbalance which was not related to the deficiency or limitation of one amino acid, but in the case of the non-essential amino acid mixture it was related apparently to the adequacy of all the essential amino acids in the diet.

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The Effect of Lactose Feeding on the Body Fat of the Rat[1]

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Young rats fed a diet containing lactose in the caloric proportion present in human milk have 40% less body fat than control animals fed a glucose diet.[2] This characteristic effect of lactose feeding on fat deposition has been long recognized. Steuber and Seifert ('28) in a study with infants calculated from the respiratory quotients that there was little fat synthesized when the fat of human milk was replaced isocalorically with lactose. A clinical impression that breast-fed infants had firmer flesh than infants fed formula with added vegetable sugar led Jarvis ('30) to compare the carcass composition of rats fed diets containing sucrose or lactose; 25% less fat was found in the lactose-fed rats. More detailed investigations by Whittier et al. ('35) with rats and pigs and by Scheunert and Sommer ('56) with rats, have also shown a lowered deposition of body fat with lactose feeding.

The mechanism by which lactose feeding reduces body fat deposition is not known. Lactose when fed in excessive amounts is toxic to the young rat. Diarrhea, enlargement of the cecum, unthrifty appearance, alopecia, and growth retardation occur, presumably as a result of the incomplete digestion and absorption of lactose since equivalent mixtures of glucose and galactose do not produce these symptoms (Riggs and Beatty, '47; De Groot and Engel, '57; Cenni and Finzi, '56). Other symptoms, attributable to absorbed galactose, include polydipsia, polyuria, galactosuria, and cataract formation. It was the purpose of the present investigation to study the mechanism by which lactose decreases deposition of body fat.

EXPERIMENTAL

Male Sprague-Dawley rats, individually caged, were used in all experiments. The basal diet consisted of casein 24,[3] carbohydrate 52, fat mixture 20,[4] salts 4,[5] and adequate vitamins.[6] The caloric proportions supplied by protein, fat, and carbohydrate were 20, 37, and 43%, respectively. Food was given ad libitum except in paired feeding studies. The experiments were of 6 weeks duration.

For total carcass analysis, the rat was cut into small pieces and added to an equal weight of powdered cellulose.[7] After standing several days in the cold in a tightly sealed jar, the contents were ground in a Fitzpatrick comminuter. Duplicate 50-gm samples were dried overnight at 100°C in a vacuum oven for moisture determination. The dried sample was ground and ash and nitrogen analysis run by conventional procedures; fat was determined by the wet ether extraction

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[2] Tomarelli, R. M., and F. W. Bernhart 1959 Carcass fat of the lactose-fed rat. Federation Proc., *18:* 548.

[3] Sheffield, High Nitrogen.

[4] Soybean, corn, coconut, and oleo oil.

[5] Hubbel, Mendel, Wakeman ('37).

[6] Five milligrams each of thiamine·HCl, riboflavin, pyridoxine·HCl, and 2-methyl naphthoquinone, 50 mg each of niacin and Ca pantothenate, 100 mg each of inositol and *p*-aminobenzoic acid, 2 mg folic acid, 0.5 mg of biotin, 50 μg of vitamin B_{12}, and 1 gm of choline chloride per kilogram diet. Fat soluble vitamins were given by dropper, 2 drops twice weekly; 30 mg of mixed tocopherols, 4400 units of vitamin A and 600 units of vitamin D per gram of corn oil.

[7] Solka Floc, Brown Company.

procedure of Bixby et al. ('54). In experiments in which only the fat content of carcass was desired, the rat was frozen, chopped in thin pieces with a meat cleaver, and transferred to a Waring blendor (gallon size) and homogenized one minute after each addition of the following solvents: 250 ml water, 250 ml water, 250 ml acid alcohol, (1 ml conc. HCl/100 ml 95% ethanol), 250 ml of water, and 250 ml acid alcohol. Fifty grams of the stable homogenate was ladled into a 250 ml centrifuge bottle, 50 ml of ethyl ether added, the mixture shaken for one minute, and 50 ml of petroleum ether added. After again shaking for one minute, the bottle was centrifuged and the upper layer transferred to a tared beaker. The extraction of the aqueous layer was repeated with 15 ml of ethanol, 50 ml of ethyl ether, and 50 ml of petroleum ether. (The proportions of solvents were similar to those used by Bixby et al. ('54) for determination of fat in liver.) The combined ether extracts were evaporated to dryness under a heat lamp and then heated for 15 minutes at 100°C in a vacuum oven. The absolute variation between duplicate analyses averaged less than 0.5%.

In most experiments the cecum was removed, the pH of the contents quickly determined, and smears made for bacteriological staining. The cecum was washed, blotted, weighed, and included with the rest of the carcass for subsequent analyses.

RESULTS

Young rats fed the lactose diet consumed less food and grew at about 70% of the rate of the glucose controls. They utilized food less efficiently; 3.15 gm of food was required for one gram of body weight gain as compared with 3.07 gm per gm for the rats fed glucose. The thermochemical efficiency, i.e., calories fed per calorie gain of tissue, is a truer measure of diet utilization in this case since the glucose-fed rats had a higher content of body fat. The rats fed the lactose and glucose diets required 7.3 and 6.1 cal. per calorie gain, respectively.

The fat content of the carcass of the rat fed lactose was about 60% that of the glucose-fed rat (table 1). The low carcass fat of the lactose-fed rat was not related to growth retardation *per se*. Glucose-fed rats that were pair-fed or restricted to the same weight gain as the lactose-fed rats did not have a lower content of body fat than did rats that had free access to the glucose diet. The lactose-fed animals had higher water and ash content; there was no difference in the protein content.

The experiment of table 2 was designed to determine whether the low carcass fat of the lactose-fed rat was caused by the galactose component of lactose. Rats fed a glucose-galactose mixture equivalent to that supplied by the lactose diet grew at a faster rate than the lactose group and had a body fat content that was not significantly different from that of the group

TABLE 1

Carcass analysis of glucose and lactose-fed rats[1]

Group[2]	Weight	Fat	Protein	H_2O	Ash
	gm	%	%	%	%
		Experiment 1			
Glucose	309	14.3 ± 1.4	20.1 ± 0.2	58.5 ± 1.0	3.3 ± 0.1
Lactose	238	8.3 ± 0.2	20.4 ± 0.1	62.8 ± 0.2	3.8 ± 0.1
Glucose-pair fed	256	14.4 ± 0.9	20.0 ± 0.3	58.7 ± 0.8	3.5 ± 0.1
		Experiment 2			
Glucose	288	13.9 ± 0.7	21.6 ± 0.6	57.8 ± 1.4	3.7 ± 0.1
Lactose	221	9.5[3] ± 1.0	21.4 ± 0.2	60.7 ± 0.4	4.0 ± 0.1
Glucose-pair weight	222	14.5 ± 0.6	20.4 ± 0.3	56.7 ± 0.3	3.6 ± 0.1

[1] Seven rats per group, 6 weeks experiments, average initial weight 55 gm in exp. 1, 51 gm in exp. 2.

[2] Food consumption for the glucose, lactose and glucose-pair fed groups of experiment 1 were 805, 596 and 569 gm, respectively. In experiment 2 the glucose, lactose and glucose-pair weight groups consumed 718, 524 and 477 gm, respectively.

[3] Six rats had values below 9.8%, the 7th was 14.0%.

TABLE 2

Lactose vs. glucose-galactose feeding[1]

Group	Body weight	Cecal pH	Cecum weight	Liver fat	Carcass fat	Statistical significance[2]
	gm		*gm/100 gm*	%	%	P
52% Lactose	288	6.52	0.68	4.35	8.9 ± 0.1	> 0.001
26% Glucose + 26% galactose	319	7.90	0.24	4.26	13.2 ± 0.7	> 0.09
52% Glucose	342	7.92	0.20	4.11	15.0 ± 0.7	

[1] Six weeks experiment, 10 rats in lactose group, 7 in the other two groups.
[2] Student's test, P = 0.05 or less considered significant.

fed the glucose diet.[8] It was evident that the intact disaccharide and not its monosaccharide constituent was responsible for both the growth retardation and the low carcass fat. The weight of the cecum and the pH of the cecal contents of the rats fed the glucose-galactose diet were similar to those found in the glucose group. The fat content of the liver did not appear to be influenced by the nature of the dietary carbohydrate.

The preceding experiment suggested the possibility that the low carcass fat of the rats fed lactose was related to events occurring in the intestinal tract. In the next experiment (fig. 1) the relationships found between dietary lactose concentration, cecum size, and carcass fat are in accord with this hypothesis. With a dietary carbohydrate mixture of ¼th of lactose and ¾ths of glucose there was little effect on body size or carcass fat and the cecum was only slightly enlarged. With equal parts of the two sugars, weight gain and carcass fat decreased and the cecum was definitely enlarged. These effects be-

[8] In a previous experiment (Tomarelli and Bernhart, '58) rats fed a mixture of glucose and galactose had weight gains almost identical with that of a glucose control.

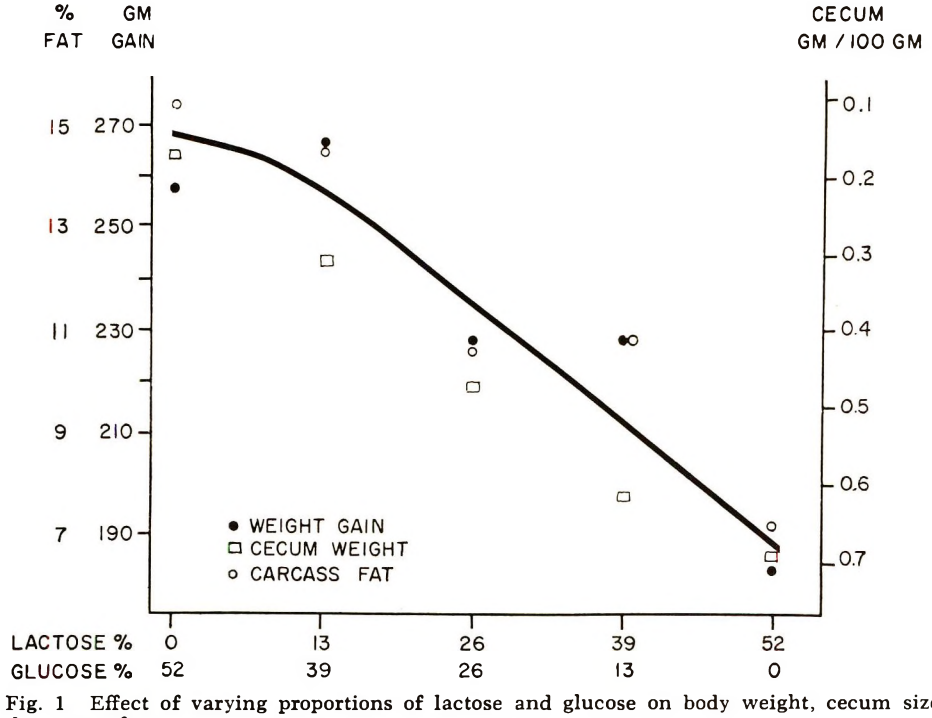

Fig. 1 Effect of varying proportions of lactose and glucose on body weight, cecum size and carcass fat.

came more pronounced as the lactose proportion was increased.

The relatively high carcass fat content attendant with glucose feeding was also found with the feeding of other readily digested and absorbed carbohydrates such as dextrin and sucrose, table 3. As expected there was no enlargement of the cecum. The addition of cellulose, 20% of the diet at the expense of glucose, did not influence fat deposition, neither was there an enlargement of the cecum, 0.19 mg per 100 gm as compared with 0.18 mg per 100 gm for the glucose control.

TABLE 3

Effect of carbohydrates on body fat[1]

Group	Body fat
	%
52% Lactose	9.8
52% Dextrin	14.3
52% Sucrose	13.0
52% Lactose	10.4
52% Glucose	15.5
32% Glucose + 20% cellulose	14.2

[1] Seven rats per group, 6 weeks experiments.

Sorbitol when fed to young rats will produce many of the symptoms of excessive lactose feeding, i.e., transient diarrhea, enlargement of the cecum, and growth retardation (Morgan and Yudkin, '57). The data presented in table 4 show that sorbitol feeding also lowered carcass fat deposition. The growth retardation, enlargement of the cecum, and the increased acidity of the cecal contents were comparable to those of lactose-fed rats.

Rats fed lactose and sorbitol, in addition to the digestive disturbance, are subject to the stress of metabolizing excessive amounts of either galactose or sorbitol. The experiment of table 5 was designed to separate the intestinal effect from a possible toxic metabolic effect. Cellobiose and raw potato starch were selected as carbohydrates that are incompletely digested

TABLE 4

Effect of lactose and sorbitol on body fat[1]

Group	Weight gain	Cecum weight	Cecal content	Body fat
	gm	*gm/100 gm*	*pH*	%
52% Glucose	257	0.17	7.31	15.3
26% Lactose + 26% glucose	228	0.47	6.69	10.7
52% Lactose	184	0.69	6.65	7.3
15% Sorbitol + 37% glucose	234	0.72	7.17	9.5
25% Sorbitol + 27% glucose	185	0.98	6.67	9.2

[1] Eight rats per group, 6 weeks experiment.

TABLE 5

Effect of carbohydrates on body fat[1]

Group	Weight gain	Cecum weight	Cecal content	Carcass fat	Statistical significance[2]
	gm	*gm/100 gm*	*pH*	%	P
52% Glucose	232	0.22	7.7	14.2 ± 0.6	
52% Lactose	195	0.74	5.9	9.8 ± 0.4	0.001
15% Cellobiose + 37% glucose	195	0.58	6.8	9.8 ± 0.6	0.001
26% Raw potato starch + 26% glucose	236	0.65	5.7	11.6 ± 0.5	0.01
26% Raw potato starch + 26% galactose	221	0.69	5.5	10.8 ± 0.5	0.001
Galactose[3]	225	0.23	7.6	13.3 ± 1.7	insig.

[1] Six weeks experiment, 7 rats per group.
[2] As compared with fat of glucose control.
[3] Diet consisted of galactose 29, casein 34, fat 33, and salt 4; galactose supplied 21% of the calories.

and consequently cause enlarged cecum (Moinuddin and Lee, '58; Fischer, '57). Unlike lactose, however, these carbohydrates yield only the physiological sugar glucose, upon hydrolysis. One group of rats was fed a mixture of raw potato starch with galactose, a combination to simulate the action of lactose in producing enlarged cecum and also forcing an increased load on the metabolic processes concerned with galactose metabolism.

The deposition of body fat was decreased in every group that had an enlarged cecum, table 5. The effect of feeding of 15% of cellobiose very closely approximated that obtained with 52% of lactose on growth retardation, cecum enlargement, and body fat content. The groups fed raw potato starch with either glucose or galactose had enlarged ceca, but there was no growth retardation. The body fat, while not as low as that of the lactose-fed group, was lower than that of the glucose control, the difference being highly significant, statistically. Galactose feeding gave results similar to those obtained with glucose feeding. The combination of galactose and potato starch did not differ in its effects from that of glucose and potato starch.

Gram stains of smears of the cecal contents from rats of the above experiment showed a bacterial picture that was characteristic of the carbohydrate fed. The flora of groups with enlarged ceca, with contents of acid reaction, were almost entirely Gram positive. Cecal bacteria of the lactose-fed group were predominantly rods, the cellobiose-fed group, cocci, and the groups fed potato starch, a mixture of rods and cocci. On the other hand, the bacterial flora of the groups fed diets containing glucose or galactose alone consisted of a mixture of rods and cocci, predominantly Gram negative.

While it appeared quite probable that the low body fat content of the lactose-fed rat is related to reactions originating in the intestinal tract no explanation for the mechanism has been obtained. Experiments attempting to correlate it with the intestinal bacteria have so far been negative. Rats fed the lactose diet with the addition of 100 mg/kg of chlortetracycline had a body fat content that was not significantly different from that of a control, 8.4 vs 9.5% (Tomarelli and Bernhart, '58). Supplementation of the lactose diet with twice the usual amount of the vitamins plus 50 mg of orotic acid, 20 mg of thioctic acid, and 50 mg of pantethine per kilogram of diet or with 10% of whole liver powder did not influence body fat content. The possibility that a bacterial product was produced which blocked glucose utilization was considered. The addition of 0.25% of tolbutamide[9] to the diet or daily injections of one unit of protamine zinc insulin had no effect on body fat of rats fed either the glucose or the lactose diet.

All the experiments described to this point have been of 6 weeks duration and have studied young animals during the most active period of growth when the energy requirements have been at a maximum. If the differences in carcass fat content were related to differences in the available energy of diets it would be expected that after growth had ceased the carcass fat content of the rats fed incompletely digested carbohydrate would approach that of a glucose control. Two groups of 5 rats each were fed the lactose and the glucose diet for 42 weeks. The growth curve for both groups had almost reached a plateau. The average weights of glucose and the lactose groups were 583 and 475 gm, respectively; the body fat contents were 31.8 and 17.5%, respectively. On a fat-free basis the two groups were almost of identical weight, 392 and 394 gm, respectively, indicating that the only difference in body development was in the deposition of carcass fat.

DISCUSSION

The characteristic low content of carcass fat in the rat fed a lactose diet appears to be associated with events occurring in the intestinal tract. The body fat was not reduced when an equivalent mixture and glucose and galactose was substituted for the lactose component of the diet, thus implicating the unhydrolyzed disaccharide as the causative agent. Feeding of other incompletely digested carbohydrates such as sorbitol, cellobiose, and

[9] Orinase, Upjohn Company.

raw potato starch also resulted in low content of body fat while sucrose and dextrin, readily digested and absorbed carbohydrates, yielded higher fat values. In every case where a low content of body fat was found there was also evidence of intestinal changes, i.e., transient diarrhea, grossly enlarged ceca, and conversion of the cecal bacteria to a predominantly Gram positive flora.

Attempts to define the mechanism by which lactose feeding influences fat deposition have so far been unsuccessful. Since many factors affect lipogenesis numerous explanations are suggested. The passage of undigested lactose into the cecum with the resulting bloat may depress the rate of feeding as well as the total amount of food consumed. The possibility that a reduced rate of feeding would result in decreased lipogenesis may be inferred from the study of Dickerson et al. ('43) who found increased lipogenic activity in the liver of rats that were trained to eat their entire daily ration in one hour. Forced feeding of rats, twice daily with the same amount of food consumed by controls eating ad libitum, also increased deposition of body fat (Cohn and Joseph, '59). No difference in the feeding habits of rats fed the glucose and lactose diets of the present study was detected; this was studied by weighing the food jars every few hours during several 24-hour periods.

The lactose-fed rats consumed less food and there was a loss of calories through incomplete digestion and by the excretion of galactose in the urine. The urine loss varied from 18 to 30% of the ingested galactose; this was about 6% of the diet energy. The loss of energy in the feces was not determined. By calculating the thermo-chemical efficiency it was found that the glucose-fed rats required only 83.5% as much food energy as the lactose-fed animals for the deposition of carcass energy. If it is assumed that this 16.5% difference was due entirely to losses of calories in the urine and feces and that this reduced amount of available energy was responsible for the low content of body fat, then a comparable reduction of caloric intake for rats fed a glucose diet would also be expected to reduce body fat content. This was not the case. In experiments 1 and 2 of table 1, the glucose pair-fed and pair-weight groups were restricted to 70 and 66%, respectively, of the energy intake of controls eating ad libitum without any effect on body composition.

The ratio of dietary protein to available energy has been shown to exert an influence on fat deposition. Hill and Dansky ('54) added oat hulls to a 20% protein basal diet for chickens and observed that as the level of indigestible material increased the carcass fat content decreased. Peterson et al. ('54) reported that the feeding of low protein diets to chickens resulted in carcasses high in fat content. When increasing levels of cellu flour[10] were included in the feed the fat content of the carcasses decreased. This effect was not found in the rat studies of Sibbald et al. ('57). Diets were fed in which 10, 20, 30 and 40% of the sucrose of the basal diet were replaced by cellulose and 4 levels of protein nitrogen were fed with each cellulose level. A tendency for more moisture and less fat in the carcass was noted as the cellulose level increased but there were no differences in fat content within each group despite a variation of protein from 10 to 25% of the diet.

An increase in the nitrogen/available energy ratio does not appear to be responsible for the low carcass fat of the lactose-fed rat. In the experiment presented in table 3, the addition of 20% cellulose at the expense of glucose did not affect body composition. The nitrogen/available energy ratio of this diet is equal to that of a lactose diet in which 40% of the carbohydrate is non-available.

Another indication that caloric intake is not primarily responsible for the low body fat of the rat fed an incompletely digested carbohydrate may be deduced from the finding that the relatively low content of body fat persists even in the adult lactose-fed rat when energy demands for growth are no longer required.

The increased proliferation and the characteristic nature of the bacteria found in cecum of rats fed lactose, and the other poorly digested carbohydrates, suggests that the microflora may play a role. The

[10] Cellu Flour, Chicago Dietetic Supply House, Chicago.

bacterial synthesis or destruction of factors influencing lipogenesis has not been indicated by preliminary experiments in which chlortetracycline was added to the diet (Tomarelli and Bernhart, '58). Increasing the amounts of the usual vitamin supplement plus the addition of other nutritional factors also yielded negative results. The degradation of carbohydrate by the intestinal bacteria may produce compounds which block glucose metabolism in a manner similar to that of deoxyglucose (Landau and Lubs, '58). In exploratory experiments the blood glucose levels of rats fed glucose and lactose diets were not significantly different nor could any effect on body fat deposition be demonstrated by daily administration of insulin or tolbutamide.

The well known ability of lactose to increase gastrointestinal absorption of calcium is also shared by other incompletely absorbed carbohydrates (Fournier, '54; Wasserman and Comar, '59). Another physiological effect of the feeding of lactose and other slowly digested carbohydrates to rats is the increased urinary excretion of organic acids of the Krebs cycle (Fournier and Digaud, '57). The further finding of a decreased deposition of body fat with poorly digested carbohydrate in the diet suggests that the completeness of digestion of carbohydrates may be a factor of general nutritional significance.

SUMMARY

1. Young rats fed diets containing lactose consumed less food, grew at a reduced rate, and had 40% less carcass fat than control rats fed diets containing glucose, sucrose, dextrin or a glucose-galactose mixture.

2. Glucose-fed rats, pair-fed, or maintained by diet restriction at the same body weight as lactose-fed rats, had carcass fat contents as high as that of rats eating the glucose diet ad libitum.

3. The ceca of the lactose-fed rats were enlarged, the contents were of acid reaction, and characterized by the predominnance of Gram positive bacteria.

4. Feeding other carbohydrates which are incompletely digested and absorbed, such as sorbitol, cellobiose, and raw patato starch also resulted in enlarged ceca and a low content of carcass fat indicating a correlation between these two factors.

5. Supplementation of the lactose diet with nutritional factors, the administration of insulin of tolbutamide had no influence on fat deposition.

6. The difference in body fat content of rats fed the glucose and lactose diets was still present after 42 weeks when the animals had reached a growth plateau.

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Amino Acid Requirements of Men and Women

II. RELATION OF LYSINE REQUIREMENT TO SEX, BODY SIZE, BASAL CALORIC EXPENDITURE AND CREATININE EXCRETION[1]

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Lysine requirements of men who consumed mixtures of purified amino acids varied from 400 to 800 mg (Rose et al., '55). A daily intake of 500 mg was considered adequate for most women (Jones et al., '56). Men and women required between 400 and 800 mg of lysine when the diet contained both cereals and amino acids (Clark et al., '57). Because of the current worldwide interest in lysine, the initial experiment has been extended to include additional subjects, requirements of men and women have been compared, and relationships between individual requirements and certain physical and metabolic measurements have been examined.

PROCEDURE

The method of investigation was essentially the same as that described earlier (Clark et al., '57). Five men and 5 women between 22 and 28 years old participated. Their basal metabolic rates were within a satisfactory range, and the subjects varied from being sedentary to very active as shown by records kept during the experiment.

The quantities and sources of amino acids consumed daily were similar to those stated in table 1 in the preceding paper (Clark et al., '57). The basal diet contained 159 gm of flour, 21 gm of cornmeal and a few other foods low in nitrogen.[3] It provided at least 400 mg of lysine. Purified essential L-amino acids were added in such quantities that the subjects consumed the equivalent of the amounts present in 20 gm of egg protein during the first two periods. Thereafter, the lysine intake of each subject was modified as necessary to determine the minimum quantity that would maintain nitrogen equilibrium. This amount then was tested in as many 6-day periods as time permitted. All subjects consumed 9.0 gm of nitrogen daily, of which cereals and other foods supplied 45%, purified essential amino acids 8%, and a mixture of glycine, glutamic acid and diammonium citrate 47%. Caloric intakes were believed to be adequate for all subjects and slightly above the actual need for a few. In addition to calories provided by foods (table 1), the amino acid mixtures supplied nearly 150 calories.

RESULTS AND DISCUSSION

Minimum lysine requirements of 5 men and 5 women. The minimum quantities of lysine that maintained nitrogen equilibrium are presented in table 1. The daily requirements of the 5 men varied from 400 to 1200 mg, those of the women from 300 to 700 mg. The nitrogen balances of some subjects were sufficiently positive to suggest that quantities of lysine below the stated requirement might have been adequate. However, negative balance was

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TABLE 1

Minimum amounts of lysine that maintained nitrogen equilibrium

Subject			Daily intake of			Nitrogen[1]		
	Body weight	Surface area	Lysine	Calories[2]	Nitrogen	In urine	In feces	Balance
	kg	m^2	*mg*		*gm*	*gm*	*gm*	*gm*
				Men				
JM	67.4	1.78	400	3170	9.01	8.06	0.70	+0.25
BC	65.2	1.86	500	3220	9.01	8.13	0.82	+0.06
WHC	72.9	1.89	700	3400	9.01	8.24	0.91	+0.06
CM	86.2	2.13	900	3500	9.02	8.11	0.92	0
HM	95.9	2.16	1200	3900	9.02	7.57	1.06	+0.39
				Women				
JK	77.4	1.97	300	2660	9.00	7.92	0.75	+0.33
BB	63.7	1.73	350	2600	8.99	8.07	0.58	+0.34
NP	49.9	1.49	450	2050	9.01	7.93	0.81	+0.27
PM	51.4	1.47	500	2400	9.01	7.90	0.64	+0.47
PC	58.0	1.64	700	2550	9.02	8.36	0.61	+0.05

[1] Mean daily values obtained in 6-day metabolism periods.
[2] From food only; 150 Cal. additional were supplied by amino acids.

induced in every subject by reducing the lysine intake 100 mg below the estimated need. For example, mean daily nitrogen balances of subject JM were + 0.25 and − 0.14 gm, respectively, when he consumed 400 and 300 mg of lysine. An intake of 500 mg of lysine was recommended for PM who retained 0.60 and 0.34 gm of nitrogen in two periods when ingesting 500 mg of the amino acid, but had balances of − 0.14 and + 0.13 gm when receiving 400 mg of lysine. Delineation of minimum requirements was difficult because sharp improvements in nitrogen balance occurred frequently when a lysine intake near the least amount that permitted equilibrium was increased by as little as 100 mg.

The requirements of CM and HM, who weighed 86 and 96 kg, were two and three times as high, respectively, as that of JM who weighed 67 kg. Nitrogen balances of CM and HM were more negative during the initial adjustment periods than those of other subjects and improved more slowly. The estimated requirement for HM was based on balances of − 0.27 and + 0.39 gm when he consumed 1100 and 1200 mg, respectively, of lysine. His caloric intake was undoubtedly adequate but absorption of nitrogen was less satisfactory than that of most subjects.

Attainment of nitrogen equilibrium by JK and BB when they consumed 300 and 350 mg, respectively, of lysine was surprising, but supporting data were obtained in successive periods and in two separate experiments. JK retained, on the average, 1.08 gm of nitrogen daily when consuming 400 mg of lysine and was in equilibrium for 4 days at the end of the experiment with as little as 230 mg. When the lysine intake of BB was increased from 350 to 450 mg, her mean daily nitrogen balance changed from + 0.34 to + 1.05 gm. Dietary records during the week prior to the experiment did not indicate inadequate intakes of protein or calories by either subject that would necessitate repletion of protein stores during the test.

Pooled data concerning lysine requirements of 10 men and 10 women. Lysine requirements established earlier (Clark et al., '57) have been confirmed and the range of requirements has been extended in the present experiment. Thus, information has been obtained in two separate series of tests concerning 10 men who weighed between 63 and 96 kg and 10 women between 45 and 80 kg. Five men and 5 women participated in each series. All subjects were between 20 and 29 years old. Heights and weights of these subjects are presented in table 2. The respective mean heights of the women and of the men exceeded by one inch and by two inches the average heights for the present adult population in the United States (LeBovit and Stiebeling, '57). Thirteen participants were within the range of

TABLE 2

Daily lysine requirements of 10 men and 10 women

Subject	Body weight	Height	Creatinine	Lysine requirement			
	kg	*cm*	*gm*	*day*	*mg/kg*	*mg/m²*	*mg/gm creatinine*	*mg/1000 basal Cal.*
				Men				
JM	67.4	178	1.76	400	6	225	230	240
EO	62.6	183	1.69	500	8	280	300	270
BC	65.2	182	1.73	500	8	270	300	310
AP	68.2	171	1.74	650	9	370	385	400
WHC	72.9	182	1.88	700	10	370	370	460
WC	85.5	182	1.84	750	9	360	420	330
LM	79.6	180	1.96	850	11	425	430	485
GN	71.7	179	1.78	900	12	460	510	390
CM	86.2	193	1.77	900	10	420	500	480
HM	95.8	183	2.23	1200	12	540	520	600
Mean	75.5	181	1.84	750	10	370	400	400
				Women				
JK	77.4	180	1.30	300	4	160	240	200
BB	63.7	171	1.40	350	5	200	240	210
NP	49.9	160	0.91	450	9	300	500	370
EG	45.2	155	0.95	500	11	360	525	430
PM	51.4	159	1.08	500	10	350	480	430
VS	64.8	165	1.12	550	9	330	510	440
DB	53.6	159	1.24	600	11	410	500	460
GM	64.8	163	1.28	650	10	390	530	590
PC	58.0	170	1.32	700	12	430	530	560
RS	79.8	173	1.47	700	9	360	470	470
Mean	60.9	165	1.21	550	9	330	450	420

desirable weights for heights stated in the Recommended Dietary Allowances of the National Research Council ('58). Subjects EO and EG were 7% below the range limit, WC and JK 4% above, and RS, GM and HM 11 to 15% above the range limit.

Minimum quantities of lysine that maintained nitrogen equilibrium in these subjects are presented in table 2. The mean daily lysine requirement of the 10 men was 750 mg. This figure would be useful in estimating needs of population groups but not of individuals because of the divergence among the values. An intake of 900 mg of lysine would fulfill the minimum needs of all except one subject studied. Some men whose weights exceeded 70 kg needed more than 800 mg, which was the highest requirement observed for men who consumed mixtures of purified amino acids (Rose et al., '55). Fecal nitrogen losses also were greater. Although the availability of lysine from cereals has not yet been compared with that of the purified form in this laboratory, Linkswiler et al. ('58) concluded that valine in corn was as well utilized as purified valine.

The mean daily lysine requirement of 10 women was 550 mg, the highest value being 700 mg. Five individuals needed more than 500 mg, which Jones and associates ('56) considered adequate for most women consuming purified amino acids. The mean lysine requirement of the women was 200 mg lower than that of the men, and the maximum value for any woman approached the mean requirement for the men. Individual requirements of 5 men and 8 women overlapped between 400 and 700 mg. As far as the authors are aware, the data presented herein provide the first direct comparison under similar conditions of the requirements of men and women for any amino acid.

The marked individual differences in amino acid requirements reported by several investigators merit consideration. Williams ('56), in discussing the concept of biochemical individuality, has emphasized the importance of turning attention to "understanding and supplying individual needs—needs that quantitatively do not apply to all humanity but are more or less distinctive and crucial for individuals." A

range of lysine requirements extending from 300 to 1200 mg raises pertinent questions concerning the utilization of lysine from various foods and combinations of foods; the effect of consuming quantities of lysine that differ from the minimum requirement; and interrelationships among quantitative requirements for all essential amino acids.

At the present time, the principal value of information concerning amino acid requirements lies in its application to the evaluation and planning of diets. Even the relatively small quantities of cereals and fruits in the basal diet used in this experiment supplied enough lysine for some individuals and at least 50% of the daily need of many others. Examination of the lysine content of foods (Orr and Watt, '57) reveals that the quantities of lysine needed by adult human subjects for maintenance of nitrogen equilibrium can be provided without difficulty unless the diet is restricted to a few foods low in lysine.

Relation of lysine requirement to body size, creatinine excretion and basal caloric expenditure. Daily lysine requirements of men and women are expressed per unit of body weight, surface area, creatinine and basal caloric expenditure in table 2. Means for men agree more closely with means for women when the data are so treated than when only mean total daily requirements are considered. Nevertheless, marked differences between subjects preclude the possibility of using any one of these mean values to estimate needs of individuals.

The conduct of certain types of experiments would be facilitated if lysine requirements of participants could be predicted with reasonable success. Therefore, the regressions of lysine requirements on certain physical and metabolic measurements that might be related to amino acid metabolism were calculated. The regressions of lysine requirement (L) of the 10 men on body weight (W), metabolic body size (M), surface area (S) and creatinine excretion (C) were highly significant ($P < 0.01$), and the regression on basal caloric expenditure (E) was significant ($P < 0.05$). The regressions of lysine requirement of men on body weight and creatinine are shown in figure 1a, b. The respective regression equations and correlation coefficients were as follows: body weight (kg), $L = 18.8W-677$, ($r = +0.86$); creatinine (gm), $L = 1166C - 1402$, ($r = +0.79$); metabolic body size ($kg^{3/4}$), $L = 74M - 1175$, ($r = +0.89$); surface area (m^2), $L = 1355S - 1903$, ($r = +0.75$); and basal calories per 24 hours $L = 0.6E - 381$, ($r = +0.56$).

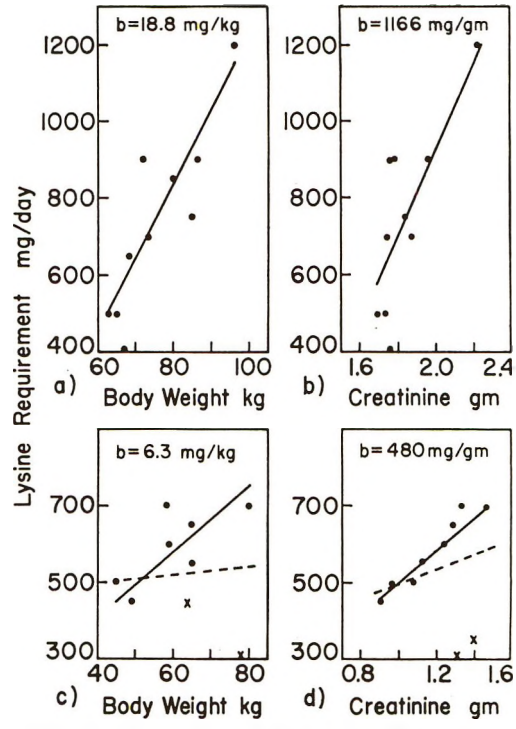

Fig. 1 Regression of lysine requirement: on body weight for 10 men (a), $L = 18.8W-677$; on creatinine excretion for 10 men (b), $L = 1166C-1402$; on body weight for 8 women (solid line in c), $L = 6.35W + 219$; for 10 women (dashed line in c), $L = 0.16W + 527$; on creatinine excretion for 8 women (solid line in d), $L = 480C + 29$; and for 10 women (dashed line in d), $L = 157C + 347$; b = coefficient of regression of lysine requirement on body weight or creatinine excretion.

In contrast, little correlation existed between lysine requirements of the 10 women and any of these measurements, as shown by correlation coefficients of 0.210 or less. When the data were plotted, the points representing JK and BB were distinctly out of line with those for the other 8 women. If these two values were excluded, the regressions of lysine requirements on body weight and creatinine excretion were highly sig-

TABLE 3

Mean daily nitrogen balances of one subject when the sequence of feeding specific quantities of lysine was reversed[1]

Period	Experiment 1		Experiment 2		Experiment 3	
	Lysine intake	N balance	Lysine intake	N balance	Lysine intake	N balance
	mg	*gm*	*mg*	*gm*	*mg*	*gm*
1	500	−2.03	1500	−0.28	500	−0.35
2	500	−0.59	1500	+0.49	600	−0.39
3	500	−0.43	1500	+0.61	750	−0.34
4	600	−0.52	1500	+0.34	1500	+0.22
5	750	−0.61	950	+0.09		
6	950	−0.91	670	+0.14		
7	—	—	600	−0.08		

[1] Daily nitrogen intake of subject AP was 9.01 gm; mean fecal nitrogen in experiments 1, 2 and 3, respectively, 0.75, 0.84 and 0.80 gm.

nificant ($P < 0.01$), on metabolic body size and surface area were significant ($P < 0.05$), and on basal caloric expenditure was nonsignificant. By means of regression equations calculated from the values for the 8 women, estimates could be made that would be applicable to most women although they would exceed the requirements of individuals such as JK and BB.

Regressions of lysine requirements of the women on body weight and creatinine excretion are shown in figure 1c, d. The regressions for all 10 women are dashed lines and for 8 women (omitting BB and JK) are solid lines. The respective regression equations and correlation coefficients for the 8 women were as follows: body weight (kg), $L = 6.35W + 219$, ($r = +0.74$); creatinine (gm), $L = 480C + 29$, ($r = +0.96$); metabolic body size ($kg^{3/4}$), $L = 21.5M + 134$, ($r = +0.73$); surface area (m^2), $L = 419S - 80$, ($r = +0.75$); and basal calories per 24 hours, $L = 0.4E + 123$, ($r = +0.45$).

No single method of predicting lysine requirements could be applied with precision to all individuals of either sex. However, a reasonably satisfactory estimate of lysine requirements could be made prior to the initiation of an experiment on the basis of body weight, and checked again during the adjustment phase when data pertaining to creatinine excretion became available.

No correlation between lysine requirement and body weight, surface area or creatinine excretion was observed in men (Rose et al., '55) or in women (Jones et al., '56). The wide range in body size and presumably in muscle mass of individuals of both sexes in the present experiment may provide a partial explanation for this discrepancy in findings, particularly since lysine participates principally in reactions related to protein metabolism.

Effect of initial lysine intake on apparent requirement. The decision to supplement the basal diet with 1000 mg of lysine during the initial standardizing interval was based on earlier observations that the response of certain individuals to a given amount of lysine was altered if the initial lysine intake was inadequate. For example, in one test the basal diet containing 500 mg of lysine was fed without supplementation during the first three periods, then was supplemented with increasing quantities of L-lysine monohydrochloride. As illustrated in table 3, subject AP, who was a man weighing 72 kg, did not attain equilibrium even when he consumed quantities of lysine that exceeded his requirement of 670 mg. Caloric intake was adequate to maintain weight. Nitrogen balances associated with a specific quantity of lysine were different when the initial intake of lysine was suboptimal and when it exceeded the minimum need, as tested in three experiments. Subject LM, a man who weighed 80 kg and required 850 mg of lysine, also responded unfavorably to a low initial intake of lysine. On the other hand, nitrogen balances of EO and GM who required 500 and 650 mg of lysine, respectively, were not affected adversely by feeding the unsupplemented basal diet in the initial phase of the same experiment in which AP and LM participated. These findings emphasize the importance of establishing

conditions at the beginning of an experiment that will allow all subjects to attain equilibrium within a reasonable time.

SUMMARY

The minimum quantities of lysine that maintained nitrogen equilibrium were estimated for 5 men and 5 women who consumed 9.0 gm of nitrogen principally as cereals, purified amino acids and diammonium citrate. Requirements of the men varied from 400 to 1200 mg, and of the women from 300 to 700 mg per day. The mean lysine requirement of 10 men was 750 mg and of 10 women 550 mg. These mean values are useful in estimating needs of population groups but not of individuals.

Regressions of lysine requirement of men on body weight, metabolic body size, surface area and creatinine excretion were highly significant, and on basal caloric expenditure was significant. Little correlation was found between lysine requirements of the 10 women and any physical or metabolic measurements, but regressions of lysine requirement of 8 women on body weight and creatinine excretion were highly significant, and on metabolic body size and surface area were significant.

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The Effect of Type of Dietary Fat on Reproductive Performance and Body Composition of the Vitamin B_6-Deficient Rat

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Much of the early work relating vitamin B_6 to fat metabolism has been reviewed by Sherman ('50). Definitive evidence of a role of vitamin B_6 in essential fatty acid metabolism was reported by Witten and Holman in 1952. Mascitelli-Coriandoli et al. ('59) investigated the effect of several types of fat on the cardiac hypertrophy of vitamin B_6 deficiency, and report the percentage of lipid content and weight of the heart to be increased as the saturation of dietary fat increased. While available evidence favors the suggestion of a synergistic relationship between polyunsaturated fatty acids and vitamin B_6, the antagonistic effect of oleic acid on growth of vitamin B_6-deficient animals observed by Sarma et al. ('47) suggests that fatty acids other than those of the polyunsaturated series also may influence the requirements for vitamin B_6.

Preliminary work in this laboratory indicated that weanling rats fed 15% of corn oil as the source of dietary fat were better able to withstand the effects of a vitamin B_6 deficiency than animals fed 15% of hydrogenated shortening.[1] The present study was undertaken to compare the effect of two commonly used fats fed with and without pyridoxine on reproductive performance of the female rat and on body composition of the mother and young.

EXPERIMENTAL

Weanling rats obtained from our stock colony were placed on synthetic diets containing either corn oil (IV 122) or a hydrogenated shortening (IV 71) as the source of dietary fat. The diet consisted in per cent of vitamin-free casein, 26; sucrose, 18.15; cornstarch, 36; fat, 15; salt mixture (Jones-Foster, '42), 4; l-cystine, 0.15; choline chloride, 0.4; inositol, 0.2; and *p*-aminobenzoic acid, 0.1. Also added in milligrams per kilogram of diet were: thiamin·HCl, 20; riboflavin·HCl, 20; niacin, 100; Ca pantothenate, 80; biotin, 0.4; vitamin B_{12} triturate (0.1% in mannitol), 40; folic acid, 4; naphthoquinone, 10; and pyridoxine·HCl, 8. Fat-soluble vitamins were administered twice weekly in 0.05 ml of corn oil and supplied weekly 20 mg of α-tocopheryl acetate, 5000 I.U. vitamin A and 480 I.U. vitamin D_3. Ad libitum feeding was allowed and weekly records were kept of food intake and weight of the animals.

When the animals were approximately 80 days old, half of each group was changed to the diet from which pyridoxine had been omitted. Three weeks later animals were mated with stock males at the appropriate time in the estrous cycle as indicated by daily vaginal smears. Mating was confirmed by the presence of sperm in the vaginal smear. The day in which sperm was found was considered day one of pregnancy.

Food intakes were recorded for each of the three weeks of gestation and animals were weighed and vaginal smears were examined daily in order to detect any gross deviation from the normal course of pregnancy. On the 22nd day, animals were killed by chloroform anesthesia and specimens obtained for analyses.

Approximately 2 ml of blood were removed by cardiac puncture and centrifuged to obtain serum for cholesterol determination. The contents of the uterus were removed and the young were weighed and frozen for later analyses. The liver was

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[1] Unpublished data.

weighed and frozen separately. The uterus and gastrointestinal tract were thoroughly cleaned and added to the remaining carcass and the total weighed, autoclaved and frozen for chemical analyses.

Analyses of maternal carcasses and livers, and of litters were made on slurries prepared by homogenizing the tissue with three times its weight of water. Moisture was determined by heating in a vacuum oven at 70°C for 24 hours. The dry sample was ashed at 600°C. Fat was determined by the A.O.A.C. ('55) acid hydrolysis method and nitrogen by the standard Kjeldahl procedure. The factor 6.25 was used for converting nitrogen to crude protein. Serum and liver were analyzed for total cholesterol by a macro modification of the method of Lowry et al. ('54) using the color reagent described by Zlatkis et al. ('53).

The data were subjected to an analysis of variance using the Duncan and Bonner test for significance (Duncan and Bonner, '54).

RESULTS AND DISCUSSION

Growth. Body weight was fairly uniform in animals maintained for 8 weeks after weaning on the basal diet containing pyridoxine (table 1). When the vitamin was omitted from the diet further growth ceased so that body weight was significantly lower ($P = 0.01$) by about 25 gm in the deficient groups at the end of the 11-week period in comparison with the controls receiving the vitamin. Animals fed corn oil were somewhat heavier than those fed hydrogenated shortening but the differences were not statistically significant.

Reproductive performance. Reproductive performance was similar in the two groups fed pyridoxine (table 2). Vitamin B_6-deficient animals produced fewer and lighter young than controls. Nelson and Evans ('51) found no reduction in average weight of young born of mothers placed on a pyridoxine-deficient diet at 90 days of age and mated two to 4 weeks later. They suggested that an adverse effect on the weight of the young might have been ob-

TABLE 1

Body weight of female rats fed two types of fat, with and without pyridoxine

Diet	No. animals	Initial weight	Weight at 8 weeks	Weight at 11 weeks
		gm	*gm*	*gm*
With pyridoxine				
Corn oil	20	58 ± 0.7[1]	243 ± 6	274 ± 8
Hydrogenated fat	22	58 ± 0.7	229 ± 5	256 ± 5
Without pyridoxine				
Corn oil[2]	21	59 ± 1.0	236 ± 5	236 ± 4
Hydrogenated fat[2]	9	58 ± 1.2	231 ± 7	232 ± 7

[1] Standard error of the mean.
[2] Pyridoxine omitted from diet at 8 weeks.

TABLE 2

Reproductive performance of rats fed two types of fat, with and without pyridoxine

Diet	No. animals mated	No. successful pregnancies	Total no. live young	Average litter size	Average fetal weight
					gm
With pyridoxine					
Corn oil	26	20	200	10.0 ± 1.0[1]	5.06 ± 0.22
Hydrogenated fat	24	22	202	9.2 ± 0.8	4.99 ± 0.17
Without pyridoxine					
Corn oil	28	21	151	7.2 ± 0.7	4.40 ± 0.19
Hydrogenated fat	28	9[2]	66	7.3 ± 0.8	3.83 ± 0.23

[1] Standard error of the mean.
[2] Excluding those of 5 females showing on autopsy fetuses in varying stages of resorption.

served if animals were fed the deficient diet at an earlier age. The results of the present study confirm this suggestion.

The number of successful pregnancies was considerably lower in vitamin B_6-deficient animals fed hydrogenated shortening than in any of the other groups. The total number of live young produced by these animals was less than half the number produced by the vitamin B_6-deficient group fed corn oil and only a little more than one-fourth the number produced by pyridoxine-fed controls. In 5 of the 28 deficient animals fed hydrogenated shortening, fetuses in varying stages of resorption were found at autopsy. These animals were excluded from the final group of pregnant animals as indicated in table 2. Failure to mate and false pregnancies also contributed to the poor reproductive performance of animals in this group. These data appear to indicate that the hormonal disturbance in vitamin B_6-deficient animals suggested by the work of Nelson et al. ('51) is more intense when hydrogenated shortening is the source of dietary fat than when corn oil is included in the diet.

Nelson and Evans ('51) have observed a reduction in number of young produced by vitamin B_6-deficient mothers, whereas Ross and Pike ('56) found no such difference between controls and animals made deficient by simple omission of the vitamin or with 0.5% of deoxypyridoxine. The first-named workers fed a diet containing 8% of hydrogenated shortening whereas the latter group fed 18% of hydrogenated fat plus 5% of corn oil. On the basis of their data Ross and Pike suggested that the type or amount of dietary fat may have accounted for the difference.

In the present study where the two fats were compared under identical conditions, the total number of live young was significantly lower when hydrogenated shortening was fed in the presence of a vitamin B_6 deficiency than when corn oil was the source of dietary fat. These data support the suggestion that the difference observed in the two studies may be due to the inclusion of 5% of corn oil in the diet used by Ross and Pike although the beneficial effect of a higher level of fat cannot be excluded altogether.

Food intake and maternal weight gain. Vitamin B_6-deficient animals ate less and gained less weight during pregnancy than did controls, but no difference was associated with type of dietary fat (table 3). In the vitamin B_6-deficient group fed hydrogenated shortening, total food intake for the three-week gestation period for each of the 5 animals that resorbed was 218 gm and total weight gain was only 16 gm. The corresponding figures for the 9 animals in this group carrying live young were 229 gm and 45 gm.

In the vitamin B_6-deficient animals only a small amount of the weight gained during gestation became a part of maternal tissue. Litter weight was approximately two thirds of the weight gain in deficient mothers whereas it was one third of the weight gain of mothers on the pyridoxine-containing diets. The type of dietary fat did not appear to affect litter weight significantly.

Maternal body composition. Analyses for gross body composition were made on

TABLE 3

Food intake, maternal weight gain and total litter weight of rats fed two types of fat, with and without pyridoxine

Diet	No. animals	Average total food intake	Average total weight gain	Average litter weight
		gm	*gm*	*gm*
With pyridoxine				
Corn oil	20	364 ± 11[1]	138 ± 7	49.3 ± 5.2
Hydrogenated fat	22	367 ± 14	136 ± 7	46.0 ± 4.3
Without pyridoxine				
Corn oil	21	226 ± 6	49 ± 4	31.2 ± 3.3
Hydrogenated fat	9	228 ± 11	45 ± 6	27.6 ± 3.1

[1] Standard error of the mean.

TABLE 4

Carcass and liver composiiton of pregnant rats fed two types of fat, with and without pyridoxine

Diet	No. animals	Weight	Moisture		Ash		Protein		Fat	
		gm	*gm*	%	*gm*	%	*gm*	%	*gm*	%
					Maternal carcass					
With pyridoxine										
Corn oil	10	312 ± 9[1]	159.2 ± 4.3	51.1 ± 0.8	8.3 ± 0.3	2.7 ± 0.1	49.1 ± 1.1	15.8 ± 0.3	97.5 ± 5.6	31.0 ± 1.1
Hydrogenated fat	15	311 ± 8	157.6 ± 3.1	50.8 ± 0.8	7.7 ± 0.3	2.5 ± 0.1	48.4 ± 1.0	15.6 ± 0.3	98.3 ± 5.3	31.3 ± 1.1
Without pyridoxine										
Corn oil	10	216 ± 5	131.6 ± 2.4	61.1 ± 0.5	8.8 ± 0.2	4.1 ± 0.2	42.1 ± 1.2	19.5 ± 0.2	34.7 ± 2.1	16.0 ± 0.6
Hydrogenated fat	6	216 ± 6	129.7 ± 2.7	60.2 ± 0.8	8.5 ± 0.3	3.9 ± 0.1	42.1 ± 1.0	19.5 ± 0.3	37.5 ± 3.0	17.3 ± 1.1
					Maternal liver					
With pyridoxine										
Corn oil	11	15.7 ± 0.7	11.3 ± 0.5	72.4 ± 0.6	0.22 ± 0.01	1.4 ± 0.04	3.19 ± 0.17	20.3 ± 0.5	0.62 ± 0.03	3.9 ± 0.07
Hydrogenated fat	14	14.9 ± 0.6	11.1 ± 0.5	73.0 ± 0.9	0.19 ± 0.01	1.2 ± 0.03	2.95 ± 0.13	19.4 ± 0.2	0.57 ± 0.02	3.8 ± 0.08
Without pyridoxine										
Corn oil	11	9.2 ± 0.4	6.7 ± 0.3	72.9 ± 0.4	0.11 ± 0.01	1.2 ± 0.03	1.85 ± 0.07	20.2 ± 0.3	0.32 ± 0.01	3.5 ± 0.10
Hydrogenated fat	5	10.4 ± 0.9	6.3 ± 0.6	73.2 ± 0.5	0.13 ± 0.01	1.2 ± 0.08	2.07 ± 0.14	20.1 ± 0.3	0.36 ± 0.03	3.5 ± 0.12

[1] Standard error of the mean.

a limited number of rats. The data as shown in table 4 indicate an altered body composition in vitamin B_6-deficient animals which is chiefly a reflection of diminished body fat. The percentage of carcass fat was almost twice as great in controls as in vitamin B_6-deficient animals. The difference in body composition is more striking, however, when viewed in terms of body weight. Controls weighed on the average approximately 100 gm more than the deficient animals, and body fat accounted for roughly 60 gm of this difference.

Diminished body fat in vitamin B_6 deficiency has been observed by others using both ad libitum and pair fed controls (Beare et al., '53; Guggenheim and Diamant, '59). Williams et al. ('59) observed a reduction in body fat in vitamin B_6-deficient animals fed 10 to 20% of cottonseed oil but not with 5 or 40% of fat as compared with pair-fed controls when the ratio of protein to food energy was kept constant. Carter and Phizackerley ('51) found no significant difference between deficient animals and pair-fed controls when dietary fat consisted of 20% of margarine and 5% of cod liver oil.

The present data indicate that while body fat is significantly reduced by even the mild vitamin B_6 deficiency produced under the conditions of this study, the type of dietary fat had little effect on the gross body composition of the deficient animals.

Liver composition. Liver weight was markedly reduced in deficient animals as compared with controls and was closely related to body weight. Liver hypertrophy has been reported both in young vitamin B_6-deficient rats (Guggenheim and Diamant, '59) and in pregnant vitamin B_6-deficient rats (Ross and Pike, '56), and we have made a similar observation in severely deficient animals in this laboratory.[2] The animals of this study were not severely deficient and this probably accounts for the different effects observed.

Liver fat, like carcass fat, was lower in the deficient animals, 3.5% of fresh liver as compared with approximately 3.9% in controls, a small but statistically significant difference. Under these conditions type of dietary fat had no effect on liver composition. It is apparent from these data that although marked changes occurred in body fat, the quantity of liver fat was much less affected by the vitamin B_6 deficiency. Similar results have been obtained by Guggenheim and Diamant ('59) who compared liver fat of deficient animals with both ad libitum and pair-fed controls.

Fetal composition. Fetal composition appeared quite similar in all groups (table 5) and reflected the high water, low fat content typical of the newborn of most species (Spray and Widdowson, '50). The small difference of only 0.1% in fat content between young of control and deficient animals was statistically significant ($P = 0.01$). Again, no difference attributable to type of dietary fat was apparent.

Comparison of maternal and fetal composition indicates that in the mother both body weight and body composition are adversely affected by maternal vitamin B_6 deficiency whereas in the fetus, body weight is affected to a much greater extent than is body composition. Goldwater and Stetten ('47) have presented evidence suggesting that fatty acids are synthesized in the fetal organism. It appears that the synthetic process in the newborn may be

[2] Unpublished data.

TABLE 5

Composition of young born of rats fed two types of fat, with and without pyridoxine

Diet	No. of litters	Moisture	Ash	Protein	Fat
		% wet weight	*% wet weight*	*% wet weight*	*% wet weight*
With pyridoxine					
Corn oil	11	86.4 ± 0.12[1]	1.64 ± 0.04	9.62 ± 0.11	1.22 ± 0.03
Hydrogenated fat	15	86.5 ± 0.16	1.64 ± 0.03	9.52 ± 0.12	1.21 ± 0.03
Without pyridoxine					
Corn oil	11	86.8 ± 0.19	1.60 ± 0.02	9.41 ± 0.15	1.13 ± 0.02
Hydrogenated fat	6	87.4 ± 0.33	1.63 ± 0.03	8.99 ± 0.22	1.08 ± 0.04

[1] Standard error of the mean.

TABLE 6

Serum and liver cholesterol of rats fed two types of fat, with and without pyridoxine

Diet	No. of animals	Serum cholesterol	Liver cholesterol	
		mg %	*mg/gm liver*	*mg/gm liver fat*
With pyridoxine				
Corn oil	20	107 ± 14[1]	2.55 ± 0.08	64.2 ± 1.2
Hydrogenated fat	22	92 ± 13	2.57 ± 0.04	68.1 ± 1.0
Without pyridoxine				
Corn oil	20	98 ± 14	2.62 ± 0.05	73.6 ± 1.6
Hydrogenated fat	9	77 ± 19	2.68 ± 0.08	73.3 ± 1.6

[1] Standard error of the mean.

impaired by a lack of vitamin B_6 in the maternal diet.

Maternal serum and liver cholesterol. Serum cholesterol levels were significantly higher in animals fed the corn oil diets than in those fed diets containing hydrogenated shortening (table 6). A reduction in serum cholesterol associated with the vitamin deficiency was observed in rats fed either fats, but this difference was of doubtful significance ($P = 0.05$). Serum cholesterol levels were lower in the deficient animals fed hydrogenated shortening than in any of the other groups.

No differences in the concentration of cholesterol in the liver were observed. Similar results have been obtained by Carter and Phizackerley ('50). However, when cholesterol was calculated as a percentage of liver fat, cholesterol constituted a greater proportion of fat in deficient animals than in controls. The difference is small but it is statistically significant ($P = 0.01$). This finding may be related to the decrease in neutral fat of liver reported by Carter and Phizackerley ('50). It appears, therefore, that the characteristics of liver fat as well as the amount may be altered in a vitamin B_6 deficiency. Kind of dietary fat, however, had no effect on liver constituents under the conditions of this study.

SUMMARY AND CONCLUSIONS

The effects of two types of fat, corn oil and a hydrogenated shortening, at 15% dietary levels, have been determined in studies of reproductive performance and body composition in vitamin B_6-deficient and control female rats, when the deficiency was initiated at 80 days of age, at least three weeks prior to mating.

No significant differences associated with type of dietary fat were observed in body or liver composition of animals at the levels of fat fed in these experiments, but the composition of liver lipids appeared to be altered by the vitamin B_6 deficiency.

The amount of carcass and liver fat was lower in deficient mothers, and carcass fat was slightly lower in young of deficient mothers than in those of controls.

Serum cholesterol levels were lower in animals fed the hydrogenated shortening than in those receiving corn oil. Cholesterol levels were lowest in the deficient group fed the hydrogenated shortening.

Vitamin B_6-deficient animals fed either fat produced fewer and lighter young than controls. Deficient animals fed hydrogenated shortening produced fewer than half as many live young as those fed corn oil. It is concluded that the more unsaturated fat exerts a protective effect in the vitamin B_6-deficient animals, and that the adverse effect of the deficiency on fertility and reproductive performance is accentuated when hydrogenated shortening rather than corn oil is the source of dietary fat.

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Nutrition and Longevity in the Rat[1]

I. FOOD INTAKE IN RELATION TO SIZE, HEALTH AND FERTILITY

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There is considerable evidence indicating that nutrition is one of the forces involved in regulating the time of onset of disease and longevity (Silberberg and Silberberg, '55). Since a great deal is known about the nutrition of the rat, this species is particularly suited for studies on diet in relation to aging. In a previous paper (Berg and Harmison, '57) a base line was established for rates of growth of rats kept under favorable standard conditions from weaning through maturity and old age. The rats were free from lung infection—a disease that interferes seriously with the validity of nutrition experiments. In other papers the principal diseases and tumors in our strain of Sprague-Dawley rats (Berg et al., '52; Berg, '56; Berg and Harmison, '57), the age of onset of lesions (Simms and Berg '57) and the survival rates (Berg, '59) were described. All of these data were obtained from rats fed ad libitum.

Extension of the rat's lifespan by extreme underfeeding was reported by McCay and co-workers ('35 and '43). Under their severe dietary restriction, growth and sexual maturity were greatly retarded. Also, their results were hampered by a high percentage of lung and middle ear infection in the rats. In man, overweight and obesity have an adverse effect on longevity (Armstrong et al., '51; Marks, '56; Olsen, '59). The questions arise as to what constitutes optimum nutrition—first, in relation to body size and health, and second, in relation to life expectancy and the onset of disease. The present paper deals with the first of these problems by comparing rats fed a restricted diet with those fed ad libitum. Since the latter became obese with advancing age (Berg and Harmison, '57), levels of food restriction were established for our restricted rats which prevented accumulation of excess body fat and yet provided for good skeletal growth. The final skeletal measurements of the restricted rats were 90 to 95% of those of unrestricted animals. The results with respect to body size, health, fertility and related findings using various levels of food intake are described in this paper. In the succeeding one (Berg and Simms, '60), longevity and onset of disease in the same rats will be discussed in relation to food intake.

METHODS AND MATERIALS

Rat selection. A total of 339 rats, 189 males and 150 females, were used. They were bred from a colony of Sprague-Dawley rats started in 1945 and were raised under especially favorable conditions (Simms and Berg, '57). Animals were weaned at 28 days of age and the young were selected so that equal numbers with the same body weight at the time of weaning were represented in each experimental group. Weaning weights ranged from 40 to 80 gm.

Colony conditions. The animal quarters were air-conditioned and were kept throughout the year at constant temperature and humidity. Rats were handled gently and cages were kept scrupulously clean. Lighting was regulated to give 12 hours of uniform indirect illumination and 12 hours of darkness each day. None of the rats had lung or middle ear infections. The diet consisted of Rockland "D free" pellets and was composed of natural foods

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containing approximately 24.3% of crude protein, 4.0% of fat and 54.2% of carbohydrate. All of the essential vitamins and minerals were present in amounts adequate for growth and maintenance. Approximate caloric values per 100 gm of diet were: protein, 78; fat, 36; carbohydrate, 217. Using this diet a high fertility rate was sustained. Mating was restricted to rats used for breeding, and breeders were discarded after three matings. For the dietary studies the rats were isolated in single cages except for a group of animals fed ad libitum.

Food intake. Twenty-eight-day-old rats of both sexes were started at the time of weaning on three levels of food intake:

1. *Ad libitum (unrestricted).* Thirty-seven males and 38 females caged singly, and 25 males and 25 females kept in cages holding three or 4 animals were fed all they would eat. Mean daily intake was calculated from weekly food consumption.

2. *Thirty-three per cent restriction.* After establishing ad libitum levels for both sexes, 48 males were given the amount eaten by unrestricted females, and 48 females were given an amount reduced correspondingly from female ad libitum levels. Thus the extent of restriction was the same for both sexes, and from 125 to 630 days of age was relatively constant at 33% of ad libitum values.

3. *Forty-six per cent restriction.* In another group of 79 male and 39 female rats, food intake was reduced empirically to levels which kept the weight of males down to that of unrestricted females and maintained the weight of females constant and equal to that of 56-day-old unrestricted females. The latter became sexually mature at this age. Reduction from ad libitum levels for both sexes between 125 and 630 days of age averaged 46%.

About one half of the unrestricted males became moribund or died before they were 760 days old, while most of the rats in the other groups (restricted males, and unrestricted and restricted females) were still alive and in good condition at this age. All rats that survived to the age of 760 to 833 days were killed with pentobarbital sodium[2] intraperitoneally injected (except for 26 males and 20 females on 46% restriction, which were kept for life-span studies). The killing of these apparently healthy animals at this age range was necessitated by the demolition of the building in which our animal colony was located.

Body measurements. Body weights were obtained weekly up to 448 days of age and then at intervals of 28 days. Tibia length was measured by precision calipers and was the distance from the spines of the head to the medial malleolus. Width of the proximal epiphyseal cartilage and

[2] Nembutal, Abbott.

TABLE 1

Final body measurements of surviving rats killed at 760 to 833 days of age receiving different levels of food intake

	Food intake		
	Unrestricted	33% Restricted	46% Restricted
	Males		
No. of rats	31	42	38
Mean daily food intake, gm[1]	19.5(100%)	13.0(67%)	10.5(54%)
Body weight, gm	448	342	275
Tibia length, cm	4.61 ± 0.12[2]	4.39 ± 0.08	4.28 ± 0.09
Body length, cm	25.0 ± 0.5	23.4 ± 0.7	22.4 ± 0.8
	Females		
No. of rats	58	22	17
Mean daily food intake, gm[1]	13.0(100%)	8.7(67%)	7.0(54%)
Body weight, gm	280	207	161
Tibia length, cm	4.00 ± 0.08	3.81 ± 0.07	3.77 ± 0.05
Body length, cm	21.1 ± 0.6	19.7 ± 0.7	18.3 ± 0.3

[1] Observations taken during the period from 125 to 630 days of age.
[2] Standard deviations.

osteogenesis were determined microscopically after preliminary decalcification of the tibia in a 5% nitric acid solution followed by staining of paraffin-imbedded sections with hematoxylin and eosin. Body length was the calipered distance from the tip of the nose to the anus. (table 1)

Fertility. Fertility of female rats on 33% restriction was tested in 24 animals, 600 days to 790 days old, and the results were compared with those obtained in 33 unrestricted females, 560 days to 730 days old, taken from our colony. The males used for matings were 110-day to 120-day-old unrestricted colony rats. Females were rated as infertile when no pregnancies occurred after three matings for 7-day periods spaced at 35-day intervals. During mating and gestation previously restricted females were allowed to eat ad libitum. Data on food intake and body weight of unrestricted females up to the time of mating are included in figures 1 and 4, but final body weight and measurements of mated animals are not included in table 1.

Male fertility was determined microscopically by the presence of normal spermatozoa in the epididymis and of spermatogenesis in the testis.

RESULTS

Food intake

Ad libitum. Curves for mean daily food consumption of singly-caged male and female rats fed ad libitum are shown in the upper and middle curves of figure 1. During the post-weaning period of rapid growth and of sexual development, peak intakes of 22.7 gm by males and 15.7 gm by females were reached between 49 and 63 days, the time when sexual maturity is attained. Afterward, there was a decline in food consumption by males to 19.5 gm at 170 days, and to 13.0 gm by females at 125 days. At these levels plateaus continued up to 630 days for males and 600 days for females. Later, there was an upturn in the curves with final values similar to those of early life. Overall intake of young rats was greater for those with high weaning weights than for those with lower initial weights, but in adults, there was considerable overlapping of the food consumption curves of rats with different weaning weights. Up

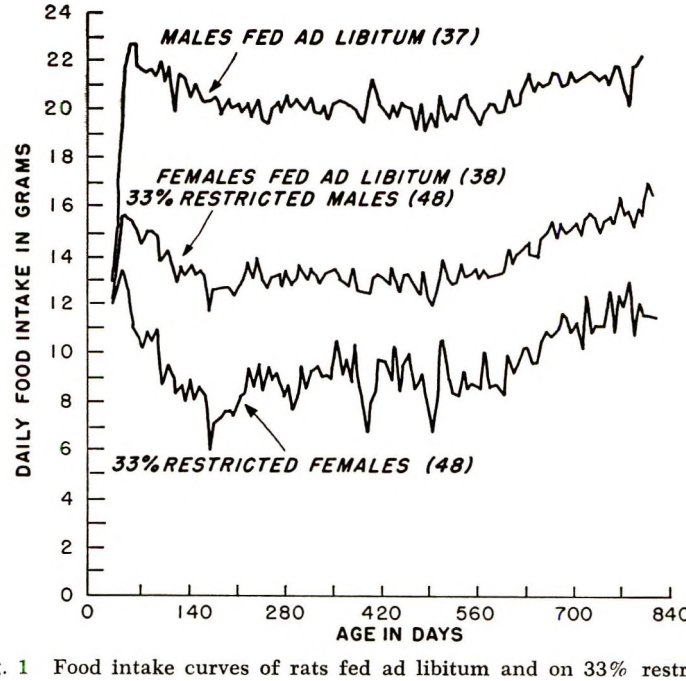

Fig. 1 Food intake curves of rats fed ad libitum and on 33% restriction.

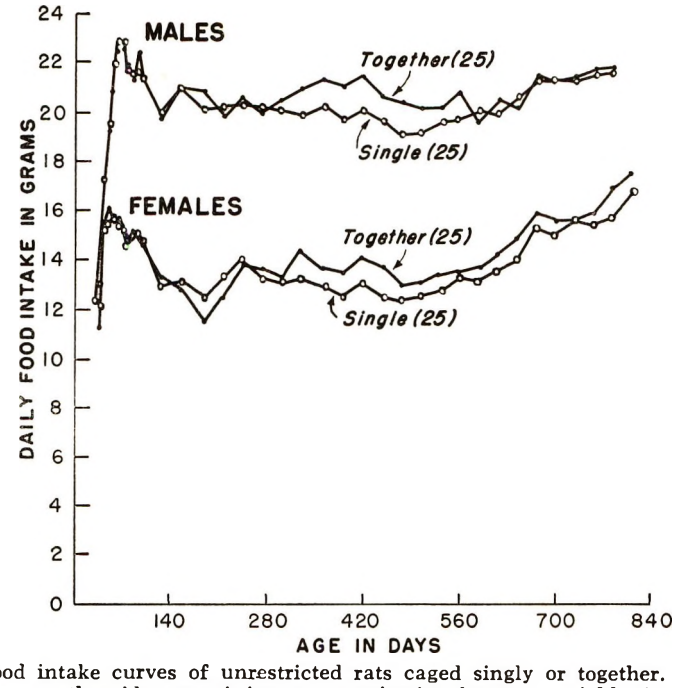

Fig. 2 Food intake curves of unrestricted rats caged singly or together. All cages had mesh windows on the sides permitting communication between neighboring rats.

to 300 days of age, food intake of rats caged singly or together was nearly the same. Afterward, the level of food consumption of singly caged animals was about 1 gm lower than that of animals kept together (fig. 2). At 560 days the curves converged in males but remained separate in females.

Thirty-three per cent restriction. The middle and lower curves of figure 1 show the dietary intake of both sexes when reduced by one-third from ad libitum levels. In rats from 125 to 630 days old, mean daily food consumption of males was lowered from 19.5 gm to 13.0 gm, and from 13.0 gm to 8.7 gm in females.

Forty-six per cent restriction. The lower two curves in figure 3 show greater food reduction from ad libitum levels. Mean daily intake from 125 days to 630 days of age was 10.5 gm for males and 7.0 gm for females. Restriction from ad libitum intake was 46% for both sexes.

Body measurements

Body weights of unrestricted rats. Curves for both sexes are shown in figure 4. Up to 100 or 140 days, body weights increased rapidly. Subsequent increments were progressively smaller, reaching a peak of 467 gm at 615 days in males, and continuing upward to 287 gm at 810 days in females. Late decline in body weight of unrestricted males was due to onset of disease which developed earlier than in females (Berg, '56; Berg and Harmison, '57; Berg, '59) or than in restricted males (Berg and Simms, '60). Final weights were similar to those recorded previously (Berg and Harmison, '57).

Body weights of adult rats with high weaning weights remained greater than those starting with low weaning weights. At 560 days of age males showed a difference of 42 gm and in females at 750 days there was a difference of 28 gm (figs. 5 and 6). Singly-caged adult males weighed about 20 gm less than males kept together, whereas females showed no significant difference (fig. 7).

Body weights of 33%-restricted rats. The shapes of the curves for restricted rats of both sexes were the same as for unrestricted rats, but at lower levels (fig. 4). After an initial steep rise, all the curves gradually leveled off after 100 days.

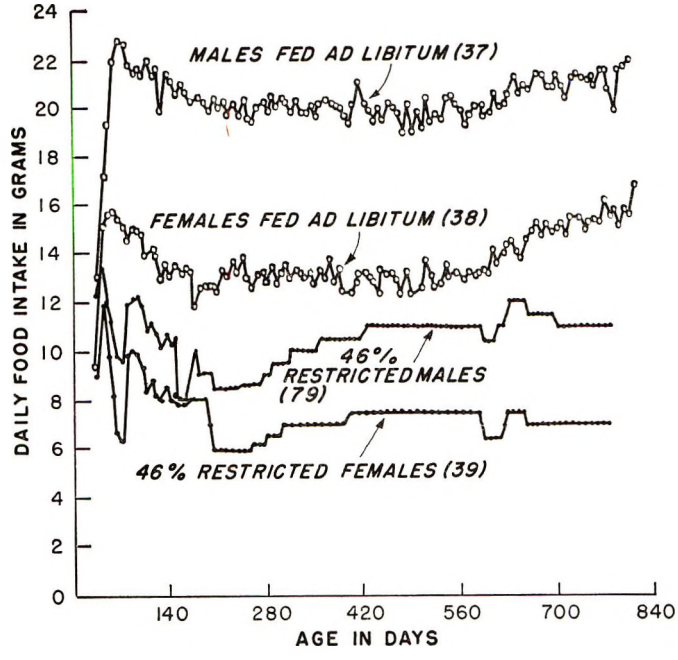

Fig. 3 Food intake curves of rats fed ad libitum and on 46% restriction.

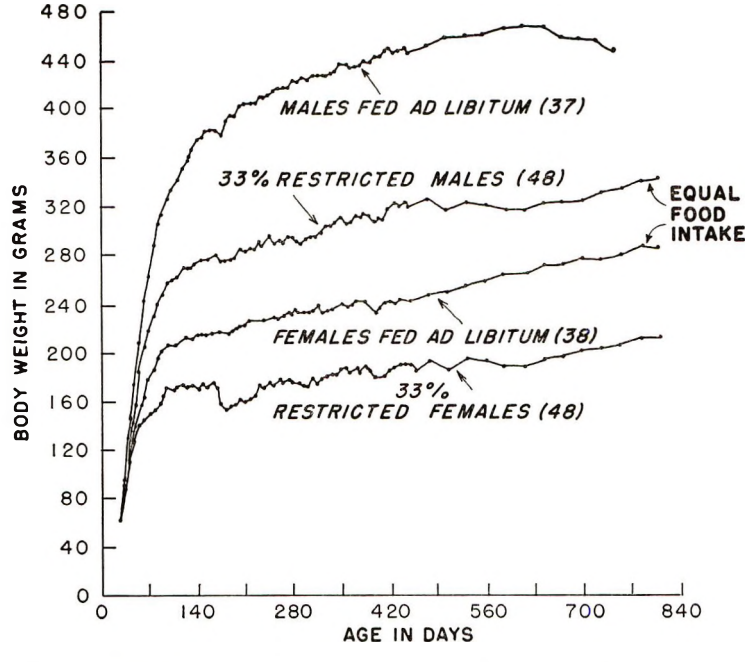

Fig. 4 Weight curves of rats fed ad libitum and on 33% restriction.

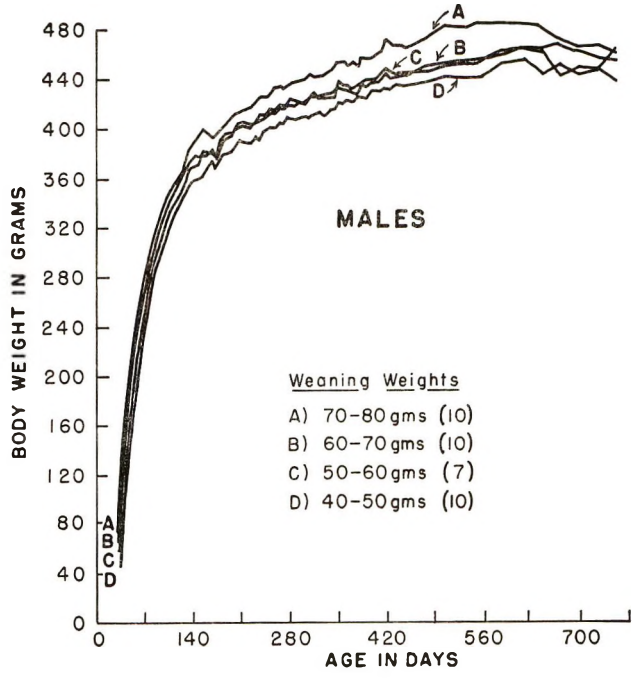

Fig. 5 Weight curves of unrestricted male rats with different weaning weights.

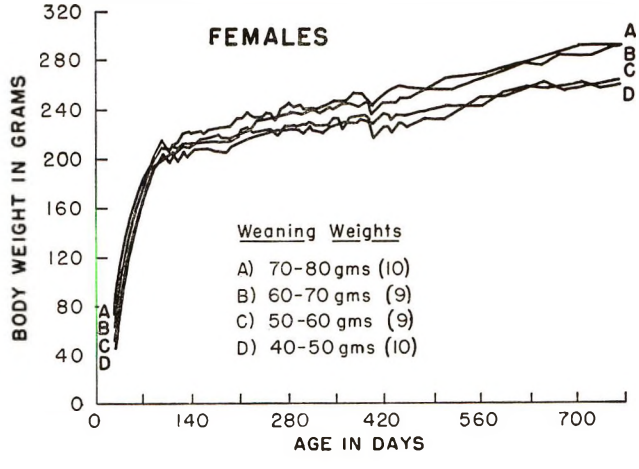

Fig. 6 Weight curves of unrestricted female rats with different weaning weights.

Weight reduction below the ad libitum levels was about 25% for both sexes.

Body weights of 46%-restricted rats. In males the weight curve was nearly identical with that of unrestricted females. In restricted females a plateau was reached at about 100 days and remained at a level of between 140 and 160 gm, which is the weight of 56-day-old unrestricted females (fig. 8). Weight reduction below the ad libitum levels was about 40% for both sexes.

Tibia length and body length. Final measurements are given in table 1. Tibia

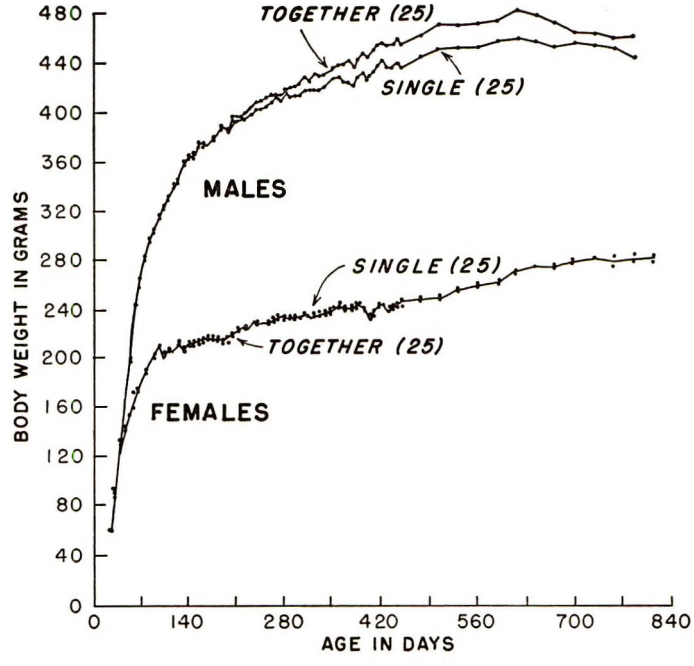

Fig. 7 Weight curves of unrestricted rats caged singly or together.

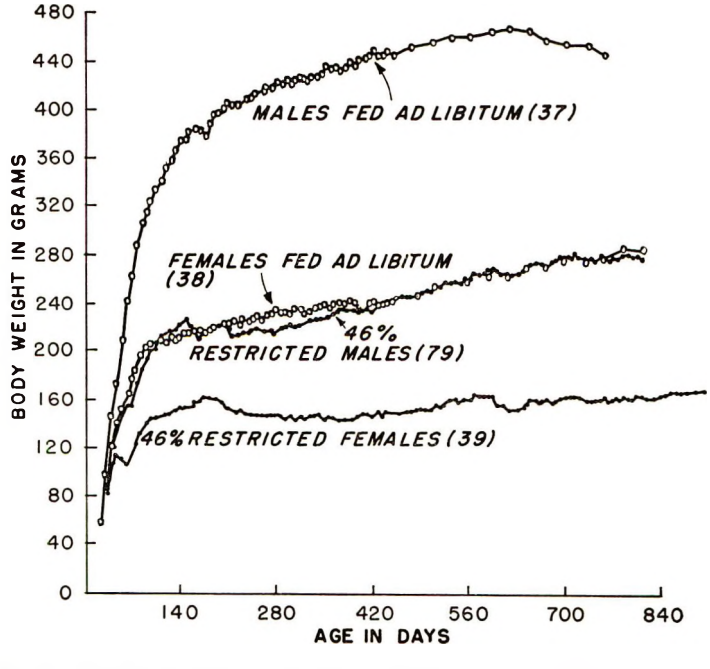

Fig. 8 Weight curves of rats fed ad libitum and on 46% restriction.

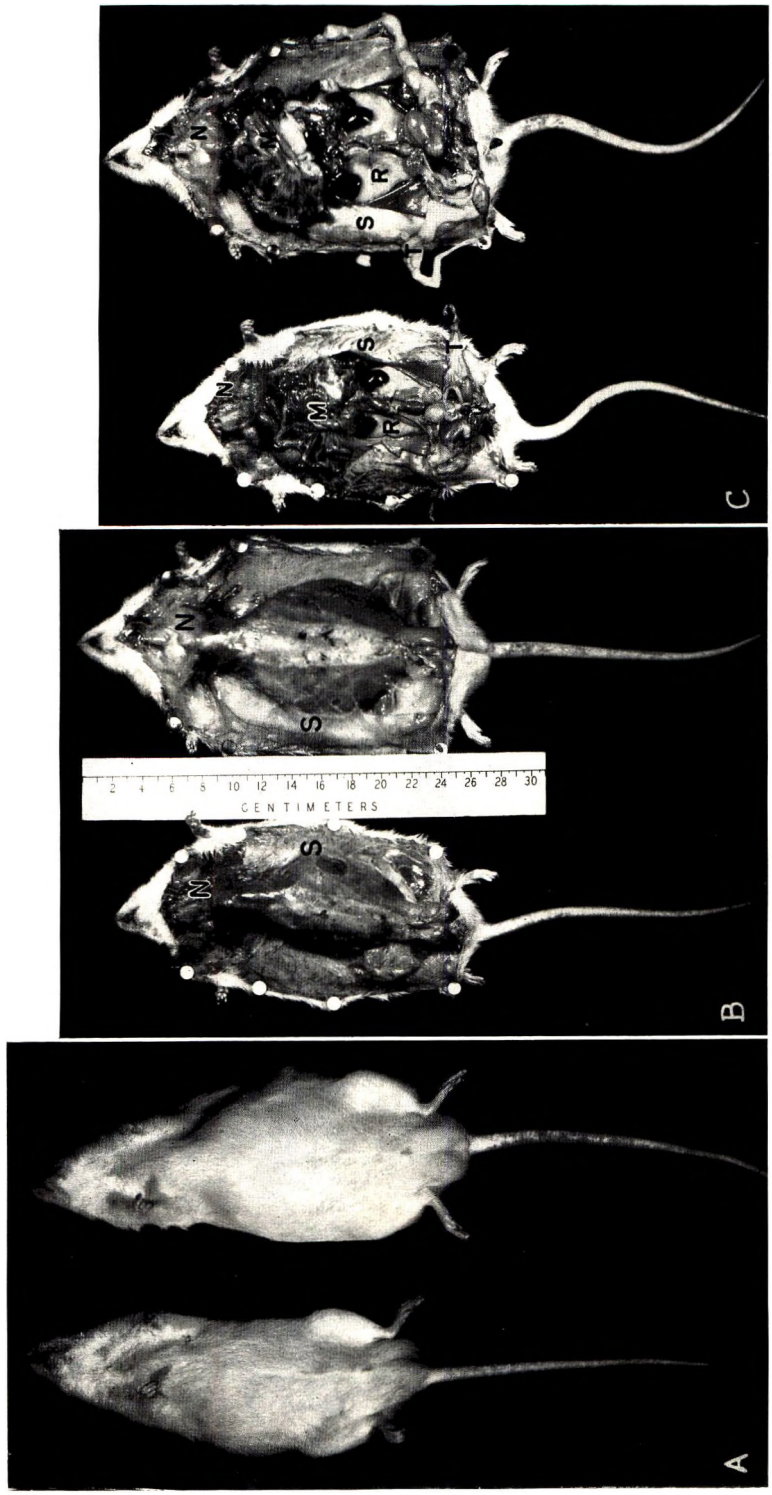

Fig. 9 A. Size of unrestricted male rat (right) weighing 534 gm at 637 days of age, compared with male rat on 33% restriction weighing 377 gm at 816 days of age. B, Excess fat in subcutaneous tissues of abdominal wall (S) and neck (N) in unrestricted rat (right); none present in 33%-restricted rat. C, Excess fat in mesentery (M), perirenal and retroperitoneal areas (R), and in testicular fat pad (T) of unrestricted rat; none present in 33%-restricted rat (left) except for normal deposit of perirenal fat (R).

length of restricted rats was 5% shorter (33%-restricted) to 7% shorter (46%-restricted), and body length was 6% less (33%-restricted) to 13% less (46%-restricted), than in unrestricted rats. Measurements of unrestricted rats were close to those obtained previously (Berg and Harmison, '57). Measurements of 33%-restricted females used for fertility studies are not included in the table but were the same as those of virgin females on 33% restriction. Comparative size of 33%-restricted and unrestricted male rats is shown in figure 9A. There was no significant difference between the skeletal measurements of unrestricted rats caged singly or together (table 2).

Measurements of unrestricted and restricted rats in relation to heterogony. According to Huxley's principle of heterogony ('32), the ratio of relative growth rates between any two parts of the body, or a part of the body and the whole, remains constant. In an earlier paper (Berg and Harmison, '57) a linear relation with points for both sexes falling on a single line was demonstrated in rats with respect to tibia length and body length. When the same measurements are plotted for restricted rats of both sexes, the points fall on the same straight lines as those of unrestricted animals (fig. 10). The same proportionality constant holds for restricted and for freely fed rats.

TABLE 2

Comparative final measurements of unrestricted rats caged singly or together

Caging	Mean age	Tibia length			Body length			Body weight		
		No.	Mean	Range	No.	Mean	Range	No.	Mean	Range
	days		*cm*	*cm*		*cm*	*cm*		*gm*	*gm*
					Males					
Single	812	11	4.56	4.38–4.75	10	25.0	24.4–25.5	11	435	390–509
Together	806	13	4.64	4.47–4.80	13	25.2	24.5–26.1	13	458	374–512
					Females					
Single	829	24	4.00	3.84–4.12	24	21.2	19.8–22.2	24	277	240–315
Together	823	23	4.02	3.88–4.18	24	21.1	20.0–22.0	24	277	189–357

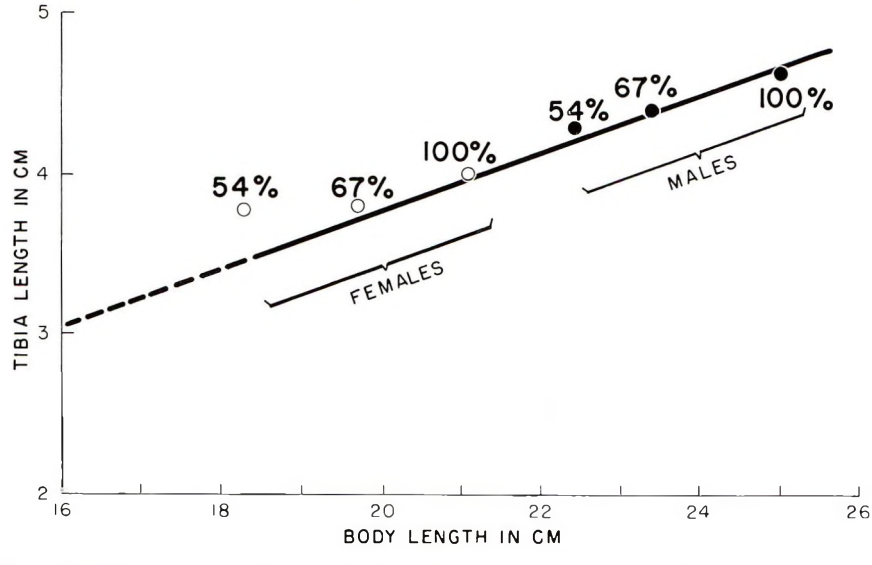

Fig. 10 Points representing tibia length plotted against body length of unrestricted (100%) and restricted (67% and 54% of the unrestricted level—also referred to in this paper as 33% and 46% restriction, respectively). The straight line passes through the origin and follows the equation: $5.37\ l_T = l_B$.

OTHER OBSERVATIONS

The proximal tibial epiphysis. Microscopic examination of this epiphysis in rats over 760 days old showed no difference between unrestricted and restricted animals. There was no evidence of osteogenesis in either group. The epiphyseal cartilage, which persists in the adult rat, was narrow and irregular, and newly formed trabeculae indicative of osteogenesis were absent. The findings showed that skeletal growth had stopped. Changes in the epiphyseal plate at different ages were described previously (Berg and Harmison, '57). Figure 11 shows an inactve epiphyseal cartilage of an 820-day-

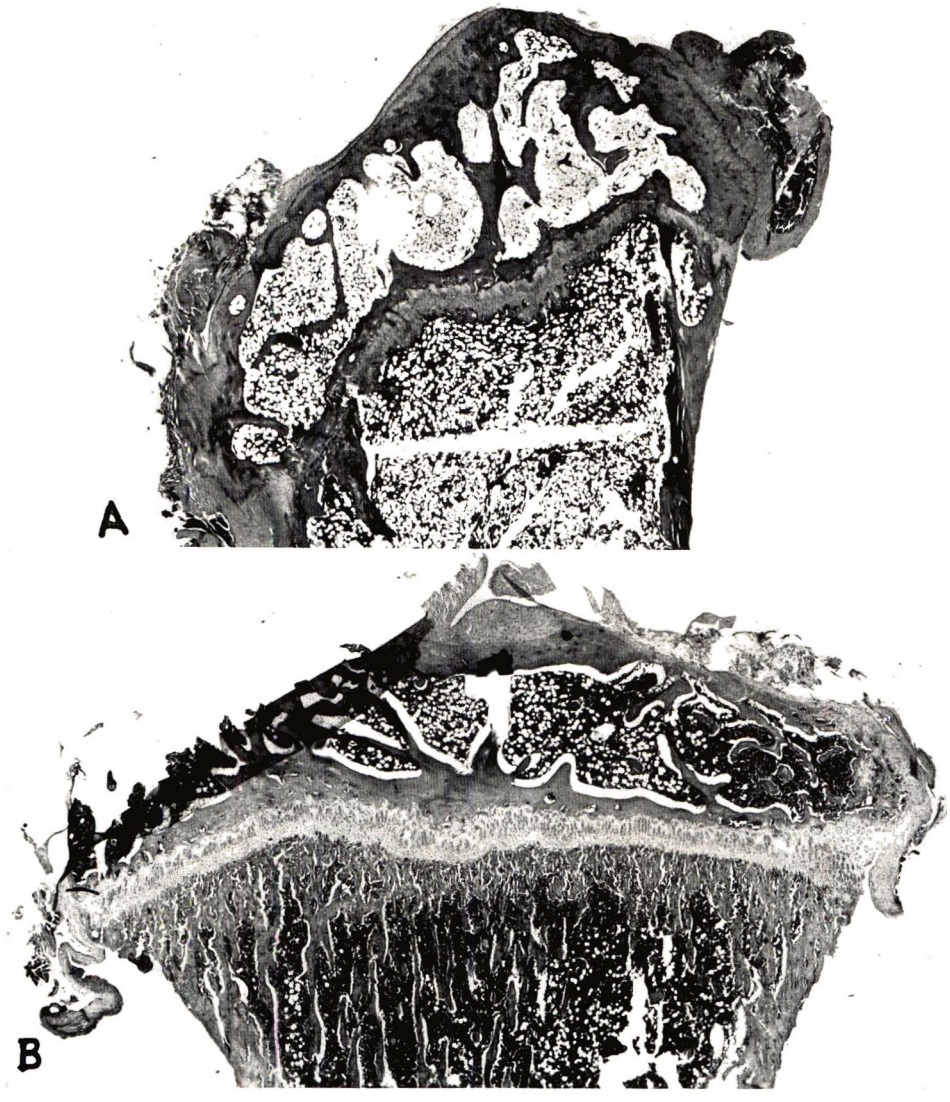

Fig. 11 A, Proximal tibial epiphysis of 820-day-old male rat on 46% restriction. Cartilage plate is narrow and irregular. There is no evidence of osteogenesis. Tibia length 4.16 cm; body weight 270 gm. Hematoxylin and eosin. Sagittal section. × 8. B, Proximal tibial epiphysis of 100-day-old male rat fed ad libitum. Cartilage plate is wide and even, and numerous primary and secondary bony trabeculae extend from the diaphyseal side of the cartilage. Osteogenesis is active. Tibia length 4.08 cm; body weight 320 gm. Hematoxylin and eosin. Frontal section. × 8.

old male on 46% restriction and also an active epiphyseal cartilage of a 100-day-old male unrestricted rat having the same tibia length.

Food utilization. In terms of body weight increments and final body measurements, food utilization was more efficient in males than in females. On 33% restriction, male rats receiving the same amount of food as unrestricted females gained more weight during early life and were heavier in adult life than females. Weight curves were 20% higher in males than in females (fig. 1) and final body weight was 342 gm for males, as compared with 280 gm for females (table 1). Also, body measurements were 10% greater in males than in females (table 1). Final tibia length was 4.39 cm and body length was 23.4 cm, while corresponding measurements of females were 4.00 cm and 21.1 cm.

Further evidence of more efficient food utilization by males was shown with 46% restriction. Here, on a 20% lower food intake male rats maintained an equal body weight with unrestricted females and had larger skeletal measurements. Tibia length was 4.28 cm and body length was 22.4 cm as compared with 3.81 and 19.7 cm respectively for females.

Our findings with respect to the differences in food utilization by the two sexes correspond to those of previous workers. Morris et al., ('33) found that utilization was more efficient in the male rat than in the female, and McCay and co-workers ('35) showed that the calorie requirements for maintenance of retarded rats at various levels of body weight were greater for females than for males.

Gross appearance, appetite, activity and body fat. Rats on food restriction had smooth, clean fur and fine hair. Their teeth showed no abnormalities. They were hungry and consumed their daily supply of pellets within one or two hours. For the rest of the 24-hour period, the hoppers were empty and, in anticipation of getting more pellets, the rats became extremely active and climbed the doors of their cages whenever an attendant entered the room. The animals were lively and aggressive and quickly devoured pellets offered to them. At autopsy there was little or no evidence of body fat (figs. 9B, 9C).

In contrast with the sleek appearance of restricted rats, the coat of unrestricted animals, particularly of males, was coarse and soiled. A dark brown pigment was often deposited on the nose and hairs of the snout, and around the eyes. The incisor teeth were elongated and were frequently fractured. Malocclusion of the incisors and perforation of the palate or lip were not uncommon. With advancing age, unrestricted rats became sluggish, slept most of the time and responded to stimuli slowly. They accepted food pellets but stored them in the rear of the cage without eating them. Examination at autopsy revealed large deposits of fat in the subcutaneous tissues, particularly in the neck, in the retroperitoneal and perirenal spaces, in the testicular fat pad, in the mesenteries of the intestine and uteri, in the perineal region, and at the site of the interscapular body (figs. 9B, 9C).

Fertility. Table 3 gives the comparative fertility of female rats on ad libitum and on 33%-restricted feeding. Out of 33

TABLE 3

Fertility of female rats receiving unrestricted and 33%-restricted food intake

No. of rats	Mean body weight at first mating	Fertile			Infertile		
		No.	Per cent	Age range	No.	Per cent	Age range
	gm			*days*			*days*
			Unrestricted				
33	284	4	12	560–620	29	88	560–760
			33% Restricted				
24	197	16	67	730–790	8	33	730–790

unrestricted females from 560 to 760 days old, only 4 (all under 700 days old) bore litters. In contrast with this low fertility rate, 16 out of 24 restricted females from 730 to 790 days old had successful matings and in 5 instances bore litters twice. Litters were small, the death rate of the newborn was high, and eating of the young was common.

Evidence of male fertility as determined by the presence of spermatozoa in the epididymis and of spermatogenesis in the testis was observed in 90% of rats fed ad libitum and in the same number with 33% restriction. Studies on 46%-restricted males were hampered by a high incidence of hydroceles which caused atrophy of the testes.

DISCUSSION

It is well known that male rats are larger than females. Previous studies on unrestricted rats (Berg and Harmison, '57) showed that rate of skeletal growth diminished after 170 days of age, but weight continued to increase for about a year in males and longer in females. To what extent the weight increase, which amounted to 20%, was due to fat accumulation had not been determined quantitatively, but gross autopsy findings indicate that the increase is attributable largely to fat deposition. In view of the fact that the proportion of excess body fat which may be called obesity is ill-defined (Mayer, '53), the use of the term obesity in this paper is arbitrary. To the extent that the additional weight is the result of food intake in excess of energy requirement, ad libitum feeding was not ideal for the cage-confined rat. Ingle ('49) produced extreme obesity by long-term confinement of rats fed ad libitum in small cages which greatly restricted their activity. On 33 or 46% restriction extra body fat was not deposited, less than 10% retardation of skeletal growth occurred, and health and female fertility were better than in unrestricted animals. Also, in the succeeding paper (Berg and Simms, '60) it will be shown that freedom from disease and longevity were enhanced without the severe stunting effect on growth produced by drastic underfeeding (McCay and associates, '43).

In the natural state, hunger is a primary instinct of the rat. Under laboratory conditions which are highly artificial for the rat, hunger is dulled by ad libitum feeding. On restricted intake hunger is sharpened and the quest for food heightens spontaneous activity. Insofar as appetite remains unsatisfied, the restricted rat appears to be in a more natural condition than the unrestricted one.

Comparative data on food intake and body measurements showed small differences between unrestricted rats caged singly or together. Provided that cages are of ample size, housing several rats together for long periods showed no demonstrable effect on their skeletal size. However, there was a small effect on the food intake (fig. 2) and on male body weight (fig. 7).

The fertility rate of 730 to 790-day-old females on 33% restriction was equal to that of young female breeders fed ad libitum, while the number of successful matings of 560 to 620-day-old females on ad libitum feeding was much smaller. The infertility of the latter could not be attributed to the absence of ovulation because estrus cycles in our colony of rats continue at this age. However, at autopsy, uterine polyps and adenocystic endometritis were found in a high percentage of unrestricted females but in relatively few restricted ones. These pathological findings could explain the difference in fertility rates.

The hyperplastic mammary changes characteristic of pregnancy did not develop in the breasts of old rats. It is probable that failure of lactation was the cause of the large number of deaths among the newborn. The pituitary glands and the ovaries of infertile females appeared to be normal microscopically.

SUMMARY

Restriction of food intake of rats to levels 33 and 46% below the ad libitum level, from weaning to 800 days of age, resulted in 5 to 7% reduction in tibia length, 6 to 13% reduction in body length, and 25 to 40% reduction in body weight. The difference in body weight was due

largely to absence of excess body fat which developed in unrestricted animals. The latter ate more than they needed to meet energy output. Health and female fertility were better in rats kept on dietary restriction than in unrestricted rats attaining maximum size.

In accord wih Huxley's principle of heterogony, measurements of tibia length and body length of rats on restricted food intake were proportional, and linear plots gave points falling on the same straight line as the corresponding measurements of animals fed ad libitum.

There was no significant difference in skeletal size of unrestricted rats caged singly or together. However, food intake and male body weight were slightly lower in singly-caged animals. Differences in body weight at the time of weaning continued through adult life in rats that were fed ad libitum.

The proximal epiphysis of the tibia of 800-day-old rats on restricted food intake showed no evidence of osteogenesis and had the same microscopic appearance as the epiphyseal cartilage of unrestricted rats of the same age.

Food utilization was more efficient in males than in females. Skeletal measurements and body weight were greater in males on 33% restriction than in unrestricted females on the same food intake. The body weight of males on 46% restriction was equal to that of unrestricted females but skeletal measurements were greater.

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Nutrition and Longevity in the Rat[1]

II. LONGEVITY AND ONSET OF DISEASE WITH DIFFERENT LEVELS OF FOOD INTAKE

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In the preceding paper (Berg, '60) it was shown that ad libitum feeding, which leads to large skeletal size and obesity, was less favorable for health, activity and female fertility of aging rats than restricted food intake which produces leaner and slightly smaller animals. The present report on the same rats deals with the onset of disease, the longevity, and the mortality, as influenced by the level of food intake.

McCay and associates ('43) extended the lifespan of rats by restricting caloric intake. However, they used drastic underfeeding which resulted in severe retardation of growth and in immaturity. Also, since the animals available at that time had a high incidence of lung and middle ear infections, this interfered seriously with nutrition experiments. In the present work, these adverse factors are excluded by using less drastic dietary restriction and by the use of rats free from infection.

METHODS AND MATERIALS

In the preceding paper (Berg, '60), details were given concerning rat selection, laboratory conditions, duration of experiments, body weight and measurements and composition of diet. Food intake was at three levels: (1) ad libitum (unrestricted), (2) one-third less than ad libitum level, designated as "33% restriction," and (3) 54% of the ad libitum level, designated as "46% restriction."

Autopsies and microscopic examinations. Rats which were moribund before, or surviving to the end of, the experimental period were killed with pentobarbital sodium[2] injected intraperitoneally. Complete autopsies were performed immediately after death. Among animals dying spontaneously, microscopic examinations of tissues were made only in rats showing no signs of autolysis. Specimens were taken from all tissues, and after fixation in Zenker's fluid, were imbedded in paraffin. Sections for microscopic study were stained with hematoxylin and eosin.

Grading of lesions. Lesions of the major diseases were graded as early (slight), moderate, or severe, according to the extent of the pathological changes. Rating was based on the interpretation of gross and microscopic findings by a single observer. Tumors were not classified according to severity. Grading was approximate but accurate enough for the purposes of this study.

Diseases selected for study

Excluding tumors, 4 major diseases developed in our colony of rats. Data on only three diseases, namely, chronic glomerulonephritis, periarteritis and myocardial degeneration are included in this report because lesions of the 4th condition, muscular dystrophy (Berg, '56) did not appear within the experimental period in sufficient numbers for comparative studies. The pathology of these lesions was described in an earlier paper (Simms and Berg, '57), but the following descriptions are more detailed:

Chronic glomerulonephritis. In the early stage of the disease the lumina of a few proximal convoluted tubules were dilated and contained casts. Lesions rated as moderately severe involved more tu-

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[2] Nembutal, Abbott.

bules, including those in the collecting system, and some of the connecting glomeruli showed hyalinization. The findings in severe lesions correspond to the changes seen in advanced glomerulonephritis. The parenchyma was honeycombed with cysts of variable size resulting from greatly dilated tubules and there was extensive hyalinization and fibrosis of the glomeruli. Fibrosis of the supporting stroma and thickening of the media of the smaller arteries were also present. Late complications were hypertension with cardiac hypertrophy (Berg, '55a, b), hydrothorax, ascites and terminal uremia with high nonprotein nitrogen blood values. Subcutaneous edema was rare.

Periarteritis. This condition is characterized by focal degenerative and inflammatory changes involving medium and small-sized arteries and giving the vessels a beaded appearance. The mesenteric and spermatic arteries were chiefly affected, but other vessels, such as the coronary, hepatic, thyroid, pulmonary, cerebral and adrenal were occasionally involved. Lesions were rarely seen in the renal arteries.

Changes were observed first in the vessels of the pancreas and of the testes. Initially there was swelling of the collagen fibers of the media and adventitia of the smaller arteries with some perivascular infiltration by leucocytes. When these changes were limited to a few vessels, they were rated as early. More advanced alterations extending to the upper mesenteric arteries were graded moderate. Characteristic findings at this stage were intense leucocytic infiltration, swelling and disruption of the media and endarteritis with partial or complete occlusion of the lumina. Lesions classified as severe involved all of the mesenteric vessels and were characterized by aneurysms filled with thrombi in various stages of organization. In the pancreas, zones of parenchyal degeneration and fibrosis surrounded diseased vessels. Ascites was frequently present, but intestinal infarction was rare despite extensive involvement of mesenteric vessels. Death due to hemorrhage from a ruptured aneurysm occurred occasionally.

Myocardial degeneration. Changes consisted of localized areas of myocardial atrophy, necrosis and fibrosis. In order of decreasing frequency, lesions occurred in the papillary muscles and adjacent myocardium of the left ventricle, at the base, at the apex, in the interventricular septum and around the branches of the coronary arteries. A small zone of degeneration with condensation of the supporting stroma in a single area was rated as early. Greater myocardial damage with replacement by connective tissue was graded as moderate. Extensive degeneration and fibrosis were classified as severe. Moderate or severe lesions often involved more than one site. There were no changes in the coronary arteries nor inflammatory reactions in areas of myocardial injury.

The myocardial, vascular and renal lesions corresponded in many respects to the changes in Sherman rats described by Wilens and Sproul ('38a, b). Chronic glomerulonephritis and periarteritis were primary causes of death while myocardial degeneration was a contributory cause.

Tumors. Many types of spontaneous tumors developed. The more common neoplasms were benign and included adenoma of the thyroid, chromophobe adenoma of the pituitary, fibroadenoma of the female breast and pheochromocytoma of the adrenal. Less common benign tumors were myxoma and fibroma of the subcutaneous tissue, islet adenoma of the pancreas, and leiomyoma of the uterus and intestine. The chief maligant neoplasms were carcinoma of the thyroid, and sarcoma of the nervous system and subcutaneous tissue. Other malignant tumors included carcinoma of the pancreas, kidney and bladder, hypernephroma and seminoma.

RESULTS

Mortality and survival rates. The mortality in different age groups is shown in table 1, as well as the percentage of rats surviving to the end of each age group. On ad libitum feeding only 48% of males were alive at 800 days as compared with 81% to 87% survival for restricted animals. Because of the lower mortality rate of the females, a comparison between the unrestricted and the restricted groups cannot be made. However, the data given in the next sections on incidence and severity of lesions of the major diseases and on ir

TABLE 1

Mortality and survival rates of rats receiving unrestricted and restricted food intake

Age range	Unrestricted (50 males, 50 females)			33% Restricted (48 males, 48 females)			46% Restricted (79 males, 39 females)		
	No. deaths	Probability of death[1]	Surviving	No. deaths	Probability of death[1]	Surviving	No. deaths	Probability of death[1]	Surviving
days		*P*	%		*P*	%		*P*	%
				Males					
200–299	0	0	100	0	0	100	0	0	100
300–399	0	0	100	1	0.021	98	0	0	100
400–499	0	0	100	0	0	98	2	0.025	97
500–599	1	0.020	98	2	0.043	94	1	0.013	96
600–699	11	0.224	76	1	0.022	92	4	0.053	91
600–699	11	0.224	76	1	0.045	87	8	0.111	81
				Females					
400–499	0	0	100	0	0	100	0	0	100
500–599	0	0	100	1	0.021	98	2	0.051	95
600–699	1	0.020	98	0	0	98	0	0	95
700–799	2	0.041	94	1	0.021	96	0	0	95

[1] P is the probability of death within a 100-day period among the rats alive at the beginning of the period.

TABLE 2

Mortality and survival rates of unrestricted rats caged either together or singly[1]

Age range	Single (25 males, 25 females)			3 or 4 in a cage (25 males, 25 females)		
	No. deaths	Probability of death	Surviving	No. deaths	Probability of death	Surviving
days		*P*	%		*P*	%
			Males			
400–499	0	0	100	0	0	100
500–599	0	0	100	1	0.040	96
600–699	5	0.200	80	6	0.250	72
700–799	9	0.450	44	5	0.278	52
			Females			
500–599	0	0	100	0	0	100
600–699	0	0	100	1	0.040	96
700–799	1	0.040	96	1	0.042	92

[1] Mesh windows in cage sides allowed communication between neighboring rats.

cidence of tumors indicate that life expectancy will be greater for restricted than for unrestricted females observed for longer periods. Included in table 1 are 24 females on 33% food restriction which were used for fertility studies, all surviving to 800 days; also 20 females and 26 males on 46% restriction spared for lifespan studies.

There were no significant differences between the mortality rates of unrestricted rats kept singly or together in cages (table 2).

Incidence of major diseases excluding tumors

Incidence of lesions in unrestricted and restricted rats is given in tables 3A and 3B. Included in the unrestricted groups are data obtained from 37 males caged singly and 22 kept together (three or 4 in a cage), and from 38 females caged singly and 25 kept together (three or 4 in a cage). The pathological findings under both types of caging were essentially the same.

Due to the small number of rats which were moribund or dead before the end of the experimental period (in all groups except the unrestricted males) comparative results with different levels of food intake were not significant below 800 days of age. Data on the 24 females with 33% restriction which were used for fertility studies were not included because food

TABLE 3A

Incidence of microscopically observed lesions of three major diseases occurring separately or in combination in unrestricted and restricted rats (males)

Mean age	No. of rats	No. with single lesions			No. with multiple lesions				Total with lesions	
		G[1]	P[2]	M[3]	G + P	G + M	P + M	G + P + M	No.	Per cent
days										
					Unrestricted					
670	13	0	0	0	3	2	0	7	12	92
747	15	1	0	0	4	2	0	8	15	100
763[4]	7	0	0	0	2	0	0	5	7	100
809[4]	24	0	0	0	1	9	0	14	24	100
					33% Restricted					
394	1	0	0	0	0	0	0	0	0	0
536	2	0	0	0	0	0	0	0	0	0
642	1	0	0	0	0	0	0	0	0	0
780	2	0	0	1	0	0	0	0	1	—
809[4]	42	8	5	7	2	5	0	0	27	64
					46% Restricted					
422	2	0	0	0	0	0	0	0	0	0
518	1	0	0	0	0	0	0	0	0	0
629	4	0	0	0	0	0	0	0	0	0
761	8	1	0	0	0	0	0	0	1	13
818[4]	38	0	0	8	0	0	1	0	9	24

[1] Glomerulonephritis.

[2] Periarteritis.

[3] Myocardial degeneration.

[4] Survivors to this age. Other rats were moribund or died.

TABLE 3B

Incidence of microscopically observed lesions of three major diseases occurring separately or in combination in unrestricted and restricted rats (females)

Mean age	No. of rats	No. with single lesions			No. with multiple lesions				Total with lesions	
		G[1]	P[2]	M[3]	G + P	G + M	P + M	G + P + M	No.	Per cent
days										
					Unrestricted					
680	1	0	0	0	0	0	0	1	1	—
746	4	1	0	0	1	0	0	2	4	—
764[4]	11	3	1	0	0	0	0	3	7	64
826[4]	47	9	0	0	7	6	0	5	27	57
					33% Restricted					
506	1	0	0	0	0	0	0	0	0	0
770	1	0	0	0	0	0	0	0	0	0
822[4]	22	0	0	1	0	0	0	0	1	5
					46% Restricted					
589	2	0	0	0	0	0	0	0	0	0
821[4]	17	0	0	0	0	0	0	0	0	0

[1] Glomerulonephritis.

[2] Periarteritis.

[3] Myocardial degeneration.

[4] Survivors to this age. Other rats were moribund or died.

TABLE 4A

Total incidence of microscopically observed lesions of three major diseases in unrestricted and restricted rats (males)

Mean age	No. of rats	Glomerulonephritis		Periarteritis		Myocardial degeneration	
		Total with lesions		Total with lesions		Total with lesions	
		No.	Per cent	No.	Per cent	No.	Per cent
days							
			Unrestricted				
670	13	12	92	10	77	9	70
747	15	15	100	12	80	10	68
763	7	7	100	7	100	5	71
809	24	24	100	15	63	23	96
			33% Restricted				
394	1	0	—	0	—	0	—
536	2	0	—	0	—	0	—
642	1	0	—	0	—	0	—
780	2	0	—	0	—	1	—
809	42	15	36	7	17	12	28
			46% Restricted				
422	2	0	—	0	—	0	—
518	1	0	—	0	—	0	—
629	4	0	—	0	—	0	—
761	8	1	13	0	—	0	—
818	38	0	—	1	3	9	24

TABLE 4B

Total incidence of microscopically observed lesions of three major diseases in unrestricted and restricted rats (females)

Mean age	No. of rats	Glomerulonephritis		Periarteritis		Myocardial degeneration	
		Total with lesions		Total with lesions		Total with lesions	
		No.	Per cent	No.	Per cent	No.	Per cent
days							
			Unrestricted				
680	1	1	—	1	—	1	—
746	4	4	—	3	—	2	—
764	11	6	55	4	36	3	27
826	47	27	57	12	24	11	23
			33% Restricted				
506	1	0	—	0	—	0	—
770	1	0	—	0	—	0	—
822	22	0	—	0	—	1	5
			46% Restricted				
589	2	0	—	0	—	0	—
821	17	0	—	0	—	0	—

TABLE 5A

Severity of microscopically observed lesions of three major diseases in unrestricted and restricted rats (male)

Mean age	No. of rats	Glomerulonephritis				Periarteritis				Myocardial degeneration			
		0[1]	E	M	S	0	E	M	S	0	E	M	S
days													
						Unrestricted							
670	13	1	0	0	12	3	0	0	10	4	0	3	6
747	15	0	0	1	14	3	0	0	12	5	1	4	5
763	7	0	0	2	5	0	1	1	5	2	1	1	3
809	24	0	1	9	14	9	1	3	11	1	2	10	11
Total	59	1	1	12	45	15	2	4	38	12	4	18	25
						33% Restricted							
394	1	1	0	0	0	1	0	0	0	1	0	0	0
536	2	2	0	0	0	2	0	0	0	2	0	0	0
642	1	1	0	0	0	1	0	0	0	1	0	0	0
780	2	2	0	0	0	2	0	0	0	1	1	0	0
809	42	27	11	4	0	35	2	4	1	30	4	2	6
Total	48	33	11	4	0	41	2	4	1	35	5	2	6
						46% Restricted							
422	2	2	0	0	0	2	0	0	0	2	0	0	0
518	1	1	0	0	0	1	0	0	0	1	0	0	0
629	4	4	0	0	0	4	0	0	0	4	0	0	0
761	8	7	1	0	0	8	0	0	0	8	0	0	0
818	38	38	0	0	0	37	0	1	0	29	5	4	0
Total	53	52	1	0	0	52	0	1	0	44	5	4	0

[1] Zero = no lesions, E = "early" (slight) lesions, M = moderate, S = severe.

TABLE 5B

Severity of microscopically observed lesions of three major diseases in unrestricted and restricted rats (female)

Mean age	No. of rats	Glomerulonephritis				Periarteritis				Myocardial degeneration			
		0[1]	E	M	S	0	E	M	S	0	E	M	S
days													
						Unrestricted							
680	1	0	0	0	1	0	0	0	1	0	0	1	0
746	4	0	0	1	3	1	0	1	2	2	1	1	0
764	11	5	2	1	3	7	0	1	3	8	1	2	0
826	47	20	7	8	12	35	0	2	10	36	3	3	5
Total	63	25	9	10	19	43	0	4	16	46	5	7	5
						33% Restricted							
506	1	1	0	0	0	1	0	0	0	1	0	0	0
770	1	1	0	0	0	1	0	0	0	1	0	0	0
822	22	22	0	0	0	22	0	0	0	21	0	1	0
Total	24	24	0	0	0	24	0	0	0	23	0	1	0
						46% Restricted							
589	2	2	0	0	0	2	0	0	0	2	0	0	0
821	17	17	0	0	0	17	0	0	0	17	0	0	0
Total	19	19	0	0	0	19	0	0	0	19	0	0	0

[1] Zero = no lesions, E = "early" (slight) lesions, M = moderate, S = severe.

restriction was stopped at the time of mating.

A high percentage of those unrestricted rats of both sexes which became moribund or died before 800 days had lesions of moderate or marked severity, whereas the few restricted animals examined at corresponding ages were practically free from major diseases.

Comparative incidence of diseases occurring separately or in combination in rats surviving to the end of experimental period (of 809 days to 826 days) showed significant differences between unrestricted and restricted animals (tables 3A and 3B). In males, frequency of lesions in unrestricted rats was 100% as compared with 64% incidence for the 33% -restricted rats. The P value for the difference was < 0.005. On 46% restriction, incidence was reduced to 24%, a clearly significant lowering from ad libitum incidence.

Though more of the females, unrestricted and restricted, survived this experimental period, the difference in percentage of lesions in the two groups was striking. Except for a single rat with myocardial changes, all female survivors on 33 or 46% restriction were free from disease, while 57% of the unrestricted ones had lesions.

At all levels of food intake, incidence of lesions in males was much higher than in females. This accounted for the greater survival rate of females. In both sexes there was a graded relation between the incidence of disease and level of food intake. On 33% restriction, frequency of lesions in males (64%) was about the same as in unrestricted females (57%). With 46% restriction the percentage was reduced to 24% in males.

Data on the total incidence of lesions of the three major diseases are presented in tables 4A and 4B. The findings parallel those recorded in tables 3A and 3B for combined incidence and show a progressive decrease with reduction of food

TABLE 6

Incidence of tumors occurring separately or in combination in unrestricted and restricted rats

Total no. of rats		Number of rats with tumors					Total number with tumors			
		Single tumors		Multiple tumors (usually two)						
Moribund or died before 800 days	Killed at 760 to 833 days	Benign	Malignant	Benign	Benign and malignant	Malignant	Benign	Malignant	Both	Per cent
				Unrestricted males						
28		17	2	1	0	1	18	3	21	75
	31	15	0	3	0	0	18	0	18	58
				33% Restricted males						
6		1	2	1	0	0	2	2	4	67
	42	13	0	1	1	0	15	1	15	36
				46% Restricted males						
15		7	3	2	0	0	9	3	12	80
	38	8	2	0	0	0	8	2	10	26
				Unrestricted females						
5		1	0	1	1	0	3	1	3	60
	58	19	1	5	0	0	24	1	25	43
				33% Restricted females						
2		0	1	0	0	0	0	1	1	—
	22	1	1	1	0	0	2	1	3	14
				46% Restricted females						
2		0	1	0	0	0	0	1	1	—
	17	2	0	0	0	0	2	0	2	12

intake. The percentage of renal lesions was greater than the percentage of vascular or myocardial lesions in unrestricted rats of both sexes.

Severity of lesions and onset of disease. In tables 5A and 5B lesions of the three major diseases are classified according to severity. The overall incidence of lesions of moderate or marked severity in unrestricted rats was about three times higher in males than in females, while the incidence in restricted rats of both sexes was much lower than in their unrestricted counterparts. Delay in onset of disease in restricted rats was shown by the low incidence of moderate or severe lesions at all ages as compared with the much higher frequency in unrestricted animals. The majority of lesions in restricted rats were early.

While the incidence of lesions in 33% restricted males was about the same as in unrestricted females, the degree of severity was lower in males. (tables 3 and 5).

Incidence of tumors. Table 6 gives the incidence of benign and malignant tumors in unrestricted and restricted rats. The data on the animals killed between 760 and 833 days were adequate for statistical analysis. In both sexes the tumor incidence was significantly lower in the restricted rats (33 and 46% combined) than in the unrestricted ones. In males, the P value for the difference between the two groups was significant at about the 1% level; in females the P value was well below 1%.

In both unrestricted and restricted males, the per cent incidence of total tumors, benign and malignant, was greater in rats moribund or dead before 800 days than in those which were killed between 760 and 833 days of age.

DISCUSSION

It is evident from these observations that maximum body size was not optimum in relation to the onset of disease and to longevity. With food intake adjusted at levels that had relatively little retarding effect on growth and prevented accumulation of excess fat (Berg, '60), the latent period preceding the onset of lesions was extended and longevity was increased. Delay in onset of lesions by dietary restriction points to a metabolic factor that influences the susceptibility of aging tissues to disease and to certain tumors. Tannenbaum and Silverstone ('53) reported a similar inhibitory effect of limited food intake on tumor development in mice.

Since food restriction was started at weaning and resulted in some retardation of skeletal growth, it would be of interest to determine whether the delaying effects on onset of disease would be the same if restriction were postponed until rats were about 170 days of age, when they have reached nearly maximum skeletal size (Berg and Harmison '57).

Hormonal influences appear to play a role not only in metabolism and growth, but also in onset of disease. In table 1 it will be noted that the 33% restricted male rats had about the same probability of death (and survival) as the unrestricted females of the same age. Both these groups received the same weight of food per rat—but since the males were heavier they received less food per 100 gm of body weight. Hence longevity appears to be affected both by food intake and by sex hormones.

Though the major diseases leading to death in the rat and in man differ in nature, the tissues affected by lesions were the same. In both species the blood vessels, heart and kidneys are involved and hypertension is a frequent complication. Also in man there is considerable evidence that overweight and obesity predisposes to shortened life expectancy and to the development of cardiovascular, renal disorders and hypertension (Armstrong et al., '51; Marks, '56; Society of Actuaries, '59). Another similarity is the greater longevity of females than of males. This is true of most species.

The observation that growth, body weight, and survival rates were essentially the same for unrestricted rats caged singly or together is important for laboratories with limited space for housing rats.

It should be noted that McCay's restricted rats ('43) were immature and had an extended life span. Our results show that the extended life span of restricted

rats is not related to immaturity since under our conditions the life span was definitely extended, but there was no evidence of immaturity. According to McCay's survival rate curves ('43), at 800 days, less than 10% of male rats on unrestricted diet and about 20% of females were alive. With underfeeding, almost 65% of males and 75% of females survived to this age. In our experiments the longevity of both unrestricted and restricted rats was greater. At 800 days, 48% of male rats fed ad libitum and 94% of females were alive, whereas 81% to 87% of males on restricted intake and 95% of females survived. The shorter survival rate of McCay's rats was probably due to lung infection.

SUMMARY

On ad libitum feeding, rats attained large skeletal size and developed obesity. When food intake was restricted by 33 or 46%, levels which prevented fat accumulation and had little retarding effect on skeletal growth, longevity was extended and onset of disease was delayed. At 800 days of age only 48% of unrestricted male rats were alive as compared with 81 to 87% survival for restricted animals. Because most of the females remained alive at this age, a comparison between unrestricted and restricted animals could not be made.

Comparative incidence of tumors and of cardiac, renal and vascular lesions at 800 days of age showed significant differences between unrestricted and restricted animals. In males frequency of lesions in unrestricted rats was 100% as compared with 64% incidence for the 33%-restricted rats and 24% for the 46%-restricted rats. Though more of the females, unrestricted and restricted, survived this experimental period, the difference in percentage of lesions in the two groups of females was striking. All of the females surviving to 800 days on restricted food intake were free from disease, while 57% of the unrestricted ones had lesions. The lower incidence and lesser severity of lesions in restricted females at 800 days indicated that life expectancy would be greater in restricted than in unrestricted females observed for longer periods.

Optimum weight and skeletal size in relation to disease and life expectancy were below maximum measurements. Also, hormonal as well as dietary factors seem to play a role in the greater longevity of the female as compared with the male. The extended longevity of the restricted rats was not associated with immaturity.

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Changes in the Fatty Acid Composition of the Depot Fat of Mice Induced by Feeding Oleate and Linoleate[1]

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It had been observed (Tove and Smith, '59) that even though the level of linoleic acid in the depot fat of mice varied from 5 to 50%, the level of the total unsaturated fatty acids remained constant. Constancy of unsaturated fatty acids was also observed by Sinclair ('35), Reiser ('51) and Tove et al. ('54). In all of these investigations the monoenoic acids were determined together; and the question remained whether the mono-unsaturated acid that changed was palmitoleic acid, oleic acid or both. In this connection, Mead ('57) found an increase in the level of palmitoleic acid in a fat-deficient rat. The recent development of gas chromatography as an analytical tool for the methyl esters of the fatty acids (James and Martin, '56; Orr and Callen, '58; Lipsky and Landowne, '58) enabled the rapid determination of each of the fatty acids in the depot fat as the linoleic acid level varied. The results of the experiments with linoleic acid prompted an extension of the investigation of the changes in fatty acid composition of the depot fat when the oleic acid level was increased by dietary means. The results of these studies are reported herein.

EXPERIMENTAL

Analysis of fatty acids. Mouse depot fat isolated as described previously (Tove and Smith, '58) was saponified with 10% alcoholic potassium hydroxide. After extraction of the non-saponifiables the fatty acids were extracted from the acidified aqueous medium with ether. The ether solution was dried with anhydrous sodium sulfate and fatty acids converted to their methyl esters by the addition of diazomethane (Arndt, '43). After evaporation of the solvent and excess diazomethane, an aliquot of the methyl esters was injected into a gas chromatography apparatus.

The apparatus consisted of an oven made of a three-foot length of three-inch steel pipe wrapped with asbestos paper around which was coiled two parallel strands of resistance wire. In the oven was inserted a Gow-Mac[2] thermal conductivity cell containing 4 tungsten filaments wired in a Wheatstone bridge. The imbalance of the bridge, i.e., amount of organic vapor in the effluent gas stream, was measured by a recording potentiometer. The sample was injected through a silicone rubber diaphragm into a small stainless steel injection port heated by a 20-watt heater made of resistance wire. The column, attached to the injection port and thermal conductivity cell, was made of 12 feet of ¼-inch copper tubing. The column packing was a mixture of celite 545[3] (70%) and succinate-ethylene glycol polyester (30%). Prior to use, the celite was size-graded by repeated suspension in a two-liter cylinder of water, discarding any material that failed to settle in three minutes, until all the celite settled within the three-minute period. The graded celite

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[2] Gow-Mac Instrument Company, 100 Kings Row, Madison, New Jersey.
[3] Celite 545, Johns-Manville Products Corporation, New York.

was ashed at 300°C for three hours; then washed with hot 7% hydrochloric acid, water, 10% methanolic potassium hydroxide and finally water. The succinic acid-ethylene glycol polyester was prepared by heating, with an electric heating mantle, a mole of succinic acid and a mole of ethylene glycol with 0.1 gm toluene sulfonic acid under a vacuum of 0.25 to 0.5 mm mercury until the reaction ceased. The polyester was dissolved in acetone and mixed with the celite on a steam bath with continuous stirring until the solvent evaporated. The column packing material was packed into the copper tubing by vibration after which it was folded to fit into the oven.

The column was operated at a temperature of 190°C with a helium flow rate of 100 ml per minute. Under these conditions the complete resolution of all of the fatty acids through arachidonic acid was obtained in less than 30 minutes.

The percentage of each acid was computed from the ratio of the area of a given peak on the chromatogram to the sum of the areas occupied by all the peaks. The areas of the peaks were obtained by triangulation (Keulemans, '57).

Because of the small amounts of linolenic and arachidonic acids present in depot fat, these acids appeared as very low broad peaks on the chromatogram, making measurement of the areas of these peaks impractical. The levels of these acids were estimated, therefore, by means of alkaline isomerization (Brice et al., '52).

Animal procedure. Mice obtained from the North Carolina Laboratory of Hygiene were housed individually and fed, ad libitum, the egg albumin-starch purified diet[4] described previously (Tove and Smith, '58). Graded levels of either safflower oil,[5] olive oil, oleic acid, or glycerol mono-oleate were added at the expense of starch. Except for the olive oil experiment in which adult male mice were used, weanling male mice were used in these studies. The fatty acid composition (table 1) of each of the oils fed was determined by gas chromatography.

RESULTS

Influence of linoleic acid on fatty acid composition of depot fat. Samples of depot fat from a group of male mice and a female group, used in the previous experiments (Tove and Smith, '59) on the kinetics of the decline of linoleic acid from the depot fat, were subjected to fatty acid analysis by gas chromatography. In the 12 samples from the female mice the linoleic acid levels ranged frm 2.5% to 12%, and in the 24 samples from the male mice the linoleic acid varied from 8 to 48%. The levels of the various fatty acids were adjusted for the wide divergence in the per cent linoleic acid by computing the ratio of the percentage of a given fatty acid to the per cent of all the fatty acids minus the per cent linoleic acid.

This ratio, expressed as relative per cent, was graphed for each acid against the per cent linoleic acid. The regression coefficients of these data were computed by least squares (Snedecor, '56). If the regression coefficient for a particular acid was not significantly different from zero ($P < 0.05$), then it was assumed that the level of the fatty acid was independent of the linoleic acid level. The values for the regression coefficients are given in table 2.

[4] We wish to acknowledge our gratitude to Merck Sharp and Dohme, Rahway, New Jersey, for the vitamins used in the diets.

[5] Supplied through the courtesy of the Pacific Vegetable Oil Corporation, San Francisco.

TABLE 1

Fatty acid composition of the oils fed

Oil fed	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic
	%	%	%	%	%	%
Safflower oil	—	7.7	—	3.9	13.6	74.8
Olive oil	—	16.1	3.2	1.5	64.4	14.8
Glycerol mono-oleate	3.5	4.2	8.0	4.1[1]	74.2	4.7
Oleic acid	4.6	4.7	13.1	2.1[1]	70.7	2.4

[1] Hydrogenation showed the stearic peak to be almost entirely a 17-carbon unsaturated acid.

TABLE 2

Relative changes in fatty acids of depot fat with increasing concentrations of linoleic acid expressed as regression coefficients

Group	No. mice	Myristic	Palmitic	Palmitoleic	Stearic	Oleic
		Regression coefficients and standard error				
Decline, males	24	−0.016 ± 0.010	0.108 ± 0.049[1]	−0.283 ± 0.043[2]	0.118 ± 0.018[2]	0.117 ± 0.059
Decline, females	12	0.012 ± 0.034	0.786 ± 0.246[2]	−0.619 ± 0.214[2]	0.269 ± 0.034[2]	−0.427 ± 0.433
Diet SO[3] (< 45% linoleic)	15	−0.004 ± 0.014	0.202 ± 0.108	−0.252 ± 0.082[2]	0.153 ± 0.043[2]	−0.109 ± 0.154
Diet SO (> 45% linoleic)	15	−0.101 ± 0.026[2]	−0.372 ± 0.113[2]	−0.214 ± 0.051[2]	0.394 ± 0.120[2]	0.292 ± 0.153

[1] Slope significantly ($P < 0.05$) different from 0.
[2] Slope highly significantly ($P < 0.01$) different from 0.
[3] SO, safflower oil.

The most striking feature of the data on linoleic acid is that only one acid, palmitoleic acid, showed a relative decrease with an increase in the depot fat level of linoleic acid. The relative levels of palmitic acid and stearic acid increased whereas myristic and oleic acids changed only randomly as the depot fat level of linoleic acid increased. The same pattern of fatty acid change is observed with both the male and female mice, although the magnitude of the regression coefficients is greater for the females.

To further examine the effect of increased depot fat levels of linoleic acid on the distribution of the other fatty acids of the depot fat, a group of male mice was fed safflower oil for 4 weeks. The dietary levels ranged from 1 to 50%. The mean fatty acid composition of the depot fats is shown in table 3 (diet SO). As expected, an increase in dietary linoleic acid resulted in an increase in the depot fat level of this acid and a decrease in the levels of the major acids of the depot fat. Linolenic and arachidonic acids tended to increase with linoleic acid. The weight of depot fat (table 3), although variable, did not exhibit any correlation with dietary treatment, thus showing that there was an actual replacement of fatty acids synthesized from carbohydrate by dietary linoleic acid. When the depot fat level of linoleic acid was plotted against the dietary level (fig. 1), two rates of incorporation were evident with a transition point occurring when the linoleic acid in the depot fat reached about 45%.

If the relative per cent values for the other fatty acids other than linoleic acid are computed, two patterns of change corresponding to the two rates of linoleate deposition are observed (fig. 2). As the depot fat increased to a level of 45%, only palmitoleic acid showed a significant decrease, whereas stearic acid increased, and myristic acid, oleic acid and palmitic acid showed only random changes (table 2 and fig. 2). As the depot fat level of linoleic acid increased beyond 45%, however, myristic acid and palmitic acid accompanied palmitoleic acid in the decrease, whereas the stearate level increased at an increased rate (table 2 and fig. 2). It should be emphasized that al-

TABLE 3

Fatty acid composition of depot fat

Diet	No. mice	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic	Arachidonic	Weight of fat
		%	%	%	%	%	%	%	%	*gm*
1% SO[1]	4	2.9	27.7	13.2	3.7	33.5	19.1	0.5	0.9	1.71
5% SO	6	2.2	24.8	7.4	5.1	33.8	26.6	0.5	0.9	1.16
10% SO	4	2.0	23.9	6.3	4.2	24.0	39.6	0.6	1.3	1.90
20% SO	3	1.4	17.7	3.5	4.1	22.4	51.0	0.6	1.0	2.02
30% SO	5	1.4	15.2	2.8	5.3	25.2	47.2	0.7	1.5	0.74
40% SO	4	0.8	13.3	2.1	4.1	22.1	57.6	0.8	1.0	2.68
50% SO	4	0.6	11.1	1.6	5.4	19.7	61.6	0.8	1.5	1.22
0	4	2.6	25.0	15.8	4.0	52.1	0.5	0.1	0.7	1.42
5% Oleic acid	4	3.3	22.1	16.5	2.5	54.4	1.0	0.3	0.6	1.91
10% Oleic acid	3	3.4	19.8	17.9	1.7	56.4	1.5	0.4	0.7	2.43
15% Oleic acid	3	3.1	15.6	12.1	2.4	65.9	1.7	0.3	0.9	1.48
20% Oleic acid	3	3.2	14.3	12.9	1.8	64.8	2.0	0.4	1.2	1.65
0	6	2.1	26.3	13.9	3.0	49.2	5.5	0.4	1.0	2.91
1% GMO[2]	6	2.4	26.7	9.9	4.1	46.0	10.8	0.7	1.3	1.72
5% GMO	5	2.4	20.2	9.6	3.6	58.6	5.6	0.5	1.6	1.00
10% GMO	6	2.7	22.1	8.9	4.1	54.2	7.9	0.7	1.1	1.34
20% GMO	5	2.2	18.6	7.1	3.5	59.2	9.5	0.7	1.7	1.99
30% GMO	6	1.8	13.1	6.8	3.3	66.6	8.4	0.6	0.8	2.04
40% GMO	6	1.8	13.7	6.2	2.7	66.4	9.1	0.5	0.8	2.04
50% GMO	6	1.7	11.3	6.5	2.8	68.9	8.8	0.5	0.6	2.86
10% Olive oil	6	1.3	17.3	6.9	3.1	62.1	9.3	0.4	1.9	1.96
20% Olive oil	7	1.1	17.2	5.5	2.4	63.1	10.8	0.5	0.9	2.70
30% Olive oil	7	1.0	15.4	5.4	2.2	65.0	11.0	0.4	1.5	2.32
40% Olive oil	6	0.7	15.0	5.3	2.3	65.1	11.6	0.4	1.0	2.86
50% Olive oil	7	0.6	15.4	4.8	2.2	65.1	11.8	0.4	1.0	2.56

[1] SO, safflower oil.

[2] GMO, glycerol mono-oleate.

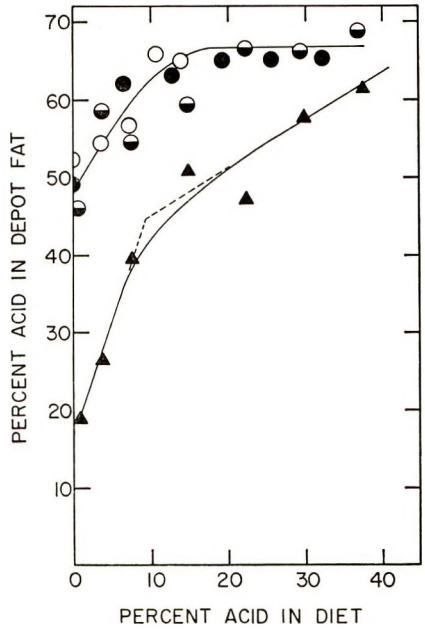

Fig. 1 Effect of dietary level of linoleate and oleate on their level in depot fat; ▲, linoleic acid, safflower oil fed; ○, oleic acid, oleic acid fed; ◒, oleic acid, glycrol mono-oleate fed; ●, oleic acid, olive oil fed. Dashed line is an extrapolation of the two slopes.

though strong trends for oleate are seen in each of the 4 cases listed in table 2, as evidenced by the large values of the regression coefficients, the individual variation is great and there is no consistency to the trend.

Influence of oleic acid on fatty acid composition of depot fat. The results with the linoleic acid experiments prompted an investigation of the effects of increased levels of oleic acid in the depot fat on the distribution of the other acids. Three dietary sources of oleic acid were chosen: oleic acid, glycerol mono-oleate and olive oil. The effect of dietary oleate on the level of oleic acid in the depot fat is shown in figure 1. A level of about 67% of oleic acid in the depot fat was reached when the dietary percentage was about 15%, and this depot fat level was not exceeded even though the diet contained as much as 37% of oleic acid.

The mean fatty acid compositions of the depot fat of the groups of mice fed the various oleate levels are shown in table 3. As with the linoleate-fed mice the levels of myristic, palmitic, palmitoleic and stearic acids decreased as the level of the dietary oleic acid increased. Moreover, since the weight of fat was not influenced by dietary treatment, the changes observed in the different fatty acid levels are the result of replacement of these fatty acids by oleate and not by dilution from dietary oleate.

The relative per cent values for myristic, palmitic, palmitoleic, stearic and linoleic acids were computed, and the regression coefficients of their change as the level of oleate in the depot fat increased are shown in table 4. With the mice fed oleic acid and glycerol mono-oleate, the pattern is completely different from the pattern of change observed with linoleate (table 2). With the two oleate-fed groups the only acid to show a relative decrease was palmitic acid. Myristic acid increased whereas palmitoleic acid showed only random changes. The increase in linoleate undoubtedly resulted from the contamination of the dietary fat with this acid (table 1). Only random changes were observed in the relative level of stearate. However the stearate peaks in the chromatograms of the depot fats of these animals were distorted by the presence of a C-17 unsaturated acid that was also in the dietary fats, and thus the nature of the change in the level of stearate in the depot fat is uncertain.

The pattern of change in the relative percentage of the fatty acids of the mice fed olive oil is different from the other oleate-fed groups. In fact the pattern resembles that of the linoleate group in that palmitoleate decreased. The olive oil group was different from the others in that adult rather than weanling mice were the experimental subjects. Therefore, the age difference might account for the discrepancy in fatty acid change. A more plausible reason, perhaps, lies in the relatively high level of linoleic acid in the olive oil (table 1). Even though the oleate was the major acid in the dietary oil, the linoleate therein probably played a dominant role in the regulation of the deposition of the non-dietary fatty acids. Additional evidence for this view arises from the fact that the slope of the change

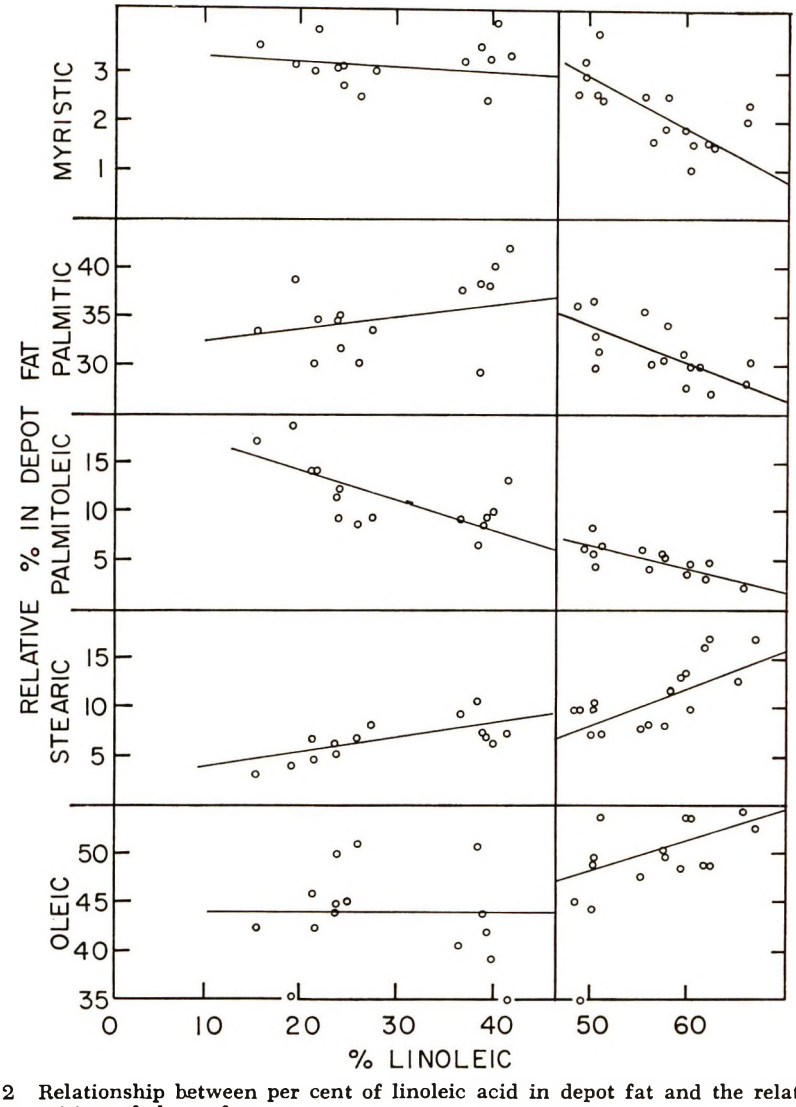

Fig. 2 Relationship between per cent of linoleic acid in depot fat and the relative fatty acid composition of depot fat.

in relative per cent linoleic acid with oleic acid was greater than unity.

DISCUSSION

The suggestion brought out in previous work (Tove and Smith, '59) that the deposition of fatty acids is not the result of a completely random process but is rather a regulated process, is certainly borne out by the data presented herein. When the depot fat level of linoleate increased, there was a relative decrease in only palmitoleic acid; there was a strong relative increase in stearate and in two of the three experiments, a relative increase in palmitate. When oleate was increased, an entirely different picture was obtained. Palmitic acid was the only acid that showed a relative decrease while there was a relative increase in myristic acid.

It is apparent that the previously observed decrease in monoenoic acids counterbalancing the increase in linoleic acid (Tove and Smith, '59) was the result of two factors: first, dilution of the oleate accompanied by an increase in stearate and palmitate; and second, an actual decrease in the relative amount of palmit-

TABLE 4

Relative changes in fatty acids of depot fat with increasing concentrations of oleic acid expressed as regression coefficients

Fat fed	No. mice	Myristic	Palmitic	Palmitoleic	Stearic	Linoleic
		Regression coefficients and standard error				
Oleic acid	17	0.235 ± 0.043[2]	−0.438 ± 0.106[2]	0.177 ± 0.154	−0.003 ± 0.069	0.249 ± 0.112[1]
Glycerol mono-oleate	40	0.034 ± 0.016[2]	−0.457 ± 0.072[2]	0.067 ± 0.058	0.085 ± 0.035	0.216 ± 0.099[1]
Olive oil	33	−0.239 ± 0.032[2]	−0.718 ± 0.884	−0.854 ± 0.228[2]	0.202 ± 0.190	1.624 ± 0.270[2]

[1] Slope significantly ($P < 0.05$) different from 0.
[2] Slope highly significantly ($P < 0.01$) different from 0.

oleic acid deposited. An increase in stearate in the depot fat of swine fed cottonseed oil was observed by Ellis et al. ('31). The results with linoleic acid confirm and extend the observations of Mead ('57) who found that palmitoleic acid comprised 4.3% of the total fatty acids of normal rats but 11% of the total fatty acids from fat-deficient rats. The similarity of physical properties between palmitoleic and linoleic acids suggested that there was a tendency on the part of the animal to maintain the desirable properties of the body lipids of an animal ingesting linoleic acid (Mead, '57).

The only fatty acids observed to decrease when the depot fat levels of linoleate and oleate increased were those with the same physical properties; i.e., palmitoleic and palmitic acids respectively. There was, in addition, an increase in a different saturated acid accompanying the increase in linoleate and oleate. Thus, the effect of increased depot fat levels of linoleate and oleate on the distribution of the other fatty acids (tables 2, 4) indicates that the distribution of fatty acids in the depot fat is adjusted such that there is a tendency to maintain a homeostasis of the physical properties of the depot fat.

That a characteristic pattern of fatty acid change is associated with the deposition of a given fatty acid leads one to speculate that the mechanism for the specificity of the process should be in specificity of the enzymes involved in fatty acid deposition. One enzyme system having a high degree of specificity for linoleic acid and palmitoleic acid, and another having a high degree of specificity for oleic acid and palmitic acid, would account for the specific decrease in palmitoleate or palmitate with increases in linoleate or oleate respectively. With such systems the presence of one member of a pair (e.g., linoleic acid) would competitively inhibit the deposition of the other (e.g., palmitoleic acid). Among the possible metabolic sites of such specificity would be: (a) the formation of the fatty acyl-CoA, (b) the formation of the phosphatidic acid from α-glycerol phosphate and the fatty acyl-CoA (Kornberg and Pricer, '53) or (c) the formation of the

triglyceride from a diglyceride and a fatty acyl-CoA (Weiss and Kennedy, '56). It should be emphasized that with the latter two systems inhibitory action could arise from the nature of the mono- or diglyceride as well as the fatty acyl-CoA. Although Kornberg and Pricer ('53) examined the fatty acyl-CoA specificity of their system, the enzyme source was a particulate fraction; and the presence of multiple enzymes with differing specificity is not precluded by their work. Similarly the particulate system of Weiss and Kennedy ('56) could also contain multiple enzymes with similar function.

In any consideration of fatty acid specificity the position of the fatty acid in the triglyceride must be taken into account. That such specificity does occur is clearly established by the work of Savary et al. ('57) and Mattson and Lutton ('58). Studies on the specificity of fatty acid deposition in each of the three positions of the triglyceride are in progress.

The inability to increase the oleic acid level of the depot fat above 67% irrespective of the amount fed (fig. 1) is suggestive that the system tends to resist forming glycerides containing more than two oleic acid moieties. Similarly the change in the rate of linoleate deposition and the associated shift in the pattern of the fatty acid distribution (figs. 1, 2) is also suggestive of different specificity patterns associated with different position in the triglyceride.

SUMMARY

When elevated depot fat levels of linoleate or oleate were produced by dietary means, specific patterns of fatty acid replacement were observed. As the linoleate level in the depot fat increased to about 45%, palmitoleic acid was the only acid to decrease relative to the other acids; and there was a relative increase in stearate. As the linoleate level increased above 45%, palmitic, palmitoleic and myristic acids decreased and stearic acid increased. As the level of oleate in the depot fat increased, palmitic acid was the only acid that decreased relative to the other acids; and there was a relative increase in myristic acid. When the diet contained 15% of oleic acid, the level of oleate in the depot fat reached 67%. This depot fat level was not exceeded even though the dietary content of oleate was more than doubled. These results indicate that the deposition of fatty acids in the depot fat is a regulated process with a marked specificity of substitution of one acid for another, and suggests that the specificity may extend to the different positions of the glycerol moiety of the triglyceride.

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The Effect of Thiamine Deficiency on the Activity of Erythrocyte Hemolysate Transketolase[1]

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With the demonstration that methylene blue specifically activated the pentose phosphate pathway in mammalian erythrocytes,[2] (Brin and Yonemoto, '58), it became feasible to study this pathway relatively free from other interfering oxidative reactions. Of interest to nutritionists was the fact that one of the enzymes in the system required thiamine pyrophosphate (TPP) as a cofactor, (Racker et al., '53). By using radioactive glucose, further studies revealed that the cyclic activity of this group of reactions was markedly depressed in thiamine deficiency (Brin et al., '58).[3] The data which were accumulated with red cells from rat and human blood work, supported that observation (Brin et al., '58; Wolfe et al., '58). The unique availability of an enzyme system which required thiamine as a cofactor and which might be sampled and assayed without detriment to the host led to its consideration as a functional test for vitamin status, and it was then applied successfully to a recognized problem in human thiamine nutrition (Wolfe et al., '58).

Despite the applicability and sensitivity of the method for thiamine deficiency (Brin et al., '58),[4] the procedure was not particularly suitable for routine evaluation of thiamine status. Described in this communication are studies directed toward modifying the procedure so that it is more readily applicable to nutritional studies.

BIOCHEMICAL BASIS

The metabolic cycle in the intact cell upon which the original method was based is shown diagrammatically in figure 1. In summary, extracellular glucose is converted to intracellular glucose-6-phosphate. It is then oxidized to pentose phosphate (in the presence of fresh, intact red cells plus methylene blue) with the release of its aldehyde carbon (carbon-1) as carbon dioxide. Two pentose phosphate molecules are then recombined, and are ultimately converted among other products to another molecule of glucose phosphate plus a tetrose phosphate. This new molecule of glucose (as intracellular glucose phosphate) is then available for another cycle. Carbon-2 of the original glucose is now the aldehyde carbon of the hexose phosphate formed after a single cycle. Therefore, the carbon dioxide released in the subsequent trip around the cycle, is derived from what was the second carbon of the original glucose.

In thiamine deficiency, the activity of transketolase was depressed in intact erythrocytes (Brin et al., '58).[5] Under these conditions the pentose formed from the initial glucose was shown to accumulate in these intact cells. A sensitive index of reduced activity was the prompt and marked depression in the recovery of

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[1] This study was initiated under Contract no. DA-49-007-MD-862 of the Surgeon General's Office at the Food and Drug Research Laboratories, Inc., and extended at Syracuse with the support of the Research Foundation of the State of New York and the Williams-Waterman Fund.

[2] Brin, M., S. S. Shohet and C. S. Davidson 1956 Effect of thiamine deficiency on mammalian erythrocyte metabolism. Federation Proc., *15:* 224 (abstract).

[3] See footnote 2.

[4] See footnote 2.

[5] See footnote 2.

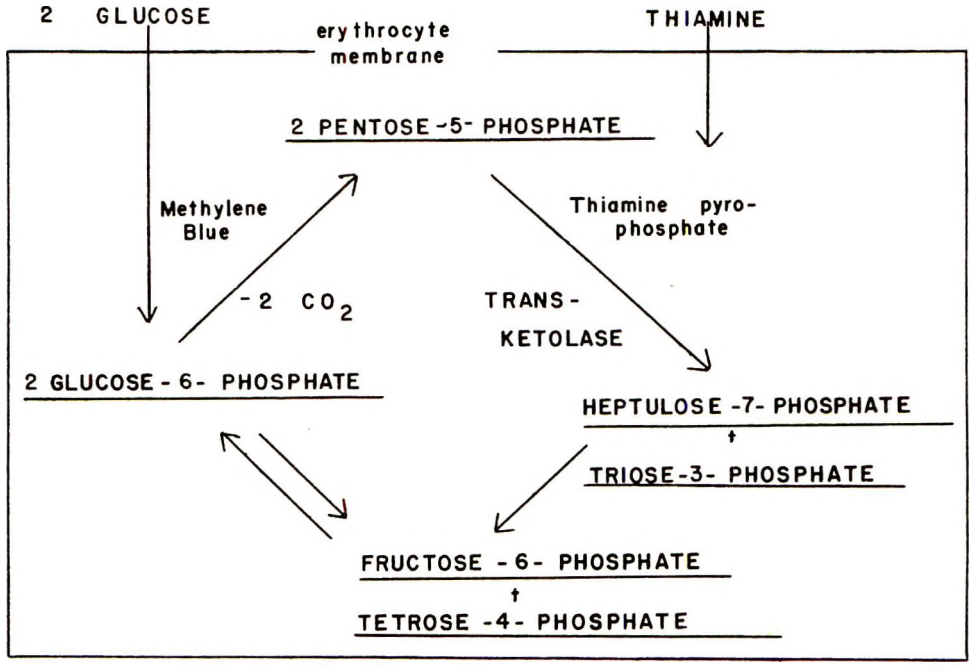

Fig. 1 A diagrammatic representation of the principal reactions involved in the glucose oxidative pathway used in the assay of transketolase as related to thiamine deficiency. Glucose and thiamine are phosphorylated when utilized as metabolic mediators within the cell. The oxidation of glucose with the concomitant production of carbon dioxide are not measured in the hemolysate procedure.

radioactive $C^{14}O_2$ (in the center well of the Warburg flask) when the glucose, which was added as substrate, was labeled with carbon 14 in the second carbon. Both measures of deficiency were partially restored to normal by the addition of thiamine to the cells, *in vitro* or by supplementing the intact animal with the vitamin (Brin et al., '58).[6]

The two factors which made it difficult to apply the method to routine studies were the absolute requirement (Brin et al., '58; Wolfe et al., '58), for freshly shed cells, and the use of isotopes in the Warburg. Unfortunately, refrigeration of the blood cells while awaiting assay was not adequate to preserve the integrity of the metabolic cycle and freezing resulted in cell rupture which destroyed oxidative activity (and the release of $C^{14}O_2$). It was thought advisable, therefore, to investigate whether the use of an intracellular non-oxidative, non-cyclic system could be derived from hemolysates which would still retain the reliability and specificity for the evaluation of thiamine deficiency. The use of hemolysates in this manner would allow for the freezing of samples until they could be assayed when convenient. Furthermore, the elimination of a membrane barrier might facilitate a more complete reaction.

Referring again to figure 1, the reaction sequence between pentose phosphate and hexose phosphate was considered. Pentose phosphate was incubated with an aliquot of hemolyzed red cells both with and without added thiamine pyrophosphate. The reaction was stopped by the addition of trichloracetic acid (TCA) and the filtrate was analyzed for pentose uptake and/or hexose production. The presence of the deficient state was indicated by decreased enzymatic reactivity, and by partial restoration in the presence of TPP.

EXPERIMENTAL PROCEDURE

Male rats, ranging in weight from 50 to 100 gm, depending on the study, were

[6] See footnote 2, p. 273.

maintained in wire-bottom cages and fed a purified thiamine-free diet[7] ad libitum. Control animals were raised similarly on a complete ration. All rats were fed ad libitum, watered daily and weighed three times weekly. Hemolysates were prepared as follows: blood was drawn by cardiac puncture from the anesthetized animal into a heparinized syringe and prepared for experimental use by centrifuging for 15 minutes at 1500 rpm in a graduated centrifuge tube, removing the plasma and buffy coat, washing the cells once with saline and discarding the supernatant. A volume of distilled water was added equal to two times the volume of cells, and the cells were resuspended to hemolyze. (Initially, the cells were hemolyzed by freezing and thawing, but this has since been shown to be unnecessary.)

The following reagents were required:[8]

1. Heparin solution: 10 mg/ml.

2. Ribose-5-phosphate: 1.62 gm of barium ribose-5-phosphate was dissolved in a minimum of 1N HCl and made up to about 50 ml with distilled water. The barium ions were removed with a concentrated solution of sodium sulfate by a process of cautious addition, centrifugation, and testing of the supernatant. After the precipitation was complete, the supernatant was separated and set aside, the $BaSO_4$ was washed twice with distilled water and the washings were added to the original supernatant. The solution was adjusted to pH 7.4 with KOH, made to a volume of 100 ml, distributed to smaller tubes and kept frozen until used. The final concentration was determined in each assay.

3. Thiamine pyrophosphate: TPP was dissolved in "B" buffer to give a final concentration of 1 mg/ml at a pH of 7.4. Analysis for thiamine before and after hydrolysis with acid phosphatase indicated an ester content of 90%.

4. Trichloracetic acid: a 7.5% solution of TCA in distilled water was stored in the refrigerator.

5. Anthrone reagent: placed in a two-liter flask were 500 mg of anthrone, 10 gm of thiourea, and one liter of 66% (by volume) sulfuric acid. This was warmed on a steam bath to 80 to 90°C with occasional shaking until dissolved. When stored in the refrigerator, the reagent was stable for two weeks.

6. Glucose standard: 1 mg/ml was dissolved in saturated benzoic acid. This was stable at room temperature, and was diluted when needed.

7. Orcinol solution: 1 gm of orcinol was dissolved with 0.2 gm of ferric alum in 100 ml of concentrated HCl. Made fresh before using.

8. Ribose standard: 1 mg/ml of D-ribose was dissolved in saturated benzoic acid. This was stable at room temperature and was diluted as needed.

9. "B" buffer: this is primarily the buffer used previously by Brin et al. ('54) and Umbreit et al. ('57) but in which potassium salts replaced sodium and vice versa, as follows: 4 parts of 0.9% NaCl, 103 parts of 1.15% KCl, 1 part of 3.82% $MgSO_4 \cdot 7H_2O$, and 20 parts of phosphate buffer (17.5 gm K_2HPO_4 plus approximately 20 ml of 1 N HCl to bring pH to 7.4, per liter).

Substrate solutions were prepared as follows:

Solution A: three volumes of ribose-5-phosphate solution were mixed with one volume of "B" buffer.

Solution B: 8 volumes "B" buffer were mixed with one volume of TPP solution. Prepared immediately before use.

Solution C: 1 ml of ribose-5-phosphate solution was mixed with 40.33 ml of 7.5% TCA.

Three samples of each hemolysate were run simultaneously, namely (a) hemolysate plus ribose-5-phosphate, (b) hemolysate plus TPP plus ribose-5-phosphate, and (c) hemolysate blank, respectively, as follows:

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[7] Where indicated, animals raised in the colony of the Food and Drug Research Laboratories, Inc. (FDRL strain) were fed ration S-55 (Hawk, P. B., Oser, B. L., and Summerson, W. H. 1954 Practical Physiological Chemistry, Blakiston, New York, p. 1375). Also, where indicated, animals obtained from Carworth Farms, New York (CFN strain) were fed rations obtained from Nutritional Biochemicals Corp., Cleveland, as "thiamine-deficient diet" and "vitamin test diet-complete ration."

[8] Heparin, ribose-5-phosphate barium, and thiamine pyrophosphate were obtained from Nutritional Biochemicals Corp. Anthrone and orcinol were obtained from Fisher Scientific Co.

(a) to 0.5 ml of hemolysate was added 0.45 ml of "B" buffer. Following incubation for 30 minutes at 38°C 0.2 ml of solution A was added. The mixture was stirred by rotating, and reincubated at 38°C for 60 minutes. The reaction was stopped by the addition of 6.0 ml of 7.5% TCA.

(b) for the TPP series, 0.5 ml of hemolysate was incubated with 0.45 ml of "B" buffer containing 25 to 50 μg of TPP, for 30 minutes at 38°C to permit unsaturated apoenzyme to combine with coenzyme in the absence of substrate. At that time 0.2 ml of solution A was added, and the incubation was continued for one hour. The reaction was terminated by the addition of 6.0 ml of 7.5% TCA.

(c) for the hemolysate blank 0.5 ml of hemolysate was incubated with 0.45 ml of "B" buffer for 30 minutes followed by the addition of 6.2 ml of solution C. All incubations were done at 38°C. The TCA suspensions were centrifuged, and the supernatants were analyzed for hexose and pentose as follows:

For pentose, the appropriate aliquots (0.2 ml of filtrates [a] and [b] and 0.1 ml of filtrate [c]) were distributed to a series of optically standardized pyrex test tubes (as cuvettes) and water was added to 3 ml. A three-milliliter aliquot of the orcinol reagent was added; the test tubes were shaken, capped with glass marbles and placed in rapidly boiling water for 45 minutes. They were read promptly in a colorimeter at 670 mμ without cooling. D-Ribose standards and a reagent blank accompanied each set of determinations.

For hexose, one-milliliter aliquots of each of the three filtrates per sample were distributed to a series of optically standardized test tubes. Ten milliliters of cold anthrone reagent were added with good mixing. After capping with glass marbles the tubes were placed in rapidly boiling water for 10 minutes after which they were removed, cooled rapidly and allowed to stand for 15 to 20 minutes in a dark cabinet. The tubes were read in the colorimeter at 620 mμ. Glucose standards and a reagent blank were analyzed with each set of determinations.

In all cases, an equal number of control and deficient animals were assayed simultaneously to minimize experimental variation. Approximately 60% of the substrate was utilized by the control hemolysates during the reaction period.

The data are presented as the number of micrograms of pentose which disappeared, and/or the number of micrograms of hexose which were formed, per milliliter of hemolysate per hour. Where adequate numbers of determinations were available, the standard error was presented with the mean value; otherwise, the range of values was given. Where a thiamine supplement was administered to the rat orally, or by injection, the size of dose and the frequency of administration were presented with the data.

RESULTS

The growth response of the rats receiving the thiamine-deficient diet was similar to that observed previously by Brin et al. ('58).[9] The data presented graphically in figure 2 show that normal growth prevailed for 10 to 14 days, at which point growth ceased and was followed by losses in body weight. Despite the cessation of growth, the physical appearance of the deficient group was normal until 18 to 21 days on test when some rats showed roughening of their coats. Nervousness was not generally evident until three to 4 weeks on the diet.

Transketolase activity, as measured by the disappearance of pentose was determined on groups of rats at intervals during the first three weeks. These data, presented in table 1, indicated that the rate of disappearance of pentose added to the erythrocyte hemolysate was depressed as early as day 9 and that the effect increased as the deficiency became more severe (12 to 43%). Whereas the addition of TPP had little effect on the activity of the control hemolysates appreciable stimulation of the disappearance of pentose in the deficient hemolysates was observed.

Another indication of reduced transketolase activity in the erythrocytes of thiamine-deficient animals was the diminished accumulation of hexose phosphate from pentose phosphate. (Hexose cannot be further oxidized to pentose by this hemolysate system.) The data for the

[9] See footnote 2, p. 273.

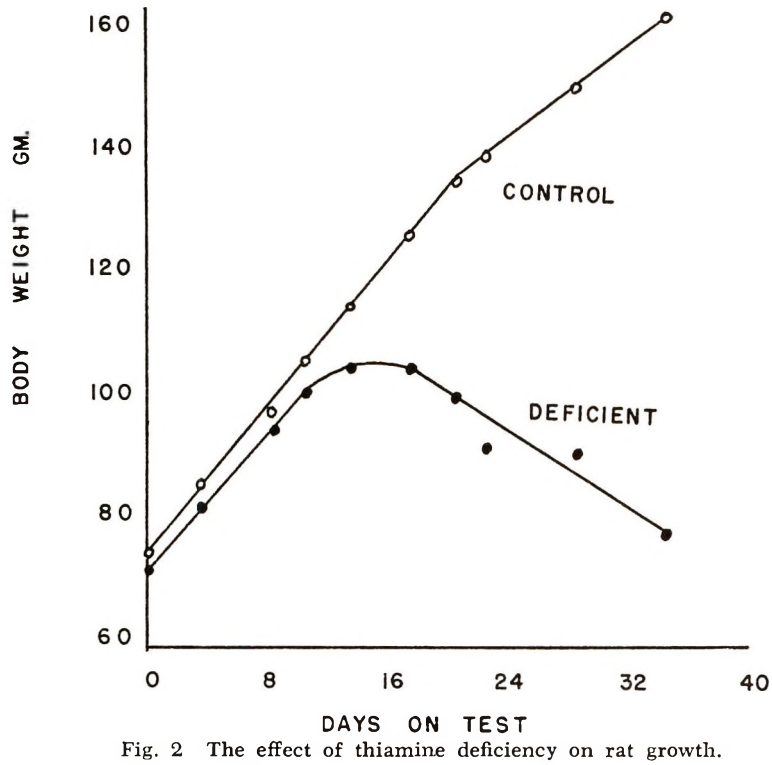

Fig. 2 The effect of thiamine deficiency on rat growth.

TABLE 1

The effect of thiamine deficiency on the disappearance of pentose in rat red cell hemolysates[1,2]

Days on test	Control rats		Thiamine-deficient rats		Depression from control
	No addition	+TPP[3]	No addition	+TPP[3]	
	μg pentose/ml/hour		*μg pentose/ml/hour*		%
9	1706 ± 81	1695 ± 28(0)[4]	1524 ± 91	1586 ± 60(4.0)[4]	12
11	—	1699 ± 51(−)	1094 ± 50	1369 ± 99(25)	35
13	1644 ± 45	1721 ± 50(4)	1236 ± 92	1397 ± 44(13)	25
15	1594 ± 46	1632 ± 61(3)	1028 ± 51	1213 ± 37(19)	35
17	1525 ± 28	1549 ± 29(1)	1176 ± 40	1357 ± 34(15)	23
20	1628 ± 53	1624 ± 49(0)	916 ± 67	1099 ± 62(20)	43

[1] These rats were FDRL strain.
[2] Each value represents the mean ± S.E. of 6 rat blood hemolysates.
[3] To each milliliter of hemolysate 100 μg TPP was added.
[4] The values in parentheses represent the percentage change due to the presence of added coenzyme.

appearance of hexose in the filtrates from control and thiamine-deficient rats, are presented in table 2. It was apparent that this was depressed in thiamine-deficient hemolysates in as early as 7 days and progressive depression was observed for at least three weeks. The addition of TPP had some stimulating effect on control hemolysates but a much greater effect was observed in the deficient system.

A variation of 10 to 15% may be observed on repeat assay of the same hemolysate with this method. To minimize this variation, all assays (control and deficient samples) for any feeding interval were performed at the same time. It is recog-

TABLE 2

The effect of thiamine deficiency on the appearance of hexose (from pentose) in rat red cell hemolysates[1,2]

Days on test	Control rats		Deficient rats		Depression from control
	No addition	+TTP[3]	No addition	+TPP[3]	
	μg hexose/ml/hour		*μg hexose/ml/hour*		%
7	897 ± 14	898 ± 44(0)[4]	680 ± 53	749 ± 36(9)[4]	24
9	859 ± 26	936 ± 34(9)	667 ± 78	776 ± 52(17)	22
11	922 ± 24	955 ± 22(3)	460 ± 50	590 ± 32(28)	50
13	922 ± 46	1017 ± 40(10)	491 ± 75	650 ± 47(33)	46
20	978 ± 17	941 ± 18(0)	371 ± 36	487 ± 28(31)	61

[1] These rats were FDRL strain.

[2] Each value represents the mean ± S.E. of 6 rat blood hemolysates.

[3] To each hemolysate 100 μg TPP was added.

[4] The value in parentheses represents the percentage change due to the presence of added coenzyme.

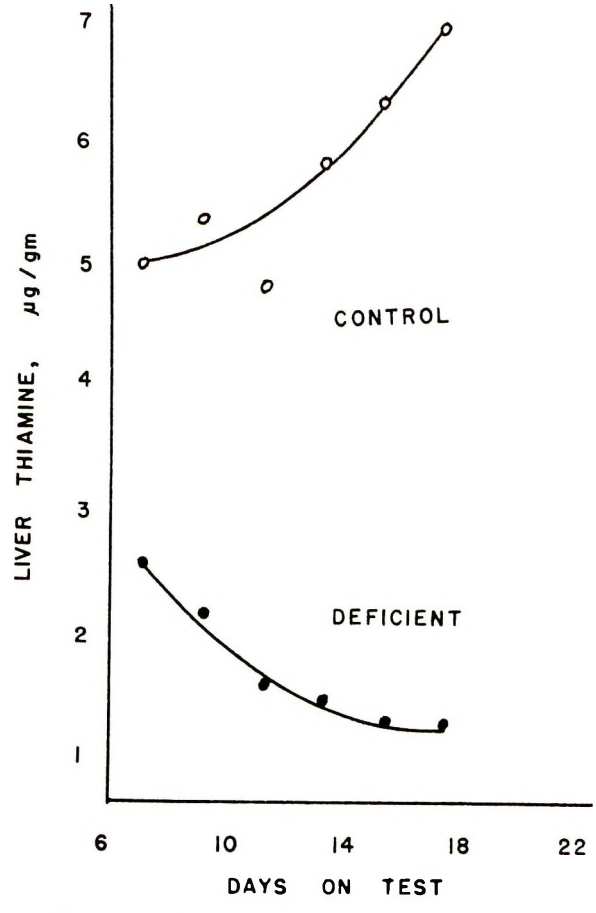

Fig. 3 The effect of thiamine deficiency on liver content of the vitamin.

TABLE 3
Repletion in vivo of thiamine-deficient rats[1]

Group	Days on test	No. rats and sex	Pentose disappearance		Hexose appearance	
			No addition	+TPP[2]	No addition	+TPP[2]
			μg/ml blood/hour		*μg/ml blood/hour*	
Control	6	5F	1207 ± 9	1203 ± 9	1119 ± 28	1099 ± 23
	16	3F	1068	1059	1456	1430
			(1054–1076)	(1018–1090)	(1339–1611)	(1263–1533)
Deficient	9	5F	540 ± 19	735 ± 42	732 ± 37	974 ± 18
	16	3F	375	590	501	805
			(344–437)	(502–696)	(443–559)	(675–947)
Injected[3]	9	3F	1164	1233	1087	1061
			(1090–1205)	(1212–1255)	(1035–1132)	(997–1073)
	12	3F	903	935	1237	1224
			(868–925)	(903–961)	(1108–1301)	(1070–1340)
Fed[4]	13	5F	1110	1149	1054	1011
			(1011–1205)	(1108–1194)	(963–1238)	(839–1330)

[1] Data are presented as mean ± S.E. or mean with range of values. CFN rats.
[2] One hundred gamma of thiamine pyrophosphate added *in vitro* to the hemolysates.
[3] Four intraperitoneal injections of 2 mg thiamine·HCl daily over a 5-day period to rats which were fed the deficient diets for 9 and 12 days.
[4] After 13 days on the deficient diet the control diet was fed for 14 days.

nized that the enzyme assay described in this paper is in fact a gross measure of the total activity of a number of interrelated enzymes. The ribose-5-phosphate must be converted to ribulose-5-phosphate and xylulose-5-phosphate by ribose isomerase and epimerase, respectively, in order to supply the two essential substrates for transketolase. A variation in the activity of either or both of these in the hemolysate might affect the availability of substrate for the transketolase, in which we are primarily interested, and in so doing, affect the rate of disappearance of pentose. The other measure, the formation of hexose, is similarly removed from transketolase by at least two enzymes, a variation in the activity of any one of which might influence the hexose value. However, in view of the fact that the enzyme activity of thiamine-deficient hemolysates was enhanced by adding TPP, it appeared that transketolase was indeed the limiting factor in the reaction chain.

The effects of thiamine deficiency on the thiamine content of the livers of these rats is shown in figure 3. It was evident that the livers were partially depleted by 7 days (averages of 6 rats per group), and more so at 6 weeks.

In view of the positive effects of adding TPP to the hemolysates from deficient erythrocytes, it was of interest to investigate the effects on the enzyme system of administering thiamine to the intact rat. This was done both by injecting thiamine hydrochloride or by placing already deficient rats on the control diet. When injected, thiamine hydrochloride was administered at a level of 2 mg per day. The enzyme data for the repletion studies are presented in table 3. It was observed, that for both time periods on the deficient diet before treatment, (9 or 12 days) the injection of thiamine resulted in marked increases of both the disappearance of pentose and the formation of hexose to values approximating 85% of normal. Animals which were deficient for 13 days, and treated by feeding the control diet for two weeks, also demonstrated markedly enhanced pentose and hexose values, having been increased at least twofold when compared with the appropriate deficient untreated group. When the per-

formance of this group was compared with control values, it was evident that the feeding of thiamine for the more extended period resulted in pentose and hexose values equivalent to normal. It was worthy of note that the addition of TPP to hemolysates from fed, or injected animals resulted in no appreciable further enhancement of transketolase activity.

DISCUSSION

The requirement of thiamine for the metabolism of pyruvate in brain has been established by Peters and Thompson ('33) and in heart muscle by Olson et al. ('48). More recently it has been demonstrated that yeast transketolase also requires thiamine pyrophosphate as a coenzyme for activity (Racker et al., '53). The availability of transketolase in the erythrocyte (Brin and Yonemoto, '58) presented a unique opportunity to determine whether this enzyme, available from an experimental subject without detriment, might reflect the thiamine status of that individual. Studies with intact erythrocytes from the rat (Brin et al., '58),[10] and human (Wolfe et al., '58) appeared to support this contention.

For purposes of broader application of the technique, it was desirable to use an erythrocyte hemolysate (rather than intact cells). With such a system, too, a biochemical defect was demonstrated in rats fed a thiamine-deficient diet before growth rate was modified. Depression of transketolase activity was evident at 7 to 9 days on test, growth ceased at 12 to 14 days, hair coat roughened at 16 to 20 days and nervous symptoms appeared in individual rats after three weeks. The defect in enzyme activity was therefore evident approximately a week before the growth rate of young rats was affected, and one to two weeks before other clinical signs were evident. Furthermore, the defect became more severe as the feeding period lengthened.

The specificity of the assay for the availability of the coenzyme form of thiamine was supported by the various repletion studies which were described. Transketolase activity of deficient hemolysates were markedly stimulated by the addition of TPP to the system *in vitro* before assay, albeit not to normal values. On the other hand, little effect was seen when TPP was added to the control samples. This observation indicated that whereas the apotransketolase in control blood was saturated with cofactor, there was a dual shortage in deficient blood, firstly, of available holoenzyme, and secondly, of sufficient coenzyme to saturate the total apoenzyme which was present, and suggested the feasibility of evaluating thiamine status in individuals. A similar situation of unsaturated apoenzyme, in this case transaminase, was seen previously in pyridoxine deficient duck heart homogenates (Brin et al., '54). In both deficiencies, thiamine and pyridoxine, the addition of cofactor to the deficient tissue resulted in increased enzyme activity, but not to normal values.

By virtue of the (a) simplicity of assay for pentose and/or hexose, (b) the ability to store hemolysates by freezing and (c) the availability of blood from an experimental subject without detriment, it appeared that the hemolysate technique was more readily adaptable to routine evaluation studies than the original intact cell, method. Despite the variations which were encountered on repeat assay the method has been applied with favorable results by a number of investigators to the evaluation of the thiamine adequacy of rats fed various foodstuffs which were preserved by ionizing radiation, Progress Reports ('58, '59).

SUMMARY

A method has been presented for the assay of the transketolase enzyme system in erythrocyte hemolysates. The assay permitted the measurement of a biochemical defect in thiamine-deficient animals before the onset of other signs of deficiency such as cessation of growth or the development of neuritis. The specificity of the assay for thiamine was demonstrated by stimulating transketolase activity in deficient hemolysates through (a) the addition of thiamine pyrophosphate (TPP) to the hemolysates *in vitro* and (b) the administration of thiamine hydrochloride to the rat *in vivo*. The application of

[10] See footnote 2, p. 273.

the method of the evaluation of thiamine status was indicated.

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Studies on the Sodium, Chlorine and Iodine Requirements of Young Pheasants and Quail

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Previous studies from this laboratory have dealt with the protein, calcium, phosphorus, vitamin D, manganese, zinc, riboflavin, niacin and choline requirements of pheasants (Scott, et al., '54, '58a, '59). The phosphorus requirements of young Bobwhite quail also have been reported (Scott, et al., '58b).

Although many studies have been made on the effects of various levels of salt in rations for chickens, little has been reported on the sodium and chlorine requirements of this species, and nothing on either salt, sodium or chlorine requirements of young pheasants and quail. Since iodine is usually supplied to practical rations in the form of iodized salt, it was considered desirable to determine not only the sodium and chlorine requirements, but also to determine whether or not the amount of iodized salt needed to supply these requirements would also supply sufficient iodine for normal growth and thyroid development in young pheasants and quail. The results of these experiments are presented in this report.

EXPERIMENTAL

All experiments were conducted at the Ithaca Game Farm of the New York State Conservation Department. Both the pheasants and quail were housed in wire pens with raised wire floors. Each pen was equipped with a thermostatically-controlled electric hover. The basic diet used in all experiments is presented in table 1. According to the A.O.A.C. ('55) method for total chlorides, the diet contained 0.048% of chlorine. Sodium was determined by flame spectrophotometry and was found

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TABLE 1

Sodium, chlorine and iodine deficient pheasant and quail starter diet

Ingredients	*pounds/ton*
Cornmeal, yellow	800
Corn oil	40
Soybean oil meal, dehulled, 50% protein	770
Corn gluten meal	200
DL-Methionine	2
Brewers' dried yeast	100
Stabilized vitamin A (5,000 I.U./gm)	5
d-α-Tocopheryl acetate[1]	0.5
Vitamin D_3-activated animal sterol (3,000 I.C.U./gm)	1
Dicalcium phosphate	40
Calcium carbonate	40
Manganese sulfate, feed grade	1
	gm/ton
Zinc sulfate	112
Aureomycin·HCl, crystalline	15
Santoquin antioxidant	112
Menadione sodium bisulfite[2]	6
Vitamin B_{12}	0.006
Niacin	40
$Ca(IO_3)_2$[3]	1

Analysis	
Protein, %	29.5
Metabolizable energy, Cal./pound	1331
ME/P	45
Fat, %	4.2
Fiber, %	2.5
Calcium, %	1.44
Phosphorus, %	0.84
Sodium, %	0.025
Chlorine, %	0.048
Iodine, ppm	0.56

[1] Myvamix, 20,000 I.U./pound, Distillation Products Industries, Rochester, New York.

[2] Klotogen F, Abbott.

[3] $Ca(IO_3)_2$ was omitted and 0.25% NaCl added when iodine requirements were determined. Under these conditions, basal diet contained 0.2 ppm of iodine.

to be present at 0.025% of the basal diet. Iodine determinations were conducted according to a modification of the Elmslie-Caldwell Method as described by the A.O.A.C. The basic diet showed a level of iodine of 0.2 ppm. Feed and water were supplied ad libitum. For the pheasant studies, each experimental lot contained 50 Ringneck pheasant chicks of mixed sex at the start of the experiment. There were 25 quail per lot. All treatments were run in duplicate. The pheasants were distributed among 32 6′ by 6′ pens; the quail treatments were distributed among 24 4′ by 4′ pens.

Pheasants

Sodium and chlorine. Experiment 1. The first experiment was conducted to compare the effects of graded levels of sodium chloride, ranging from 0.25 to 2.0% in the diet of young pheasants to 4 weeks of age. Sodium bicarbonate at 0.36% (sodium equivalent to that in 0.25% sodium chloride) and 0.32% of potassium chloride (chloride equivalent to that in 0.25% sodium chloride) were also fed singly and in combination. The experimental plan and results of experiment 1 are presented in table 2. Exceedingly poor growth was observed in the pheasants receiving the basal diet without any supplemental salt. The results further showed that the basal diet was severely deficient in sodium and somewhat deficient in chlorine. Since the lowest level of salt (0.25%) added to the basal diet produced approximately maximum growth, it is not possible from this experiment to determine the exact sodium chloride requirement of pheasants receiving this diet.

Experiment 2. The second experiment was conducted to determine more precisely the salt requirement, and also to attempt to determine both the sodium and the chlorine requirements. Sodium chloride was fed in graded levels from 0.1 to 0.25%. In an effort to determine the sodium requirement, several lots were fed graded levels of sodium bicarbonate in the presence of 0.32% of potassium chloride. Studies on the chlorine requirement were conducted by feeding graded levels of potassium chloride in the presence of 0.36% of sodium bicarbonate. The experimental outline and results of experiment 2 are presented in table 3.

These results show that when the diet contained sufficient chlorine, the sodium requirement was met by the addition of approximately 0.06% of sodium to this diet. Since the basal diet contained 0.025% sodium, the total sodium requirement of pheasant chicks, under the conditions of this experiment, was approximately 0.085% of the diet. The sodium requirement was met either by the addition of 0.15% of sodium chloride or by the addition of 0.216% of sodium bicarbonate with adequate chlorine supplied in the form of potassium chloride.

When sodium bicarbonate was the only supplement to the diet, pheasant weights were reduced due to a lack of chlorine. The addition of the lowest level of KCl (0.128%) appeared to furnish sufficient chlorine, thereby indicating that the chlorine requirement is no higher than that

TABLE 2

Results of first experiment on sodium and chlorine requirements of pheasants

Treatment	Av. weight, 4 weeks	Mortality
	gm	%
Basal diet (no salt)	87 (85–88)[1]	6
Basal diet + 0.25% salt (NaCl)	193(178–208)	5
Basal diet + 0.50% salt	204(198–209)	7
Basal diet + 1.00% salt	208(203–213)	4
Basal diet + 2.00% salt	206(199–213)	11
Basal diet + 0.36% sodium bicarbonate (Na^+ equivalent to that in 0.25% salt)	176(174–178)	7
Basal diet + 0.32% potassium chloride (Cl^- equivalent to that in 0.25% salt)	104(103–105)	13
Basal diet + 0.36% $NaHCO_3$ + 0.32% KCl	203(201–204)	10

[1] Figures in parentheses show range of values for the duplicate lots on each treatment.

TABLE 3

The sodium, chlorine and total salt requirements of pheasants

Treatment	Av. weight, 4 weeks
	gm
Basal diet + 0.1% NaCl	195(189–201)[1]
Basal diet + 0.15% NaCl	226(225–227)
Basal diet + 0.20% NaCl	231(229–232)
Basal diet + 0.25% NaCl	230(225–234)
Basal diet + 0.32% KCl	76(73– 78)
Basal diet + 0.32% KCl + 0.144% $NaHCO_3$ (0.04% Na)	169(166–171)
Basal diet + 0.32% KCl + 0.216% $NaHCO_3$ (0.06% Na)	227(223–231)
Basal diet + 0.32% KCl + 0.288% $NaHCO_3$ (0.08% Na)	225(224–226)
Basal diet + 0.32% KCl + 0.36% $NaHCO_3$ (0.1% Na)	232(228–236)
Basal diet + 0.36% $NaHCO_3$	176(174–178)
Basal diet + 0.36% $NaHCO_3$ + 0.128% KCl (0.066% Cl)	216(208–223)
Basal diet + 0.36% $NaHCO_3$ + 0.192% KCl	224(223–224)

[1] Figures in parentheses show range of values for the duplicate lots on each treatment.

obtained when 0.066% of supplementary chlorine was added to this diet. The basal diet contained approximately 0.048% of chlorine. Therefore, the total chlorine requirement of pheasant chicks under these conditions was more than 0.048% but no more than 0.11%. More than 0.11% of chlorine was supplied when the diet was supplemented with 0.15% of sodium chloride, which was the amount needed to meet the sodium requirement. Thus, this level of salt satisfied both the sodium and the chlorine requirements of pheasant chicks receiving this basal diet.

Experiment on salt toxicity. An experiment was conducted to determine the amount of salt which is toxic to pheasants, and also to determine the effects of high levels of salt upon the moisture content of the droppings in pheasants.

The experimental plan and results of this experiment are presented in table 4. These results show that only when the salt content of the diet reached 7.5% was growth markedly depressed and mortality increased. The moisture content of the droppings increased appreciably as the salt content of the diet increased.

TABLE 4

Studies on salt toxicity in pheasants

Salt levels in diet	Average pheasant weight, 4 weeks	Mortality	Moisture content of feces
%	*gm*	%	%
0.25	230(225–235)[1]	4	74
1.0	232(229–234)	5	78
2.0	223(218–227)	3	86
3.0	219(217–220)	6	88
4.0	218(215–221)	5	89
5.0	197(196–197)	7	—
7.5	165(158–171)	23	—

[1] Figures in parentheses show range of values for the duplicate lots on each treatment.

Iodine. One experiment was conducted with pheasants to determine the iodine requirement. For this experiment the basal diet presented in table 1 was modified by the omission of the calcium iodate and the addition of 0.25% of NaCl.

Since iodized salt contains 0.007% of iodine and is usually added to poultry rations at a level of 0.25% of the diet, this amount of salt would supply approximately 0.18 mg of iodine per kg of diet (0.18 ppm). In view of this, the experiment on iodine requirements of pheasants was designed so that one lot of pheasants received 0.18 mg of iodine per kg of diet; other lots received levels of iodine above and below this level. The results of the experiment, (table 5) show that iodine supplementation of the basal diet had no effect upon growth of pheasant chicks to 4 weeks of age. On the other hand, the average thyroid weights of the pheasants receiving the basal diet were significantly larger than those of the pheasants receiving iodine supplementation. Among the iodine-supplemented groups, including and above that receiving 0.09 mg of added iodine per kg of diet, the thyroid weight bore no significant relationship to the level of iodine added. Microscopic examination of the thyroids showed practically normal follicle development in all of the pheasants. Measurements of epithelial heights of the

TABLE 5

Studies on the iodine requirement of pheasants

Treatment	Av. pheasant weight, 4 weeks	Thyroids	
		Average weight	Epithelial height
	gm	*mg*	*μ*
Basal diet	217(214–220)[1]	14.7	3.60
Basal diet + 0.0625 mg $Ca(IO_3)_2$/pound (0.045 mg I_2/kg [ppm])	213(212–214)	13.6	3.76
Basal diet + 0.125 mg $Ca(IO_3)_2$/pound (0.09 mg I_2/kg [ppm])	213(210–215)	10.7	3.47
Basal diet + 0.25 mg $Ca(IO_3)_2$/pound (0.18 mg I_2/kg [ppm])	207(203–211)	11.1	3.03
Basal diet + 0.50 mg $Ca(IO_3)_2$/pound (0.36 mg I_2/kg [ppm])	213(211–214)	9.9	3.13
Basal diet + 0.75 mg $Ca(IO_3)_2$/pound (0.45 mg I_2/kg [ppm])	207(207–207)	11.9	3.33

[1] Figures in parentheses show range of values for the duplicate lots on each treatment.

TABLE 6

Results of first experiment on sodium and chlorine requirements of quail

Treatment	Av. quail weight, 4 weeks
	gm
Basal diet (no salt)	27.0(26.5–27.6)[1]
Basal diet + 0.25% salt (NaCl)	56.9(56.6–57.2)
Basal diet + 0.50% salt	55.1(53.0–57.2)
Basal diet + 1.00% salt	61.7(58.4–65.0)
Basal diet + 2.00% salt	60.8(60.6–61.0)
Basal diet + 0.36% sodium bicarbonate (Na^+ equivalent to that in 0.25% salt)	49.0(47.3–50.7)
Basal diet + 0.32% potassium chloride (Cl^- equivalent to that in 0.25% salt)	32.0(31.1–32.6)
Basal diet + 0.36% $NaHCO_3$ + 0.32% KCl	58.5(57.2–59.7)

[1] Figures in parentheses show range of values for the duplicate lots on each treatment.

thyroids (table 5) showed no consistent relationship between epithelial heights and level of iodine supplementation. Since the basal diet contained 0.20 mg of iodine per kg of diet, it appears that the total iodine requirement of pheasants under the conditions of this experiment was not greater than approximately 0.30 mg of iodine per kg of diet.

Quail

Sodium and chlorine. Experiment 1. The sodium and chlorine requirements of quail were determined by the same procedures used to determine the requirements for pheasants. The experimental plan and results of the first experiment are presented in table 6. These results were very similar to those obtained with the pheasants. The omission of salt or the inclusion of only sodium bicarbonate or only potassium chloride alone resulted in decreased growth of the quail, indicating that the basal diet was deficient in both sodium and chlorine for quail. Furthermore, it appeared that the level of 0.25% of added salt was sufficient to produce near maximum growth in quail receiving this diet.

Experiment 2. The second experiment was conducted to determine more precisely the salt requirements of quail, and also to attempt to deterine both the sodium and chlorine requirements. The experimental outline and results of this experiment are presented in table 7. While

the results with 0.1% of sodium chloride indicated this level to be sufficient for maximum growth, the results obtained when increasing levels of sodium bicarbonate were added to the diet in the presence of 0.32% KCl, indicated that the amount of added sodium needed was approximately 0.06% which is the amount of sodium supplied by 0.15% of sodium chloride. Thus the sodium and the salt requirements of quail appear to parallel very closely those of the pheasants, the total sodium requirement being approximately 0.085% of the diet.

The slight reduction in growth obtained when sodium bicarbonate alone was added to the diet indicated that this diet was slightly deficient in chlorine for quail. The addition of 0.128% of potassium chloride, which supplied 0.066% of chlorine, was sufficient to produce maximum growth, indicating, therefore, that the level of 0.15% of sodium chloride also would supply sufficient chlorine for quail receiving this diet, and that the chlorine requirement of quail is no higher than 0.11% of the diet.

An experiment on salt toxicity in quail. An experiment was conducted to determine the amount of salt which is toxic to quail. The experimental plan and results of the experiment are shown in table 8. These observations also are similar to those obtained with the pheasants and indicate that young quail can tolerate levels of salt up to and including 5% before showing signs of toxicity. A definite decrease in growth and an increase in mortality was caused by 7.5% of salt. Although fecal moisture determinations were not made, it was noted that the droppings showed an increasingly moist consistency as the level of salt was increased.

Iodine requirements of quail. One experiment was conducted on the iodine requirement of quail. The experimental plan and weights of the quail receiving the various iodine levels are shown in table 9. It is apparent from these results that the omission of iodine from the basal diet had

TABLE 7

The sodium, chlorine and total salt requirements of quail

Treatment	Av. weight, 4 weeks
	gm
Basal diet + 0.10% NaCl	54.4(53.8–54.7)[1]
Basal diet + 0.15% NaCl	53.4(52.6–53.7)
Basal diet + 0.20% NaCl	55.1(54.8–55.3)
Basal diet + 0.25% NaCl	55.0(53.2–56.7)
Basal diet + 0.32% KCl	22.8(21.9–23.7)
Basal diet + 0.32% KCl + 0.144% $NaHCO_3$ (0.04% Na)	46.3(44.7–48.0)
Basal diet + 0.32% KCl + 0.216% $NaHCO_3$ (0.06% Na)	55.6(55.0–56.2)
Basal diet + 0.32% KCl + 0.288% $NaHCO_3$ (0.08% Na)	53.7(51.3–56.0)
Basal diet + 0.32% KCl + 0.36% $NaHCO_3$ (0.1% Na)	54.0(53.0–55.0)
Basal diet + 0.36% $NaHCO_3$	49.0(47.3–50.7)
Basal diet + 0.36% $NaHCO_3$ + 0.128% KCl (0.066% Cl)	55.3(55.0–55.6)
Basal diet + 0.36% $NaHCO_3$ + 0.192% KCl	54.1(52.3–56.2)

[1] Figures in parentheses show range of values for the duplicate lots on each treatment.

TABLE 8

Studies on salt toxicity in quail

Salt levels in diet	Average quail weight, 4 weeks
	gm
Basal diet (no salt)	24.9(23.2–26.7)[1]
Basal diet + 0.1% NaCl	59.4(58.0–61.3)
Basal diet + 0.15% NaCl	60.8(60.2–61.3)
Basal diet + 2.5% NaCl	59.9(59.7–60.0)
Basal diet + 5.0% NaCl	60.0(57.2–62.7)
Basal diet + 7.5% NaCl	54.2(51.3–57.0)

[1] Figures in parentheses show range of values for the duplicate lots on each treatment.

TABLE 9

Studies on the iodine requirements of quail

Treatment	Average quail weight, 4 weeks	Thyroid weight 4 weeks[1]
	gm	*mg*
Basal diet	62.2(62.1–62.2)[2]	3.16
Basal diet + 0.125 mg $Ca(IO_3)_2$/pound (0.09 mg I_2/kg [ppm])	60.7(59.3–62.0)	2.83
Basal diet + 0.25 mg $Ca(IO_3)_2$/pound (0.18 mg I_2/kg [ppm])	59.8(59.3–60.2)	2.70
Basal diet + 0.50 mg $Ca(IO_3)_2$/pound (0.36 mg I_2/kg [ppm])	60.9(58.7–63.0)	2.80

[1] Thyroid weights average of 6 quail per treatment.
[2] Figures in parentheses show range of values for the duplicate lots on each treatment.

no effect on quail growth. These results also are similar to those obtained with pheasants. Based upon thyroid weights (table 9), it appears that the first level of added iodine produced normal thyroid glands. Therefore, according to these experiments, the iodine requirement of quail also appears to be no greater than 0.3 ppm, which is that supplied by the lowest level of iodine supplementation.

DISCUSSION

Mitchell and Carman ('26) reported that chicks fed a cereal ration containing no added salt showed retarded growth with decreased efficiency of feed utilization. They concluded that the retarded growth was due to a deficiency of sodium rather than of chlorine. These results have been confirmed by Prentice ('33) and Burns et al. ('53). Burns and associates found that the minimum amount of sodium required by chicks was between 0.1 and 0.3% whereas the minimum amount of chlorine required was less than 0.06%. Neither the requirement for sodium nor that for chlorine has been established with any degree of precision.

With the improved methods of flame spectrophotometry it has been possible to determine accurately the sodium content of the basal ration used in the present studies. Therefore, it appears that the sodium requirement of pheasants and quail of 0.085% is precise under the conditions of these experiments.

Under various conditions in previous experimental work, evidence has been obtained from time to time indicating an interrelationship between sodium requirements and potassium levels in the diet. It should be noted, therefore, that the sodium requirements observed here were determined using a practical diet naturally high in potassium. However, one should also note that the sodium requirement obtained upon supplementing the diet with potassium chloride and sodium bicarbonate was approximately the same as that obtained by supplementing the diet with sodium chloride. Thus, the increased potassium level in the presence of potassium chloride did not appear to change the sodium requirement.

The iodine requirement of chicks, according to Patton et al. ('39), Wilgus et al. ('41) and Gassner and Wilgus ('40) was originally estimated to be approximately 1 mg of iodine per kg of diet, for optimum growth and prevention of goiter. These workers observed congenital goiter in baby chicks hatched from hens receiving 0.025 mg of iodine per kg of breeder ration. On the other hand, Godfrey et al. ('53) found the iodine requirement of chicks to be between 0.03 and 0.15 mg per kg of diet. Later, Creek et al. ('54) reported that 0.05 mg of iodine per kg of diet or less failed to support normal growth, while 0.075 mg per kg of diet was sufficient for normal growth. Somewhat higher amounts, however, were considered to be required for completely normal thyroid histology, since slight abnormalities were noted when the level was below 0.3 mg of iodine per kg of diet.

It is possible that some of the discrepant results obtained by these various

workers are due to variations in precision of the various methods of iodine analysis. On the other hand, they may be due to variations in stability and/or availability of the iodine in the iodine supplements used. Griem et al. ('42) showed that tests of a number of samples of iodized salts (presumably containing potassium iodide) showed losses of iodine up to 76%. Calcium iodate[1] was used in the present studies because it is one of the most stable iodine compounds.[2]

SUMMARY

Studies have been presented which indicate that, under practical conditions, the sodium requirement of both pheasants and quail is approximately 0.085%; the chlorine requirement is between 0.048 and 0.11%; and the iodine requirement is no more than 0.3 ppm of diet.

The results of these studies indicate that the sodium and chlorine requirements of both pheasants and quail are satisfied by the addition to a practical ration of 0.15% of sodium chloride. If iodized salt is used, containing 0.007% of iodine, this level of 0.15% of salt also appears to satisfy the iodine requirement of both pheasants and quail.

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[2] Mallinckrodt Technical Information Unit X-367, code 2404.

Erythropoiesis in Ducks with Various B-Vitamin Deficiencies[1]

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The initial step in the biosynthesis of protoporphyrin involves the condensation of glycine with succinyl coenzyme A to form δ-aminolevulinic acid (ALA)[2] (Shemin and Kumin, '52; Kikuchi et al., '58; Gibson et al., '58). Pyridoxal-5′-phosphate was found to be involved in porphyrin synthesis prior to ALA formation (Schulman and Richert, '57) and its role as a cofactor in ALA synthesis was established in isolated systems (Kikuchi et al., '58; Gibson et al., '58). Succinyl coenzyme A is derived from the citric acid cycle within erythrocytes since ALA and protoporphyrin are formed *in vitro* from such succinate precursors as α-ketoglutarate, citrate, and D-isocitrate (Shemin and Kumin, '52; Granick, '58; Brown, '58).

Several of the cofactors (α-lipoic acid, coenzyme A, diphosphopyridine nucleotide [DPN]) that are involved in the formation of succinyl coenzyme A from intermediates of the citric acid cycle have been found to stimulate ALA formation by red cell preparations (Gibson et al., '58; Brown, '58; Granick, '58). Furthermore, normal Tetrahymena vorax cells formed more porphyrin from glycine than cells deficient in pyridoxal, pantothenic acid, niacin, thiamine, lipoic acid or riboflavin (Lascelles, '57). However, the formation of porphyrin from ALA was the same in normal and deficient cells (except riboflavin), suggesting that these vitamins were involved in the formation of ALA.

Animals deficient in these vitamins sometimes become anemic. The purpose of our previous studies was to see whether heme synthesis *in vitro* was retarded in red cells derived from vitamin-deficient ducks; if true, this might provide a theoretical basis for the anemia. The incorporation of glycine into heme by blood from ducklings deficient in vitamin B_6 and pantothenic acid was decreased (Schulman and Richert, '57). However, it was found later that the reticulocyte count was also decreased in the blood of the deficient animals (Richert and Schulman, '59). Similarly, blood from niacin-, riboflavin-, and thiamine-deficient ducks also contained fewer reticulocytes and had a lower rate of heme synthesis. Only in the case of vitamin B_6 deficiency was it possible to implicate the coenzyme form of the vitamin, i.e., pyridoxal-5′-phosphate, in the process of heme synthesis, since the *in vitro* addition of pyridoxal-5′-phosphate to the deficient blood stimulated heme formation from labeled glycine. Since the synthesis of heme from glycine takes place mostly, if not entirely, in immature erythrocytes, and since stimulation by the missing coenzyme form of the vitamin failed in all cases except pyridoxal-5′-phosphate, this approach was not suitable for further study of the effects of B vitamins on heme synthesis.

Since the administration of phenylhydrazine to normal ducks increases the number of immature erythrocytes and the synthesis of both heme and globin (Kassenaar et al., '57), it was of interest to determine whether a reticulocytosis could be induced in the vitamin-deficient ducks by this hemolytic agent. This was found to be the case and thus favorable condi-

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[2] The abbreviation ALA is used for δ-aminolevulinic acid, DPN for diphosphopyridine nucleotide and FAD for flavin adenine dinucleotide.

tions were provided for studying the rates of heme synthesis in reticulocyte-rich blood from vitamin-deficient ducks.

PROCEDURE

The composition of the synthetic control diet is given in table 1. Each vitamin-deficient diet was prepared in the same way except that the appropriate vitamin was omitted.

Day-old White Pekin ducklings were fed a commercial starter diet[3] 4 or 5 days and then either the vitamin-deficient or control diets for 9 to 12 days. Six of the 12 birds in each group were injected subcutaneously with 1.4 mg of phenylhydrazine (1% solution in 0.9% NaCl) per 100 gm of body weight on two successive days, and the ducklings were killed on the 4th day following the first injection.

Jugular blood was collected in heparin under ether anesthesia. Hemoglobin was determined by the method of Schultze and Elvehjem ('34); total erythrocyte counts were made in Gower's solution; microhematocrits were measured in sealed capillary tubes, and blood smears were stained by the procedure of Manwell and Feigelson ('48). All measurements, with the exception of ALA biosynthesis, were made on blood from individual birds. Samples from two or more birds were pooled when necessary to obtain sufficient blood for the study of ALA formation. All measurements were made on the day of sacrifice.

TABLE 1

Composition of the basal diet

Constituent	Amount
	gm
Corn starch[1]	53.1
Casein[2]	25.0
Gelatin	10.0
Corn oil[3]	3.5
Choline chloride	0.2
Inositol	0.1
Salts[4]	8.1
	mg
Thiamine	0.5
Riboflavin	0.8
Pantothenate	2.5
Pyridoxine·HCl	0.4
Niacin	12.0
p-Aminobenzoic acid	2.0
Mixed tocopherols (34%)	7.2
Folic acid	0.2
Menadione	0.05
Biotin	0.02
Vitamin B_{12}	0.005

[1] Argo.
[2] Labco.
[3] Mazola.
[4] The salt mixture, based on the one described by Reid et al. ('56), consisted of 481 gm $CaCO_3$, 1428 gm $CaHPO_4$, 180 gm KCl, 172.8 gm $MgSO_4 \cdot 7H_2O$, 150 gm NaCl, 24.3 gm $MnSO_4 \cdot H_2O$, 48 gm iron citrate powder, 0.39 gm $CuSO_4$, 1.2 gm $ZnCl_2$, 1.2 gm KI, 60 mg $Na_2MoO_4 \cdot 2H_2O$ and 15 mg $CoCl_2 \cdot 6H_2O$.

The incorporation of glycine-2-C^{14} into heme was measured as follows: 2 ml of whole blood were incubated at 37° in air in a Dubnoff shaker with 20 μmoles of glycine-2-C^{14} (22,500 cpm per μmole) and 0.1 mg of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. The incorporation of glycine into heme by erythocytes from phenylhydrazine-treated ducks is enhanced by iron (Kassenaar et al., '57). After two hours the vessels were chilled, 5 ml of hemoglobin carrier (725 mg of commercial hemoglobin in 0.9% NaCl) were added, hemin crystals were prepared from hot glacial acetic acid by a modified Fischer ('55) procedure (Vogel et al., '60) and counted as described previously (Schulman and Richert, '57).

Measurement of ALA biosynthesis in washed insoluble particles of red cell hemolysates (Gibson et al., '58; Brown, '58) was made by the procedure described elsewhere (Vogel et al., '60). Briefly, the particulate fraction was prepared by the procedure of Laver et al. ('58), except that the entire insoluble mass was retained. It was suspended in 0.1 M phosphate buffer, pH 6.8, with which it was diluted to half the volume of the original blood sample. Two milliliters of the suspension, equivalent to 4 ml of blood (Brown, '58) were incubated at 37° for two hours with 37 mg of glycine, 146 mg of sodium citrate·$2H_2O$, 50 mg of $MgCl_2 \cdot 2H_2O$, 0.25 mg of pyridoxal-5′-phosphate·H_2O[4], 1 mg of coenzyme A, 1 mg of DPN·$4H_2O$, 1 mg of α-lipoic acid, 1.7 mg of glutamine, and sufficient buffer to bring the total volume to 6 ml. Determination of ALA was made by

[3] Purina Duck Startena, Ralston Purina Company, St. Louis.
[4] Pyridoxal phosphate and thiamine pyrophosphate were obtained from Nutritional Biochemicals Corporation, Cleveland; coenzyme A from Pabst Laboratories, Milwaukee; and α-lipoic acid, DPN (Cozymase "90") and FAD (80%) from Sigma Chemical Company.

the procedure described by Gibson et al. ('58).

RESULTS

Peripheral erythrocytes

As found previously, the reticulocyte counts were low in the various vitamin-deficient ducks not treated with phenylhydrazine (2.4 to 4.5% compared with 6.9 to 10.2% in the controls) (table 2). Total erythrocyte counts were not decreased. Following the administration of phenylhydrazine, the reticulocyte counts were increased to 35% or more in both the control and the vitamin-deficient ducks. Although the high degree of reticulocytosis made it appear that the deficient animals were capable of producing normal numbers of cells, this was not so. While the control ducks treated with phenylhydrazine had hemoglobin levels of over 9% and about two million erythrocytes per mm^3, the hemoglobin and cell counts were significantly below this for all the deficient animals except those lacking niacin. On the basis of body weight the latter group was not as deficient as those fed the other diets; this may have accounted for the relatively small decreases in hemoglobin and erythrocytes. In the other deficiencies, differences in cell counts and hemoglobin values between the deficient and control ducks (also treated with phenylhydrazine) were significant at $P < 0.01$ except for the hemoglobin data in thiamine deficiency and the red cell counts in riboflavin deficiency; in these instances the values still differed distinctly from the control but at $P = 0.03$.

The mean cell volumes of the erythrocytes from phenylhydrazine-treated ducks, whether vitamin-deficient or not, were increased over those from the non-treated ducks. The mean cell hemoglobin concentrations were decreased. This was probably due to the presence of the large numbers of immature cells. Small cells were found only in the vitamin B_6-deficient blood, the mean cell volume being 123 μ^3. Hegsted and Rao ('45) reported a microcytic anemia in vitamin B_6-deficient ducks. The mean cell hemoglobin concentration also fell to low levels in some of their birds. Under our conditions the mean cell hemoglobin concentration was not significantly low, although the 23.9% value was slightly less than 25.2 and 26.2% found in the control groups.

It is concluded that the deficient ducks were incapable of producing comparatively normal numbers of erythrocytes under the stress of a hemolytic agent. Since the mean cell volumes and mean cell hemoglobin concentrations were not decreased and heme synthesis was not impaired under these conditions, except in vitamin B_6 deficiency (see below), it appears that the difficulty in maintaining normal blood hemoglobin concentrations by the deficient ducks was not the synthesis of heme *per se*, but rather the production of normal numbers of erythrocytes; the main hematologic defect appeared to be an inability to form enough cells to compensate for the destruction by the drug.

The low reticulocyte counts in the deficient ducks not treated with phenylhydrazine were at first thought to result from an inability of the ducks to form or release young cells into the peripheral blood. The results with phenylhydrazine administration show that the deficient ducks produced fewer erythrocytes than the control ducks. However, even with the reduced capacity for synthesizing new cells, more reticulocytes appeared following the treatment with phenylhydrazine than would have been necessary to maintain a normal level of about 10% in the non-treated deficient ducks. For example, 0.2 million reticulocytes per mm^3 of blood would provide the difference between a control 10% and a deficient 2% reticulocyte count if the erythrocyte count were 2.5 million per mm^3 of blood. The amounts of immature cells found following the administration of phenylhydrazine (erythrocyte count $\times$ % reticulocytes) were 0.63, 0.82, 0.42, 0.60 and 0.92 million per mm^3, respectively, in the niacin-, riboflavin-, thiamine-, pantothenic acid-, and vitamin B_6-deficient ducks.

The fall in reticulocyte counts in the deficient ducks might be related to the retarded growth which results in a diminished expansion of blood volume and therefore a diminished need for new cell formation. Alt ('38) found that the loss of weight by young rats given a diet either deficient in trypotophan or in total food energy was accompanied by a rapid de-

TABLE 2

Effect of the administration of phenylhydrazine on the red blood cells of some B vitamin-deficient ducklings[1]

Diet	Phenyl-hydrazine	Body[1] weight	Hemoglobin	Hematocrit	Reticulocytes	Erythrocytes	Mean corpuscular Hb concentration	Mean corpuscular volume
		gm	%	%	%	*millions/mm³*	%	μ^3
Control 1	−	399	10.9 ± 0.14[2]	35.5 ± 0.13	10.2 ± 0.80	2.48 ± 0.07	30.7	143
Control 1	+	366	9.1 ± 0.21	35.8 ± 0.96	37.5 ± 1.02	1.99 ± 0.12	25.4	180
Control 2	−	376	11.1 ± 0.42	33.7 ± 1.27	6.9 ± 0.90	2.24 ± 0.09	33.0	150
Control 2	+	356	9.2 ± 0.31	35.0 ± 1.36	44.8 ± 2.46	2.28 ± 0.06	26.2	154
Niacin-deficient	−	249	11.1 ± 0.20	34.5 ± 0.81	2.8 ± 0.22	2.31 ± 0.11	32.1	149
Niacin-deficient	+	249	8.4 ± 0.31	31.3 ± 1.03	34.3 ± 2.23	1.85 ± 0.23	26.8	169
Riboflavin-deficient	−	199	10.8 ± 0.45	32.2 ± 1.62	4.5 ± 0.43	2.40 ± 0.16	33.5	134
Riboflavin-deficient	+	184	5.5 ± 0.25	23.2 ± 1.23	52.3 ± 1.37	1.58 ± 0.10	23.7[3]	147
Thiamin-deficient	−	185	13.4 ± 0.18	38.2 ± 2.13	2.4 ± 0.40	2.82 ± 0.11	35.0	135
Thiamine-deficient	+	188	7.1 ± 0.83	25.2 ± 2.41	36.4 ± 7.40	1.61 ± 0.19	28.1	157
Pantothenic acid-deficient	−	201	10.0 ± 0.55	30.0 ± 1.51	3.0 ± 0.73	2.21 ± 0.06	33.3	136
Pantothenic acid-deficient	+	223	6.1 ± 0.32	22.3 ± 1.38	43.8 ± 2.32	1.37 ± 0.11	27.3	163
Vitamin B_6-deficient	−	137	10.0 ± 0.24	30.2 ± 0.52	2.7 ± 0.46	2.58 ± 0.16	33.1	117
Vitamin B_6-deficient	+	140	4.3 ± 0.70	18.0 ± 1.91	63.2 ± 5.19	1.46 ± 0.12	23.9	123

[1] Six ducks were used in each experiment. Control 1, niacin-deficient, and riboflavin-deficient groups were run simultaneously. These ducks were maintained on Purina Duck Startena for 5 days and then fed the synthetic diets for 9 days. Control group 2 and the thiamine-, pantothenic acid-, and vitamin B_6-deficient groups were started together and were fed the Startena for 4 days. The synthetic diets were fed for 11, 12, 12 and 9 days respectively. The body weights were taken on the day of sacrifice.

[2] Standard error of the mean.

[3] In a separate trial, using 12 riboflavin-deficient ducks, the mean corpuscular hemoglobin concentration was 26.9% and therefore comparable with the control values.

crease in the reticulocyte level in the blood. Rats receiving the diet deficient in tryptophan showed a normal reticulocyte response to bleeding, indicating that the reticulocytopenia was not primarily related to faulty nutrition in the hemopoietic system. Von Euler and Malmberg ('39) also found a fall in the reticulocyte count in young rats fed diets deficient in vitamin A or in the vitamin B complex. When vitamin A was added to the diet of the vitamin A-deficient rats, the reticulocytes increased along with growth.

Heme synthesis

Except in vitamin B_6 deficiency, the radioactive glycine was incorporated into heme as well by the bloods derived from the phenylhydrazine-treated deficient ducks as by the treated controls (table 3). The specific activities of the hemin crystals are recorded in column 1. Since hemoglobin concentrations of the incubated bloods (and therefore total amounts of heme carrier) were variable, radioactivity was calculated for the total amount of hemin in each vessel (from 725 mg of added hemoglobin carrier plus the hemoglobin contained in 2 ml of the whole blood, assuming that hemoglobin yielded 3.94% of hemin). The total radioactive counts are shown in column 2 and are expressed in terms of uniform erythrocyte counts (1 million per mm^3 of blood) and uniform reticulocyte counts (100%) to correct for variations in the number of red cells and immature cells in the various bloods:

$$\frac{\text{cpm/mg hemin} \times \text{total mg hemin}}{\text{RBC count} \times 10^{-6} \times \%\ \text{reticulocytes}} \times 100.$$

The counts in the hemin derived from phenylhydrazine-treated, vitamin B_6-deficient ducks were only 8,500 compared with control values of 24,100 and 18,500. The addition of pyridoxal-5′-phosphate restored the activity to 24,600 cpm. So the vitamin B_6-deficient ducks not only produced fewer erythrocytes, but those that were formed exhibited impaired heme synthesis. In contrast with these results, values of 22,600 to 28,000 cpm were found in the other deficiencies. This indicated that heme synthesis in each cell that was newly formed under conditions of vitamin deprivation was as rapid as in control duck cells produced in response to phenylhydrazine treatment. These values also compared favorably with those obtained with blood from control ducks not treated with phenylhydrazine (20,600 and 28,300 in two groups with 10.2 and 6.9% reticulocytes, respectively). This indicated that phenylhydrazine *per se* did not interfere with the processes of heme synthesis. It might be anticipated that phenylhydrazine would react with the carbonyl group of pyridoxal-5′-phosphate and inhibit heme synthesis, but obviously this was not the case. The only possibility that these comparisons may not be completely valid is that reticulocytes produced with phenylhydrazine appear less mature than the reticulocytes of the non-treated ducks on the basis of appearance and staining characteristics of the cells.

Although the cells that were formed under conditions of vitamin deprivation appeared normal metabolically when evaluated by heme formation from glycine, ALA

TABLE 3

Incorporation of glycine-2-C^{14} into heme following the administration of phenylhydrazine

Diet[1]	Counts per minute per mg hemin	Total cpm per vessel (calculated for one million RBC count and 100% immature cells)
Control 1	503 ± 38	24,100
Control 2	514 ± 28	18,500
Niacin-deficient	485 ± 22	26,900
Riboflavin-deficient	571 ± 17	22,700
Thiamine-deficient	479 ± 73	28,000
Pantothenic acid-deficient	407 ± 33	22,600
Vitamin B_6-deficient	246 ± 50	8,500
Vitamin B_6-deficient + pyridoxal-5′-phosphate, *in vitro*[2]	692 ± 77	24,600

[1] Same groups of animals as in table 2.
[2] 0.25 mg of pyridoxal-5′-phosphate·1 H_2O per vessel.

TABLE 4

δ-Aminolevulinic acid (ALA) synthesis by isolated particles of erythrocytes from ducks treated with phenylhydrazine

Diet	μg ALA/4 ml blood/two hours (calculated for 1 × 10^6 RBC count and 100% immature cells)[1]					
	Regular incubation medium[2]	Additions to (+) or omissions from (−) the incubation medium				
		−Pyridoxal-5′-phosphate	−CoA	+TPP (0.4 mg)	−DPN	+FAD (0.25 mg)
Control 1	326	[3]			296	289
Control 2	240		214	216		
Vitamin B_6-deficient	116	15				
Pantothenic acid-deficient	169		94			
Thiamine-deficient	183			195		
Niacin-deficient	140				138	
Riboflavin-deficient	88					84

[1] These values are expressed on the basis of uniform red cell and immature cell counts because ALA synthesis by particulate fractions prepared from normal blood is dependent upon the reticuloyte concentration of the blood (Vogel et al., '60).

[2] Includes pyridoxal-5′-phosphate, coenzyme A (CoA) and diphosphopyridine nucleotide (DPN), but not thiamine pyrophosphate (TPP) or flavin adenine dinucleotide (FAD).

[3] Preparations from control ducks were not tested without pyridoxal-5′-phosphate in this series. In another experiment the fractions from control ducks treated with phenylhydrazine formed about three times as much ALA with pyridoxal-5′-phosphate as without it.

synthesis was impaired to varying degrees in the insoluble particulate fractions derived from the red cells of the vitamin-deficient ducklings treated with phenylhydrazine (table 4). Impaired heme synthesis from glycine was evident only in the vitamin B_6-deficient cells and in these cells the particulate fractions formed only 15 μg of ALA. This was much less than seen in the other deficiencies and apparently accounts for the impairment in heme synthesis. Addition of pyridoxal-5′-phosphate restored the activity to 116 μg of ALA. This was sufficient to restore heme synthesis by whole cells to control levels, but was still less than half of the amount of ALA formed by fractions from ducks fed the control diet.

Fractions derived from pantothenic acid-deficient blood synthesized 94 μg of ALA in the absence of added coenzyme A. The control blood fractions formed 214 μg. Addition of coenzyme A stimulated the deficient fractions 80% (94 vs. 169) and the control fractions only 12% (214 vs. 240). This suggests that the concentration of coenzyme A as well as that of pyridoxal phosphate was decreased in the vitamin deficiencies; only the pyridoxal phosphate levels were low enough to cause a decrease in the rate at which glycine was incorporated into heme. It is conceivable that a more prolonged pantothenic acid deficiency or the use of another species would decrease the coenzyme A level sufficiently to actually influence heme, and therefore hemoglobin, synthesis. Such an effect might account for the microcytic anemia seen by Carter et al., ('45) in pantothenic acid-deficient rats.

It can also be seen in table 4 that ALA synthesis was impaired in the fractions from niacin- and riboflavin-deficient ducks. The activity was not increased by the addition of DPN or of FAD, respectively. It is possible that the decreased ALA synthesis observed in niacin or riboflavin deficiency was due to a fall in the level of the electron transfer system which is coupled to the functions of the citric acid cycle within erythrocytes. However, since the additions of DPN or FAD were not stimulatory, and since actual measurements of the specific enzyme activities were not made, such conclusions would be premature. Use of caution in the interpretation of the results is indicated by the report of Burch et al. ('56) that DPNH dehydrogenase activities in brain, liver, kidney and heart of riboflavin-deficient rats were remarkably stable even in prolonged omission of riboflavin from the diet.

The lack of correlation between the effect of the deficiencies on ALA synthesis

by isolated particles and on the conversion of glycine to heme by intact cells is not clear. If the rate of ALA formation within the cells were not limiting, then the rate of conversion of glycine to ALA could be decreased without affecting the rate of conversion of glycine to heme.

SUMMARY

Phenylhydrazine was administered to ducklings deficient in niacin, riboflavin, thiamine, pantothenic acid or vitamin B_6 as well as to control ducklings. Both the deficient and control ducks responded to the drug by producing increased percentages of immature red cells in the peripheral blood. The phenylhydrazine treatment produced lower blood hemoglobin concentrations in the vitamin-deficient ducks than in the controls. This could be accounted for primarily by the presence of fewer total red cells in the deficient animals; the mean corpuscular volumes and mean corpuscular hemoglobin concentrations were comparable in both groups treated with phenylhydrazine, except in vitamin B_6 deficiency where the cells were microcytic.

The red cells obtained from phenylhydrazine-treated control and deficient ducks showed comparable rates of heme synthesis *in vitro* from glycine-2-C^{14} except in vitamin B_6 deficiency. In this deficiency heme synthesis was decreased to about one third of the control level, but addition of pyridoxal-5′-phosphate to the deficient cells restored heme synthesis to control values.

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Amino Acid Balance and Imbalance

IV. SPECIFICITY OF THREONINE IN PRODUCING AN IMBALANCE IN DIETS DEFICIENT IN NIACIN AND TRYPTOPHAN[1]

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Amino acid imbalances may be produced in two ways: they may be produced consistently by adding a fairly large amount of a severely unbalanced protein to a diet containing a low to a moderate amount of protein; they may be produced occasionally by adding a relatively small supplement of one or two amino acids other than the most limiting amino acid to a diet that is low in protein (Harper, '58, '59; Salmon, '58). Growth retardation as a result of such dietary additions is taken as evidence of imbalance. In a previous paper in this series, Kumta et al. ('59) reported that amino acid imbalances could be produced in both ways in diets low in fibrin: an imbalance caused by the addition of an amino acid mixture lacking histidine could be demonstrated using a wide range of fibrin levels in the diet; another, involving a very delicate balance among the amino acids in fibrin, resulted from the addition of 0.2% of DL-methionine and 0.3% of DL-phenylalanine and caused a growth retardation which could be corrected only by the addition of leucine, isoleucine, valine and histidine. Further, the growth retardation caused by the addition of methionine and phenylalanine could be demonstrated only with a level of 6% of fibrin in the diet.

Imbalances of both types have also been produced in diets low in casein and lacking niacin. The addition of tryptophan-deficient proteins such as gelatin, acid-hydrolyzed casein, oxidized casein, or amino acid mixtures lacking tryptophan to low casein diets consistently cause imbalances that result in severe growth retardations (Lyman, '51a; Salmon, '54). A more delicate type of imbalance can be demonstrated using niacin-free diets low in casein to which a small quantity of DL-threonine (0.18%) has been added (Hankes, '48). In this imbalance the levels of both tryptophan and threonine are critical. Henderson et al. ('53) using diets containing acid-hydrolyzed casein found that if the level of tryptophan was raised a little above 0.1% of the diet, the addition of threonine would not cause an imbalance, presumably because sufficient tryptophan was available in the body for both protein and niacin synthesis.

There has been no methodical investigation of the specificity of individual amino acids in causing these more delicate imbalances. Hankes et al. ('48) found that if either threonine or phenylalanine were added to a low-casein diet a growth depression would occur, and that threonine was as effective in causing a growth depression when injected intraperitoneally but phenylalanine was not. Lyman ('51a) investigated the growth retarding effects of gelatin and found that certain amino acid mixtures would cause a growth depression. All of these mixtures contained both threonine and phenylalanine as well as a number of other amino acids. As such a small quantity of threonine alone will cause an amino acid imbalance in a niacin-free diet containing a low level of casein the specificity of threonine in producing an imbalance in such diets has been studied. The effects of small additions of other amino acids in increasing the severity of the imbalance by altering the dietary amino acid pattern have also been studied. In all instances threonine was required to produce a growth depression, but the depres-

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sion could be enhanced by the addition of other amino acids.

EXPERIMENTAL

Male weanling rats of the Holtzman strain weighing 40 to 50 gm were used in these experiments. They were housed in individual suspended cages and fed the basal diet ad libitum for two to three days to allow them to adapt to the environment. The animals were then separated into groups of 5 which did not differ in their average initial weights by more than 1 gm. These groups were then offered the various experimental diets ad libitum and weighed three times weekly during the course of the experiment. The percentage composition of the basal diet was as follows:[3] casein, 8.0; DL-methionine, 0.3; salt mixture, 5.0 (Harper, '59); corn oil containing fat soluble vitamins, 5.0; choline chloride, 0.15; water-soluble vitamin mix (less niacin) in sucrose, 0.25 (Harper, '59); and sucrose to make 100. All additions to the diet as indicated in the tables of results were made at the expense of sucrose.

At the end of the two week experimental period the effects of niacin or tryptophan in reversing the growth depression were examined by giving the animals supplements of nicotinic acid or L-tryptophan in the diet, or intraperitoneal injections of a solution containing 250 μg of nicotinic acid on alternate days. The control animals received corresponding injections of a 0.9% NaCl solution and the treatment was continued for one week.

Although growth retardation could be readily demonstrated in rats fed diets containing either 8 or 9% of casein to which gelatin had been added, a comparable retardation did not occur in all animals in groups fed a 9% casein diet to which threonine had been added. Henderson et al. ('53) had indicated that the level of tryptophan in the diet was critical in producing this imbalance; so, to reduce the tryptophan content of the diet and yet maintain an adequate growth rate, the effect of adding threonine to diets containing 7, 8 and 9% of casein was studied. Threonine caused a fairly severe depression in the rate of weight gain of rats fed 7 and 8% casein diets, and as previously observed, those fed the 9% casein diet showed a variable growth response to threonine. Therefore, although diets containing 9% of casein were used in a few instances, 8% casein diets were used in the majority of the experiments in these studies.

The differences in the growth rates of the various control groups perhaps calls for some comment. Low-protein diets of this type have been used in this laboratory for the past 15 years and in experiments run at different times the growth rates of control groups fed the 9% casein diet have ranged from 10 to almost 20 gm per week. Since higher growth rates are accompanied by increased food consumption, it is obvious that some unrecognized factor affecting the food intake of rats fed these low protein diets is not rigidly controlled. As the relative growth rates of similar experimental groups remain the same, such variations do not affect comparisons made within each experiment.

RESULTS

The growth rates of rats fed diets containing 8% of casein and 0.3% of DL-methionine to which had been added 0.36% of DL-threonine or 6% of gelatin are shown in table 1; the effects of supplements of niacin and tryptophan are also shown. Neither niacin nor tryptophan stimulated the growth of rats fed the basal diet, thus indicating that the diet was not primarily deficient in these components. Growth was depressed when either threonine or gelatin was added to the basal diet, and the depression in both cases could be prevented by supplementation with either niacin or tryptophan. The addition of both together did not give any further increase in growth.

A comparison of the effects on growth of additions of 6 and 12% of gelatin to the basal diet is presented in table 2. The growth rates of groups receiving 6 and 12% of gelatin were approximately the same. Niacin, which completely prevented the growth depression caused by the addition of 6% of gelatin, was only partially effective when the diet contained 12% of gelatin. Doubling the niacin content of the diet had no further effect, but the

[3] Crystalline vitamins were kindly provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

addition of tryptophan increased the growth rate substantially.

The possible growth retarding effects of additions of single amino acids to low-casein diets were studied next; an equivalent quantity of each amino acid being studied was added to the basal diet containing 9% of casein. A comparison was first made among the amino acids threonine, phenylalanine, valine and lysine. The results are shown in table 3. Although a retardation of growth was obtained with both 0.2 and 0.4% of DL-threonine, no depression was evident with 0.4% of DL-phenylalanine, L-lysine·HCl or DL-valine.

The other essential amino acids and glutamic acid were also tested separately with 8% casein diets, and the growth rate of each group was compared with that of rats fed a diet containing additional threonine. In an attempt to increase the severity of the imbalance caused by the addition of threonine and in an effort to produce an imbalance with other amino acids, a mixture (A.A. 1) of phenylalanine, lysine and histidine, in the proportions and quantities found in 12% of gelatin, was added with each of the amino acids tested individually. This combination of amino acids was used since in low-casein diets to which methionine, threonine and tryptophan have been added, the amino acids that should be next in the sequence of limiting amino acids (by calculation) are phenylalanine,

TABLE 1

Effect of niacin and tryptophan on the weight gain of rats fed diets containing 8% of casein and 0.3% of DL-*methionine, and 0.36% of* DL-*threonine or 6% of gelatin*

Supplements		Weight gain		
Nicotinic acid	L-tryptophan	Basal	Threonine 0.36%	Gelatin 6.0%
mg %	*%*	*gm/2 weeks*	*gm/2 weeks*	*gm/2 weeks*
—	—	15 ± 11[1]	4 ± 1	5 ± 1.2
2.5	—	13 ± 0.8	35 ± 3	27 ± 1.2
—	0.1	13 ± 1	32 ± 3.1	32 ± 6
2.5	0.1	14 ± 3.5	39 ± 3.1	33 ± 3.1

[1] Standard error of the mean.

TABLE 2

Effect of niacin, tryptophan and gelatin on the body weight-gain of rats fed a diet containing 8% of casein and 0.3% of DL-*methionine*

Supplements		Weight gain		
Nicotinic acid	L-Tryptophan	Basal	6% Gelatin	12% Gelatin
mg %	*%*	*gm/2 weeks*	*gm/2 weeks*	*gm/2 weeks*
—	—	36 ± 1.8[1]	7.8 ± 1.7	5.2 ± 1.9
2.5	—		36.8 ± 3.8	29.4 ± 2.1
5.0	—			22.8 ± 2.1
2.5	0.1			59.6 ± 7.8

[1] Standard error of the mean.

TABLE 3

Growth of rats fed diets containing 9% of casein, lacking in niacin and supplemented with methionine and other amino acids

Group	Supplement	Weight gain
		gm/2 weeks
1	Basal (9% casein–0.3% DL-methionine)	18.8 ± 1.9[1]
2	Basal + 0.2% DL-threonine	7.2 ± 1.1
3	Basal + 0.4% DL-threonine	9.4 ± 2.4
4	Basal + 0.4% DL-phenylalanine	22.6 ± 3.7
5	Basal + 0.4% L-lysine·HCl	20.0 ± 0.6
6	Basal + 0.4% DL-valine	25.4 ± 4.3

[1] Standard error of the mean.

lysine and histidine. The results are shown in table 4. Again, only when threonine was present was the growth rate severely depressed. The depression was somewhat greater when the amino acid mixture was added with threonine. The imbalance produced was corrected by nicotinic acid injection. The average gains, during the third week in which rats received threonine with and without nicotinic acid, were 25.3 gm and 2.3 gm respectively, and for the other groups 15.5 ± 1.2 gm with nicotinic acid and 15.5 ± 1.5 gm without.

As the growth retardation seemed to be more severe when the amino acid mixture was added along with threonine, various levels of mixture A.A. 1 were added with threonine to see whether the severity of the growth retardation increased as the level of this mixture in the diet was increased. The results of the growth studies are shown in table 5. No retardation of growth occurred when this amino acid mixture was added alone at the level of 0.63%, but when it was added in combination with threonine growth was retarded. The degree of severity of growth retardation increased with each increase in the level of the amino acid mixture added up to 1.26%.

For the addition of threonine to cause an imbalance in these low-protein diets a sulfur-containing amino acid must be added (Hankes, '49) but in some studies (Lyman, '51a) cystine seemed to cause a more severe imbalance than methionine. Both were tested under the experimental conditions used here and proved to be

TABLE 4

Effect of various individual amino acids and an amino acid mixture in producing a growth depression in rats fed 8% of casein and 0.3 of DL-*methionine*

Supplemental amino acids, 0.36% level	Weight gain	
	−A.A. 1[1]	+A.A. 1
	gm/2 weeks	*gm/2 weeks*
DL-Threonine	10.4 ± 1.9	6.0 ± 0.7
L-Leucine	25.2 ± 2.1	18.8 ± 2.5
DL-Isoleucine	21.8 ± 3.0	17.2 ± 2.4
DL-Valine	22.0 ± 3.5	17.0 ± 2.2
L-Arginine	18.4 ± 2.5	19.8 ± 3.3
L-Glutamic acid	28.8 ± 1.0	19.6 ± 1.9

[1] A.A. 1 indicates 1.26 gm of amino acid mixture 1 (0.52 gm DL-phenylalanine, 0.6 gm L-lysine·HCl, 0.14 gm L-histidine·HCl).

TABLE 5

Effect of threonine and amino acid mix 1 on the weight gains of rats fed diets containing 8% of casein and supplemented with DL-*methionine or* L-*cystine*

Group	Supplements		Weight gain
	DL-Threonine	Amino acid mix[1]	
			gm/2 weeks
		With 0.3% DL-methionine	
1	—	—	36.6 ± 5.1[2]
2	0.36	—	24.6 ± 6.1
3	—	0.63	35.0 ± 1.5
4	0.36	0.63	18.4 ± 4.3
5	0.36	1.26	5.2 ± 1.7
6	0.36	1.89	8.6 ± 2.4
		With 0.3% L-cystine	
7	0.36	—	27.6 ± 2.8
8	—	0.63	35.2 ± 4.7
9	0.36	0.63	18.0 ± 5.6
10	0.36	1.26	13.0 ± 1.6
11	0.36	1.89	15.0 ± 2.1

[1] A.A. 1 indicates 0.63 gm of amino acid mixture 1 (0.26 gm DL-phenylalanine, 0.3 gm L-lysine·HCl, 0.07 gm L-histidine·HCl), equaling the quantity in 6% gelatin.
[2] Standard error of the mean.

TABLE 6

Effect of amino acid combinations on threonine-induced growth depression in rats fed 8% of casein and 0.3% of methionine

Supplements				Weight gain	
A.A. 1[1]	A.A. 2	A.A. 3	Arginine	−Threonine	+Threonine
%	%	%	%	*gm/2 weeks*	*gm/2 weeks*
—	—	—	—	25.8	14.0
1.26	—	—	—	19.2	12.2
—	1.26	—	—	29.4	12.0
—	—	1.26	—	21.1	10.8
—	0.91	—	—		10.0
—	—	4.93	—		7.4
—	—	—	0.9	24.0	10.0

[1] A.A. 1 indicates amino acid mixture 1 (0.52 gm DL-phenylalanine, 0.6 gm L-lysine·HCl, 0.14 gm L-histidine·HCl); A.A. 2 indicates amino acid mixture 2 (0.38 gm L-leucine, 0.19 gm DL-isoleucine, 0.34 gm DL-valine); A.A. 3 indicates amino acid mixture 3 (1.21 gm L-glutamic acid, 1.09 gm DL-alanine, 2.63 gm glycine), equaling quantities found in 12% of gelatin.

equally effective in diets to which threonine or threonine and a low level of mixture A.A. 1 had been added.

Groups 5 and 6, and 10 and 11, which had received the higher levels of the amino acid mixture were regrouped and given nicotinic acid or tryptophan in the diet and their weight gains determined after 10 days. Since the animals in these groups did not have a high food intake, 5.0 mg% of nicotinic acid or 0.2% of L-tryptophan were added to insure that the animals would get adequate amounts to correct the growth retardation. Growth was restored equally by both supplements, indicating that the severity of these imbalances was less than that of the imbalance with 12% of gelatin, which could only be corrected by tryptophan. The methionine-supplemented animals gained 52.2 ± 3.1 gm per 10 days with nicotinic acid and 50.0 ± 2.1 gm with L-tryptophan. The corresponding weight gains for the cystine-supplemented groups were 48.4 ± 1.9 and 51.6 ± 4, respectively.

Other combinations of amino acids were also tested with threonine to see if a specific effect of certain amino acids in increasing the severity of the imbalance caused by threonine would be detected. The mixtures contained amino acids in amounts equivalent to the amounts of the L-isomers in 12% of gelatin and consisted of amino acid mixture 2 (essential amino acids, L-leucine, DL-isoleucine, DL-valine) and amino acid mixture 3 (non-essential amino acids, L-glutamic acid, DL-alanine, glycine). These mixtures were also added at a level of 1.26 gm/100 gm diet, equivalent to the weight of mixture A.A. 1 used in the previous experiment. Arginine, which is not limiting in the diet, but which is found in high quantities in gelatin was also tested. The results are shown in table 6. Only when threonine was present in the diet was there a marked depression in growth rate. The addition of any of the amino acid combinations slightly increased the depression, although by themselves they did not appear to have any depressing effect. At the end of the two-week growth experiment, a nicotinic acid solution was injected into the animals to correct the imbalance condition. The growth responses for groups with no added threonine in the diet were 15.2 gm per week with nicotinic acid and 16.2 gm per week with saline. For groups with threonine in the diet, however, the growth rates increased after injection of nicotinic acid (18.9 gm per week) but not after injection of saline (2.1 gm per week).

DISCUSSION

The results obtained when 0.36% of DL-threonine and 6% of gelatin were added to diets containing 8 or 9% of casein are in agreement with the results of the earlier studies (Krehl et al., '46; Hankes et al., '48) in that growth was severely retarded by these additions but the retardation could be prevented by the addition of either niacin or tryptophan. That a delicate balance between tryptophan and threonine exists in these diets, as suggested by Henderson et al. ('53) was indicated by the

difficulty in obtaining a consistent depression in growth rate when the basal diet contained 9% of casein. The difficulty in obtaining the imbalance in some animals in a group, while others in the same group showed a severe growth retardation, might be caused by individual differences in pre-experimental intake and storage of niacin.

Addition of 12% of gelatin to low-casein diets caused a growth depression which could not be completely corrected by niacin but only by tryptophan. This observation confirms results obtained by Salmon ('54). In this case utilization of the limiting amino acid tryptophan, may be so severely depressed, as he suggested, that the addition of niacin cannot spare enough tryptophan to completely correct the imbalance.

Phenylalanine, added alone or added as one of the components of amino acid mixture 1 in combination with other amino acids did not cause a retardation of growth. Only when threonine was present with the phenylalanine was the growth rate depressed. Hankes et al. ('49) had suggested that the depression caused when phenylalanine was added to low casein diets was attributable to the D-form. In their studies the additions of 0.204% of D-phenylalanine caused a depression in growth rate. Similar results were not obtained in the present studies when DL-phenylalanine was used at a level of 0.4% of the diet. Lyman et al. ('51b) encountered difficulty in obtaining growth retardation consistently with the addition of phenylalanine to low-casein diets. They attributed the retardation of growth which they obtained, at least in part, to a concurrent deficiency of thiamine.

The present study indicates that the effect of threonine in producing an imbalance of amino acids in low casein diets lacking in niacin is quite specific. Growth was retarded when threonine was added alone to 8% casein diets, and the effect was enhanced somewhat by the addition of any one of several combinations of amino acids. Other amino acids did not cause growth retardations when they were added alone to low casein diets or as components of amino acid mixtures unless threonine was also present.

In these studies, the addition of threonine, which is the second most limiting amino acid in the diet, caused a more severe deficiency of the most limiting amino acid, tryptophan. Other examples of amino acid imbalances produced by the addition of the second most limiting amino acid for growth have been reported. Among these are an imbalance in a diet containing fibrin caused by the addition of methionine and phenylalanine (Deshpande et al., '58); one in a diet containing egg albumen which was primarily deficient in histidine, caused by the addition of lysine (Winje et al., '54); and one in rice diets caused by the addition of lysine (Deshpande et al., '55). A recent study by Rosenberg et al. ('59) indicates that when the levels of threonine and lysine in rice diets are balanced so that both are equally limiting for growth, the further addition of either one may cause a growth depression which can be corrected by supplementation with the other. The effect was most consistent when the growth depression was caused by the addition of too much lysine. Similar observations were made by Koeppe and Henderson ('55) using amino acid diets, in which they were able to alter the second most limiting amino acid in the diet. They found that supplementation with the second most limiting amino acid caused a growth retardation, which could be corrected by a supplement of tryptophan, the most limiting amino acid in the diet.

Although the number of observations is insufficient to warrant any general conclusion, the high degree of specificity of threonine in causing an amino acid imbalance in the present study, together with the observations that amino acid imbalances occur under other conditions when the second limiting amino acid is added to a diet low in protein, suggest that control of food intake (Harper and Kumta, '59), protein synthesis or some equally vital function is particularly sensitive to the balance of the two most limiting amino acids in the diet.

SUMMARY

An imbalance of amino acids can be produced in diets containing 8% of casein and L-cystine or DL-methionine by the addition of 0.36% of DL-threonine. This imbalance causes a depression in the growth rate of rats which can be corrected with

either niacin or tryptophan. In the present studies, of a variety of amino acids tested, only threonine was effective in causing a growth depression. Phenylalanine, previously found to cause growth depression, was not effective, nor were leucine, lysine, valine, isoleucine, glutamic acid or arginine unless they were in combination with threonine.

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The Effect of Dietary Fat and the Repeated Withdrawal of Small Samples of Blood on Plasma Cholesterol Levels in the Rat[1,2]

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Extensive studies of the effects of dietary fat on the blood cholesterol levels of man have produced conclusive evidence that certain fats of terrestrial animal origin are hypercholesteremic, while other fats, mostly of vegetable origin, depress plasma cholesterol values (Ahrens et al., '54; Beveridge et al., '55, '56). The active principles of these fats responsible for this divergence in blood cholesterol response in man have not been isolated and adequately characterized. Investigations leading to such characterization would be greatly facilitated by use of a small test animal the response of which to dietary fat parallels that of the human subject. The present study on the effects of different types of dietary fats on the plasma cholesterol level of the rat was initiated to determine if its response was sufficiently like that of man to permit its use as a test animal.

The literature supplies conflicting claims for the action of dietary fats on the plasma cholesterol level of the rat. Some investigators (Kim et al., '52; Swell et al., '53; Mead et al., '54; Deuel et al., '55; Greenbaum et al., '57; Okey et al., '57; Avignan et al., '58) have reported that increase in the level of fat in the diet leads to altered blood cholesterol levels, while others have found that it was unaffected both by the level and kind of dietary fat (Bose et al., '50; Hegsted et al., '57; Best et al., '58; Buttner et al., '58). Such lack of agreement indicated that the relationship of dietary fat and blood cholesterol level in the rat required further study before a decision on the suitability of the animals could be made. This report deals with the effect of the nature and level of dietary fat on plasma cholesterol levels in the rat, together with some findings on the effect of changing the routine of blood sampling in both frequency and extent. The results may offer some explanation for the conflicting reports quoted above.

EXPERIMENTAL

The basal diet,[4] essentially free from fat, was similar to that used in human studies (Beveridge et al., '56). Protein supplied in the form of skim-milk powder was kept constant at 16.9% of calories. Dietary fat was added to provide 28.4% (low fat) and 58.5% (high fat) of calories, these alterations being made by substituting the fat for an isocaloric amount of carbohydrate in the form of maltose and dextrins.[5] Diets were mixed in 3-kg batches and stored at 5°C in tins with tightly fitting lids. Fresh food was supplied daily so that diets were not exposed to air or the high temperature of the animal room for longer than 24 hours.

Male Sprague-Dawley rats were used in the first 5 experiments; rats from the Holtzman Company were used in the last experiment. The animals were caged individually and allowed food and water ad libitum. On days of blood sampling, food cups were removed 7 hours before bleeding to ensure a fasting period of at least this duration. Blood samples were taken by sectioning the tail using heparin as an

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[3] Present address: Defence Research Board, Kingston Laboratories, Kingston, Ontario.
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[5] Dextri-Maltose.

in vitro anticoagulant. Total plasma cholesterol was determined in all experiments by the method of Sperry and Webb, ('50). Except for the first experiment a micro-modification of this procedure was used which was more suited to the low concentrations of cholesterol in rat plasma.

RESULTS

1. *Effect of the level and nature of dietary fat.* Nine groups of 14 animals each, matched for weight distribution (average body weight about 280 gm) were transferred from a fox chow ration to diets in which butter, beef dripping, coconut oil and corn oil supplied 28.4 or 58.5% of calories. Two diets were supplemented with an amount of cholesterol equal to that supplied by the rations containing butter fat. A group of rats was maintained on fox chow. The resulting changes in plasma cholesterol during the experimental period of 5 weeks are given in table 1, and the data for butter fat, corn oil and fox chow for the first 8 days are plotted in figure 1.

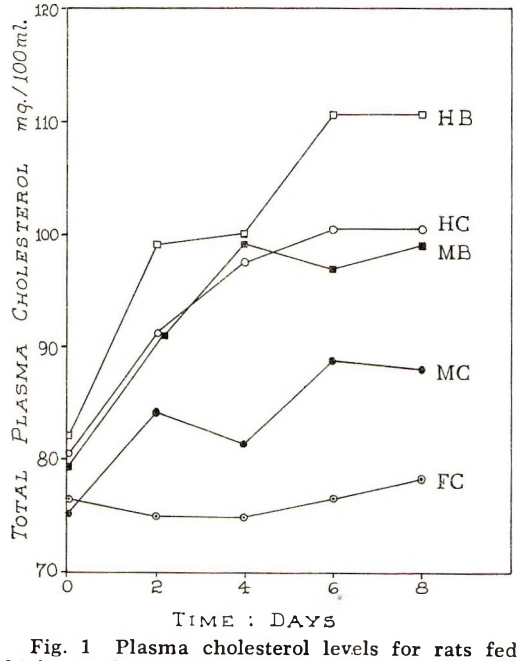

Fig. 1 Plasma cholesterol levels for rats fed high, moderate and low-fat diets during the first 8 days of experiment 1. H.B. and M.B. indicate groups fed diets of 58.5 and 28.4% of calories as butter fat; H.C. and M.C. indicate groups fed the same levels of corn oil and F.C. is the group fed fox chow.

TABLE 1

Effect of dietary fat on the plasma cholesterol level of the rat[1]

Dietary fat	Total calories	Total plasma cholesterol mg/100 ml[2]						
		Day 0	Day 2	Day 4	Day 6	Day 8	Day 14	Day 21	Day 35
	%								
Beef dripping	58.5	81.4 ± 8.6	104.5 ± 13	105.4 ± 10.3	105.3 ± 12.6	106.6 ± 12.7	100 ± 18	99.7 ± 15	97.7 ± 9.2
Butter	58.5	82.3 ± 4.9	99.4 ± 7.4	100.6 ± 11.6	111.2 ± 9.5	111.9 ± 9.1	104.3 ± 8.5	100.8 ± 14	101.9 ± 11
Corn oil	58.5	80.5 ± 6.9	91.4 ± 7.0	97.7 ± 6.4	100.6 ± 5.9	100.8 ± 9.6	103.1 ± 11.6	106.9 ± 12.1	96.5 ± 12.3
Coconut oil	58.5	72.3 ± 9.5	97.9 ± 12	96.3 ± 9.8	91.7 ± 12	101.7 ± 13	97.9 ± 6.8	100.1 ± 9.4	99.6 ± 11
Butter	28.4	79.9 ± 7.2	91.3 ± 12.2	99.5 ± 8.8	97.5 ± 11.9	99.0 ± 10.9	87.0 ± 10.2	90.0 ± 7.3	89.7 ± 11.5
Corn oil	28.4	75.7 ± 9.3	84.4 ± 8.9	81.2 ± 8.0	89.0 ± 8.1	88.2 ± 8.3	94.2 ± 17	90.0 ± 7.6	86.2 ± 9.9
Corn oil + cholesterol[3]	58.5	82.7 ± 8.2	89.4 ± 8.2	99.5 ± 12	99.5 ± 12	105.2 ± 11	102.6 ± 7.7	101.1 ± 10.3	94.4 ± 10.8
Corn oil + cholesterol[3]	28.4	75.5 ± 10.7	83.7 ± 8.3	89.0 ± 10.2	93.5 ± 7.5	87.2 ± 9.1	95.6 ± 6.2	93.0 ± 11.5	92.4 ± 7.4
Fox chow	—	76.4 ± 9.5	75.1 ± 6.7	74.8 + 8.3	76.4 ± 10	78.5 ± 8.7	74.1 ± 9.4	78.3 ± 8.2	76.6 ± 7.8

[1] Groups of 14 male rats each were fed diets in which fat supplied 28.4 and 58.5% of total calories. Plasma cholesterol values for the diet period of 35 days are compared with a control group fed fox chow. Blood samples of 0.6 to 0.8 ml were taken after fasting for 7 hours with analysis by the standard procedure of Sperry and Webb ('50).

[2] Mean and standard deviation.

[3] Cholesterol added to equal intake in butter rations.

With diets high in animal fat, the plasma cholesterol level rose to a peak value within the first week, decreasing somewhat at two weeks and remaining essentially constant thereafter. Using diets high in vegetable fat, increases occurred more slowly, but by the third week the responses were indistinguishable. A similar pattern was seen in the case of the rations providing 28.4% of calories as fat but the degree of hypercholesterolemia was less. Supplementation of the vegetable fat with the amount of cholesterol provided by butter did not alter this pattern.

Analysis of variance of the plasma cholesterol values of the rats fed butter and corn oil at high-fat intakes revealed that the values of the former group were significantly higher ($P < 0.01$) at the 6th day of the feeding period. On low-fat intakes, a significant difference was observed also on the 6th day. These differences in response to butter and corn oil had disappeared by the 14th day.

Results appear to indicate that, contrary to observations in human trials, both butter fat and corn oil produce an increase in rat plasma cholesterol, but differ significantly from each other in that butter produces a more rapid increase. It was felt that this property, although not parallel to the results in man, might be utilized in an assay aimed to elucidate the hypercholesterolemic factors in butter. This attempted application is described in the following section.

2. *Effect of butter fat fractions on rat plasma cholesterol.* Butter fat was separated into the following fractions: unsaponifiable matter, fatty acids, saturated and unsaturated triglycerides. The first two fractions were obtained using the method of Heilbron et al. ('32); the latter two by the low-temperature precipitation of saturated triglycerides from acetone according to Hilditch et al. ('38). The fat fractions were fed in amounts equivalent to that supplied by a ration providing 28.4% of calories as butter.

Since the unsaponifiable matter supplied no calories as fat and since, at the time this work was performed, we knew of no fat that could be unequivocally classed as being "neutral," it was decided to use the unsaponifiable matter by recombining appropriate amounts with part of the free fatty acids. This permitted a test of whether the process of fractionation had destroyed the hypercholesterolemic factor in the original butter fat.

In table 2 the mean plasma cholesterol of the 6 groups of 10 rats at day zero and 6 are recorded. It is pertinent to point out that because of the use of the microprocedure for cholesterol analysis, blood samples not exceeding 0.3 ml were withdrawn. Contrary to the results found in experiment 1, none of the diets produced an increase in the plasma cholesterol levels. The only difference in the conditions of these two experiments that appeared to offer any rational basis of explanation for the ap-

TABLE 2

Total plasma cholesterol value of rats fed butter fractions[1]

Group	Diet	Total plasma cholesterol mg/100 ml		
		Day 0	Day 6	Significance[2]
1	Butter	69.4 ± 11.9	77.7 ± 8.2	Non-sig.
2	Corn oil	72.1 ± 9.5	78.8 ± 9.8	Non-sig.
3	Butter fatty acids	73.8 ± 10.6	81.1 ± 11.1	Non-sig.
4	Butter saturated triglycerides	68.2 ± 10.9	77.2 ± 10.6	Non-sig.
5	Butter unsaturated triglycerides	81.5 ± 11.0	76.5 ± 16.1	Non-sig.
6	Butter unsaponifiable	69.5 ± 8.0	73.4 ± 13.7	Non-sig.

[1] Groups of 10 rats each were fed diets supplying 28.5% of calories as fat for 6 days after being fed fox chow for two weeks. Blood samples of 0.3 ml were taken in the fasting state on day 0 and day 6 with analysis for total plasma cholesterol by micromodification of the method of Sperry and Webb. Groups 1 and 2 were fed butter and corn oil respectively and served as controls for the remaining groups, fed fractions of butter fat. For groups 3, 4 and 5, the butter fractions were incorporated into the diets as directly substituting on an equal weight basis for the butter control diet. Group 7 consisted of the unsaponifiable (0.3%) and the fatty acids of butter.

[2] Significance by analysis of variance.

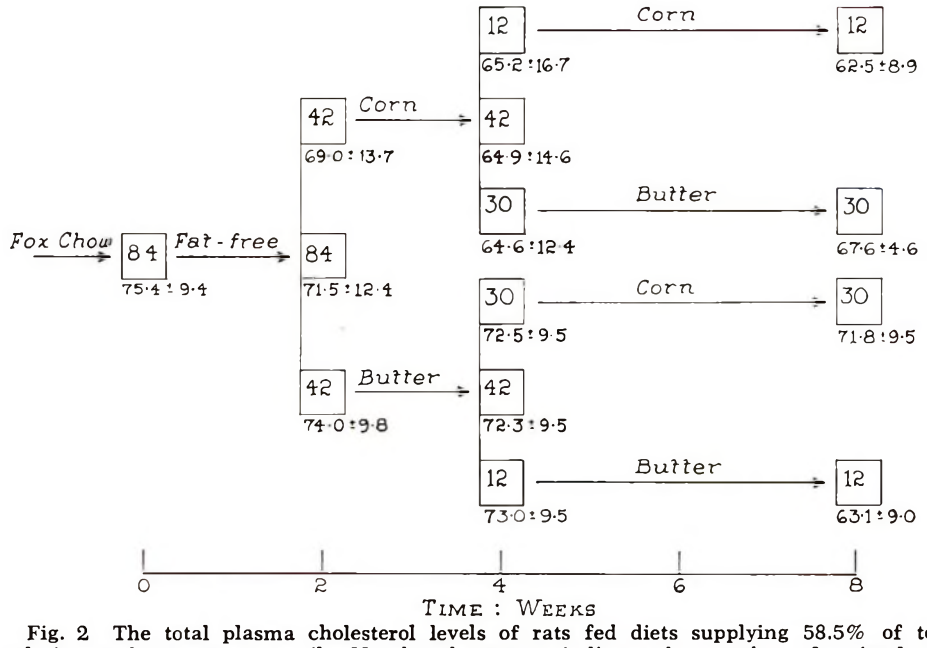

Fig. 2 The total plasma cholesterol levels of rats fed diets supplying 58.5% of total calories as butter or corn oil. Numbered squares indicate the number of animals per group; the mean and standard deviation of the plasma cholesterol level in mg/100 ml are shown beneath the squares. Duration of diet periods in weeks is proportional to scale. Sequence of the introduction of diets, identified by the arrows, proceeds from left to right. Subdivision of the groups is indicated by vertical displacement.

parently divergent results, was the difference in the extent and frequency of blood sampling. This was at first, regarded as unlikely, since no reports could be found in the literature concerning hemorrhage causing lipemia in the rat similar to that described for the rabbit and guinea pig.

Blood samples of 0.6 to 0.8 ml were taken from animals in the first experiment on alternate days for the first 8 days. In the second experiment only 0.3 ml was taken on day 0 and day 6. This result made it necessary to re-examine the effect of dietary fat on plasma cholesterol levels in the rat under widely differing routines of blood sampling.

3. *Dietary fat and plasma cholesterol with low bleeding stress.* Eighty-four rats of average weight, (440 gm), were placed for two weeks on the fat-free basal diet before dividing them into two equal groups when they were given rations providing 58.5% of total calories in the form of butter fat or corn oil. At the conclusion of two weeks' feeding, 12 animals fed each diet were allowed to continue for a period of 6 weeks. The remaining 30 animals were changed for 4 weeks to rations containing the other fat. The blood samples, not exceeding 0.3 ml were taken at intervals of two weeks. Further bleeding after the sample had been taken was effectively controlled by application of a pressure pad. The experimental design and plasma cholesterol values are given in figure 2.

The differences in the means were tested by analysis of variance. None of the differences observed was significant. Thus, neither high-corn-oil nor high-butterfat diets produced a detectable change in the plasma cholesterol levels. Also, the nature of the antecedent diet did not alter the response, nor was there any detectable trend in period of feeding for two to 6 weeks.

4. *Dietary fat and plasma cholesterol with moderate bleeding stress.* Rats of the previous experiment of average weight, 463 gm, were distributed randomly into three groups of 20 animals each. One

group was placed on a fat-free diet, the others on rations providing 58.5% of total calories from butter or corn oil. After two weeks, blood samples of 0.6 and 0.8 ml were withdrawn. This procedure was repeated on the second, 4th and 6th days. The plasma cholesterol values are shown in table 3.

The animals on high-fat diets did not show significantly different plasma cholesterol values from those on a fat-free diet during the first two weeks of feeding. However, during the period when blood was taken on alternate days, the plasma cholesterol values of the groups on high fat diets rose steadily and at the 4th bleeding highly significant increases were observed. Those on a fat-free diet did not show any significant change throughout this period.

5. *Dietary fat and plasma cholesterol with high bleeding stress in young and old rats.* Three groups of 15 rats, average weight 450 gm, over a year in age, and three groups of 14 rats, average weight 205 gm and about two-months old, were placed on one of the following dietary regimens: butter fat (58.5% Cal.), corn oil (58.5% Cal.) fat-free in the case of the old rats, and fox chow in the case of the younger ones.

Blood samples amounting to 2.5 to 4% of blood volume, 1.2 to 1.4 ml and 0.6 to 0.8 ml for the old and young animals respectively, were taken on day 0, 2, 4 and 6. Plasma cholesterol values for day 0 and day 6 are reported in table 4.

In the case of the old rats, plasma cholesterol values of the groups fed butter or corn oil rose from 62.2 and 67.2 mg/100 ml to 141.2 and 150.1 mg/100 ml respectively by day 6. These increases were highly significant but there was no significant difference between the effects of the two fats on cholesterol levels. Smaller but highly significant increases were also noted in the case of the young animals fed the high-fat rations. Since the extent of bleeding was approximately equivalent for both age groups, apparently young animals resist the development of this type of hypercholesterolemia. One must qualify this conclusion, however, because of the fact that the old rats were obtained from the Sprague-Dawley colony and the young animals from the Holtzman colony, both of Madison, Wisconsin.

The group of young rats fed the fox chow diet also responded with a highly significant increase which may be contrasted with the result obtained in experiment 1, no 1. Presumably the disparity in

TABLE 3

The effect of moderate bleeding stress and high-fat diet on the plasma cholesterol of the rat[1]

Diet	Mean total plasma cholesterol in mg/100 ml				
	Day 14	Day 16	Day 18	Day 20	Significance
Fat-free	71.8 ± 11.7	70.9 ± 11.7	72.0 ± 11.1	78.8 ± 15.7	Non-sig.
High butter fat	74.7 ± 9.7	77.0 ± 10.9	83.6 ± 13.5	97.8 ± 19.6	Highly sig.
High corn oil	73.7 ± 11.1	79.3 ± 19.5	85.0 ± 12.4	96.6 ± 10.7	Highly sig.

[1] Two groups of 20 rats each were fed diets supplying 58.5% of calories as butter fat or corn oil, one group was fed the fat-free diet. Animals were given diets for 14 days before bleeding when 0.6 to 0.8 ml of blood was withdrawn on alternate days for the ensuing 6 days. Significance of the difference of plasma cholesterol values on the 14th and 20th day was calculated by analysis of variance.

TABLE 4

Dietary fat and plasma cholesterol with high bleeding stress in young and old rats

Diet	Plasma cholesterol mg/100 ml			
	Old rats		Young rats	
	Day 0	Day 6	Day 0	Day 6
Butter fat	62.2 ± 19.5	141.2 ± 25.2	82.7 ± 10.4	105.4 ± 11.3
Corn oil	67.2 ± 14.3	150.1 ± 18.0	83.1 ± 13.6	105.5 ± 10.8
Fat-free	67.6 ± 12.8	107.7 ± 27.2	—	—
Fox chow	—	—	81.8 ± 13.01	97.3 ± 8.0

extent of bleeding accounts for the difference. That this factor is of great importance is obvious from a consideration of the results from the group of old rats fed the fat-free basal diet. In comparison with the previous experiment in which this diet was used, on this occasion a highly significant increase in plasma cholesterol occurred. This response indicates that dietary fat is not a prerequisite for the production of a hypercholesterolemia if the repetitive bleeding is sufficiently severe.

DISCUSSION

In the absence of bleeding stress, as in experiments 2 and 3, where the volume of blood taken was small and the period between sampling was kept as long as feasible experimentally, there was no indication that any of the fats used had an influence on the plasma cholesterol level of the rat. These results are in agreement with those of Bose and Subrahmanyan, ('50), Best et al. ('58) and others, but do not agree with those reported by Swell and Flick, ('53) and others, (Deuel et al., '55; Mead et al., '54).

The increase in plasma cholesterol of the rat in response to high bleeding stress and high fat diets has not, to the authors' knowledge, been previously reported. It is in some respect similar to the elevation in all plasma lipids which can be observed following repeated blood-letting in certain species, namely, the rabbit (Boggs et al., '09; Horiuchi, '20) and the guinea pig (Bloor, '21). However, since other lipids than cholesterol were not estimated, the term "lipemia" has not been applied to the response of the rat. Until the involvement of other lipid fractions of the blood is established, the term "hypercholesterolemia" is more appropriate.

The mechanism of the hypercholesterolemia is at present unknown, but some information on the phenomenon has been obtained. From experiments 4 and 5, the plasma cholesterol increase is shown as being proportional to the degree of bleeding stress imposed. From experiments 1, 4 and 5 the degree of elevation of the plasma cholesterol is shown to be dependent upon the fat level in the diet, but not upon the type of fat. However, as demonstrated in experiment 5 under conditions of high bleeding stress, even the presence of dietary fat is not obligatory for the hypercholesterolemic response. Spitzer et al. ('55) has recently investigated the lipemia produced by repetitive bleeding in the rabbit, and has concluded that the response is not due to a decrease in red blood cells, a decrease in plasma volume, or a change in the level or distribution of albumin or globulin of the plasma. He was able to demonstrate the lack or absence of "clearing factor" from the serum of serially bled rabbits after heparin injections. Associated also was an increase in the lipoproteins of the S_f74-300 class. No mention is made of any increase in the plasma cholesterol but presumably this occurred. This finding would indicate that the abnormality in the blood of serially-bled animals is centered either in the lipo-protein lipase system described by Korn, ('55, '55a) or in the accessory "acceptor factors" for the fatty acids liberated by this enzyme.

There would appear to be a difference between the rabbit and rat. Spitzer's rabbits were bled approximately 10 to 15% of their blood volume on successive days in order to elicit the response. The rats of this study did not lose more than 4% of their blood volume by bleeding on alternate days. Spitzer also described the rabbit as recovering from the lipemia in about the same length of time required to induce it. From the work of experiment 1 the increases in plasma cholesterol due to the combined effect of bleeding and the consumption of fat were maintained for 4 weeks after the termination of the initial period of bleeding stress. Although no substantiated explanation of this phenomenon can be advanced at the present time, the authors wish to draw it to the attention of other workers in this field as a possible explanation for some of the conflicting reports on the effect of dietary fat on the plasma cholesterol level of the rat.

SUMMARY

The influence of the nature and level of dietary fat on the plasma cholesterol level of the white rat has been investigated, with emphasis placed on the response to butter fat and corn oil. With diets varying in fat content from 0.6 to 58.5% of total calories, there was essen-

tially no effect on plasma cholesterol level. When blood samples amounting to 1 to 1.5% of the total blood volume were taken on alternate days for 6 days, there occurred a highly significant increase in plasma cholesterol in all groups fed rations containing fat. When the degree of hemorrhage was increased to 2.5 to 4% of the total blood volume, even rats fed a fat-free diet responded with increases in plasma cholesterol. Young rats were more resistant to the hypercholesterolemic effect of hemorrhage than old rats.

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Sequence in which Indispensable and Dispensable Amino Acids Become Limiting for Growth of Rats Fed Diets Low in Fibrin[1]

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Detailed information is needed about the sequence in which the amino acids of the proteins used in studies of amino acid balance and imbalance become limiting for growth. The sequence can be calculated from a knowledge of the amino acid requirements of the experimental animal and the amino acid composition of the dietary protein but this method has definite limitations. Inadequate information about the biological availability of amino acids, the limited accuracy of some of the values for the amino acid compositions of proteins and the limited accuracy of some of the measurements of amino acid requirements are among the sources of error (see also Bender, '54). Therefore it is important that any calculated sequence be confirmed by the results of growth experiments. The sequence in which the amino acids of casein become limiting for the growth of the rat, as determined experimentally, differs from that determined by calculation (Harper, '59a).

Since a complex amino acid imbalance involving 6 amino acids was studied using diets containing low levels of fibrin (Deshpande et al., '58a,b; Kumta and Harper, '58; Harper and Kumta, '59), the sequence in which the amino acids in this protein become limiting for the growth of the rat has been determined experimentally and is reported below. In a previous study (Kumta and Harper, '60) the growth of rats was suboptimal when fed a diet containing 6% of fibrin as the only source of protein, but supplemented to contain the indispensable amino acids in quantities sufficient to meet the accepted amino acid requirements. Growth appeared to be limited by a lack of some other amino acid or acids. Similar results were obtained when rats were fed a diet containing 6% of casein supplemented with indispensable amino acids and only when a supplement of dispensable amino acids such as glutamic acid, glycine, alanine and aspartic acid was provided was the gain in weight optimal (Harper, '59a). Therefore, the effects of adding various dispensable amino acids to diets containing low levels of fibrin have also been determined and in addition the effects of adding such nonspecific nitrogen sources as ammonium acetate, ammonium citrate and urea. These substances are known to stimulate the growth of rats fed diets containing only the indispensable amino acids (Rose et al., '49; Lardy and Feldott, '50; Frost and Sandy, '51; and Rechcigl et al., '57).

EXPERIMENTAL

Male weanling rats of the Holtzman strain, weighing 40 to 50 gm were used in these experiments. The rats were housed in individual suspended cages and fed the basal diet (described below) ad libitum. After a preliminary period of 4 to 5 days to allow the rats to adapt to the new environment, those which showed a progressive gain in weight were selected and divided into groups of 5 rats each. The average initial weights of the groups did not differ by more than one gram. These groups were then fed the various experimental diets ad libitum and were weighed

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at least twice weekly during the two-week experimental period.

The basal diet used was essentially that described earlier (Kumta et al., '58). This contained fibrin, 6; salt mixture, 5 (Harper, '59b); corn oil, 5; vitamin supplements,[2] 0.25 (Harper, '59b); choline chloride, 0.15 and dextrin as the carbohydrate to make up 100%. All diets were refrigerated. Additions of amino acids and nonspecific nitrogen compounds, as indicated in the tables of results, were compensated for by adjusting the percentage of dextrin.

RESULTS

An estimate of the sequence in which the amino acids in fibrin become limiting for growth is presented in table 1. Methionine and phenylalanine are apparently most limiting, followed by histidine, valine, leucine, lysine and isoleucine, in that order. Threonine is limiting only to the extent of 18% whereas neither tryptophan nor arginine appear to be limiting in diets containing 6% of fibrin. However, it appeared from growth experiments that leucine, isoleucine, valine and histidine were all equally limiting for the growing rat and that methionine and phenylalanine were somewhat less limiting (Deshpande et al., '58a). If the amounts of cystine and tyrosine in fibrin are included in the calculations, then, methionine plus cystine and phenylalanine plus tyrosine become limiting to the extent of 58 and 35%, respectively.

The sequence in which the amino acids in diets containing 6% of fibrin become limiting for the growth of the rat can be determined from the results presented in table 2. When methionine and phenylalanine are added to the basal diet containing 6% of fibrin, the rate of gain is depressed and this is prevented only when leucine, isoleucine, valine and histidine are included in the diet. This indicates, as shown previously (Deshpande et al., '58a,b; Kumta et al., '58), that the addition of methionine and phenylalanine causes an amino acid imbalance. That leucine, isoleucine, valine and histidine and not methionine and phenylalanine are the most limiting amino acids is evident from the weight gains of groups 10 and 11. The omission of leucine, isoleucine, valine and histidine from the complete mixture of amino acids caused a greater depression of weight gain than the omission of methionine and phenylalanine.

The position in the sequence occupied by lysine can also be determined from the results reported in table 2. Supplementation of the diet containing the 6 limiting amino acids with lysine caused further gain in weight (group 4) and a complete mixture of amino acids, including arginine, threonine, tryptophan and glutamic acid (group 5), but from which lysine was omitted, caused no growth response. Further, when lysine was included and each of the amino acids arginine, threonine,

[2] Crystalline vitamins were kindly provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

TABLE 1

Calculation of sequence in which amino acids become limiting for growth of the rat when 6% of fibrin is the source of dietary protein

	Amino acid requirements[1]	Amino acids in 6 gm fibrin[2]	Deficit
	gm/100 gm diet		%
Methionine (cystine)	0.6	0.14(0.25)	75(58)
Phenylalanine (tyrosine)	0.9	0.25(0.58)	72(35)
Histidine	0.4	0.16	60
Valine	0.7	0.31	55
Leucine	0.8	0.40	50
Lysine	1.0	0.53	47
Isoleucine	0.5	0.28	44
Threonine	0.5	0.41	18
Tryptophan	0.2	0.21	—
Arginine	0.2	0.41	—

[1] Rose et al. ('49).
[2] Data obtained from Block and Bolling ('51).

TABLE 2

Growth of rats fed diets containing 6% of fibrin supplemented with various amino acids

Group	Amino acids added to diets containing 6% fibrin											Gain in weight
	DL-Methionine 0.4%	DL-Phenylalanine 0.6%	L-Leucine 0.4%	DL-Isoleucine 0.4%	DL-Valine 0.6%	L-Histidine 0.2%	L-Lysine 0.5%	L-Arginine 0.1%	DL-Threonine 0.3%	DL-Tryptophan 0.1%	L-Glutamic acid 1%	
												gm/2 weeks
1	+	+	+	+	+	+	+	+	+	+	+	35 ± 3.2[1]
2	+	+	+	+	+	+	+	+	+	+	+	20.8 ± 2.4
3	+	+	+	+	+	+	+	+	+	+	+	38 ± 5.4
4	+	+	+	+	+	+	+	+	+	+	+	51 ± 5.8
5	+	+	+	+	+	+	+	+	+	+	+	41 ± 2.7
6	+	+	+	+	+	+	+	+	+	+	+	50.4 ± 5.5
7	+	+	+	+	+	+	+	+	+	+	+	55 ± 3.6
8	+	+	+	+	+	+	+	+	+	+	+	53.4 ± 4.9
9	+	+	+	+	+	+	+	+	+	+	+	48.2 ± 3.5
10	+	+	+	+	+	+	+	+	+	+	+	10.4 ± 1.8
11	+	+	+	+	+	+	+	+	+	+	+	21.4 ± 1.5

[1] Standard error of the mean for 5 rats.

tryptophan and glutamic acid was omitted in turn, growth was not retarded, an indication that threonine, arginine, tryptophan and glutamic acid did not limit the growth of the rats fed these diets.

The question of whether the cystine and the tyrosine content of fibrin should be taken into account in calculating the sequence in which the amino acids in this protein become limiting for growth is important because they can be substituted for part of the requirement for methionine and phenylalanine (Womack and Rose, '34, '41; Armstrong, '55; Grau and Steele, '54). The results in table 3 show that the addition of cystine and tyrosine, unlike that of methionine and phenylalanine, to a diet containing leucine, isoleucine, valine and histidine, did not stimulate growth. Also, the addition of cystine and tyrosine to the basal diet did not cause a growth depression such as is typically observed when methionine and phenylalanine are added to this diet.

Attempts made to devise a diet containing only 6% of fibrin and which would support a rate of growth equivalent to that of rats fed a high-protein diet are summarized in table 4. The percentages of some of the indispensable amino acids were increased above those used earlier on the basis of the results of experiments not reported in which the effects of graded levels of several of the indispensable amino acids were tested. Each amino acid was included at the concentration that gave maximum growth response yet the greatest weight gain was only 62 gm in two weeks. Increasing the quantity of the entire amino acid mixture by 33% was ineffective in augmenting growth (group 3). However, when a mixture of amino acids consisting of glutamic acid, glycine, aspartic acid and alanine was added the rate of gain increased. Further experiments (group 6) showed that addition of 2% of glutamic acid alone was as effective as the addition of the mixture. If the least limiting amino acids, arginine, tryptophan, and threonine were omitted from the diet glutamic acid was without effect (group 10).

The results of experiments in which 3% of glutamic acid was replaced by isonitrogenous quantities of ammonium acetate, ammonium citrate or urea are presented in table 5. The addition of each of these

TABLE 3

Effects of adding methionine and phenylalanine or cystine and tyrosine to a diet containing 6% of fibrin

	Amino acids added					Gain in weight
	DL-Methionine 0.4%	DL-Phenylalanine 0.6%	L-Cystine 0.4%	L-Tyrosine 0.6%	Amino acid mixture[1]	
						gm/2 weeks
1	−	−	−	−	−	31.8 ± 0.7[2]
2	+	+	−	−	−	17.4 ± 1.2
3	−	−	+	+	−	30.2 ± 2.5
4	+	+	−	−	+	50.4 ± 1.7
5	−	−	+	+	+	27.2 ± 2.2

[1] This contained L-leucine, 0.4%; DL-isoleucine, 0.4%; DL-valine, 0.6%; and L-histidine, 0.2%.

[2] Standard error of the mean for 5 rats.

compounds was beneficial (compare groups 4, 5, and 6 with group 2) but none was as effective as glutamic acid. A supplement of ammonium acetate and α-ketoglutaric acid (group 7) was as effective as glutamic acid. This effect was obtained in two separate experiments but 0.4% of α-ketoglutaric acid was less effective.

DISCUSSION

The results of the present experiments and of those with diets containing casein (Harper, '59a) amply demonstrate the importance of confirming by feeding experiments the reliability of calculations of the sequence in which the amino acids in a protein become limiting for growth. The methods based on chemical analyses are certainly satisfactory for determining the most limiting amino acid in many proteins as has been amply proven by the relatively high correlation between chemical score and biological value (Block and Mitchell, '46; Mitchell, '54; Bender, '54). This is particularly true for unbalanced proteins in which one or two amino acids are usually present in relatively low concentrations. In contrast, in well balanced proteins such as albumin and fibrin, and among the amino acids that are not obviously low in proteins such as casein and wheat gluten, there is no sharp distinction in the order in which the amino acids become limiting and much greater accuracy is required to predict the sequence from analytical data. Deviations from the theoretical sequence have been observed by several authors who have used different biological methods (Bender, '54; Winje et al., '54; Deshpande et al., '55; Harper, '59a; Longenecker and Hause, '59).

The results presented here also illustrate how the sequence calculated from amino acid composition differs from that determined by growth experiments. Thus methionine and phenylalanine which were calculated to be the most limiting amino acids do not occupy this position. Instead, leucine, isoleucine, valine and histidine are most limiting. If the maximum percentage deficits of the amino acids in 6% fibrin are calculated from the amino acid requirements recently reported by Rama Rao et al. ('59), the various indispensable amino acids become limiting in the following order: methionine, 72; phenylalanine, 65; isoleucine, 50; leucine, 42; valine, 43; histidine, 20; and threonine, 18. Clearly, this sequence also does not coincide with the sequence determined from growth experiments.

The failure of cystine and tyrosine to give responses comparable to those obtained with methionine and phenylalanine, respectively, under the conditions of these experiments raises another difficult problem in amino acid supplementation studies. To what extent are methionine and phenylalanine spared by cystine and tyrosine, respectively, when the dietary levels of methionine and phenylalanine are well below the minimal requirements? Without such information the accuracy of calculations of the deficits of methionine and phenylalanine in low protein diets is limited, and the true values might fall anywhere between the two extremes shown in table 1. This problem is also encountered in esti-

TABLE 4

Attempts to obtain optimum growth of rats by supplementing diets containing 6% of fibrin with amino acids

Group	Amino acids added to diets containing 6% fibrin												Gain in weight
	DL-Methionine 0.4%	DL-Phenylalanine 0.6%	L-Leucine 0.6%	DL-Isoleucine 0.4%	DL-Valine 0.4%	L-Histidine 0.4%	L-Lysine 0.6%	L-Arginine 0.2%	DL-Threonine 0.4%	DL-Tryptophan 0.2%	Amino acid mixture[1]	L-Glutamic acid	
													gm/2 weeks
1	−	−	−	−	−	−	−	−	−	−	−	−	31.2 ± 2.2[2]
2	+	+	+	+	+	+	+	+	+	+	−	−	62.0 ± 4.2
3	+[3]	+	+	+	+	+	+	+	+	+	−	−	60.4 ± 5.3
4	+	+	+	+	+	+	+	+	+	+	+	−	79.2 ± 2.3
5	+	+	+	+	+	+	+	+	+	+	−	1.0%	67.2 ± 4.5
6	+	+	+	+	+	+	+	+	+	+	−	2.0%	82.4 ± 3.1
7	+	+	+	+	+	+	+	+	+	+	−	4.0%	75.6 ± 2.5
8	+	+	+	+	+	+	+	−	−	−	−	−	50.6 ± 4.3
9	+	+	+	+	+	+	+	−	−	−	−	−	64.6 ± 2.0
10	+	+	+	+	+	+	+	−	−	−	−	2.0%	63.2 ± 4.3

[1] This contained L-glutamic acid, 1.5%; glycine, 0.5%; DL-aspartic acid, 0.5%; and DL-alanine, 0.5%.

[2] Standard error of the mean for 5 rats.

[3] ++ indicates level of each amino acid increased by 33%.

mating nutritive values of proteins by the Chemical Score method.

The growth promoting effects of dispensable amino acids in diets containing low levels of fibrin and casein merit special attention. Although a growth response to glutamic acid has been noted in experiments in which diets containing only amino acids were used (Rose et al., '49; Rechcigl et al., '57), no optimum ratio of dispensable to indispensable amino acids has been established. An estimate of this ratio can be made from the relative proportions of the two groups of amino acids in the diets used in the present study. Diets containing 12% of fibrin provide 6.2 gm of dispensable amino acids and 5.6 gm of indispensable amino acids, whereas diets simulating 12% fibrin (6% fibrin and indispensable amino acids) contain 3.1% of dispensable amino acids and 6.8% of indispensable amino acids. Since with the latter diets, optimum growth is obtained only when 2% of the dispensable amino acids are added, it would seem that the dispensable amino acid content of the diet must be between 35 and 40% for optimum growth. Similar figures have been obtained for both rats and chicks fed diets in which amino acids have been substituted for protein.[3] It is interesting to note, however, that in diets containing fibrin, the growth response to glutamic acid is not obtained unless the least limiting amino acids, threonine, arginine and tryptophan are added.

The greater effectiveness of glutamic acid than ammonium salts observed in the present experiments is in agreement with previous observations of Rose et al. ('49) and Rechcigl et al. ('57). Glutamic acid would probably serve as a better source than ammonium salts for the net synthesis of dispensable amino acids. Whether glutamic acid spares some of the indispensable amino acids from being deaminated, or replenishes the supply of keto acids for active transamination and protein synthesis remains to be investigated. The growth response obtained with low levels of α-ketoglutaric acid (0.2%) in diets containing ammonium acetate (group 7, table

[3] Stucki, W. P., A. E. Harper and C. A. Elvehjem 1960 The importance of dispensable amino acids for normal growth of chicks. Federation Proc., *19:* 12 (abstract).

TABLE 5

Comparative growth response of rats fed diets containing 6% of fibrin supplemented with nonspecific nitrogenous sources isonitrogenous with 3% of glutamic acid

Group	6% Fibrin	Amino acid mixture[1]	Additions	Gain in weight
				gm/2 weeks
1	+	−	—	34.8 ± 1.6[2]
2	+	+	—	59.6 ± 3.7
3	+	+	3% Glutamic acid	78.8 ± 3.0
4	+	+	1.75% Ammonium acetate	65.6 ± 4.2
5	+	+	2.32% Diammonium citrate	71.2 ± 3.6
6	+	+	0.61% Urea	67.8 ± 2.9
7	+	+	1.75% Ammonium acetate + 0.2% L-ketoglutaric acid	78.6 ± 3.3
8	+	+	1.75% Ammonium acetate + 0.4% α-ketoglutaric acid	70.4 ± 3.8
9	+	−	6% Fibrin	77.6 ± 2.1

[1] This contained in percentage: DL-methionine, 0.4; DL-phenylalanine, 0.6; L-leucine, 0.6; DL-isoleucine, 0.4; DL-valine, 0.4; L-histidine, 0.4; L-lysine·HCl, 0.6; L-arginine·HCl, 0.2; DL-threonine, 0.4; and DL-tryptophan, 0.2.

[2] Standard error of mean for 5 rats.

5) tends to support the latter alternative. However, as higher levels of α-ketoglutaric acid (0.4%) were less effective further experiments are necessary to explore this possibility.

SUMMARY

The results of growth experiments indicate that in diets containing 6% of fibrin as the only source of protein leucine, isoleucine, valine and histidine are all equally the most limiting amino acids for the growth of the rat; methionine and phenylalanine are equally next limiting; lysine is the third limiting amino acid and threonine, arginine and tryptophan are least limiting.

Diets containing 6% of fibrin supplemented with a mixture of the indispensable amino acids to satisfy the accepted amino acid requirements of the rat do not support optimal growth. Addition of a mixture of dispensable amino acids containing glutamic acid, glycine, aspartic acid and alanine or addition of glutamic acid alone stimulates growth considerably.

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The Cariogenic Property of Cereal Foods[1,2]

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The development of smooth surface caries in white rats fed a diet consisting principally of 4 cereal foods and 18% of commercial glucose[3] was reported by McClure ('52). These particular cereal foods were rye bread and white bread (dried); and rolled oats and yellow corn grits (cooked and dried). In a later study, McClure ('58) reported that wheat flour when autoclaved with the commercial glucose and white bread after toasting, showed increased cariogenicity. A dietary supplement of 2.0% L-lysine significantly decreased the cariogenic effect of diets containing the processed cereal foods (McClure, '55, '58).

This prior evidence concerning a relation of cereal foods to experimental dental caries prompted this current survey of the cariogenic properties of 4 common cereals, namely, wheat, corn, rice and oats. Each cereal was fed in its natural state, and also in three processed forms which are commercial products.

EXPERIMENTAL

The experimental rats were the Sprague-Dawley strain, bred in this laboratory from a colony which originated from National Institutes of Health breeding stock. Thirty rats, weighing 40 to 55 gm, were placed on each diet at weaning. Littermates, without regard to sex, were assigned to the 4 forms of each cereal. The animals were housed two per cage and received water and food ad libitum. The food intakes were recorded twice a week, and the rats weighed weekly. The studies on the wheat cereals deviated from this plan in that due to the poor survival of the first group of animals, the experiment was repeated. However, the data from the two studies did not differ significantly and are combined.

The cereals studied were as follows:

1. Natural raw wheat, Wheatena (enriched), Wheaties (enriched) and Shredded Wheat Biscuit.
2. Raw yellow corn, yellow corn meal (enriched), corn flakes (enriched) and Corn Kix (enriched).
3. Raw "natural" rice, "Minute" rice (enriched), Rice Krispies (enriched) and Rice Chex (enriched).
4. Raw oats, "Mother's" rolled oats (regular, enriched), "Mother's" rolled oats (quick, enriched) and Cheerios (enriched).

A 5th series of experiments provided a direct comparison of raw wheat, corn, rice and oats by using litter-mated rats.

All of the cereal foods were finely ground before mixing in the diets. The diets were fed dry. The cereal food was the only variable component except for a double quantity of liver powder added at the expense of glucose in the rice diets to more nearly equalize the protein content. With this exception, the diets contained 18% of commercial glucose, 2.0% of liver powder, 78.52% of the cereal food, 0.88% of $CaHPO_4$ and 0.60% of $CaCO_3$. A vitamin A, D and E supplement was given orally to each rat once a week. The diets were analyzed for nitrogen, ash, calcium, phosphorus and lysine. The nitrogen was determined by the micro Kjeldahl method, lysine by microbiological assay, and calcium and phosphorus by standard procedures (Kirk, '50; Gee and Dietz, '53). The analyses of the diets appear in table 1.

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[3] Cerelose.

TABLE 1

Analyses of diets

Cereal components	Protein (N × 6.25)	Lysine	Ash	Ca	P	Ca/P
	%	*mg/gm*	%	%	%	
Wheat						
Raw—whole grain	10.2	2.8	2.77	0.49	0.54	0.91
Wheatena	12.2	3.2	5.04	0.66	0.48	1.38
Wheaties	10.5	2.2	2.78	0.63	0.54	1.17
Shredded Wheat	9.6	2.8	2.92	0.53	0.57	0.93
Corn						
Raw—whole grain	9.1	2.4	2.40	0.49	0.50	0.98
Corn meal	8.2	1.6	1.70	0.50	0.32	1.56
Corn flakes	8.8	1.5	2.70	0.50	0.31	1.61
Corn Kix	9.1	1.7	2.47	0.50	0.36	1.39
Rice						
Raw—whole	8.9	3.4	2.28	0.51	0.47	1.05
Minute Rice	9.0	3.7	1.86	0.59	0.35	1.69
Rice Krispies	8.0	2.3	3.48	0.55	0.33	1.36
Rice Chex	6.6	2.1	3.17	0.53	0.30	1.77
Oats						
Raw—whole	10.6	3.8	3.52	0.58	0.51	1.14
Mother's—regular	15.0	5.3	2.75	0.54	0.57	0.95
Mother's—quick	15.6	5.3	2.85	0.56	0.58	0.97
Cheerios—puffed	11.9	3.4	4.78	0.67	0.54	1.06
Raw cereals						
Wheat	9.1	2.9	2.54	0.54	0.50	1.08
Corn	7.7	2.4	2.21	0.44	0.42	1.05
Rice	8.9	3.6	2.35	0.45	0.48	0.94
Oats	10.6	3.8	3.29	0.47	0.52	0.90

The protein content of all diets was low. As a group, the rice diets had the lowest protein content, 6.6 to 9.0%; the oat diets the highest, 10.6 to 15.6%; corn and wheat diets had intermediate values. As determined by microbiological assay, the lysine content of the oat diets was the highest, 3.4 to 5.3 mg/gm; of the corn diets the lowest, 1.5 to 2.4 mg/gm. The lysine in the rice and wheat diets was intermediate. The Ca/P ratio varied from 0.91 to 1.77.

After 60 days on experiment, the rats were killed and the heads autoclaved to facilitate removal of the jaws. The lower molar teeth were examined for smooth surface caries as described by McClure ('58). As will be noted, some occlusal or fissure caries were observed.

RESULTS

In table 2 are the data showing the food intake, average daily gain and dental caries experience which occurred as a result of these different cereal-diet regimens. The percentage of carious rats per group, the number of carious teeth per rat, smooth surface and fissure caries severity scores per rat, describe this caries experience. Of the 4 cereals and their food products, wheat and its three commercial foods were most cariogenic, followed by corn, rice and oats and their products. Since this comparison of the 4 cereals and their products was not based on littermated rats, these results may be open to question. To substantiate this comparison, a 5th series of experiments utilized litter-mated rats to compare directly raw wheat, corn, rice and oats. In this case, the wheat was again the most cariogenic in terms of smooth surface caries severity, followed by corn flour and "natural" rice flour which were approximately the same. Oat flour was least cariogenic (see table 2). The rice flour developed the most occlusal caries.

Special interest in the planning of this study pertained to the processed foods particularly as compared with the raw cereals. The caries data, in general, do not indicate any increased cariogenic ef-

TABLE 2

Effect of high cereal diets on development of dental caries—percentage of carious rats; average number carious teeth; smooth surface caries severity score and fissure caries severity score per rat; average growth rate and food intake

Diets	Rats	Carious rats	Av. no. carious teeth/rat	Caries severity score/rat		Av. growth	Av. food intake
				Smooth surface	Fissure		
	No.	%				*gm/rat/day*	*gm/rat/day*
Wheat							
Raw—whole grain	51	100.0	4.8	11.5 ± 0.82[1]	0.08 ± 0.04[1]	0.78	8.3
Wheatena	44	97.7	3.7	8.1 ± 1.13	1.1 ± 0.29	0.36	6.2
Wheaties	47	89.4	3.7	8.9 ± 1.45	1.4 ± 0.37	0.07	4.5
Shredded Wheat	48	75.0	2.4	5.0 ± 0.99	0.7 ± 0.26	1.00	9.1
Corn							
Raw—whole grain	31	87.9	2.6	3.9 ± 0.59	0.8 ± 0.19	0.82	8.1
Corn meal	34	77.8	2.7	3.3 ± 0.76	1.5 ± 0.48	0.42	6.3
Corn flakes	32	79.4	2.2	4.1 ± 0.76	0.2 ± 0.12	0.06	3.9
Corn Kix	32	82.4	1.9	2.7 ± 0.57	0.1 ± 0.09	0.12	4.2
Rice							
Raw—whole grain	29	93.1	3.5	3.2 ± 0.67	2.4 ± 0.49	1.47	9.6
Minute Rice	30	46.7	0.6	0.4 ± 0.11	0.3 ± 0.12	1.14	8.8
Rice Krispies	30	73.3	2.0	3.0 ± 0.70	0.3 ± 0.15	0.17	4.3
Rice Chex	29	69.0	1.9	2.7 ± 0.49	0.4 ± 0.20	0.47	5.8
Oats							
Raw—whole	28	39.3	0.6	0.7 ± 0.24	0	0.84	7.8
Mother's—regular	27	48.1	1.4	1.9 ± 0.71	0.4 ± 0.28	0.93	7.7
Mother's—quick	28	67.8	2.6	4.2 ± 0.96	0.3 ± 0.14	1.21	7.4
Cheerios—puffed	30	53.3	1.2	2.1 ± 0.70	0	1.01	6.6
Raw cereals							
Wheat	30	96.7	3.4	6.5 ± 0.83	0.4 ± 0.14	0.83	7.7
Corn	32	87.5	3.1	4.1 ± 0.73	1.3 ± 0.41	0.71	7.5
Rice	32	100.0	4.3	4.8 ± 0.83	3.2 ± 0.44	1.25	8.9
Oats	31	67.7	1.6	2.4 ± 0.55	0.3 ± 0.03	0.82	7.6

[1] Standard error.

fect due to commercial processing. Thus, shredded wheat produced the least caries in the wheat series. In comparison with whole wheat, this difference in caries was statistically significant at the 1% level. In comparison with Wheaties and Wheatena, the difference was probably significant at the 5% level. Our result with shredded wheat agrees with the previous observation by McClure ('58), namely, that this processed cereal did not show increased cariogenicity over and above whole wheat flour.

In the corn series, although there were no significant differences in surface caries severity scores, the processed Corn Kix diet was the least cariogenic.

The rice-fed rats' severity scores were all low for smooth surface caries. Minute rice, processed for quick cooking, resulted in an average severity score of less than 1.0, which was significantly lower than the other three scores of this series.

The oat cereals, excepting for "Quick Mother's" oats, resulted in relatively low severity scores (much of the order of the rice series) and the raw oats diet resulted in the lowest severity. The difference in caries produced by the raw form versus "Quick Mother's" oats, was highly significant (1%). Between the raw product and the regular "Mother's" oats, and Cheerios (puffed), the caries produced was statistically different at the 5% level of significance. The difference in caries produced by the less-processed form of "Mother's" oatmeal over the highly processed was significant at the 5% level.

There was an irregular development of fissure caries in the rats receiving these diets (table 2). The greatest occurrence was with the raw rice diets. The corn, corn meal, Wheatena and Wheaties diets were also responsible for fissure caries but to a lesser extent. Both fissure and smooth surface caries were present in 50 rats fed the raw rice diets, and in 24 rats receiving the raw corn diets.

The rate of growth with these various diets varied considerably and was subnormal (table 2). The diet intakes also varied widely, and in general paralleled the weight gains. Rate of growth was most uniform in the oats series. Inspection of the protein level and the lysine content of the diets indicates little relation to the rate of growth. Incidence of caries and severity scores was not reflected in weight gains.

DISCUSSION

A lysine deficiency of these heat-processed cereal foods was anticipated as a possible cariogenic factor in light of previous studies (McClure, '55, '58). However, no consistent loss of lysine due to processing was apparent nor was there an increase of caries severity with the processing. This problem of lysine deficiency could be answered for these diets satisfactorily only by a direct observation on the caries inhibitory effect of a lysine supplement in the diet.

A factor which was somewhat variable among the different cereal foods was their physical characteristics. Although all were ground to a fine powder, their potential for retention on tooth surfaces may have differed and could possibly explain a variation in cariogenicity. With respect to the development of fissure caries, the relative hardness and the size of particles was most likely a determining factor.

Attention is called to the relatively low cariogenicity of the oat diets because of the observation by Taketa and Phillips ('57) that oat hulls contain an anticariogenic factor for the cotton rat. It was observed also that rats receiving the raw oat diets had abnormally-developed mandibular bones, characterized by an extensive outward bowing of the shelf of the bone where the teeth are attached. The jaws were not examined prior to autoclaving and thus there was no evaluation of the condition of the periodontal attachment.

The cariogenicity of the diets which contained the natural raw cereals in which no effect of commercial processing was involved is of interest, particularly in the case of wheat. Further studies are being undertaken to throw light particularly on wheat flour and its caries potential.

SUMMARY

Albino rats were maintained on high cereal diets of raw wheat, corn, rice, oats and three processed counterparts of these 4 grains.

Smooth surface caries were produced on all diets. There was no indication that the processed cereals produced increased caries, incidence or severity.

The smooth surface caries developed by these cereal foods was in decreasing order: wheat, corn and rice, then oats. This was true in general of the raw as well as processed foods. The relatively extensive cariogenic property of wheat and its products is noted.

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Effect of Hepatic Coccidiosis Infection in Rabbits on Tissue Levels of Vitamins A and E[1]

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Although a voluminous literature exists concerning the effect of various vitamins on the course of infections, relatively little is known about the effect of infectious diseases on the vitamin status of the host. A few reports indicate that parasitic infections may lead to a serious depletion of vitamin A reserves. Very low vitamin A levels were found in chicks with coccidiosis (Davies, '52), in lungworm-infected cattle and guinea pigs (Soliman, '53) and in *Ascaridia galli*-infested poultry (Pande and Krishnamurty, '59). It has been reported from this laboratory that rabbits with hepatic coccidiosis, kept on a vitamin E-free diet but supplied with normally sufficient oral supplements of α-tocopheryl acetate, had extremely low tissue tocopherol levels and that some showed symptoms of muscular dystrophy.[2] In order to investigate further the interrelationship of hepatic coccidiosis and vitamin status of the rabbit, vitamin E and A levels of infected animals kept on regular rabbit chow have been studied. In view of the very widespread occurrence of liver coccidiosis among commercially raised rabbits, such an investigation appeared valuable.

METHODS

Twenty-six White New Zealand rabbits of mixed sex, approximately 4 weeks old when purchased, were placed individually in wire cages and fed commercial pelleted rabbit chow ad libitum. This diet contained 900 I.U. of vitamin A, 3.5 mg of carotene, 0.8 mg of α-tocopherol and 2.2 gm of total fats per 100 gm. Nine animals were infected with sporulated *Eimeria stiedae* oocysts by stomach tube. Intentionally-infected and non-infected animals were kept in separate rooms. The animals died or were killed at one to 10 weeks after arrival. Tissue samples were stored in the deep freeze until analyzed for vitamins E and A.

Rabbits were found frequently to harbor *E. stiedae* even if not intentionally infected. Therefore, in reporting the results of the vitamin analyses, the animals were classified on the basis of microscopic examination of the gallbladder contents and gross appearance of the liver. Group A includes rabbits with liver lesions and with oocysts and merozoites in the gallbladder. Of 11 animals in this group 7 had been intentionally infected with *E. stiedae*. The other 4 animals were either infected when purchased or were infected in the laboratory regardless of the precautions taken. Rabbits in group B harbored the merozoite stage of *E. stiedae* but showed neither liver lesions nor oocysts. Two of the 6 animals in this group had been intentionally infected. Rabbits in group C exhibited no signs of coccidiosis infection. No animal in this group had been intentionally infected.

Two of the rabbits in group A died, one at 4 weeks, the other at 8 weeks after intentional infection. Liver coccidiosis appeared to be the cause of death in both cases. No other diseases were noticed during the course of the experiment in any of the rabbits. Food consumption was approximately the same in infected and non-infected animals. Average weight gain was 35 gm per day in all three groups. Only in the terminal stage of the disease, a few days before death, did the

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[1] This investigation was aided by research grant no. A-3294 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.
[2] Diehl, J. F. 1959 Vitamin E deficiency in hepatic coccidiosis of the rabbit. Federation Proc., *18:* 523 (abstract).

infected animals of group A take less food and stop gaining weight or actually lose weight. All animals in groups B and C gained weight up to the day they were killed. Rabbits of different age and purchased from different dealers were distributed through all three groups. Differences in average vitamin levels of the three groups therefore can not be due to age differences or to previous dietary history.

Total tocopherols in liver and skeletal muscle were determined on 10-gm samples by the method of Swick and Bauman ('52). The procedure described by Ames et al. ('54) was used in the determination of vitamin A in livers, using 5-gm samples of tissue. Dry weight and lipid content of livers were determined by lyophilization of liver homogenate and petrolether extraction in the Soxhlet apparatus for 4 hours.

RESULTS

Vitamin E. The data presented in table 1, column 1, indicate that the average tocopherol content per gram of liver was lowered in coccidiosis-infected animals to about one third of normal, even if no gross liver damage was seen at necropsy. Analysis of variance (Snedecor, '56) shows that this decrease is highly significant. As livers of coccidiosis-infected rabbits sometimes become enlarged, it appeared desirable to take liver and body weights into consideration. The second column in table 1 shows the ratio of total liver-tocopherol content to body weight. Again, a highly significant decrease was seen in animals of groups B and A. Within group A, the rabbits which showed severe liver damage at necropsy invariably were found to have a very low liver-tocopherol content. Within group B, no clear correlation was found between number of merozoites in the gallbladder and tocopherol content of the liver.

Tocopherol content per gram of dry liver and per gram of liver lipid (table 2) was also much lower in infected than in non-infected rabbits. The tocopherol content of skeletal muscle of 4 non-infected rabbits was 5.0, 7.7, 3.3 and 5.6 μg per gm of tissue (average, 5.4 μg per gm) and of 4 infected rabbits of group A was 2.7, 3.4, 2.5 and 2.5 μg per gm (average 2.8 μg per gm).

Vitamin A. Non-infected rabbits (group C) had such widely differing liver vitamin A levels that it is difficult to recognize a possible effect of infection on the vitamin A status of the rabbits. By analysis of variance, the decrease of average liver vitamin A levels in infected animals shown in table 1 is not statistically significant. The lower minimum and the lower maximum vitamin A levels, however, were found in infected animals. Within group A, rabbits with extensive liver damage always exhibited low vitamin A reserves. When expressed on a dry weight and lipid weight basis (table 2), liver vitamin A values again appeared low in infected animals. Liver lipid and

TABLE 1

Average liver tocopherol and vitamin A content (± standard deviation) of coccidiosis-infected and non-infected rabbits

	Tocopherol /gm liver	Tocopherol in whole liver/100 gm body weight	Vitamin A /gm liver	Vitamin A in whole liver/100 gm body weight
	μg	μg	I.U.	I.U.
Group A, 11 infected animals with liver lesions	4.0 ± 2.8 (0–7.6)[1]	19.9 ± 18.9 (0–55.6)	62 ± 29 (26–114)	305 ± 165 (117–585)
Group B, 6 infected animals without liver lesions	4.8 ± 3.9 (1.6–11.9)	18.4 ± 15.6 (5.2–44.7)	105 ± 88 (32–238)	389 ± 254 (183–728)
Group C, 9 non-infected animals	14.6 ± 9.0 (6.5–29.4)	56.7 ± 44.6 (27.2–104)	195 ± 153 (48–468)	582 ± 494 (169–1610)

[1] Figures in parentheses indicate extreme values.

TABLE 2

Average liver tocopherol and vitamin A content of coccidiosis-infected and non-infected rabbits (expressed on dry weight and lipid weight basis[1])

	Liver dry weight in % of fresh weight	Tocopherol/ gm dry liver	Vitamin A/ gm dry liver	Lipid content of liver	Tocopherol/ gm liver lipid	Vitamin A/ gm liver lipid
		μg	*I.U.*	%	*μg*	*I.U.*
Group A, infected animals with liver lesions	24	14.6	173	1.49	235	2790
Group B, infected animals without liver lesions	33	12.4	346	3.13	131	3640
Group C, non-infected animals	29	62.1	556	1.77	1030	9100

[1] Average of three animals in each group.

dry weights were not determined on a sufficiently large number of animals to permit statistical evaluation on this basis.

The correlation coefficient between tocopherol and vitamin A content per gram of liver in all 26 animals was $r = 0.69$.

DISCUSSION

In an ideal experimental setup for the study of the effect of an infectious disease on the nutritional status of the host, the experimental animals would have to be infected at a specified date and any changes in the nutritional status would have to be studied in regular intervals until death or recovery.

In the present study this has been impossible, due to the difficulty of obtaining coccidiosis-free rabbits. We have purchased rabbits from 7 dealers in different parts of the country. Of these dealers, 4 labeled their rabbits as "coccidiosis-free" or as "coccidiosis-free for all practical purposes." Sample animals of each shipment were killed immediately after arrival. Microscopic examination of the gallbladder content revealed the presence of *E. stiedae* oocysts or merozoites in almost every case. In several instances the "coccidiosis-free" animals even exhibited gross liver lesions. Inspection for coccidiosis infection is commonly made by fecal examination for presence of oocysts. However, if fecal examination is carried out at a time when *E. stiedae* has reached only the merozoite stage in its life cycle, no oocysts will be found in the feces even if an abundance of merozoites is present in the liver. The designation "coccidiosis-free by fecal examination" does therefore not exclude the possibility that the animal harbors *E. stiedae*. The only reliable way to ascertain absence of infection is, in our experience, microscopic examination of the gallbladder contents. Even then the detection of merozoites requires some experience as they are quite translucent and much more difficult to discover than the oocysts.

We are thus unable to say which rabbits harbored *E. stiedae* at the beginning of this experiment and which did not. But the fact that classification of animals into infected and non-infected ones at termination of the experiment gave such a striking correlation with their liver vitamin E reserves, justifies the conclusion that liver coccidiosis did have an effect on the vitamin E status of the host. Unexpectedly, rabbits which harbored only the merozoite stage of *E. stiedae* and showed neither gross liver damage nor oocysts, were found to have liver vitamin E levels as low as the more severely infected animals. It thus appears possible that many rabbits used in laboratory studies and designated as "coccidiosis-free by fecal examination" may be in a state of hypovitaminosis E.

Literature values for tocopherol content of rabbit tissues vary widely and differences in diets and analytical methods used by various authors presumably account for this variation. The average liver content of 14.6 μg per gm of tissue found for non-infected animals in this study compares with 9.2 μg per gm reported by Hines and Mattill ('43) and 9.0 to 14.0

μg per gm reported by Kofler ('45). Rosenkrantz ('57) found 29 ± 9 μg per gm of dry tissue by one method and 14 ± 3 μg per gm of dry tissue by another method or approximately 8.7 and 4.2 μg per gm of fresh tissue, respectively (assuming 70% water in fresh liver).

The average muscle tocopherol content of 5.4 μg per gm tissue of non-infected rabbits compares with 8.0 μg per gm reported by Hines and Mattill ('43) and 1.3 ± 3.3 μg per gm reported by Kofler ('45). Rosenkrantz found 17 ± 9 and 31 ± 17 μg per gm of dry tissue by the two methods or approximately 4.2 and 7.8 μg per gm of fresh tissue, respectively (assuming 75% water in fresh muscle).

Liver vitamin A contents showed a less pronounced trend toward lower values in infected animals, chiefly because of wide variations in liver vitamin A reserves of non-infected rabbits. The fact that severely infected animals always showed a low liver vitamin A content appears to indicate an effect of coccidiosis infection on the vitamin A status. Work with a larger number of animals will be required to ascertain whether or not coccidiosis-infected animals without gross liver lesions have significantly lower liver vitamin A levels than non-infected animals. The average vitamin A content of 195 I.U. per gm of liver tissue of non-infected animals compares well with the average of 186 I.U. found by Harms[3] who used 74 rabbits.

On the basis of the present data, no conclusion is possible as to the reasons for the lowered vitamin levels in coccidiosis-infected rabbits. The possibilities of malabsorption, defect of storage and increased demand are under investigation. Lowered vitamin A reserves may be a secondary phenomenon, due to the lack of vitamin E as antioxidant. The role of vitamin E as a protective agent for vitamin A has been established by other authors (Davies and Moore, '41; Dam et al., '52). The correlation coefficient between liver vitamin E and A found in the present investigation is consistent with this idea. However, the possibility of a direct effect of coccidiosis on the vitamin A status can at present not be excluded.

The question arises as to whether coccidiosis infection might have an effect on the other fat soluble vitamins. No vitamin D and K determinations were carried out. Blood clotting time was normal in the infected animals. Rickets has never been reported in rabbits.

As this work has been carried out on growing animals, we do not know what effect coccidiosis infection might have on the vitamin status of adult rabbits.

SUMMARY

1. Average vitamin E levels in liver and skeletal muscle of young rabbits with hepatic coccidiosis were significantly lower than in non-infected animals.

2. Low liver vitamin E reserves were found in infected animals even if no gross liver lesions or oocysts of *Eimeria stiedae* were present. The practical significance of this finding is discussed.

3. Liver vitamin A reserves were always low in infected rabbits with extensive liver damage.

[3] Harms, F. 1942 Vitamin A and animal health. Thesis, Hannover, p. 32, as quoted by T. Moore, 1957 Vitamin A, Elsevier Publishing Company, Amsterdam, p. 460.

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The Amino Acid Composition and the Nutritive Value of Proteins

II. AMINO ACID MIXTURES AS A DIETARY SOURCE OF NITROGEN FOR GROWTH[1]

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The minimum essential amino acid and total protein (N × 6.25) requirements of the growing rat have been reported from our laboratory[2] (Rama Rao et al., '59). Diets containing half of their protein as casein (5%) and half as amino acids (hereafter called casein plus amino acid diet) were used in those studies, while the adequacy of pure amino acid diets for rat growth and maintenance have been investigated by a number of workers (Rose and Womack, '46; Ramasarma et al., '49; Wretlind, '48). Rose and Womack ('46) observed that rats fed a ration containing 18% of casein supplemented with 0.2% of methionine gained 5.1 gm per day, whereas rats fed a diet containing amino acids in place of protein gained 3.5 gm per day. Ramasarma et al. ('49) showed that improved rations containing 18 amino acids providing a nitrogen level of 2.5% resulted in an average growth of 4.1 and 4.4 gm per day under conditions of ad libitum and forced, paired feeding, respectively, the latter rate of growth being 80 to 90% of that obtained on an isonitrogenous casein diet. The present communication deals with the formulation of an amino acid mixture capable of supporting with ad libitum feeding a growth rate of 4.4 gm per day in weanling rats with a nitrogen level of 1.55%. The effects of the type of carbohydrate and the carbohydrate and fat content of the diet on the adequacy of the ration have also been studied. The minimum essential amino acid requirements reported earlier[3] served as the basis for the essential amino acid levels used in these diets.

METHODS AND MATERIALS

Male weanling rats of the Sprague-Dawley strain, weighing 45 to 55 gm, were used. They were housed in individual cages and fed ad libitum for 21 days, except where indicated otherwise. Daily weight gains and food consumption records were kept. The composition of the diets used are given in tables 1, 2, and 3.

RESULTS AND DISCUSSION

Rat growth with amino acid diets and influence of carbohydrate. The results in table 4 show that growth rates of 4.9 and 3.9 gm per day were obtained on diets 12B and 12D, respectively. Diet 12B is a 10% casein diet supplemented with amino acid mixture M_1; diet 12D provides all the amino acids (in their L-forms) at the same levels as in the casein diet 12B; and diet 8A provides the amino acids at their determined requirement levels (Rama Rao et al., '59) from casein and L-amino acids, glycine serving as added non-essential nitrogen source. Rats fed diet 8A grew 5.15 gm per day. When glycine was replaced by a mixture of non-essential amino acids, the growth was improved to 5.7 gm per day (diet 12F). However, when a mixture of essential and non-essential amino acids was used in place of casein (diet 12G) a growth rate of only 3.2 gm per day was obtained. The growth rate was improved to 4.4 gm per day by changing the carbohydrate from starch to dextrin (diet 12H).

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[1] These studies were supported in part under contract no. DA-49-007-MD-544, with the Office of the Surgeon General, Department of the Army. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of the Army.

[2] Rama Rao, P. B., V. C. Metta and B. C. Johnson 1957 Essential amino acid and protein requirements of the growing rat. Federation Proc., *16:* 397 (abstract).

[3] See footnote 2.

This is 77% of the gain (5.7 gm per day) obtained on the comparable casein plus amino acid diet (12F). Ramasarma et al. ('49) showed that on rations containing 18 amino acids to provide a total nitrogen level of 2.5%, average growth rates of rats were 4.1 and 4.4 gm per day under conditions of ad libitum and forced, paired feeding, respectively. Greenstein et al. ('57) obtained growth rates of 4.1 gm per day using a water-soluble, chemically defined diet containing approximately 20% of

TABLE 1

Amino acid mixtures

Amino acid	M_1[1]	M_2[2]	M_3[3]	M_4[4]	M_5
	gm	*gm*	*gm*	*gm*	*gm*
L-Lysine·HCl	0.063	1.063	1.125	0.594	—
L-Histidine·HCl monohydrate	—	0.432	0.325	0.054	—
L-Arginine·HCl	—	0.508	0.268	—	—
L-Tryptophan	0.02	0.13	0.15	0.085	—
L-Phenylalanine	—	0.63	0.70	0.385	—
L-Methionine	0.11	0.35	0.50	0.325	—
L-Threonine	0.05	0.45	0.50	0.275	—
L-Leucine	—	1.00	0.70	0.200	—
L-Isoleucine	—	0.75	0.55	0.175	—
L-Valine	—	0.77	0.55	0.165	—
L-Cystine	—	0.04	—	—	—
L-Tyrosine	—	0.64	—	—	—
L-Glutamic	—	2.30	—	—	41.6
L-Aspartic	—	0.70	—	—	12.7
DL-Serine	—	0.68	—	—	12.3
DL-Alanine	—	0.33	—	—	5.9
L-Proline	—	1.31	—	—	23.7
Glycine	—	0.21	—	—	3.8
Total	0.243	12.293	5.368	2.258	100.0

[1] A supplement to casein when fed at 10% level to meet the established amino acid requirements (Rama Rao et al., '57); see footnote 2, p. 327.

[2] Amino acids as in casein based on Block and Bolling ('51).

[3] Amino acid mixture based on determined requirements; see footnote 2, p. 327.

[4] A supplement to 5% casein diet to bring the total essential amino acids to the established amino acid requirements; see footnote 2, p. 327.

TABLE 2

Diets

	8A	8B	8C
	gm	*gm*	*gm*
Whole egg protein	—	—	19.8
Casein	5.6	—	—
Amino acid mixture	2.258	7.520(M_3)	—
Glycine	2.14	7.439	—
Vitaminized glucose[1,2]	5.0	5.0	5.0
Sucrose	10.0	2.0	2.0
Glucose[1]	10.0	2.0	2.0
Starch	46.002	5.31	12.2
Lard	10.0	34.0	50.0
Wheat germ oil	0.5	0.5	0.5
Cod liver oil	1.5	1.5	1.5
Salts 446	4.0	4.0	4.0
Sodium chloride	1.0	1.0	1.0
Cellulose[3]	2.0	2.0	2.0
Total	100.0	72.269	100.0
Protein (N × 6.25%)	10%	14.9	14.45
Energy, K cal/gm	4.33	6.45	6.44

[1] Cerelose.

[2] Rama Rao et al. ('59).

[3] Woodflock, Brown Company.

TABLE 3

Diets (percentage composition)

Ingredient	12A	12B	12C	12D	12F	12G	12H
Casein[1]	11.2	11.2	—	—	5.6	—	
Amino acid mixture M_1	0.243	0.243	0.243	0.243	—	—	
Amino acid mixture M_2	—	—	12.293	12.293	—	—	
Amino acid mixture M_3	—	—	—	—	—	5.368	5.368
Amino acid mixture M_4	—	—	—	—	2.258	—	—
Non-essential amino acid mixture M_5	—	—	—	—	3.822	8.0	8.0
Glucose[2]	15.0	15.0	15.0	15.0	15.0	15.0	—
Vitaminized sucrose[3]	10.0	10.0	10.0	10.0	10.0	10.0	15.0
Lard	50.0	0.5	50.0	0.5	5.0	5.0	—
Corn oil	—	—	—	—	5.0	5.0	2.0[4]
Wheat germ oil	0.5	0.5	0.5	0.5	0.5	0.5	—
Cod liver oil	1.5	1.5	1.5	1.5	1.5	1.5	—
Salts 446[5]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sodium chloride	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cellulose[6]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Starch	4.557	54.057	3.464	52.964	44.320	42.632	—
Dextrin	—	—	—	—	—	—	62.632

[1] Vitamin-Free Casein, Labco.

[2] Cerelose.

[3] The composition of vitaminized sucrose is as follows: thiamin·HCl and riboflavin, 10 mg, each; Ca pantothenate, 50 mg; pyridoxine·HCl, 5 mg; nicotinic acid, 20 mg; folic acid, 2 mg; *p*-aminobenzoic acid, 50 mg; menadione, 0.1 mg; biotin, 0.1 gm; vitamin B_{12} in mannitol, 1 gm; inositol, 330 mg; choline chloride, 660 mg; and sucrose to 100 gm.

[4] Fat-soluble vitamin mixture in 2 gm corn oil (vitamin A 400 I.U.; vitamin D 200 I.U.; and α-tocopherol 10.0 mg).

[5] Spector ('48).

[6] Woodflock, Brown Company.

TABLE 4

Rat growth with protein and pure amino acid diets (21 days)

Diet	Average weight gain[1]	Average food intake	PER[2]
	gm/day	*gm/day*	
12B	4.9 ± 0.18[3]	11.0	4.5
12D	3.9 ± 0.14	9.1	4.25
8A	5.2 ± 0.12	10.9	4.6
12F	5.7 ± 0.11	12.4	4.6
12G	3.2 ± 0.19	8.3	3.95
12H	4.4 ± 0.21	9.3	4.73

[1] Five rats fed diet 8A and 6 in all other groups.

[2] Protein efficiency ratio: $\frac{\text{weight gain (gm)}}{\text{total protein intake (gm)}}$.

[3] Standard deviation of the mean.

amino acids providing a total N level of 2.52%. Diet 12H (table 3) provides 13.4% of amino acids for a total nitrogen level of 1.6% (equivalent to 10% of protein [N × 6.25]). An average growth rate of 4.4 gm per day was obtained using this diet (table 4). No previous reports have been found of weight gains of over 4 gm per day with amino acid diets at any total nitrogen level.[4]

Replacing starch by dextrin in diet 12H has clearly resulted in improved food intake and weight gain of the rats, and from the protein efficiency ratio data (table 4) it can be seen that dextrin also increased the efficiency of protein utilization.

The beneficial effect of dextrin has been reported by Marshall and Womack ('54), who observed that corn dextrin in some

[4] Since this manuscript was submitted the paper of Wachter and Berg ('60) has appeared reporting a gain of 4.3 gm/rat per day over a 28-day period with a diet supplying 12.1% of an L-amino acid mixture.

TABLE 5

Effects of fat on growth using protein and amino acid diets

Diet	Fat	Protein (N × 6.25)	Average weight gain (21-day trial)	Average weight gain 26th–40th day
	%	%	*gm*	
8A	12	10	108 ± 3.2	
8B	50	14.9	− 15	
8C	52	14.45	75 ± 3.5	
		In 26 days		*gm*
12A	52	10	93 ± 3.1	41
12C	52	10	82 ± 4.5	−16

[1] Five rats in groups 8A, 8B, and 8C; 6 rats in 12A and 12C.
[2] Standard deviation of the mean.

fashion facilitates the utilization of protein for liver protein formation, resulting in a lower nitrogen excretion and retarding the accumulation of liver fat, while Harper and Spivey[5] showed that the type of carbohydrate in the diet influences the food intake.

Rat growth using high-fat amino acid diets. The data in table 5 show that the rats fed diet 8A, which provided 12% of fat, and 10% of protein (N × 6.25) as casein plus an amino acid mixture (Rama Rao et al., '59), gained an average of about 5 gm per day. Since it has been observed repeatedly[6] that the rats fed a pure amino acid diet consumed only 7 to 8 gm of diet per day as compared with a food intake of 11 to 12 gm per day by rats fed comparable diets providing intact protein, diets 8B and 8A were formulated to provide equal intakes of calories and nitrogen, assuming that the rats fed diet 8B (the amino acid diet) would consume on an ad libitum basis 70% of the intake of rats receiving diet 8A (the control diet). The data on this pure amino acid high-fat diet (8B) show that the rats consumed only about 4 gm of diet per day and growth failure was accompanied by symptoms reminiscent of riboflavin deficiency. The riboflavin requirement has been shown to increase with the fat content of the diet (Czaczkes and Guggenheim, '46); however the addition of large excesses of riboflavin or of a mixture of all the known B-vitamins did not bring about any improvement in growth or any alleviation of symptoms. With the diet containing whole egg (8C) the food intake was about 11 gm per day and a growth rate of 3.4 gm per day was obtained, although diet 8C provided the same percentage of protein and calories as diet 8B. In a preliminary pair-feeding experiment comparing diets 8B and 8C, growth failure ensued also with the whole egg-high fat diet (8C), since the food intake was decreased to 4 gm per day to equate it with the intake on diet 8B. However, apart from growth failure, there were no other symptoms over a period of three weeks with diet 8C, while at the same time three out of 5 rats fed the pure amino acid high-fat diet (8B) died.

In diets 8A and 8B, glycine provided the extra non-essential amino acid nitrogen. Toxicity of glycine has been observed[7] (Rama Rao et al., '60), and it was thought that the toxicity of glycine coupled with the reduced intake using the high-fat diet (8B) might be more deleterious to rats fed an amino acid diet than a diet containing "intact protein" (8C). Diets 12A and 12C contained 52% of fat and were identical except that 12A contained intact casein and 12C contained an amino acid mixture based on casein. Thus diet 12C contained a mixture of non-essential amino acids, in contrast with diet 8B, which contained glycine as non-specific N source. The results given in table 5 again showed growth failure, decreased food intake and deficiency symptoms on the amino acid diet

[5] Harper, A. E., and H. E. Spivey 1957 Effect of type of carbohydrate on protein utilization. Federation Proc., *16:* 387 (abstract).

[6] Rama Rao, P. B., and B. C. Johnson 1958 Amino acid mixture as dietary source of nitrogen for rat growth. Federation Proc., *17:* 489 (abstract).

[7] Rama Rao, P. B., V. C. Metta, H. W. Norton and B. C. Johnson 1960 The amino acid composition and the nutritive value of proteins. III. The total protein requirement and the role of non-essential amino nitrogen. Unpublished data.

(12C), while the rats on the intact casein + high-fat diet (12A) continued to grow at the rate of over 3 gm per day. These results extend the observations of Borman et al. ('46) that better gains are obtainable with amino acid diets containing smaller proportions of fat. Yoshida et al. ('57) have reported that the fat content of the diet did not influence the nitrogen retention or calorie utilization with rations containing intact protein (10% of casein) and had only a transitory beneficial effect on food consumption in the first week in which the protein/calorie ratio was held constant. The causative factors for the deleterious effect of a high-fat content in amino acid rations are at present under study.

SUMMARY

Male weanling rats of the Sprague-Dawley strain were used in growth studies on protein and amino acid rations, and the effects of fat content and the type of carbohydrate were studied.

An amino acid diet containing 50% of fat resulted in growth failure and a diseased appearance not curable by extra oral supplementation with B-vitamins.

An amino acid diet providing the minimum requirements of the essential amino acids plus non-essential amino acids and fed at a 10% protein level (N × 6.25) was found to promote a growth rate of 4.4 gm per day when fed ad libitum.

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Energy Requirements of the Adult Rat Fed an Amino Acid Diet[1]

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A higher caloric requirement (22 to 29%) for the maintenance of nitrogen equilibrium in adult man has been observed when an adequate mixture of amino acids or a protein hydrolyzate fortified with tryptophan was fed than when an isonitrogenous amount of protein was used (Rose et al., '54). This observation in the human seems to have been difficult to explain, according to the authors, who stated: "No satisfactory explanation can be offered to account for these unexpected observations. One might anticipate that amino acids would be absorbed from the alimentary tract more rapidly when ingested in the free state than when they must first be liberated from proteins by the process of digestion. Conceivably, this might lead to a temporary flooding of the organism, thereby inducing either a spillage into the urine or an accelerated catabolism. That wastage through excretion does not occur to a significant extent is demonstrated by the daily determinations of urinary α-amino nitrogen." Limitations imposed by the experiment had necessitated the inclusion of several racemic amino acids in the simulated-casein amino acid mixture; however, the distribution of the L-isomers was essentially the same as that of the casein control diet. The D-amino acids other than methionine contributed about 25% nitrogen of the complete mixture.

In view of the established principle that the protein requirements for maintenance among adult animals of different species are closely related to the basal metabolism, (Terroine and Sorg-Matter, '27; Smuts, '35) it seems justified to express protein requirements of adult animals as a percentage of the diet or dietary calories. But if the observation that 22 to 29% higher dietary energy is needed for adult man when the protein fraction of the diet is replaced by the corresponding amino acids should be true for other animals, the customary practice of expressing amino acid requirements may have to be revised. As the adult rat is extensively used in nutritional investigations, it was felt of value to determine whether the energy requirements of the rat to maintain nitrogen equilibrium will change if protein in the diet is replaced by a mixture of the corresponding amino acids or by the protein hydrolyzate.

The purpose of this paper is to report that, in the case of the adult rat, replacing the dietary protein by a mixture of the corresponding amino acids or the protein hydrolyzate does not increase the energy requirement for maintenance of the nitrogen balance. These findings with the rat are in disagreement with those for the human, indicating a species difference in the relationship of energy metabolism and dietary amino acids.

MATERIALS AND METHODS

Two experiments were conducted with adult male rats. In the first experiment, the effect on nitrogen metabolism of replacing casein in the diet with its corresponding L-amino acids was studied. In the second experiment, the effect on nitrogen balance of replacing the casein by its hydrolyzate or its corresponding amino acids was studied.

The daily energy requirements of the adult rat were estimated from Brody's generalized equation for basal energy, $Q_{cal.} = 70.4\ W_{kg}^{0.734}$ (Brody and Proctor,

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[1] Preliminary results of the investigation were reported in the Division of Biological Chemistry of the 132nd American Chemical Society meeting held in New York, September 8–13, 1957.

'32), to which was added 25% for activity increment. The metabolizable energy values of the diets were assumed to be 90% (Metta and Mitchell, '54). Casein-nitrogen required for nitrogen equilibrium was estimated using the value of 4.24 mg of absorbed nitrogen per basal calorie (Mitchell, '59); a digestibility coefficient of 0.98 was assumed for casein. The test diets were then formulated and mixed in such a way that, when offered in appropriate amounts, they would simultaneously provide the required amounts of nitrogen and energy to rats.

Experiment 1

Diets. Two diets, one a casein diet and the other an amino acid diet simulating casein, were formulated (see table 1 for composition) and mixed so that they were identical in composition except for the nitrogen source. Barium sulfate was included in the diets to serve as roughage. The first diet was composed of 8.68% of casein[2]; the second diet contained 10% of the amino acid mixture, simulating casein. This mixture was composed of 18 L-amino acids in the proportion found in casein, as is reported in summary table 8 of Block and Bolling ('51). No DL-amino acids were used; 8.68 gm of casein and 10 gm of this amino acid mixture were found by analysis to be isonitrogenous.

Twelve Sprague-Dawley male rats, over 6 months old and weighing approximately 470 gm each, were distributed at random into two equal groups. They were individually housed in wire-bottom cages during the 10-day prefeeding period and were transferred to glass metabolism cages during the following 9-day collection period, to enable separate and accurate collection of urine and feces. Each rat was offered and consumed 14 gm a day of the diet over an entire feeding period of 20 days, and maintained approximately his initial body weight during this period.

Experiment 2

Diets. Three diets were used in this experiment: a casein diet, an L-amino acid diet simulating casein, and a casein-hydrolyzate diet to which tryptophan was added. These three diets were similar in composition except for the source of nitrogen (see table 1) and were isocaloric and isonitrogenous. Diets 1 and 2 were similar to diets used in experiment 1. Diet 3 contained 9.7% of casein hydrolyzate fortified with 1.3% of L-tryptophan.

[2] Labco, Borden Company.

TABLE 1

Composition of the experimental diets

	Diet 1	Diet 2	Diet 3
Casein[1]	8.7	—	—
L-Amino acid mixture[2]	—	10.0	—
Casein hydrolyzate + L-tryptophan[3]	—	—	9.7
Starch	47.3	46.0	46.3
Sucrose	20.0	20.0	20.0
Lard	10.0	10.0	10.0
Vitaminized glucose[4]	5.0	5.0	5.0
Cod liver oil	1.5	1.5	1.5
Wheat germ oil	0.5	0.5	0.5
Sodium chloride	1.0	1.0	1.0
Mineral mixture (446)[5]	4.0	4.0	4.0
Barium sulfate	2.0	2.0	2.0
Chemical analysis of diets after mixing:			
Total nitrogen (%)	1.310	1.317	1.351
Gross energy (Cal./gm)	4.27	4.34	4.33

[1] Labco, Borden Company.
[2] L-Amino acids, purchased from the California Corporation for Biochemical Research, to simulate casein (Block and Bolling, '51).
[3] Hydrochloric acid hydrolyzate, purchased from Nutrition Biochemicals Corp., Cleveland; 1.3% L-tryptophan was mixed with the hydrolyzate.
[4] Vitaminized Cerelose, Metta and Mitchell ('54).
[5] Spector ('48).

Twelve Sprague-Dawley female rats, 6 months old and weighing 300 gm, were divided at random into three groups. They were individually fed the test diets in amounts to satisfy their nitrogen and energy requirements, as described above. Following a 10-day prefeeding period, individual collections of the excreta were made for a period of 10 days.

Chemical analysis

Diets and their nitrogen-components were analyzed for total nitrogen by the Kjeldahl method. Gross energy (heat of combustion) was determined by burning the samples in an oxygen bomb calorimeter.

Feces and urine from each rat were collected separately every day and pooled for the collection period. Urines were preserved under toluene at 4°C and feces were collected in small bottles, loosely stoppered.

Separation of hair from rat feces. Adult rat feces are contaminated by hair which the rat sheds or licks. If the feces are not freed from hair before making nitrogen determination by the usual Kjeldahl method, the nitrogen values will be exaggerated. Fecal material is freed from hair as follows:

Feces are suspended in a few ml of 0.2 N HCl in a beaker for a day, in a refrigerator free from ammonia. With a thick rubber policeman or a rubber stopper the feces are then worked through a 40-mesh sieve, placed on a funnel. Warm water is squirted on the feces from time to time while working with the rubber policeman. The fecal matter passes down the sieve and collects in a beaker placed below the funnel, while the rat hair stays on the screen. Complete separation of feces from hair is indicated when the hair on the sieve appears clean and free from any marker that may have been used. The fecal suspension, free from hair, is now made to a volume and aliquots are taken for nitrogen and for heat of combustion determination.

Gross energy on urine and feces suspensions was determined in the bomb calorimeter after absorbing the sample on a cellulose block and drying at a low temperature.

RESULTS AND DISCUSSION

Results of the first experiment are given in table 2. Rats on the casein-simulated L-amino acid diet consumed almost the same amounts of nitrogen and calories, as the rats on the casein control diet. Sixty

TABLE 2

The effect on nitrogen balance of feeding casein versus L-*amino acids to adult rats. Results expressed on a 9-day basis; mean values with standard deviations of the mean*[1]

	Casein diet	L-Amino acid diet
Diet intake, gm	126	126
Energy intake, gross Calories	538	547
Nitrogen intake, gm	1.650	1.659
Fecal energy, Calories	21.6 ± 1.14	23.4 ± 0.99
Urinary energy, Calories	10.9 ± 0.62	10.3 ± 0.33
Fecal nitrogen,[2] mg	215 ± 13.7	155[3] ± 6.1
Urinary nitrogen, gm	1.112 ± 0.012	1.186[4] ± 0.025
Nitrogen balance, mg	313 ± 17	351 ± 39
Digestibility of food energy, %	96.0 ± 0.20	95.7 ± 0.17
Metabolizable energy of the diet consumed,[5] Calories	504 ± 1	511 ± 1
Metabolizable energy of the diet, %	93.6 ± 0.19	93.5 ± 0.01
Hair from feces, mg	147 ± 30	114 ± 19
Nitrogen of fecal hair, mg	21 ± 4.4	16 ± 2.7
Initial body weight of the rat, gm	471 ± 2	472 ± 2
Final body weight of the rat, gm	475 ± 2	482 ± 1

[1] Six rats per treatment.
[2] All hair contaminating the feces was removed before analyzing for total nitrogen.
[3] $P < 0.01$.
[4] $P > 0.02 < 0.05$.
[5] Metabolizable energy was determined after correcting for nitrogen balance (Metta and Mitchell, '54).

dietary calories per day (gross energy) were found adequate to maintain the body weights of these rats. The data demonstrate that adult male rats consuming an amino acid mixture, isonitrogenous with protein, are able to maintain body protein stores, with all rats remaining in a positive nitrogen balance. An increase in energy requirement above maintenance did not ensue using the amino acid as compared with the protein-containing rations.

The latter phenomenon was observed in studies on the amino acid requirements of man, negative nitrogen balances being consistently observed when the casein in a diet was replaced by an isonitrogenous amount of an adequate amino acid mixture or a casein hydrolyzate supplemented with tryptophan. Even a simulated-casein amino acid mixture failed to restore nitrogen equilibrium as long as the energy intake was kept constant. However, Rose et al. ('54) found it possible to restore nitrogen equilibrium in subjects receiving amino acids by increasing the energy intake by 21%.

The results of the second experiment are given in table 3. Variation in nitrogen or energy intake had no effect on nitrogen or energy digestibility or on nitrogen balance, according to covariance analysis. By analysis of variance, no significant difference in digestibility of nitrogen, or nitrogen balance was found as the result of type of nitrogen source. Thus, when casein in the diet is replaced by an isonitrogenous amount of the corresponding L-amino acids or an acid-hydrolyzate of casein fortified with L-tryptophan, female rats continue to remain in positive nitrogen balance, indicating that the amino acid mixture and the hydrolyzate are as efficiently utilized in metabolism as is casein. Furthermore, this observation in the female rat confirms the finding in the male rat that the simulated-casein L-amino acid mixture is as efficient as casein itself in nitrogen metabolism when fed in isocaloric diets. The observation that the casein hydrolyzate is as efficient as casein as a nitrogen source for the rat, and that, when fed, it does not necessitate an increased energy requirement in the rat for maintenance of the nitrogen status, is different from the finding in man, where it was observed that a negative nitrogen balance ensued in two individuals when casein was replaced by an isonitrogenous amount of its acid hydrolyzate fortified with tryptophan.

In experiments 1 and 2 the amount of casein-nitrogen and food-energy offered to the rats to maintain them in nitrogen and energy equilibrium seems to be slightly in excess of the requirements under the experimental conditions used. Male rats fed the casein diet gained an average of 0.4 gm a day, whereas those on an isocaloric and isonitrogenous amino acid diet gained 1.1 gm. The latter value is significantly larger ($P < 0.01$) and is perhaps indicative of better dietary energy utilization with an amino acid diet, assuming the composition of the body gains to be similar to those consuming the casein diet. However, the possibility of water retention in the tissues with amino acid diets cannot be excluded.

A positive nitrogen balance of 35 to 39 mg of nitrogen a day, which was observed in experiment 1, or of 44 to 51 mg in ex-

TABLE 3

The effect on nitrogen balance of feeding casein, casein-hydrolyzate and an amino acid diet to the adult female rat.[1] *Mean values; results expressed on per day basis*

Energy intake	Nitrogen intake	Digestibility of dietary nitrogen	Nitrogen balance
Cal.	*mg*	*%*	*mg*
	Casein diet (diet 1)		
49.2	151	92.1	+47
	Simulated casein L-amino acid diet (diet 2)		
48.3	146	91.7	+51
	Casein-hydrolyzate plus tryptophan diet (diet 3)		
47.0	147	91.1	+44

[1] Four rats per treatment.

periment 2, merits consideration. Nitrogen equilibrium as a criterion for assessing the adequacy of dietary nitrogen is recognized to be inadequate, as it fails to accredit the nitrogen of the integumental structures, such as hair, wool, epidermis, nails, and claws, which grow throughout life (Holt and Albanese, '44). In the case of the adult rat, hair is an important integument and is found to contain 15.9% of nitrogen. In the first experiment, the adult rat on the casein diet, on an average, shed daily 41 mg and voided daily 16 mg of hair mixed with feces. The shed hair represented 5.9 mg of nitrogen, and the hair which passed through the gastrointestinal tract, 2.3 mg of nitrogen. Thus, 8.2 mg of hair-nitrogen, if added to the fecal nitrogen values, may introduce an error of considerable magnitude. Nitrogen balance, by definition, is the difference between nitrogen intake and nitrogen excreted, and thus excludes completely the nitrogen of the integuments. Hence, it becomes imperative to separate all hair from adult rat feces before analyzing for total nitrogen.

SUMMARY

The energy requirement of the adult rat does not increase (or decrease) if the protein of the diet is replaced by its natural forms of the amino acids or by the protein-acid-hydrolyzate plus tryptophan.

A method to separate hair from rat feces is described, and the significance of hair nitrogen in computing nitrogen balances is discussed.

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